BIOTECH SCHOLARS



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Introduction to Biotechnology

Orange County Biotechnology Education Collaborative

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Lab Manual: Synthetic Biology Protocols

Nathan Reyna, Ruth Plymale, & Kristine Johnson

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University of Iowa

Genetics, Agriculture, and Biotechnology

Walter Suza and Donald Lee

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Doing Research

Celia Brinkerhoff

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The Ohio State University

Choosing and Using Sources - A Guide to Academic Resear

Cheryl Lowry

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BIOTECH SCHOLARS (SDSU)

Welcome to the Biotech Scholars at San Diego State University Home Page



BioTech Scholars will embark on a year-long program with a learning community of peers, faculty, and graduate students in the life sciences. Scholars will improve their study strategies and academic success and build skills in writing resumes, interviewing, and networking. Scholars will participate in a two-week intensive lab research experience, network with professionals from the BioTech industry, and gain assistance securing summer internships in BioTech.

1: Learning Communities

1.1: Welcome Session

1.2: Why STEM?

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3. Meet our 23-24 Scholars

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1: LEARNING COMMUNITIES

Overview

The **learning community sessions** engage scholars in biweekly professional learning opportunities. Each week will consist of pre-, during- and post- session activities designed to facilitate scholar's skills within STEM.

The session links below are in order of occurrence and will take you to a page for each session

1.1: Welcome Session

1.2: Why STEM?

1.3: Biotech Panel

1.4: Building a Resume

1.5: Navigating Internships

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2.3 Biotech Panel 2

Wrapping Up

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1.1: Welcome Session

Session Overview

This welcome session introduces participants to the Biotech Scholars at San Diego State University Program, promotes community building, provides a case study of biotechnology in action, and orients participants to the Libretext platform that will be used for asynchronous content delivery.

Learning Outcomes

This session will be used to

- Make you feel like you belong to this community of BT@SDSU Scholars
- Motivate you to fully engage with the program
- Show our interest in you and your experiences
- Make clear what you can expect of the program and what's expected of you
- Help you learn about your peers in the program and the leaders
- Provide information about a hot topic in biotech: lab-grown meat

Key Terms

- Biotechnology
- Lab-cultivated meat
- Synchronous/asynchronous delivery

Active Learning/Formative Assessment Strategies

- Large group Activity
- Small group Activity
- Brainstorming
- Directed- and random-call report out
- Reflection

Pre-Session

Background

Biotech Scholars will embark on a year-long program with a learning community of peers, faculty, and graduate students in the life sciences. Scholars will improve their study strategies and academic success and build skills in writing resumes, interviewing and networking. Learning communities will occur biweekly.

Tasks

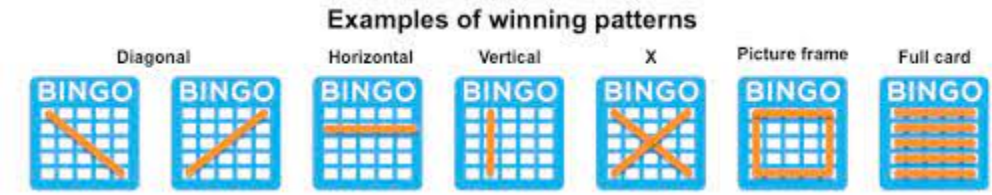
Please complete the following tasks prior to the workshop:

1. Join our GroupMe at https://groupme.com/join_group/95539409/TGx19dUf.

During Session

Activity

1. Human Bingo



2. Cultivated Meat Case Study

Jeff Rodriguez, a junior biology major, was feeling nervous as he drove to pick up his date for the evening. Meeting Jordan in Chemistry lab last Monday had felt like a scene straight out of a movie.

“I guess you can call me adenine because I got paired with you”, Jordan had said to him, approaching the lab bench with a smile.

“You’re sodium funny”, Jeff had responded, making Jordan laugh.

Even though it seemed they had quite the chemical bond, Jeff caught himself wiping the sweat off his palms on his pants as they parked in front of a new local restaurant.

As the two were seated, the server explained the evening’s special, “We have a treat this evening! We are currently serving our certified cultured beef steaks. This is a new line of beef being offered to only the finest restaurants. It is truly the latest meat product and is supposed to be more nutritious than traditional steaks as it is biologically cultured.”

“I saw a news story on this last week”, Jordan says. “The FDA just approved for lab-grown meat to be sold for public consumption. Do you think you are going to try one?”

Curious, but unsure, Jeff asks the server for another minute to look over the menu.

3. Logistics and Questions

After Session

By next meeting, each GROUP is expected to submit a brief (approx. 1 double spaced page) report on your findings. Using the information you have collected, decide as a GROUP whether you think Jeff should try the burger. Everyone should be prepared to discuss what they found and what they decided by next meeting.

Please submit your reports here: https://drive.google.com/drive/folders/1JGPsn9sKMgLt4xj2yJiw_gz4xRDiBHYM?usp=share_link

---

References

Cultivated Meat Case Study adapted from Hains, B. Hains, D. & Balschweid, M. Certified Cultured Beef: Raising Beef Without the Cow. Youth Development and Agricultural Educaiton. Purdue University.

Drive Links

Session Slides:

https://docs.google.com/presentation...f=true&sd=true

Cultivated Meat Case Study:

https://docs.google.com/document/d/1...f=true&sd=true

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1.2: Why STEM?

Session Overview

This session is designed to help garner student awareness of how values are tied to STEM research. Students will reflect on how both personal and social values were reflected in their decisions for/against choosing to try lab-cultivated meat in the week 1 case study. They will learn of the different types of value and share about their own values as they relate to their STEM research journey.

Learning Outcomes

This session will be used to

- Help scholars identify and articulate personal and social values influencing STEM research
- Increase motivation for STEM research careers
- Establish a community of student scholars and mentors

Key Terms

- Value
- Utility Value
- Intrinsic Value
- Attainment Value
- Social Influencers

Active Learning/Formative Assessment Strategies

- Directed- and random-call report out
- Reflection
- Large group activity
- Debriefing

Pre-Session

Background

For this week, we are going to take a deeper dive into our "why". Why exactly is STEM research important to each of us? Take a moment to reflect on this question. Think about the particular people, places, times, experiences, beliefs, etc. that have brought you to this moment in your career. What has been most important in getting you this far? Why is STEM important to you?

Tasks

Please complete the following tasks prior to the session:

1. Submit After-session Activity from Week 1.
2. Now that you have reflected, we want you to select the **ONE** reason that you think has been **MOST** important to your "why" that you would feel comfortable sharing with the group. You may find it hard to pick just one, but that is normal. As we found out from last week, there are always a lot of factors to consider when making decisions! Now pick an **object** to represent this reason. For example, you might choose to represent a specific person by their favorite flower, your family by a favorite meal,

or the time you broke your arm by lizard sticker your best friend gave you to feel better. Be creative! Once you have decided on your object, send a picture of that object to Shannon (mallory.clark@uga.edu). You do not need to provide any context to the picture, and DO NOT share your picture with the group.

During Session

Activities

1. Cultivated Meat Case Study Reflection

2. STEM Values Activity

3. STEM Values Reflection

Post-Session

1. Next session we will be hosting panelists from various Biotech companies. Please submit at least 2 questions you would like to have answered by the panelists to the following document: https://docs.google.com/spreadsheets...it?usp=sharing

2. Between now and next learning community, your goal is to meet up with another scholar or PI. Go for coffee, schedule a zoom meeting, participate in a study group, or even just catch up between classes in the hallway - the activity is yours to choose! In the GROUP Me, let us know what you did (pictures are always appreciated!)

References

STEM Values Reflection based on Cultural Artifact Activity adapted from Stonehill College (2012). Practicing Incusion: Icebreakers and Team Builders for Diversity.

Eccles, J. S., & Wigfield, A. (2020). From expectancy-value theory to situated expectancy-value theory: A developmental, social cognitive, and sociocultural perspective on motivation. *Contemporary Educational Psychology, 61,* Article 101859.

Session Slides

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1.3: Biotech Panel

Session Overview

This session will introduce scholars to individuals currently working within the Biotech Industry.

Learning Outcomes

This session will

- Provide access to STEM career resources for scholars
- Build social capital through connections with STEM professionals in the biotech industry
- Increase motivation for STEM research careers

Key Terms

Networking

Active Learning/Formative Assessment Strategies

- Panel discussion

Pre-Session

Background

Networking, or interacting with other individuals within in a field, is a key way to exchange knowledge and develop professional contacts. Interactions can be more formal, such as participating in internships or attending research conferences, or less formal, such as sharing ideas or resources with a colleague or classmate. Whether you talk about research ideas, job opportunities, project collaboration, or hobbies outside of work - the important part is to make connections!

Tasks

Please complete the following tasks prior to the workshop:

1. Submit week 2 post-session activities.
2. Familiarize yourself with our panelists by reading the information at the following link: https://docs.google.com/document/d/1...it?usp=sharing

---

During Session

Activities

1. Biotech Panel

Post-Session

No post-session activities. Please see pre-session activity on next page.

References

Session Slides

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1.4: Building a Resume

Session Overview

This session will assist scholars in finding internships within the field of biotechnology and developing resumes for these positions.

Learning Outcomes

Scholars will

- Learn about the different parts of a resume and their purpose
- Reflect on their own unique strengths and experiences
- Prepare a professional resume that reflects their knowledge, skills, and education

Key Terms

- Resume
- Objective Statement
- Cohesion
- Tailoring
- Internship

Active Learning/Formative Assessment Strategies

- Workshop
- Independent writing

Pre-Session

Background

Tasks

1. Complete the following questions prior to our next session: https://docs.google.com/document/d/1...f=true&sd=true

---

During Session

Activities

1. Logistics
2. Resume Workshop
3. Session Activity

    a) Find a summer biotech internship for which an undergraduate STEM major with basic skills/training would be qualified to apply. Fill in the following spreadsheet with the information found about the internship.

https://docs.google.com/spreadsheets...usp=share_link

b) Pick one of the internships and begin constructing a draft of your resume for that internship.

Post-Workshop

- Please submit your resume draft to the appropriate folder to be reviewed. All resumes must be submitted by **5 p.m. October 26, 2023**.

https://drive.google.com/drive/folde...usp=drive_link

References

Perdue University. (2023). Resumes and CVs. https://owl.purdue.edu/owl/job_searc...tas/index.html

Lasek-White, C. (2023). English 4001: Careers for English Majors [Syllabus].

Session Slides

https://docs.google.com/presentation...f=true&sd=true

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1.5: Navigating Internships

Session Overview

This session will introduce scholars to career services available on SDSU campus. Students will learn how to use Handshake to locate and apply for internships.

Learning Outcomes

Scholars will be able to

- Navigate Handshake as a career resource for locating and applying for internships
- Develop professional online profiles via Handshake
- Access resources provided by SDSU career services

Key Terms

- Handshake
- Artificial Intelligence Scanning
- Internship
- Professional Profile

Active Learning/Formative Assessment Strategies

- Workshop

Pre-Session

Background

1. Prior to attending next section,

    a. Log into Handshake via the SDSU Career Services webpage https://sdsu.joinhandshake.com/login

    b. Begin updating your user profile within handshake. Remember this is a professional website which may be seen by potential employees.

During Session

1. Welcome and Logistics

2. Career Services Workshop presented by Alejandro Rodriguez, Career Counselor, SDSU

Post-Session

1. Cover Letter Activity

a. Explore resources on how to write a good cover letter. (Here are a few to help you get started, but feel free to find additional ones!)

https://www.youtube.com/watch?v=hAKbTiIoQXA

https://www.youtube.com/watch?v=rEmoFGlq8lM

https://www.biospace.com/article/how...cientist-job-/

https://tgilifesciences.com/blog/5-t...aceutical-job/

b. Draft your own cover letter and submit it to the same folder as your resume (link below). **Drafts are due by 5 p.m. Nov. 9.**

https://drive.google.com/drive/folde...h9?usp=sharing

References

Session Slides

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1.6: Finalizing Internship Documents

Session Overview

During this session, scholars will utilize feedback from peers and PI's to finalize cover letters and resumes so they are ready to apply for biotech internships

Learning Outcomes

This session will

- Provide scholars with constructive feedback on internship documents
- Demonstrate the process of collaborating with peers
- Allow scholars to finalize resumes and cover letters
- Help scholars identify and apply for additional biotech internships

Key Terms

- Peer feedback
- Constructive feedback

Active Learning/Formative Assessment Strategies

- Small group discussion
- Review and Resubmit
- Application submissions

Pre-Session

Background

Collaboration is central to the scientific process. It is important to include in each step - from designing an experiment all the way to publishing results. Discussing work with both peers and mentors can provide new perspectives and strengthen independent work. Collaboration also supports the opportunity to build professional networks and expand research teams.

Tasks

1. Identify and add another biotech internship to the spreadsheet.

https://docs.google.com/spreadsheets...it?usp=sharing

Reminder: If you find an internship with a deadline before Nov. 15 and would like your resume and cover letter reviewed before then, please email Shannon.

During Workshop

Tasks

1. Review feedback from PI's on your resume and cover letter.
2. Collaborate with both peers and PI's to make appropriate updates to resume and cover letter.
3. Select one interhsip that you plan to apply for.

Post-Workshop

Tasks

It is good practice to receive feedback on application materials before applying. When you actually apply to an internship, we want to review your materials to help you build this practice. Therefore, all scholars must apply to at least one internship by **December 31**.

To receive feedback on your application materials prior to winter break, please submit them to the following folder no later than **December 13.**

https://drive.google.com/drive/folders/1Zgmzw5MRlyrZm9UHYIOhlFiddV1bZRGW?usp=drive_link

If you have an application that needs to be submitted prior to December 13, please notify Shannon no less than 10 days before the application deadline to organize feedback on your application materials.

References

Session Slides

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1.7: Creating Balance

Session Overview

During this session, scholars will

Learning Outcomes

This session will

- Provide program feedback
- Shape spring program goals

Key Terms

- Work-life balance

Active Learning/Formative Assessment Strategies

- Large group activity
- Reflection
- Goal setting

Pre-Session

Background

Whether it's making sure to always eat breakfast before your 8 a.m. class, finishing a tough assignment, or acing your finals - it is important to celebrate successes along your journey. During this session, we will celebrate the successes of the semester by reflecting on what we have learned and what goals we have for the spring semester.

During Workshop

Activities

1. Feedback Survey https://ugeorgia.ca1.qualtrics.com/j...8lMn30qvkVRFiK
2. Large group reflection.

Post-Workshop

- Submit items for feedback by December 13.
- Enjoy your break!

Session Slides

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1.8 Interviewing

Session Overview

During this session, scholars will learn how to prepare for professional interviews

Learning Outcomes

This session will

- Describe the important components of a good interview
- Determine how to prepare for an interview

Key Terms

- Professional Interviews

Active Learning/Formative Assessment Strategies

- Small group discussion
- Workshop

Pre-Session

Background

Interviewing is a two-way street. Employers use interviews to better understand the qualifications, character, and chance of success of potential applicants. At the same time, applicants should use the interview process to determine their own fit within the company.

Tasks

1. Access the zoom link for today: https://zoom.us/j/93153524945

During Workshop

Tasks

Interviewing Workshop

- Preparing for the interview
- During the interview
- After the interview

Post-Workshop

Tasks

Link to Materials: https://drive.google.com/drive/folde...usp=drive_link

1. Complete questions 1-5 of the reading guide.

Important Links

Summer Internship List: https://docs.google.com/spreadsheets...it?usp=sharing

---

Session Slides

Interviewing Slides: https://drive.google.com/file/d/1tDYlbkl8FrwRDeUjTUfNIV4CCpg3wUzA/view?usp=drive_link

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1.9 Analyzing Scientific Literature

Session Overview

During this session, scholars will learn how how to read, understand, critique, and summarize scientific literature.

Learning Outcomes

This session will

- Practice summarizing the information provided in scientific literature
- Improve skills in navigating scientific literature, e.g., making sense of what you read, what to do when you don't understand something you read

Active Learning/Formative Assessment Strategies

- Article Critique
- Guided Reading
- Think-Pair-Share

Pre-Session

Background

Reading, understanding, critiquing, and summarizing scientific literature are each important skills for anyone in the biotech industry. In addition, you may be asked to share research findings with a variety of audiences - from fellow experts in your field to children just learning about all the possibilities that the biotech industry has to offer. These skills. are challenging and take practice, so we will be dedicating the next few sessions to building them.

Tasks

1. Submit post assignment to the Google drive: https://drive.google.com/drive/folde...usp=drive_link

---

During Workshop

Tasks

1. Discussion of guided reading assignment Questions 1-5.

Post-Workshop

Tasks

1. Sign up for a quick zoom check-in with Shannon. **Please sign up no later than Thursday Feb. 8.**

https://docs.google.com/spreadsheets...f=true&sd=true

2. Complete questions 6-12 of the reading guide.

Link to Materials: https://drive.google.com/drive/folde...usp=drive_link

Important Links

Summer Internship List: https://docs.google.com/spreadsheets...it?usp=sharing

Session Slides

https://docs.google.com/presentation...f=true&sd=true

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2.0 Analyzing Scientific Literature

Session Overview

During this session, scholars will learn how how to read, understand, critique, and summarize scientific literature.

Learning Outcomes

This session will

- Practice summarizing the information provided in scientific literature
- Improve skills in navigating scientific literature, e.g., making sense of what you read, what to do when you don't understand something you read

Active Learning/Formative Assessment Strategies

- Article Critique
- Guided Reading
- Think-Pair-Share

Pre-Session

Background

Reading, understanding, critiquing, and summarizing scientific literature are each important skills for anyone in the biotech industry. In addition, you may be asked to share research findings with a variety of audiences - from fellow experts in your field to children just learning about all the possibilities that the biotech industry has to offer. These skills. are challenging and take practice, so we will be dedicating the next few sessions to building them.

Tasks

1. Submit post assignment to the Google drive: https://drive.google.com/drive/folde...usp=drive_link

---

During Workshop

Tasks

1. Discussion of guided reading assignment Questions 6-12.

Post-Workshop

Tasks

Imagine your interviewing for an internship. One of the interview questions is: Tell me about your lab experience in the BT@SDSU

program. Prepare a 2-minute FlipGrid video with a response to that question.

Keep in mind that your potential employer will want to see that you can clearly articulate what you did, why you did it, and what you learned. Your potential employer will also use your response to gauge your professionalism, for example, your skills as a communicator and as a team member.

Access flip grid and post your response at the link below no later than **February 28, 2024.**

https://flip.com/fd0ebfce

Note: Only the PI's will be able to see your videos when you submit them.

Important Links

Summer Internship List: https://docs.google.com/spreadsheets...it?usp=sharing

Session Slides: https://docs.google.com/presentation...f=true&sd=true

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2.1 Summarizing Reserach

Session Overview

During this session, scholars will learn how to succinctly describe research experiences in preparation for interviews and presentations.

Learning Outcomes

This session will

- Practice describing your lab-intensive experience to someone else
- Practice handling followup questions about your research
- Gain confidence as a researcher

Active Learning/Formative Assessment Strategies

- Interviewing Role Play

Pre-Session

Background

During interviews or networking events, it's common to be asked about your research experiences or current projects. Therefore, it is good to practice answering this question *clearly and concisely*. You can think of your response like a sales pitch or elevator pitch - long enough to answer the question, short enough not to lose your audience, and compelling enough that people want to know more. The best way to achieve this perfect balance? PRACTICE!

Tasks

1. Watch your fellow scholar's submissions to FlipGrid. Comment on 2 of your fellow scholar's responses.

During Workshop

Tasks

1. Feedback on Interview Practice.

2. Interview role play.

Post-Workshop

Tasks

1. Complete the flipgrid post assignment from last week (if not already completed)

2. Watch your fellow scholar's video.

3. Provide feedback on at least two videos (if not already completed).

4. Complete the sequencing assignment located in the folder with your name on it in preparation for next learning community. Link: https://drive.google.com/drive/folde...usp=drive_link

---

Important Links

Summer Internship List: https://docs.google.com/spreadsheets...it?usp=sharing

Session Slides: https://docs.google.com/presentation...f=true&sd=true

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2.2 Analyzing Results

Session Overview

During this session, scholars will anylyze reuslit, draw conclusions, make predictions, and determine next steps for their data obtained during the lab intensive.

Learning Outcomes

This session will

- Analyze results from experiments
- Draw conclusions from data
- Make predictions regarding novel gene fucnction
- Determine what experiments should be conducted next based on results

Active Learning/Formative Assessment Strategies

- Data Sharing
- Small group discussion
- Large group discussion

Pre-Session

Background

You've conducted the experiment - now it's time to analyze the results. This process can be one of the most rewarding - and sometimes frustrating - parts of conducting research. Yet, whether your original hypotheses are supported or the results fail to support your original predictions - you have added new knowledge to the world. And that is something to be proud of and to share.

Tasks

1. Submit your sequencing results.

During Workshop

Tasks

1. Discuss sequencing results from lab intensive

Submit your Genotyping Data HERE: https://docs.google.com/spreadsheets/d/1RzbcXg6mpFT3Qmkw8T4x7iTovwojW0MV4imIjmF0JAg/edit?usp=sharing

---

Post-Workshop

Tasks

1. Submit two questions for our panelists visiting on the 17th. We are still finalizing guests, so feel free to make these questions applicable to anyone in biotech.

    Link: https://docs.google.com/spreadsheets...it?usp=sharing

2. Fill out the excel sheet in your folder labeled "Internship Application Summary"

    Link to folders: https://drive.google.com/drive/folde...l3?usp=sharing

3. Enjoy your spring break!

Important Links

Summer Internship List: https://docs.google.com/spreadsheets...it?usp=sharing

Session Slides: THESE WILL BE ADDED AFTER THE SESSION TODAY

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2.3 Biotech Panel 2

Session Overview

This session will introduce scholars to individuals currently working within the Biotech Industry.

Learning Outcomes

This session will

- Provide access to STEM career resources for scholars
- Build social capital through connections with STEM professionals in the biotech industry
- Increase motivation for STEM research careers

Key Terms

Networking

Active Learning/Formative Assessment Strategies

- Panel discussion

Pre-Session

Background

Networking, or interacting with other individuals within in a field, is a key way to exchange knowledge and develop professional contacts. Interactions can be more formal, such as participating in internships or attending research conferences, or less formal, such as sharing ideas or resources with a colleague or classmate. Whether you talk about research ideas, job opportunities, project collaboration, or hobbies outside of work - the important part is to make connections!

Tasks

Please complete the following tasks prior to the workshop:

1. Complete Year 2 Interest Survey: https://ugeorgia.ca1.qualtrics.com/j...31pOyZjgCvqNfw
2. Locate the folder with your name on it at the following link: https://drive.google.com/drive/folde...l3?usp=sharing
3. Complete the form entitled "Internship Application Summary".

During Session

Activities

1. Biotech Panel

Name: Ali McCarthy

Company/Position: Sr Talent Partner, Element Biosciences

What you do at your job: Support all business functions (Technical and non-technical) from a talent and performance management perspective. I help with hiring, career and organizational development, employee relations, etc.

If you have any affiliation with SDSU: n/a

Name: Nicole Jensen

Company/Position: Novartis, Principal Scientist I

What you do at your job: Oncology drug discovery & development

If you have an affiliation with SDSU: BSc Biology 2012, MSc Molecular Biology 2016; performed my undergraduate research in Dr. Waters lab

Name: Grace DeSantis, PhD

Company/Position: R&D Leadership (ex ONI, ex illumina). VP and Director level

What you do at your job: I build and lead scientific teams focused on making life science tools and diagnostic products. My academic training is in Biochemistry and Chemistry.

If you have any affiliation with SDSU: My son did a summer internship at SDSU. He plans to apply as a transfer student to SDSU or UCSD after community college. I worked with Dr Liz Waters at Driversa Inc.

Post-Session

If you have not completed the pre-session tasks, then please do so!

References

Session Slides

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Wrapping Up

Session Overview

Congratulations to our inaugural class of biotech scholars!

Learning Outcomes

This session will

- Allow scholars to provide feedback on the spring semester of the biotech scholars program

Key Terms

CELEBRATION!

Active Learning/Formative Assessment Strategies

- Survey
- Informal discussion.

Pre-Session

Background

You did it!

During Session

Activities

Complete the following surveys

1. https://ugeorgia.ca1.qualtrics.com/j...oo7y8KFGy5Tkfs

2. https://ugeorgia.ca1.qualtrics.com/j...L2uAceL01qqWKG

Ensure that the following items are complete and/or updated:

1. Complete Year 2 Interest Survey: https://ugeorgia.ca1.qualtrics.com/j...31pOyZjgCvqNfw
2. Locate the folder with your name on it at the following link: https://drive.google.com/drive/folde...l3?usp=sharing. Complete the form entitled "Internship Application Summary". If you have already completed this form, please make sure that the information is current. Some of you were still awaiting decisions from different applications the last time I checked! :)
3. Sign up for an exit interview with me at the following link: https://docs.google.com/spreadsheets/d/1qrZaKUgQwLuUaTQeK9A3856M5ZNrHxcBi-X6SvQvVgU/edit?usp=sharing

Post-Session

Mark your calendars with your exit interview times.

Have a wonderful summer! :)

References

Session Slides

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Day 1

Advance Preparation

1. Be sure you know where to find lab schedule, reading materials, lab protocols, and data sheets for the next two weeks! https://drive.google.com/drive/folde...tj?usp=sharing
2. There is always time for your questions. Ask about anything. If you are curious or confused, no doubt one or more of your colleagues are also. Ask! Don't assume you should know.

Lab Partner Plant Experiments

	Benae & Dishant	
	Lillian & Juwayria	
	Adryan & Arlene	
	Gisele & Alexia	
	Nika & Eric	
	Tram & Alex	
	Lia & Jasmine	

Activities

Introductions and start of Experiments 1 & 2

Morning:

1) Introduction to the National Science Foundation (NSF) and the ATAD3 research project that is supporting you.

2) Outline of our two weeks together.

3) Where to find information and where to record your data/observations.

4) Explanation of first experiment (heat stress sensitivity of plants) & meet your team-mate for this experiment.

5) Sterilize seeds for first experiment. (Protocols --> Experiment 1_Heat Tolerance of Photosynthesis)

Afternoon:

1) Place seeds individually and sterilely on plates (Protocols --> Experiment 2_Heat Tolerance of Seedling Elongation and Root Growth)

2) Foil wrap plates (no light leaks!). Place plates in a vertical position at room temperature to grow for heat stress treatment on Friday.

3) Explanation of experimental design (Why are we testing the seeds we are testing?)

4) Debrief and discuss activities for Day 2.

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Day 2

Advance Preparation

Review materials from Day 1.

Activities

Continuation of Experiment 1

Morning:

1) Heat stress treatments (Protocols --> Experiment 1_Heat Tolerance of Photosynthesis)

2) Introduction to chlorophyll fluorescence.

Afternoon:

1) Measurement of chlorophyll fluorescence (Protocols --> Experiment 1_Heat Tolerance of Photosynthesis)

2) Introduction to Physcomitrium

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Day 3

Advance Preparation

1. Watch the short video that describes CRISPR. https://www.jax.org/personalized-medicine/precision-medicine-and-you/what-is-crispr
2. Download Snapgene Viewer https://www.snapgene.com/snapgene-viewer

Activities

Begin CRISPR Construct Experiment and Introduction to Bioinformatics

Morning

1) Access CRISPR Construct Experiment protocol (Protocols --> CRISPR Design Lab)

2) Introduction to CRISPR.

3) Find good protospacer sequences.

4) Design and order oligonucleotides for each protospacer. (Be sure to record information requested in eLabNotebook.)

Afternoon

1) Introduction to Bioinformatics

2) Using bioinformatics tools, discover 5-10 facts about your assigned protein. (Add this information to your eLabNotebook)

3) Discussion

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Day 4

Advance Preparation

Review Day 2-3 Activities

Activities

Continuation of Experiments 1, 2, and CRISPR Design Lab

Morning:

1) Observe heat stressed plates. Note any phenotypes. (Protocols -->Experiment 1_Heat Tolerance of Photosynthesis)

2) Obtain more chlorophyll fluorescence data.

3) Analyze data on chlorophyll fluorescence.

4) Before lunch: Move plates for Experiment 2 to a growth chamber in vertical position. (Protocols --> Experiment 2_Heat Tolerance of Seedling Elongation and Root Growth)

Afternoon:

1) Get more info about the gene. (Protocols --> CRISPR Design Lab)

2) Element Biosciences visit.

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2: LAB INTENSIVE

Overview

This will be a two-week laboratory skills building course

Day 1

Day 2

Day 3

Day 4

Day 5

Day 6

Day 7

Day 8

Day 9

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Day 5

Advance Preparation

Be sure to watch the videos that review DNA replication, introduce Hi-Fi assembly, and bacterial transformation.

DNA replication: https://www.youtube.com/watch?v=TNKWgcFPHqw&t=207s

Hi-Fi assembly 1: https://www.neb.com/en-us/tools-and-resources/video-library/introduction-to-nebuilder-hifi-dna-assembly?autoplay=1

Hi-Fi assembly 2: https://www.neb.com/en-us/tools-and-resources/video-library/nebuilder-hifi-dna-assembly-bridging-dsdna-with-a-ssdna-oligo?autoplay=1

Hi-Fi assembly 3: https://www.neb.com/en-us/tools-and-resources/video-library/construction-of-an-sgrna-cas9-expression-vector-via-an-ssoligo-bridge?autoplay=1

Bacterial Transformation: https://www.youtube.com/watch?v=w_fZmiIAdco

Activities

Morning:

1. Introduction to Hi-Fi assembly.
2. Introduction to bacterial transformation.
3. Measure chlorophyll autofluorescence and take pictures of plates (Protocols --> Heat Tolerance of seedling elongation and root growth)

Afternoon:

1. Dilute Oligos (Protocols --> CRISPR Construct Experiment).
2. Set up Hi-Fi Assembly reaction.
3. Bacterial transformation

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Day 6

Advance Preparation

1. Review Day 5 Material

2. Complete the following form: https://docs.google.com/document/d/1...usp=drive_link

Activities

Morning:

1. Take pictures of bacterial transformation plates. Add to eLabNotebook. (Protocols --> CRISPR Construct Experiment)
2. Introduction to PCR.
3. Introduction to genotyping.

Afternoon:

1. Extract DNA from moss plants (Protocols --> Genotyping Experiment).
2. Set up PCR reaction.
3. Inoculate bacterial cultures for minipreps the next day (Protocols --> CRISPR Construct Experiment).

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Day 7

Advance Preparation

Review Day 6 Materials

Activities

Morning:

1. Pour Gel (Protocols --> Genotyping Experiement)
2. Spin down over night cultures. Remove supernatant and store at -20°C until the afternoon.
3. Run gel.
4. Image gel and add image to eLabNotebook.
5. Identify plants that have edits. Save that genomic DNA for sequencing PCR tomorrow.

Afternoon:

1. Miniprep the DNA. (Protocols --> CRISPR Construct Experiment)
2. Measure the concentration of the DNA, record in eLabNotebook.
3. Set up sequencing reactions.

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Day 8

Advance Preparation

1. Complete the following course. It is REQUIRED in order for you to receive payment: https://research.sdsu.edu/research_affairs/human_subjects/training

2. Email your certificate of completion to mailto:eric.gelbart@sdsu.edu

3. Review Day 7 Materials.

Activities

Morning:

1. Set up PCR for sequencing plants that were identified in the competition PCR experiment. (Protocols --> Genotyping Experiment)
2. Pour gel.

Afternoon:

1. Run 5 µL of the PCR reaction on a gel. (Protocols --> Genotyping Experiment)
2. Take a picture of the gel and record in eLabNotebook.
3. Perform PEG purification.

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Day 9

Advance Preparation

Review Day 8 Materials.

Activities

Morning:

1. Group Discussions

Photosynthesis Data: Arlene, Alex, Benae, Nika,

Link to Class Data: https://docs.google.com/spreadsheets...usp=drive_link

Genotyping Data: Lillian, Eric, Adryan, Gisele, Lia

CRISPR Genes Data: Tram, Juwayria, Dishant, Alexia, Jasmine

a) Purpose of the experiment

b) Basic Procedure

c) Possible Outcomes

d) Next Steps

2. Measure concentration of PCR products (Protocols --> Genotyping Experiments).

3. Paula virtual visit.

Afternoon:

Survey: https://ugeorgia.ca1.qualtrics.com/j...iaSqvWSecijQx0

Wrapping Up

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3. Meet our 23-24 Scholars



Back row (L to R): Dishant Vandra (sophomore kinesiology major), Alex Sandoval (junior biology major), Gisele Chhoeuy (junior biology major), Arlene Alvarez (senior biology major), Benae Tabancura (sophomore biology major), Adryan Aquino (sophomore kinesiology major), Juwayria Hussein (senior biology major)

Middle row (L to R): Dartmouth College biological sciences professor Magdalena Bezanilla, SDSU biology professor Liz Waters, University of Georgia biochemistry and molecular biology professor and Associate Dean of the Franklin College of Arts and Sciences Paula Lemons, University of Massachusetts Amherst biochemistry and molecular biology Distinguished Professor Emerita Elizabeth Vierling, Jasmine Pooni (sophomore biology major), SDSU graduate assistant Olivia Baldwin, University of Georgia graduate assistant Shannon Clark

Front row (L to R): Eric Lee Hosler (sophomore biology major), Lia Escober (junior biology major), Alexia Carranza (junior biology major), Trâm Nguyen (senior biology major), Nika Recto (senior biology major), Lily Sanchez (junior biology major)

Photo Credit: Bryana Quintana/SDSU

To read more about the program: https://www.sdsu.edu/news/2024/05/ns...iotech-careers

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2: RESOURCES AND READING MATERIALS

Overview

Sources here will be used to support your learning both during and after your time as a scholar.

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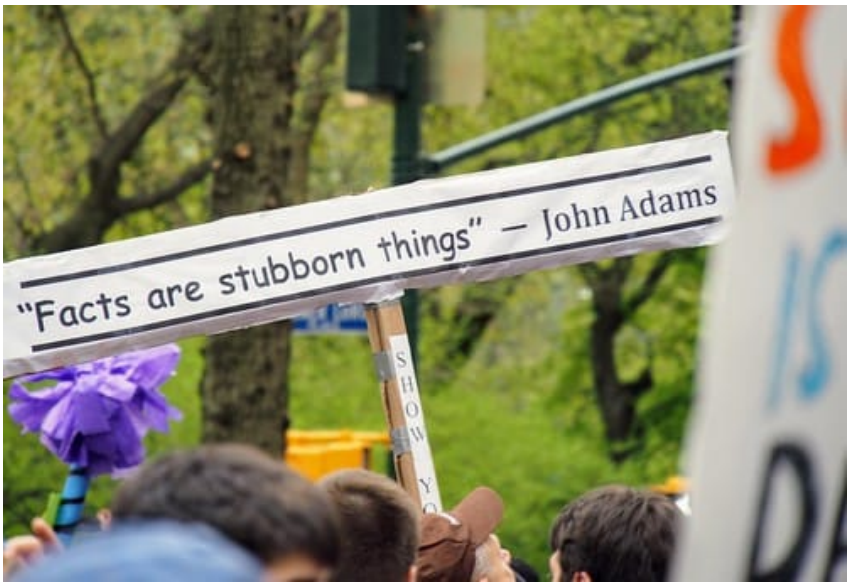
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2.1.1.1: Introduction

The Scientific Method

Scientists use a methodology for systematically investigating natural phenomena. This method uses existing information or observations to acquire new information or validate previous knowledge. These knowledge types come from empirical (experiential) or measured information. Empirical and measured data (or knowledge) are referred to as **observations**. While **empirical** data comes from experiences, science has developed into a mode of inquiry using **experimentation**. Experimental science uses the pre-existing base of knowledge to ask a testable question called a **hypothesis**. As a youngster, we're incorrectly taught that a hypothesis is ~~*an educated guess*~~. Formulating previous observations and measurements into a cohesive line of inquiry requires no guessing. People often have "theories" on something, when they actually have hypotheses based on their observations and assumptions.



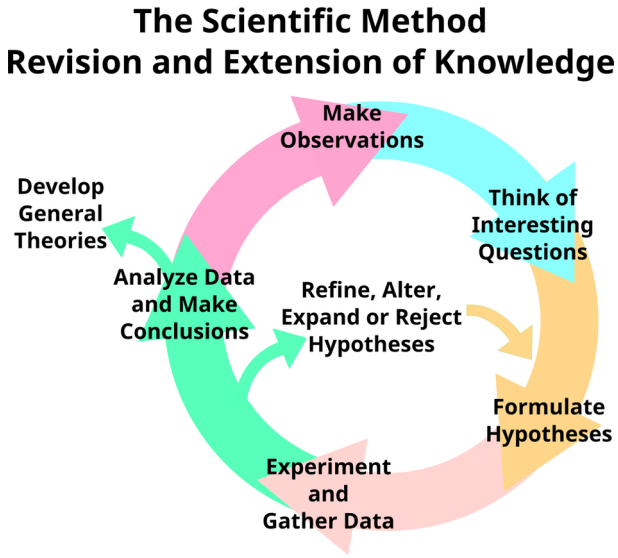


Experimental Science

Hypothesis testing is the means by which experimental science is conducted. Experimental science is designed to enhance the understanding of a problem and removing biases from the interpretation. The goal of hypothesis testing is to try every way possible to disqualify the validity of the hypothesis. By doing so, the experimenter removes any biases in the experimental design. If the experimenter is unable to invalidate the hypothesis, the hypothesis becomes more valid and better able to act as a predictor of phenomena.

Experiments utilize **controls**. In a controlled experiment, there is a positive and negative control. These controls act as references in the experiment. A **positive control** is an experimental condition where the expected outcome that is tested will be produced. This control is necessary to assess the validity of a test or treatment. There can be multiple instances used as a positive control to examine the sensitivity of the experiment. A **negative control** is an experimental condition where the expected outcome is known not to occur. This type of control sometimes comes in the form of a sham or mock treatment such as giving someone a sugar pill (a **placebo**).

Through the use of experimental science and hypothesis testing, an increased refinement of existing knowledge can aid in designing new hypotheses. Hypothesis testing is re-iterative. That is to say, we use new knowledge to continue to enhance our understanding of the universe.



The scientific method is a reiterative process based on testing and revising knowledge. (CC-BY-NC-SA Jeremy Seto)

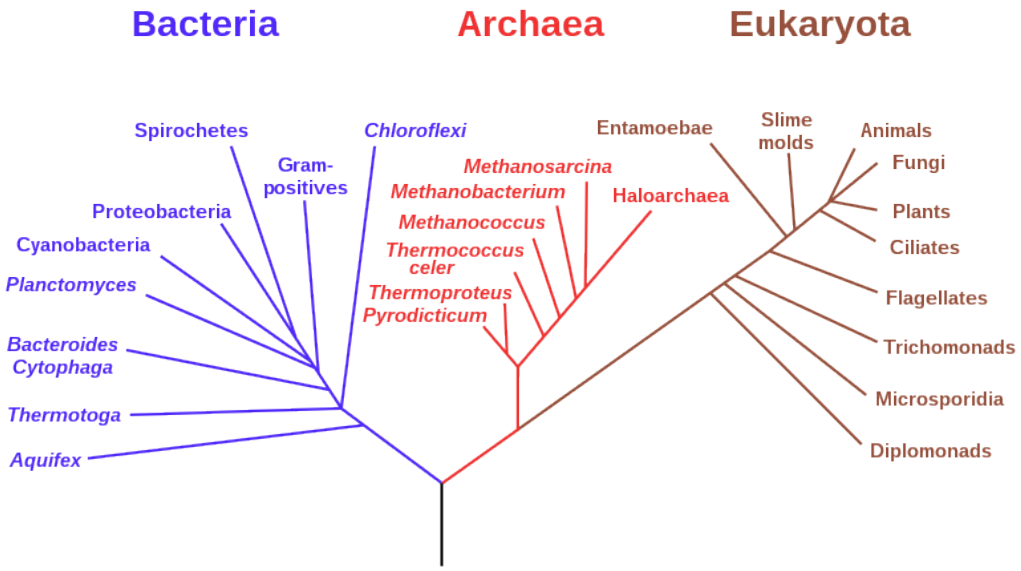
Theories

A **scientific theory** comes from repeated substantiation of multiple tested hypotheses. That is to say, confirmed hypotheses, observations, and experiments permit scientists to formulate a cohesive idea that integrates multiple substantiated pieces of evidence. As with hypotheses, theories are designed to be predictive and falsifiable. In the common language, we often hear the word theory to mean a conjecture, and as already discussed, conjectures based on evidence can be formulated into testable hypotheses.

When a theory is accepted by a predominant population of the specialists, it is referred to as a **scientific principle**. An example of a scientific principle is the ***theory of evolution by natural selection***. Numerous tested hypotheses have been confirmed that lead to the understanding of natural selection as a method of evolution. This theory allows scientists to understand the underlying relatedness of all living things on the planet. Additionally, it unifies the disparate fields of Biology that can utilize the theory in a predictive manner. It is therefore also referred to as a **unifying principle** of Biology.

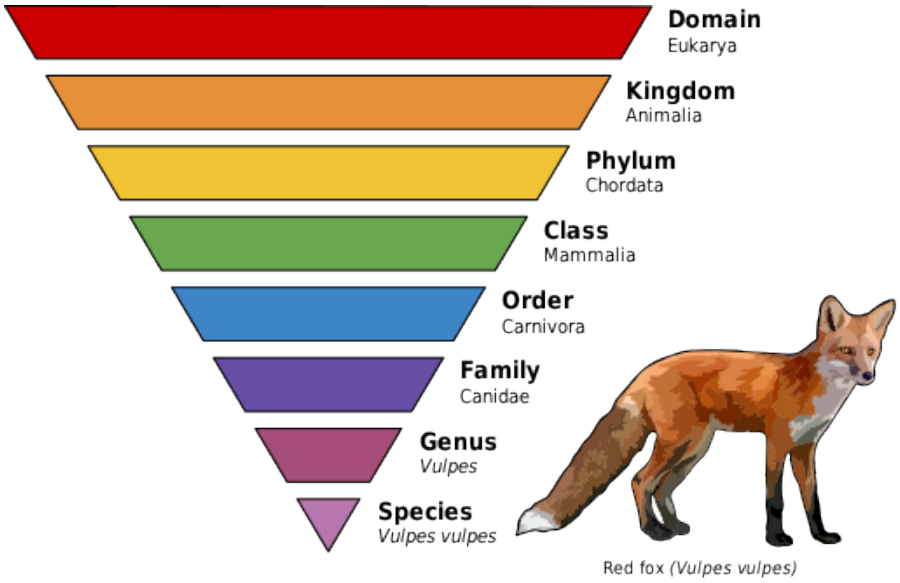
Classification of Life

All living things on Earth share a relationship. The rules that govern life processes can be generalized across all **organisms** (living things), as well as non-living biological entities (viruses). The relatedness of organisms is often visualized as a **phylogenetic tree**. This tree is a hierarchical classification system that groups organisms together based on common features and used these similarities to name them. This is referred to as **taxonomy** with the broadest category is called a **domain**.



The phylogenetic tree of life describing the inter-relatedness of all living things on Earth.

Three domains exist, **Archaea**, **Bacteria,** and **Eukarya**. Archaea and Bacteria are also grouped together as **Prokaryotes** (*pro–* before; *karya–* nucleus). Eukarya (*eu–* true; *karya–* nucleus), or eukaryotes, is a group of organisms that have nuclei. The second most inclusive or broad category is **Kingdom**. Humans are in the Kingdom of animals. The third most inclusive or broad category is **Phylum**. Humans are in the phylum called Chordata. Each level of organization can be further subdivided and you may be more familiar with the subphylum called Vertebrata. Within this division, humans fall in the **class** of mammals. Amongst the mammals, humans are in the **order** of primates. Humans are categorized into a narrower group of organisms in the **family** of great apes or hominids. Within this family, humans fall into the **genus** of *Homo*. Biologists use a method of identifying specific organisms called binomial nomenclature. Binomial nomenclature uses the most specific groupings of taxonomy (genus and **species**) as a two-part name. While humans are of the species sapiens, the species name of humans using binomial nomenclature is *Homo sapiens*.



Taxonomic ranking of the red fox illustrating the inclusiveness of Domain and the exclusiveness of the Species level of categorization.

Credit: Annina Breen (CC-BY-SA 4.0)

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2.1.1.2: Microscopy

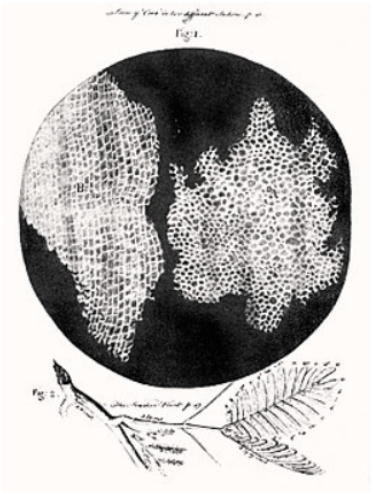
The Light Microscope

Hooke's Cell

In 1665, Robert Hooke published *Micrographia*, a book that illustrated highly magnified items that included insects and plants. This book spurred on interest in the sciences to examine the microscopic world using lenses but is also notable for Hooke's observations of cork where he used the word "cell" in a biological sense for the first time.

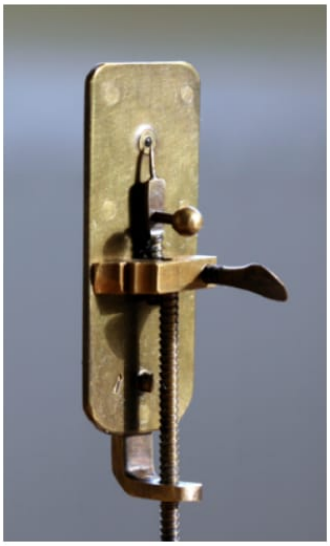


Illustration of Hooke's microscope



The empty chambers of cork inspired Hooke to coin the term "Cell"

The Father of Microbiology: van Leeuwenhoek



van Leeuwenhoek's microscope

The Dutch tradesman Antonie van Leeuwenhoek used high power magnifying lenses to examine the parts of insects and to examine the quality of fabric in his drapery business. He began to experiment with pulling glass to generate lenses and developed a simple microscope to observe samples. Using a simple single lens with a specimen mounted

on a point, he was able to identify the first microscopic “animalcules” (*little animals*) that will be later known as protozoa (*original animals*).

Though van Leeuwenhoek’s apparatus was simple, the magnifying power of his lenses and his curiosity enabled him to perform great scientific observations on the microscopic world. He was ridiculed for fabricating his observations of protists at first. Ever the scientist, van Leeuwenhoek examined samples of his own diarrhea to discover *Giardia intestinalis*. While he did not make the connection of the causative nature of this microorganism, he described the details of the way this organism could propel itself through the medium in great detail.



Modern micrograph of Giardia intestinalis (Kingdom Protista)



Modern Compound Microscope

Unlike van Leeuwenhoek’s single-lens microscope, we now combine the magnifying power of multiple lenses in what is called a compound microscope.

1. Ocular lens or eyepiece
2. Nose Piece/ Lens Carousel
3. Objective lens
4. Course Focus Knob
5. Fine Focus Knob
6. Stage

7. Lamp
8. Condenser
9. Stage control

Using the Light Microscope

Microscopic World



Daphnia magna, the freshwater flea is a small crustacean (scanning magnification)



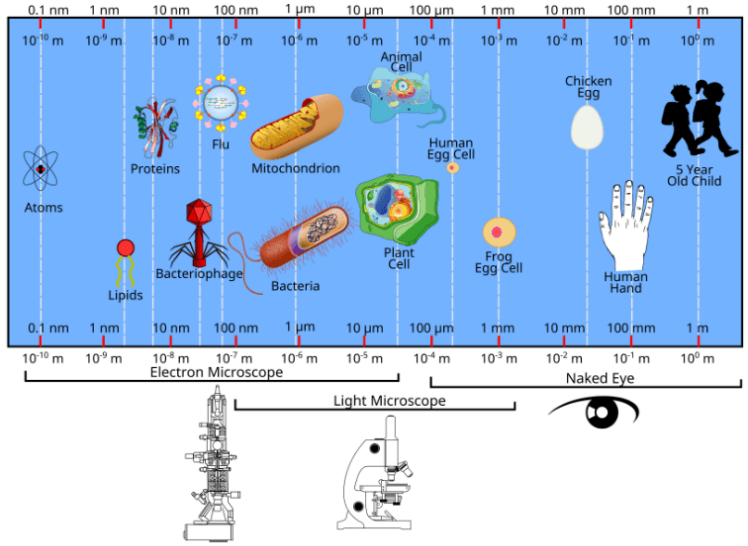
Low magnification of Daphnia magna





Protozoa. Top: Amoeba Center: Euglena Bottom: Paramecium

Scale



Credit: Jeremy Seto and derived from works by IgniX, Gringer, Jiver, Ninjatacoshell, Ali Zifan (CC-BY-SA)

Magnification

Magnification is the process of enlarging the appearance of an object. We calculate the magnification of an object by indicating the fold change in size. So if something appears to be double the size of the real item, then it is obviously magnified 2X. Because there is a magnification by the eye-piece (**ocular lens**), as well as the **objective lenses**, our final magnification of an item is the product of those two lenses.

The lowest magnification objective lens (usually 4X or 5X) is referred to as a **scanning lens**. There is also usually a low power lens at 10X and a higher magnification lens at 40X. There may be a higher magnification lens at 100X but these usually require oil to function properly and are often reserved for microbiology labs.

- What is the power of the ocular lens?
- We can calculate that as:
  $\text{Magnification}_{\text{total}} = \text{Magnification}_{\text{objective}} \times \text{Magnification}_{\text{ocular}}$
- With this in mind, fill in the following table:

	Objective Magnification	Ocular Magnification	Total Magnification
Scanning			
Low Power			
High Power			
Oil Immersion	100X		

Field of View (FOV)

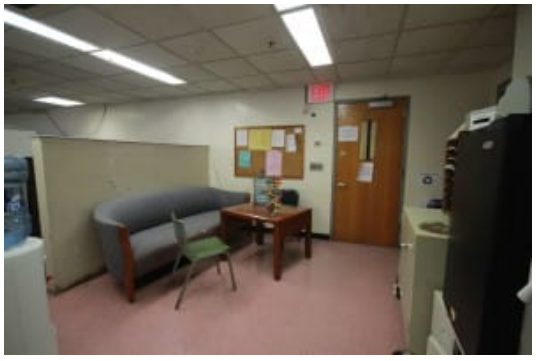
In a microscope, we ordinarily observe things within a circular space (or field) as defined by the lenses. We refer to this observable area as the **field of view (FOV)**. Understanding the size of the FOV is important because actual sizes of the object can be calculated using the Magnification of the lenses.

FOV can be described as the area of a circle:

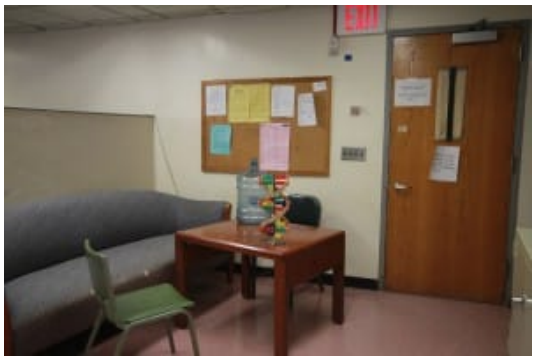
File:Circle Area.svg

$$Area = \pi r^2$$

What are the effects of magnification on FOV?



1) Lowest Magnification



2) Low Magnification



3) High Magnification



4) Highest Magnification

In image 1, we can see a model of DNA on a table with a water bottle and a large area of the room. Image 2 displays less of the room in the background but the DNA model is larger in appearance because the magnification is greater. In image 3, we no longer see evidence of a door and the DNA model is much larger than before. In image 4, we no longer see the table the model and water bottle rest upon. While the last image is largest, we see less of the surrounding objects. We have higher magnification at the cost of the field of view. FOV is inversely related to the magnification level.

Field of View Calculation

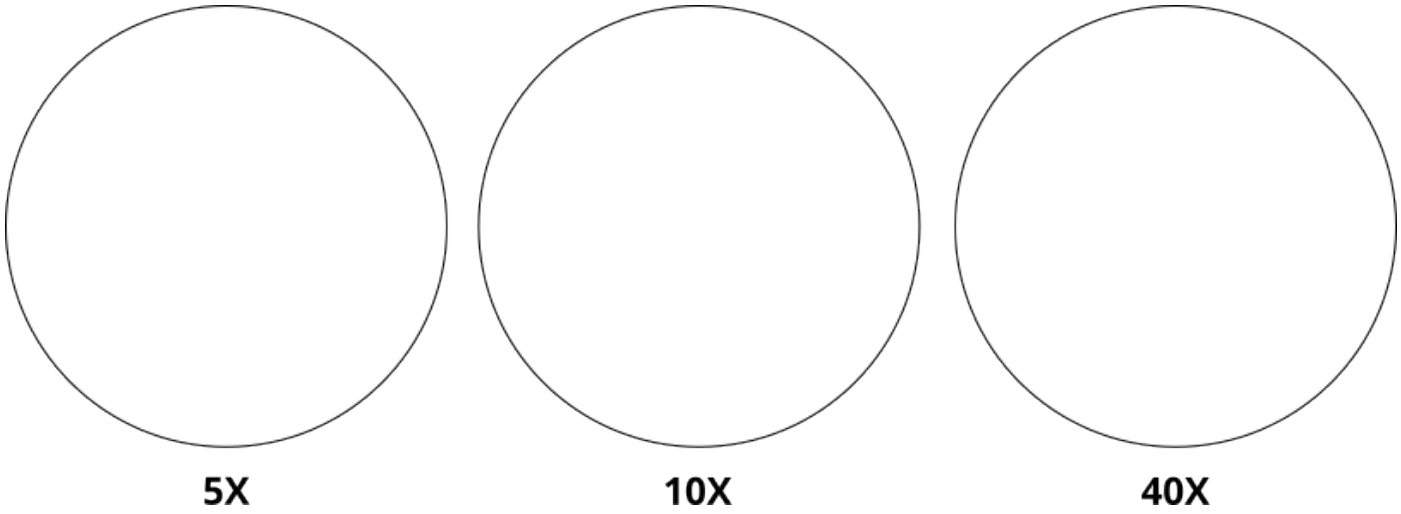
1. Examine a ruler under scanning magnification
    - Measure the diameter in mm.
    - Diameter= _____
    - Radius= _____
    - Calculate the field of view at this magnification= _____
2. Examine a ruler under low magnification (10x)
    - Measure the diameter in mm.
    - Diameter= _____
    - Radius= _____
    - Calculate the field of view at this magnification= _____
3. What is the relationship in the between the magnification and field of

view?_____

4. What is the proportion of change in the field of view when doubling the magnification?_____

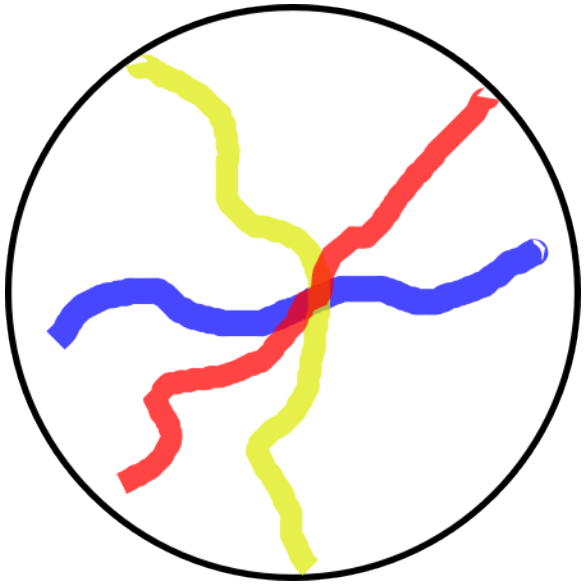
The Letter “e”

1. Orient a slide with the Letter “e” so that it is read as an “e” without magnification
2. Draw the “e” at scanning, low and high magnification



Depth of Field

1. Examine the slide of colored threads under scanning power so the cross-point of the threads is at the center of the field.



2. Raise the magnification to the low power objective

- What do we notice about the threads and the focus?
- How can we explain this observation with respect to the threads?
- Close the diaphragm so allow a pinpoint of light through the slide. What effect does this have on the image?

We notice that when we observe 3 overlapping threads of different color under a microscope, we can focus on one thread at a

time. Similarly, when we zoom in a great deal on the DNA model below, we notice that the print on the water bottle is not sharp.

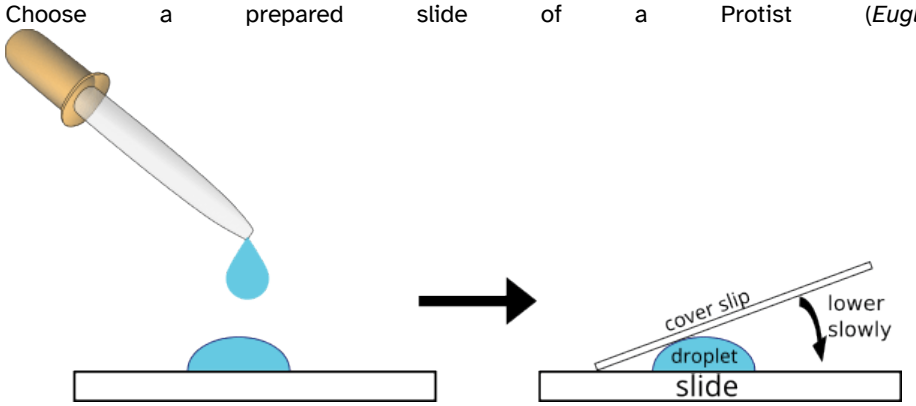


Highest Magnification with shallow depth of field. Notice how the label on the water bottle is blurry while the lettering on the DNA model is sharp.

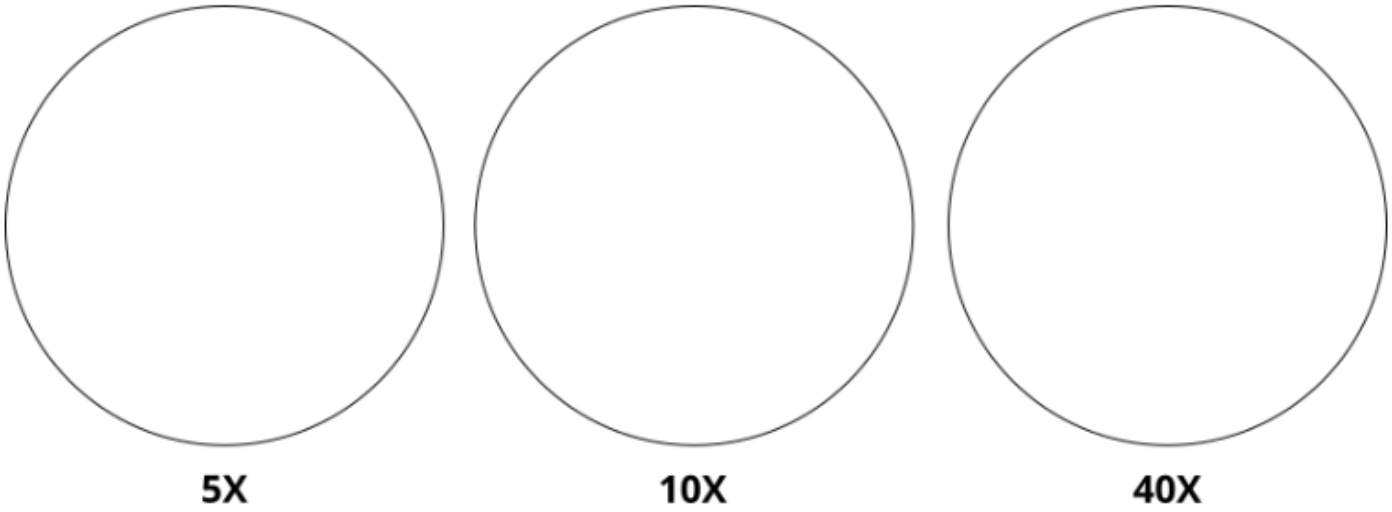
We know that the water bottle is behind the DNA molecule. Under the microscope, the threads of differing color are also stacked on top of each other. We recognize that they are on different planes because they are three dimensional. Each thread has depth and does not occupy the same exact space. If we focus on the print of the water bottle on the image above, we would no longer see the lettering on the DNA molecule sharply. We refer to this concept as **Depth of Field (DOF)**. Under the microscope, at low magnification, we can make out fewer finer details. However, most items appear on the same plane in this case and or comparably sharp. But as we increase the magnification and see finer details, the distances between the various planes in view become more apparent. We can see a similar phenomenon at low magnification of the DNA model. At the low magnification, we may not be able to read the print on the water bottle, but the bottle and DNA molecule are of a similar distance from our view that the small difference in apparent depth is not as noticeable. We can still draw on other visual cues to know that the bottle is behind the model, but the sharpness of both items are equivalent.

Examining Cells

1. Choose a prepared slide of a Protist (*Euglena*, *Amoeba*, *Paramecium*)



2. Prepare a wet mount of a drop of pond water and place a coverslip over the drop.
3. Swab the inside of your cheek
    1. Roll the swab across a slide
    2. Drop some methylene blue onto the slide
    3. Place a coverslip over the drop
    4. Visualize and draw your cheek cells
4. Document your observations by drawing the cells and by using your phone to snap an image.



Real Biological Examples

We can see the concepts of FOV and DOF in the following pictures.





In an even more extreme close-up (higher magnification), we would have difficulty focusing on both the eyes and beak since there is depth and distance between those features.

How Do We Use Microscopes

In our lab, we look at some pond water. What do we see? Why is this significant? How does the microscope help us study these items? What is the utility of the concepts of magnification, FOV, and DOF when we use microscopes to study biological samples?

alt

Interactive Content

This interactive element is not available in this format. Visit the link or scan the QR code to access it online.

View this Interactive Content online

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2.1.1.3: Units of Measure

The Metric System

The **metric system** is an internationally agreed-upon measurement system based on decimals or powers of 10. Scientists use a refined version called the **International System of Units** (abbreviated **SI**). In biology, you will often find a need to describe measurements of length, volume, mass, time, temperature or amount of substance.

International System of Units

BASIC SI UNITS		
MEASURE	**SI UNIT**	**SYMBOL**
length	meter	**m**
mass	kilogram	**kg**
time	second	**s**
temperature	Kelvin *(Celsius is used in Biology)*	**K** *(°C)*
quantity	mole	**mol**
current	Ampere	**A**
luminosity	candela	**cd**

Metric Units

- length: meter (**m**)
- volume: liter (**L**)
- mass: gram (**g**)
- time: second (**s**)
- temperature: Celsius (**°C**)
  - Kelvin (**K**) is a unit of thermodynamic temperature and is the SI unit. The Kelvin scale in the same as the Celsius or centigrade scale but offset by 273.16
  - Biology uses Celsius predominantly because of the range in which organisms live.
- amount of substance: mole (**mol**)
  - A mole is a number representing 6.022×10^{23} of something
  - Just as a pair of shoes equals 2 shoes, a mole of shoes is 6.022×10^{23} shoes
  - Just as a dozen eggs equals 12 eggs, a mole of eggs is 6.022×10^{23} eggs

METRIC PREFIXES IN EVERYDAY USE			
PREFIX	SYMBOL	SCIENTIFIC NOTATION	FACTOR
tera	T	10^{12}	1 000 000 000 000
giga	G	10^{9}	1 000 000 000
mega	M	10^{6}	1 000 000
kilo	k	10^{3}	1 000
hecto	h	10^{2}	100
deca	da	10^{1}	10
BASE UNIT	(none)	10^{0}	1
deci	d	10^{-1}	0.1
centi	c	10^{-2}	0.01
milli	m	10^{-3}	0.001
micro	μ	10^{-6}	0.000 001
nano	n	10^{-9}	0.000 000 001
pico	p	10^{-12}	0.000 000 000 001

Strategy for Conversions

1. What unit is being asked for?
   - 500ml = ____L → liters
2. What unit are you starting from?
   - 500ml = ____L → milliliters
3. Which unit is larger? By how much is that unit larger?
   - Liters are the larger unit. Liters are 1,000X (10^3) greater than milliliters.
4. Which direction are we moving?
   - Since we are moving to a larger unit, our value will be smaller. In this case, the value is smaller by 1,000X
   - In other words, the value is 1/1000 or 0.001 the value.
   - So what is the answer?

Factoring Out

Using the idea of factors of ten, you can assess the difference between the two units and cancel out the original unit algebraically to reach the desired final unit.

- 500ml=____L
  - $1ml = \frac{1}{1000}L$ or, $\frac{1L}{1000ml}$
    - which states 1000 milliliter in every 1 liter
  - $500ml \times \frac{1L}{1000ml} = \frac{500L}{1000} = 0.5L$
    - pay attention to the units and how we've canceled out the ml in the numerator of 500ml and in the denominator in the conversion of 1L in 1000ml

Additional Resources

Accuracy and Precision

Accuracy refers to how closely a measured value agrees with the correct or target value.
Precision refers to how closely individual measurements agree with each other and reflects the repeatability in those measurements.

File:High accuracy Low precision.svg

This illustrates accuracy. Measurements are on target.

File:High precision Low accuracy.svg

This illustrates precision. Measurements are very close to each other and repeatable.



This illustrates Accuracy AND Precision. Each measurement is on target and also highly repeatable.

Instruments have a finite amount of accuracy and it is important to report measurements within that level of accuracy. **Significant figures**, report the number of digits that are known to some degree of confidence with the measuring device. With the increased sensitivity of the equipment, the number of significant figures increases.

Pipetting Basics

Types of Micropipettors

Pipettors are made by many different manufacturers and thus all do not look the same. Learning to correctly use one type of pipettor will provide you the knowledge to use others as they share the same method of distributing small volumes. This lab will illustrate the Rainin Pipetman® micropipettors.

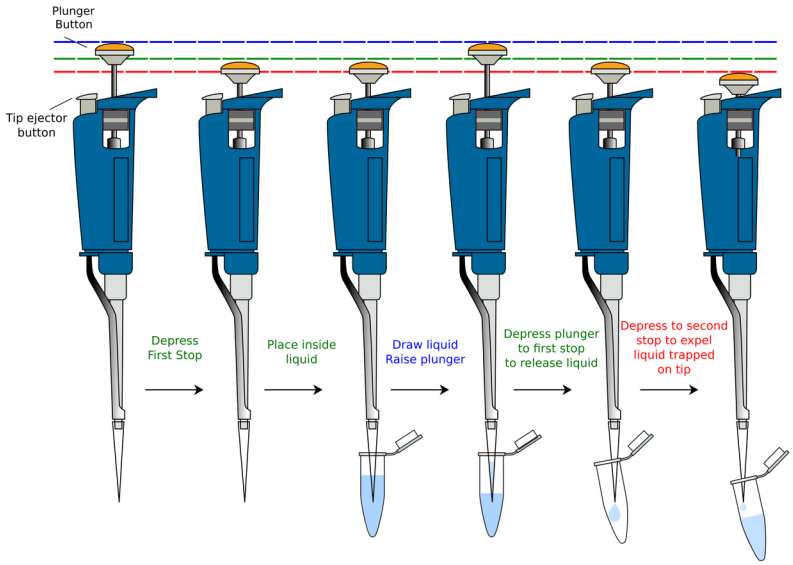
The top of the plunger shows the pipettor size for the Pipetman models. Each pipettor has its own volume range and it is **CRITICAL** to use a pipettor only in its proper volume range. The “P-number” represents the maximum volume in µL that the pipettor can measure. Pipettors are more accurate in the *upper* part of their range. 20 µL should be measured with a P20 rather than with a P200. The four pipettor sizes (P10, P20, P200, P1000) used in our lab will measure from 1 µL – 1000 µL as shown below.

Correctly Adjusting the Pipettors

	P10	P20	P200	P1000
Volume Range (µL)	1-10	2-20	20-200 50-200 *	200-1000
	tens 0 ones 4 tenths 7 4.7µl	tens 1 ones 7 tenths 8 17.8µl	hundreds 1 tens 5 ones 4 154µl	thousands 0 hundreds 8 tens 6 860µl

Gilson Pipetman pipetting ranges chart. Note that the P200 officially has a range from 50-200µl

Tutorial on Proper Usage



Pipetting sequence. Ensure the plunger is depressed outside of the liquid to displace air and avoid blowing bubbles into solution. Carefully draw the plunger up slowly and follow the liquid to avoid drawing air. Depress the plunger in destination tube to the first stop. Depress to the second stop if fluid remains in the tip to expel full volume.

https://www.youtube.com/watch?v=uEy_NGDfo_8

Rules For Use of the Micropipettors

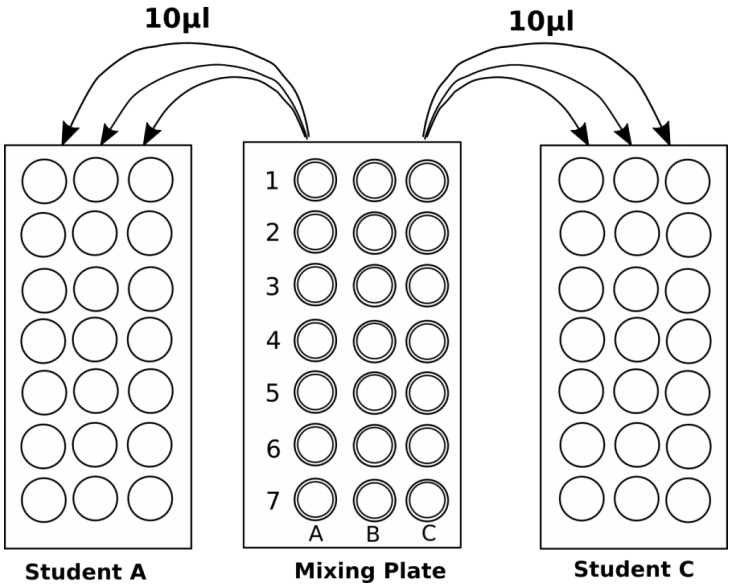
These are precision instruments which can easily be damaged. Treat them with respect and care. They are essential for your success in this course and shared amongst numerous students.

1. Never measure higher or lower than the range of the pipettor allows.
    - An exception to this rule is the P200 labeled 50-200 µl.
    - While we have P100 pipettors for this range, they appear too similar to P20 that they are often confused.
    - Originally, P200 were labeled 20-200 µl and we know that the lower range is less precise on these.
2. Never turn the volume adjuster above or below this range indicated on the pipettor or you risk breaking the instrument.
3. Never allow liquid to get into the micropipettor.
    - This causes contamination.
    - This weakens the seal on the o-rings and can damage them.
4. Never use the micropipettor without a tip.
5. Never invert or lay down the micropipettor with liquid in the tip.
    - Liquids will roll into the piston this way.
6. Never let the plunger snap back when filling or ejecting liquid.
7. Never immerse the barrel in fluid.
    - This causes contamination.
8. Never set the micropipettor on the edge of the bench; this may result in the micropipettor falling or being knocked onto the floor.

Exercise: Pipetting Practice

1. Prepare seven dye mixtures as illustrated in the table below.
    1. One student mix samples in column A and a second mixes in column C.
    2. Column B is left empty and used if one student makes a mistake.
2. Each dye mixture prepared in the first well to reach a total volume of 45 µl.
3. Pipet 10 µl in triplicate from each well of the mixing plate into the center of the appropriate circles on the target card.

Well #	Buffer (µl)	Red (µl)	Blue (µl)	Yellow (µl)	Glycerol (µl)	Alcohol (µl)	Total (µl)
1	40	5	-	-	-	-	**45**
2	15	-	-	10	10	10	**45**
3	20	5	10	10	-	-	**45**
4	30	-	15	-	-	-	**45**
5	22	13	-	-	-	10	**45**
6	25	-	10	-	10	-	**45**
7	20	6	-	6	13	-	**45**



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2.1.1.4: Quantitative Skills

Variables

Experimental science looks at cause and effect types of relationships. Controlled experiments vary one of the factors or traits to observe the effect on another factor or trait. These factors are called **variables**. A **dependent variable** is something that is observed and expected to change as a result of modifying another factor in the experiment. That is to say, the outcome *depends* on another factor. Another name for dependent variable is **responding variable**. The **independent variable** is the factor or condition that is changing or being changed by the experimenter. Sometimes waiting is the condition that is changing, making the independent variable: time. Since we change the independent variable, it is also called the **manipulated variable**.

Graphing a Line

File:Linear Function Graph.svg

A graph displaying 2 lines and their equations

A line can be described mathematically by the equation:

$$y = mx + b$$

This is referred to as the **slope-intercept form**. The 2 variables **y** and **x** refer to coordinates along each axis. The term **m** refers to the change in the y-values over the change in the x-values. This is referred to as the **slope of the line**. The term **b**, the **y-intercept**, is the y-value where the line crosses the y-axis.

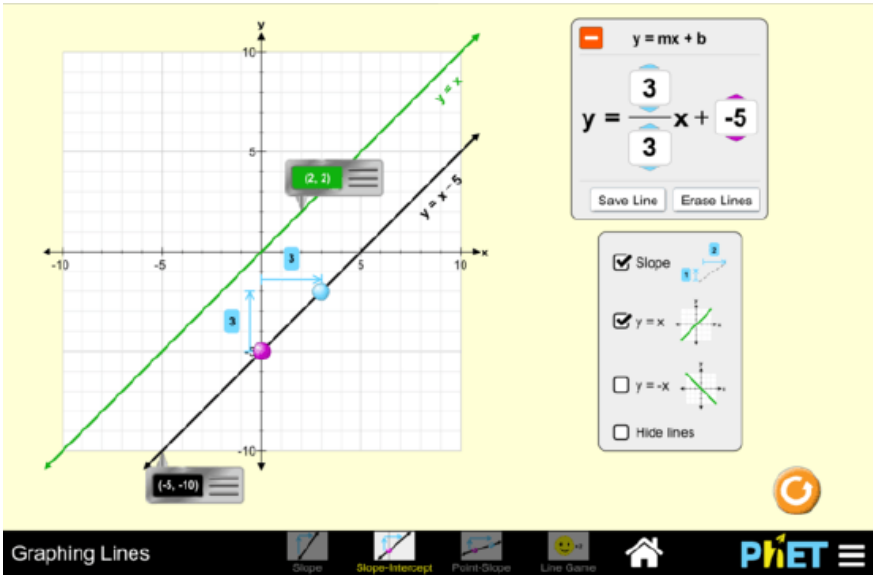
$$m = \frac{y_2 - y_1}{x_2 - x_1} \text{ OR } m = \frac{\Delta y}{\Delta x}$$

This is how the slope of a line (m) is determined.

The slope of the line indicates the relationship between the two variables, x and y. The equation of the line $y = mx + b$ indicates to us that “y” occurs as a function of changes to “x”. Sometimes this is represented by the equation $f(x) = mx + b$. Since “y” depends on “x”, “y is the dependent variable and “x” is the independent variable. As “x” changes, how does “y” change in response? This is what the slope reveals to us.

For more review, visit the following link.

Slope Activity



Click here to run the simulation.

Data Plotting Activity

1. Use the following data from the USDA to plot a **scatterplot** and generate a trend-line.
2. Use the info from the table below or download (a text file that can be opened in notepad/textedit)
3. Follow a tutorial on how to graph a scatterplot with line of best fit (trend-line)
4. Or use this tutorial in plot.ly
    - Show the equation of the trend-line on the graph
    - Ensure that "lbs. Mozzarella" is the y-axis
    - Ensure that "Year" is the x-axis
    - Remember not to use a line graph

Year	lbs. Mozzarella
2000	9.33
2001	9.70
2002	9.66
2003	9.65
2004	9.94
2005	10.19
2006	10.52
2007	11.02
2008	10.57
2009	10.63

What Do We Learn?

1. What does this scatterplot tell us about the relationship between consumption of mozzarella in relationship to years?
2. How would this graph influence the way you invest in a mozzarella cheese company? Can you predict anything about the

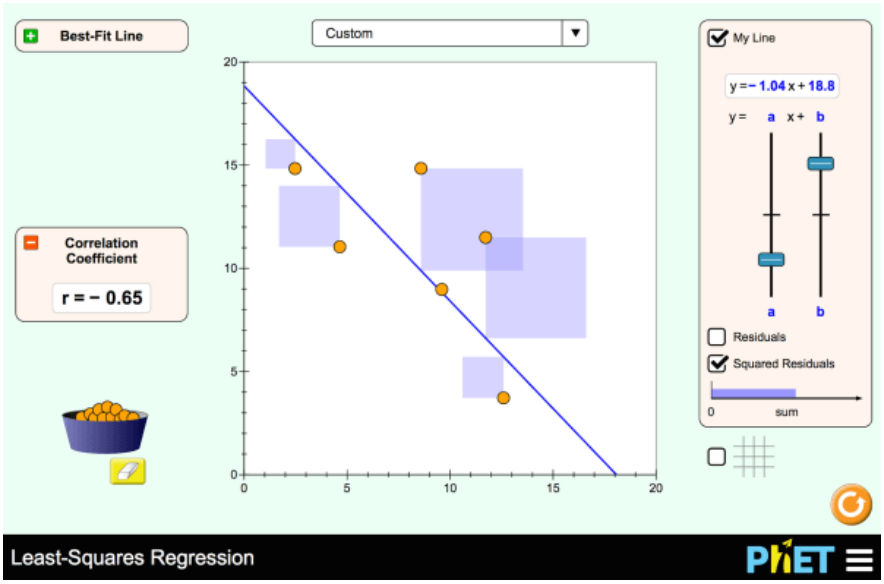
future of cheese consumption?

3. What does the slope of this line indicate to you?
   a. Use mathematics to illustrate this point.
   b. The slope has a unit related to “lbs.” and “year”, what is this unit?

Creating a Line of Best Fit

Not all points collected will fall on a straight line. A **Line of Best Fit** or a **Trendline** approximates the average of those points through a mathematical process called the **least-squares** method. While one could “eyeball” this line, the least-squares method uses the data to minimize the distance from all those points to the line to have an averaging effect.

1. Create a column of data for “x” values, “y” values, x^2 and xy
2. At the bottom of these columns, sum the data. Σx, Σy, $Σx^2$, Σxy
3. Calculate the slope from these values
   - $m = \frac{N(\Sigma xy) - \Sigma x \Sigma y}{N(\Sigma x^2) - (\Sigma x)^2}$ (where N=number of entries in a column)
4. Calculate the y-intercept from these values
   - $b = \frac{N(\Sigma y) - m(\Sigma x)}{N}$
5. Which provides use the function $f(x) = mx + b$



Click here to run the simulation.

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2.1.1.5: Reporting On Science

Lab Reports

Title

A description of the main idea or question of the lab. This can also highlight a key finding or question.

Abstract

A brief summary of the main question, methods, and findings. This is usually the last thing written but the first thing presented in order to grab the attention of the reader.

A rough breakdown of an abstract would contain about:

- 3 sentences worth of introduction with the key question
- 2 sentences of major methodology
- 3-6 sentences of the major results and conclusions drawn from them

Lengths will vary, but using this framework, you will not deviate too far from having a reader lose interest.

Introduction

Introduce the background that is relevant for forming the hypotheses being tested. What were the previous observations or prior knowledge used to come to these ideas? State the actual hypotheses to be tested and how it will further the understanding of the issue.

Materials and Methods

This section is a little like a cooking recipe. The main steps taken should be summarized as standard prose in a manner that anyone could follow and repeat. This is written in the past tense and 3rd person. Do not write in the first person as *you* have nothing to do with the experiments. Explain “What was done with which reagents?”

Results

This section is descriptive of what was observed. Figures and tables serve as a summary of the results to illustrate the data. They also serve as guides to outline the text of the section. Slowly describe each figure or table. Expand these points into sentences and paragraphs. Present the data as fully as possible, including stuff that at the moment does not quite make sense. This is written in the past tense and 3rd person. Conclusions are not provided in this section as they are made from analyzing the information and synthesizing the results.

Discussions

Discussions are the conclusions made by analyzing the results. At this time, you will be able to re-emphasize the original hypotheses made in the introduction. Indicate whether or not the hypotheses were demonstrated sufficiently. If this is not the case, offer alternatives and interpretations. Can you improve or modify your hypotheses? Explain how multiple lines of evidence corroborate each other and help to further the understanding of the problem.

References

Prior knowledge requires demonstration of strength or validity. The references should be from primary sources and should

illustrate the point of the statement. The section is presented in numerous formats as a bibliography, but citations are inserted near the text where knowledge or statements are displayed. Use a reference Manager to insert citations and format the bibliography. An excellent free reference manager with a plug-in to common word processors is Zotero.

Additional Resources

- MIT OCW

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2.1.2: CHEMISTRY

2.1.2.1: Introduction

2.1.2.2: Water

2.1.2.3: Biologically Important Macromolecules

2.1.2.4: Chromatography

2.1.2.5: pH

2.1.2.6: pH (Activity)

2.1.2.7: Carbohydrates

2.1.2.8: Lipids

2.1.2.9: Proteins

2.1.2.10: Nucleic Acids

2.1.2.11: Biological Molecules (Concept)

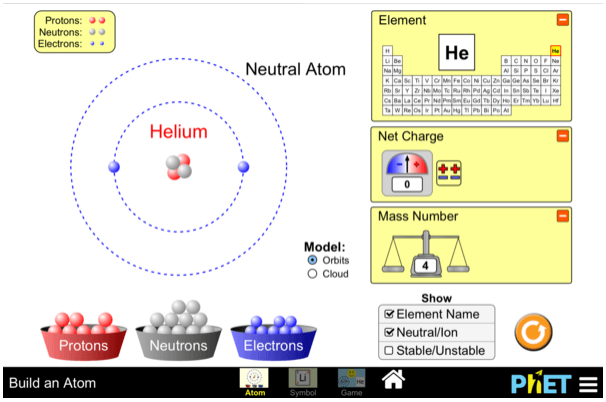
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2.1.2.1: Introduction

Chemistry

Self-Guided activity: http://phet.colorado.edu/files/activities/3807/BAA-Student-Handout.doc



Follow the self-guided activity for a review of basic chemistry before continuing to the organic chemistry section.

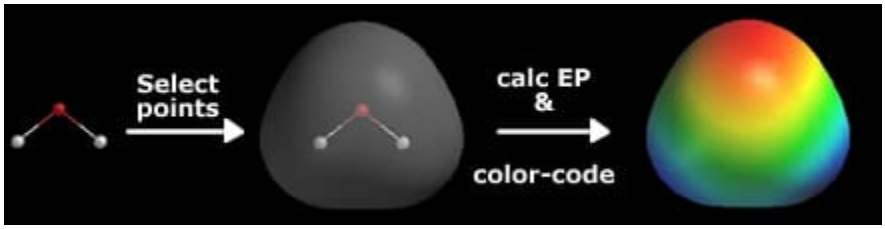
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2.1.2.2: Water

Polar Covalent Bonds

H_2O is a polar covalent molecule. The Bonds between the H atoms and the O atom arise from sharing electrons. These shared electrons form to satisfy the **octet rule**. However, oxygen is a "selfish" sharer. This **electronegative** aspect of oxygen means that the electrons of the H_2O molecule preferentially associate near the oxygen atom, creating partial charges. We indicate this by placing a δ^-near the O and δ^+'s near the H atoms. These partial charges make the H_2O **polar**.



The electron cloud around a water molecule lingers around the oxygen molecule to render it partially negative. Red illustrates the partial negative end of the molecule while blue indicates the partial positive.

Because of this polarity, H_2O molecules arrange in a highly structured way. Use the following **simulation to explore polarity of molecules**.

File:3D model hydrogen bonds in water.svg

These weak associations that arise from the polar: polar attractions are referred to as **Hydrogen Bonds (H-bonds)**. While independently weak, the summation of all the H-bonds is very strong. These associations give rise to the special properties of water: **surface tension**, **cohesion**, **adhesion**, and **high specific heat capacity**.

- **Hydrogen Bond Simulation**

Polar materials mix with polar materials. Things that can dissolve in water are also polar and referred to as being **hydrophilic** (*hydro* = water, *philic* = liking). Non-polar substances do not interact or mix with polar solvents and are referred to as **hydrophobic** (*hydro* = water, *phobic* = hating). Since carbon and hydrogen share electrons equally, organic compounds are non-polar. Oil is a hydrocarbon that does not mix well with water or vinegar. Vinegar, however, is a polar compound that interacts with water. Detergents are called **amphiphilic** (*amphi* = both; *philic* = liking) because they have portions that are non-polar and portions that are very polar. Detergents can, therefore, dissolve in hydrocarbons and water. Water alone cannot effectively remove oil from your skin but a detergent can dissolve the oil and carry it away in water.

File:Sodium laurylsulfonate V.1.svg

The common detergent in soap – Sodium Lauryl Sulfate

Surface Tension

Surface tension presents as an invisible film that encompasses the surface of water. The attractive forces arising from the intermolecular **cohesion** holds the surface of water together.

File:Surface tension March 2009-3.jpg

This paperclip would sink if it broke through the surface of the water.

File:Wasserläufer bei der Paarung crop.jpg

This water strider is not on top of the water because it is light. It has not broken through the surface of the water and is, therefore, on top of the water.

Solutions

Water is an excellent solvent of other polar compounds. Table salt (NaCl) ionizes readily in water. The δ^- O associate around Na^+ while the δ^+ H associate with the Cl^-. If NaCl is dissolved in H_2O, what do you think happens to the intermolecular interactions between water molecules? What do you think would happen to the H-bonds? Would you expect there to be a difference in the surface tension? How do you think this explains the difference of boiling or freezing?

Running on Water



Basiliscus plumifrons (green basilisk)



Basiliscus basiliscus (common basilisk)

Lizards of the genus *Basiliscus* have the nickname “Jesus Christ lizard” for their very special adaptation regarding water.

In the face of danger, these lizards run on their hind legs across the water to escape predation. Their hind legs have long toes that help in increasing surface area to distribute their weight so they can propel themselves on the surface of the water. They do not sink because the surface tension of the water is not broken by the large surface covered by their feet. After about 4.5m, they lose sufficient momentum to propel themselves on the water surface and break through. H-bonds enable this adaptation.

Video from National Geographic

What If?

The namesake of these lizards walked on water but was also able to turn water into wine. Wine is a solution of ethanol (11%) in water. Ethanol has a polar end and a non-polar end.

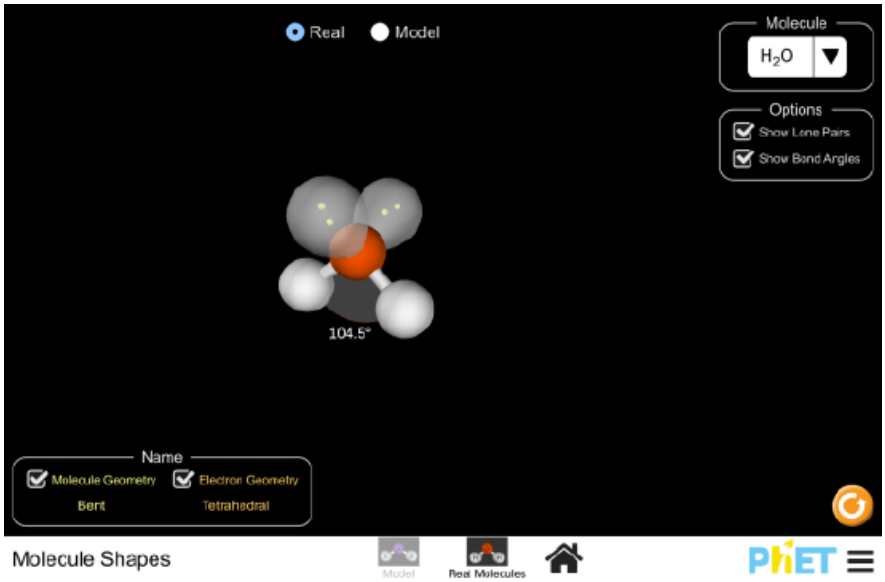
- How successful would the basilisk run on wine?
- Why?
- How could you test this without necessarily using a lizard?

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2.1.2.3: Biologically Important Macromolecules

Understanding Chemistry in 3D



1. Click on the image above to launch the simulation.
2. Choose “Real Molecules”.
3. Click on “Show Bond Angles”.
    - You may also deselect “Show Lone Pairs”.
4. Drag the atoms to understand the 3-D shape.
5. Choose CH_4 from the drop-down menu of molecules.
    - CH_4 is methane, the simplest organic molecule.
    - Notice the restraints on the physical space occupied by the atoms as you drag the atoms around.
    - Remember these bond angles and constraints when we explore organic chemistry.
    - Toggling between “Model” and “Real” will illustrate if the position of the atoms are influenced by other factors we don’t see.
6. Choose NH_4 from the drop-down menu.
    - NH_4 is ammonia, a polar solvent.
    - NH_4 is **NOT** an organic molecule.
    - Toggle the lone pairs to see how these electrons play a role in the positioning of atoms.
7. Choose CO_2 from the drop-down.
    - Explore this non-organic molecule to visualize the effects of double bonds on geometry.

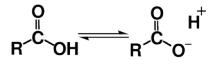
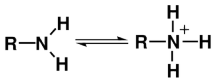
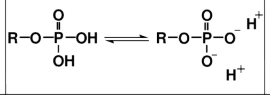
Organic Chemistry

Living things are composed of **organic molecules** primarily made up of the elements carbon and hydrogen. Molecules of hydrogen and carbon (referred to as **hydrocarbons**) have the property of being **nonpolar**. Yet 70- 90% of cells are composed of water (a **polar** compound). Polar substances mix with other polar substances. Likewise, non-polar substances interact with other non-polar compounds. Polar and non-polar compounds are immiscible (unable to mix).

So How do Cells Keep From Falling Apart in a Liquid Environment?

Functional groups are clusters of atoms in a group that impart a new “function” to the compound they are attached to. Hydrocarbons in cells have functional groups attached to them that permit them to interact with the water environment of the

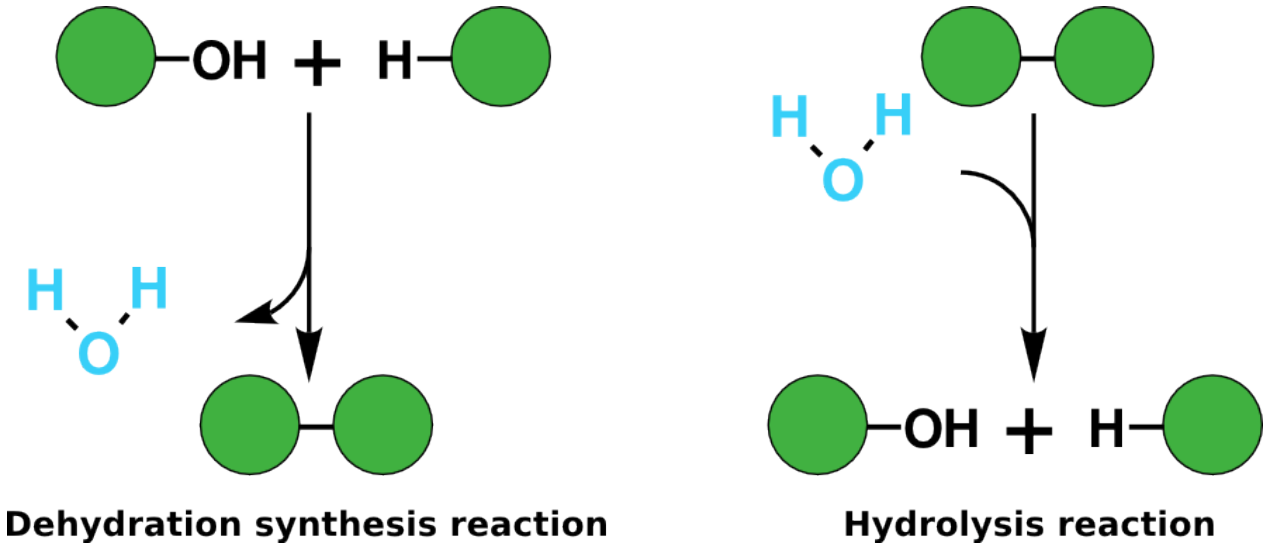
cell. These functional groups also define the type of molecule it is based on the characteristics of those groups.

Group Name	Structure	Compound Name	Property
Hydroxyl	R—OH	alcohol	polar
Carbonyl		Aldehyde (R1=H) Ketone (R1=C)	polar
Carboxyl		acid	polar acidic
Amino		amine	polar basic
Sulfhydryl	R—SH	thiol	polar
Phosphate			polar acidic

Functional Groups. *Functional groups commonly found in organic compounds. R is a placeholder chemical that can be anything (like a hydrocarbon chain). The functional groups in this table are those that add polar or charged properties to the hydrocarbon chains. Bi-directional arrows indicate that those functional groups dynamically ionize and reach an equilibrium in solution.*

How are Macromolecules Assembled?

The common organic compounds of living organisms are **carbohydrates**, **proteins**, **lipids**, and **nucleic acids**. Each of these are macromolecules or **polymers** made of smaller subunits called **monomers**. The bonds between these subunits are formed by a process called **dehydration synthesis**. This process requires energy; a molecule of water is removed (dehydration) and a covalent bond is formed between the subunits. Because a new water molecule is formed, this is also referred to as *condensation*. The opposite where water and energy are used to break apart polymers into simpler monomers is called **hydrolysis** (***hydro***– water, ***lysis***– to break or split).



Polymer formation. Dehydration reactions join monomers. Hydrolysis splits polymers.

Where Do We Find Macromolecules?

File:US Nutritional Fact Label 2.svg

A nutrition label illustrates the breakdown of chemical components of Macaroni and Cheese. This is not limited to the macromolecules discussed here. Items like Iron and Sodium are ions that are important for the function of the cell.

Additional Resources

- Carbohydrates http://www.visionlearning.com/en/library/Biology/2/Carbohydrates/61
- Lipids http://www.visionlearning.com/en/library/Biology/2/Lipids/207/reading
- Fats and Proteins http://www.visionlearning.com/en/library/Biology/2/Fats-and-Proteins/62

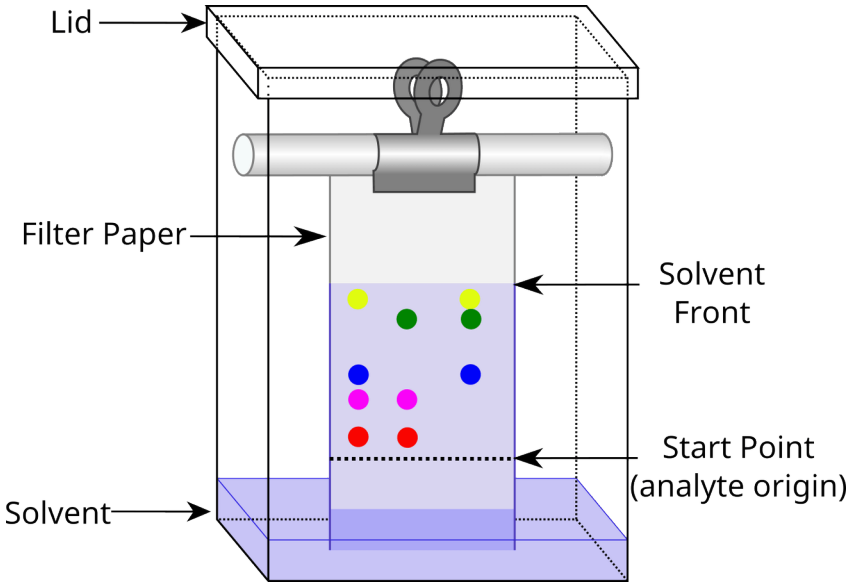
Vocabulary

• Carbohydrates • Reducing sugar • Polymer • Monomer • Monosaccharide • Disaccharide • Polysaccharide	• Dehydration synthesis • Hydrolysis • Reduction • Aldehyde • Ketone • Lipid

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2.1.2.4: Chromatography



Credit: Theresa Knott [CC BY-SA 3.0 or GFDL]

Chromatography is a collective term for a set of analytical techniques used to separate mixtures. *Chroma* means color and *graph* means to write or draw. Paper chromatography is an analytical technique used to separate mixtures of chemicals (sometimes colored pigments) using a partitioning method. The paper in this method is called the **stationary phase** because it does not move and serves as a substrate or surface for the separation. **Analytes** (substances being analyzed) are separated from each other based on a **differential affinity** to a solvent. The solvent dissolves and carries the analytes along the matrix of the stationary phase. Since the solvent moves through a wicking action, it is called the **mobile phase**.

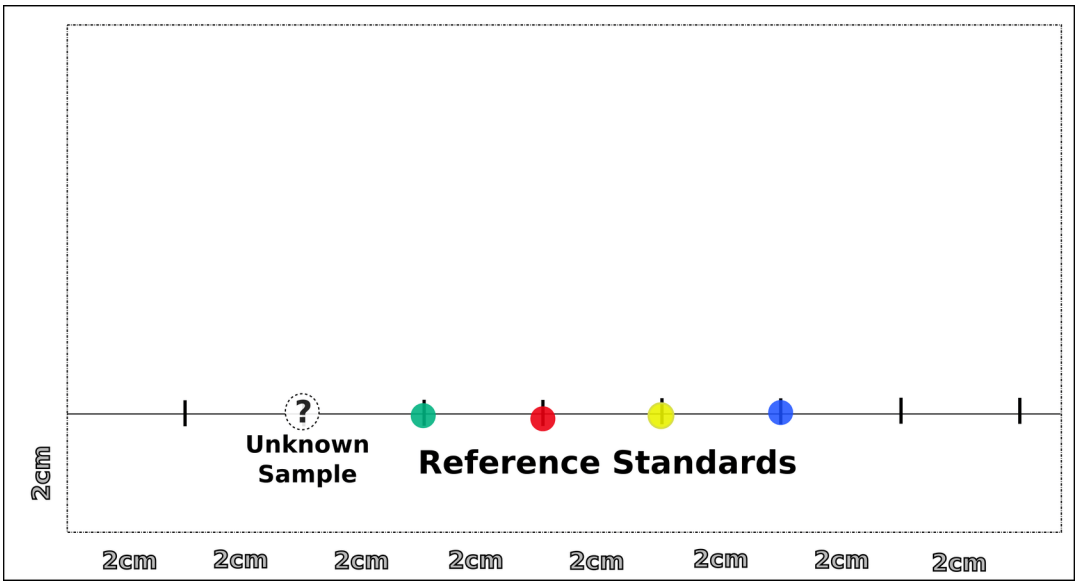
The distance that the analyte migrates along the paper related to the total distance that the solvent or mobile phase moves is called the **Retention Factor** or $\mathbf{R_F}$.

$$R_F = \frac{D_A}{D_S} = \frac{migrationdistanceofanalyte}{migrationdistanceofsolvent}$$

Are The Food Colorings Used in Colored Candy the Same as the FD&C Approved Chemicals?

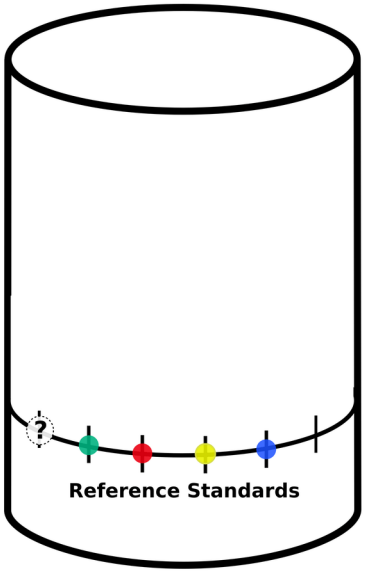
How many colored spots do you expect to see for each reference standard?

1. Obtain a 25 cm square piece of chromatography paper that will fit into the beaker that will serve as the chromatography chamber.
2. Draw a pencil line across the lower end of the chromatography paper about 2 cm from the bottom.
3. Draw additional vertical tick marks along this line every 2 cm.
4. Place colored candy in a flask with 2 ml ethanol until the color dissolves into the solution.
5. Using an applicator, create a very small spot on a tick mark and allow to dry.
6. Repeat application on the spot to make a very small and dark spot.
7. Continue to spot reference standards along other tick marks. These reference standards are food coloring.

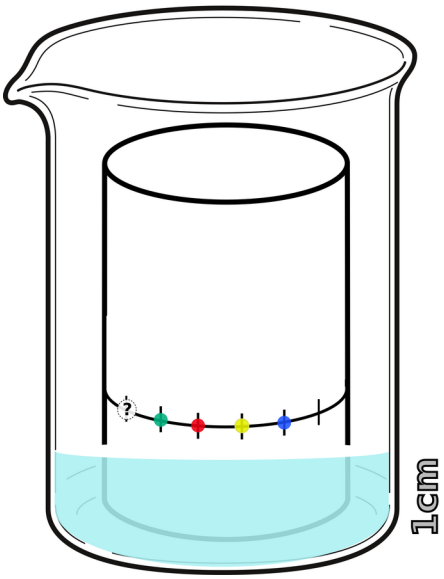


8. Place approximately 1 cm of mobile phase solution (a very polar saltwater solution) into the beaker.

9. Roll the filter paper into a cylinder and fix with staples.



10. Place cylinder into the beaker and cover for 20 minutes **or** until the mobile phase reaches 2 cm from the top of the paper.



11. Mark the final distance of the mobile phase and dry the filter.

12. Measure the distance of each spot from the starting point.

1. Each spot is a separate analyte.
2. Some spots separate into multiple analytes.
3. Measure **EACH** one.

13. Measure the distance from the starting point to the final point that the solvent reached.

14. Calculate R_f values and tabulate results.

Table of Reference Standards

Reference Standard	Distance (cm)	R_F
Green	D_1= D_2= D_3=	R_{F1}= R_{F2}= R_{F3}=
Red	D_1= D_2= D_3=	R_{F1}= R_{F2}= R_{F3}=
Yellow	D_1= D_2= D_3=	R_{F1}= R_{F2}= R_{F3}=
Blue	D_1= D_2= D_3=	R_{F1}= R_{F2}= R_{F3}=

Table of Unknowns

Unknown	Distance (cm)	R_F
Brown Candy	D_1= D_2= D_3=	R_{F1}= R_{F2}= R_{F3}=
Blue Candy	D_1= D_2= D_3=	R_{F1}= R_{F2}= R_{F3}=
Red Candy	D_1= D_2= D_3=	R_{F1}= R_{F2}= R_{F3}=
Green Candy	D_1= D_2= D_3=	R_{F1}= R_{F2}= R_{F3}=
Orange Candy	D_1= D_2= D_3=	R_{F1}= R_{F2}= R_{F3}=
Yellow Candy	D_1= D_2= D_3=	R_{F1}= R_{F2}= R_{F3}=

Reflect

1. Compare and average the R_F values of each analyte across the entire class.

2. Did you predict the number of spots that would appear from each analyte (reference or candy)?

3. Assuming all the dye molecules are of the same mass, what influenced the migration patterns of each spot?

4. Were the colors used in the candy the same as the references?

5. What does it mean if the candy color didn't match anything from the food colors from the cake decorating set used as references?

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2.1.2.5: pH

pH

Acids and Bases

We can call any compound that adds H^+ ions (a free proton) into solution an **acid**. Along with this, we would expect that any compound that would decrease the concentration of free H^+ of a solution as a **base**. **pH** is the *power of* H^+ of a solution. We define this *power* as a **molar concentration** of H^+ in solution. This concentration invariably ends up being a relatively small number (though great in absolute numbers) and is expressed as a decimal number. Because the range of the concentrations is so great, we express these numbers as logarithmic numbers to avoid writing many 0's after the decimal and to facilitate communicating the concentration. Since these numbers are so (relatively) small, we use the negative logarithm to describe this concentration.

Mathematically defined, $pH = -\log_{10}[H^+]$

The pH scale ranges so that anything below pH 7 is acidic and anything above pH 7 is alkaline. So a smaller number is more acidic. But didn't we just state that something acidic contains more H^+ ions? Remember, because we are dealing with a negative Logarithm, this means the concentration is higher.

Logarithmic Scales

If we have a quantity that is 10^2, we know that translates into 100. Just as if we have a quantity of 10^4, we know that translates into 10000. Just as it becomes inconvenient to keep writing all those 0's, it's really impractical to write many many 0's after a decimal. It's really hard to talk about too! So we likewise will express numbers like 0.0001 as 10^{-4}. A logarithm is the reverse function of an exponent. Therefore:

- $\log_{10}(0.0001) = -4$
- $\log_{10}(10^{-4}) = -4$

So how do we define a solution that is pH 2? Well, we already decided that this solution is below pH 7 — making it an acid. But what does this mean in terms of H^+ ion concentration?
Let's work this out algebraically:

- $pH = -\log_{10}[H^+]$ Let's bring the (-) over to the other side
- $-pH = \log_{10}[H^+]$ Now let's reverse the Log → base 10
- $10^{-pH} = [H^+]$ Plug in the pH → molar concentration of $[H^+]$

As we can now see, a solution of pH 2 is acidic because the molar concentration of $[H^+]$ is 10^{-2}mole/L or 0.01M

Dissociation of Ions: That Number is Small!

It's not a small number. Remember that a mole is 6.022 X 10^{23}. That's a very large number! Think about it! A solution of pH 4 is acidic, but if we plug in the formula, we realize that this is equal to 0.0001M H^+ – less than pH 2 at 0.01M!

But let's compare it to the $[H^+]$ content of H_2O. Now I'm going to sound crazier! Water can be thought of as being in an equilibrium where some of the molecules are ionizing and deionizing. We can express this in 2 ways:

- $H_2O \Leftrightarrow H^+ + OH^-$

- $2H_2O \diamond H_3O^+ + OH^-$

So at any given point, a liter of H_2O at neutral pH (7) has 10^{-7} moles of H^+ ions. Incidentally, it also has 10^{-7} moles of OH^- in solution. The second expression indicates the formation of a hydronium ion (H_3O^+) instead of a free proton in solution. So something that is pH 2 is a stronger acid than pH 4, right? Nope. That just indicates the amount of free protons in solution. It is more acidic but acid strength means something else. When we talk about **strong acids**, it means that it is more likely to donate a proton to the solution because it is more likely to ionize. Let's look at the following:

- $HA \diamond H^+(aq) + A^-(aq)$ Where HA is an acid dissociating in solution

If this dissociation is very high, then we say that it is a strong acid. Similarly, a compound like NaOH readily dissociates completely in solution and provides OH^- ions that can readily remove H^+ from the solution—a strong base! We speak of dissociation in terms of rates and we express this as the acid dissociation constant, $\mathbf{K_a}$. This is calculated using the concentrations of $[H^+]$ (proton), $[A^-]$ (conjugate base) and [HA] (non-dissociated) at equilibrium:

- $$K_a = \frac{[H^+][A^-]}{[HA]}$$

Just like the orders of magnitude we have when discussing pH, the rates of dissociation are more conveniently communicated on a logarithmic scale.

- $$pK_a = -\log_{10} K_a$$

Think about it this way, if the concentration of the dissociated ions is very high, the numerator in the rate is very high → K_a is great. In other words, at equilibrium, the dissociation reaction looks more unidirectional than bi-directional as the compound is readily ionized:

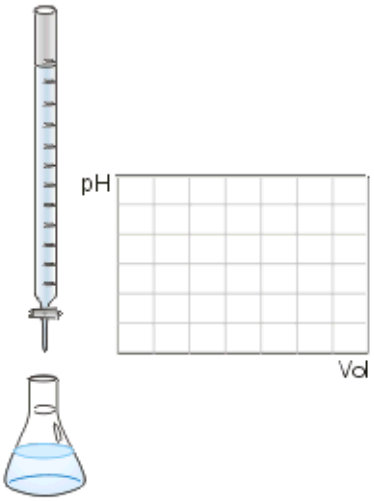
- $HA \rightarrow H^+(aq) + A^-(aq)$

On this scale, we refer to anything with a $pK_a < -2$ as a strong acid since it will readily dissociate in solution. This form of the dissociation constant is extremely useful in estimating the pH of **buffered solutions** and for finding the equilibrium pH of the acid-base reaction (between the proton and the conjugate base). We can estimate the pH by utilizing the Henderson-Hasselbalch Equation:

- $$pH = pK_a + \log_{10}\left(\frac{[A^-]}{[HA]}\right)$$

Buffered Solutions

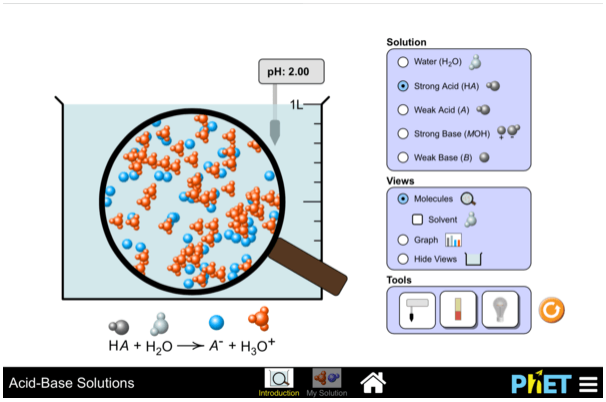
A **buffer** is something that resists change. A **buffered solution** is one that consists of a weak acid or weak base that will control the pH of a solution. Imagine a buffer to be a reservoir of available H+ or OH- ions. If a buffered solution is pH 2, adding a basic solution to it will not cause a drastic change to the pH because the reservoir of H+ will continuously neutralize the base. Eventually, this store or reservoir of H+ will be depleted. When this happens, the pH will suddenly change. The range in which acid or base is added without a significant change in pH is called the buffered zone or the **buffering capacity**. When this store of H+ or buffering capacity is expended, we have reached the **equivalence point** that describes the point at which the base has completely neutralized the weak acid.



File:Titration of weak acid with strong base.PNG

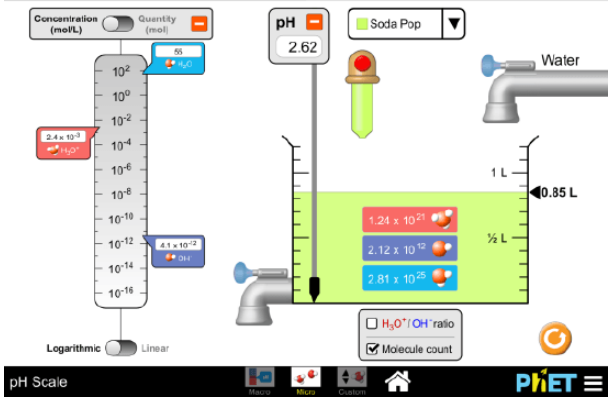
Titration of an acid by a base The solution has a good buffering capacity between pH 3 and pH 5

Acids and Bases Simulation



Click here to run the simulation on Acids and Bases

pH simulation



Click here to run the simulation on pH scales

Use the table below to indicate whether an item is an acid or a base and what you predict the pH to be. We can determine these in class through measuring.

Solution	Acid or Base	Predicted pH	Actual pH
Coffee			
Cola			
Distilled H_2O			
Detergent			
Bleach			
Apple Juice			
Antacid Solution			

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2.1.2.6: pH (Activity)

Inquire!

- What is the mechanism of action for antacids?
- Do they all antacids have the same efficacy?

Explore: Determine the pH of Common Solutions

1. Use the table below to indicate whether an item is an acid or a base and what you predict the pH to be. We can determine these in class through measuring.

Solution	Acid or Base	Predicted pH	Actual pH
Coffee			
Cola			
Distilled H_2O			
Detergent			
Bleach			
Apple Juice			
Antacid Solution			

2. Using the pH meter, measure the pH and validate your predictions.

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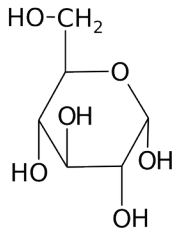
2.1.2.7: Carbohydrates

Introduction: Carbohydrates

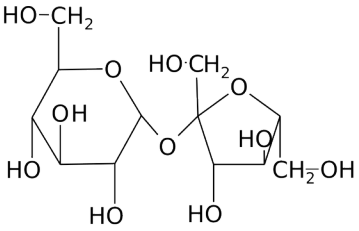
Carbohydrates serve 2 major functions: energy and structure. As energy, they can be simple for fast utilization or complex for storage. Simple sugars are monomers called **monosaccharides**. These are readily taken into cells and used immediately for energy. The most important monosaccharide is glucose ($\mathbf{C_6H_{12}O_6}$), since it is the preferred energy source for cells. The conversion of this chemical into cellular energy can be described by the equation below:

$$\mathbf{C_6H_{12}O_6\ (s) + 6\ O_2\ (g) \rightarrow 6\ CO_2\ (g) + 6\ H_2O\ (l) + energy}$$

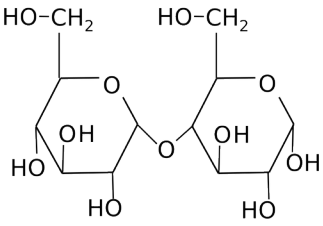
Long polymers of carbohydrates are called **polysaccharides** and are not readily taken into cells for use as energy. These are used often for energy storage. Examples of energy storage molecules are amylose, or **starch,** (plants) and **glycogen** (animals). Some polysaccharides are so long and complex that they are used for structures like **cellulose** in the cell walls of plants. Cellulose is very large and practically indigestible, making it unsuitable as a readily available energy source for cells.



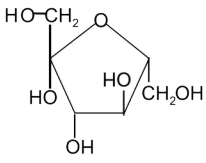
Glucose, a monosaccharide



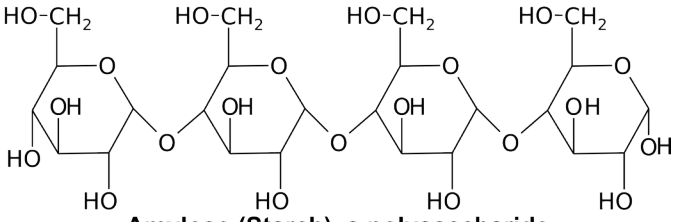
Sucrose, a disaccharide



Maltose, a disaccharide



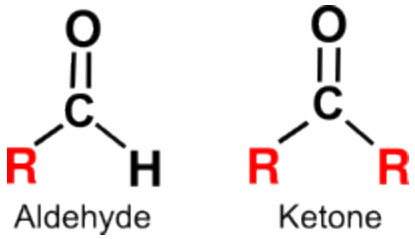
Fructose, a monosaccharide



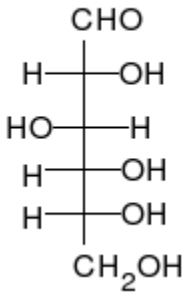
Amylose (Starch), a polysaccharide

Carbohydrates: *Carbohydrates are composed of sugar units referred to as -saccharides.*

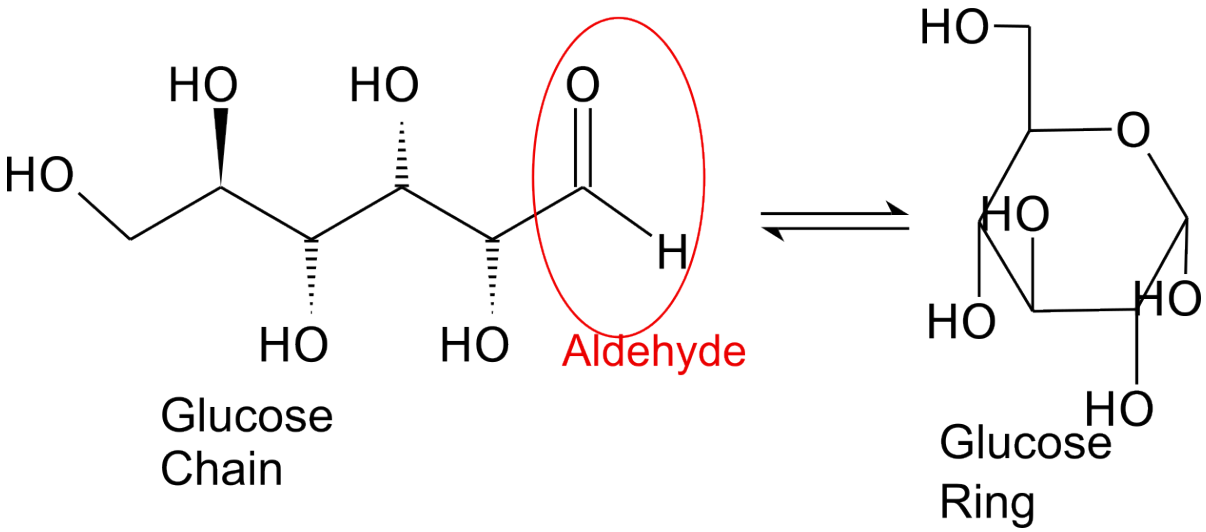
Many monosaccharides such as glucose and fructose are **reducing sugars,** meaning that they possess free **aldehyde** or **ketone** groups that reduce weak oxidizing agents such as the copper in Benedict's reagent. The double bond in the carbonyl group is a source of electrons that can be donated to something else. That is to say, those electrons can be "*lost*" by the sugar and "*gained*" by another chemical. **Benedict's reagent** contains cupric (copper) ion complexed with citrate in alkaline solution. Benedict's test identifies reducing sugars based on their ability to reduce the cupric (Cu^{2+}) ions to cuprous oxide (Cu^{+}) at basic (high) pH. Cuprous oxide is green to reddish-orange. Roughly speaking, **reduction** is a type of chemical reaction that is paired with **oxidation**. In oxidation/reduction reactions (**RedOx**), some chemical loses electrons (oxidized) to another chemical that gains them (reduced). We remember whether a compound is reduced or gained by using the pneumonic: LEO goes GER or **L**oss of **E**lectrons is **O**xidation & **G**ain of **E**lectrons is **R**eduction.



Monosaccharides contain a carbonyl group. The carbonyl is a source of electrons (the double bond on the oxygen). These electrons can be donated (or lost and oxidized) to reduce another compound (that gains those electrons).



Glucose is the preferred carbohydrate of cells. In solution, it can change from a linear chain to a ring.



Monosaccharides are capable of **isomerizing**. This means they alternate in structure from a linear chain to a ring form in solution. In the chain form, the aldehyde is free to donate (lose) electrons to reduce another compound. When monosaccharides undergo dehydration synthesis to form polymers, they can no longer isomerize into chains with free aldehydes and are unable to act as reducing sugars. Green color indicates a small amount of reducing sugars, and reddish-orange color indicates an abundance of reducing sugars. Non-reducing sugars produce no change in color (i.e., the solution remains blue).

Oxidized Benedict's reagent (Cu^{2+}) + Reducing sugar (R-C=O)
(blue)

Heat
High pH

Reduced Benedict's reagent (Cu^{+}) + Oxidized sugar (R-COOH)
(green to reddish orange)

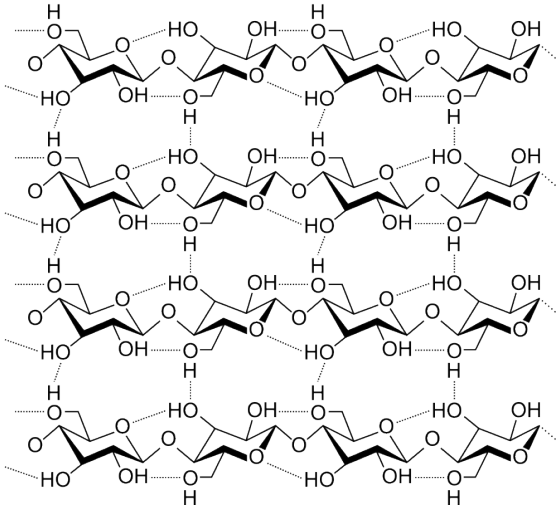
Note: Cu^{2+} has fewer electrons than Cu^{+}.

When monosaccharides undergo dehydration synthesis to form polymers, they can no longer isomerize into chains with free aldehydes and are unable to act as reducing sugars. Green color indicates a small amount of reducing sugars and reddish-orange color indicates an abundance of reducing sugars. Non-reducing sugars produce no change in color (i.e., the solution remains blue).

Structural Carbohydrates

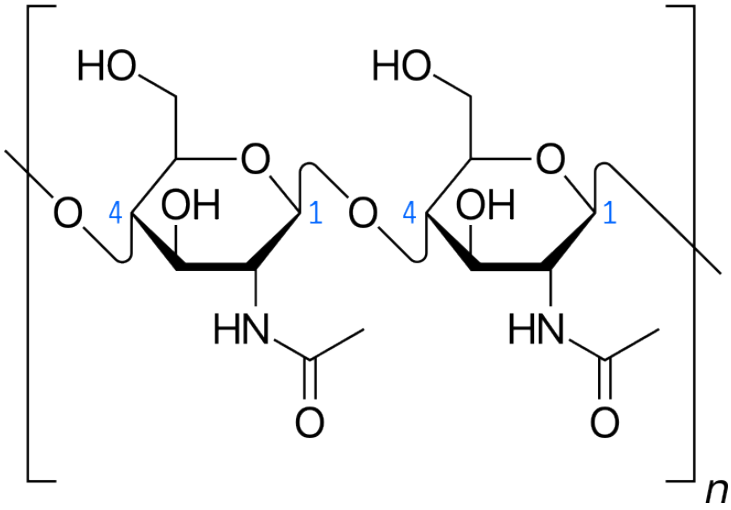
In food, more complex carbohydrates are derived from larger polysaccharides. These larger carbohydrates are fairly insoluble in water. Dietary fiber is the name given to indigestible materials in food most often derived from the complex carbohydrates from vegetable material. Some of this material serves the plants as a structural component of the cells and is completely insoluble. **Cellulose** is the major structural carbohydrate found in plant cell walls. Similarly, animals and fungi have structural carbohydrates that are composed of the indigestible compound called **chitin**. We will not be testing for these items.

Cellulose (plant structural)



Cellulose is a complex carbohydrate of glucose molecules. It is the major structural component of plant cell walls. It' structural durability is enhanced by intramolecular hydrogen bonds.

Chitin (animal and fungi)



Chitin is a structural carbohydrate found in animal shells or fungi cell walls. The polymer contains amide groups that differentiate it from other carbohydrates composed of glucose.

File:Lyristes plebejus.jpg

A cicada molting from its shell made of chitin.

Test Your Knowledge

- http://www.visionlearning.com/en/library/Biology/2/Carbohydrates/61/quiz

Detection of Carbohydrates (Activity)

Materials:

• potato juice • apple juice • urine sample 1 • urine sample 2 • reducing sugar solution • starch solution • Benedict's Reagent	• Sucrose solution • glucose solutions • distilled water • hot plates • beakers of water • test tubes • test tube rack

Stop and Think

- Use your senses and previous observations/experiences about the qualities of the experimentals.
- Formulate some hypotheses about the carbohydrate content of the experimentals or unknowns.
  - Identify if the sample is experimental or control before making a hypothesis.

Question: Are There Simple Reducing Sugars in my Juice? Are There Simple Reducing Sugars in my Urine?

Diabetes mellitus is a disease that refers to the inability of the cells to take in glucose. The word diabetes refers to urination and mellitus refers to sweetness. Since the cells of diabetics cannot remove glucose from the blood, there is an excess of glucose circulating that is eliminated in the urine. The traditional method of diagnosing someone with diabetes mellitus was to taste the sweetness of the patient's urine. Let's use Benedict's test for the detection process instead of the unhygienic alternative.

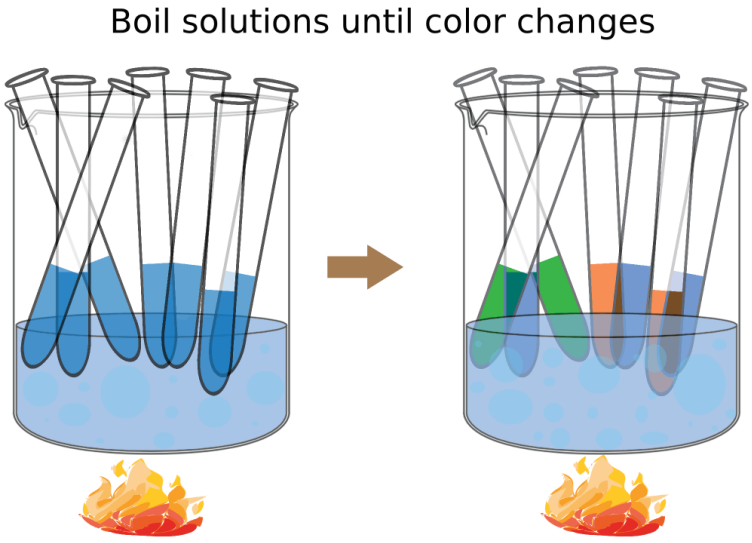
Make a hypothesis and ask what we would predict from a Benedict's test if testing a urine sample of someone with diabetes mellitus.

Benedict's Test For Reducing Sugars

1. Obtain 9 test-tubes and number them 1-9.
2. Add to each tube the materials to be tested. Your instructor may ask you to test some additional materials. If so, include additional numbered test tubes.
3. Indicate in the table whether the sample you are testing is positive control, a negative control, or an experimental.
4. Before you begin the heating of the samples, predict the color change (if any) for each sample. (use the sample type to aid in your prediction)
5. Add 40 drops (or 2 ml) Benedict's solution to each tube.
6. Place all of the tubes in a boiling water bath for 3 min or until a noticeable color change and observe colors during this time.
7. After 3 minutes, remove the tubes from the water bath and let them cool to room temperature. Record the color of their contents in the Table.

Table:Solution And Color Reactions For Benedict's Test For Reducing Sugars

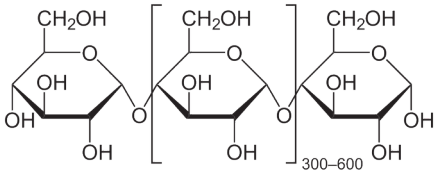
Tube	Solution	Type of Sample (unknown, + control, - control)	Hypothesized Color	Benedict's Test Color
1	10 drops apple juice			
2	10 drops potato juice			
3	10 drops sucrose solution			
4	10 drops glucose solution			
5	10 drops distilled water	- **Control**	**Blue**	
6	10 drops reducing sugar	+ **Control**	**Red**	
7	10 drops starch solution			
8	10 drops Urine sample 1			
9	10 drops Urine sample 2			



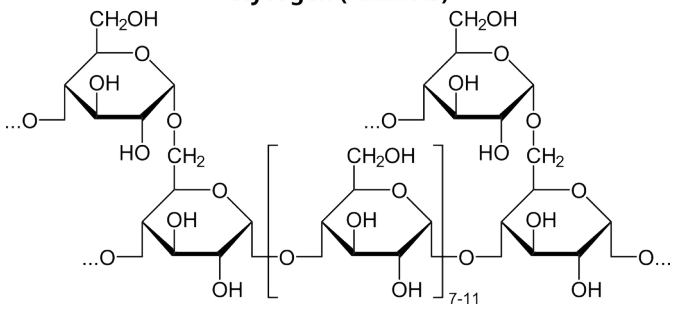
Iodine Test for Starch

Carbohydrates that are used for energy storage are not reducing sugars since they are polymers that lack free aldehydes. Plant cells store energy in the form of starches like amylose or pectin. Since these molecules are larger than monosaccharides or disaccharides, they are not sweet to the taste and are not very soluble in water. **Iodine** (iodine-potassium iodide, I_2KI) staining distinguishes starch from monosaccharides, disaccharides, and other polysaccharides. The basis for this test is that starch is a coiled polymer of glucose — iodine interacts with these coiled molecules and becomes bluish-black. Iodine does not react with other carbohydrates that are not coiled and remains yellowish brown. Therefore, a bluish-black color is a positive test for starch, and a yellowish-brown color (i.e., no color change) is a negative test for starch. Notably, glycogen, a common energy storage polysaccharide in animals, has a slightly different structure than does starch and produces only an intermediate color reaction.

Amylose or Starch (Plants)



Glycogen (Animals)



Plants store carbohydrates as a simple repeating polymer of glucose called starch. Amylose is a type of starch. Animal cells store glucose into a storage polymer called glycogen which is slightly more complicated than amylose.

Activity: Iodine Test For Starch

1. Obtain 7 test-tubes and number them 1-7.

2. Hypothesis Testing: Indicate in the table if the sample is experimental or control. Predict your expected color changes for each sample.
3. Add to each tube the materials to be tested as indicated in the table below. Your instructor may ask you to test some additional materials. If so, include additional numbered test tubes.
4. Add 10 drops of iodine to each tube. This test does **NOT** require boiling.
5. Record the color of the tubes' contents in the table below.

Table: Solution And Color Reactions For Iodine Test For Starch

Tube	Solution	Type of Sample (unknown, + control, - control)	Hypothesized Color	Iodine Test Color
1	20 drops apple juice			
2	20 drops potato juice			
3	20 drops sucrose solution			
4	20 drops glucose solution			
5	20 drops distilled H_2O	- **Control**	Yellow	
6	20 drops reducing sugar			
7	20 drops starch solution	+ **Control**	**Black-Purple**	

Questions for Reflection

1. In the Benedict's test, which of the solutions is a positive control? Which is a negative control?

2. Which is a reducing sugar, sucrose or glucose?

3. Which contains more reducing sugars, potato juice or onion juice?

4. Is there a difference between the storage of sugars in onions and potatoes?

5. Which patient sample likely comes from a diabetic patient and how do we know this?

6. In the Iodine test, which of the solutions is a positive control? Which is a negative control?

7. Which is more positive for the iodine test: onion juice or potato juice?

8. What can you infer about the storage of carbohydrates in onions? In potatoes?

9. Describe the half-reaction $\mathbf{Cu^{2+}} \rightarrow \mathbf{Cu^{+}}$ as oxidation or reduction.

10. Describe the half-reaction $\mathbf{Cu^{+}} \rightarrow \mathbf{Cu}$ as oxidation or reduction.

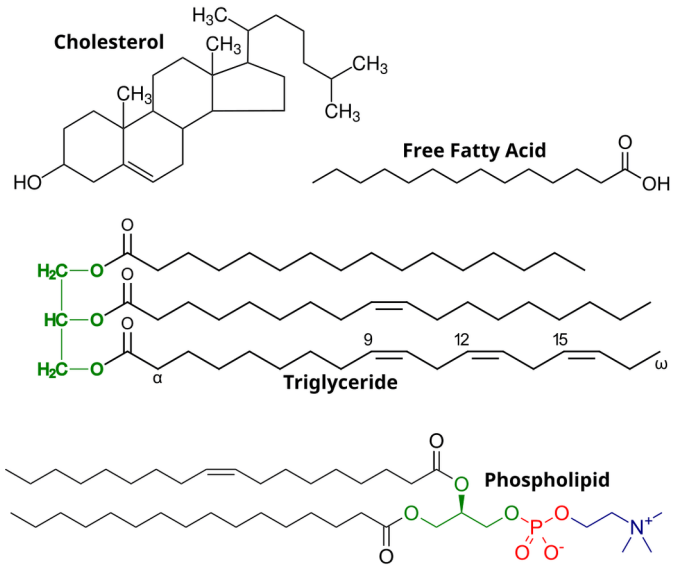
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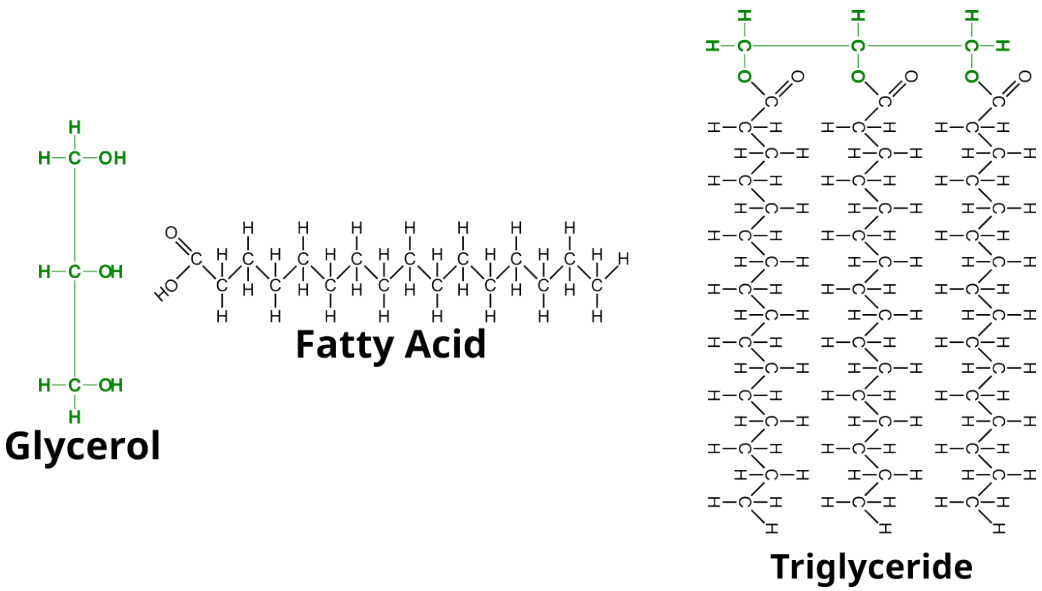
2.1.2.8: Lipids

Structure of Lipids

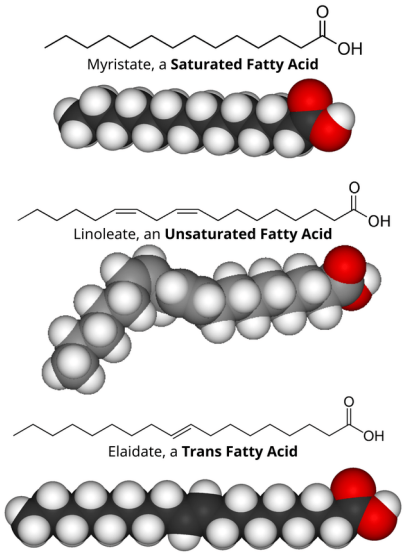
Lipids are the class of macromolecules that mostly serve as long-term energy storage. Additionally, they serve as signaling molecules, water sealant, structure, and insulation. Lipids are insoluble in polar solvents such as water and are soluble in nonpolar solvents such as ether and acetone.



Fats or triglycerides are made of glycerol and three fatty acid chains. They form through 3 dehydration synthesis reactions between a hydroxyl of the glycerol and the carboxyl group of the fatty acid.



Saturated versus Unsaturated Fats



- A saturated fatty acid. The molecule takes up little space in three dimensions. Many molecules can stack upon each other. Saturated fats are solid at room temperature.
- A polyunsaturated fatty acid. A kink from the double bond increases the amount of three-dimensional space that the molecule fills. Unsaturated fats tend to be liquid at room temperature.
- A trans-fatty acid. Despite an unsaturated bond, the molecule fills as much space as a saturated fatty acid and is solid at room temperature. Trans fats usually arise from artificial saturation techniques.

File:TriglycerideDairyButter.png

Butterfat is almost completely saturated. Notice how molecules can stack very closely.

File:Western-pack-butter.jpg

Because butterfat can stack together very closely, it is dense and found as a solid at room temperature. Credit: Steve Karg (CC BY 2.5)

Testing for Lipids

Tests for lipids are based on a lipid's ability to selectively absorb pigments in fat-soluble dyes such as Oil Red O or Sudan IV.

Tube	Solution	Control (+/-) or Experimental	Reaction Description
1	1 ml vegetable oil + water		
2	1 ml vegetable oil + Oil Red O		
3	1 ml water + Oil Red O		
4	1 ml Lipid Solution + Oil Red O		

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2.1.2.9: Proteins

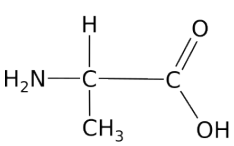
Proteins are Polymers of Amino AcidsFile:AminoAcidball.svg

Proteins provide much of the structural and functional capacity of cells. Proteins are composed of monomers called amino acids. **Amino Acids** are hydrocarbons that have an **amino group** (-NH_2) and an acidic **carboxyl group** (-COOH). The R group represents a hydrocarbon chain with a modification that alters the properties of the amino acid. 20 universal amino acids are used to construct proteins. The variation in functional groups along the amino acid chain gives rise to the functional diversity of proteins.

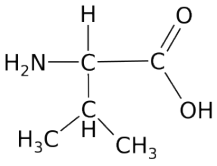
File:Amino Acids.svg

20 amino acids and their properties. A 21st amino acid on this table represents the non-universally found selenocysteine.

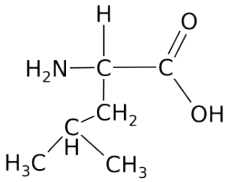
Monomers bond together through a dehydration synthesis reaction between adjacent amino and carboxyl groups to yield a **peptide bond**.



Alanine

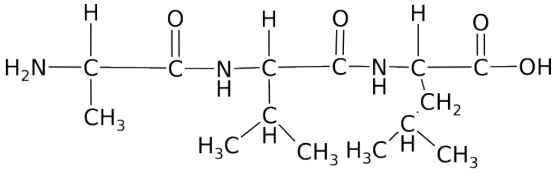


Valine



Leucine

Three Common Amino Acids



Tripeptide
The beginnings of a protein

Three amino acids bound into a tripeptide.

How Amino Acids Interact with Each Other and the Environment

Use the following simulation to test how a polypeptide chain with fold based on the type of solution it is in and the composition of the amino acids.

- Protein Folding Simulation (CC BY 4.0 Concord Consortium)

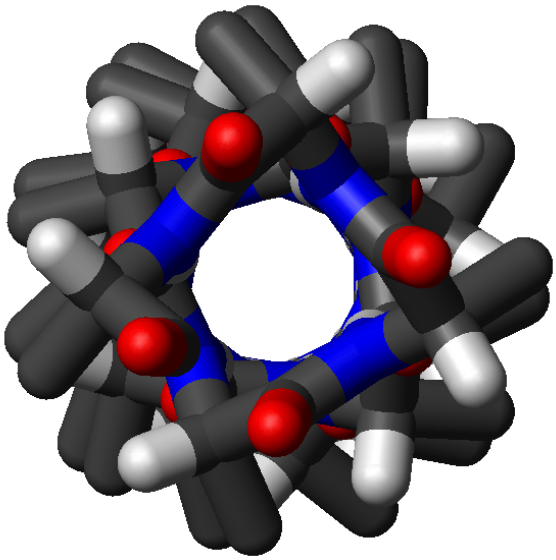
Levels of Structure

File:Main protein structure levels en.svg

- **Primary Structure (1°)**: The sequence of amino acids read from the Amino or N-terminal end of the molecule to the Carboxyl or C-terminal end
  - Tyr-Cys-Arg-Phe-Leu-Val-....
- **Secondary Structure (2°)**: local three-dimensional structures that form from interactions of amino acids, like hydrogen bonding
  - **Alpha Helix** – coils occurring from the H-bonds between N-H and C=O groups along the backbone of the protein

File:Alpha helix neg60 neg45
sideview.png

Side view of α-helix illustrating H-bonds in magenta between carboxyl oxygen (red) and amine nitrogen (blue)



Top-down view of an α-helix

File:1gzm opm.png

Side view of ribbon diagram of α-helices traversing a membrane.

- **Beta Sheets** – laterally connected strands or sheets of amino acids occurring from the H-bonds between N-H and C=O groups along the backbone of the protein

File:Beta sheet bonding antiparallel-color.svgFile:Beta sheet bonding parallel-color.svg



Ribbon diagram of β-sheets

- **Tertiary Structure (3°)**: overall 3-D structure of the peptide chain
- **Quaternary Structure(4°)**: multimeric protein structure from assembling multiple peptide subunits

Diversity of Proteins

File:Protein structure examples.png

Learn more about the complexity of protein structures at the Protein Data Bank.

Protein Detection (Activity)

Protein Detection Theory:

Proteins can be detected through the use of the **Biuret test**. Specifically, peptide bonds (C-N bonds) in proteins complex with Cu^{2+} in Biuret reagent and produce a violet color. A Cu^{2+} must complex with four to six peptide bonds to produce a color; therefore, free amino acids do not positively react. Long polypeptides (proteins) have many peptide bonds and produce a positive reaction to the reagent. **Biuret reagent** is an alkaline solution of 1% $CuSO_4$, copper sulfate. The violet color is a positive test for the presence of protein, and the intensity of the color is proportional to the number of peptide bonds in the solution.



File:Biuret-reaction.pdf

Biuret Test

1. Examine the table below. Indicate if the sample is a negative control, positive control or an experimental.
2. Predict the color change of the solution.
    - Formulate a hypothesis about the components of the experimentals.
3. Obtain 6 test tubes and number them 1-6.
4. Add the materials listed in the table.
5. Add 3 drops of Biuret reagent (1.0% $CuSO_4$ with NaOH) to each tube and mix.
6. Record the color of the tubes' contents in Table.

Tube	Solution	Control (+/-) or Experimental	Predicted Color Change	Actual Color Change
1	2 ml urine sample 1			
2	2 ml urine sample 2			
3	2 ml egg albumin			
4	2 ml amino acid solution			
5	2 ml distilled water			
6	2 ml protein solution			

Conclusions about the Urine Samples

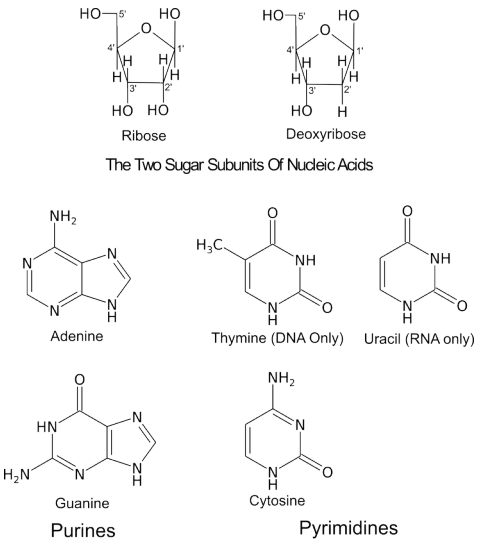
Based on the results of the Benedict's test and the Biuret test, can we make any conclusions?

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2.1.2.10: Nucleic Acids

Nucleic Acids



The Five Nitrogenous Bases of Nucleic Acids

Nucleic acids are composed of linked nucleotides. DNA includes the sugar, deoxyribose, combined with phosphate groups and combinations of thymine, cytosine, guanine, and adenine. RNA includes the sugar, ribose with phosphate groups and combinations of uracil, cytosine, guanine, and adenine.

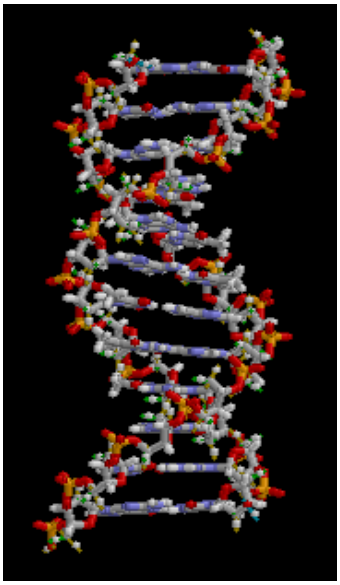
DNA and RNA are nucleic acids and make up the genetic instructions of an organism. Their monomers are called **nucleotides**, which are made up of individual subunits. Nucleotides consist of a 5-Carbon sugar (a pentose), a charged phosphate and a **nitrogenous base** (Adenine, Guanine, Thymine, Cytosine or Uracil). Each carbon of the pentose has a position designation from 1 through 5. One major difference between DNA and RNA is that DNA contains deoxyribose and RNA contains ribose. The discriminating feature between these pentoses is at the 2′ position where a hydroxyl group in ribose is substituted with a hydrogen.

File:DNA chemical structure.svg

DNA has a double-helical structure. Two antiparallel strands are bound by hydrogen bonds.

The following video illustrates the structure and properties of DNA.

DNA is a double helical molecule. Two antiparallel strands are bound together by hydrogen bonds. Adenine forms 2 H-bonds with Thymine. Guanine forms 3 H-bonds with Cytosine. This AT & GC matching is referred to as **complementarity**. While the nitrogenous bases are found on the interior of the double helix (like rungs on a ladder), the repeating backbone of pentose sugar and phosphate form the backbone of the molecule. Notice that phosphate has a negative charge. This makes DNA and RNA, overall negatively charged.



There are 10 bases for every complete turn in the double helix of DNA.

Nucleic Acids: DNA Extraction and Dische's Diphenylamine Test (Activity)

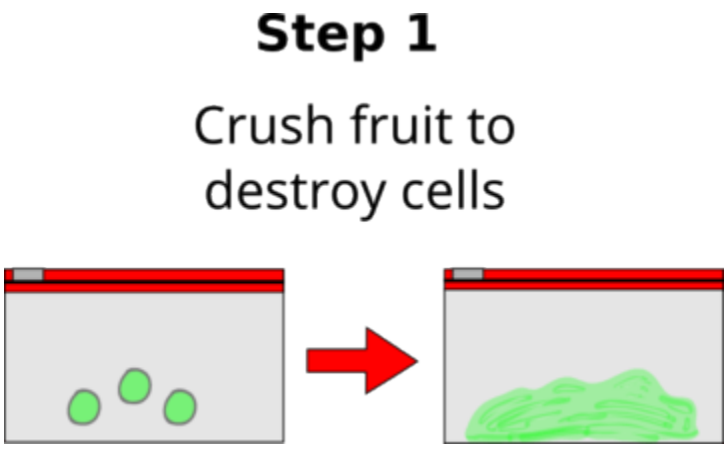
Prelab Questions

1. What are fruits?
   a. Where do they come from?
   b. What are they made of?
2. Use phylogeny to classify plants (DKPCOFGS)
3. Where is DNA located within the fruits? Where is it located in you?
4. Why would you want to extract DNA from an organism?
5. What class of molecule is DNA?

Extraction of DNA from fruit

single panel instructions can be found at https://github.com/jeremyseto/bio-oer/blob/master/figures/chemistry/DNA/fruitdnaisolation.svg

1. Mash about 10g or 3cm of over-ripe banana OR 3 grapes OR 1 strawberry in zip-top bag.



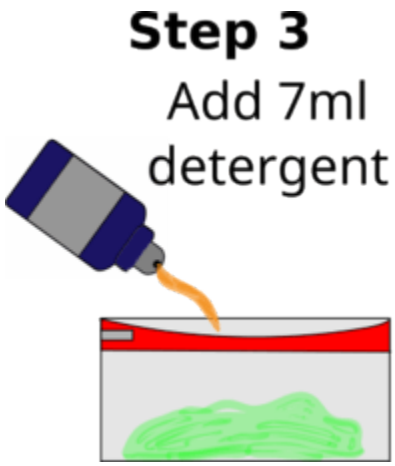
- Over-ripe banana is best since the cell walls are already decomposing
- Physical mashing continues to break up the cell walls

2. Add 7ml of salt solution.



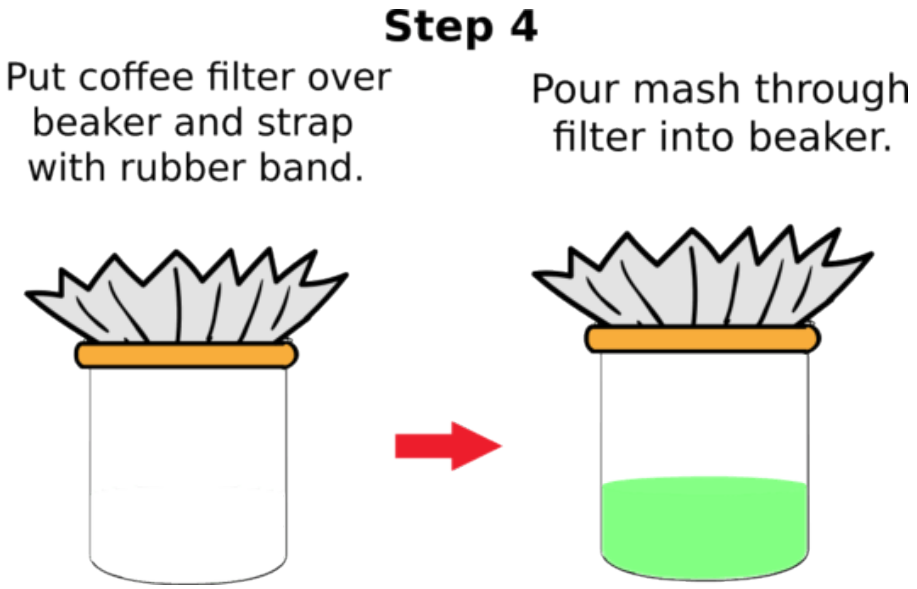
- The salt solution helps the DNA to aggregate (clump together).

3. Add 7ml of liquid detergent and mix.



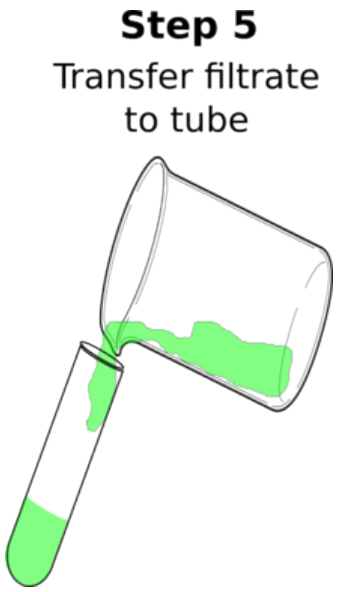
- Dissolves the lipids in the cell and nuclear membranes
- Releases DNA into the salt solution

4. Place a coffee filter over a cup or beaker and fasten with an elastic band.

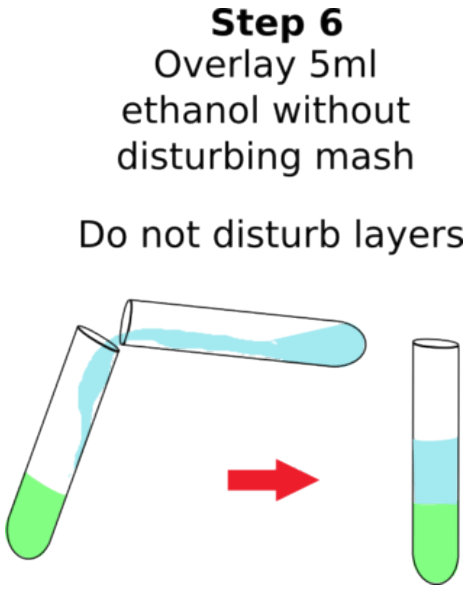


- Pour mash through the filter into a beaker

5. Pour about 5ml of filtrate into a test tube.

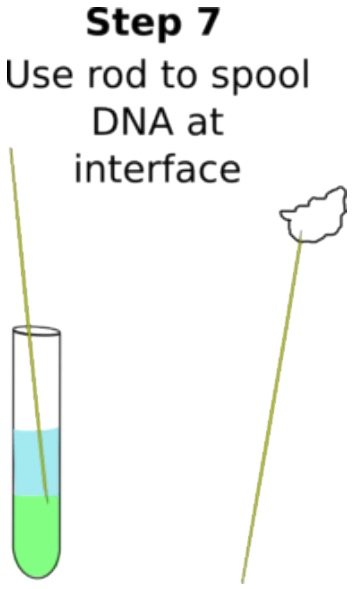


6. Slowly pour an **EQUAL** volume of cold ethanol down the side of the tube to form a layer on top of the fruit fluid.



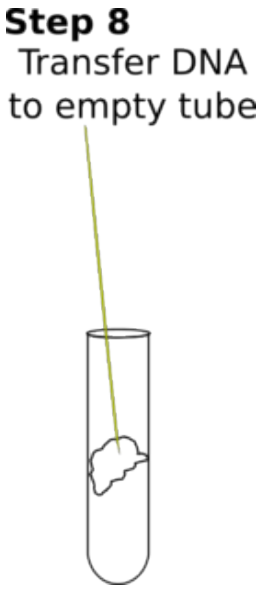
- Carefully run the alcohol down the side to form a separate layer on top of the fruit solution.
- Do not mix the alcohol and banana solution.
- Ice-cold 100% ethanol works best.

7. Spool the DNA: use a plastic loop or glass rod to gently swirl at the interface of the two solutions.



- The interface is where the two solutions meet.
- DNA is not soluble in alcohol.
- Bubbles may form around a wooly substance (this is the DNA).

8. Transfer the DNA.



Dische Diphenylamine Test For DNA

DNA can be identified chemically with the **Dische diphenylamine test**. Acidic conditions convert deoxyribose to a molecule that binds with diphenylamine to form a blue complex. The intensity of the blue color is proportional to the concentration of DNA. The Dische's Test will detect the deoxyribose of DNA and will not interact with the ribose in RNA. The amount of blue corresponds to the amount of DNA in solution.

File:Dische's test.svg

The diphenylamine compound of the Dische's test interacts with the deoxyribose of DNA to yield a blue coloration.

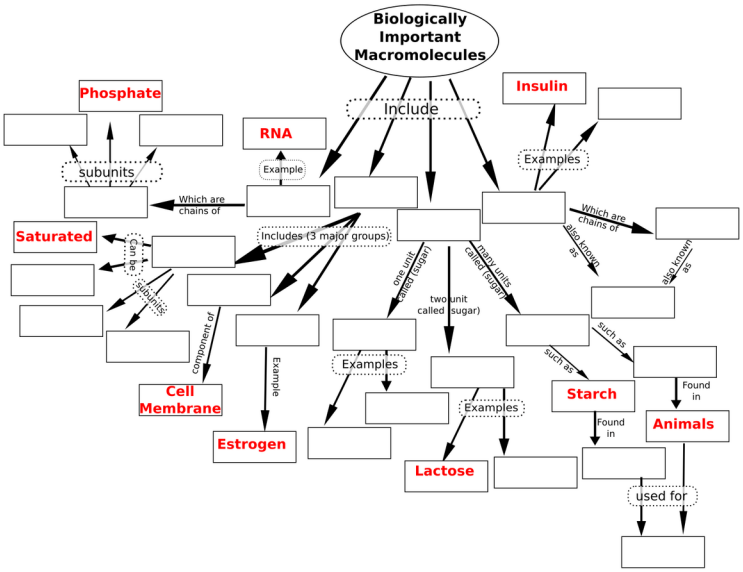
1. Obtain 3 test tubes and number them 1-3.
2. Suspend the spooled DNA in 3 ml of distilled water. MIX.
3. Add to tubes:
    1. 2 ml of DNA solution
    2. 1 ml of DNA solution with 1 ml H_2O
    3. 2 ml of H_2O
4. Add 2 ml of the Dische's diphenylamine reagent to each tube and mix thoroughly.
5. Place in a boiling water bath for 10 minutes.
6. Evaluate your results. A clear tube indicates no nucleic acids. A blue color indicates the presence of DNA. A greenish color indicates the presence of RNA.

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2.1.2.11: Biological Molecules (Concept)

Macromolecules Concept Map



Download the PDF.

Summarizing Macromolecule Detection:

File:US Nutritional Fact Label 2.svg

A nutrition label illustrates the breakdown of chemical components of Macaroni and Cheese. This is not limited to the macromolecules discussed here. Items like Iron and Sodium are ions that are important for the function of the cell.

Fill in the table below to illustrate how molecules in the above nutrition table were detected. What would you use as a Positive control? What would be the outcome of that Positive Control (color)? What about negative controls? Using the nutrition label above or a similar one, indicate what the test result would be if using the individual tests and indicate if that molecule is absent or present. Remember, if the sugars (simple reducing sugars) and the dietary fiber don't add up to the total carbohydrates, the remainder is starch.

Table 1. Determination of Biological Macromolecules in **Some Food Item**							
Test Name	**Test Purpose**	**Positive Control**		**Negative Control**		**Food Item**	
		Control substance	Color Result	Control substance	Color Result	Color Result	Present or Absent
Benedict's							
Iodine							
Biuret							
Oil Red O							

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2.1.3: QUANTITATIVE DETERMINATIONS

2.1.3.1: Introduction

2.1.3.2: Enzymes

2.1.3.3: Exploring Beer's Law (Virtual)

2.1.3.4: Quantitative Detection of Protein (Activity)

2.1.3.5: Quantitative Detection of Proteins (SpectroVis Plus)

2.1.3.6: Enzyme Kinetics (Activity)

2.1.3.7: Enzyme Kinetics with Spectrovis

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2.1.3.1: Introduction

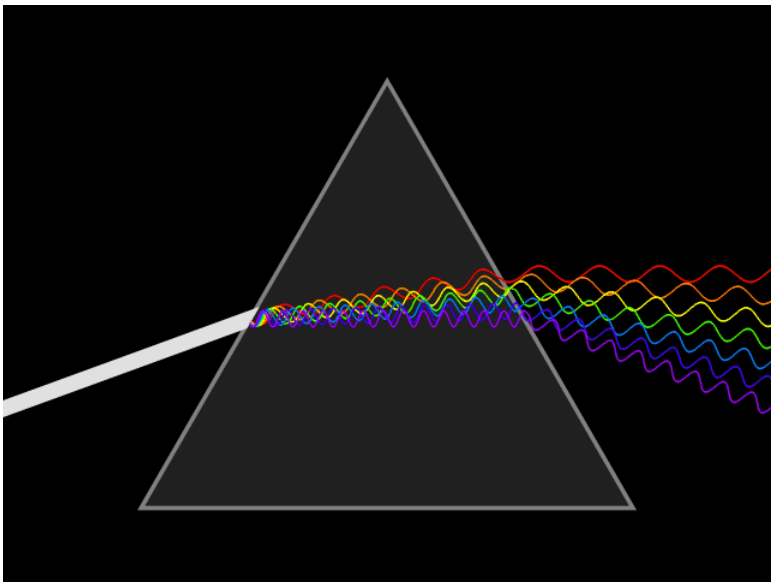
Properties of Light

File:EM Spectrum Properties reflected.svg

Credit: Inductiveload, NASA (CC-BY-SA 3.0)

Light is a type of energy that travels as a wave-particle. The **wavelength** of light is the distances between peaks in the waves as light travels. Wavelengths are measured in nanometers (nm) and different wavelengths of light represent differing colors. White light is a mixture of the visible light **spectrum**. Light of long wavelengths (infra-red) and very short wavelengths (ultraviolet) are invisible to humans but can be observed by other organisms. As the wavelength decreases, the energy of the light is increased.

Diffraction of light through a prism exposes the components wavelengths of light.



Spectrophotometry

Spectrophotometers (***spectro***-image/color; ***photo-****light****; meter***-measure) are used for chemical analysis of solutions based on properties of absorption or transmission.

File:Spetrophotometer-en.svg

Schematic of a spectrophotometer. The monochromator is a prism that splits the light. A single wavelength of light is focused through the aperture to pass through the solution in the cuvette. GYassineMrabetTalk [CC-BY-SA].

Transmittance refers to the amount of light that passes through the solution.

$$T = \frac{I}{I_0}$$

The transmittance of a light source through a cuvette. The intensity of light, $\mathbf{I_0}$, decreases as it passes through the solution. The light detected by the sensor, $\mathbf{I}$, reflects the transmittance of the solution.

If the light is being absorbed by chemicals in the solution, this results in a lower transmission. **Absorbance** is therefore inversely related to transmittance as expressed by the equation:

$$A = -\log_{10} T$$

Follow the virtual demonstration at http://www.virtual-labs.leeds.ac.uk/pres/spectrophotometry/ (CC-BY-NC-SA) for a more in-depth explanation of spectrophotometry.

Beer's Law

Beer's Law is a relationship between the concentration or amount of a dissolved substance in a solution that is reducing the amount of transmitted light due to the absorption of the radiant energy. **Lambert's Law** states that the reduction of transmittance was related to the length of the path of light. As the light path increases through a substance, there is a reduction in transmittance. Collectively, these ideas are referred to as **Beer-Lambert Law**, but most observers will control the path length and simply refer to it as Beer's Law.

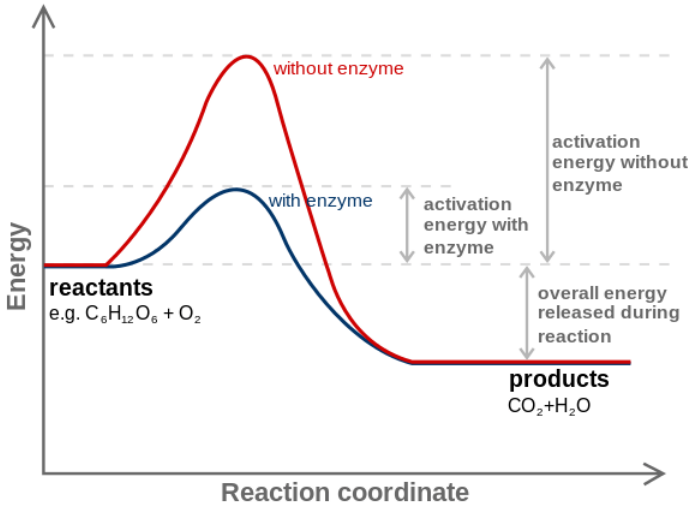
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2.1.3.2: Enzymes

Energy and Catalysts

In biological systems, **energy** is roughly defined as the capacity to do work. Molecules are held together by electrons. Breaking and building these bonds requires an input of energy. The energy needed to initiate such reactions is referred to as **activation energy** (***E_A***). Sometimes the necessary energy to initiate a reaction is so large that it greatly limits the likelihood of the reaction ever occurring. **Catalysts** are chemicals that take part in facilitating reactions by reducing the energy of activation. If the activation energy is reduced, the likelihood of a reaction occurring is greatly enhanced. In cells, the catalysts are often made of proteins and called **enzymes**.



*Reaction coordinate of an **exothermic** reaction with and without an enzyme. The enzyme reduced the **E_A** to facilitate the likelihood that the reaction occurs. This **catabolic** reaction breaks complex things down, thus increasing entropy and releasing energy into the system.*

Enzymes

Reactants in enzymatic reactions are called **substrates**. They have an imperfect fit to a binding domain of the enzyme called the **active site**. Substrate binding to this active site induces a change in the shape of the protein that coordinates the substrate into a transition state that will reduce the amount of E_A required for the reaction to go to completion. The **induced fit** of the protein also aids in coordinating other cofactors or **coenzymes** that will aid in the reaction.

File:Two substrates.svg

***Induced fit** model of enzymes and substrates. The active site of the protein is an imperfect match for the substrate. Intermolecular interactions between the enzyme and substrate induce a new fit that facilitates the formation of a **transition state** and results in the catalysis of the reaction.*

The reaction follows the standard flow where the Enzyme (E) and the Substrate (S) interact to form an Enzyme-Substrate Complex (ES). The ES then dissociates into Enzyme and the resultant Product (P).

$$E + S \Rightarrow ES \Rightarrow E + P$$

The induced fit of the enzyme-substrate complex coordinates the transition state to facilitate the reaction. This induced fit occurs

through non-covalent means that result in a tugging on the molecules (an application of energy) while molecules are coaxed into the reactions.

File:Hexokinase induced fit.svg

Hexokinase enzyme interacts with an ATP and a hexose. These interactions alter slightly the structure of the enzyme (induced fit). This pulling on the enzyme and the substrates aids in catalyzing the reaction through coordinating the molecules, sometimes with the aid of cofactors and coenzymes. The yellow sphere represents the cofactor Mg^{2+}.

Coenzymes can be covalently linked to amino acid side chains of the enzyme and are also referred to as **prosthetic groups**. While prosthetic groups are organic in nature, they may also involve the coordination of metal ions, like the heme group which binds to iron. These prosthetic groups enhance the repertoire of the amino acids to provide additional functioning to the entire protein. Early coenzymes were described as being vital to normal functioning and were characterized as organic molecules with amine groups. Because of this coincidence, they were referred to as **vitamins** (for *vital amine*s) though not all vitamins have amine groups. The trace metal ions that work with these groups are also required and represent the minerals on food items.

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2.1.3.3: Exploring Beer's Law (Virtual)

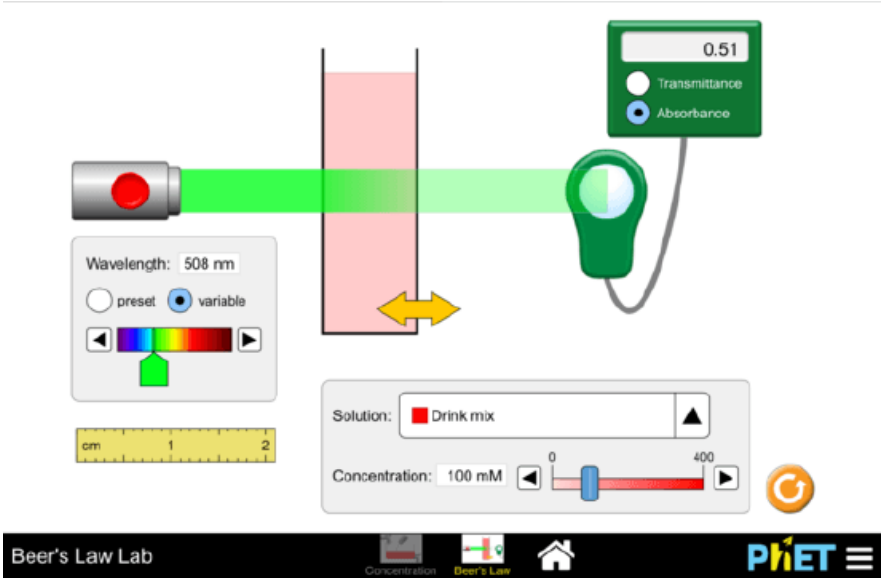
Use the link below to launch a simulation where you can alter the properties involved in spectrophotometry and examine the Beer-Lambert Law. Increase and decrease the concentration slider in the simulation:

1. What happens to the contents in the cuvette?
2. How does this change the Transmittance and Absorbance readings?
3. Click “variable” and use the slider. What happens to the readings when the Wavelength of the laser is a similar color as the solution in the cuvette?
4. Consult the color star below and find the color wavelength that is opposite of the color of the solution. Set the laser to this color using “variable” and the slider.

File:Color star-en.svg

1. What is the effect on Transmittance and Absorbance with this color?

2. Using the previous observations (using variable wavelength slider), how would you use the relationship on Transmittance/ Absorbance to best measure the concentration of a solution?



Click on image above to begin Beer’s Law Simulation

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2.1.3.4: Quantitative Detection of Protein (Activity)

Experimental Background

Bovine Serum Albumin (BSA) is a protein that circulates in the blood of cows. Purified BSA can be used with Biuret solution in serial dilutions to generate a **Standard Curve**. The standard curve will illustrate the relationship between concentration (the **dependent variable**) and absorbance at 540 nm (the **independent variable**). We can then use this curve to estimate the concentration of unknown samples.

1. On a graph, do you remember which axis is the dependent and which is the independent variable?

2. In the table below, can you identify which samples are the **negative controls** and which are the **positive controls**?

3. What is the prediction of the absorbance or color intensity of the different tubes?

Dilute BSA Standards

1. Label 9 tubes 1-9.
2. Combine the components of the table below to generate the appropriate concentration of solutions.

	Blank	BSA Standard Dilution						Unknowns	
	A 0 mg/ml	B 1 mg/ml	C 2 mg/ml	D 4 mg/ml	E 6 mg/ml	F 8 mg/ml	G 10 mg/ml	H ? mg/ml	I ? mg/ml
ml BSA	0	0.1	0.2	0.4	0.6	0.8	1	---	---
ml H2O	1	0.9	0.8	0.6	0.4	0.2	0	---	---
ml unknown	---	---	---	---	---	---	---	1	1
ml Biuret	4	4	4	4	4	4	4	4	4

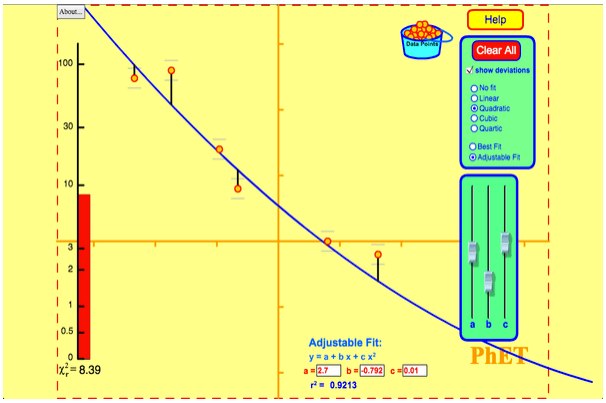
3. Place tube 1 (**blank**) into a cuvette and measure the absorbance (**A**) in the spectrophotometer at 540 nm.
4. Calibrate the spectrophotometer to read 0 at $\mathbf{A_{540nm}}$.
5. Sequentially read each sample at $\mathbf{A_{540nm}}$ and record values in the table below.

Tube #	BSA (mg/ml)	A_{540nm}
A	0	
B	1	
C	2	
D	4	
E	6	
F	8	
G	10	
Do NOT plot the values below. Use the plot to estimate the concentration of the samples.		
H		
I		

6. Plot each BSA dilution in a spreadsheet program like Excel as a scatterplot.
7. Generate best-fit line for these standards with the equation of the line.
8. Use the equation of the line to estimate the concentration of the unknown sample.

Curve Fitting

Run the simulation below to understand how you can use the standard dilution series to estimate your sample concentrations.



Click on the image above to begin simulation on curve fitting.

Scatterplot Tutorial

Use the tutorial below and watch at 1.25X to plot your own data.

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2.1.3.5: Quantitative Detection of Proteins (SpectroVis Plus)

Experimental Background

Bovine Serum Albumin (BSA) is a protein that circulates in the blood of cows. Purified BSA can be used with Biuret solution in serial dilutions to generate a **Standard Curve**. The standard curve will illustrate the relationship between concentration (the **dependent variable**) and absorbance at 540 nm (the **independent variable**). We can then use this curve to estimate the concentration of unknown samples.

1. On a graph, do you remember which axis is the dependent and which is the independent variable?

2. In the table below, can you identify which samples are the **negative controls** and which are the **positive controls**?

3. What is the prediction of the absorbance or color intensity of the different tubes?

Dilute BSA Standards

1. Label 9 tubes 1-9
2. Combine the components of the table below to generate the appropriate concentration of solutions

	Blank	BSA Standard Dilution						Unknowns	
	A 0 mg/ml	B 1 mg/ml	C 2 mg/ml	D 4 mg/ml	E 6 mg/ml	F 8 mg/ml	G 10 mg/ml	H ? mg/ml	I ? mg/ml
ml BSA	0	0.1	0.2	0.4	0.6	0.8	1	---	---
ml H2O	1	0.9	0.8	0.6	0.4	0.2	0	---	---
ml unknown	---	---	---	---	---	---	---	1	1
ml Biuret	4	4	4	4	4	4	4	4	4

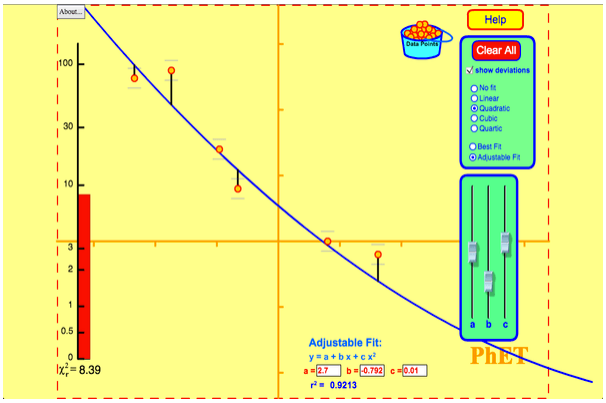
3. The instructor will begin to set-up the units for distribution.
    1. Connect SpectroVis Plus to LabQuest2.
    2. Power on.
    3. Select the LabQuest App.
    4. On the “Meter” tab, tap on **Mode.**
        1. Change the mode to “**Events with Entry**“.
        2. Enter the Name: Concentration.
        3. Enter Units: mg/ml.
        4. Select “OK”.
        5. If a message appears about saving run, choose Discard.
        6. Tap on the red area showing Absorbance to trigger menu items
            1. Select “**Change Wavelength**“.
            2. Enter 540.
        7. Tap on the red area showing Absorbance to trigger menu items.
            1. Select “Calibrate”.
            2. Select “Warm Up”.
            3. Select Finish Calibration.
4. Place tube 1 (**0 mg/ml**) into a cuvette for measuring the absorbance (**A**) in the SpectroVis Plus.
    - Tap on the file cabinet icon to store this data.
5. Sequentially read each sample at the stored wavelength ($\mathbf{A_{540nm}}$)and record values in the table below.

Tube #	BSA (mg/ml)	A_{540nm}
A	0	
B	1	
C	2	
D	4	
E	6	
F	8	
G	10	
Do NOT plot the values below. Use the plot to estimate the concentration of the samples.		
H		
I		

1. Plot each BSA dilution in plot.ly as a scatterplot (Log-on using Facebook/Google/Twitter credentials for free).
2. Generate best-fit line for these standards with the equation of the line.
3. Use the equation of the line to estimate the concentration of the unknown sample.

Curve Fitting

Run the simulation below to understand how you can use the standard dilution series to estimate your sample concentrations.



Click on the image above to begin simulation on curve fitting.

LabQuest2 and SpectroVis Tutorial

Scatterplot Tutorial

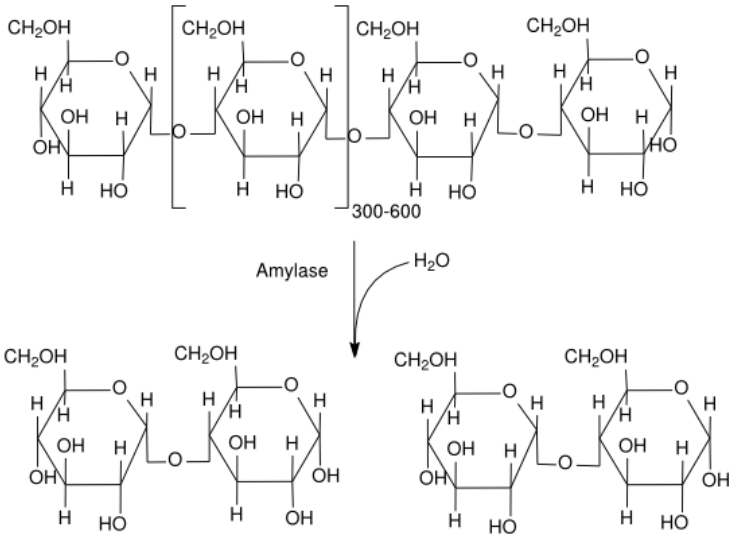
You can watch this tutorial at 1.25X and pause when needed.

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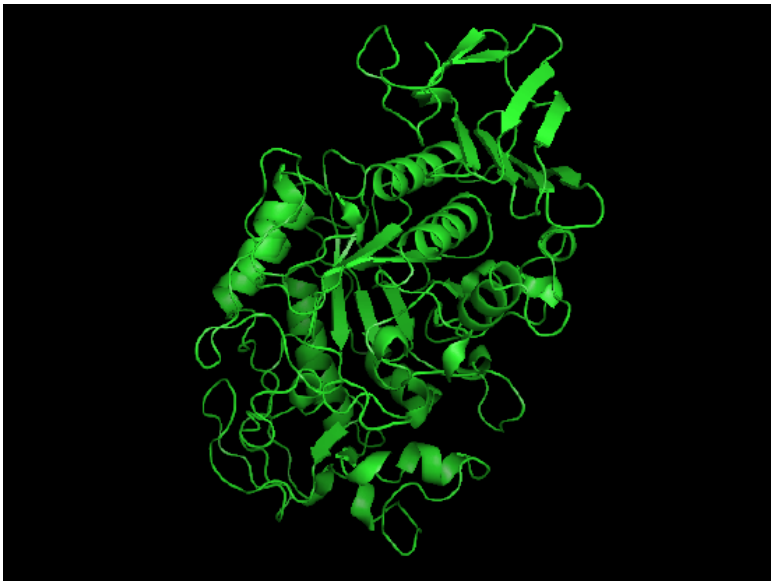
2.1.3.6: Enzyme Kinetics (Activity)

The Enzyme

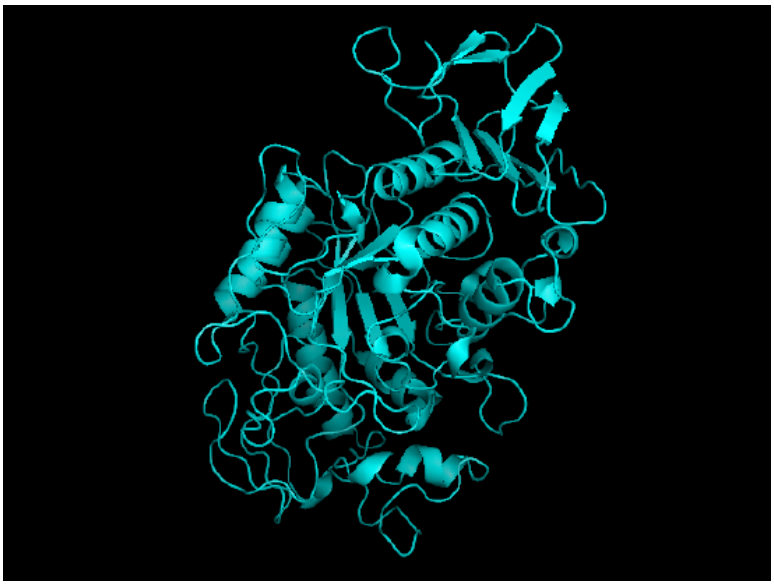


Amylase is an enzyme that breaks down amylose (starch) into glucose molecules.

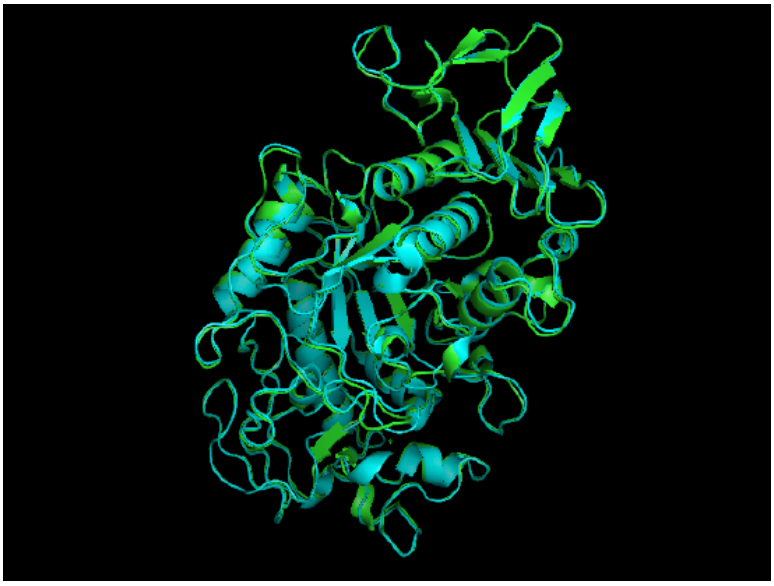
1. What test can be used to indicate the presence of Starch?
2. What parameters would influence the ability of the enzyme to facilitate the rate of the reaction?
3. What is the role of an enzyme in a chemical reaction and what is it made of?
4. What test can be used to indicate the presence of glucose?



Salivary amylase is produced in the mouth, where digestion begins.



Pancreatic amylase is produced in the pancreas and is supplied to the duodenum of the small intestines.



*Overlay of salivary (**green**) and pancreatic (**teal**) amylase molecules.*

Effect of Temperature on Enzyme Activity

1. Add 5 ml of H_2O to a tube (this is the BLANK).
2. Add 5 ml of starch (substrate) to 3 separate tubes.
   - One on ice (0°C), one on the bench (25°C), and one in a 40°C water bath
3. Add 2 drops of iodine to each tube and mix: Blank, 0°C, 25°C, and 40°C.
4. Read the Blank in the spectrophotometer and calibrate it to 100% transmittance at 560nm.
5. Read each tube in the spectrophotometer. This is time 0 minute.
6. Add 35 drops of amylase solution to each tube simultaneously. Mix and ensure incubation is occurring at the correct temperature.
7. At 2 minute intervals, quickly read **ALL** tubes in the spectrophotometer and immediately return the tubes to the proper temperature.
8. Continue reading the samples every 2 minutes until you reach 22 minutes on the table below.

Time (min)	0°C % Trans	25°C % Trans	40°C % Trans
0			
2			
4			
6			
8			
10			
12			
14			
16			
18			
20			
22			

Effect of pH on Enzyme Activity

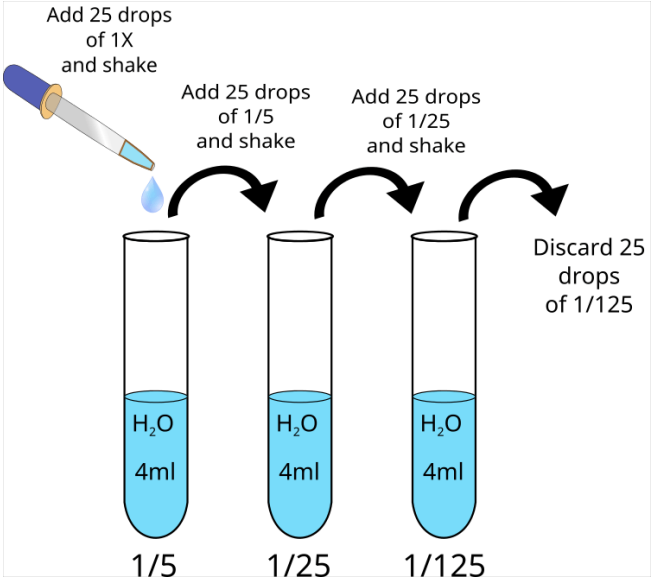
1. Add 5 ml of water to an empty tube (this is the BLANK)
2. To 3 separate tubes, add 2.5 ml of buffer pH 3, pH 5 or pH 7
    1. Add 2.5 ml of starch (substrate) to each of these tubes (excluding BLANK)
3. Add 2 drops of iodine to each tube and mix: Blank, pH 3, pH 5, pH 7
4. Read the Blank in the spectrophotometer and calibrate it to 100% transmittance at 560nm
5. Read each tube in the spectrophotometer. This is time 0 min.
6. Add 35 drops of amylase solution to each tube simultaneously, mix to homogeneity.
7. At 2 minute intervals, quickly read **ALL** tubes in the spectrophotometer.

8. Continue reading the samples every 2 minutes until you reach 22 minutes on the table below.

Time (min)	pH 3 % Trans	pH 5 % Trans	pH 7 % Trans
0			
2			
4			
6			
8			
10			
12			
14			
16			
18			
20			
22			

Effect of Enzyme Concentration on Enzyme Activity

1. Add 5 ml of water to an empty tube (this is the BLANK)
2. Add 5 ml of pH 7 buffer to 3 separate tubes.
3. Follow the dilution scheme below:



Dilution scheme for amylase (enzyme)

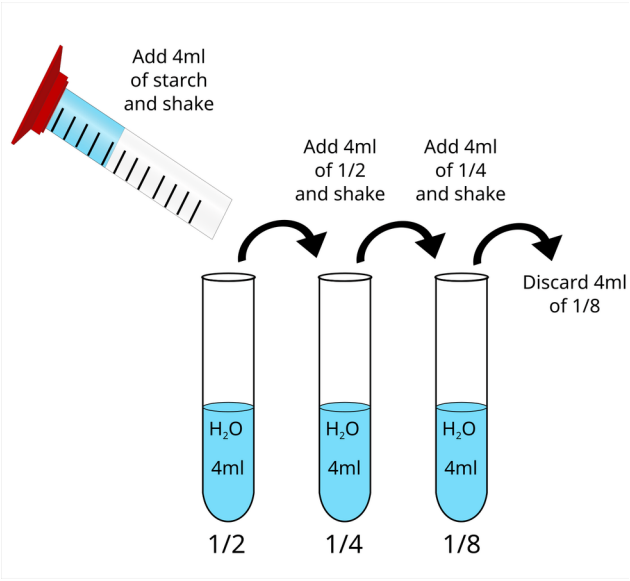
4. In 4 separate tubes, ADD 4 ml of starch solution.
   - Label them 1x, 1/5x, 1/25x, 1/125x
5. Add 2 drops of iodine to each starch tube and the Blank.
6. Read the Blank in the spectrophotometer and calibrate it to 100% transmittance at 560nm.
7. Read each tube in the spectrophotometer. This is time 0 minute.
8. Add 35 drops of diluted amylase solutions to the appropriately labeled tubes simultaneously and mix.
   - Each tube receives a different Amylase dilution.
9. At 2 minute intervals, quickly read **ALL** tubes in the spectrophotometer.
10. Continue reading the samples every 2 minutes until you reach 22 minutes on the table below.

Time (min)	1 X % Trans	1/5 X % Trans	1/25 X % Trans	1/125 X % Trans
0				
2				
4				
6				
8				
10				

12				
14				
16				
18				
20				
22				

Effect of Substrate Concentration on Enzyme Activity

1. Add 5 ml of water to an empty tube (this is the BLANK)
2. Add 5 ml of pH 7 buffer to 3 separate tubes
3. Follow the dilution scheme below:



4. In 4 cuvettes, ADD 4 ml of the diluted starch solution.
   - Label them 1x, 1/2x, 1/4x, 1/8x.
5. Add 2 drops of iodine to each starch tube and the Blank.
6. Read the Blank in the spectrophotometer and calibrate it to 100% transmittance at 560nm.
7. Read each tube in the spectrophotometer. This is time 0 minute.
8. Add 35 drops of amylase solution to each tube simultaneously and mix.
9. At 2 minute intervals, quickly read **ALL** tubes in the spectrophotometer.
10. Continue reading the samples every 2 minutes until you reach 22 minutes on the table below.

Time (min)	1 X % Trans	1/2 X % Trans	1/4 X % Trans	1/8 X % Trans
0				
2				
4				
6				
8				
10				
12				
14				
16				
18				
20				
22				

Plot the Results

1. Using a plot.ly, plot the data on the same chart.
2. Calculate the line of best fit of each dataset.
3. The slope represents the activity of the enzyme in each condition. What is the unit of this activity?

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2.1.3.7: Enzyme Kinetics with Spectrovis

Enzyme Kinetics of Turnip Peroxidase

Hydrogen peroxide (H_2O_2) is a strong oxidizing agent that can damage cells and is formed as a by-product of oxygen consumption. Fortunately, aerobic cells contain peroxidases that break down peroxide into water and oxygen. This enzyme reduces hydrogen peroxide into H_2O by oxidizing an organic compound (*AH_2 -> A*).

$$H_2O_2 + AH_2 \xrightarrow{\text{peroxidase}} 2H_2O + A$$

This exercise uses turnip extract as a source of peroxidase. This turnip extract requires a source of electrons (a reducing agent) in order for the reaction to occur. In this case, a colorless organic compound called **guaiacol** is used. Guaiacol is oxidized in the process of converting the peroxide and becomes brown. The enzymatic activity can then be traced using a spectrophotometer to measure the amount of brown being formed.

Set-up and Calibrate LabQuest with SpectroVis Plus

1. Set-up the cuvette to serve as a Blank.
    1. Add 10 drops 0.02% hydrogen peroxide.
    2. Add 5 drops 0.2% guaiacol.
    3. Add 20 drops of pH 7 buffer.
    4. Add 10 drops extraction buffered.
2. Connect a LabQuest2 to a SpectroVis plus and start the data collection program.
3. Calibrate the SpectroVis.
    1. Change Mode.
        1. Select “Time-Based” from the dropdown menu and press OK when done.

    - Rate: 0.5 samples/s
    - Interval: 2 s/sample
    - Duration: 200 s

2. Press the Play button (green arrow).

    1. Perform Warm-up for 90 seconds.

        1. Choose "Finish Calibration".

        2. Press "OK".

    2. Press the "Stop" button (Red Square).

4. Select “Meter Icon” (top left most of the display).

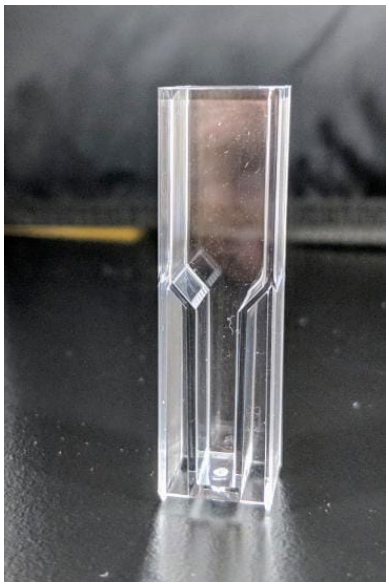
    1. Press the large red area showing the current absorbance reading.
    2. Choose “Change Wavelength”.
    3. Set Wavelength to 500nm.

Effect of pH on Peroxidase Activity

1. Set-up the cuvette for pH 3.
    1. Add 10 drops 0.02% hydrogen peroxide.
    2. Add 5 drops 0.2% guaiacol.
    3. Add 20 drops of pH 3 buffer.

4. Add 10 drops turnip extraction last.
5. Quickly invert the cuvette and place the cuvette into the Spectrovis Plus.
6. Press play button to begin recording data (choose “discard data” if prompted).
    1. After 200s, remove SpectroVis from USB.
    2. Insert a USB flash drive and wait 1 minute.
    3. Press File → Export → choose USB icon and rename the file and add “.csv” to the end of the file name.
    4. Press OK.

2. Sequentially repeat the experiment exchanging the pH buffer with pH 5, 7, 10.



Effect of Temperature on Peroxidase Activity

1. Set-up the cuvette for 0°C
    1. Add 10 drops 0.02% hydrogen peroxide (incubated at 0°C for 10 minutes).
    2. Add 5 drops 0.2% guaiacol.
    3. Add 20 drops of extraction buffer at 0°C.
    4. Add 10 drops turnip extraction (incubated at 0°C for 10 minutes) last.
    5. Quickly invert the cuvette and place the cuvette into the Spectrovis Plus.
    6. Press play button to begin recording data (choose “discard data” if prompted).
        1. After 200s, remove SpectroVis from USB.
        2. Insert a USB flash drive and wait 1 minute.
        3. Press File → Export → choose USB icon and rename the file and add “.csv” to the end of the file name.
        4. Press OK.
2. Sequentially repeat the experiment exchanging the buffers stored at 20°C, 40°C, 60°C.

Effect of Substrate Concentration on Peroxidase Activity

1. Set-up the cuvette for 1X substrate.
    1. Add 10 drops 0.02% hydrogen peroxide.
    2. Add 5 drops 0.2% guaiacol.
    3. Add 20 drops of extraction buffer.
    4. Add 10 drops turnip extraction last.
    5. Quickly invert the cuvette and place the cuvette into the Spectrovis Plus.
    6. Press play button to begin recording data (choose “discard data” if prompted).
        1. After 200s, remove SpectroVis from USB.
        2. Insert a USB flash drive and wait 1 minute.
        3. Press File → Export → choose USB icon and rename the file and add “.csv” to the end of the file name.
        4. Press OK.

2. Sequentially repeat the experiment with different amounts of buffer and peroxide:
    1. Repeat the experiment with 0.2X substrate and export data to a USB drive.
    2. Repeat the experiment with 2X substrate and export data to a USB drive.
    3. Repeat the experiment with 3X substrate and export data to a USB drive.

alt

Effect of Enzyme Concentration on Peroxidase Activity

1. Set-up the cuvette for 1X enzyme.
    1. Add 10 drops 0.02% hydrogen peroxide.
    2. Add 5 drops 0.2% guaiacol.
    3. Add 20 drops of extraction buffer.
    4. Add 10 drops turnip extraction last.
    5. Quickly invert the cuvette and place the cuvette into the Spectrovis Plus.
    6. Press play button to begin recording data (choose “discard data” if prompted).
        1. After 200s, remove SpectroVis from USB.
        2. Insert a USB flash drive and wait 1 minute.
        3. Press File → Export → choose USB icon and rename the file and add “.csv” to the end of the file name.
        4. Press OK.
2. Sequentially repeat the experiment with different amounts of buffer and extract:
    1. Perform the experiment on 1X enzyme and export data to a USB drive.
    2. Repeat the experiment with 0.2X enzyme and export data to a USB drive.
    3. Repeat the experiment with 2X enzyme and export data to a USB drive.
    4. Repeat the experiment with 3X enzyme and export data to a USB drive.

	0.2X enzyme	1X enzyme	2X enzyme	3X enzyme
Drops Peroxide	10	**10**	10	10
Drops Guaiacol	5	**5**	5	5
Drops Extraction Buffer	28	**20**	10	0
Drops Extract	2	**10**	20	30

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2.1.4: OSMOSIS AND DIFFUSION

2.1.4.1: Introduction

2.1.4.2: Why Are Cells Small? (Activity)

2.1.4.3: Do Larger Things Diffuse Faster? (Activity)

2.1.4.4: Gummy Bears- Tonicity (Activity)

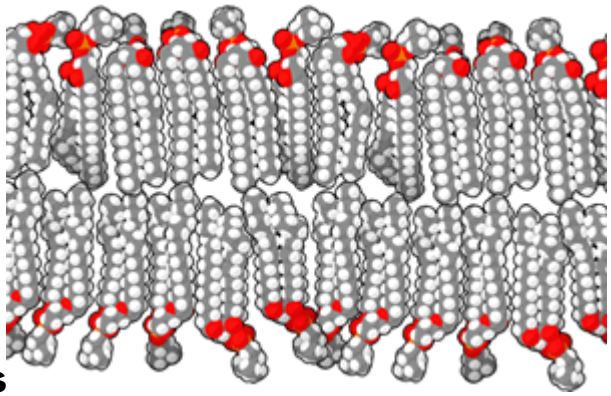
2.1.4.5: Observing Osmosis (Activity)

2.1.4.6: Agar Cubes (Preparation)

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2.1.4.1: Introduction



Understanding Membranes

The cell membrane is the barrier that separates the cytoplasm from the external world. The cell membrane consists primarily of phospholipids in a bilayer. Phospholipids are amphipathic with a polar head (phosphate group) and a hydrophobic tail (2 hydrocarbon chains). Due to the chemical properties of the heads being attracted to water and the tails having a desire to avoid water, phospholipids self-assemble into micelles. Cell membranes form from a phospholipid bilayer where the lipid tails interact with each other and the phosphate heads face the external water environment or the internal cytoplasm of the cell.
The cell membrane does not solely consist of phospholipids but also have proteins and cholesterol inserted into the bilayer. As the image of the bilayer above indicates, the molecules are constantly moving and flow in a lateral motion. Cholesterol modulates the fluidity of this motion. Proteins associated with the membrane may sit on either side (**peripheral proteins**) of the membrane or pass through both layers of the membrane (**transmembrane proteins**). The model that describes the components of the cellular membrane is referred to as the **Fluid Mosaic Model**. This model states that the cell membrane is a mosaic of 1) Phospholipids 2) Proteins 3) Cholesterol that moves about in a side to side motion.

File:Cell membrane detailed diagram en.svg

The fluid mosaic of phospholipids, proteins, and cholesterol that create the selective barrier between the interior and the exterior of the cell.

Small uncharged molecules pass through the double layer of phospholipids. Polar, charged or large molecules have great difficulty passing through the membrane and require the aid of transmembrane proteins. An example of a transmembrane protein that facilitates the movement of a polar substance is aquaporin, which permits the free movement of water.

File:Blausen 0213 CellularDiffusion.png

Diffusion

Diffusion is the net movement of a substance from high concentration to low concentration. This difference in concentration is referred to as a **concentration gradient**. This movement does not require any external energy but uses the free energy intrinsic to the system.

- Temperature Effects on Diffusion
  - Temperature/Diffusion Simulation
- Molecular Mass Effects on Diffusion
  - Size/Mass/Diffusion Simulation

File:Blausen 0315 Diffusion.png

Concentrated dye diffuses along the concentration gradient until reaching equilibrium (no net movement).

Osmosis

Osmosis is a special case of diffusion. Instead of observing the net change in solute, osmosis follows the net movement of a solvent across a **semipermeable membrane**. Since a semi-permeable membrane permits specific things to pass through, some solutes are partitioned.

- Simulation of diffusion through a semipermeable membrane (CC-BY 4.0 Concord Consortium)

File:Osmose en.svg

A semi-permeable membrane allows the solvent to pass but not this red salt molecule. The water moves along the concentration gradient (of water) . This movement of water causes an osmotic pressure.

A cell lacking a cell wall is affected greatly by the tonicity of the environment. In a **hypertonic** solution where the concentration of dissolved solute is high, water will be drawn out of the cell. In a **hypotonic** solution where the concentration of dissolved solute is lower than the interior of the cell, the cell will be under great **osmotic pressure** from the environmental water moving in and can rupture.

File:Osmotic pressure on blood cells diagram.svg

Plants have rigid cell walls composed of cellulose. These cell walls permit for maintenance of cellular integrity when the external environment is **hypotonic** (less dissolved substances). In this situation, the water moves into the cell. Without the cell wall, the cell would burst open from the excessive water pressure entering the cell. This state of swelling is referred to as turgid, resulting from **turgor pressure**.

File:Turgor pressure on plant cells diagram.svg

Cell walls of a plant retain the shape of the cell despite the state of external tonicity.

When the exterior environment is **hypertonic** (greater amount of dissolved substances), the reverse condition occurs whereby the cellular fluid exiting the cell reduces the size of the cytoplasm. This condition is referred to as **plasmolysis**.

Active Transport Mechanisms

File:Endocytosis types.svg

File:Phagocytosis.svg

File:Scheme sodium-potassium pump-en.svg

Further Reading

- http://www.visionlearning.com/en/library/Biology/2/Membranes-I/198/reading
- http://www.visionlearning.com/en/library/Biology/2/Membranes-II/204
- http://www.biologycorner.com/bio1/notes_diffusion.html (CC-BY-NC)

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2.1.4.2: Why Are Cells Small? (Activity)

Introduction

1. Take 3 blocks of agar of different size (1cm, 2cm, 3cm) → these are our cell models.
2. Measure the length, width, and height of each cube using a ruler.
3. Calculate the area of each face of the cubes and add all the areas together for a single cube.

- A cube has 6 faces → the total surface area is the same as the area of one side multiplied by 6.

4. Calculate the volume of each cube.
5. Report the surface area-to-volume in the table below.

Data Table: Calculating Surface Area-to-Volume Ratio

Cell Model (Cube)	Length	Width	Height	Total Surf
1				
2				
3				

Stop and Think

- Which cube has the greatest surface area:volume ratio?
- Which cube has the smallest surface area:volume ratio?
- **Hypothesize**: In an osmosis or diffusion experiment, which cube size would have the greatest diffusion rate?

Procedures

1. Each group will acquire three agar cubes: A 3cm cube, a 2cm cube, and a 1cm cube. **CUT AS ACCURATELY AS POSSIBLE**. *(This may be already completed for you.)*
2. Place cubes into a beaker and submerge with 200 ml NaOH.
3. Let the cubes soak for approximately 10 minutes.
4. Periodically, gently stir the solution, or turn the cubes over.
5. After 10 minutes, remove the NaOH solution.
6. Blot the cubes with a paper towel.
7. Promptly cut each cube in half and measure the depth to which the pink color has penetrated. Sketch each block's cross-section.
8. Record the volume that has remained white in color.
9. Do the following calculations for each cube and complete the following data table:

Data Table: Calculation of Diffusion Area-to-Volume

Cube Size	Cube volume (cm^3) (V_{total})	Volume white (cm^3) (V_{white})	Sketch of each Cube	Volume of the diffused cube ($V_{total} - V_{white}$) = ($V_{diffused}$)	Percent Diffusion ($V_{diffused}/V_{total}$)	Surface Area: Volume (from the previous table)
1 cm.						
2 cm.						
3 cm.						

Conclude

1. Which cube had the greatest percentage of diffusion?

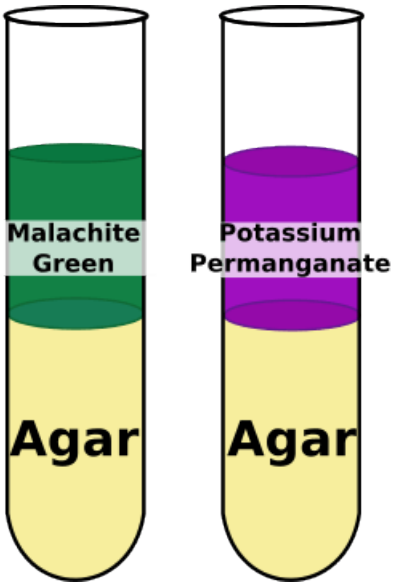
2. Did this meet your expectations with your hypothesis?

3. If you designed a large cell, would it be a large sphere or something long and flat?

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2.1.4.3: Do Larger Things Diffuse Faster? (Activity)



Introduction

Agar is a gelatinous substance derived from a structural carbohydrate found in seaweed. It is often used in cooking as a vegetarian alternative to gelatin and can be used as a thickener. Microbiologists pour plates of agar containing nutrients in order to isolate and grow bacteria and other microbes. As with gelatin, the long fibery nature of this structural carbohydrate permits it to be melted and tangled together in a mesh-like network where the spaces between molecules are filled with solution. Altering the amount of fluid solution will change the pores between fibers. More fluid will create a looser gel that has larger spaces between molecules. Reducing the fluid solution volume will conversely create a stiffer gel with smaller spaces between fibers.

1. Take 2 tubes of agar and a solution of Malachite green (365 g/mole) and a solution of Potassium permanganate (164 g/mole).
2. Mark the top of the agar on the outside of the tube (the starting point).
3. Add 10 drops of malachite green to one tube and 10 drops of Potassium permanganate to the other.
4. Take note of the time.
5. At 20 minute intervals, measure the distance from the top that the agar has moved. Do this for at least 1 hour.
6. Plot the data and compare the trends. Describe the rate of diffusion for each.

Stop and Think

- **Hypothesize** which solution will move faster through the agar and provide a reason.

	DIFFUSION SPEED OF DYE MOLE
DYE	**MOLECULAR WEIGHT**
Malachite Green	365 g/mole
Potassium Permanganate	164 g/mole

Conclude

- Which solution actually moved faster?_____
- Did this meet your expectations?_____
- Propose a reason why a certain dye moved faster._____
- **Review through simulation**

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2.1.4.4: Gummy Bears- Tonicity (Activity)

Introduction

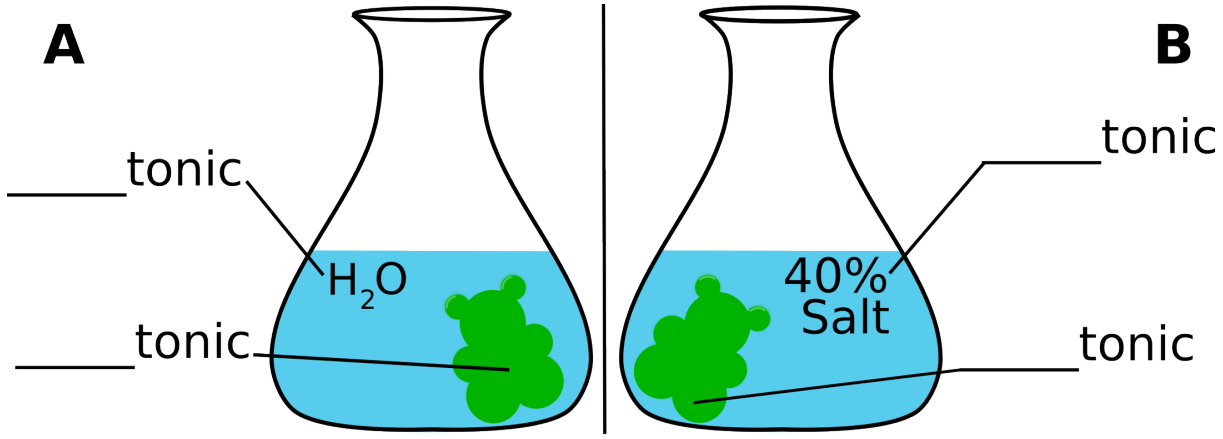
We're all familiar with gelatin (like the Jello brand). Gummy candies are made of gelatin. Gelatin is a protein that exists as long fibers. When gelatin is dissolved in a liquid and cooled, the gelatin fibers tangle together in a mesh-like network. The space in between the gelatin molecules is filled with the fluid it was dissolved in. Gummy candies are considerably more firm than the gelatin molds we have as desserts because they contain a lot less fluid. Nonetheless, gummy candies are filled with a sugary solution with coloring. Like a cell, a gummy candy placed in solution will be affected by the properties of osmosis when submerged in different solutions.

Stop and Think

- Is distilled water hypertonic, hypotonic, or isotonic compared to the sugar solution inside a gummy candy?
- Based on your answer, hypothesize if a gummy candy submerged in distilled water or 40% salt solution will swell faster? Label the diagram below with your hypothesis.

Procedures

1. Obtain 2 gummy bears and place them in 2 different small flasks.
2. Drown 1 bear in distilled water and drown the other in 40% salt solution.
3. At the end of the lab session, remove the bears from the solution and document the size difference with your mobile phone.



Hypothesized swelling of the bear based on tonicity

Condition	Tonicity Inside Bear Relative to the Solution	Outside
A		
B		

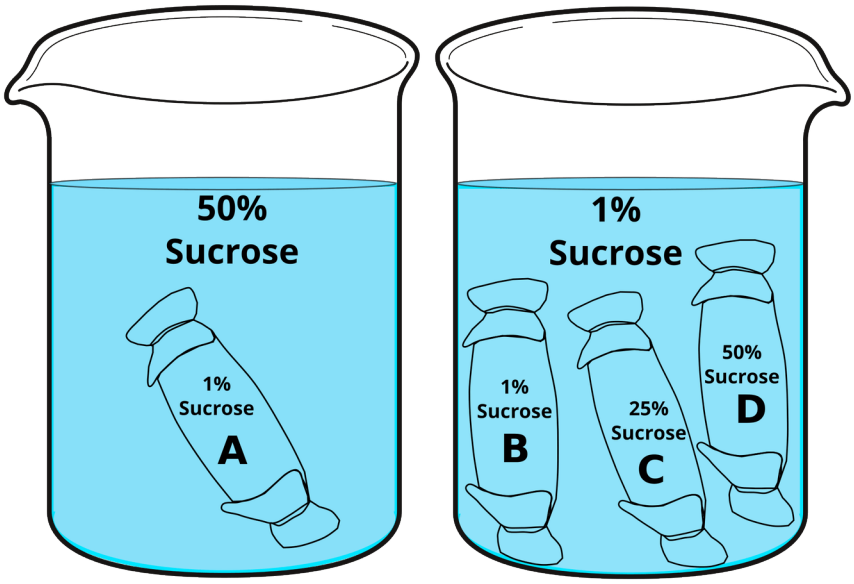
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2.1.4.5: Observing Osmosis (Activity)

Observe Osmosis Along A Free Energy Gradient

1. Obtain four pieces of water-soaked dialysis tubing 15 cm long and eight pieces of string. Seal one end of each tube by tying it into a knot.
2. Open the other end of the tube by rolling it between your thumb and finger.
    1. Write A, B, C, D on 4 pieces of paper.
    2. Insert the labels into individual bags.
3. Fill the bags with the contents shown in the figure below with 10 ml of solution.
    A. **Bag A** 10 ml 1% Sucrose
    B. **Bag B** 10 ml 1% Sucrose
    C. **Bag C** 10 ml 25% Sucrose
    D. **Bag D** 10 ml 50% Sucrose
4. For each bag, loosely fold the open end and press on the sides to push the fluid up slightly and remove most of the air bubbles. Tie the folded ends securely, rinse the bags, and check for leaks.
5. Blot excess water from the outside of the bags and weigh each bag to the nearest 0.1 gram.
6. Record the weights in **Data Table 1: Weight of Dialysis Bags**.
7. Place bags B, C, and D in a beaker or large bowl filled with 1% sucrose. Record the time.
8. Place bag A in an empty beaker and fill the beaker with just enough 50% sucrose to cover the bag. Record the time.
9. Remove the bags from the beakers at 10-minute intervals for the next hour, blot them dry, and weigh them to the nearest 0.1 g. Handle the bags delicately to avoid leaks, and quickly return the bags to their respective containers. For each 10-minute interval record the total weight of each bag and its contents in **Data Table 1**. Then calculate and record in **Data table 2: Change in Weight of Dialysis Bags** the change in weight from the initial weight.



Stop and Think

- Define the tonicity of the solution inside the bag relative to the outside
- Based on your definitions, **hypothesize** the direction the solution will move (in or out of the bag) and fill in the table below

Hypothesized Movement of Solution based on Tonicity

Bag	Tonicity Inside Bag Relative to the Solution	Tonicity of Outside Solution relative to th
A		
B		
C		
D		

Data Table 1: Weight of Dialysis Bags

Bag	0 Min	10 Min	20 Min	30 Min	40 Min	50 Min	60 Min
A							
B							
C							
D							

Data Table 2: Net Weight Change of Dialysis Bags

Bag	0 Min (WT_0-WT_0)	10 Min (WT_{10}-WT_0)	20 Min (WT_{20}-WT_0)	30 Min (WT_{30}-WT_0)	40 Min (WT_{40}-WT_0)	50 Min (WT_{50}-WT_0)	60 Min (WT_{60}-WT_0)
A	**0**						
B	**0**						
C	**0**						
D	**0**						

Present Your Data

- Plot your data using only the Change in Weight. (subtract the Initial Weight at 0 minutes from the Total Weight at each time point)
  - Using a computer, create a scatter plot of the data from Table 2 and calculate the equation of the line
- Change in Weight = Total $Weight_{(current\_time)}$ – Initial $Weight_{(time\_0)}$

Conclude

- Did your results match your hypotheses?_____
- What do the slopes of the lines generated from plotting Change Weight indicate to you?_____
- Can you analyze and articulate in words what has occurred with respect to these slopes?_____
  _____
  _____
  _____

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2.1.4.6: Agar Cubes (Preparation)

Procedures

1. Prepare a 2% solution of agar.
   - Mix 20 g of agar with 1 L of distilled water.
2. Heat almost to a boil. Stir frequently until the solution is clear.
3. Remove from heat and add 10 mL of 1% phenolphthalein solution.
   - 1 g phenolphthalein in 100 mL 95% ethanol (If pink, titrate with HCl until clear.)
4. Pour agar into a shallow tray to a depth of 3 cm and allow it to set (overnight).

Silicone Ice-cube trays can be used. It is difficult to find them in the appropriate metric measurements. 3 cm cubes are approximately 1.2 inches and there are molds for this.

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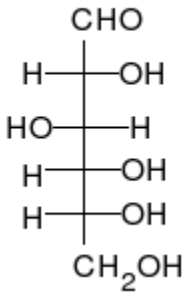
2.1.5: CELLULAR RESPIRATION

2.1.5.1: Introduction
2.1.5.2: Fermentation
2.1.5.3: Cell Respiration (Concept)

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2.1.5.1: Introduction

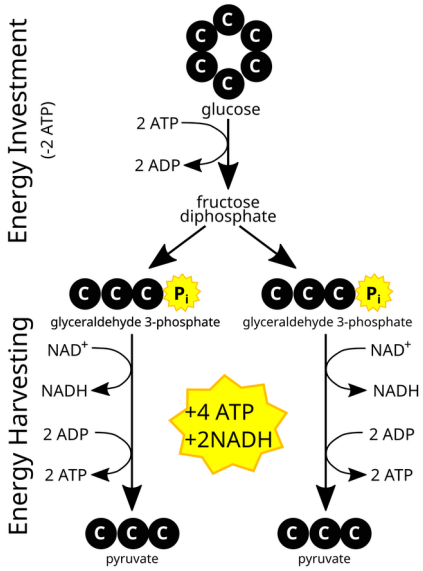


Glucose is the preferred carbohydrate of cells. In solution, it can change from a linear chain to a ring.

Energy is stored in the bonds of the carbohydrates. Breaking these bonds releases that energy. Crushing sugar crystals creates tiny electrical fields that give off invisible ultraviolet light. The wintergreen chemical (methyl salicylate) gets excited by these excited electrons and fluoresces in a visible blue wavelength. This phenomenon is called triboluminescence.

Glycolysis

Glucose is the preferred carbohydrate of cells. **Glycolysis** (***glyco*** – sugar; ***lysis*** – splitting) is a universal process of all cells that occurs in the cytosol whereby the glucose (a 6-carbon sugar) is split into two pyruvates (a 3-carbon molecule) molecules to generate ATP and reduced NADH. **ATP** (adenosine triphosphate) is the energy currency of the cell that stores chemical energy in 3 high energy phosphate bonds. **NADH** (reduced nicotinamide adenine dinucleotide) is a high energy electron carrier that acts as a coenzyme in reactions and as a rechargeable battery of sorts. The uncharged state that is not carrying high energy electrons is called **NAD^+**.



Glycolysis is the splitting of glucose into 2 pyruvate molecules to generate 2 NADH and 2ATP molecules.

File:Adenosintriphosphat protoniert.svg

ATP contains 3 high energy phosphates and acts as cellular energy currency.

File:NAD oxidation reduction.svg

NADH is the reduced form of NAD+. The High energy electrons associated with the reduced form come with a Hydrogen atom.

Fermentation

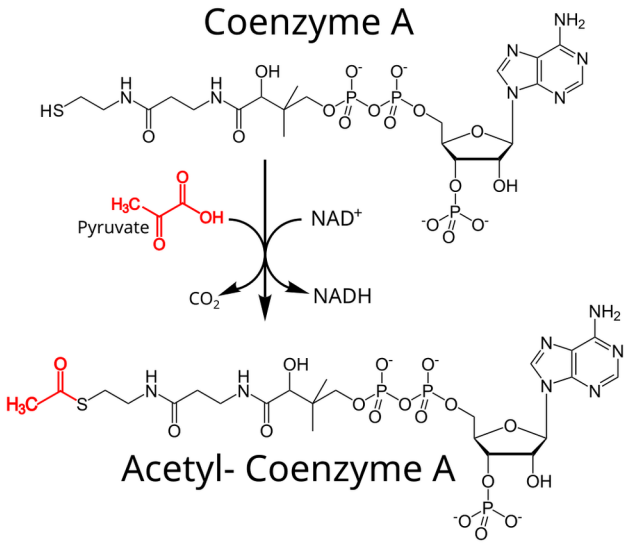
In the absence of oxygen, cells may decide to utilize the pyruvate from glycolysis to rapidly generate additional ATP molecules in a process called fermentation. **Fermentation** is the anaerobic process of reducing pyruvate to generate ATP. This process uses the NADH generated from glycolysis as the reducing agents. Fermentation is a familiar process that occurs in yeast to generate ethanol. In other organisms, like humans, fermentation results in the production of lactic acid. Both lactic acid and ethanol are toxic, but this aids the cells in generating ATP when energy is required rapidly. Fermentation also generates CO_2 as a waste molecule as pyruvate is broken down into a 2-carbon compound.

File:Ethanol fermentation-1.svg

Fermentation in yeast generates ATP in the absence of oxygen but yields little ATP at the cost of the reduced NADH. Credit: Davidcarmack (CC-BY-SA)

The Preparatory Reaction

In the presence of O_2, aerobic organisms will use a reaction of pyruvate decarboxylation in the cytosol. This reaction generates a molecule of **Acetyl-CoA** from the Coenzyme A which can enter the mitochondria.



Coenzyme A (CoA) is charged with an Acetyl group (2 carbon compound) to generate Acetyl-CoA and a CO_2.

When there is an excess of carbohydrates, the Acetyl-CoA is used as a starting point for long-term energy storage in lipid synthesis.

Mitochondria

Mitochondria are the power station of eukaryotic cells. They are derived from a process described by the **endosymbiotic theory** whereby aerobic prokaryotes were engulfed by a proto-eukaryote. In this mutualistic arrangement, the prokaryote detoxified the deadly O_2 gas in the environment and used it to fully break down glucose to yield many ATP molecules. Evidence for this theory comes from the independent replication of the mitochondria, the bacterial-like mitochondrial DNA, the bacterial-like mitochondrial ribosomes, the bacterial lipids found in the inner membrane and the eukaryotic nature of the outer membrane. Mitochondria are genomically similar to bacteria of the order Rickettsiales. Some bacteria of this order are still free-living and

some are intracellular pathogens.

File:Mitochondrion structure.svg

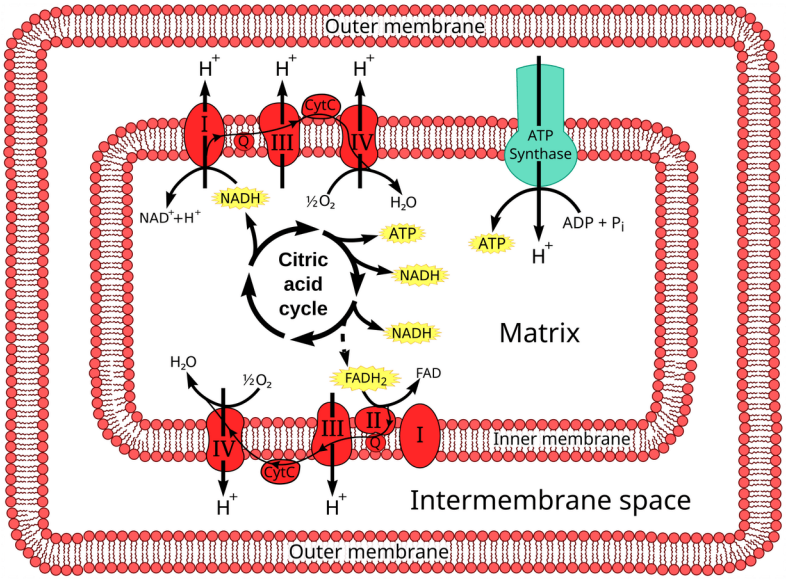
Credit: Kelvinsong (CC-BY-SA 3.0)

Aerobic Respiration

File:CellRespiration.svg

Cellular Respiration. Left side is glycolysis (anaerobic). The Right side is what occurs in the presence of oxygen in eukaryotes. The aerobic reactions occur inside the mitochondria after being fed Acetyl-CoA molecules from the cytoplasmic preparatory reaction. Credit: RegisFrey (CC-BY-SA 3.0)

Acetyl-CoA enters the mitochondrial matrix where it is used in the **Krebs Cycle** (aka Tricarboxylic acid cycle (TCA), aka Citric acid cycle). For each pyruvate, there are 2 turns of the cycle where additional NADH and another high energy electron carrier **$FADH_2$** (flavin adenine dinucleotide) are generated. The electrons stored by NADH and $FADH_2$ are transferred to proteins called **cytochromes** that have metal centers for conducting these electrons. In the process of moving these electrons, the cytochromes in this **Electron Transport Chains** (ETC) power the movement of protons into the intermembrane space. The terminus of these electrons is an O_2 molecule that is reduced into 1/2 H_2O molecules. This apparent movement of water molecules from the chemical synthesis is termed **chemiosmosis**. A channel in the membrane called **ATP synthase** acts as a gateway for the H^+ back into the matrix, but use this motion to convert ADP into ATP.



*Closeup of the **Electron Transport Chain** (ETC) that takes place on the inner membrane of mitochondria. This is where oxygen is utilized as the final electron acceptor. Reduction of 1/2 O_2 results in the generation of a water molecule (**chemiosmosis**). Credit: Jeremy Seto (CC-BY-NC-SA 3.0)*

Metabolic Pool

The catabolic pathways involved in the glycolysis and the Krebs cycle constitute the **metabolic pool** that supplies building blocks for other anabolic reactions in the cell. An excess of carbohydrates can result in an accumulation of Acetyl-CoA molecules. If there is a great excess of Acetyl-CoA, the acetyl groups can be committed to fatty acid synthesis for long-term energy storage. Glycolytic products can also be the starting point for amino acid synthesis. 3-phosphoglycerate can be used to synthesize glycine, cysteine and serine. Pyruvate can be used to generate alanine, valine, and leucine. Oxaloacetate from the Krebs cycle can be used as a starting point for aspartate, lysine, asparagine, methionine, threonine, and isoleucine. Glutamate and glutamine are synthesized from α-ketoglutarate formed during the Krebs cycle. While most of the 20 amino acids can be synthesized *de novo*, there are 9 **essential amino acids** in humans that can not be synthesized in sufficient quantity and

therefore must be gained from the diet. These essential amino acids include histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine.

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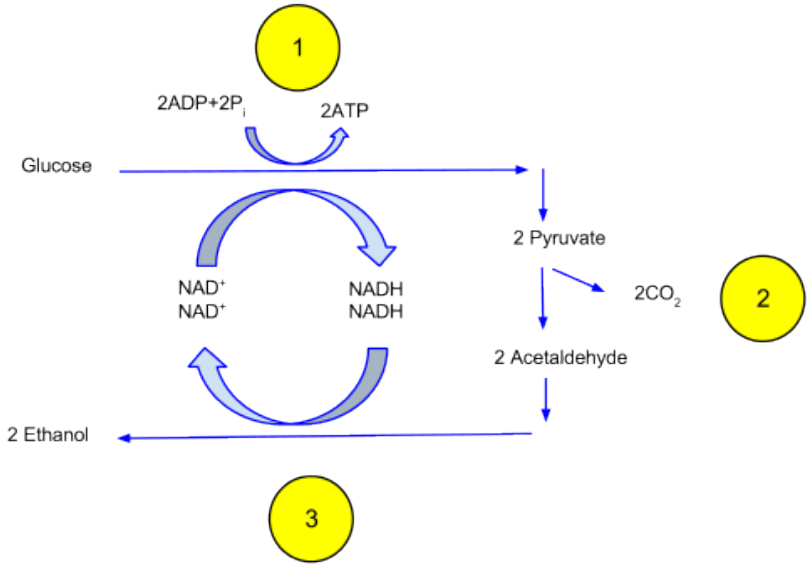
2.1.5.2: Fermentation

Introduction

Yeasts are single-celled fungi. The species called *Saccharomyces cerevisiae* is commonly called Baker's or Brewer's yeast. Like other eukaryotes with mitochondria, yeast can use oxygen to generate ATP in the process of **oxidative phosphorylation**. These yeast are **facultative aerobes** which means they can also switch to an anaerobic mechanism of ATP production called fermentation.

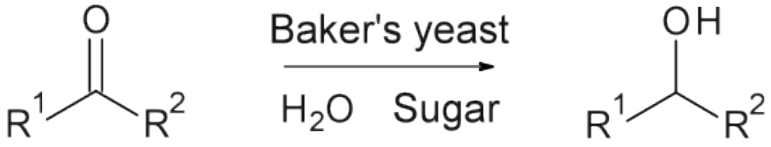
File:S cerevisiae under DIC microscopy.jpg File:20100911 232323 Yeast Live.jpg

In all organisms, the process of glycolysis occurs anaerobically in the cytoplasm to produce two pyruvate molecules from a single glucose. This process produces 2 new ATP molecules and reduced nicotinamide adenine dinucleotide (NADH).



In ethanol fermentation. One glucose molecule breaks down into two pyruvates via glycolysis (1). The energy from these exothermic reactions is used to bind inorganic phosphates to ADP and convert NAD+ to NADH. The two pyruvates are then broken down into two Acetaldehyde and give off two CO2 as a waste product (2). The two Acetaldehydes are then reduced to two ethanol, and NADH is oxidized back into NAD+ (3).

Fermentation is an anaerobic process that occurs in the cytoplasm and quickly generates an additional ATP through the reduction of pyruvate. NADH is the source of electrons in this process that is oxidized to NAD+. Many organisms will ferment to generate lactic acid and CO_2 from the pyruvate in order to generate ATP. Yeast fermentation produces **ethanol**.



Fermentation Set-up

1. Mix the solutions in the table below in a fermentation tube.
2. Eliminate the air bubble in the sealed end of the fermentation tube.
3. Place rubber stopper into the open end of the fermentation tube and place at the appropriate temperature for an hour.

4. Predict the amount of CO_2 generated in the last column of the table using +, – or +++.
    - After an hour, measure the headspace created by the bubbles and compare with your predictions.

TUBE	YEAST	SUGAR	ADDITIVE	TEMP.	CO_2 generation
1	10 ml yeast	None	20 ml H_2O	25°C	
2	10 ml yeast	10 ml glucose	10 ml H_2O	25°C	
3	10 ml yeast	10 ml glucose	10 ml H_2O	37°C	
4	10 ml yeast	10 ml lactose	10 ml H_2O	37°C	
5	10 ml yeast	10 ml sucrose	10 ml H_2O	37°C	
6	10 ml yeast	10 ml maltose	10 ml H_2O	37°C	
7	10 ml yeast	10 ml glucose	10 ml 0.1M $MgSO_4$	37°C	
8	10 ml yeast	10 ml glucose	10 ml 0.1 M NaF	37°C	
9	10 ml yeast	10 ml lactose	10 ml H_2O + LactAid pill	37°C	

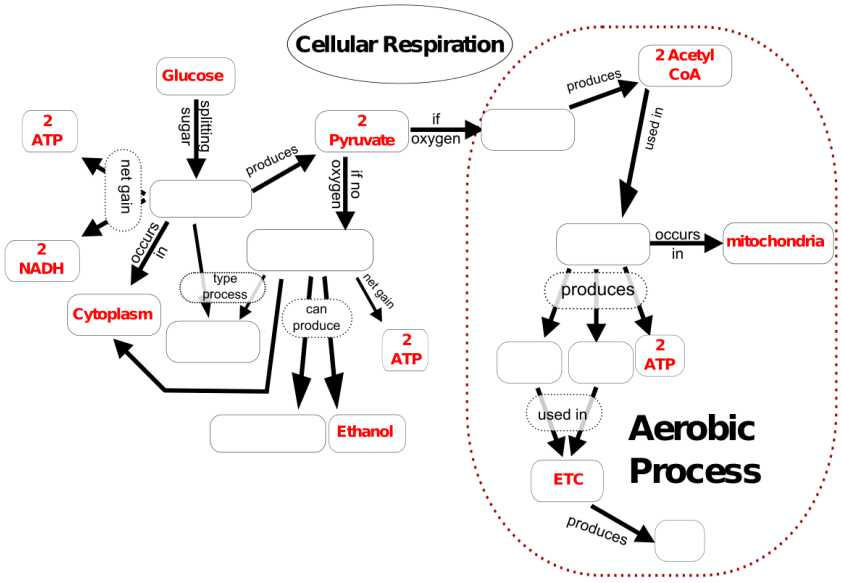
Questions to Direct Hypothesis Formation

1. What is the preferred energy source of all cells?
2. What types of sugars are being used in each tube (monosaccharide, disaccharide, etc)?
3. What effect should temperature have on the fermentation reactions?
4. What does CO_2 indicate in these tubes?
5. What do you think the additives do? What effect will they have?

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2.1.5.3: Cell Respiration (Concept)



Cellular Respiration concept map.

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2.1.6: PHOTOSYNTHESIS

2.1.6.1: Introduction
2.1.6.2: Photosynthetic Pigments
2.1.6.3: Absorbance Spectra of Photosynthetic Pigments
2.1.6.4: Photosynthesis (Concept)

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2.1.6.1: Introduction

Chloroplasts

File:Chloroplast-cyanobacterium comparison.svg

Credit: elvinsong [CC-BY-SA 3.0]

Chloroplasts arose through a second endosymbiotic event in plants and various protists. These light-harvesting organelles share a similarity in structure and genome to photoautotrophic cyanobacteria.

Light-Harvesting

File:Thylakoid membrane 3.svg

The thylakoid membranes of chloroplasts and cyanobacteria provide additional surface area for energy capture of light to occur. The light-dependent reactions in chloroplasts utilize two protein complexes referred to as **Photosystem I** (PSI) and **Photosystem II** (PSII) located on the thylakoid membranes. At the center of each photosystem complexes are **photopigments** optimized to absorb specific wavelengths of light. When light is absorbed in a photosystem, an electron is excited and transferred to the electron transport chain. In PSII, the electron is regenerated by splitting of two water molecules into $4H^+ + 4e^- + O_2$. As the electrons move through the ETC, protons are pumped into the thylakoid space. The ETC leads to the reduction of a high energy electron carrier $NADP^+$ to **NADPH**. Since this pathway uses consumes water in a chemical reaction, the apparent loss of water in the thylakoid space is referred to as chemiosmosis.

PSI is also known as the cyclic pathway since the excited electron runs through a closed circuit of the ETC to regenerate the lost electron. This closed circuit also generates a proton gradient through powering of a proton pump but does not lead to the reduction of NADPH. As with the ETC-powered proton pump in mitochondria, the proton gradient is used to power ATP-synthase in producing ATP molecules.

Light Independent Reactions

File:Calvin-cycle4.svg

Credit: Mike Jones [CC-BY-SA 3.0]

The light-independent reactions are also known as the dark reactions or **Calvin Cycle** and utilize the ATP and NADPH from the light-dependent reactions to fix gaseous CO_2 into carbohydrate backbones. Photosynthesis is often simplified into $6CO_2 + 6H_2O$ + light –> $C_6H_{12}O_6 + 6O_2$. However, the true product is 3-phosphoglycerate that can be used to generate longer carbohydrates like glucose. The starting point of **carbon fixation** is the carbohydrate Ribulose 1,5-bisphosphate. The enzyme Ribulose Bisphosphate Carboxylase (**RuBisCO**) captures a CO_2 molecule onto Ribulose 1,5-bisphosphate to generate 2 molecules of 3-phosphoglycerate which can enter the process of **gluconeogenesis** to generate glucose. ATP from the light reactions can then facilitate the conversion of 3-phosphoglycerate to 1,3 bisphosphoglycerate which can be reduced by NADPH to glyceraldehyde-3-phosphate (G3P). G3P can then be used to regenerate Ribulose 1,5-bisphosphate.

File:Calvin cycle step 1.svg

1: Carbon fixation by RuBisCO

File:Calvin cycle step 2.svg

2: Reduction by NADPH

File:Calvin cycle step 3.svg

The Great Oxygenation Event

File:Oxygenation-atm-2.svg

Two estimates of the evolution of atmospheric O_2. The upper red and lower green lines represent the range of the estimates. Stage 1 (3.85–2.45 Ga) represents the primordial reducing atmosphere. Stage 2 (2.45–1.85 Ga) coincides with the emergence of oceanic cyanobacteria where O_2 was being absorbed by the oceans and sediment. O_2 escaped the oceans during Stage 3 (1.85–0.85 Ga). O_2sinks filled in Stage 4 (0.85–0.54 Ga) and Stage 5 (0.54 Ga–present) leading to atmospheric accumulation.

File:Black-band ironstone (aka).jpg

Banded iron formations in 2.1 billion-year-old rock illustrate the oxidation of dissolved oceanic iron that precipitated in response to accumulating O_2 concentrations.

File:Carbon cycle.jpg

The Carbon Cycle illustrates carbon sequestration and release between various carbon sinks.

File:M15-162b-EarthAtmosphere-CarbonDioxide-FutureRoleInGlobalWarming-Simulation-20151109.jpg

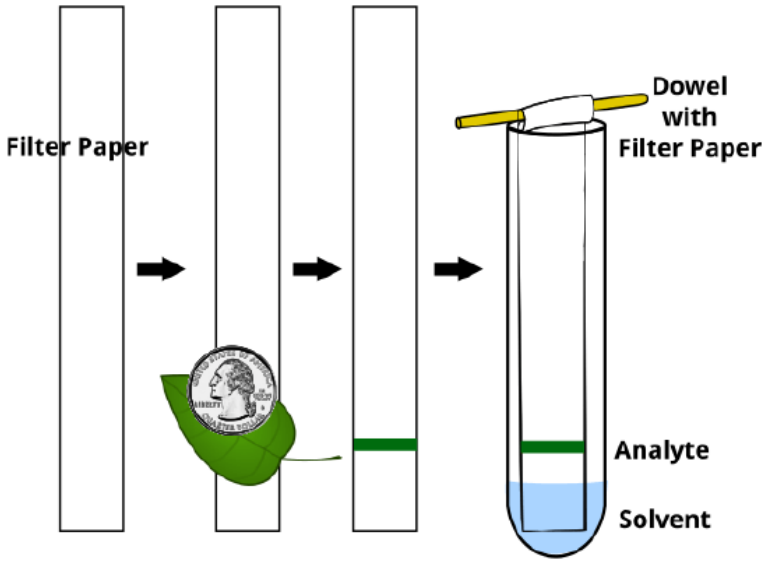
Projection of atmospheric CO_2 accumulation without reduction of fossil fuel reduction by NASA.

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2.1.6.2: Photosynthetic Pigments

Extract and Separate the Pigments



1. Lay a strip of filter paper on the bench.
2. About 2 cm from the bottom of the strip, place a fresh spinach leaf and rub a coin across the leaf to transfer pigment to the strip.
3. The instructor will be provided with a spoonful of *Spirulina* powder that has been soaked in 10ml acetone overnight.
    - On a separate strip, the instructor will apply the Spirulina extract approximately 2cm from the bottom of the strip.
4. Suspend the strips by a dowel or paper clip in a tube with about 3ml chromatography solution *(2 isooctane: 1 acetone: 1 diethyl ether)*.
5. Develop the strips until the solvent reaches about 2 cm from the top.

Chromatography Analysis

1. How many different pigments separate from the spinach extract? From the spirulina?
2. Are all pigments represented between the two extracts?
3. The mobile phase is non-polar. What are the properties of each pigment?
4. Measure the R_f of each pigment.

Pigment Color	Distance (cm)	R_F
	D_A= D_S=	
	D_A= D_S=	
	D_A= D_S=	
	D_A= D_S=	

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2.1.6.3: Absorbance Spectra of Photosynthetic Pigments

Prelab Exercise

1. Fill the *Color* field in the table below
2. Use plot.ly to create a **line** graph with the 3 samples below (A, B, C)
    - Plot *% Reflectance* on the Y-axis and *Wavelength (nm)* on the X-axis

Color	nm
	400
	425
	450
	475
	500
	525
	550
	575
	600
	625
	650

	675
	700

Stop and Think: Reflectance

A sign of plant health is viewed through the near infra-red. While we cannot see this spectrum of light with our eyes, we can use other sensors to detect this light. Compare the images of the Black & White with the Infra-red image. What differences can you see in the 2 images that will help you understand how this is a useful measure of plant health? How do you think this corresponds to the table above?



The English Garden

Reflectance Slider shows an overlay of the next images.



The English Garden (black & white)



The English Garden (near infra-red)

Visible light wavelengths (between 400nm-700nm) are strongly absorbed by the pigments in leaves (Chlorophylls, Xanthophylls, and Carotenoids). These pigments utilize the energy of these wavelengths to take part in the light reactions. The cellular structure of leaves does not absorb wavelengths longer than these wavelengths (>700nm in the infra-red range). By comparing the amount of visible light to the amount of near infra-red lights that are reflected, one can gauge the relative health of leaves, forests, or jungles. This is the rough description of the Normalized Differential Vegetation Index (NDVI) that scientists use in conjunction with satellite imagery to assess the health of vegetation.

The Role of Light in Carbohydrate Synthesis

1. Pick a leaf from a geranium exposed to light and one kept in the dark for 48 hours.
    - Keep the stem on the leaf grown in the light.
    - Remove the stem from the leaf grown in the dark.
2. Hydrolyze the cell walls of the geranium leaves by boiling in a water bath for 5 minutes or until it looks like over-cooked vegetables).
3. ***"Bleach"*** the leaves by removing the pigments. Place the leaves in hot alcohol for 7 minutes or until they turn white.
    1. Save this green solution for Absorbance Spectrum exercise.
4. Remove the leaves and place it in a petri dish.
5. Add iodine to the dish. If starch is present, the leaf will turn a deep bluish-black color.
6. Photograph the leaf with your phone to document the effects of light on carbohydrate storage.

Measuring the Absorbance

1. Connect the Spectrovis to the LabQuest2.
2. Turn on the Labquest2 units.
3. Choose the Labquest app.
4. Select the icon that looks like X|Y.
5. Press the green Play button on the bottom left.
6. Press OK to calibrate.
7. Let the machine calibrate for 90 seconds.
8. Choose "Finish calibration".
9. Insert the Geranium pigment from the bleaching reaction.
    1. Do NOT use Acetone in these plastic cuvettes since it will frost over the plastic.
10. Press the Red Stop button.

11. Students should record the absorbance values at every 10 nm from 380nm-700nm.
12. The professor will prepare *Spirulina* extract diluted in ethanol in a cuvette and obtain the continuous absorbance spectrum.
13. Plot Relative Absorbance against *wavelength* using a line graph and compare the absorption spectrum of the extracts.
    1. Relative Absorbance sets the maximum value in each dataset as a denominator.
    2. Every value is divided by this maximum value.

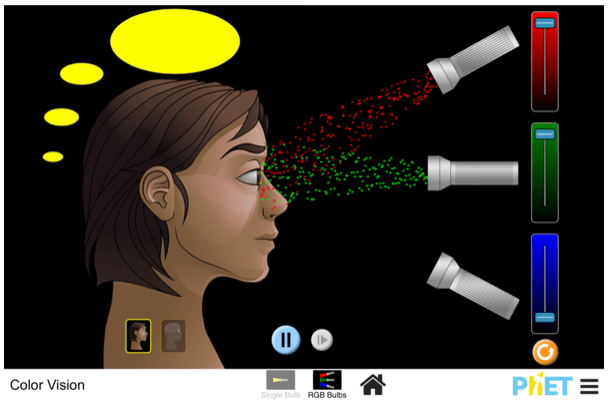
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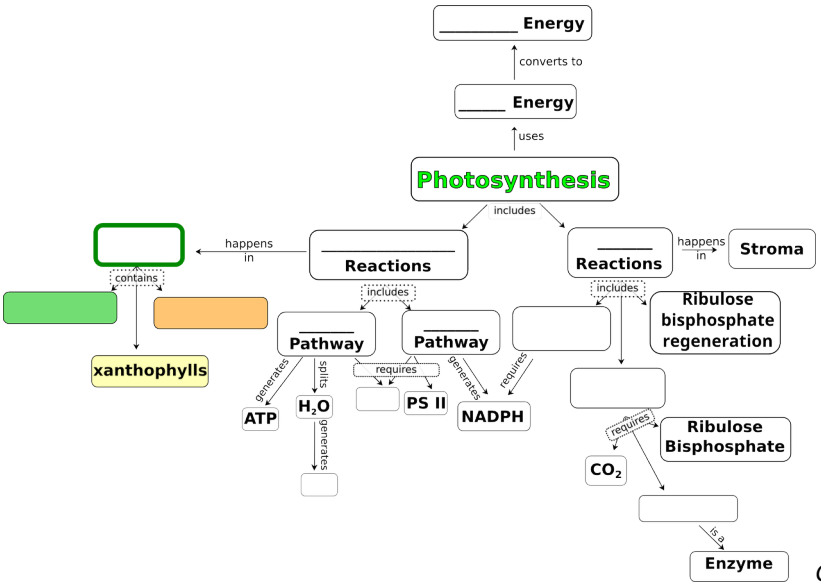
- 6.3: Absorbance Spectra of Photosynthetic Pigments by Bio-OER is licensed CC BY-NC-SA 4.0.

2.1.6.4: Photosynthesis (Concept)

Exploring Light

Run the simulation below to understand how white light, specific wavelengths of light, and filtered light work.





Concept map for Photosynthesis. Download PDF

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2.1.7: CELL DIVISION

2.1.7.1: Introduction

2.1.7.2: Mitosis (Activity)

2.1.7.3: Modeling Mitosis and Meiosis (Activity)

2.1.7.4: Chromosomes and Karyotypes

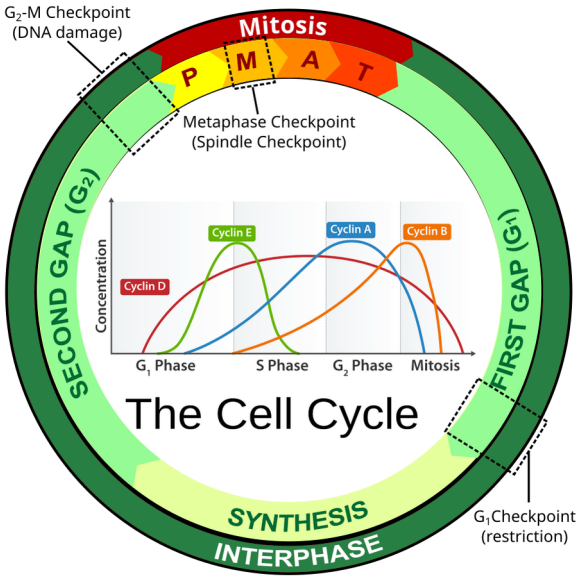
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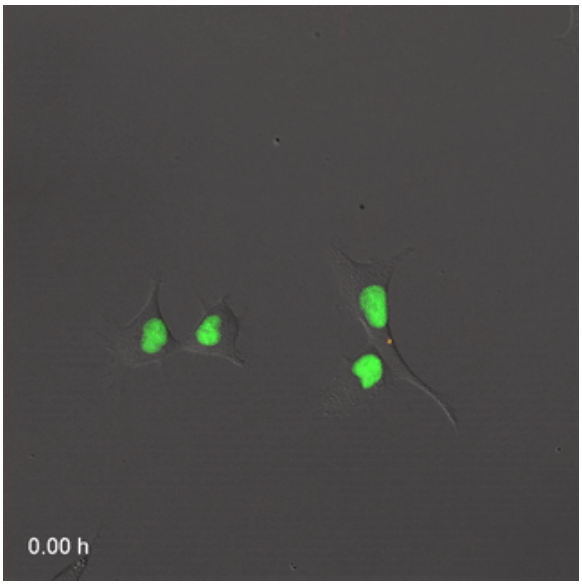
2.1.7.1: Introduction

Introduction: The Cell Cycle and Mitosis

The cell cycle refers to a series of events that describe the metabolic processes of growth and replication of cells. The bulk of the cell cycle is spent in the “living phase”, known as **interphase**. Interphase is further broken down into 3 distinct phases: G_1 (Gap 1), S (Synthesis) and G_2 (Gap 2). **G_1** is the phase of growth when the cell is accumulating resources to live and grow. After attaining a certain size and having amassed enough raw materials, a checkpoint is reached where the cell uses biochemical markers to decide if the next phase should be entered. If the cell is in an environment with enough nutrients in the environment, enough space and having reached the appropriate size, the cell will enter the S phase. **S phase** is when metabolism is shifted towards the replication (or synthesis) of the genetic material. During S phase, the amount of DNA in the nucleus is doubled and copied exactly in preparation to divide. The chromosomes at the end of G_1 consist of a single **chromatid**. At the end of S phase, each chromosome consists of two identical **sister chromatids** joined at the **centromere**. When the DNA synthesis is complete, the cell continues on to the second growth phase called **G_2**. Another checkpoint takes place at the end of G_2 to ensure the fidelity of the replicated DNA and to re-establish the success of the cell’s capacity to divide in the environment. If conditions are favorable, the cell continues on to mitosis.



Eukaryotic cell cycle is governed by the expression of cyclin proteins along with their activity. Credit: Jeremy Seto (CC-BY-SA)



Mammalian cells in culture going through the Cell Cycle. Green marker proteins expressed during the G1 phase. Red marker proteins are expressed during S/G2/M. During the G1 to S transition, fluorescence disappears as the marker proteins also transition in expression.

Mitosis is the process of nuclear division used in conjunction with cytokinesis to produce 2 identical daughter cells. **Cytokinesis** is the actual separation of these two cells enclosed in their own cellular membranes. Unicellular organisms utilize this process of division in order to reproduce asexually. Prokaryotic organisms lack a nucleus, therefore they undergo a different process called binary fission. Multicellular eukaryotes undergo mitosis for repairing tissue and for growth. The process of mitosis is only a short period of the lifespan of cells. Mitosis is traditionally divided into four stages: **prophase**, **metaphase**, **anaphase,** and **telophase**. The actual events of mitosis are not discreet but occur in a continuous sequence—separation of mitosis into four stages is merely convenient for our discussion and organization. During these stages, important cellular structures are synthesized and perform the mechanics of mitosis. For example, in animal cells, two microtubule-organizing centers called **centrioles** replicate. The pairs of centrioles move apart and form an axis of proteinaceous microtubules between them called **spindle fibers.** These spindle fibers act as motors that pull at the centromeres of chromosomes and separate the sister chromatids into newly recognized chromosomes. The spindles also push against each other to stretch the cell in preparation of forming two new nuclei and separate cells. In animal cells, a contractile ring of actin fibers cinches together around the midline of the cell to coordinate cytokinesis. This cinching of the cell membrane creates a structure called the **cleavage furrow**. Eventually, the cinching of the membrane completely separates into two daughter cells. Plant cells require the production of new cell wall material between daughter cells. Instead of a cleavage furrow, the two cells are separated by a series of vesicles derived from the Golgi. These vesicles fuse together along the midline and simultaneously secrete cellulose into the space between the two cells. This series of vesicles is called the **cell plate**.

File:Mitosis Stages.svg

File:Prophase eukaryotic mitosis.svg

File:Metaphase eukaryotic mitosis.svg

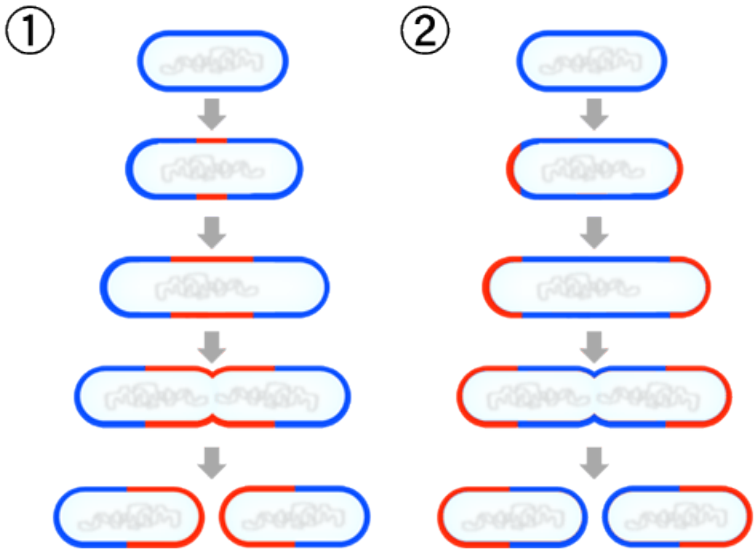
File:Anaphase eukaryotic mitosis.svg
File:Telophase eukaryotic mitosis.svg
File:Cytokinesis eukaryotic mitosis.svg

Mitosis Review

- Khan Academy Review
- Mitosis Flash Card Review
- Mitosis Sequence Review

Binary Fission

Mitosis refers to nuclear division, which happens to coincide with cytokinesis. Prokaryotes do not have nuclei, therefore they do not undergo a process of mitosis. Instead, prokaryotes undergo the process of binary fission. Growth of new membranes during the cellular growth and expansion in a prokaryote occurs by 2 mechanisms **1)** along the center of the cell or **2)** apically.



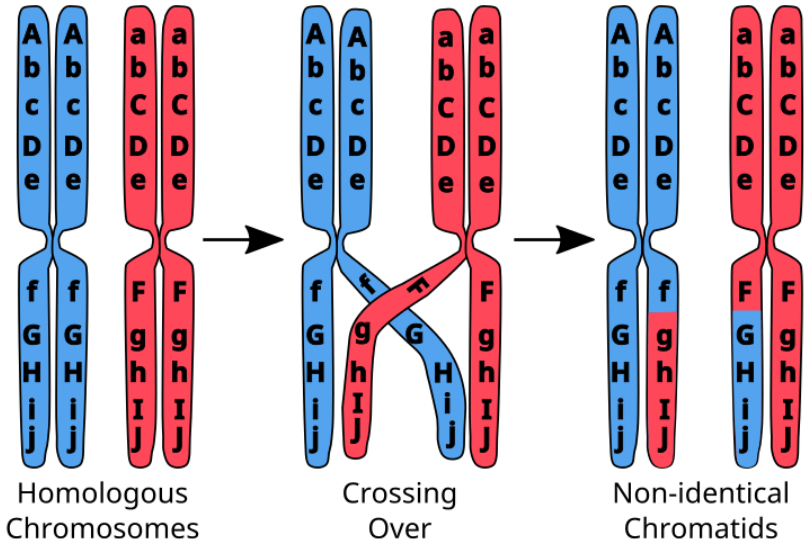
The chromosome of prokaryotes is organized in a circular form. A mechanism called the **rolling circle model** describes the replication of the chromosome that accompanies binary fission. This is also the mechanism utilized by extrachromosomal DNA called plasmids.

File:Rolling-circle replication.png

Introduction: Meiosis

Meiosis is a process of nuclear division that reduces the number of chromosomes in the resulting cells by half. Thus, meiosis is sometimes called “reductional division.” For many organisms, the resulting cells become specialized “sex cells” or **gametes**. In organisms that reproduce sexually, chromosomes are typically **diploid** (**2N**) or occur as double sets (**homologous pairs**) in each nucleus. Each homolog of a pair has the same sites or loci for the same genes. You might recognize that you have one set of chromosomes from your mother and the remaining set from your father. Meiosis reduces the number of chromosomes to a **haploid** (**1N**) or single set. This reduction is significant because a cell with a haploid number of chromosomes can fuse with another haploid cell during sexual reproduction and restore the original, diploid number of chromosomes to the new individual. In addition to reducing the number of chromosomes, meiosis shuffles the genetic material so that each resulting cell carries a new and unique set of genes in a process of **independent assortment**.

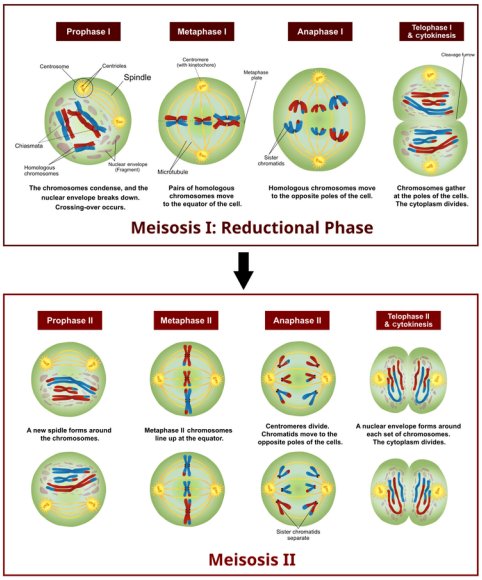
As in mitosis, meiosis is preceded by replication of each chromosome to form two chromatids attached at a centromere. However, reduction of the chromosome number and production of new genetic combinations result from two events that don’t occur in mitosis. First, meiosis includes two rounds of chromosome separation. Chromosomes are replicated before the first round, but not before the second round. Thus, the genetic material is replicated once and divided twice. This produces half the original number of chromosomes.



Crossing over between chromatids of homologous chromosomes increases genetic diversity during meiosis I. Synapsis occurs during prophase I as the homologous chromosomes begin to pair up. Credit: Jeremy Seto (CC-BY-NC-SA)

Second, during an early stage of meiosis each chromosome (comprised of two chromatids) pairs along its length with its homolog. This pairing of homologous chromosomes results in a physical touching called **synapsis**, during which the four chromatids (a tetrad) exchange various segments of genetic material. This exchange of genetic material is called **crossing-over** and produces new genetic combinations. During crossing-over there is no gain or loss of genetic material. But afterward, each chromatid of the chromosomes contains different segments (alleles) that is exchanged with other chromatids.

Stages and Events of Meiosis



Stages of Meiosis. Credit: ***Ali Zifan*** *(CC-BY 4.0)*

Although meiosis is a continuous process, we can study it more easily by dividing it into stages just as we did for mitosis. Indeed, meiosis and mitosis are similar, and their corresponding stages of prophase, metaphase, anaphase, and telophase have much in common. However, meiosis is longer than mitosis because meiosis involves two nuclear divisions instead of one. These two divisions are called Meiosis I and Meiosis II. The chromosome number is reduced (**reductional division**) during Meiosis I, and chromatids comprising each chromosome are separated in Meiosis II. Each division involves the events of prophase, metaphase,

anaphase, and telophase.

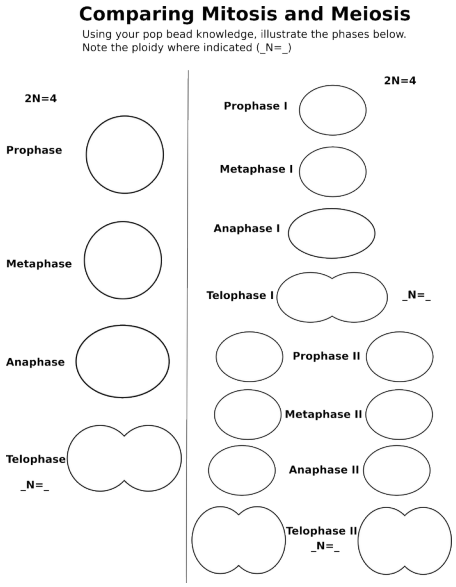
Advanced Video Overview of Meiosis

Oogenesis

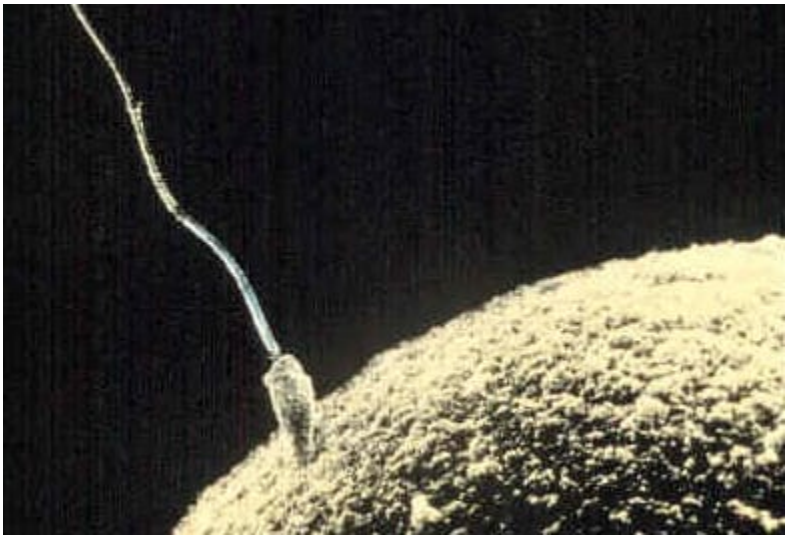
File:Gray5.svg

Spermatogenesis

File:Figure 28 01 04.jpg



Fertilization



Summary of Cell Division		
	Mitosis	Meiosis

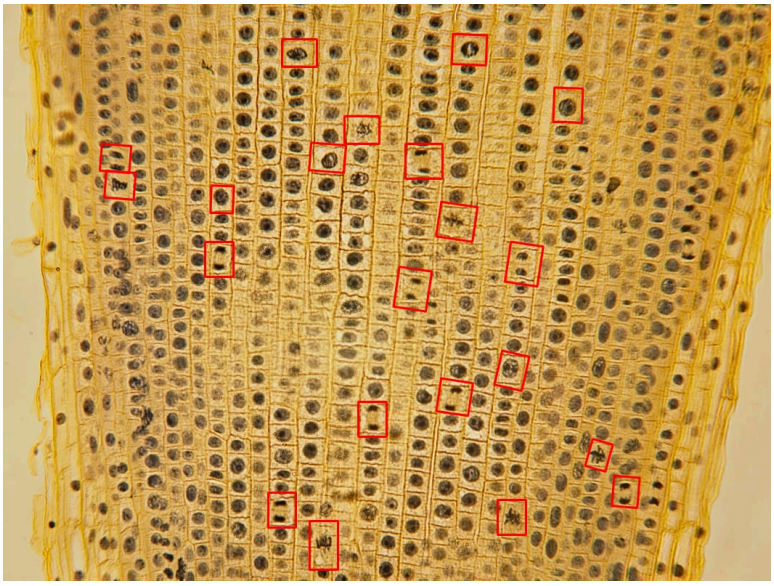
Number of Cells at the Start		
Number of Cells at the End		
Number of Cell Divisions		
Chromosome Number (N)	Start:_____ End:_____	Start:_____ End:_____
Number of Chromosomes in the Cell at the Start of the Process (Human Cell)		
Number of Chromosomes in the Cell at the End of the Process (Human Cell).		
Daughter Cells (N or 2N)		
Daughter Cell Genetics (Identical or Non-identical)		
Purpose of Division		

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2.1.7.2: Mitosis (Activity)

Identifying Mitotic Phases



Identifying phases of mitosis in the onion root tip. Click on the image to view a larger image.

The original figure without red boxes.

Activity: Estimating the Time Spent in the Phases of Mitosis

Cells in your body reproduce at different rates. Skin cells reproduce frequently (about once per day); liver cells reproduce rarely (about once per year). Some specialized cells like nerve and muscle cells almost never reproduce and are in a special stage called G_0. The whole process of mitosis, prophase to telophase, takes approximately 90 min. In plants, an area of rapid growth is the tips of roots. This exercise uses onion root tips to illustrate the amount of time spent in each phase of mitosis.

1. Work as a team to look at onion root tips under the microscope. This area of the root is undergoing rapid cell reproduction.
    - If time is short, use the slides below
        - slide 1
        - slide 2
        - slide 3
        - slide 4
        - slide 5
        - slide 6
        - slide 7
2. Identify the phases of the cell cycle for 20 randomly chosen cells. Record this information in the table.
3. Trade results with 3 other people.
4. In an onion root tip, the entire cell cycle takes about 12 hours or 720 minutes
5. Calculate the percentage of time spent in each phase by counting the total number of cells in each phase (total in interphase, in prophase, etc.) and dividing each by the total number of cells you counted.
6. Multiply the percentage of time in each phase by the total time of the cell cycle (720 minutes) and this gives you an estimate of the time spent in each phase.

Number of Cells in each phase

	Interphase	Prophase	Metaphase	
You (25)				
Partner 1 (25)				
Partner 2 (25)				
Partner 3 (25)				
Totals				

Estimate of time spent in each phase of the cell cycle.

	Interphase	Prophase	Metaphase	
% of cells in each phase				
Estimated Time				

Test Yourself at Home

Use the following resource bio.rutgers.edu/~gb101/lab2_mitosis/section1_frames.html to test yourself and practice without a microscope.

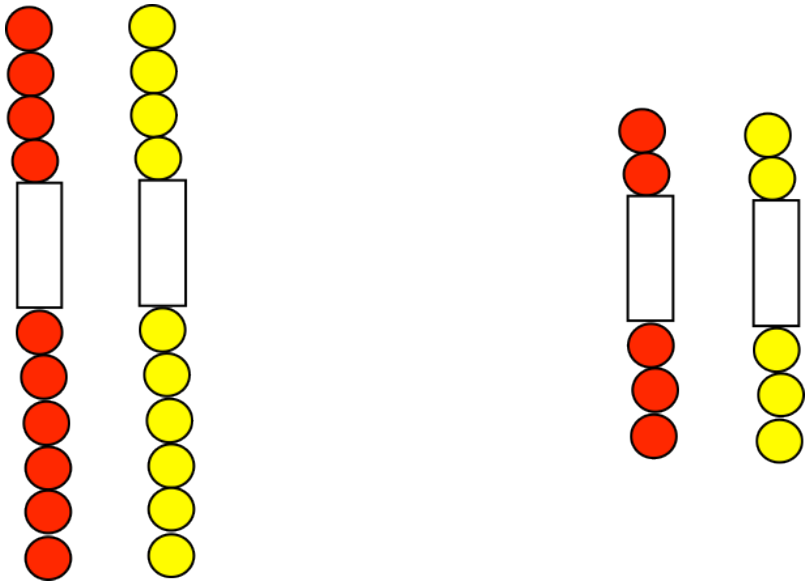
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2.1.7.3: Modeling Mitosis and Meiosis (Activity)

Mitosis Simulation with Pop-it Beads

Pop-beads are small, colored beads that can be joined together to simulate chromosome strands. We will use the pop-it beads to simulate the process that chromosomes undergo during cell division. Imagine that the beads represent long stretches of DNA that comprises the genetic instructions for the cell. Start with a cell with a chromosome number of 4 (4 chromosomes, or 2 homologous pairs). We will use red to identify the chromosome from the mother and yellow for the chromosome from the father.



Pop-it Bead Chromosomes (2N=4)

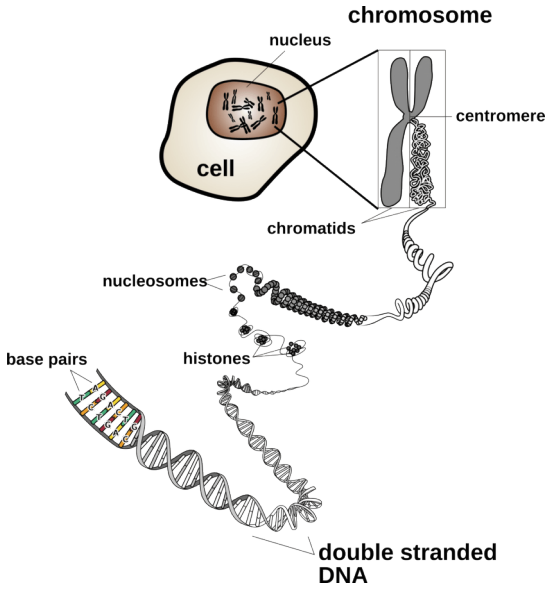
1. Make 4 chromosomes: two long chromosomes (one red and one yellow) and two short chromosomes (one red and one yellow). The two long chromosomes should each have the same number of beads, as should the two short chromosomes. The two long chromosomes are one homologous pair; the two short chromosomes are the second homologous pair.
2. *Simulate S phase*: Replicate your chromosomes by making an identical set of pop-it bead chromosomes (you should have a total of eight pop-it bead strands; four long and four short). Attach the identical replicas (chromatids) by their magnetic centromeres.
3. *Simulate Mitosis*: Move the “chromosomes” through each of the four stages of mitosis. Draw and label the pop- bead chromosomes for ONE of the phases on a separate sheet. It is not necessary to draw each individual bead.
4. Draw a large circle on the paper to represent the cell and the nucleus with a pencil.
5. Place your chromosomes in this and walk through each stage of Meiosis.
6. Erase the outlines as you go along and re-draw the boundaries of nuclei.
7. Document each stage with your cell phone:
    1. Prophase
    2. Metaphase
    3. Anaphase
    4. Telophase

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- 7.3: Modeling Mitosis and Meiosis (Activity) by Bio-OER is licensed CC BY-NC-SA 4.0.

2.1.7.4: Chromosomes and Karyotypes

Chromosomal Features

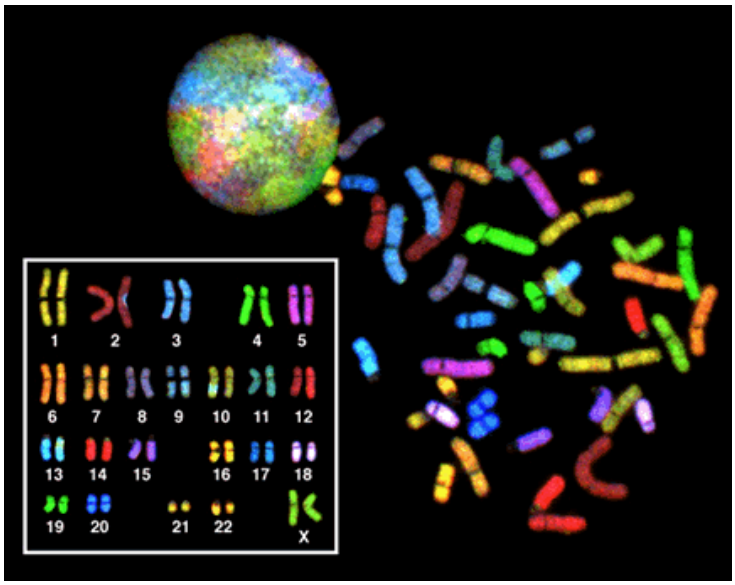


Chromosomes are made of double-stranded DNA molecules wound about histones and condensed into the familiar X-shape. Under regular functioning, these chromosomes are decondensed in the nucleus and not recognizable.

Chromosomes in Interphase are not visible individually. In preparation for nuclear division (mitosis or meiosis), they begin to organize tighter and condense in preparation for movement to subsequent daughter nuclei. The animation below illustrates the process of histone packaging and the molecular visualization of DNA replication. **Histones** are proteins that aid in the packaging of the chromosomes into organized coils that give rise to the recognizable chromosomes during metaphase.

Large-scale genomic rearrangements result in genetic abnormalities. Biologists utilize a technique called a **chromosome spread** followed by a **karyotype** or **karyogram**. To make a chromosome spread, one blocks the progression of mitosis at metaphase where chromosomes are condensed into the structures we are familiar with. A karyotype analysis is an arrangement of the chromosome spread into the homologous pairs of chromosomes.

Events associated with the improper separation of chromosomes during metaphase results in an alteration of chromosome number in the subsequent generation of cells. Using the Pop-beads, we can understand better how the timing of these events will lead to differences in the karyotype.



A “spectral” karyotype of a female nucleus. Each homologous pair is “painted” to differentiate them.

Abnormal Karyotypes

Down’s Syndrome is a common genetic abnormality referred to as Trisomy 21. Instead of having the complement of 46 chromosomes of 22 homologous pairs plus 2 sex chromosomes, there are 47 chromosomes consisting of an additional Chromosome 21.

File:Karyotype.png

Standard Human Karyotype with 46 chromosomes. Both XX and XY are also shown here.

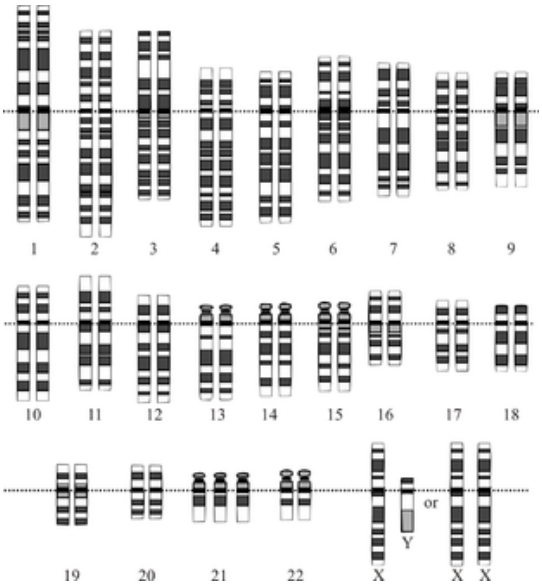
The appearance of extra or missing chromosomes arises during meiosis in an event called **nondisjunction**. After fertilization, a zygote with an improper chromosome complement occurs.

File:Mitotic nondisjunction.png

File:Nondisjunction Diagrams.svg

From left to right, the ploidy of the resultant zygotes: 2N, 2N, 2N+1, 2N-1; 2N+1, 2N+1, 2N-1, 2N-1

Nondisjunction can occur during Meiosis I or Meiosis II to yield **aneuploid** states of 2N+1 or 2N-1.



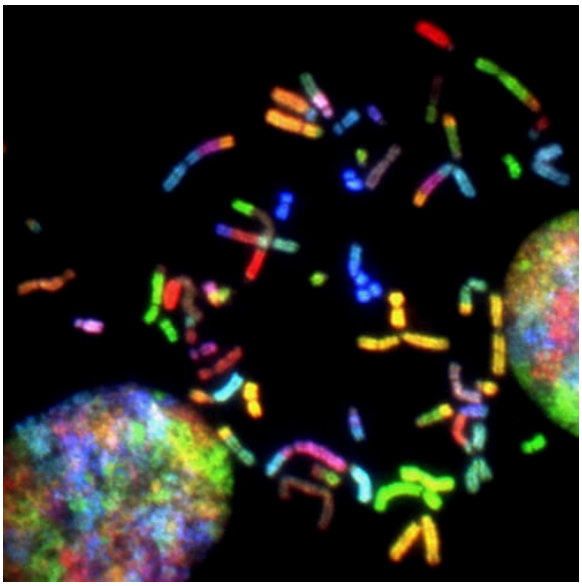
Down's syndrome karyotype showing the traditional trisomy 21.

Translocation

Translocation is the movement of a piece or a whole chromosome onto another chromosome. The acrocentric nature of Chr 21 and it's small size makes it prone to an event called **Robertsonian Translocation** whereby two acrocentric chromosomes fuse.

File:Robertsonian translocation rob(14;21).png

Down's Syndrome from a 14:21 translocation.



A spectral karyogram of a brain cancer (glioblastoma) illustrating multiple translocation problems and chromosome instability.
Credit: Thomas Reid, NCI (CC-BY-NC)

Abnormalities in mitosis also occur and can result in diseases from translocations.

File:Chromosomal translocations.svg

Polyploidy

Some organisms and cells have entire sets of chromosomes additional to the standard 2N diploid. Cells that have extra sets in the formula of 3N are called **triploid**. If they are 4N, they are called **tetraploid**. This is different than the case of Down's syndrome, which has a chromosome complement of 2N+1. Any time there are abnormal numbers of chromosomes, cells are referred to as **aneuploid**. A special case of aneuploid occurs from having entire sets more of chromosomes—**polyploid**. Plants are especially robust in the regard of polyploidy and often have different species arise in such a way. Some plants become sterile in the case of polyploidy and will not produce seeds properly.

File:Inside a wild-type banana.jpg

Wild bananas or plantains (Musa acuminata or Musa balbisiana) are deemed inedible because of their large seeds.

Have you ever seen a banana with these large seeds? The answer is most likely "No!" since these are not regarded as being edible. However, due to selective breeding practices, most edible plantains and bananas are hybrids of the two species *Musa acuminata* or *Musa balbisiana* that are 3N or 4N. In this case, the fruits are sterile and the seeds don't develop. Other seedless fruits are also developed this way and require propagation through clonal means.

Advanced View of Chromosome Segregation

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2.1.8: ANALYZING DNA

2.1.8.1: Introduction

2.1.8.2: DNA Replication

2.1.8.3: Variable Number Tandem Repeats

2.1.8.4: Propagating DNA in Bacteria

2.1.8.5: Restriction Enzymes

2.1.8.6: DNA Fingerprinting (RFLP)

2.1.8.7: Cheek Cell DNA Extraction

2.1.8.8: D1S80 VNTR (Genotyping)

2.1.8.9: DNA Miniprep by Alkaline Lysis (Activity)

2.1.8.10: Sanger Sequencing of DNA

2.1.8.11: Next Gen Sequencing

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2.1.8.1: Introduction

Agarose Gel Electrophoresis

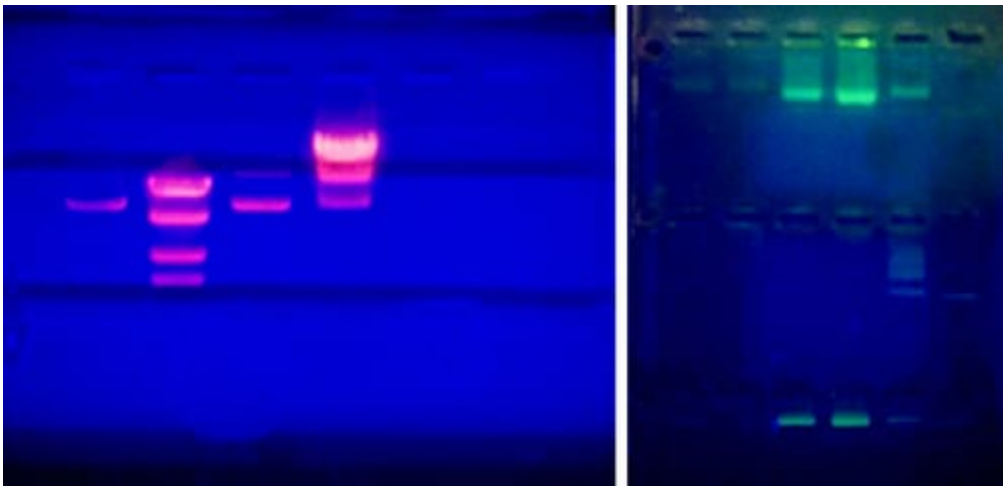
File:Agarose polymere.svg

Agarose is a linear carbohydrate polymer purified from the cell walls of certain species of algae. Agar is a combination of the crude extract that contains agarose and the smaller polysaccharide agaropectin. When dissolved and melted in liquid, agarose strands become tangled together to form a netting that holds the fluid in a gel. Reduction of the fluid creates a higher percentage of the gel that is firmer and contains smaller pores within the netting.

File:Two percent Agarose Gel in Borate Buffer cast in a Gel Tray (Front, angled).jpg

Placing a **comb** within the melted agarose creates spaces that allow for the insertion of samples when the gel is solidified. Molecules can traverse through the pores as they are drawn by electrical currents. Charged compounds will migrate towards the electrode of opposite charge but migration rate will be influenced by the size of the molecules. Smaller compounds can easily traverse through the webbing while larger items are retarded by the pore size. Follow this simulation to get a better idea of how we use **Agarose Gel Electrophoresis** in molecular biology to study DNA fragments.

DNA molecules are not readily visible when **resolved** (separated) on an agarose gel. In order to visualize the molecules, a DNA dye must be administered to the gel. In research labs, a **DNA intercalating agent** called Ethidium Bromide is added to the molten gel and will bind to the DNA of the samples when run. Ethidium Bromide can then be visualized on a UV box that will fluoresce the compound and reveal bands where DNA is accumulated. Since Ethidium Bromide is known as a carcinogen, teaching labs will use a safer DNA intercalating agent known as Sybr Green. This can be visualized in a similar fashion but will fluoresce a green color instead.



Agarose gels visualized on a UV transilluminator. Left shows a gel with Ethidium bromide. Right shows a gel with Sybr Green.

Agarose gels are made of and bathed in a buffered solution, usually of Tris-Borate-EDTA (**TBE**) or Tris-Acetate-EDTA (**TAE**). Regardless of the buffered solution, the buffer provides necessary electrolytes for the current to pass through and maintain the pH of the solution.

DNA samples are prepared in a buffer similar to the solution that it will be run in to ensure that the phosphate backbone of the DNA remains deprotonated and moves to the positive electrode. Additionally, **glycerol** or another compound is added to this buffer in order for the solution to sink into the wells without spreading out. A dye is often included in this loading buffer in order to visualize the loading in the wells and to track the relative progression of gel.

Agarose Gel Set-up

File:OSC Microbio 12 02 AgaroseGE.jpg

Click here to watch the "Assembling the Rig & Loading/Running The Gel" video:

External Resources

- Gel Electrophoresis Simulation
- The Structure of DNA http://www.nature.com/scitable/topicpage/discovery-of-dna-structure-and-function-watson-397
- http://learn.genetics.utah.edu/content/science/forensics/

Electrophoresis of Dyes (Activity)

1. Prepare a 1% agarose gel by adding 60ml Tris-Borate-EDTA buffer (TBE) to 0.6g agarose in an Erlenmeyer flask
2. Place the flask in a microwave or on the heat until agarose is melted.
    - Stop periodically and swirl solution and do not permit to boil over.
3. Assemble the casting tray by blocking the ends with tape or plastic gaskets.
4. Place the comb into the center of the casting tray.
5. You may place the casting trays inside a refrigerator and pour the solution into the tray.
6. Wait until the gel is solidified.
7. Carefully separate the gaskets from the tray.
8. Remove the comb and place the casting tray into an electrophoresis chamber.
9. Cover the gel with TBE buffer.
10. Using a micropipettor, load 40-50µl dye samples sequentially into the wells.
11. Cover the electrophoresis chamber with the lid and ensure good contact between electrodes.
    - It is conventional that the **POSITIVE** side of the tank is nearest to you.
    - With the **POSITIVE** side nearest to you, load the samples from left to right.
12. Set the power supply to 100-120V and press the Run button (you should see bubbles at each electrode) and allow to run for at least 40 minutes.
13. After 40 minutes, stop the current and remove the gel in the casting tray.
14. Place tray on a white background and document your gel.

Activity Follow-up

1. What colors were the dyes originally before loading into the wells?

2. How many separate bands of dye are in each well following the run?

3. What does it mean that there are multiple bands in a lane? What does it mean that there is only one band in a lane?

4. What does the length of migration illustrate to us about the properties of the dye molecules?

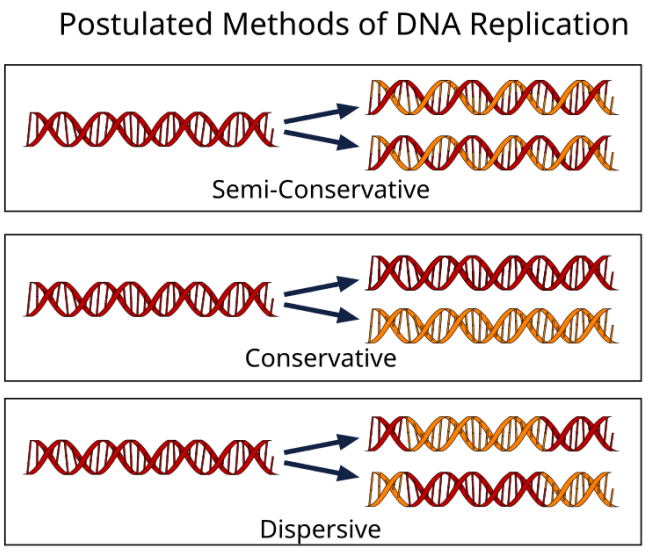
5. In which direction did the dye molecules migrate? What does the direction of migration indicate about the analytes?

6. Are there lanes where there are multiple bands of the SAME color?

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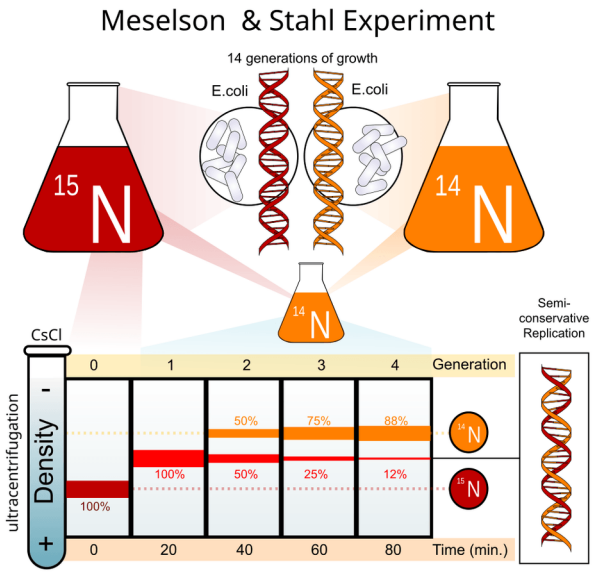
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2.1.8.2: DNA Replication



Francis Crick proposed 3 models for how DNA might replicate. Credit: Jeremy Seto (CC-BY 3.0)

Meselson & Stahl Experiment



Meselson & Stahl revealed semi-conservative replication as the method through use of radiolabeling DNA in bacteria. Credit: Jeremy Seto (CC0)

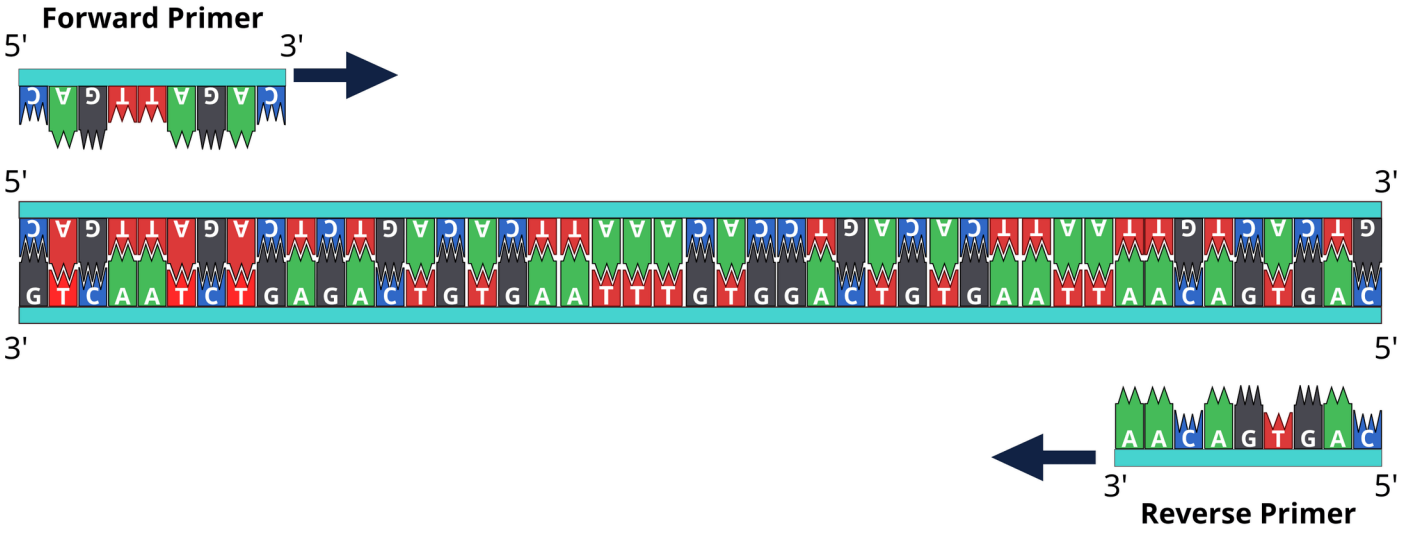
Replication Machinery

File:DNA replication split.svg

File:OSC Microbio 11 02 RepFork.jpg

Polymerase Chain Reaction (PCR)

The **Polymerase Chain Reaction** (**PCR**) is a method of rapidly amplifying or copying a region of DNA in a tube. As the name implies, the technique uses a thermostable **DNA Polymerase** enzyme to mimic in a tube what happens within a cell during DNA replication. The chain reaction permits us to rapidly copy DNA from very minute source material in an exponential way. This technique is used in forensic science, genetic testing and cloning of rare genes. Because of the exponential copying process, a stray cell left behind can provide enough genetic material to make billions of copies of this DNA. The process of PCR can be observed in an animation found at Cold Spring Harbor Laboratory's DNA Learning Center website (http://www.dnalc.org/resources/3d/19-polymerase-chain-reaction.html).



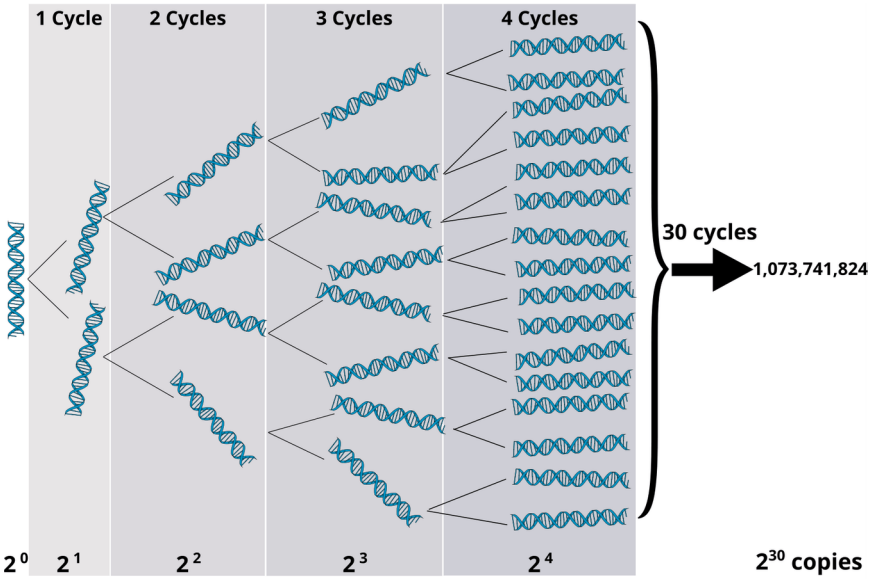
Primers are reverse complementary to one of the 2 strands of DNA. They flank the area of interest and become incorporated into the replicated product. Credit: Jeremy Seto (CC-BY 3.0)

As with any DNA replication process, one needs to start off with a **template**. The template is the source material that is meant for duplication. In this process, scientists are not interested in copying the entirety of the genome, just a small segment of interest. DNA polymerases require primers to begin the polymerization process. **Primers** are designed as small oligonucleotide segments that flank the area of interest. These are short strands of DNA that reverse complement to the DNA area of interest so that the DNA polymerase has a starting point and is guided only to the DNA segment of interest. These primers tend to be about 18-24 bases long.

File:Polymerase chain reaction-en.svg

Polymerase chain reaction However, a double-stranded DNA molecule is already base-paired together into a double helix so our primers can not interact. The first step of PCR is to separate the double-stranded DNA molecule by **denaturing** the H-bonds using high heat (95°C). The primer concentrations are much higher than the original template. The next step of PCR is called **annealing**. During this step, the temperature is reduced to a temperature of about 55°C. This temperature is still hot by our standards but is necessary to enhance the stringency of the correct base pairing of the primers to their targets on the template. The DNA Polymerase used in this process is derived from a bacteria that lives in very high temperatures and does not denature as other proteins would under such conditions (thermostable). The original enzyme was isolated from an organism called *Thermus aquaticus*, so we call the enzyme Taq polymerase or just **Taq** for short. This bacteria lives in hot springs where the temperatures are about 50°C but it thrives at a range between 50-80°C. The temperature is raised again to a higher temperature of 72°C for the polymerase to **extend** (also called **elongation**) or continue the polymerization step from the primer.

PCR is accomplished by cycling rapidly between these three steps: denature, anneal, and extension. The rate-limiting step is the extension which limits the length of DNA to be copied. If the original template is only a single copy, we would have 2 copies after the completion of a cycle. The subsequent cycle would have 4 copies, then 8, then 16, then 32, and so on. The doubling process is exponential so from 1 copy undergoing 30 cycles; we would have 2^{30} or 1,073,741,824 copies. This is over a billion copies in a few hours of time.



Exponential amplification by PCR. Credit: Jeremy Seto (CC-BY 3.0)

External Resources

- Walkthrough of the steps in PCR
  - www.dnalc.org/view/15924-Making-many-copies-of-DNA.html

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2.1.8.3: Variable Number Tandem Repeats

Polymorphisms

The difference in nucleotide sequences between humans lies between 0.1-0.4%. This means that people are greater than 99% similar. But when you look at your classmates around the room, you can see that that small difference amounts to quite a bit of variation within our species. The bulk of these differences aren't even within the coding sequences of genes but lie outside in regulatory regions that change the expression of those genes. Imagine if there were mutations to the coding sequences, this could be very deleterious to the well-being of the organism. We say that the coding sequences of genes that ultimately lead to proteins have **selective pressure** to remain the same. The areas outside of the coding sequences have a reduced and sometimes non-existent selection pressure. These areas are allowed to mutate in sequence and even expand or contract. Areas of changes or differences are called **polymorphic** (many forms). If you were to read a repetitive set of sequences and count the repetition, you' would make mistakes and lose count. Likewise, DNA polymerase will make errors or stutter in areas of repetitiveness and produce polymorphic regions.

Tandem Repeats

A type of polymorphism occurs due to these repeats expanding and contracting in non-coding regions. These regions are called variable number tandem repeats (**VNTRs**)or sometimes short tandem repeats (**STRs**). Any region or location on a chromosome is referred to as **locus** (loci for plural). Scientists use polymorphic loci that are known to contain VNTRs/STRs in order to differentiate people based on their DNA. This is often used in forensic science or in maternity/paternity cases. Any variation of a locus is referred to as an **allele**. In standard genetics, we often think of an allele as a variation of a gene that would result in a difference in a physical manifestation of that gene. In the case of STRs, these alleles are simply a difference in the number of repeats. This means the length of DNA within this locus is either longer or shorter and gives rise to many different alleles. VNTRs are referred to as minisatellites while STRs are called **microsatellites**.

CoDIS

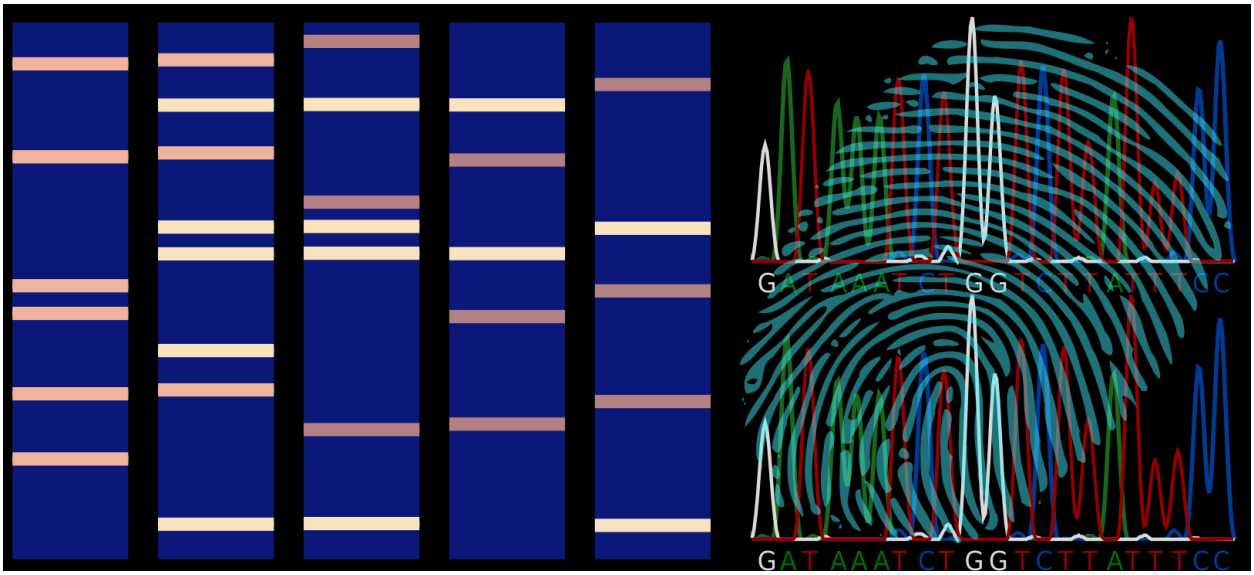


Figure 2.1.8.3.1: DNA fingerprinting. Credit: Helixitta, The Photographer and Jeremy Seto (CC-BY-SA 3.0)

The FBI and local law enforcement agencies have developed a database called the Combined DNA Index System (**CoDIS**) that gathers data on a number of STRs. By establishing the number of repeats of a given locus, law enforcement officials can differentiate individuals based on the repeat length of these alleles. CoDIS uses a set of 20 loci that are tested together. As you would imagine, people are bound to have the same alleles of certain loci, especially if they were related. The use of 20 different loci makes it statistically improbable that 2 different people could be confused with each other. Think about this in terms of

physical traits. As you increase the number of physical traits used to describe someone, you are less likely to confuse that person with someone else based on those combinations of traits. Using the CoDIS loci increases the stringency since there are many alleles for each locus. The twentieth locus in CoDIS (called AMEL) discriminates between male and female.

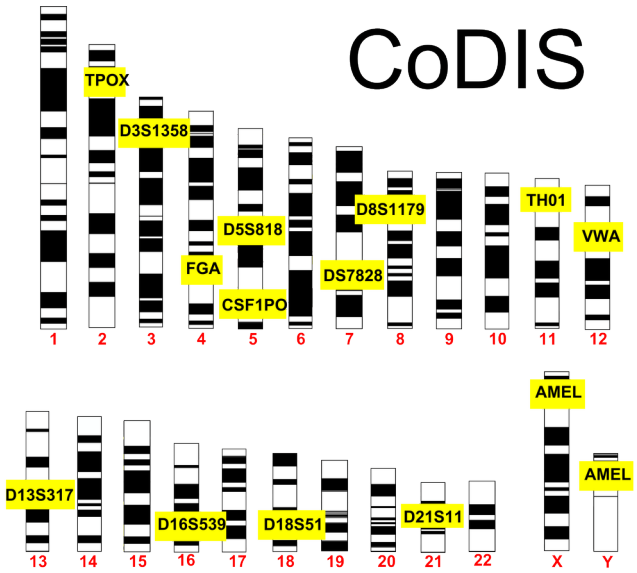


Figure 2.1.8.3.2: **CoDIS STRs:** The FBI utilizes 20 different loci to discriminate between people. AMEL discriminates by gender and is located on the X & Y. Credit: Jeremy Seto (CC-BY-NC-SA)

Crime Scene Investigation

This lab uses a CoDIS locus called TH01. TH01 is a locus on chromosome 11 that has a repeating sequence of TCAT. There are reported to be between 3-14 repeats in this locus. With the exception of X and Y in a male, all chromosomes have a homologous partner. Therefore, each individual will have 2 alleles for each CoDIS locus.

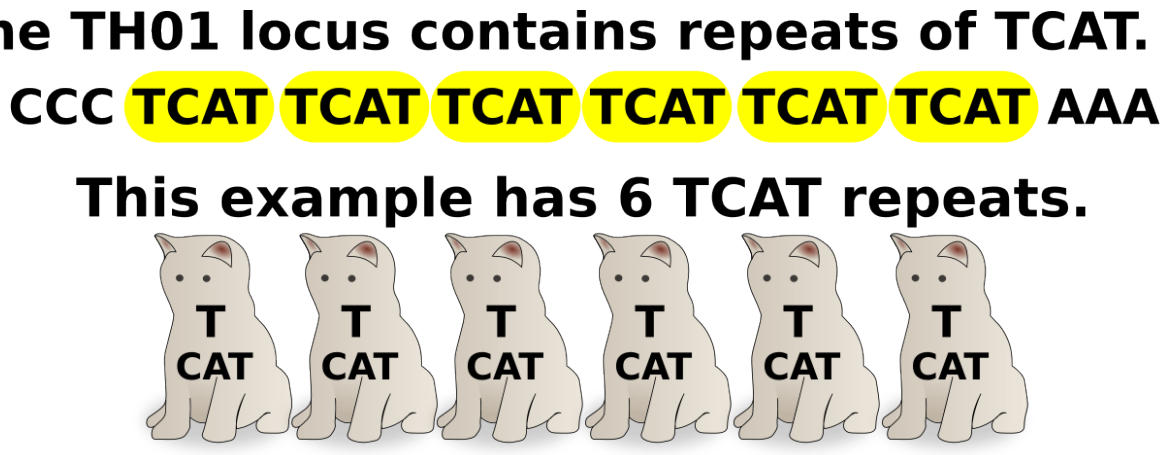


Figure 2.1.8.3.2: **TH01 STR:** Outside of the STR, there are flanking areas of known sequence. The primers that amplify TH01 in PCR recognize these flanking sequences to amplify the TCAT repeats. Credit: Jeremy Seto (CC-BY-NC-SA)

At a crime scene, criminals don't often leave massive amounts of tissue behind. Scant evidence in the form of a few cells found within bodily fluids or stray hairs can be enough to use as DNA evidence. DNA is extracted from these few cells and amplified by PCR using the specific primers that flank the STRs used in CoDIS.

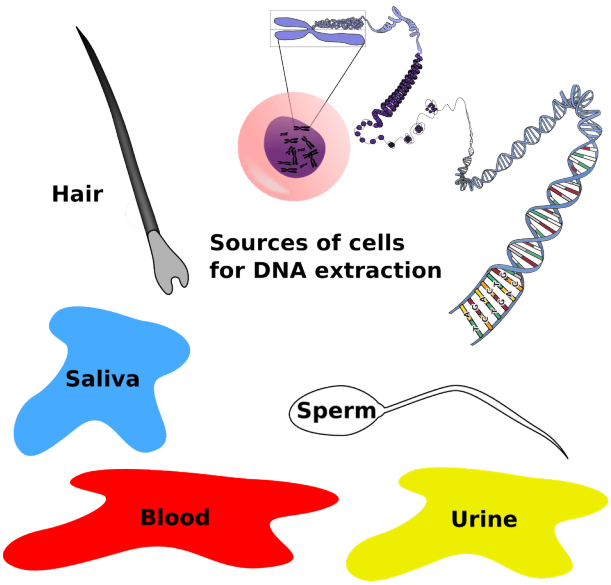


Figure 2.1.8.3.4: **DNA evidence from a crime scene:** DNA can be extracted from cells found from various sources at a crime scene. PCR can amplify this small amount of DNA. Credit: Jeremy Seto (CC-BY-NC-SA)

Amplified DNA will be separated by gel electrophoresis and analyzed. Size reference standards and samples from the crime scene and the putative suspects would be analyzed together. In a paternity test, samples from the mother, the child, and the suspected father would be analyzed in the same manner. A simple cheek swab will supply enough cells for this test.

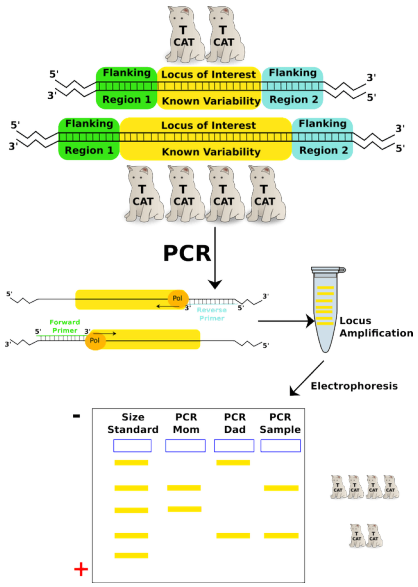


Figure 2.1.8.3.5: **TH01 locus used in a Paternity/Maternity test:** Individual PCR reactions are run for each sample (mom, dad, and child). The TH01 primer pair specifically amplifies the locus. Each amplified sample is run on the same gel to resolve the different alleles of TH01 from each individual. From this test, the sample could be the offspring from these 2 parents but the use of more STRs would make it more definitive. Count the TCATs. Credit: Jeremy Seto (CC-BY-NC-SA)

External Resources

- Flash animation walking through what an STR is: http://www.dnalc.org/view/15981-DNA-variations.html

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2.1.8.4: Propagating DNA in Bacteria

Complementation

George Beadle and Edward Tatum first described the concept that each gene corresponded to an enzyme in a metabolic pathway by exposing the yeast *Neurospora crassa* to mutagenic conditions (Beadle & Tatum, 1941). Following these procedures, Joshua Lederberg continued these studies with Tatum where they generated two mutants strains in *Escherichia coli*. These bacteria were **auxotrophs**, unable to generate some basic nutrients necessary to sustain their growth. The two strains were described as met^- bio^- Thr^+ Leu^+ Thi^+ (Strain A) and Met^+ Bio^+ thr^- leu^- thi^- (Strain B). Strain A can sufficiently synthesize the amino acids threonine, leucine, and the cofactor thiamine while deficient in producing the cofactor biotin and the amino acid methionine while the converse was true of Strain B. When either of these two strains was plated onto minimal media, no growth occurred. Supplementing minimal media with methionine and biotin permitted Strain A to grow as normal. When the two strains were mixed together and plated on minimal media, there was a growth of bacteria. The two strains were capable of complementing each other in some way as if a sexual exchange of genetic material had occurred (Lederberg & Tatum, 1946).

Bacteria are equipped with all the necessary capacities to replicate DNA. Common bacterial species have bee adapted for use in the lab to carry DNA and propagate it for uses in biotechnology. In addition to chromosomal DNA of the bacterial genome, bacteria also have extrachromosomal DNA called **plasmids**. These plasmids replicate independently of the bacterial chromosome and can occur in a high copy. These circular pieces of DNA are modified in labs to carry specific pieces of DNA so they can be studied or used for expression into proteins. Plasmids can naturally carry important traits, including antibiotic resistance. Plasmids are relatively small, ranging in size from 1000 bases to 1,000,000 bases long (1kb-1000kb).

File:Plasmid (english).svg

*Bacterial DNA usually exists as a large **circular chromosome** (red). **Plasmids** are extrachromosomal and autonomously replicating pieces of DNA (blue).*

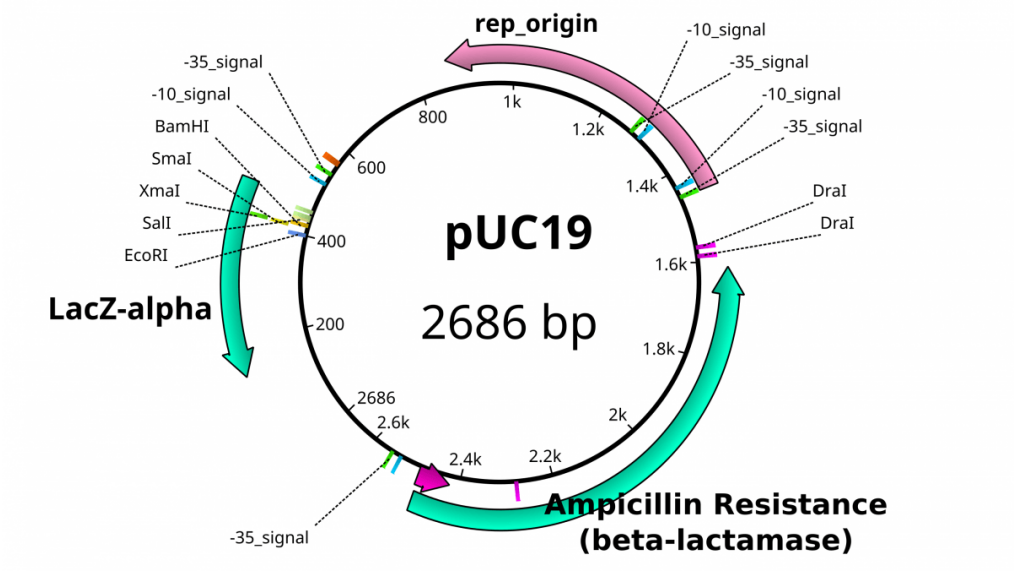
Through a process called **conjugation**, bacteria can “sexually” transfer genetic material to another by passing plasmids through a structure called a **conjugation pilus**.

File:Conjugation.svg

Conjugation process between a plasmid bearing donor and a plasmid-less recipient. The donor creates a conjugation pilus to create a cytosolic bridge with the donor where the plasmid is replicated into the recipient through the rolling circle method of replication. The recipient then becomes competent to act as a donor.

Features of Plasmids

Plasmids that are designed by Biologists to shuttle pieces of DNA for study are referred to as **vectors** because they *move* a piece of DNA.



These plasmid vectors have the same hallmarks as traditional plasmids with the capacity to replicate independently of the bacterial genome. The feature that allows these DNA's to replicate is called an **origin of replication** (ori) that is usually rich in A's and T's. However, these plasmid vectors have the additional properties that make them easy to work with and distinguishable from bacterial plasmids; a selection marker and a multiple cloning site. A **selection marker** usually comes in the form of a gene that encodes resistance to a specific antibiotic. In the pictured plasmid, Ampicillin resistance granted by the β-lactamase gene. The **multiple cloning site** (**MCS**), also known as the polylinker, is the location in which the DNA of interest is incorporated into the vector. MCSs are defined by a set of unique sites where the DNA can be cut by **restriction endonucleases** (**RE**). As the name implies, restriction enzymes are "restricted" in their ability to cut or digest DNA. The restriction that is useful to biologists is usually **palindromic** DNA sequences. Palindromic sequences are the same sequence forwards and backward. Some examples of palindromes: RACE CAR, CIVIC, A MAN A PLAN A CANAL PANAMA. With respect to DNA, there are 2 strands that run antiparallel to each other. Therefore, the reverse complement of one strand is identical to the other.

File:EcoRI restriction enzyme recognition site.svg File:SmaI restriction enzyme recognition site.svg

EcoRI *generates sticky of cohesive ends* ***SmaI*** *generates blunt ends*

Restriction enzymes hydrolyze covalent phosphodiester bonds of the DNA to leave either "sticky/cohesive" ends or "blunt" ends. This distinction in cutting is important because an *EcoRI* sticky end can be used to match up a piece of DNA cut with the same enzyme in order to glue or ligate them back together. While endonucleases cut DNA, **ligases** join them back together. DNA digested with *EcoRI* can be ligated back together with another piece of DNA digested with *EcoRI*, but not to a piece digested with *SmaI*. Another blunt cutter is *EcoRV* with a recognition sequence of GAT | ATC.

By "cutting and pasting" DNA into vectors, we can introduce foreign or exogenous DNA into bacteria. This type of DNA is now called **Recombinant DNA** and is the heart of biotechnology.

Recombinant DNA Technology

File:OSC Microbio 12 01 MolCloning.jpg

File:Gene cloning.svg

Additional Resources

1. http://www.dnalc.org/resources/3d/20-mechanism-of-recombination.html
2. http://www.dnalc.org/view/16705-Animation-34-Genes-can-be-moved-between-species-.html

Questions for Thought

1. Why do you think that the origins of replication are made of A's and T's?

2. What is different about the types of bonds holding the double strands together versus phosphodiester bonds of the DNA backbone?

3. Can DNA be digested with *SmaI* be ligated to DNA digested with *EcoRV*?

4. If so, which enzyme will be able to digest this new DNA?

- *Beadle, G. W.; Tatum, E. L. (1941).* "Genetic Control of Biochemical Reactions in Neurospora". *Proceedings of the National Academy of Sciences.* **27** (11): 499–506. doi:10.1073/pnas.27.11.499. PMC 1078370. PMID 16588492
- *Lederberg J, Tatum EL (1946). "Gene recombination in E. coli". Nature.* ***158*** *(4016): 558. doi:10.1038/158558a0*

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2.1.8.5: Restriction Enzymes

DNA can be cut by **restriction endonucleases** (**RE**). Endonucleases are enzymes that can hydrolyze the nucleic acid polymer by breaking the **phosphodiester** bond between the phosphate and the pentose on the nucleic acid backbone. This is a very strong covalent bond while the weaker hydrogen bonds maintain their interactions and double strandedness.

As the name implies, restriction endonucleases (or restriction enzymes) are "*restricted*" in their ability to cut or digest DNA. The restriction that is useful to biologists is usually **palindromic** DNA sequences. Palindromic sequences are the same sequence forwards and backwards. Some examples of palindromes: RACE CAR, CIVIC, A MAN A PLAN A CANAL PANAMA. With respect to DNA, there are 2 strands that run antiparallel to each other. Therefore, the reverse complement of one strand is identical to the other. Molecular biologists also tend to use these special molecular scissors that recognize palindromes of 6 or 8. By using 6-cutters or 8-cutters, the sequences occur throughout large stretches rarely, but often enough to be of utility.

File:HindIII Restriction site and sticky ends vector.svg

Restriction enzymes hydrolyze covalent phosphodiester bonds of the DNA to leave either "sticky/cohesive" ends or "blunt" ends. This distinction in cutting is important because an *EcoRI* sticky end can be used to match up a piece of DNA cut with the same enzyme in order to glue or ligate them back together. While endonucleases cut DNA, **ligases** join them back together. DNA digested with *EcoRI* can be ligated back together with another piece of DNA digested with *EcoRI*, but not to a piece digested with *SmaI*. Another blunt cutter is *EcoRV* with a recognition sequence of GAT | ATC.

File:EcoRI restriction enzyme recognition site.svg File:SmaI restriction enzyme recognition site.svg

EcoRI *generates sticky of cohesive ends* ***SmaI*** *generates blunt ends*

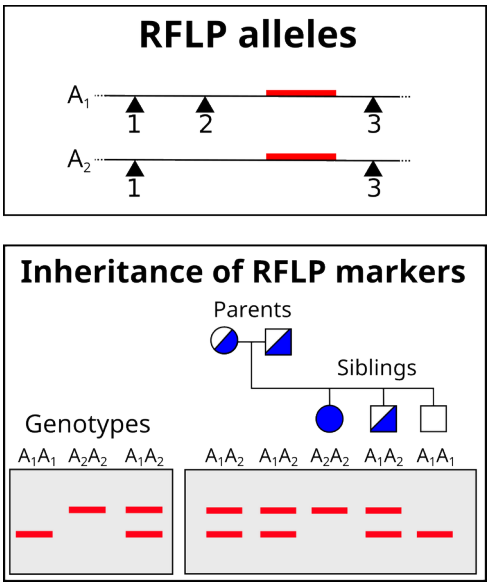
Restriction Digestions

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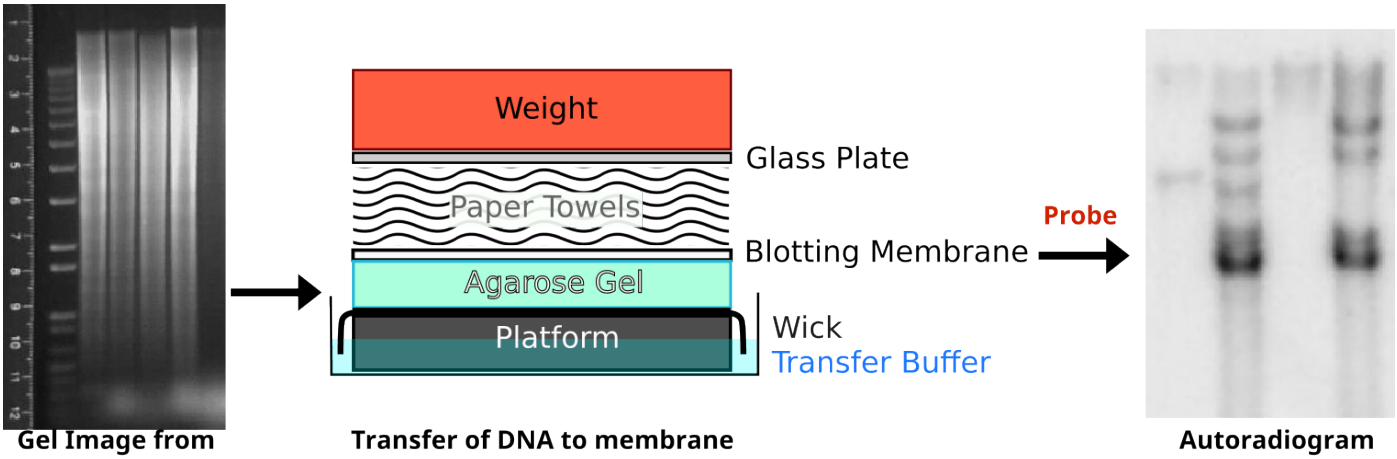
2.1.8.6: DNA Fingerprinting (RFLP)

Restriction fragment length polymorphism (**RFLP**) is a technique that exploits variations in DNA sequences. DNA from differing sources will have variations or **polymorphisms** throughout the sequence. Using **Restriction Enzymes**, these differences in sequences may be teased out. However, if one were to take the entirety of the human genome and chop it up with a restriction enzyme, many indecipherable fragments would be made. In fact, the resulting agarose gel would simply show a large smear of DNA. RFLP analysis requires that a **probe** to a specific area of DNA be used to identify specific locations. Agarose gels would be transferred to a membrane or filter where they would be **hybridized** to these radioactive probes.



Homologous chromosomes with restriction sites noted by triangles. the rectangle sitting on the chromosomes correspond to a probe locus. Credit: Jeremy Seto (CC0)

RFLP analysis was designed for forensic science to discriminate between people. Since people are 2N, they have pairs of homologous chromosomes with the same loci. However, these loci may contain different alleles. In this case, the phenotype for these alleles is the actual sequence that may or may not contain restriction sites. The presence or absence of a restriction site may arise from **single nucleotide polymorphisms** (**SNPs**) that reveal the natural variation between people.
The schematic below illustrates a comparison of restriction profiles between two sources. Note that the probe overlaps a restriction site in one of the alleles. This probe will be able to bind to both fragments given sufficient sequence overlap. Upon resolving on an agarose gel, genomic DNA that does not hybridize with the probe will obscure the locus of interest as a large smear. A filter is placed on top of the agarose and pressed against it to transfer the DNA in a process called **Southern Blotting**. Following a lengthy transfer, the filter is denatured to and incubated with the radioactive probe. To visualize this probe hybridization, a film is exposed to the filter and processed.



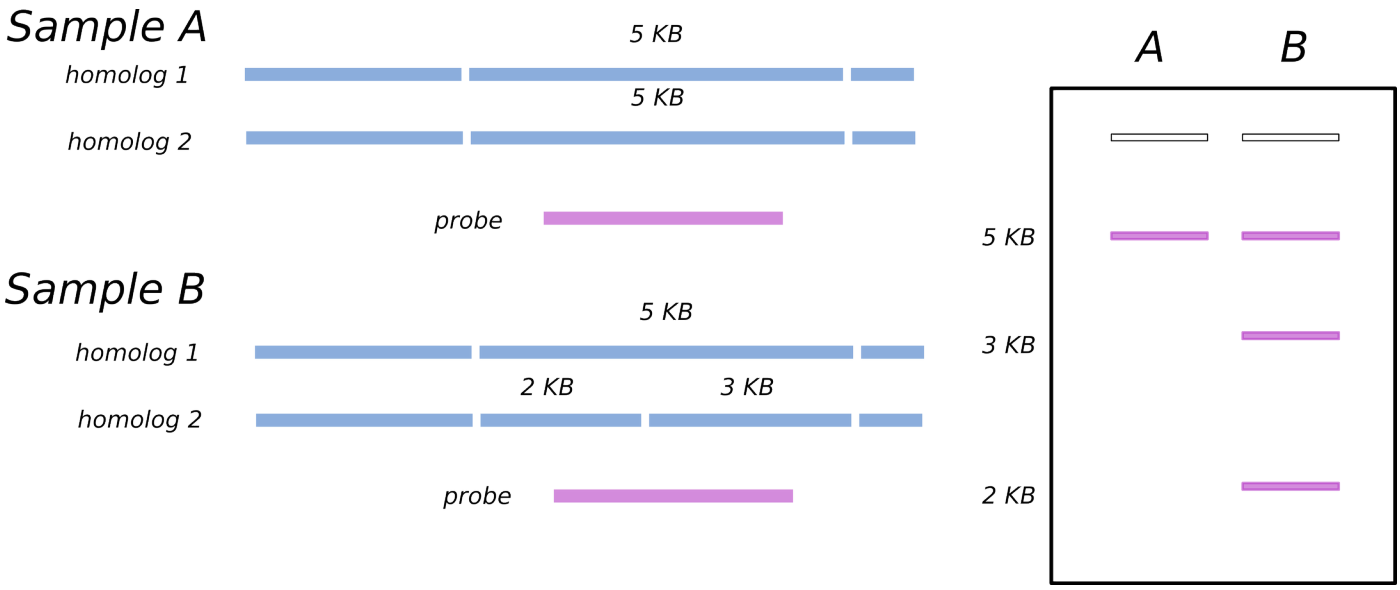
Gel Image from transilluminator

Transfer of DNA to membrane through cappilary action

Autoradiogram

Following restriction digestion, the samples are resolved on an agarose gel. Digestion of genomic DNA will result in a large smear. Following transfer of the DNA onto a membrane through capillary action, the membrane is probed with radioactive probe DNA. The probe binds selectively to complementary sequences to reveal a series of distinct bands. An interactive demonstration of the first DNA fingerprinting.

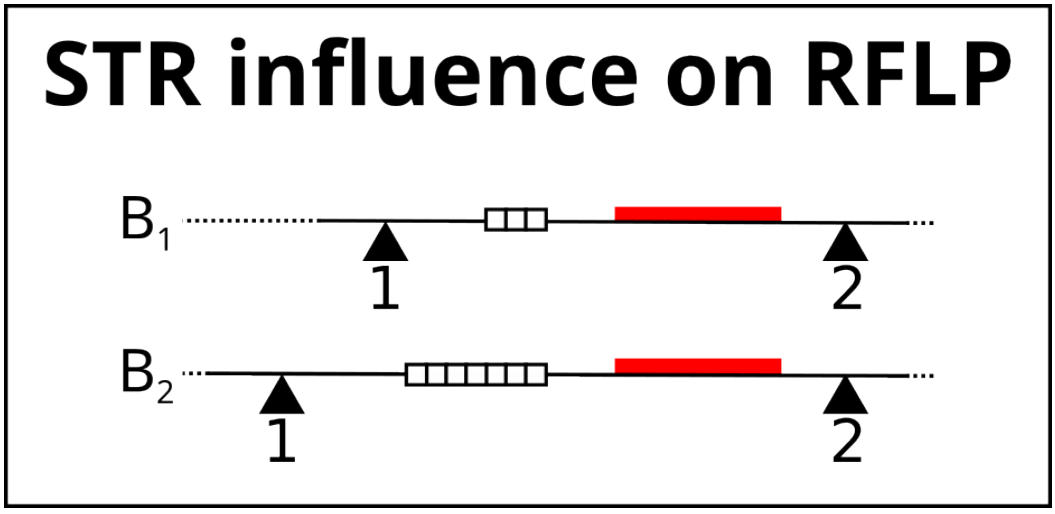
Credit: Oder Zeichner: abigail [or CC-BY-SA-3.0] /Autoradiogram



Sample A only reveals one band after processing because this person is homologous for the same allele. Sample B is heterozygous and reveals three bands.

Credit: Retama (CC-BY-SA 4.0)

RFLPs represent inheritable markers and can reveal relationships between different individuals. A pedigree can illustrate the relationship of the inherited alleles. The technique can be more informative if using multiple probes simultaneously for different loci or to use **multi-locus probes** that hybridize to multiple locations.



RFLPs may arise from differences in the STR/VNTR repeats between restriction sites. Credit: Jeremy Seto (CC0)

While RFLPs can arise from SNPs, they may also be caused by the expansion or contraction of repeated elements between restriction sites. These repeated elements of DNA are referred to as Variable Number Tandem Repeats (VNTR) and illustrate polymorphisms that normally occur in non-coding regions of the genome.

Related Pages

- **Restriction Enzymes**
- **Analyzing DNA**
- **VNTR**
- **Genetics**

External Resources

- Using Tandem Repeats for DNA Fingerprinting (Interview)
- The first DNA fingerprint (Flash Simulation)

DNA Fingerprinting (Activity)

1. The origin of the DNA samples for this exercise will be explained by the Instructor as numerous scenarios may be used (Edvotek Cat. #109).
2. Prepare a 1% agarose gel by adding 60ml Tris-Borate-EDTA buffer (TBE) to 0.6g agarose in an Erlenmeyer flask.
3. Place the flask in a microwave or on the heat until agarose is melted.
    - Stop periodically and swirl the solution. Do not permit to boil over.
4. Assemble the casting tray by blocking the ends with tape or plastic baskets.
5. Place the comb into the casting tray at the **NEGATIVE** end.
6. The instructor will add 6µl Sybr Safe to his/her own gel solution at this time.
7. You may place the casting trays inside a refrigerator and pour the solution into the tray.
8. Wait until the gel is solidified.
9. Carefully separate the gaskets from the tray ensuring not to tear apart the wells made by the comb.
10. Remove the comb and place the casting tray into an electrophoresis chamber.
11. Cover the gel with TBE buffer.
12. Using a micropipettor, load 40-50µl dye samples sequentially into the wells.
13. Cover the electrophoresis chamber with the lid and ensure good contact between electrodes.
    - It is conventional that the **POSITIVE** side of the tank is nearest to you.
    - With the **POSITIVE** side nearest to you, load the samples from left to right.
14. Set the power supply to 100-120V, press the Run button (you should see bubbles at each electrode), and allow to run for at

least 40 minutes.
15. After 40 minutes, stop the current and remove the gel in the casting tray.
16. Slide the gels into the staining solution if they do not include Sybr Safe for visualization the subsequent meeting time.
17. The instructor will slide the gel onto a UV transilluminator behind a shield and show the results to the class.
    - Document the findings of the gel by photographing with your phone.
    - The instructor will discuss the results and ask for you to interpret the findings.

Activity Follow-up

1. Why are the samples loaded at the negative side of the gel?

2. What is the role of the dye in these samples? Should we be alarmed that the samples are all the same color?

3. What does it mean that there are multiple bands in a lane? What does it mean that there is only one band in a lane?

4. What can we conclude from the banding patterns in this forensics or paternity case? Is this sufficient data for these conclusions?

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2.1.8.7: Cheek Cell DNA Extraction

Using Cytobrush

1. Use a sterile cytobrush and insert into mouth.
2. Brush the cytobrush on the inside of your cheek 25 times.
3. Swirl the cytobrush in 100 µl of Chelex suspension (10% w/v).
4. Place a centrifuge tube with Chelex and cell suspension on a 100 °C heat block for 10 minutes.
5. Centrifuge the tubes at maximum speed for 5 minutes.
6. DNA is in the supernatant. Avoid beads at the bottom.
7. Store DNA at -20 °C.

PCR with PCR Beads

1. Add 22 µl of the primer mix (forward and reverse) to beads.
2. Ensure that the bead is dissolved.
3. Add 3 µl of DNA.

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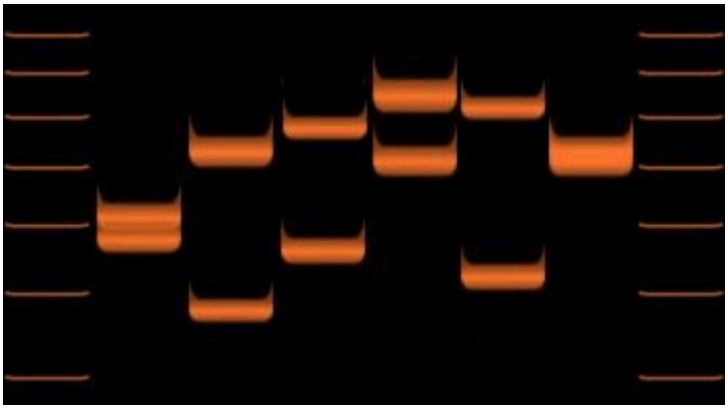
2.1.8.8: D1S80 VNTR (Genotyping)

The minisatellite marker D1S80 is located at 1p35-p36. This VNTR is 16 bases long. With a variation of alleles between 3-24 repeats, the locus displays enough diversity to aid in distinguishing between people. Although this is not a CoDIS marker, the use of multiple loci is required to definitively identify samples. The large repeat (16bp) permits the use of standard agarose gel electrophoresis to explore the diversity of this locus in our lab. PCR products range from 430bp to 814bp long.

- D1S80-for: 5′-GAAACTGGCCTCCAAACACTGCCCGCCG-3′
- D1S80-rev: 5′-GTCTTGTTGGAGATGCACGTGCCCCTTGC-3′

1. PCR the DNA samples extracted from cheek cells using the PCR Beads.
2. Pour 2% agarose into casting apparatus in the refrigerator.
    - 2 gels per class need to be made → 100ml of TBE with 2g agarose
    - Add 5µl SYBR safe solution into the molten agarose before casting.
    - Place 2 sets of combs into the gel → at one end and in the middle
3. Load the gel with a DNA ladder.
    - The sample is from the PCR.
4. Run the gel at 120V for 20 minutes.
5. Visualize on the UV transilluminator.

Example Results



1. How many alleles are visible in each lane?

2. Are the genotypes distinguishable between individuals?

3. Are any of the alleles common between individual samples?

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2.1.8.9: DNA Miniprep by Alkaline Lysis (Activity)

Alkaline Lysis

Once DNA is introduced and carried in bacteria, we would like to isolate the DNA again for further manipulation. In order to do so, bacteria containing the plasmid of interest is grown in a liquid culture of nutrient-rich broth made of yeast extract called Luria-Bertani Broth (**LB**). These cultured bacteria are grown until they are of a high concentration overnight. They are harvested through centrifugation and the broth is removed. The resulting pellet of bacteria is resuspended in a physiological buffer containing the chelator EDTA. A **chelator** is a chemical that removes divalent cations like Ca^{2+} or Mg^{2+} from solution. This is significant because divalent cations are necessary for DNA digesting enzymes to be active. By chelating the ions, the DNA we ultimately wish to purify will be safe from degradation.

After resuspension of the bacteria, an alkaline solution of 0.1N NaOH is mixed into the bacterial mix. This solution also contains an ionic detergent called sodium dodecyl sulfate (**SDS**) that aids in denaturing proteins and disrupting their interactions with the DNA. The mixture becomes viscous as the bacteria burst open and their contents leak into the solution. This basic solution is then neutralized with a potassium acetate buffer at pH5.5. As the solutions mix together, the pH approaches 7 and the potassium interacts with the SDS to cause the precipitation of the genomic chromosomal DNA and proteins. In order to separate the precipitate from the solution, the mixture is centrifuged at high speed to pellet the genomic DNA and protein. The **supernatant**, or solution, is transferred to a column containing a **silica membrane**. Under high salt conditions, DNA adheres to glass or silica. By passing the solution through this column, the plasmid DNA in the supernatant is trapped onto the silica membrane and removed from the solution. Additional washes are used to removed stray contaminants and remove the excessive salt. Plasmid DNA is finally removed from the column through **elution** by a low salt buffer. This low salt buffer is Tris pH 8 with EDTA (**TE**). Plasmid DNA can be stored stably in the TE buffer in the freezer for extended periods.

Exercise 1: Plasmid DNA Mini-Prep by Alkaline Lysis

1. **Inoculate** 2 ml of rich medium (LB, YT, or Terrific Broth) containing the appropriate antibiotic with a single colony of transformed bacteria. Incubate the culture overnight at 37°C with vigorous shaking. (This is what you were provided)
    1. Each group should take 2 cultures
2. **Centrifuge** culture tubes directly at maximum for 5 minutes.
    1. If incapable of spinning in these tubes, transfer 1.5 ml of the culture into a microfuge tube (Eppendorf Tube).
    2. Centrifuge at maximum speed for 30 sec.

3. When centrifugation is complete, pour the broth solution into a container of bleach.
4. **Resuspend** the bacterial pellet in 250 µl of ice-cold P1 solution by vigorous shaking and transfer back into a microcentrifuge tube.

- P1 is a physiological solution of 50mM Tris at pH 8.
- P1 contains a Chelator called EDTA.
    - Chelators bind up excess divalent cations that are required for DNAse activity.

5. **Lyse**: Add 250µl of P2 solution to each bacterial suspension. Close the tube tightly, and mix the contents by inverting the tube gently five times. Do not vortex! Store the tube on ice.

- This is the lysis buffer containing the detergent Sodium Dodecyl Sulfate and NaOH.

6. **Neutralize:** Add 350 µl of ice-cold P3 solution. Close the tube and disperse lysis solution by inverting the tube several times. Store the tube on ice for 3-5 minutes.

- This is the neutralization buffer containing Potassium Acetate.
- Neutralization restores pH to near 7 and also causes the precipitation of genomic DNA and proteins into a gloopy mess (snot-like).

7. Centrifuge the bacterial lysate at maximum speed for 5 minutes in a microfuge.

- Snot-like substances should be tightly packed into a pellet at the bottom of the tube after this step.
- The solution or supernatant contains the plasmid DNA.

8. **Column Purification of DNA**: Transfer the supernatant to a fresh tube with a silica-membrane column

- DNA likes to bind to glass under high salt conditions.
- The white membrane is made of glass fiber.

File:Qiagen Mini Spin Column.svg

9. Centrifuge the supernatant through the column for 1 minute at maximum speed in a microfuge.

- The DNA will be bound to the membrane on the column (silica)

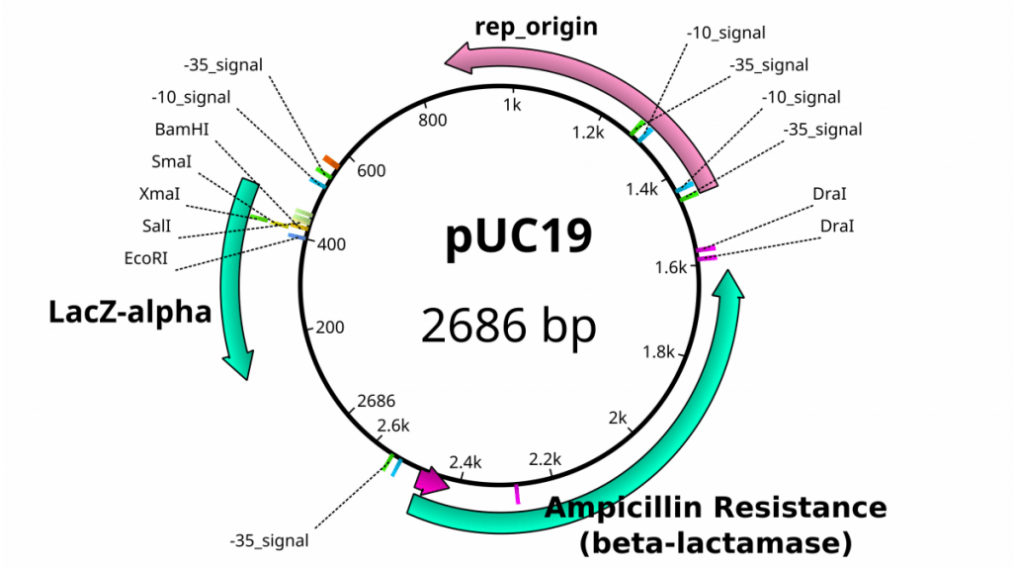
10. **Wash**: Discard flow-through and place column back into the waste tube. Wash column with 500 µl PE. Centrifuge the supernatant through the column for 1 minute at maximum in a microfuge.

- PE is a solution that helps to wash away the non-specifically bound substances

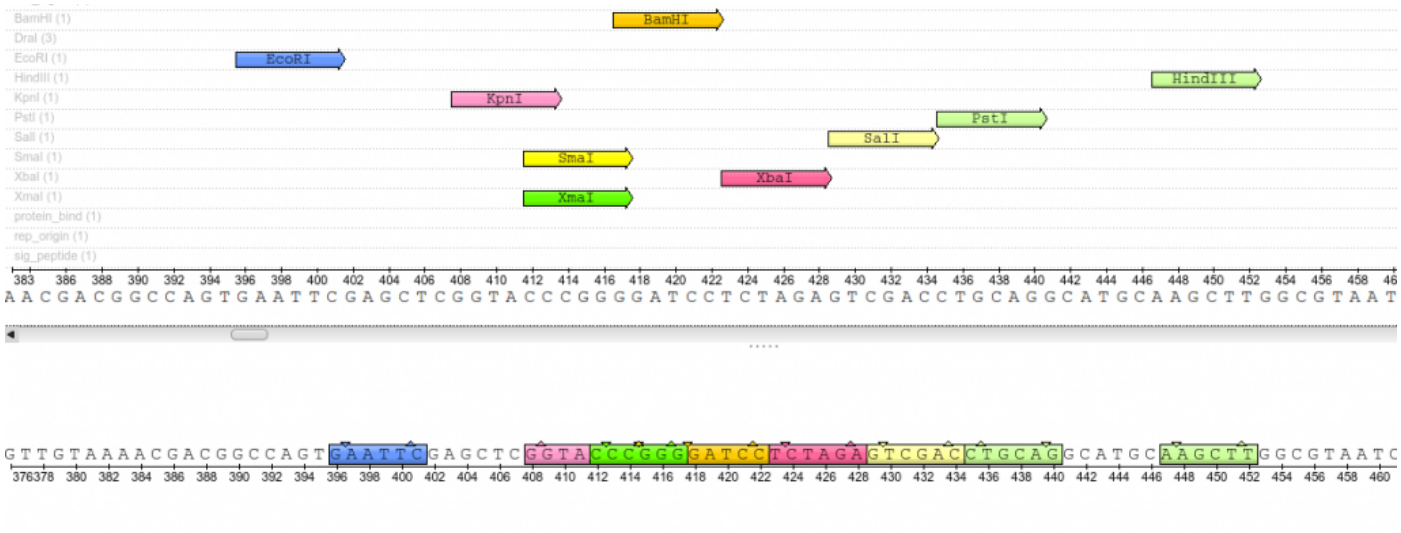
11. Discard flow-through and place column back into the waste tube. Wash Column with 700 µl PE. Centrifuge the supernatant through the column for 1 minute at maximum in a microfuge. Discard flow-through and repeat spin to dry column.
12. Place column in a fresh centrifuge tube and **Elute** the nucleic acids in 50 µl of TE (pH 8.0) by binding for 1 minute and spinning at maximum speed for 1 minute.

Identification of Plasmid DNA

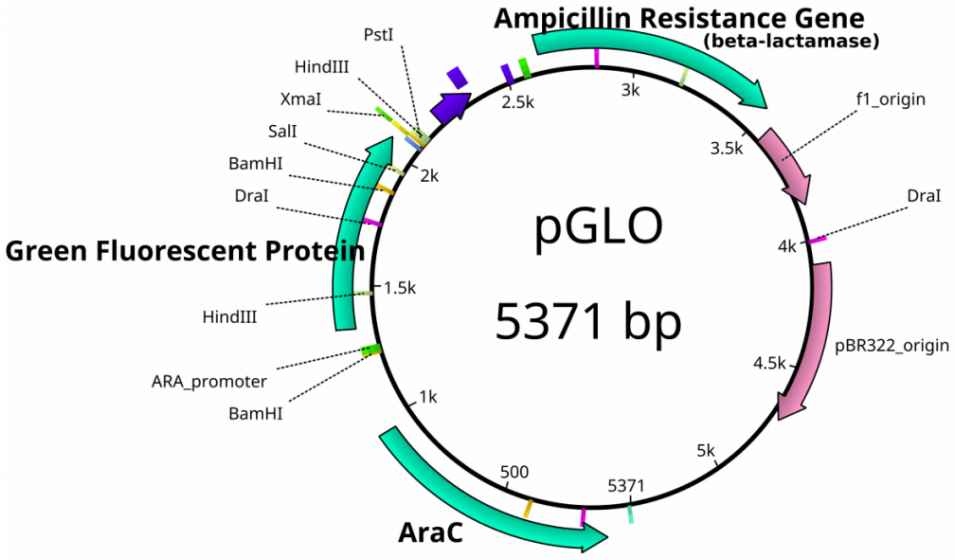
Once plasmids are isolated, they require identification. Plasmid vectors have known sequences and are mapped of their major features. Knowing the sequence of these pieces of DNA means knowing the locations of RE digestion sites. By using Res, digesting plasmids into known sizes aids in the verification of plasmid identity without the need to have the entire plasmid re-sequenced. A common plasmid is called pUC18 or pUC19. The “p” stands for plasmid, the “UC” stands for University of California (where it was designed) and 18 or 19 refer to the difference in the MCS. This plasmid is 2,686 base pairs or ~2.7kb (kilobase) long with a single *EcoRI* site in the MCS. Another plasmid of interest in learning Molecular Biology is called pGlo. This plasmid has a jellyfish gene in the MCS that codes for a protein that will fluoresce green when expressed under UV light. pGlo is 5.4kb long and contains a single *EcoRI* site. Once digested, by an enzyme, these plasmids can be identified based on size separation on an agarose gel. Usually, it is best to identify by using 2 different REs. Digestion is important before size comparisons since circular DNA migrates through agarose differently than linear DNA. Additionally, circular DNA can sometimes be “super-coiled” and lead to very rapid migration despite the size.



Feature map of pUC19 including the locations of some restriction enzymes.



Close up view of the pUC19 Multiple Cloning Site (MCS). These restriction sites appear only once throughout the entirety of the plasmid sequence.

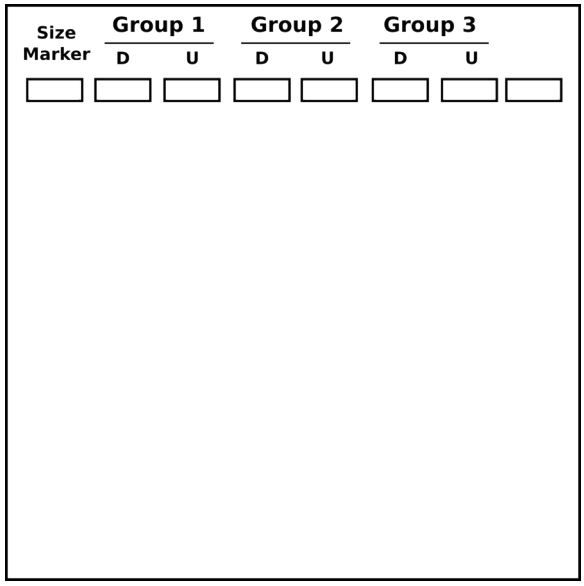


Plasmid feature map of pGlo. This is not a cloning vector, so an MCS does not exist.

Exercise 2: Restriction Digestion Identification of Plasmids

1. The class should prepare 2X 0.8% agarose gels by preparing 0.4g agarose in 50ml TBE buffer.
    1. Melt agarose solution by microwaving for 1 minute.
    2. Add 5µl Sybr Safe solution into the 100ml gel solution.
    3. Pour this solution into a casting tray inside the refrigerator.
    4. Insert a comb.
2. To a new tube add 2µl of plasmid DNA to 8µl of *Eco*RI fast digest mixture.
    1. 1µl Fast Digest Buffer
    2. 1µl Fast Digest *Eco*RI enzyme
    3. 6µl H_2O
3. Incubate at 37°C for 10 minutes.

4. Add 2µl of loading buffer to digestion mixture.
5. In a separate tube, combine 3µl plasmid DNA with 2µl loading buffer and 7µl of H_2O.
6. Load gel with an appropriate size ladder in the first lane, load the Digested plasmid in the next lanes, then the undigested plasmid.
    - 3 groups can load onto one gel
    - D=digested plasmid
    - U=undigested plasmid
7. Run the gel at 110V for 30 minutes and visualize on a UV transilluminator.
8. Document with your camera.



Additional Resources

- For help on this problem, please try out the *In silico* digestion activity.

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2.1.8.10: Sanger Sequencing of DNA

Radioactive Chain Termination

The polymerization of nucleic acids occurs in a 5′ → 3′ direction. The 5′ position has a phosphate group while the 3′ position of the hexose has a hydroxyl group. Polymerization depends on these 2 functional groups in order for a dehydration synthesis reaction to occur and extend the sugar-phosphate backbone of the nucleic acid. In the 1970s, Fred Sanger's group discovered a fundamentally new method of 'reading' the linear DNA sequence using special bases called **chain terminators** or **dideoxynucleotides**. The absence of a hydroxyl group at the 3′ position blocks the polymerization, resulting in termination. This method is still in use today and is called "Sanger dideoxynucleotide chain-termination method". This method originally used a radioactively labeled primer to initiate the sequencing reaction. Four reactions take place where each reaction is intentionally "poisoned" with a dideoxy chain terminator. For example, one reaction will have all 4 dNTPs (deoxynucleotide triphosphates) with the addition to a small amount of ddATP (dideoxyadenosine triphosphate). This reaction will result in a series of premature terminations of the polymerization specifically at different locations where an Adenine would be incorporated.

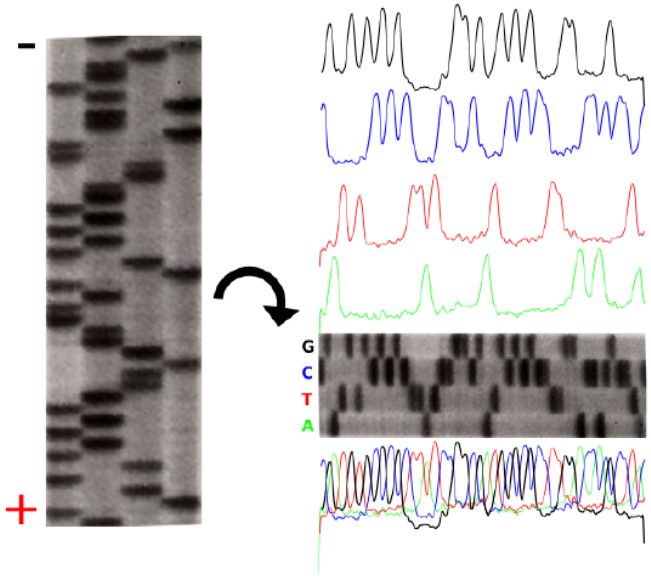
File:Adenosintriphosphat protoniert.svg

dATP is a natural monomer used in the polymerization of DNA. The 3′-OH is the attachment point of the next subsequent nucleotide.

File:Dideoxyadenosine triphosphate.svg

The lack of a 3′-OH in this molecule of ddATP makes it a chain terminator that will prohibit the addition of another nucleotide to the DNA polymer.

The product of these 4 separate sequencing reactions is run on a large polyacrylamide sequencing gels. The smallest fragments run through the gel the fastest and create a ladder-like pattern. This can be visualized through the use of an x-ray film that is sensitive to the radioactivity. Each lane of the gel corresponds to one of the four chain-terminating reactions. The bases are read sequentially from the bottom up and reveal the sequence of the DNA.



The sequencing gel can be manually scored. The profiles of each lane have been created using ImageJ to illustrate the banding pattern and subsequent sequence.

Credit: John Schmidt & Jeremy Seto (CC-BY-SA 3.0)

Fluorescent Chain Termination and Capillary Electrophoresis

File:Sanger-sequencing.svg

Credit: Estevezj (CC-BY-SA 3.0)

Radioactivity is dangerous and undesirable to work with so chain terminators with fluorescent tags were developed. This method synthesizes a series of DNA strands that are specifically fluorescent at the termination that is passed through a capillary electrophoresis system. As the fragments of DNA pass a laser and detector, the different fluorescent signal attributed to each ddNTP is identified and generates a chromatogram to represent the sequence. Fluorescent Chain Terminators are now used in reactions and run through a small capillary. The smallest fragments run through first and are detected to reveal a chromatogram.

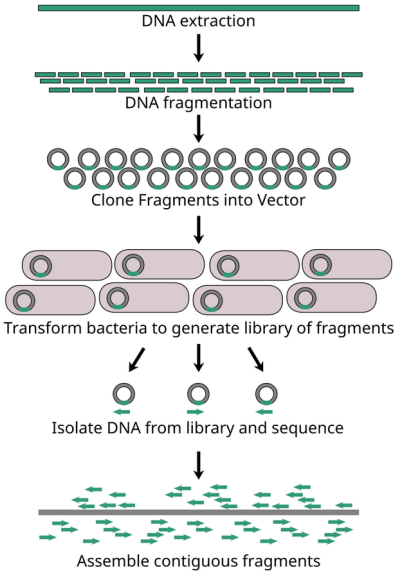
Fluorescent Chromatograms are used to score the nucleotide chain termination. The amplitude of each peak corresponds to the strength or certainty of the nucleotide call. Chromatogram files are usually provided alongside the sequence file with the extension ***.ab1** while the sequence files are provided as a text file in the **fasta** format. More about these files can be found **here**. The ab1 files are extremely important to analyze when there are ambiguity or sequencing errors. These ab1 files can also be used to ascribe a quality score on the base call.

File:DNA sequencing interferogram.png

When there is too much ambiguity in the signal because of multiple peaks, you will often find an **N** in place of one of the 4 nucleotides (A, T, C, and G).

This video (source: www.yourgenome.org CC-BY) illustrates the mechanism of fluorescent chain termination and capillary electrophoresis.

Sequencing Genomes



Credit: Jeremy Seto (CC-BY-NC-SA 3.0)

Traditional sequencing of genomes was a long and tedious process that cloned fragments of genomic DNA into plasmids to generate a genomic DNA library (**gDNA**). These plasmids were individually sequenced using Sanger sequencing methodology and computational was performed to identify overlapping pieces, like a jigsaw puzzle. This assembly would result in a draft scaffold.

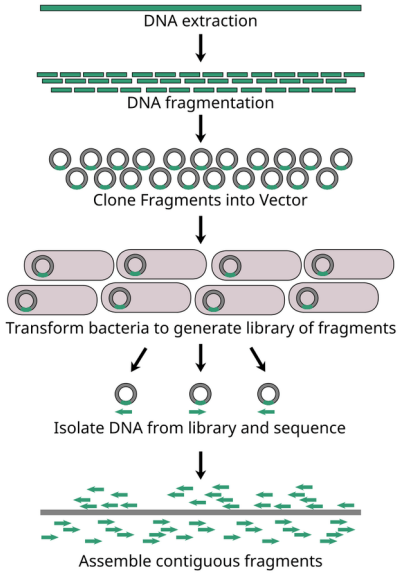
The video below is taken from yourgenome.org (CC-BY) and illustrates the sequencing of the human genome through the shotgun sequencing approach.

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2.1.8.11: Next Gen Sequencing

Expansion of Sequencing Technology



Credit: Jeremy Seto (CC-BY-NC-SA 3.0)

Traditional sequencing of genomes was a long and tedious process that cloned fragments of genomic DNA into plasmids to generate a genomic DNA library (**gDNA**). These plasmids were individually sequenced using Sanger sequencing methodology and computational was performed to identify overlapping pieces, like a jigsaw puzzle. This assembly would result in a draft scaffold.

As technology improved, the cost of sequencing genomes became less expensive. This technology outpaced Moore's Law, a semiconductor projection about the speed of computers as time progressed. A dramatic price decrease in the cost of genome sequencing occurred around 2008 due to technical advances.

File:Cost per Genome.png

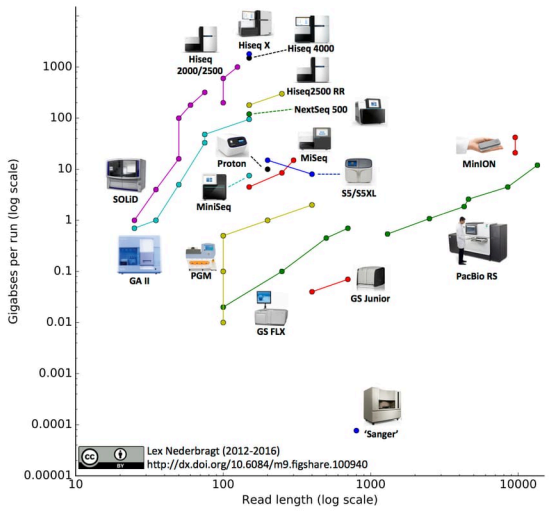
As the cost of genome sequencing decreased, a dramatic increase in genome deposition into Genbank was observed. These deposits reflected small genomes of bacteria and archaea.

File:Number of prokaryotic genomes and sequencing costs.svg

Credit: Estevezj (CC-BY-SA 3.0)

The decrease in per nucleotide sequencing cost came from the parallelization of sequencing. Whereas Sanger Sequencing is capable of sequencing one stretch at a time, a parallel assembly of sequencing reactions has led to the high throughput sequencing often dubbed Next Generation Sequencing (**NGS**).

The Next Generation of Sequencing: High-Throughput Technologies



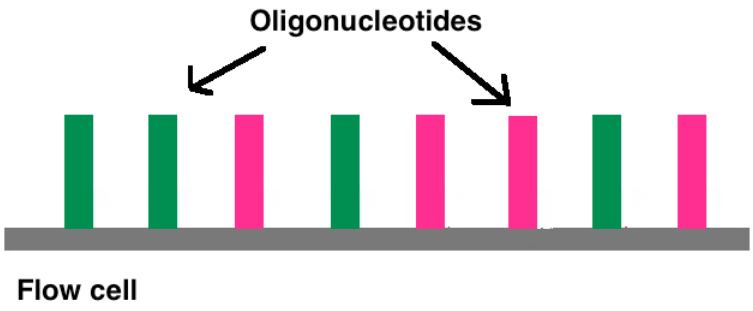
Credit: Lex Nederbragt (CC-BY 3.0)

High Throughput Sequencing Applied to Genome Sequencing (TEDed CC BY-NC-ND 4.0)

Short Read Sequencing by Synthesis

Illumina

Illumina short-read sequencing uses flow cell technology where oligonucleotides complementary to adapter primers are physically seeded.



Flow cell surface with the adapter oligonucleotides. Credit: DMLapato (CC-BY-SA 4.0)

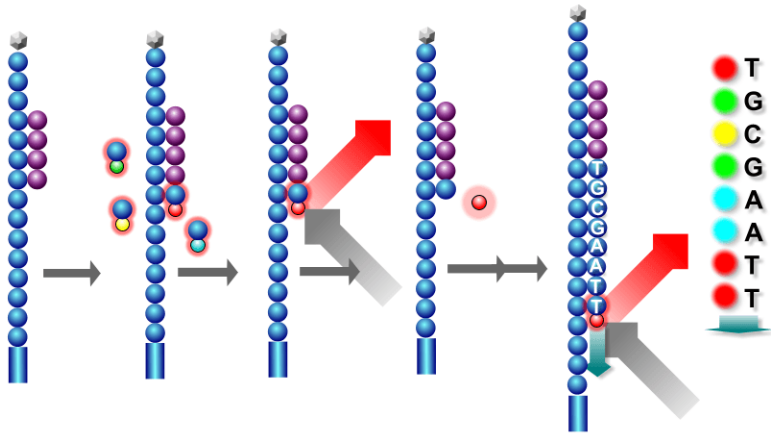
Fragmented DNA sequences are adapted with primers through ligation and hybridized to the flow cell. To increase the signal from sequencing, the short DNA sequences are amplified through a process called bridge amplification or cluster generation.

File:Cluster Generation.png

Cluster generation through bridge amplification. A low numb er of PCR cycles is used. Cluster generation aids in subsequent signal/noise determination.

Credit: DMLapato (CC-BY-SA 4.0)

The flow cell undergoes successive rounds of flooding with a fluorescent nucleotide, permitted to incorporate with a DNA polymerase and washed away. After each flood/wash cycle, fluorescent signals are measured to indicate the incorporation. Specific locations of fluorescence are tracked and consolidated to indicate the sequence at each registered point.



Each flow cycle introduces a fluorescent nucleotide for incorporation. Credit: Abizar Lakdawalla (CC-BY 3.0)

Ion Torrent

Fragmented DNA is ligated to adapter sequences and adhered onto microbeads. The beads are embedded into microwells on a semiconductor. Ion Torrent performs the sequencing reactions in an unbuffered solution since the semiconductor acts as a pH meter to identify nucleotide incorporation. Standard nucleotides are flooded onto the chip and incorporated. Because nucleotide incorporation creates a proton (H^+), a microenvironment of low pH is detected in the unbuffered solution.

Single-Molecule Real-Time Sequencing

Pac Bio

Pac Bio uses nano wells with covalent-bonded DNA polymerase to sequence individual molecules of DNA. Fluorescent nucleotides are incorporated during synthesis reactions and real-time incorporation can be measured. Pac Bio sequencing has the advantage of sequencing fragments of 10-20kb, in stark contrast to the short-read methods.

Oxford Nanopore

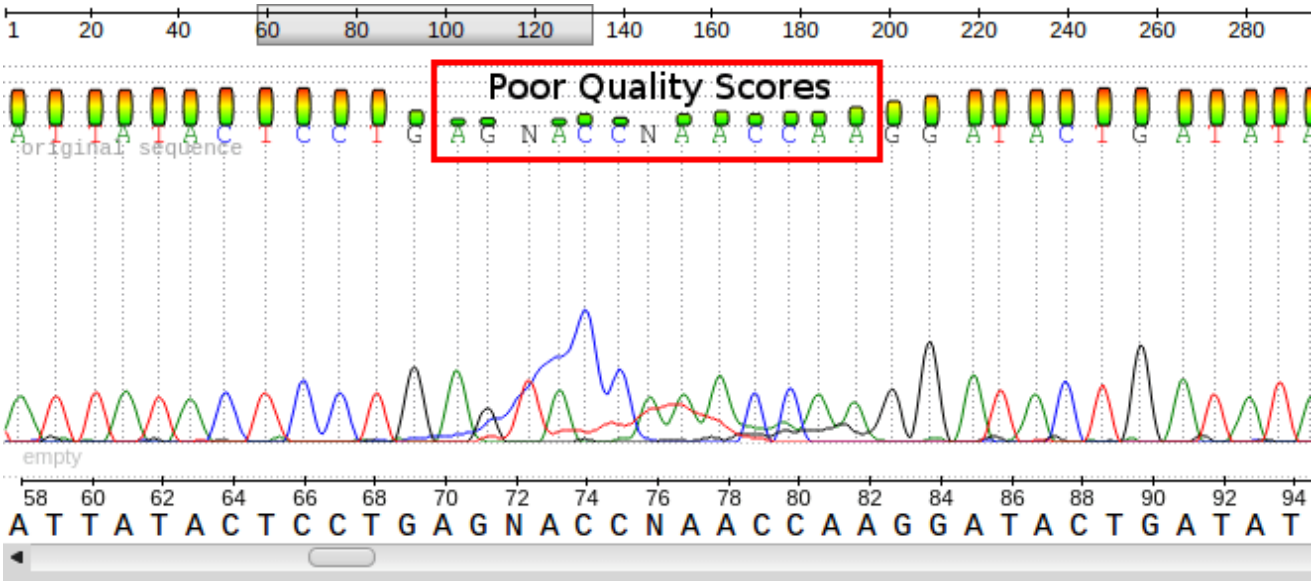
File:AHL DNA.GIF

Credit: George Church (CC-BY 3.0)

Oxford Nanopore utilizes the protein alpha-hemolysin integrated onto a semiconductor chip. The pore size of the protein is the correct ssize for a single DNA molecule to fit through. A DNA Polymerase molecule is linked to the opening of the pore where the replicated DNA is fed through. As the DNA traverses the pore, the voltage changes are measured and mapped to the qualities of specific bases.

Link: youtu.be/BNz880V52rQ

Sequence Output



A sample ab1 file displaying the base calls, the chromatograms and the quality scores for each base. Notice the poor quality in the red box and the corresponding peaks/bases.

The output file of the next-generation sequencing methods utilizes the **fastq** format. Like a **fasta** file, there is a header that describes the sequence. The first line is the header or title line which begins with '**@**' (remember that fasta begins with '**>**'). The second line is the actual raw sequence (once again similar to fasta). The third line has no meaning while the fourth line is filled with symbols as long as the sequence line. This last line is the quality score of the base call. As with the Sanger sequencing, there may be ambiguity with the base call of the sequence and the certainty is maintained in the quality score.

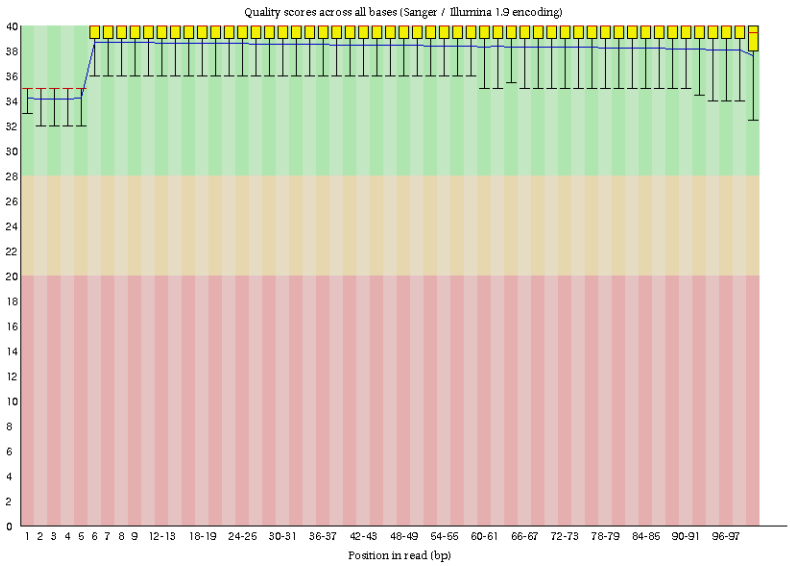
```
@GWZHISEQ02:207:H3VYHBCXX:1:1101:1465:1989 1:N:0:GTGGCC
AGAAGCCAAACGCATCATGGAAAAGCAGTTGCTCGAAGAACTCGAGCGACAGAGACAAGCTGAGCTTGCAGCACAAAAAGCCAGAGAGGAGGAAGAGAGAG
+
DBDDDIHIHHIIIHIHHHHIIIIIGHIIIIIIIIIIHIHHHEHHIIHIIIIIIIHIHIIIIIHHIIIHHHIIIHIHIHIHIIHIHHH?@HHHIIHIHHHIGG
@GWZHISEQ02:207:H3VYHBCXX:1:1101:1715:1978 1:N:0:GTGGCC
NTACAGCCACGTGGAACCTGAGCACCATGAAGTCCCCCAAACCAGTGGGTTTTAAAGCAATCACCACAAAACTCCCTTTTTAGCTTTTGCTTGGGGTGGGG
+
#<DDDIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIHIIIHIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII
@GWZHISEQ02:207:H3VYHBCXX:1:1101:1695:1987 1:N:0:GTGGCC
GCCAAGCAAGCCTGCCCCTCAGCCCCCAGAGTGCACCCAATGACCCCCCTCCCTTGGGGTGACTCCTGATGAAGCACAACTCCCCACAGGGCCCCTAACCC
+
@BDADHIIIHIHIIIIHHIHIIHIIICHDHIIHIGEHIIHGFEHHHIIIIIIIIHHHHCHDIHHHIIHHIHIICCHFEHI?FGHIGIGGHGHHHHHIHHHH
@GWZHISEQ02:207:H3VYHBCXX:1:1101:1915:1948 1:N:0:GTGGCC
NAGTGTCTTTATTATTTATATTTTTTAAAAGCCAAACAATTCCAGCATACTCGTGATAACTTTGAGGATTAGATACATTTTCTTAACATTTTTTTCCCTTT
+
#<<<DGHIIIGIIIIII1GHIIIIIIHIIIHEHHIIHHIIIHGEHIHIIIIIGIIIIIIFHHEHIIIIGHIEEHIIIIIIIIIIHIHHHHIIIIIGHIHII
@GWZHISEQ02:207:H3VYHBCXX:1:1101:2116:1940 1:N:0:GTGGCC
NGAGGGATAATCAGGCCGGACCTGAAGGGAGCGGAGCTGCAGCTACCGGAGTTGGATCGGTCATGGGACTTTGCAAGTGCCCCAAGAGGAAGGTGACGAAC
+
#<DBDIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIHIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII
```

Sample fastq file displaying 5 short-read sequences.

Phred scores were developed to assess the quality of the base calls arising from fluorescent Sanger sequencing during the Human Genome Project. The phred program scans the peaks of the chromatogram and scores based on the certainty or accuracy of the call. The scores are logarithmically based and scores greater than 20 represent greater than 99% accuracy of the base call.

Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%
60	1 in 1,000,000	99.9999%

Using the phred scores embedded in the last line of fastq files, poor quality reads can be removed. Using a program like FastQC permits the assessment of the reads and produces the graphical representation of quality.



FastQC quality output illustrating the Phred score for each base call.

This short read sequence of about 100 nucleotides has all bases made at greater than 30, or > 99.9% accuracy.

Assembly and Alignment

Sequences from short reads must be assembled into a usable sequence. To do so, a **reference genome** may aid in the assembly after adapter sequences are trimmed using automated methods. In the case that there is no reference genome, a related species may be used or a more computationally intensive process of ***de novo* assembly** must take place. With *de novo* assembly, it may be useful to have some long reads performed with PacBio to create scaffolds for generating the assembly into contiguous sequences, or **contigs**.

File:Mapping Reads.png

RNA-Seq

File:Journal.pcbi.1004393.g002.png

Credit: Malachi Griffith, Jason R. Walker, Nicholas C. Spies, Benjamin J. Ainscough, Obi L. Griffith (CC-BY 4.0) https://doi.org/10.1371/journal.pcbi.1004393

File:RNA-Seq-alignment.png

Credit: Rgocs (CC-BY)

RT-PCR and **RT-qPCR** can be used to measure the abundance of specific transcripts in a fairly low throughput way. Leveraging the concept of Reverse Transcription and coupling that to high-throughput sequencing technologies, transcripts can be sequenced and mapped to a genome to depict the quantity of transcripts as represented by the number of reads.

Given sufficient read coverage, novel splice isoforms can also be identified as different exon-exon junctions are identified.

The general workflow of RNA-Seq analysis follows:

File:RNASeqWorkflow2016.png

Credit: Salubrious Toxin (CC-BY-SA 4.0)

Advanced Video of Variant Calling from NGS to Decipher a Genetic Susceptibility

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2.1.9: GENETICS

2.1.9.1: Introduction
2.1.9.2: Bitter Taste (Activity)
2.1.9.3: Sex-linked Genes
2.1.9.4: Probability and Chi-Square Analysis
2.1.9.5: Non-Mendelian Genetics
2.1.9.6: Hardy-Weinberg and Population Genetics

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2.1.9.1: Introduction

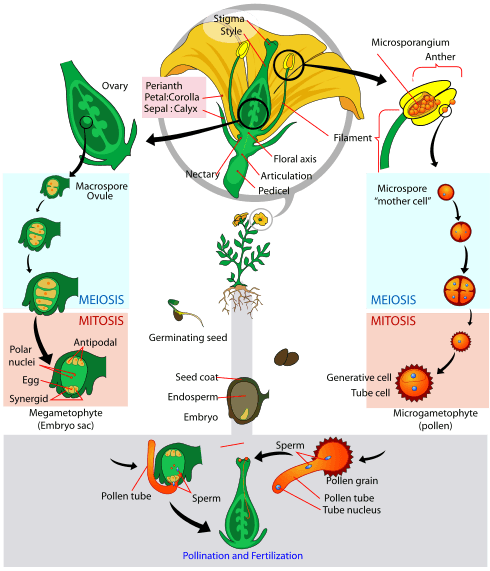


Writing the Rules of Heredity:

In the mid-1800s, an Augustinian friar named Gregor Mendel formalized quantitative observations on heredity in the pea plant. He undertook hybridization experiments that utilized purebred or ***true-breeding*** plants with specific qualities over many generations to observe the passage of these traits. Some of these physical traits included: seed shape, flower color, plant height, and pod shape.

File:Snow pea flowers.jpg The pea plant (*Pisum sativum*) offered a great advantage of being able to control the fertilization process and having large quantities of offspring in a short period of time. In a simple experiment of tracking the passage of a single trait (**monohybrid cross**) like flower color through multiple generations, he was able to formulate rules of heredity. In this case, pea plants either produced white flowers or purple flowers for many generations (true-breeding purple flower or true breeding white flower). These true-breeding plants are referred to as the **Parental Generation** (**P**). By removing the male parts of the pea flower (anthers containing pollen), Mendel was able to control for self-pollination. The hybridization came from applying the pollen from one true-breeding plant to the female part (the pistil) of the opposite true-breeding plant. The subsequent offspring are referred to as the **First Filial Generation** ($\mathbf{F_1}$). In the first generation, all the flowers are purple. Permitting self-pollination generates a **Second Filial Generation** ($\mathbf{F_2}$). This generation sees the re-emergence of the white-flowered plants in an approximate ratio of 3 purple-flowered to 1 white-flowered plants.

Pea flowers



Male and female parts of flowers. Mendel removed the anthers containing pollen to prohibit self-pollination and selectively applied the pollen to stigmas in order to control the "hybridization".

The loss of one variant on the trait in the F_1 plants with the re-emergence in the F_2prompted Mendel to propose that each individual contained 2 hereditary particles where each offspring would inherit 1 of these particles from each parent. Furthermore, the loss of one of the variants in the F_1 was explained by one variant masking the other, as he explained as being **dominant**. The re-emergence of the masked variation, or **recessive** trait, in the next generation was due to both particles being of the masked variety. We now refer to these hereditary particles as **genes** and the variants of the traits as **alleles**.

File:Mendel seven characters.svg

Mendel's Rules of Segregation and Dominance:

The observations and conclusions that Mendel made from the monohybrid cross identified that inheritance of a single trait could be described as passage of genes (particles) from parents to offspring. Each individual normally contained two particles and these particles would separate during the production of gametes. During sexual reproduction, each parent would contribute one of these particles to reconstitute offspring with 2 particles. In the modern language, we refer to the genetic make-up of the two "particles" (in this case, alleles) as the **genotype** and the physical manifestation of the traits as the **phenotype**. Therefore, Mendel's first rues of inheritance are as follows:

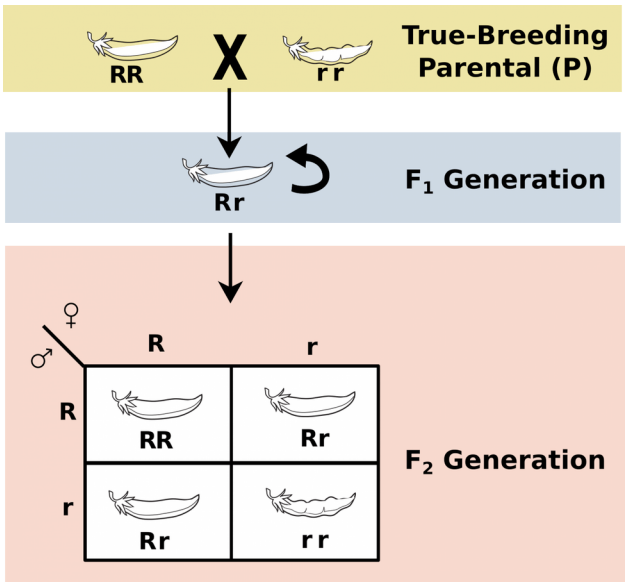
1. **Law of Segregation**
    - During gamete formation, the alleles for each gene segregate from each other so that each gamete carries only one allele for each gene
2. **Law of Dominance**
    - An organism with at least one dominant allele will have the phenotype of the dominant allele.
    - The recessive phenotype will only appear when the genotype contains 2 recessive alleles. This is referred to as **homozygous recessive**
    - The dominant phenotype will occur when the genotype contains either 2 dominant alleles (**homozygous dominant**) or one dominant and one recessive (**heterozygous**)

File:Punnett square mendel flowers.svg

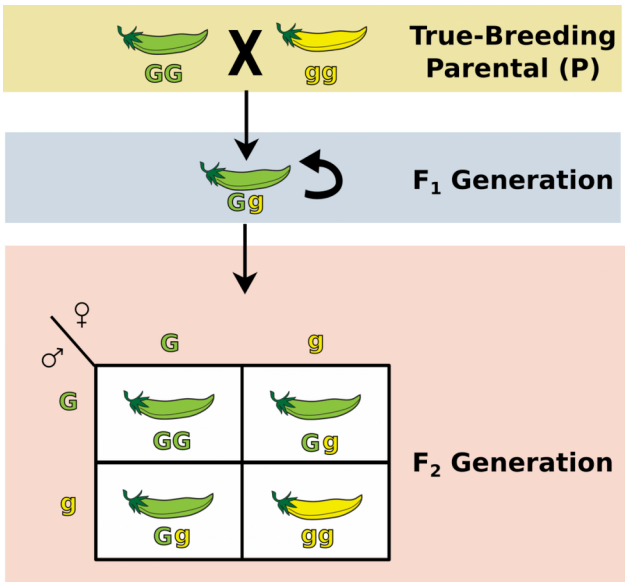
The F_1 cross (Punnett square) illustrating flower color inheritance in the F_2

Punnett Square is a tool devised to make predictions about the probability of traits observed in the offspring in the F_2 generation and illustrate the segregation during gamete formation.

The Single Trait Cross (Monohybrid Cross):



Monohybrid cross (one trait cross) observing the pod shape of peas.



Monohybrid cross (on trait cross) observing the pod color of peas.

Corn Coloration in an F_2 Population (Activity):

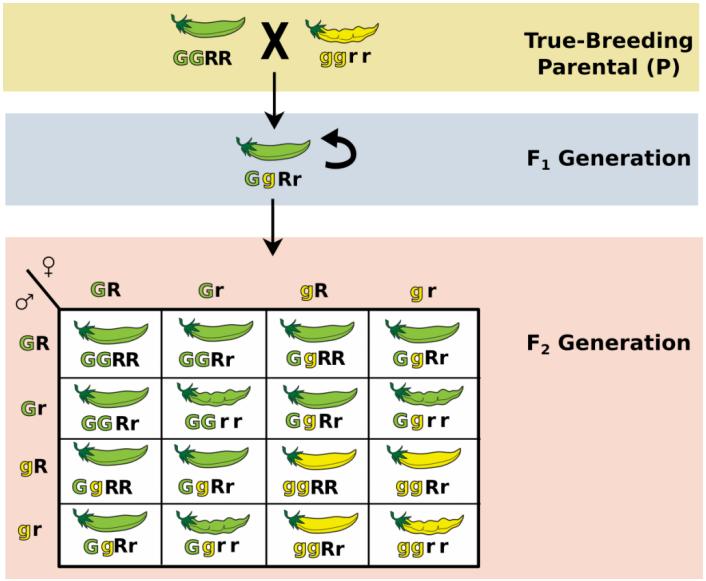


A corn cob contains hundreds of kernels. Each kernel is a seed that represents an individual organism. In the cob, we can easily see kernel color as a phenotype.

1. Retrieve an F_2 corn cob
2. Count a total of 100 kernels
    1. Tally the number of Yellow Kernels within that 100 (in the dried state, anything yellow or honey-colored counts as yellow)
    2. Tally the number of Purple Kernels within that 100 (in the dried state, purple colored kernels may appear brown)
    3. Ignore any speckled kernels that may have yellow and purple within them
3. Compare numbers with the class as a whole
4. From the numbers:
    1. Is there a dominant color?_____
    2. Which is dominant, if there is?_____
    3. Create a Punnett square to illustrate the expected number of each color in a simple dominant:recessive paradigm.

The Two Trait Cross (Dihybrid Cross):

Mendel continued his experimentation where he looked at two traits. These two trait crosses are called **dihybrid crosses**. While the monohybrid cross would yield 3:1 ratio of the phenotypes, the dihybrid crosses would yield 9:3:3:1 ratio of all the combinations of each phenotype.



Mendel's Rule of Independent Assortment:

The dihybrid cross revealed another law of inheritance to Mendel. By observing the 9:3:3:1 ratio, Mendel concluded that traits were not tied to each other. That is to say, if a pea pod was yellow, it could still be either smooth or wrinkled in texture. This lack of linkage between genes yielding different characteristics was dubbed the **Law of Independent Assortment**. Genes for different traits can segregate independently during the formation of gametes.

Kernel Coloration and Texture in an F_2 Population (Activity):



1. Retrieve a dihybrid F_2 corn cob
2. Count a total of 200 kernels
    1. Tally the number of Yellow Kernels that are rounded and smooth in texture
    2. Tally the number of Yellow Kernels that are shriveled and wrinkly in texture (honey-colored)
    3. Tally the number of Purple Kernels within that rounded and smooth in texture
    4. Tally the number of Purple Kernels within that are shriveled and wrinkly in texture
    5. Ignore any speckled kernels that may have yellow and purple within them
3. Compare numbers with the class as a whole

4. Each kernel constitutes an individual organism (a seed that can give rise to a whole new plant). From the numbers:
    1. Is there a dominant texture (smooth or shriveled)?_____
    2. Which is dominant, if there is?_____
    3. Is there a color that always pairs with a texture or do these characteristics *assort independently*?_____
    4. Create a Punnett square to illustrate the expected number of each color/texture combination in a simple dominant:recessive paradigm.

Modern Study of Genetics:

Human variation and diversity: SNPs

Epigenetics: Beyond the DNA

Epigenetics: Environment Shaping Expression

Additional Resources:

- Mendelian Inheritance http://www.visionlearning.com/en/library/Biology/2/Mendel-and-Inheritance/129
- Independent Assortment http://www.visionlearning.com/en/library/Biology/2/Mendel-and-Independent-Assortment/145
- Genetics and Statistical Analysis http://www.nature.com/scitable/topicpage/genetics-and-statistical-analysis-34592
- Genetic Linkage http://www.nature.com/scitable/topicpage/discovery-and-types-of-genetic-linkage-500
- DNA as the Genetic Material http://www.nature.com/scitable/topicpage/discovery-of-dna-as-the-hereditary-material-340
- What is a gene? http://www.nature.com/scitable/topicpage/what-is-a-gene-colinearity-and-transcription-430

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2.1.9.2: Bitter Taste (Activity)

Genetics leaves a bad taste in my mouth... or not

Some of our personal preferences arise from the way we were brought up. Culture plays a role in our likes and dislikes. Likewise, our experiences play a role in how we respond to certain stimuli. Another major factor that plays a role in our preferences comes wired in our genome. The DNA in our cells is the instruction manual for who we are. We are programmed to seek out things of nutritive values in order to acquire raw materials like carbohydrates, proteins, and lipids. In our search for nutritive compounds, we have learned to avoid things that don't taste good. Bitter things have a tendency to be associated with toxic compounds in nature. When eating a food item for the first time, molecules hit our tongue and stimulate multiple sensations: sweet, sour, salty, savory, and bitter. Attributed to these multiple taste types are a diverse family of receptors that bind to the molecules that result in our perception of these sensations. Something bitter might make us learn to avoid this food item in the future.

One type of bitter receptor senses the presence of a chemical called phenylthiocarbamide (**PTC**). This chemical chemically resembles toxic compounds found in plants but is non-toxic. The ability to taste PTC comes from a gene called *TAS2R38*. This gene encodes a protein that on our tongues that communicates the bitterness of this chemical. There are two common alleles of this gene with at least five more uncommon variants. Within the two common forms, a **single nucleotide polymorphism** (**SNP**) is responsible for changing one amino acid in the receptor. It's this difference of one amino acid that results in the ability of the receptor to either respond or not respond to PTC. We inherit one copy of the gene from our father and one copy from our mother. How our parents' gametes formed and what alleles we received during the fertilization event determines how we respond to this chemical. Because we each have 2 copies of this gene, we can utilize simple Mendelian genetics to understand which allele is dominant or recessive.

1. Place a piece of "Control" paper on the tongue and indicate if there is a taste.
2. Place a piece of "PTC" paper on the tongue and indicate if there is a taste and the taste severity.
3. Fill out the table for the class to identify how many non-tasters, tasters, or super-tasters there are.
4. Indicate if you believe the trait is dominant or recessive (ability to taste or not taste).
5. Assign a descriptor allele for the dominant (a capital letter) or the recessive (a lowercase letter) and draw a Punnet square for the F_2 generation of 2 Heterozygous parents.
6. Compare the class tally of tasters and non-tasters in the class and discuss with your instructor if there is a clear dominance of this trait.

Table: PTC Tasting Tally

Phenotypes	Number	% Total
PTC Tasters (Dominant or Recessive)		
PTC Non-tasters (Dominant or Recessive)		
Total		

Questions:

1. How do you explain the presence of those who can't taste PTC, those who can taste it and those who really can't stand the taste of it?

2. This chemical is non-toxic and doesn't exist in nature. Do you think there is a **selective pressure** that confers an advantage to those who do taste it?

Exercise: Coding Bitterness:

Prior to this exercise, review the Central Dogma.

The full coding sequence of ***TAS2R38*** is 1,002 bases (334 amino acids) long. A segment of the gene is shown below where the SNP (in red) occurs. Variant 1 is the version of the gene that encodes for the ability to taste PTC. Variant 2 is the version of the gene that is unable to bind to PTC. This SNP mutation is called a **missense** mutation because it changes the amino acid. Some mutations cause the insertion of a premature stop codon. This **nonsense mutation** results in a truncated protein and can be disastrous to the function. We already know that the simple substitution of one nucleotide translates to a change in one amino acid and determines the ability to taste PTC. Imagine if a large group of amino acids from the protein was missing.

With the template strand ("Complement") information:

1. Write the sequence of the coding strand.
2. Write the sequence of the mRNA
3. Use the Genetic Code Chart to translate the amino acid sequence

Variant 1
Coding Strand : 5′-
Complement : 3′-TTC TCC GTC CGT GAC TCG-5′
mRNA : 5′-
Amino Acid :

Variant 2
Coding Strand : 5′-
Complement : 3′-TTC TCC GTC GGT GAC TCG-5′
mRNA : 5′-
Amino Acid :

File:Aminoacids table.svg

PCR Genotyping the TAS2R38 PTC receptor:

- 5'-CCTTCGTTTTCTTGGTGAATTTTTGGGATGTAGTGAAGAGGCGG-3' (Forward Primer)
- 5′-AGGTTGGCTTGGTTTGCAATCATC-3′ (Reverse Primer)

1. PCR the DNA samples extracted from cheek cells using the PCR Beads.
2. Pour 2% agarose into casting apparatus in refrigerator.
   - 2 gels per class need to be made → 100ml of TBE with 2g agarose.
   - Add 5µl SYBR safe solution into the molten agarose before casting.
   - Place 2 sets of combs into the gel → at one end and in the middle.
3. Digest PCR product with *Hae* III.
   1. Remove 10µl of PCR product into a fresh tube.
   2. Add 1µl of *Hae*III enzyme into the tube.
   3. Incubate for 10 minutes at 37°C.
4. Load gel with DNA ladder, Digested and Undigested.
   - the undigested sample is from the original PCR.
5. Run gel at 120V for 20 minutes.
6. Visualize on the UV transilluminator.

SNP Detection:

The longer primer ends with the sequence "GG". Both alleles at this locus will amplify equally well with this primer set, however, one allele will have the sequence "GGGC" and another "GGCC". "GGCC" is the restriction site for the enzyme *Hae*III. The digestion of this amplified DNA will be digestible for one allele and yield a DNA fragment the size of the large primer (44 bp) as well as the remainder of the amplicon. Because of this difference in digestion profile of the amplicon, we can identify the 2 alleles at this locus.

Analysis Questions:

1. What is the size of the PCR product?
    - Perform an *in silico* PCR on the *Tas2R38* gene and identify the size of the amplicon.
2. The long primer is 44bp. If the amplicon of the allele digests, what are the sizes of fragments expected following *Hae*III?
3. Which allele is the one that can be identified through *Hae* III digestion?
    - Use the results of the PTC paper test.
4. Some lanes contain 3 bands instead of 1 or 2. Can you explain this?

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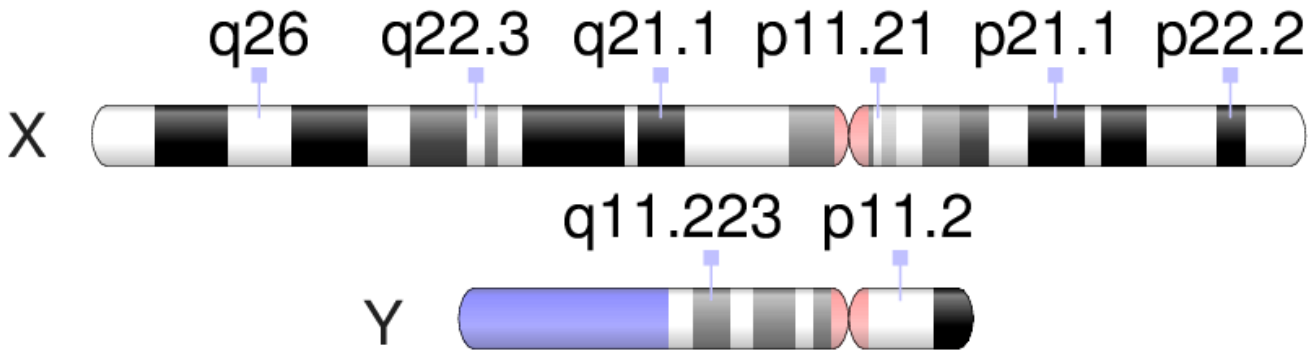
2.1.9.3: Sex-linked Genes

Sex Chromosomes

For the most part, mammals have gender determined by the presence of the Y chromosome. This chromosome is gene-poor and a specific area called sex-determining region on Y (**SRY**) is responsible for the initiation of the male sex determination.

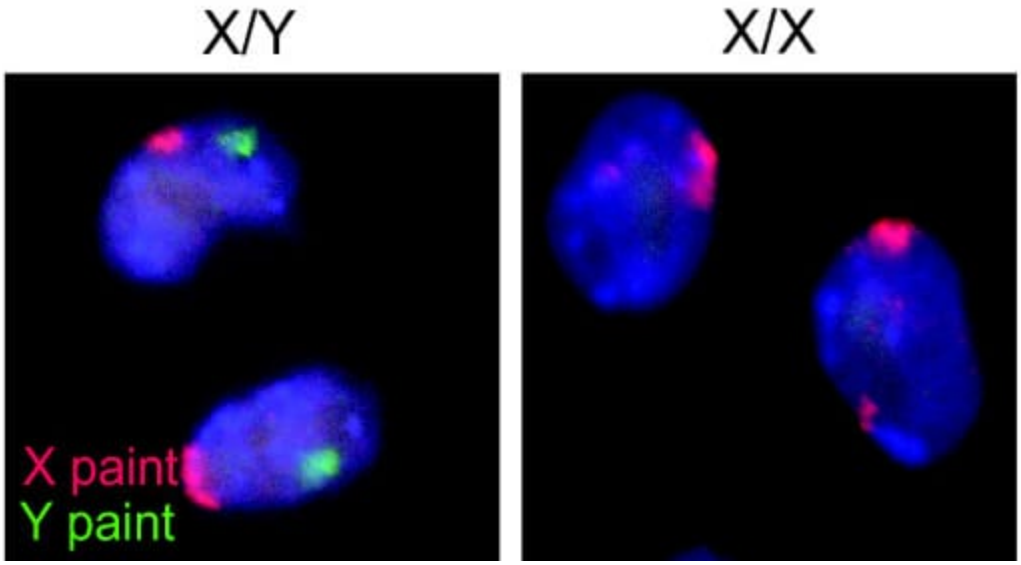


The X-chromosome is rich in genes while the Y-chromosome is a gene desert. The presence of an X-chromosome is absolutely necessary to produce a viable life form and the default gender of mammals is traditionally female.



Human X and Y chromosomes with G-Banding.

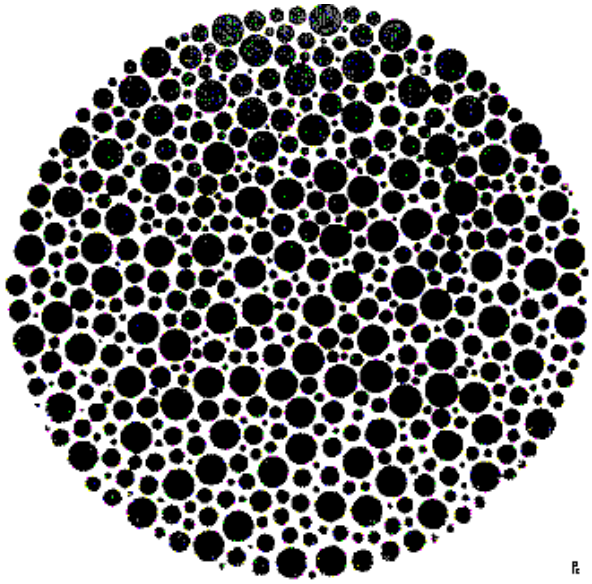
Chromosomal painting techniques can reveal the gender origin of mammalian cells. By using fluorescent marker sequences that can hybridize specifically to X or Y chromosomes through Fluorescence In Situ Hybridization (FISH), gender can be identified in cells.



The male cells have an X and a Y while the female cells have X and X combination. Credit: Janice Y Ahn, Jeannie T Lee [CC BY 2.0]

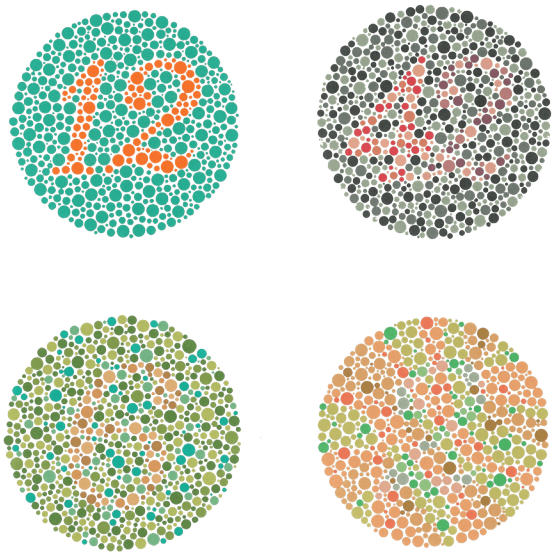
Ishihara Tests (Activity)

The genes encoding photoreceptor proteins for the long wave-length (reds) and middle wavelengths (greens) reside on the X chromosome at Xq25. Since the Y-chromosome is not homologous, any mutation to either of these genes that render them non-functional results in an inability to perceive either of those colors. Men are more susceptible to the condition of red-green colorblindness since they are **hemizygous**. This means that there is no corresponding gene that could complement a deficient red or green photoreceptor gene.



Monochromatic representation of Ishihara test to a colorblind individual as it emerges to something visible to a color-sighted individual.

Dr. Shinobu Ishihara published his test for color perception in 1917 and this test is widely used to detect deficits in color perception. Below are examples of the Ishihara plates. Record the number that you perceive in each plate and discuss with the rest of the class.



Ishihara Test

1. As you go through the plates above, note the number that you see (if any).

2. The genes for the Red and Green receptors are on the X-chromosome, who are most affected by mutation? Create a Punnett Square to illustrate how this works.

3. Can women be color-blind for red/green?

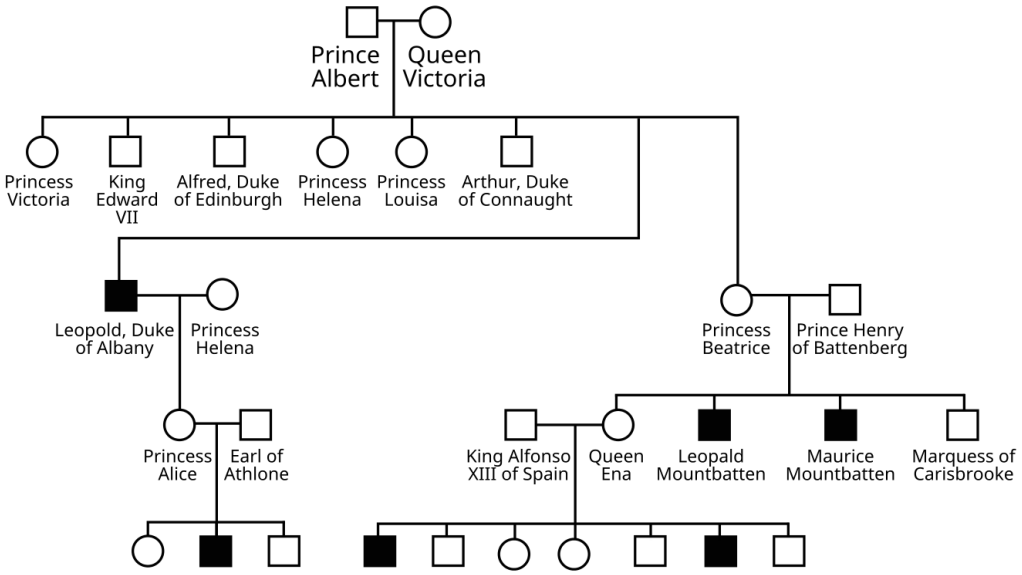
4. Humans have 3 color light receptors and have trichromatic vision. Some women are described as possibly having tetrachromatic vision (seeing 4 colors) and being able to discriminate colors invisible to the rest of us. Describe a mechanism for why this could happen. Why is there a possible gender bias?

Sequence Links:

- Opsin 1 Long Wave sensitive gene (OPN1LW) – red photoreceptor (human)
- Opsin 1 Medium Wave sensitive gene (OPN1MW) – green photoreceptor (human)

The Case of Queen Victoria

Hemophilia literally translates to *blood-loving*. This is a description of a series of disorders where an individual has an inability to clot blood after a cut. In modern times, clotting factors may be administered to an afflicted individual, but a prior treatment involved blood transfusions. A very famous family had a genetic predisposition to hemophilia and due to the proliferative nature of this family, we have some statistical power to verify predictions on the probabilities of passing the disease state. Below is a partial pedigree for Victoria, Queen of the United Kingdom of Great Britain and Ireland and Empress of India. The filled-in shapes represent individuals who suffered from hemophilia.



Credit: Jeremy Seto (CC-BY-SA)

Questions:

1. From the pedigree above, what can you say about this form of hemophilia with respect to dominance?

2. From this pedigree, can you comment on the probable chromosome where the deficiency occurs?

3. Assign genotypes for Prince Albert and Queen Victoria and perform a Punnett Square to illustrate if their offspring reflect your statements on dominance and chromosome location.

4. Albert and Victoria were 1st cousins. Do you believe this had anything to do with the propagation of this disease? What does your Punnett Square tell you?

5. Highlight the definitive carriers of the disease gene in the pedigree above.

Additional Resources:

- The full case study can be acquired at the National Center for Case Study Teaching in Science.
- Human Factor IX mRNA sequence

X-Inactivation

The mammalian X-chromosome contains significantly more genetic information than the Y-chromosome. This gene dosage is controlled for in females through a process called **X-inactivation** where one of the X-chromosomes is shut down and highly condensed into a **Barr body**. Inactivation of the X-chromosome occurs in a stochastic manner that results in females being cellular mosaics where a group of cells has inactivated the paternal X-chromosome and other patches of cells have inactivated the maternal X-chromosome. The most striking example of **mosaicism** is the calico cat. A calico cat (tortoiseshell cat) is always a female. One of the genes that encodes coat color in cats resides on the X-chromosome and exist as either orange or black alleles. Due to the stochastic inactivation, the patterning of orange and black fur is a distinctive quality of calicos.



Credit: Howcheng [CC-BY-SA-3.0]

While the genetic information for the orange or black coat color exists in all cells, they are not equally expressed. This type of heritable trait in spite of the presence of the genetic material (DNA) is called **epigenetic** to imply that it is "above" (epi) genetics.

Drosophila: Thomas Hunt Morgan

Around 1908, Thomas Hunt Morgan began to explore the genetics of what was to become a model organism, *Drosophila melanogaster* (Fruit fly). This small organism had a relatively short life cycle, great fecundity and was easily managed. From these flies that normally have red eye coloring, he and his students found white-eyed mutants. The lab noted that white-eyed flies were almost exclusively male. This gender imbalance led Morgan to believe that the trait was sex-linked.

File:EyeColors.jpg

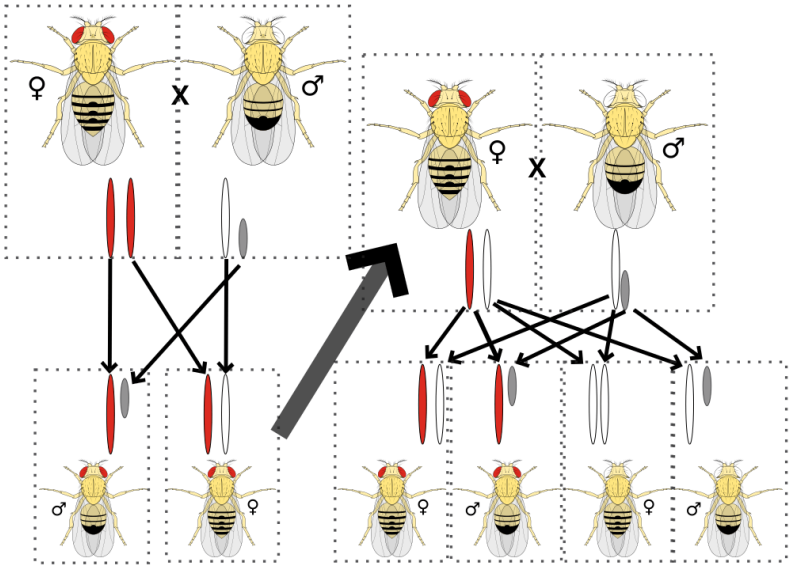
Eye colors (clockwise): brown, cinnabar, sepia, vermilion, white, wild. Also, the white-eyed fly has a yellow body, the sepia-eyed fly has a black body, and the brown-eyed fly has an ebony body. White-eyed flies have a gender imbalance and occur mostly in males.

In 1911, Morgan published a paper that described the inheritance patterns of 5 eye-colors in *Drosophila* (Morgan, 1911).

File:Drosophila XY sex-determination.svg

Drosophila follows a sex determination based on the ratio of X:A chromosomes and not by the presence of a Y as in mammals. A 1:1 ratio results in a female and a 1:2 ratio results in a male where the Y is ignored. The standard 2N = 8. Credit: GYassineMrabet [CC-BY-SA 4.0]

While DNA was not yet known as the source of genetic information, Morgan's studies revealed that the location of genes most likely resided on the chromosomes. By cataloging many mutations in the lab, he was able to construct a map of gene locations. His 1922 paper specifically stated that some traits were sex-linked and therefore residing on the sex chromosome. When performing crosses of white-eyed males to wild-type females, he continued to find white-eyed trait only in males. However, in the subsequent cross of females from that generation with white-eyed males, the presence of white-eyed males and females were revealed. This indicated that the white-eyed trait was recessive and resided on the X chromosome.



Analysis of the transmission of "White-Eyed" color in Drosophila. Credit: Jeremy Seto (CC-BY-SA)

Morgan received the Nobel Prize in Physiology or Medicine in 1933 for his inference of chromosomes being a physical mechanism for packaging genetic information in the cells.

- **Morgan** TH. THE ORIGIN OF FIVE MUTATIONS IN **EYE** COLOR IN **DROSOPHILA** AND THEIR MODES OF INHERITANCE. **Science**. 1911 Apr 7;33(849):534-7

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2.1.9.4: Probability and Chi-Square Analysis

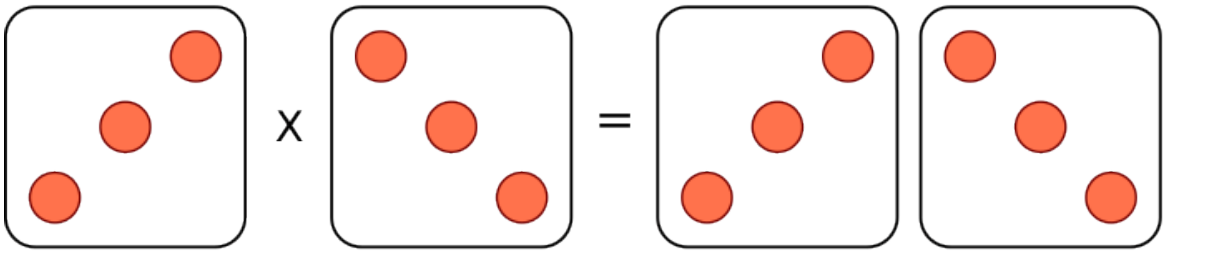
Mendel's Observations

Parental Cross	F_1 Phenotypes	F_2 Phenotypes	F_2 Ratios
Round x Wrinkled Seeds	All Round	5474 Round: 1850 Wrinkled	2.96:1
Yellow x Green Seeds	All Yellow	6022 Yellow: 2001 Green	3.01:1
Purple x White Flowers	All Purple	705 Purple: 224 White	3.15:1
Tall x Dwarf Plants	All Tall	787 Tall: 227 Dwarf	2.84:1

Probability: Past Punnett Squares

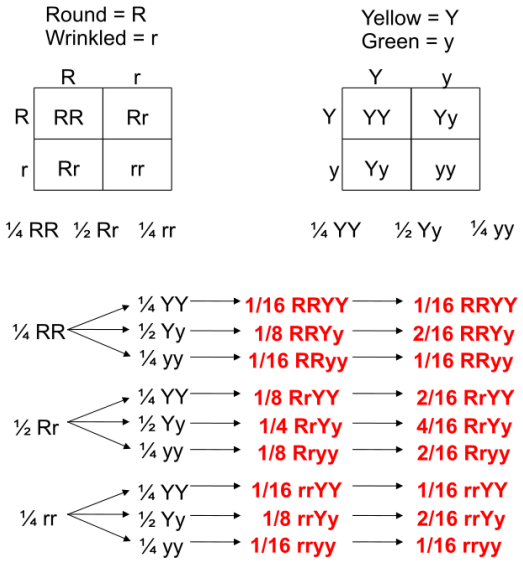
Punnett Squares are convenient for predicting the outcome of monohybrid or dihybrid crosses. The expectation of two heterozygous parents is 3:1 in a single trait cross or 9:3:3:1 in a two-trait cross. Performing a three or four trait cross becomes very messy. In these instances, it is better to follow the rules of probability. **Probability** is the chance that an event will occur expressed as a fraction or percentage. In the case of a monohybrid cross, 3:1 ratio means that there is a $\frac{3}{4}$ (0.75) chance of the dominant phenotype with a $\frac{1}{4}$ (0.25) chance of a recessive phenotype.

$$1/6 \text{ X } 1/6 = 1/36$$



A single die has a 1 in 6 chance of being a specific value. In this case, there is a $\frac{1}{6}$ probability of rolling a 3. It is understood that rolling a second die simultaneously is not influenced by the first and is therefore independent. This second die also has a $\frac{1}{6}$ chance of being a 3.

We can understand these rules of probability by applying them to the dihybrid cross and realizing we come to the same outcome as the 2 monohybrid Punnett Squares as with the single dihybrid Punnett Square.



This forked line method of calculating probability of offspring with various genotypes and phenotypes can be scaled and applied to more characteristics.

The Chi-Square Test

The χ^2 statistic is used in genetics to illustrate if there are deviations from the expected outcomes of the alleles in a population. The general assumption of any statistical test is that there are no significant deviations between the measured results and the predicted ones. This lack of deviation is called the **null hypothesis** (**H_0**). X^2 statistic uses a distribution table to compare results against at varying levels of probabilities or **critical values**. If the X^2 value is greater than the value at a specific probability, then the null hypothesis has been rejected and a significant deviation from predicted values was observed. Using Mendel's laws, we can count phenotypes after a cross to compare against those predicted by probabilities (or a Punnett Square).

Critical values for χ^2 test				
p-val / DF	0.1	0.05	0.01	0.005
1	2.706	3.84	6.64	7.88
2	4.605	5.99	9.21	10.60
3	6.251	7.82	11.35	12.94
4	7.779	9.49	13.28	14.86
5	9.236	11.07	15.09	16.75
6	10.65	12.59	16.81	18.55
7	12.02	14.07	18.48	20.28
8	13.36	15.51	20.09	21.96
9	14.68	16.92	21.67	23.59
10	15.99	18.31	23.21	25.19

In order to use the table, one must determine the stringency of the test. The lower the p-value, the more stringent the statistics. **Degrees of Freedom** (**DF**) are also calculated to determine which value on the table to use. Degrees of Freedom is the number of classes or categories there are in the observations minus 1. DF=n-1

In the example of corn kernel color and texture, there are 4 classes: Purple & Smooth, Purple & Wrinkled, Yellow & Smooth, Yellow

& Wrinkled. Therefore, DF = 4 – 1 = 3 and choosing p < 0.05 to be the threshold for significance (rejection of the null hypothesis), the X^2 must be greater than 7.82 in order to be significantly deviating from what is expected. With this dihybrid cross example, we expect a ratio of 9:3:3:1 in phenotypes where 1/16th of the population are recessive for both texture and color while $\frac{9}{16}$ of the population display both color and texture as the dominant. $\frac{3}{16}$ will be dominant for one phenotype while recessive for the other and the remaining $\frac{3}{16}$ will be the opposite combination.

With this in mind, we can predict or have expected outcomes using these ratios. Taking a total count of 200 events in a population, 9/16(200)=112.5 and so forth. Formally, the χ^2 value is generated by summing all combinations of:

$$\frac{(Observed - Expected)^2}{Expected} \tag{2.1.9.4.1}$$

Chi-Square Test: Is This Coin Fair or Weighted? (Activity)

1. Everyone in the class should flip a coin 2x and record the result (assumes class is 24).
2. Fair coins are expected to land 50% heads and 50% tails.
   - 50% of 48 results should be 24.
   - 24 heads and 24 tails are already written in the “Expected” column.
3. As a class, compile the results in the “Observed” column (total of 48 coin flips).
4. In the last column, subtract the expected heads from the observed heads and square it, then divide by the number of expected heads.
5. In the last column, subtract the expected tails from the observed tails and square it, then divide by the number of expected tails.
6. Add the values together from the last column to generate the X^2 value.
7. Compare the value with the value at 0.05 with DF=1.
   - There are 2 classes or categories (head or tail), so DF = 2 – 1 = 1.
   - Were the coin flips fair (not significantly deviating from 50:50)?

Heads or Tails	Expected Number	Observed Number	$\frac{(\text{Observed-Expected})^2}{\text{Expected}}$
Heads	24		
Tails	24		
$\chi^2 = \sum \frac{(\text{Observed-Expected})^2}{\text{Expected}} \rightarrow$			

Let’s say that the coin tosses yielded 26 Heads and 22 Tails. Can we assume that the coin was unfair? If we toss a coin an odd number of times (eg. 51), then we would expect that the results would yield 25.5 (50%) Heads and 25.5 (50%) Tails. But this isn’t a possibility. This is when the X^2 test is important as it delineates whether 26:25 or 30:21 etc. are within the probability for a fair coin.

Chi-Square Test of Kernel Coloration and Texture in an F_2 Population (Activity)

1. From the counts, one can assume which phenotypes are dominant and recessive.

2. Fill in the “Observed” category with the appropriate counts.
3. Fill in the “Expected Ratio” with either 9/16, 3/16 or 1/16.
4. The total number of the counted event was 200, so multiply the “Expected Ratio” x 200 to generate the “Expected Number” fields.
5. Calculate the $\frac{(Observed-Expected)^2}{Expected}$ for each phenotype combination
6. Add all $\frac{(Observed-Expected)^2}{Expected}$ values together to generate the X^2 value and compare with the value on the table where DF=3.
7. Do we reject the Null Hypothesis or were the observed numbers as we expected as roughly 9:3:3:1?
    - What would it mean if the Null Hypothesis was rejected? Can you explain a case in which we have observed values that are significantly altered from what is expected?

Phenotype	Expected Ratio (9/16, 3/16,1/16)	Expected Number (Total # x Ratio)	Observed Number	$\frac{(\text{Observed-Expected})^2}{\text{Expected}}$
Purple & Smooth				
Purple & Wrinkly				
Yellow & Smooth				
Yellow & Wrinkly				
		$\chi^2 = \sum \frac{(\text{Observed-Expected})^2}{\text{Expected}} \rightarrow$		

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2.1.9.5: Non-Mendelian Genetics

Co-Dominance and Multiple Alleles

Co-dominance is said to occur when there is an expression of two dominant alleles. The prototypical case for this is the human ABO blood grouping.

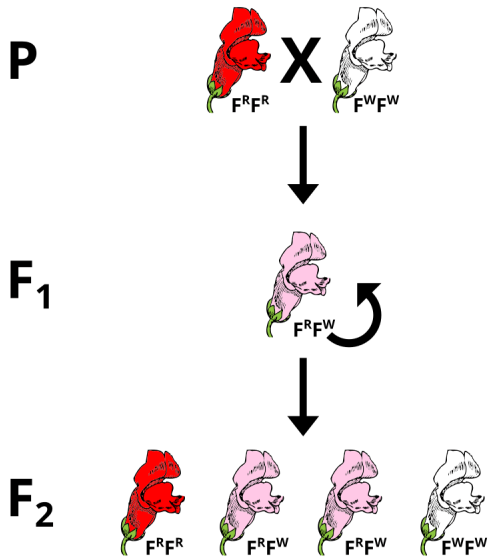
File:ABO blood type.svg

Three alleles exist in the ABO system: A, B and O. This results in four blood types: A, B, O and the blended AB.

File:ABO system codominance.svg

Incomplete Dominance

During Mendel's time, people believed in a concept of blending inheritance whereby offspring demonstrated intermediate phenotypes between those of the parental generation. This was refuted by Mendel's pea experiments that illustrated a Law of Dominance. Despite this, non-Mendelian inheritance can be observed in sex-linkage and co-dominance where the expected ratios of phenotypes are not observed clearly. **Incomplete dominance** superficially resembles the idea of blending inheritance, but can still be explained using Mendel's laws with modification. In this case, alleles do not exert full dominance and the offspring resemble a mixture of the two phenotypes.



Incomplete dominance in snapdragon flowers superficially appears like blending inheritance. Credit: Jeremy Seto (CC-BY-NC-SA)

The most obvious case of a two allele system that exhibits incomplete dominance is in the snapdragon flower. The alleles that give rise to flower coloration (Red or White) both express and the heterozygous genotype yields pink flowers. There are different ways to denote this. In this case, the superscripts of R or W refer to the red or white alleles, respectively. Since no clear dominance is in effect, using a shared letter to denote the common trait with the superscripts (or subscripts) permit for a clearer denotation of the ultimate genotype to phenotype translations.

Problem: Incomplete Dominance

If pink flowers arose from blending inheritance, then subsequent crosses of pink flowers with either parental strain would continue

to dilute the phenotype. Using a Punnet Square, perform a test cross between a heterozygous plant and a parental to predict the phenotypes of the offspring.

Epistasis and Modifier Genes:



The interplay of multiple enzymes in a biochemical pathway will alter the phenotype. Some genes will modify the actions of another gene.

Credit: Jeremy Seto (CC0)

Genes do not exist in isolation and the gene products often interact in some way. **Epistasis** refers to the event where a gene at one locus is dependent on the expression of a gene at another genomic locus. Stated another way, one genetic locus acts as a modifier to another. This can be visualized easily in the case of labrador retriever coloration where three primary coat coloration schemes exist: black lab, chocolate lab, and yellow lab.

File:3labradorcols.jpg

Chocolate lab (top), Black lab (middle), Yellow lab (bottom) coat colorations arise from the interaction of 2 gene loci, each with 2 alleles. Credit: Erikeltic [CC-BY-SA 3.0]

Two genes are involved in the coloration of labradors. The first is a gene for a protein called TYRP1, which is localized to the melanosomes (pigment storing organelles). Three mutant alleles of this gene have been identified that reduce the function of the protein and yield lighter coloration. These three alleles can be noted as "***b***" while the functioning allele is called "***B***". A heterozygous (Bb) or a homozygous dominant individual will be black coated while a homozygous recessive (bb) individual will be brown.

File:Labrador Retrievers blackandchocolate.jpg

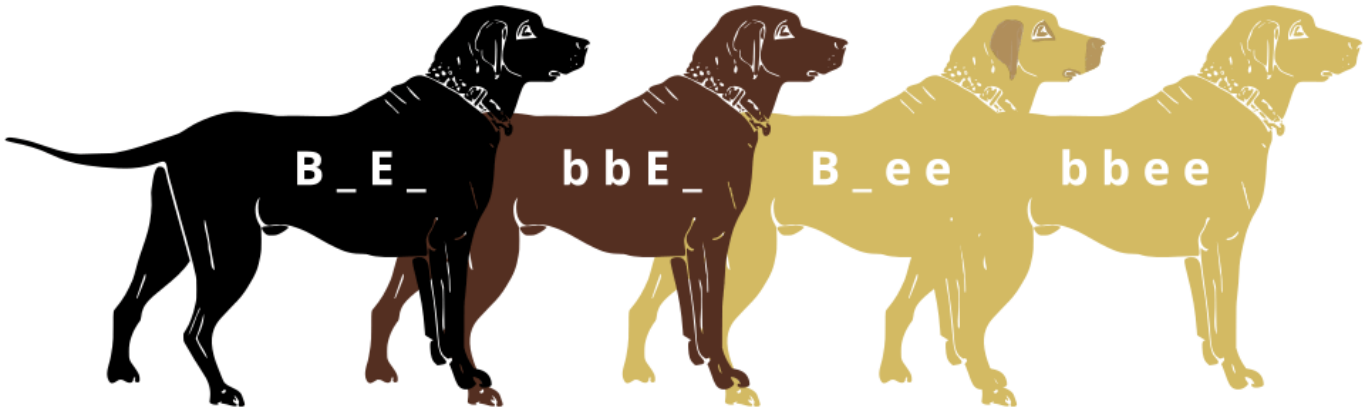
Black lab (BB or Bb) and Chocolate lab (bb). Credit: dmealiffe[CC BY-SA 2.0]

The second gene is tied to the gene for Melanocortin 1 Receptor (MC1R) and influences if the eumelanin pigment is expressed in the fur. This gene has the alleles denoted "***E***" or "***e***". A yellow labrador will have a genotype of either *Bbee* or *bbee*.



Black lab (EE or Ee) and Yellow lab (ee) [CC0]

The interplay between these genes can be described by the following diagram:



Black lab (B_E_, Chocolate lab (bbE_), Yellow lab with dark skin where exposed (B _ee) and Yellow lab with light skin where exposed. Credit: Jeremy Seto (CC-BY-SA 3.0)

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2.1.9.6: Hardy-Weinberg and Population Genetics

Hardy-Weinberg Principle

The **Hardy-Weinberg principle** is a mathematical model used to describe the equilibrium of two alleles in a population in the absence of evolutionary forces. This model was derived independently by G.H. Hardy and Wilhelm Weinberg. It states that the allele and genotype frequencies across a population will remain constant across generations in the absence of evolutionary forces. This equilibrium makes several assumptions in order to be true:

1. An infinitely large population size
2. The organism involved is diploid.
3. The organism only reproduces sexually.
4. There are no overlapping generations.
5. Mating is random.
6. Allele frequencies equal in both genders
7. Absence of migration, mutation, or selection

As we can see, many items in the list above can not be controlled for but it allows for us to make a comparison in situations where expected evolutionary forces come into play (selection etc.).

Hardy-Weinberg Equilibrium

The alleles in the equation are defined as the following:

- Genotype frequency is calculated by the following:

$$\text{genotype frequency } = \frac{\#\text{ individuals of given genotype}}{\text{total }\#\text{ individuals in population}} \tag{2.1.9.6.1}$$

- Allele frequency is calculated by the following:

$$\text{allele frequency } = \frac{\#\text{ of copies of an allele in a population}}{\text{total }\#\text{ of alleles in population}} \tag{2.1.9.6.2}$$

- In a two allele system with dominant/recessive, we designate the frequency of one as ***p*** and the other as ***q*** and standardize to:
  - $p =$ Dominant allele frequency
  - $q =$ recessive allele frequency
- Therefore the *total frequency of **all alleles*** in this system equal 100% (or 1)

$$p + q = 1 \tag{2.1.9.6.3}$$

- Likewise, the *total frequency of **all genotypes*** is expressed by the following quadratic where it also equals 1:

$$p^2 + 2pq + q^2 = 1 \tag{2.1.9.6.4}$$

  This equation is the Hardy-Weinberg theorem that states that there are no evolutionary forces at play that are altering the gene frequencies.

Calculating Hardy-Weinberg Equilibrium (Activity)

This exercise refers to the PTC tasting exercise. One can test for selection for one allele within the population using this example. Though the class size is small, pooling results from multiple sections can enhance the exercise. Remember to surmise the dominant/recessive traits from the class counts.

Phenotype	Genotype	Genotype Frequency
Super Taster		
Taster		
Non-taster		

1. What is the recessive phenotype and how can we represent the genotype?

2. What is the dominant phenotype and how can we represent the genotypes?

3. What is the frequency of recessive genotype? (q^2)

4. What is the frequency of the recessive allele? (q)

5. What is the frequency of the dominant allele? (p=1-q)

6. Use Hardy-Weinberg to calculate the frequency of heterozygotes in the class. (2pq)

7. Use Hardy-Weinberg to calculate the frequency of homozygotes in the class. (p^2)

8. Using an aggregate of multiple sections, compare the local allelic and genotypic frequencies with what the Hardy-Weinberg would predict.

9. With this small number in mind, we can see that there are problems with the assumptions required for this principle. The instructor will perform the following simulation in class to illustrate the effects on multiple populations with the effects of selection and /or population limitations. A coefficient of fitness can be applied to illustrate a selective pressure against an allele.

- **Population Genetics Simulation of Alleles**

10. In the case of selective pressure, a **fitness coefficient** (***w***) can be introduced. A research article **http://www.jci.org/articles/view/64240** has shown that the Tas2R38 receptor aids in the immune response against *Pseudomonas*. Imagine a situation where there is an epidemic of antibiotic-resistant *Pseudomonas*. This would
show that the dominant allele will have a selective advantage.

- Modify the fitness coefficient in the Population Genetics Simulator and describe the effects this would have over many successive generations.

A Case Study of Evolution: Population Genetics at Work

Additional Resources/Experiments:

- Population Genetics Simulation of Alleles (Schaffer)
- Population Genetics Simulation of Alleles (Sheehy)

- *Lee RJ*, *Xiong G*, *Kofonow JM*, *Chen B*, *Lysenko A*, *Jiang P*, *Abraham V*, *Doghramji L*, *Adappa ND*, *Palmer JN*, *Kennedy DW*, *Beauchamp GK*, *Doulias PT*,*Ischiropoulos H*, *Kreindler JL*, *Reed DR*, *Cohen NA*. **T2R38 taste receptor polymorphisms underlie susceptibility to upper respiratory infection.** *J Clin Invest*. 2012;122(11):4145-4159. doi:10.1172/JCI64240.

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2.1.10: DNA AS THE GENETIC MATERIAL

2.1.10.1: Introduction
2.1.10.2: Bacterial Transformation (Activity)

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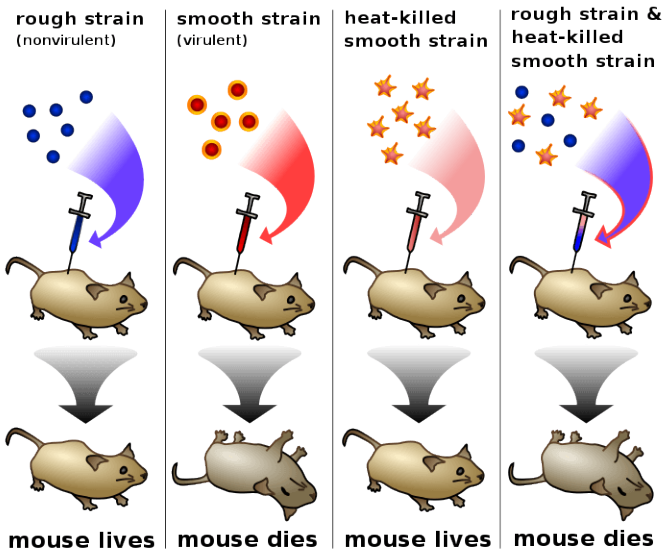
2.1.10.1: Introduction

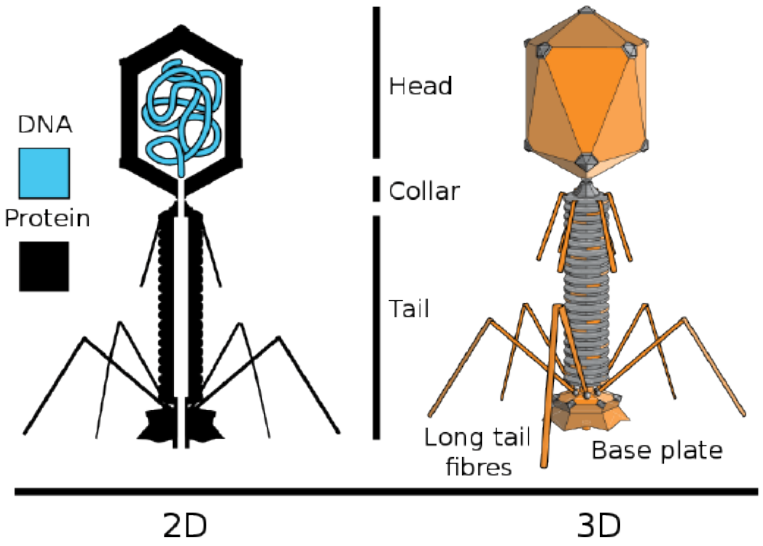
History of Genetic Transformation

Any uptake of genetic information from the external environment into cells that results in the expression of new traits is called **genetic transformation**. This process can occur naturally. Some bacteria are referred to as being “competent” to indicate that they are capable of taking DNA into the cell from the environment. This is referred to as **natural competence**. Bacteria are also capable of receiving DNA through the process of conjugation where plasmids from one bacteria are sent to another through the **conjugation pilus**. Other methods of introduction of foreign DNA include direct injection into the cytosol or through the use of viruses in a process called **transduction**. In eukaryotic cells, we refer to the introduction of DNA as **transfection**.

Frederick Griffith and the Transforming Agent

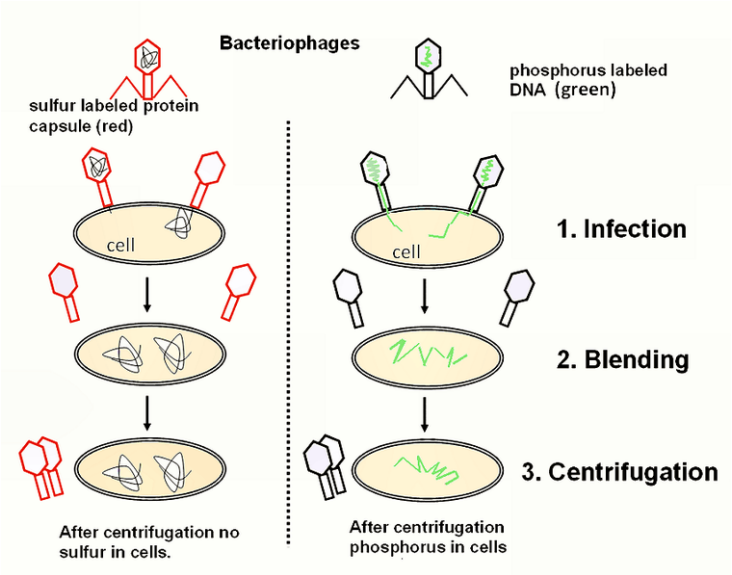
At the beginning of modern biology, the source of genetic material was not known to be DNA. In fact, many scientists thought DNA was too simple to perform this job. Scientists believed that proteins, with their 20 varied amino acids, were the carriers of genetic information. In an attempt to develop a vaccine for bacterial-induced pneumonia, Frederick Griffith was the first to describe the process of genetic transformation by accident in 1928. Griffith took a virulent strain of bacteria (smooth in appearance) that caused pneumonia and injected them into mice. This would result in the death of the mice. He also observed that injection of a rough bacteria did not cause any disease. After heat-killing the smooth bacteria, he discovered that living bacteria of the virulent strain was required for the disease to progress. Finally, he observed that injecting the heat-killed virulent bacteria with living bacteria of the non-virulent strain resulted in pneumonia and death in the mice. From this experiment, a **transforming agent** with the capacity to pass on a trait was found to be within the contents of those dead cells. But no one knew this agent to be DNA at that point.





Hershey and Chase

Hershey and Chase studied bacteriophage (phage=eater). Phages are bacterial viruses that infect bacteria and cause lysis of the cells. They have a very simple structure of a proteinaceous head/collar/tail and a DNA core. It was known that bacteria infected with phage were resistant to additional infection. In 1952 Hershey and Chase grew bacteriophage in conditions that would specifically label either the DNA or the protein with radioactivity. They subsequently took phage with radiolabeled DNA and infected bacteria. In parallel, they took phage with radiolabeled protein and infected another set of bacteria. After just enough time for infection, the bacterial cultures were placed into a blender to separate the bacteriophage from the bacteria.



Solutions were centrifuged to isolate bacteria from the phage. Bacteria were radioactive only when the phage grown in conditions to radiolabel DNA infected the bacteria to indicate that DNA might be the transforming agent.

Questions to Think About

1. What isotope would be used to label protein?
2. What isotope would be used to label just DNA?

Avery, McCarty, and MacCleod

In 1944, Oswald Avery, Colin MacCleod and Maclyn McCarty repeated Griffith's experiment. Instead of using heat-killed bacteria, these scientists isolated protein, carbohydrates, lipids and nucleic acids from the virulent strain and co-injected with the non-virulent bacteria. Carbohydrate extracts were ineffective at transforming bacteria. Protein extracts were incapable of causing transformation. Lipid injections were unable to result in virulence. Only nucleic acid samples treated with RNase were capable of transforming bacteria. When co-injecting with DNase, bacteria were not transformed. Along with Hershey-Chase, this definitively illustrated that DNA was the transforming agent capable of transferring genetic information.

More Resources

- http://www.dnalc.org/view/16375-Animation-17-A-gene-is-made-of-DNA-.html

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2.1.10.2: Bacterial Transformation (Activity)

Bacterial Transformation

Escherichia coli are commensal gram-negative bacteria found in the guts of humans. They have the capacity to double every twenty minutes and make a favorable carrier of recombinant DNA. Plasmid DNA can be introduced into *E. coli* easily after making them competent. One method to achieve this is through chemical competence with heat shock. In this process, the bacteria are incubated in $CaCl_2$ solution on ice. The cold serves to slow down the molecular motion of the plasma membrane while the Ca^{2+} ions remove the charge-charge repulsion between the phospholipids and the negatively charged DNA seeking to gain entry into the cell. Cells are placed for a short period of time at 42°C to induce **heat shock**. This heat shock results in the cell taking up the DNA. This method is very low efficiency so many bacteria do not take in any DNA. Cells are allowed to recover from heat shock at 37°C in rich nutrient broth to allow for the production of the antibiotic resistance proteins encoded on the vector as a selection marker. Transformed cells are then spread across an agar plate containing the antibiotic which will then kill all non-transformed cells. Only the bacteria containing the vector with the antibiotic resistance gene will survive and replicate to form small colonies on the surface of the agar.

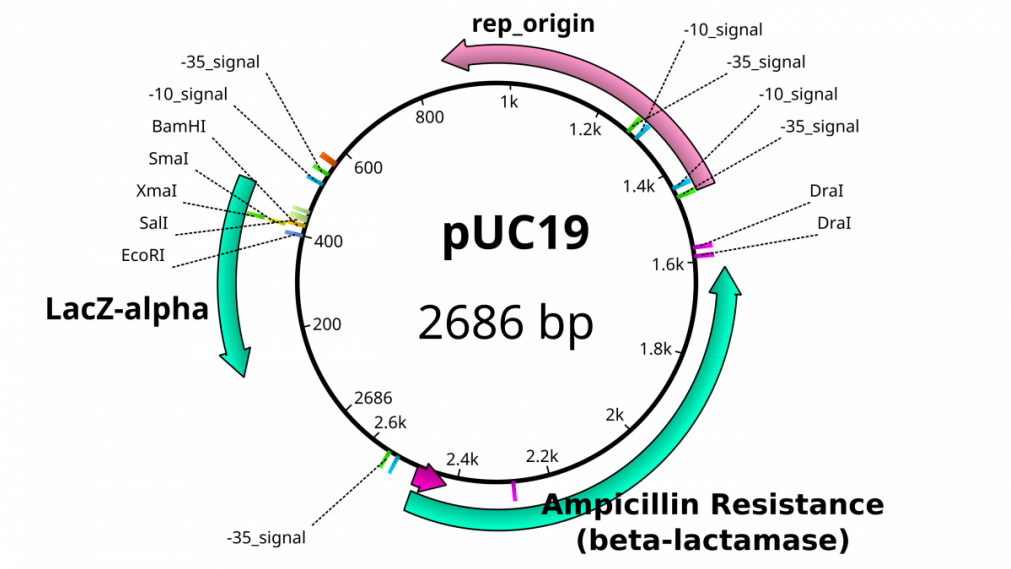
- http://www.dnalc.org/view/15918-Transformation.html
- http://www.dnalc.org/view/15916-DNA-...formation.html

Exercise: Transformation of Bacteria with RE Identified Plasmids

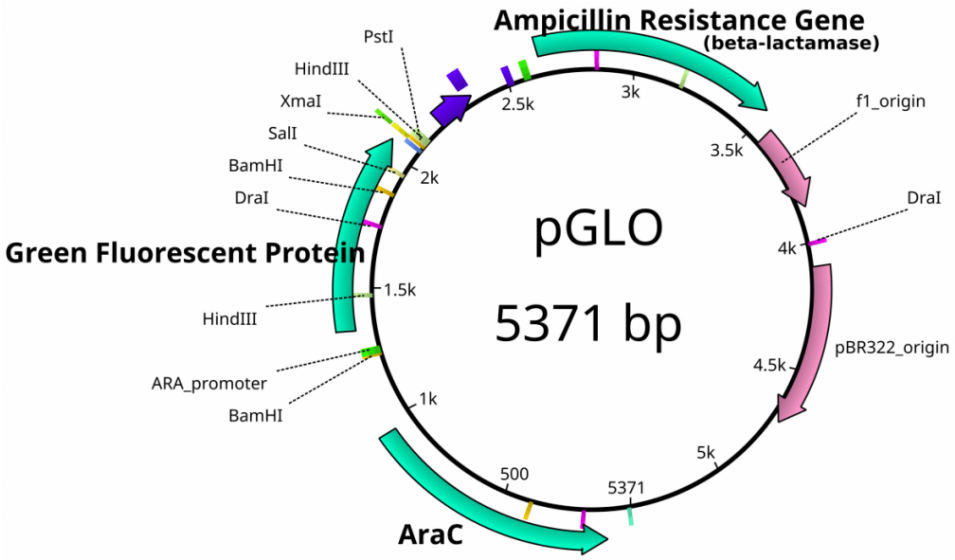
1. Each group retrieves the 2 mini-prepped plasmids from the previous week in the freezer and allow to thaw on ice.
2. Bring 2 agar plates to room temperature
    - 2 plates will contain antibiotic, X-Gal, and arabinose
3. For each plasmid, obtain 250µl of transformation buffer (50mM $CaCl_2$) in microfuge tubes and place on ice for 10 minutes.
4. Take an inoculating loop and remove a single colony of bacteria from a freshly streaked plate grown overnight.
5. Swirl bacteria in each tube containing transforming solution to distribute bacteria throughout the solution.
6. Pipette 5 µl of the plasmid into the tube and incubate on ice for 10 minutes.
7. During this incubation, flip the warmed plates and label them with your group names.
8. Place transformation tubes into 42°C heat block for 1 minute to heat shock the cells.
9. Add 500µl fresh SOC media (or LB) and incubate at 37°C for 15 minutes.
10. Pipette 150µl of transformation solution onto each plate and spread across the plate.
11. Turn plates agar side up and place them into 37°C incubator overnight. (your instructor will retrieve them and place them into the refrigerator).

Hypothesize: What Will I Expect of My Transformed Cells?

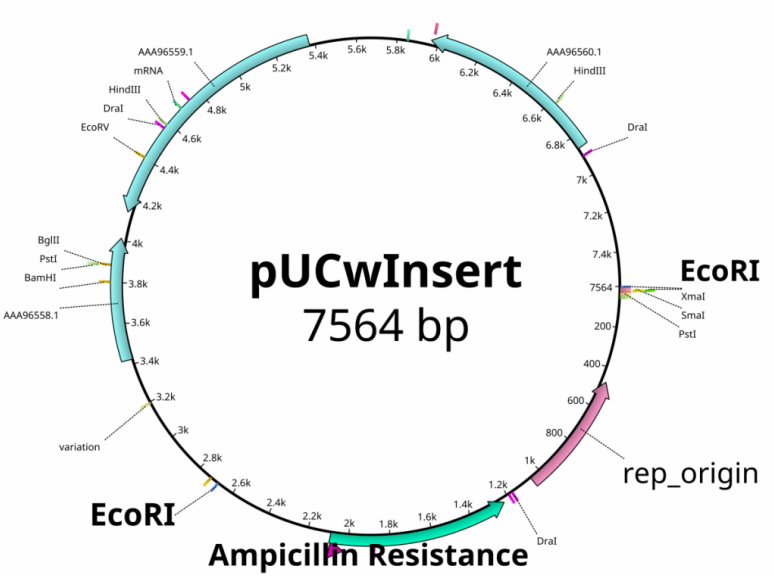
From the previous lab, we can identify our plasmids. The plasmids are either pGlo, pUC18/19 or pUC18/19 with a 6kb insert disrupting the LacZ gene. pGlo contains a gene that encodes the protein GFP that will fluoresce green under UV light and is 5.4kb. pUC is typically 2.7kb in size. LacZ is a gene encoding the protein β-Galactosidase, the enzyme that hydrolyzes lactose into the monosaccharides galactose and glucose. X-Gal is a chemical resembling lactose. However, upon hydrolysis, the molecule deposits a blue coloring into the cell.



pUC19 genbank file

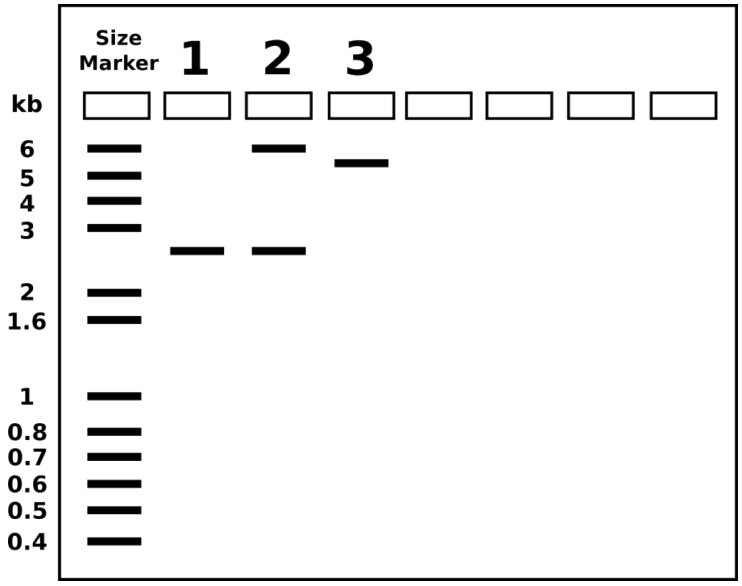


pGlo genbank file



pUC with insert file

1. If the previously mentioned plasmids were digested by *EcoRI*, label the lanes below with the appropriate plasmid (pGlo, pUC, and pUC-inserted)
2. Predict if your transformants will be green under UV, white in all conditions or blue.
3. For additional help on this problem, utilize the *In silico* digestion activity.



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2.1.11: GENE EXPRESSION

2.1.11.1: Introduction

2.1.11.2: Prokaryotic Transcription

2.1.11.3: RNA Miniprep (Activity)

2.1.11.4: Quantitative Nucleic Acid Measurement

2.1.11.5: Eukaryotic Transcription

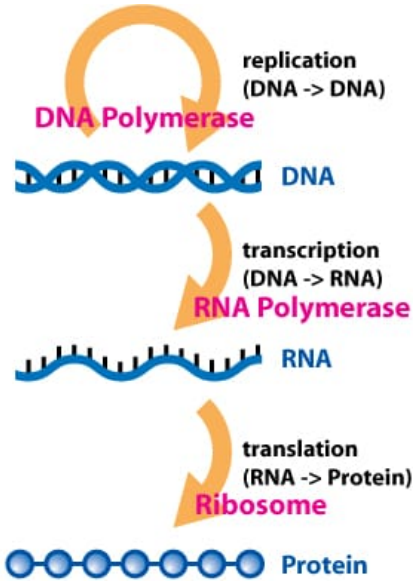
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2.1.11.1: Introduction

The Central Dogma

DNA was described as a molecule consisting of 2 anti-parallel strands in a double helix by Francis Crick and James Watson. The elegant model illustrated the intrinsic redundancy that made DNA a suitable data storage vessel for genetic information. Francis Crick later posited a notion of how this information went from storage to an actual program that runs cells. Crick first posited it as a "sequence hypothesis". This idea of information flow is called the **Central Dogma of Molecular Biology**. DNA stores the information that is expressed as an intermediate message of RNA. This RNA is then translated into amino acids to yield proteins.



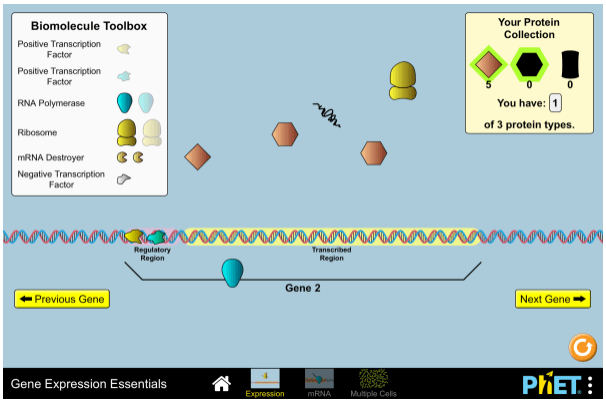
*The flow of information in cells. DNA serves as a template for copying itself (**replication**). DNA can also serve as a template for RNA (**transcription**). RNA is decoded into amino acids to generate proteins (**translation**). Credit Daniel Horspool (CC-BY-SA 3.0)*

Transcription

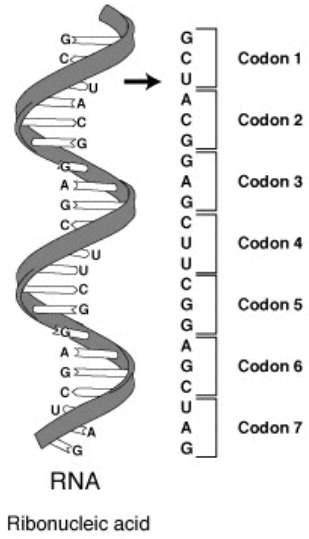
DNA is simply a storage vessel of genetic information. It sits in the nucleus and must be called upon through a process of **transcription** where an enzyme called **RNA Polymerase**"reads aloud" the stored information into a molecule called messenger RNA (**mRNA**). Since DNA is double-stranded in an **anti-parallel** fashion, we automatically know the sequence of the second strand by knowing the first. The mRNA is made through complementary base-pairing to the **template strand**, which is the reverse complement of the coding strand. The **coding strand** is the strand that reads identically in sequence to the mRNA with the exceptions of T's being replaced by U's.

File:Simple transcription initiation1.svg
File:Simple transcription elongation1.svg

Transcription Simulation



Translation



This coding strand is later decoded by the **ribosomes** with the help of transfer RNA's **tRNA**'s) that act as a decoder of the information and protein assembler in a process called **translation**. The ribosome scans along the mRNA and recognizes nucleotides in batches of 3 . These batches of 3 can be translated into an amino acid and are known as **codons**. Since there are 4 types of bases and they are read as groups of 3, there are 4^3 (or 64) combinations of these codons. However, there are only 20 amino acids used to build proteins. This indicates that there is room for redundancy. Three of these codons tell the ribosome to stop, like a period in a sentence. These are called **stop codons**. There is one special codon that performs double duty: ATG. The codon (ATG) that encodes the amino acid Methionine also acts as a **start codon** that tells the ribosome where to start reading from. Like nucleic acids, proteins have a polarity and are synthesized in an amino to carboxyl direction. We abbreviate this by terming the beginning of the protein sequence, N-terminal, and the ending of the sequence as the C-terminal.

File:Ribosome mRNA translation en.svg

Ribosomes are large complexes of enzymes that coordinate the decoding of mRNA into amino acids to generate proteins.

File:RNA-codons-aminoacids.svg

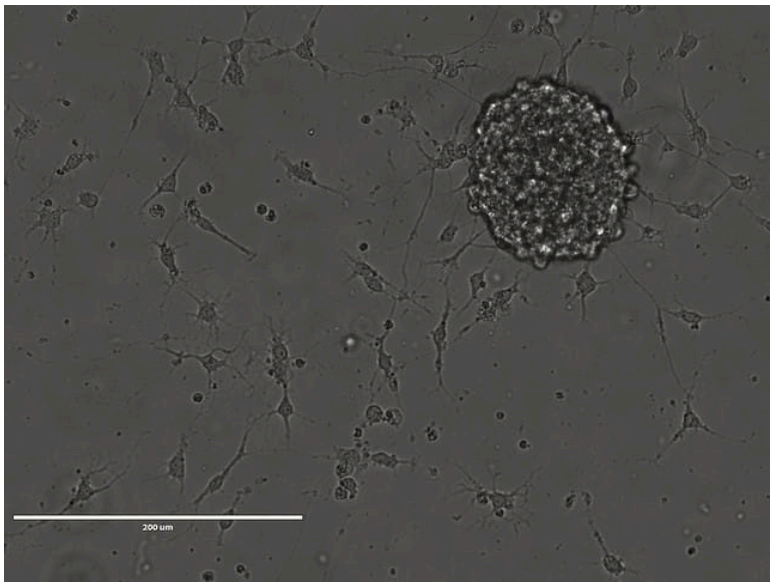
File:Aminoacids table.svg

The standard genetic code.

Advanced Video of Translation

Decisions... decisions...

What kinds of decisions are made for stem cells to differentiate into different cell types? What types of regulation occur during this process?



A cluster of neuronal progenitor cells (neurosphere) dissociates and differentiates into neurons.

Video Review

- Transcription Simulation (MIT License)
- **Transcription (CC-BY-NC-ND Cold Spring Harbor Lab – DNA Learning Center)**
  https://www.dnalc.org/content/c15/15510/transcription_basic.mp4
- **Translation (CC-BY-NC-ND Cold Spring Harbor Lab – DNA Learning Center)**
  https://www.dnalc.org/content/c15/15501/translation_basic.mp4

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2.1.11.2: Prokaryotic Transcription

The Lactose Intolerance of Bacteria

File:Bacterial growth en.svg

The standard growth kinetics of E. coli are described by the curve. Credit: Michał Komorniczak (CC-BY-SA 3.0)

Glucose is the preferred energy source of cells. François Jacob and Jacques Monod sought to understand how bacteria made decisions to switch between different sugars as sources of energy. Jacob and Monod found that if glucose and lactose were presented as food for bacteria, there would be a **biphasic growth** pattern.

File:Lactose etc.png File:OSC Microbio 11 07 Diaux.jpg

Credit: CNX OpenStax (CC-BY 4.0)

Jacob and Monod came to understand that the glucose would first be utilized (preferred source) and the lactose would be digested after the depletion of glucose. This occurred because under normal situations the bacteria would not have access to lactose and would waste energy by creating enzymes to digest it. The enzyme **β-galactosidase**, which is responsible for digesting lactose to the monomers galactose and glucose would only be induced under the conditions of low glucose and high lactose. Monod found that when lactose was the sole sugar, the expression of the enzyme β-galactosidase was induced and displayed a monophasic growth with a delay.

The Lac Operon

Jacob and Monod later found that the genes involved in utilizing lactose were clustered together in proximity under a coordinated control mechanism. This became known as the **Lac operon**.

File:Lac operon1.png

A schematic of the Lac Operon. LacZ, LacY, and LacA are transcribed as a single mRNA.

The usage of lactose as a source of energy is preferred by bacteria when glucose is not present. In the presence of abundant glucose, it would be a waste of energy and cellular resources to commit to synthesizing the mRNA and the protein for β-galactosidase. Unless lactose is present, a protein binds to a portion of the Lac promoter referred to as the **operator**. This **repressor** protein is encoded by another gene (***LacI***) outside of the gene cluster. Occasionally, the repressor unbinds to the operator and RNA Polymerase is permitted to transcribe the ***LacZ*** gene (β-galactosidase), ***LacY*** gene (permease), and ***LacA*** gene (acetylase). This “leakiness” of expression is important since the permease protein is needed on the surface of the cell to allow lactose into the cell if it is present in the environment. The presence of lactose causes the repressor to fall off the operator to grant RNA pol access to the DNA. When glucose is low, a protein called **CAP** (Catabolite Activated Protein) binds to the Lac promoter and works as a recruiter of RNA pol. The coordinated effects of CAP activation and Lac Repressor inactivation yield high transcription of the operon.

File:Lac operon-2010-21-01.png

Credit: G3pro (CC-BY 2.0)

File:Annotated Theoretical Model of Bound Tetrameric Lac Repressor.png

LacI bound to 2 DNA operator sequences.

Credit: SocratesJedi (CC-BY-SA 3.0)

File:Gene structure prokaryote 2 annotated.svg

Lac Operon Simulation

Launch the simulation below to explore the coordinated activation of the Lac Operon.

LacZ as a Reporter Gene

pUC19 contains LacZ DNA as a reporter gene to illustrate the presence of the functioning gene. Transcription of this gene is driven by the binding site for the RNA Polymerase subunit called **σ factor**. The σ factor binding site determines the directionality of the RNA polymerase since there is an option of transcribing in 2 directions. The standard σ factor binding site is often denoted as **-35 TTGACA...TATAAT -10** from the transcription initiation.

The multiple cloning site within the plasmid provides a convenient location to shuttle a foreign piece of DNA. When no foreign DNA is inserted to this space, the LacZ gene product β-galactosidase is functional. Disruption of the reading frame for this gene likewise disables the functional product from being translated. By using chemical reporters, the integrity of this gene can be confirmed through enzymatic activity.

File:Hydrolysis of lactose.svg

Hydrolysis of lactose to galactose and glucose

Two chemical reporters used to reveal the presence of a functioning LacZ are **X-Gal** (5-bromo-4-chloro-3-indolyl-β-D-galactoside5-bromo-4-chloro-3-indolyl-β-D-galactoside) and **ONPG** (orthonitrophenol).

File:X-Gal.svg

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) yields a blue color when cleaved by β-galactosidase

File:ONPG.svg

ONPG (orthonitrophenol) yields a yellow color upon cleavage by β-galactosidase

As in the case of the Lac operon, the *LacI* (repressor protein) will occupy the operator. This operator happens to be overlapping the -35 & -10 sequences. In order to fully activate these genes, the Lac repressor must be removed by binding to a lactose analog. In this case, the chemical **IPTG** (Isopropyl β-D-1-thiogalactopyranoside) is used since it is a non-cleavable analog that will perpetually bind to the Lac repressor.

File:IPTG2.svg

IPTG

Blue-White Screening

File:Blue-white test.jpg

Blue-White Screening reveals a phenotype of transformed bacteria based on the ability of X-Gal conversion by β-lactamase
Credit: Stefan Walkowski (CC-BY-SA 4.0)

References

- JACOB F, MONOD J. **Genetic regulatory mechanisms in the synthesis of proteins**. *J Mol Biol. 1961 Jun;3:318-56.*

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2.1.11.3: RNA Miniprep (Activity)

Exercise: RNA Miniprep

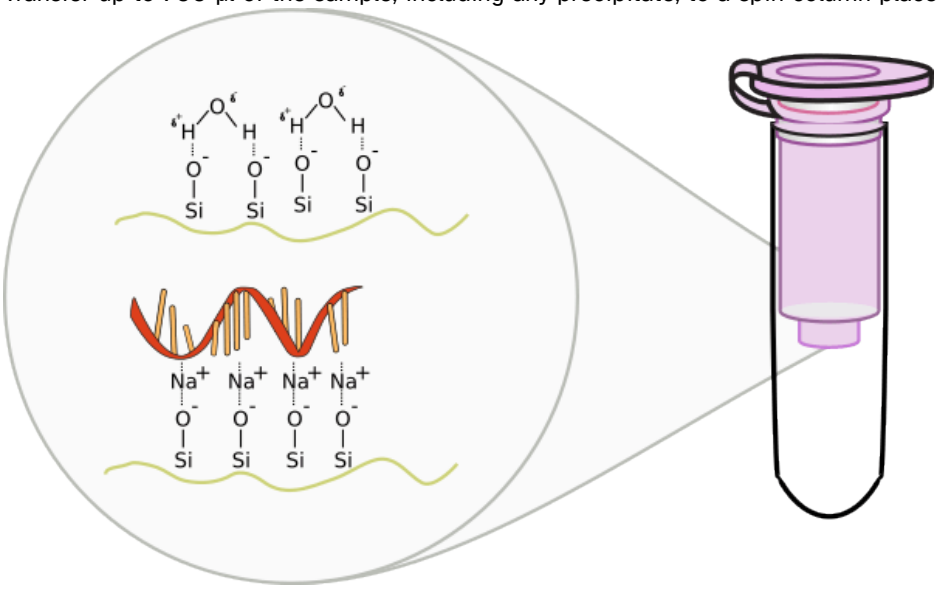
RNA purification occurs similar to DNA preparations. A silica-based column is used where DNA is excluded from binding based on size and through an additional DNA digestion step using the enzyme DNase I. RNA is extremely fragile and prone to degradation. Because of this, separate pipettes and plastics are usually used in labs to reduce the amount of exposure to environmental or experimental RNase. When handling RNA, be extremely careful of contaminating the buffers or samples. Always wear gloves as skin carries RNase enzymes. Refrain from talking as to not contaminate the area with RNase found in saliva.

RLT = RNA lysis buffer: contains guanidine, a harsh denaturant
RW1 = RNA wash buffer
RPE = Second RNA wash buffer with Ethanol
RDD = DNase digestion buffer

1. **Harvest** a maximum of 1 x 10^7 cells, as a cell pellet or by direct lysis in the vessel. Add the appropriate volume of Buffer RLT and vortex vigorously.

- If < 5 x 10^6 cells → 350 µl RLT (< 6cm plate)
- If ≤ 1 x 10^7 cells → 600 µl RLT (6-10cm plate)

2. Add 1 volume of 70% ethanol to the lysate, and mix well by pipetting. Do not centrifuge. Proceed immediately to the next step.
3. Transfer up to 700 µl of the sample, including any precipitate, to a spin column placed in a 2 ml collection tube (supplied).

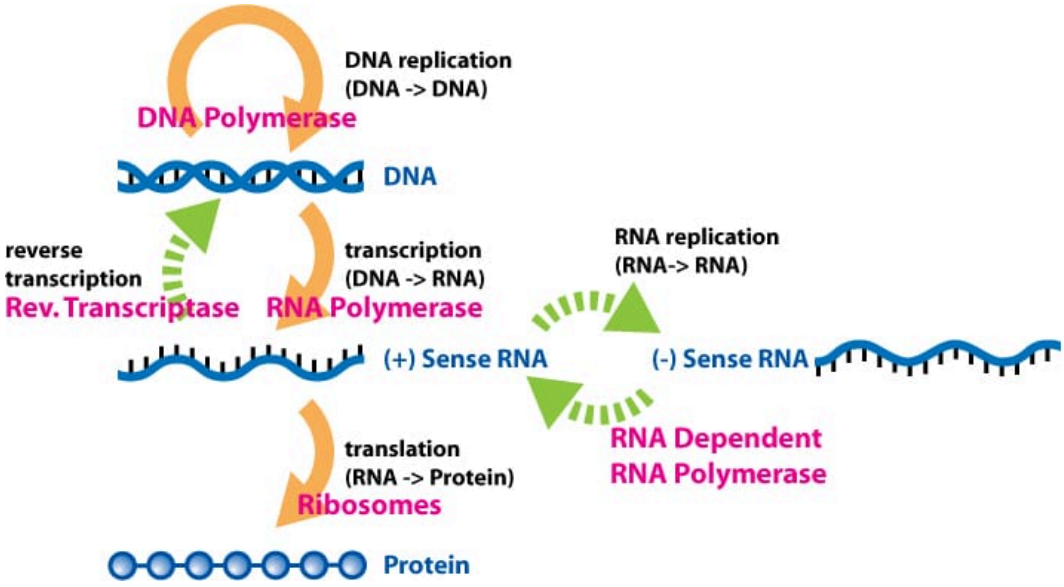


    1. Close the lid, and centrifuge for 15 s at ≥8000 x g.
    2. Discard the flow-through.
4. **Wash**: Add 350 µl Buffer RW1 to spin column, close lid, centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard flow-through.
5. Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently inverting the tube.
6. **Remove DNA** (optional): Add DNase I incubation mix (70 µl) directly to spin column membrane, and place on the benchtop (20–30°C) for 15 minutes.
7. **Wash**: Add 350 µl Buffer RW1 to spin column, close lid, centrifuge for 15 s at ≥8000 x g. Discard flow-through.
8. Add 700 µl Buffer RW1 to the spin column. Close the lid, and centrifuge for 15 s at ≥8000 x g. Discard the flow-through.
9. Add 500 µl Buffer RPE to the spin column. Close the lid, and centrifuge for 15 s at ≥8000 x g. Discard the flow-through.
10. Add 500 µl Buffer RPE to the spin column. Close the lid, and centrifuge for 2 min at ≥8000 x g.
11. Discard all flow-through and centrifuge at full speed for 1 min to dry the membrane.

12. Place the spin column in a new 1.5 ml collection tube. Add 30 µl RNase-free water directly to the spin column membrane.
13. Close the lid, and centrifuge for 1 min at ≥8000 x g to elute the RNA.
14. Add 30 µl RNase-free water directly to the spin column membrane. Close the lid, and centrifuge for 1 min at ≥8000 x g to elute the RNA.

Reverse Transcription

The **Central Dogma** of Molecular Biology was proposed by Francis Crick, the co-describer of the double-stranded helical structure of DNA. This "dogma" was a statement to describe the flow of genetic information to show that DNA houses or stores data that is transcribed into RNA that is subsequently translated from nucleotides into amino acids through the machinery of the ribosomes. Since DNA is relatively static in its ability to store genetic information, the expression of this stored data into the intermediate RNA or to the final protein product is of great significance. Imagine that the DNA in the nucleus of your cheek cells is identical to the DNA of the nucleus of cells in your liver. While the instructions are identical, these are clearly different cells that have a difference in the expression of proteins. Imagine a hard drive on a computer that stores information as 1's and 0's. These 1's and 0's do not have meaning until specific programs are called upon to act on this information. Likewise, different programs are called to use the instructions of your DNA to make a cheek cell different than a liver cell.



Credit: Daniel Horspool (CC-BY-SA 3.0)

In 1970, Howard Temin and David Baltimore independently isolated an enzyme from the Rous Sarcoma Virus and Murine Leukemia Virus, respectively. This enzyme was capable of violating the Central Dogma. The genomes of these viruses consist of RNA, not DNA. During the infection process, this enzyme is responsible for converting the RNA into DNA in a process called **reverse transcription**. This enzyme is logically called **reverse transcriptase** (RT). This discovery was rewarded with Nobel Prize in 1975. Later on, more viruses were discovered that were composed of RNA genomes that utilized this process, including HIV. Other enzymes within cells were also recognized to have reverse transcriptase activity, such as telomerase and retrotransposons. In molecular biology, these enzymes are used to convert mRNA into complementary copies of DNA called cDNA. The sum total of everything that is transcribed into RNA is referred to as the **transcriptome**. Synthesis of cDNA from any transcribed RNA can then be used for transcriptome analysis.

Exercise: Reverse Transcription of Eukaryotic mRNA

mRNA from eukaryotes are modified with 3′ polyadenylated tails. Oligo-dT primers can be used to prime the reverse transcription process of all mRNAs. All solutions should be kept on ice.

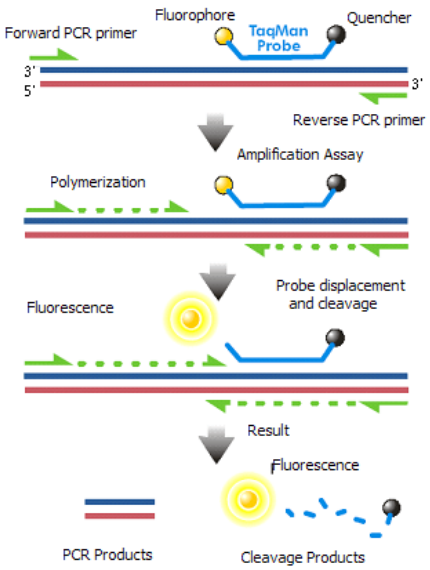
1. Determine the concentration of total RNA.
2. Adjust the concentration of RNA to 0.1mg/ml using Rnase free water.
3. Combine 10 ml RNA, 1 ml Oligo-dT (50µM), and 1 ml dNTP Mix (10 mM each).

4. Denature mixture at 65°C for 5 minutes and then place on ice.
5. Combine the following in a separate tube:
    1. 4µl Buffer 5X → contains all salts and pH buffer
    2. 2µl 0.1 M DTT → a reducing agent to mimic the cellular environment
    3. 1µl RNaseOUT (40U/µl) → an RnaseA inhibitor
    4. 1µl SuperScript III RT → the reverse transcriptase enzyme
6. After the denatured mixture has been sufficiently cooled, add 8µl enzyme mixture.
7. Incubate 45°C for 1 hour.
8. Deactivate enzyme by incubating 75°C for 10 minutes.
9. Store your cDNA in the freezer.

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2.1.11.4: Quantitative Nucleic Acid Measurement



Quantitative PCR (qPCR)

Measurements can be made of individual genes of interest through PCR of those specific genes. A process known as Real-Time PCR or quantitative PCR (**qPCR**) is used to measure individual genes using fluorescence measurements. An intercalating agent that binds only to double-stranded DNA called **Sybr Green** is used in a qPCR machine that is measuring fluorescence after each cycle of PCR indirectly indicates the amount of amplified product. However, non-specific products of amplification may also be measured and not discriminated from the authentic amplicon.

An alternative to Sybr Green is exemplified by the TaqMan technology. With TaqMan, a third primer (**TaqMan probe**) is designed in the middle of the area to be amplified. This middle primer is designed with a hairpin self-complementarity so that the 5′ and 3′ ends are in close proximity. At one end, a **fluorescent reporter** is attached while the other terminus has a **quencher** that absorbs any fluorescence signal. Under normal circumstances, measurements of fluorescence will be very low. When PCR extension occurs, the Polymerase hydrolyzes this middle primer, thereby separating the quencher and reporter. The name TaqMan is a play on words since it is imagined that the polymerase is chewing up the probe like Pacman. With increased distance between quencher/reporter, the fluorescence signal from this probe can now be measured. This method is much more specific than Sybr Green. However, the use of specific probes increases the cost considerably.

Threshold Cycles (C_t)

File:Qpcr-cycling.png

Credit: Zuzanna K. Filutowska (CC-BY-SA 3.0)

Fluorescence measurement early during the PCR process will be very low due to the small number of dsDNA molecules (Sybr Green) or most TaqMan primers being quenched. During this exponential DNA production, a threshold will be reached in which the fluorescence will linearly increase. A specific point where the fluorescence is clearly measurable is called the **Threshold Cycle** (**C_t**) is used as a reference point to compare expression values.

Looking at the example of Sybr Green qPCR above, it can be observed that samples exponentially increasing at a lower cycle number (C_t) has a higher level of mRNA expression (towards the left) of that gene than samples with higher cycle number (towards the right). Notice that the fluorescence eventually plateaus and stops increasing. This is due to the depletion of raw materials for DNA production like dNTPs.

Since the PCR reactions theoretically represent a doubling of DNA after each cycle, the C_t values can be interpreted on a base 2

system. If there is a difference in C_t between two samples (ΔC_t) of 5 cycles, this corresponds to 2^5 or 32 fold difference. We can control for variations in the RNA preparation through comparing the fluorescence values of our gene of interest to a housekeeping gene like actin. The use of a house-keeping gene to normalize the initial input to the reactions and comparison between samples is referred to as **Relative Quantification**.

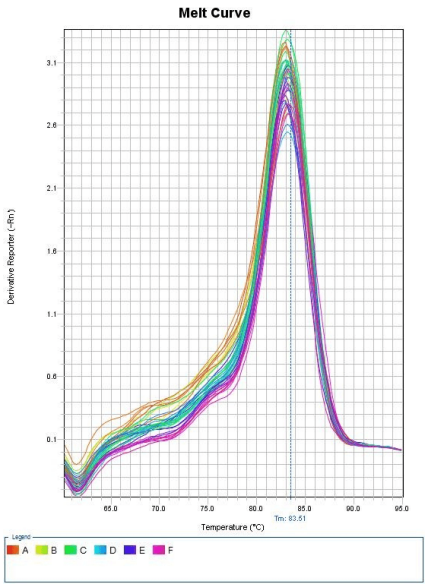
Melt Curves for Sybr Green

File:Melting Curve Analysis Graphs.svg

The top panel illustrates the decrease in fluorescence as the temperature increases due to the dissociation of double-stranded DNA. The bottom panel illustrates the first derivative plot. Each peak in this example illustrates a different allele. The double peaks represent the presence of the 2 distinct alleles in the amplification products.

Credit: Seans Potato Business (CC-BY-SA 3.0)

When using Sybr Green, we need to ensure that the PCR is specific so that the fluorescence measurement truly reflect amplification of our gene of interest. At the end of each qPCR run (~40 cycles), a melt curve is performed. A **melting curve** (or dissociation curve) comes from constant measurements as the temperature is increased. As temperature increases, the DNA strands start to denature and fluorescence will begin to decrease. After complete separation of DNA strands, the fluorescence will again remain constant. The way this curve is viewed is through a derivative plot where the inflection in fluorescence reading is reported as the **melting temperature (Tm)**.



This melt curve illustrates each sample contains the same specific product with a melting temperature of 83.51°C.

Any peaks in this plot refer to a specific PCR product. If multiple peaks appear, the results will not be valid as they do not directly measure a single product.

Expression Measurements

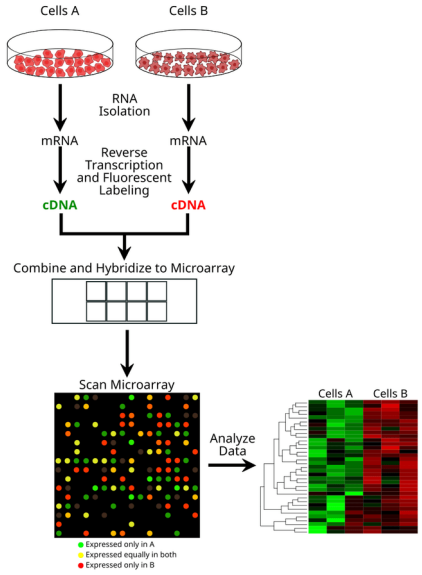
Differential gene expression refers to transcriptional programs activated by the cell under various conditions. “Differential” refers to a comparison of two or more states or timepoints. Using mRNA as an indirect measurement of protein, one can ascertain which proteins are linked to these different states. In eukaryotes, this can be assessed by enriching total RNA for polyA-containing mature mRNA. Through the use of oligo-(dT) containing resin, mRNA can be separated from non-protein encoding RNA. Likewise, performing a reverse-transcription using an oligo-(dT) primer will create a stable complementary DNA (cDNA) molecule that can be used with PCR. Using qPCR in this way is called **RT-PCR** or reverse-transcription polymerase chain reaction where specific primer pairs are used to amplify a small portion of a known gene.

Hybridization-Based Methods and Microarrays:

File:Northern Blot.jpg

Credit: FrozenMan (CC-BY-SA 4.0)

Prior to RT-PCR, the expression of individual genes was assessed through a hybridization-based approach. This method called for running RNA on an agarose gel and transferring the size-fractionated RNAs onto a membrane through a method called “blotting”. This transferred RNA was then hybridized to a radioactively labeled probe for a specific gene (corresponding to the reverse complementary sequence) and visualized by exposure to X-ray film in a process called **Northern Blotting**. The intensity of the band would be proportional to the amount of mRNA corresponding to the gene of interest. Re-probing with a housekeeping gene like actin would be used as a loading control to illustrate that a similar amount of total RNA was loaded into each well. Differences in sizes of the mRNA on the Northern Blot also revealed differences in splice variants of mature mRNA in the different states.



Credit: Jeremy Seto (CC-BY-NC-SA)

This technique was later adapted using non-radioactive methods. Using these non-radioactive methods, the reverse protocol was developed to measure multiple gene targets. By systematically immobilizing gene-specific probes onto a membrane or a microscope slide, an array of targets can be produced. In the simplest paradigm of having 2 states (control or experimental), cDNA from each sample can be used to generate fluorescent RNA that can hybridize to immobilized probes. Using 2 different fluorescent markers allows for the competitive hybridization onto the array whereby the fluorescent signal in each channel can reveal the differential gene expression of the two states in a 2-color **microarray**.

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2.1.11.5: Eukaryotic Transcription

Eukaryotic Gene Expression

File:Gene structure eukaryote 2 annotated.svg

The Central Dogma in Eukaryotes. Genomic DNA of genes often contain introns that are spliced out when an RNA matures to an mRNA. This excision of introns can result in splice variants of the same gene with variants of the same protein. Credit: Thomas Shafee (CC-BY 4.0)

Unlike prokaryotic genes, the expression of genes in eukaryotic cells has complex systems of transcription factors that act on promoters to recruit RNA polymerases. Additionally, **enhancer elements** may reside many kilobases upstream of the promoter. These enhancers strengthen the transcription of the gene. In this case, **transcription activator proteins** or trans-activators augment the promoter activity.

File:Enhancer Nucleotide Sequence.svg

1. DNA 2. Enhancer 3. Promoter 4. Gene 5. Transcription Activator Protein 6. Mediator Protein 7. RNA Polymerase

Credit: Jon Cheff (CC-BY-SA 4.0)

Mediator proteins (**coactivators**) form a multiprotein complex with the activators to recruit RNA polymerase to the promoter.

Eukaryotic mRNA

File:MRNA.svg

Credit: Kelvinsong (CC-BY-3.0)

Eukaryotic genes may often contain **introns** (non-coding sequences) that are spliced out from the **exons** (coding sequences). This complexity permits for an increased variety of gene products. Mature eukaryotic mRNAs contain a 5′-methyl-Guanine followed by an untranslated leader sequence (**5′-UTR**), the coding sequences (**cds**), a 3′-untranslated region (**3′-UTR**) and a long stretch of Adenines (polyA tail).

File:Alternative polyadenylation.svg

Expression is the most easily measured with RNA since nucleic acid manipulation is fairly simple with 4 different nucleotides. In eukaryotes, the messenger RNA (mRNA) intermediate that is transcribed from DNA contains a polyA tail that is used to separate these messages from other types of RNA that are abundant within cells (like ribosomal RNA). Through the use of an enzyme called **reverse transcriptase** (RT) and primers composed of deoxy-Thymidines (**oligo-dT** or dT_{18}), mRNA can be converted into a single strand of DNA that is complementary to the mRNA. This complimentary DNA is called **cDNA**. cDNA is very stable compared to the highly labile mRNA and is used for subsequent processing.

Advanced Video of Eukaryotic Transcription Regulation

The first video describes the discovery of transcription factors that regulate the expression of eukaryotic genes.

The second video describes the complexity of gene expression that involves chromatin remodeling and enhancers. This video explores the roles and outcomes of differential gene expression.

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2.1.12: TRACING ORIGINS

2.1.12.1: Introduction
2.1.12.2: Maternal Lineage
2.1.12.3: Maternal Lineage (Activity)
2.1.12.4: Alu Insertion (Activity)
2.1.12.5: Transposons

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2.1.12.1: Introduction

Being Human

File:Lions painting, Chauvet Cave (museum replica).jpg

Drawings dating to approximately 30,000 years ago in the Chauvet Cave.

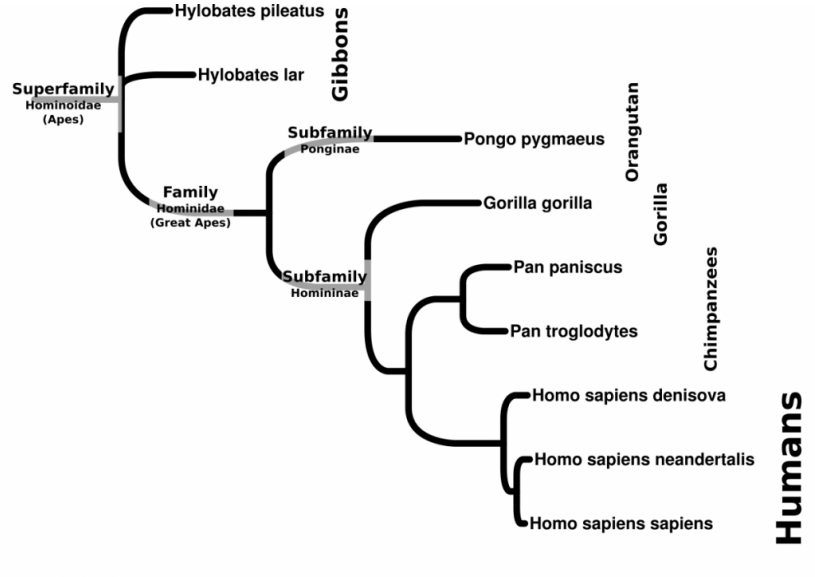
What constitutes being human? Many will point at cultural identity and leaving long-standing remnants of that culture. Such prehistorical artifacts like cave drawings and tools provide an anthropological framework for identifying what it is to be human, but the biological identity remains locked in the history of our DNA.

File:Clovis Rummells Maske.jpg

Spear points of the Clovis Culture in the Americas dating to approximately 13,000 years ago.

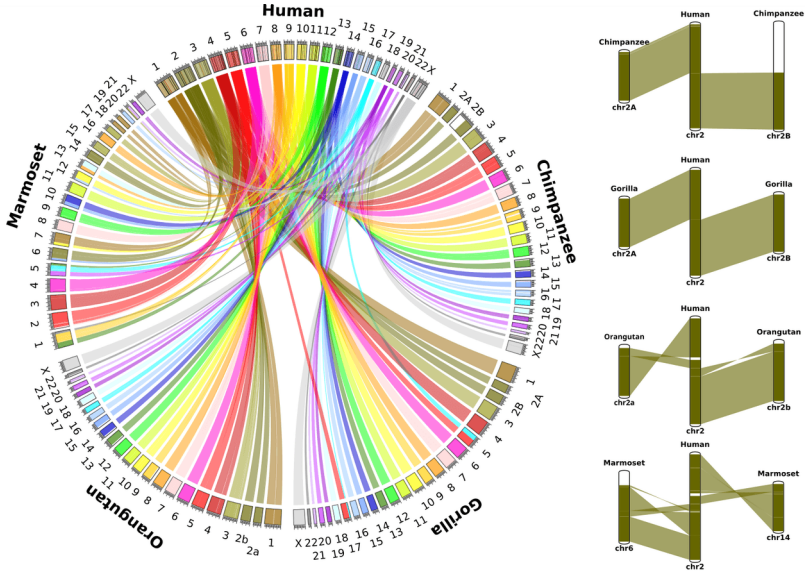
Credit: Bill Whittaker [CC-BY-SA 3.0]

The Great Apes



Phylogenetic tree generated with Cytochrome Oxidase I (COI) genes.

Homo sapiens represent a branch of primates in the line of Great Apes. The family of Great Apes consists of four extant genera: *Homo, Pan, Gorilla, Pongo*. Karyotype analysis (Yunis *et al.*, 1982) reveals a shared genomic structure between the Great Apes. While humans have 46 chromosomes, the other Great Apes have 48. Molecular evidence at the DNA level indicates that Human Chromosome 2 is a fusion of 2 individual chromosomes. In the other Great Apes, these 2 Chromosomes are referred to as 2p and 2q to illustrate their synteny to the human counterpart.



Synteny map of Human, Chimpanzee, Gorilla, Orangutan, and Marmoset (non-ape primate). Mapping of chromosome 2a and 2b in the apes compared to 6 and 14 in the marmoset illustrates the relatedness of the chromosomal structure of the apes. Minor inversions are apparent in the orangutan chromosome.

Credit: Jeremy Seto [CC-BY-NC-SA]

Chimpanzees (*Pan*) are the closest living relatives to modern humans. It is commonly cited that less than 2% differences in their nucleotide sequences exist with humans (Chimpanzee Sequencing and Analysis Consortium, 2005). More recent findings in comparing the complement of genes (including duplication and gene loss events) now describe the difference in genomes at about 6% (Demuth JP, et al., 2006).



The Pan-Homo divergence. A display at the Cradle of Humankind illuminates the skulls of two extant Hominini with a series of model fossils from the Hominina subtribe of Australopithecina and Homo. Credit: Jeremy Seto [CC-BY-NC-SA] https://flic.kr/p/SmhHTd

The Genus *Homo*



An underground lake at inside the Sterkfontein Cave system at the Cradle of Humankind (South Africa) Credit: Jeremy Seto [CC-BY-NC-SA] https://flic.kr/p/RczrEg

The rise of the human lineage is thought to arise in Africa. Fossils of *Australoptihs* (southern apes) found in death traps, like those at the Cradle of Humankind, reveal a historical record of organisms inhabiting the landscape. The breaks in the ceiling of the caves provide opportunities for animals to fall inside these caves to their death. The limestone deposits of the caves serve as an environment for fossilization and mineralization of their remains. An abundance of fossilized hominids in these caves including *Australopithecus africanus, Australopithecus prometheus, Paranthropus boisei,* and the newly discovered *Homo naledi* continue to reveal the natural history of the genus *Homo* from 2.6 million to 200,000 years ago.



The entrance to the archaeological site at Sterkfontein, Cradle of Humankind (South Africa). Credit: Jeremy Seto [CC-BY-NC-SA] https://flic.kr/p/ULs2Sv

Ancient DNA of Humans

File:Spread and evolution of Denisovans.jpg

In 2008, a piece of a finger bone and a molar from a Siberian Cave were found that differed slightly from that of modern humans and Neandertals. The cave, called Denisova Cave, maintains an average temperature of 0°C year-round and the bones were suspected to contain viable soft tissue. An initial mitochondrial DNA analysis revealed that these hominids represented a distinct line of humans that overlapped with them in time (Krause *et al.*, 2010). Analysis of the full nuclear genome followed and indicated that interbreeding existed between these Denisovans, Neandertals and modern humans (Reich *et al.*, 2010). Furthermore, analysis of DNA from a 400,000-year-old femur in Spain revealed that these three lines diverged from the species *Homo heidelbergensis* with Denisovans closest in sequence similarity (Meyer *et al.*, 2016).

Between modern humans, markers found in the mtDNA can be used to trace the migrations and origins along the maternal line. Similarly, VNTRs found on the Y chromosome have revealed migration patterns along paternal lines within men. Other markers, like the insertion points of transposable elements, can be used to further describe the genetics and inheritance of modern humans while providing a snapshot into evolutionary history.

Other Resources

- **Great Ape Mitochondrial Sequences**
- http://media.hhmi.org/biointeractive/click/Origins/01.html
- Yunis JJ, Prakash O. The origin of man: a chromosomal pictorial legacy. Science.1982 Mar 19;215(4539):1525-30.
- Chimpanzee Sequencing and Analysis Consortium. Initial sequence of the chimpanzee genome and comparison with the human genome. Nature. 2005 Sep 1;437(7055):69-87.
- Demuth JP, De Bie T, Stajich JE, Cristianini N, Hahn MW. **The evolution of mammalian gene families**. PLoS One. 2006 Dec 20;1:e85.
- Krause, Johannes; Fu, Qiaomei; Good, Jeffrey M.; Viola, Bence; Shunkov, Michael V.; Derevianko, Anatoli P. & Pääbo, Svante (2010), "The complete mitochondrial DNA genome of an unknown hominin from southern Siberia", Nature, **464** (7290): 894–897, doi:10.1038/nature08976, PMID 20336068
- Reich, David; Green, Richard E.; Kircher, Martin; Krause, Johannes; Patterson, Nick; Durand, Eric Y.; Viola, Bence; Briggs, Adrian W. & Stenzel, Udo (2010), "Genetic history of an archaic hominin group from Denisova Cave in Siberia", *Nature*, **468** (7327): 1053–1060, doi:10.1038/nature09710, PMID 21179161
- Meyer M, Arsuaga JL, de Filippo C, Nagel S, Aximu-Petri A, Nickel B, Martínez I, Gracia A, Bermúdez de Castro JM, Carbonell E, Viola B, Kelso J, Prüfer K, Pääbo S. **Nuclear DNA sequences from the Middle Pleistocene Sima de los Huesos hominins.** Nature. 2016 Mar 24;531(7595):504-7. doi: 10.1038/nature17405. PMID:26976447

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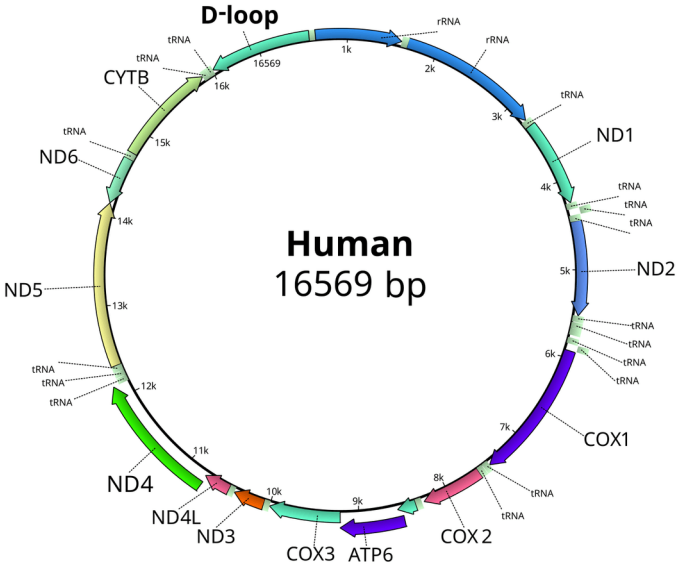
2.1.12.2: Maternal Lineage

Mitochondrial and Maternal Inheritance

In addition to the 23 chromosomes inherited from mother and 23 chromosomes inherited from father, humans have an additional genome that is only inherited from the mother. This genome comes from the **endosymbiotic** organelle, the mitochondrion.

File:Mitochondrial DNA lg.jpg

Mitochondria are thought to have arisen in the eukaryotic line when bacteria capable of detoxifying the deadly effects of atmospheric oxygen were engulfed by a eukaryote that did not proceed to consume it. Over the course of time, these formerly free-living bacteria became dependent on the eukaryotic cell environment while providing the benefit to the host cell of aerobic respiration. Hallmarks of this endosymbiotic event include the inner prokaryotic membrane surrounded by the outer eukaryotic membrane, the presence of prokaryotic ribosomes and most significantly, and the circular prokaryotic chromosome. Mitochondria still replicate independently of the host cell but can not survive outside of this cellular environment. Animal mitochondria have the simplest genomes of all mitochondrial genomes, ranging from 11-28kb. The human mitochondrial genome consists of 37 genes which are almost all devoted to processing ATP through oxidative phosphorylation.



Human mitochondrial genome

The human mitochondrial genome (genbank file) consists of 16,569 nucleotides (16.6kb). While most of this 16.6kb genome consists of protein-encoding genes, approximately 1.2kb non-coding DNA takes part in signals that control the expression of these genes and replication processes. It is the area of DNA where the double-strandedness is displaced and having the name **D-loop** (displacement loop). Mutations in this area generally have very little effect on the functioning of the mitochondria. Because of this reduced selection pressure on this area, this **control region** is also referred to as the **hypervariable region**. This hypervariable region actually has 10 times more SNPs than the nuclear genome. Due to this abundance of mutations, it is possible to track down the maternal line of an individual. Why just maternal? The human oocyte contains many mitochondria while sperm cells only contain mitochondria that power the flagellar motion. Upon fertilization, the flagellum and the associated mitochondria are lost, leaving the zygote with only maternal mitochondria.

The cluster of SNPs found in the mitochondrial control region are linked and are always inherited together. Because of the lack of paternal contribution, this linkage is referred to as a **haplotype**, or “half-type”. Tracking these polymorphic haplotypes, a family tree of humans was developed in the 1980s which concluded that humans arose from a metaphorical “Mitochondrial Eve” 200,000 years ago. As a metaphor to the Biblical Eve, this alludes to an origin but unlike the Biblical event, this does not mean that it was a single woman that gave rise to all of modern humanity. On the contrary, the metaphor merely indicates that a series

of females; sisters and cousins, of this line gave rise to modern humans.

File:World map of prehistoric human migrations.jpg

Migration map of mitochondrial haplogroups. Numbers represent 1000 years ago.

The use of mitochondria for this analysis provides great flexibility, especially from ancient sources. Unlike the nuclear genome which only has 2 copies of DNA per cell, the mitochondria are abundant in number and provide many copies of genome per cell. Ancient sources of DNA in fossils will most often have degradation of the DNA. The mitochondrial genome is just as likely to undergo degradation over time. However, the high copy number allows for gaps to be filled in easily. SNPs do not alter the overall size of the hypervariable region, therefore amplification by PCR can not resolve these differences based on agarose gel migration. However, **amplicons** (amplified copies) can be sent for sequencing whereby each nucleotide can be called out in succession and reveal the specific SNPs.

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2.1.12.3: Maternal Lineage (Activity)

The PCR Amplification of the Mitochondrial Control Region

There are 2 hypervariable regions within the control region of the mitochondria. This exercise amplifies just one of these. For more definitive results, both should be amplified and sequenced. This exercise will permit us to have a rough idea of the origins of our maternal line and we will be able to attribute ourselves to various tribes throughout the world. The human mitochondrial genome (genbank file).

Forward Primer 5'-TTAACTCCACCATTAGCACC-3'

Reverse Primer 5'-GAGGATGGTGGTCAAGGGAC-3'

1. PCR the previously extract DNA samples.

- Pour 2% agarose into casting apparatus in refrigerator.
- 2 gels per class need to be made → 100ml of TBE with 2g agarose
- Add 5µl SYBR safe solution into the molten agarose before casting.
- Place 2 sets of combs into the gel → at one end and in the middle.

2. Load the gel with DNA ladder and PCR.
3. Run the gel at 120V for 20 minutes.
4. Visualize on the UV transilluminator.
5. Document with a camera to verify amplification.
6. The instructor will submit viable reactions for sequencing.
7. **Analyze data during Bioinformatics Lab session.**
    1. Using NYCCT email address, register for an account at https://dnasubway.cyverse.org/.
    2. Retrieve reference mitochondrial sequences.
    3. Perform multiple sequence alignment using MUSCLE.
    4. Draw phylogenetic trees using PHYLIP and visualize using a FigTree.

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2.1.12.4: Alu Insertion (Activity)

Alu's are unique SINEs that appear in the primate lineage and reveal the lineage and diversification of primates. While retrotransposons can disrupt gene (as in some cases of hemophilia), they often land outside of genes or within introns without effect. One example of a non-disruptive Alu element in humans is found in the location called **PV92** on chromosome 16. This element is of the youngest subfamily of Alu, called **Ya5**.

Since PV92 does not cause any deleterious effects, it can be used as a non-selected marker to illustrate lineage. Some people have an *Alu* element int his location while others do not. The presence or absence of this marker is viewed as an allele. This lab uses a primer that flanks the location of the *Alu* insertion that span 416 bp. If an *Alu* is present, the amplified DNA will be 300bp larger (the size of an *Alu*) at 731bp.

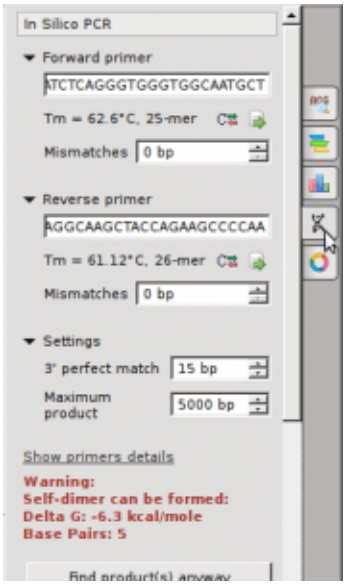
Exercise: In Silico PCR of PV92

Forward primer: 5′ GGATCTCAGGGTGGGTGGCAATGCT 3′
Reverse primer: 5′ GAAAGGCAAGCTACCAGAAGCCCCAA 3′

1. Perform Virtual PCR **Informatics Exercise/Discussion.**
2. Visit BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch
3. Paste both primers: GGATCTCAGGGTGGGTGGCAATGCT GAAAGGCAAGCTACCAGAAGCCCCAA
4. Choose "Somewhat Similar"
    1. Locate the locus of the product and the size.

5. Find the PCR fragments in Ugene

    1. Download the sample FASTA file: PV92 sample
    2. Open the file in Ugene and select option "As Separate Sequences in Viewer"
    3. Select the "In Silico PCR" button on the far right (double helix button) and insert the primers



4. A PCR product should be noted for one of the sequences after pressing "Find Products anyway"

5. Click on the second sequence in the viewer and Press "Find Products anyway"

Exercise: PCR Genotype PV92 Locus

1. PCR the individual samples.
2. Pour 2% agarose into casting apparatus in the refrigerator.
    - 2 gels per class need to be made → 100ml of TBE with 2g agarose.
    - Add 5µl SYBR safe solution into the molten agarose before casting.
    - Place 2 sets of combs into the gel → at one end and in the middle.
3. Load DNA ladder and PCR samples.
4. Run gel at 120V for 30 minutes.
5. Visualize on UV transilluminator.
6. Score gels for the presence/absence of the alleles to determine genotype frequency in the class.

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2.1.12.5: Transposons

Transposable Elements

Mobile genetic elements called **transposable elements** or **transposons** are located throughout the genome. These elements were first described in maize by Barbara McClintock at the Cold Spring Harbor Laboratory where she observed a disruption of coloring in corn kernels that did not follow simple Mendelian inheritance.

McClintock's Corn Kernels



Each kernel represents a distinct new individual organism. Kernel color is described through simple Mendelian inheritance where purple is dominant over yellow. Dr. McClintock noticed that some kernels contained spots. She noticed that the coloration disruption could later reverse in subsequent generations. She described the phenomenon of this break in the Mendelian characteristics as a "genetic instability". Over time, she would come to realize that the spots in these kernels arose from the insertion of DNA into the area of genes that were involved in controlling kernel coloration.

Dr. McClintock's description of this phenomenon and the underlying mechanisms was extremely unpopular as it violated what was already known about the *fixity* of genetics. Though initially skeptical, biology has found that these "jumping genes" are found in every taxa including prokaryotes (where they are often associated with genes conferring antibiotic resistance) and she was later awarded the Nobel Prize. Approximately half of the human genome consists of transposons, making up the bulk of what was previously referred to as "junk DNA". **(See Animation: http://www.dnaftb.org/32/animation.html)**

Advanced Video of Gene Disruption by Jumping Genes

Classes of Transposons

Transposons can be autonomous or non-autonomous. **Autonomous transposons** encode their own **transposase** enzyme that facilitates the jumping of the gene while **non-autonomous transposons** require the transposase activity of another transposable element. Functional DNA transposons are autonomous and work through a "*cut and paste*" mechanism. DNA transposons are delineated by flanking terminal repeats that mark the location that the transposase excises the DNA. These DNA elements then re-integrate at a different location within the genome. The excision from DNA leaves marks of these flanking repeats that can be used to study the rate and level of DNA transposition events within a genome. The insertion of these transposons can affect the expression of nearby genes and can completely disrupt genes they land into as evidenced in the speckled corn kernels that

McClintock described.

RNA transposons are called **retrotransposons** because they are transcribed into an mRNA and require a reverse transcription to integrate into the genome. The most common mobile element in the human genome is the Long Interspersed Nuclear Elements (**LINEs**) and the Short Interspersed Nuclear Elements (**SINEs**). These retrotransposons are most abundantly represented by the autonomous LINE1 (L1) and non-autonomous *Alu* elements, respectively. *Alu* elements rely on the expression of the L1 in order to be reverse-transcribed and integrated into the genome. These retrotransposons work in a "*copy and paste*" mechanism and are responsible for genomic expansion. As their classifications signify, LINEs are longer than SINEs. This is due to the presence of a second reading frame that encodes the transposase.

File:PLoSBiol3.5.Fig7ChromosomesAluFish.jpg

The Human Genome is littered with "junk" DNA. Green coloration corresponds to Alu sequences in this karyogram of the human genome.

Credit: Andreas Bolzer, Gregor Kreth, Irina Solovei, Daniela Koehler, Kaan Saracoglu, Christine Fauth, Stefan Müller, Roland Eils, Christoph Cremer, Michael R. Speicher, Thomas Cremer (CC-BY 2.5)

- Human L1 sequence (fasta)
- Human Ya5 Alu sequence (fasta)

Advanced Video of Classification of Transposons and the Evolution of Genomes

Further Reading

- http://www.nature.com/scitable/topicpage/barbara-mcclintock-and-the-discovery-of-jumping-34083
- http://www.nature.com/scitable/topicpage/functions-and-utility-of-alu-jumping-genes-561

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2.1.13: DNA BARCODING

2.1.13.1: Introduction
2.1.13.2: Barcoding (Activity)

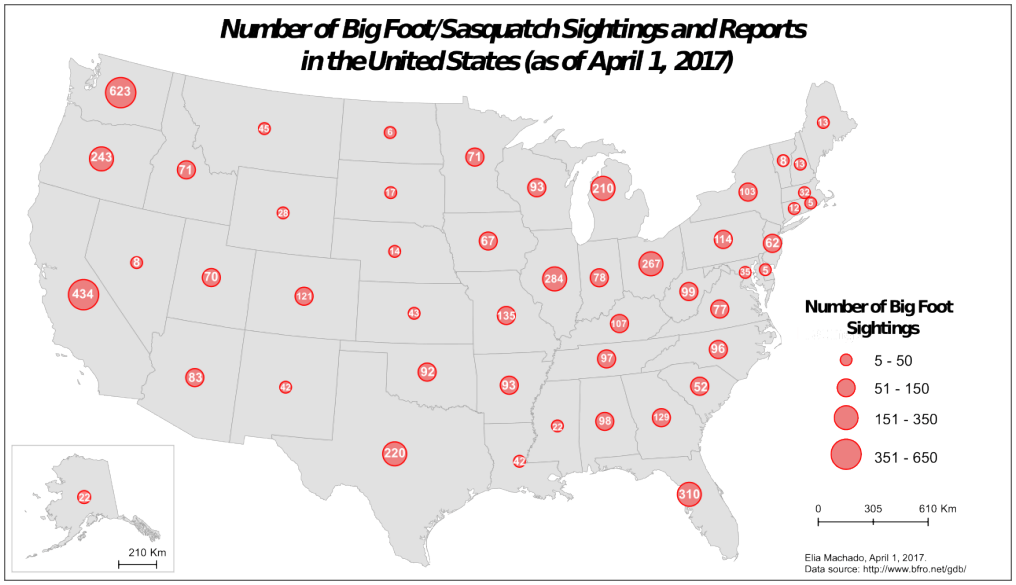
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2.1.13.1: Introduction

Cryptozoology

Cryptozoology is a pseudoscience centered around the description of animals that have little or no evidence of existing. These mythical beasts include Bigfoot, Yeti, Sasquatch, jackalope, Loch Ness Monster, and chupacabra. Little evidence exists to illustrate their existence other than folklore.



Sightings of Bigfoot in North America. Credit: Elia Machado (CC-BY-NC-SA)

Sometimes, physical evidence is left behind like hair or feces. With DNA evidence, we can help to confirm the existence of these unknown creatures. Below features a table from Sykes et al. displaying results on supposed cryptic Apes (Bigfoot/Yeti) and what DNA evidence has revealed them to be.

ref. no.	location	attribution	GenBank sequence match	common name
25025	Ladakh, India	*yeti*	*U. maritimus*	polar bear
25191	Bhutan	*yeti/migyhur*	*U. maritimus*	polar bear
25092	Nepal	*yeti*	*Capricornis sumatraensis*	serow
25027	Russia	*almasty*	*U. arctos*	brown bear
25039	Russia	*almasty*	*Equus caballus*	horse
25040	Russia	*almasty*	*Bos taurus*	cow
25041	Russia	*almasty*	*Equus caballus*	horse
25073	Russia	*almasty*	*Equus caballus*	horse
25074	Russia	*almasty*	*U. americanus*	American black bear
25075	Russia	*almasty*	*P. lotor*	raccoon
25194	Russia	*almasty*	*U. arctos*	brown bear
25044	Sumatra	*orang pendek*	*Tapirus indicus*	Malaysian tapir
25035	AZ, USA	*bigfoot*	*P. lotor*	raccoon
25167	AZ, USA	*bigfoot*	*Ovis aries*	sheep
25104	CA, USA	*bigfoot*	*U. americanus*	American black bear
25106	CA, USA	*bigfoot*	*U. americanus*	American black bear
25081	MN, USA	*bigfoot*	*Erethizon dorsatum*	N. American porcupine
25082	MN, USA	*bigfoot*	*U. americanus*	American black bear
25202	OR, USA	*bigfoot*	*U. americanus*	American black bear
25212	OR, USA	*bigfoot*	*C. lupus/latrans/domesticus*	wolf/coyote/dog
25023	TX, USA	*bigfoot*	*Equus caballus*	horse
25072	TX, USA	*bigfoot*	*Homo sapiens*	human
25028	WA, USA	*bigfoot*	*U. americanus*	American black bear
25029	WA, USA	*bigfoot*	*C. lupus/latrans/domesticus*	wolf/coyote/dog
25030	WA, USA	*bigfoot*	*Bos taurus*	cow
25069	WA, USA	*bigfoot*	*Odocoileus virginianus/hemionus*	white-tailed/mule deer
25086	WA, USA	*bigfoot*	*Bos taurus*	cow
25093	WA, USA	*bigfoot*	*C. lupus/latrans/domesticus*	wolf/coyote/dog
25112	WA, USA	*bigfoot*	*Bos taurus*	cow
25113	WA, USA	*bigfoot*	*C. lupus/latrans/domesticus*	wolf/coyote/dog

Genetic analysis of hair samples attributed to yeti, bigfoot and other anomalous primates
Bryan C. Sykes, Rhettman A. Mullis, Christophe Hagenmuller, Terry W. Melton, Michel Sartori
Published 2 July 2014.DOI: 10.1098/rspb.2014.0161 CC-BY

Cryptozoological samples of hair believed to arise from legendary animals like Bigfoot, Sasquatch, Yeti, etc.

Table taken from http://rspb.royalsocietypublishing.org/content/281/1789/20140161 CC-BY

The Need for Barcoding

Taxonomy of living things was created by Carl von Linné, who formalized it by using a binomial classification system to differentiate organisms. Binomial nomenclature was used to describe a genus and a species name to each organism to provide an identity. These days, classifications of organisms is becoming increasingly important as a measurement of diversity in the face of habitat destruction and global climate change. There is no consensus on how many life forms exist on this planet, but the estimation of extinction rates is about 1 species per 100-1000 million species. Classification in Linné's day was mostly performed by morphological differences. This was carried on in fossils. However, morphology has many drawbacks, especially in sexually dimorphic species or species with multiple developmental morphologies.



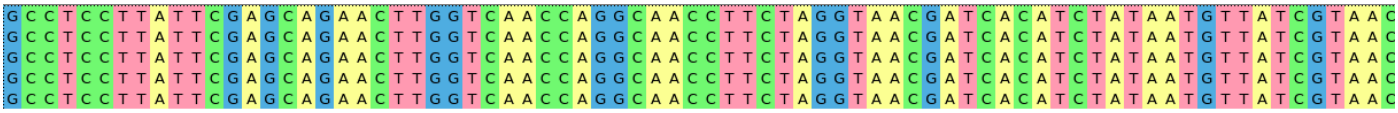


Larva (top) of the Green Lacewing and the adult (bottom)

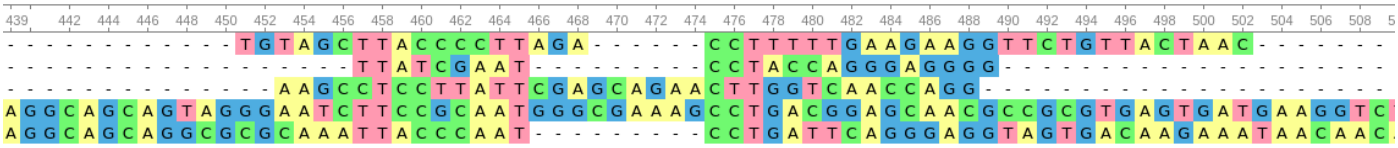
Molecular biology and DNA technologies have revolutionized the classification system of living things especially in providing the ability to match relatedness of these species. **DNA barcoding**, like the name implies, seeks to utilize DNA markers to differentially identify organisms. But what DNA markers should be used? What criteria do we use to develop barcodes? Discrimination, Universality, and Robustness are the criteria used to define the usefulness of barcodes.



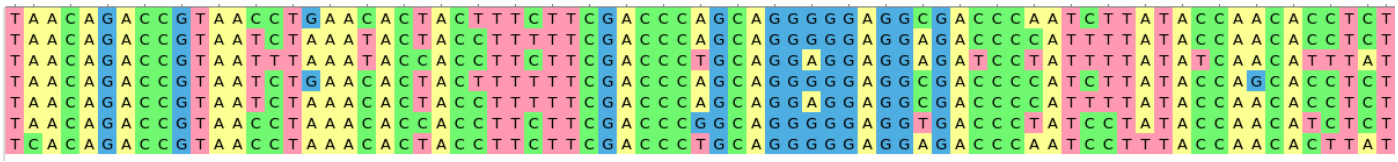
Since the goal of barcoding is to define specific organisms, discrimination is the *primary objective*. **Discrimination** refers to the difference of sequences that occur between species. However, science is easier when there is some universality in the locus used for discrimination. As it sounds, **universality** is an attempt to use the same locus in disparate genomes. While discrimination is about the uniqueness of sequences, universality seeks to use a single set of PCR primers that will be able to amplify that same distinct region with variable sequence similarity. If some region of DNA has absolutely no sequence deviation between species, this has great universality but poor discrimination. But if a sequence has very low sequence similarity, this is great for discrimination but has absolutely no universality and can not be amplified with the same set of primers. **Robustness** refers to the reliability of PCR amplification of a region. Some regions of DNA just don't amplify well or it is too difficult to design appropriate and unique primers for that locus.



A case where there is universality for designing primers, but not an area where discrimination can occur.



While discrimination of different organisms can occur in this situation, the lack of similarity in sequence would make it difficult to design primers. That is, the lack of universality in sequence would also make this PCR not robust.



Enough variability in these sequences gives us the ability to discriminate between species. The high similarity provides us the universality required to design primers that may be robust enough to amplify by PCR.



Sometimes, species are so similar for one sequence that a second marker is required. Just as the standard UPC barcode has a series of vertical lines of different spacing and width, a 2-dimensional barcode adds that second dimension of information into a square of dots like in a QR code (Quick Response code). We can also utilize the second, the third, or the fourth set of loci that will aid in increased discrimination just as CoDIS utilizes multiple STR sites to define individual people. In animals, the most

commonly used barcode is the mitochondrial gene, Cytochrome Oxidase I (**COI**). Since all animals have mitochondria and have this mitochondrial gene, it offers high universality. It is a robust locus that is easy to amplify and has high copy number with enough sequence deviation between species to discriminate between them.

Animal mitochondrial genomes vary from 16kb-22kb. However, plants, fungi, and protists have wildly different and larger mitochondrial genomes. For plants, we use a chloroplast gene, ribulose-bisphosphate carboxylase large subunit (**rbcL**) or maturase K (**matK**) (Hollingsworth et al. 2011). Prokaryotes are often discriminated by their **16s** rRNA gene while eukaryotes can be identified by **18s** rRNA. COI (a maternally transmitted gene) will not create a clear picture of species identity in the case of hybrid animals (mules, ligers, coydogs, etc.). Sometimes, closely related species are also indistinguishable by a single barcode, so the inclusion of 18s with COI may be necessary to define the identity of the species. Since it is so difficult to meet the three criteria (robustness, universality, and discrimination) for all species, having these multiple barcodes is important. Fungi prove to be difficult in identification by COI, so another marker called the internal transcribed spacer (**ITS**) is used to aid in their identification. We must also remember that not everything with chloroplasts are plants and, therefore, additional markers are used to identify protists.

Mixtures of Organisms



Lichens are composite organisms composed of cyanobacteria or other algae with fungi. In this case, a single barcode would incorrectly identify the species.

File:Kefirpilze.jpg

Kefir granules represent colonies of mixed microbes that are used to generate kefir. Credit: A. Kniesel (CC-BY-SA 3.0)

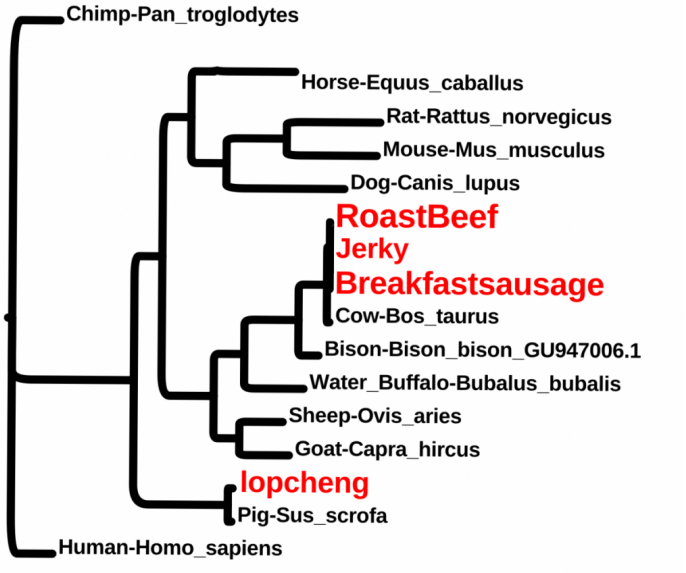
File:SCOBY mushroom.jpg

A symbiotic colony of bacteria and yeast is used to ferment kombucha. As the name implies, this is a complex composite colony of multiple species that contribute to the qualities of the kombucha. Credit: Lukas Chin (CC-BY-SA 4.0)

Metabarcoding and Microbiomes

Class Results

Students wanted to check some food items. These included breakfast sausage from a Halal cart, “beef jerky” from the vending machine, roast beef from the cafeteria, and Chinese sausage (lopcheng).



For more class results, please visit https://openlab.citytech.cuny.edu/dna-barcodes/

Further Resources

- Reference Sequences
  - Gorilla COI: FASTA
  - Apple rbcL: FASTA
  - *Staphylococcus* 16S: FASTA
  - *Toxoplasma* 18S: FASTA
  - Porcinni mushroom ITS: FASTA
- Sykes BC, Mullis RA, Hagenmuller C, Melton TW, Sartori M. Genetic analysis of hair samples attributed to yeti, bigfoot and other anomalous primates. Proc Biol Sci. 2014 Aug 22;281(1789):20140161. doi: 10.1098/rspb.2014.0161.
- Hollingsworth PM, Graham SW, Little DP (2011) Choosing and Using a Plant DNA Barcode. PLOS ONE 6(5): e19254. https://doi.org/10.1371/journal.pone.0019254
- http://www.dnabarcoding101.org/

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2.1.13.2: Barcoding (Activity)

DNA Barcoding of Samples

1. Place sample in a clean 1.5 mL tube.
2. Add 100 µl of nuclear lysis solution to tube.
    - Twist a clean plastic pestle against the inner surface.
3. Add 500 µl more nuclear lysis solution to tube.
4. Incubate the tube in a water bath or heat block at 65°C for 5-15 minutes.
5. [Optional] Add 200 µl of protein precipitation solution to each tube incubate on ice for 5 minutes.
6. Centrifuge for 4 minutes at maximum speed to pellet protein and cell debris.
7. Transfer 600 µl of supernatant to a clean labeled tube.
8. Add 600 µl of isopropanol.
9. Centrifuge for 2 minutes at maximum speed to pellet the DNA.
10. Pour off the supernatant and add 600 µl of 70% ethanol to wash the pellet.
11. Centrifuge the tube for 2 minutes at maximum speed and carefully remove the solution.
12. Air-dry the pellet for 10 minutes and add 100 µl of the DNA rehydration solution (TE).
13. Incubate the DNA at 65°C for 5-10 minutes to dissolve.
14. Obtain a PCR tube containing Ready-To-Go PCR Bead. Label the tube with your identification number.
15. Use a micropipette with a fresh tip to add 23 µL of one of the following primer/loading dye mixes to each tube. Allow the beads to dissolve for 1 minute.
    - Plants: rbcL primers (rbcLaF / rbcLa rev)
    - Fish: COI primers (VF2_t1/ FishF2_t1/ FishR2_t1/ FR1d_t1)
    - Insects: (LepF1_t1/ LepR1_t1)
16. Add 2 µl of your DNA directly into the appropriate primer/loading dye mix.
17. Place tubes in a Thermal cycler.
18. Pour 2% agarose into casting apparatus in the refrigerator.
    1. 2 gels per class needed to be made → 100ml of TBE with 2g agarose
    2. Add 5 µl SYBR safe solution into the molten agarose before casting.
    3. Place 2 sets of combs into the gel → at one end and in the middle.
19. Load DNA ladder and PCR samples.
20. Run the gel at 120V for 30 minutes.
21. Visualize on the UV transilluminator.
22. Document with a camera.
23. Send amplicons of verified samples for sequencing.

- Plant rbcL gene
    - rbcLaf 5'- ATGTCACCACAAACAGAGACTAAAGC-3' (forward primer)
    - rbcLar 5'- GTAAAATCAAGTCCACCRCG-3' (reverse primer)
- Animal coi gene
    - lepF1 5'- ATTCAACCAATCATAAAGATATTGG -3' (forward primer)
    - lepR1 5'- TAAACTTCTGGATGTCCAAAAAATCA-3'(reverse primer)
    - vf1f 5'- TCTCAACCAACCACAAAGACATTGG-3' (forward primer)
    - vf1r 5'- TAGACTTCTGGGTGGCCAAAGAATCA-3' (reverse primer)

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2.1.14: GENETIC MODIFICATION

2.1.14.1: Introduction

2.1.14.2: GMO Food (Activity)

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2.1.14.1: Introduction

Genetic Manipulation (Selection)

Genetic modification of organisms has been occurring through human manipulation since the beginning of agriculture. Humans selectively bred crops and livestock to propagate desirable traits in a process termed **artificial selection**. The original grass that gave rise to domesticated corn called teosinte hardly resembles what we think of when imagining modern maize.

File:Maize-teosinte.jpg

Teosinte, the progenitor of maize. Corn came about due to selective breeding. Credit: John Doebley (CC-BY)

Variation: Crop Domestication

Selective breeding can yield a variety of features even within the same species. Below is a selection of vegetables of the species *Brassica oleracea* that have been developed into different varieties over the course of agricultural history.

File:Starr 070730-7852 Brassica oleracea var. capitata.jpg

Cabbage: Brassica oleracea var. capitata Credit: Forest & Kim Starr ***(CC-BY 3.0)***

File:Brocoli 02.jpg

Broccoli: Brassica oleracea var. italica Credit: Coyau (CC-BY-SA 3.0)

File:Chou-rave 01.jpg

Kohlrabi: Brassica oleracea var. gongylodes Coyau (CC-BY-SA 3.0)

File:Romanesco Brassica oleracea Richard Bartz.jpg

Romanesco: Brassica oleracea var. botrytis Credit: Richard Bartz (CC-BY-SA 2.5)

Variation: Animal Domestication

File:Dog morphological variation.png

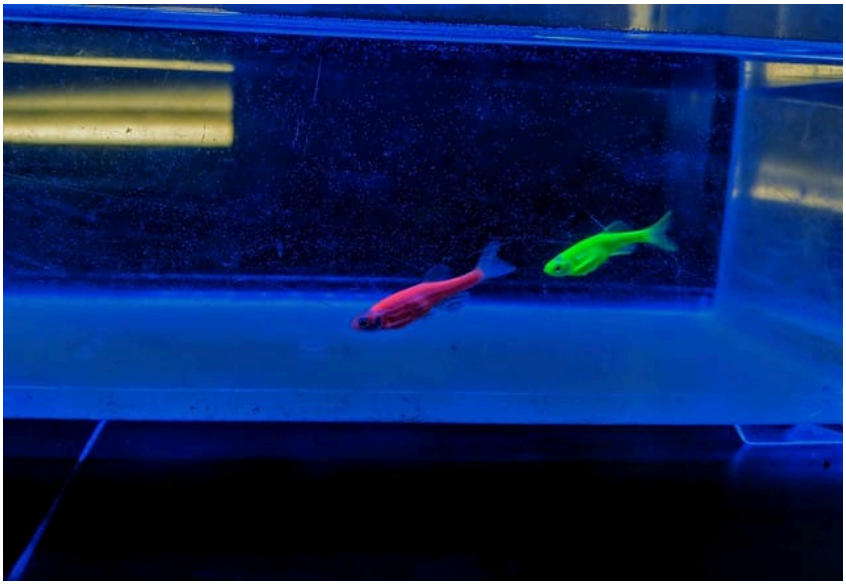
Credit: Mary Bloom, American Kennel Club (CC-BY-SA 4.0)

File:Collage of Nine Dogs.jpg

Companion animals like dogs underwent thousands of years of domestication and selection for traits that were desirable for different circumstances. A high degree of morphological diversity exists between dog breeds and their ancestral grey wolf progenitor.

Genetic Manipulation (Engineered)

Artificial selection takes multiple generations over a long period of time. With the advent of recombinant DNA and biotechnology, scientists can now genetically modify organisms through the introduction of foreign genes to provide desirable characteristics within one generation. This process does not require traits to naturally arise in a species.



File:Zebrafisch.jpg

GloFish are transgenic zebrafish (Danio rerio) expressing variants of GFP. Bottom features a wild-type fish. Credit: Azul (CC-BY)

GloFish® are novelty pets that have the insertion of various cnidarian fluorescent protein genes into the genome. These fish were released in the United States in 2003 and have subsequently been developed in red, orange, and blue varieties. Black tetras and tiger barbs are also now available.



Black tetra (Gymnocorymbus ternetzi) GloFish Credit: https://www.flickr.com/photos/fergy08/ (CC-BY 2.0)

File:BlackTetraCloseUp.JPG

Wild-type Black Tetra Credit: Fernandograu (CC-BY-SA 3.0)

Genetic Engineering in Plants

With the advent of agribusiness, agriculture has become a profit-driven venture independent of food production. In this case, high production is paramount. Whereas traditional agriculture or artificial selection was slow and methodical, genetic modification in

the context of agribusiness is instantaneous through **genetic engineering**. The ***objective*** of genetic engineering is to transfer the DNA encoding a useful or favorable gene from an organism that carries that gene to one that does not. Simply inserting DNA into an organism does not result in expression. An appropriate promoter for the transgenic organism must be upstream of the gene of interest in order to drive transcription. In mammals, a strong promoter that will result in expression in every cell is the CMV promoter that is derived from cytomegalovirus. Likewise in plants, a strong promoter that works in every cell is derived from viral promoters like the **CaMV promoter** from cauliflower mosaic virus from the **35S gene** (a ribosomal RNA). (CaMV 35S sequence on NCBI)

Examples of useful traits include:

- Degrade herbicides
- Kill agricultural pests
- Synthesize critical nutrients
- To improve color and taste
- Resist damage during transit or prolonged storage.
- Increase in size
- Reduce time to market (more rapid growth or maturation)

Genetically Modified foods have become a hot topic of contention in recent times. These crops are generated through the infection of plant cells by a bacterium called ***Agrobacterium tumefaciens***. *Agrobacterium* is a gram-negative alphaproteobacterium of the family Rhizobiaceae which includes symbiotic nitrogen fixers found in legumes. Unlike those symbionts, *Agrobacterium* is a pathogenic soil bacterium known as a causative agent of crown galls (tumors).

File:Crown-gall detail.jpg

Crown gall on a Kalanchoë infected with Agrobacterium tumefaciens Credit: Bhai (CC-BY-SA 3.0)

The tumors are caused by the infection of plant cells by the bacterium and the subsequent insertion of the **T-DNA** ("Transfer DNA") that has a tumor inducing capability (**Ti**). Through the engineering of the T-DNA in a plasmid, selected genes can be delivered to plant cells through infection of transformed bacteria.

File:Ti plasmid.svg

Ti plasmid has the T-DNA region replaced by the transgene. Ti is transformed into Agrobacterium which transduces the DNA into plant cells.

A modified Ti plasmid called **pGreen** was engineered to provide an MCS and selection marker for insertion of foreign genes of interest. In order for these genes to be expressed, they are driven by strong plant promoters like those from the CaMV 35S gene.

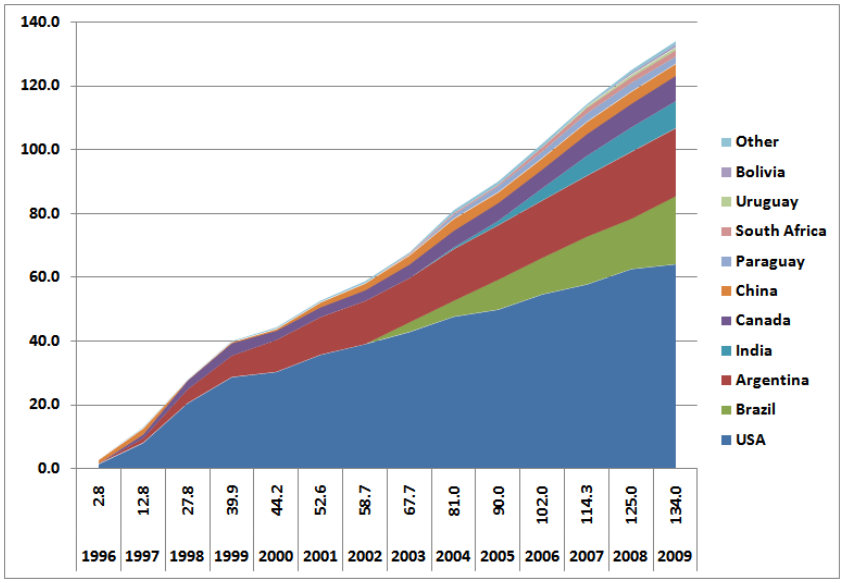
pGreen

The plant to be engineered is cultured and infected with the transformed *Agrobacteria* that will then induce cysts that eventually root. The strong promoter of the CaMV 35S will constitutively express the gene in all cells of the plant.

File:Transformation with Agrobacterium.JPG

Transformation of wild potato in culture using agrobacterium Credit: Seb951 (CC-BY-SA 3.0)

Growth of GMO Crops



Land area used in millions of hectares.

Damage Resistance

The first genetically modified crop FDA approved for sale was known as the Flavr Savr tomato. Tomatoes are prone to damage during shipping and are therefore picked before ripening. However, vine-ripened tomatoes have a richer flavor. Calgene developed the Flavr Savr and sold it to market between 1994-1997 in the United States. Flavr Savr was modified by inserting antisense into the genome that knocked-down the expression of polygalacturonase. Polygalacturonase degrades pectin in the cell walls of the fruit that results in softening, proclivity to damage, and eventual rotting.

Herbicide Resistance

File:Glyphosate.svg

Roundup is the trade-name of the herbicide **glyphosate** used in agriculture to control weed populations developed and patented by Monsanto. Glyphosate is absorbed through the foliage of plants and interferes with the enzymes that aid in the production of tyrosine, phenylalanine, and tryptophan. Plants and lower organisms generate aromatic amino acids through an enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase which is the target of this compound. A series of Roundup Ready crops were designed by the insertion of the *Agrobacterium* EPSP gene driven by the CaMV 35S promoter. This version of the gene is inherently resistant to glyphosate poisoning.

File:Glyphosate USA 2013.png

The Emergence of Superweeds



Palmer amaranth (Amaranthus palmeri), commonly referred to as pigweed, is a pest species in cotton and soy fields that have become glyphosate-resistant.

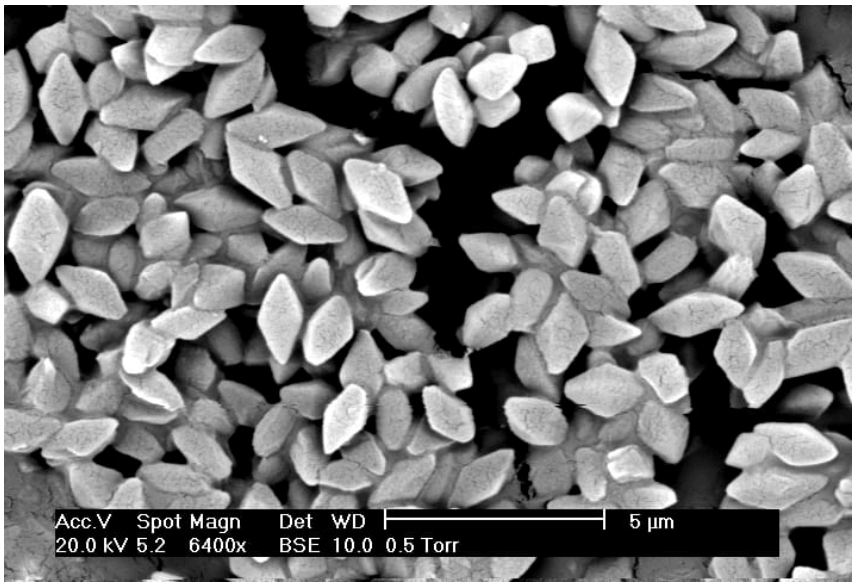
Credit: Pompilid (CC-BY-SA 3.0)

Pest Resistance

File:Helicoverpa zea larva.jpg

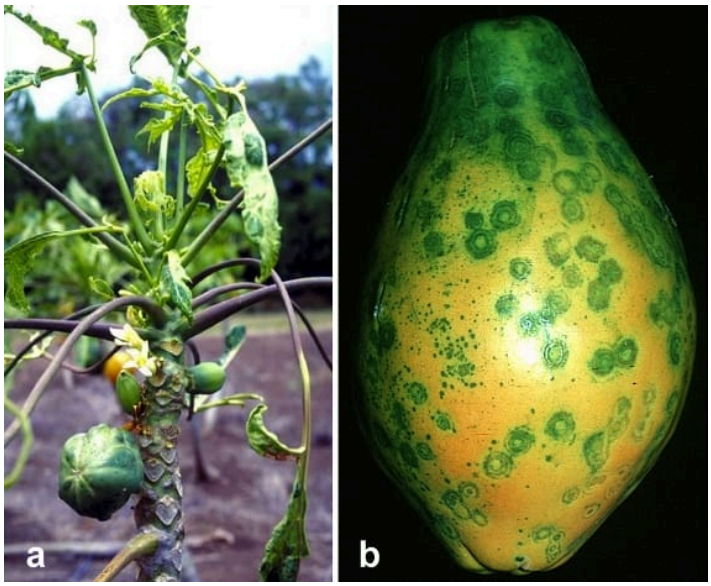
Credit: Cyndy Sims Parr (CC-BY-SA)

Crystals from the *Bacillus thuringiensis* (**Bt**), called ***Cry*** protein, are toxic to various insects: moths & butterflies, beetles, ants, wasps, flies & mosquitoes, bees, nematodes. The insertion of this gene into plants like corn (Bt-Corn) was designed to be resistant to pests.



Disease Resistance

Papayas in the United States primarily come from Hawaii. A virus known as Papaya Ringspot Disease threatened the papaya crop in Hawaii. To combat this, papayas were genetically engineered to block viral entry into the papaya cells. Papayas purchased in Latin markets are most likely unmodified and usually come from Mexico where the ringspot disease is not yet a problem.



Plum Pox Virus is a threat to the genus *Prunus*. A genetically modified plum plant has been developed called C5. The cells in these plants silence the expression of plum pox coat protein if infected to block propagation of the virus.

File:Plum pox in apricot.jpg

An apricot infected with plum pox.

File:C5 plum pox resistant plum.jpg

C5 resistant plums

Nutritional Engineering

File:Carotenoidsynthesis.svg

Credit: en:user: Petaholmes, (CC-BY-SA 3.0)

Golden rice is a genetically engineered rice that is meant to address Vitamin A deficiency. It introduces enzyme genes from other species involved in the biosynthetic pathways for β-carotene production, a vitamin A precursor. It is estimated that millions of deaths and irreversible blindness occur in the third world each year due to Vitamin A deficiency and creating this rice was meant to address the problem.

Rice is a staple in many cultures, therefore it is a good delivery system. Many controversies exist surrounding Golden Rice due to anti-GMO sentiment (from patenting systems), cultural sensitivities (white rice revered in certain cultures), and Vitamin A content/ conversion doubts.

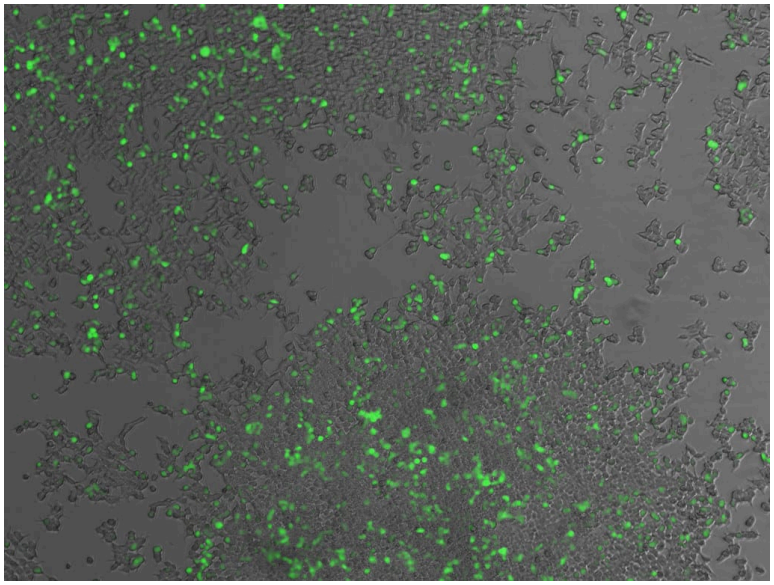
File:Golden Rice.jpg

Explore the Debate

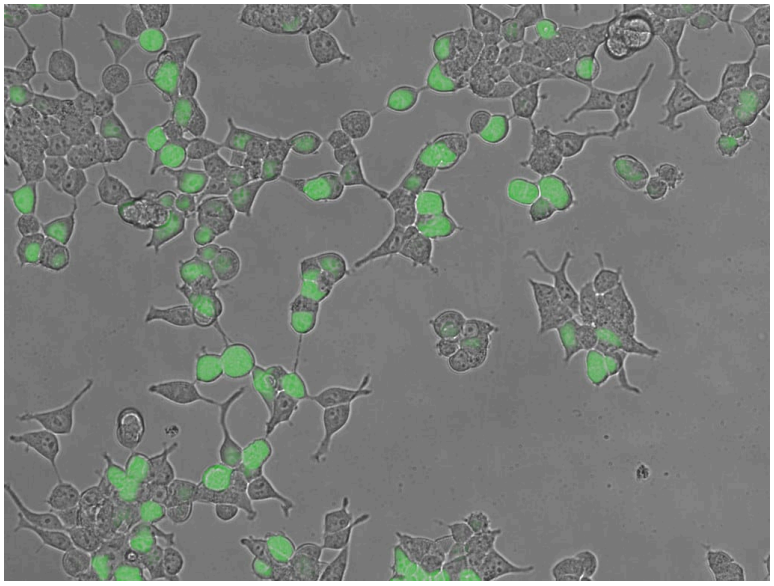
- Case studies reveal the complex truths behind GM crop myths.

- Case studies: A hard look at GM crops. Gilbert N. Nature. 2013 May 2;497(7447):24-6. doi: 10.1038/497024a

Heterologous Expression in Tissue Culture

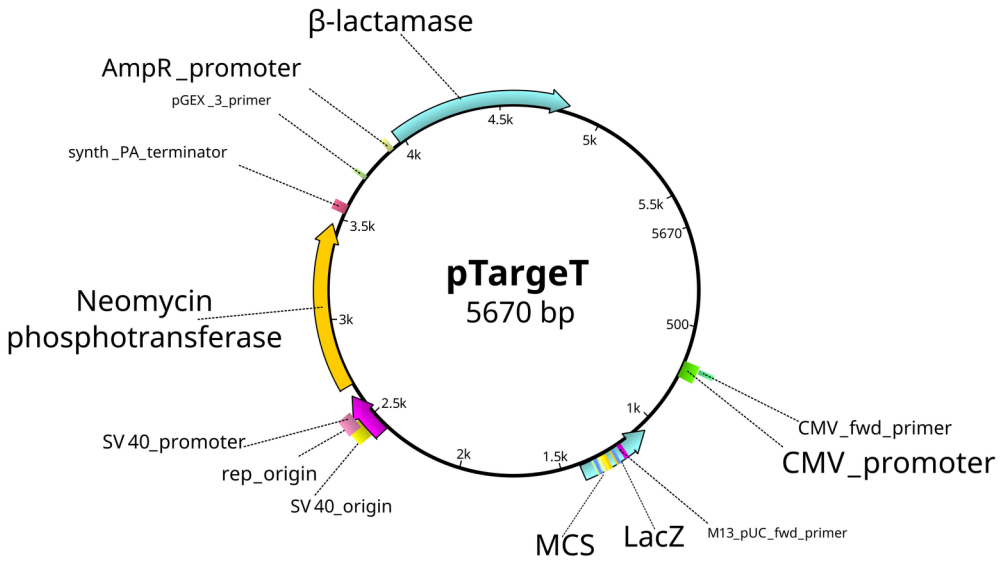


Human Embryonic Kidney cells (HEK293T) expressing the green fluorescent protein.



Higher magnification of HEK293T expressing the green fluorescent protein.

Plasmid Structure



pTarget mammalian expression vector. The MCS is inside the LacZ gene which permits for blue/white screening of the bacteria after cloning. The LacZ runs in the opposite orientation as the CMV promoter which will drive the gene transcription when inside a mammalian cell. SV40 origin servers as a plasmid replication origin if the cell line also expresses SV40 large T antigen, such as HEK293T cells.

Mammalian expression vectors contain the same hallmark features as bacterial plasmids: bacterial replication of origin and bacterial antibiotic resistance gene (β-lactamase or **Amp_R**). General bacterial plasmid features allow for the carrying and the propagation of the plasmid in a bacterial cell. Mammalian expression vectors additionally include a strong mammalian promoter (like **CMV** from the cytomegalovirus immediate early promoter) upstream of a multiple cloning site (**MCS**). Plasmids transfected into cells are transient in nature unless the DNA is selected for. The inclusion of a mammalian antibiotic resistance gene, like neomycin phosphotransferase (**Neo_R**), allows for the integration of the plasmid into the genome of the cell by using high concentrations of Neomycin or the analog G418.

Lipofection

Cationic lipids can encapsulate plasmid DNA in liposomes. The cationic portions interact with the negatively charged plasma membrane to deliver the DNA into cells.

Calcium Phosphate Transfection

Calcium chloride solution can be used to incubate with plasmid DNA. When this solution is mixed with a HEPES-buffered saline solution (HeBS) containing phosphate ions, the solution precipitates onto the surface of mammalian cells where they are taken up with the DNA.

Knock-Out & Transgenesis

In the laboratory, model organisms are modified in order to understand the basic mechanisms of genes. The transformation of recombinant DNA into bacteria is an example of a genetic modification. Other model organisms, like mice, are used to study genes. Through recombinant DNA scientists can selectively ablate a gene, or create a **knock-out** (KO).
Embryonic stem (**ES**) cells are pluripotent cells with the capacity to differentiate into other cell types. Cultured ES cells can be transfected with plasmid DNA in order to genetically alter them. Linearized vectors containing a disrupted gene can homologously recombine with the native gene to replace it. Selection of cells with the disrupted gene by an antibiotic (like G418) enables the isolation and propagation of engineered ES cells.

File:Knockout mouse production 2.svg

Credit: Kjaergaard (CC-BY- 3.0)

File:Knockout mouse breeding scheme.svg

Credit: Kjaergaard (CC-BY- 3.0)

ES cells can be injected into mouse blastocysts and partially contribute to the subsequent mouse upon implantation into a mouse. These first mice are referred to as **chimeras** because they arise from mixtures of cells from 2 genetic sources. Germline transmission of the modified cells is desired and breeding of the chimera reveals heterozygous offspring of the engineered background. Full knock-out mice can be generated in the subsequent generation of breeding.

File:ChimericMouseWithPups.jpg

File:Breeding transgenesis cisgenesis.svg

Credit: Smartse (CC-BY-SA 3.0)

Scientists can also overexpress or heterologously express foreign genes in what is termed **transgenic** organisms. As the name sounds, transgene refers to a gene from one place brought across into another.

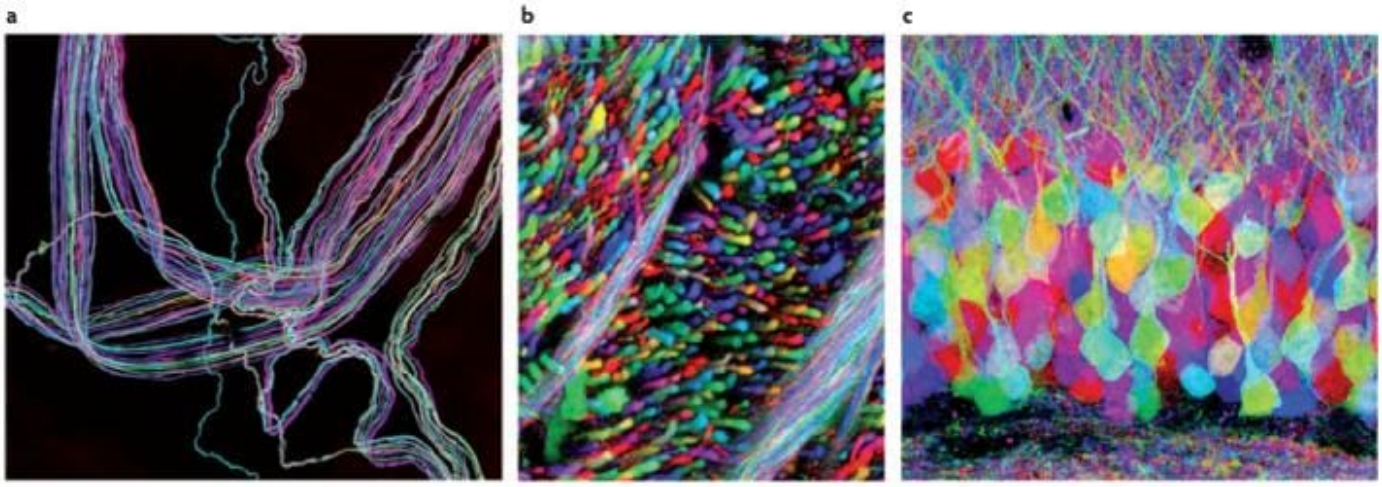
Transgenic and KO models permit scientists to study the roles of genes inside the organism and understand basic functioning.

Through mutagenesis, derivatives of the green fluorescent protein (GFP) have been produced to provide a palette of colors. Additionally, the subsequent discovery of similar genes from other cnidarian species have aided biotechnology by providing tracer molecules within developing organisms or within cells.

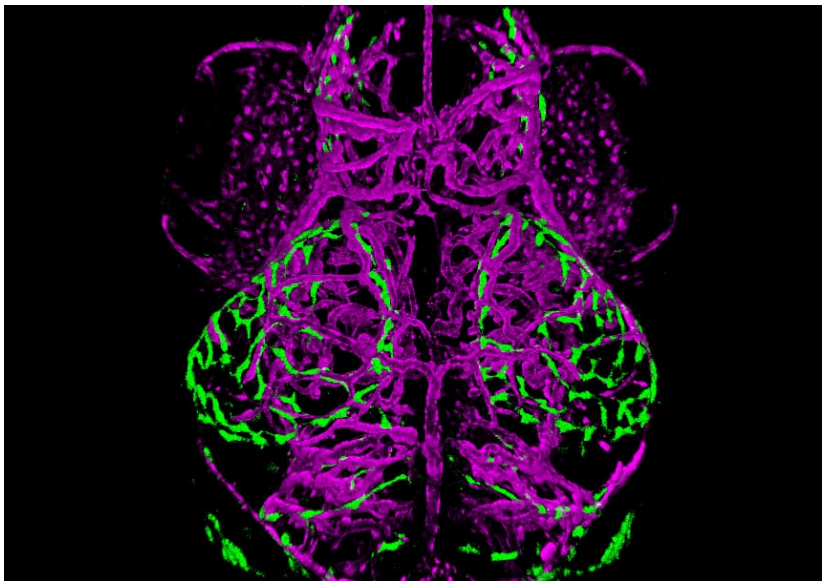
File:FPbeachTsien.jpg

Bacteria expressing various GFP derivatives on agar from the lab of Nobel Laureate Roger Tsien.

While commercial organisms like GloFish are a novelty, directed insertion of GFP and the variants into the genome under different promoter systems allow scientists to understand the cell-specific functioning or contribution to the organism. An example of this can be found in developmental neurobiology where individual axons can be traced.



A "brainbow" is a system where a cassette of GFP variant genes are placed downstream of a neuronal promoter to permit the tracing of individual neurons and their axons in mice. Credit: Jeff W. Lichtman and Joshua R. Sanes (CC-BY 3.0)



Brain of a 10-day old double-transgenic zebrafish. Blood vessels are shown in magenta (Kdrl:mcherry) and a novel population of perivascular endothelial cells are shown in green (MRC1a:eGFP).

Further Reading

- CRISP-Cas9 https://www.neb.com/tools-and-resources/feature-articles/crispr-cas9-and-targeted-genome-editing-a-new-era-in-molecular-biology

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2.1.14.2: GMO Food (Activity)

PCR Detection of GM Food

Briefly, genomic DNA will be isolated from food items derived from vegetation. Genetic modification will then be identified by PCR of the plant promoter used in genetic engineering, **CaMV 35S**. As a **positive control** for the appropriate extraction of DNA, PCR for **plant-specific tubulin** will be used.

1. Add 100 µL of lysis buffer to each tube containing the plant or food material.
2. Twist a clean plastic pestle against the inner surface of the 1.5-mL tube to forcefully grind the plant tissue or food product for 1 minute.
3. Add 900 µL of lysis buffer to each tube containing
4. Boil the samples for 5 minutes in a water bath
5. Spin for 2 minutes to pellet cell and food debris.
6. Transfer 350 µL of each supernatant to a fresh tube
7. Add 400 µL of isopropanol to each tube
8. Mix and leave at room temperature for 3 minutes.
9. Spin for 5 minutes.
10. Carefully pour off and discard the supernatant from each tube. Air-dry pellet.
11. Add 100 µL of TE buffer to each tube. 5 min at room temperature then keeps on ice.

PCR for 35S Promoter

1. Label one tube “35S FP” for food product.
2. Label one tube “35S WT” for wild-type soy: Negative control.
3. Label “35S RR” for Roundup Ready® soy plant: Positive control.
4. Different groups will only do one control.
5. Add **22.5 µL** of the 35S primer/loading dye mix to each tube containing PCR bead.
    - 5′-CCGACAGTGGTCCCAAAGATGGAC-3′ (Forward Primer)
    - 5′-ATATAGAGGAAGGGTCTTGCGAAGG-3′ (Reverse Primer)
6. Add **2.5 µL** of food product DNA to the reaction tube marked “35S FP.”
7. Add **2.5 µL** of wild-type or Roundup Ready® soybean DNA to the appropriate reaction tube marked “35S WT” or “35S RR.”

PCR for Tubulin: (Positive Control for DNA Quality and PCR Conditions)

1. Label one tube “T FP” for food product.
2. Label one tube “T WT” for wild-type soy.
3. Label “T RR” for Roundup Ready® soy plant.
4. Different groups will only do one control.
5. Add **22.5 µL** of the Tubulin primer/loading dye mix to each tube containing PCR bead.
    - 5′-GGGATCCACTTCATGCTTTCGTCC-3′ (Forward Primer)
    - 5′-GGGAACCACATCACCACGGTACAT-3′ (Reverse Primer)
6. Add **2.5 µL** of food product DNA to the reaction tube marked “T FP.”
7. Add **2.5 µL** of wild-type or Roundup Ready® soybean DNA to the appropriate reaction tube marked “T WT” or “T RR.”

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2.1.15: PROTEIN PRODUCTION

2.1.15.1: Introduction
2.1.15.2: Protein Purification (Activity)

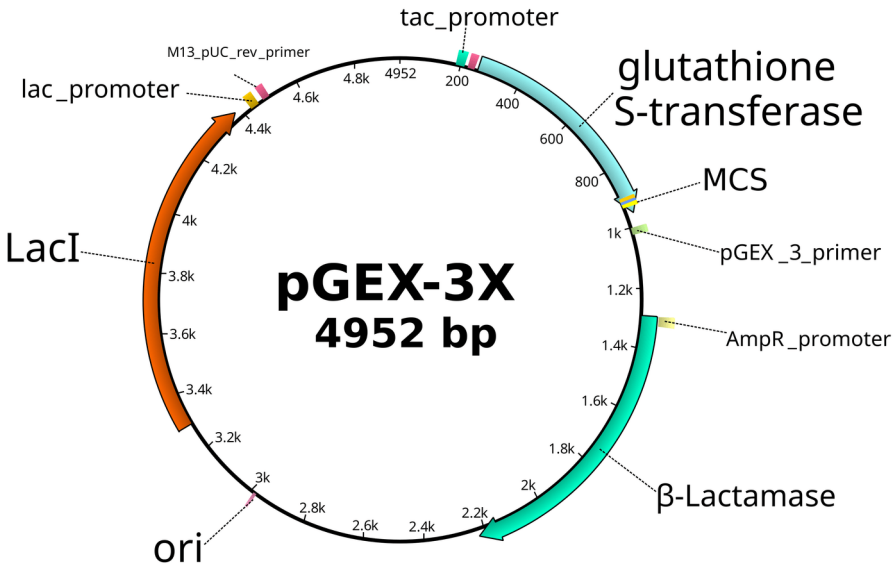
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2.1.15.1: Introduction

Protein Expression

Recombinant DNA technology has many uses in basic scientific research to better understand the nature of living things. As a tool, recombinant DNA technology can be used to express proteins towards medical applications. Prior to biotechnology, type I diabetes (insulin-dependent) was treated by injection of insulin isolated from the pancreas of pigs. With the ability to express human proteins inside bacteria, yeast, and other cells, sacrificing pigs for porcine insulin is no longer necessary.



*Bacterial expression vector **pGEX-3X** contains the AmpR gene, the origin of replication, MCS downstream of the hybrid lac/trp promoter (tac) and the coding sequence for glutathione-S-transferase (GST). GST acts as a tag that is fused directly with the protein from the gene of interest and used to purify the protein with a glutathione resin.*

Bacteria or other cells can be engineered to express proteins through the process of cloning and transformation. Bacteria are advantageous because of their rapid life cycle and ease of growth. A bacterial expression vector contains the basic plasmid features: the origin of replication as well as antibiotic resistance gene. Often, an **affinity tag** will be used to aid in the purification of the protein. An example in the vector above shows the **GST** (glutathione-s-transferase) tag that can be purified with glutathione resin. Expression is only the first problem since bacteria are also synthesizing proteins that are required for the bacteria to grow and divide. Injecting these proteins in addition to insulin would cause an immune reaction that could be deadly. Therefore, it is required that overexpressed proteins be purified and isolated from other undesirable proteins.



Credit: Stewart EJ, Madden R, Paul G, Taddei F (CC-SA 3.0).

Criteria for Choosing an Expression System

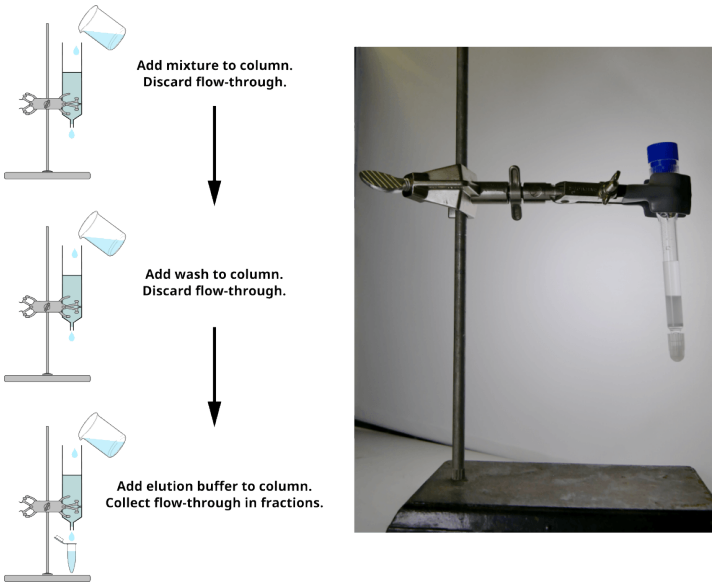
Desired characteristics	Bacteria	Yeast	Insect (baculovirus)	Mammalian cell culture (COS, CHO, HEK)
Cell growth	Rapid	Rapid	Slow	Slow
Complexity of growth medium	Minimum	Minimum	Complex	Complex
Cost of growth medium	Low	Low	High	High
Expression level	High	Low to high	Low to high	Low to moderate
Extracellular expression	Secretion to periplasm	Secretion to medium	Secretion to medium	Secretion to medium
Protein folding	Refolding usually required	Refolding may be required	Proper	Proper
Glycosylation	No	Yes	Some	Yes

Gene Expression Systems. Using nature for the art of expression (Fernandez, J.M. & Hoeffler, J.P., eds), Academic Press, San Diego, 1999.

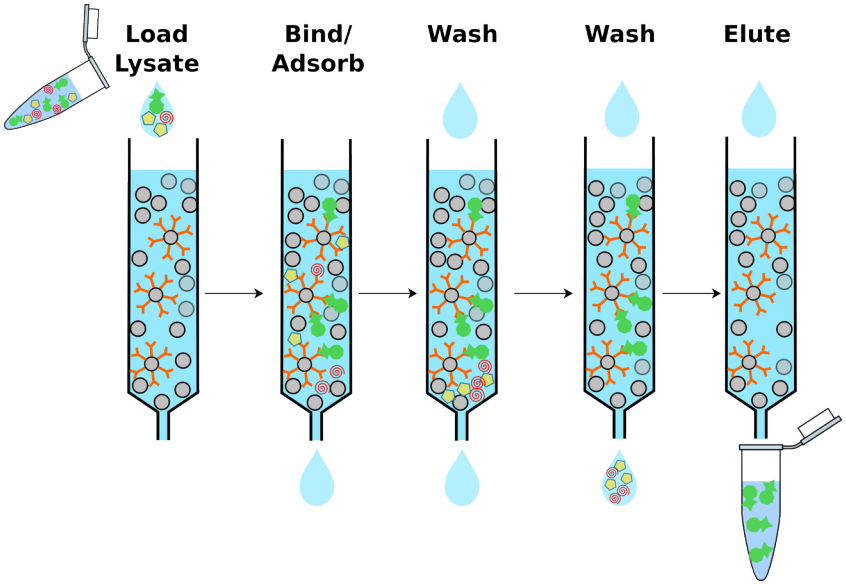
Protein expression systems have inherent advantages and disadvantages. The table above summarizes the comparison of the various cellular systems of production (Fernandez & Hoeffler, 1999).

Purification

Different methods of isolation can be applied depending on the properties of the protein. **Ion exchange chromatography** is useful if the protein of interest has a specific charge that will interact with a resin packed with the opposite charge.



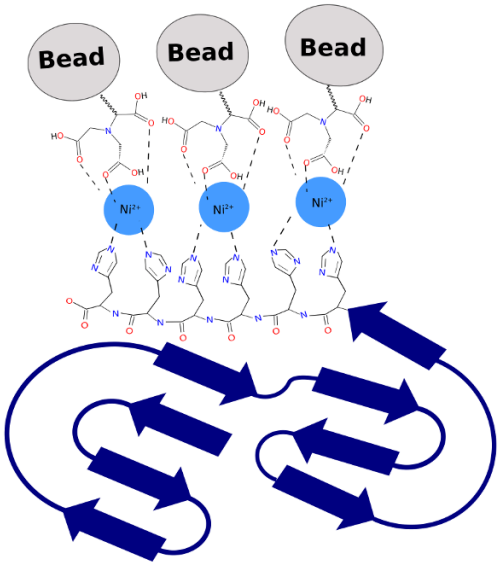
Immunoprecipitation



Immunoprecipitation: Column is packed with Protein-A agarose which binds to antibodies. Cell lysates are then loaded onto the columns where they flow through and are allowed to interact with the antibody. Washes are performed to remove the non-specifically bound proteins. An elution buffer is used to disrupt the interaction of the antibody to the protein target.

Affinity Purification

Affinity purification employs the use of specific antibodies that bind to the protein of interest very tightly to retain it on a column. With these techniques, the protein retained on the resin is washed numerous times to remove other proteins that are non-specifically sticking. A change in pH or ionic conditions then is used to disrupt the interaction with the resin and **elute** the proteins from the column. Proteins that are engineered to contain tags can be purified by antibodies specific to those tags. Also, the addition of 6 or more consecutive Histidine residues to the end of a protein makes them susceptible to purification with Nickel-NTA resin or Cobalt purification. In these cases, the 6XHis tag associates with these metal ions on the resin are selectively adhered.



Nickel NTA resin coordinating the capture of a 6His tagged protein.

Size Exclusion

File:Activated Charcoal.jpg

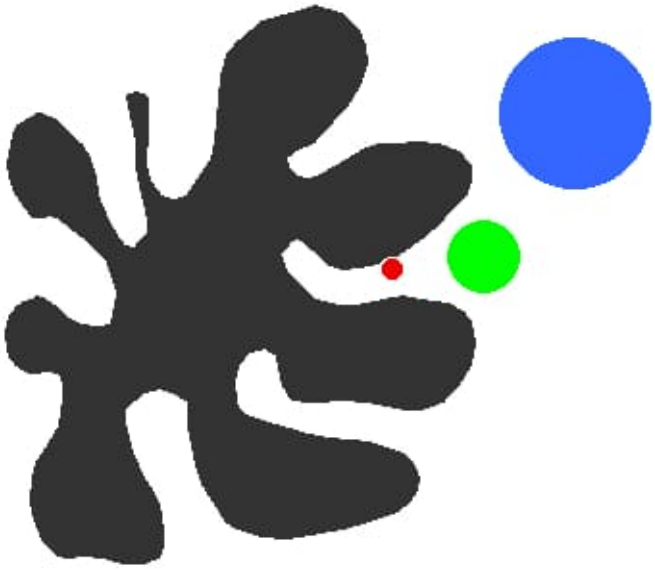
Credit: Mydriatic (CC-BY-SA 3.0)

File:SizeExChrom.png

Credit: Takometer(CC-BY 2.5)

Most of you are familiar with water purification filters. Before using these filters, you soak them in water and dark residue leaks out. This dark residue is activated charcoal. The activated charcoal has tiny microscopic pores that trap small items like ions and other particles. The primary goal of these filters is to remove metals and chlorine that are found in tap water. The porous nature of activated charcoal renders it useful for trapping molecules in water purification systems.

The process used to trap these small particles is called **size exclusion**. Unlike agarose gel electrophoresis where the smaller particles navigate through the matrix faster, size exclusion resins trap the smaller molecules.



The smaller the molecule, the longer they spend within the pores as they traverse through the matrix.

Significance of Purification

File:Limulus polyphemus (aq.).jpg

Credit: Hans Hillewaert (CC-BY)

All injectable drugs must be clean of endotoxins from bacteria. Purification of the protein of interest from bacterial lysates removes the dangerous pathogenic materials from that would otherwise activate host immune reactivity.

The horseshoe crab (*Limulus polyphemus*) performs a special function in the ecosystem by providing eggs for migratory birds to feed on. This organism also houses a special cell type in its hemolymph. The Limulus amoebocyte lysate (**LAL**) test is the most sensitive assay of detecting endotoxins from bacteria. Amoebocytes are collected from these organisms for use on testing batches of injectable drugs to ensure proper purification and safety.

References

- Joseph M. Fernandez and James P. Hoeffler. Introduction: SO MANY POSSIBILITIES: HOW TO CHOOSE A SYSTEM

TO ACHIEVE YOUR SPECIFIC GOAL, In Gene Expression Systems. Academic Press, San Diego. 1999, Pages 1-5, ISBN 9780122538407. http://dx.doi.org/10.1016/B978-012253840-7/50001-8.

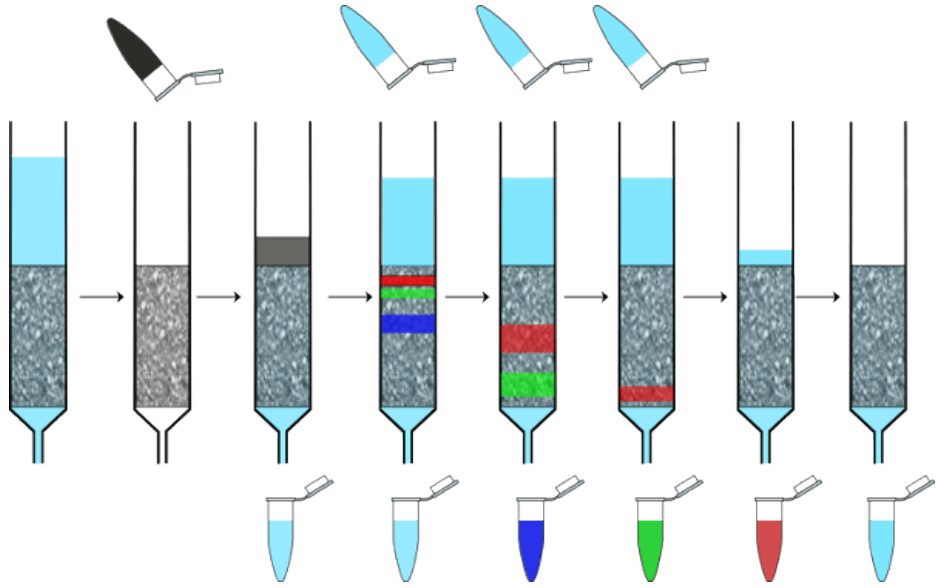
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2.1.15.2: Protein Purification (Activity)

Size-Exclusion of Dye Molecules

As a demonstration, the instructor may illustrate the concept of size exclusion on a set of mixed food coloring.

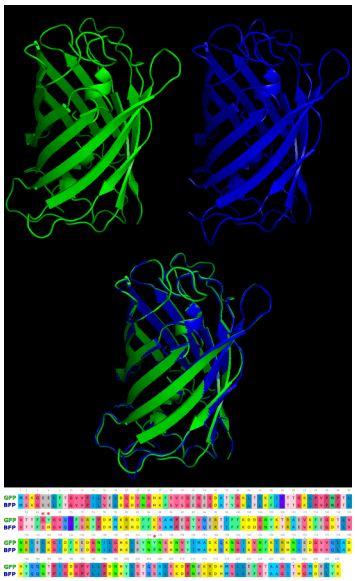


Size exclusion chromatography of food coloring.

1. Let the column empty over a beaker.
2. Carefully load 0.2 ml of food coloring mixture onto the column.
3. Place 10 tubes on a rack under the column.
4. Place a 1 ml buffer on the column and collect 0.5 ml fractions.
5. Continue to add buffer 1 ml at a time until all fractions have been collected.

Size-Exclusion of Proteins

This exercise seeks to purify Green Fluorescent Protein (GFP) or Blue Fluorescent Protein (BFP) from the bacterial lysate. These proteins have a specific size of 238 amino acids and are 40,000 daltons (40kD). Based on their specific size, they will have a specific rate of migration through the size-exclusion resin. Remember that the bacterial lysate is full of additional proteins that are not your protein of interest that we are attempting to isolate.



*3D model of **GFP** (Top Left), **BFP** (Top Right), structural alignment of GFP and BFP (Center) and the sequence alignment (Bottom) illustrating the 3 amino acid changes to produce the alternative protein. Red asterisks indicate the location of mutations.*

Drops of fluid will be collected in fractions. The fractions containing the fluorescent proteins will be found only in specific fractions that will be visible under UV illumination.

1. Vertically mount the column on a ring stand. Make sure it is straight.
2. Slide the cap onto the spout at the bottom of the column.
3. Mix the slurry (molecular sieve) thoroughly by swirling or gently stirring.
4. Carefully pipet 2 ml of the mixed slurry into the column by letting it stream down the inside walls of the column.
5. Place an empty beaker under the column to collect wash buffer.
6. Remove the cap from the bottom of the column and allow the matrix to pack into the column.
7. Label eight microcentrifuge tubes #1-8.
8. Slowly load the column with 0.2ml of the GFP extract. Allow the extract to completely enter the column.
9. Add 1ml of the elution buffer on top of resin without disturbing the resin.
    - Add buffer slowly (several drops at a time) to avoid diluting the protein sample.
    - Using the graduated marks on the sides of the tubes, collect 0.5ml fractions in the labeled microcentrifuge tubes.
    - Continue to add 1ml buffer and collect fractions until all tubes are full.
10. Check all fractions by using long wave U.V. light to identify tubes that contain the fluorescent GFP or BFP proteins.
11. Further purification may be performed with a different resin with the few fractions containing the protein of interest.
12. Protein samples should be run on an acrylamide gel and stained against all proteins to check the purity of the sample or fluorescence measurements taken.

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2.1.16: BIOINFORMATICS

2.1.16.1: Introduction
2.1.16.2: Sequence Analysis
2.1.16.3: In Silico Restriction
2.1.16.4: In Silico PCR
2.1.16.5: Primer-BLAST
2.1.16.6: Morphometic Analysis
2.1.16.7: Sequence Alignment and Tree Building

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2.1.16.1: Introduction

FASTA Format

Biological sequences are passed to software in a standardized format referred to as **FASTA**. FASTA is a plain text format that can be read in any text editor (TextEdit, Notepad, VIM, TextWrangler, etc.). Nucleic acids (DNA and RNA) and Proteins are represented by single-letter nucleotides (A, T, C, G) or single letter amino acid (20 amino acids). FASTA sequences begin with a **>** character in the first line followed by some descriptive information about the sequence, like a sequence name. The next line consists of the sequence information. A FASTA file can contain multiple sequence entries all demarcated by a new line and a title line beginning with **>**.

Example FASTA File

> Made-up nucleic acid sequence
ATATAGGGATTAGGATTAGAGGATAGAGGGGATTGCGCCG
> Another nucleic acid sequence in the same file
GGGTCGGGCTAGCGGAATCGGATTCGGCATTCGGATATTCGGATTCGGAT

FASTA files are plain text but usually have an extension indicating it as a sequence file: .fasta, .fa, .fna or even .txt

A list of single-letter codes for nucleic acids follows below:

Nucleic Acid Code	Meaning
A	A
C	C
G	G
T	T
U	U
R	A or G
Y	C, T, or U
K	G, T, or U
M	A or C
S	C or G
W	A, T, or U
B	not A (i.e. C, G, T, or U)
D	not C (i.e. A, G, T, or U)
H	not G (i.e., A, C, T, or U)
V	neither T nor U (i.e. A, C, or G)
N	A, C, G, T, U
X	masked
-	Gap of indeterminate length

Graphical Sequence Manipulation

The exercises described here regarding bioinformatics will utilize a free and open-source software called Unipro UGENE.

- Okonechnikov K, Golosova O, Fursov M, the UGENE team. **Unipro UGENE: a unified bioinformatics toolkit**.*Bioinformatics* 2012 28: 1166-1167. doi:10.1093/bioinformatics/bts091
- Golosova O, Henderson R, Vaskin Y, Gabrielian A, Grekhov G, Nagarajan V, Oler AJ, Quiñones M, Hurt D, Fursov M, Huyen Y.**Unipro UGENE NGS pipelines and components for variant calling, RNA-seq, and ChIP-seq data analyses**. *PeerJ 2014* 2:e644. doi:10.7717/peerj.644

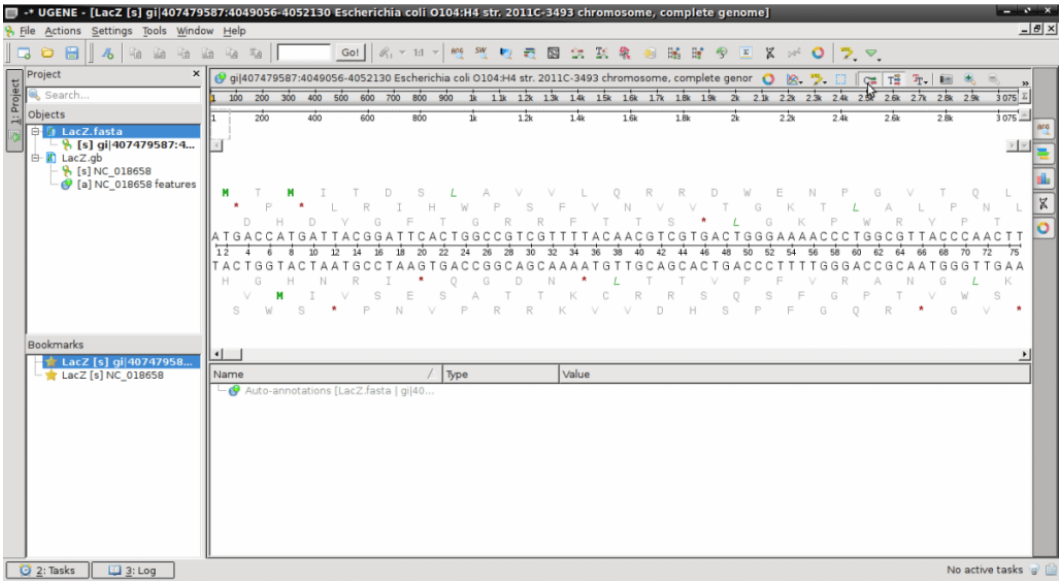
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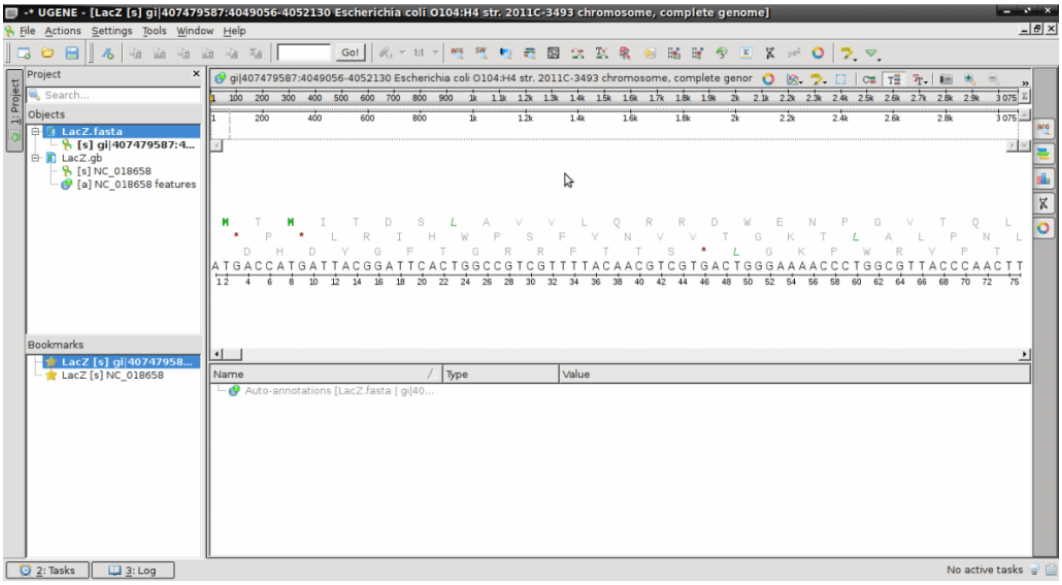
2.1.16.2: Sequence Analysis

1. Download the file **LacZ.gb** and open in a text editor.
   - This is a **Genbank format** file that contains the sequence following the word '**ORIGIN**' and terminating with '**//**'.
   - Prior to the sequence is a batch of descriptive information including references, organism, and database cross-reference identifiers. While these don't mean much to you, the appropriate database within Genbank can be queried to reveal more information about the sequence.
2. Download the file **LacZ.fasta** and open in a text editor (NotePad).
   - Notice the simple structure of the **fasta** file beginning with the '>' and description of the sequence.
   - This is a DNA sequence. ***But DNA is usually double-stranded!*** We can assume the sequence of the second strand because it will be complementary to this one.
     - By convention: we know that this sequence is **5' → 3'.**
   - This text contains a portion of the *E. coli* genome that includes a gene called LacZ.
   - This file does not contain any annotation to indicate where the gene sequence actually begins or ends.
3. Launch UGENE and open both files. They will appear on the left side "Objects" pane.

- The default display automatically shows the reverse complement of the DNA strand and all 6 **Open Reading Frames** (ORFs).

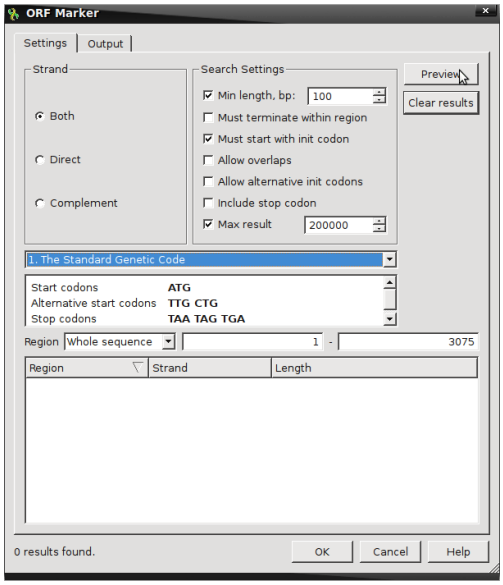


- To simplify the view, click on the '**C**' to remove the complementary strand (look at the cursor in the image).



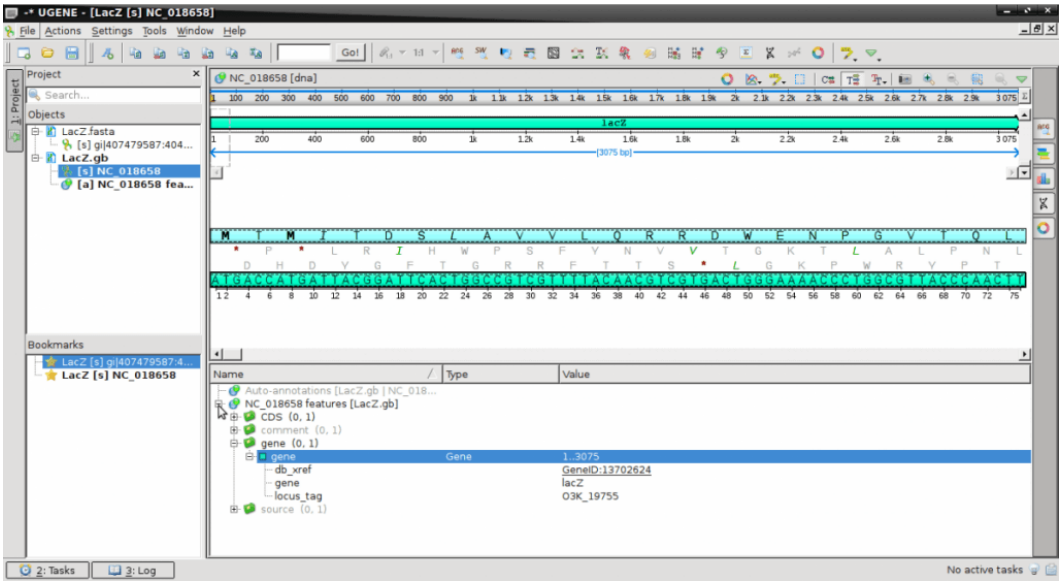
4. **Count the ORFs:**

- Find ORFs by right-clicking on the sequence and select "**Analyze → Find ORFs**"
- Default setting looks for ORFs on both strands with a minimum length of 100 nucleotides
- The **Open Reading Frame** here is defined as something beginning with initiation or start codons from the Standard Genetic Code (**ATG**) and two additional alternative start codons (**TTG** & **CTG**) that is terminated by any one of the three standard stop codons (**TAA**, **TAG**, **TGA**)
- Selecting Preview will provide the number of possible ORFs fitting these criteria.

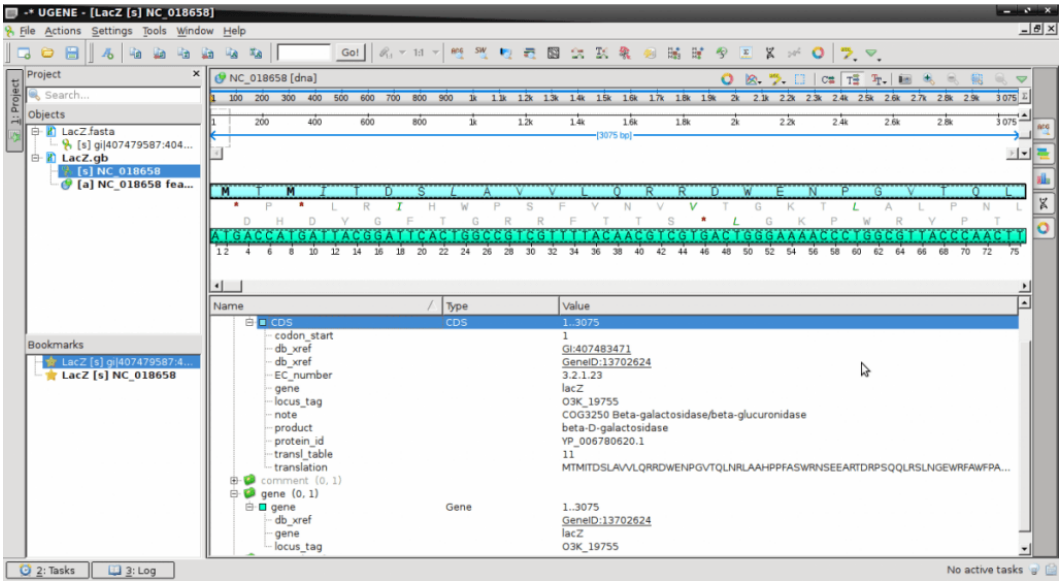


5. Double click on the **LacZ.gb** in the **Objects** panel to activate the view.

- This file now shows the same sequence with information about the DNA.



- Expand the various features in the Annotations pane at the bottom to explore the sequence features.



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2.1.16.3: In Silico Restriction

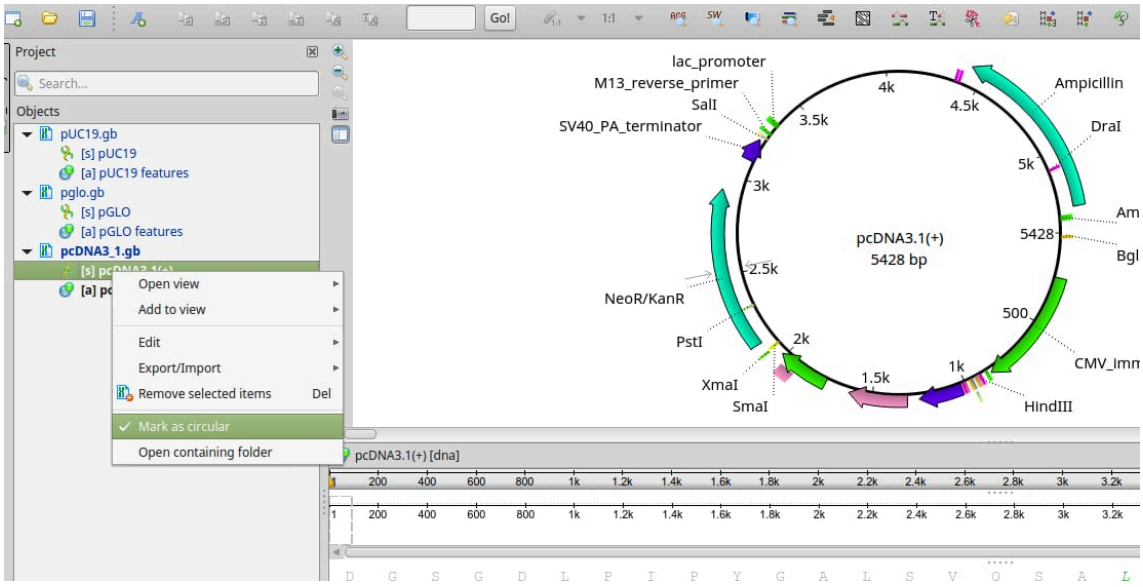
Concept

Restriction enzymes act like molecular scissors. The ones we use in Molecular Biology are those that cut within known sequences that occur often enough, yet rare enough to cut our DNA into analyzable fragments. Molecular Biologists often use 6-cutters. This means that the site of digestion is “restricted” to a recognition sequence of 6 nucleotides. These nucleotides are usually palindromic as discussed before.

Imagine a linear piece of DNA as a piece of string. When cutting the string once, you result in 2 pieces. Now consider a plasmid. This was already discussed to be a circular piece of DNA. With a circle, there are no ends. cutting the plasmid once results in 1 piece of DNA as opposed to 2. Keep this in mind when digesting circular plasmids. In this case, nucleotide 1 is adjacent to and contiguous with the last nucleotide of the sequence.

In Silico Digestion

1. This activity is meant to supplement Identification of DNA (activity)
2. Launch UGENE and open the following files:
   - **pGlo.gb**
   - **pUC19.gb**
   - **pcDNA3.1.gb**
   - **pUC with Insert**
3. In the **Objects** menu, right-click on the sequences and select “**Mark as circular**”
   - The sequences will now be treated as circular DNA.
   - The first nucleotide and the last nucleotide become adjacent as a continuous sequence this way.



4. From the top menu, select **Actions → Analyze → Find restriction sites.**

- This will load a set of default restriction enzymes
- If none are loaded, select them individually (found in alphabetical order)
  - Choose: BamHI, BglII, ClaI, DraI, EcoRI, EcoRV, HindIII, KpnI, PstI, SalI, SmaI, XbaI, XmaI, NotI

5. **Actions → Cloning → Digest into fragments**

- Choose your enzyme.
- Try *Hind* III for each plasmid.
- Fragments will be added to the circular view and annotations for these fragments will be added.
- You can use this information to calculate how many fragments come from enzymes based on how many times they cut and by the nucleotide coordinates found in the annotation of the fragment.

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2.1.16.4: In Silico PCR

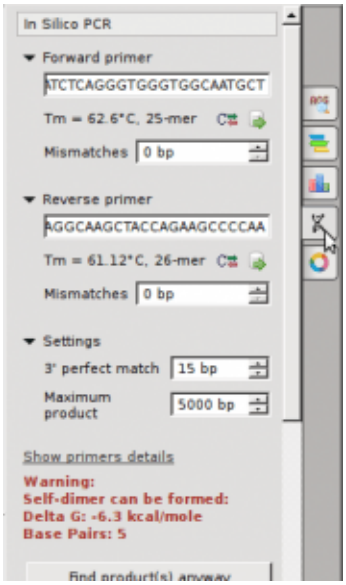
Using the primer sequences, one can determine the size and/or location of a PCR product. This can be done using BLAST or with a program like UGENE.

Using BLAST

1. Primers for the PV92 insertion.
   - Forward primer: 5′ GGATCTCAGGGTGGGTGGCAATGCT 3′
   - Reverse primer: 5′ GAAAGGCAAGCTACCAGAAGCCCCAA 3′
2. Visit BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch.
3. Paste both primers:
   - GGATCTCAGGGTGGGTGGCAATGCTGAAAGGCAAGCTACCAGAAGCCCCAA
   - Remove the 5′ and 3′ numbers.
4. Choose "**Somewhat Similar**".
   - Locate the locus of the product and the size.

Using Ugene

1. Exercise using a human D-Loop Primers.
   - Forward Primer 5'-TTAACTCCACCATTAGCACC-3'
   - Reverse Primer 5'-GAGGATGGTGGTCAAGGGAC-3'
2. Download the sample Genbank file: Human Mitochondrial Genome.
3. Open the file in Ugene.
4. Select the "**In Silico PCR**" button on the far right (double helix button).
   - Insert forward and reverse primers in the appropriate spaces.



5. A PCR product should be noted for one of the sequences after pressing "**Find Products anyway**".

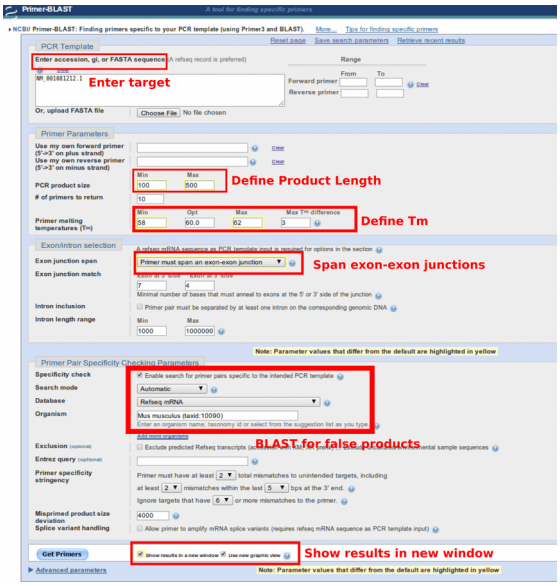
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2.1.16.5: Primer-BLAST

Primer-BLAST is a combination of a program called **Primer3** that aids in the design of primers with specific properties and BLAST. Primer-BLAST allows for the construction of primers for qPCR where the user can specify the melting temperature, reduce the amount of self-priming, and span exon-exon junctions in order to avoid amplification of contaminating genomic DNA. After the design of primers, each primer pair is sent into BLAST to identify if similar products within the genome of the model organism will also be primed and amplified. This process ensures that the primers designed fall within your design parameters and most likely only amplify your gene of interest.

1. Enter the sequence OR the NCBI accession number for the gene of interest.
2. Define the PCR product length.
    1. Limiting the product between 100-500 permits for good efficiency in qPCR.
    2. Longer products may not be efficiently replicated depending on your cycling protocol.
3. Define the desired melting temperature ($\mathbf{T_m}$) of the primers (the minimum, optimal, maximum, difference between the set).
    1. 60°C is fairly high and will aid in the enhanced specificity of the primer with the target during amplification to avoid false priming.
    2. Try to have the T_m as close as possible so that they are annealing about equally.
4. Choose the option “Primer must span an exon-exon junction”.
    1. This aids in amplifying cDNA and not genomic DNA that may be contaminating.
    2. Do not select this if it a single exon gene as this will fail.
5. Select Refseq mRNA as the database to search against.
    1. **Refseq** provides sequences to naturally occurring sequences.
    2. Things like plasmid sequences or vector constructs do not show up in Refseq.
6. Select the organism you are BLASTing against.
    1. There are options for model organisms as well as cell lines.
    2. If you are using something like PC12 cells, you may use *Rattus norvegicus* or PC12 genome since that is also an option in the database.
7. Evaluate the location of the primers and the other parameters. We generally choose primers at the 3′ end of the RNA since RT reactions often have a 3′ bias in eukaryotes by using oligo-dT priming in the reverse transcription.



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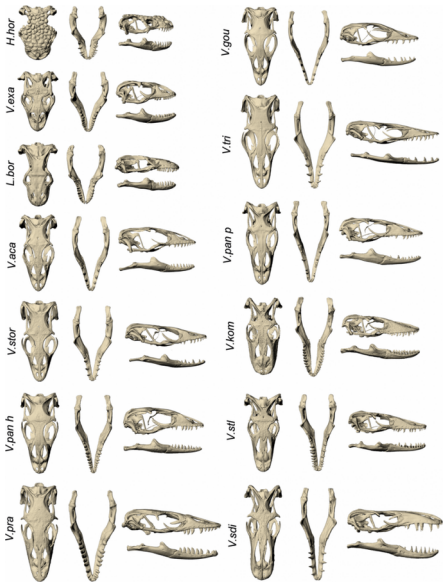
2.1.16.6: Morphometic Analysis

Morphometrics and Physical Markers

Morphometrics (*morpho*– shape; *metrics*– measurements) is the use of physical measurements to determine the relatedness of organisms. With extinct organisms that have died out long ago, DNA extraction proves to be difficult. Likewise, prior to DNA technologies to analyze species, **Linnean taxonomy** was ascribed to organisms based on similarities in features.

Describing Species and Variation of Morphologies

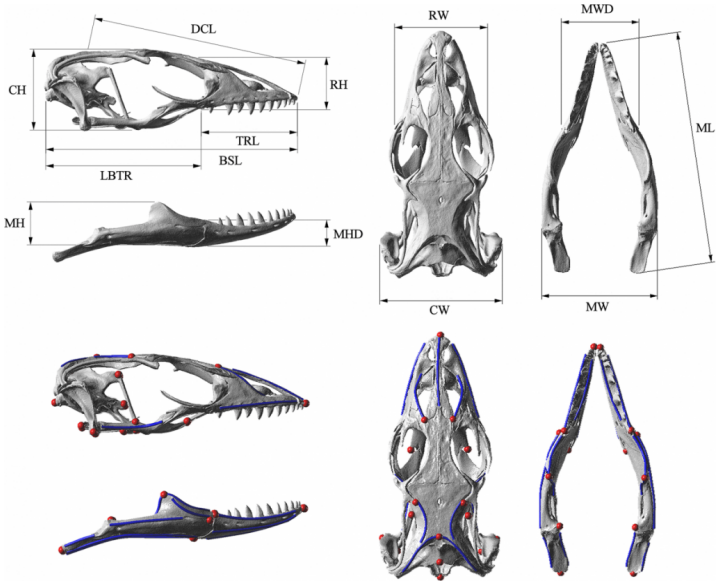
Below are images of skull landmarks of the lizard family Varanidae. This family includes monitor lizards and Komodo Dragons. As can be seen below, the general morphology of the skulls is similar enough that they all retain the same landmarks. The figure below also illustrates the diversity in these lizards that illustrate a large variation between species.



Skulls of the species involved in this analysis. McCurry et al. (2015) *(CC-BY)*

Landmarks Standardize Measurements

Having a set of shared landmarks provides the opportunity to make systematic measurements of morphometric features.



Landmarks and measurement metrics for the morphometric analysis of skulls. McCurry et al. (2015) (CC-BY)

Euclidean Distance to Measure Relatedness

Euclidean distance is a measurement derived from Pythagorean geometry that describes the shortest distance (d) between 2 points (A and B) as a straight line using triangulation. In a Cartesian space, the points can be defined:

$$A = (x_A, y_A)$$

and

$$B = (x_B, y_B)$$

Standard Pythagorean theorem can be expressed as:

$$x^2 + y^2 = d^2$$

To find the distance between the 2 points, we utilize algebra to calculate for d.

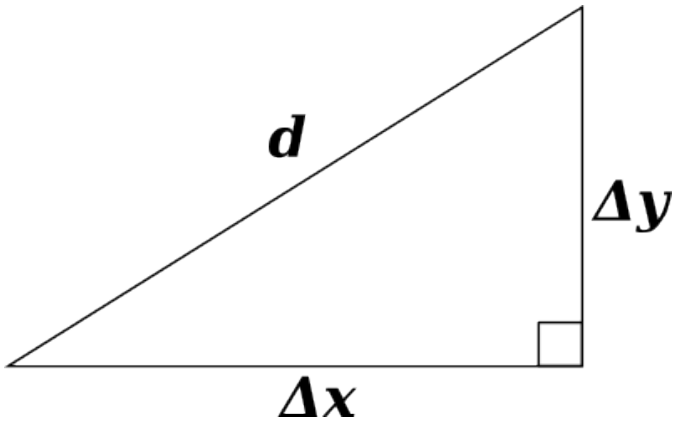
$$d = \sqrt{x^2 + y^2}$$

In this case, we expand to comparing the coordinates of the two points:

$$\Delta x = x_B - x_A$$

and

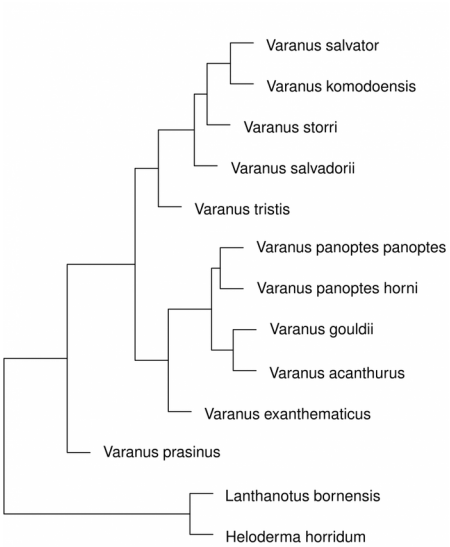
$$\Delta y = y_B - y_A$$



We can then expand this idea to include the differences in data points that describe the comparisons of multiple measurements.

$$d\left(\mathbf{X}_\mathrm{i}, \mathbf{X}_\mathrm{j}\right) = \sqrt{\sum_{k=1}^{p} \left(X_{ik} - X_{jk}\right)^2}$$

Calculating Distance with R



Varanoid Dendrogram from R

1. Download the **dataset** (McCurry *et al.* 2015) associated with this activity (a Comma Separated Value .csv file). This can be used in a spreadsheet or in a text editor. This data can be imported into R to determine the Euclidean distances of landmarks.
2. The following code in R will download the data set into a variable called “varanoid”, measure Euclidean distance and save a plot into a PDF file in a directory called “/tmp”.

```
## install curl for fetching from internet if it isn't
install.packages('curl')
## Load the curl library
library(curl)
## read the data of measurements and assign it to a variable 'varanoid'
varanoid = read.csv(curl('https://raw.githubusercontent.com/jeremyseto/bio-oer/master/
## set the row names to the Species column
row.names(varanoid) = varanoid$Species
## remove the first column of the table to have purely numeric data
varanoid_truncated = (varanoid[,2:14])
## calculate distance using euclidean as the method
dist_measure = dist(varanoid_truncated, method='euclidean')
## display dist_measure to look at the comparisons
dist_measure
varanoid_cluster = hclust(dist_measure)
## open PDF as a graphics device to save a file in the '/tmp' directory
pdf(file='/tmp/varanoid_tree.pdf')
plot(varanoid_cluster)
dev.off()
## close the device to save the plot as pdf
```

DNA Analysis

Before starting this activity, review **bioinformatics** and **sequence analysis**.

1. Search NCBI for mitochondrial sequences from the species involved in McCurry 2015. The data has been submitted by Ast (2001).
2. Find the sequences and identify/extract elements that are common to all.
3. Assemble the shared sequences in a text editor as a single FASTA file where each species is separated by a header (“>Species A”).
   - Notepad on Windows (but it’s better to download notepad++)
   - Textedit on Mac (but probably better to download TextWrangler)
   - Gedit on Linux
4. Save the file as “something.fasta”.
5. Perform a multiple sequence analysis using **UGENE.**
6. Generate a phylogenetic tree using UGENE. For this exercise, use Maximum Likelihood (PhyML) as the algorithm. File the tutorial below.
7. Compare the DNA with the morphometric analyses. What problems could we imagine arise if we rely solely on morphometry?

References

- McCurry MR, Mahony M, Clausen PD, Quayle MR, Walmsley CW, Jessop TS, Wroe S, Richards H, McHenry CR. (2015) **The Relationship between Cranial Structure, Biomechanical Performance and Ecological Diversity in Varanoid Lizards.** *PLoS ONE* 10(6): e0130625. doi: 10.1371/journal.pone.0130625
- Ast, Jennifer C. (2001) **Mitochondrial DNA Evidence and Evolution in Varanoidea (Squamata**). *Cladistics* 17(3): 211–26. http://www.sciencedirect.com/science/article/pii/S0748300701901690
- Fisher, R.A. (1936) **The use of multiple measurements in taxonomic problems.** *Annals of Eugenics,* 7: 179–188. doi:10.1111/j.1469-1809.1936.tb02137.x

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2.1.16.7: Sequence Alignment and Tree Building

UGENE

The following video illustrates the tree-building process using MUSCLE and PhyML in **UGENE**.

Command Line

The following requires:

- A UNIX-like environment like Linux or MacOS
- **MUSCLE** to perform a multiple sequence alignment
- **PhyML** to generate Maximum Likelihood
- **FigTree** to manipulate the tree

Download the example file **oranges**. In the download directory, perform the following:

```
unzip orange.zip
cd orange
cat ./*txt >> oranges.fasta ## merges all files into a single fasta file
muscle -in oranges.fasta -phyiout oranges.phy
## -phyiout tells muscle to use the interleaved phylip format for output
phyml -i oranges.phy -m HKY85
## -m is for method and HKY85 is the default nucleotide method we used in UGENE
mv oranges.phy_phyml_tree.txt oranges.nwk
## change the name of the output to reflect it is a nwk file
```

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2.2.1: Lab Safety and Laboratory Notebook

Learning Objectives

Goals:

- Understand the importance of a well-kept notebook.
- Learn how to enter information and maintain a legal scientific notebook.
- Familiarize students with basic safety rules.
- Allow students to become familiar with the safety equipment in the laboratory.

Student Learning Outcomes::

Upon completion of this lab, students will be able to:

- Record information into a notebook in a legally acceptable format.
- Format a table of contents.
- Make appropriate corrections to their notebook.
- Engage in good laboratory practices.
- Properly use Personal Protective Equipment (PPE).
- Identify the location and proper use of emergency equipment.
- Recognize the meaning of common laboratory safety signs.
- Interpret data on the safety diamond.

Introduction

Regardless if you work at a biotechnology company or in an academic research lab, keeping a lab notebook is a requirement. The Food and Drug Administration's (FDA) handbook states, "if it isn't written down, it wasn't done." The lab notebook is considered a legal document and can be used in court to settle patent disputes or to report a specialist's finding in paternity suits or criminal cases. Often times, the lab notebook is used as a starting point for other scientists who work in the lab. Lab notebooks are always maintained for the following reasons:

- To record the steps an individual has carried out and to document their observations
- To establish ownership in case of a patent dispute or other legal issues
- To establish guidelines used to evaluate the process in which a product was made and to evaluate the product itself
- To follow the production of a product through the manufacturing process
- To create a contract between a company and consumers and/or between a company and regulatory agencies
- To demonstrate a procedure was done correctly
- To develop, follow, and evaluate standard operating procedures (SOP)

Without documentation, even quality work is worthless. In the event of an experiment failing, the documentation allows for a scientist to review their protocol and make adjustments for future experiments. As stated above, in industry, lab notebooks are legal documents. They are used to determine product quality, patent rights, and liability. Notebooks are always treated as if they will be used in court because the consequences of not doing so could be devastating to a company.

Part I: Setting Up a Legal Scientific Notebook

A notebook should contain what equipment and materials were used and what steps were followed to show that the equipment and materials were validated before use. In case of an audit by government regulatory agencies, a company must be able to produce documentation that proves that they are following **Good Manufacturing Practices (GMPs).** If they find that a notebook was missing information or was not legible, then the company may be fined or held liable for damages in a product lawsuit. The importance of a well-kept notebook cannot be emphasized enough.

Guidelines for Setting Up a Legal Scientific Notebook

*For the purpose of this course, pages suitable for a lab notebook are included in the appendix. Your instructor may direct you to print a specific number of pages and bind them with staples along the long left edge to serve as a low-cost substitute for an official bound notebook.

1. Obtain a **bound notebook**, such as a composition book. Spiral notebooks are not acceptable.
2. Use only blue or black pen to make entries. **NO PENCIL ALLOWED.**
3. Label the outside of your notebook with the information:

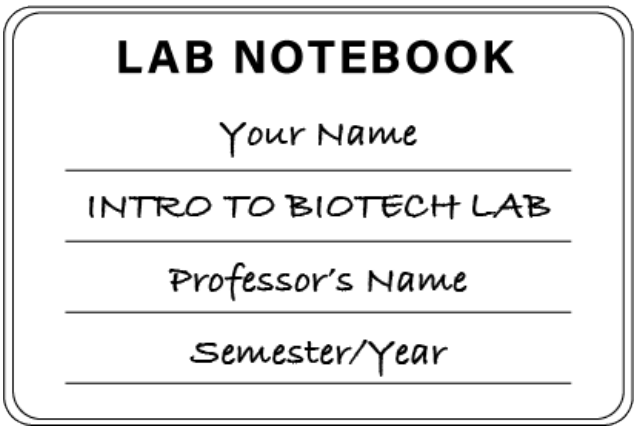


Figure 1. Lab notebook cover setup: Row 1: Name, Row 2: Intro to Biotech Lab, Row 3: Professor's Name, Row 4: Semester/Year.

4. Make pages 1-4 the Table of Contents. Write “Table of Contents” at the top of each of these pages. Record the title of each lab or entry and the page number for each.
5. Each page in the notebook must be numbered. Number each page of the notebook in the upper corner with the first page of the notebook being “page 1.” The back of the first page will be “page 2” and so on.
6. Record the date of each entry at the top of each page.
7. Sign and date the bottom of each page.
8. For each experiment (or activity) that you perform, you should include the purpose for the experiment, the materials and methods you use, any data tables, figures or graphs properly labeled with a title, and a conclusion.
9. Do not erase errors. Just draw a single line through an erroneous entry, then add your initials. Enter the correct entry nearby.
10. Never leave blank spaces. Draw a diagonal line through all open or blank space.
11. If an entry continues on a different page, include “go to page ___” on the bottom right hand of the page to let the reader know where the rest of the information is located.
12. Graphs and other small sheets of paper can be pasted into the notebook using a glue stick.
13. Always include enough details for someone else to successfully duplicate the work you have recorded.
14. Label all figures, tables and calculations. Figures should be labeled on the **bottom left** and tables are labeled on the **top left.**
15. Never remove pages from your notebook.

Activity: Analysis of Sample Lab Notebook Entries

1. Using blue or black pen complete steps A-E of the “guidelines for setting up a legal scientific notebook.”
2. Enter a title in your lab notebook page (and in Table of Contents) as “Analysis of Sample Lab Notebook Entries.”
3. Label the top of page 5 Lab A: Analysis of Sample Lab Notebook Entries.
4. Each student should obtain a copy of the “Sample student notebook entries.”
5. Review each of the three samples by yourself. You may choose to use the notebook scoring rubric provided in the appendix of this manual. Once you have reviewed the entries you can discuss your opinions with your group members. Make a list of what the student did well and what the student could have improved upon.
6. **Draw 3 tables into your notebook, one for each student entry you will be evaluating.**

Score: ___ / 10 pt

Table 1. Review of Sample Lab Notebook: Entry #1

What did student do well?	Improvements Needed?

7. If you were grading these lab entries, what categories/criteria would you use to assign points? If you were the grader, what overall score (out of 10 points) would you assign the samples?

Part II: Lab Safety

Introduction

Laboratory safety involves all the measures taken by the laboratory worker, laboratory owner, institution and regulatory agencies to eliminate potential harm to human health and well-being. Although steps are taken to reduce risks in the workplace, safety is a matter of **personal responsibility**. A biotechnology lab may have several safety hazards that must be known and understood by all students or employees working in the lab. It is the responsibility of each person in the lab to know and follow basic laboratory safety rules, to understand how to safely operate equipment, understand the hazards of materials they are working with and to work to reduce potential risks. In the event of an accident, it is critical to know the location and use of emergency equipment. Having this knowledge should help to prevent accidents and minimize damage that might occur in the event of an accident.

A. Laboratory Safety Equipment

Draw Table 2 in your lab notebook. Fill in the location of the following lab safety items:

Table 2. Laboratory Safety Equipment and Location

Safety Equipment	Location
Telephone and Campus Emergency Number	
Fire Alarm	
Fire Extinguisher	
Eye Wash	
Shower	
Glass Waste Container	
Biohazard Waste Container	

Safety Equipment	Location
Lab Coats	
Goggles	
Gloves	
Disinfectant	
First Aid Kit	
Broom and Dust Pan	
Emergency Class Evacuation site	

Laboratory Safety Guidelines and Contract

General Rules

- No guests are allowed in the lab.
- Know emergency procedures, use and location of emergency equipment (emergency exits, fire extinguishers, fire blanket, eye wash station, first aid kit, and broken glass container).
- In case of fire, evacuate the room and assemble outside the building.
- Report all accidents, no matter how insignificant they appear, to a laboratory supervisor
- Be aware of your surroundings and potential dangers created by others.

Personal Protection

- Do not smoke, eat, drink, chew gum, or apply cosmetics in a laboratory.
- Wear protective clothing such as long pants, closed-toe shoes, a lab coat, and goggles.

- Tie long hair up or behind the shoulders. Do not wear long, dangling jewelry or scarfs.
- Dispose of gloves in the laboratory trash. Do not wear lab coats and gloves into public areas. You will need to dispose of gloves and take off your lab coat before leaving the lab.
- Cover cuts or scrapes with a sterile, waterproof bandage before entering a lab.
- Wash skin immediately and thoroughly if contaminated by chemicals or microorganisms.
- Wash your hands regularly, with soap and water, especially after working with bacteria.
- If you are pregnant or ill, please let your lab instructor know immediately.
- Let your lab instructor know before leaving the classroom.

Handling Chemicals

- Keep all containers capped with the appropriate lid. Clearly label items produced in the lab.
- If a chemical is splashed into the eyes or skin, flush for 15 minutes.

- Clean up spills and broken glass immediately. Use broom and dustpan to pick up broken glass.
- Keep chemicals away from direct heat or sunlight. Keep containers of alcohol, acetone, and other flammable liquids away from flames.
- Read labels carefully. Be aware of hazardous chemicals and precautions for safe use.
- Follow instructions about proper disposal of lab reagents.

Handling Equipment

- Keep your work area clean and clutter-free.
- Be aware of your potential impact on others.
- Notify lab supervisor of malfunctioning equipment.
- If you do not know how to use an instrument or equipment, then do not touch it.
- Do not use laboratory equipment without first receiving instruction in its use.
- Keep balances clean and dry, always use weigh paper/boats.
- Never leave heat sources unattended. Be careful when using hot plates or burners. Note that there is often no visible flame, glow or sign that objects are hot.

I have read the lab safety guidelines, found all lab safety equipment, and understand the procedure about emergency class evacuation. I will conduct myself in a safe and conscientious manner and take proper care in the use of all lab equipment.

Signature _____ Date _____

B. Lab Safety Video

1. While watching the lab safety video, take notes on as many safety hazards as you notice.
2. When the video is over, discuss the hazards you observed with your group and what can be done to work safely using good laboratory practices.
3. Make Table 3 in your notebook and organize your observations.

Table 3. Laboratory Hazards and Corrections

Laboratory Hazard Observed	Correction/Good Laboratory Practices (GLP)

C. Chemical Hazards Labeling Activity

Hazard Communication Standard (HCS)

A quick assessment of a chemical's hazards is visible on its container on a Hazard Communication Standard (HCS) label. HCS labeling does not replace the more detailed Safety Data Sheet (SDS) but rather gives the following information in brief:

- Name, Address, and Phone Number of Responsible Party (i.e., the manufacturer or distributor)
- Chemical Identification - chemical name and code or batch number that matches the information found in Section 1 of the chemical's SDS
- Signal Word - "DANGER" for more severe hazards or "WARNING" for less severe hazards
- Hazard Statements - a brief description of the hazard(s)
- Precautionary Statements (optional) - prevention, response, storage, and disposal
- Hazard Category Numbers 1-4 (optional) - "1" for the most severe hazard to "4" for the least severe
- Pictograms

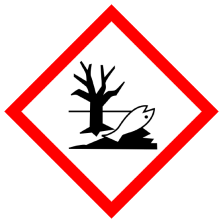
Pictograms are red-bordered, diamond shapes that frame a black graphic on a white background, and these symbols depict the type of hazard(s). HCS labels are required by the Occupation Safety and Health Administration (OSHA) and are standardized,

having been adopted from the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) set by the United Nations.

HCS Pictogram Guide

Pictogram	Hazard Class	Hazard Type	Example Signal Word & Hazard Statement
	Flammables Self-Reactives Self-Heating Pyrophorics Emits Flammable Gas Organic Peroxides	Physical	DANGER Heating may cause a fire
	Explosives Self-Reactives Organic Peroxides	Physical	WARNING Fire or projection hazard
	Gases Under Pressure	Physical	WARNING Contains gas under pressure; may explode if heated
	Corrosive to Metals Corrosive Skin Corrosion/Burns Eye Damage	Physical Health	DANGER Causes severe skin burns and eye damage

Pictogram	Hazard Class	Hazard Type	Example Signal Word & Hazard Statement
	Oxidizer (gases) Oxidizers (solid or liquid)	Physical Health	WARNING May intensify fire; oxidizer
	Acute Toxicity (fatal or toxic)	Health	DANGER Fatal if swallowed
	Carcinogen Mutagenicity Respiratory Sensitizer Reproductive Toxicity Target Organ Toxicity Aspiration Toxicity	Health	DANGER May cause cancer
	Irritant (skin and eyes) Dermal Sensitizer Acute Toxicity (harmful) Narcotic Effects Respiratory Tract Irritation Hazardous to Ozone Layer (optional)	Health Other	WARNING Causes skin irritation

Pictogram	Hazard Class	Hazard Type	Example Signal Word & Hazard Statement
	(optional label) Environmental Toxicity Aquatic Toxicity	Environment.	WARNING Toxic to aquatic life

Procedure

1. Walk around the room and locate two items that display the Hazard Communication Standard label.
2. Draw a sketch of the pictogram in your lab notebook and carefully copy the chemical identification information of the reagent that it is describing.
3. Write down the label's signal word and any hazard statements, and comment on the characteristics and hazards of the two items.

Chemical Hazards and Waste Disposal

Laboratory waste must be disposed of safely and appropriately. Labs must be aware of the school, state and federal guidelines for waste disposal. Many chemicals can NOT be poured down the sink. Be sure you know which chemicals are hazardous, require special storage and must be placed into properly labeled waste containers kept in fume hoods until sent to the proper hazardous waste disposal. Know which materials are considered biohazards and the proper area or container to place them so they can be autoclaved.

Table 4. Medical and Biohazardous Waste Treatment and Disposal Chart, U. California

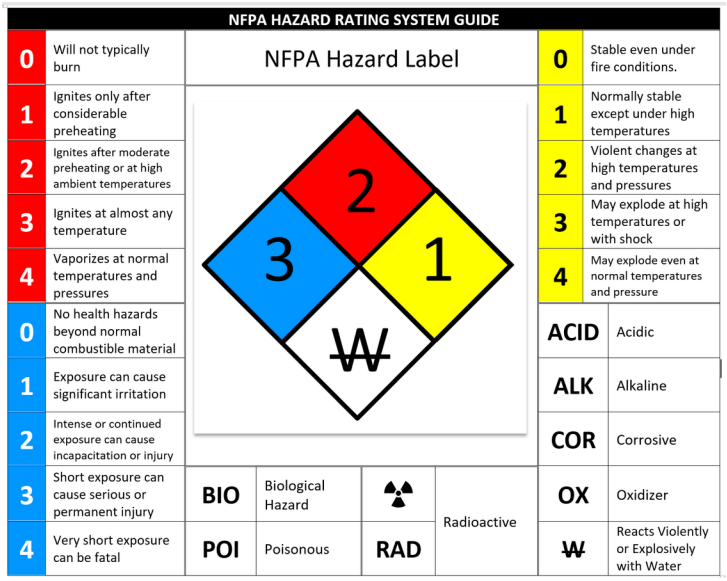
Type of Waste	Definition	Required Treatment
Chemical Waste	Any solid, liquid, or gaseous material generated in the laboratory that poses a danger to human health or the environment.	This will vary depending on the chemical. The institution you are in will have specific requirements to meet regulatory code. The aim is to reduce hazards and minimize environmental impact. Follow your instructor's directions.
Solid Biohazardous Waste	Materials such as pipettes, petri dishes, or other culture flasks disposable or glass that do not contain liquid but were in contact with cultures of cells or human or animal-derived materials.	Disposable waste is placed in red biohazard bags. Treat and replace bags when they are halfway full. Reusable glass materials must be placed in autoclavable trays. To treat waste, the autoclave must be properly loaded and set for a minimum temperature of 121°C (250°F) for 60 minutes at 15 psi. Autoclave tape must be placed on the bag or tray to indicate it has passed through the cycle. Material should be autoclaved as soon as possible, but at a maximum of 7 days after it is generated

Type of Waste	Definition	Required Treatment
Liquid Biohazardous Waste	Broth cultures or cell culture media or contaminated liquid media.	Decontaminate using 10% bleach solution for a minimum 2 hours contact time. Dispose down the drain with water. Alternatively, racks of used culture tubes can be autoclaved using the same temperature and time indicated above but set for a slow exhaust liquid setting. Use autoclave tape as above. Treat waste as soon as possible but not longer than 7 days post generation.
Sharps Waste	Any object that is capable of piercing or damaging human skin that is contaminated with chemical, or biohazardous waste e.g., scalpels, blades, needles, broken glass, etc.	Place in the marked sharps container. Remember that broken glass that is not contaminated will have a separate disposal container within the laboratory.

D. National Fire Protection Association (NFPA) Hazard Rating System

The National Fire Protection Association (NFPA) Hazard Rating System was designed for emergency workers such as fire responders to be able to swiftly get basic information about the hazards of a chemical from a simple label. The diamond-shaped label is divided into four color-coded squares, and inside each square is printed a ratings code or number from 0-4, with "0" as the least severe hazard to "4" as the most severe.

Inside the red square, the number indicates the flammability rating; blue indicates the health hazard rating; yellow indicates the chemical's instability; and the white square indicates any special hazards.



PROCEDURE

1. Walk around the room and find a label with the NFPA safety diamond. Read the explanations in the table above. Write down

the chemical name and draw its NFPA diamond.
2. Think of a chemical used in a lab or find a second container without an NFPA label already affixed and then write down its name. Use an internet or in-class resource to draw that chemical's NFPA ratings.
3. Compare the NFPA 704 label with OSHA's Hazard Communication label from the previous activity. How are they the same? How do they differ? What are the pros and cons of each type of label?

E. Safety Data Sheets (SDS)

A Safety Data Sheet (previously called Material Safety Data Sheet) for each chemical is required by the Occupational Safety and Health Administration (OSHA). Sections 1-8 contain information about the identification, hazards, composition, safe handling practices, and emergency control measures. Sections 9 through 11 and 16 contain technical and scientific information, such as physical and chemical properties, stability and reactivity, toxicology, and exposure control.

Draw table 1.3 in your lab notebook and answer the following.

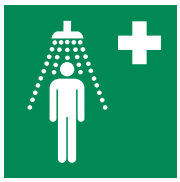
Using the SDS for sodium hydroxide provided to your group, determine what should be done in the event that the reagent: a) got on your skin b) splashed into your eyes c) was inhaled

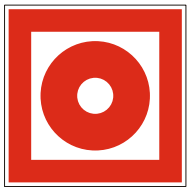
Table 5. Safety Procedures for Sodium Hydroxide (NaOH)

Adverse Event	Response
a) NaOH in eyes	
b) NaOH on skin	
c) NaOH inhaled	

F. Science Laboratory Safety Signs

Table 6 Write the meaning of each symbol.

Study Questions

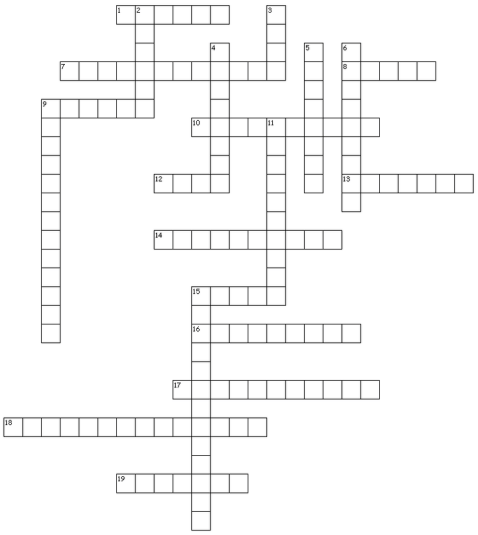
1. Review lab safety signs and know their meanings.
2. What are the types of PPE required in a laboratory?
3. How should you dispose of glass waste?
4. How should you dispose of biohazard waste?
5. What should you do if a chemical gets in eyes?
6. Given a laboratory hazard, be able to describe the correction of GLP needed in the lab.
7. What do the 4 diamonds in the NFPA represent?
8. Why must a scientist keep a lab notebook?
9. In industry, a lab notebook is a legal document. What can it be used to determine?
10. What is GMP?
11. What are the Do's and Don'ts of keeping a legal notebook?

Puzzle: Lab Safety and Lab Notebooks

Terms

Alkaline, Biohazardous, Broom, Contents, Corrosive, Diamond, Flammability, Four, Glass, Health, Instability, Laboratory, Legal, Manufacturing, OSHA, Oxidizing, PPE, Sharps, Special

Puzzle: Lab Safety and Lab Notebooks



ACROSS	DOWN
1. broken test tubes and beakers should be placed in the ___ waste container	2. the lab notebook is considered a ___ document and can be used in court
7. hazard that the yellow color on the NFPA sign indicates	3. abbreviation for Personal Protective Equipment
8. governmental agency that regulates chemical safety data sheets	4. shape of sign that lists the chemical ratings from the National Fire Protection Association
9. broken test tubes/beakers should be swept with a ___ and dust pan	5. hazard that the white color on the NFPA sign indicates
10. COR on the NFPA sign	6. table found at the front of a scientific lab notebook
12. instrument used to write in a scientific lab notebook	9. type of waste containing recombinant DNA and tissue culture dishes
13. used razors, scalpels and needles should be placed in the ___ waste container	11. OX on the NFPA sign
14. SOP stands for Standard _____ Procedures	15. hazard that the red color on the NFPA sign indicates
15. rating number for the most dangerous hazards in the NFPA sign	
16. ALK on the NFPA sign	
17. GLP stands for Good ____ Practices	
18. GMP stands for Good ____ Practices	
19. hazard that the blue color on the NFPA sign indicates	

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- No eating or drinking sign via Wikimedia Commons; public domain
- Nonpotable water sign via Wikimedia Commons; public domain
- Do not enter sign via Wikimedia Commons; public domain
- No smoking sign via Wikimedia Commons; public domain
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- Poisonous substances sign via Wikimedia Commons; public domain
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2.2.2: Metrics and Measurements

Learning Objectives

Goals:

- Review the metric system.
- Learn to convert between metric units.
- Use various instruments found in the biotechnology lab.
- Measure mass and volume with precision and accuracy.
- Pipet with precision and accuracy.
- Learn how to use a micropipette to measure very small volumes.

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Convert between metric units for mass, volume and size.
- Use a gram balance to obtain the mass of an object.
- Make accurate and precise measurements with a graduated cylinder and serological pipette.
- Calculate percent error for a given measurement.
- Read, set, and operate a micropipette.
- Determine which pipette should be used to measure a specific volume.
- Determine how accurately you can measure with each micropipette.

Part 1: Metrics

Introduction to Metrics

Working in a biotechnology lab requires knowledge of the metric system. The metric system uses standardized units of measurement for length, mass, and volume, ensuring measurements are reproducible and easily made. Appropriate instruments are used to make these measurements. For example, balances measure mass in grams and graduated cylinders measure volume in milliliters.

The metric system has base measurements. The meter is used to measure distances; the liter measures volume; and the gram measures mass. A measurement must always consist of a number and a unit, for example, 2 m, conveys the length is twice that of the base unit of length, the meter. The abbreviations are permitted when expressing measurements. The metric system allows for easy conversion between units as everything is base 10. This means you will either multiply or divide by ten as you convert from one unit to another. For example, one decameter is 10 times larger than a meter. Therefore, you need 10 meters to equal a decameter. A kilometer is 1000 times larger than a meter. Therefore, you need 1000 m to equal one kilometer.

Base Units of Measure

- Length: meter (m)
- Mass: gram (g)
- Volume: liter (L)
- Time: seconds (s)
- Temperature: Celsius (C)

Metric Prefixes

Prefix	Unit	Multiplier	Scientific Notation
Kilo-	k	1,000	10^3
Hecto-	h	100	10^2
Deca-	da	10	10^1
One	base (m, L, g)	1	10
Deci-	d	0.1 = $1/10$	10^{-1}
Centi-	c	0.01 = $1/100$	10^{-2}
Milli-	m	0.001 = $1/1,000$	10^{-3}
Micro-	μ	0.000001 = $1/1,000,000$	10^{-6}

Converting Metric Units

Memorize the table above and know how to use metric prefixes. You can use the helpful mnemonic below.

Mnemonic for remembering metric conversions

Mnemonic	King	Henry	Does	Usually	Drink	Chocolate	Milk	
Prefix	Kilo-	Hecto-	Deca-	Base units	Deci-	Centi-	Milli-	Micro
unit	k	h	da	m, L, g	d	c	m	μ

When you are converting a smaller unit to a larger unit, you move the decimal point to the left the appropriate number of steps. Keep in mind each time you move the decimal point you are dividing by 10.

When you are converting from a larger unit to a smaller, you will move the decimal point to the right. This means each time you move the decimal point you are multiplying by 10.

Steps for Converting Metric Units

1. Write down the number you are converting for example (100 cm). Then right in the decimal point. It is always right after the ones place to the right of the number.

100 = 100

2. If you want to convert 100 cm to meters (m) you would now look at your chart and determine how many “steps” you have to move the decimal to the right or left. From centimeter to meter you have to take 2 steps to the left. That means you must move your decimal 2 places to the left.
   100. cm = 1.00 meters

Metric Conversion Practice

Using the steps above, complete the following problems in your lab notebook.

1. 50 mm = X cm
2. 50 cm = X km
3. 700 mL = X L
4. 30 m = X µm
5. 3 dm = X m
6. 15 kg = X cg
7. 55 L = X mL
8. 52 mg = X µg

Part 2: Measuring Using the Metric System

A. Taking Linear Measurements with a Ruler

Linear measurements in science are in metric units. The basic unit is the meter (m). The rulers you will be using today are centimeter (cm) rulers. There are 100 cm in a meter. If you look at the ruler, you will see 10 hatch marks between each centimeter marking. Each hatch mark represents a millimeter (mm). There are 10 millimeters in a centimeter.

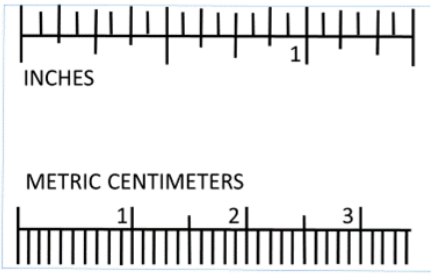


Figure 1. Ruler: centimeter scale bottom. Inch scale top.

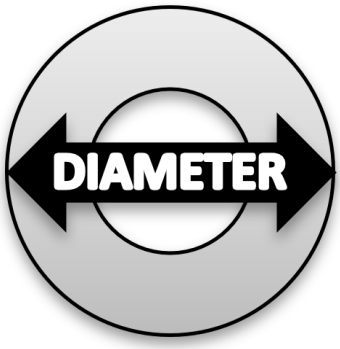


Figure 2. diameter of washer, measured in a straight line across the center of the washer from outer edge to outer edge.

Materials

- 5-6 washers of various sizes

- Centimeter ruler

Procedure

1. Obtain 5 washers from your instructor.
2. Order the washers on a piece of paper from the smallest diameter to the largest, labeling them #1-5.
3. Using a centimeter ruler, record the diameter of each washer in centimeters. See Figure 2 for and image on how to measure the diameter.
4. Record your results in Table 1.
5. Convert all your washer diameter measurements to millimeters and meters. Record in Table 1.
6. Keep your washers in order as you will be using them later.

Results

Draw the following table in your lab notebook including the title of the table.

Table 1. Diameter Measurements of Washers with Unit Conversions

Washer #	Diameter of Washer (cm)	Diameter of Washer (m)	Diameter of Washer (mm)
1			
2			
3			
4			
5			

B. Taking Mass Measurements with an Electronic Balance

Weight measurements in science are also in metric units. The basic unit is the gram (g). The electronic balances you will be using today are gram balances. The model you will be using will accurately measure to 0.01 gram. There are 1000 grams in a kilogram. One of the most common units used is the milligram (mg). There are 1000 milligrams in a gram. If you need a very small amount of something, you measure it in micrograms (µg). There are 10^6 µg in a gram. Some conversions are indicated below:

1000 g = 1 kg
1 g = 1000 mg
1 g = 1,000,000 µg (106 µg)
1 mg= 1000 µg

Materials

- 5 washers of various sizes that were previously measured.
- Gram balance

Procedure

1. Press the on button and wait for the balance to display zeros on screen.
2. If the screen doesn't display zeros, press the "zero" or "tare" button.
3. Once the machine displays zeros (0.00 g), place your washer on the center of the platform.
4. Wait for the scale to achieve a stable reading (numbers are not fluctuating).
5. Record your mass in grams in Table 2 for each washer starting from smallest to largest.

Results

1. Draw the following table in your lab manual including the title.
2. Record your results in grams (g) and then convert those masses to kg and mgs.

Table 2. Weight Measurements of Washers with Unit Conversions

Washer #	Diameter of Washer (cm)	Diameter of Washer (m)	Diameter of Washer (mm)
1			
2			
3			
4			
5			

C. Volumetric Measurements

The metric unit for volume is the LITER (L). There are 1000 milliliters (mL) in one liter. Another common unit in volume is the microliter (µL). There are 106 µL in one liter and 1000 µL in one milliliter. Some common conversions are shown below:

1 L = 1000 mL
1 L = 1,000,000 µL (106 µL)
1 ml= 1000 µL

You will need to become familiar with the different types of instrumentation and glassware that you will be using throughout this semester. Today, we will focus on glassware and devices that measure larger volumes of liquid. You will also determine when a particular device is appropriate to use based on the volume that you are dispensing. The types of measuring devices are very different if you want to measure and dispense a liter vs. a milliliter!

Graduated Cylinder

You will use this to dispense large volumes that are more than 10 mL. You will be using various size graduated cylinders, ranging from 20mL – 2000 mL (2L), in this class.

Serological Pipet

These pipettes accurately dispense volumes of 1mL to 10mL and can be used for volumes up to 50 mL. You will be using mostly 5mL and 10mL serological pipettes in this class.



Figure 3. Graduated Cylinders



Figure 4. Serological Pipettes



Figure 5. Beakers

Materials

- 1 - 50 ml beaker
- Squirt bottle with diH20
- 1 - 50 ml graduated cylinder
- 1 - gram scale
- 1 - 5 ml serological pipette
- 1 pipette pump or electronic pipet aide

Procedure

Graduated Cylinder Measurements

1. Draw a table 3 in your lab manual as shown on the following page.
2. Obtain a 50 mL beaker. Weigh and record the weight in grams on Table 3 under “Weight of container”. This is the container you will use to weigh your water. It is not what you will use to measure in this experiment.
3. The **target** amount of water you will be measuring using a graduated cylinder is 42 mL. This has been recorded in Table 3.
4. Using a squirt bottle, squirt 42 mL of water into a graduated cylinder. Be sure to read from the bottom of the meniscus.
5. Pour the 42 mL from the graduated cylinder into the weighed beaker.
6. Weigh the beaker with the water and record on Table 3 under “weight of container and water.”
7. Determine the weight of the water and record as “weight of water only”.
8. Convert this weight to mL. Water has a density of 1g/mL. Because water has a density of 1g/mL, then the number g=mLs (50ml=50g) Record this number as “actual volume dispensed”.
9. Determine the % error for each of your measurements as follows: ∖[" id="MathJax-Element-5-Frame" role="presentation" style="position:relative;" tabindex="0"> % Error = [(Target amount of water - Actual volume dispersed) / Target amount of water] * 100 ∖]" id=
10. If you are not with in an error range of +/- 5% then try again.

Serological Pipet

1. Dry the 50 ml beaker you used previously. You already weighed this container in the previous exercise. Write the weight of the
2. Obtain a 5 mL serological pipet and a pipet pump. Put on the pipet pump. Do not shove the pipet way up into the pump. Use th
3. Dispense the water into the weighed beaker dialing in the opposite direction. To get the last bit out of the pipet, quickly dial or
4. Dispense the 3.6 mL into the weighed beaker and determine its mass (g). Record this value in your table as “weight of containe
5. Determine the weight of the water and record. If you accurately dispensed 3.6 mL, the weight difference should be very close t
6. Convert the weight to mL and record.
7. Determine the % error as described in the previous section and record on Table 3.
8. If you are not with in an error range of +/- 5% then try again

Results

Table 3. Determining the Accuracy of Measurements of Water Volumes

Device used	Attempt	Target volume dispensed (mL)	Weight of container (g)	Weight of container a
50 mL graduated cylinder	trial #1	42		
50 mL graduated cylinder	trial #2 (if needed)	42		
5 mL serological pipet	trial #1	3.6		
5 mL serological pipet	trial #2 (if needed)	3.6		

D. Accuracy and Precision

When making measurements, both precision and accuracy are extremely important. **Precision** of a measurement refers to the **clos**

Precision can be affected by the measuring instrument. If a student uses a thermometer calibrated to the nearest degree to measu

Accuracy, however, is dependent upon both the **precision of the measuring instrument** and the **technical skills** of the individua

To calculate the "**mean" or "average"**, you will add all the measurements and then divide by the number of measurements. For exa

Materials

- 1 - 50 ml beaker
- Squirt bottle with diH20
- 1 - 25 ml graduated cylinder
- 1 - gram scale
- 1 - 10 ml serological pipette
- 1 pipette pump or electronic pipet aide

Procedure

1. Obtain a 50 mL beaker. Place the beaker on the scale and hit "tare." This will cancel out the weight of the beaker.
2. Obtain a second 50 mL beaker and use it to measure 10 mL of deionized water. Pour the measured 10 mL of water into the 50
3. Dispose of the water in the beaker that is on the scale. Dry the beaker between measurements. Repeat steps 1-3 for a total of 4
4. Obtain a 25 mL graduated cylinder and measure 10 mL of deionized water. Pour the measured 10 mL of water into the 50 mL b
5. Dispose of the water in the beaker that is on the scale. Repeat steps 4-5 for a total of 4 trials using a graduated cylinder to mea
6. Obtain a 10 mL serological pipet and measure 10 mL of deionized water. Pour the measured 10 mL of water into the 50 mL be
7. Dispose of the water in the beaker that is on the scale. Repeat steps 6-7 for a total of 4 trials using a serological pipet to meas
8. When finished, dump out all the water and place your used glassware on the cart in the front of the room.
9. Determine the average and the range for your data for each measuring device and record in table 4-6.
10. Record your averages for each measuring device in Table 2.1-2.6 for the class data.
11. Record your ranges for each measuring device in Table 2.1-2.7 for the class data.

Results

Table 4. Actual Volume Dispense for 4 trials (mL)

Measuring Device to Measure 10 mL	1	2	3	4	Average	Range
beaker						
Graduated cylinder						
Serological pipet						

Table 5. Class Average Actual Volume Dispensed for 4 Trials

Measuring Device to Measure 10 mL	1	2	3	4	Class Average
Beaker					
Graduated cylinder					
Serological pipet					

Table 6. Class Range of Actual Volume Dispensed for 4 trials (mL)

Measuring Device to Measure 10 mL	1	2	3	4	Class Range
Beaker					
Graduated cylinder					
Serological pipet					

Conclusion

Use your data and the questions below to write your conclusion in your lab notebook

1. For the volume measurements, which glassware used to measure 10 mL was the most precise?
2. Which measuring device was the least precise? Explain in detail why you think that particular device is the most precise.
3. For the glassware used to measure 10 mL, which instrument was the most accurate?
4. Was the instrument that was most precise the same one that is the most accurate?
5. Why or why not (in your answer be sure to include which measuring device was the most precise and which one was the most a
6. What can contribute to a larger % error when making a measurement? If you had to repeat a measurement in Table 3, be sure

Study Questions

1. Convert the following:
   a. 345 mL = X µl
   b. 0.34 dag = X kg
   c. 5.2 km = X mm
2. Which equipment would you use to measure 10 mL, 5 mL, 1 mL?
3. Be able to calculate your percent error.

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2.2.3: Micropipetting

Learning Objectives

Goals:

- Use various instruments found in the biotechnology lab.
- Measure volume with precision and accuracy.
- Pipet with precision and accuracy.
- Learn how to use a micropipette to measure very small volumes.

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Make accurate and precise measurements with micropipettes and serological pipettes.
- Calculate percent error for a given measurement.
- Read, set, and operate a micropipette.
- Determine which pipette should be used to measure a specific volume.
- Determine how accurately you can measure with each micropipette.

Introduction to Micropipetting:

The ability to measure very small amounts, **microliters (µl)**, of liquid chemicals or reagents is a fundamental skill needed in the biotechnology or research lab. Scientists use a device called a **micropipette** to measure these very small volumes with **accuracy**. This activity introduces the technique of micropipetting. Remember, as with all fine motor skills, this new skill will require practice and determination. Be sure to operate the micropipette slowly and carefully.

Part I: Choosing and Setting the Micropipette

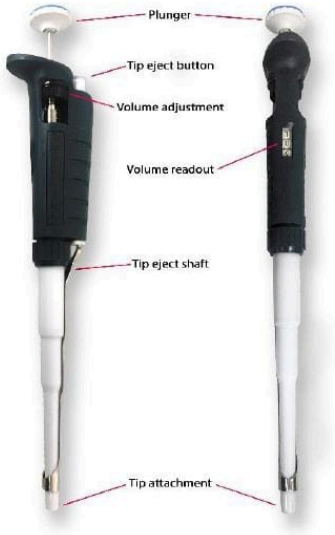


Figure 1. Labeled parts of a micropipette, front and back.

There are several sizes of micropipettes used in the biotechnology lab. Today, you will be using the P-1000, P-200, and P-20. The P-1000 measures volumes between 100-1000 µl, the P-200 measures volumes between 20-200 µl, and the P-20 measures

volumes in the 2-20 µl range. It is important to always pick the correct micropipette for the volume to be measured.

Looking at Figure 3.1, you can see that each micropipette has a similar but different display window. For the P1000, the red number indicates the thousands place, followed by the hundreds, tens, and the ones displayed as small vertical lines. Each line represents 2 µl. The P-200 is reads differently. The display from the top down reads, hundreds, tens, ones, and the vertical lines are considered 0.2 µl. Finally, the P-20 can be read from the top down tens, ones, and the red tenths.

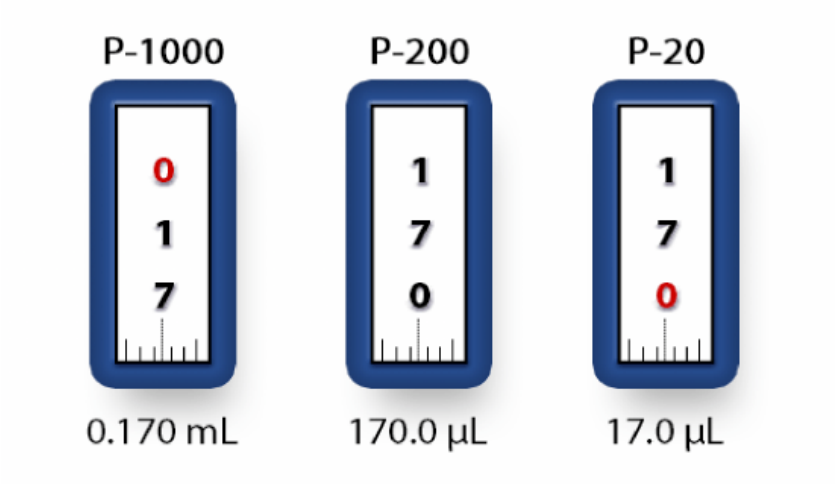


Figure 2. Reading a micropipette at various sizes.

A. Choosing your Micropipette

For each amount listed below, indicate the correct micropipette needed to measure the volume accurately then set the pipette to the indicated amount and show your partner.

Table 1. Pipette Size Choice

Amount	Pipette Needed	Partner Observation
1. 567 µl:		
2. 160 µl:		
3. 700 µl:		
4. 25 µl:		
5. 15 µl:		

B. Setting your Micropipette

Materials

- P-20 micropipette
- P-20 tips

- Waste container
- Tube of red dye in tube rack
- Laminated sheet for pipetting

Procedure

1. Each student will load 5, 10, 15, and 20 µl of red dye onto the laminated sheet.
2. Locate the p-20 and set the dial to 5 µl.
3. Hold the micropipette in your dominant hand, and gently but securely place the end of the micropipette into the proper size tip. Once the tip is on, be careful **not to touch the tip on anything**! If your tip touches the bench, lab coat etc. eject the tip into the waste container and get a new clean pipet tip.
4. With your other hand, open the cap of the tube of red dye and bring the tube of red dye to eye level,
5. Push the micropipette plunger down to **the first stop and hold your thumb in this position.**
6. Place the pipet tip into the red dye solution.
7. Gently release your thumb from the plunger to draw fluid into the tip.
8. Confirm that the tip has liquid and that no bubbles are present within the tip.
9. Close the tube of red dye and place back in tube rack.
10. Gently touch the tip to the center of the circle labeled 5 µL and slowly push all the way down (to second stop) on the plunger to dispense the liquid.
11. Repeat this process for the remaining volumes.
12. Be sure to watch your groupmates to provide feedback and help with their technique.

Results

Take a picture or draw a picture of your spots and include this in your lab notebook as Figure 1. Make sure the figure has a title.

Conclusion

1. Observe if your spots were similar in size to your groupmates.
2. Which volume had the most variability?
3. **What could have contributed to your spot being too large or small?**

Part II: Pipetting Practice

A. Microplate Art

Materials

- p20 pipette (1)
- p200 micropipette (1)
- P-20/P-200 tips
- Microplate art set (design cards, colored dyes, and 96-well microplate) (1)
- Analytical or electronic balance

Procedure

1. Obtain a 96-well microplate, a design card, and tubes of colored dyes.
2. Write the Microplate Art Design number in your lab notebook.
3. Using the gram balance, obtain the weight of your 96 well microplate and record in your notebook.
4. Using the p200 micropipette with tip, dispense 50 µl of dye into the wells written on the design card.
5. Once you have finished pipetting, weigh your completed microplate, and record in your lab notebook.

Results

1. Be sure to record your weight in grams of your microplate pre/post pipetting in your lab notebook.
2. Using these values, calculate your percent error of the microplate you just created. Include the calculation in your lab notebook.
3. Take a picture of your microplate design and include this in your lab notebook.

CONCLUSION

1. Was your percent error below +/- 5%? If your percent error was above this range, elaborate on the potential causes.
2. Did your pattern look correct? How could you avoid errors in the future?

B. Micropipette Practice Matrix

Materials

- p20 pipette (1)
- p200 micropipette (1)
- 1.5ml microfuge tubes (3)
- Permanent marker
- Analytical or electronic balance

Procedure

1. Label three microfuge tubes: 1, 2, 3,
2. Weigh each tube before placing any liquid inside.
3. Draw table 2 in your lab notebook and use it to record your data.

Results

Table 2. Calculating Accuracy for Micropipetting

Tube #	Weight of tube (g)	Weight of tube + dye (g)	Theoretical weight of dye	Actual weight of dye	% Error
1					
2					
3					

4. Deliver the volumes indicated in Table 3 into each of the 3 labeled tubes.

Table 3. Volumes to be Pipetted into each Tube

Tube #	Micropipette	Red Dye (µl)	Blue Dye (µl)	Green Dye (µl)
1	P1000	210	435	332
2	P200	110	153	67
3	P20	15	17	10

5. Weigh each tube after pipetting.
6. Determine the theoretical weight of the dye using the information about the weight of a mL of the dye solution at room temperature provided by your instructor.
7. Determine the % error for each tube.

Conclusion

Based on your data comment on the following in your lab notebook:

1. Which micropipette gave the most precise measurement?
2. Which micropipette gave the most accurate measurement?
3. **What may have contributed to higher percent errors?**

Study Questions

1. Convert the following:
   - 345 mL = _____ µl
   - 0.54 mL = _____ µl
   - 5.2 L = _____ mL
2. Which micropipette would you choose to measure 550µl? 17µl? 167µl?
3. Make 3 suggestions that other biotechnologists can use to improve micropipetting accuracy.
4. Assuming that the density of water is 1 gram per milliliter, how much should 550 µL of water weigh?
   - 17 µL of water?
   - 167 µL of water?
5. What is the formula to calculate percent error?
6. What is the maximum volume you can set for each micropipette (P-1000, P-200, P-20)?

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2.2.4: The Scientific Method

Learning Objectives

Goals:

- Utilize the steps in the scientific method to design, collect and interpret scientific data.

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Formulate a hypothesis based on an observation.
- Design their own experimental method including proper controls.
- Collect results and describe colony growth (morphology) present.
- Determine if data collected supports their hypothesis

INTRODUCTION

Microbes are all around us. In this lab, you will be introduced to this as well as to the scientific method. Throughout the semester, you will learn how to aseptically work with bacteria; meaning learning how to culture bacteria without contaminating yourself and keeping a pure sample of bacteria free from other unwanted bacteria. During this lab exercise, you are going to swab an area of your choice to see what bacteria and/or fungi are present there. You will also test the effect of some disinfectants on the bacterium *Escherichia coli* (*E. coli*) Using the scientific method, you will design an experiment, collect data, and interpret your results.

The **scientific method** is a generalized tool used to aid in asking and answering a scientific question by making observations and performing experiments. There are steps that are generally followed when conducting and designing an experiment. First, an initial **observation** is made. An observation can involve noting any event (a pattern, an action, a behavior, or a reaction). After making an observation, a question can be asked about the event. Once a question is asked, then research regarding what is already known relating to this question (finding background material) can be discovered to better understand the observation. This background information typically comes from publications in scientific literature, such as journal articles and reviews. Once the background information is understood, a **hypothesis** can be formed. This gathering of information and its application to a solution is an example of inductive reasoning. The hypothesis is then either supported or rejected depending on the analysis of the results of well-designed experiments. Each experiment needs **dependent** and **independent variables**. The value of the dependent variable is determined and is a function of the independent variable. In an ideal experimental setup, the independent variable is something over which we have some control and changes in some predetermined way, while changes in the dependent variable are observed and measured. A hypothesis must include both of these variables. A hypothesis can be generated by creating an “if-then” statement. For example, “If I treat cancer cells with drug x then they will die. “

Part I: Disk Diffusion Method to Evaluate Disinfectants

For this portion of the lab, you will be provided the protocol/instructions but you will choose the substances to test. You will develop and test your hypothesis.

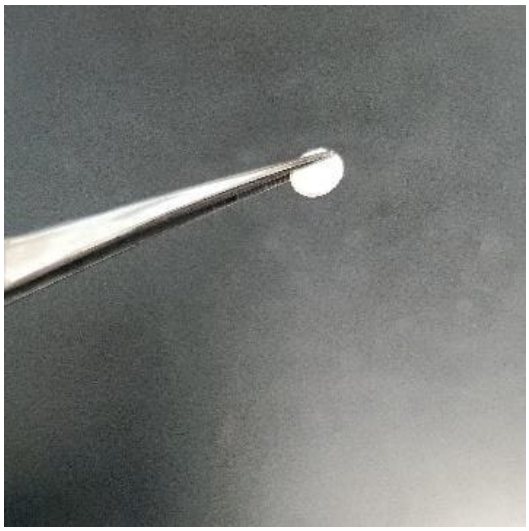


Figure 1. Forceps with sterile disk.

Materials

- 1 culture of *E. coli*
- sterile swabs (1 swab needed per plate)
- sterile absorbent paper disks
- sterile water (negative control)
- 10% bleach (positive control)
- 30% hydrogen peroxide (positive control)
- 4 test disinfectant solutions
- 1 petri plate (containing sterile nutrient agar)

Method

1. Plan your experiment. In addition to the controls, which solutions would you like to test?
2. Answer parts A, B, and C below to help with your planning.
3. Dip the sterile swab into the *E. coli* solution then spread it over the entire surface of the NA plate by rubbing the swab over the entire surface. You want to coat the entire surface with the bacteria so do not leave spaces that have not been in contact with the swab. Be careful to only open the lid of the plate enough to work (like a clamshell). If you open the lid all the way, you risk contaminating the surface with unwanted bacteria/fungi from the environment.
4. Dispose of the swab in the appropriate waste container.
5. Using sterile forceps (tweezers) dedicated to the solution to be tested, dip sterile disks one at a time into the following solutions and place them onto the agar surface that has been inoculated with *E. coli*. Be sure not to allow the tweezers themselves to come in contact with the agar because that will cause them to become contaminated with bacteria.
6. **Include all answers to your questions in your lab notebook along with your procedure for testing the disinfectants.**

Observation

- Based on your experience and observations, which solutions do you think will inhibit the growth (or kill) *E. coli* the most? Which solutions are you interested in testing?

Hypothesis

- Based on your observations, write the hypothesis you wish to test.

Experimental Design

Work with your group to write a protocol for your experiment based on the questions below. Start with the instructions and insert the necessary details such that a person with no knowledge of your project would be able to read your protocol and fully understand what to do.

1. Based on your hypothesis, which solutions will have the largest **zone of inhibition** around the disks?
2. What will you include as your experimental controls? (Which solutions WILL or will NOT inhibit the bacteria)?
3. How will you set up your experiment? (I recommend writing a map on your plate on the agar side where you will place the disks and then making a key to the map in your lab notebook).

Example Protocol

1. On the bottom of your NA agar plate (the side with the agar, NOT the lid!!), label the plate using a permanent marker with your initials, "Biotech Lab", the date, *E.* coli test, and where you are placing each disk.
2. Take the sterile swab and dip it in the *E. coli* culture. (**Don't** place the lid for the *E. coli* on the desk or it will now be contaminated!) Place the labeled plate on the desk in front of you with the lid side of the plate up. With one hand, open the lid (only open it a little bit so that you can have access to the agar; think of a clam shell) and use the swab to spread the bacteria all over the plate. Make sure to move the swab around to cover the entire plate.
3. Cover the plate with the lid and discard the swab in the appropriate waste container
4. Using dedicated forceps, dip a sterile paper disk into a solution to be tested and then place the disk onto the *E.coli*-inoculated surface. Open the plate like a clamshell each time you place a disk then close the lid immediately when finished.

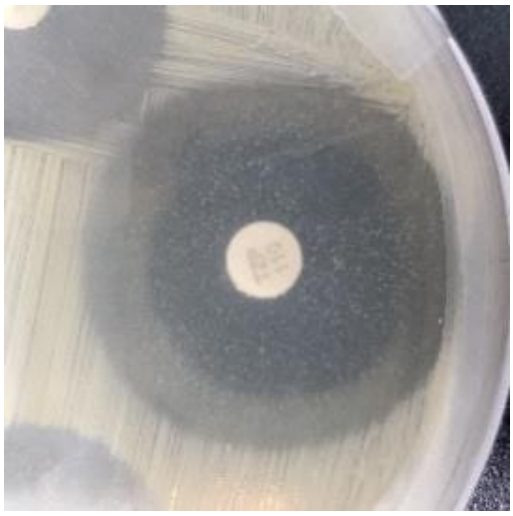


Figure 2. Zone of inhibition

5. Place plate in a 37^0 C incubator, with the agar side up. (note: you can place it into the same 32^0 C incubator as the next experiment)
6. The plate will grow in the **incubator** for 48 hours.
7. When incubation is complete, measure the diameter of any zones of inhibition using a millimeter (mm) scale. Report data in the table below.

Results

1. **Remove your plates from the incubator. DO NOT OPEN THE PLATES!**
2. Take a picture of your plates and include them in your lab notebook. Be sure to clearly label each portion of the plate.
3. Make the following table in your notebook and record your data.

Table 1. Comparison of Zones of Inhibition for Tested Solutions

Solution Tested	Diameter of Inhibition Zone (mm)

Conclusion

1. Which solution did you use as a **negative control**? Did this control provide the expected result?
2. Based on your observations, which solution had the greatest effect on the *E. coli*? Which has little or no effect?

Part II: Environmental Sampling

For this portion of the lab, you will develop and test your hypothesis as well as design the method to test it.

Materials

- 1 tube of sterile water
- sterile swabs (1 swab for each sample to be collected)
- Luria broth (LB) or nutrient agar (NA) plates (1 plate for each sample to be collected)

Method

1. Working with your group, determine your experimental design for this lab.
2. Complete parts A, B, and C below to help with your planning.
3. Include all answers to your questions in your lab notebook along with your procedure for collecting your samples.

Observation

- Based on your observations of the world around you what surfaces do you think are most “dirty” or “clean”? Which surfaces are you interested in testing?

Hypothesis

- Based on your observations write the hypothesis you wish to test in your lab notebook.

Experimental Design

1. Based on your hypothesis, how many surfaces/samples will you test?
2. What will you include as your experimental control?
3. How will you perform your experiment? (I recommend dipping your sterile swab into the sterile water and then swabbing your sample).
4. How long and in which pattern will you swab your samples? (roll, zigzag, etc.)
5. How many plates will you need and how will you section them? (you can use a sharpie to label the bottom of the plate and draw sections if needed).

Based on these questions: Work with your group to write a protocol for your experiment. Include enough detail that a person with no knowledge of your project would be able to read your protocol and fully understand what to do. Below is a general protocol for this lab to help you get started.

Example Protocol

1. On the bottom of your NA agar plate (the side with the agar, NOT the lid!!), label the plate using a permanent marker with your initials, “Biotech Lab”, the date, and where you are choosing to swab.
2. Take the sterile swab and dip it in the sterile water (don’t place the lid for the sterile water on the desk or it will now be contaminated!). Then touch the wet swab to whatever surface you would like to test in order to pick up the bacteria. Place the labeled plate on the desk in front of you with the lid side up. With one hand, open the lid (only open it a little bit so that you can have access to the agar; think of a clam shell) and use the swab to spread the bacteria all over the plate. Make sure to move the swab around to cover the entire plate.
3. Cover the plate with the lid and discard the swab.
4. Place plate in a 32^0 C incubator, with the agar side up. (Some organisms in the environment do not grow well at 37^0 C.)
5. The plate will grow in the incubator for 48 hours.

Results

1. **Remove your plates from the incubator. DO NOT OPEN THE PLATES!**
2. Take a picture of your plates and include them in your lab notebook. Be sure to clearly label each portion of the plate.
3. Make tables 2 and 3 in your notebook; record your data.

Table 2. Comparison of Bacterial Colony Growth on Open Surfaces

Sample Collected	Number of Colonies Present

Table 3 Morphology of Colony Growth on Open Surfaces

Sample Collected	Morphology of Colonies Present

Use the image below to help you describe the morphology of the colonies present on your plates.

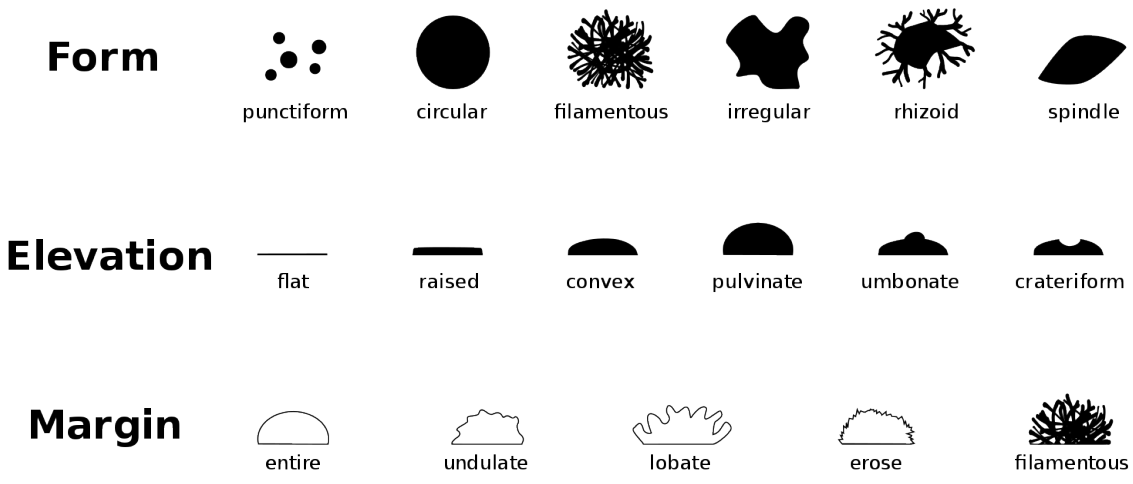


Figure 3. Morphologies of colony growth [by Madeco (CC-BY-SA) via Wikimedia Commons].

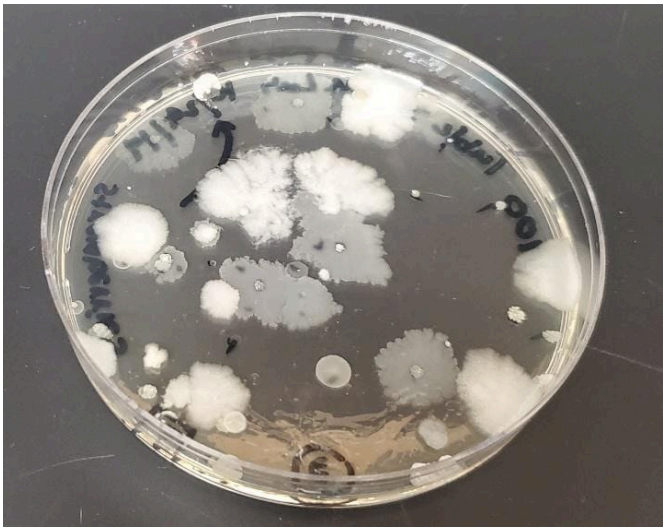


Figure 4. Nutrient agar media plate with various colony types growing on it

Conclusion

1. Based on your experimental data, which surfaces had the most bacterial/fungal growth?
2. Which surface had the most diverse number of bacteria/fungi?
3. Based on your observations, what types of bacteria/fungus do you think were present on your plate?

Study Questions

1. What are the steps of the scientific method?
2. Be able to write a hypothesis based on a given observation.
3. What is the purpose of an experimental control?
4. What is the definition of an independent variable? A dependent variable?
5. Why do we incubate plates upside down?
6. Why do we label the agar side of the plate?
7. What is the purpose of incubating the plates?
8. What is the purpose of the LB or NA in the plate?
9. Given a set of data, be able to formulate a conclusion based on the results given.

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Detailed Licensing

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2.2.5: Microscopy

Learning Objectives

Goals:

- Properly use and care for a sensitive scientific instrument.
- Learn the techniques required to prepare cells for viewing with a microscope.
- Gain a sense of the size of cells.

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Identify the parts of a microscope and their functions.
- Properly carry, use, and store a microscope.
- Prepare a wet mount slide.
- View and focus specimens under a microscope.
- Determine total magnification of a specimen.
- Locate a specimen if given a slide.

Introduction

In Biology, the compound light microscope is a useful tool for studying small specimens that are not visible to the naked eye. The microscope uses bright light to illuminate through the specimen and provides an inverted image at high magnification and resolution. There are two lenses that magnify the image of the specimen – the objective lens on the nosepiece and the **ocular lens** (or eyepiece). To determine the **total magnification** of the specimen, you must multiply the objective lens magnification with the ocular lens magnification.

Scientists and technicians often use light microscopes to study cells. **Prokaryotic cells** are very simple and lack a **nucleus** or membrane bound **organelles** and are small in size. On the other hand, **eukaryotic cells** are more complicated in that they contain a nucleus and many specialized organelles. A cell's structure dictates its function; thus, each eukaryotic cell looks very different from the next. This is why a cardiac cell looks completely different from a **neuron** (brain cell).

It is very important to learn how to handle and use a microscope properly. Review the following rules and tips for using and handling your microscope.

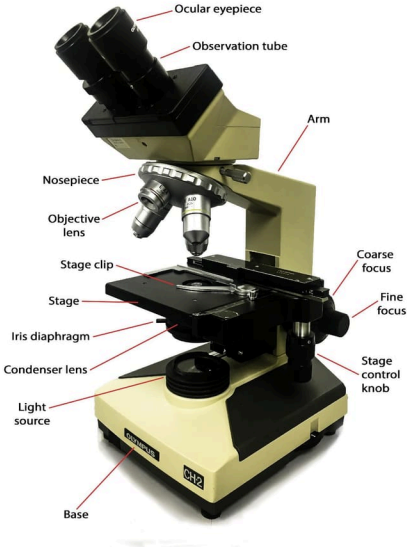


Figure 1. Labeled parts of a microscope.

General Rules

- Always START and END with the low power lens when putting on OR taking away a slide.
- Never turn the nose piece by the objective lens.
- **Do not get any portion of the microscope wet - especially the stage and objective lenses.**
- **Use only lens paper to clean microscope lenses.**

Cleaning the Microscope

If needed, obtain a small square of lens paper (and ONLY lens paper) and gently wipe the microscope lenses directly across, in this order:

1. the lower surface of all the objective lenses
2. the ocular lens
3. the condenser lens and the light housing

Part I: Finding the Letter

Materials

- Microscope
- Lens paper
- Letter “E” slide
- Stage Micrometer Slide

Procedure

1. Always use one hand around the microscope arm and one hand under the microscope base.
2. Carry it in a vertical position without swinging, tipping, dropping or bumping the microscope.
3. Place the microscope gently on the lab bench with the arm toward you. Never place the microscope near the edge, and never slide it across the table.

Identify the following microscope parts with a partner. Check off each part as you go. If you are unsure about a component, consult your instructor.

- Eyepiece (ocular lens)
- Nosepiece Ring (turret)
- Objective Lenses (low, medium, high power)
- Stage
- Stage Controls
- Iris Diaphragm Condenser Lens
- Light Source
- Light intensity knob (rheostat)
- Coarse Focus Adjustment Knob
- Fine Focus Adjustment Knob

4. Carefully plug in and position electric cord to avoid tripping or having the microscope pulled off the table.
5. Turn on the microscope and rotate the **nosepiece ring (turret)** to snap the **10x objective** lens in place. Do not use the objective lens to rotate!
6. Turn the **light control (rheostat)** halfway to adjust the amount of light.
7. The total magnification you observe when looking through a microscope is the magnification of the ocular lens multiplied by the magnification of the objective lens. Fill out Table 5.1 to indicate the total magnification achieved by each lens.

Table 1. Total Magnification Achieved Using Various Objectives Lenses of a Compound Light Microscope

Lens Name	Objective Lens	Ocular Lens	Total Magnification

8. On the side of the microscope are two knobs, one on top of the other. The larger of the two knobs is the **coarse focus adjustment knob**. Turn the knob so that the stage goes down as far as it can.
9. Clean all lenses with lens paper. Never use paper towels or kimwipes or shirt!
10. Obtain a letter “e” slide from your instructor. Draw the “e” in Table 5.2 as you view it with your eyes (not through the microscope).

Table 2. The letter “e” viewed at different magnifications using a light microscope

Letter “e” as seen...	Drawing
with the naked eye	
100X total magnification	
400X total magnification	
1000X total magnification	
1000X total magnification	
In what direction does the “e” move (as you look into the microscope), when you move the stage to the right?	

11. Place the slide on the stage and secure it with the **stage clip**.
12. Use the coarse focus knob to move the stage as high as it can go.
13. Use **stage adjustment knobs** to center the “e” so that the light from the **light source** can pass through it.
14. Looking through the ocular lenses, lower the stage with the coarse focus adjustment knob until the “e” comes into view.
15. Use the **fine focus adjustment knob** to make the image as clear as you can.
16. At this point there are different adjustments you can make to improve the quality of the image:

17. The **rheostat** on the side of the microscope controls the intensity of the light. If it is too bright or dim at any time, use this knob to adjust the light.
18. The **condenser** will also adjust the light intensity. The condenser gathers and focuses the light to illuminate the specimen. Only use this if the rheostat failed to improve your image. Move the condenser with the condenser adjustment knob so that it touches the stage. Slowly lower it to improve lighting on your sample. Usually it should be about ½ inch below the stage.
19. The **iris diaphragm** adjusts the aperture of the opening and controls the amount of light that exits the condenser (or illuminates the specimen). You can open and close this aperture, for most purposes it should be fully open, but sometimes partially closing it will increase contrast in the image.
20. Draw the letter “e” as it appears through the microscope in Table 4-2. Note the change in orientation. Notice that the LEFT eyepiece can be rotated, but the ocular scale (known as a reticle) stays in the middle of the ocular lens. Note that the RIGHT eyepiece can be rotated, to move the position of the pointer.
21. Move the stage slowly to the right. Note what direction the “e” moves as you look through the microscope and record in Table 4-2.
22. Move the slide back to the left to re-center the “e”.
23. Once the “e” is re-centered and in focus, turn the nosepiece to the 40x objective lens and snap it into place. Use the fine focus to make the image clear. Only if needed, make light adjustments with the rheostat, condenser, or diaphragm. Draw everything you see through the microscope in Table 4-2.
24. Once the “e” is re-centered and in focus turn the nosepiece to the 40x objective lens and snap it into place. Use the fine focus to make the image clear (**NEVER** use the coarse focus at this or any higher magnification or you risk snapping the slide or worse, snapping the lens!!!) Only if needed, make any light adjustments with the rheostat, condenser, or diaphragm. Draw everything you see in the microscope in Table 4-2. Answer the questions below.
25. To use the oil immersion lens, rotate the nosepiece BETWEEN the 40x and the 100x lenses so that the wand containing the oil can reach the slide. Place a generous drop of oil on the slide and snap the 100x objective lens into place. The lens will slide into the drop of oil.
26. Use the fine focus to make the image clear. Only if needed, make light adjustments with the rheostat, condenser, or diaphragm. Draw what you see in Table 4-2.
27. **NEVER return to the 40X objective lens after there is oil on the slide**. If you are having trouble focusing using the oil immersion lens, you must go back and use the 10x lens to re-center (turn the nosepiece so that the 40x objective lens is NOT dragged through the oil on the slide). Then go directly back to the 100x lens. If this doesn’t work, the slide must be wiped clean and you should start over.

When finished with the slide, lower the stage and remove the slide. (Do not lower the stage if you are going to view a different slide). Clean the oil off the slide and return it to your instructor.

Part II: Diameter of the Field

Microscopes are for magnification of images too small to be seen with the naked eye. However, they can be used as a tool to estimate the size of the object being viewed. In order to do this, you must know the diameter of each viewing field with each objective lens. You can then estimate how much of the field your object takes in the field and compare this to the measured diameter. For example, let’s say the diameter of field using the 40x objective lens is 0.10 mm. You then view an object using that lens that takes up ¼ of the field of view. You can then estimate that object is ¼ (0.10mm) long or 0.025mm. To determine the field diameter, you will use a stage micrometer slide, which is basically a very fine ruler (usually 2 mm) that is etched onto a microscope slide.

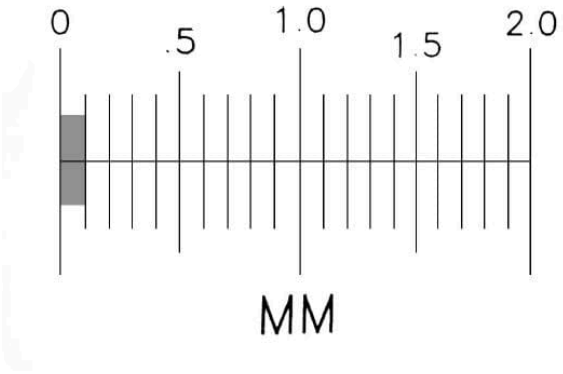


Figure 2. Stage micrometer

Procedure

1. Obtain a stage micrometer slide. BE VERY CAREFUL. A stage micrometer slide is costly, so please treat it with respect! Place the stage micrometer slide on the stage and focus on the millimeter markings using the 10x objective lens. Record the field diameter in mm when you use this lens in Table 3.
2. Switch to the 40x objective lens and focus. Determine the field diameter in mm with the micrometer and record in table 3. Repeat the same steps for the other objective lenses.
3. Convert the diameters to micrometers (µM). All cell and organelle measurements will be done in µM. Clean the slide carefully and place it back in its tray on the demo table.

Table 3. Determining the Field of View Using a Stage Micrometer

Lens Used	Total Magnification	Diameter of Field (mm)	Diameter of Field in (µm)

Part III: Making Wet Mounts

Materials

• Slides (in alcohol jar) • Cover slip (in alcohol jar) • Paper towels • Tray or Beaker to wash slides • Forceps • Transfer pipets • Knife and cutting boardToothpicks	• Onion • Iodine (dropper bottle) • Methylene blue (dropper bottle) • Elodea Leaf (in beaker of water) • Pond water (in beaker) • 20% salt water (dropper bottle) • Deionized water (dropper bottle)	**Prepared Slides:** • Paramecium • Spirogyra • Human Blood Smear • Human Sickle Cell Blood • Amphibian Blood Smear

Procedure

Human Cheek Cells

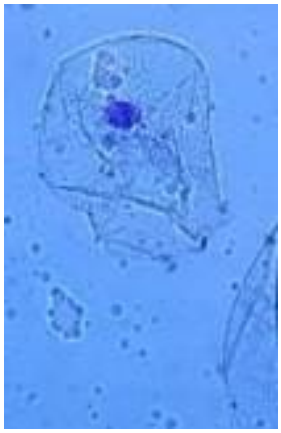


Figure 3. Human cheek cell at 400x zoom.

The human cheek is lined with **epithelial cells**. They will be used today for you to observe a eukaryotic animal cells and its nucleus. You will scrape and stain a sample of your cheek cells with the dye methylene blue. The dye will allow you to clearly stain the nuclei of the cells. Be careful with the dyes used for the wet mounts as they will stain your skin and clothes. Also, the slides and coverslips you will use are stored in alcohol. Make sure to dry off the slides and coverslips with paper towels (not the expensive lens paper) before preparing your wet mount slides.

1. Get a dry microscope slide and cover slip.
2. Put a drop of methylene blue on the slide.
3. Gently scrape the inside of your cheek with a toothpick and swirl it in the dye on the slide.
4. Place a cover slip on the suspension and view at 1000X total magnification
5. Draw 1-3 cells large enough to show the detail that you see in your lab manual. Label its **cell membrane, cytoplasm** and **nucleus.** Be sure to indicate the magnification used and specimen name. Also indicate the estimated cell size in micrometers under your drawing. See the example (which is missing the labels).

Elodea Leaf Wet Mount

The cell membrane is not visible on the *Elodea* leaf because of its proximity to the much thicker cell wall. In order to view the membrane, you will add salt to the *Elodea*. Water will flow out of the Elodea cells by osmosis, shrinking the cell membrane away from the stiff cell wall (plasmolysis).

1. Get a microscope slide. Place 2 drops of dI water on the left and 2 drops 20% salt on the right.
2. Obtain a leaf from a stalk of Elodea and cut the leaf in half. Place a half leaf in each solution.
3. Wait 3-5 minutes and then place a cover slip over each leaf (dab off excess water).
4. View at between 400X total magnification.
5. Look for cells that have undergone plasmolysis. If none are found, prepare the slide again.
6. Draw 2-3 connected cells large enough to show the detail that you see. Label the **cell wall, cell membrane, cytoplasm, and chloroplasts** in your lab manual. Be sure to indicate the magnification used and specimen name. Also indicate the estimated cell size in micrometers under your drawing.

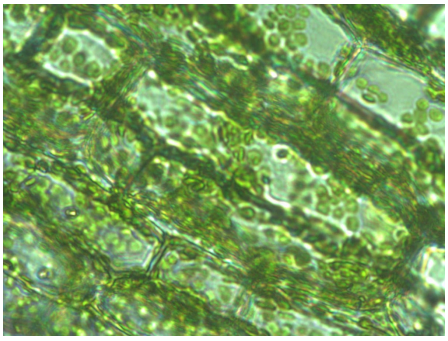


Figure 4. *Elodea* cells at 400x

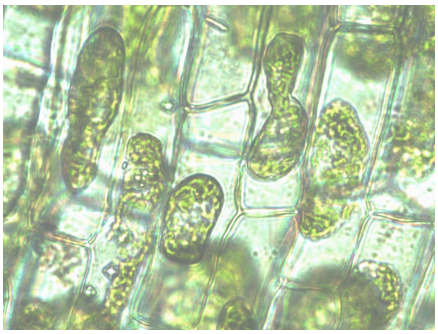


Figure 5. *Elodea* cells
undergoing plasmolysis at 400x

Onion Membrane Wet Mount

Onion bulbs are actually swollen leaves that form an underground structure. Although not a good source for viewing chloroplasts, they are an excellent source for viewing eukaryotic plant nuclei.

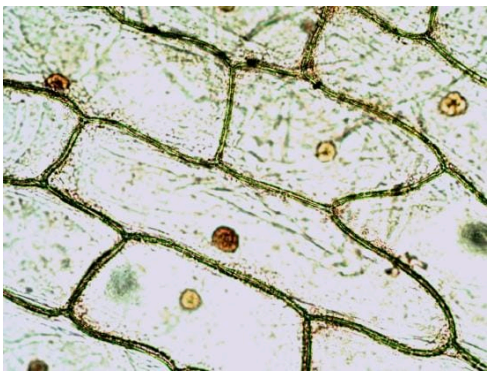


Figure 6. Onion cells at 400x

1. Get a dry microscope slide and cover slip.
2. Cut a tiny square of one layer of the onion. Use forceps to peel the thin, white, transparent membrane from the inner concave side of an onion section (you only need a small piece, about the size of a pencil eraser) and place on slide. Try to smooth out the transparent onion membrane as flat as possible.
3. Add a drop of iodine to the membrane and wait 30 seconds. Cover the membrane with a coverslip. Place the slide inside folded paper towel and pat gently for 1 second to remove excess dye.
4. View at either 100X or 400X total magnification, so that you can see 2-3 cells.
5. Draw 2-3 connected cells large enough to show the detail you see. Label the **cell wall, nucleus, and cytoplasm**. Be sure to indicate the magnification used and specimen name. Also indicate the estimated cell size in micrometers under your drawing.

Pond Water Wet Mount

You will prepare a wet mount of one of the following protists that can be found in pond water: *Euglena, Spirogyra, Paramecium*, and/or *Amoeba*.

1. Get a depression slide and dry it off. The depression slide has a curved indent in the middle of the slide, which allows the living creatures to move around and not get squished.
2. Put a drop of methyl cellulose and a drop from the pond water or cultures.
3. Carefully put on a coverslip. If you have too much liquid on the slide, then gently use a corner of a paper towel to absorb the excess liquid.
4. View at 100X or 400X total magnification.

6. Draw the organisms that you see. Be sure to indicate the magnification used and specimen name. Also indicate the estimated cell size in micrometers under your drawing.

Prepared Slides

You will look at various prepared slides including *Paramecium, Spirogyra*, Human Blood Smears, Human Sickle Cell Red Blood Smears, Frog Blood Smears, and possibly others. View under the microscope using the highest magnification for the best cellular details and draw what you see. Be sure to indicate the magnification used and specimen name. Also, indicate the estimated cell size in micrometers under your drawing.

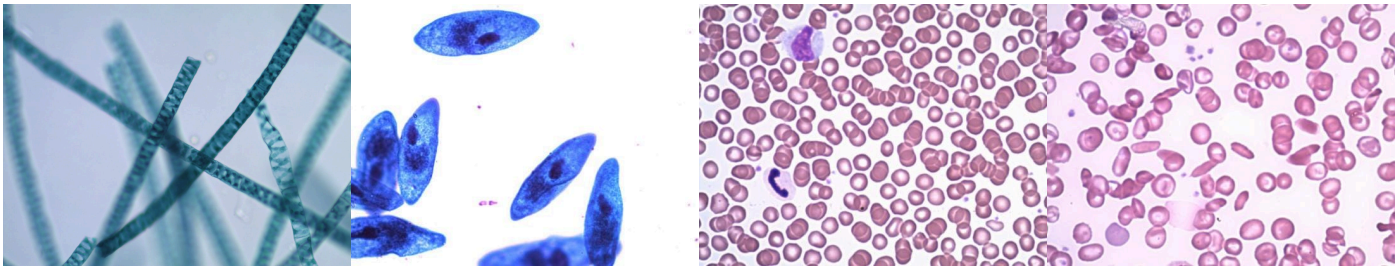


Figure 7. Specimens at 400x Magnification.From left to right: *Spirogyra*, *Paramecium*, Human Blood Smear, Human Sickle Cell.

Part IV: Lab Check-out

Check off each task when complete. The instructor must sign before storing your microscope.

Returning Compound Microscope

- Rotate low power objective lens in position
- Use coarse focus to raise nosepiece to the top
- Remove slide from stage
- Be sure that the bar from stage clips does not stick out
- Turn rheostat to lowest before turning off the light
- Unplug and wrap power cord according to instructor's instructions
- Carry microscope properly to cabinet and return to the correct shelf

Cleaning Wet Mounts

- Rinse slide in a beaker of water, remove and return cover slip and slide into the correct jars
- Put prepared slides back onto the correct tray on the demo table
- Tighten all reagent bottle caps.
- Clean up the demo table
- Wipe off all tables with wet sponge

Instructor Signature

Study Questions

1. On the next page, label the parts of the microscope and list their function.
2. How is a microscope properly carried?
3. How is the microscope properly put away?
4. What is the magnification power of:
    - High-power objective lens?
    - Medium-power objective lens?
    - Low-power objective lens?
    - Ocular lens?
5. How is the total magnification of a specimen determined?
6. When the magnification increases, how does the size of the field of view change?
7. Why is it important to place the medium on a slide before selecting the specimen to be mounted?
8. Name one way to be sure the specimen will be found in the field of view when you change magnification.
9. What are the distinguishing characteristics of a plant cell versus an animal cell?
10. Knowing the size of the field of view using one of the three magnification lenses, be able to determine the size of a specimen being observed.



Figure 8. Blank microscope to label parts.

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2.2.6: Spectrophotometry

Learning Objectives

Goals:

- Identify the main features on the spectrophotometer and define their functions.
- Use a spectrophotometer to obtain an absorbance spectrum.

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Identify the parts of a spectrophotometer and their related functions.
- “Blank” a spectrophotometer.
- Obtain an absorbance spectrum for a molecule.
- Use the wavelength absorption scans to determine the dyes in colored skittles.

Introduction

Spectrophotometers are one of the most frequently used tools by scientists to determine both the presence and concentration of dissolved chemicals. As radiant energy (visible light) strikes matter, molecules will absorb certain wavelengths of light and transmit or reflect others based on the nature of their chemical bonds. For example, proteins and nucleic acids absorb wavelengths in the visible light range of 240-300 nanometers (nm), pigments and dyes absorb light in the 400-770-nm range, and other organic molecules absorb wavelengths above 770-nm. Each chemical has a distinctive atomic arrangement and bonding pattern, and thus absorbs or transmits different wavelengths of visible light in a pattern that is unique for that chemical. This unique pattern of light absorption and transmittance creates a “fingerprint” for that chemical. In this exercise you will determine the unique “fingerprint” for a colored molecule and use a spectrophotometer to measure the concentration of a chemical in a given sample.

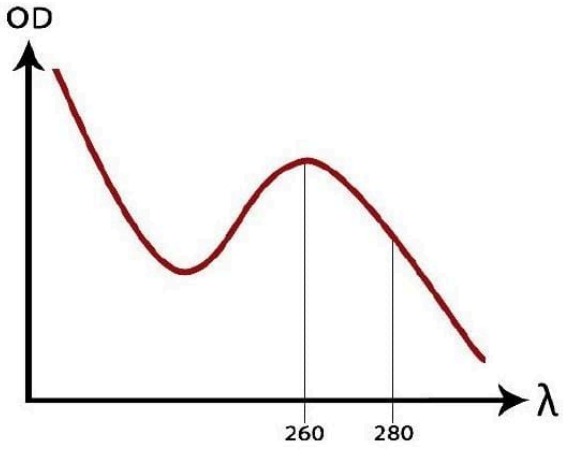


Figure 1: Curve of light absorbance and transmission

Spectrophotometers are instruments designed to detect the amount of light energy that is absorbed or transmitted by molecules dissolved in a solution. Since molecules have wavelengths unique to their structure, different chemicals and their concentrations can be identified based on their absorbance or transmittance.

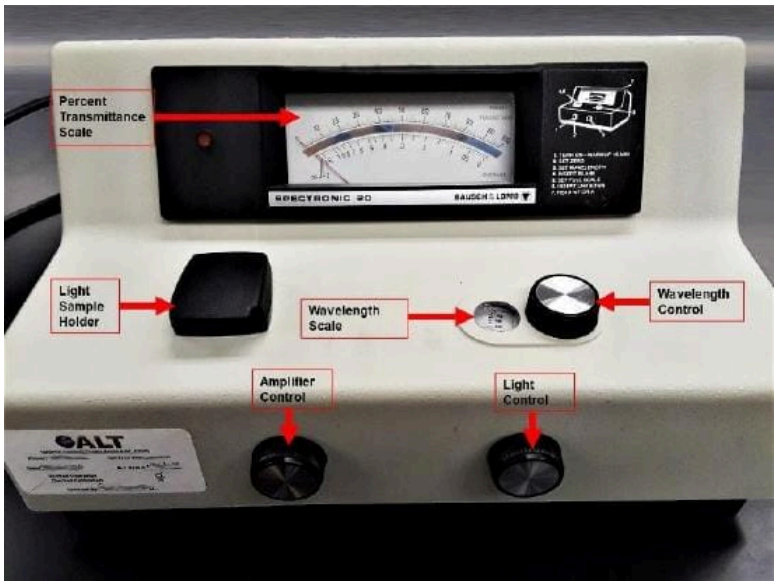


Figure 2. Labeled parts of a spectrophotometer

A spectrophotometer is an instrument used for detecting the presence of any light-absorbing particles dissolved in a solution and for measuring the concentration of those particles. A light source inside the spectrophotometer emits a full spectrum of white light towards a compartment where a sample liquid is placed. The samples are prepared in cuvettes that are made using specialized plastics or quartz so that they do not absorb any light and will not affect our measurements. Before the light passes through the sample in the cuvette, an adjustable prism and diffraction grating filters the light so that only a single wavelength of light can be selected and allowed to pass through the sample. All molecules differ in how strongly they absorb each wavelength of light in the visible spectrum because of differences in their molecular structure and composition. This allows us to use a specific wavelength of light to detect the presence of, and quantify, one molecular compound in a simple or complex liquid mixture. Spectrophotometers are also calibrated by using a "blank" solution that we prepare containing all of the components of the solution to be analyzed except for the one compound we are testing for so that the instrument can zero out these background readings and only report values for the compound of interest.

Light passing through a sample solution will partially be absorbed by molecules present in the sample. The amount of light unable to pass through a sample is measured as the absorbance value. Absorbance is directly proportional to the concentration of the molecules and is measured on a logarithmic scale from 0 to infinity. The amount of light that is not absorbed is transmitted or passed through the sample. Compared to the amount of light entering the sample, the amount that exits is measured as a percentage of the light transmitted. Percent transmittance is inversely proportional to the concentration of the molecules in the sample and is measured on a linear scale from 0% to 100%.

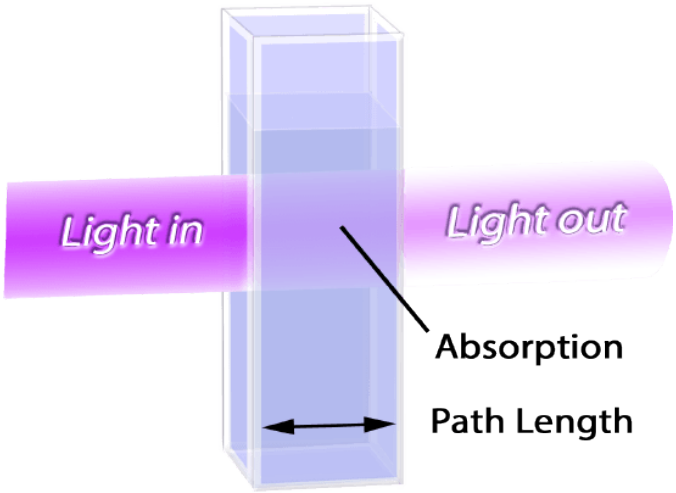


Figure 3: Light is absorbed and transmitted through the spectrophotometer cuvette and solution

A photodetector on the other side of the sample compartment converts the intensity of the light it receives into an electrical signal. The instrument can then calculate and display the absorbance and % transmittance values by measuring the difference between the intensity of light of the selected wavelength entering and exiting the sample.

The absorbance scale reflects the measurement of the amount of light absorbed and converted into absorbance (A) units by the spectrophotometer. Absorbance units are calculated by using the following equation:

Equation 1

$$\text{Absorbance}(A) = \log_{10}\left(\frac{1}{T}\right)$$

where "T" is the decimal form of "$\%T$"

$$T = \frac{\%T}{100}$$

Example

If a solution containing a given dye is found to transmit 10% of the light when placed in a spectrophotometer, its absorbance then would be calculated as follows:

Transmittance (T) = 10% = 10%/100 = 0.10

Absorbance (A) = log10 (1 / 0.10) = 1.0

Part I: Identifying Food Dyes in Candies

Many foods, drugs and cosmetics are artificially colored with federally approved food dyes (FD & C dyes). These dyes include Red 40, Red 3, Yellow 5, Yellow 6, Blue 1, and Blue 2. Since each dye has an identifiable absorption spectrum and peak, a spectrophotometer may be used to identify the types of FD & C dye used in a product.

Pigments may be extracted from foods and drinks that contain one or more of these dyes. An absorption spectrum of that extract can then determine what dyes are in that food or drink by comparing the peaks of maximum absorbance with information in the table below. If the absorption spectrum of a food extract has a peak at 630 nm and one at 428 nm, you can assume the food contains both Blue #1 and Yellow #5. The following table gives the wavelength of peak absorbance for each of these dyes.

Table 1. Wavelength of Maximum Absorbance of Commonly Used FD & C Dyes

FD & C Dye	Name	Wavelength (nm) of Maximum Absorbance
Blue #1	Brilliant Blue FCF	630
Green #3	Solid Green FCF	625
Blue #2	Indigo Carmine	610
Red #3	Erythrosine	527
Red #40	Allura Red AC	502
Yellow #6	Sunset Yellow FCF	484
Yellow #5	Tartrazine	428

Materials

- Spectrophotometer

- Cuvette
- Skittles
- KimWipes
- Test tubes

Procedure

A. Extracting Dye from Candy (Your Instructor will do this for you)

You will need one test tube and one cuvette for each color to be tested. Measure 4 mL water into one tube. Place 2-4 candies of the same color in a test tube with the water. Gently swirl, and wait one minute. After, pour approximately 1 mL of liquid into a microcentrifuge tube. Spin the microcentrifuge tube at max speed for 60 seconds. Make sure the centrifuge is balanced before spinning. Transfer the clear liquid (supernatant) into a cuvette. Make sure to leave behind the particulates (pellet).

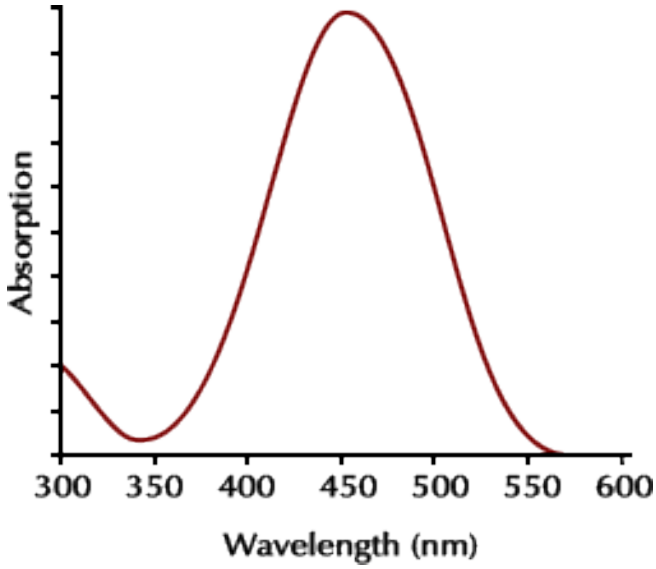


Figure 4. Curve of absorbance

B. Measuring Absorbance with Spectrophotometer

1. Turn on the spectrophotometer. Let it warm up for 15 minutes.
2. Select wavelength scan.
3. Fill a cuvette 2/3 full with DI water to serve as the “BLANK” cuvette.
4. Calibrate the Spectrometer

1. 1. Wipe the outside of the BLANK cuvette with a KimWipe
   2. Place cuvette into the machine so that the clear portions of the cuvette are oriented left to right. (The light needs to pass through clear area on cuvette).
   3. Press "Zero"
   4. Remove the blank.

5. Determine optimum wavelength absorbance and set up data collection mode.

1. 1. Place a cuvette with a sample into the spectrophotometer
   2. Press "read"
   3. Set cursor mode to peak and valley under "Options" > "More" > "Cursor Mode"
   4. Press the left and right arrows to the right of the graph to select the highest peak.
   5. Record the wavelength and absorbance in Table 6.2.

6. Decide which dyes were used to make each color. Enter the Wavelength of that dye in the last column of table 6.2. Explain

your reasoning for each choice in your lab notebook.

Results

Table 2.

Color skittle	Maximum Absorbance	Wavelength (nm) at Maximum Absorption	Proposed Dye	Wavelength (nm) of Maximum Absorption for Proposed Dye
Red				
Orange				
Yellow				
Green				
Purple				

Instructions for Cleaning Cuvettes

1. Discard solutions to sink
2. Rinse with tap water once
3. Rinse with DI water two times
4. Place in tube rack, allow to air dry

Study Questions

1. Name the parts of the spectrophotometer and identify their function.
2. What is the difference between % transmittance and absorbance?
3. How did you determine which wavelength was absorbed at the highest level? How is this process useful in determining the identity of a molecule?
4. How do you clean a cuvette?

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2.2.7: pH and Buffers

Learning Objectives

Goals:

- Accurately measure the pH of solutions using pH indicator strips and a pH meter.
- Create buffer solutions and test the effects of adding acid and base to each.

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Describe the pH scale.
- Correctly use pH indicator strips and a pH meter.
- Explain the function and composition of a buffer.

Introduction

The pH of solutions is an important characteristic. Cells must maintain a constant pH so that the enzymes and processes taking place inside the cells will continue as needed. Chemical and enzymatic reactions are typically dependent on a specific pH range. Thus, it is important to understand pH and be able to determine the pH of various solutions.



Figure 1. The pH meter

The pH scale is a familiar concept for students who study science. The pH value of a solution reflects the relative concentration of hydrogen ions (H+) or protons to the concentration of hydroxide ions (OH-) in a solution. Solutions with a pH value less than 7 are acidic and those with a value greater than 7 are basic, or alkaline. The value 7 is neutral meaning the amount of H+ in a solution is equal to the amount of OH- in a solution. Pure water H_2O, which can dissociate naturally into H^+ and OH^- ions, would have a value of 7.

Equation 1

$$H_2O \rightleftharpoons H^+ + OH^-$$

Table 1. The pH Scale

[H+] in mol/L	pH	[OH-] in mol/L	pH Classification
1.0	**0**	10^{-14}	**Acidic**
0.1	**1**	10^{-13}	**Acidic**
0.01	**2**	10^{-12}	**Acidic**
0.001	**3**	10^{-11}	**Acidic**
10^{-4}	**4**	10^{-10}	**Acidic**
10^{-5}	**5**	10^{-9}	**Acidic**
10^{-6}	**6**	10^{-8}	**Acidic**
10^{-7}	**7**	10^{-7}	**Neutral**
10^{-8}	**8**	10^{-6}	**Basic**
10^{-9}	**9**	10^{-5}	**Basic**
10^{-10}	**10**	10^{-4}	**Basic**
10^{-11}	**11**	0.001	**Basic**
10^{-12}	**12**	0.01	**Basic**
10^{-13}	**13**	0.1	**Basic**

[H+] in mol/L	pH	[OH-] in mol/L	pH Classification
10^{-14}	**14**	1.0	**Basic**

Pre-lab Reading Assignment:

Chemistry Review

In a chemical equation, variables that are surrounded by brackets "[" and "]" are expressions of concentration, or the specific amount of a molecule in a given volume of solution. For example, if you see "[H+]" in an equation, this is read as "the concentration of hydrogen ion".

The concentration of a solution is often expressed in units of moles per liter (mol/L). Just as one "dozen" represents a quantity of 12 items, one "mole" represents a quantity of approximately 6.022 X 10^{23} items.

one dozen molecules = 12 molecules

one mole of molecules = 602,200,000,000,000,000,000,000 molecules!

Note: "***n***" is used in equations to indicate a quantity measured in moles. For example if you see "n_{Acid}" in an equation, this is read as "moles of acid".

The term "Molarity" indicates that a solution's concentration is in units of moles per liter. A one molar solution (1 M) contains one mole of solute within each liter of that solution. Reagents used in the laboratory will often be labeled with their concentrations expressed in terms of molarity.

The relative concentration of H+ or OH- may change very dramatically in solutions, so a logarithmic scale (called pH) instead of a linear scale is used to express concentration. Equations 2 and 3 can be used to calculate the pH based on hydrogen ion concentration or vice versa.

Equation 2

To calculate pH based on hydrogen ion concentration $[H^+]$:

$$\mathbf{pH = -\log [H^+]}$$

Equation 3

To calculate hydrogen ion concentration $[H^+]$ based on pH:

$$\mathbf{[H^+] = 10^{-pH}}$$

Buffers

A buffer is a mixture of a weak acid (HA) and its salt (e.g., NaA), and is sometimes referred to as a **conjugate acid-base pair**. As mentioned above, buffers have a major role in stabilizing the pH of living systems. Vertebrate organisms maintain the pH of blood using a buffer composed of a mixture of carbonic acid (H_2CO_3) and sodium bicarbonate ($Na^+HCO_3^-$). The weak acid in this buffer

is carbonic acid and the salt is sodium bicarbonate. When dissolved in water, sodium bicarbonate disassociates completely into sodium ions (Na^+) and bicarbonate ions (HCO_3^-). The H_2CO_3 is the conjugate acid of HCO_3^- and the HCO_3^- is the conjugate base of H_2CO_3 . Together, this conjugate acid-base pair functions as the bicarbonate buffer system.

Buffer systems are also of particular importance to experimental cell biology.

The pH of a buffer solution may be calculated as follows:

Equation 4

The pH of a buffer solution may be calculated as follows:

$$pH = pK_a + log\frac{n_A}{n_{HA}}$$

Where pK_a = dissociation constant of the acid, n_A = initial number of moles of salt in the buffer, and n_{HA} = initial number of moles of acid in the buffer.

If you know these values, it is possible to accurately calculate the pH of a buffer system before you create it!

The pK_a of acetic acid (used in today's experiment) is 4.75

Equation 5

To find the volume of the conjugate base or conjugate acid:

$$\boldsymbol{n_A} = \textit{volume of conjugate base (mL)} \times \frac{1\ L}{100\ mL} \times \textit{concentration of conjugate base (mol/L)}$$

$$\boldsymbol{n_{HA}} = \textit{volume of conjugate acid (mL)} \times \frac{1\ L}{100\ mL} \times \textit{concentration of conjugate acid (mol/L)}$$

Use of pH Indicator Strips

The pH of a solution can be roughly approximated using strips of paper treated with color changing indicator reagents. The strips are dipped into the solution to be tested for several seconds and then removed. The color of the indicator strip is then compared to a reference chart, often printed on the side of the strip's container. The reference color on the chart that most closely matches the color of the reacted strip will have a pH value printed below it and that will be the approximate pH. One advantage to using pH indicator strips is that they are relatively inexpensive, easy to use, and are adequate for determining pH where an error of +/- 1 pH unit is acceptable. A more accurate method of determining pH is to use a calibrated pH meter, which can determine the exact pH to one or more decimal places depending on the quality of the device.

Use of a pH Meter

The pH meter measures the acidity of a solution. It is a scientific instrument that uses electrodes to measure the hydrogen ion (proton) concentration of water-based solutions. Essentially, the pH meter is a voltmeter that will measure the difference between two electrodes. The probe you place into the solution contains a reference electrode and a detector electrode. The reference electrode is not affected by the solution being measured and is in contact with a solution of potassium chloride. The detector electrode comes in contact with the test solution. The hydrogen ions in the test solution interact with the electrode and the difference in electrical potential between the two electrodes is detected and reported as millivolts or converted to a pH value.

For accurate measurements, it is important to calibrate your pH meter before use with buffer solutions of known values. It is best to calibrate your meter with buffer solutions that are near the anticipated or desired pH of your test solution. You should also blot the probe with laboratory wipes in between solutions to avoid contamination but avoid rubbing. Rubbing the probe may cause a

static electricity charge to build up on the electrode which will cause inaccurate readings to occur. Accidentally letting the probe dry out will also cause it to stop working so always keep the end of the probe immersed in a holding solution when not taking measurements. Remember to return it to the storage solution as soon you are finished with the experiment.

Calibrate the pH meter for pH 4, 7, and 10 before taking measurements. If calibrated properly, your pH meter should produce measurements with an accuracy of +/- 0.06 pH units. Always test your meter after calibration using the standard buffers and recalibrate the meter if necessary before proceeding.

Your instructor will demonstrate the proper calibration, care, and use of the meter. Be sure to take good notes!

Activity 1: Measuring pH

Materials

Per group of 4:

- 1 Set of 4 unknown solutions (in 30 mL tubes with screw top lids)
- 1 container of pH indicator strips and color reference chart
- 1 pair of forceps
- 1 pH meter (calibrated – See instructor for directions)

Procedure

1. Obtain a set of unknown solutions from instructor.
2. Measure the pH of each solution using the pH indicator strips first. Hold the strips with the forceps. **Use a new strip for each solution!**
3. Record your data in Table 1.
4. Measure the pH of each solution using the pH meter. **Be sure to rinse the tip of the probe with DI water before putting the probe into each sample! (Ask the instructor for instructions if you are not sure how to properly calibrate and use the pH meter).**
5. Record your data in Table 2.

Table 2. Measured pH values of Known Test Solutions

Unknown Solution	pH value measured using indicator strips	pH value measured using pH meter	Expected pH value (Ask Instructor)
A			
B			
C			
D			

Data Analysis

- How do your pH indicator strip values compare to your pH meter values?
- Check your measured pH values with those of the other teams. Are your values similar?

- Check with your instructor to see what the actual pH values should be. How accurate were you?

Activity 2: Preparation of an Acetate Buffer

Materials

Per Class:

- 1 bottle stock solution of 0.1 M acetic acid (CH_3COOH)
- 1 bottle stock solution of 0.1 M sodium acetate ($Na^+CH_3COO^-$)

Per Group of 4:

- 6 clean 30 mL plastic tubes
- 2 clean 5 mL serological pipettes
- 2 pipette pumps (10 mL capacity)
- 1 Sharpie Marker

Procedure

1. Using a sharpie marker, label the two 30 mL tubes - one as “Acetic Acid” and the other “Sodium Acetate”. Fill each tube up with the correct stock solution.
2. Using a sharpie marker, label each of the two 5 ml pipettes - one as “AA” and the other as “SA”. To avoid contamination, DO NOT dip pipettes into stock solution bottles and ONLY use the designated pipette to transfer either acetic acid or sodium acetate from your group’s labeled tubes.
3. Using a sharpie marker, label a clean 30 mL tube as “Buffer 1”, another as “Buffer 2”, the third as “Buffer 3”, and the fourth as “H2O”. Each student in your group will take one tube. If there are only 3 students, one of you can also take the “H2O” tube. Write your names into the first column of table 2 next to the tube(s) you will be working with.
4. Create the acetate buffers using your marked serological pipettes and the specified volumes of acetic acid and sodium acetate in Table 2.
    - **Be sure to accurately pipet the volumes indicated to get good results! Review proper pipetting technique with your instructor if necessary.**
    - For the “H2O” tube, simply fill the tube about a third full with pure deionized water
5. Close the lids and gently shake each tube for about 20 seconds or more to mix the contents.
6. Measure the pH of each solution with the pH meter using proper technique and enter your measurements in table 2.

Activity 3: Effects of Adding Acid and Base to Acetate Buffer

Materials

Per Group of 4:

- **Everything From Activity (2 above)**
- 30 mL dropper bottle of 0.1 M HCl (Hydrochloric Acid)
- 30 mL dropper bottle of 0.1 M NaOH (Sodium Hydroxide)

Procedure

1. Add a single drop of HCl to each of your team’s 4 tubes. Close the lids and gently shake the tubes to thoroughly mix the contents.
2. Measure the pH of each solution and enter the pH values in table 4.
3. Continue adding drops of HCl according to the table, measuring pH, and recording values.
4. When you have completed Table 3, you will now start adding drops of NaOH (base) to your tubes according to table 5.
5. Be sure to shake the tubes to mix the contents thoroughly before measuring pH and entering the values in Table 5.

6. Look at your results and compare the pH changes in your 4 tubes. What do you notice about the pH changes when you compare them?
7. Compare your pH values to those of the other teams. Ask your instructor for the expected values.

Table 3. Experimental Acetate Buffers Mixing Chart

Student	Tube	Volume of Acetic Acid (mL)	Volume of Sodium Acetate (mL)	Measured pH	Expected pH (Ask Instructor)
	Buffer 1	5.0	5.0		
	Buffer 2	7.0	3.0		
	Buffer 3	3.0	7.0		
	H_2O	None	None		

Table 4. Effect of Adding 0.1 M HCl (acid) to Acetate Buffers and Water

Tube	pH after 1 drop HCl added	pH after 2 drops HCl added	pH after 3 drops HCl added
Buffer 1			
Buffer 2			
Buffer 3			
H_2O			

Table 4. Effect of Adding 0.1 M NaOH (base) to Acetate Buffers and Water

Tube	pH after 1 drop NaOH added	pH after 2 drops NaOH added	pH after 3 drops NaOH added
Buffer 1			
Buffer 2			
Buffer 3			

Tube	pH after 1 drop NaOH added	pH after 2 drops NaOH added	pH after 3 drops NaOH added
H_2O			

Study Questions

1. What range of pH values indicates that a solution is acidic? Basic?
2. In general, how does the relative concentration of hydrogen ions [H+] compare to that of hydroxide ions [OH-] in a neutral, acidic, and basic solution?
3. Based on your observations, how would you describe what a buffer does?
4. What factors determine the accuracy of a reading with a pH meter?
5. What is the pH of a solution that has a hydrogen ion concentration of 2.46×10^{-5} M?
6. What is the expected pH of a buffer made from 25.7 mL of 2.0 M Acetic acid and 0.0492 L of 0.90-M Sodium acetate?

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2.2.8: Serial Dilutions and Standard Curve

Learning Objectives

Goals:

- Prepare solutions starting with a solid.
- Perform a serial dilution.
- Use the spectrophotometer to measure the absorbance of solutions.
- Generate a standard curve and use the standard curve to determine the concentration of a solution.

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Determine the mass of solute needed to make at %(w/v) solution.
- Make a buffer of the appropriate concentration.
- Make a stock solution of the appropriate concentration.
- Create a series of solutions of decreasing concentrations via serial dilutions.
- Use the spectrophotometer to measure the absorbance of a solution.
- Use excel and make a standard curve and use the R2 value to evaluate the quality of the standard curve.
- Use the standard curve to calculate the concentration of a solution.

Introduction

A Serial dilution is a series of dilutions, with the dilution factor staying the same for each step. The **concentration factor** is the initial volume divided by the final solution volume. The **dilution factor** is the inverse of the concentration factor. For example, if you take 1 part of a sample and add 9 parts of water (solvent), then you have made a 1:10 dilution; this has a concentration of 1/10th (0.1) of the original and a dilution factor of 10. These dilutions are often used to determine the approximate concentration of an enzyme (or molecule) to be quantified in an assay. Serial dilutions allow for small aliquots to be diluted instead of wasting large quantities of materials, are cost-effective, and are easy to prepare.

Equation 1.

$$concentration factor = \frac{volume_{initial}}{volume_{final}}$$

$$dilution factor = \frac{1}{concentration factor}$$

Answer available at the end of this section.

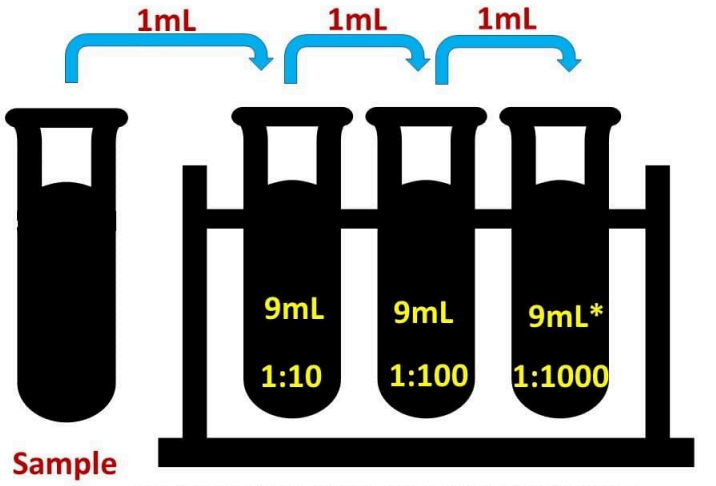


Figure 1. A ten-fold serial dilution, which can also be called a 1:10 dilution, or a series with dilution factor of 10. To determine the concentration at each step of the series, you divide the previous concentration by the dilution factor.
*Dilution tubes begin with 9-mL. 1-mL is added and mixed, then 1-mL is transferred to the next tube. The ending volume in the last tube would be 10-mL

Key considerations when making solutions:

- Make sure to always research the precautions to use when working with specific chemicals.
- Be sure you are using the right form of the chemical for the calculations. Some chemicals come as **hydrates,** meaning that those compounds contain chemically bound water. Others come as **"anhydrous"** which means that there is no bound water. Be sure to pay attention to which one you are using. For example, anhydrous $CaCl_2$ has a MW of 111.0 g, while the dehydrate form, $CaCl_2 \bullet 2\ H_2O$ has a MW of 147.0 grams (110.0 g + the weight of two waters, 18.0 grams each).
- Always use a graduate cylinder to measure out the amount of water for a solution, use the smallest size of graduated cylinder that will accommodate the entire solution. For example, if you need to make 50 mL of a solution, it is preferable to use a 50 mL graduate cylinder, but a 100 mL cylinder can be used if necessary.
- If using a magnetic stir bar, be sure that it is clean. Do not handle the magnetic stir bar with your bare hands. You may want to wash the stir bar with dishwashing detergent, followed by a complete rinse in deionized water to ensure that the stir bar is clean.
- For a 500 mL solution, start by dissolving the solids in about 400 mL deionized water (usually about 75% of the final volume) in a beaker that has a magnetic stir bar. Then transfer the solution to a 500 mL graduated cylinder and bring the volume to 500 mL
- The term "bring to volume" (btv) or "quantity sufficient" (qs) means adding water to a solution you are preparing until it reaches the desired total volume
- If you need to pH the solution, do so BEFORE you bring up the volume to the final volume. If the pH of the solution is lower than the desired pH, then a strong base (often NaOH) is added to raise the pH. If the pH is above the desired pH, then a strong acid (often HCl) is added to lower the pH. If your pH is very far from the desired pH, use higher molarity acids or base. Conversely, if you are close to the desired pH, use low molarity acids or bases (like 0.5M HCl). A demonstration will be shown in class for how to use and calibrate the pH meter.
- Label the bottle with the solution with the following information:
  - Your initials
  - The name of the solution (include concentrations)
  - The date of preparation
  - Storage temperature (if you know)
  - Label hazards (if there are any)

Lab Math: Making Percent Solutions

Equation 2.

Formula for weight percent (w/v):

$$\frac{\text{Mass of solute (g)}}{\text{Volume of solution (mL)}} \times 100$$

Example

Make 500 mL of a 5% (w/v) sucrose solution, given dry sucrose.

1. Write a fraction for the concentration

$$5\,\%\,(\frac{w}{v}) = \frac{5\ g\ sucrose}{100\ mL\ solution}$$

2. Set up a proportion

$$\frac{5\ g\ sucrose}{100\ mL\ solution} = \frac{?\ g\ sucrose}{500\ mL\ solution}$$

3. Solve for g sucrose

$$\frac{5\ g\ sucrose}{100\ mL\ solution} \times 500\ mL\ solution = 25\ g\ sucrose$$

4. Add 25-g dry NaCl into a 500 ml graduated cylinder with enough DI water to dissolve the NaCl, then transfer to a graduated cylinder and fill up to 500 mL total solution.

Activity 1: Calculating the Amount of Solute and Solvent

Calculate the amount (include units) of **solute** and **solvent** needed to make each solution.

A. Solutions with Soluble Solute and water as the solvent

1. How many grams of dry NaCl should be used to make 100 mL of 15% (W/V) NaCl solution?
2. How many grams of dry NaCl should be used to make 300 mL of 6% (W/V) NaCl solution?
3. How many grams of dry NaCl should be used to make 2L of 12% (W/V) NaCl solution?
4. How many grams of dry NaCl should be used to make 300 mL of 25% (W/V) NaCl solution?
5. How many grams of dry NaCl should be used to make 250 mL of 14% (W/V) NaCl solution?

B. Solutions with Insoluble Solutes in Cold Water

1. Calculate how to prepare 200 mL 1.2% (w/v) agarose in 1X SB buffer, given dry agarose and SB buffer.
2. Calculate how to prepare 300 mL 2.5 % (w/v) agarose in 1X SB buffer, given dry agarose and SB buffer.
3. Calculate how to prepare 50 mL 1.5 % (w/v) agarose in 1X SB buffer, given dry agarose and SB buffer.
4. Calculate how to prepare 60 mL 0.8 % (w/v) agarose in 1X SB buffer, given dry agarose and SB buffer.
5. Calculate how to prepare 150 mL 1.8 % (w/v) agarose in 1X SB buffer, given dry agarose and SB buffer.

Note

For dry chemicals that cannot dissolve in cold water (such as agarose and gelatin), pour the dry solute directly into an Erlenmeyer flask, measure the total volume of solvent in a graduated cylinder, then add the total volume of solvent into flask. Microwave the solution as recommended until solute is dissolved.

Part I: Solution Prep of 30-mLs of 13.6% Sodium Acetate

Sodium Acetate Buffer solutions are inexpensive and ideal to practice your skills. Your accuracy can be verified by taking a pH reading.

MATERIALS

Reagents

- Sodium Acetate (Trihydrate) solid
- DI H2O
- Stock bottle of verified 1 Molar Acetic Acid solution

Equipment

- pH meter
- Stir plate
- Electronic balance and weigh boats
- 50-mL graduated cylinder
- 50-mL conical tubes (Falcon tubes)
- P-1000 Micropipettes with disposable tips (or 5 mL Serological pipettes with pumps)

Calculations

- Calculate the amount of sodium acetate needed to make 30 mL of 13.6% sodium acetate solution.

Procedure

1. **Make sure to wear goggles and gloves.**
2. Measure _____ g of solid sodium acetate in a weigh boat on an electronic balance.
3. Transfer the sodium acetate into a 50 mL conical tube.
4. Add about 20 mL of DI water into the conical tube.
5. Secure the cap on the tube and invert to mix the contents until the solute is completely dissolved.
6. Pour out all of the solution into a 50 mL graduated cylinder.
7. Add DI water to bring the total volume to 30.0 mL.
8. Transfer all of the solution back into your 50 mL conical tube and secure the cap.
9. Invert the tube several times to thoroughly mix the contents.
10. Label the tube with contents (13.6% Sodium Acetate), initial, and date.

Verify your work by creating a buffer solution

1. Pipette exactly 5.0 mL of your sodium acetate solution into a clean 15 mL conical tube (or 25 mL glass test tube).
2. Pipette exactly 5.0 mL of 1M acetic acid solution into your conical tube (or 25 mL glass test tube).
3. Secure the cap on the conical tube (or a piece of parafilm over the test tube opening).
4. Invert several times to thoroughly mix the 10 mL of solution into an acetate buffer.
5. Measure the pH of the test buffer solution using a calibrated pH meter.
6. If you were accurate in all of your work, the test buffer should have a pH of 4.75 (+/- 0.06).
7. Check in with your instructor and report the pH of your test buffer.

8. **If your test buffer pH is within the expected range**, then congratulations! You have verified that the sodium acetate solution you made earlier has a concentration of 13.6%. Give your 50 mL tube of remaining sodium acetate solution to your instructor to save for use in a future lab.
9. **If your test buffer pH is far outside of the expected range** then something went wrong during the preparation of your sodium acetate solution and you should mark the tube with an "X" and give it your instructor to set aside.

Part II: Preparation of a Standard Curve

In this part of the lab, we will be preparing solutions of known concentrations. These then will be used to create a standard curve. Standard curves (also known as calibration curves) represent the relationship between two quantities. The standard curve will be used in part 3 of the lab to determine the concentrations of unknown solutions of methylene blue.

Materials

Reagents

- Stock 1% (w/v) methylene blue solution – (500 microliter (µL) aliquots in 1.5 mL microcentrifuge tubes)
- DI H_2O

Equipment

- P-20 Micropipettes and disposable tips
- P-1000 Micropipettes and disposable tips
- Spectrophotometer

Glassware

- 10 mL serological pipettes and pumps
- 1.5 mL microcentrifuge tubes
- 15 mL plastic conical tubes with screw-top caps
- 50 mL plastic conical tubes with screw-top caps

Calculations

1. Calculate the volume of stock 1% methylene blue solution needed to make 40 mL of 0.0005 % methylene blue solution.
2. This new percentage concentration is equivalent to **5.0 micrograms per milliliter (µg/mL)** and will be the concentration of our working solution for the next 2 parts of the lab exercise.

Procedure

Prepare Stock Solution of Methylene Blue

Prepare 40 mL of 5.0 µg/mL Methylene Blue Working Solution

1. **Make sure to wear goggles and gloves.**
2. Very accurately pipette 40.0 mL of DI water into a 50 mL conical tube.
3. Very accurately micropipette _____ µL of 1% stock methylene blue into the DI water in your tube.
4. Secure the cap on the tube and invert repeatedly to thoroughly mix the solution.
5. Label your tube as "5.0 µg/mL Methylene Blue", your name, and date.

Prepare Known Concentrations of Methylene Blue Working Solution via Dilution

Prepare 80% Methylene Blue Working Solution

1. Pipette 8.0 mL of 5.0 µg/mL methylene blue working solution into a 15 mL conical tube.

2. Pipette 2.0 mL DI H2O into the tube to make 10.0 mL of total solution.
3. Seal the tube and invert repeatedly to mix.
4. What is the concentration of your new solution? Label the tube _____ µg/mL methylene blue.

Prepare 60% Methylene Blue Working Solution

1. Pipette 6.0 mL of 5.0 µg/mL methylene blue working solution into a 15 mL conical tube.
2. Pipette 4.0 mL DI H2O into the tube to make 10.0 mL of total solution.
3. Seal the tube and invert repeatedly to mix.
4. What is the concentration of your new solution? Label the tube _____ µg/mL methylene blue.

Prepare 40% Methylene Blue Working Solution

1. Pipette 4.0 mL of 5.0 µg/mL methylene blue working solution into a 15 mL conical tube.
2. Pipette 6.0 mL DI H2O into the tube to make 10.0 mL of total solution.
3. Seal the tube and invert repeatedly to mix.
4. What is the concentration of your new solution? Label the tube _____ µg/mL methylene blue.

Prepare 20% Methylene Blue Working Solution

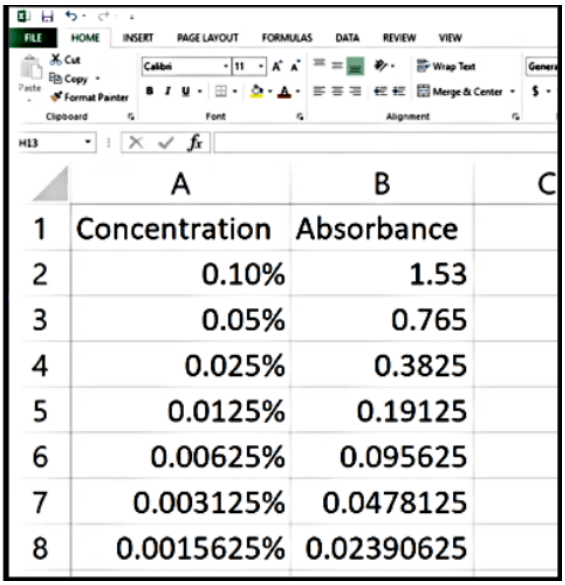
1. Pipette 2.0 mL of 5.0 µg/mL methylene blue working solution into a 15 mL conical tube.
2. Pipette 8.0 mL DI H2O into the tube to make 10.0 mL of total solution.
3. Seal the tube and invert repeatedly to mix.
4. What is the concentration of your new solution? Label the tube _____ µg/mL methylene blue.

Measuring Absorbance of Methylene Blue Working Solutions

1. Turn on the spectrophotometer and let it warm up for at least 10 minutes.
2. Place 1 mL of DI water into a clean cuvette. This is your blank.
3. Place 1 mL of your methylene blue solutions into clean cuvettes. These are your samples.
4. Set the wavelength of the spectrophotometer to 664 nm.
5. Place the blank into the spectrophotometer.
6. Press the “Zero” button and wait for the Absorbance to read “0.00”
7. Take out the blank and set aside.
8. Place your first sample into spec and record the absorbance reading. **Do not press any buttons.**
9. Repeat with each sample and record into lab notebook

Results

Complete **Data Table 1.** based on your results. Put in your notebook



	A	B	C
1	Concentration	Absorbance	
2	0.10%	1.53	
3	0.05%	0.765	
4	0.025%	0.3825	
5	0.0125%	0.19125	
6	0.00625%	0.095625	
7	0.003125%	0.0478125	
8	0.0015625%	0.02390625	

Figure 1. Completing the Excel table

Note this is an example: do not use these values for your concentration or absorbance

Table 1. Absorbances of Methylene Blue at Various Concentrations

Percentage of Working Solution Conc.	Methylene Blue Concentration (µg/mL)	Absorbance @ 664 nm
100%	5.0	
80%		
60%		
40%		
20%		

Making a Standard Curve

1. Enter the data into Excel in adjacent columns.
2. Select the data values with your mouse. On the Insert tab, click on the Scatter icon and select Scatter with Straight Lines and Markers from its drop-down menu to generate the standard curve.

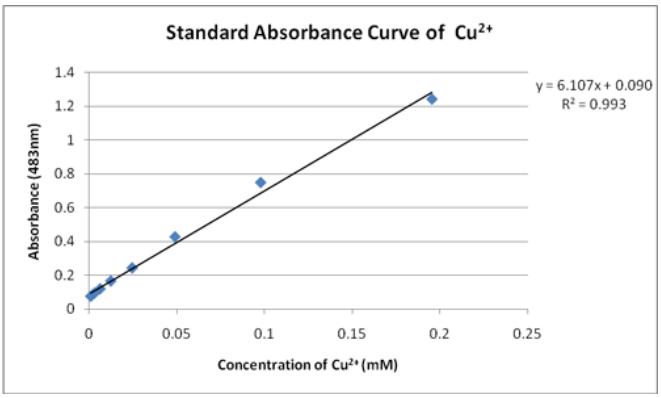


Figure 2. An example of a standard curve

1. To add a trendline to the graph, right-click on the standard curve line in the chart to display a pop-up menu of plot-related actions. Choose *Add Trendline* from this menu. Select “display equation on chart” and “display R-squared value on chart”. Ideally, the R2 value should be greater than 0.99.
2. Use the equation to determine the concentration of the sample solution by entering the absorbance for y and solving for x.
3. Print the standard curve and add to your notebook.

Part III: Determining Concentrations

Serial dilutions are quick way of making a set of solutions of decreasing concentrations. In this part of the lab we will make a series of dilutions starting with the Methylene Blue solution prepared in part 2 of this lab. Then, we will us the spectrophotometer to determine the absorbance of each solution. Once we know the absorbance, we will use the equation from your standard curve prepared in part 2, to determine the actual concentrations of each of your solutions.

Materials

Reagents

- 5.0 µg/mL Methylene Blue Working Solution
- DI H2O

Equipment

- P-20 Micropipettes and disposable tips
- P-1000 Micropipettes and disposable tips
- Spectrophotometer
- 5 mL serological pipettes and pumps
- 15 mL plastic conical tubes with screw-top caps

Preparation of Methylene Blue Solutions

Using the remainder of your 5.0 µg/mL methylene blue working solution from part 2, perform a set of 1:2 serial dilutions to make the following concentrations of the solution (50.0 %, 25.0 %, 12.5 %, 6.25 %, 3.125 %, and 1.5625 %).

Diagram of 1:2 Serial Dilutions

In your notebook, draw a diagram showing the serial dilutions for the 6 methylene blue solutions you are preparing. In the diagram, indicate the volume being withdrawn from the concentrated solution, the volume of water added, the concentration of the new solution, and the total volume.

Procedure

Preparation of Methylene Blue Concentrations via Serial Dilutions

Making 1:2 dilutions

- Pipette 5.0 mL of the 5.0 µg/mL methylene blue working solution into a 15 mL conical tube.
- Pipette 5.0 mL of DI water into the tube for a total of 10 mL of solution.
- Cap and mix well.
- Label this tube “50.0% MB”

Making 1:4 dilution

- Pipette 5.0 mL of the 50.0% MB solution into a new 15 mL conical tube.
- Pipette 5.0 mL of DI water into the tube for a total of 10 mL of solution.
- Cap and mix well.
- Label this tube “25.0% MB”

Making 1:8 dilution

- Pipette 5.0 mL of the 25.0% MB solution into a new 15 mL conical tube.
- Pipette 5.0 mL of DI water into the tube for a total of 10 mL of solution.
- Cap and mix well.
- Label this tube “12.5% MB”

Continue with this process to make the 1:16, 1:32, and 1:64 serial dilutions.

Write the procedures you used to make the solutions in your lab notebook.

Measuring absorbance

1. Follow the procedures in part 2 to prepare the spectrophotometer
2. Measure the absorbance values of the diluted solutions
3. Record the absorbance values and concentrations in your lab notebook in a table as shown below.

Data Table 2

Dilution Factor	% of Working Solution Concentration	Absorbance @ 664 nm	Methylene Blue Conc. (µg/mL)
1:2	50.0%		
1:4	25.0%		
1:8	12.5%		
1:16	6.25%		

Dilution Factor	% of Working Solution Concentration	Absorbance @ 664 nm	Methylene Blue Conc. (µg/mL)
1:32	3.125%		
1:64	1.5625 %		

Calculations

Use the equation from your standard curve in part 2 and the absorbance values of your solutions from Part 3, to determine the actual concentration of your solutions.

Study Questions

1. Describe how you would prepare 50.0-mL a 0.10% NaOH solution. In your description, include a calculation and step by step procedures including glassware.

2. It is common for solutions that are used often in a lab (or which are time consuming to prepare) to be intentionally prepared to be many times more concentrated than needed. For example, if a 1.36% sodium acetate is often used in the lab, then the 13.6% sodium acetate solution prepared in part 1 can be labeled as "10X" sodium acetate solution because the concentration is 10 times greater than needed. This way, you can save on storage space for the solution and you can quickly and easily dilute any desired amount of this to the correct concentration right before use.
3. Describe how you would prepare 100.0 mL of 10X sodium acetate solution. In your description, include a calculation and step by step procedures including glassware. Make sure to include steps to verify your solution by checking the pH.
4. Describe how you would prepare 100.0 mL of 1X sodium acetate solution from the 10x sodium acetate solution prepared in the questions above. In your description, include a calculation and step by step procedures including glassware.
5. Using a serial dilution, describe how you would prepare 10 mL of a 1%, 0.1% and 0.01% solution of NaOH. The stock solution of NaOH is 10%. Draw diagram as part of your description.
6. Using the standard curve below, calculate the concentration of an unknown solution if its absorbance is 0.55.

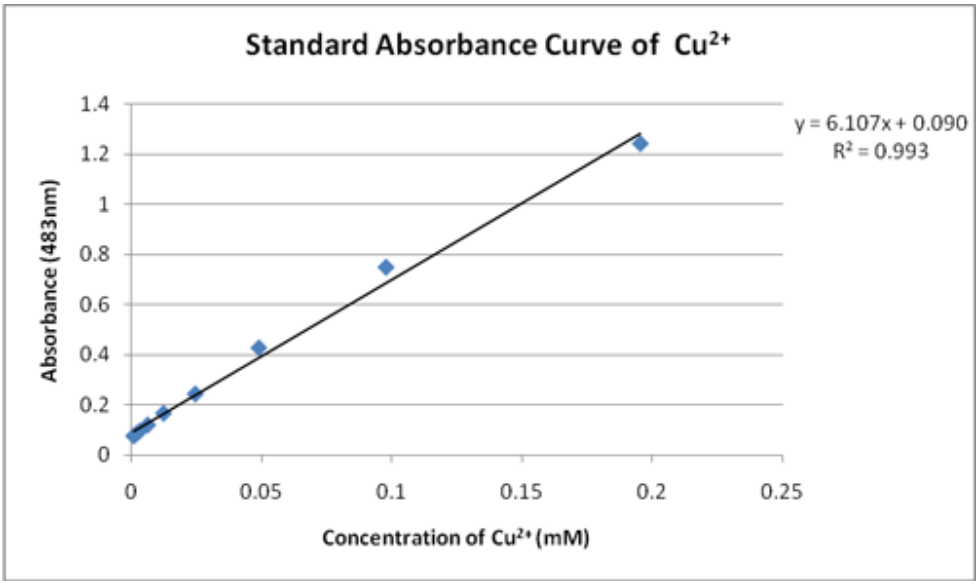


Figure 3. A standard absorbance curve of Copper II

7. Evaluate the quality of the standard curve above by using the R2 value.

Answers

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2.2.9: Biomolecule Detection

Learning Objectives

Goals:

- Employ indicators to discover characteristics of a solution.
- Use indicators to determine contents of an unknown solution.
- Employing positive and negative controls to validate a test.

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Describe the properties of some important biomolecules.
- Explain important characteristics of proteins and carbohydrates.
- Perform tests to detect the presence of carbohydrates and proteins.
- Explain the importance of a control in biochemical tests.
- Use a biochemical test to identify the presence of a molecule in an unknown solution.

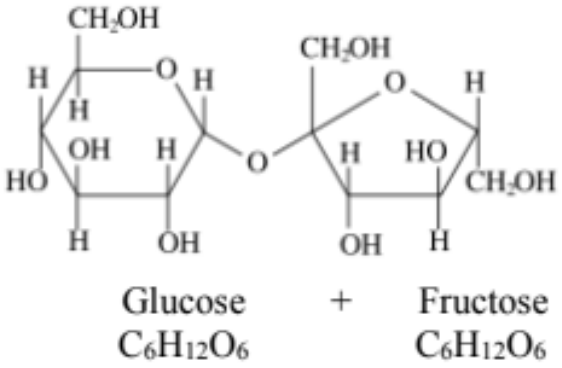
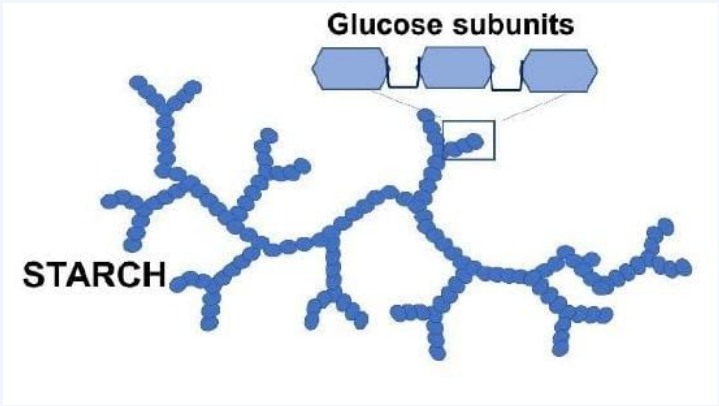
INTRODUCTION

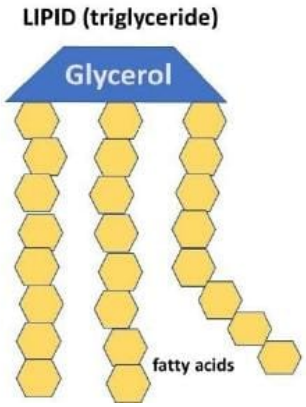
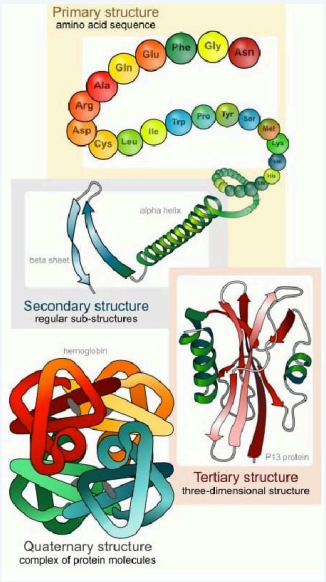
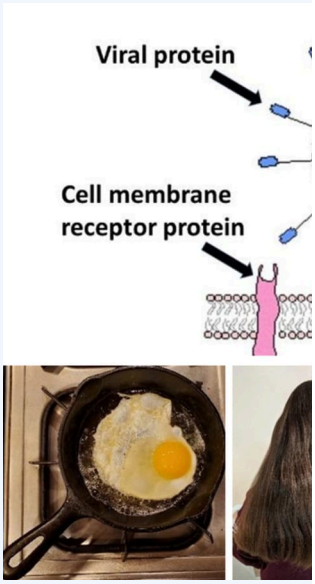
The Macromolecules of Life: Proteins, Carbohydrates, and Lipids

The cells of living organisms are composed of large molecules (macromolecules) sometimes also referred to as organic molecules because of the presence of the element carbon. Very many of the organic molecules found in living organisms are **carbohydrates, proteins, lipids**, and **nucleic acids**. Each of these macromolecules is made of smaller subunits. The different molecules have different chemical properties. For example, monosaccharides such as glucose will react with a chemical agent called Benedict's solution but disaccharides, like sucrose, and polysaccharides, like starch will not. Similarly, proteins will react with a mix of potassium hydroxide and copper sulfate but free amino acids, carbohydrates, and lipids will not.

Today, we will focus on three of these molecular types: lipids, proteins and carbohydrates. You will work with nucleic acids in another lab. You may want to review the properties of the biomolecules of life.

Figure 1: The molecular and macro structures of sucrose, starch, lipids, and proteins.

Molecular Type	Molecular Structure	Macro St
Sucrose		*Common sourc* 
Starch		*Common so* 

Molecular Type	Molecular Structure	Macro St
Lipids		*Common sourc* 
Proteins		*Common sources: cell rec* 

Part I: Controlled Experiments to Identify Organic Compounds

Indicators are chemicals that change color when chemical conditions change, such as pH, or when a chemical reaction takes place producing a colored molecule. There are many biochemical procedures that can be used to detect the presence of important molecules. In this exercise, you will test various solutions in order to detect the presence of these molecules. We will

employ **controls** as we test the solutions. Controls provide results to compare to the solution being tested. Controls should give predictable results. By comparing the test solution result with the controls, you can determine the result of the test solution.

A **positive control** contains the variable for which you are testing. When the positive control is tested, it reacts in an expected manner. If, for example, you are testing for a type of carbohydrate in unknown solutions, then an appropriate positive control is a solution known to contain that type of carbohydrate. The resulting reaction, when properly performed, will demonstrate that the reagents work as expected and shows what the result should look like if the test solution is positive. If the positive control does not react as expected, your test is not valid. Perhaps your test reagents are not working properly.

A **negative control** does not contain the variable for which you are testing. Often a negative control contains only water. It will not react with the indicator reagents. Like the positive control, the negative control solution shows you what a negative result looks like and verifies that the detecting reagent is working properly. If the negative control does react, your test result is not valid. Perhaps the control solution or reaction tube was contaminated with the test variable.

I. Carbohydrates

Benedict's Test for Monosaccharides

Molecules made of the atoms carbon (C), hydrogen (H), and oxygen (O), in a ratio of 1:2:1 are carbohydrates. For example, glucose, one of the most important carbohydrates for living cells, has the chemical formula $C_6H_{12}O_6$. Simple sugars also known as **monosaccharides** are carbohydrates. Paired monosaccharides form **disaccharides**. A common example of a disaccharide is the table sugar, sucrose. It is composed of the monosaccharides glucose and fructose linked to fructose. Similarly, linking three or more monosaccharides forms a polysaccharide. Starch, glycogen, or cellulose are polysaccharides important to cells and have many monomers of glucose linked together in different ways.

Starch

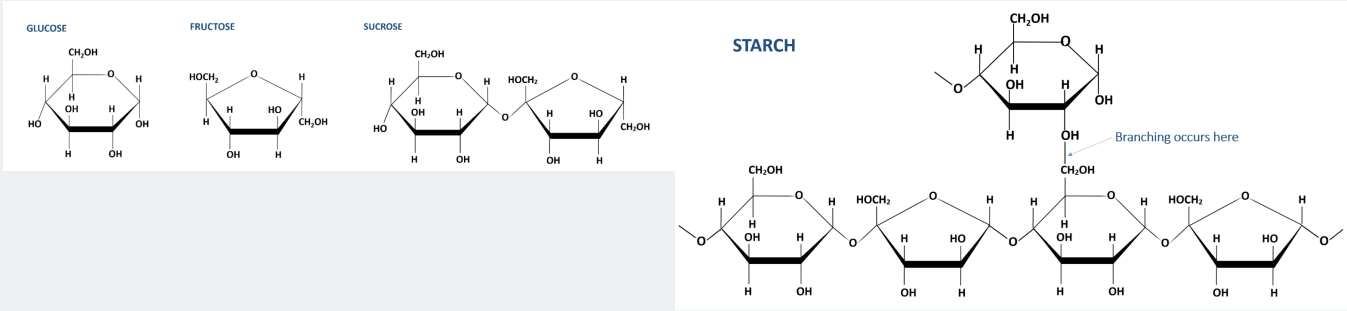


Figure 2. Monosaccharides (glucose, fructose), Disaccharide (sucrose), and Polysaccharide (starch)

Benedict's reagent is the indicator we use to detect monosaccharides. When monosaccharides are mixed with Benedict's and heated, a color change occurs. If there is a small amount of monosaccharide in the solutions, a greenish solution is produced. If the solution contains a large amount of monosaccharide, an orangish precipitate results. A precipitating solution means small particles settle out of the solution.

Reaction 1

Monosaccharides + Benedict's reagent + Heat ⇒ Green to Orange

II. Proteins

The cell relies on proteins for very many functional reasons. Proteins may be enzyme catalysts, form channels for molecules to pass across membranes, form structures and more. The subunit of protein molecules are monomers of amino acids. The bond that forms between amino acids to form protein is called a **peptide bond.**

Peptide bonds can be detected by using two chemical reagents, potassium hydroxide (KOH) and copper sulfate ($CuSO^4$). Potassium hydroxide causes a protein to break apart so that copper sulfate can react with the peptide bonds. The resulting color is purple. The more protein, and hence more peptide bonds, in the solution, the darker the resulting purple will become.

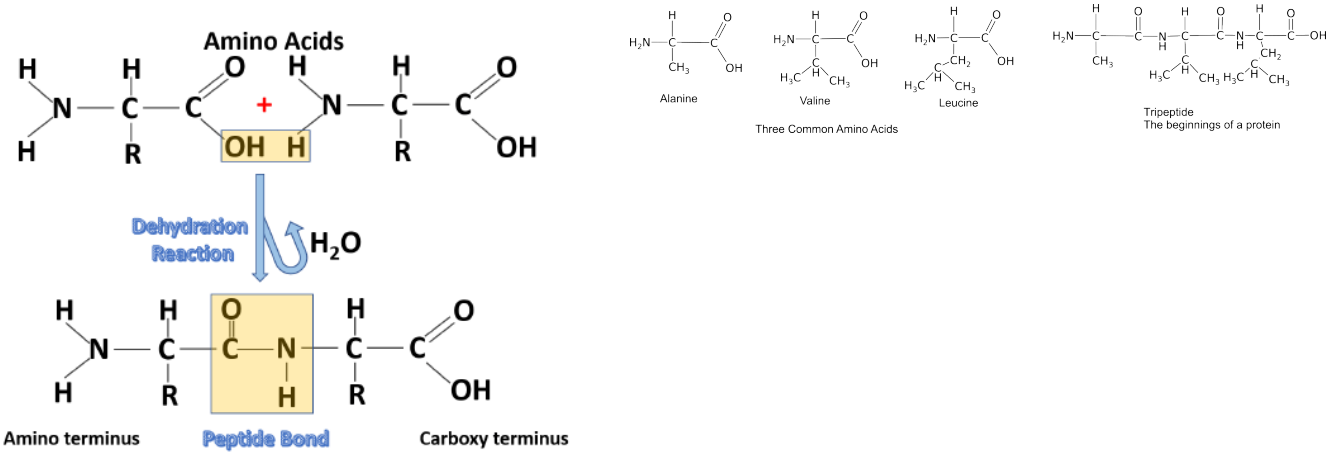


Figure 3. Amino acids forming a peptide bond.

Testing for Monosaccharides with Benedict's Reagent

Reaction 2

Proteins + KOH + $CuSO_4$ ⇒ Purple

Answer available at the end of this section.

Materials

1. Test tubes labeled with the contents you will add to each tube
2. Beaker with water and hot plate (water heated to near boiling)
3. Metric ruler
4. Marker
5. Deionized water and carbohydrate solutions
6. Appropriate tool to remove hot tubes from water

Procedure

1. Obtain 5 test tubes and number them 1 – 5.
2. Use a marker to indicate 2.5 cm from the bottom and another mark at 5cm from the bottom.
3. Fill each test tube to your 2.5 cm mark with the appropriate solution:
   1. Distilled water 2. Concentrated glucose solution 3. Diluted glucose solution 4. Sucrose solution 5. Starch solution
4. Add Benedict's solution to each tube to the 5 cm mark.
5. Place all of the tubes in a hot (90°C) water-bath for 2 min, and observe color-changes during this time.
6. After 2 min, remove the tubes from the water-bath and record the color of their contents in the table below. Also observe your classmate's reactions.

Observations

Perform the Benedict's test for monosaccharides. Reproduce this table in your lab book and complete it with your observations.

Data Table 2.

Tube Contents	Color after reaction	Presence of monosaccharide?
1. Water		
2. Concentrated glucose		
3. Diluted glucose		
4. Sucrose solution		
5. Starch solution		

Instructions to clean up

** Clean tubes are very important. Contaminated tubes may influence results of future tests.*

1. When your observations are complete, carefully wash and rinse the tubes following the instructions in part 2. You may leave the markings on them until the final clean up procedure of the day.

Data Analysis

1. Which of the above solutions serve as your positive control? Negative control?
2. Examine your test and your classmates test solutions. Which solutions were positive for monosaccharides?
3. **Which contains a higher concentration of monosaccharides, potato juice or onion juice? How do you know?**
4. **Which solutions did not react with the Benedict's solution?**

Testing for Peptide Bonds (Protein)

Materials

- Four clean test tubes labeled with the contents you will add to each tube
- deionized water, and test solutions
- Indicator reagents potassium hydroxide (KOH) and copper sulfate ($CuSO^4$)

Procedure

Perform the Peptide Bond test for Protein

Caution!



Do not spill the KOH – it is extremely caustic. Rinse your skin if it comes in contact with KOH.

1. Use your four clean test tubes from the previous procedure. They still need to be numbered and marked at 2.5 and 5 cm from the bottom.
2. Fill each test tube to the 2.5 cm mark with the appropriate solutions indicated below
   a. Water
   b. Protein Solution
   c. Amino Acid Solution
   d. Test Solution
3. Add potassium hydroxide (KOH) to the 5cm mark on each test tube.
4. Add five drops of copper sulfate ($CuSO_4$) to tube and mix well.
5. Record the color of the tubes' contents in the table below. Also observe your classmate's reactions.
6. When finished dump the contents of the tubes and wash them. Rinse with distilled water.

Observations

Perform the Protein Test: Reproduce this table in your lab book and complete it with your observations.

Data table 3.

Tube Contents	Color after reaction	Presence of protein?
Water		
Protein solution		
Amino acid solution		
Unknown solution		

Instructions to clean up

**Clean tubes are very important. Contaminated tubes may influence results of future tests.*

When your observations are complete, carefully wash and rinse the tubes following the instructions in **Part I.**

Data Analysis

1. Which of the solutions is a positive control? Which is a negative control?
2. Do individual amino acids have peptide bonds? How do you know this to be true?
3. What type of solution did you test as your unknown? Did it contain protein?

4. Observe your classmates reactions and describe which unknown solutions contain the most and the least protein. How can you tell?

III. Lipids

Lipids are a class of molecules that are not soluble (do not dissolve) in water. They are composed of the molecular building blocks of glycerol and three fatty acids. Fatty acids come in two major types, saturated and unsaturated. This difference is due to the presence of particular types of bonds within the fatty acid molecule (see figure) and affect the shape and characteristics of the overall lipid containing these fatty acids. You may want a review of lipids.

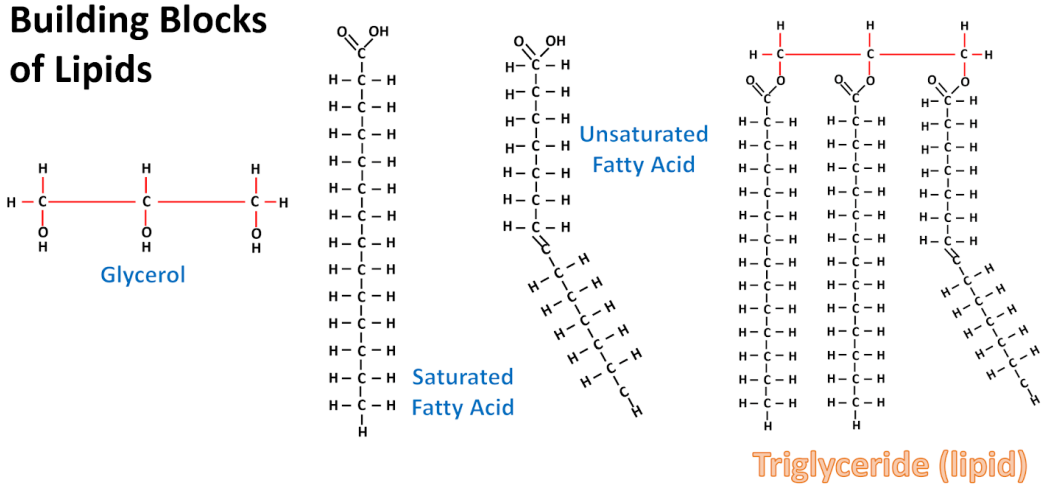


Figure 4. Saturated and unsaturated fatty acid and a lipid (triglyceride)

Testing for Lipid with Sudan IV

Caution!

Use gloves and avoid contact with Sudan IV as it is considered a possible carcinogen. Immediately wash your skin with soap and plenty of water if you come in contact with the solution.

Materials

- Filter paper (small enough to fit in the petri dish) and pencil with areas labeled for test substances
- clean empty petri dish
- solution of 0.2% Sudan IV
- Gloves (see safety warning)
- Dedicated transfer pipettes or micropipettes with tips.
- Solutions of deionized water, vegetable oil, and test solutions (cream, dairy milks, coconut milk, soy milk etc.)
- optional- hairdryer

Procedure

1. Obtain filter paper and on the far edge mark with pencil which solutions will be placed toward the interior of the mark.
2. Drop a small amount of solution near the appropriate mark. 1. Distilled water 2. Vegetable oil 3-6. Test solutions

3. Allow to dry. Use a hairdryer to speed up this process.
4. While the paper is drying, answer the Data Analysis questions below.
5. Soak the paper in the petri dish containing 0.2% Sudan IV. (handle with gloved hands)
6. Rinse the paper in distilled water and allow to dry.
7. Record the color of the spots in the table below. Also observe your classmate's reactions.

Observations

Sudan IV test for lipid: Reproduce this table in your lab book and complete it with your observations. The darker the stain, the more lipid is present.

Data table 4.

Spot Contents	Color after reaction	Relative amount of lipid?
1. Water		
2. Vegetable oil		
3.		
4.		
5.		
6.		

Instructions to clean up:

When your observations are complete, carefully dispose of any remaining Sudan IV solution in the container provided by your instructor. Always use gloves and do not move the container if there is a danger of spilling.

Data Analysis

1. Which of the above solutions serve as your positive control? Your negative control?
2. Hypothesize which solutions will contain the greatest amount of lipid. Why do you believe this to be true?
3. Which solutions contained the greatest amount of lipid?
4. Did your observations support your hypothesis? Were you surprised by some of the results? Explain.

Part II. The Saga of the Soda Dispenser

Enrique was a new employee. This was his first job and he had only been on the job for a couple of weeks and was still on "hiring probation." He liked the crew he worked with and the paycheck that would come every few weeks. He wanted to stay. Today, there was a problem and he had to figure out something fast to solve it. He knew that if he did, the manager would be really pleased and his job was guaranteed.

Someone was complaining that the soda dispenser was dispensing "regular" cola from the "diet cola" dispenser. The customer

claimed to be on a reduced-calorie diet and was not happy about the extra calories consumed. There was more at stake than one unhappy customer, though. The manager told Enrique that many of their customers were diabetic and consuming sugar-laden soda could alter their blood-sugar chemistry in a dangerous way. They could not allow those customers to be harmed.

Scope of the Problem

If the diet soda dispenser did have regular soda, then did the regular soda dispenser have diet? What about the Dr. Pepper dispenser? That, at least, tasted like Dr. Pepper, so it was OK- or was it? What a mess! Should they throw all the soda in the dispenser out and start again? Or was there some way of determining if the soda was being dispensed correctly? If they could determine what the problem was, they could save the business money and not waste the soda products.

Enrique's Attempt to Solve the Mystery

Enrique knew that most soda had high fructose corn syrup in it but diet soda had sugar substitutes in it: Substitutes that were not sugar but fooled your taste buds into believing it was.

Questions for your lab book:

1. Does the regular soda have high fructose corn syrup in it? Look at the label determine if it does or doesn't. Write your observation in your lab book.
2. Does the diet soda have high fructose corn syrup in it? Look at the label determine if it does or doesn't. Write your observation in your lab book.
3. Determine whether fructose is a monosaccharide, disaccharide or polysaccharide.
4. Can we do a test?

Just the other day, in science lab, Enrique had run some tests on solutions in order to determine their compositions. One of the tests was for detecting monosaccharides in solution! He knew his science teacher would still be in the classroom at this time and the school was barely a 5 minute walk from the restaurant. He could solve the mystery in under 30 minutes! Enrique quickly told his manager his plan and grabbed some cups of soda, which he labeled, so he could tell which dispenser they came from, then headed out. Enrique quickly ran to the school lab and got permission to run his experiment. Help Enrique set up an experiment to test the soda.

More questions for your lab book:

1. Would it be a good idea to include controls? If so, which solutions?
2. Which detector reagent(s) will you use?
3. What colors will you look for to indicate the presence of the "regular" soda?
4. How many test tubes do you need? How will you label them?

Testing Unknown Soda Solutions

Materials

1. Clean test tubes labeled with the contents you will add to each tube
2. deionized water, and solutions to test
3. Indicator

Procedure

Perform the test for monosaccharides:

1. Obtain the needed number of clean test tubes and mark them at 2.5 and 5 cm as before. Code them as to the contents (numbers corresponding to your solutions- which you record below)
2. Obtain the unknown solutions from your instructor.
3. Fill the tubes to the 2.5 cm mark with the control and test substances.

4. Fill the tubes to the 5 cm mark with indicator and treat was needed.
5. Reproduce this table in your lab book and complete it with your observations, then answer the questions regarding the soda saga.

Observations

Perform the Appropriate Test: Reproduce this table in your lab book and complete it with your observations.

Data table 5.

Tube Contents	Color after reaction	Presence of fructose?	Diet or regular?
1.			
2.			
3.			
4.			
5.			
6.			

Instructions to clean up:

Caution!



DO NOT allow ethanol to come in contact with the hotplate. Ethanol is very flammable.

**Clean tubes are very important. Contaminated tubes may influence results of future tests.*

1. When your observations are complete, carefully wash and rinse the tubes following the instructions in part 1.
2. At the end of the lab period be sure all labels are removed from the tubes using a small piece of paper towel and ethanol.

Final Conclusion

1. What does Enrique tell his manager? Is the soda dispenser messed up or not?
2. What, if any, soda needs to be changed?

Study Questions

1. Why should you always include controls in each procedure?
2. What serves as a good negative control and why?
3. Describe a positive control.
4. If you run a test for monosaccharide on what you believe is “regular” lemon lime-flavored soda, but the solution is sky-blue after heating with Benedict’s what does this tell you?
5. What if only AFTER running your test, you read the label of the lemon-lime soda and notice that the ingredients do not contain fructose but does contain sucrose. Is your test procedure faulty or is there another explanation for your result?

Attributions

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3. Amino Acids forming a peptide bond (bottom image) by OpenLab at CitiTech CC-BY-NC-SA

Answers

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2.2.10: Paternity Case with Electrophoresis

Learning Objectives

Goals:

- Run agarose gel electrophoresis on samples.
- Learn how to analyze DNA fingerprints.

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Separate molecules by electrophoresis.
- Analyze bands resulting from gel electrophoresis.
- Determine the paternity of offspring using DNA fingerprint analysis.

Part I: Agarose Gel Electrophoresis

Introduction

Gel electrophoresis is a very common and useful technique for separating DNA, RNA, and protein molecules on the basis of molecular size and charge. Agarose is a polysaccharide, found in seaweed, that forms a gel matrix (meshwork of various-sized holes). When an electric current is passed through the buffer and the gel, the molecules in the sample move toward the electrode with the opposite charge. DNA, RNA and protein molecules typically have a negative overall charge and will move toward the positive electrode. Smaller molecules can move through the matrix of the gel faster than larger molecules.

DNA fingerprinting uses band patterns that result from specific treatment of DNA samples to identify the relationship of a sample to reference samples. In this lab, you will run a simulation of a DNA fingerprinting activity in order to become familiar with this process. You will load samples on a gel and separate the bands in each sample to create specific patterns using gel electrophoresis.

Preparing Agarose Gels

There are various running buffers that can be used to separate DNA. A few of the commonly used buffers are called TAE (Tris Acetate EDTA), TBE (Tris Borate EDTA), and SB (Sodium Borate). Always use the same buffer to make the agarose gel and run the electrophoresis.

MATERIALS

- Agarose powder
- Spatula
- Weigh boat
- 20x stock
- Graduated cylinders
- PipetAid
- Serological pipet
- Flask with vented cap

EQUIPMENT

- Balance

- Microwave

PROCEDURE

A. **Prepare 500 ml of 1X Sodium Borate buffer from a 20X stock solution.**
   1. Measure 25 mL 20X Sodium Borate buffer stock in a 25 mL graduated cylinder and pour into a 500 mL container.
   2. Measure 475 mL DI-water in a 500 mL graduated cylinder and pour into the same container.
   3. Cap tightly and mix.

B. **Prepare 50 ml 0.8% (w/v) agarose in 1X Sodium Borate buffer** to pour four Mini One gels. Note that agarose (and gelatin) are two substances that are prepared in a special way, as they can only dissolve in boiling liquids. So, we NEVER put agarose (and gelatin) powder into graduated cylinders. Instead we pour the powder into the final container (Erlenmeyer flask).
   1. Measure 50 mL 1X buffer with a 50 mL graduated cylinder and pour into an Erlenmeyer flask.
   2. Measure 0.4 g of agarose powder and pour into the same flask. This will look like an opaque slurry.
   3. Use a vented cap if possible (or no cap). Place the flask in a microwave oven and turn on (for 1 minute) at high setting. Keep a close watch - do not allow the liquid to boil over!
   4. Watch for bubbling of the liquid. As soon as the liquid starts to bubble, stop the microwave, use mitts or silicone “Hot Hands” to swirl the flask 2-3 times.
   5. Then replace and turn on the microwave again for the second boil.
   6. As soon as the liquid starts to bubble, stop the microwave, use mitts or “Hot Hands” to hold the flask to the ceiling light. Look carefully for any specks or crystals in the liquid. When there are no more specks visible in the clear solution, then the agarose has completely melted.
   7. Place the flask on the table and allow to cool to 60oC. When you are first able to hold the flask with bare hands, then the agarose is cool enough to pour into trays. **Do not pour agarose too hot, as the casting gels will warp and crackle. Do not cool agarose too long, as the gel will not polymerize evenly.**
   8. Prepare the casting trays and practice loading gel samples while you wait.

Casting Agarose Gels (MINI ONE EMBITEC GEL SYSTEM)

1. Place two clear acrylic casting trays into the white casting stand.
2. Insert the 9-well comb into the proper slot of the casting stand.
3. It is best to use a Pipet-Aid and 25 mL Serological Pipet to measure and transfer 12.5 mL of melted agarose solution into each casting tray. Or pour agarose solution until 1/3 up the comb.
4. Quickly move or pop air bubbles with a pipet tip or gel comb. Insert the gel comb into the proper slot.
5. Do not move or bump the gel tray until the gel has solidified in about 15 minutes.
6. Store extra agarose solution in flask, covered with parafilm or cap, at 4oC for later use.

Practice Loading Gel Samples

Loading gel samples is a skill that takes practice to learn! The actual agarose gel that you will be using for electrophoresis is very delicate and can easily be punctured. The practice gel cannot be punctured, but provides the same size wells to dispense your samples. As DNA is clear, usually a colored loading dye is added to the DNA samples. High-density glycerol is also added to the loading dye, so that the DNA samples will fall to the bottom of the well quickly.

Lab Tip: When loading samples into the gel wells, only push the micropipette plunger to the first stop, do NOT push to the second stop. Be sure to keep your thumb pressed down on the plunger, until the micropipette is completely out of the buffer tank.

1. Obtain a practice urethane gel.
2. Cover the gel with deionized water. If there are bubbles in the wells, use a plastic transfer pipet to remove the bubbles.
3. Use a pipet tip on a P20 micropipette and set dial to 10.0 uL.
4. Push and hold micropipette plunger to the first stop.
5. Pick up 10 µL of the practice red dye and slowly release your thumb.
6. Hold the micropipette vertically (see Figure 1) over the practice gel, such the tip is below the water level and just above the well. There is no need for tip to enter into the well, as the heavy glycerol will pull the sample down into the bottom of the well.

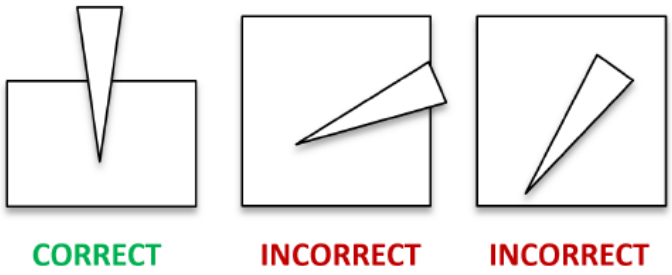


Figure 1. The correct positioning of a micropipette tip

7. Push the plunger to the FIRST STOP only and keep your thumb there. (Optional: try pushing to the second stop to see what happens.)
8. Move your entire arm up, so that the micropipette is out of the water, then allow your thumb to come off the plunger. (Optional: try releasing your thumb while the pipette is still in the water to see what happens).
9. Practice loading 10.0 µL of the red dye into three or more wells of the practice gel.
10. You should be able to see the colored sample drop down to the bottom of the wells.

Lab Tip: If done correctly, all of the sample will stay in the well. Check if there is any colored dye floating away from the top of the well, which means that the sample may contaminate another well. Check if there is any dye leaking out from the bottom of well, which means that you punctured the well and the sample may not enter the gel.

PART II: Paternity Case: Who is the Father of My Kittens?

Mary has a white cat named “Honey” who was lost for two days about three months ago. She now has four kittens (photo 1) and Mary wants to know if the two neighboring cats, “Tom” or “Butch,” could be the father of each kitten. To analyze their DNA fingerprint, Mary has collected hair follicles from each adult cat and kitten, extracted DNA, and amplified DNA using the polymerase chain reaction.



Figure 2: Left to right. Honey and her four kittens (in order): Cream, Molasses, Ginger, Sugar; potential fathers Tom and Butch.

Hypothesis

Using the photo, complete table 1 with your prediction of the father of each kitten.

Table 1. Hypothesis on Paternity of Kittens

Kitten	Potential Father	Reasoning
Cream		
Molasses		
Ginger		
Sugar		

Materials

Reagents

- Pre-cast 0.8% agarose gel
- Seven samples in microfuge tubes. One for each feline
- 135-150 mL 1X sodium borate running buffer (enough to submerge the agarose gel)

Equipment and Supplies

- Electrophoresis system such as MiniOne or other brand with the gel chamber and power supply
- P-20 micropipettes and appropriate tips
- Cell phone or camera to photograph the gel to document results

Procedure

1. NOTE: The following is completed when the electrophoresis chamber has been prepared with an 0.8% agarose gel and 1x Sodium Borate buffer in the chamber. Do not forget to document your procedure appropriately in your laboratory notebook.
2. Obtain the “DNA samples” – there are seven microfuge tubes labeled P- V.
3. Using a P-20 micropipettor and a pipet tip, measure 10µL from Tube P and transfer into the first well of the agarose gel. Be sure to follow the gel loading order noted in Column 1 – *Well*.
4. Using a new tip for each sample, transfer 10µL of each sample into new wells of the gel.
5. Be sure to keep track of your sample loading, if you do not follow the table below. If there were any problems with the loading (punctured gel, not enough sample), be sure to write in the NOTE column.

Table 2. ***Loading notes:*** *Include any deviations or notes in your laboratory notebook*

Well	Tube	DNA Sample 10µL	Notes for loading
1	P	Tom (male)	
2	Q	Cream (kitten)	

Well	Tube	DNA Sample 10µL	Notes for loading
3	R	Molasses (kitten)	
4	S	Honey (female)	
5	T	Ginger (kitten)	
6	U	Sugar (kitten)	
7	V	Butch (male)	

6. If using a MiniOne electrophoresis system, run the gel for 15 minutes, until color bands separate. If using another electrophoresis system, run the gel at 135V until the dye front is.
7. For best viewing of the results, pick up the casting tray (with gel) out of the buffer tank, slide the gel onto a white laminated paper, label the samples (and your team name) and take a photo.
8. **Because DNA samples will diffuse through the agarose gels, you should always record results quickly once the electrophoresis has been turned off.**

ANALYSIS

1. Use colored pencils to record the band patterns (color the appropriate blocks) in the Data Table below.

Table 3. Colored "DNA" Bands Separated by Agarose Gel Electrophoresis

Tube	P	Q	R	S	T	U	V
Band	**Tom** (Male)	**Cream**	**Molasses**	**Honey**(Female)	**Ginger**	**Sugar**	**Butch** (Male)
Blue #1							
Blue #2							
Pink #1							
Purple #1							
Yellow #1							

Tube	P	Q	R	S	T	U	V
Yellow #2							

2. Carefully consider each band of all four kitten samples and determine whether the band matches Tom, Honey or Butch. For the kitten samples (columns QRTU) in Data Table 3, write (within the colored blocks) who matches that band -- Tom, Honey, or Butch.
3. Draw your conclusions based on the “DNA evidence”.
4. Fill in the 2nd and 3rd column in table 4 below. Compare your hypothesis in table 1 where you guessed the father for each kitten based on appearances to your conclusion regarding the father based on the DNA evidence. Was your hypothesis correct for each kitten?
5. What is the specific evidence that justifies your conclusion determining each kitten’s father? Fill your responses to these questions in the table below.

Table 4. Comparison of Hypothesis and Conclusion backed by Experimental Evidence

KITTEN	FATHER based on visual	FATHER based on DNA	Evidence
Cream			
Molasses			
Ginger			
Sugar			

Study Questions

1. During gel electrophoresis, DNA will migrate toward which electrode?
2. If you had DNA molecules that were small, medium, long, and extra long, which would be closest to the bottom of the gel after electrophoresis?
3. What do you know about the pattern of bands that result from an offspring as they relate to the mother and father?
4. If you ran DNA fingerprint analysis but the band pattern for the offspring was not matching either parent, what would you conclude?

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2.2.11: Restriction Digest with Gel Electrophorisis

Learning Objectives

Goals:

- Understand the function of restriction enzymes.
- Conduct analysis of DNA fragments by gel electrophoresis.

STUDENT LEARNING OUTCOMES:

Upon completion of this lab, students will be able to:

- Read a plasmid map to determine restriction sites and fragment sizes.
- Determine if restriction enzyme recognition sequences are palindromes.
- Predict the sizes of DNA fragments formed after a restriction digest.
- Compare gel electrophoresis bands to determine DNA sizes.

Introduction

Recombinant DNA technology is possible due to several tools useful for manipulating DNA molecules and transforming cells -- including plasmids, restriction enzymes and DNA ligase. This lab introduces you to plasmids and restriction enzymes, as well as the lab technique of gel electrophoresis. Later lab experiments will introduce you to the other tools of biotechnology.

Restriction enzymes (also called **restriction endonucleases**) are proteins made by many bacterial species, to defend against viral infections. Each restriction enzyme moves along a DNA molecule until it finds a specific **recognition sequence** in the DNA. The enzyme cuts the double-stranded DNA, resulting in DNA fragments. Over 3000 restriction enzymes that recognize short (4-8 bp) palindromic sequences have been discovered.

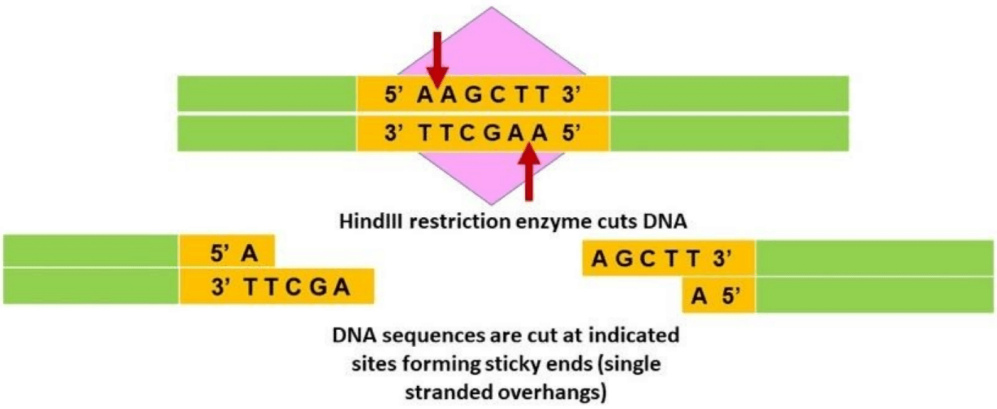


Figure 1. Recognition sequence for enzyme Hind III

Figure 1 shows the recognition sequence for restriction enzyme Hind III. Notice that the recognition sequence is a **palindrome**, and reads the same going forwards and backwards. The Hind III enzyme makes a **staggered** cut of the DNA, and produces fragments that have single stranded areas called “sticky ends”. Figure 2 shows the recognition sequence of two other restriction

enzymes Sca 1 and Pst 1. Enzyme Pst 1 makes a staggered cut of the DNA at its recognition sequence. But restriction enzyme Sca I makes a **blunt** cut at its recognition sequence to generate DNA fragments with no sticky ends.

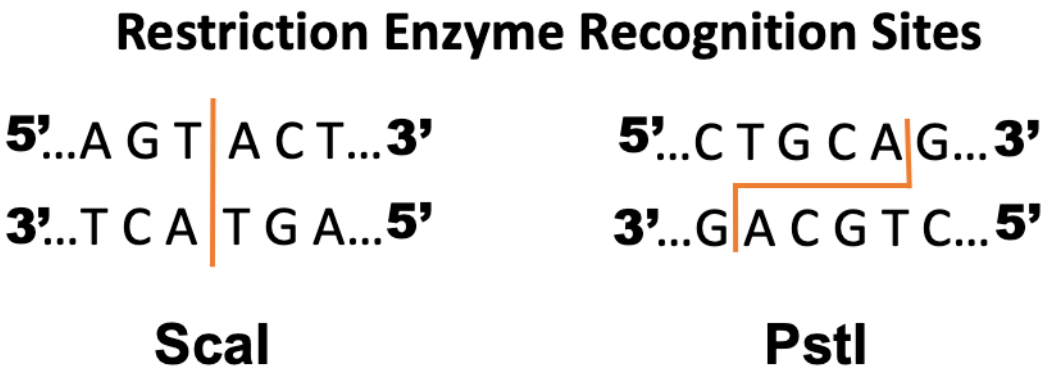


Figure 2: Restriction enzyme recognition sites.

Bacterial cells have all of their genes (genome) in a single circular chromosome. But bacterial cells can also carry non-essential pieces of DNA called **plasmids**. A plasmid is a small circular DNA that is able to replicate itself, and can carry a few genes from cell to cell. Scientists are able to design recombinant plasmids to carry specific genes into a target host cell.

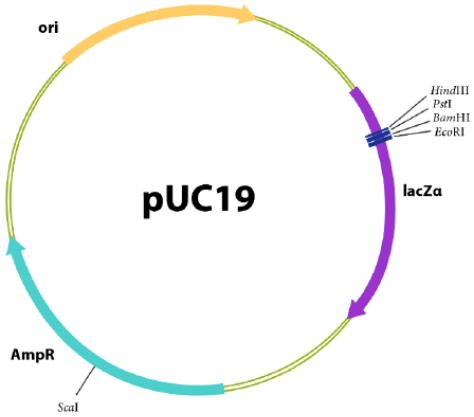


Figure 3: Plasmid map of pUC19.

The genetic map of a plasmid "pUC19" is shown in Figure 3. The total size of the plasmid is 2686 bp. There is a Pst I recognition site at position 439, Hind III recognition site at position 447, and Sca I recognition site at 2179. If one restriction enzyme is used to cut pUC19 plasmid, what would be produced?

Determine what DNA fragments are produced when two restriction enzymes are used to cut pUC19 plasmid DNA. imagew216amph1amprev1ampac1ampparent1gk1-qezbOd_EasksNT7LvbYaQLJ41OZ6

Table 1. Predicted DNA Fragments from Restriction Digest of pUC19 Plasmid

Cut with Restriction enzymes	Sca I and Pst I	Sca I and Hind III	Pst I and Hind III
Resulting DNA fragment sizes			

Part I: Restriction Digest

Agar is a polysaccharide derived from red algae. The agar powder is first dissolved in a boiling liquid, and then cooled to form a gelatinous solid matrix. As microbes cannot digest agar, this material is used commonly in laboratories to hold the nutrients that bacteria need.

Materials

- P-20 micropipette
- Box of disposable pipette tips
- Clean microfuge tubes
- Microfuge tube rack
- Permanent marker
- Waste container for used tips and microfuge tubes
- Microfuge tubes containing:
- pUC19 plasmid DNA
- pPUS2 plasmid DNA
- Pst 1 Restriction enzyme
- Sca 1 Restriction enzyme
- Restriction buffer
- Deionized water

Equipment

- Microcentrifuge
- 37°C water bath (or dry bath or incubator)

Method

1. Use a Sharpie to label the top and side of 3 clean microfuge tubes A B C and your group name.
2. Follow the reagent table below and dispense the proper amounts of reagents to the labeled tubes. Use new tips for different reagents. Add reagents to the solution at bottom of tube. Always check that your pipet tip is empty after dispensing the reagent.

Table 2. Volumes of Reagents to Add to Each Tube

Tube	DNA	Pst 1 RE	Sca 1 RE	Restriction Buffer	Water
A	4 µL pPUS2	2 uL	none	4 uL	none
B	4 µL pUC19	2 uL	2 uL	2 uL	none

Tube	DNA	Pst 1 RE	Sca 1 RE	Restriction Buffer	Water
C	4 µL pUC19	2 uL	none	4 uL	none
D	4 µL pUC19	none	none	none	6 uL

3. Cap tubes tightly Place two tubes directly across from each other in the microcentrifuge.
4. Spin for five seconds to bring all the reagents to the bottom of each tube.
5. Place tubes into a floating rack in the 37°C water bath for at least one hour (but no more than two hours.)
6. After the incubation period is finished, you will analyze the contents by gel electrophoresis in Part IV.

Part II: Casting Agarose Gel

Agarose is a complex carbohydrate found in seaweed. If agarose is dissolved in a boiling liquid and then cooled, the solution converts into a solid gel matrix. The agarose solution will be poured into a casting tray to form the desired gel shape. A gel comb has teeth that is used to form the “wells” or holes for loading the samples. You will be prepare and cast a 1% agarose gel with electrophoresis buffer.

Materials

- Agarose powder
- Weigh boat
- Spatula
- Masking tape
- 100 Graduated cylinder
- 250 mL Erlenmeyer flask
- Electrophoresis Gel Casting tray
- Gel comb
- Deionized or distilled water
- 1X Electrophoresis buffer
- Heat-resistant silicone mitts or tongs
- Electronic or analytical balance
- Microwave

Method

Note

- Note: If you have concentrated electrophoresis buffer stock, you must dilute the stock to 1X working concentration before preparing agarose solutions or running gel electrophoresis.
- For 20X stock, combine 25 mL 20X stock with 475 mL deionized water to make 500 mL 1X buffer.
- For 50X stock, combine 10 mL 50X stock with 490 mL deionized water to make 500 mL 1X buffer.

1. Using the graduated cylinder, measure 100 mL of the 1X electrophoresis buffer.
2. Using an electronic scale, measure 1.0 g of agarose powder.
3. Pour some of the measured buffer into an 250 mL Erlenmeyer flask. Pour in the measured agarose powder. Pour some of the measured buffer into the agarose weigh boat, and pour into flask. Repeat until all agarose has been transferred to flask. Pour rest of buffer into flask.
4. Cover the opening of the flask with plastic wrap. Use a pipette tip to poke a small hole in the plastic wrap.

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5. Place the covered flask in a microwave and heat on high. When you see bubbles form in the solution, pause the microwave, use oven mitts to gently swirl the flask a few times.
6. Continue microwaving the flask until the liquid starts to bubble again. Using oven mitts, hold the flask to the light and swirl the solution. Look carefully to check that there are no specks or swirls of agarose suspended in the liquid. If liquid is clear, then the agarose is dissolved. Wait five minutes for the agarose to cool. Note: Instructor will announce if you have a casting stand system and do not need to tape each tray.
7. Prepare the acrylic electrophoresis gel trays for casting. You may need to tape the two open ends of each tray. Be sure to press tape firmly along the entire edge of the tray with your fingernail. If using masking tape, you can see a difference in the tape translucence.

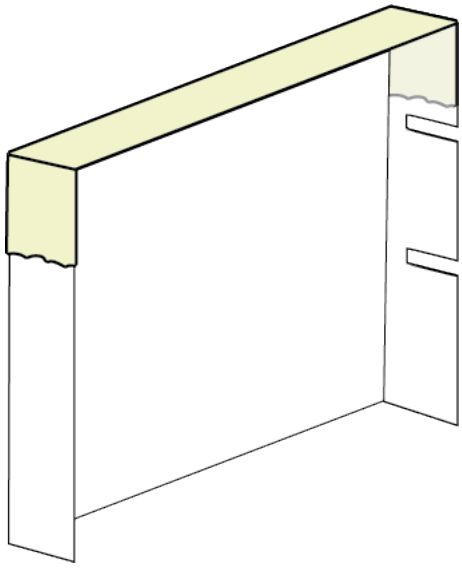


Figure 4. Casting tray with tape

8. Place a comb in each tray before adding the agarose solution.
9. When the agarose solution has cooled to the point that you can safely touch the bottom of the flask (~60°C; about five minutes), pour agarose solution into each casting tray, so that the solution covers about 2 mm of each comb. Note: Each Mini One gel requires 12.5 mL agarose solution, each casting tray holds two gels = 25 mL total.
10. Once the gels solidify (which will take around 30 minutes), pull the comb out of each gel. Pull it straight out without wiggling it back and forth; this will minimize damage to the front wall of the well.

Part III: Practice Pipetting

While you are waiting for the restriction digest to incubate, you can practice loading samples into a practice gel. As agarose gels are very easy to puncture through, it is important to have good technique for loading the samples. As the gel wells are small, only push the micropipette to the FIRST stop to dispense the sample. Purified DNA looks like water, so a colored dye is added to ensure that you can see the sample loading into the well. Glycerol, a viscous liquid, is added to the loading dye to ensure that the DNA sample will sink to the bottom of the well.

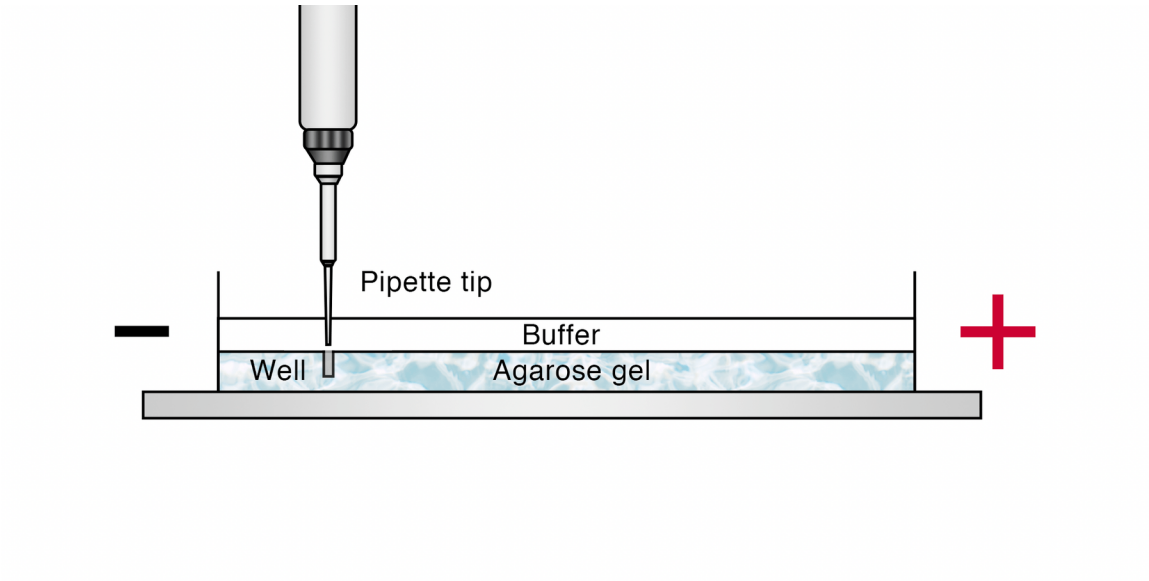


Figure 5. Pipetting a sample into the well of an agarose gel

Materials

- P-20 micropipette
- Box of disposable pipette tips
- Waste container for used tips
- Tube of colored dye/glycerol
- Agarose or polyurethane practice gel
- Agarose powder
- Weigh boat
- Spatula
- Masking tape

Method

1. Watch the video or instructor demonstration.
2. Submerge the practice gel with water or buffer.
3. Practice loading 5 µL and 10 µL colored dye into several wells of a practice gel. Do not change tips for this practice.
4. To steady your pipette hand, place your elbow on the table. You may also use your other hand to support and steady your pipette hand.

Part IV: Gel Electrophoresis

Gel electrophoresis is a technique to use electrical current to separate a mixture of molecules such as DNA, RNA, and proteins. The electrophoresis buffer contains ions to conduct electric current. As DNA molecules are negatively charged, they will migrate towards the positive electrode (red). The solidified agarose gel matrix will have pores of various sizes (similar to a sponge), so the size, shape and charge of the molecules can affect the rate of travel through the agarose gel. Smaller molecules move faster than the larger molecules.

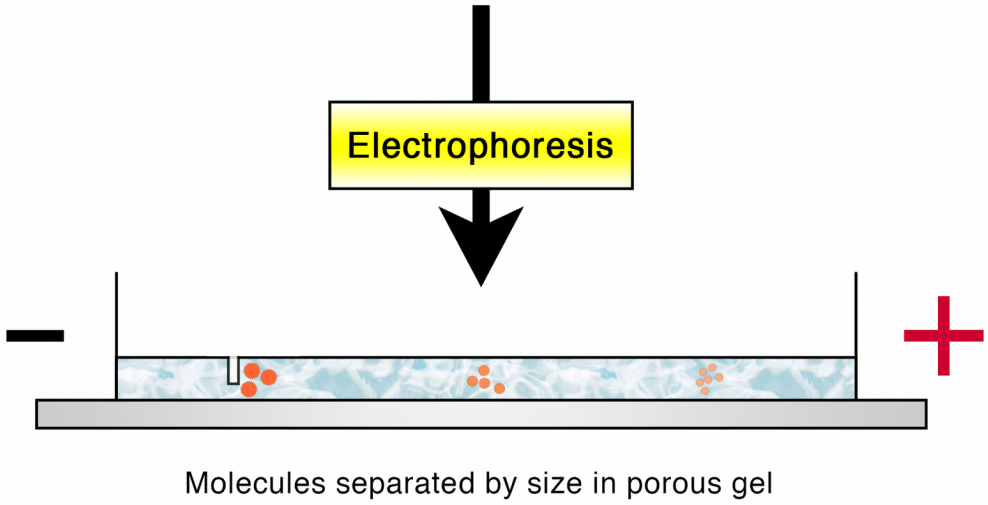


Figure 6. Electrophoresis causes the molecules to separate by size in the porous gel

DNA can be visualized with various dyes. Scientists typically use ethidium bromide (either inside the agarose gel or as post-stain after the gel run). As ethidium bromide is mutagenic, we will not be using that in our class. Instead, we will use gel green stain, which is compatible with the blue LED transilluminators (eg. MiniOne). The alternative stain is gel red, which works with the uV transilluminators.

Materials

- Agarose powder
- Weigh boat
- Spatula
- Masking tape
- 100 Graduated cylinder
- 250 mL Erlenmeyer flask
- Electrophoresis Gel Casting tray
- Gel comb
- Deionized or distilled water
- 1X Electrophoresis buffer
- Heat-resistant silicone mitts or tongs
- Electronic or analytical balance
- Microwave

Method

Setting Up the DNA Samples

1. Find your tubes from the restriction digest (Part 1).
2. Add 2 µL of Gel green Loading dye into each of the sample tubes. Pipet up and down twice to mix the liquid.
3. Place tubes in a balanced configuration in a MicroCentrifuge and spin for five seconds.

Setting Up the Electrophoresis System

1. Watch a demo or assigned videos and follow instructions for placing the gel tray into the electrophoresis buffer tank.
2. Fill the buffer tank with 1X Electrophoresis buffer, ensuring that the entire gel is completely submerged. You want about 1 mm liquid layer above the gel, but not too much buffer as that can build up resistance.
3. Check that the gel is oriented with sample wells closest to the negative electrode (black). Check that the power cord can

reach easily. Check that the gel box will not need to be moved for 30 minutes.
4. Draw and label in your notebook how the samples will be loaded in the gel. Check whether you will be sharing the gel with another group.
5. Using a new tip for each sample, load the DNA samples carefully into the gel wells.
6. After all the samples are loaded, place the cover over the electrophoresis box. [Note: Gel green is especially sensitive to light, so do not leave the Mini One light on during the electrophoresis].
7. Connect the electrical leads to the power supply. Connect both leads to the same channel, with the negative (-) cathode to cathode (black to black) and the positive (+) anode to anode (red to red). [Note: Mini One system must have orange hood in place to turn on].
8. Turn on the power supply and set the voltage to 130–135 V. [Note: Mini One systems do not have adjustable voltage].
9. After turning on the power on the gel boxes, look for bubbles forming on the negative electrode (to show electric current) and that dyes are moving toward the correct direction. If running the wrong way, wait until dyes are inside the agarose gel, then turn the gel 180o and restart the run..
10. Do not allow the loading dye to run off the gel. Be sure to turn off the power switch and unplug the electrodes from the power supply. Do this by grasping the electrode at the plastic plug, NOT the cord.
11. Carefully remove the cover from the gel box and pick up the gel tray. Slide the gel onto plastic wrap on top of the appropriate transilluminator. Take a photo.
12. Compare the sizes of the DNA ladder to the pUC19 fragments. The pPSU1 cut with Pst1 has fragments of 4100, 2000, 1000, 900, 800, 700, and 500. The pPSU2 cut with Pst I have sizes of 4100, 1500, 600, 500, 400, 300, 200, 100, 50.
13. What sizes are the pUC19 DNA fragments?

RESULTS

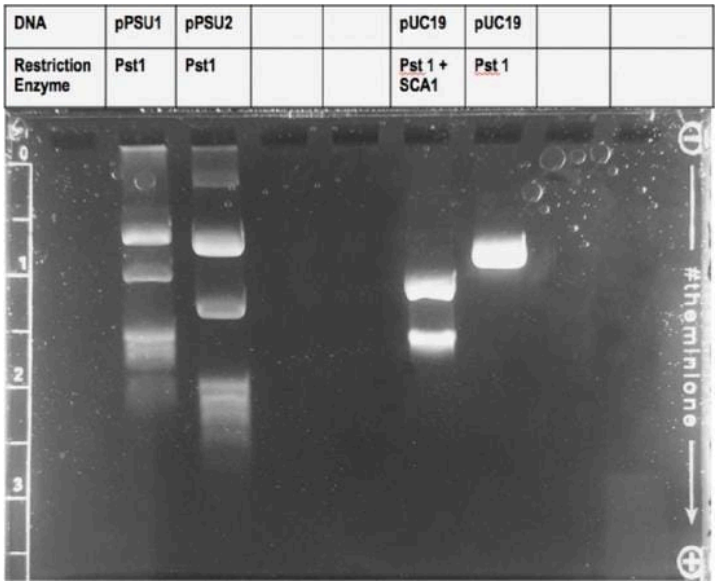


Figure 7: Restriction digests after electrophoresis

Study Questions

1. In nature, what is the function of restriction enzymes?
2. What is a palindrome? How does that relate to restriction enzymes?
3. Why do molecular biology research labs always have microwaves?
4. Why should you not ever eat in a molecular biology research lab?
5. How do you dispense samples into an agarose gel?

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2.2.12: Transformation

Learning Objectives

Goals:

- Explain how the information encoded in a gene is expressed as a *trait*
- Describe the role of transformation in cloning genes
- Explain the purpose of each control in the transformation experiment

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Carryout a transformation
- Predict the growth results for the negative control and plasmid containing reaction on both antibiotic-containing and nutrient agar only media
- Explain your reasoning, if predicted growth results don't match actual growth results

Introduction

Genetic engineering or DNA technology has been useful for producing large quantities of a specific protein to treat human diseases. For example, patients with diabetes, hemophilia, or anemia require treatments with insulin, clotting factor, and growth factor proteins. Targeted genes (DNA) can be cut with restriction enzymes and joined with other DNA with the enzyme ligase. A cloning vector is used to carry the recombinant DNA into living cells, so that the cells can synthesize the encoded proteins. The best cloning vectors are small in size, able to replicate its DNA, contain restriction enzyme recognition sites, and have a marker gene (usually antibiotic resistance gene). In this lab, we will use a recombinant plasmid as the cloning vector. This recombinant plasmid contains (1) a *promoter* that enables **transcription** of desired gene, (2) a sequence for the initiation of DNA replication (*ori* site), and (3) an antibiotic resistance gene.

Transforming Bacteria with Recombinant Plasmid

Inserting a gene into a plasmid vector is an important first step in the gene cloning process. However, if the ultimate goal is to produce a large amount of a particular protein, the plasmid must replicate to make sure that there are many copies of the gene and the gene of interest must be *expressed,* meaning the gene is utilized to produce the encoded protein. Both activities can only occur inside a cell. Therefore, in this lab we will put a **recombinant** plasmid into *E. coli* bacteria through a process that is called *transformation*, so named because it changes the DNA content of the bacteria.

The plasmid will be taken up by bacteria where it replicates, and its genes will be expressed using the bacterial cellular machinery. If a gene of interest has been inserted into the plasmid vector, the bacteria produces the product encoded by that gene.

In this exercise, you will carry out the transformation of ***E. coli*** bacteria using a recombinant plasmid that contains a gene that produces colored proteins.

Bacterial Transformation

Once a recombinant plasmid is made that contains a gene of interest, such as insulin, the plasmid can enter bacterial cells by a process called transformation. **Figure 13.1** illustrates transformation. The uptake of DNA from the environment of a bacterial cell occurs with a very low efficiency in nature. *E. coli* bacteria have complex plasma membranes that separate the external environment from the internal environment of the cell and carefully regulate which substances can enter and exit the cell. In addition, the cell wall is negatively charged and repels negatively charged DNA molecules.

Cells that have been treated to become *competent* are more efficient at taking in DNA from their surrounding environment. Competent cells can be made by treating the bacteria with a calcium solution. Calcium ions are positively charged, and will neutralize the negatively charged outer membrane on the *E. coli* bacteria. With the positive charge now coating the membrane, the inherently negatively charged DNA molecules will move through the plasma membranes and into the cell. The transformation efficiency can be further increased by stressing the cells in a heat shock. By changing the temperature of the cells drastically from cold to warm, the plasma membranes become more fluid and create pores in them. The plasmid DNA can travel from the environment through these pores and enter the cell. The cells are then plunged back into a cold temperature, which causes the pores to close and the plasmid DNA to remain inside the cell.

However, even competent cells do not always uptake the plasmid. For some plasmid DNA molecules, only about 1 in 10,000 cells will be transformed. When so few cells have taken in the plasmid, how will you be able to identify transformed cells? When designing a recombinant plasmid, one of the requirements is to add a gene for an antibiotic resistance. This way, the bacteria can be grown in the media with an antibiotic added to it, and only cells that have the resistance gene, such as those that express the recombinant plasmid, will be able to grow.

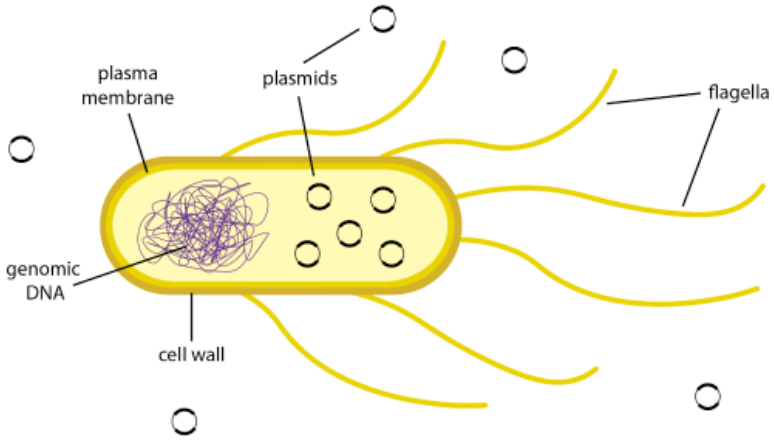


Figure 1. Bacterial transformation.

From Plasmid DNA to Protein

After a recombinant plasmid enters a bacterial cell, the cell begins to express the genes on it. DNA polymerase locates the *ori*- the origin of replication, and starts to replicate the plasmid using the bacterial cell's machinery. Multiple copies of the recombinant plasmid can enable the bacterial cell to express large amounts of a protein. Usually, a bacterial cell will only make the protein of interest, after it is induced to do so by adding a chemical which will promote the transcription of the gene. Recall that to express the gene encoding the protein on the recombinant plasmid, DNA is transcribed to mRNA, which is then translated to protein (Figure 13.2). The expressed proteins may affect the visible traits when observing the bacteria colonies.

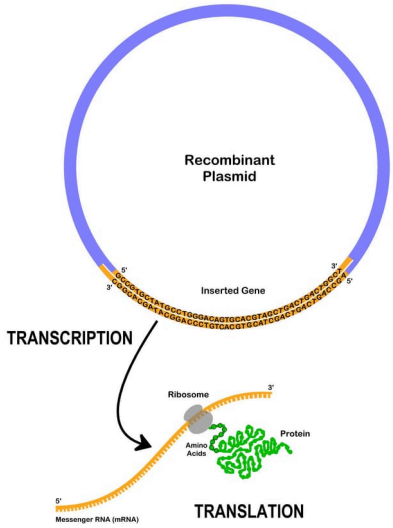


Figure 2. Gene expression from a plasmid in the bacterial cell

Recombinant plasmids and other forms of genetic engineering is possible because all living organisms use DNA as a platform to encode genetic information. Genes from different organisms can be expressed in other organisms like bacteria since they are encoded in DNA. The DNA instructions can be transferred, and other organisms can express foreign traits.

Proteins have many different functions inside and cells. They are made up of smaller subunits, **amino acids**, which are encoded by DNA **nucleotides**. A specific three nucleotide sequence that codes for a single amino acid is called a **codon.** For example, the codon TTG codes for the amino acid tryptophan, whereas the codon AAG codes for the amino acid lysine. In many cases, more than one codon can encode the same amino acid. For example, AAA is also a codon for lysine. In addition, there are informational codons, such as the *start codon* (ATG) and the *stop codon* (TTA), which show where in the DNA sequence the code for the protein begins and ends.

Transforming Bacteria with Plasmids

In this laboratory experiment you will transform *E. coli* bacteria cells with plasmids. You will be using *E. coli* that has been made competent with a calcium chloride treatment, and form two different testing groups: a negative control cell group that does not have plasmids added to it, and the experimental group that has the plasmids added. After the cells are heat-shocked, they will be grown under various testing conditions:

- The control group on nutrient agar (a type of growth media that bacteria thrive on).
- The control group on nutrient agar with an antibiotic added.
- The experimental group on nutrient agar.
- The experimental group on nutrient agar with an antibiotic.
- The experimental group on nutrient agar, antibiotic and an inducer (such as IPTG).

By examining the growth of bacteria under these conditions, you can verify that your procedure worked, and you can identify the bacteria transformed with the added plasmid. How will you know if you are successful? In the examples for plasmids we have recommended for this exercise, the recombinant bacteria will have a new and highly visible trait: It will now produce colored protein, which makes the cells themselves colored! As the bacteria multiply on the media, they form visible collections of cells called colonies. Each colony represents the decedents of the original bacterial cell that landed on that spot on the medium and began to replicate. Thus colonies are clones (exact copies) of the cell that began the replication process.

The relevant components of your plasmid are the gene for the colored protein, the inducible promoter, and the ampicillin resistance gene (*ampR*). The *ampR* gene confers resistance to the antibiotic ampicillin. (Biotechnologists call these genes *selectable markers* because only bacteria having the gene will survive in the presence of an antibiotic.) If the inducer is present in the bacteria, the promoter will be “turned on” so RNA polymerase can transcribe the gene of interest. This will allow protein to be produced.

Prelab Questions

Discuss the following amongst yourselves. Be ready to share your thoughts with the rest of the class.

1. Ampicillin is a derivative of the antibiotic penicilliin. It disrupts cell wall formation in bacterial cells which kills the cells. However, our recombinant plasmid contains a gene that provides antibiotic resistance by producing a protein that breaks down ampicillin. Why do we include ampicillin in the test medium?
2. What will happen if the transformed cells do not grow in the presence of the chemical inducer?
3. In the experiment, you will add the control and experimental groups of cells onto different media combinations. What do you predict for each condition? Fill in **Table 1** by indicating if you predict growth or no growth, and if growth, will there be minimal growth or lots of growth.

Read through the Procedures below and outline the steps, using words and a flowchart in your lab notebook.

Table 1. Predictions for your experiment; transformation of E. coli

Medium	No plasmid control	Treatment with plasmid
Nutrient Agar		
Nutrient Agar + Ampicillin		
Nutrient Agar + Ampicillin + Inducer		

Transforming *E. coli*

MATERIALS

Reagents

- Plasmid – in microfuge tube
- Nutrient Broth (NB) – in microfuge tube
- Competent E. coli cells (CC) – in microfuge tube (Always keep CC tube on ice)
- 3 agar plates:

Plate 1: NA

Plate 2: NA/amp

Plate 3: NA/amp/ind

Supplies and Equipment

- P-20 micropipette
- P-200 micropipette
- Pipette tip box (for P-20, P-200)
- Microfuge tube rack
- Two 1.5 mL microfuge tubes
- Permanent marker
- Disposable gloves
- Crushed ice in a Styrofoam cup (fill cup first with ice before taking CC tube.)

- Pack of cell spreaders (do not remove spreaders from pack until directed to do so)
- Timer or clock
- Floating microfuge tube rack
- 42°C water bath
- 37°C incubator
- Tape
- Waste container
- Biohazard bag (for supplies that handle cells)

SAFETY

Check your protocol and follow all safety measures and wear proper attire prior to conducting the experiment.

Practice *aseptic technique* while using *E. coli* or other live specimens in a laboratory setting. Aseptic technique is the practice of taking precautions to limit potential contamination to both the person performing the experiment, and to the sample/s. Please note the following:

- Wear gloves when working with bacteria.
- Avoid touching contaminated areas which includes anything that has touched bacteria. Notify your instructor ASAP if an accident takes place, such as a spill.
- Put all supplies that have been exposed to bacteria into either a biohazard bag, or a designated biowaste container. These contaminated supplies may include pipette tips, cell spreaders, and microfuge tubes.
- Keep agar plates closed at all times after removing them from the incubator.
- Always wash your hands for 20 seconds with soap and water before leaving the lab.

PROCEDURE

1. Make sure you have all the reagents in a tube rack.
2. Retrieve a chilled CC tube and put in the cup of crushed ice. **Keep the competent cells cold at all times. Hold the tube by the rim, not the bottom.**
3. Label the top and sides of two new microfuge tubes with “P-” and “P+”.

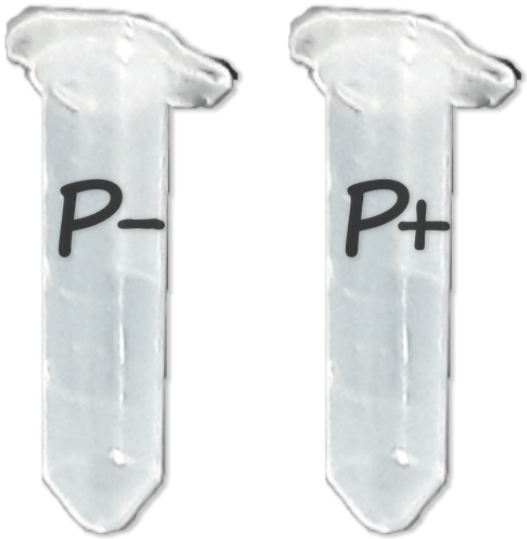


Figure 3. Labeled microfuge tubes

4. Put the P– and P+ tubes with the CC tube on ice.
   To ensure the best results possible, it is crucial that each step is followed exactly. Limit any possible contamination to the materials, yourself, and surroundings.
5. Add *E. coli* competent cells (CC tube) to both the P- and P+ tubes.
6. Take the P-200 pipette, set to 50 µL and put on a tip.

7. Holding the CC tube by the rim, gently resuspend the cells by slowly pumping between the first and no stop with the pipette (a gentle downward plunger motion to the first resistance point then a gentle upward motion to the top plunger position).
8. Add 50 µL of cells to the P+ tube and place the P+ tube on ice immediately. Discard the tip into the sharps container.
9. Grab a new pipette tip. Repeat for the P- tube. Discard the tip. Both P- and P+ tubes should be on ice and contain 50 µL of competent cells.
10. Add plasmid to the P+ tube only.
    a. Take the P-20 pipette, set to 10 µL and put on a tip.
    b. Remove 10 µL of the plasmid and add it to the P+ tube. Mix together by slowly pumping between the first and no stop 2-3 times then place the tube back on ice.
11. Put the P- and P+ tubes on ice for 15 minutes.
12. While the tubes are chilling, obtain your agar plates and marker. Do not open the plates during this step
    a. Each agar plate contains different media- one of Nutrient Agar only (NA), one of Nutrient Agar + ampicillin (NA/AMP), and one of Nutrient Agar + ampicillin + inducer (NA/AMP/IND). These may be labeled with a stripe pattern or written on the plate
    b. Do not open the plates. Turn every plate upside-down; the agar should be on top. Label the agar side with 1) date 2) group number 3) class period. Try to write small along the bottom edge of the plate.
    c. Next, draw a line down the middle of the NA and NA/AMP plates. One half is labeled as “P-” and the other is “P+”. The NA/AMP/IND is only labeled “P+”. They should look similar to Fig 4

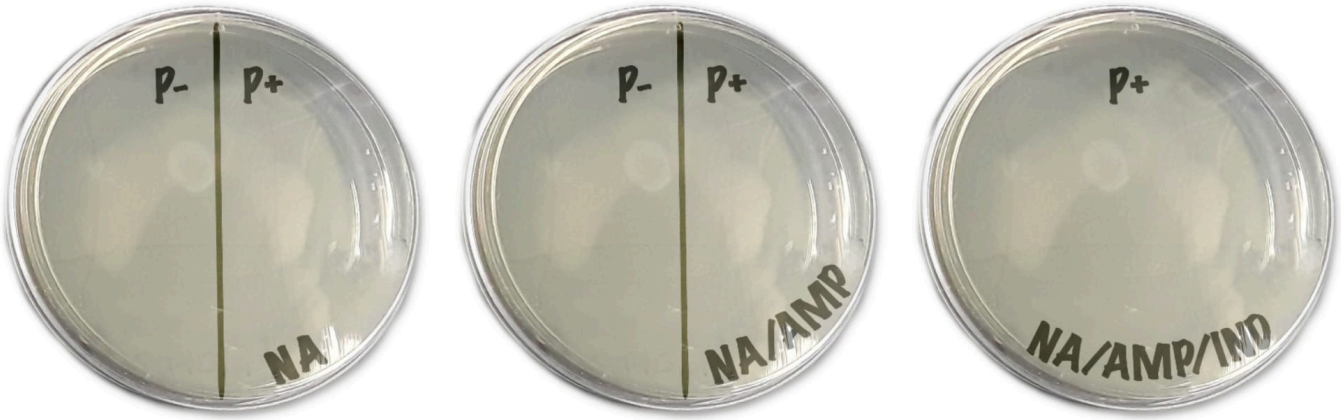


Figure 4, Labeled plates

13. After the P- and P+ tubes have been on ice for 15 minutes, keep the tubes on ice and bring the ice cup over to the 42°C water bath. You will also need your timer/clock. Put both tubes into a floating microfuge tube rack, then place it into the water bath for precisely **45 seconds.**
14. As soon as the 45 seconds pass, immediately put the tubes back into the ice cup and keep on ice for a minimum of 1 minute.
15. Add Nutrient Broth (NB) to the P- and P+ tubes.
    1. Take the P-200 pipette, set to 150 µL and put on a tip.
    2. Remove 150 µL of NB and add to the P- tube. Mix together by slowly pumping between the first and no stop with the pipette. Discard the tip into the biowaste container.
    3. Get a new pipette tip. Repeat the same process for the P+ tube.
16. Keep the tubes at room temperature for 15 minutes. If you are running short on time, this step can be shortened.
17. Add cells from the P– tube onto your NA and NA/amp plates. Keep the plates right side up so the agar is on the bottom. You add cells to the surface of the agar (not the plastic lid).
    a. Take the P-200 pipette, set to 50 µL and put on a tip
    b. Take the P- tube. Slowly pump the pipette between the first and no stop with the pipette to resuspend the cells. Remove 50 µL of the cells.
    c. Lift the lid of the NA plate like a “clamshell” to leave a large enough gap to deliver the cells, while lowering the risk of airborne contamination. Add the 50 µL of cells to the P- half of the plate. Close the plate and prepare to spread the cells.
    d. Repeat this for the NA/AMP plate by resuspending the P- tube cells with the pipette and same tip. Add 50 µL of the cells to P- side of the NA/AMP plate using the clamshell method again. Discard the tip into the sharps container.

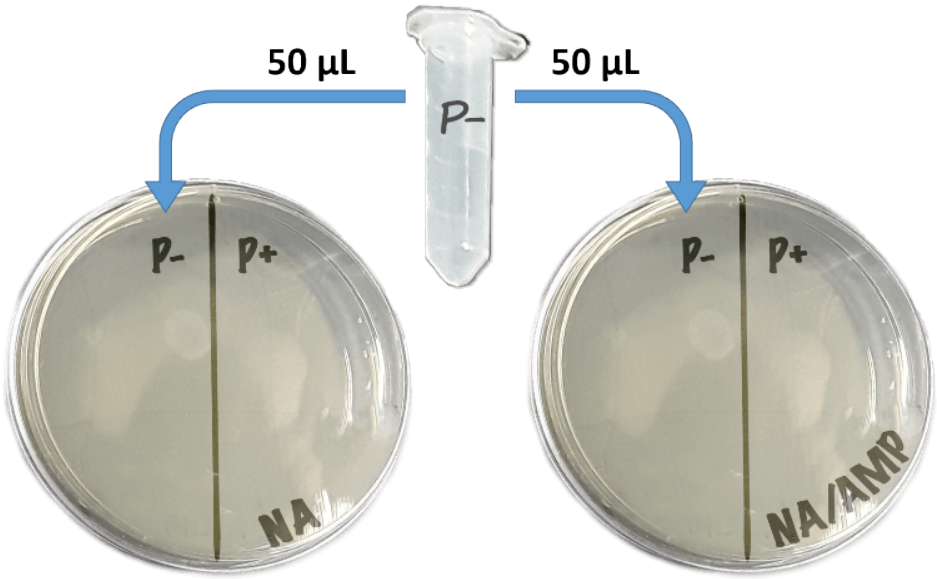


Figure 5. How to distribute the no-plasmid control treatment on the media

18. Spread the cells from the P– tube on your NA and NA/amp plates. You must spread your plates in this order.
    a. Open the sterilized cell spreader package. Take a single spreader out and hold it only by the end you removed it by. Be careful not to touch the other end of the spreader to anything but the cells and agar. Close the package.
    b. Using the "clamshell" method, open the NA lid and spread the cells on the P- half of the plate. Gently hold the spreader flat against the surface of the agar; treat it gently as if you were handling gelatin. Close the plate.
    c. Repeat the same technique for the NA/AMP plate on the P- side using the same spreader. Once you are finished, discard the spreader into the designated biowaste container.
19. Add cells from the P+ tube to your NA, NA/amp, and NA/amp/ind plates:
    a. Take the P-200 pipette, double check it is set to 50 µL and put on a tip.
    b. Take the P+ tube. Slowly pump the pipette between the first and no stop with the pipette to resuspend the cells. Remove 50 µL of the cells.
    c. Open the NA plate again like a clamshell to deliver 50 µL of cells to the P+ half of the plate. Close the plate.
    d. Repeat this for the NA/AMP plate by resuspending the cells with the pipette and same tip. Add 50 µL of cells to the P+ half of the plate. Close the plate.
    e. Repeat this for the NA/AMP/IND plate by resuspending the cells with the pipette and same tip. Add 50 µL of cells **two times** to the entire plate. There is a total of 100 µL of P+ cells added to the plate and must cover the entire surface when spread. Close the plate.

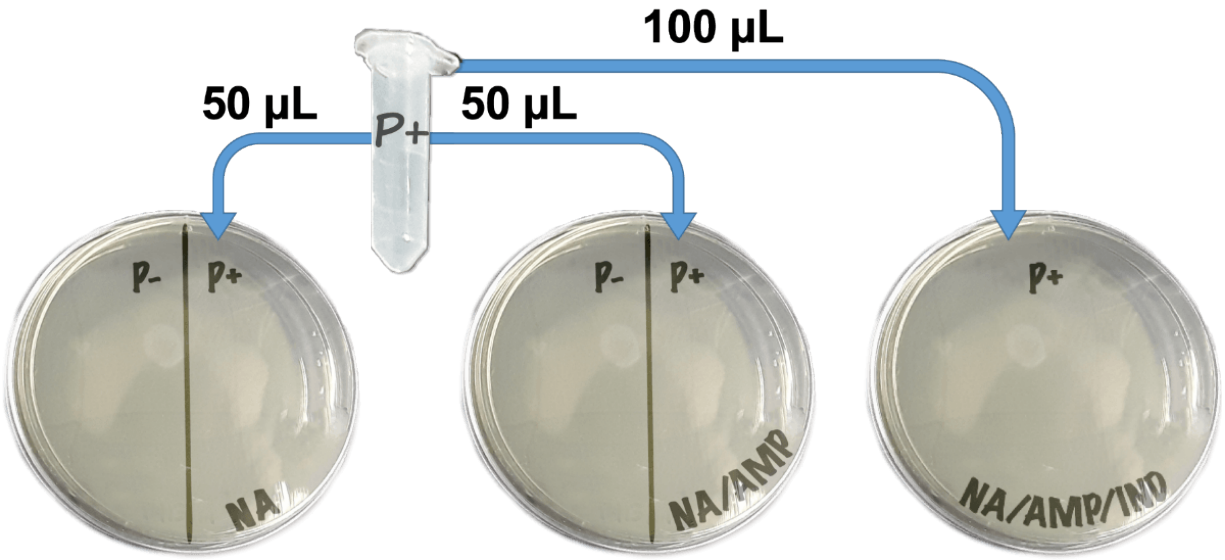


Figure 6: How to distribute the plasmid treatment onto media.

20. Spread the P+ cells on the NA, NA/AMP and NA/AMP/IND plates. You must spread your plates in this order.
    a. Open the cell spreader package again and take out a single spreader, only touching the handle. Do Be careful to not touch the other end of the spreader to anything but the cells and agar.
    b. Using the clamshell method, open the NA lid and spread the cells on the P+ half of the plate. Gently hold the spreader flat against the surface of the agar; treat it gently as if you were handling gelatin. Close the plate.
    c. Using the same spreader, repeat the same technique for the NA/AMP plate on the P+ side .
    d. Repeat the same technique for the NA/AMP/IND plate except the cells must be spread across the entire plate, not just one half. Rotate the plate gently to evenly disperse the cells with the spreader. Once you are finished, discard the spreader into the designated biowaste container.
21. Keep all plates right side up for 5 minutes until the liquid is fully absorbed into the plate. Stack the plates together and tape them, labeling the tape with your class period, group number and date.
22. Put the plates upside down (agar side on top) into the 37°C incubator to prevent condensation from forming and falling on the cells.
23. Put anything that touched the cells into the biohazard bag, including pipette tips, microfuge tubes and cell spreaders. Wipe down tabletops with disinfectants and wash hands.
24. Incubate the plates at 37°C for 24-36 hours and look for growth. Keep the plates closed.
25. Put the agar plates into the biohazard bag when told to do so.

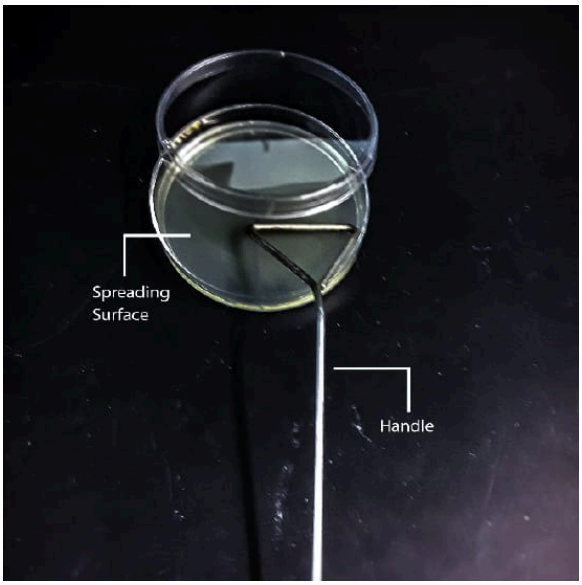
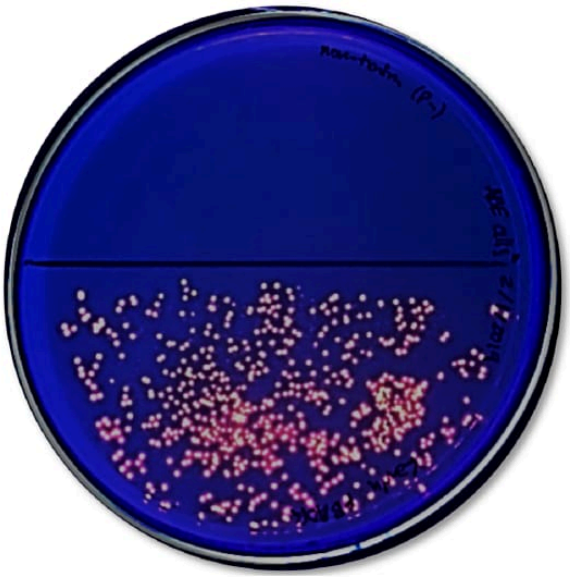


Figure 7. Spreading the *E. coli* onto the medium surface


Figure 8. *E. coli* transformed with a plasmid containing a gene encoding fluorescent protein growing on NA+AMP+IND

Analysis

1. Look at the results of your transformation. Fill out **Table 2** with observations on whether you see growth or not on the different media.
2. Do your actual results match your predicted results? If not, what differences do you see and what are some explanations for these differences?
3. How many colored colonies were present on your NA/AMP/IND plate?

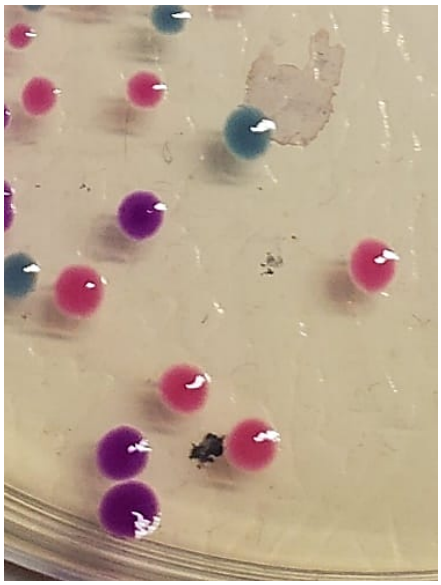


Figure 9. Transformed *E.coli* producing colored molecules on NA+AMP+IND

Table 2. Transformation of E. coli

Medium	No plasmid control	Treatment with plasmid
Nutrient Agar		
Nutrient Agar + Ampicillin		
Nutrient Agar + Ampicillin + Inducer		

Study Questions

1. Why would colored colonies form on the NA/AMP/IND plate and not the NA/AMP plate?
2. What are some possible reasons for colored colonies to form on the NA/AMP plate?
3. Extrachromosomal DNA in bacteria, like a recombinant plasmid, can replicate within the cell without the rest of the cell's DNA replicating. This can lead to multiple plasmids within a cell. Why is this important?
4. Previously, you learned about the interactions between DNA, RNA, proteins and traits. Explain how an inserted gene such as one in plasmid DNA, is expressed as a trait.
5. How can bacteria make proteins from foreign DNA, such as human insulin or a protein from a jellyfish like green fluorescent protein (GFP)?
6. What would happen if we grew the transformed bacteria in the presence of a different antibiotic such as kanamycin, instead of ampicillin?

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2.2.13: Column Chromatography

Learning Objectives

Goals:

- Explain the conditions for bacterial growth and relate it to the goal of collecting protein.
- Explain what is meant by protein folding.
- Describe the relationship between a protein's *conformation* (three-dimensional shape) and function of the protein.
- Use column chromatography to separate proteins.

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Lyse bacterial cells to release protein.
- Purify GFP via hydrophobic interaction column.
- Identify the location of GFP throughout the purification process.

Introduction

Proteins are biological molecules made of chains of amino acids that take on a three-dimensional shape called a **conformation**. A specific conformation is achieved by folding of the amino acid chain in a way that the protein can perform a specific job or function for the cell. If the protein does not fold in the specific way, it cannot do the job it otherwise would. Proteins can be purified and used as therapeutic agents (drugs) to treat patients with specific conditions. Because purifying specific human proteins can be difficult or impossible from human or animal tissues, we can employ cells such as bacteria like *E. coli* to do the job. These cells are genetically engineered to make the proteins of interest in large amounts that are more easily purified.

An example of a therapeutic protein is insulin, used to treat diabetes. This was the first genetically engineered protein to be introduced as a therapeutic agent. The gene for human insulin was engineered into a plasmid and this recombinant plasmid was introduced into *E.coli*. The bacterial cells made insulin which was purified to be used for treating diabetes.

Similarly, green fluorescent protein (gfp) can be produced and purified. Prior to this lab, the *gfp* gene was introduced by the process of genetic engineering into a recombinant plasmid capable of allowing *E. coli* to produce protein from the genetic instructions. Producing protein from a gene is termed **expression**. This recombinant plasmid was introduced into *E. coli* using the process of transformation. This laboratory uses the process of obtaining purified green fluorescent protein to model how therapeutic proteins are purified for human treatments.

You will be provided a culture of bacteria that have been grown and induced (stimulated) to produce gfp. You will treat the bacteria with a solution that will lyse (break open) the cells to release the protein. Then you will employ the process of column chromatography, the focus of this laboratory. Column chromatography is a method of allowing a solution to flow over a substance that will selectively bind and separate the components of the solution. In this case, tiny beads are packed into a tube-like column and the solution obtained through bacterial lysis is allowed to flow over the beads. As you pass other solutions over the column, you will be able to collect some of the solution flowing through the beads which will contain a much more concentrated and pure gfp.

Growing Bacterial Cells

The pattern of growth for bacterial cells is well understood. In the laboratory setting, the goal is to grow cells and have them produce the protein we are interested in purifying. When using optimal conditions to grow *E. coli,* four phases of growth will occur (see Figure 1)

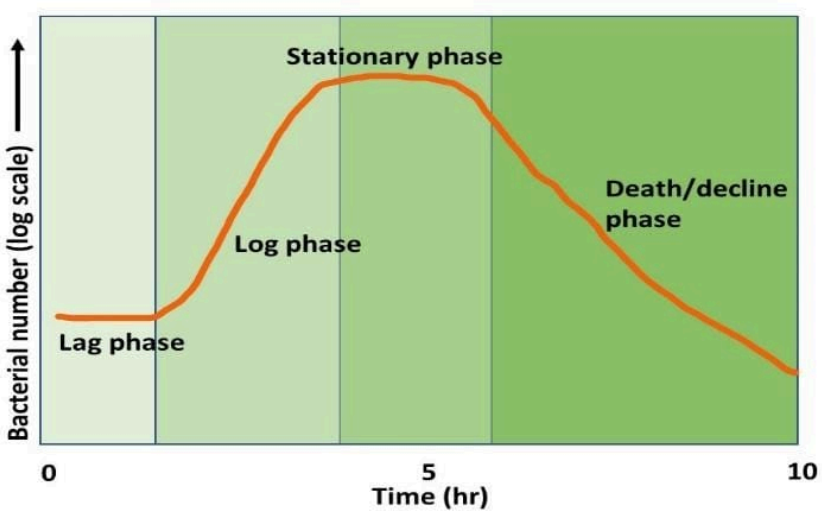


Figure 1. Bacterial growth curve outlining change in growth over time

1. During *lag phase* no cell division occurs. Cells are preparing to divide by making new enzymes and proteins as well as copies of their DNA. Cells enlarge.
2. In the *log phase,* a doubling of the population is occurring. This is termed logarithmic growth. Cells undergo binary fission (cells divide in half) approximately every 20 minutes for *E. coli* under optimal conditions. Other types of bacteria, and *E. coli* under less than ideal conditions, will divide at different rates.
3. The *stationary phase* occurs after the log phase when there is no overall change in the population size. During this period, cell division is equivalent to cell death and happens as resources such as nutrients and oxygen are depleted, and waste products are building up in the environment.
4. During the decline or death phase of a bacterial culture, cells are dying faster than they replicate. The cell population is decreasing. This occurs as waste builds further and the food supply is exhausted.

CONSIDER: If the gene of interest is controlled by an **operon**, such as the lac operon, when is the best time to turn on the gene? Keep in mind:

- Production of the protein takes energy away from the processes of cell growth and cell division.
- A greater number of cells will produce more protein
- **Proteins can degrade over time**

Pre-lab Activity: Think Pair Share - Discuss What You Already Know

Prior to the lab period you should read the material, take some notes on your thoughts on the following questions (Think). In lab, you will be asked to discuss with your partner your thoughts and hear theirs (Pair). Take additional notes on new insights during this small discussion. Finally, you may be asked to discuss your ideas with the class (Share). It is not expected that you know all the answers before the class discussion but by the end of the activity, you should have the questions answered completely and correctly.

1. What is the term for bacterial reproduction? Describe this process.
2. What roles do proteins play within cells?
3. What happens to protein function if a protein loses, or never correctly achieves, the prescribed conformation?
4. How does the order of amino acids relate to protein conformation and thus protein function?
5. Since we can control when to “turn on” or express our gene, when is the best time to do so? To help you decide ask yourself:
    a. Would producing protein take energy?
    b. Would a greater number of cells produce more of your protein of interest than fewer cells?
    c. Can proteins degrade if left too long?
6. Compare your flow charts with each other prior to beginning lab. Note any differences and attempt to resolve which flow chart(s) has/have more accurate information. Adjust your own flow chart as necessary.

Protein Purification

Transformed bacteria can multiply in culture and produce the protein of interest. If this protein is to be used therapeutically, it will need to be purified. This means that other cellular components, including other proteins, must be separated from your protein. Column chromatography is a common method to separate proteins.

Proteins are made of amino acids. Individual amino acids have different properties such as hydrophobicity (water-hating) or hydrophilicity (liking water), ionic charge, or the ability to form weak or strong bonds with other amino acids. When a protein is first made in a cell, it is a long chain of amino acids in an order determined by the gene. The order of the amino acids in a protein determines how the chain will fold to produce a three dimensional protein conformation (See Figure 2). This specific conformation will have different amino acids interacting with each other in specific ways. Amino acids facing the environment in a folded protein can interact with other molecules. Specific groups of amino acids near each other can form binding sites to interact with other specific molecules. Overall, these relationships determine protein structure and thus protein function. Imagine proteins involved in enzymatic reactions, as channels in membranes, in transporting other molecules, or for binding DNA. These proteins all have very specific binding interactions determined by amino acids in specific locations in a folded protein.

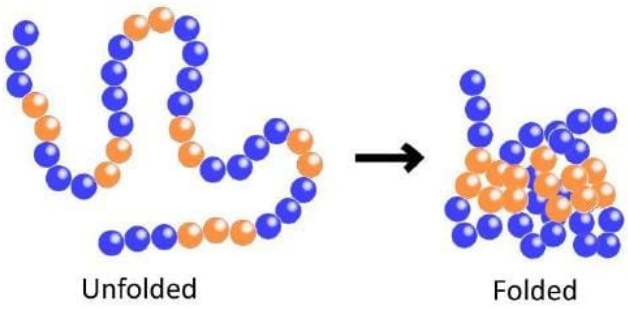


Figure 2. The folding of a protein

QUESTION: If individual amino acids are swapped or deleted in an amino acid chain, do you imagine this would affect the function of a protein?

The rules for protein folding are not perfectly understood and is an area of active scientific investigation. However, a few basic rules have been discovered. Factors that cause proteins to fold in specific ways include:

1. Weak bonds will form between amino acids with a negative and a positive charge.
2. Strong (covalent) bonds will form between sulfur-containing amino acids. These are called disulfide bridges.
3. Hydrophilic amino acids locate to the outer surfaces of proteins because they interact with the cell environment, which is mostly water. Hydrophobic amino acids hide on the inside of proteins or embed within cell membranes to avoid contact with the water in the environment.

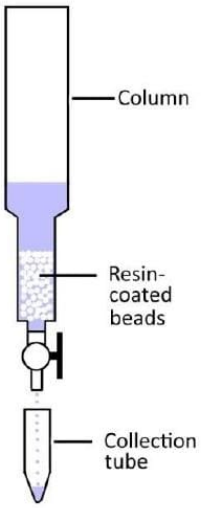


Figure 3. Liquid column chromatography

Depending on the content of amino acids in a specific protein, overall it will take on a hydrophobic or hydrophilic character. Column chromatography can separate hydrophilic and hydrophobic proteins from the rest of the cell contents. Small beads coated with a material called a resin are packed into a column. The resin attracts proteins which will bind to the resin as other cell contents flow past. For hydrophobic proteins to stick, they must be treated to expose the typically buried hydrophobic amino acids. Buffer solutions can be used to cause proteins to denature (unfold) and expose the amino acids that will be attracted to the resin.

Different buffers are passed over the column in an order determined to best separate the proteins of interest from the rest of the cell contents. Figure 14.4 shows three solutions used to separate green fluorescent protein from the rest of the cell. The binding buffer denatures proteins so that the hydrophobic amino acids stick to the resin. The wash buffer removes loosely adherent proteins and material from the column leaving the more strongly attached protein of interest. Finally, the elution buffer, which has a low buffer concentration, causes the protein to begin to refold to hide the hydrophobic amino acids which releases the protein from the resin coated beads in the column. The portion or *fraction* of fluid exiting the column that contains your protein can be captured in a container and saved.

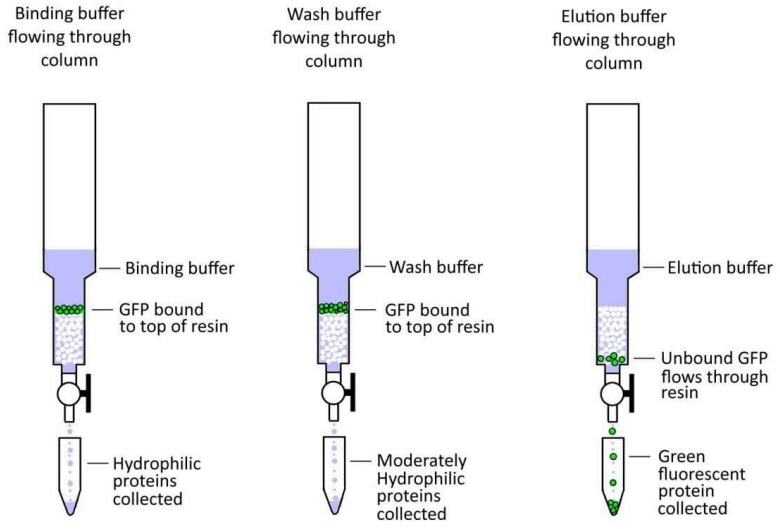


Figure 4. Separation of green fluorescent protein by hydrophobicity using column chromatography

QUESTION: Do you believe that all types of protein would use the same types of resin-coated beads and the same types of buffers to become purified? Explain your answer.

Part I: Lysis of Bacterial Cells

Previously, bacteria were transformed with a recombinant plasmid capable of expressing gfp when cells were induced. The reason the cells can be induced to produce protein is that within the plasmid, in front of the gene for gfp, there is a special sequence of DNA that will respond if a chemical is placed in the media. This chemical is called an inducer (ind) and signals that the gene should be “turned on” and messenger RNA should be transcribed from the DNA instructions and the protein should be produced. The plasmid also contains the selectable ampicillin (amp) resistance gene to ensure that the cells growing in your culture are cells that contain your plasmid.

Prior to this lab period, cells were grown in the presence of ampicillin until late in log phase. The cells in culture divide and each cell contains many copies of the plasmid. At late log phase, the chemical inducer was added to the medium to turn on the *gfp* gene and the cells were allowed to continue to grow and produce gfp.

First, you will collect your cells and break them open. This process is called cell lysis. After the cells are lysed, you will use column chromatography to purify gfp.

MATERIALS

Reagents

- Microfuge tube rack with the following tubes:
  - LB/amp/ind culture of *E. coli* cells (EC)
  - Elution buffer (EB)
  - Lysis buffer (LyB)
- Extra 1mL of the EC culture – from the instructor

Equipment and Supplies

- P-200 micropipette
- Pipette tip box
- Permanent marker
- Microcentrifuge (shared with class)
- Vortex mixer (shared with class)
- Long wave UV light
- Liquid waste container
- Sharps container
- Biohazard bag for materials that come into contact with *E. coli* cells (shared with class)

Safety Reminders

Appropriate safety precautions should be used at all times. These will be reviewed by your instructor and can be found in the beginning of your laboratory manual which you should refer to before you begin this procedure. Aseptic technique is required when handling *E.coli* and materials that have come in contact with the bacterial culture. Remember that aseptic technique are the procedures used to protect your culture and samples from contamination but also protect you.

1. Disinfect your work area and wash your hands before beginning an experiment.
2. Never touch anything that has come in contact with the *E.coli*. This includes pipettes, spreaders, and the interior of tubes. Pipet tips should never touch anything except the material to be transferred. Spreaders and pipettes should only be handled from the end that will not touch bacteria.
3. When handling petri dishes, only open the lid enough to work with the agar surface and then close the lid immediately. This will avoid contamination, such as fungal spores from the air, landing on your agar plate.
4. If something becomes accidentally contaminated, speak to your instructor to inquire if a replacement is appropriate and available.
5. Avoid spills. If one occurs, notify your instructor immediately for help in cleaning it appropriately.
6. Contaminated waste such as used microfuge tubes and cell spreaders will be placed in the biohazard bag. Pipet tips will be placed in a sharps container.
7. Only when directed to do so will you dispose of your used petri dishes in the biohazardous waste.

8. Be sure to clean your work area and wash your hands before exiting the lab.

Procedures

1. Take a long wave UV light and look at the EC tube, record your observations.
2. Weigh your EC tube. Look for another tube with a similar weight; +/- 0.1g or create a balance tube for the microcentrifuge.
3. Spin the EC tube for 5 minutes at 13,000 rpm (or as high speed as possible) in a microcentrifuge. **Make sure to balance the tubes correctly.**
4. Very carefully take out the EC tube from the microcentrifuge. Avoid disturbing the cell pellet at the bottom of the tube.
5. Take the P-200 micropipette, set it to 200.0 µL and get a tip. Press to the first stop before going into the supernatant (liquid layer) and gently pull out the old liquid growth media. Do not disturb the cell pellet when doing so.
6. Discard the liquid into the liquid waste container, and the tip in sharps container.
7. Bring your cell pellet (the EC tube) to your instructor to dispense 1 ml of the same culture into your tube.
8. Repeat steps 2-6, so spin down the cells for 5 min again and remove the supernatant. Record the color of the supernatant and pellet at this step.
9. Take the P-200 micropipette and a new tip and carefully try to fully remove all the liquid from the pellet without taking up the cells. Discard the tip in sharps.
10. Set the P-200 to 150.0 µL and get a new tip. Add 150 µL of elution buffer (EB) to the EC tube. Discard the tip.
11. Firmly close the EC tube and resuspend the cell pellet with a vortexer. If one is unavailable, drag the tube quickly across an empty microfuge tube rack. This should cause the cell pellet to dislodge from the bottom of the tube and the buffer should become turbid. Repeat this movement until the entire pellet is gone.
12. Take the P-200 and get a new tip. Add 150 µL of lysis buffer (LyB) to the EC tube. Mix the tube contents with a vortexer or the microfuge tube rack method like previously.
13. The EC tube will incubate in the lysis buffer overnight at room temperature. Label your tube with class period and group number and give to your instructor to do this step.
14. Clean your work area and discard all contaminated tubes and tips into the appropriate biohazardous waste.

Part II: Using Column Chromatography to Separate the Green Fluorescent Protein

Materials

Reagents

- Microfuge tube rack with the EC tube that contains cells, elution buffer, and lysis buffer
- Bottles of:
- Binding buffer (BB) = 4.0 M Ammonium sulfate solution
- Equilibration buffer (EQ) = 2.0 M Ammonium sulfate solution
- Wash buffer (WB) = 1.3 M Ammonium sulfate solution
- Elution buffer (EB) = 10 mM Tris, 1 mM EDTA, pH 8.0 solution
- 20% Ethanol solution (For cleaning and storing resin in columns at the end of lab)

Equipment and Supplies

- P-1000 micropipette
- Pipette tip box
- 2-3, 1.5 mL microfuge tubes
- Chromatography column
- Microcentrifuge (shared)
- Liquid waste container
- Biohazardous waste container
- Sharps container

Procedure

1. Divide the work by assigning one person to do step 2-3, another 4-5, and a third do 6-7.
2. Verify that you have all the necessary materials.
3. Label one microfuge tube as “SUPER” and another as “GFP”.
4. Set up your column as directed, always maintain it in an upright position. **Do not ever allow the column resin to go completely dry**.
5. To set up the column:

    - Take off the caps at the top and bottom of the column. Do not confuse the bottom cap with the stopcock.
    - Place the column liquid waste container at the base of the column.
    - Turn the stopcock valve to a vertical position and drain the column until 1-2mm of liquid remains above the column resin. Turn the stopcock to a horizontal position to close the valve.
    - Take the P-1000 micropipette, attach a tip, and set to 1000 µL. Add 1000 µL of equilibration buffer (EQ) gently down the side of the column, trying to disturb the resin bed as little as possible.
    - Drain the column until 1-2mm of liquid remains above the column resin then close the valve.
    - Using the same tip, add another 1000 µL of equilibration buffer (EQ) gently down the side of the column, trying to disturb the resin bed as little as possible. Discard the tip afterwards.
    - Drain the column until 1-2mm of liquid remains above the column resin then close the valve.
    - Double check that the liquid stopped draining when the valve is closed.

6. Weigh the EC tube, find a balance tube, and spin the tube down for 5 min at 13,000 rpm (or the highest speed) in a microcentrifuge. **Be sure to balance the microcentrifuge**.
7. Carefully remove the tube and bring it back to your workspace. Record your observations when using a long wave UV light to examine the tube. What do you observe regarding the pellet and supernatant?
8. Take the P-1000 micropipette, set to 250 µL and get a tip. Very carefully, try to remove as much of the supernatant and transfer into the labeled “SUPER” tube. If the pellet is disturbed during this step, spin down the tube again and repeat this step. Discard the empty tip in sharps.
9. Get a new tip and add 250 µL of binding buffer (BB) to the SUPER tube. Gently pipette up and down two times to mix the solution. There should be approximately 500 µL of liquid total now.
10. With the same tip, add 200 µL of the SUPER tube solution to the column **two times**. The entire contents of the SUPER tube should be added to the column. Slowly drip the solution down the sides of the column, so the resin bed is disturbed as little as possible. Discard the tip in sharps.
11. Turn the stopcock valve and drain the liquid from the column until there is 1-2 mm of liquid above the resin.
12. Use the long wave UV light to examine the column and record your results. Where is the green fluorescent protein located in this step?
13. Take the P-1000 micropipette and set it to 1000 µL and get a new tip. Add 1000 µL of wash buffer (WB) gently down the side of the column, again trying to disturb the resin bed as little as possible. Discard the tip in sharps.
14. Drain the wash buffer until there is 1-2 mm of liquid above the resin.
15. Use the long wave UV light to examine the column and record your results. Where is the green fluorescent protein located in this step?
16. With a new tip for the P-1000 pipette, add 1000 µL of elution buffer **two times** to the column (a total of 2 mL of elution buffer). Slowly add the buffer down the sides of the column. Discard the tip in sharps.
17. Hold your “GFP” tube under the column stopcock. Another person should shine the UV light on the column so GFP can be located. Open the stopcock and collect the GFP into the GFP tube. OPTIONAL: For this step, if you have an extra microfuge tube, you can collect the fainter fluorescent liquid in one and the stronger glow in another to concentrate the GFP.
18. Once the GFP is mostly collected, drain the column into the waste container until 1-2 mm of liquid is above the resin.
19. With a new tip, add 1000 µL of storage solution- 20% ethanol **three times** down the side of the column.
20. Drain the column until there is **1 cm** of liquid above the resin bed.
21. Put the caps back onto the top and bottom of the column for storage.
22. Dispose of the column flow through waste container by pouring the contents down the drain in the sink.

Data Analysis

While under the UV light, compare your GFP tube with GFP tubes from other groups. Record any observed differences in the color intensity in your lab book.

Study Questions

1. What characteristics of amino acids are important for protein conformation?
2. How is protein conformation related to protein function?
3. Did you notice if your lysed cell solution looked more or less bright than the fraction you collected from the column? If you observed a difference, why might that be the case?
4. Why are you able to use the column to separate your green fluorescent protein from the other cellular components?
5. For this procedure, what could be adjusted to increase purity of your protein in the sample?

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2.2.14: SDS-PAGE

Learning Objectives

Goals:

- Prepare protein samples from transformed bacterial cells and perform a PAGE.
- Analyze PAGE products and identify proteins by molecular weight.

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Explain how SDS-PAGE works.
- Run and analyze the results of a SDS-PAGE.

Introduction

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) is probably the most common analytical technique used to separate and characterize proteins. A solution of **acrylamide** and bisacrylamide is polymerized. Acrylamide alone forms linear polymers. The bisacrylamide introduces crosslinks between polyacrylamide chains. The 'pore size' is determined by the ratio of acrylamide to bisacrylamide, and by the concentration of acrylamide. A high ratio of bisacrylamide to acrylamide and a high acrylamide concentration cause low electrophoretic mobility. Polymerization of acrylamide and bisacrylamide **monomers** is induced by ammonium persulfate (APS), which spontaneously decomposes to form free radicals. TEMED, a free radical stabilizer, is generally included to promote polymerization.

The gels are usually prepared with the top portion of the gel under the sample wells made less dense than the remainder of the gel below that is intentionally made denser. The top portion is referred to as the “stacking gel” and the lower portion is termed the “running gel” or “separating gel”. The purpose of the stacking gel is to concentrate all of the different sized proteins into a compact horizontal zone by sandwiching them between a gradient of glycine molecules above and chloride ions below. This way most of the proteins will enter the denser resolving gel simultaneously before they begin to migrate downwards at different rates based on their size. This way, the bands are much clearer and better separated for visualization and analysis. Without the stacking gel, the proteins will produce a long smear through the resolving gel instead of tight distinct bands for us to analyze.

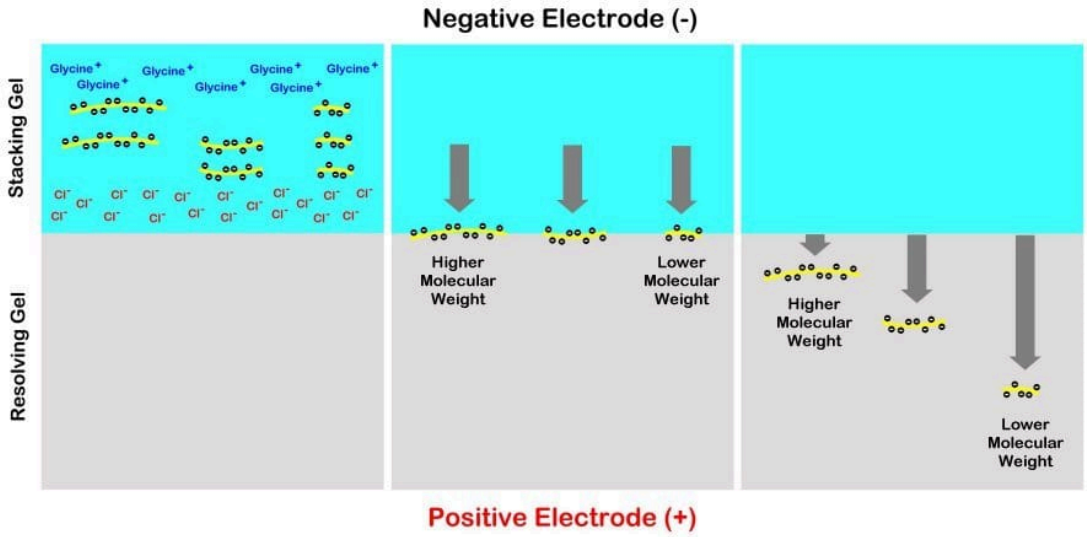


Figure 1. PAGE gel. A protein first runs through the stacking gel, where the samples spread out. Once a protein reaches the separating gel, the proteins pack together in tight bands. As they move through the resolving gel they separate by size.

SDS-PAGE

Sodium dodecyl sulfate (SDS) is an **amphipathic** detergent. It has an anionic head group and a **lipophilic** tail. It binds non-covalently to proteins, where roughly one SDS molecule is attracted to every two amino acids. SDS causes proteins to denature and disassociate from each other (excluding covalent cross-linking) and essentially unravel into linear molecules. It also confers negative charge. In the presence of SDS, the intrinsic charge of a protein is masked. During SDS-PAGE, all proteins migrate towards the anode (the positively charged electrode). SDS-treated proteins have very similar charge-to-mass ratios, and similar shapes. During PAGE, the rate of migration of SDS-treated proteins is effectively determined by their unfolded length, which is related to their molecular weight.

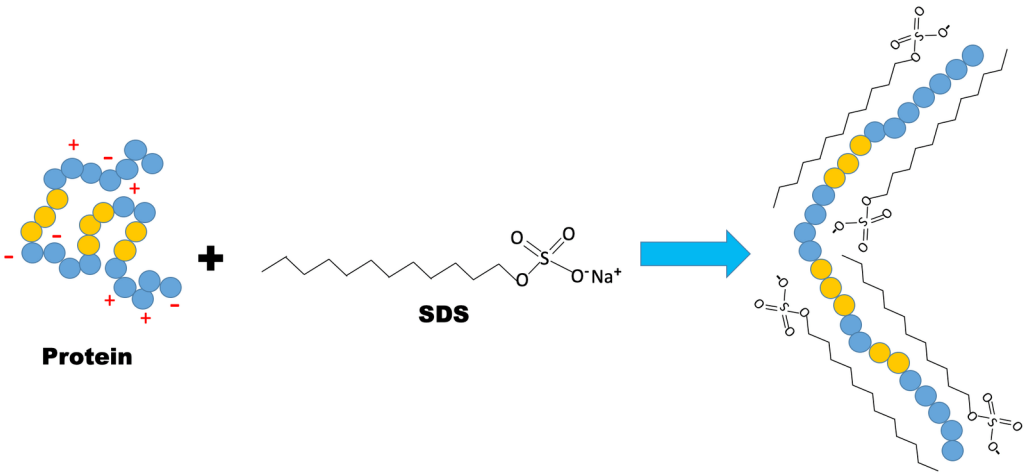


Figure 2: A protein surrounded by the SDS molecules.

Part I: SDS-PAGE

Materials

- Vertical gel electrophoresis chambers and gel cassette assembly (Bio-Rad Mini PROTEAN)
- Tris/Glycine/SDS Running Buffer
- Power supply
- Bio-Rad 10% precast polyacrylamide Mini PROTEAN TGX stain-free gels (8.6 X 6.7 cm)
- Gel loading guide
- Micropipettes with gel loading tips
- Protein samples
- Bio-Rad 2X Laemmli Sample Buffer (contains SDS and either sucrose or glycerol) and 2-Mercaptoethanol (reduces disulfide bonds, disrupts protein cross-links) and loading dye
- Prestained protein molecular weight standards (already prepared in sample buffer)

Procedure

Sample Preparation

1. Be sure to wear gloves.
2. Prepare a hot water bath (100°C). Place some water in a 600 mL or larger beaker and microwave or leave on a hot plate to boil. (This can take 15 minutes or more.)
3. Combine 10 µL of each protein sample with 20 µL of Laemmli sample buffer/Loading Dye in labeled screw-top microcentrifuge tubes.
4. Boil the samples for no more than 5 minutes to fully denature the proteins.
5. After boiling, leave the sample tubes at room temperature until ready to load onto the gel.

Preparation of the Gel and Electrophoresis Chamber

1. Be sure to wear gloves.
2. Remove the pre-cast gel from the packaging. Carefully remove the green strip from the bottom of the gel.
3. Open the two green side clamps on the vertical gel cassette assembly.
4. Place the pre-cast gel on one side of the cassette and use the clear buffer dam on the other side of the cassette. Then carefully close the green side clamps.
5. Insert the cassette into the vertical gel chamber matching the color of the electrodes (red and black) with the color guides at the sides of the chamber.
6. Fill the inside of the cassette with 1X Tris Glycine SDS PAGE buffer until the wells are submerged.
7. Fill the bottom of the vertical gel chamber with 1X Tris Glycine SDS PAGE buffer up to the mark on the side for 1 to 2 gels.

Loading the samples into the Gel

1. Place the yellow gel loading guide on the top of the cassette.
2. Using gel loading tips, micropipette 10 µL of prepared protein MW standard into the first (#1), fifth (#5) and last (#10) lanes
3. Using gel loading tips, micropipette 10 µL of each protein sample into each of the remaining wells (2-4; 6-9) of the gel. Note which sample is in which lane in your notebook.

Electrophoresis

1. Place the lid on the vertical gel chamber
2. Insert the red and black wires into the correct matching colored terminals on the power supply
3. Plug in the power supply and turn on the power switch
4. Select “Constant Voltage” and then adjust the voltage to 300 volts
5. Press the run button

6. Set a timer for 10 minutes
7. If the smallest band of the protein marker has traveled down to 1 cm from the bottom edge of the gel, turn off the power and stop the run, otherwise continue the run until this is the case
8. Unplug the power supply and wires from gel chamber
9. Disassemble gel chamber and carefully remove gel
10. Pour used buffer into a used buffer container – Do not pour down the sink!
11. The gel can now be imaged on a gel documentation camera system or it has to go through stain/destain.

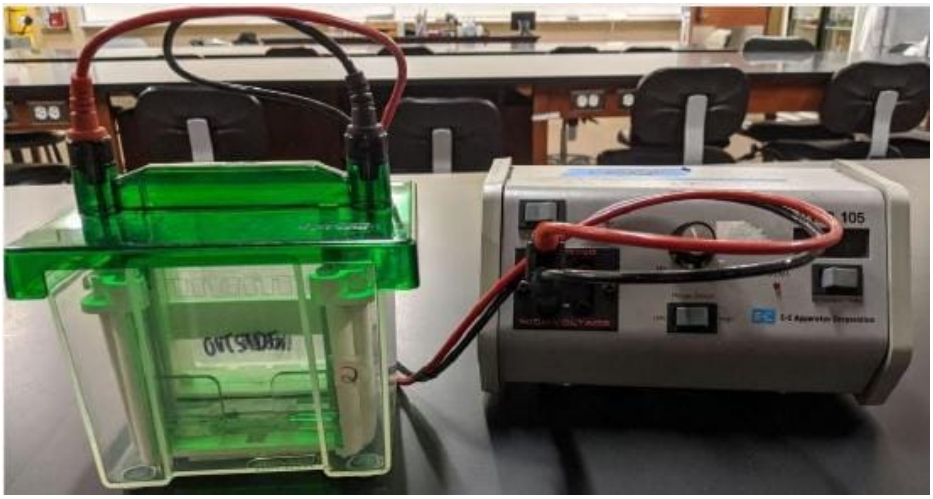


Figure 3: SDS PAGE apparatus with a power supply

Study Questions

1. What is SDS and why is it added to a protein sample prior to running a PAGE?
2. Why is the protein heated for 5 minutes before being loaded into a gel?
3. Which electrode does a protein run toward in a SDS-PAGE and why?
4. What is the difference between a stacking gel and a separating gel?
5. Given a gel, be able to analyze it using the molecular weight standard?

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2.2.15: A Taste of Genetics - PTC Taster

Learning Objectives

Goals:

- To understand basic PCR and gel electrophoresis
- To understand basic DNA mutation detection
- To learn how restriction enzymes are incorporated in biotechnology

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Understand SNPs
- Know how to perform a DNA extraction, PCR, and restriction digest
- Know how to interpret a DNA gel after electrophoresis

Introduction

Every organism on Earth has a different way to perceive the world due to their individual life experiences as well as their genetic make-up. Humans are no different; every individual has their own experiences that shapes their world perception but so too does their DNA. You may be surprised to learn that, 99.9% of the human genome is identical from one individual to the next, and it is the 0.1% difference that makes each individual unique.

Some of these differences can affect our **sensory systems** and how we perceive the natural world. For example, over time we have learned which things taste good and are good for us while simultaneously learning which things taste bad or are bad for us. Specifically, bitter compounds are closely associated to toxic substances in nature. The way we know things taste bitter, or any other flavor for that matter, is because we have special chemical receptors in our mouth and nose that bind molecules in our food and send signals to the brain telling it what the food tastes like.

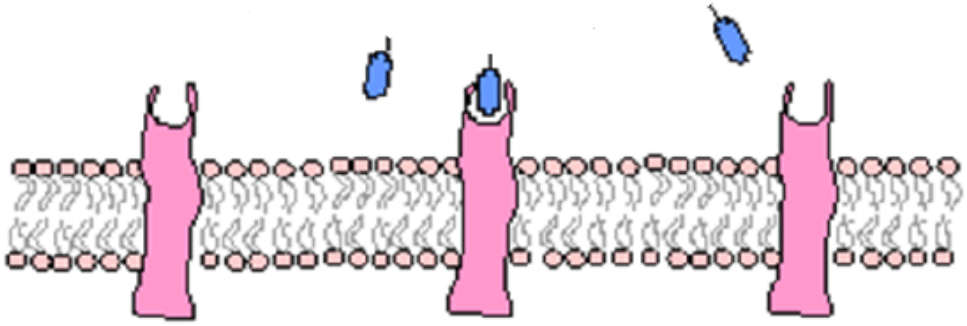


Figure 1: A chemical binding a membrane receptor

One type of bitter receptor in our mouth senses the presence of a chemical called phenylthiocabamide, or PTC. PTC is a non-toxic chemical but it very closely resembles toxic compounds often found in food. The unique thing about PTC is that not everyone can taste it! We first learned this in the 1920s when Arthur L. Fox and C. R. Noller were working with PTC powder and Noller complained about the extremely bitter taste while Fox tasted nothing at all. This lead to experimentation where scientists ultimately discovered the ability to taste PTC was hereditary; it was in our DNA!

Before we talk about the genetics of PTC tasting, we first need to understand some terminology. The observable trait, such as the ability to taste PTC, is called a **phenotype**. The genetic information that codes for that phenotype is called a **genotype**. The genes that make up a genotype come from the parents in the form of **alleles**; one allele from the mother and one allele from the

father. The two copies can be the same allele, **homozygous**, or the two copies can be different, **heterozygous**.

The ability to taste PTC comes from the gene *TAS2R38* which encodes one of the chemical receptors in our mouth that binds to PTC. By comparing PTC tasters to non-tasters, scientists have found three **single nucleotide polymorphisms (SNPs)** that differentiate the taster allele (T) from the non-taste allele (t). A SNP is a genetic mutation where one nucleotide in DNA is different from one individual to the next. The word mutation sounds scary but a mutation is not always bad; there are nearly 10 million SNPs in humans which means SNPs are common. The three SNPs (see table 1) found in the *TAS2R38* gene leads to changes in the amino acid sequence which can potentially change the proteins function.

Table 1. SNPs Present in Tasters vs Non-Tasters for PTC

Nucleotide position (bp)	Nucleotide Change		Codon Change		Amino Acid Change	
phenotype	**Non-Taster**	**Taster**	**Non-taster**	**Taster**	**Non-taster**	**Taster**
145	G	C	**G**CA	**C**CA	Alanine	Proline
785	T	C	G**T**T	G**C**T	Valine	Alanine
886	A	G	**A**TC	**G**TC	Isoleucine	Valine

Before you figure out your tasting ability, lets first understand the genetics of the alleles. Individuals who are tasters can be TT (homozygous dominant) or Tt (heterozygous). Individuals who are non-tasters will always be tt (homozygous recessive). To understand how the genes are inherited, examine table 2 below where the potential offspring of two heterozygous parents are analyzed. There is a 75% chance of having children that are tasters for PTC and a 25% chance of having children that are non-tasters.

Table 2. Sample Inheritance Pattern for PTC Tasting

Parent Alleles	T	t
T	**TT** (homozygous taster)	**Tt** (heterozygous taster)
t	**Tt** (heterozygous taster)	**tt** (homozygous non-taster)

We will figure out your genotype today using three very commonly used assays in the field of biotechnology. The first is **polymerase chain reaction (PCR)** which is used to selectively amplify a specific region of DNA of interest. PCR allows us to take one or two copies of DNA and make millions of them making it easier for us to analyze the results. Then we will perform a **restriction digest** with **restriction enzymes**. Restriction enzymes are like “molecular scissors” because they cut DNA at specific nucleotide sequences called **recognition sites**. For this lab, you will be using the restriction enzyme called HaeIII, which recognizes the sequence GGCC. When HaeIII comes across the recognition sequence, the enzyme will cut the DNA between G and C nucleotides producing two different size DNA strands. In order to visualize the DNA, we will run **gel electrophoresis**, our

third assay, which allows us to separate DNA molecules based on their size. See Figure 2 below for the expected results.

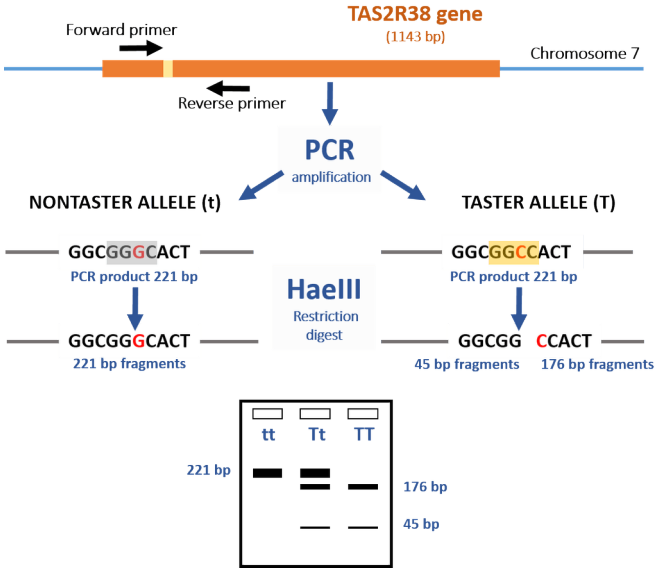


Figure 2:. Expected gel electrophoresis results post-restriction digest

Part I: Day 1

Materials



Figure 3. Day 1 Reagents

Reagents

- PTC and control paper strips
- 2 Small microcentrifuge/PCR tubes
- 0.9% saline solution
- Extraction solution
- Taq master mix
- Primer mix

Equipment

- P-20 and P-200 micropipettes and disposable tips
- Microcentrifuge
- Thermocycler
- Ice bucket
- Freezer -20°C
- BioWaste container (for saliva, tips, test strips, PCR tubes)
- Rack for PCR tubes (microtiter plate or empty p_200 tip boxworks as susbstitute)

Procedure (per manufacturer guidelines)

1. Place one strip of PTC paper on the tip of your tongue and record whether it taste bitter or not. Discard the used PTC paper in biological waste
   - Bitter
   - Not Bitter
2. Tally the students in the class to determine the number of tasters and non-tasters and place that information in the box below:

Table 3. Class Data

Phenotypes	Number of Students	% Total
PTC Taster		
PTC Non-taster		
Total		

3. Label 2 PCR tubes and a cup of saline solution with your own identifier/initials.
4. Pour the 0.9% saline solution into your mouth and swish vigorously for 2 minutes to dislodge the cells in your mouth. This is where the DNA will be coming from in our experiment.
5. Pipette 200µL of your saliva/saline mix into one of the labeled PCR tubes and close the PCR tube tightly.
6. Centrifuge the PCR tube containing the saliva/saline at 8,000RPM for 3 minutes. (Be sure to counterbalance the tubes).
7. Look for the white cell pellet at the bottom of the tube. Carefully remove the supernantant using a micropipette (do not disturb the pellet) and discard into a biological waste container. Be careful not to disturb the cell pellet!
8. Add 50µl of the extraction solution to the PCR tube with the cell pellet. Resuspend the cells by mixing using the micropipette and continue to do so until the cell pellet is broken up and there are no longer large clumps of cells.
9. You need to incubate your tube at 95°C for 5 minutes to break open the cells and release the DNA into solution, followed by cooling it until ready to use. You can place a tube on ice to chill it. If using the MiniOne system, place the tube in the PCR machine. Using the mobile device with MiniOne PCR mobile app, program the PCR machine using the constant temperature mode to incubate the samples at 95°C for 5 minutes. Enter 4°C for final incubation temperature. This will keep your samples cold until you are able to pick them up. (**Table 4**)

   Table 4 Cell Lysis Program

Step	Duration	Temperature
Cell Lysis	5 mins	95^{0} C
Final Incubation	∞	4^{0} C

10. Retrieve PCR tubes and centrifuge for 1 minute at 8,000 RPM to collect cell debris at the bottom of the tube. Your DNA will now be found in the supernatant of the tube.
11. Without disturbing the pellet at the bottom, carefully pipette 5µL of the DNA containing supernatant into your 2nd labeled PCR tube.
12. To your PCR tube containing DNA, add 10µL of *Taq* Master Mix and 5µL primer mix. Make sure to avoid placing a bubble at

the bottom of the PCR tube as this can affect the PCR reaction.

13. Cap the tube tightly, gently flick tube to mix, then centrifuge for 15 second at 8,000RPM to bring all the liquid to the bottom of the tube.
14. Place the PCR tube in the thermocycler. When all samples are loaded, close the lid and follow instructor's direction to set up the PCR protocol as seen in **Table 16.5**.
15. Once the protocol is complete, remove your sample from the thermocycler and place at -20^{0}C until next class period.

*Table 5. PCR Program (optimized for MiniOne PCR System)**

Step	Duration	Temperature	Cycles
Initial Denaturation	30 sec	94^{0}C	
Denaturation	5 sec	94^{0}C	30 Cycles
Annealing	10 sec	66^{0}C	
Extension	15 sec	66^{0}C	
Final Incubation	∞	4^{0}C	

Part II: Day 2



Figure 4. Day 2 Reagents

MATERIALS

Reagents

- Stored frozen sample from previous period
- New PCR tube
- HaeIII restriction enzyme

- Dilution buffer
- Agarose gel with Gel Green
- Loading dye
- Running buffer (TBE)
- DNA marker

Equipment

- Water bath or programed **thermocycler**
- Gel casting tray and comb
- Gel electrophoresis unit
- Microcentrifuge
- Blue light box
- Photo documentation equipment

Procedure

1. Obtain your PCR tube from the previous lab session
2. Split your reaction into two by pipetting 10µL of your PCR product into a clean PCR tube. Label one “U” for undigested and the other “D” for digested.
3. Add 5µL of HaeIII restriction enzyme to the “D” tube and 5µL enzyme dilution buffer to the “U” tube. Cap the tubes and gently flick with your fingers to mix. Centrifuge your tubes for 15 seconds at 8,000 RPM to collect all liquid to the bottom of the tube.
4. Place your tubes in a suitable water bath or thermocycler using the settings in Table 5. When using the MiniOne PCR System, set up the incubation for the restriction digest at 37^{0}C for 15 minutes using the constant temperature mode. Enter 4^{0}C for the final incubation. (See table 6)

   Table 6. HaeIII Digest Program

Step	Duration	Temperature
HaeIII Incubation	15 mins	37^{0}C
Final Incubation	∞	4^{0}C

5. While you wait for your digest, prepare an agarose gel. You will need a dye such as gel green included to visualize DNA in the gel. Lab 11 has more detailed instructions if you are not using the kit. For the MiniOne kit, the gel green is included in a premeasured amount of agarose. Poke a small hole in the plastic on top of the gel cup to allow for steam to escape. Microwave gels for 20 second increments until the gel is completely dissolved and in a liquid state. Pour your gel in the casting tray using the 9-well side of the comb. Allow your gel to solidify (it will be somewhat opaque when dry).
6. When incubation is complete, retrieve your samples. Add 3µL of loading dye to each of your tubes containing DNA. Cap your tube and flick gently to mix reagents. Centrifuge your tubes for 15 seconds at 8,000RPM to bring liquid down to the bottom of the tube.
7. Obtain your electrophoresis unit. For the MiniOne Gel Tank, ensure the black platform is in the tank to aid in visualization. Place your gel in the tank and ensure the wells are on the negative end of the gel box.
8. Pour TBE running buffer into the tank and ensure the gel is completely submerged by the buffer. Incomplete submersion of gel will lead to pour results in the gel electrophoresis.
9. Turn on the low intensity blue light and load 10 µL of your undigested sample and 10 µL of your undigested sample into two adjacent wells of the gel. Make sure your group also loads a DNA marker into one of the wells. Your group may use 10 µL of the MiniOne® DNA Marker. Use table 6 below to keep track of which samples are loaded into which wells.

Table 7. Loaded Samples

Well	1	2	3	4	5	6	7	8
Sample								

10. Turn on the MiniOne Electrophoresis System by placing the orange cover onto machine and pressing the power button. The green light should turn on and small bubbles should be visible in the buffer solution. Run samples for 20 minutes to allow proper separation of bands. If using a different electrophoresis system run the gel at 135V until the bands separate sufficiently and the dye front has traveled about 70% down the gel.
11. At the end of the run, turn on the high intensity blue light and use your phone, camera, or gel documentation system to take a picture of the gel. The blue light make the gel green that is incorporated into the DNA molecules fluoresce so they can be visualized.
12. Analyze the gel based on the information provided in the introduction of this lab.
13. Dispose of your gel and TBE buffer according to instructor instructions.

Study Questions

1. If someone can taste PTC, what is/are their possible genotype(s)?
2. If someone is homozygous for a trait versus heterozygous, when comparing their results on gel electrophoresis, what differences, if any, do you expect to see.
3. When you used PCR to amplify the *TAS2R38* gene, what component of the reaction makes it specific for that gene in your genome and not another gene?
4. Restriction enzymes recognize very specific sequences in the DNA. They read the same forward and reverse. What are these types of sequences called?
5. If you did not see any bands in your reaction after electrophoresis, what might have gone wrong? List two possible reasons for this result.
6. After comparing your bands to those of the marker DNA bands, did your bands and those of your classmates match the expected size bands?
7. Do your results in the DNA band analysis match your phenotype as a taster or non-taster based on the paper taste? What did you expect to see for the different phenotypes in the class?

Attributions

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2.2.16: ELISA

Learning Objectives

Goals:

- Demonstrate the power of an ELISA as a biomedical diagnostic tool.
- Perform an ELISA.
- Analyze the results of the ELISA and present a diagnosis based on the results.

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Describe how an ELISA works.
- Given a set of data, interpret the results of the ELISA.

Introduction

ELISA (Enzyme-Linked ImmunoSorbent Assay) is an immunologic technique used to detect the presence and concentration of an **antigen** or **antibody** in a sample. The power of an ELISA is based on the extreme specificity of the antigen-antibody interaction. ELISAs have wide-ranging applications, especially as medical diagnostic tools.

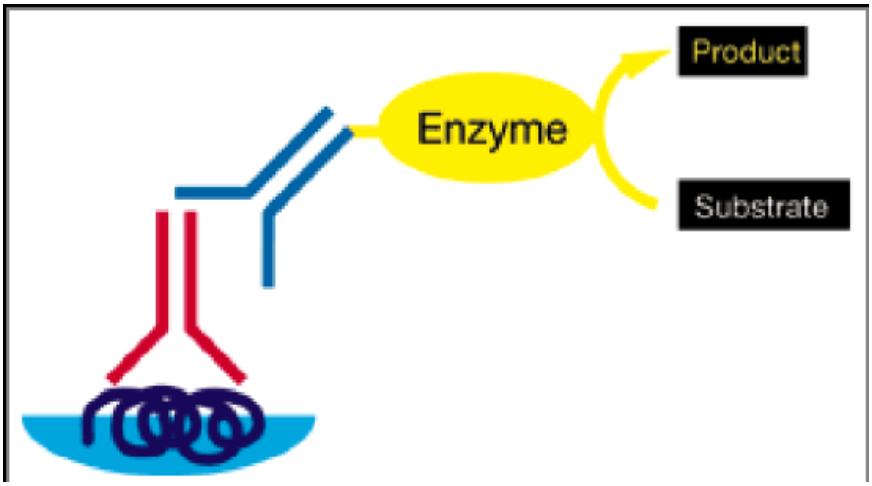


Figure 1. ELISA reactions

This lab is a simulation of an ELISA performed on patients to determine if they may have been exposed to the HIV virus. Patients exposed to the virus (foreign antigen) will develop antibodies to the HIV virus, and the antibodies circulate in the bloodstream. By testing the patient blood samples, the presence or absence of these antibodies can be measured using the ELISA.

In the ELISA conducted for this lab, the antigen (from HIV virus) is adsorbed to the surface of the plastic wells (on the 8-well strip or 96-well plate). Patient blood serum samples (which may contain antibodies to the antigen) are added. If antibodies are present, then antigen-antibody complexes form (ImmunoSorbent Process). The detection of these complexes is accomplished by the addition of a secondary antibody that detects all human antibodies. For easier detection, the secondary antibody has been covalently linked to an enzyme. When the enzyme binds to its **substrate**, a reaction occurs to create a colored product. In summary, for patients with HIV, the antibodies in their blood bind to the HIV antigen, the secondary antibody will bind to the human antibodies, and the enzyme will produce a colored product that is easy to visualize. For patients that do not have antibodies to the HIV antigen, no antibodies bind in the first stage and no colored product is produced in the end.

Clinical Application

Scenario: You work in a clinic and two patients come in who have had possible exposure to HIV. ELISA is the first screening method for HIV antibodies because it uses less costly materials and machinery than other diagnostic procedures (i.e. Western Blot or PCR). You take a blood sample and **centrifuge** it to separate the blood serum from the red blood cells and will now be performing an ELISA, testing the serum for the presence of HIV antibodies.

Materials

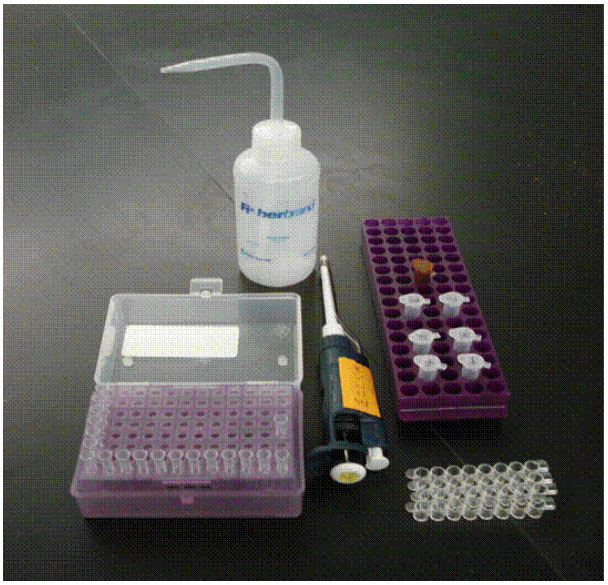


Figure 2. ELISA lab materials

Reagents

Samples in Microfuge tubes

- (GREEN) – *Positive Control:* Serum with Antibodies to HIV antigen
- (YELLOW) – *Negative Control:* Serum with no Antibodies to HIV antigen
- (PINK) – *Patient A's Blood Serum* (potential primary antibody)
- (BLUE) – *Patient B's Blood Serum* (potential primary antibody)
- (CLEAR) – *Secondary Antibody:* Anti-Human Immunoglobulin linked to an enzyme
- (AMBER) – *Substrate:*
- Tetramethylbenzidine (TMB) chromogenic substrate
- 0.25M sulfuric acid (optional)

Equipment

- HIV protein-coated 8-well ELISA strips (antigen)
- P200 micropipette
- Box of P200 pipette tips
- Microcentrifuge tube rack with samples
- Squirt bottles with PBS Wash Buffer
- Waste bucket for tips
- Pan for washing strips
- Paper towels
- Microtiter plate reader with 450nm filter (optional)

Procedure

Step 1: Coat Antigen to Plate (Inactivated HIV Proteins) *This step has been completed for you*

1. 200-µL Inactivated HIV Proteins (antigens) were added to each well of the 8-well ELISA strip
2. The strip was covered with plastic wrap and incubated at room temperature for 1 hour.

Step 2: Block Non-specific Binding of Antibodies *This step has been completed for you*

1. The contents of the 8-well strip were emptied by turning upside-down and flicking until no more liquid was present.
2. Blocking Solution was added to each well. This step will prevent non-specific binding of the antibodies.

Step 3: Add the Sample (with possible Primary Antibody)

Begin the experiment here: Your antigen-coated ELISA strip is now ready for you to add the samples*

Note

IMPORTANT: Before adding the samples, mix the solutions by inverting the tubes. Remember to change pipette tips between each solution.

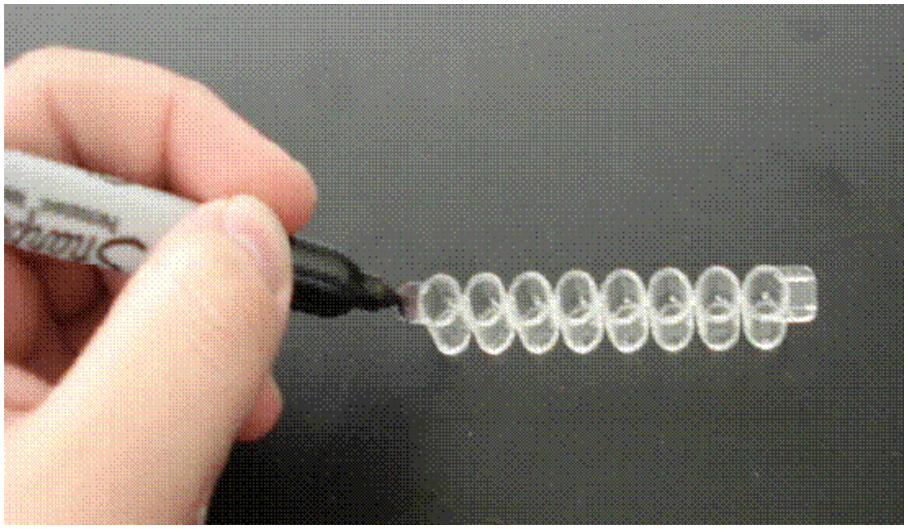


Figure 3. Marking to orient the well strip

1. Mark one side of the strip with a lab marker to keep it oriented during the procedure.
2. Add 100-µL of Positive Control (GREEN tube) to wells 1 & 2.
3. Add 100-µL of Negative Control (YELLOW tube) to wells 3 & 4.
4. Add 100-µL of Patient A's Blood Serum (PINK tube) to wells 5 & 6.
5. Add 100-µL of Patient B's Blood Serum (BLUE tube) to wells 7 & 8.
6. Let sit for at least 5 minutes.
7. Empty the contents of the 8-well strip by turning upside-down and flicking until no more liquid leaves the strip. Blot gently on paper towel to remove any remaining liquid.
8. Wash. Fill wells to the top with PBS Buffer. Empty the contents as above.
9. Repeat this wash step 3 more times.



Figure 4. Washing the wells

Step 4: Add the Secondary Antibody

1. Add 100-µL Secondary Antibody (CLEAR tube) to all wells. Let sit for 5 minutes.
2. Empty the contents of the 8-well strip by turning upside-down and flicking until no more liquid leaves the strip. Blot on a paper towel before washing.
3. Wash. Fill wells to the top with Buffer. Empty the strip and blot on paper towels. imagew200amph1amprev1ampac1ampparen
4. Repeat this wash step 3 more times.

Step 5: Detect Presence of Antigen-Antibody Reaction

1. Add 100-µL Substrate (AMBER tube) to all wells. After a few minutes, some wells may begin to change color. A color change to blue indicates the presence of the antigen-antibody complex. The more antibody bound to antigen, the bluer the solution will be.
2. Fill in the presence of color in the chart below and answer the questions on the results page.

Step 6: (optional) Stop the Enzymatic Reaction and Read the Well on a Microtiter Plate Reader

1. Add 100µL 0.25M sulfuric acid (color will turn from blue to yellow)
2. Place your strip into the plastic frame carefully and read the absorbance (optical density) set to 450nm on the microtiter plate reader

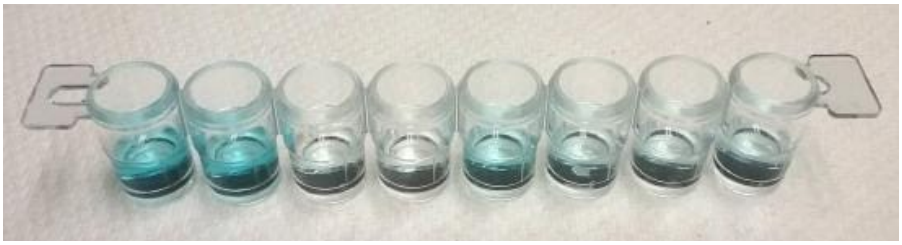


Figure 5. Positive wells (varying shades of blue) and negative wells (no color)

Results

Using the following color key, record your results in the table below:

0 = no color + = very light blue (or yellow) ++ = light blue (or yellow) +++ = dark blue (or yellow)

Data Table 1. Presence of Antigen-Antibody Complex

Well #	1	2	3	4	5	6	7	8
Sample Added								
Color								
Absorbance (optional)								

Data Analysis

1. Did your positive control exhibit color change? If not, how could this have occurred?
2. Did your negative control remain clear? If not, how could this have occurred?
3. Compare patient A to the positive and negative control. What can you deduce about patient A's condition?
4. Compare patient B to the positive and negative control. What can you deduce about patient B's condition?

Study Questions

1. Describe an ELISA assay.
2. Draw the Positive Control Well after Step 2.
3. Draw and label a diagram of the positive control well at the end of the procedure. What color is the product?
4. Draw the Negative Control Well at the End of the procedure.

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2.2.17: Serial Dilutions and Standard Curves with a Microplate Readers

Learning Objectives

Goals:

- Perform several serial dilutions.
- Use spectrophotometry to measure the absorbance of solutions.

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Create a series of solutions of decreasing concentrations via serial dilutions.
- Measure the absorbance of solutions with a microplate reader.
- Generate standard curves in Excel.
- Evaluate the quality of standard curves by their R2 value.

Part I: Serial Dilutions

Introduction

A **serial dilution** is a series of dilutions made sequentially, using the same dilution factor for each step. The **concentration factor** is the initial volume divided by the final solution volume; the **dilution factor** would be the inverse of the concentration factor. For example, if you take 1 part of a sample and add 9 parts of water (solvent), then you have made a 1:10 dilution; this is 1/10th (0.1) of the concentration of the original solution and has a dilution factor of 10. These serial dilutions are often used to determine the approximate concentration of an enzyme (or molecule) to be quantified in an assay. Serial dilutions allow for small aliquots to be diluted instead of wasting large quantities of materials, are cost-effective, and are easy to prepare.

Equation 1

$$\text{concentration factor} = \frac{\text{volume}_{\text{initial}}}{\text{volume}_{\text{final}}}$$

$$\text{dilution factor} = \frac{1}{\text{concentration factor}}$$

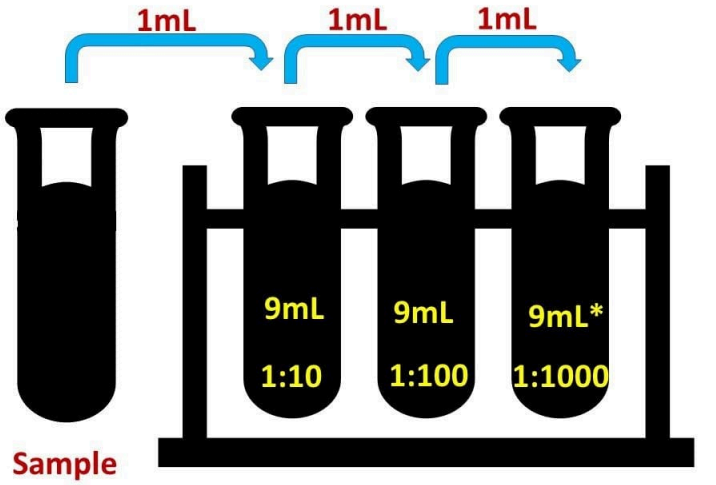


Figure 1. A ten-fold serial dilution, which can also be called a 1:10 dilution, or a series with dilution factor of 10. To determine the concentration at each step of the series, you divide the previous concentration by the dilution factor. *Dilution tubes begin with 9-mL. 1-mL is added and mixed, then 1-mL is transferred to the next tube. The ending volume in the last tube would be 10-mL.

Diagram of 1:2 Serial Dilutions

In your notebook, draw a diagram showing the serial dilutions for the 6 colored solutions you are preparing. In the diagram, indicate the volume being withdrawn from the concentrated solution, the volume of water added, the concentration of the new solution, and the total volume.

Practice Calculations

Problem 1. Assume the original sample used in Figure 1 contained 400 g/L of Reagent X.

a. Then the first 1:10 dilution tube would have a concentration of 400/10 = _____
b. Then the second 1:10 dilution would have a concentration of _____

Problem 2. Assume the original sample used in Figure is considered 100% concentration.

a. Then the first 1:10 dilution tube would have a ____ % concentration.
b. The second 1:10 dilution tube would have a ____ % concentration.

Problem 3. To make a serial dilution with a dilution factor of 5, you would need to add 1 part of the reagent plus ___ parts of water to make a total of 5 parts. This five-fold serial dilution would have concentrations of 100%, _____% in first diluted tube, ____% in second diluted tube, _____% in third diluted tube.

Problem 4. Suppose the third diluted tube of a two-fold serial dilution has a concentration of 300 g/L.

a. That means that the second diluted tube has a concentration of _____
b. The first diluted tube has a concentration of _____
c. The original tube has a concentration of _____
d. What formula could you use to calculate the concentration of the original tube from the problem statement?

Part 1: Making Serial Dilutions

Materials

Reagents

- Blue (or other color) food dye
- DI H_2O
- 96 well microplate (dry)

Supplies

- P200 Micropipette
- Box of P200 Pipet tips

> Note
>
> Use one pipet tip for each serial dilution.

Procedure

Preparing Two-Fold Serial Dilution (Dilution Factor of Two)

1. Obtain a clean, dry 96 well microplate, always touching the edges only. Use a dry clean paper towel to wipe off any fingerprints on the bottom of the plate.
2. Hold plate up to the light and check that there are no dirty spots on the three rows that you will use.
3. (Optional) You can scan the plate with no liquid, to find out the baseline absorbance of the plastic.
4. Pipet 100 µL of DI-water into the first 5 wells of row A (A1-A5).
5. Pipet 100 µL of the original blue dye into the first well (A1). Carefully pipet up and down twice to mix.
6. You do not need to change the pipet tip. But make sure that you released all liquid into the first well.
7. Transfer 100 µL of the mixture into the next well (A2). Mix carefully and release all liquid.
8. Transfer 100 µL of the mixture into the next well (A3). Mix carefully and release all liquid.
9. Transfer 100 µL of the mixture into the next well (A4). Mix carefully and release all liquid.
10. Transfer 100 µL of the mixture into the next well (A5). Mix carefully and release all liquid.
11. Transfer 100 µL of the mixture into the next well (A6). This ensures that all well A1-A5 have the same volume.
12. Take a photo of the wells on top of a white paper.

Preparing Four-Fold Serial Dilution (Dilution Factor of Four)

1. Using a new pipet tip, pipet 150 µL of DI-water into the first 5 wells of row B (B1-B5).
2. You do not need to change the pipet tip. Pipet 50 µL of the original blue dye into the first well (B1). Carefully pipet up and down twice to mix. Then make sure that you released all liquid into the first well.
3. Transfer 50 µL of the mixture into the next well (B2). Mix carefully and release all liquid.
4. Transfer 50 µL of the mixture into the next well (B3). Mix carefully and release all liquid.
5. Transfer 50 µL of the mixture into the next well (B4). Mix carefully and release all liquid.
6. Transfer 50 µL of the mixture into the next well (B5). Mix carefully and release all liquid.
7. Transfer 50 µL of the mixture into the next well (B6). This ensures that all wells B1-B5 have the same volume.
8. Take a photo of the wells on top of a white paper.

Preparing Five-Fold Serial Dilution (Dilution Factor of Five)

1. Using a new pipet tip, pipet 160 µL of DI-water into the first 5 wells of row C (C1-C5).
2. You do not need to change the pipet tip. Pipet 40 µL of the original blue dye into the first well (C1). Carefully pipet up and down twice to mix. Then make sure that you released all liquid into the first well.
3. Transfer 40 µL of the mixture into the next well (C2). Mix carefully and release all liquid.

4. Transfer 40 µL of the mixture into the next well (C3). Mix carefully and release all liquid.
5. Transfer 40 µL of the mixture into the next well (C4). Mix carefully and release all liquid.
6. Transfer 40 µL of the mixture into the next well (C5). Mix carefully and release all liquid.
7. Transfer 40 µL of the mixture into the next well (C6). This ensures that all wells C1-C5 have the same volume.
8. Take a photo of the wells on top of a white paper.

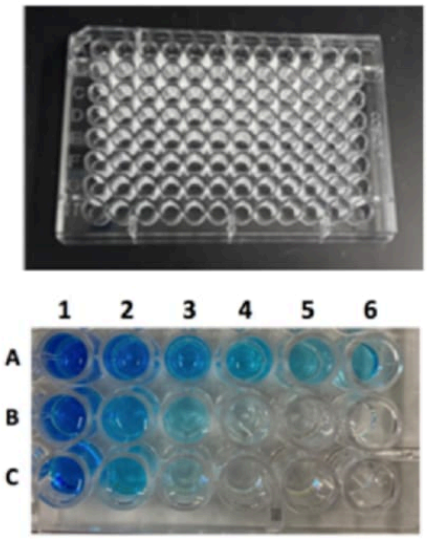


Figure 2: Microplate and serial dilutions.

Part II: Measuring Absorbance

A microplate reader is a spectrophotometric instrument that can measure the absorbance of 96 different samples at one time. Does that save time compared to working with individual cuvettes and a spectrophotometer? We will use a microplate with 96 wells, so that you can perform all of your serial dilutions onto one plate and scan the entire plate with the microplate reader once. The microplate has rows marked A-H and columns marked #1-12. Using blue dye, you will make a 1:2 serial dilution on row A, make a 1:4 serial dilution on row B, and a 1:5 serial dilution on row C.

Materials:

- Equipment
- Microplate Reader and cables
- Laptop computer with program installed to run microplate reader

Procedure:

1. Attach the cable from laptop computer to microplate reader.
2. Power “ON” the laptop computer and the microplate reader.
3. Open the computer program to run the microplate reader.
4. Push the button to open the microplate reader and expose the microplate loading platform.
5. Place your microplate securely into the holder area, ensuring that well A1 is at the top left corner.
6. Push the button to close the microplate reader.
7. On the computer program, start “read new plate” using wavelength 595 nm (for blue dye). You might use a different wavelength if instructed to do to correspond to the best absorbance for the dye you are using.
8. With the computer mouse, highlight the cells corresponding to the microplate wells that you used. Take a photo of the computer screen and note the Absorbance data in the tables below.
9. Calculate the dye concentration for each well, by dividing the dilution factor for each step of the serial dilution. The original dye is 100% concentration.

Data Table 1. Absorbance Measurements of Two-Fold Serial Dilution with Microplate Reader

Well	A1	A2	A3	A4	A5
Absorbance @ 595 nm					
Dye Concentration					

Data Table 2. Absorbance Measurements of Four-Fold Serial Dilution with Microplate Reader

Well	A1	A2	A3	A4	A5
Absorbance @ 595 nm					
Dye Concentration					

Data Table 3. Absorbance Measurements of Five-Fold Serial Dilution with Microplate Reader

Well	A1	A2	A3	A4	A5
Absorbance @ 595 nm					
Dye Concentration					

Part III: Standard Curves

Introduction

Standard curves (also known as calibration curves) show the relationship between two quantities. The standard curves are most often used to determine the concentration of “unknown” samples by comparing them to reference samples with “known” concentrations. Later in the course, we will use standard curves to measure amounts of extracted protein and to determine the size of DNA molecules. In today’s lab, you made three serial dilutions and should be able to calculate the concentrations for each dilution. Using Excel, you will prepare standard curves for each serial dilution and determine if your standard curve is accurate. Then you will determine the concentration of unknown samples, using your standard curves.

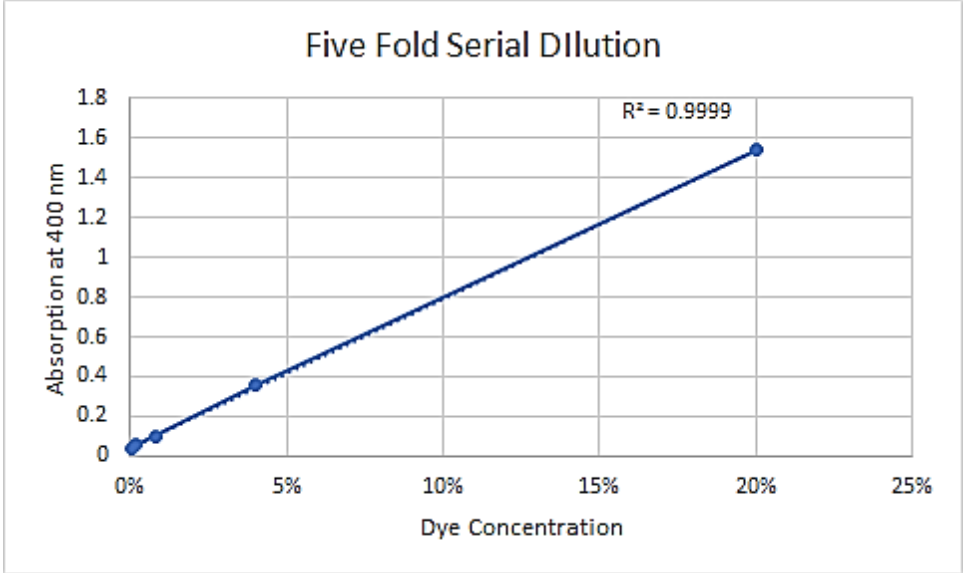


Figure 3: Standard curve of a five-fold serial dilution

The R-squared value (R2) is the correlation coefficient or the square of the correlation. For the standard curve, this value measures how strong the linear relationship is between the reagent concentration (X-axis) and the absorbance value (Y-axis). If the R2 value = 1, then that shows a perfect positive relationship. Since your standard curves are generated from the serial dilutions you pipetted, the R2 values can also show how accurate your pipetting skills are.

Activity A: Making a Standard Curve for Each Serial Dilution

1. Enter the data into Excel.
2. Select the data values with your mouse. On the Insert tab, click on the Scatter icon and select Scatter with Straight Lines and Markers from its drop-down menu to generate the standard curve.
3. Be sure to add graph title and labels for X and Y axes.
4. To add a trendline to the graph, right-click on the standard curve line in the chart to display a pop-up menu of plot-related actions. Choose Add Trendline from this menu.
5. Select “display equation on chart” and “display R-squared value on chart”. Ideally, the R2 value should be greater than 0.99.
6. Print the standard curves and add to your notebook.

Activity B: Determining the Concentration of “Unknown” Samples

1. Your instructor will have several unknown samples.
2. Determine the absorbance values of each sample.
3. On your standard curve, use the graph equation to solve for the corresponding concentration of these samples. Or estimate from the line graph.

Data Table 4. Concentration Compared to Serial Dilution Graphs

Unknown Sample	Absorbance	2-fold	4-fold	5-fold

Study Questions

1. Using a serial dilution, describe how you would prepare 10 mL of 1.0%, 0.1% and 0.01% solutions of NaOH. The stock solution is 10% NaOH. Draw diagrams as part of your descriptions/protocols.
2. Using the depicted standard curve, calculate the concentration of an unknown solution if its absorbance value is 0.55.
3. Evaluate the quality of the standard curve (see diagram) by using the R2 value.

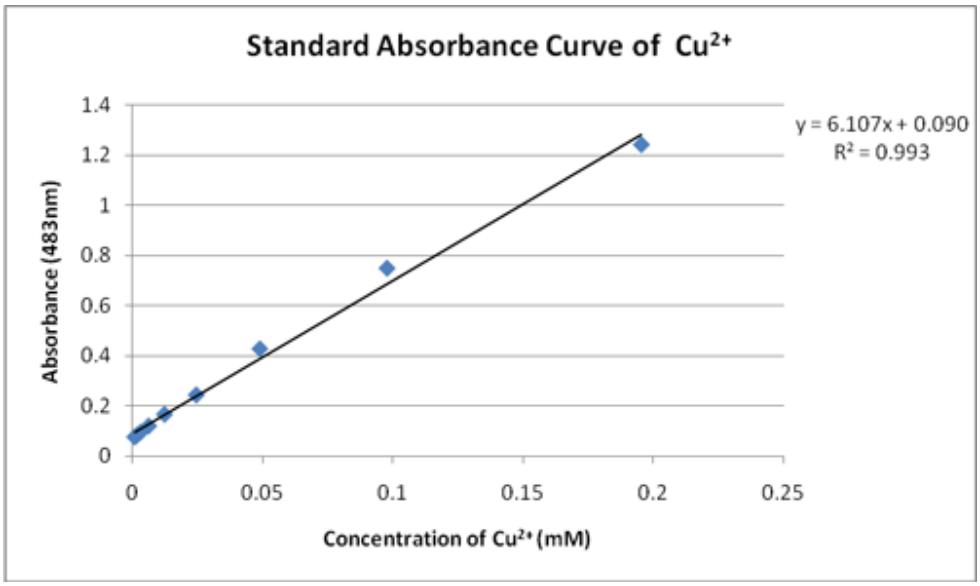


Figure 4. Standard curve of absorbance of Copper II

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2.2.18: Pouring Agar Plates

Learning Objectives

Goals:

- Learn the basics of aseptic technique.
- Learn to prepare sterile agar plates for growing bacteria

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Practice aseptic technique.
- Sterilize and pour agar plates by hand

Introduction

Microbes are all around us. In lab 4, our class sampled various surfaces and found that microbes were easily found everywhere in the environment. We used sterile agar plates that provided the nutrients and correct pH for bacteria to grow. In this lab, you will be learning to produce these sterile agar plates.

Agar is a polysaccharide derived from red algae. The agar powder is first dissolved in a boiling liquid, and then cooled to form a gelatinous solid matrix. As microbes cannot digest agar, this material is used commonly in laboratories to hold the nutrients that bacteria need.

The main instructions for pouring agar plates are presented here. But there are many different recipes to prepare growth media for bacteria, as some bacterial species require different combinations of nutrients. Some types of common agar include blood agar, Luria Bertani (LB) agar, MacConkey agar, nutrient agar (NA), and tryptic soy agar (TSA). Follow the specific package instructions regarding the amounts of powder and water to use for the growth media you are making. The recipe to make 1-liter LB agar is 9.1 g tryptone 4.6 g yeast extract, 4.6 g NaCl and 13.7 g agar. If an antibiotic additive is needed in the medium recipe, that is added after the sterilized agar has cooled to 60oC to avoid denaturation.

An **autoclave** is a high-pressure apparatus that is used by laboratories, dentists, and hospitals to sterilize equipment, instruments, glassware, growth media, liquids and biohazardous waste. The autoclave applies high pressure (15 psi) and saturated steam at 121oC (250oF) for 15-20 minutes to kill microbes and spores. After the media has gone through this cycle, it is sterile. Cool to 60-65oC before adding any antibiotics and pouring into sterile Petri dishes.



Figure 1. Digital Balances and Weigh Boats

Materials

- disinfectant spray
- paper towels
- petri dishes
- gloves
- LB agar powder
- Weigh boat
- Stir bar
- 500 mL autoclavable bottle
- Autoclave tape
- Sharpie marker (colored)
- Deionized or distilled water
- Electronic balance
- Autoclave or pressure cooker
- Metal tray
- Ampicillin or arabinose



Figure 2. Some supplies for preparing plates with media

Procedure

Preparing the media

1. Label a clean glass autoclavable 500 mL autoclavable bottle with media name, date, and initial.
    - Note: only fill bottle halfway, to avoid overflow during the heating process in the autoclave.
2. For a 500 mL bottle, calculate the needed weight of powdered media to make 250 mL. Subtract that from 250 to determine the volume of water to add.
3. Add __ mL of distilled water to the bottle.
4. Add __ g of media with agar powder to the same bottle. (your total should be 250 mL)
5. Add a stir bar (optional). Stir or shake until fully mixed and check that there are no lumps.
6. Add a piece of autoclave tape to the cap or bottle and loosen the cap a half-turn. If using a container with no cap, then cover loosely with aluminum foil

Setting up the autoclave

1. Place the prepared media bottles into a metal tray.
2. Add distilled water until it covers the bottom of the tray; about 1-2 cm deep.
3. Place into the autoclave.
4. Autoclave at 121°C for 15 minutes at 15 psi.
5. Once the cycle is complete, wear heat-resistant gloves to remove the tray and bottles from the machine.
6. Allow bottles to cool to approximately 60°C.

Prepping the workspace:

1. To keep as sterile of an environment as possible to avoid contaminating the media and plates, don a lab coat and gloves, and use a disinfecting agent or wipes to wipe down all surfaces.
2. This includes tabletops and edges, gloves, scissors, permanent markers, etc.
3. Make sure to clean your gloves if they have touched another surface that is not disinfected (eg.if you touched face, arm, chair, etc.).
4. If available, use Bunsen burners and carefully pour near the open flame to better prevent airborne contaminants.
5. Once the area is disinfected, bring out the sterile Petri dishes. Keep the sterile dishes closed.
6. Stand a bag up vertically.
7. To conserve the plate bag to reuse for storage, ignore any “Tear Here” markings, and snip a small corner off the top of the bag. Insert half of the scissors into this opening and cut along the crease of the bag.
8. Flip the entire stack of plates upside down, with the cut opening at the very bottom of the stack.
9. Gently apply a small amount of pressure downward while simultaneously rolling the bag up.
10. Fold or roll up the empty bag and put aside to use for re-bagging.

Striping the plates

1. To quickly differentiate between plates that have a similar appearance, a stripe code can be used. A combination of different colored permanent markers can signify different additives to a plate. For example, a green stripe may mean ampicillin, a type of antibiotic, was added to the media in that plate. Striping codes are specific to an individual lab, so always double check the code key.
2. To stripe a plate, the top AND bottom must be labeled with the code.
3. Take the marker with the color in the key, and with the unbagged stack of plates, apply a gentle amount of pressure with your hand onto the top of the stack.
4. Draw a line straight down from the top edge to the base of each plate in the stack.
5. If done quickly, you may need to go back in and redraw the line on the bottom base.
6. If the stripe code has another line or color, repeat the process by adding another line.
7. The spacing of the second line should be within 1 cm from the first line.
8. Repeat the process until the stripe code is complete.

Setting out the plates

1. Begin to unstack the plates. Make sure the plate tops and bottoms do not separate.
2. Place individual plates around the edge of the table (not in stacks), to create a line or chain of plates.

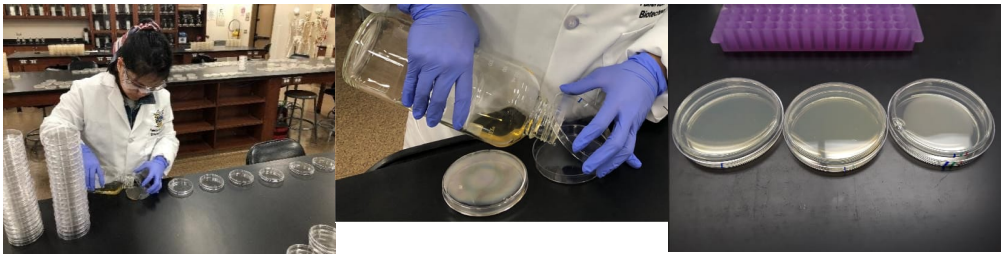


Figure 3: Pouring Plates

Pouring the Plates

1. Once the media has cooled to 60 o C, the liquid solution is ready to be poured.
2. At this time, an antibiotic (ex: ampicillin) may be added to the media and gently swirled or stirred to mix. Note: do not add the antibiotic if the liquid is hotter than 60 o C, as the antibiotic would be denatured.
3. Uncap the media bottle and hold the bottle in your dominant hand. Note: once the bottle is opened, do not talk. Talking will allow bacteria from your mouth to become airborne and may contaminate the media.
4. The cap can be held in the same hand (between fingers) as your bottle or can be placed on a disinfected surface.
5. Grab a plate with your other hand and slide it towards the edge of the table, while keeping it closed.
6. Once the plate is at the edge, open the lid as if there is an imaginary hinge at one end; so the plate opens like a clamshell.
7. Pour the media into the bottom of the plate until it just covers the surface. Do not over fill.
8. Close the lid and allow to cool. The media will be solid.
9. Leave the plates out for a day if possible so the condensation will evaporate from the plate. You may place the plates in a 25C incubator overnight.
10. Stack plates with the same type of media and slide the plastic sleeve over the top.
11. Flip the stack over and seal the plastic sleeve with masking tape.
12. Label the tape with the type of media, date produced, and name of individual that produced the stack.
13. Store sealed stacks in the refrigerator until use.

STUDY QUESTIONS

1. What is the typical amount of agar included in 1 L of media?
2. Why must one take such precautions to disinfect the space and avoid actions that may cause bacteria and mold to become airborne? What is the term applied to these precautions and procedures?
3. Does agar provide nutrients for bacteria?
4. Why can't you include ampicillin in the media before it is autoclaved?
5. Why must you leave solidified plates out for a day before you seal them and refrigerate them?

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2.2.19: Good Manufacturing Practices (GMPs)

Learning Objectives

Goals:

- Make a batch of Good Manufacturing Practice (GMP) popcorn within a specified timeframe (1.5 hours).
- Use Standard Operating Procedures (SOPs) to accomplish the manufacturing task.
- Understand the complexity of the GMP process.
- Understand Quality Control (QC) and Quality Assurance (QA).
- Understand the need for team members to complete individual jobs appropriately to accomplish goals.

Student Learning Outcomes:

Upon completion of this lab, students will be able to:

- Explain the role of Standard Operating Procedures (SOPs) and batch/lot Records.
- Employ the principles of a quality systems approach to manufacturing.
- Learn the difference between Quality Control (QC) and Quality Assurance (QA).
- Discuss the value of teamwork.

Part I: Review Terms and Background Information

Introduction

In everyday speech, quality is a relative attribute like beauty. However, in the Life Sciences or any other manufacturing-related industry quality means **compliance to specifications. Quality systems** are comprised of the policies, processes, and procedures for maintaining a product with specifications.

This lab exercise will utilize the manufacturing of a popcorn snack as an example where **good manufacturing practices (GMPs)** can be followed. The exercise used the components of a Quality System. Students are the employees with tasks to accomplish within the defined QA/QC parameters.

Quality Management Systems (QMS)

Quality is the business of the entire company. Generally, companies build their quality systems by first making a commitment by management to design and deliver a 'quality' product. The **term quality management system** refers to the organizational resources, processes, and procedures to implement quality management. In the case of pharmaceuticals, diagnostics and medical devices, these requirements mean the product is EXACTLY what was tested in clinical trials.



Figure 1: Symbiotic relationship between the Quality System, Quality Assurance, and Quality Control

Quality product management is usually implemented through two departments; **quality assurance (QA)** and **quality control (QC).** The QA department plans activities and develops the processes that guarantee the accuracy and precision of outputs. QC performs the actual tests during the process and on the final product to ensure that specifications are met. Quality Assurance is process oriented and focuses on defect prevention, while quality control is product oriented and focuses on defect identification**.** QC refers to a measuring process, or to check a result and provide assurance that all activities are performing within predetermined limits.

Often, QMS systems are explained in this simple statement: **"*Say what you do, do what you say, document it.*"**

Say What You Do = SOPs

Standard Operating Procedures (SOPs) and documentation are essential components of a **QA program**. The management team is responsible for ensuring adherence to the QA plan and SOPs. Essential to this process is documentation of the proposed quality system and processes used by the company.

Perhaps the most important form of documentation is SOPs. SOPs are validated methods that are at the technical core of the product or service. To determine whether methods are fit for their intended purpose, the selected methods must have established accuracy, precision, calibration and limits of detection and quantification. All methods must be fully validated for accuracy and precision before a company starts to sell its product or service.

Do What You Say = Follow the Process (SOPs)

Quality systems are implemented in many sectors of the life sciences industry including manufacturing, a testing laboratory, clinical trials or even Research and Development. In all cases the SOPs must be followed. In the example of a manufactured product, all manufacturing employees follow the defined process and document that they have done so. The QC department provides the process checks and balances. For example, they manage the acceptance and release of raw materials, in–process checks and testing of the final product.

Document It = Evidence of Quality

If you didn't write it down = you didn't do it!

No documentation = no **PROOF** you did it!

No documentation = **BAD** product and lost money for your company

Current Good Manufacturing Practices (cGMPs)

The general principles that all QMS have in common are:

- ***Say what you do.*** Quality, safety, and effectiveness must be designed and built into the product, not tested or inspected into the product.
- ***Do what you say.*** Each step in the manufacturing process must be documented and controlled to ensure the finished product meets design and regulatory specifications.
- ***Document it***. Process documentation provides evidence of compliance with **cGMPs.**

Statistical Process Control

Another aspect of a quality program it use of **statistical process control (SPC),** which utilizes statistical methods to evaluate variability in procedures. In the design of the product, multiple data sets are obtained to establish accuracy and precision criteria. These criteria are documented in the **Master Record**. QC testing procedures ensure SPC through instrument calibration and validation, in process testing, final product testing, raw material testing etc. They use process control charts to monitor the process and procedures in order to manage variability.

The purpose of the quality systems is to ensure quality. Since quality is defined as compliance to specifications, a statistical process control (SPC) must be established to ensure this compliance. The SPC requires understanding of both the accuracy and precision required in the product.

Accuracy is the 'right answer' or 'bulls-eye". Accuracy is not always known but when it is, it is part of the process. **Precision** is reproducibility. Whatever the process or procedure, the quality system is there to ensure reproducibility.

The ideal situation is when precision and accuracy are on target: Expected and observed values are close and the results are consistently reproducible. If results are reproducible but the results are not close enough to the expected value, one has good precision but poor accuracy. Poor precision but good accuracy occurs when the results are not close to each other in value but they all fall within an acceptable target range. This situation often can be improved upon with the appropriate action. Poor precision and poor accuracy is typically the most problematic result since neither reproducibility nor an expected target value have been achieved (Figure 2).

Corrective and Preventative Actions (CAPA)

The purpose of the Corrective and Preventative Actions (CAPA) system is to serve as a feedback loop to identify and investigate all quality problems. CAPA policies are central to a quality system. Part of the QC testing that takes place on every lot of product, is to assure compliance to specifications. If the specifications are not met, it is important to determine what is the root cause of the problem. Until the root cause is used to identify the cause of problems with accuracy and precision, product quality cannot be assured.

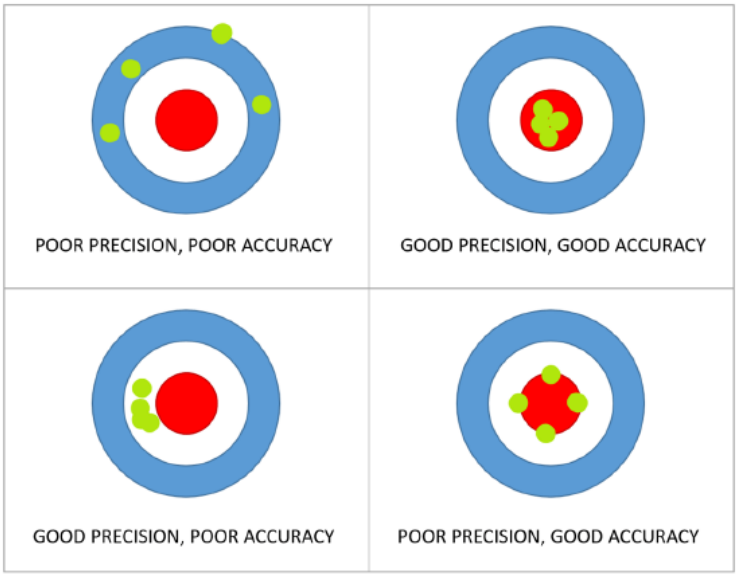


Figure 2. Accuracy and precision

Quality Assurance and Quality Control (QA/QC)

Table 1. Quality Assurance Versus Quality Control

Element	Quality Assurance (QA)	Quality Control (QC)
Definition	the activities focused on the processes for preventing mistakes and producing products free of defects	a set of activities concerned with monitoring and verifying resulting products meet the defined standards and specifications for quality
Purpose	Verification: Answer the question "Am I building the product correctly"? Prove the system meets all specified requirements at a particular stage.	Validation: Answer the question "Am I building the right product"? Ensure that the product meets customer expectation.
Type of process	Proactive	Reactive
Goal	Plan to prevent problems	Identify defects in the finished product Identify and correct source of product defects
Tools	Statistical Process Controls: Control Charts Run Charts	Statistical Quality Controls: Random Acceptance Sampling Range Charts Histograms Means Standard Deviation
Responsibility	Everyone	Quality Control Group

Part II: Train and Prepare for a Production Run

A. Lab Activity: Review the Lab Flow

Construct a flow diagram of the following overview of the training and production of your component for the snack product today.

Scenario: Your company, Awesome Snacks Company (ASC), needs you to produce popcorn for their seasonally available Summer Snack Mix. Your job will be to take the raw material and produce the popcorn that goes to the Snack Mixing Department.

Materials needed:

microwave popcorn bags, microwave, digital balance, forms ASC001-004

1. Employee Overview

- Supervisor (teacher) – Welcomes Employees (students) to Awesome Snack Company.
- Overview of QMS and why it's important to the company.

2. Group Formation

- Divide employees up into Quality Assurance Teams (5 employees each team)

3. Training

A. Supervisor gives training form (see figure below, in QA packet) and packets to team.

ASC TRAINING
—COMPLETE BEFORE STARTING PRODUCTION—

Figure 3. Heading of training document in packet

B. Team Assigns Jobs to Members
   1. Material Control Tech.
   2. Quality Control Tech.
   3. Manufacturing Tech. I
   4. Manufacturing Tech II
   5. Quality Assurance Tech.

C. QA Tech Reads SOP Standards 11-001 to team

D. QA Tech distributes packets to each team member
   - Members read through their training packet

E. Team completes ASC Training Document

F. The completed ASC Training Document must be turned in to the supervisor (instructor) to receive material to start the production run.

4. Production

A. Each technician performs their role

B. Document everything on company forms

C. Put the following calculation from ASC Form 004 into your laboratory notebook:
   - Percent weight loss for each of the three bags.

D. Evaluate: Does batch meet specification?
   - Statistical Process Control: Mean, Standard Deviation, and Coefficient of Variation

E. Post Production Product Specifications: Release or Reject
   - Are you accepting or rejecting this batch? Why?

F. Once everything is completed, QA will turn forms into Supervisor

5. Corrective Action Plan

A. Class discussion of Statistical Process Control
B. Corrective and Preventative Action plan is constructed.

B. Activity: Training Using Good Manufacturing Practices (GMP)

PURPOSE: Every employee must be trained in conformance with GMP (Good Manufacturing Practices).

MATERIALS

- 1 official SOP Standards for Employee Safety and Conduct: STANDARDS 11 -001 (see below)
- 1 official Awesome Snack Company (ASC) Documentation of Training Document (see below)
- 1 Set of the Standard Operating Procedures (SOPs) for Production: MAT-11-001, MAN-11-001, MAN-11-002, MAN-11-003, QA-11-001, QC-11-001
- 1 set of production forms: ASC 001, ASC 002, ASC 003, ASC 004

PROCEDURE

Form Groups, Assign Roles, Complete Training

1. The ideal group size is 5. Form groups and assign roles (jobs) to each member of Production Team. Jobs: Quality Assurance (QA), Quality Control (QC), Manufacturing Tech I, Manufacturing Tech II, Material Control (MC). Note: If the team is comprised of a group less than 5, 1 member must take multiple roles.
2. Follow the training instructions below. After training, the documentation of training document (Figure 4) must be turned in to the supervisor (instructor) to receive material to start your production run.

AWESOME SNACK COMPANY
Standard Operating Procedure (SOP)

SOP: STANDARDS-11-001	TITLE: STANDARDS FOR EMPLOYEE SAFETY & CONDUCT		PAGE 1 OF 1
AREA: MAN/QUAL	REVISION: 4.3	EFFECTIVE DATE: 1/25/17	SUPERCEDES: 4.2.1
PREPARED BY: ASC TEAM			

Figure 4. The heading of SOP 11-001 from your packet

Instructions:

1. **Quality Assurance (QA) Technician** will read SOP Standards-11-001 to the Team.
2. **QA Technician** distributes signs, SOPs and ASC Forms to each team member.
3. Each team member reviews the scope and ASC guidelines, signs the page and receives a supervisor (or their designee) signature (see below)
4. Each team member reads the SOP for his/her job to identify their primary activity. (Ex. Weigh unpopped popcorn (UPP))
5. **QA** fills out table 2 below. In the order presented, **QA** lists the activity and asks the name of who will do it.

SCOPE

- ALL ASC employees must be trained, acknowledge training, and receive sign-off from a supervisor.
- Not following the stated ASC guidelines may result in **written warning and/or termination** of employment

The *ASC Guidelines* include but are not limited to the following:

1. All hair that is shoulder length or longer must be tied back or contained in a hair net
2. Fingernails must be neatly trimmed. Fingernails should not protrude past the ends of the fingertips more than ¼ inch
3. Clothing and other personal belongings must be stored in designated areas
4. All jewelry is prohibited in all manufacturing areas with the exception of one smooth ring (no stones) on finger. This includes and is not limited to watches, multiple rings, ear, nose, tongue, eye, belly piercings or rings...etc. are not allowed
5. No items are allowed in pockets or affixed to clothing above the waist in the manufacturing area. The exception is for PPE (Personal Protective Equipment)
6. Electronic devices such as cell phone, iPods, blue tooth, radio, pager, MP3, handheld games, etc. These items may be stored in lockers. Employees may use these devices only in designated areas during breaks, lunch, before and/or after work
7. Areas must be left in a clean and organized manner at the end of each shift
8. No food (including gum, candy, nuts and/or similar snacks) and/or drinks can be stored or consumed in manufacturing areas
9. Every glass or plastic breakage in any manufacturing area must be reported to the Shift Supervisor or Safety Coordinator and a Quality Incident Report generated
10. Use the following format for dates: Day-Month-Year (ex. 05-Mar-2019)
11. When Initialing, use THREE initials, to include your middle initial

I, _____ , certify that I have read and understand the *ASC Guidelines*.

Employee Full Name

Employee Signature		**Supervisor Signature**
Date		**Date**

Table 2. Assignment of Production Roles

ACTIVITY	**WHO DOES THIS JOB? (NAME)**	**SOP/FORM**
Inspect and/or Release, Reject UPP*		MAT-11-001 / ASC 002
Start Lot/Batch Record		QA-11-001 / ASC 001
Prepare and clean microwave/ balance		MAN-11-001, MAN-11-002, / ASC 003
Inspect Cleaning; OK production start		QC-11-001 / ASC 003

Weigh UPP		MAN-11-003 / ASC 004
Produce IPP*		MAN-11-003 / ASC 004
Place popcorn in Reject or Release		MAT-11-001 / ASC 002
Complete Lot/Batch Record		QA-11-001 / ASC 001
If necessary, initiate CAPA		

*UPP= unpopped popcorn; IPP= in process popcorn

12. Print name and Initial on the OFFICIAL Documentation of Training document (looks like below) **after** training on Standards and SOPs is complete. Be sure to include your middle initial
13. Once completed, Material Control turns in the document in to Supervisor (Instructor) to receive the starting material (UPP) and start production

Table 3. Documentation of Training

JOB TITLE	**NAME** (Print)	**INITIALS** *(Include Middle Initial)*
Material Control Tech 		
Quality Control Tech 		
Manufacturing Tech I 		
Manufacturing Tech II 		

Quality Assurance Tech		

This is similar to the official document to be used and completed during the exercise.

Part III: Production and Documentation

Perform Roles and Document Appropriately Using Company Forms

Procedure

1.
    1. Use official company SOP documents to perform the assigned roles in Table 1.
    2. Document appropriately on the “official” Awesome Snack Company (ASC) forms as listed in Table 1

Results

ASC form 004 (IPP production report) is to be completed by your team and turned in to your supervisor (instructor) along with ASC forms 001, 002, 003.

Put the following calculations from this form into your laboratory notebook:

1. Percent weight loss for each of the three bags. Are you **accepting** or **rejecting** this batch? Why?
2. Statistical Process Control:
    - Mean:
    - Standard Deviation:
    - Coefficient of Variation:
3. Post Production Product Specifications: **Release** or **Reject**

Study Questions

1. Did you need to implement CAPA? Explain the deviance that caused this procedural process to begin and explain the corrective and preventative process you implemented. This will be shared with the class during discussion.
2. The Ishigawa fishbone diagram (Figure 4) is a tool used to determine the root cause of a problem. It enables a team to brainstorm and categorize all the potential problems that could be the cause of a problem so they can be systematically eliminated until the real cause or causes is/are identified.
3. With your team come up with all the potential reasons your popcorn may fail to meet quality specifications because it is burnt. Redraw the diagram, including the title, and fill it out in your laboratory notebook.

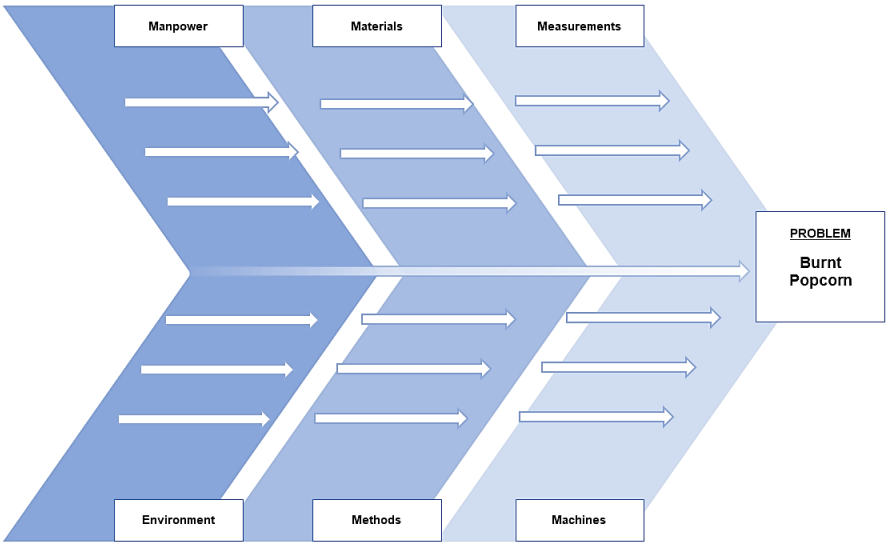


Figure 4. Blank Ishigawa fishbone diagram.

Attribution:

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2.2.20: BioFuel Project

Learning Objectives

Goals

- Convert cellulose biomass to usable biofuel.

Student Learning Outcomes

- Test predictions about how variables such as biomass type or grinding affect conversion into sugars and ethanol.
- Trace the transformation of cellulose into glucose and then ethanol.
- Infer the action of cellulase enzymes on cellulose based upon sugar readings.
- Measure the conversion of sugars to ethanol using ethanol sensors.
- Use sugar and ethanol readings to evaluate initial predictions and draw conclusions about the effects of treatment variables.

Introduction

Biomass is any organic material that comes from organisms, such as plants. **Plant biomass** contains energy that can be used for food or fuel depending on what part of the plant is used. **Cellulosic biomass** is the part of the plant that most people cannot digest such as tough fibrous or woody grass, leaves, stems, flowers, corn stalks, wood, or paper products. Although cellulosic biomass cannot be used for food, it contains a large amount of energy that can be used as fuel for transportation. Cellulosic biomass, also referred to as cellulose, is the primary component of plant cell walls. Without cellulose, plants would not be able to stand upright. **Cellulose** is one of the most abundant molecules on Earth and represents a huge potential pool of renewable energy if we can find a way to easily convert it into transportation fuel.

In this lab, you will investigate the challenge of converting cellulosic biomass into ethanol. You will use some of the same strategies used by scientists and engineers in biotechnology companies. The process involves three key steps: Pretreatment, hydrolysis (enzymatic digestion) and fermentation.

Biofuel Pipeline Overview

The process outlined below provides an overview of the steps and describes the chemical changes occurring as cellulosic biomass is converted to sugar and then ethanol. We call this a "**biofuel production pipeline**" because the products generated from one step are used in the next step until ethanol is produced.

In the **pretreatment stage**, the goal is to loosen the cell wall structure so that the cellulose is exposed. Plant cell walls are made up of three primary components: **cellulose, hemicellulose**, and **lignin**. These molecules must be separated so that enzymes can reach the cellulose. Heating and grinding are effective pretreatment methods.

Cellulose is actually made up of long chains (polymers) of **glucose** molecules. In the **hydrolysis** stage, the goal is to break the long cellulose molecules down into individual glucose molecules. Special enzymes called **cellulases** are able to cut up the cellulose strands into glucose. Glucose is a simple sugar that can be used as food by many organisms.

In the final step of the pipeline, yeast is added to the enzymatically digested biomass mixture. Without oxygen, yeast consumes the glucose and produces ethanol through a process called fermentation. The yeast used in this process is the same single-celled organisms used to bake bread or brew beer.

The process outlined below provides an overview of the steps and describes the chemical changes occurring as cellulosic biomass is converted to sugar and then ethanol. We call this a "**biofuel production pipeline**" because the products generated from one step are used in the next step until ethanol is produced.

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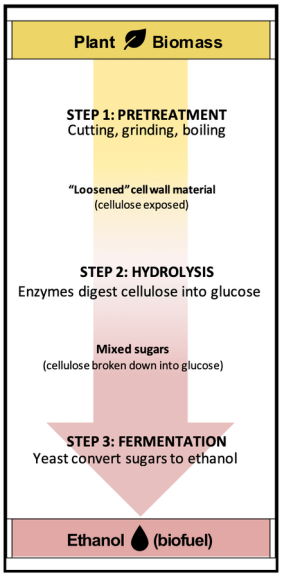


Figure 1: Biofuel pipeline

Cellulose is actually made up of long chains (polymers) of **glucose** molecules. In the **hydrolysis** stage, the goal is to break the long cellulose molecules down into individual glucose molecules. Special enzymes called **cellulases** are able to cut up the cellulose strands into glucose. Glucose is a simple sugar that can be used as food by many organisms.

In this investigation, you and your research team will pick a cellulosic biomass sample to first convert into sugars, and then into ethanol through the process described above. You will track the conversion process by measuring sugar (glucose) and ethanol levels at key stages. The data you and your classmates collect will help you determine which biomass sources and pretreatment methods are most effective for producing sugars and ethanol and develop explanations for why some samples produce more sugar and ethanol than others.

Part 1: Prelab Work

Before the Lab

The goal of this lab is to convert a cellulosic biomass sample into sugar and then ethanol. Your lab group should select a biomass sample and cutting/grinding treatment that you think will effectively produce ethanol. Your group will prepare both an experimental and a control treatment to evaluate the effects of the enzyme on the production of sugar and ethanol.

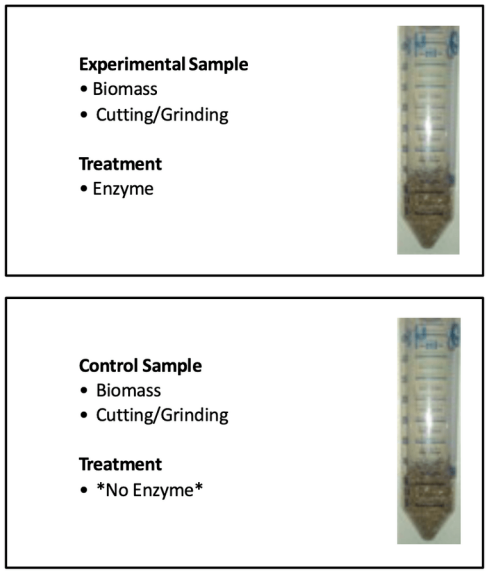


Figure 2: Experimental and control samples of biomass

Experimental Sample

- Biomass
- Cutting/Grinding

Treatment

- Enzyme

Control Sample

- Biomass
- Cutting/Grinding

Treatment

- *No Enzyme*

Table 21.1.

Table 21.1 Project Timeline

Lab Research Stage	Duration	Activities
1. **Experimental Design and Planning**	1-2 days	• Develop research plan • Choose biomass type and/or pretreatment options (cutting or grinding)

Lab Research Stage	Duration	Activities
2. Sample Prep and Pretreatment	1 day	• Set up experiment • Cut, grind and/or boil biomass • Measure initial sugar levels
3. Hydrolysis (Enzyme Digestion)	1 day	• Add cellulase enzymes • Measure sugar levels (after 24 hours)
4. Fermentation	1 day	• Measure initial ethanol levels • Add yeast • Measure final ethanol levels (after 24 hours)
5. Data Analysis, Conclusions, and Discussion	1-2 days	• Graph final results • Summarize conclusions and communicate findings to class • Write up final results based on evidence from your other lab group results

Experimental Design and Planning

In your group, discuss and decide which biomass and grinding options will be best for producing ethanol. As instructed by your teacher, write down why you think it will produce the most ethanol. Be prepared to explain what you think will happen in this experiment and pinpoint what evidence you will use to determine whether your prediction was accurate.

The goal of this lab is to produce as much ethanol as possible from 1-gram of biomass mixed with 25-mL of water. Based upon the options provided by your teacher, work with your lab group to decide what biomass type and grinding option you would like to convert into ethanol. Answer the questions below and be prepared to share your answers with the class.

1. What biomass did you choose? Explain why.

2. If applicable, what grinding option did you choose? Explain why.

3. What evidence will you gather from this experiment to determine whether your biomass is effectively converted into ethanol?
4. At what stage in the lab (pretreatment, enzyme digestion, fermentation) and in which treatment (control or experimental) do you expect to measure the highest glucose levels? Explain.
5. At what stage (pretreatment, enzyme digestion, fermentation) and in which treatment (control or experimental) do you expect to measure the highest ethanol levels? Explain.

Materials

• Vernier or Pasco data-collection interface	4/class
• Vernier or Pasco Ethanol Probe	4/class
• 50mL Conical Centrifuge Tubes	2/group
• TRUE balance Blood Glucose meter	1/group
• Blood glucose test strips	~10/group
• Wax paper or parafilm	1 sheet/group
• Cellulosic biomass: sawdust, straw, corn stover, switchgrass, cardboard, etc.	~50 grams of each
• Weight boats	1/group
• Electronic balance	1/group
• Grinder for biomass samples	4/class
• Scissors, saws, shears, etc. for cutting biomass	~ 1/group
• 25 or 50mL graduated cylinder	1/group
• 600mL beakers	1/group
• Hot plates	1/group
• Pens or tape to mark Falcon tubes	1/group
• Pipettes	1/group

• Thermometer	1/group
• Water bath or incubator with racks to hold tubes	1/class
• Cellulase enzyme (Celluclast: available from Sigma)	~10mL/class
• Yeast (standard dry active baker's)	1/2 tsp/group
• 1/4 teaspoon measurer	1/group

Safety

- All appropriate safety precautions and attire required for a science laboratory should be used, including safety goggles. Please refer to your teacher's instructions.
- Wash your hands well with soap after completing the lab.

Part II: The Experiment

Procedure

STEP 1: Sample Preparation and Pretreatment

GOAL

Break down plant cell walls to release the cellulose fibers

1. Label two 50-mL conical tubes and caps with your team initials, date, and sample description (biomass source and any pre-treatment)
   - The labels in **Figure 3**. are examples. Every group will have 2 tube setups with the same biomass.
   - If any pre-treatment is required, do so (cutting, grinding, drying, etc.).
2. Measure 1.0 gram of your biomass samples and put the 1.0 gram into the corresponding 50-mL falcon tube.
3. Test the initial glucose concentration using the blood glucose test monitor and test strips, record this data. Describe the biomass (ex: Appearance, odor).
4. Test the initial ethanol concentration using the ethanol probes; record this data. Describe the biomass (ex: Appearance, odor).

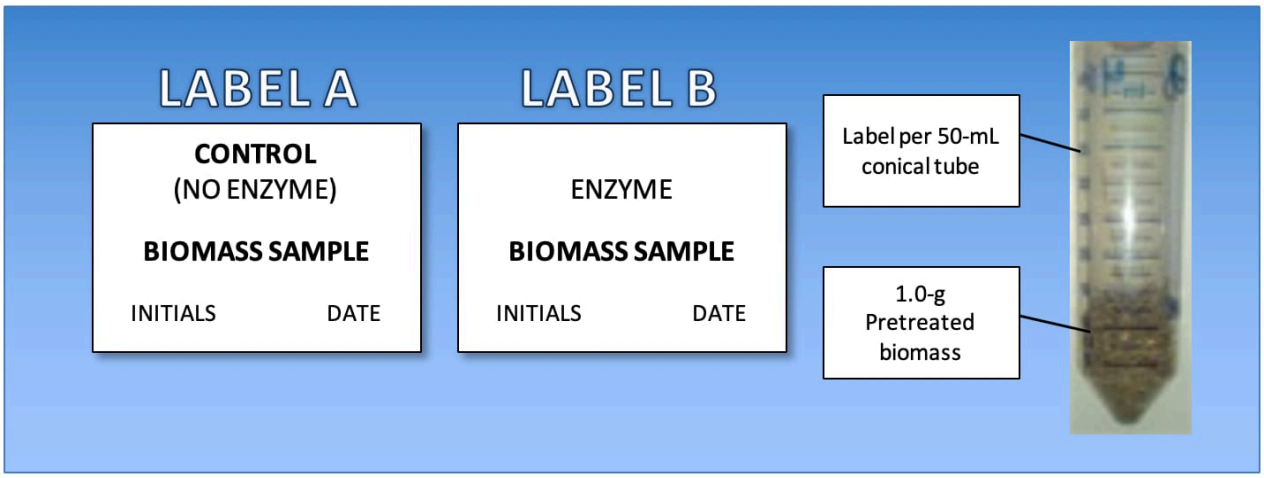


Figure 3: Example labels and diagram of sample preparation.

Hot Water Pretreatment

1. Start the hot plate to bring approximately 400-mL water to a gentle boil in a 500-mL glass beaker. Use pre-heated water to fill your beaker.
2. Set up a conical tube holder (i.e. chicken-wire screen or aluminum) foil for your 500-mL beaker as directed by your teacher. If you are partnering with another group, you can pack 4 tubes in a beaker without setting up a holder.

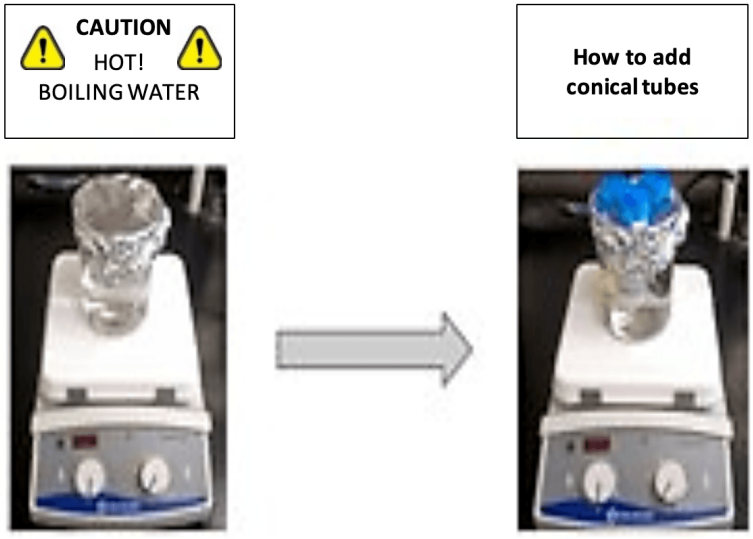


Figure 4: How to setup hotplate and tubes.

3. Add 25-mL of distilled water to the to all three of your labeled 50-mL conical tubes.
4. Swirl to mix the biomass and the water. Let it sit for 1 minute.
5. Loosely screw the cap onto the conical tube; DO NOT tighten all the way.

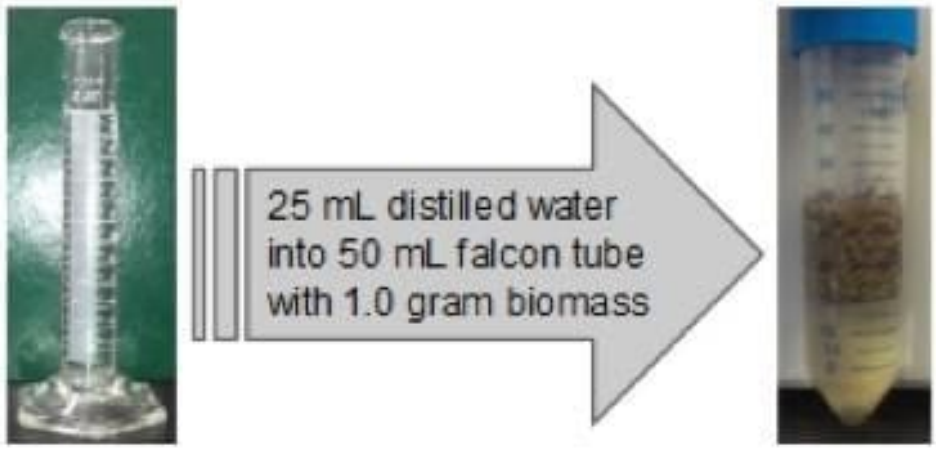


Figure 5: Contents of the falcon (conical) tube.

6. Wait for water in your beaker to come to a gentle boil on your hot plate.
7. Gently push your two conical tube samples into the beaker though the aluminum foil or wire screen. If you are using the 4-tube method pack the 4 tubes into the 500-mL beaker. Make sure that the biomass samples and the liquid are completely submerged below the surface of the boiling water in the beaker.
8. Leave tubes in the water for 10 to 25 minutes depending on how much time you have. The longer the time period, the higher the potential yield of ethanol will be.
9. Turn off the hot plate and remove your samples. Allow them to cool to room temperature. **Lab Tip:** Use a cold-water bath to make the tubes cool more quickly.
10. Test the glucose concentration using the blood glucose test monitor and test strips; record this data. Describe any detectable changes in the biomass (ex: Appearance, odor).
11. Test the ethanol concentration using the ethanol probes; record this data. Describe any detectable changes in the biomass (ex: Appearance, odor).
12. If samples will not be used in the next 2 days, refrigerate or freeze them immediately. This

will suppress microbial growth.

Step 2: Enzymatic Digestion (Hydrolysis)

GOAL

Digest the cellulose fibers into glucose (sugar)

1. Remove samples from refrigerator or freezer and bring to room temperature.
2. Make sure the common water bath or the incubator is at 50°C.
3. Add 1.0 mL of Celluclast™ cellulase enzyme product to each test tube that is undergoing hydrolysis. The control will not have any enzyme added.
4. Screw caps on tightly. Mix gently.
5. Place both conical tubes in a common water bath or incubator at 50°C.
6. Leave the tubes in the water bath for 24 hours.
7. After 24-hour hydrolysis data collection: Use the blood glucose test monitor and test strips to test post-enzyme glucose concentration of the sample. Record this data. Describe any detectable changes in the biomass (ex: Appearance, odor).
8. Test the ethanol concentration using the ethanol probes. Record this data. Describe any detectable changes in the biomass (ex: Appearance, odor).

NOTE: For more accurate ethanol readings, allow samples to reach room temperature before taking measurements.

9. If fermentation will not begin at this stage, freeze or refrigerate samples to prevent microbial contamination.

Step 3: Fermentation

GOAL

Convert glucose (sugar) into ethanol (fuel)

1. Make sure the common water bath or incubator is at 37°C.
2. Add ¼ teaspoon or 1.0 gram of active yeast to each tube. These measurements are roughly equivalent.
3. Gently mix in the yeast. The yeast will grow more quickly if evenly mixed.
4. Loosely screw on the cap to the tubes. It is important that the tubes not be air-tight for the fermentation. Yeast will produce CO2 and will build up pressure in the tube unless the gas is allowed to escape.
5. Place conical tubes upright in the 37°C water bath or incubator. Use a test tube rack or similar apparatus (chicken wire) to keep the tubes upright.

OPTIONAL: After 30 minutes measure ethanol and glucose concentration, record data and other observations about changes occurring in the tubes.

6. Return your tubes to the 37°C common water bath or incubator for 24 hours of fermentation.
7. After 24 hours, remove your tubes from the 37°C water bath.

NOTE: If 24-hour measurement does not fit with class schedule, instructor can remove samples from water bath and refrigerate or freeze until final measurements can be taken.

8. Take final glucose readings: Use the blood glucose test monitor and test strips to test post-enzyme glucose concentration of the sample. Record this data. Describe any detectable changes in the biomass (appearance, odor?).

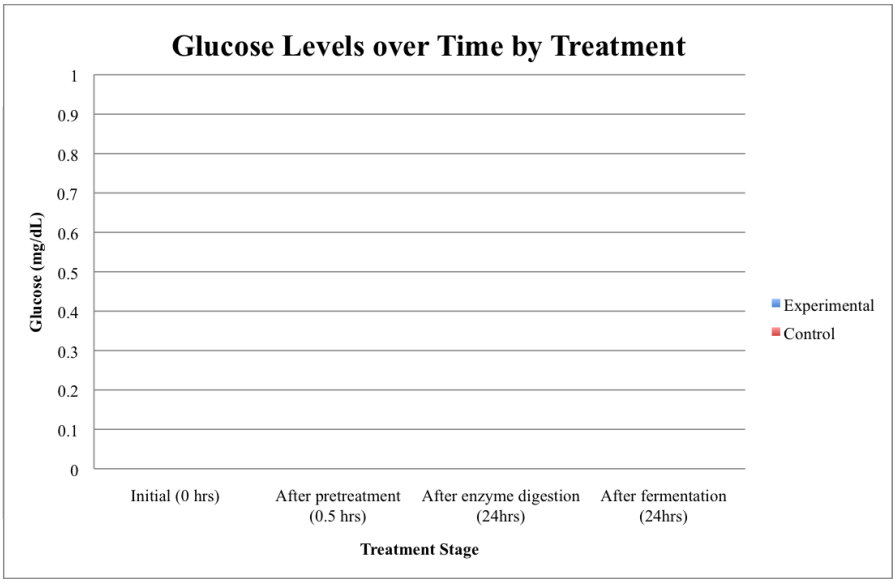
9. Take final ethanol readings. Test the ethanol concentration using the ethanol probes.
10. Record this data. Describe any detectable changes in the biomass (Appearance, odor). NOTE: For more accurate ethanol readings, allow samples to reach room temperature before taking measurements.
11. Clean tubes and lab area as instructed by your teacher.

DATA ANALYSIS

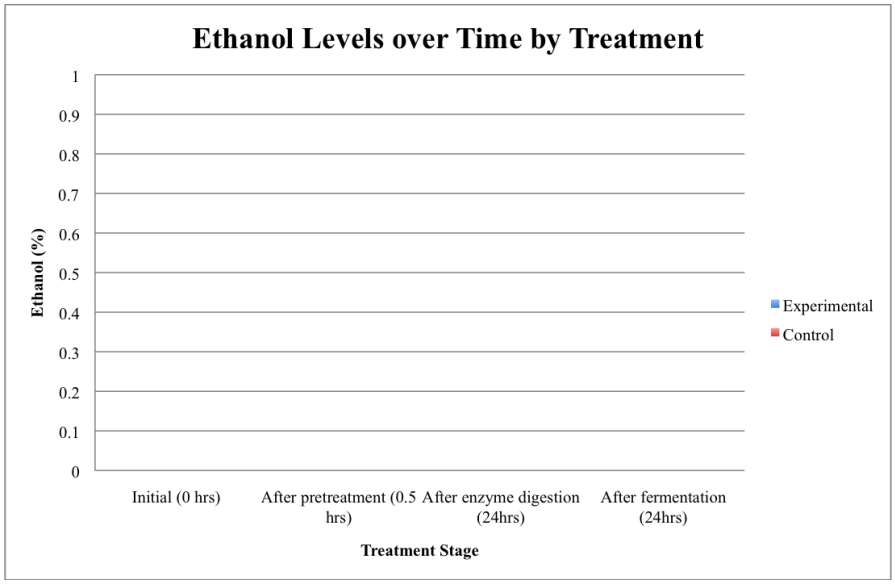
To organize and draw conclusions form your data, it is helpful to compare changes in glucose and ethanol levels over time using bar graphs. Using a computer program such as Microsoft Excel (or by hand), create two bar graphs to summarize your results (Graphs 21.1-2). The empty graphs below can serve as a guide (full sized are available on page 21-9).

Discuss the graphs with your lab group:

1. Do these results match your initial prediction? Why or why not?
2. How do you explain your results? Summarize and communicate results as instructed by your instructor.



Graph 21.1.



Graph 21.2.

RESULTS

Use your graphs and lab notebook data to answer these questions about the results of this experiment. Be prepared to share your answers with the class.

1. Did you observe any changes in glucose and ethanol levels after the enzyme digestion stage (hydrolysis)? Explain why or why not.
2. Where does the glucose come from in this experiment?
3. Did you observe any changes in glucose and ethanol levels after fermentation? Explain.
4. Why do you think that glucose levels went up then then went down in over the course of this experiment?
5. Did your observed results match what you expected would happen? Explain why or why not.

CONCLUSION

Share your results and initial conclusions with the class. Learn from your classmates' results and observations so you can determine what might be the most effective ways to convert biomass into ethanol.

STUDY QUESTIONS

1. Of all of the samples tested in your class, what biomass treatment produced the most glucose and ethanol? Explain why you think this treatment was most effective.
2. Of all of the samples tested in your class, what biomass treatment produced the least glucose and ethanol? Explain why you think this treatment was least effective.
3. If you were to try this experiment again to produce more ethanol what would you do differently? Explain why.
4. Explain how you would design an experiment to determine whether the boiling pretreatment had an effect on how much glucose and ethanol is produced.

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2.2.21: Enzyme Function

Learning Objectives

Goals:

- Carryout a colormetric assay to monitor amylase activity.

STUDENT LEARNING OUTCOMES:

Upon completion of this lab, students will be able to:

- Describe how an enzyme works.
- Explain the effect temperature has on the rate of chemical reactions.
- Explain the effect of pH on enzyme activity.

Introduction

Living organisms sustain the activities of life by carrying out thousands of chemical reactions each minute. These reactions do not occur randomly, but are controlled by biological catalysts called **enzymes**. Enzymes accelerate the rate of chemical reactions by lowering the **activation energy** needed to trigger the reaction. Without enzymes, chemical reactions would not occur fast enough to support life. Enzymes are typically proteins and each is composed of a specific sequence of amino acids. Hydrogen bonds form between specific amino acids and help create the 3-dimensional shape that is unique to each enzyme. The shape of an enzyme, particularly its **active site**, dictates **catalytic specificity** of a particular enzyme. Each enzyme will only bind with specific molecules, as these molecules must fit with the active site on the enzyme like a lock and key.

A molecule that binds with an enzyme and undergoes chemical rearrangement is called a **substrate**. The enzyme “E” combines with the substrate molecule(s) “S” at the active site and forms a temporary **enzyme-substrate** complex “ES”, where the specific reaction occurs. The modified substrate molecule is the product “P” of the reaction. The product separates from the enzyme and is then used by the cell or body. The enzyme is neither consumed nor altered by the reaction and can be used in other catalytic reactions as long as additional substrate molecules are available.

Reaction 1

$$\mathrm{E} + \mathrm{S} \longrightarrow \mathrm{ES} \longrightarrow \mathrm{E} + \mathrm{P}$$

An individual enzyme molecule may facilitate several thousand catalytic reactions per second, and therefore only a small amount of enzyme is needed to transform large amounts of substrate molecules into product. The amount of a particular enzyme found in a cell at any given time is relative to the rate at which the enzyme is being synthesized compared to the rate at which it is degraded. If no enzyme is present, the chemical reaction catalyzed by that enzyme will not occur at a functional rate. However, when the concentration of the enzyme increases, the rate of the catalytic reaction will increase as long as the substrate molecules are accessible.

Various factors can inactivate or **denature** enzymes by altering their 3-dimensional shape and inhibiting their substrate binding efficiency. Many enzymes function best within a narrow temperature and pH range as substantial changes in temperature or pH disrupt their hydrogen bonds and alter their shape. Change in enzyme shape typically alters the shape of the active site, and affects its ability to bind with substrate molecules. It is the unique structural bonding pattern of an enzyme that determines its sensitivity to change in temperature and pH.

In the following exercise you will explore the effects of pH and temperature on the activity of the enzyme amylase. Amylase is found in the saliva of humans and other animals that consume starch as part of their diet. Starch is a plant polysaccharide composed of many glucose molecules bonded together. Amylase controls the initial digestion of starch by breaking it down into

disaccharide maltose molecules. Maltose is ultimately broken down into glucose molecules in the small intestine when other enzymes are utilized.

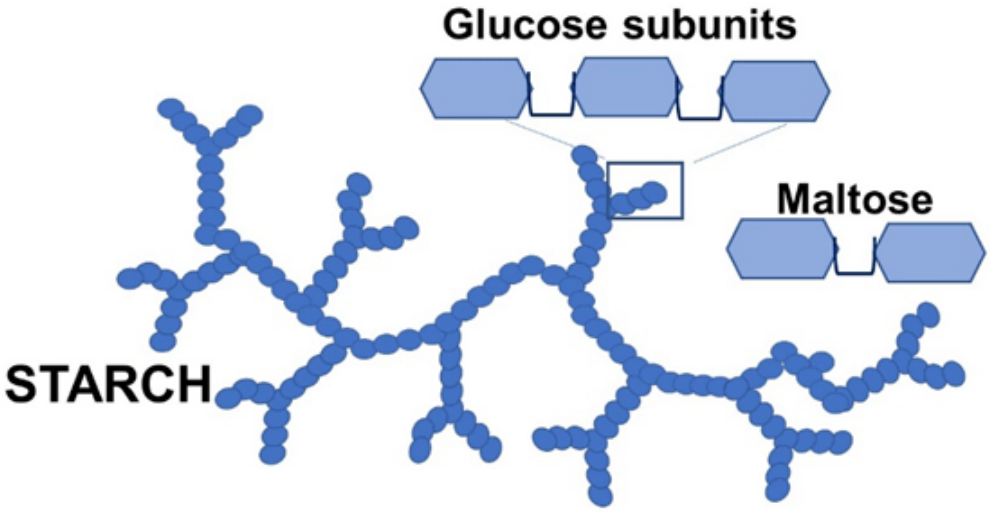


Figure 1: Branching structure of starch.

The rate at which starch is digested into maltose is a quantitative measurement of the enzymatic reaction. The rate of starch degradation is relative to the rate at which maltose is produced, however it is easier to test for the presence of starch than it is to measure the rate of maltose production. I_2KI will be used as indicator for the presence of starch. When starch is present, I_2KI turns a blue-black color. In the presence of maltose, I_2KI will not react and remains an amber color.

Reaction 2

$$Starch + I_2KI \rightarrow \text{Dark Blue-Black color}$$

Reaction 3

$$Maltose + I_2KI \rightarrow \text{Amber color}$$

Your group will be assigned to conduct one or more of the following activities (in part or whole):

I. The effect of pH on amylase activity

II. The effect of temperature on amylase activity

Results from each exercise will be presented to the class and students will be responsible for the information and results from all exercises.

Part I: The Effect of pH on Amylase Activity

Each enzyme has an optimal pH at which it is the most active or effective. A change in pH can alter the bonds of the 3-dimensional shape of an enzyme and cause the enzyme to change shape, which may slow or prohibit binding of the substrate to the active site. You will determine how pH affects amylase activity in this exercise.

Before beginning this experiment, formulate a hypothesis you wish to test and a prediction to evaluate your hypothesis by and write these into the data sheet at the end of the exercise.

Materials

- Test tubes
- Buffers (pH: 1.0, 5.0, 10.0)
- Micropipettes
- Sterile pipette tips
- 1% starch solution
- I_2KI solution
- Well plate
- 0.5% amylase solution
- Timer

Procedure

1. Label 3 test tubes #1 - #3. Beginning with tube #1 and pH 1, mark one tube with each of the following buffer pH: 1.0, 5.0, and 10.0 (See Table 1 below). After you mark the test tubes, use a P-1000 micropipette and add 4.0 mL of the appropriate buffer to each test tube (4.0 mL pH 1.0 buffer to tube #1, 4.0 mL of pH 5.0 buffer to tube #2, etc). **Be sure to place a new tip on the micropipette for each buffer.**



Figure 2. Well plates

2. Using a P-1000 micropipette, add 2.0 mL of the 1% starch solution to each tube and mix by gently swirling the tube and tapping the bottom of the tube against your palm.
3. Place 2 drops of I_2KI into the compartments of several rows of the test plate so that 24 compartments have clear amber liquid in them.
4. Starting with Tube #1 only for now, do the following:
5. Using a **clean new tip** on a P-200 micropipette, draw 50.0 microliters of liquid from the test tube and dispense it into the first compartment of the test plate containing I_2KI. **The mixture should turn dark blue or black.** (This confirms that starch is present in your test tube before the enzyme is added to the tube.)
    - **Do not touch the I2KI with the Pipette Tip!**
    - If you do, eject the tip and change to a new clean one.
6. Add 400.0 µL of 0.5% amylase solution using a P-1000 micropipette. **Begin recording the time in seconds the moment the amylase is added**. Mix the tube contents by swirling the tube and gently tapping the bottom of the tube against your palm. Proceed to the next step immediately (within 20 seconds).
7. **At exactly 20 seconds**, transfer 50.0 µL (using your P-200 micropipette) of the reaction mixture from tube #1 into the next new compartment containing I_2KI on the test plate.
    - **Do not touch the I2KI with the Pipette Tip!**
    - You can continue using the same tip for step 8 below as long as it remains uncontaminated.
8. **Repeat step 7 every 20 seconds** using the next new compartment on the test plate. Continue until a blue-black color is no longer produced and the I_2KI solution remains amber (indicating that no starch remains). Then count the total number of compartments that did change colors plus on that did not change colors and multiply by 20. Record the time required for the complete digestion of the starch in Table 1.

If the color of the I_2KI continues to change to a darker color after a total of 8 minutes of testing, stop testing that reaction mixture, record 480 seconds as the time in the data table, and proceed with step 9.

9. Repeat steps 5 - 8 using tube #2 then #3. You may need to clean your test plate by rinsing it with DI water, tapping it dry, and then adding fresh I_2KI to the compartments.

Data Table 1

Tube	pH	Time of Starch Digestion (sec)
1	1	
2	5	
3	10	

Data Analysis

1. How would you interpret the results shown in Table 1?

Part II: The Effect of Temperature on Amylase Activity

Chemical reactions speed up as temperature increases. A 10° C rise in temperature typically results in a two- to threefold increase in the rate of reaction. However, at high temperatures proteins can be irreversibly denatured and substrate binding is prohibited. The activity of an enzyme is dependent on its proper structure, and the optimum temperature for activity may vary depending on the structure of the enzyme.

Before beginning this experiment, formulate a hypothesis you wish to test and a prediction that can be used to evaluate your hypothesis. Write these into the data sheet at the end of the exercise.

Materials

- Test tubes
- Micropipettes
- Sterile pipette tips
- DI water
- Hot water bath (80°C and 37 $^{\circ}$C)
- Ice bath (4°C)
- 1% starch solution
- I_2KI solution
- Well plate
- 0.5% amylase solution
- Timer

Procedures

1. Label 3 test tubes #1-#3.
2. Using a P-1000 micropipette, add 1.0 mL of 1% starch solution to each tube.
3. Using a new tip on your P-1000 micropipette, add 3.0 mL DI water to each tube.

4. Using a new tip on your P-1000 micropipette add 1.0 mL of pH 5.0 buffer to each tube.
5. Place the test tubes as follows: Tube #1 in 80°C water bath, Tube #2 in 37°C water bath (or incubator), Tube #3 in crushed ice (4°C).
6. Let all 3 tubes sit in the specified environments for at least 15 minutes.
7. Place 2 drops of I_2KI into the compartments of several rows of the test plate so that 24 compartments have clear yellow liquid in them.
8. Starting with tube #1, do the following:
9. Using a **CLEAN NEW TIP** on a P-200 micropipette, draw 50.0 microliters of liquid from the test tube and dispense it into the first compartment of the test plate containing I_2KI. The mixture should turn dark blue or black. (This confirms that starch is present in your test tube before the enzyme is added to the tube.) DO NOT TOUCH THE I_2KI WITH THE PIPETTE TIP! If you do, eject the tip and change to a new clean one.
10. Add 400.0 µL of 0.5% amylase solution using a P-1000 micropipette. **Begin recording the time in seconds the moment the amylase is added.** Mix the tube contents by swirling the tube and gently tapping the bottom of the tube against your palm. Proceed to the next step immediately (within 20 seconds)

IMPORTANT! Leave the tubes in their temperature environments as they are being tested!

11. **At exactly 20 seconds**, transfer 50.0 µL (using your P-200 micropipette) of the reaction mixture from tube #1 into the next new compartment containing I_2KI on the test plate.
    - **Do not touch the I2KI with the Pipette Tip!**
    - You can continue using the same tip for step 8 below as long as it remains uncontaminated.
12. **Repeat step 11 every 20 seconds** using the next new compartment on the test plate. Continue until a blue-black color is no longer produced and the I_2KI solution remains amber (indicating that no starch remains). Then count the total number of compartments that did change colors plus on that did not change colors and multiply by 20. Record the time required for the complete digestion of the starch in Table 2.
13. If the color of the I_2KI continues to change to a darker color after a total of 8 minutes of testing, stop testing that reaction mixture, record 480 seconds as the time in the data table, and proceed with step 9.
14. Repeat steps 9 - 13 using tube #2 then #3. You may need to clean your test plate by rinsing it with DI water, tapping it dry, and then adding fresh I_2KI to the compartments.

Data Table 2

Tube	Temperature (°C)	Time of Starch Digestion (sec)
1	80	
2	37	
3	4	

Data Analysis

How would you interpret the results shown in Table 10.2?

Study Questions

1. How does an enzyme speed up a chemical reaction?
2. What factors can denature a protein? How?
3. What reaction does amylase catalyze?
4. Explain the colormetric assay used to monitor amylase activity.

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https://docs.google.com/document/d/1-jEu3cud3t2D1qj9EyVyLrjVFOHRrq9N/edit

The protocols and instructor guide included in this lab manual are part of a semester-long Synthetic Biology undergraduate research course (CURE) designed by the authors. The lab presented in this manual is designed for a Genetics or Biotechnology class and has been used at both two-year and four-year institutions. Students are tasked with designing and then engineering a biological device in a bacterium (*E. coli*) using standardized DNA parts called Bio-Bricks from the iGEM (www.iGEM.org) registry. The process of engineering the device requires iteration of standard molecular biology cloning techniques which are outlined in the lab manual, some including video protocols. Additionally, provided are recipes for reagents, a supply/resources list, and instructor recommendations. A series of faculty training videos were created to further help with the implementation of the SynBio CURE.

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2.3.1.1: Making Media (LB Broth and Agar)

LB Agar:

Making 1 L of LB Agar:

- 5 g Yeast extract
- 10 g Peptone
- 10 g NaCl
- 12 g Agar

1. Mix this with 1 L of Distilled H2O
2. Autoclave at 121 °C for 30 minutes (If there is a setting, set it to slow/liquid)
3. Let cool before pouring into plates (approximately 25 mL of agar in each plate)

This recipe makes about 40 plates and can be adjusted as needed!

LB Broth:

Making 1 L of LB Broth:

- 25 g pre-mixed broth powder

This powder contains 10 g tryptone, 5 g yeast extract and 10 g NaCl

1. Mix this with 1 L of Distilled H2O
2. Autoclave at 121 °C for 30 minutes (If there is a setting, set it to slow/liquid)
3. Let cool before pouring into **STERILE** test tubes with lids (approximately 5 mL of broth per test tube)

This recipe makes about 200 tubes of broth and can be adjusted as needed!

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2.3.1.2: Adding Antibiotics

Color coding LB-agar plates is recommended to simplify which plate contains which antibiotic

Addition of antibiotics to media is done after autoclaving, when the media has cooled to 50-55oC

**Ampicillin (Amp/A): ***Working concentration 100 ug/mL freezer stock 100 mg/mL (in water)

- Add 1 mL of 100 mg/mL ampicillin (dissolved in water) into 1 L of LB broth or agar

Chloramphenicol (Chlor/C): *Working concentration 35 ug/mL freezer stock 35 mg/mL (in EtOH)

- Add 1 mL of 35 mg/mL chloramphenicol (dissolved in ethanol) into 1 L of LB broth or agar

Kanamycin (Kan/K): *Working Concentration 50 ug/mL freezer stock 50 mg/mL (in water)

- Add 1 mL of 50 mg/mL kanamycin (dissolved in water) into 1 L of LB broth or agar

**Tetracycline (Tet/T): ***Working concentration 10 ug/mL freezer stock 10 mg/mL (in water)

- Add 1 mL of 10 mg/mL tetracycline (dissolved in water) into 1 L LB broth or agar

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2.3.1.3: Making Linearized Backbone PCR Protocol

PCR Protocol for Linearized Backbones

All procedures copied or modified from iGEM's linear backbone protocol and Q5® Hot Start High-Fidelity 2X Master Mix Protocol

1. Grow up part BBA_J04450 from the iGem plasmid. Grow 1 part for each different backbone (**psB1C3**, **psB1K3**, **psB1T3**, **psB1A3**)
2. Isolate the DNA from culture and obtain DNA concentration (via DeNovix/Nanodrop/Qubit)
3. Make PCR reaction mixture for plasmid amplification using the following table:

**Keep everything on ice and label PCR tubes before starting. Always add the DNA polymerase last as it will sink to the bottom. Use the 50μL reaction for linear backbones. Usually, 1µL of DNA is sufficient. **

Component	25 uL Reaction	50 uL Reaction	Final Concentration
Q5 High-Fidelity 2X Master Mix	12.5 uL	25 uL	1X
10 uM Forward Primer (SB-prep-3P-1)	1.25 uL	2.5 uL	0.5 uM
10 uM Reverse Primer (SB-prep-2Ea)	1.25 uL	2.5 uL	0.5 uM
Template DNA (psB1C3, A3, K3, T3)	Variable	10 ng	< 1,000 ng
Distilled Water	up to 25 uL	up to 50 uL	

Primers for linear backbone:

SB-prep-3P-1 gccgctgcagtccggcaaaaaa,

SB-prep-2Ea atgaattccagaaatcatccttagcg

4. Place tube in the thermocycler, making sure there are **no bubbles** and the lids are closed tight

5. Set the thermocycler to the following settings for plasmid amplification:

Step	Temperature	Time
Initial Denaturation	98°C	30 seconds
25-35 Cycles	98°C	10 seconds
	66°C	30 seconds
	72°C	3 minutes
Final Extension	72°C	1.5 minutes
Hold	4°C	

6. Run 1 uL of the **unpurified** PCR product on a gel to verify correct band size and concentration
a. **psB1C3**= 2077 bp
b. **psB1A3**= 2155 bp
c. **psB1K3**= 2204 bp
d. **psB1T3**= 2461 bp

7. Use a PCR cleanup kit to purify the samples (Qiagen or ZymoPure recommended)

8. Test concentration of purified PCR Product (via DeNovix/Nanodrop/Qubit) **Expected yield should be 40 ng/uL or higher*

9. Run a digestion with the purified PCR product

1. **Digestion:**
    1. **4 uL 10X NEB Buffer 2.1**
    2. **1 uL EcoRI-HF**
    3. **1 uL PstI**
    4. **1 uL DpnI *(Used to digest any template DNA from production)***
    5. **25 uL PCR Linearized plasmid product (*use total amount from purified PCR product)***
    6. **Digest at 37°C for 3 hours**
    7. **Heat kill at 80°C for 20 minutes**

10. PCR purify using the same kit as before, quantify DNA concentration, and adjust final concentration of the plasmid to 25 ng/uL using distilled water

11. Ligate each purified backbone to another plasmid part. Also set-up one control tube for each backbone. *(This tube will not have ANY insert added and will just have water added in place. This tests how often the backbone just closes on itself instead of ligating the insert inside).*

Choose a part with a relatively large insert that you know is correct and you have plenty to spare. Digest 300 ng of the plasmid with EcoRI and PstI according to the Double Digestion instruction. Heat inactivate the sample

1. **Ligation:**
    1. 1 uL purified E/P digested plasmid backbone (25 ng)
    2. Equimolar amount of E/P digested fragment (2 uL)
    3. 1 uL T4 DNA Ligase Buffer
    4. 0.5 uL T4 DNA Ligase
    5. Distilled water up to 10 uL
    6. **Ligate at 16°C for 30 minutes**
    7. **Heat kill at 80°C for 20 minutes**

12. Transform 25 uL of bacteria (JM109 recommended) with 1-2 uL of ligation product

See "Cell Transformation: Zippy Transformation of Z-competent Cells" protocol above for information on how to perform a transformation

13. Plate on the appropriate antibiotic plate according to backbone resistance gene

> *Any colonies on the backbone-only control plates represent background to the three antibiotic assembly process.* ***If there are too many, this could present a problem during the student projects.***
>
> *Many colonies on the backbone+insert plates indicate that the backbones are amplified and cut effectively to be ligated to E/P cut inserts.*

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2.3.1.4: DNA Extraction Supplies

DNA Extraction:

While many vendors offer mini-prep kits that will work for this step, Zymo provides an excellent kit (Cat#: D4210) with great results at a reasonable price.

Materials	**Amount Needed** **(Per Reaction)**
ZymoPURE™ P1 (Red)	250 uL
ZymoPURE™ P2 (Green)	250 uL
ZymoPURE™ P3 (Yellow)	250 uL
ZymoPURE™ Binding Buffer	275 uL
ZymoPURE™ Wash 1	800 uL
ZymoPURE™ Wash 2	1000 uL
ZymoPURE™ Elution Buffer	25 uL
Zymo-Spin™ II-P Columns **(must have purple ring)**	1
Collection Tubes	1
Serological Pipets	2 (at least 2 mL)
Microcentrifuge Tubes	3
Centrifuge	1 (used by class)

Micropipettes and Tips	100-1000 uL, 10-100 uL, 1-10 uL
Ice Bucket	1
UV Spectrophotometer	1 (used by class)

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2.3.1.5: Double Digestion Supplies

Double Digestion:

Enzymes and buffers are indicated from New England Biolabs.

Materials	**Amount Needed** **(Per Reaction)**
Distilled Water	25 uL-(Sum of DNA+NEB+Enzyme1+Enzyme2)
DNA Sample	Determined by spectrophotometer (300ng/DNA concentration)
10x NEBuffer 2.1 (Cat#: B7202S)	2.5 uL
Restriction Enzyme 1 (EcoRI [Cat#: R0101S]) or SpeI [Cat#: R0133S])	1 uL
Restriction Enzyme 2 (XbaI [Cat#: R0145S] or PstI [Cat#: R0140S])	1 uL
Water/Bead Bath (37C)	1 (used by class)
Heat Block (80C)	1 (used by class)
Microcentrifuge Tube	1
Micropipette and Tips	1-10uL, 2-20uL
Microcentrifuge	1 (used by class)

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2.3.1.6: Gel Loading/Running Supplies

Gel Loading and Running:

Materials	**Amount Needed (Per Reaction)**
Agarose	Varies depending on gel percentage
1x TBE	Approximately 165 mL (to make gel an
SybrGreen, SybrSafe, or GelRed (Cat#: S33102)	2-3 uL
Digested DNA	5 uL
DNA Ladder (Quick-Load Ladder Cat#: N0550S)	5 uL
6x DNA Loading Dye (Cat#: B7024S)	1 uL
Microwave	1 (used by class)
Flask	1 (at least 50 mL in size)
Micropipette and Tips	1-10 uL
Gel Rig (MiniOne; Cat#s: M1000, M1002, M1010, M1012)	1 (with all necessary parts included witl
Weigh Tray	1
Scale	1 (used by class)

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2.3.1.7: DNA Ligation Supplies

DNA Ligation:

Materials	**Amount Needed (Per Reaction)**
Distilled Water	5 uL (up to 10 uL)
10X DNA Ligation Buffer (Cat#: B0202S)	1 uL
Linearized Plasmid Backbone (Amp,Chlor,Kan,Tet)	1 uL
Double Digested DNA insert fragments	1 uL
T4 DNA ligase (Cat#: M0202S)	1 uL
Ice Bucket	1
Microcentrifuge Tube	1
Micropipette and Tips	1-10 uL
Heat Block (80C)	1 (used by class)

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2.3.1.8: Transformation Supplies

Transformation:

Materials	**Amount Needed** **(Per Reaction)**
Competent Bacteria Cells (**JM109=** Cat#: T3003)	25-50 uL
Ligation Mixture	1-5 uL
LB Agar with Antibiotics (either Kan, Chlor, Amp, or Tet)	1 plate (depending on backbone)
SOC Media (Cat#: 15544034)	200 uL
Sterile Glass Beads	Approximately 4-8 beads
Ice Bucket	1
Microcentrifuge Tube	1
Shaking Incubator (37C)	1 (used by class)
Plate Warmer (37C) (Can be the same as the Shaking Incubator)	1 (used by class)
Incubator	1 (used by class)

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2.3.1.9: Inoculation Supplies

Inoculation:

Materials	Amount Needed (Per Reaction)
Sterile Culture Tube (with cap)	1
Sterile Toothpick	1
LB Broth	5 mL
Lab Hood	1 (used by class)
Shaking Incubator	1 (used by class)
Antibiotics (Amp, Chlor, Kan, or Tet)	Optional (see Appendix I or Instructor's Notes)

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2.3.1.10: Glycerol Stock Supplies

Glycerol Stock:

Materials	**Amount Needed (Per Reaction)**
80% Glycerol	500 uL
Cryovial (Cat#: 5000-0020)	1
Vortex	1 (used by class)
Micropipette and Tips	100-1000 uL

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2.3.1.11: Linearizing Backbone Supplies

Linearizing Backbones:

Materials	**Amount Needed: (Per Backbone Reac**
Q5 High-Fidelity 2X Master Mix (Cat #: M0492S)	Up to 25 uL
10 uM Forward Primer (SB-prep-3P-1)	Up to 2.5 uL
10 uM Reverse Primer (SB-prep-2Ea)	Up to 2.5 uL
Template DNA (psB1C3, A3, K3, T3)	10 ng
Distilled Water	Up to 60 uL
Thermocycler	1
UV Spectrophotometer	1
Micropipette and Tips	1-10 uL, 0.2-1 uL, 20-200 uL
Heat Block (80C)	1
Gel Rig	1
PCR Clean-up Kit (Cat #: D4033)	1
10X NEB Buffer 2.1	4 uL
Digestion Enzymes (EcoRI, PstI, DpnI)	1 uL of each

Competent Bacteria Cells (**JM109=** Cat#: T3003)	25 uL
LB Agar Plate with Antibiotic (Chlor, Amp, Tet, Kan)	1 plate (depending on backbone)
Sterile Glass Beads	Approximately 4-8 beads
SOC Media (Cat#: 15544034)	200 uL
Shaking Incubator (37C)	1
T4 DNA ligase (Cat#: M0202S)	0.5 uL
T4 DNA Ligase Buffer(Cat#: B0202S)	1 uL

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2.3.1.12: Tips for Organizing the Lab for Large Classes

Preparing for a Class

Example of a Lab Room

Pre-established Freezer Boxes:

It can be helpful to have some already stocked freezer boxes ready for your students before they get into the lab. This can work both for individual students as well as teams. These freezer boxes can be marked via color and assigned to students when the lab has started. The boxes can be pre-stocked with the various reagents your students will need so they will be able to have ready access to them.

This CryoSafe Cooler Box (Cat# 03-410-497) should consist of the following reagents:

- Restriction enzymes (EcoRI, SpeI, XbaI, PstI)
- 10x NEBuffer 2.1
- 10x DNA Ligation Buffer
- T4 DNA Ligase

It is helpful to have another color coded cardboard freezer box that is empty alongside the reagent box. This way your students will ideally have no problem storing their parts as they move through the protocol as well as ensuring fewer mixups between the various student parts as they are stored.

Pre-established Student/Team Work Stations:

Much like the freezer boxes, pre-stocked work stations allow for you to be able to provide your students with access to tools/materials such as micropipettes and microcentrifuge tubes. It is helpful to color coordinate a student/team work station with a freezer box.

This work station should consist of:

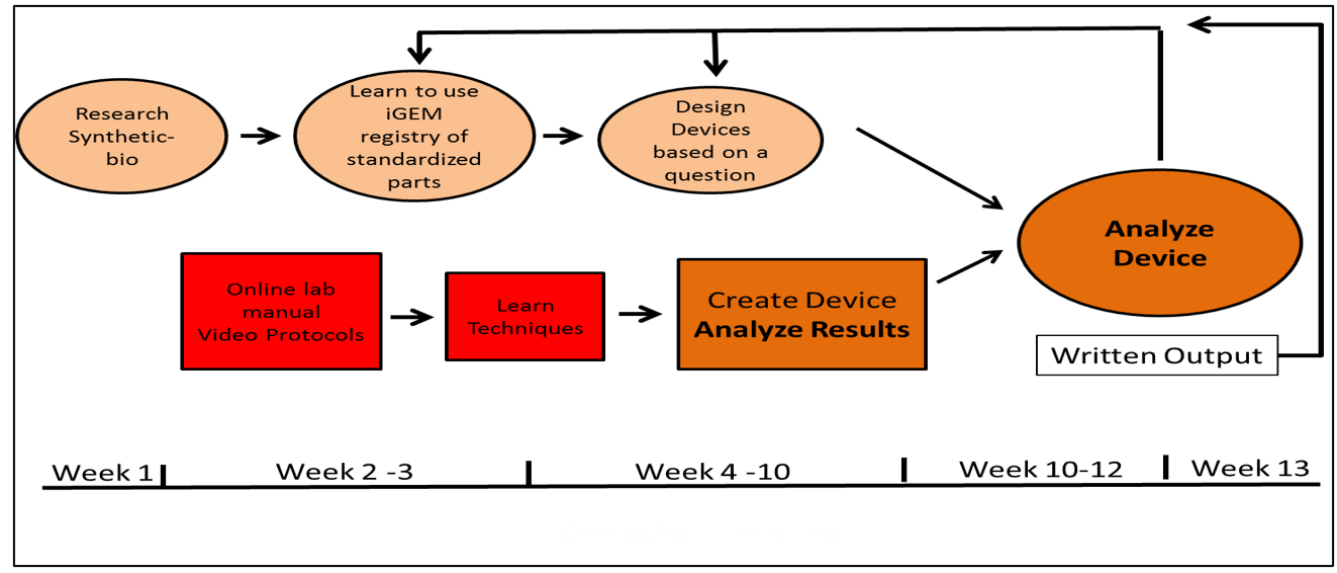
- A set of micropipettes (ranging from 1-10 uL to 100-1000 uL) per student
- At least one tube rack
- A container of microcentrifuge tubes
- A Zymo Mini-Prep kit (this can be the kit itself or pre-aliquoted amounts of the reagents)
- Sharpie
- Pipet tips (a box per type of micropipette)
- Any other room temperature reagents you would want easily accessible

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2.3.1.13: Before you begin a semester overview

The protocols included in this lab manual are part of a Synthetic Biology undergraduate research course (CURE) designed by the authors. The lab presented in this manual is designed for a Genetics or Biotechnology class and has been used at both two-year and four-year institutions. The workflow for the semester long course is shown below. The lab is based off the iGEM genetic engineering competition (iGEM.org). Students are tasked with designing and then engineering a biological device in a bacterium (*E.coli*) using standardized DNA parts called Bio-Bricks from the iGEM registry. The process of engineering the device requires iteration of standard molecular biology cloning techniques which are outlined in this lab manual, some including video protocols. Additionally, provided are appendices with recipes for reagents, supply/resources list, and instructor recommendations. **Instructors are encouraged to watch a series of implementation videos created for the Arkansas-CURE Projects Synthetic Biology faculty training workshop.**



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2.3.2: STUDENT LAB MANUAL- PROTOCOLS

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2.3.2.2: Plasmid DNA Extraction (Mini-Prep)

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2.3.2.1: Visual Representation of the Protocol

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2.3.2.2: Plasmid DNA Extraction (Mini-Prep)

Zymo-Pure Plasmid DNA extraction (Micro-Centrifuge Method)

Learning Objective

- Isolation of Plasmid DNA from overnight cultures in LB.

This method is spin column-based and purifies up to 100 μg of ultra-pure endotoxin-free plasmid DNA in less than 15 minutes. The result is plasmid DNA suitable for transfection, restriction endonuclease digestion, bacterial transformation, PCR amplification, and DNA sequencing. (ZymoPURE Plasmid Miniprep Kit)

Note

Watch the Youtube video for questions about the Miniprep protocol

This interactive element is not available in this format. Visit the link or scan the QR code to access it online.

View this YouTube Video online

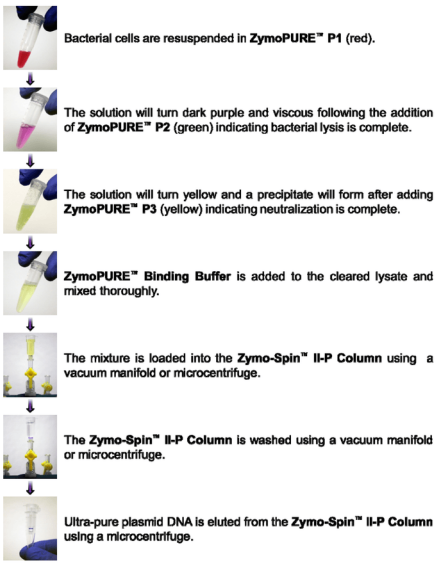
ZymoPURE Plasmid Miniprep video

Note

The P1 reagent is temperature sensitive due to RNase being present, and should be kept in the fridge or on ice at all times. All other reagents will be stored at room temperature.

Reagent name and Code	Storage Temperature
ZymoPURE™ P1 (Red)	4°C
ZymoPURE™ P2 (Green)	Room Temp.
ZymoPURE™ P3 (Yellow)	Room Temp.
ZymoPURE™ Binding Buffer	Room Temp.
ZymoPURE™ Wash 1	Room Temp.
ZymoPURE™ Wash 2	Room Temp.
ZymoPURE™ Elution Buffer	Room Temp.
Zymo-Spin™ II-P Columns **(must have purple ring)**	Room Temp.
Collection Tubes	Room Temp.

Visual Procedure of Zymo Plasmid Mini-Prep



TIPS BEFORE STARTING

The first couple of times you do the Mini-Prep, on the protocol indicate/LABEL **WHERE your DNA is** for each of the steps! (e.g., DNA is in the supernatant/liquid OR DNA is in the pellet). Each step below has a "circle one" option asking where the DNA is! (pellet or supernatant).

Note

If you *understand* exactly where your DNA is in each one of the steps, you won't LOSE your DNA!!

Protocol: (make sure you have all reagents before you begin... get ice!!!)

1. Using a serological pipet, pipet out **1.0 to 3 mL** (preferably 3 ml) of bacterial culture into a 1.5 ml microcentrifuge tube. Centrifuge this microcentrifuge tube at **full speed** for **20 seconds**. *You will see a small pellet form at the bottom of the tube*. (You must **repeat** steps 1-2 to get more than 1 ml worth of cells

   Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

2. Remove as much media as possible by pouring it off into the Biohazard bag. **Do not disrupt the cell pellet**! (**Repeat** steps 1-2 to increase cell concentration: However, 1-2ml of culture should be enough plasmid.)

   Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

3. Add **250 μL** of **ZymoPURE™ P1 (Red)** to the bacterial cell pellet and vortex or pipet the pellet to resuspend it completely. (**P1 is stored in the fridge at 4°C. Keep on ice when not in use.)** *This step involves RNase to purify the DNA by destroying any RNA in the sample.*

   Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

4. Add **250 μL** of **ZymoPURE™ P2 (Green)** and **immediately mix** by gently inverting the tube **6-8 times**. *Do not vortex!* Let sit at room temperature for **2-3 minutes**. Do not let sit for longer than 3 minutes. (*Cells are completely lysed when the solution appears clear, purple, and viscous.)*

   Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

5. Add **250** μ**L** of ice cold **ZymoPURE™ P3 (Yellow)** and mix thoroughly by inversion. Do not vortex! Invert the tube an **additional 3-4 times** after the sample turns completely yellow. (*The sample will turn yellow when the neutralization is complete and a yellowish precipitate will form.)*

   Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

6. Incubate the neutralized lysate in the microcentrifuge tube on ice for **5 minutes**

   Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

- Centrifuge the neutralized lysate for **5 minutes** at 16,000 *xg (*or **Max speed***)*. (Do not disrupt the white/pelleted cell debris).

   Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

8. Transfer **600** μ**L** of supernatant from step 7 into a clean 1.5 ml microcentrifuge tube. *Be careful not to disturb the yellow pellet and avoid transferring any white cellular debris to the new tube.*

   Where is your DNA? ☐ **Pellet or** ☐ **Supernatant**

9. Add **275** μ**L** of **ZymoPURE™ Binding Buffer** to the cleared lysate from step 8 and mix thoroughly by inverting the capped tube **8 times**.
10. Place a **Zymo-Spin™ II-P Column** (**Purple ring**) in a Collection Tube and transfer the entire mixture from step 9 by pouring or pipetting it into the Zymo-Spin™ II-P Column.
11. Incubate the **Zymo-Spin™ II-P/Collection Tube** assembly at room temperature for **2 minutes** and then centrifuge at 5,000 *xg* for **1 min**. Discard the flow through in the Biohazard bag.

12. Add **800** μ**L** of **ZymoPURE™ Wash 1** to the Zymo-Spin™ II-P Column and centrifuge at 5,000 *xg* for **1 min**. Discard the flow through.
    (The collection tube will hold 900ul of liquid. It is important that the flowthrough does not touch the bottom of the column!)

13. Add **800** μ**L** of **ZymoPURE™ Wash 2** to the Zymo-Spin™ II-P Column and centrifuge at 5,000 *xg* for **1 min**. Discard the flow through.
14. Add **200** μ**L** of **ZymoPURE™ Wash 2** to the Zymo-Spin™ II-P Column and centrifuge at 5,000 *xg* for **1 min**. Discard the flow through.
15. Centrifuge the Zymo-Spin™ II-P Column at $\geq$ 10,000 *xg* (**full speed**) for **1 min** in order to remove any residual wash buffer.
16. Transfer the Zymo-Spin™ II-P Column into a clean 1.5 ml microcentrifuge tube and add **25** μ**L** of (warmed 50$^{\circ}C$- optional) **ZymoPURE™ Elution Buffer** directly to the column matrix. Incubate at room temperature for **2 minutes**, and then centrifuge at $\geq$ 10,000 xg (**full speed**) for **1 minute** in a microcentrifuge. (*warmed buffer and a 5 min incubation may increase plasmid concentrations*)

17. Determine the concentration of your sample using a spectrophotometer (E.g. Denovix, NanoDrop, Qubit). (*See appendices II, III, IV on how to use one of the aforementioned machines.)*

    DNA Purity: Eluted DNA is ultrapure, endotoxin-free, and well suited for transfection, transformation, sequencing, restriction endonuclease digestion, *in vitro* transcription, and other sensitive applications.
    Typical: Abs 260/280 $\geq$ 1.8 and Abs 260/230 $\geq$ 2.0

18. Clearly label each plasmid tube; store it at -20 degrees and record it in the lab notebook!

    What should be on your label?

Top label:

Part # (Bba_J0176)

Side label:

Team Name/Initials (SV/JH Team)

Part Name (RBS: GFP)
Concentration (ng/µL) 260/280 ratio

Plasmid Backbone (Antibiotic Resistance) / psB1C3 (Chlor)
(Date) 6/14/2021

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2.3.2.3: DNA Double Digestion

DNA Double Digestion Protocol

Two restriction enzymes are used simultaneously to digest DNA in a single reaction.

Reagent name	Storage Temperature
Distilled Water	Room Temp
DNA Sample	4°C
10x NEBuffer 2.1	-20°C
Restriction Enzyme 1 (EcoRI or XbaI)	-20°C
Restriction Enzyme 2 (SpeI or PstI)	-20°C

TIPS BEFORE STARTING:

- As you add each reagent to a 1.5 ml microfuge tube, stir it in well with the pipet tip and always change your tip in between steps - **NEVER double dip your tip!**
- ***VERY IMPORTANT!*** Do not dispense the liquids onto the sides of the tube. Always dispense the volumes into the very bottom of the tube!
- Enzymes are stored in glycerol and will never freeze and are ready to be used immediately. They never should be out of the table freezer block or an ice bucket. However, **the buffer must be completely thawed before use**. (Gently flick the tube and spin the contents to the bottom). You can store the buffers at 4°C but they will only last two weeks. *You must keep up with how long your buffer has been at this temperature!*

Procedure: (Take your time! Make sure you're doing each step correctly!)

1. Pipette the following into a 1.5 ml microfuge tube: **Reagents must be added in the specified order (water is always the first reagent to add!)**.

25uL final volume reaction system

If your DNA concentration is too low you can increase the reaction volume to 40-50 ul.

X μL of water to get **final reaction volume of 25** μL
X is equal to *25 - (NEB+DNA+Enzyme 1+Enzyme 2)*
2.5 μL NEB buffer 2.1
Y μL DNA
(usually ~300 ng-Use spectrophotometer to determine concentration and calculate volume needed).
Y is equal to 300 ng/DNA Concentration(ng/uL)

1 μL BioBricks enzyme 1 (either EcoRI or SpeI)
1 μL BioBricks enzyme 2 (either XbaI or PstI)

2. Mix well by pipetting slowly up and down approximately **5x**. **Be gentle, and do not vortex**. Spin the samples for **5 seconds** in a balanced microcentrifuge, or flick them to collect the mixture at the bottom of the tube.

3. Incubate at 37 degrees for at least 1 hour. Samples can be stored at -20 degrees at this point, but DO NOT forget about step 4 before ligation.

4**.** After digestion, **incubate your samples for 80°C for 20 minutes (in heat block)**. For 3A assembly it is important you heat

inactivate your samples after digestion. **THIS MUST BE DONE or NOTHING ELSE WILL WORK!**

5. Run 5 μL of your digested sample in an agarose gel in order to check that your digestion worked. (*See Section 1.4 for making/ pouring of agarose gel as well as running a gel electrophoresis).* Make sure you run the proper controls with your samples on the gel: (1) a small volume of uncut DNA for each plasmid digested. (2) **Also, always run a lane with DNA Ladder**!

Be sure to clearly label and freeze the remainder of your digestion. This will be used for the ligation step.

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2.3.2.4: Gel Loading and Running

Gel Loading and Running using MiniOne Instructions

Using agarose gel electrophoresis for the visualization of digested DNA.

Video example of pouring and running a gel

Reagent name	Storage Temperature
Agarose	Room Temp
1x TBE	Room Temp
SybrGreen/SybrSafe/GelRed	Room Temp (Keep in dark place)
Digested DNA	$4^{\circ}C$
DNA Ladder	Room Temp
Loading Dye	Room Temp

TIPS BEFORE STARTING:

Start by making a bottle of 500 ml stock of 1x TBE (*C1V1=C2V2*) for your team's running buffer and to make agarose gels. (You can store this for future use- label the bottle clearly!) You will utilize either 10X, 25X or 50X TBE stock buffer– Pay attention to what stock solution you have been provided with!

Procedure:

1. You will make 30ml of agarose that is 1X TBE and usually 0.75-1.5% agarose. (**See Appendix V for suggestions on what percentage to use.**)

a. Measure ___ g of agarose (0.75-1.5%)

b. Add ___ mL of 1x TBE buffer.

1. Heat gel for **1+ min** in microwave – (**Use plastic flasks!**). Swirl to make sure all crystals are in solution!
2. Let the gel cool for 1 minute.
3. Add **2-3** μL of SybrGreen, SybrSafe or GelRed *(ask your instructor where this is stored; keep tube in the dark at all times)*
4. Let the gel cool for another minute - Do not pour HOT!!!!!
5. Split warm agarose between the two gel containers (you will only need one). Do not forget the combs.
6. Load **5** μL of your DNA ladder per gel lane. (*Your DNA ladder should preferably be in the first lane of your gel).*
7. Load your samples into the wells of the gel, and mark down in your notebook the order in which you loaded the lanes. a. Loading your samples: **5** μL of DNA mixed with **1** μL of 6x loading dye. Load this directly in the well.
8. Run gel for **20 min** in the Gel rig (set up according to diagrams below). While the gel is running, simulate the digest(s) in your lab (physical/digital) notebook, adding the *Quick-Load Purple 1 kb Plus DNA Ladder* as your ladder and paste the image of the expected gel in your notes. Take a picture of your actual gel results and paste them into your lab notebook.

The gel can be visualized in the gel rig (blue light) or for more sensitivity on the UV light box. WARNING: UV light is dangerous. Do not use unless you have talked to your professor!

See Appendix V for any questions you might have about the agarose gel or the electrophoresis protocol

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2.3.2.5: Ligation of Products

Ligation of Products

Joining of 2 DNA molecules catalyzed by DNA ligase

This process forms a phosphodiester bond between the 3' hydroxyl and 5' phosphate of adjacent DNA strands, creating recombinant DNA. (NEB)

Video example of performing a ligation

Materials	Storage Temperature
Water	Room Temp
10X T4 DNA Ligation Buffer	-20°C
Linearized Plasmid Backbone (Amp,Chlor,Kan,Tet)	-20°C
Double Digested DNA insert fragments	-20°C
T4 DNA Ligase	-20°C

TIPS BEFORE STARTING:

An equal (1:1) molar quantity of each insert is best. However, 1 μL of each digested insert is commonly used for the ligation and gets consistent results. **If your parts are drastically different in size, you may want to adjust for molar quantity. (***If you are not getting good results/colonies upon transformation, start looking at changing the molar ratio of your inserts. Use NEBioCalculator to calculate molar ratios.)*

Procedure

1. Thaw 10X T4 DNA **Ligation Buffer** at room temperature and then store it on ice until needed. Leave **DNA ligase (enzyme)** in the freezer block until immediately before it is needed; always keep cold!

2. **Always add water and buffer first**. Buffer must be fully thawed and mixed before use:

5 μL of Water *(water up to 10ul, if insert volumes are adjusted)*
1 μL of **10X DNA ligation buffer**
1 μL (25ng) **linearized plasmid (destination) backbone**
1 μL of **insert A** *(digested with appropriate enzymes / denatured)*
1 μL of **insert B** *(digested with appropriate enzymes / denatured)*
1 μL of **T4 DNA ligase** *(actual enzyme)*

3. Incubate at room temperature for **1hr to 2hr.** *Ligations can go overnight or longer if stored at 4 °C (refrigerated). This works the best.*

4. Heat inactivate the reaction at **65°C** (or 80°C) for **10 minutes**. *(Optional but company recommended.)*

5. Samples can be stored at -20°C or used immediately in the Zippy transformation protocol. It is best if you immediately transform the **5** μL of your reaction into a tube of zippy cells. (*Ligation buffer can reduce transformation efficiency. Using more than 5ul may actual decrease transformation efficiency)*

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2.3.2.6: Cell Transformation

Cell Transformation: Zippy Transformation of Z-competent Cells

Introducing foreign DNA into E. coli bacteria

Bacterial transformation is when cells take up foreign DNA from the environment. Cells may express the genetic information they received from this foreign DNA. (ThermoFisher)

TIPS BEFORE STARTING:

- Depending on the situation, particularly when you get to the analysis phase and you request a different type of bacterial chassis, you will use a variety of competent cells. Be sure you know what cells you are using and if there is a specific protocol for the type of cell. (The competent bacteria described in this protocol are Zippy Competent JM109, ZymoResearch)
- Most plasmids carry a marker gene for a specific antibiotic resistance. By supplementing the growth medium with the antibiotic of choice, only cells containing the plasmid of interest will propagate. **You must know the antibiotic resistance associated with your plasmid backbone!! (See Appendix I)**

All competent cells are stored in the -80 freezer. Ask the instructor for help when you are ready to get the competent cells. You must have ICE, first!

Materials	Storage Temperature
Zippy Competent Bacteria Cells	-80°C
Ligation Mixture	fresh / -20°C
LB Agar plate w/Antibiotic	Room Temp / 37°C
SOC	4°C / Room Temp
Sterile Glass Beads	Room Temp

Procedure: Read every step before you begin!

1) Make sure you have a warm plate. Pre-warm by placing it in the **37°C** incubator or by taking it out at room temperature before starting the procedure. *See color coding (Appendix I) to ensure you have appropriate LB+antibiotic plate type!*

2) Get ice, then request cells / Let the cells thaw for **1-2 minutes on ice**. – The 50 μL of cells can be split into **2 tubes of 25** μL each for two different transformations if used immediately to transform different plasmids. *(If you don't have two plasmids to transform, consider sharing with another team.)*

3) **Before** the cells are completely thawed add **1-5** μL of ligation (plasmid) mixture directly to the cells (do not add to the side of the tube). For best results add the plasmid ligation to the cells while they are still slushy.

4) Incubate on ice for **5 minutes**.

5) Add **200** μL of **SOC media** with no antibiotic to the cell-ligation mix.
(SOC amount = ~4X volume of cell + plasmid mix; thus only 120ul needed for 25ul transformation)

6). Incubate with gentle shaking (200-300 RPM) for **1-2 hrs at 37°C**. *(1 hr will work well but you get a higher transformation efficiency with 2 hr out-growths).* **Skip this step if using Ampicillin resistance and proceed directly to step 7.**

7) Spread transformed cells using glass beads onto a **LABELED** warmed (22-37°C) plate containing antibiotic. Allow plates to dry

(37°C) before flipping plates upside down. *(You do not want media/bacteria dripping onto the lid. You can flip plates early the next day, if needed.)*

8) Incubate overnight (18-20 hrs) at **37°C**. The next day, remove plates from the incubator, seal the lids with parafilm and store at 4°C.

The next day: All colonies should be about the same size (diameter). It is not uncommon with 3A assembly to see only 1 to 10 colonies.

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2.3.2.7: Inoculation

Inoculation of Overnight Cultures

Small sample of bacteria is taken from a plate and placed in LB broth to be used in ZymoPure miniprep or for long term storage.

Materials	Storage Temperature
Sterile Toothpicks	Room Temp
LB Broth	Room Temp
Isolated Colony of Transformed Bacteria	Room Temp

Procedure:

Using sterile technique, pick an isolated colony from a fresh plate (less than seven days old) and inoculate LB-Miller medium.

1. You will use a **sterile toothpick** to pick the colony, and you can simply drop the entire toothpick into the liquid culture tube.
2. **Sterile 5 mL** of **LB** broth will be provided for you for this step. You may add the required antibiotic to the LB prior to inoculating with your colony (**optional**).
3. Incubate with shaking for **8–16 hours at 37°C** before harvesting. This results in maximum yields of a high-copy-number plasmid.

(Typically, after overnight incubation, the absorbance of a tenfold dilution of the culture at a wavelength of 600nm (A600) with a 1cm path length should range from 0.10–0.35.)

These cultures can now be used in the Mini-prep procedure or stored long term in glycerol. (See Appendix VI for how to make your own glycerol stock.)

Growth of Overnight Cultures – Helpful Hints if you are having difficulty.

Different culture media will also have a profound effect on the growth of different bacterial strains. Most plasmid DNA purification systems are appropriate for bacterial cultures grown in 1X Luria-Bertani (LB) medium. However, use of a LB-Miller medium containing more NaCl will produce significantly greater yields and is highly recommended. Richer media such as 2X YT, CIRCLEGROW® or Terrific Broth may be used to increase plasmid yields by increasing the biomass for a given volume of culture. Keep the biomass in a range acceptable for the plasmid isolation system used, as overloading may result in poor purity and yield of the plasmid DNA

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2.3.3: APPENDICES- EQUIPMENT USE

2.3.3.1: Appendix I- Antibiotics and Color Coding

2.3.3.2: Appendix II- Using the Denovix

2.3.3.3: Appendix III- Using the Nanodrop

2.3.3.4: Appendix IV- Using the Qubit

2.3.3.5: Appendix V- Common Agarose Gel Questions and Tips

2.3.3.6: Appendix IV- Preparing Glycerol Stocks

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2.3.3.1: Appendix I- Antibiotics and Color Coding

LB Plates plus antibiotics (color coding)

***Green* = LB + Ampicillin^{100*}**

***Black* = LB + Chloramphenicol^{35**}**

***Blue* = LB + Kanamycin^{50***}**

***Red* = LB + Tetracycline^{10****}**

Antibiotic Selection

Ampicillin (Amp/A) A derivative of penicillin that kills growing cells by interfering with bacterial cell wall synthesis. The resistance gene (*bla*) specifies a periplasmic enzyme, β-lactamase, which cleaves the β-lactam ring of the antibiotic.

*Working concentration 100µg/mL; freezer stock 100mg/mL (in water)

Chloramphenicol (Chlor/C) A bacteriostatic agent that binds to the 50S subunit of the ribosome and blocking peptidyl transferase and inhibiting peptide bond formation. The resistance gene, chloramphenicol acetyltransferase (*cat*), specifies an enzyme that renders the antibiotic incapable of binding to the ribosome.

**Working concentration 35µg/mL; freezer stock 35mg/mL (in EtOH)

Kanamycin (Kan/K) A bactericidal agent that binds to 70S ribosomes and causes misreading of messenger RNA. The resistance gene (*kan*) specifies an enzyme (aminoglycoside phosphotransferase) that modifies the antibiotic and prevents its interaction with ribosomes.

***Working concentration 50µg/mL; freezer stock 50mg/mL (in water)

Tetracycline (Tet/T) A bacteriostatic agent that binds to the 30S ribosome and disrupts codon and anticodon interactions. The resistance gene (*tet*) specifies a multimeric transporter protein that participates in the rapid efflux of tetracycline from the bacterial cell rendering it incapable of accessing the ribosomes.

****Working concentration 10µg/mL; freezer stock 10mg/mL (in water)

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2.3.3.2: Appendix II- Using the Denovix

How to Use the Denovix

Watch the YouTube videos regarding questions for the DeNovix.

(https://www.youtube.com/user/denovixvideos/videos)

The Denovix allows you to measure the concentration of DNA, RNA, and protein in a couple of microliters of the solution. It is best to use **1.5**μL **to 2**μL for accurate measurements. It also has the ability to measure fluorescently labeled molecules.

Startup Denovix by touching the screen. Set up parameters for measuring **nucleic acid/double stranded DNA** (dsDNA).

1. **Clean Denovix**. Pipette **5-10** μL of water on the pedestal (metal part that was under the arm with a hole in the middle), close arm, and then wipe off water with a **Kim-wipe(no paper towels)**.
2. You will now need to **measure a blank**. *(This should be the same liquid your sample is dissolved in)*. Use the **elution buffer** you use in your Zymopure miniprep. Lift metal arm and pipette **2**μL of your blank liquid onto the pedestal. (*Avoid introducing bubbles*.)

4. **Gently** lower arm and click on the **blank button** on the screen**.**

5. When complete, lift the arm and simply wipe clean with a Kim-wipe (**do not** use a paper towel!).
You are now ready to measure multiple samples.
(*It is recommended you re-blank the instrument every 30 min.)*

6. Add **1.5** or **2**μL of your DNA sample and click **measure** on the screen.

*Note: You do **not** have to clean with water between samples. Simply wipe the pedestal dry when you are ready for the next sample. Repeat for all samples.*

7. Record DNA concentration (ng/μL), 260/280 and 260/230 ratios for all samples.
260/280 = a ratio of ~1.7- 1.80 is considered pure DNA (**most important value**)
260/230 ratios are commonly in the range of 2.0-2.2 (can vary a lot)

8. **Clean Denovix**. Pipette **10** μL **of water** on the pedestal, close arm, and then wipe off water with a Kim-wipe....then close out the program. (Press home button.)

What do my values mean?

Link to DeNovix Website to learn more: https://www.denovix.com/tn-130-purity-ratios-explained/

- 260/280 Nucleic Acid Purity Ratios

"260/280 ratios are routinely used to determine the purity of nucleic acid measurements. This ratio is most commonly used to determine the presence of protein and or phenol in the isolated nucleic acid sample. Table 1 describes a general acceptable range for these ratios; however, they are not a guarantee of sample purity."

- 260/230 Nucleic Acid Purity Ratios

"The 260/230 ratio is used to indicate the presence of unwanted organic compounds such as Trizol, phenol, Guanidine HCL and guanidine thiocyanate. Generally acceptable 260/230 ratios are in the range of 2.0 – 2.2. Values higher than this may indicate contamination with the aforementioned compounds."

*A 260/280 ratio below 1.5 **does not** render the DNA unsuitable for any application, but lower ratios indicate more protein contaminants are present which may interfere with downstream steps. 260/230 measure residual contaminants from the*

purification process. Low ratios may indicate impure DNA that will be less successful in downstream steps.

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2.3.3.3: Appendix III- Using the Nanodrop

How to Use the NanoDrop

Watch the YouTube videos regarding questions for the NanoDrop.

(https://www.youtube.com/watch?v=y3ICiycWeCA&t)

The NanoDrop allows you to measure the concentration of DNA, RNA, and protein in a couple of microliters of the solution. It is best to use **1.5**μL **to 2**μL for accurate measurements. Some models have the ability to measure fluorescently labeled molecules.

Procedure:

1. Wash the NanoDrop pedestal. Lift the upper arm of the NanoDrop and remove the wipe.
   Add 4-5 μL of purified water to the lower pedestal, then lower the arm.
   Wait 30-60 seconds.
   Lift the upper arm and use the wipe to vigorously scrub both the upper and lower pedestals.

2. Open the NanoDrop software on the computer by double-clicking the "ND-1000" icon that looks a bit like an hourglass

3. Initialize the NanoDrop:
   Click on the "Nucleic Acid" button in the NanoDrop software. This brings up q dialog box; **DO NOT click "okay" until you have added water.**
   Add 2 μL of purified water to the lower pedestal, then lower the upper arm.
   Click "okay" on the computer and wait about 20 seconds while the NanoDrop initializes
   When it is done, lift the upper arm and dry it off with a wipe

4. Blank the NanoDrop:
   Add 2 μL of the buffer your sample is in (most likely elution buffer).
   Lower the upper arm of the NanoDrop and click the "Blank" button on the software.
   Wait about 20 seconds for the blank measurement to be made.
   Lift the upper arm and dry the pedestal with a wipe

5. Measure your Sample:
   Add 2 μL of your sample to the lower pedestal, then lower the upper arm.
   In the "Sample ID" box, type the name of your sample.
   Click the "Measure" button on the software and wait about 20 seconds for the measurement to be complete.
   Lift the upper arm and dry the pedestal **OR** lift the upper arm and carefully pipet up as much of the sample as you can to retain for further use.

6. Collecting Data:
   Write down any measurements you are interested in.
   Click the "Print Screen" button to print the complete spectrum if desired.
   When finished making all measurements, click "Print Report" to get a table of all data.

7. Cleaning the Pedestal:
   Add 4-5 μL of purified water to the lower pedestal, then lower the arm.
   Wait 30-60 seconds.
   Lift the upper arm and use a wipe to vigorously scrub both the upper and lower pedestals.
   Place a new folded lab wipe on the lower pedestal and close the upper arm.

8. Shut down and close the NanoDrop computer.

More information on what these NanoDrop values mean:

https://tools.thermofisher.com/content/sfs/brochures/T123-NanoDrop-Lite-Interpretation-of-Nucleic-Acid-260-280-Ratios.pdf

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2.3.3.4: Appendix IV- Using the Qubit

How to Use the Qubit Fluorometer

Watch the YouTube video regarding questions for the Qubit Fluorometer.

(https://www.youtube.com/watch?v=z5g6pAFAWEc)

The Qubit allows you to measure the concentration of DNA, RNA, and protein in a couple of microliters of the solution. It is best to use **1.5**μL **to 2**μL for accurate measurements. Some models have the ability to measure fluorescently labeled molecules.

Procedure:

1. Calibration:
    1. On the “Home Screen,” choose the type of assay for which you want to run the standards. Standards Screen is automatically displayed.
    2. The screen will prompt you to choose between reading new standards and using the previous calibration. Press “Yes” to read new standards.
    3.
    4. Insert Standard #2, and press “Read.” Ensure that you are using the Standard #2 appropriate for the assay you are performing.
    5. Calibration is complete. The new standards graph with data points for standards connected by a line appears on the screen.
2. Reading Samples:
    1. Choose “Sample” to go on the Sample Screen
    2. Insert a sample into the Sample Chamber and press “Read.” The measurement takes approximately 3 seconds. Upon the completion of the measurement, the result is displayed on the screen. The number displayed is the concentration of the nucleic acid in the assay tube.
    3. To read the next sample, remove the sample from the Sample Chamber, insert the next sample, and press “Read Next Sample.”
    4. Repeat until all samples are read.

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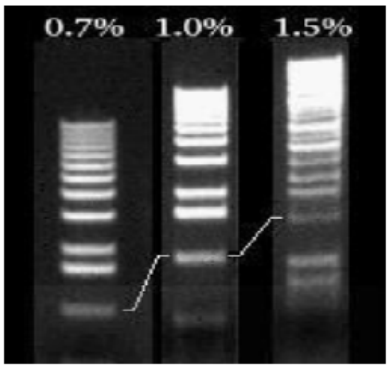
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2.3.3.5: Appendix V- Common Agarose Gel Questions and Tips

Common Agarose Gel Related Questions and Tips
(http://www.lifetechnologies.com/us/en/home/life-science/pcr/elevate-pcr-research/agarose-content-with-tips-and-tricks.html#2)

1. What Percent Agarose is best?

The standard percentage of agarose used to run a DNA gel is usually around 1.0%. A higher agarose percentage enhances the resolution of smaller bands; conversely, a lower agarose percentage gives better resolution and separation of higher-molecular-weight bands. If the wrong percentage is used, it can be difficult to visualize the DNA bands reliably



1KB ladder run in different % agarose gels. Arrow indicates 1kb DNA fragment. Notice clarity of different size bands on each gel.

% Gel	Optimum Resolution/ Linear DNA (kb)
0.5	30 to 1.0
0.75	**12 to 0.8**
1.0	**10 to 0.5**
1.2	7 to 0.4
1.5	3 to 0.2

For very small parts, 2% gel may be an option. Talk to your instructor about it!

2. How does gel casting affect the band resolution?

The recommended thickness for agarose gel is **3–4 mm**; a gel thicker than 5mm will result in fuzzy bands and higher staining background. Similarly, the amount of running buffer to cover over the gel in an electrophoresis apparatus is 3–5 mm. Too much buffer will decrease DNA mobility and cause band distortion. The thickness of the comb is also important and significantly affects

resolution. A thin comb (1 mm) gives very well-defined bands, while a thick comb gives thick bands leading to reduced resolution.

3. How much DNA to run (Less is best)

The minimal quantity of DNA that may be detected is dependent on the stains used. The maximum quantity of DNA in a band that is still clear and well-defined is approximately 100 ng.

4. What is the right buffer for your agarose gel electrophoresis?

The two most popular types of buffers for running agarose gels are Tris-acetate with EDTA (TAE) and Tris-borate with EDTA (TBE). **For small DNA (<1000 bp), and if there is no plan to extract the DNA, then 1x TBE buffer is recommended.** TBE buffer has a high ionic strength and buffering capacity. TAE buffer, together with low field strength (1–2 V/cm), is preferred for separating large DNA (12–15 kb). TAE buffer interacts with agarose, resulting in lower electroendosmosis, larger apparent pore size, and lower field strength compared to agarose gels in TBE buffer. **Buffer must be added to the GEL.**

5. Why do I see so many bands?

Uncut DNA will typically appear to run faster than fully linearized plasmids. Isolated, uncut plasmid should be in the supercoiled form. The use of restriction enzymes will linearize our plasmids.
(http://bitesizebio.com/13524/how-to-identify-supercoils-nicks-and-circles-in-plasmid-preps/)

How to identify supercoils, nicks and circles in plasmid preps

http://arbl.cvmbs.colostate.edu/hbooks/genetics/biotech/gels/supercoils.jpg

How different forms of plasmid run on agarose

DNA Ladder (*Quick-Load Purple 1kb Plus DNA Ladder, New England Biolabs)*

https://www.neb.com/products/-/media/catalog/gel-photos/n3200_thumb.gif

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2.3.3.6: Appendix IV- Preparing Glycerol Stocks

Making a Glycerol Stock

ONLY if you have confirmed that your NEW composite part *(newly built plasmid)* is **CORRECT.** This means that after conducting a Zymo Miniprep and restriction digest, the new plasmid now contains the parts that you have ligated together.

How to make a glycerol stock?

1. Using sterile technique, aliquot **500µL** of 80% glycerol into a cryovial (This may or may not have been done already, ask your instructor)
2. Label the cryovial clearly on top or on the side with the part number, the antibiotic resistance (amp, chlor, tet, kan), as well as any other labeling standard your lab uses ***(LABELING IS VERY IMPORTANT!!)***
3. Vortex your culture
4. Carefully add about **500uL** (0.5mL) of your culture to the cryovial. Vortex and place the vial on ice.

Store the vial in a -80 freezer that has been designated for long term storage.

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2.4.1: Mitosis and Meiosis

Learning Objectives

- Describe the cell cycle.
- Explain the events of all stages of mitosis.
- Track chromosome and chromatid number through all stages of mitosis.
- Explain the events of all stages of meiosis.
- Track chromosome and chromatid number through all stages of meiosis.
- Describe the role of meiosis in gamete formation and how it relates to inheritance.

Introduction

Multicellular organisms such as plants and animals are composed of millions to trillions (1,000,000,000) of cells that work together. The cells that make up different tissues have different shapes and perform different functions for the plant or animal. Even though they have diverse functions, each **somatic cell** in the organism normally has the same chromosomes and therefore the same genetic makeup. Furthermore, the millions of cells that makeup a mature organism originated from a single cell formed when the male and female gametes from the parents of the organism fused. This single cell established the life of the organism. Understanding multicellular organisms requires an understanding of the lifecycle of the cells that make up the organism.

The Cell Cycle

Let us think about the cell cycle from a personal point of view. Your age plus about nine months ago you were a **zygote**, a single cell formed when the sperm and egg from your biological parents fused in a fallopian tube (or possibly in a test tube if in vitro fertilization factored into your birth). You have come a long way since then, progressing one cell cycle at a time. The cell cycle is the life cycle of a single cell. We depict the cell cycle in a circular diagram although the cells in your body do not actually go around in circles. The main idea is that when new cells are made from existing cells, the new cells start their lifecycle, and the old cells end theirs.

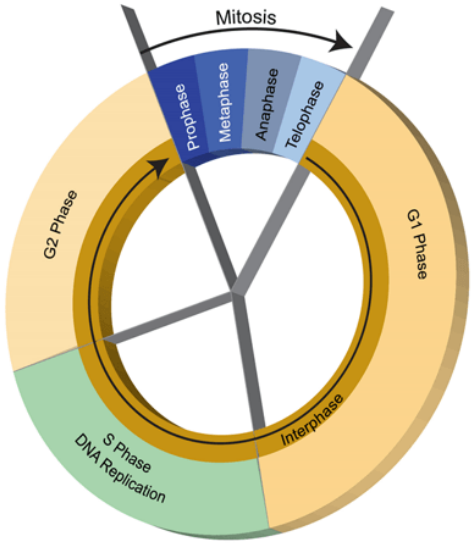


Figure 1. The cell cycle depicts the stages in the life of a cell. Image by NIH-NHGRI.

The first part of the cell cycle is the G1 phase. Cells can go through growth and development in this phase. Some cells differentiate into specialized cells and then never leave this phase. However, a **zygote** does not grow in size, but instead continues the cell cycle so it can quickly give rise to more cells.

The second part of the cell cycle is the S phase (synthesis phase). Here, the cell replicates its chromosomes so that it has a copy of each chromosome to pass on to daughter cells. The third part of the cell cycle is the G2 phase where the cell prepares for division. The G1, S and G2 phases together are called interphase. The M phase completes the cell cycle. 'M' could be mitosis or meiosis depending on the type of cell. For the zygote, the goal is to make more somatic cells. Therefore, it goes through mitosis and gives rise to two daughter cells. This completes the life cycle of the zygote and starts the lifecycle of the new cells. Rounds of the cell cycle continued over and over to form the body you have today. As long as you live, some of your cells must be able to complete the cell cycle.

From a cytogenetics point of view, you have two types of cells in your body. You have cells with 46 **chromosomes** (**somatic** or body cells) and cells with 23 chromosomes (**gamete** or sex cells). Since you started as a single cell with 46 chromosomes, there must be two types of cell division taking place in your body to accommodate both somatic and gamete cells. Mitosis and meiosis are the two types of cell division.

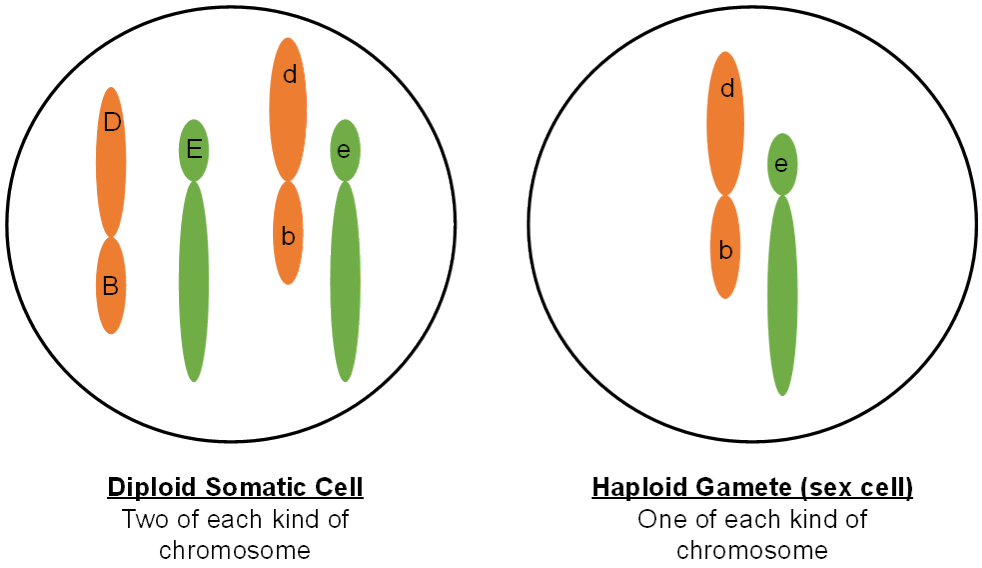


Figure 2. Multicellular organisms that sexually reproduce have diploid and haploid cells. Image by Marjorie Hanneman.

Mitosis: Somatic cell division

The objective of mitosis is to make two genetically identical cells from a single cell. In the cells of our body, we start with 46 chromosomes in a single cell and end up with 46 chromosomes in two cells. Obviously, replicating the chromosomes is a prerequisite to mitosis. Remember, replication takes place during interphase when the chromosomes are dispersed structures in the nucleus. Mitosis is an organized procession of activity in the cell that allows the replicated chromosomes to be properly divided into two identical cells. Chromosomes are important because they contain genes. Therefore, we will include **genes** on our chromosome diagrams and slide show. These pictures depict the four stages of mitosis. We will describe the events at each stage that are important in understanding the distribution of genes during cell division.

Mitosis: Prophase

Prophase is the beginning of mitosis (Figure 3).

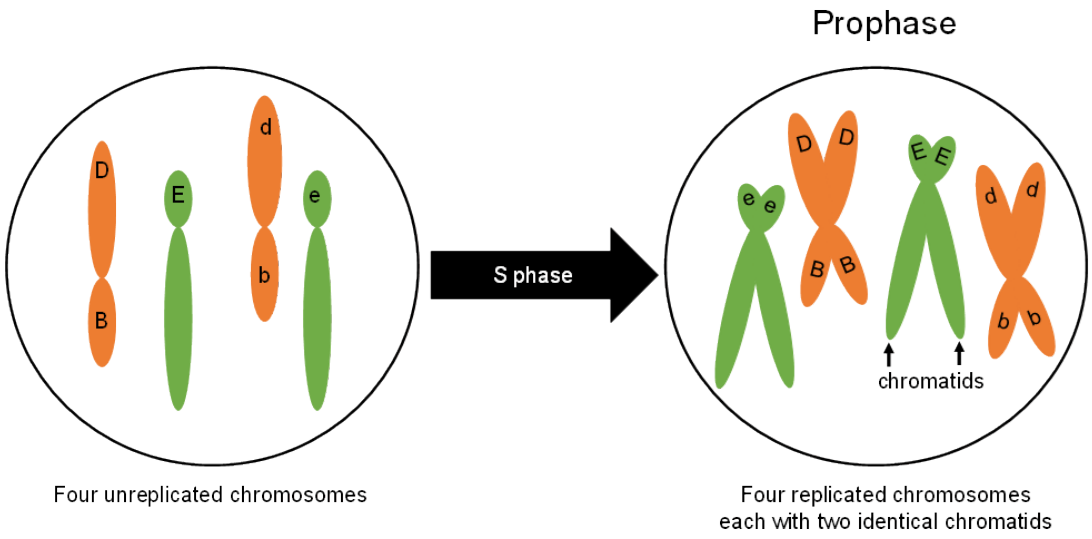


Figure 3. Prophase of mitosis. Image by Marjorie Hanneman.

During interphase, the chromosomes look like a plate of spaghetti in the nucleus. It is difficult to pick out an individual chromosome because they are each so spread out. The chromosomes in the nucleus change from being loosely dispersed to becoming more condensed. This change in chromosome structure makes them easier to move around the cell, an important issue for what is about to happen. As the chromosomes condense, they get shorter and thicker and can be seen through the microscope as individual structures (Figure 4). The chromosomes at prophase will consist of two identical parts called sister chromatids that stay connected at the centromere. It is now clear that the chromosomes have been replicated. Chromosome replication occurs during the S-phase of interphase (Figure 1). Next, the nucleus is dissolved. At the end of prophase the **replicated** chromosomes are moved by the spindle apparatus to the center of the cell.

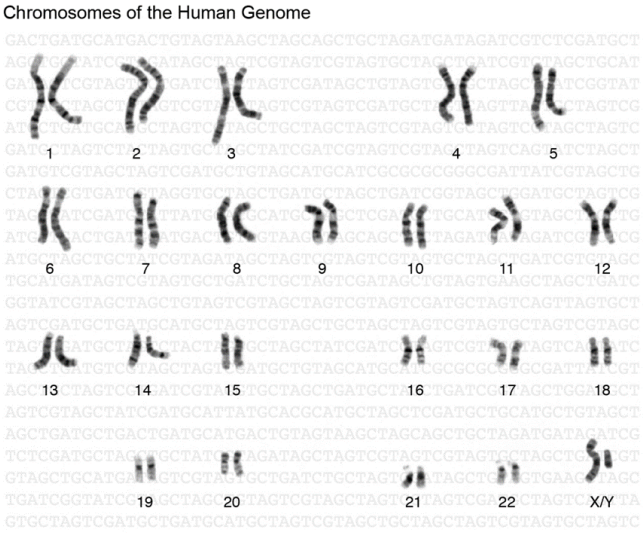


Figure 4. Chromosomes of the human genome. Image by NIH-NHGRI.

Mitosis: Metaphase

When the chromosomes are maneuvered to the center of the cell, metaphase begins (Figure 5). The spindle fiber network connects the centromere of the replicated chromosome to the outer part of each cell. The chromosomes now resemble the line of scrimmage of a football team, poised and waiting for some cellular signal to begin the next phase.

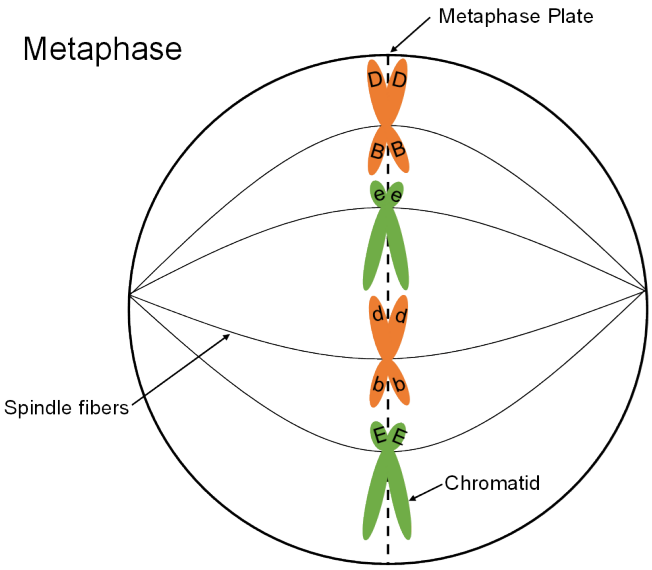


Figure 5. Metaphase of Mitosis. Image by Marjorie Hanneman.

Mitosis: Anaphase

At anaphase (Figure 6) the chromatids making up each chromosome are pulled apart and begin to move away from each other under the control of the spindle fibers. Once they are pulled apart, the chromatids are now considered individual chromosomes. As anaphase progresses, the chromosomes on each end of the cell are pulled into a bundle. When they stop moving, telophase begins.

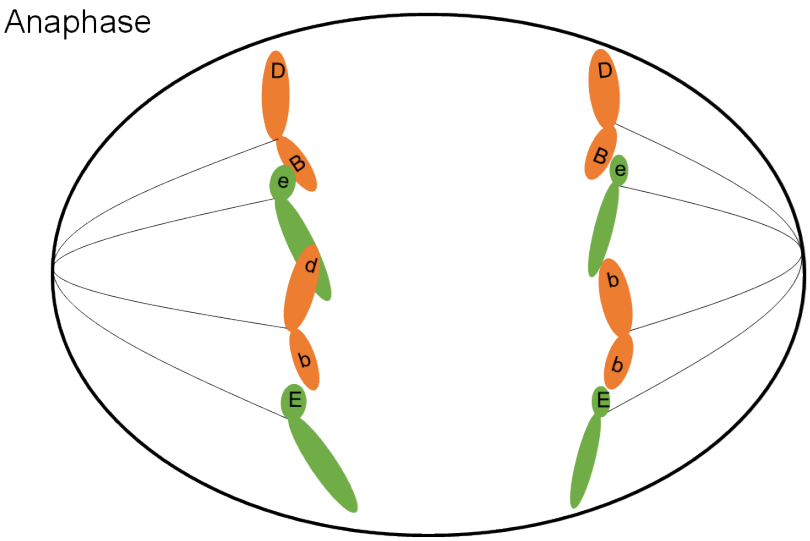


Figure 6. Anaphase of mitosis. Image by Marjorie Hanneman.

Mitosis: Telophase

The final stage of mitosis is telophase (Figure 7). A nuclear envelope will form around each bundle of chromosomes. The cell will undergo cytokinesis and the cytoplasm is split between the two identical daughter cells. Cell membranes (and cell walls in plants) form between the two cells. The chromosomes begin to decondense. They loosen up and spread around the nucleus because they no longer will need to move around. The new cells have now begun their lifecycle.

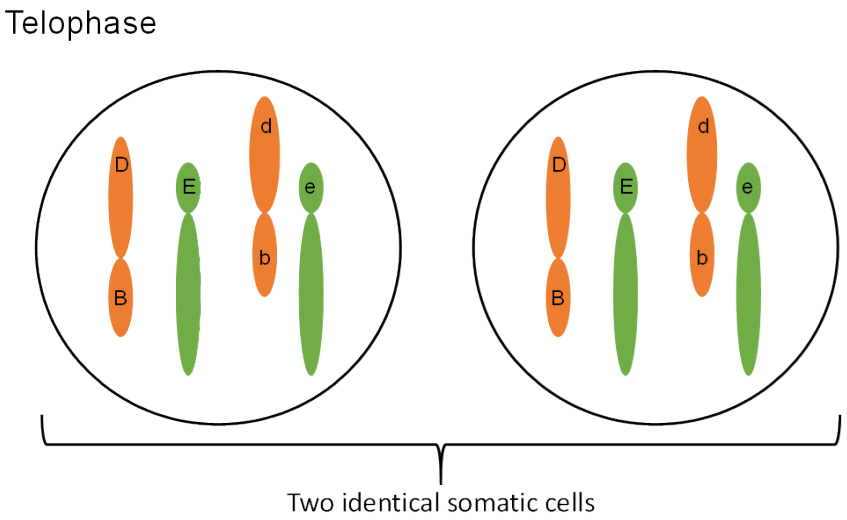


Figure 7: Telophase of Mitosis. Two cells produced from one cell. Image by Marjorie Hanneman.

Meiosis: Gamete formation

The objective of meiosis is to make four cells from a single somatic cell. The four cells each have half the chromosome number found in the somatic cell. In our human bodies, the four gametes will each have 23 chromosomes which means the 46 chromosomes in the somatic cell must replicate during interphase prior to meiosis just as they would before mitosis. Meiosis occurs in specialized cells of the body called germline cells.

To appreciate meiosis and gamete formation it is important to first understand two ideas, chromosome sets and homologous chromosomes.

Chromosome sets: The 46 chromosomes you have consist of two sets. You are a diploid organism ('di' means two and 'ploid' means sets). One set of chromosomes came from each parent when their gametes fused. Therefore, human gametes are haploid (one set).

Homologous chromosomes: The 46 chromosomes in a somatic cell can be arranged into 23 homologous or similar pairs. One chromosome from each pair came from the male parent, the other from the female. Homologous chromosomes have the same genes although in heterozygous people the genes would be different alleles (**A,a**). The exception to this would be the sex (X and Y) chromosomes. Passing on a complete set of human genes requires one chromosome from each pair to end up in each gamete.

There are several key differences between meiosis and mitosis that are summarized in the following table:

Table 1. The key events that happen in each of the stages of meiosis are summarized.

Mitosis

Meiosis

Chromosome number stays the same

Chromosome number is halved

One division occurs to make two cells. Four stages of this division.

Two divisions occur to make four cells. Eight stages in these divisions.

Similar or homologous chromosomes do not pair.

Homologous chromosomes pair during prophase I. Pairing is called synapsis.

Crossover exchanges between homologous chromosomes is rare.

Synapsis allows crossing over between homologous chromosomes.

Two cells made are genetically identical.

Four cells made are genetically different.

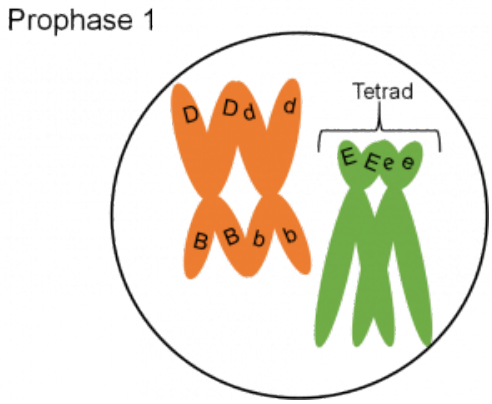


Figure 8. Prophase I of meiosis. Images by Marjorie Hanneman.

Meiosis: Prophase I

This stage starts meiosis and is the same as prophase of mitosis with one important change. As the chromosomes condense, they form groups of four chromatids called tetrads or bivalents. Close inspection reveals that each chromosome is replicated and consists of two sister chromatids. The two chromosomes in each cell that are homologous and have the same genes (but perhaps different alleles if the organism is heterozygous) will pair closely. This close association, or synapsis, allows the homologous chromosomes to crossover and exchange identical parts. The impact of crossing over is that genes that are on the same chromosome (Figure 8) can be recombined so that they are not always inherited together. The tetrad or bivalent formed during synapsis remains assembled as prophase progresses. The tetrad therefore moves as a unit to the center of the cell.

Meiosis: Metaphase I

Metaphase I starts when the tetrads are at the center of the cell (Figure 9). The tetrads have stayed together which insures that during the first division, each cell will get one chromosome from each homologous pair. The chromosomes remain at the center of the cell until the homologous pairs are ready to move away from each other.

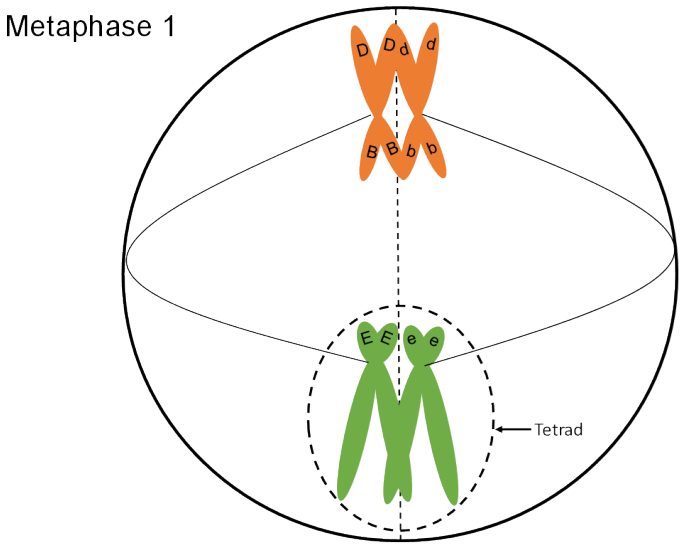


Figure 9. Metaphase I of meiosis. Image by Marjorie Hanneman.

Meiosis: Anaphase I

The chromosomes that make up each tetrad separate during anaphase I (Figure 10). However, the sister chromatids will stay connected at the centromere. Anaphase I proceeds until the chromosomes are pulled into a bundle at opposite ends of the cell.

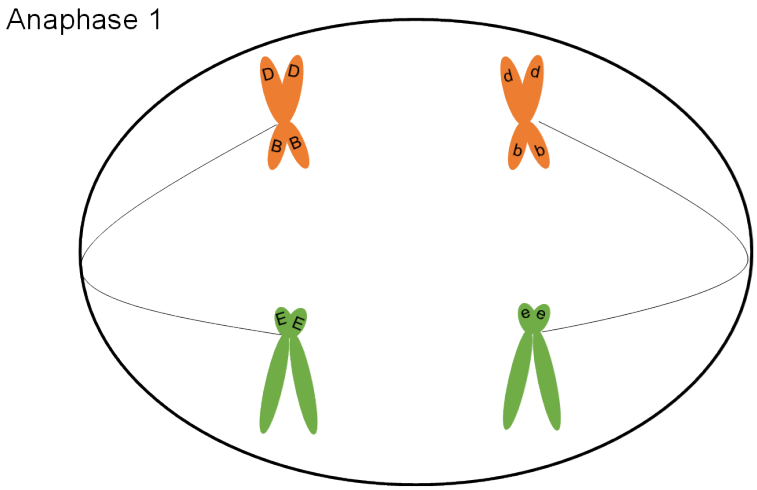


Figure 10. Anaphase l of meiosis. Image by Marjorie Hanneman.

Meiosis: Telophase l

The cell divides into two cells during telophase I (Figure 11). The bundle of chromosomes may have a nuclear envelope develop around them. The germline cells in some organisms such as human females, go through the first four stages of meiosis prior to birth. The **germline cells** remain at telophase I for some time. The second round of division occurs when the gamete is needed for reproduction. In other situations, telophase I is an abbreviated stage, and the second round of division proceeds without delay.

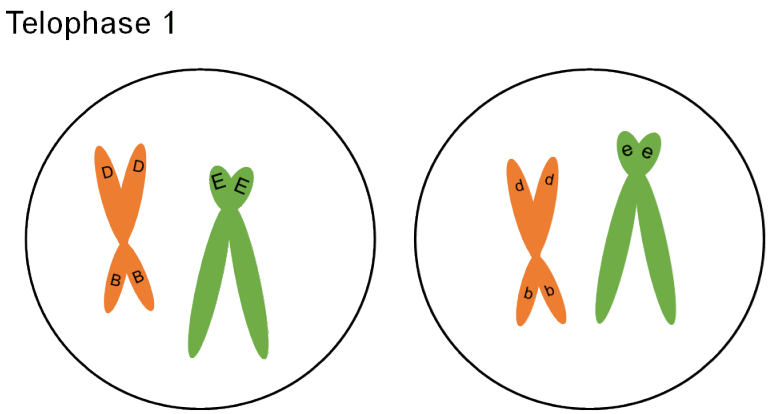


Figure 11: Telophase l of meiosis. Image by Marjorie Hanneman.

Meiosis: Prophase II

If the chromosomes became dispersed in telophase I, they will condense again at prophase II. The spindle apparatus moves the chromosomes to the middle of the cell. Look! The centromeres are still holding the sister chromatids together (Figure 12).

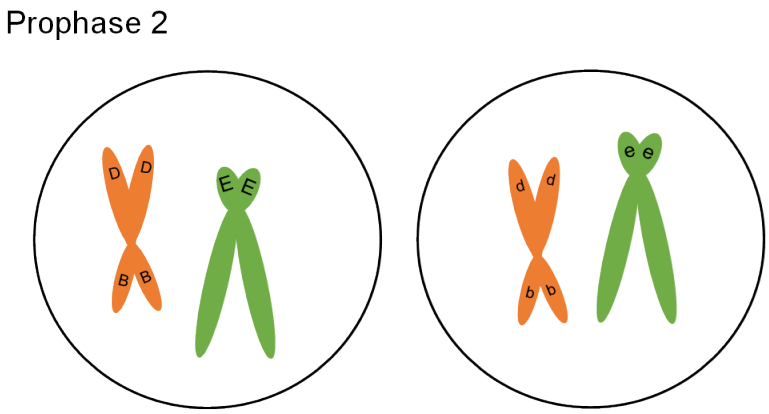


Figure 12. Prophase II of meiosis. Image by Marjorie Hanneman.

Meiosis: Metaphase II

In metaphase II the chromosomes are aligned at the center of the cell (Figure 13). This time there are not homologous chromosomes to be paired with. This metaphase looks similar to metaphase of mitosis but there is a key difference. What is the difference? (Compare Figures 5 and 13).

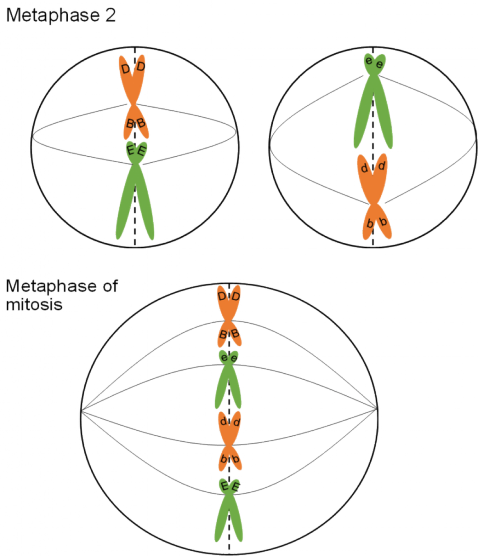


Figure 13. Metaphase II of meiosis and Metaphase of mitosis. Image by Marjorie Hanneman.

Meiosis: Anaphase II

During anaphase II, the chromatids are pulled apart by the spindle fibers. Now they are classified as chromosomes, not chromatids. The chromosomes move apart to opposite ends of the cell (Figure 14).

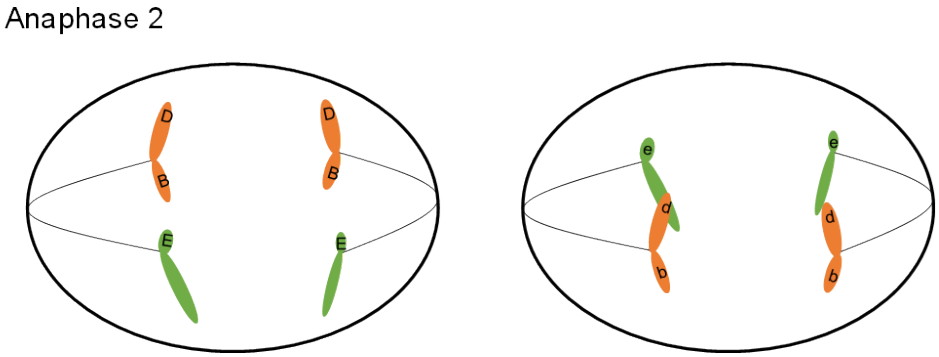


Figure 14. Anaphase II of meiosis. Image by Marjorie Hanneman.

Meiosis: Telophase II

In the final stage of meiosis, telophase II, the nucleus forms around the bundle of chromosomes (Figure 15). The cell divides. Now four cells exist that originated from one germline cell. Each cell is a gamete with half the number of chromosomes and genes as a somatic cell.

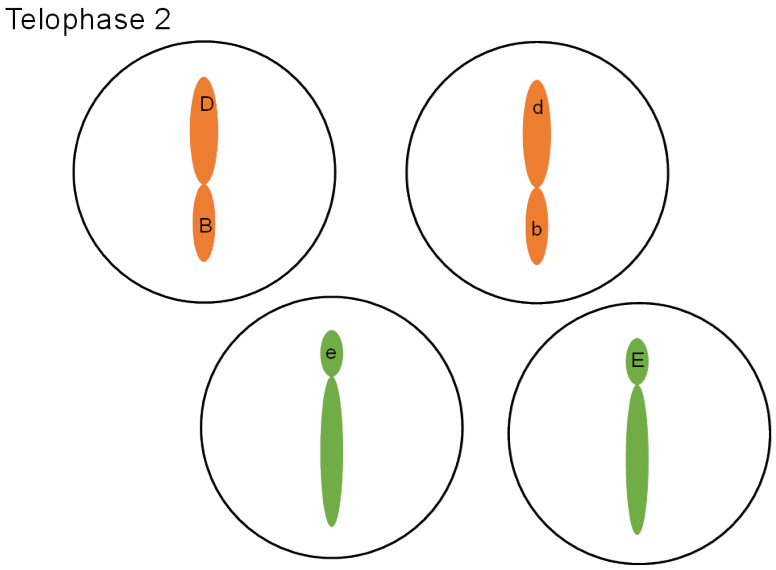


Figure 15. Telophase II of meiosis. Image by Marjorie Hanneman.

Parallel Behavior and the Chromosome Theory

The successful completion of mitosis or meiosis requires the cell to move large objects with precision and control many detailed events. The process surpasses any engineering accomplishments of NASA. The importance of mitosis and meiosis to an organism is obvious when we consider that genes are a part of the chromosome and the genes must be copied and distributed properly to produce viable daughter cells. The mechanisms of these events are far from being completely understood. From our current understanding, we can appreciate how the principles of segregation and independent assortment are controlled by the mechanics of meiosis.

When cell division was first observed and described by cytogeneticists, biologists were just beginning to accept the idea that genes were tiny objects that controlled traits and existed in the cells of living things. Two biologists, a German named Boveri and an American graduate student named Sutton, recognized that chromosome behavior during meiosis matched Mendel's principles of gene behavior. Both scientists proposed the idea that while genes had not yet been directly observed, they must be a part of the chromosome. Sutton and Boveri are both given credit for proposing this chromosome theory; genes are a part of chromosomes.

Segregation predicts gene behavior that matches the chromosome behavior observed by cytogeneticists. Genes are in pairs because chromosomes are in pairs. The gene pairs associate during gamete formation when the homologous chromosomes pair in prophase I. The gene pairs separate when the homologous pairs separate in anaphase I followed by chromatid separation in anaphase II. Thus, chromosome behavior dictates the gene's segregation behavior.

Independent assortment of gene pairs also correlates with chromosome behavior. Let us consider a dihybrid individual with the genotype **BbEe** (Figure 16). How many kinds of gametes can it make? The four possible combinations (**BE**, **bE**, **Be** and **be**) are made at equal frequencies. We can understand why this occurs if we think about what happens to chromosomes at metaphase I of meiosis. The chromosome pair that carries the '**E**' and '**e**' genes will be moved independently from the chromosome pair with the '**B**' and '**b**' chromosomes. When the chromosomes arrive at the center of the cell at metaphase I, the two tetrads may be aligned so that the '**E**' and '**B**' genes move to one cell and the '**e**' and '**b**' genes move to the other during the first division. In the other half of the cells that go through meiosis, the tetrads will line up so that the '**E**' genes will be passed on with the '**b**' genes and the '**e**' genes will go with the '**B**' genes. Keep in mind that organisms that make gametes make thousands of them. The chance alignment of the tetrads at metaphase I therefore dictates the overall frequency of gametes with different combinations of genes when the genes are on separate chromosomes.

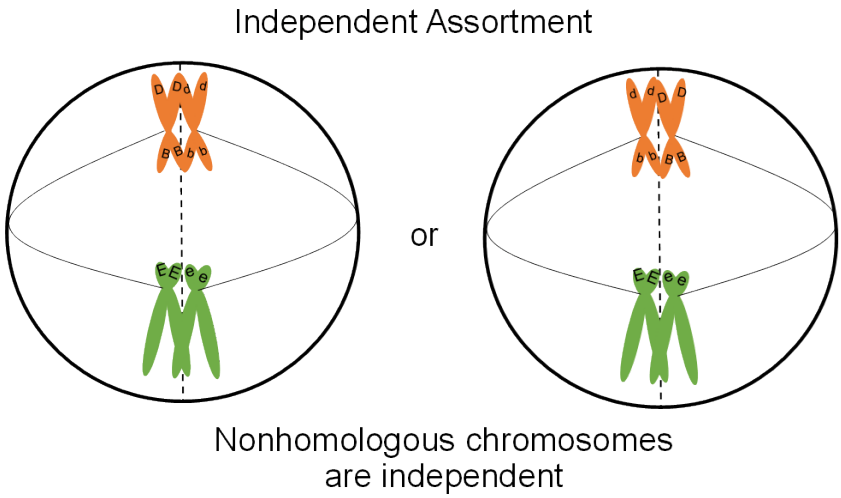


Figure 16. Alignment of chromosomes during metaphase I will determine the gene combinations that segregate into gametes. Image by Marjorie Hanneman.

When more gene pairs are considered, the same scenarios described above will be true as long as the genes are on separate chromosomes. When genes are on the same chromosome, the role of crossing over on gene inheritance needs to be considered in more detail. We will cover the inheritance of genes on the same chromosome in a later lesson.

Learning Activities

1. Watch this video about Mitosis from Daily Med Ed
2. Watch this video about Meiosis from Daily Med Ed

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This textbook provides an introduction to plant genetics and biotechnology for the advancement of agriculture. A clear and structured introduction to the topic for learners new to the field of genetics, the book includes: an introduction to the life cycle of the cell, DNA and how it relates to genes and chromosomes, DNA analysis, recombinant DNA, biotechnology, and transmission genetics.

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2.4.2: DNA- The Genetic Material

Learning Objectives

- Detail the molecular nature of a gene.
- Recognize when and where replication of the DNA that is packaged in an organism's chromosome is happening for growth, maintenance, and reproduction.
- Describe how chromosome replication speed can be increased with multiple origins of replication and bidirectional replication.
- Explain the role of each of the primary DNA replicating enzymes in conducting replication of a chromosome. Include the role of these enzyme in ensuring the accuracy of the replication process.
- Predict how the semiconservative replication of a double stranded DNA will happen given the structure of the molecule and the specific function of the DNA replicating enzymes.

Introduction

The genetic (hereditary) material for all living things is composed of DNA (deoxyribonucleic acid). The structure of DNA must enable this substance to store coded information that control the biological function of cells. The genetic material transmits this hereditary information in a stable form for the cell and organism through accurate **replication** of DNA. Although DNA is capable of change (we will discuss how this happens when we talk about DNA mutations), the replication process ensures high accuracy in copying the genetic information so that all progeny cells receive the same information. The DNA from all chromosomes in a human cell would be more than 6.5 feet long! Therefore, to fit inside the nucleus of a cell, DNA is packaged into chromosomes.

Read this article about Watson and Crick for more insight on the experimental facts discovered by chemists and biologist which contributed to the determination of the structure of DNA.

Chemical Structure of DNA Subunits

DNA is a polymer made of nucleotide subunits. A **nucleotide** consists of 3 chemical groups; a sugar, a phosphate and a nitrogenous base (Figure 1). In the case of DNA, the sugar is deoxyribose.

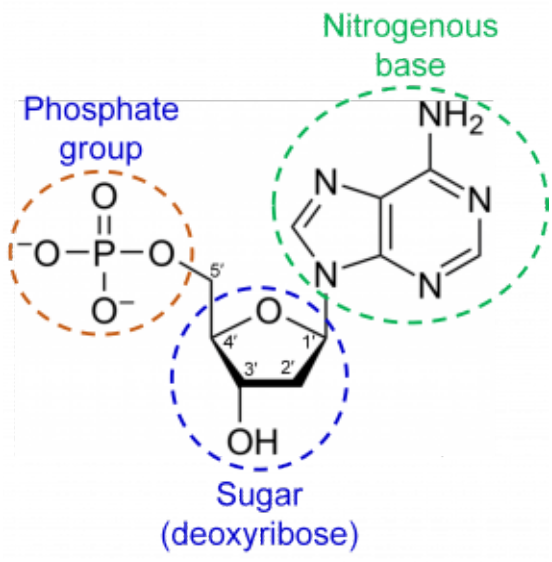


Figure 1. A nucleotide consists of three chemical groups. Image adapted from Wikipedia.

There are 4 different nucleotides in DNA that contain 4 different **bases** (Figure 2). These bases are named adenosine (A), cytosine

(C), guanine (G) and thymine (T).

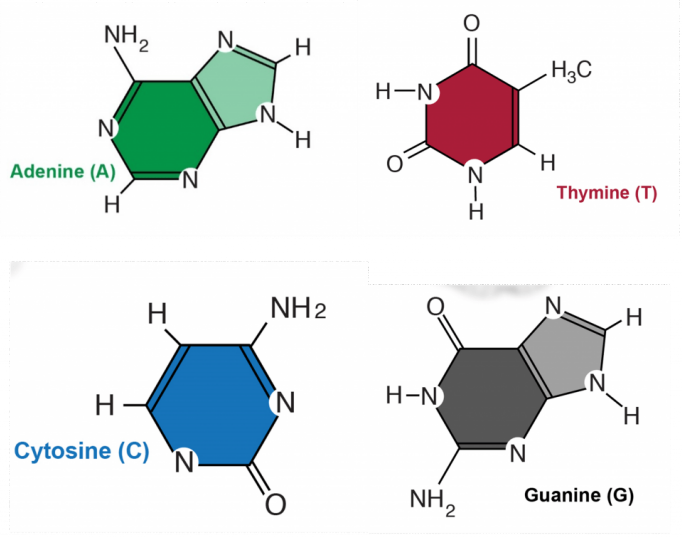


Figure 2. Adenine, Thymine, Cytosine, and Guanine are the nitrogenous bases of DNA. Adapted from NIH-NHGRI.

Complementary, antiparallel DNA strands

DNA codes information by ordering the sequence of the A,T,G and C nucleotides in a long polymer. The nucleotides are connected by phosphate bonds to form a strand. Two strands form a double stranded molecule (Figure 3). Note that in Figure 1, the carbon atoms on the deoxyribose sugar are numbered. The phosphate bond connects carbon #3 (the 3 prime or 3′ carbon) of one sugar to the 5′ carbon of the next. This gives a DNA strand directionality because the 5′ carbon faces one way and the 3′ carbon faces the other.

The bases on one strand form hydrogen bonds with the bases on the other. This is called "**base pairing**" and it is very specific, A only pairs with T and C only pairs with G. Thus, the sequences on the two strands of DNA are "**complementary**"; whenever there is an "A" on one strand, there is a "T" in the corresponding position of the complementary strand.

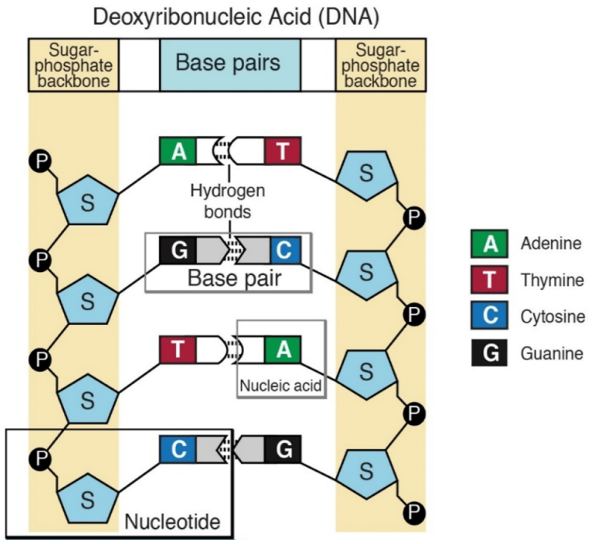


Figure 3. DNA is a double-stranded molecule. Illustration by NIH-NHGRI.

In addition to containing complementary nucleotide sequences, the strands are in opposite orientations. The 5′ end of one strand is oriented toward the 3′ end of the other. For this reason, the strands are referred to as "**antiparallel**".

Double helix

The final feature of the molecular structure is that DNA assumes a helical conformation (Figure 4). This is the most stable configuration that accommodates all the molecular structures and chemical bonds that make up DNA.

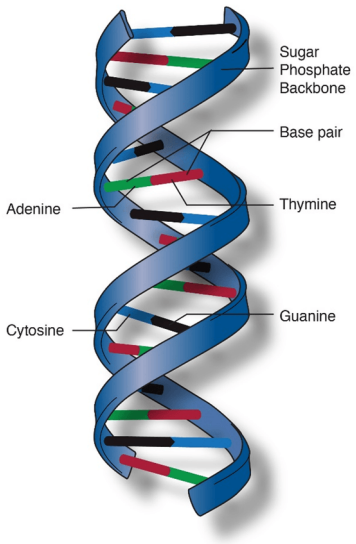


Figure 4. DNA is a double helix. Illustration by NIH-NHGRI.

Activity

Given the following sequence of a DNA strand, predict the sequence and orientation of the complementary strand.

5′-G-A-C-C-G-T-A-A-T-C-G-C-3′

Show Answer

Answer: 3′-C-T-G-G-C-A-T-T-A-G-C-G-5′

Packaging of DNA into chromatin and chromosomes

Chromosomes are long double stranded DNA molecules. Over 150,000,000 nucleotide pairs make up the human X chromosome. The complete replication and orderly transfer of something this large requires the chromosome to be packaged for stability and organization which creates genetic stability in the cells of a species. Every cell in a diploid organism contains two copies (2n) of every chromosome present in that organism (Figure 5). For example, humans have 46 chromosomes in their body, 23 were inherited from the father and 23 from the mother. **Gametes**, the reproductive cells of an organism, (egg or sperm), have only one set (1n) of chromosomes. When the two gametes unite, they form a living embryo with two sets of genetic information. Therefore, we actually have two copies of the genetic information for each trait. Sometimes one copy controls **trait expression**, and other times both copies influence a trait. As a result, the offspring will have characteristics of both the mother and the father.

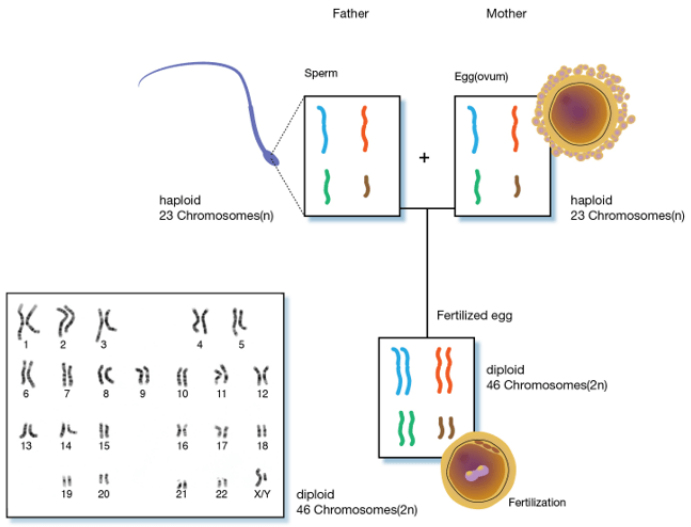


Figure 5. Human non-sex cells are diploid (2n) and contain 46 chromosomes. On the other hand, human sex cells or gametes (sperm and egg) are haploid (n) and contain single set of 23 chromosomes. Image from NIH-NHGRI.

Inside cell nuclei, DNA is assembled, or **packaged**, into material called chromatin, which is composed of both DNA and proteins (Figure 6). Chromatin functions to protect and regulate DNA, as well as to efficiently store the very long DNA molecules that make up chromosomes within the limited space of the nucleus.

DNA from higher eukaryotic organisms is about 10⁹ to 10¹⁰ bp. Human and maize chromosomal DNA is about 10⁹ bp, which is equivalent to 1.8 m if all chromosomal DNA were stretched end to end in a linear manner. The diameter of the nucleus is only about 4-6 μm making it a challenge to fit chromosomal DNA inside the cell nucleus. This is accomplished by the ability of DNA to assume a very condensed structure to fit inside the nucleus. Therefore, the organization of eukaryotic DNA into chromatin is an important aspect of DNA packaging.

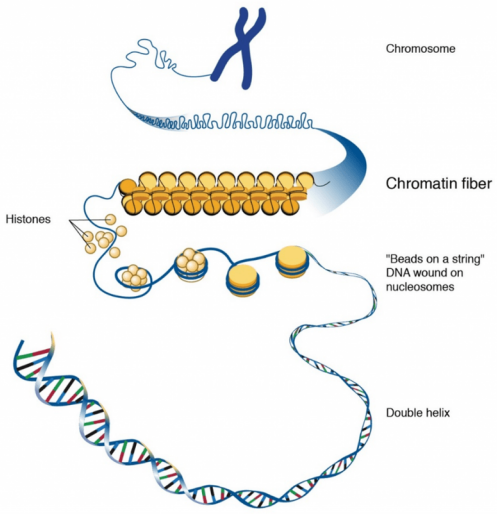


Figure 6. DNA is packed into chromatin and chromosomes. Illustration by NIH-NHGRI.

Nucleosomes and histones

The ordered coiling of chromosomal DNA around a histone protein core forms the **chromatin**. Chromatin is made up of **nucleosomes** (Figure 6) which represent the association of chromosomal DNA with histone proteins. A nucleosome is made up of about 145-147 base pairs of DNA coiled around each histone octamer, for about two complete turns. A histone octamer consists

of two copies of each core histone. The total mass of the histones in the nucleus approaches that of the DNA – 2 molecules of each core histone to approximately 200 bp of DNA.

Higher order structures

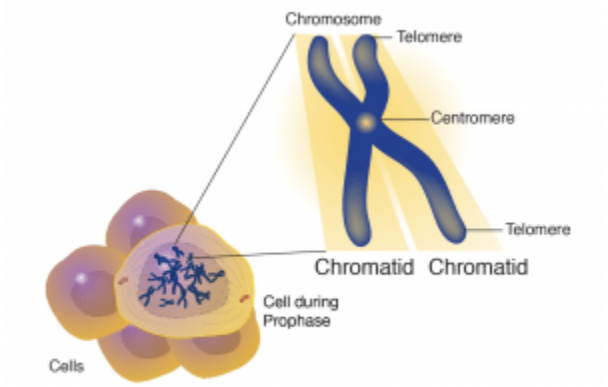


Figure 7. Chromosomes have centromeres and telomeres. Illustration by NIH-NHGRI.

If the higher order chromatin structure is disrupted, electron microscopy reveals the appearance of “beads on a string” with a diameter of about 10 nm. The “beads” represent the DNA wrapped around histones. Nucleosome formation results in a DNA fiber that is about 10 nm and a packaging ratio of about 7. The higher order chromatin structure results when the 10 nm fiber is coiled into a solenoid. The result of nucleosome coiling is a chromatin fiber of 30 nm that is observed by electron microscopy.

Centromeres

The centromere is a chromosomal region that controls chromosome segregation at mitosis and meiosis (Figure 7). Centromeres connect to microtubules of the spindle apparatus, which directs their movement to opposite poles (daughter cell nuclei) during cell division. Scientists were successful in isolating these centromeric sequences from yeast and engineered brewer’s yeast (*Saccharomyces cerevisiae*) plasmids that were able to replicate like the chromosomes. Through these efforts scientists were able to point centromeric function to a DNA stretch of about 120 bp that was resistant to DNAse and bound to a single microtubule.

Telomeres

The telomere lies at the end of the chromosome and confers stability by “sealing” the end of a chromosome (Figure 7). Telomeres consist of long series of short repeated DNA sequence that occurs in tandem arrays and are added to the end of the chromosome during DNA replication by an enzyme called telomerase. In most plant species the sequence **TTTAGGG** constitutes a conserved telomere motif.

The knowledge about the structure and function of centromeres and telomeres has had a profound impact in molecular genetics. For example, yeast artificial chromosomes (YACs) and bacterial artificial chromosomes (BACs) have proven useful in the physical mapping of plant genomes requiring the cloning and multiplying large (>100 kb) DNA fragments in yeast or bacterial cells.

DNA Replication

In 1953, J.D. Watson and F.H.C. Crick published a note in NATURE; one of the world’s most widely read research journals. The note cited just six references.

Watson and Crick’s writing had limited chemistry details given the title of their note, “A Structure for Deoxyribonucleic Acid”. A later article would share more of the chemistry behind their proposed double helix structure for Deoxyribonucleic Acid (DNA). This note in Nature was written for a broader audience, including biologists who recognized that the structure of DNA was a prerequisite for understanding the function of the genetic material. They included a diagram to allow readers to visualize this unique double helix structure. To emphasize the structure determines function importance, Watson and Crick included this statement in their note...

> "It has not escaped our notice that the specific pairing we have postulate immediately suggests a possible copying mechanism for the genetic material."

Accurate replication prior to cell division is one of the three key functions of the genetic material. While later experiments would reveal the details of how cells replicate DNA, Watson and Crick were obviously impressed that the proposed double helix structure would fit with the concept that living cells are needed to produce more living cells and the transmission of a complete set of biological instructions. The genetic material must have a structure that lends itself to being copied. Using old to help build new leads to a proposed semiconservative model for replicating the double stranded DNA molecule (Figure 8).

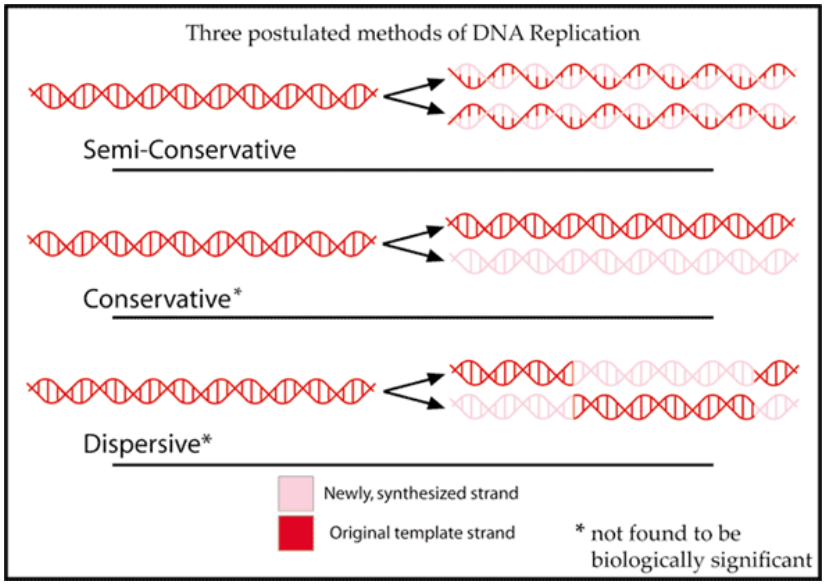


Figure 8. Watson and Crick predicted that the double helix molecule could be replicated in a living cell by the semi-conservative method. Each strand of the old double stranded molecule is read to build a new strand. Early experiments on replication confirmed this prediction. Image Source: Adenosine, CC BY-SA 2.5, via Wikimedia Commons.

Prior to both mitosis and meiosis cell division in multicellular organisms, and cell division in prokaryotes, the DNA inside the cell must be replicated. (see lesson Mitosis and Meiosis). This replication process generates the genetic information needed for either two cells, genetically identical with the original cell or in sexually reproducing organisms, four gamete cells with half of the original cell's genetic information.

When and Where:



Figure 9. Plant root tip. Image Source: SuperManu, CC BY-SA 2.5, via Wikimedia Commons.

DNA replication will happen before cells divide. In hours old embryos that are developing into multicellular organisms, all the cells are replicating their DNA and dividing into new cells. Hours or days later, multicellular organisms have developed specific meristem regions where new cell production occurs. A visual example of meristem cells where DNA replication is needed is the tip of a plant root (Figure 9). New cells made at the root tip allow the root to grow. The root must also replace the cells that are damaged as the growing root moves through soil. In Figure 8, the meristem region (1) contains meristem cells that are replicating their chromosomes, then dividing into two identical cells by mitosis. Cells on the tip part of the meristem specialize in root cap cells (2,3) with the job of protecting the meristem. The fate of these cells is to die (4), and they need to be replaced by new cells made in the meristem. Cells made on the other half of the meristem (5) can elongate and eventually specialize. These root cells will then function for the plant, all season for annual plants, years for perennial plants. Replication of DNA will only happen in the meristem cells.

Speed of chromosome replication

Fifty-two years after Watson and Crick's Nature note, a group of about 50 molecular geneticists published the complete DNA sequence of the human X chromosome (Figure 5). This discovery revealed that X chromosome is one double-stranded DNA molecule that is about 155 million nucleotide pairs long. Every time a human cell divides, one or two of these 155,000,000 nucleotides must be replicated. This must be done with speed and accuracy.

The main DNA replication enzyme (DNA polII, see below) works fast. Estimates of its replicating speed are around 750 nucleotides per second.

Activity

Let us do the math.

155,000,000 nucleotides / chromosome X 1 second / 750 nucleotides

= 206,666 seconds per X chromosome

Let us convert these seconds to hours.

1 hour / 3600 seconds X 206,666 seconds/chromosome = 547 hours per chromosome

That means one replication enzyme would take 547 hours or about 24 days to replicate an X chromosome. What are the biological ramifications of this 24 days? If you cut yourself, it would take 24 days to replicate the chromosomes before the skin cells could divide. The formation of new cells needed for healing cuts in our skin would keep us in a wounded state.

To speed up the chromosome replication process, two tactics are used by living cells. Both tactics are shown in Figure 10. First, replication will not start on just one end of the chromosome. Instead, there are hundreds of origins of replication (ori) along the chromosome. In addition, the replication process moves bidirectionally from the ori. This bidirectional replication creates forks of replication that move toward each other as the chromosome is replicated.

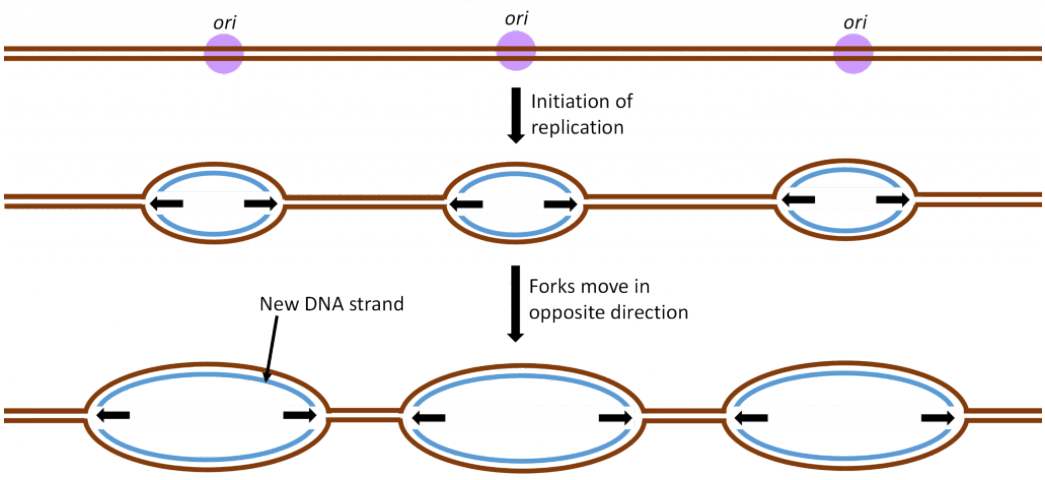


Figure 10. Origin of replication and replication forks. Image by Walter Suza.

As a result of these two tactics, a large chromosome can be replicated in hours rather than weeks and cell division can happen quickly when it is needed for growth or cell replacement.

The Chromosome Replicating Team

Like all processes in living cells, a collection of specific proteins works together to perform functions which control and complete chromosome replication. All the proteins involved in DNA replication make and break bonds, so they are enzymes. The function of the main DNA replicating enzymes is described below.

Five of the main DNA replicating enzymes

- **Helicase**: This is one of the enzymes that unwinds the double stranded DNA molecule. The unwound single strands no longer hydrogen bond to their complementary strand but the sugar-phosphate bonds remain intact.
- **Topoisomerase**: The unwinding work of Helicase creates tension in the double stranded DNA ahead of the replication fork. Topoisomerase relieves this tension by catalyzing a series of sugar phosphate bond breaking and making steps. Without this tension relief, the Double stranded DNA could break and the intact chromosome cannot finish replicating.
- **DNA Polymerase III (DNA pol III)**: This is the main DNA synthesizing enzyme. The enzyme reads the single strand as a template and places in the complementary deoxyribonucleotide. DNA pol III reads the template in the 3′ to 5′ direction and builds the new strand in the 5′ to 3′ direction, adding the next nucleotide to the 3′ end by catalyzing sugar-phosphate bonding. DNA pol III illustrates the specificity of enzymes several ways; it reads and proofreads the placement of new nucleotides to insure accurate replication. It can only read and build in one direction. DNA pol III can only add nucleotides to a free 3′ end. This last specification means that DNA pol III cannot start the replication process on the single stranded template. Another enzyme needs to be part of the in vivo replication team to prime the work of DNA pol III.
- **Primase**: Biochemists named this enzyme to describe its role in starting or priming the replication process. DNA (or RNA) primase is a special RNA polymerase. The enzyme reads the single stranded DNA template 3′ to 5′ and adds ribonucleic acid (RNA) nucleotides in the 5′ to 3′ direction. Once a few hundred RNA nucleotides are added, primase falls off the template strand and leaves the 3′ end that DNA Pol III needs to continue the process.
- **DNA Polymerase I (DNA pol I)**: The DNA polymerase specializes in the removal of the RNA primers and replacing them with DNA nucleotides. The enzyme works with the same 3′ to 5′ reading and 5′ to 3′ building.
- **DNA Ligase**: After the five enzymes described above have completed their work, some sugar phosphate bonds need to be made to complete the double stranded molecule. DNA ligase has this backbone bond sealing assignment.

Now that we have introduced the replication enzymes, we can describe the step-by-step action of this team. We will focus on the action that happens at one origin of replication.

Step 1: Initiating replication at the Ori

Helicase enzymes bind to the ori sequences and start to unwind the double stranded DNA. This establishes two replication forks (one shown in Figure 10a) and a helicase enzyme will be working to unwind the DNA at each fork as the forks move away from each other. The unwinding exposes single strands of DNA that are the template for building a new strand. Topoisomerase can be seen working to relieve tension ahead of the replication fork in Figure 10a.

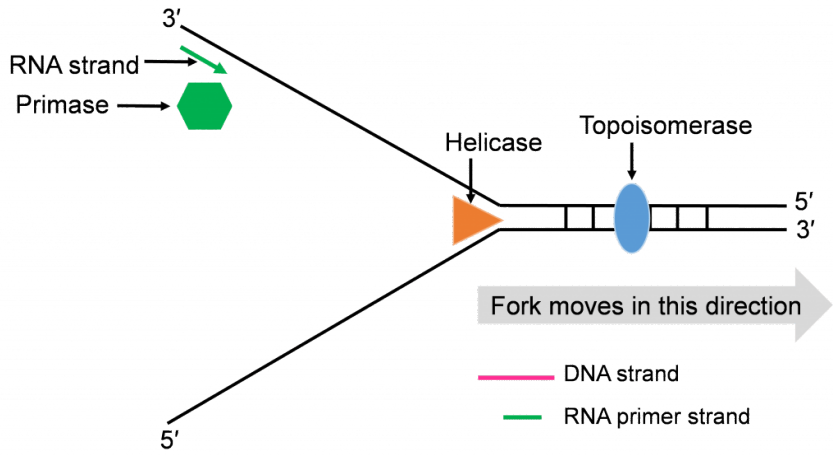


Figure 10a. Helicase and Topoisomerase work together to expose single strand DNA templates. Primase adds RNA nucleotides, reading the old DNA single strand template 3′ to 5′. Image by Walter Suza.

Step 2: Priming DNA with RNA

The process of priming with primase and then replicating with DNA pol III happens at both replication forks (Figure 10a and b). DNA pol III enzymes can replicate new strands as fast as the DNA is unwound at each replication fork. The unwinding creates two template strands at each fork so primase enzyme must work to prime both old strands.

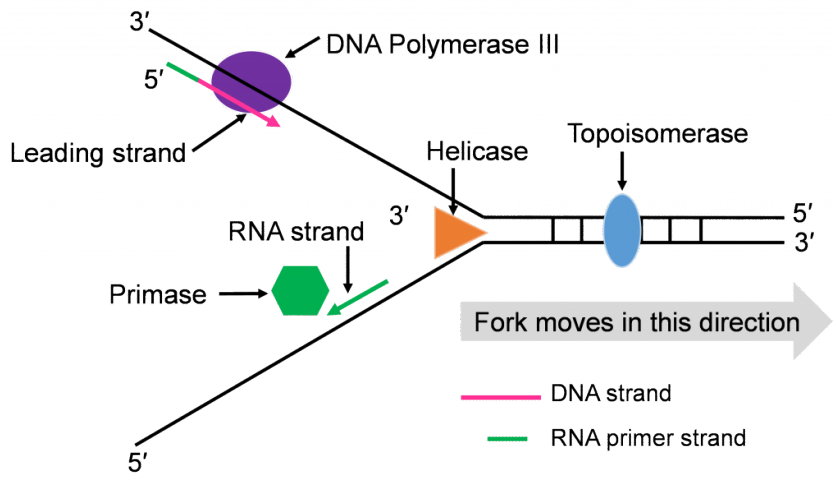


Figure 10b. DNA Polymerase III can add to the 3′ end of the RNA primer and start to read the template 3′ to 5′. Primase can prime the other template strand at this replication fork. Image by Walter Suza.

Step 3: Synthesis of leading and lagging strands

The double strands of DNA are anti-parallel and 'run' in opposite directions and DNA pol III can only work in one direction (Figure 10c). Thus, there is continuous and discontinuous replication happening at each replication fork. The strand that can be replicated as helicase unwinds is the leading strand. The DNA pol III must replicate the other strand in the opposite direction so this strand will lag behind. (Figure 10c).

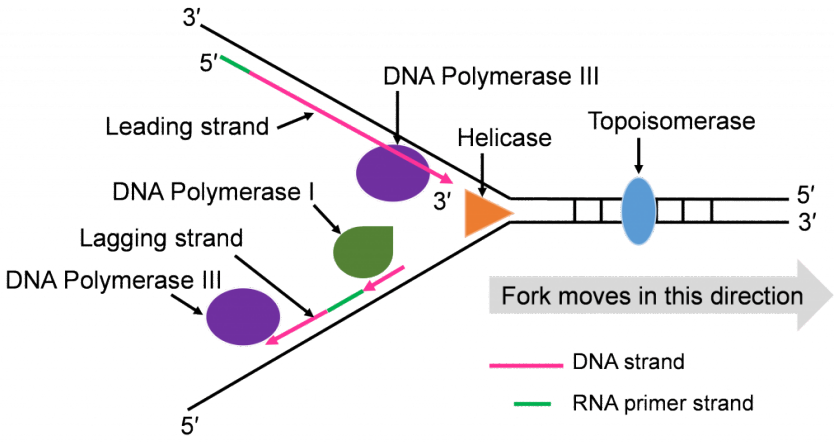


Figure 10c. Both leading and lagging strands are being made at this replication fork. DNA polymerase I removes RNA primer nucleotides and replaces them with DNA. Image by Walter Suza.

There is more priming needed to replicate the lagging strand and the priming leaves RNA-DNA hybrid stretches in the new double stranded molecule. This means there is work for the enzyme DNA pol I. This enzyme will be part of the removal of the RNA nucleotide primers, and replacement with DNA nucleotides (Figure 10c and d).

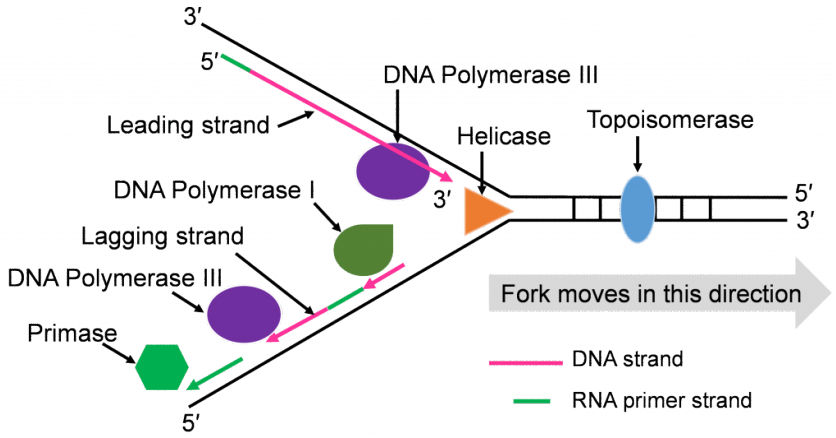


Figure 10d. Lagging strand synthesis must be primed multiple times with primase enzyme creating discontinuous replication. Leading strand just needs to be primed one time. Image by Walter Suza.

Both replication forks have a leading and a lagging strand of new DNA. The DNA nucleotide stretches between the red RNA primers would be the Okazaki fragments.

One of the first scientists to provide evidence to support this model of DNA replication was the team of Reiji and Tsuneko Okazaki in the 1960s. They used radioactive nucleotides to track the newly replicating fragments and found that many of the fragments were short. This fits with the discontinuous model.

As the four enzymes just described attend to their specific roles in replication, two double stranded DNA molecules with an old strand and a new strand are built. If the DNA pol III makes a mistake and adds the wrong, non-complementing nucleotide, the enzyme can proof read its work and replace these replication mistakes. When replication is perfect, the two double stranded DNA molecules will have identical sequences.

Step 4: Ligation of Okazaki fragments

The fifth enzyme that has a final role in completing replication is DNA ligase (not shown in Figure 10d and e). This enzyme will seal the sugar phosphate bond between the last replacement nucleotide added by DNA pol I and the first nucleotide that had been added after priming by DNA pol III. With this bond sealing work, the replication process is complete.

The two double stranded DNA molecules that get made will have identical sequences unless rare mistakes happen as the enzymes conduct their work.

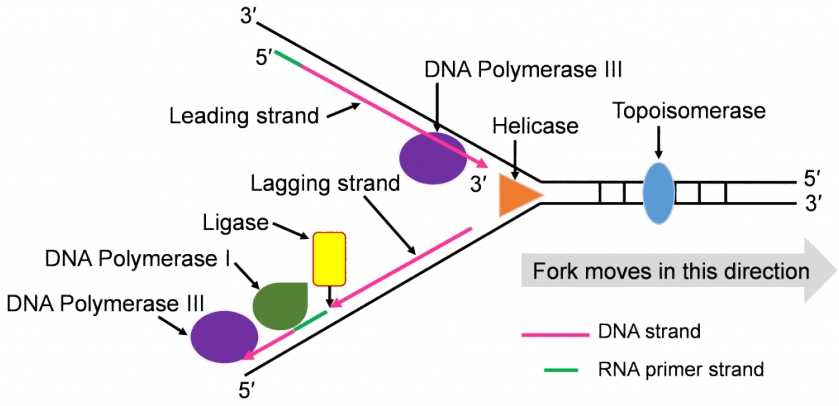


Figure 10e. Ligase must form a sugar-phosphate bond between the last nucleotide placed by DNA pol I and the first nucleotide placed by DNA pol III. Image by Walter Suza.

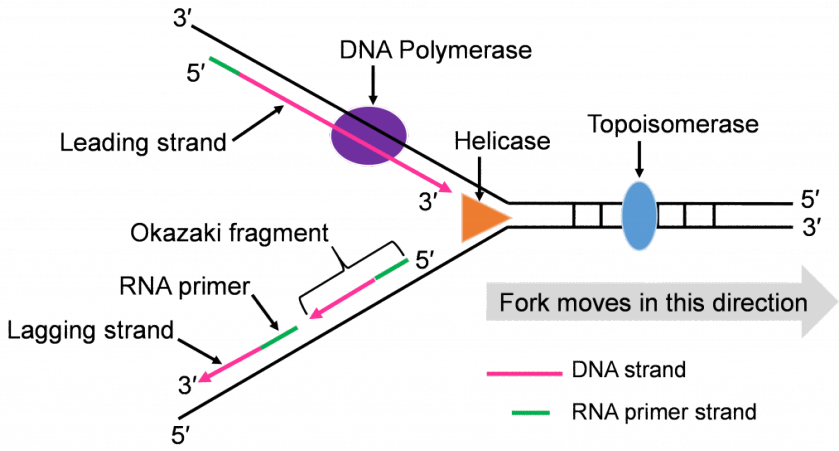


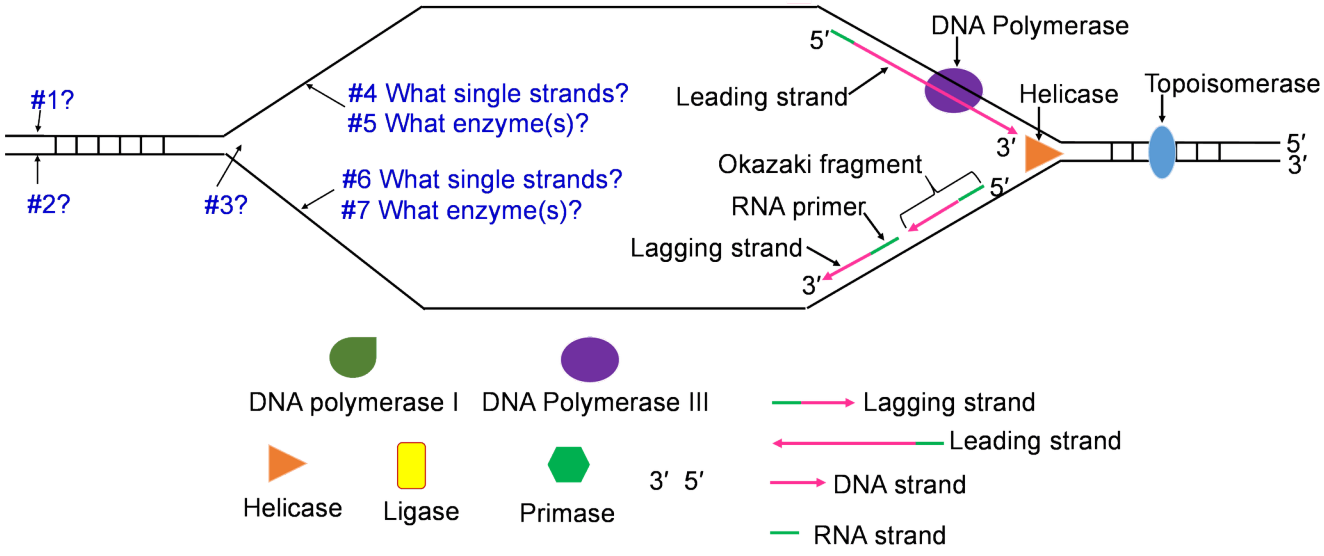
Figure 10f. Short fragments made by discontinuous replication were discovered by the Okazaki team and are called the Okazaki fragments. Image by Walter Suza.

Lesson Summary:

DNA is composed of nucleotides. Nucleotides are connected together by phosphate bonds to form a strand. The bases on one strand form hydrogen bonds with the bases on the other to form a double stranded molecule. The final feature of the molecular structure is that DNA assumes a helical conformation. To fit inside the nucleus, DNA assumes a very condensed structure. Therefore, chromosomal DNA is coiled around a histone protein core to form chromatin. The tight packaging of DNA in chromatin must be modified to allow DNA replication and transcription. In the process of replication, the two DNA strands separate and act as templates for the synthesis of complementary daughter strands.

Learning Activities

Place the single strands, replication enzymes, and 3′ / 5′ labels in the appropriate places (#1-#7)



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2.4.3: DNA Mutations

Learning Objectives

- List the types of mutation.
- Predict the effects of specific mutations in gene coding regions on the protein encoded by the gene.
- Describe ways in which mutations arise, naturally and experimentally.

Introduction

Crop improvement relies on genetic variation, which is synonymous with DNA variation. Mutations are the source of genetic variation that is the driver for trait improvement. In general, mutations are rare events, and most are deleterious and get selected against during the course of evolution. However, occasionally mutations create genetic variation that is beneficial. As such, it is often of interest for plant improvement or for genetic studies to induce genetic mutations by exposing seed or plant tissue to certain agents, called mutagens.

By definition, a mutation is a heritable change in DNA sequence. This can happen in several ways: substitution of a DNA base, insertion or deletion of one or more DNA bases, or by large-scale chromosomal rearrangements, the latter of which will not be considered here. It has also become clear in recent years that chromatin carries additional information to the DNA sequence in the form of base modifications such as cytosine methylation. This is known as **epigenetic** modification and can impact gene function and plant traits.

A. DNA base substitutions

The simplest type of mutation is a substitution of one base for another in the DNA sequence. Substitutions most often arise as errors during DNA replication or repair. The most common type is the **transition**, where one pyrimidine may be substituted by the other, or one purine by the other. The less common type is the **transversion**, in which a purine may be substituted by a pyrimidine or vice versa. As described in the next section, these can have various effects on gene function depending on where they occur in a gene.

B. Insertions & deletions



Figure 1. Example of DNA base substitution. In this example, the base G is substituted for T. Image by NIH-NHGRI.

Insertions or deletions of DNA are other types of mutations that occur quite frequently. They can vary in size from one to thousands or more nucleotide bases. Their effects on gene function depend on the size of the insertion or deletion, and the location relative to the gene. As described below, insertions and deletions that are in multiples of 3 are referred to as in-frame insertions and deletions and result in the addition or deletion of amino acids from the protein sequence. When not in multiples of three, insertions and deletions lead to **frameshift mutations**, altering the amino acid sequence of the protein encoded by the mutated gene.



Figure 2. Example of deletions. In this example, the base A is deleted. Image by NIH-NHGRI.

A common source of inserted DNA is from **transposons**, sequences of DNA that are able to move from one genomic site and insert into another. Transposons were discovered in maize by Barbara McClintock. For this discovery, she was awarded the Nobel Prize in Physiology and Medicine in 1983.

Some transposons copy themselves and multiply in number within a genome. Transposons can cause wide scale chromosome rearrangements and gene mutations. A feature of mutations caused by many plant transposons is that they are unstable. Excision of a transposon from the mutated gene can often restore it to wild type or create a new stable mutant allele. Transposons are found in every living organism studied and are a major driving force in genome evolution.



Figure 3. Example of a DNA insertion. In this example, the base A is inserted between T and C. Image by NIH-NHGRI.

Effects of mutations on gene function

A. Coding region mutations

Silent mutation

A silent mutation is a mutation that results in the change of a codon without a change in the amino acid represented by the codon. Because of the redundancy of the genetic code, DNA base substitutions may not lead to the incorporation of an incorrect amino acid in the protein.

Missense mutation (amino acid substitution)

A missense mutation is a single nucleotide base substitution that alters a codon such that it codes for a different amino acid that is incorporated into the encoded protein. The amino acid substitution may affect the function of the protein if it occurs in a critical portion of the protein.

Frameshift mutation

Due to the triplet nature of the genetic code, an insertion or deletion can change the **reading frame** for the entire subsequent sequence. For example, if a particular sequence is read sequentially (Figure 4).

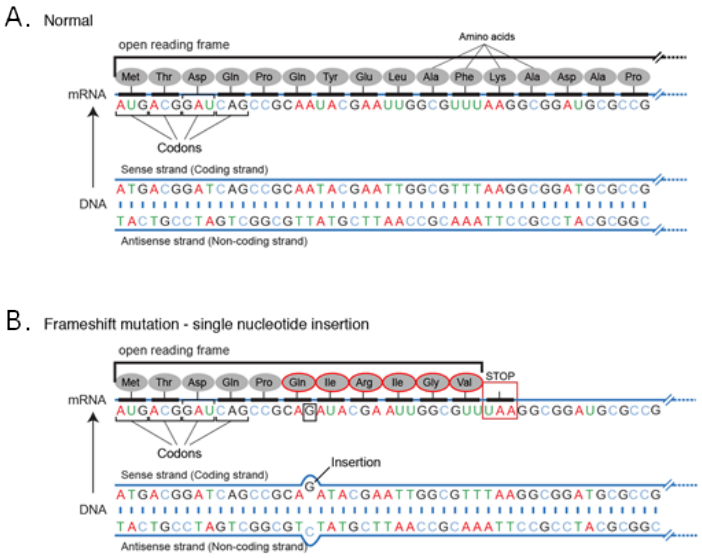


Figure 4. Impact of frameshift mutation on gene function. The normal gene sequence is shown in part A. The insertion of the base G shown in part B, results in a new protein. Image by NIH-NHGRI.

Because the new sequence of codons is different from the original, the entire amino acid sequence is changed from the point of insertion. A similar effect is seen for deletions. This effect is seen whenever the number of nucleotide bases inserted or deleted is not a multiple of 3 and it usually will result in the loss of the function of the protein.

Amino acid additions or deletions

On the other hand, if insertions or deletions are in multiples of 3, amino acid additions or deletions can arise. Consider the following 3-base insertion (XXX):

UUU CCC XXX AAA GGG (codons)

aa1 aa2 aa5 aa3 aa4 (amino acids)

If such insertions or deletions occur in important regions of a protein, they might impair function.

Nonsense mutation

A nonsense mutation arises when a functional codon is changed to a stop codon. Nonsense mutations may cause premature termination of translation.

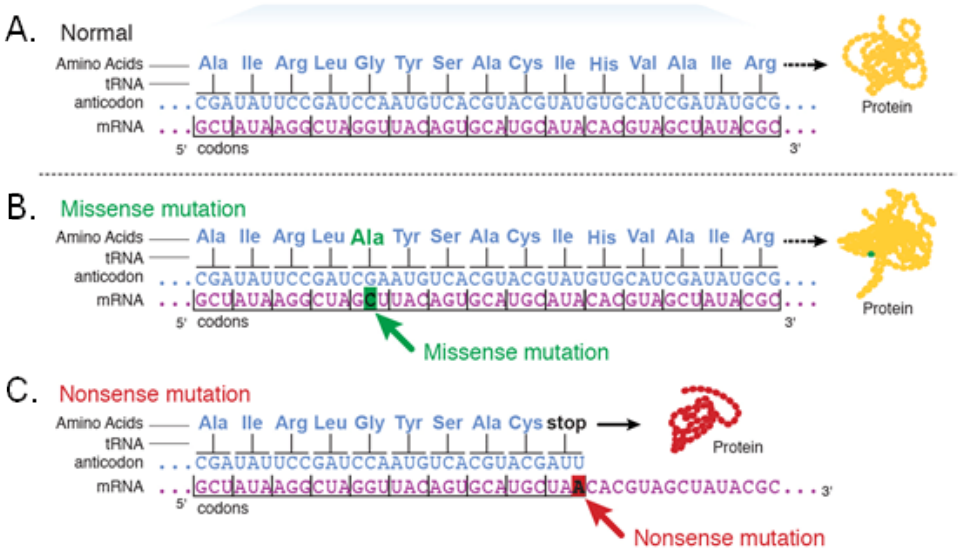


Figure 5. Impact of missense and nonsense mutations on gene function. The normal gene sequence is shown in part A. In part B, a missense mutation leads to replacement of the Gly by Ala. In part C, a nonsense mutation results in premature stop codon and shortening of the protein. Adapted from NIH-NHGRI.

B. Regulatory region mutations

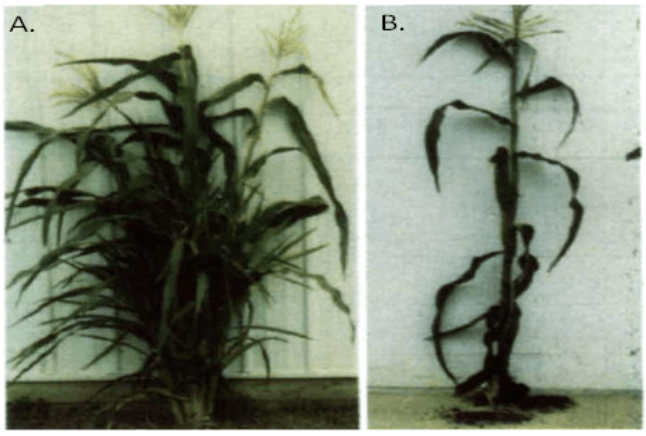


Figure 6. Regulatory region mutation of the tb1 gene converted teosinte (A) to modern maize (B). Image courtesy of John Doebley.

Promoters and **enhancers** control temporal, spatial and quantitative aspects of gene expression. As such, mutations in these regulatory regions of a gene can alter the regulation of the gene's expression. Genes can become expressed at inappropriate times, places or levels, which can affect a plant's phenotype.

Teosinte, the progenitor of modern maize, produces multiple branches (Figure 6A) due to reduced apical dominance. In teosinte and modern maize, apical dominance is under the control of a gene called *teosinte branched1*, ***tb1*** (Doebley et al. 1997). An insertion of a transposon in the regulatory region of the ***tb1*** gene in modern maize causes the gene to be strongly expressed (Doebley et al. 1997; Studer et al. 2011), resulting in enhanced apical dominance, and suppression of excessive branching (Figure 6B).

C. Recessive vs. dominant mutations

Recessive mutations

Recessive mutations most commonly result from a loss of function. A loss of function mutation causes a decrease in the function of a gene. A complete loss of function is known as a **null mutation** while partial loss of function mutants are often referred to as

hypomorphs or “leaky”. Loss of function mutations can affect gene expression or the function of the resultant protein. Most loss of function mutations are recessive because in a heterozygote, the normal allele can provide the necessary function.

Dominant mutations

Dominant mutations can affect genes in several different ways. One general class is gain of function mutations. These encompass several different types.

1. **Overexpression mutants**. As the name implies, the affected gene is expressed at levels that are inappropriately high, causing too much of a gene product to be produced.
2. **Neomorphic mutations** arise when a mutation causes a qualitatively new effect not seen with normal alleles. This can occur when a mutation affects the regulation of a gene such that it becomes expressed in a new time or place in the plant. It can also happen when a mutation alters the properties of the encoded protein. For example, a transcription factor could be mutated to recognize a different promoter sequence, or an enzyme could recognize a new substrate
3. **Dominant negative mutations** alter a gene product such that the mutant gene product interferes with the function of the normal one. This can occur at the protein level. For example, a protein might function as a multimeric complex and the presence of one mutant subunit could compromise the function of the entire complex. Dominant negatives can also occur at the RNA level for example if an insertion, deletion or rearrangement causes the wrong strand to be transcribed, creating an antisense or RNAi suppression effect.

Effects of mutations on traits

Example of a loss of function mutation – gene function is required for a trait

Sweetness is an important quality trait in maize (corn). One of the loss of function mutants used to improve sweetness in corn is ***sugary1*** (***su1***). The Su1 gene is expressed in the endosperm (Lertrat and Pulam, 2007), and it encodes and enzyme that is required for normal starch biosynthesis (Rahman et al. 1998). In the ***su1*** mutant, with defective starch biosynthesis, the concentration of sugar is 3 times higher than wild type making this mutant valuable for sweet corn (Lertrat and Pulam, 2007). In addition, ***su1*** kernels accumulate a highly branched polysaccharide called phytoglycogen, which gives the kernels a creamy texture. New commercial sweet corn hybrids are developed using a combination of starch biosynthesis mutants (Nelson and Pan, 1995; Lertrat and Pulam, 2007).

Example of a gain of function mutation – gene function is sufficient for a trait

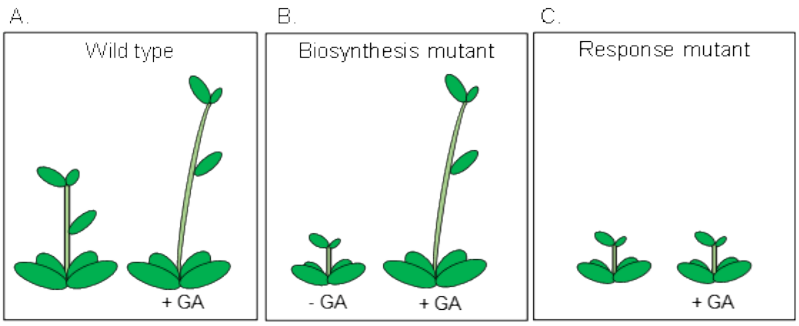


Figure 7. GA promotes plant growth. (A) When applied to wild type plants, GA induces height. (B) A mutation that blocks GA biosynthesis will result in short plants, and exogenous addition of GA to a biosynthetic mutant restores growth. (C) However, a mutation that affects GA signaling leads to failure to sense GA. Thus, exogenous addition of GA does not restore growth of a GA response mutant (C). (Image informed by Peng et al. 1999).

The plant growth hormone, gibberellin (GA) regulates plant height, thus, a plant that fails to produce GA, or does not respond to GA will have a dwarfed phenotype (Figure 7). At the molecular level, the cell response to GA involves several processes, including the destruction of repressor proteins, referred to as DELLAs.

The “green revolution gene” ***Reduced height-1*** ***(Rht-1)*** from wheat encodes a DELLA repressor of GA signaling (Peng et al. 1999). A stretch of five amino acids referred to as the DELLA domain is required for the destruction of the repressor in response to GA. Mutations in the DELLA domain of ***Rht-1*** genes result in a protein product that is resistant to degradation, leading to failure to

signal a GA response. The failure to signal the presence of GA in the ***rht-1*** mutants is responsible for their short stature. When the mutant ***rht-1*** gene is transformed into rice, the result is also short plants, which is proof that the new function of the DELLA protein is sufficient to induce dwarfism (Peng et al. 1999).

Generation of mutations

Because mutations are the ultimate source of genetic variation for breeding and genetic studies, it is sometimes of interest to generate new mutations. New DNA mutations (Figure 8) can arise spontaneously or they can be induced by experimental methods.

Spontaneous mutations arise “naturally”

That is, no particular effort was made on the part of the scientist to increase the mutation rate. Spontaneous mutations generally occur at very low rates, on the order of 1 per gene per million gametes.

Mistakes in DNA replication

Very rarely, incorrect bases are incorporated or bases omitted from a DNA strand during synthesis. Such mistakes can lead to spontaneous substitutions, insertions or deletions. For example, strand slippage due to the formation of a loop.

Environmental mutagens

There are several chemical agents present in the environment with potential to damage DNA and create mutations. These include naturally occurring compounds as well as man-made pollutants. Some modify DNA nucleotide bases through deamination (e.g., nitrous acid), while others promote oxidative reactions that may damage DNA (e.g., ozone).

Radiation is another type of environmental mutagen. Ionizing radiation (e.g., X-rays) can shatter DNA sequences and promote chromosome rearrangements. The less powerful ultraviolet rays can penetrate the cell and promote the formation of pyrimidine dimers which may inhibit replication and transcription.



Figure 8. Mutations can arise spontaneously or they can be induced by experimental methods. Illustration by NIH-NHGRI.

Experimental mutagenesis involves applying a mutagenic agent to a plant or plant part to generate a mutation. For the mutation to be useful, it must be heritable, which imposes limitations on what tissues can be treated. Following the mutagenic treatment, a subsequent breeding scheme must be implemented to generate pure breeding lines of appropriate genotypes to display mutant phenotypes. These will depend on the species and the design of the mutagenesis.

Chemical mutagenesis

A commonly used chemical mutagen for experimental plant biology and mutation breeding is ethyl methanesulfonate (EMS). The compound is known as an alkylating agent and induces a CG to TA transition.

Seed Mutagenesis

This is the most common method of chemical mutagenesis in most species. Seeds are soaked in EMS solution, planted and allowed to flower and set seed. During EMS mutagenesis, every cell in the embryo will be independently mutagenized and the

resultant seed and subsequent plant will be chimeric. These seeds can be sown and plants screened for traits of interest. For self-pollinating species, the required breeding is very simple, whereas for dioecious species it would be much more elaborate and labor-intensive.

Pollen Mutagenesis

For a few species such as maize, pollen mutagenesis is the method of choice. Pollen are treated with EMS and applied to silks of an acceptor plant. The benefit is that the resultant seed is not chimeric but rather all the cells of the embryo carry the same mutagenized paternal genome inherited from the pollen grain.

Insertional mutagenesis

The idea behind insertional mutagenesis is that a mobile or introduced piece of DNA can sometimes insert into a gene, thereby inactivating or modifying the function of the gene. The approach is useful in gene cloning because the inserted DNA serves as a "tag" that can be used to locate the gene of interest, and subsequently isolate that gene.

Another strategy used by researchers is mutagenesis using T-DNA from the ***Agrobacterium tumefaciens*** Ti plasmid. A larger number of plants (up to 10,000 for Arabidopsis) are transformed with T-DNA to generate mutants at random.

Similar to the example of transposon mutagenesis above, a gene can be "tagged" by a T-DNA and the tag used to isolate the gene. However, a T-DNA insertions are stable and thus disrupt the gene permanently.

In recent years, insertional mutagens have been engineered in many innovative ways. For example, strong promoters have been engineered into transposons such that if the transposon inserts near a gene, it can cause strong transcriptional expression of that gene. Such "activation tagging" approaches have been successful in identifying many genes that were not amenable to other mutational approaches.

Summary

Mutations alter A-T and G-C base pairs in DNA. A mutation in a coding sequence may alter the sequence and function of the protein product. A frameshift mutation changes the reading frame through insertions or deletions to produce an entirely novel product. A point mutation on the other hand alters only the amino acid represented by the codon in which the mutation exists. Mutations in regulatory regions might alter the expression of a gene. Mutations have been powerful tools for genetic studies as well as to study biological functions, for example, growth and development. The natural occurrence of mutations can be enhanced experimentally by applying agents referred to as mutagens. Researchers have used certain mutagens to induce mutations to study gene function and apply new traits for crop improvement.

Activity 1

For this activity, it may be helpful to refer to the genetic code chart.

For the following DNA sequence:

5′ ATG TTG GAG AAG GTT GAA ACT TTC 3′

Write the coding strand

3′ TAC AAG CTC TTC CAA CTT TGA AAG 5′

Write the mRNA sequence from the given DNA sequence

AUG UUC GAG AAG GUU GAA ACU UUG

Write the resulting peptide sequence

Met-Phe-Glu-Lys-Val-Glu-Thr-Leu

If the **third** triplet of the original DNA sequence is mutated to GA<u>A</u> to result in the following sequence

5′ ATG TTG GAA AAG GTT GAA ACT TTC 3′

What will be the mRNA sequence from the mutated DNA sequence?

AUG UUC GAA AAG GUU GAA ACU UUG

What will be the peptide sequence from the mutated DNA sequence?

Met-Phe-Glu-Lys-Val-Glu-Thr-Leu

What type of coding region mutation is this? Give a reason for your answer.

1. Silent
2. Amino acid substitution
3. Frameshift
4. Nonsense

If the **sixth** triplet of the original DNA sequence is mutated to TAA to result in the following sequence

5′ ATG TTG GAG AAG GTT TAA ACT TTC 3′

What will be the mRNA sequence from the mutated DNA sequence?

AUG UUC GAG AAG GUU UAA ACU UUG

What will be the peptide sequence from the mutated DNA sequence?

Met-Phe-Glu-Lys-Val

What type of coding region mutation is this? Give reason for your answer.

1. Silent
2. Amino acid substitution
3. Frameshift
4. Nonsense

The original DNA sequence is mutated to result in the following sequence

5′ ATG TTG GAG AAG GTT CGA AAC TTT C 3′

What will be the mRNA sequence from the mutated DNA sequence?

AUG UUC GAG AAG GUU CGA AAC UUU C

What will be the peptide sequence from the mutated DNA sequence?

Met-Phe-Glu-Lys-Val-Arg-Asn-Phe

What type of coding region mutation is this? Give a reason for your answer.

1. Silent
2. Amino acid substitution
3. Frameshift
4. Nonsense

Activity 2

1. A hypothetical plant gene encodes a protein with the following amino acid sequence:

Met-Leu-Lys-Thr-Phe-Val-Glu

A mutation of a single nucleotide alters the amino acid sequence to:

Met-Tyr-Asp-Pro-Gly-Ala-Lys-Ile-Arg

Describe the type of the mutation and the position of the codon that is affected.

2. For the following DNA sequence

3′ **A**TG GCC GGC AAT CAA CTA TAT TG**A** 5′

1 24 (nucleotide number)

a. Write the coding strand

b. Write the mRNA strand

c. Write the protein strand

d. Give the altered amino acid sequence of the protein for each of the following mutations:

i. A transition at nucleotide 11

ii. A transition at nucleotide 13

iii. A single nucleotide deletion at nucleotide 7

iv. A **T** to **A** change at nucleotide 15

v. An addition of ACC after nucleotide 6

vi. A transition at nucleotide 9

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2.4.4: PCR and Gel Electrophoresis

Learning Objectives

At the completion of this PCR lesson, learners will be able to:

- List the 5 chemical components of a PCR reaction and describe their roles.
- List the functions of the 3 temperature cycles which are repeated during a PCR reaction.
- Describe the process of observing results and interpreting results of a PCR experiment.
- List possible uses of PCR in genetic testing and in research.

Overview

The polymerase chain reaction laboratory technique is used in a variety of applications to make copies of a specific DNA sequence. This lesson describes how a PCR reaction works, what it accomplishes, and its basic requirements for success. Examples of interpreting results are given. PCR's strengths, weaknesses, and applications to plant biotechnology are explained.

The Discovery of PCR

In 1983, Kary Mullis was driving along a Californian mountain road late one night. As a molecular biologist, Dr. Mullis was imagining a better way to study **DNA**. This late-night thinking led to a revolutionary way to make laboratory copies of DNA molecules (Saiki et al. 1985, Mullis 1990). In the decades since, the polymerase chain reaction or **PCR**, has become the standard method used for detecting specific DNA or RNA sequences. Selling the equipment and reagent kits for PCR is a multi-billion-dollar business because DNA and RNA detection is critical information in many applications.

In Vitro vs. *In Vivo* Replication

PCR is an *In Vitro* process; a series of chemical reactions that happen outside of a living cell. This laboratory technique is modeled after an In vivo process, the living cell's natural ability to replicate **DNA** during normal cell cycles (see Lesson on DNA: The Genetic Material). Every living cell makes a duplicate copy of each **chromosome** before the cell is ready to divide. Figure 1 below illustrates the key parts of *In vivo* DNA replication that are the basis for PCR success.

There are other enzymes that play an important role in in vivo replication. However, PCR works as an *in vitro* DNA replication process by using just one of these enzymes. Mullis imagined a chemical reagent and a temperature change step in the method that could perform the work of the other two enzymes. It should be noted that because Dr. Kerry Mullis had learned about the details of *in vivo* DNA replication, he could create this science changing *in vitro* method.

Before reading the description of PCR components and processes, watching this video can help you visualize the importance of each step,

Name and Chemical Components of PCR

The name 'Polymerase Chain Reaction' represents the nature of the process. 'Polymerase' because DNA Polymerase III is required for constructing new DNA strands, just like in a living cell. 'Chain Reaction' describes repeating cycles of replication which target a specific segment of a chromosome and use a "copy the copies" progression each cycle that doubles the amount of **DNA copies of a specific segment of DNA** present each cycle. In just 20 cycles of the chain reaction, over one million (2^{20}) copies of that specific segment of DNA can be produced. This is enough DNA to see with your naked eye. The goal of PCR is to make millions of copies of a specific segment of DNA that all originate from a single DNA sample.

The five chemical components that must be added to a test tube for the PCR reaction to work, include a **DNA template, DNA polymerase III enzyme**, single stranded DNA primers, nucleotides, and reaction buffer.

1. The DNA template is a sample of DNA that contains the target sequence of DNA for copying.
2. DNA pol III. There are two requirements for a suitable DNA polymerase enzyme for PCR. First, the enzyme must have a good activity rate around 75°C. Second, the enzyme should be able to withstand temperatures of 95-100°C without denaturing and losing activity.
3. Two primers. Primers are short oligonucleotides of DNA, usually around 20 base pairs in length. Because the purpose of PCR is to amplify a specific section of DNA in the genome, such as a known gene, then primers of specific sequences must be used. The geneticist planning the PCR reaction will design a forward primer to bind to one strand and a reverse primer that complements and binds to the other strand. The **primer design** process to select forward and reverse primers is requiring appropriate genetics thinking and is describe later in this reading.
4. The four different deoxyribonucleotide triphosphates (dNTPs). Adenine (A), guanine (G), cytosine (C), and thymine (T) are needed to provide the building blocks for DNA replication. DNA polymerase will add each **complementary** base to the new growing DNA strand according to the original strand's sequence following normal A-T and C-G pairings.
5. Finally, a reaction buffer. This creates a stable pH and provides the Mg^{2+} cofactor needed for DNA pol III activity.

Three Temperature Cycles

A key insight to the success of PCR as an in vitro DNA replication process which generates millions of specific sequence copies was a three-temperature cycle which accomplish three parts of DNA replication: **denaturation** of the double stranded template, **annealing** of the primers to the single strands and **extension** of new strand synthesis by DNA pol III.

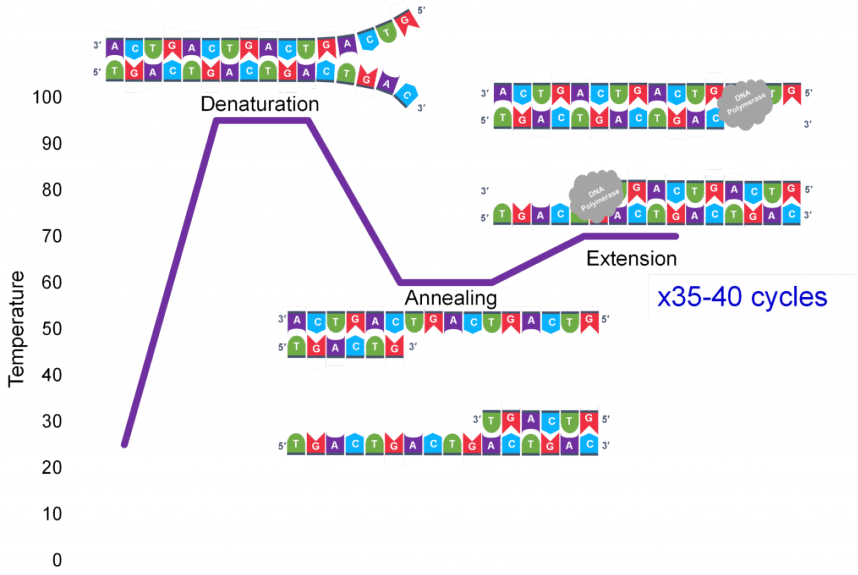


Figure 1. The three temperature steps in a PCR cycle. Image by Marjorie Hanneman.

In general, a single PCR run will undergo 25-35 cycles. The first step for a single cycle is the denaturation step, in which the double-stranded DNA template molecule (Figure 2) is made single-stranded(Figure 3). The temperature for this step is typically in the range of 95-100°C, near boiling. The high heat breaks the hydrogen bonds between the strands but does not break the sugar-phosphate bonds that hold the nucleotides of a single strand together (Figure 3).



Figure 2. DNA template in the PCR reaction test tube is a double stranded molecule. The targeted sequence of nucleotides for this illustration is shown. The template DNA could be fragments from the chromosome of various lengths. The 5′ and 3′ orientation of both strands is shown. Image by Marjorie Hanneman.

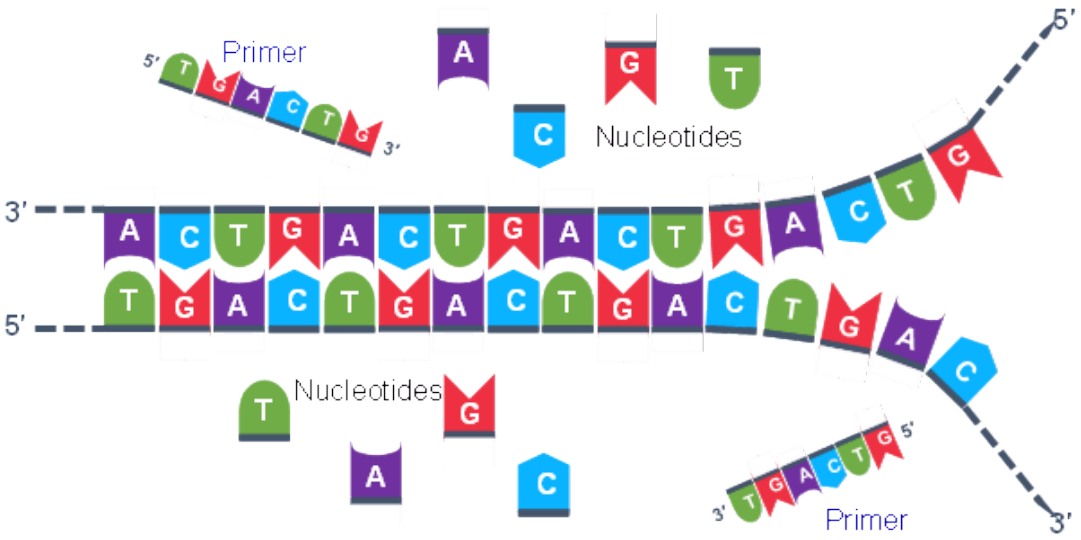


Figure 3. Denaturation: The hydrogen bonds holding the strands of the double stranded template together are broken by heating the test tube. Image by Marjorie Hanneman and Donald Lee.

Thousands of copies of the single stranded primers and the individual nucleotides were added to the test tube prior to beginning the cycles. Both the primers and nucleotides will become part of the new DNA strands. The second step in the PCR reaction is to cool the temperature in the test tube to 45-55°C. This is the primer annealing step in which the primers bind to **complementary** sequences in the single-stranded DNA template. The two primers are called the forward and the reverse primer and are designed because their sequences will target the desired segment of the DNA template for replication (Figure 4).

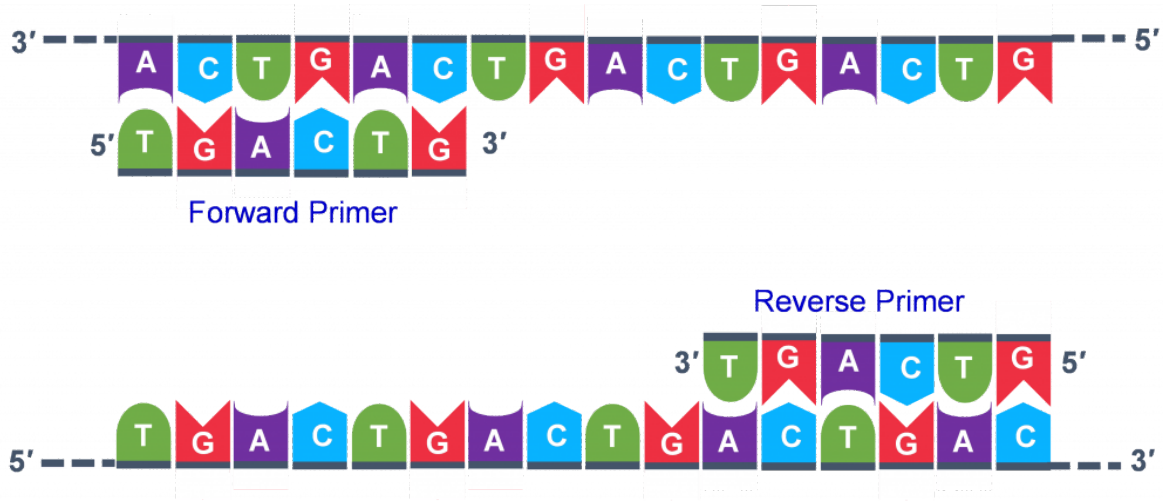


Figure 4. Annealing: The second step of PCR is to cool the test tube which allows the forward and reverse primer to bind to the template. Image by Marjorie Hanneman and Donald Lee.

The geneticist planning the PCR analysis must “design” the forward and reverse primers and then buy them from a vendor who can synthesize single stranded DNA that has a specific sequence and length. The two most important criteria for primer design are the following.

1. One primer must have a sequence that complements one of the template strands and the other primer must be complementary to the other strand. BOTH strands need to be primed for the replication process.
2. The primers must bind so that their 3′ ends are ‘pointing’ in the direction of the other primer. This ensures that the sequence between the primers is replicated in the PCR cycles.

Extension: The final PCR step is when the **DNA polymerase enzyme** reads the template and connects new nucleotidesto the primer’s 3’ end, extending a new complementary strand of DNA (Figure 5). Completion of the final step and the first cycle of PCR, will make two double stranded DNA copies from the original template DNA, doubling of the amount of DNA present. The test tube

is heated to around 75°C, optimizing DNA pol. III activity and the newly synthesizing DNA strand is extended as the template strand is read by DNA pol. III. The Extension step will run for a few minutes and this step completes one PCR cycle.

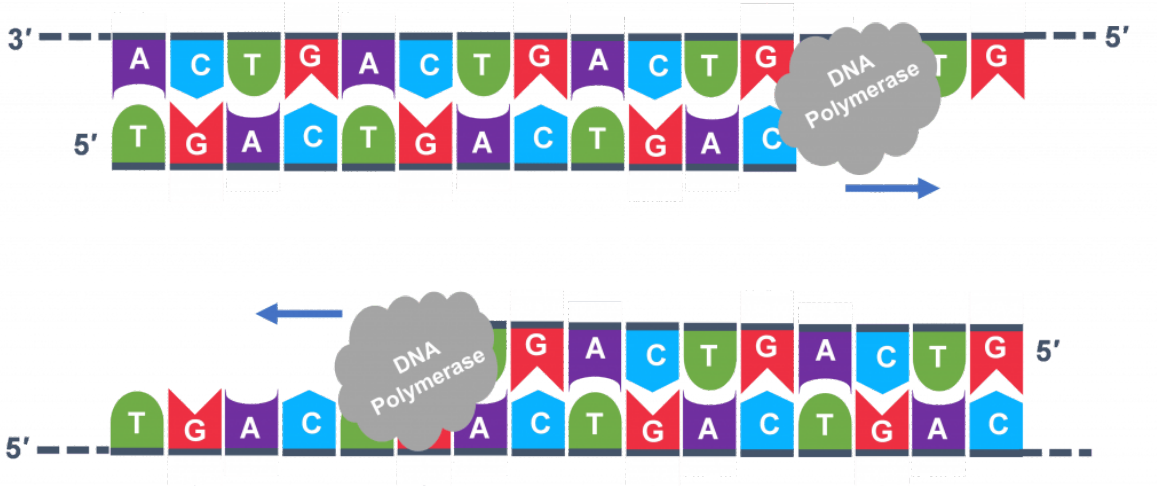


Figure 5. Extension: This step is catalyzed by DNA pol III. The time allowed for this step determines how much of the template DNA pol III can read and replicate. Image by Marjorie Hanneman.

For cycle 2, the denaturation, annealing, and extension steps are repeated (Figure 6 a, b, c). This time, though there will be twice as many DNA template molecules compared to what there was at the beginning of cycle 1. Copies are being made by reading the original template and copies are made by reading the copies made in the previous cycle. Therefore, if everything is working correctly, the DNA replication in the test tube is a chain reaction where at the end of a cycle, there is double the amount of that DNA sequence as what was found at the beginning of the cycle.



Figure 6a. Cycle 1 is complete; two double stranded DNA molecules are made from the original double stranded template. Image by Marjorie Hanneman and Donald Lee.

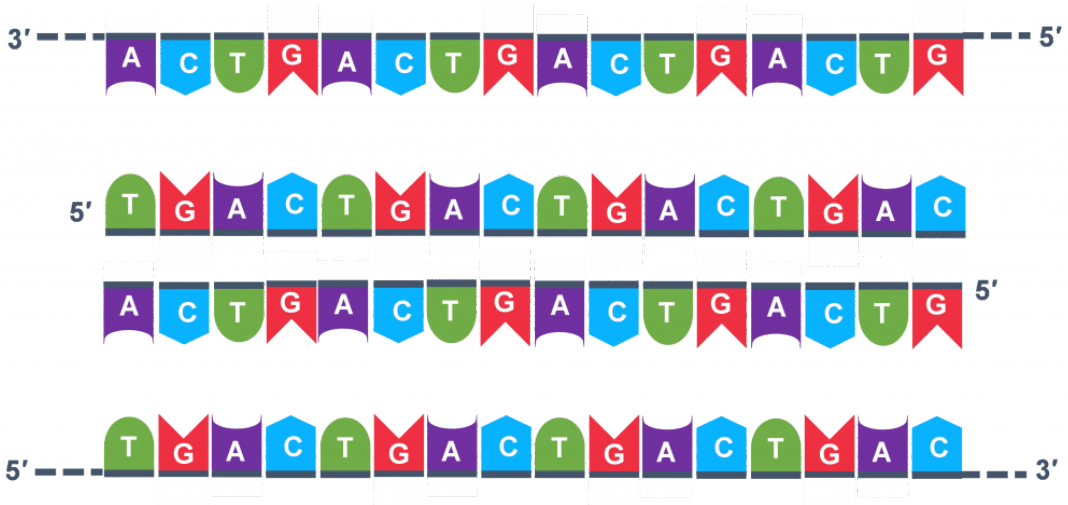


Figure 6b. Denaturation step generates four single strand templates. Image by Marjorie Hanneman

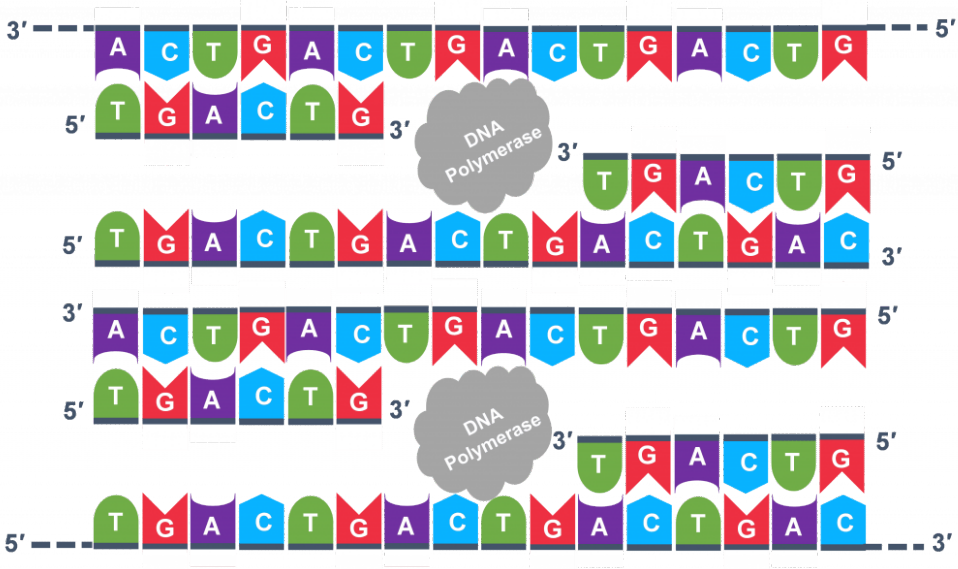


Figure 6c. Annealing and Extension. All templates are primed and four 3′ ends provide a place of DNA pol III extension. Image by Marjorie Hanneman

Because thousands of copies of the forward and reverse primer are added at the start of PCR, all the single strand templates, both the original, the copies in cycle 3 and beyond, and the copies of the copies made from previous cycles will be primed for the extension step of the cycles.

Thermal cycler



Figure 7. The thermal cycler is used to carry out PCR reaction. Image by Walter Suza.

When PCR was first invented, scientists used water baths set at different temperatures and the hand transfer of test tubes at timed intervals to run the PCR reaction. Once the technique became a proven technology for DNA analysis, engineers went to work to create PCR machines. The instrument which heats and cools the DNA samples is called a **thermal cycler** (Figure 7). Each small tube or sample well in a plate contains all the chemical components needed for a PCR reaction. Adding a specific sample to the reaction mix provides the template DNA. A thermal cycler can be programmed for specific temperatures and the amount of time spent at each temperature. The engineered design of thermal cyclers to maximize the accurate replication of the targeted DNA in a small sample volume with the minimum amount of time can be critical in many applications of PCR.

Taq DNA polymerase

When Dr. Kerry Mullis ran the first PCR experiments, he needed to add a new sample of DNA pol III after each denaturation step. This was because the high temperature needed to denature the double stranded DNA template also denatured the DNA pol III protein structure. The DNA pol III enzyme commonly available to molecular geneticists was from E. coli bacteria and this enzyme had no stability at near boiling temperatures. Fortunately, biologists had been investigating Thermus aquaticus, (Taq) a thermophilic eubacterium found in hot springs (Chien et al. 1976). The Taq version of DNA pol III does not easily denature in the hot temperatures required in PCR; plus, it has a good efficiency, able to add 60 base pairs/sec at 70°C. Like all other DNA polymerases, Taq DNA pol III cannot begin DNA replication without the addition of a starting primer. Thus, the discovery of Taq DNA pol III and the commercial availability of this enzyme made PCR a more reliable and doable technology which hastened its application to science investigation and diagnostic testing.

Visualizing the Results with Electrophoresis

Once a PCR reaction has been completed, we need to be able to see the results. To do this, a sample of the **PCR** mixture is loaded into an agarose gel for electrophoresis. The agarose gel contains a matrix of pores which enables it to separate DNA fragments based on their sizes. For details about setting up and running an electrophoresis gel, see Electrophoresis: How Scientist observe fragments of DNA

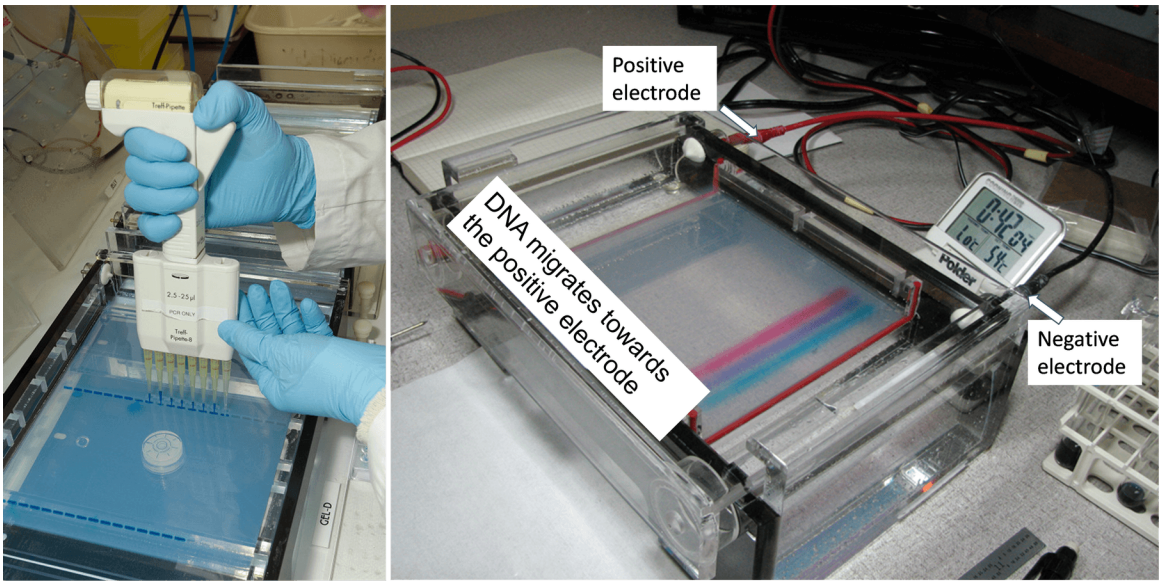


Figure 8. Electrophoresis: A gel electrophoresis set-up with agarose gel with DNA and loading dye on the left and the power supply on the right. Image Source: Michael, CC BY 2.0, via Wikimedia Commons and U. S. Department of Agriculture, CC BY 2.0, via Wikimedia Commons.

Figure 8 shows a picture of a gel electrophoresis gel that is running. The box on the right contains DNA loaded in the agarose gel. The gel placed in an aqueous solution of electrolytes. Depending on the type of dye used, color bands are a dye that was added to the PCR sample before it was loaded into the sample well. This allows for the tracking of the DNA's progression through the gel. Hooked up to this gel unit is an electrical power source which provides the force to move the DNA through the gel. Since DNA molecules are negatively charged, they will migrate towards the red, positive electrode. Shorter DNA fragments move faster than longer fragments through the pores in the gel.

After the gel is run, the DNA is stained with a chemical that binds specifically to DNA molecules and then will either reflect a specific color of visible light or fluoresce a specific color when viewed with ultraviolet light. A single 'band' contains 1000s of individual DNA fragments, all of the same length. Figure 10 illustrates the visual information that can be obtained from an electrophoresis gel after it has run. The electric current uniformly moves all the DNA fragments through a gel in the same direction. The sample wells at the top of the gel image thus establish lanes for the DNA samples to move.

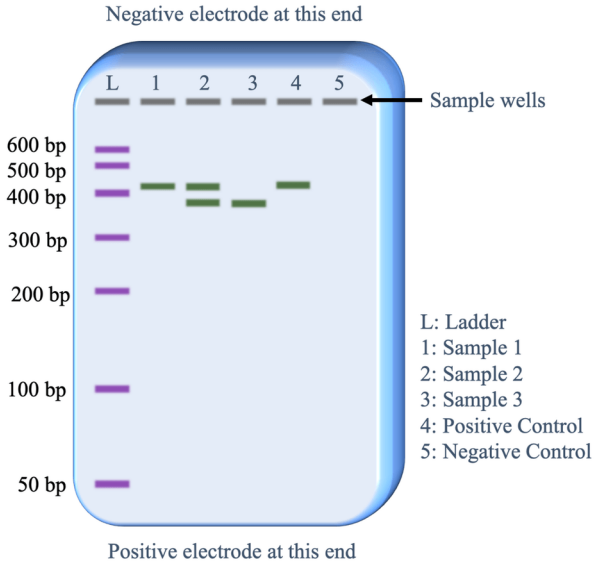


Figure 9. Depiction of an electrophoresis gel with six sample wells that were loaded with either a DNA size ladder (lane L) or a sample from a PCR run (1-5.) The gel was subjected to a DNA staining dye. Image by Marjorie Hanneman.

Below is a description of what information is revealed from each lane.

- Lane L: This was loaded with the DNA size ladder that contains copies of seven different lengths of DNA fragments. Commercial vendors of the DNA size ladder provide information on the lengths of each fragment in base pairs (bp). Running this lane provides an estimate of the DNA fragment lengths in the sample lanes (1-5).
- Lane 1: The PCR sample loaded in this lane has copies of a single length of DNA. The length is slightly more than 400 base pairs.
- Lane 2: The PCR sample loaded in this lane has copies of two lengths of DNA. One fragment is the same length as the fragments in lane 1 and the second fragment is slightly less than 400 bp.
- Lane 3: The PCR sample loaded in this lane has copies of one fragment that is the same length as the shorter fragments in lane 2.
- Lane 4: The positive control worked as predicted. The sample was set up with all the same reagents as the other PCR samples plus a DNA template that was known to contain the sequence targeted by the PCR primers that would generate a 410 base pair fragment.
- Lane 5: The negative control worked as predicted. The sample was set up with all the same reagents as the other PCR samples except no template DNA was added. Therefore, we would not expect the PCR reaction to work and the absence of a band of DNA fragments is as expected.

Because the positive and negative controls worked as expected, the biologist can be confident that the bands of DNA observed in lanes 1,2 and 3 reveal genetic information about the individual providing that DNA sample.

Advantages of PCR

PCR quickly became the method of choice for many types of DNA analysis because of several advantages over other DNA detection methods. The first; it is a simple procedure to set up and run. Fewer steps save time in getting a DNA analysis result. The second is the sensitivity. A very small amount of template DNA in the sample can be detected. Even just a few skin cells from one human hair contain enough DNA, making PCR useful in forensics. The third is that it can be designed to accurately differentiate genetic samples that are different by as little as single nucleotide in the targeted sequence. Finally, in application where large numbers of samples need to be subjected to the same PCR analysis, automation and robotic assistance allow the processing of many samples in a very short time. For example, an automated PCR would be critical for testing large numbers of people in hours during a pandemic.

Limitations of PCR

There are some drawbacks of using PCR that one should be aware of as well. First, the sequence of the gene or chromosome region being targeted is required. This limitation is rapidly diminishing as gene and genome sequencing technology and sharing of this sequence through Internet data bases has emerged as the norm in genetic analysis. Because of the size of the genomes of living things and a high conservation of gene sequences in many organisms, primers designed for a PCR test must be empirically tested with the proper controls. Biologists can easily generate false positive DNA from PCR that is caused by contamination or lack of specificity in primer design. There may also be a need to optimize concentrations of each chemical component. For example, changing the amount of DNA template, MgCl2 and Taq polymerase can affect both the quantity and quality of bands produced. Some studies have shown that even the brand of Taq polymerase can affect results (Holden et al. 2003). Likewise, the temperature cycles may need to be fine-tuned for a specific PCR test. Finally, as an in vitro DNA replication method, PCR cannot replicate entire chromosomes.

Summary

To briefly highlight the topics of this lesson, remember that **PCR** is a relatively easy lab technique which amplifies the amount of **DNA** present, much like living cells do in the beginning stages of a cell cycle. There are 5 chemical components of a PCR reaction: a **DNA template**, a **DNA polymerase**, primers, **nucleotides**, and a buffer. The 3 temperature steps for one cycle are the denaturation, primer annealing and extension steps. There are now many variations and uses of PCR ranging from forensics to genomic studies to identifying transgenic crops.

Lesson Activities

Watch these videos to learn more about PCR:

- Polymerase Chain Reaction (PCR) from DNA Learning Center
- Polymerase Chain Reaction (from UNL)

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2.4.5: Gene Expression- Transcription

Learning Objectives

- Describe the roles that the promoter, coding region and untranslated regions of a gene play in gene expression.
- Describe mRNA processing steps.
- Draw the process of transcription and include the following in your drawing. DNA template and non-template strands, RNA polymerase, new RNA strand, and direction of RNA synthesis.
- Draw the process of mRNA processing and include the following in your diagram, Gene (DNA), promoter, coding region, introns, exons, pre-mRNA, mature mRNA, poly A tail, cap.

Introduction

Genes are DNA sequences that control traits in an organism by coding for proteins (Figure 1). Organisms such as plants and animals have tens of thousands of genes. The impact that a single gene's information can have on an organism, however, is tremendous. Furthermore, organisms have all their genes in each of their cells, but they only need to use the information from a subset of these genes, depending on the type of cell and the cell's stage of development. Therefore, the key to gene function is controlling its expression.

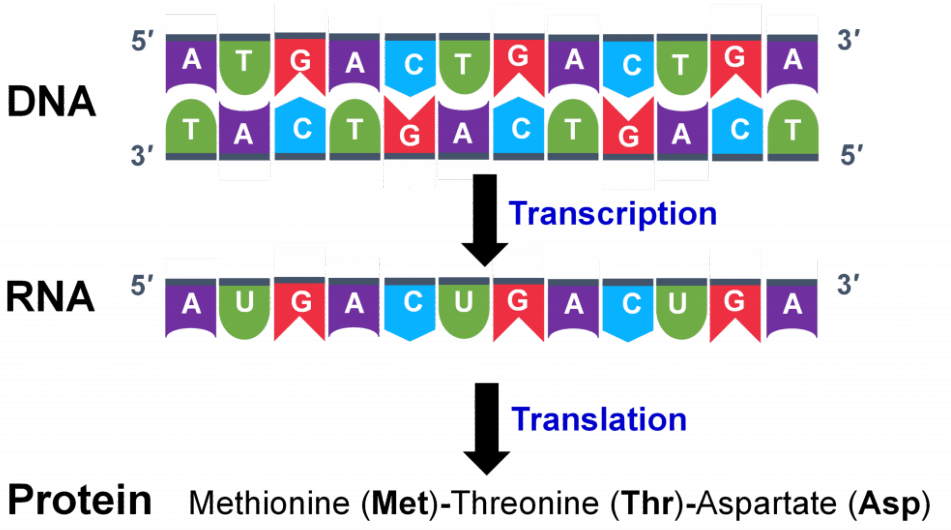


Figure 1. The central dogma of molecular genetics. Image by Marjorie Hanneman and Walter Suza.

Gene structure and transcription

The DNA sequence contains the information to control all biological functions, including the manifestation of traits important to agriculture (yield, drought tolerance, disease resistance, etc., etc.) How is the information contained in DNA sequences converted into the cellular activities necessary for plants and other organisms to function? DNA sequences are used to direct the synthesis of other molecules that actually perform these cellular functions.

Most typical genes encode proteins. The production of a protein from a gene involves several different processes (Figure 1). Transcription involves the copying of the DNA nucleotide sequence into an intermediate nucleotide molecule called RNA (ribonucleic acid). The primary RNA molecule is processed into a mature messenger RNA (mRNA) which then provides the information for the synthesis of a protein through the process of translation. Proteins are composed of amino acids connected by peptide bonds. The sequence of amino acids is determined by the sequence of nucleotide bases in the mRNA. The 20 amino acids have different chemical and physical properties and the sequence of amino acids determine the structure and function of the protein.

Gene structure

Only particular regions of chromosomal DNA are transcribed. A **gene** can be considered as the region of transcribed DNA, along with associated regions of DNA important for the regulation of transcription (Figure 1). A gene has several parts that are each important to the function of the gene.

The regulatory region (also known as the promoter) contains DNA sequence involved in the control of where and when the genes will be turned on to produce mRNA. The coding region is the part of the gene that is used as template to produce RNA molecules in a process called **transcription**. Some RNA molecules perform cellular functions directly while many others (messenger RNAs) are used to direct the synthesis of proteins in a process called **translation**.

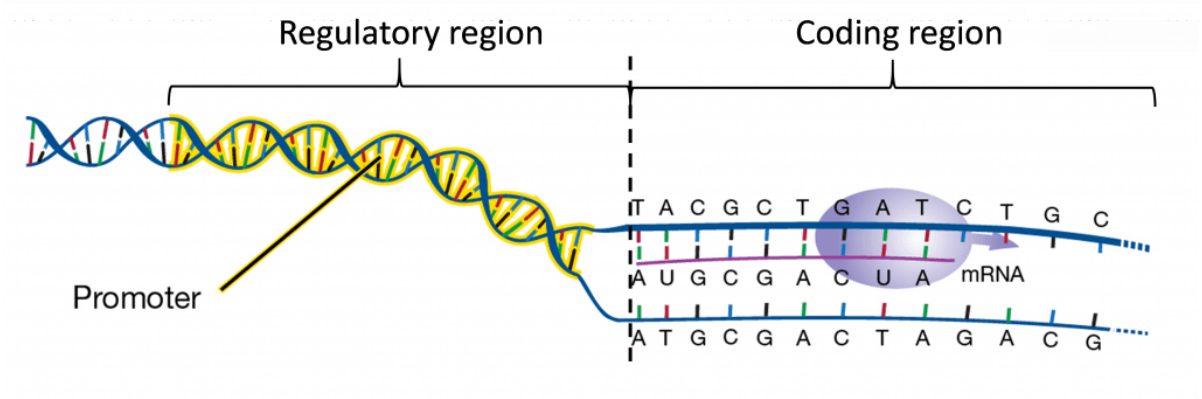


Figure 2. Every gene has a promoter and a coding region. Adapted from NIH-NHGRI.

a. Gene Promoter

The signals for starting and stopping transcription are located within DNA sequences. Specific nucleotide segments called promoters are recognized by RNA polymerase to start RNA synthesis. After the transcription of full-length RNA strand is completed, a second segment of DNA called terminator invokes termination of RNA synthesis and the detachment of RNA polymerases from the DNA template.

b. Protein-coding region

The protein-coding region of a gene is composed of the sequence of nucleotides that codes for **amino acids**. As described further in the section on **translation**, the coding region begins with an ATG **start codon** (AUG in RNA) and then ends with one of three **stop codons**. These sequences include only exons, but not all exonic sequences are protein coding as they may include **untranslated regions**.



Figure 3. The protein-coding region of a gene contains nucleotides that codes for amino acids. The 5′ and a 3′ UTR sequences do not code for amino acids but contain regulatory sequences that influence gene expression. In mRNA, translation start is AUG and translation stop can be UAA, UAG, and UGA. Image by Walter Suza.

c. Untranslated regions (UTRs)

Mature transcripts contain some sequences that do not code for amino acid sequences in proteins. These are referred to as untranslated regions or UTRs. Most mRNA transcripts contain a 5′ and a 3′ UTR. The 5′ UTR contains sequences toward the 5′ end of the mRNA sequence, before the **start codon**. These sequences can often be important for translational regulation,

and sometimes other functions. The sequences following the **stop codon** are the 3′ UTR. The 3′ UTR may also have important functions regulating transcript stability or directing transcript localization within cells, or sometimes even transport (trafficking) between cells.

UTRs and introns are often useful in genetic studies. Protein coding regions are under strong selective pressure to produce functional proteins and so sequence variation is relatively rare. UTRs and introns on the other hand are under less stringent selection and are therefore sources of sequence variants that can be used to develop genetic markers.

Transcription

The genetic information of DNA is transferred to an intermediate molecule called RNA that is often translated to amino acid sequences used to build proteins. RNA is a nucleic acid, like DNA, but with some important differences. RNA contains a ribose sugar group instead of the deoxyribose found in DNA. RNA molecules are single stranded, instead of being double stranded. RNA contains a uridine (U) base and does not contain a thymidine base. The other bases (A, C, G) are contained in both RNA and DNA. U has the property of base-pairing with A.

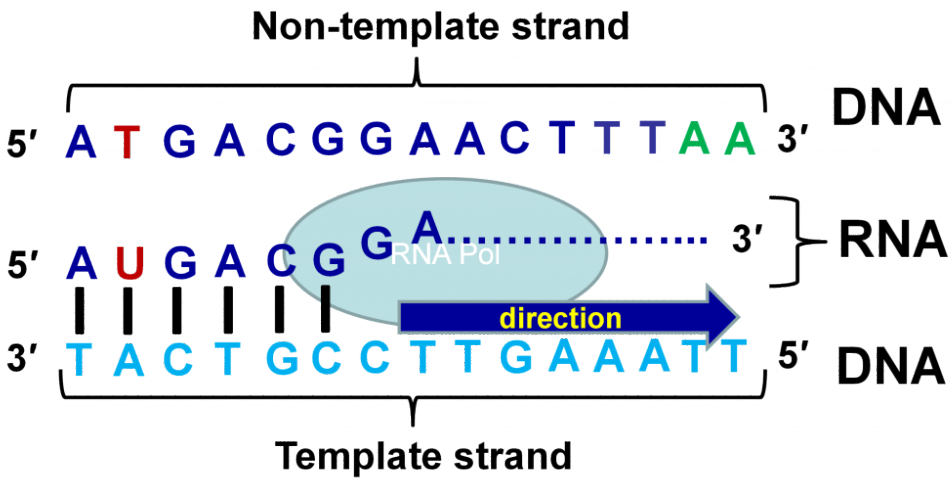


Figure 4. The enzyme RNA polymerase (RNA Pol) uses the template strand to synthesize RNA in the 5′ to 3′ prime direction. The base T is replaced with U in RNA. Image by Walter Suza.

RNA synthesis is directed by a DNA **template** in a process called **transcription**. A protein complex containing the enzyme **RNA polymerase** synthesizes an RNA molecule by adding nucleosides to the 3′ end of a growing chain. The principle of base pairing is used again and each nucleotide base added is complementary to the corresponding base on the DNA template. Thus, the RNA is complementary in sequence to the **template strand** of DNA, which is also referred to as “antisense” or “negative” strand (Figure 4). The RNA is identical in sequence (except U replaces T) to the other strand, which is called the “sense” or “positive” strand. Because RNA molecules are produced by the process of transcription, they are often referred to as **transcripts**.

RNA processing

Coding (transcribed) region

This is the region that is transcribed by RNA polymerase, also known as the RNA coding region. As described below, it may include **introns**, sequences that are removed from the mature RNA molecule during **RNA processing**. The transcribed region is demarcated by promoter and terminator sequences.

Introns and exons

As mentioned, and described in detail below, **introns** are sequences that are removed from transcripts during RNA processing. Sequences that are retained in mature transcripts are called **exons**. The corresponding stretches of DNA are typically referred to with the same terms. Introns are commonly found in genes of eukaryotes but are rare in prokaryotic organisms.

Intron splicing

The process of transcription produces pre-mRNA that contains both introns and exons. The process of splicing involves removal of introns from pre-mRNA and joining together the exons. A complex group of proteins that form a spliceosome perform the splicing reaction.

Introns sometimes serve as boundaries for sequences encoding functional protein domains, leading to possibility for new and variant proteins by exon shuffling. Also, introns can provide possibility for productions of variant RNA forms through alternate splicing allowing more than one gene product from a single gene. Some introns result from the insertion of transposable elements and may be spliced perfectly of imperfectly, offering more possibility for new genetic diversity.

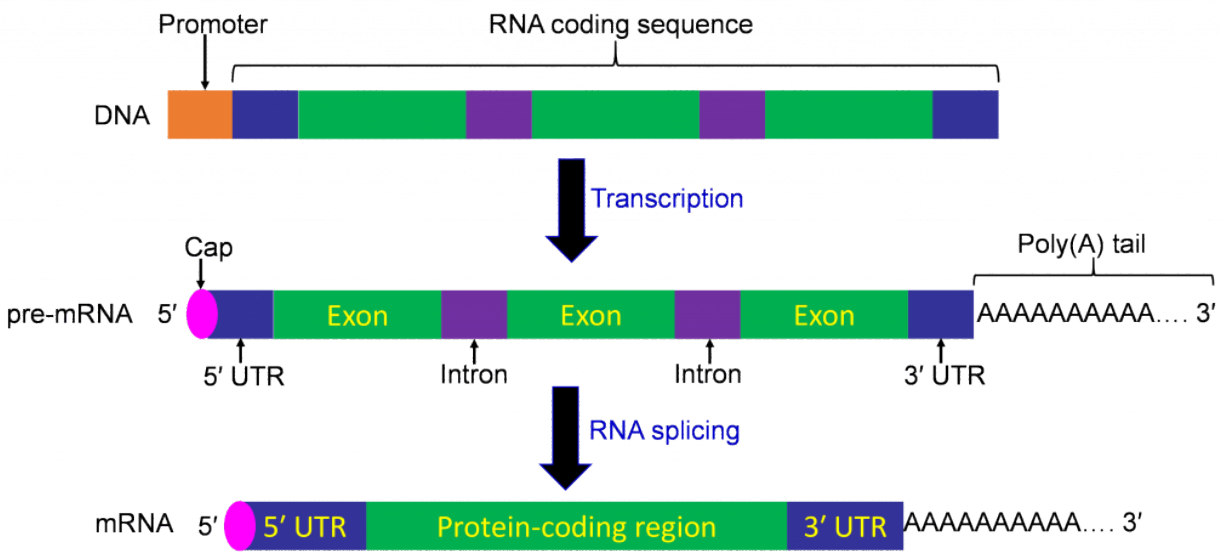


Figure 5. DNA (gene) transcription produces precursor-mRNA (pre-mRNA) that contains both introns and exons. The 5′ cap is 7-methyl guanidine. The enzyme poly(A) polymerase adds the poly(A) tail. The process of splicing involves removal of introns from pre-mRNA and joining together the exons to form mature mRNA. Image by Walter Suza

5′ Capping

The 5’ capping is the addition of a 7-methyl guanidine to the first nucleotide of mRNA molecule, usually and adenine or guanidine. The phosphodiester linkage between 7-methyl guanidine and the target nucleotide is 5′-5′ instead of 5′-3′, and 3 phosphates rather than 1 are retained in the linkage. The cap stabilizes the 5′ end of the mRNA and plays a role in translation initiation.

Poly adenylation

The transcription of a gene may proceed beyond what ends up as 3′ end of mature mRNA. Thus the 3′ end of mRNA is formed after transcription. The enzyme poly(A) polymerase adds numerous adenosines to the 3′ end to result in what is called the poly(A) tail. The poly(A) tail is necessary for proper processing and transport of mRNA to the cytoplasm. The poly(A) tail is also important for the stability of mRNA, and initiation of translation in eukaryotic organisms.

Summary

The genetic information of DNA is transferred through transcription to an intermediate molecule called RNA. The signals for starting and stopping transcription are located within the DNA sequence and referred to as promoter and terminator sequences. The coding region of a gene is composed of a sequence of nucleotides that are transcribed into RNA. These sequences include exons and introns. Exons are the sequences that code for proteins. The coding region of a gene contains exons and introns. Also, pre-mRNA contains both introns and exons. The introns in pre-mRNA are removed through a process called intron splicing. The mRNA is processed by 5′ capping and addition of a poly(A) tail.

Learning Activities

Learning Activity 1

Given the following sequence of double-stranded DNA, predict the sequence of the RNA strand.

Learning Activity 2

Watch This Video: *Transcription Detail*

A video element has been excluded from this version of the text. You can watch it online here: https://iastate.pressbooks.pub/genagbiotech/?p=24

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2.4.6: Gene Expression- Translation

Learning Objectives

- Determine the amino acid sequence of a protein given the **nucleotide** sequence of a gene.
- Recognize the biological connection among mRNA, tRNA, amino acids, and proteins.
- Understand the concept of protein structure and its relation to protein function.

Introduction

The importance of gene expression is evident when you observe the changes plants go through during their lifecycle or during a season. Trees and bushes, for example have dormant buds through the winter. Environmental signals affiliated with the coming of spring induce genes in the buds to turn on and drive the dramatic changes of leaf development and flowering. The genes were always present in those bud cells but were controlled to turn on at the proper time. Understanding gene expression thus requires an examination of two processes, the activation of **gene expression** to make a "message" and the reading of this message to build a specific protein.

Amino acids are used to make proteins

Amino acids are the building blocks of proteins. Except for the amino acid proline, they all consist of a central carbon atom covalently bonded to an amino group, a hydrogen atom, a carboxyl group, and a variable R group (Figure 1). In the physiological range, both the carboxylic acid and amino groups are completely ionized allowing amino acids to act as either an acid or a base. Thus, amino acids do not assume a neutral form in an aqueous environment.

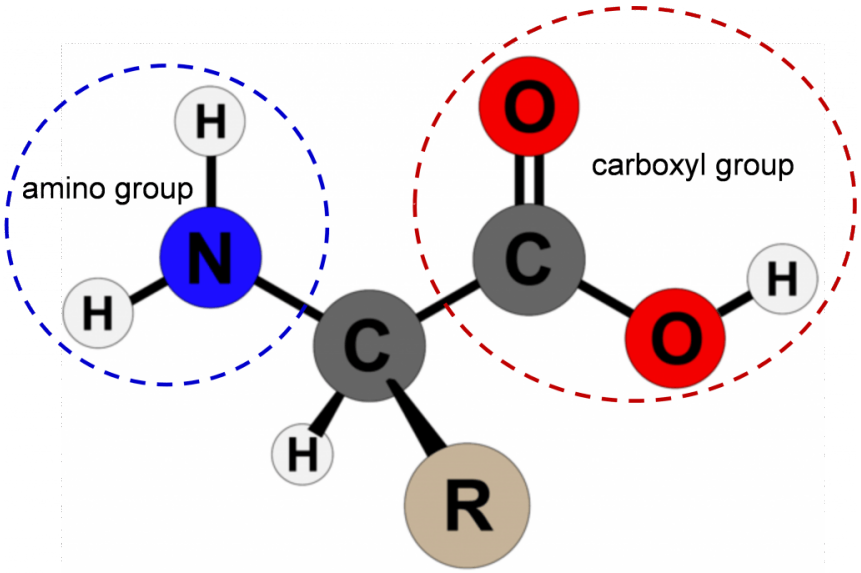


Figure 1. The structure of an amino acid. The amino (-NH2) and carboxyl (-COOH) are the same for all amino acids but the side chain (R group) is specific for each amino acid. Image Source: Techguy78, CC BY-SA 4.0, via Wikimedia Commons.

The chemical and physical properties of the side chains provide the functionally important properties to amino acids. Some of the important properties of side chains include acidic, basic, or neutral, hydrophobic vs. hydrophilic, as well as the size of side chains. They are categorized according to their side groups as nonpolar, polar, positively, or negatively charged or aromatic. All these properties and others affect how each amino acid contributes to the structure and function of a mature protein.

There are 20 different amino acids making up the subunits of **proteins** (Table 1). All proteins are made from differing combinations of the amino acids (Figure 2). Therefore, if you are going to make proteins you need to either make amino acids

first (Figure 3) or consume amino acids in your diet. Animals consume some of their amino acids (the essential amino acids), but plants and bacteria are able make all their own amino acids.

Table 1. Amino acids and their abbreviations[1]

Full Name	Abbreviation (3 Letter)	Abbreviation (1 Letter)
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

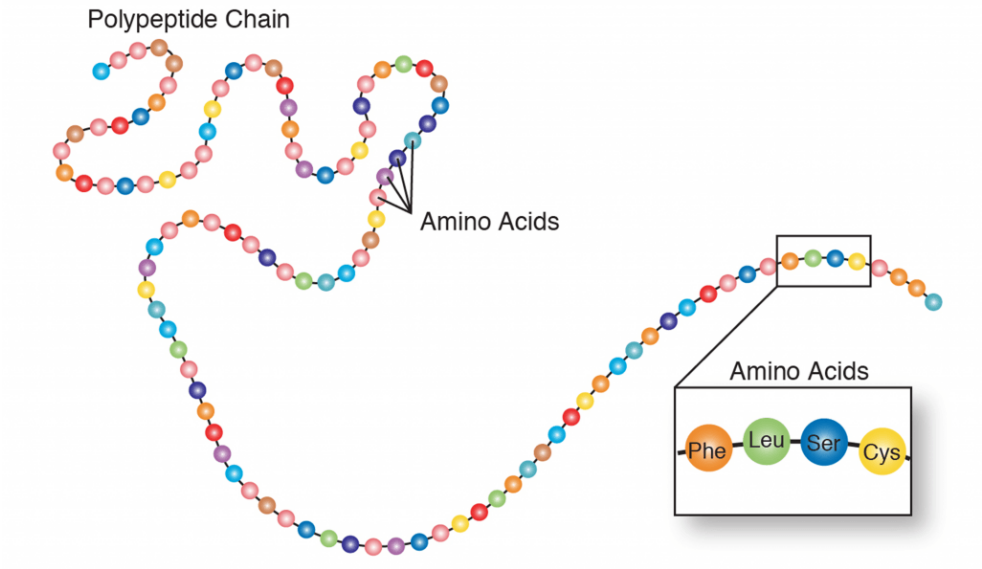


Figure 2. Proteins are polypeptides of different amino acids. Adapted from NIH-NHGRI.

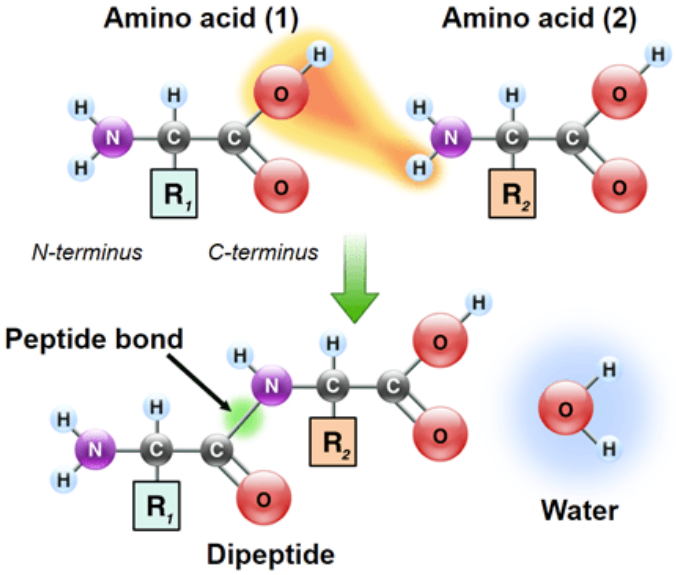


Figure 3. Proteins or polypeptides are made by connecting amino acids together with peptide bonds. The formation of peptide bond is a dehydration reaction that releases water with each new bond that is formed.

Codons and the Genetic Code

The search for the genetic code (Table 2) revealed that genetic information is stored in nucleotide triplets referred to as **codons**. The genetic code is degenerate because many amino acids are specified by more than one codon. Sixty one of the 64 possible combinations of the three bases in a codon are used to code for specific amino acids. Three **stop codons**, UAA, UAG, and UGA do not code for any amino acids but specify the termination of peptide chain synthesis during translation. The AUG **start codon** is used to initiate polypeptide synthesis and codes for the amino acid methionine.

Table 2. The genetic code. The 20 common amino acids are listed in their three and one-letter formats Stop codons are UAA, AUG and UGA.

First Position	Second Position	Third Position

	U	C	A	G	
U	Phe (F)	Ser (S)	Tyr (Y)	Cys (C)	U
					C
	Leu (L)		Stop	Stop	A
			Stop	Trp (W)	G
C	Leu (L)	Pro (P)	His (H)	Arg (R)	U
					C
			Gln (Q)		A
					G
A	Ile (I)	Thr (T)	Asn (N)	Ser (S)	U
					C
	Met (M)		Lys (K)	Arg (R)	A
					G
G	Val (V)	Ala (A)	Asp (D)	Gly (G)	U
					C
			Glu (E)		A
					G

Why a Triplet Code?

Prior to understanding the details of **transcription** and **translation**, geneticists predicted that DNA could encode **amino acids** only if a code of at least three nucleotides was used. The logic is that the **nucleotide** code must be able to specify the placement of 20 amino acids. Since there are only four nucleotides, a code of single nucleotides would only represent four amino acids, such that A, C, G and U could be translated to encode amino acids. A doublet code could code for 16 amino acids (4 x 4). A triplet code could make a genetic code for 64 different combinations (4 X 4 X 4) genetic code and provide plenty of information in the DNA molecule to specify the placement of all 20 amino acids. When experiments were performed to crack the genetic code, it was found to be a code that was triplet. These three letter codes of nucleotides (AUG, AAA, etc.) are called codons.

The genetic code only needed to be cracked once because it is universal (with some rare exceptions). That means all organisms use the same codons to specify the placement of each of the 20 amino acids in **protein** formation. A codon table can therefore be constructed and any **coding region** of nucleotides read to determine the amino acid sequence of the protein encoded. A look at the genetic code in the codon table below reveals that the code is redundant meaning many of the amino acids can be coded by four or six possible codons. The amino acid sequence of **proteins** from all types of organisms is usually determined by sequencing the gene that encodes the protein and then reading the genetic code from the DNA sequence

Ribosomes, tRNA and Anti-codons

The ribosomes are a large molecular complexes containing an assembly of several ribosomal RNAs (rRNA) and many proteins. Ribosomes are the machinery of protein synthesis, facilitating the ordered addition of amino acids to a nascent polypeptide chain under the guidance of an mRNA template (Figure 5A). Prior to the initiation of protein synthesis the ribosome occurs in two separate subunits of size 60s and 40s ("s" stands for Svedberg units and is a measure of a particles sedimentation rate during centrifugation).

The meaning of a codon for a specific amino acid is determined by the tRNA (transfer RNA). Each tRNA (Figure 5B) contains a triplet of nucleotides referred to as an anticodon, which is complementary to a specific codon. For each of the 20 amino acids, a specific enzyme (aminoacyl-tRNA synthetase) catalyzes its linkage to the 3′ end of its specific tRNA adapter. The function of aminoacyl-tRNA synthetase is critical as it provides a check for accuracy in protein synthesis by adding a specific amino acid to a specific tRNA molecule. In this way, one particular amino acid is targeted to each codon triplet of mRNAs.

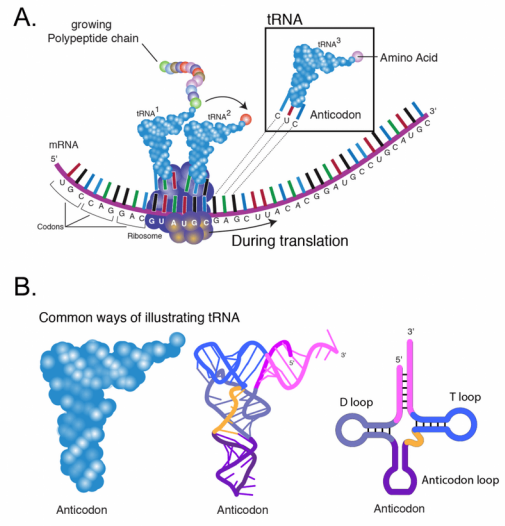


Figure 5: The key molecules needed for translation are the mRNA, ribosome, tRNA, and amino acids. Illustration by NIH-NHGRI.

Peptide chain formation

Protein synthesis is initiated by the 40s subunit through attachment it's to the mRNA, and recognition of the AUG codon. This is followed by the attachment of the 60s subunit to the 40s-mRNA complex to provide the structure necessary to align each successive aminoacyl-charged tRNA for transfer of its amino acid to the growing polypeptide. Successive amino acids are

attached to the growing polypeptide by the formation of **peptide bonds** that form between the carboxyl group of one amino acid and the amino group of the next.

Like nucleic acids, polypeptides also have molecular directionality. One end of a polypeptide chain will have a free amino group and the opposite end will have a free carboxy group. As such, these ends are referred to as the amino-terminus or carboxy-terminus, respectively. During translation, mRNA templates are read from the 5' end toward the 3' end. Proteins are synthesized beginning at the amino terminal end going toward the carboxy terminus.

Protein structure and function

Proteins assume complex 3-dimensional structures that are essential for their various functions as enzymes, regulatory factors, or structural proteins. Overall structure is determined by complex physico-chemical interactions among amino acid side groups.

Several levels of structure are considered (Figure 6). The primary structure of a protein is the amino acid sequence of its polypeptide chains. The secondary structure is derived through the interactions between neighboring amino acids to form local structural elements such as beta sheets or alpha helices. The tertiary structure refers to the three-dimensional structure of an entire polypeptide and is determined by the diverse properties of amino acid side groups. For example, in an aqueous environment, hydrophobic amino acids will often interact at the core of a protein structure while hydrophilic groups may be exposed on the protein surface.

Many proteins are multimeric, composed of several polypeptide chains called "subunits", which associate non-covalently or in some cases via disulphide bonds. Such higher order spatial associations among polypeptides are also the result of interactions among amino acids and result in a quaternary structure. Some multimeric proteins consist of multiple copies of the same polypeptide.

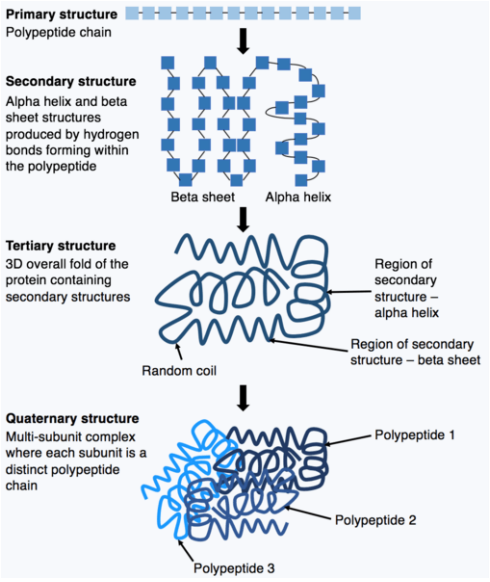


Figure 6. Levels of protein structure. Image Source: Kep17, CC BY-SA 4.0, via Wikimedia Commons.

Summary

The genetic information of DNA is transferred to through transcription to an intermediate molecule called RNA. The signals for starting and stopping transcription are located within the DNA sequence, and referred to as promoter and terminator sequences. The coding region of a gene is composed of a sequence of nucleotides that are transcribed into RNA. These sequences include exons and introns. Exons are the sequences that code for proteins. The coding region of a gene contains exons and introns. Also, pre-mRNA contains both introns and exons. The introns in pre-mRNA are removed through a process called intron splicing. The mRNA is processed by 5' capping and addition of a poly(A) tail. Mature RNA is then translated to amino acids used to build proteins.

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1. Table Source: Donald Lee, University of Nebraska-Lincoln ↩

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2.4.7: Gene Expression- Applied Example (Part 1)

Learning Objectives

At the completion of this lesson you should be able to:

1. Define the roles of DNA and proteins in cell development and metabolism
2. Determine the amino acid sequence of a protein given the **nucleotide** sequence of a gene.
3. Describe the roles that the **promoter**, **coding region** and **termination sequence** of a gene play in gene expression.
4. Recognize the differences between the structure of proteins, **amino acids**, **genes**, and **nucleotides**.
5. Draw the process of gene expression and include the following in your drawing. Gene, **RNA polymerase**, promoter, coding region, termination sequence, cell, **nucleus**, cytoplasm, **mRNA**, tRNA, ribosome, anticodon, codon, amino acid, protein, peptide bond.

You have learned this fundamental biology fact: genes provide the information to instruct cells how to build specific proteins and these proteins control cell development and metabolism. Some biologists refer to this fact as the “Central Dogma”. We prefer to refer to this process as gene expression. This lesson will integrate this biology fundamental with some gene expression examples. These examples emphasize an additional fundamental biology idea that life is a continuous cycle. In order for living cells to live, they require the genes, RNAs, and proteins that came from prior existing living cells.

Example #1: Gene Expression of the Plant Acetolactate Synthase Enzyme Gene

We have selected this gene because the protein the gene encodes is the target site for several types of herbicides and thus is an important example in agriculture. The gene also illustrates an important circle of life concept. All living things need amino acids to build proteins and some living things need proteins to build amino acids.

The ALS **gene** is not found in animals but is found in plants and bacteria. Plants and bacteria are able make all their own amino acids. Animals must consume a subset of the 20 amino acids (see Lesson on Translation) in their diet. One gene all plants and bacteria must have to live encodes the enzyme acetolactate synthase (ALS). ALS enzyme allows plants to make their own supply of three of the 20 amino acids; leucine, isoleucine, and valine. The ALS protein is an **enzyme** and is needed to catalyze a reaction in the chemical pathway that makes these three **amino acids**. Let us detail the information transfer steps needed for gene expression: use the genetic code of DNA sequences in the gene to encode the amino acid sequence of the ALS enzyme.

Three parts of the Gene and a Two-Step Process

The ALS gene consists of several thousand nucleotides of DNA and like all genes has three main parts, the **promoter**, **coding region**, and termination sequences (Figure 1, gene sequence abbreviated for demonstration).

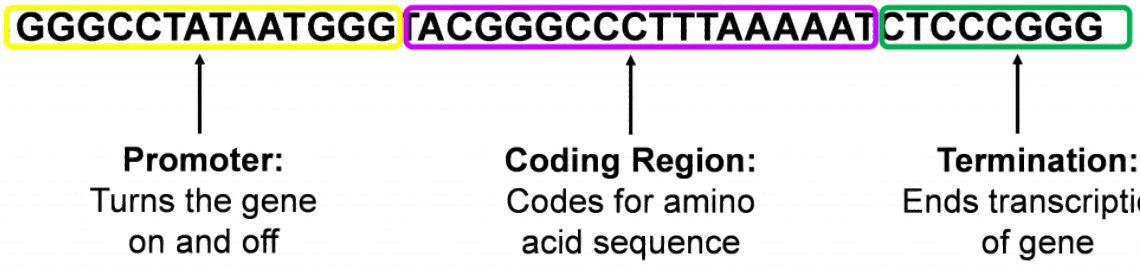


Figure 1. The parts of the ALS gene; the promoter and termination sequences control transcription while the coding region encodes the protein. Image by Marjorie Hanneman.

Each part of the ALS gene plays a role in controlling gene expression and this is a two-part process. The promoter is the on/

off switch of the gene, the coding region determines the amino acids sequence of the **protein** that will get made and the termination sequence signals where the gene information ends. The DNA sequence that makes up a gene is a part of a much larger chromosome of continuous DNA. The gene sequence is organized to make an RNA (ribonucleic acid) copy of the gene's nucleotide sequence to serve as a mobile message for protein building. RNA building is called transcription because DNA nucleotides encode RNA nucleotides.

Step 1: Transcription: control from the promoter and termination sequence

The **promoter's** role is to selectively turn on the ALS gene in cells that will need the ALS enzyme. The promoter accomplishes this by its specific nucleotide sequences that react with regulatory molecules in the cell. We will discuss the regulation of gene expression in more detail later. What cells in a plant will need the ALS **enzyme**? All cells that are making proteins. This will be all cells in the plant at some stage of development. Therefore, the ALS gene has a promoter sequence that allows the gene to be turned on in all types of cells. When the ALS **gene** is turned on, transcription can take place. Like most chemistry in the cell, enzymes will be needed for **gene expression**. **RNA polymerase** is the *transcription* enzyme (Figure 2) which will bind to the DNA sequences in the ALS promoter and interact with one strand of the gene (the coding strand).

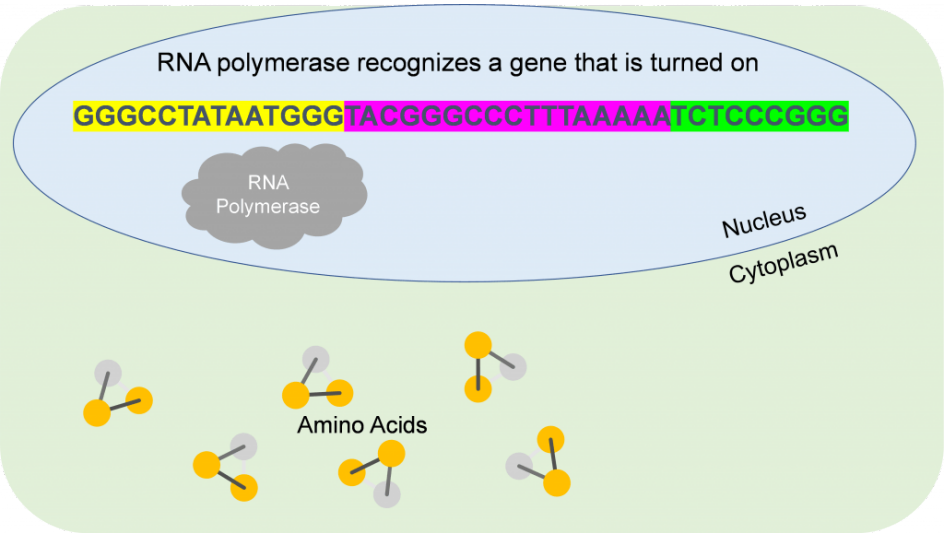


Figure 2. RNA Polymerase binds to the promoter of the ALS gene to initiate transcription. Image by Marjorie Hanneman.

RNA polymerase works by moving along the gene promoter until it encounters a series of A and T nucleotides called the TATA box (Figure 3). The TATA box signals RNA polymerase that it has reached the end of the promoter. Now the enzyme has the "green light" to read the coding strand **DNA** and make RNA. The procedure works much like DNA replication. RNA polymerase will read the DNA in a 3' to 5' direction and build the RNA 5' to 3'. Unlike DNA replication though, only one DNA strand is read and the transcription process must end once the RNA polymerase reaches the end of the ALS gene. If RNA polymerase kept going along that strand of the DNA making up the chromosome, other genes that are on the same **chromosome** might be expressed in the wrong cells or at the wrong times.

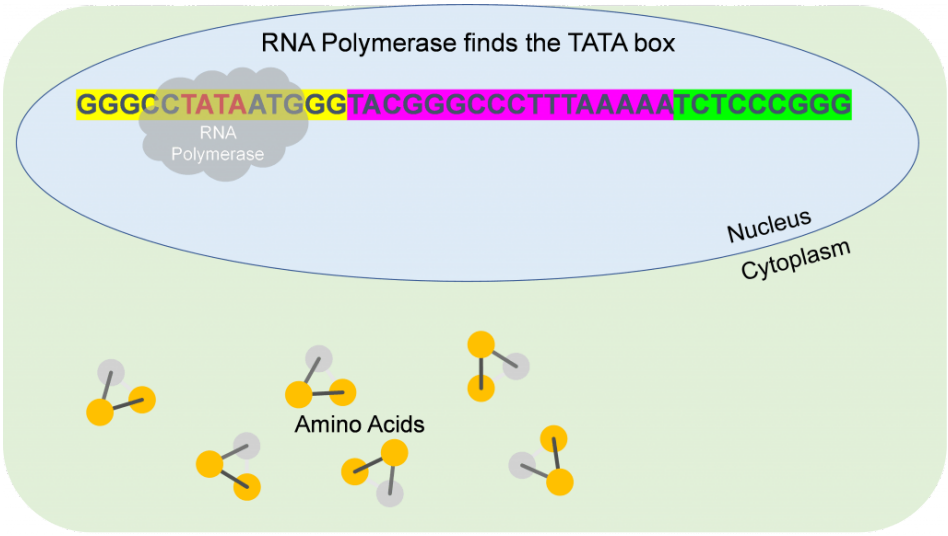


Figure 3. RNA polymerase encounters the TATA box at the end of the promoter. This signals where to begin the reading of the coding region. Image by Marjorie Hanneman.

How is the RNA polymerase signaled that it has reached the end of the gene? That is the role of a **termination sequence** in the gene (Figure 4). The termination sequences signal the end of the gene. One transcription termination strategy will be described here. The **nucleotides** that make up the termination sequence of the ALS gene could be ordered to form a palindrome. This means that the nucleotides being placed in the RNA can fold back on themselves and form a hairpin loop (Figure 5).

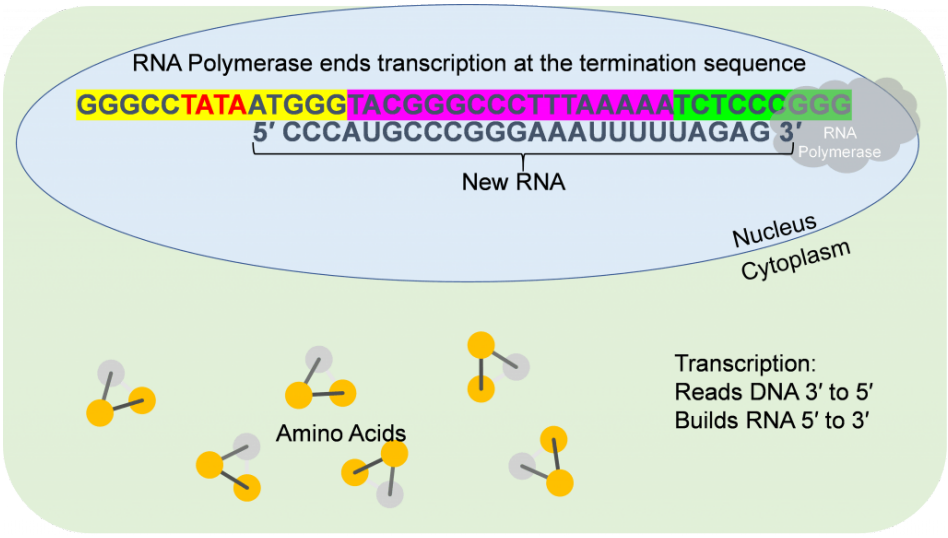


Figure 4. When RNA polymerase reads the termination sequence it is signaled to quit reading the gene's coding strand. Image by Marjorie Hanneman.

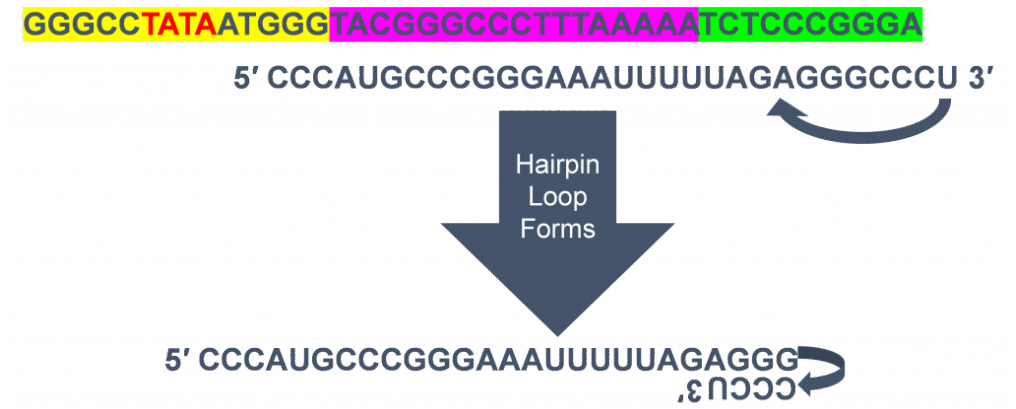


Figure 5. The termination sequence is palindromic. The RNA being made has a sequence that can form a hairpin loop. The formation of the hairpin in the newly made RNA disrupts the RNA polymerase, halting transcription. Image by Marjorie Hanneman.

While no geneticist has actually viewed this hairpin loop formation in action, it is believed that the hairpin formation snaps the RNA polymerase off the DNA coding strand and releases the RNA message. Transcription of an RNA that has the **coding region** information is now completed. Each time an RNA polymerase goes through the process, one copy of the RNA is made by reading the DNA template. The type of RNA made from transcription of the ALS gene is called a messenger RNA or mRNA for short. Some **genes** can encode transfer RNA (tRNA) or ribosomal RNA (rRNA). These RNA molecules have special roles in the next part of gene expression called translation. Once an **mRNA** is transcribed, some modifications will occur in the nucleus. We will describe some of the post-transcriptional processes later. For now, let's follow the ALS mRNA onto the **translation** step of gene expression.

Video Examples

Watch this video from UNL for more information about the Transcription process

Step 2: Translation: reading the code to make proteins

The **translation** process requires a conversion of information from a chain of **nucleotides** in RNA to a chain of **amino acids** in **protein**. The translation term was coined based on the idea that we are converting to a new molecular language at this stage of **gene** expression. The conversion of information in translation is a little more complex than **transcription** and requires several biomolecules to interact and work together (Figure 7). We will describe the ribosomes and tRNAs molecules first and then describe how they work together to translate a **mRNA**.

- **tRNA:** The 't' prefix means transfer. The tRNA is a small RNA (70 to 80 nucleotides) and has a sequence that allows hairpins to form which gives a secondary structure to the molecule. Their small size makes them mobile to provide the cells means to transfer amino acids to the site of protein building. Their structure also makes them specific in how they deliver their cargo of a single, specific amino acid. A tRNA is about 50 times larger than an amino acid.

- **Ribosomes:** Ribosomes are macromolecules that are made of both RNAs (rRNAs) and proteins. These components assemble into a workbench for the translation process to occur. The ribosome is a big macromolecule: it can hold three tRNAs in position for the translation work and can catalyze bond breaking and bond formation.

We will use a simple representation of the mRNA, tRNA, ribosome, and amino acid to show how they work together (Figure 6).

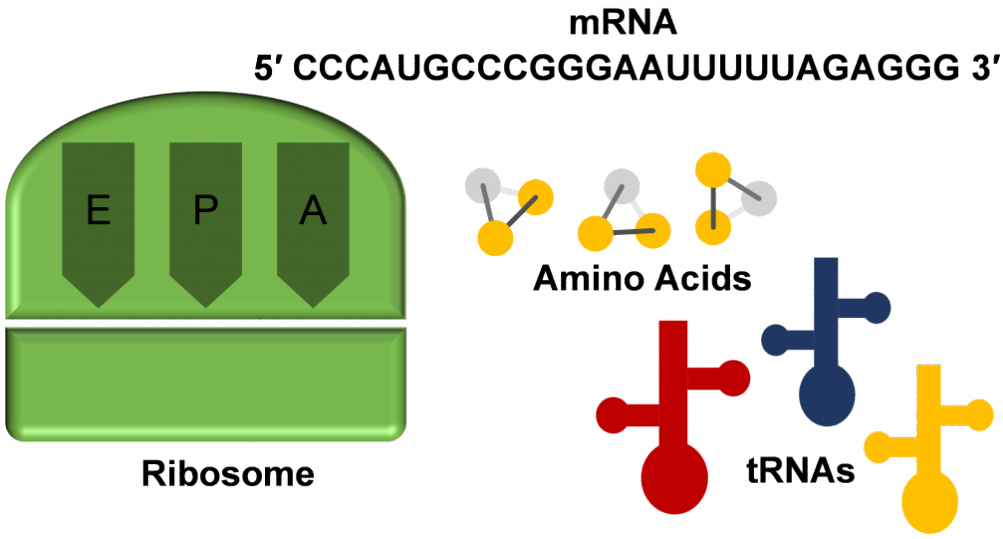


Figure 6. The key molecules needed for translation are the mRNA, ribosome, tRNA, and amino acids. Image by Marjorie Hanneman.

The tRNAs shown in Figure 6 are not ready for translation. The structure of the tRNA is recognized by special enzymes in the cell that attach the proper amino acid to the tRNAs. The tRNAs that are bound to their designated amino acid are called charged tRNAs (Figure 7). The near the middle of the 70-80 nucleotides in a tRNA are three nucleotides called the anticodon. When the tRNA folds, the anticodon becomes one end of the molecule and the other end will be attached to a specific amino acid. In the figures for this lesson, the tRNA is shown with its secondary structure and only the anticodon sequence is shown (Figure 7).

Let's describe how the key players get protein building started, how they keep it going, and how they end it. Active ribosomes are assembled from small and large subunits when the ribosome "workbench" is ready to translate a mRNA (Figure 7). When mRNAs are present in the cytoplasm, the small and large ribosome subunits assemble at the 5' end of these messages and are ready to read mRNAs like blueprints to build specific proteins.

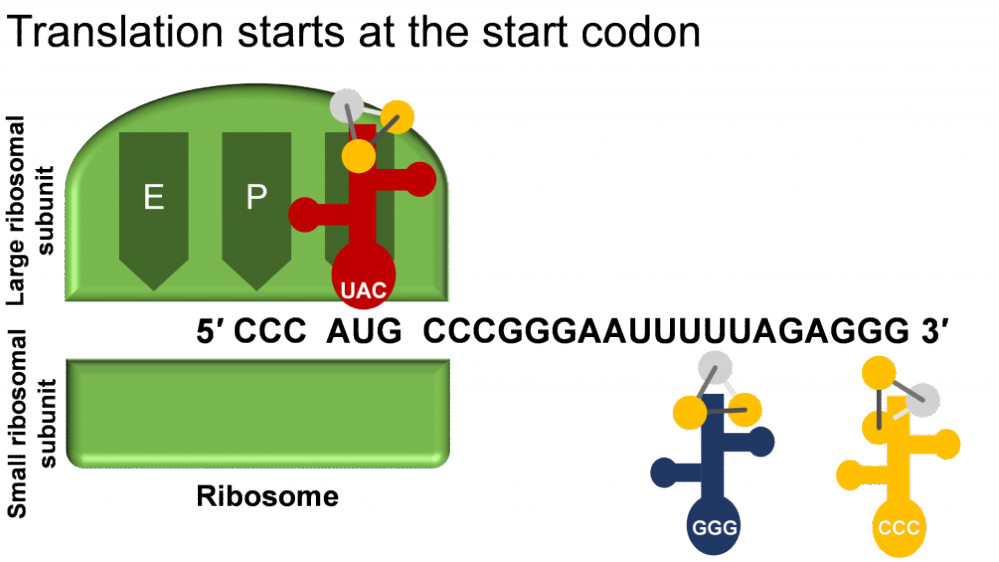


Figure 7. The ribosome translates the mRNA 5' to 3' and starts by finding an AUG sequence. The ribosome allows a tRNA with the UAC anticodon to bind and bring in an amino acid. The tRNA is bound to the A site of the ribosome. Image by Marjorie Hanneman.

The ribosome reads the mRNA quickly and accurately, but it must determine where to start. As the ribosome moves in the 5' to 3' direction along the mRNA it "looks" for the sequence AUG (Figure 7). This sequence is called the start codon and signals the ribosome that this is where to begin building the protein sequence.

Video Examples

Watch this UNL video on Translation for more information about these processes.

The Reading Frame, Codons and Anticodons

The mRNAs, tRNAs, ribosomes, and **amino acids** are now in position to combine their specific functions into the building of a protein. The AUG start codon establishes the beginning of what is called a reading frame on a mRNA. The nucleotides in the mRNA must have the specificity to code for 20 different amino acids to build the protein. The genetic code is read as a three-letter code which provides this specificity. The ribosome will move along and read the sequence in the mRNA one codon (three nucleotides) at a time to follow the reading frame and build the correct protein (Figure 8).

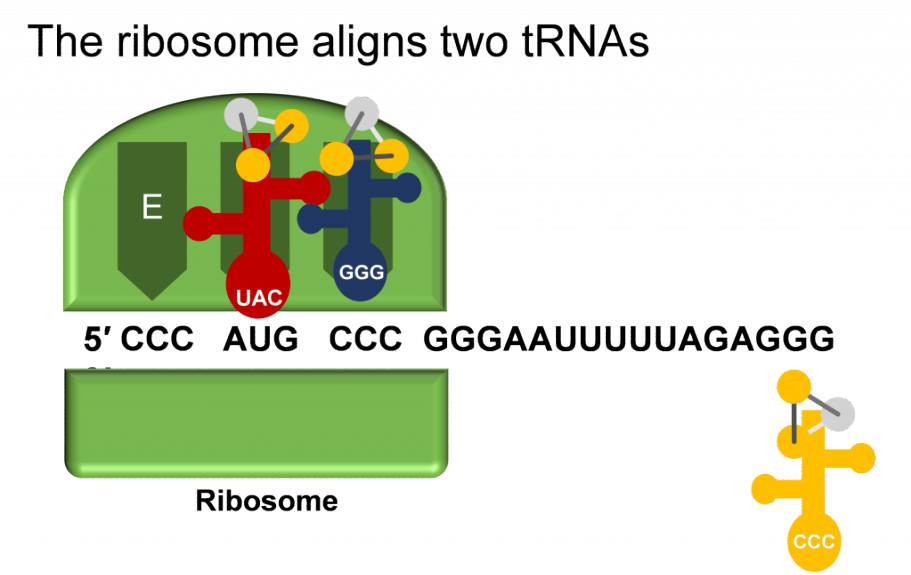


Figure 8. The ribosome allows two tRNAs with complementing anticodons to enter the 'A' and 'P' sites and bring in their amino acids. Image by Marjorie Hanneman.

Getting Translation Started with the Start Codon

The AUG start codon signals the ribosome to place in the amino acid methionine. The A, P and E sites on the ribosome site works somewhat like a vice on the workbench, holding stuff in place so it can be worked on. In Figure 9, the ribosome has moved in the 5' to 3' direction on the mRNA .mRNA. This allowed a tRNA with the anticodon GGG to move into the A site and placed two amino acids very close to each other on the ribosome. The ribosome will now move one more codon in the 5' to 3' direction which moves the two tRNAs now to the E and P sites (Figure 9). Now the ribosome is ready to start binding together amino acids with peptide bonds to start to form a protein. A third tRNA (with the CCC anticodon) can now arrive at the open A site, bringing the amino acid designated by the GGG codon.

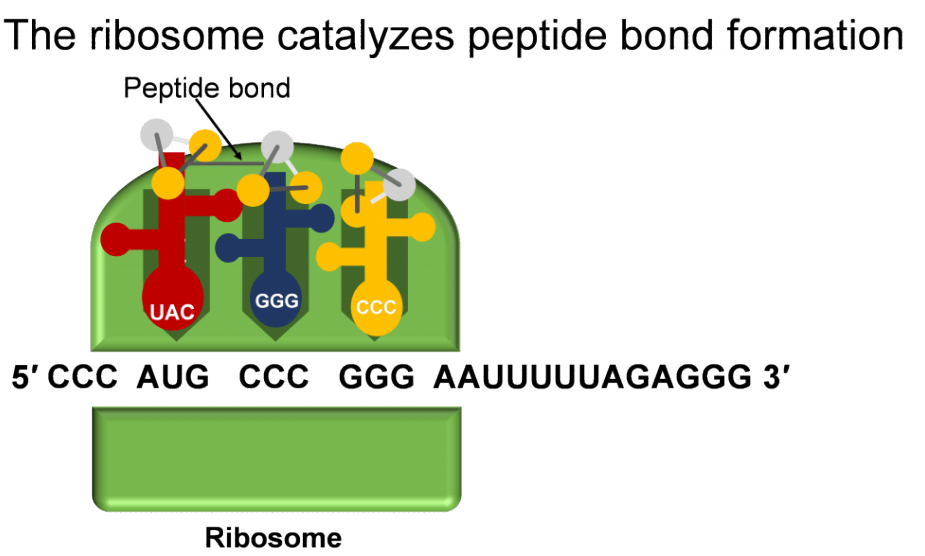


Figure 9. The ribosome can link two amino acids together with peptide bonds because the tRNAs holding them are next to each other at the 'A' and 'P' sites of the ribosome. Image by Marjorie Hanneman.

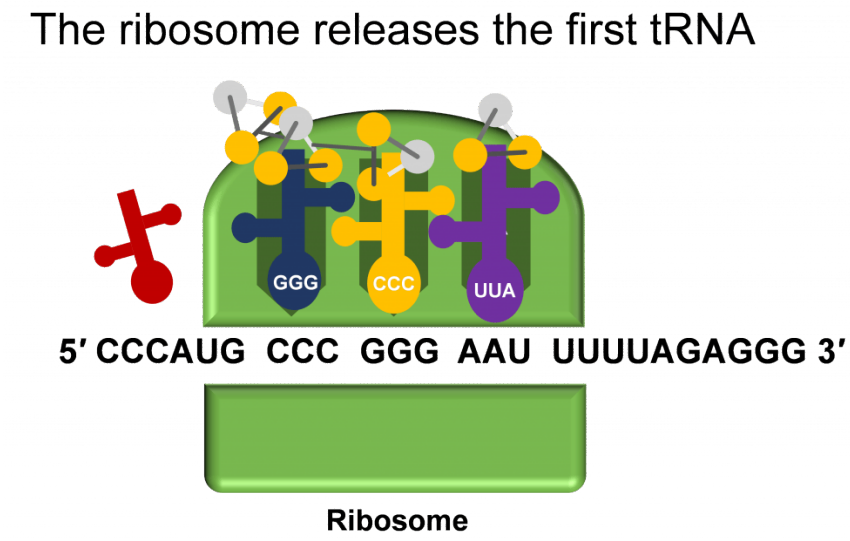


Figure 10: The ribosome moves over to the 'GGG' codon, releases the tRNA with the 'UAC' anticodon and is ready for the next tRNA. Image by Marjorie Hanneman.

Peptide Bond Formation and Protein Building

As you can see, a ribosome "workbench" has catalytic tools to go along with its 'P' and 'A' "vice sites". The ribosome has enzymatic functions that allow it to break and form bonds. The ribosome will break the bond that binds the amino acid to the tRNA at the 'E' site. Simultaneously the ribosome forms a peptide bond between the second and the third **amino acids** that were brought in by the tRNAs. The close proximity of these amino acids at the ribosome sites provides the opportunity for peptide bond formation and then release of the tRNA in a smooth continuous process (Figure 10 and 11). Peptide bonds always bind the acid end of one amino acid with the amino end of the next amino acid. Peptide bonds are strong covalent bonds which keep the amino acids connected during and after **translation**. The tRNA at the 'E' site has now accomplished its task by bringing in the first amino acid. This tRNA will then move off the ribosome (Figure 11). The tRNA released from a ribosome is recycled by the cell. It can be charged again by binding another amino acid in the cytoplasm and contribute to the synthesis of another protein. The protein. The first three amino acids in the **protein** have now been put together.

The synthesis of our protein is far from over. The ribosome continues to shift one codon in the 5' to 3' direction as a tRNA leaves the E site .site. A tRNA can now come into the A site if it has the anticodon that is complementary to the next codon on the **mRNA**.

Proteins are hundreds of **amino acids** long. In our example, the steps described continue until the complete mRNA information is read to build the 667 amino acid long ALS enzyme.

The End of Translation: stop codons looking for something they cannot find

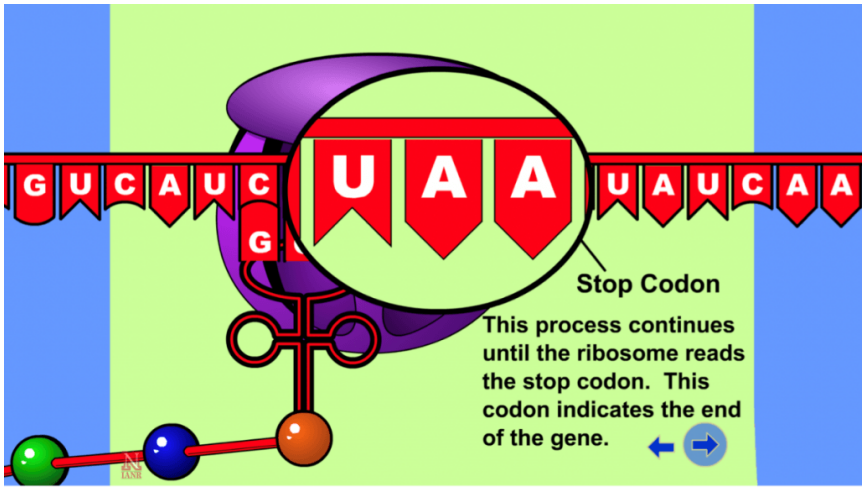


Figure 11: Ribosome encounters one of the three sequences in the genetic code that function as stop codons. Image by Donald Lee.

Start codons start **translation** so it is logical that stop codons stop translation (Figure 11) How do stop codons do this? There are 61 tRNAs with different anticodons (see Appendix 1: Codon Table). That means there are three codons that do not have corresponding tRNAs with complementary anticodons. These three codons serve as stop codons. When a ribosome encounters a stop codon on a **mRNA** it will wait for a tRNA with the right anticodon to come over. It will not skip the codon or shift over one **nucleotide** to form a new reading frame. The ribosome waits for the right tRNA, but it does not wait for long. A stalled ribosome will quickly cleave off the bound tRNA with the newly built **protein** chain and then move on to translate another mRNA. No tRNAs in the cell have anticodons that complement any of the three possible stop codons. Therefore, stop codons are able to end the translation process when the completed protein is made.

Sequence, structure, and function

In the case of our ALS protein, the assembly of all 667 amino acids into the polypeptide or protein chain in the correct order is important because the sequence of the amino acids determines how the protein folds. The folded structure of the protein determines its function. If some amino acids are changed and the structure changes, the ALS will not function properly as an enzyme in plant or microbe cells.

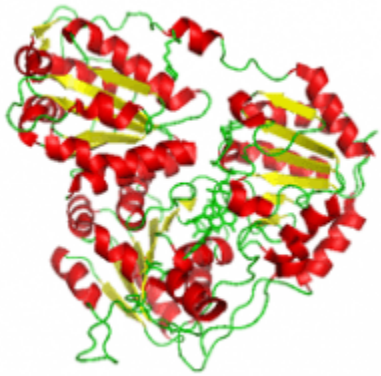


Figure 12. ALS enzyme structure. The red and green stretches depict different kinds of protein structure features that provide the enzyme its ability to function as a specific enzyme. Image Source: Rj Joplin, CC BY-SA 3.0, via Wikimedia Commons.

Summary

Organisms use the process of **transcription** to make **mRNA** as a temporary molecule to transport **protein** coding information from a **gene**. The **translation** process reads the mRNA and makes the intended protein, one amino acid at a time. This process is controlled by the **promoter** of the gene to ensure that **proteins** are made in the right cells at the right time to drive growth, development, metabolism, stress response and other processes needed to complete the organism's life cycle.

Video Examples

Watch this video from UNL for an overview of Transcription and Translation.

Circle of Life and Gene Expression

Let us revisit the idea that gene expression works because living cells give rise to more living cells. Imagine yourself when you were just a single cell zygote with a copy of all the genes in the human genome from your biological mother and a copy from your biological father. What else did you need to get from your parents before you could express your first gene and build your first protein? You needed to already have an RNA polymerase in the nucleus of the cell to transcribe your first gene and you needed to have ribosomes, tRNAs and amino acids ready for translating that mRNA in the cytoplasm. Luckily, the egg cell from your mother had the 23 human chromosomes plus all of these gene expression biomolecules. Later, you could turn on your own genes for RNA polymerase, tRNAs and ribosomes but your mother's egg cell was a living environment that allowed you to start your life.

A plant treated with a herbicide which binds to and blocks the action of the ALS enzyme likewise demonstrates that gene expression has a cycle of dependency on living cells. Small plants in an environment for rapid growth will have their growth halted by the ALS binding herbicides. The plants do not die from the herbicide, they just stop growing. The reason for this is that plants need to build new proteins to grow and to build new proteins they need the ALS enzyme to be working and building new leucine, isoleucine, and valine amino acids. ALS herbicide treated plants stop making these three amino acids and translation stops for every protein in the cell that requires at least one of these amino acids. Living things need amino acids to build proteins and plants and microbes need the ALS enzyme to build three of the amino acids. The next lesson (**Gene expression part 2**) will apply the gene expression principles from this lesson to the observation that some plants can be resistant to ALS inhibitor herbicides.

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2.4.8: Gene Expression- Applied Example (Part 2)

Learning Objectives

At the completion of this lesson you should be able to...

- Explain how a mutation in a single gene can control resistance to herbicides such as ALS inhibitors.
- Predict the impact of a specific mutation on gene expression and the eventual trait observed in a plant.
- Distinguish between the molecular and classical definitions of an allele.
- Describe the molecular basis of dominance or a lack of dominance between alleles.

This lesson describes how changes in the DNA sequence of a gene can alter the synthesis of a protein and thus influence traits such as herbicide resistance. In this lesson we will describe how changes in the gene can alter the gene expression process and influence traits in an organism. The specific example of ALS-inhibitor herbicide resistance is used to demonstrate the impact of genetic change on trait expression in a plant.

Sequence Determines Structure, Structure Determines Function

Transcription and translation are fundamental to the production of all proteins found in all living things. That is why discovering the sequence of a gene can allow geneticists to predict the amino acid sequence of the protein that gene encodes. Remember, a completely synthesized protein will be hundreds of amino acids long and thus an average gene will have two or three thousand nucleotides in its sequence. As more is learned about how amino acid sequence dictates protein function, the value of gene sequence information to biologists increases. In this lesson, we will apply our understanding of the gene expression process to understanding the genetic basis of resistance to ALS herbicides in plants.

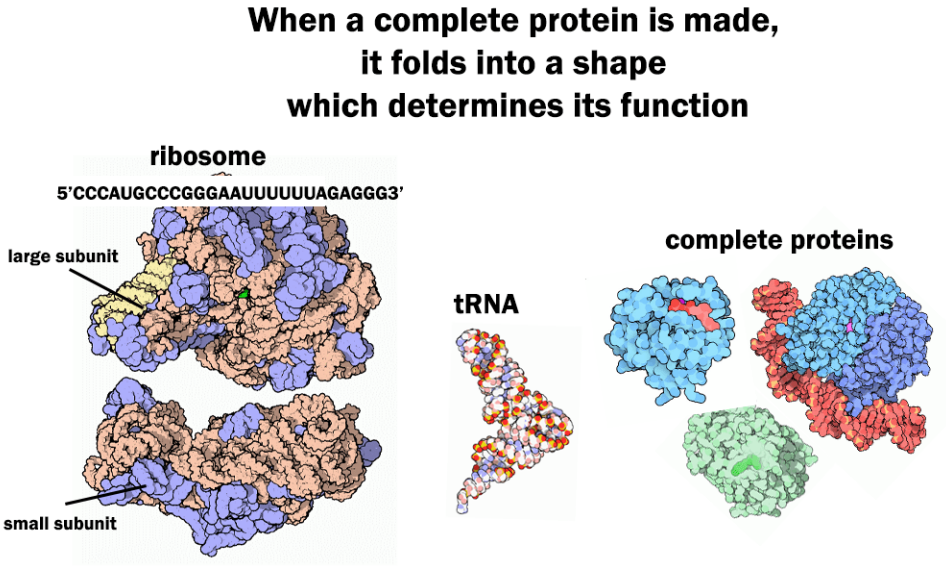


Figure 1. A protein's function is dependent upon how it folds into a shape. The folding is a result of the sequence of amino acids present after translation. Based on an image by Donald Lee. Adapted by Abbey Elder with figures taken from PDB-101: "Molecule of the Month" collections, available under a CC BY 4.0 license.

The ALS enzyme is about 800 **amino acids**. The specific order of amino acids in the protein chain dictates how the molecule can be folded up into a secondary structure (Fig. 1).[1] This is because each amino acid has a different chemistry that influences its interaction with other amino acids within the protein chain. The protein's secondary structure determines the function of the protein. We can relate this principle to functional objects that are more familiar to us. Why would someone need a set of wrenches? Every tool in the set is made of the same materials. However, the specific shape and dimensions of each tool allow it to perform a specific function. The ALS enzyme functions to catalyze reactions for amino acid synthesis in the chloroplast in a

plant cell. Therefore, the plant ALS **enzyme** structure signals the cell to transport the enzyme to the **chloroplast** and then allows the enzyme to catalyze the formation of a bond between amino acid precursor molecules. Clearly it was important to assemble the amino acids properly when building the ALS enzyme to allow it to function properly.

ALS and Weed Control

The function of the ALS **enzyme** in plants is also an interesting issue in the area of weed control. Herbicides have been discovered with sulfonyl urea or imidazolinone chemistries that will bind to ALS enzymes in plants and disrupt their catalytic functions (Figure 2). Because the herbicides bind tightly to ALS and ALS is not found in high copies in the cell, the herbicides will effectively halt the synthesis of the **Leucine, Isoleucine, and Valine amino acids** (Leu, Ile, Val). How will plants respond to the shortage of these three amino acids? They quit growing because they cannot make three of the twenty amino acids.

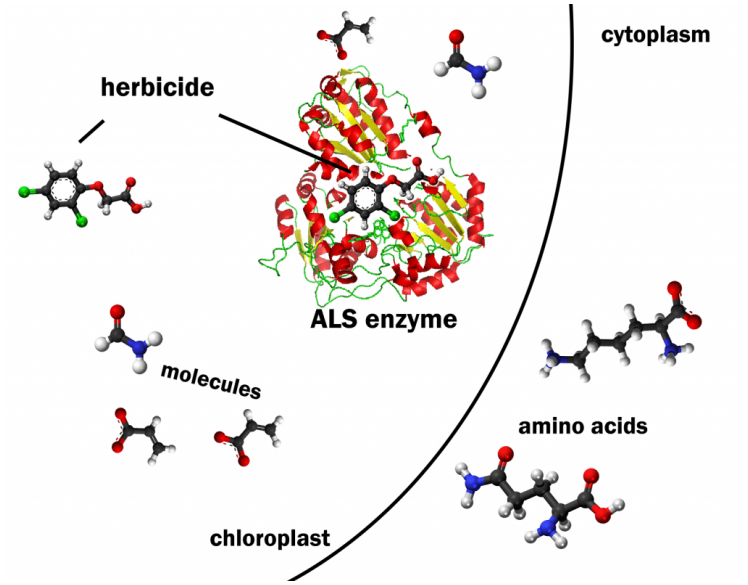


Figure 2. The ALS enzyme functions as a part of a metabolic pathway in the chloroplast that takes simple molecules and synthesizes amino acids. Herbicides can bind to the enzyme and block the amino acid synthesis. Based on an original image by Donald Lee, adapted by Abbey Elder. ALS image by Rj Joplin is used under a GNU General Public license. The amino acid Lysine representation by Ben Mills is used under a CC BY SA 4.0 license.

The **proteins** that need these amino acids will be only partially synthesized. The partial proteins will not work because they lack the structure needed for their function. The plant cells cannot grow and divide and the plant's growth and development are halted. They will not necessarily die but they will no longer be able to compete for light and other resources if nearby plants are not affected by the ALS inhibitors. If the nearby plant that is not affected by the ALS inhibitors is a crop species such as corn or soybean, a valuable post emergence weed control strategy can be implemented.

ALS Alleles

Post emergence weed control by ALS inhibitors works in corn and soybean genotypes with altered ALS genes. The altered genes code for a slightly modified ALS enzyme which is resistant to ALS inhibitors. The molecular basis of this alteration can be determined and help us understand what alleles of a gene are. Even without molecular analysis, geneticists discovered alleles of the ALS gene that would make plants resistant to the ALS herbicides. How were these alleles discovered?

Corn breeders knew that ALS **resistance** did not naturally occur in their breeding lines. This discovery was easy to make. They planted thousands of different lines in short rows in the field, sprayed the field with the herbicide and observed the reaction of the lines. All of the lines tested had significant stunting. Variation did not exist in their lines so they decided to try to induce variation by mutagenesis. Pollen was collected from plants that had been exposed to a chemical mutagen. The chemical induced mistakes in DNA replication during pollen formation. The pollen was viable but carried mutant alleles. This pollen was applied to the silks of other corn plants to produce thousands of seeds. This seed was planted and the field test for ALS resistance was repeated. This time some plants resistant to stunting from ALS were observed, tagged and self-pollinated. From this experiment, the IT (immi tolerant) allele was discovered. The IT allele appeared to be dominant over the normal ALS allele because plants with one copy of

the IT allele per cell (heterozygotes) had the same level of resistance as plants that had two copies of the IT allele (homozygotes). Molecular analysis of these plants was done to reveal how the IT allele was different for the original version.

The IT Allele

The IT allele of the ALS **gene** has a change in the coding region sequence. This was discovered by making a gene library from the DNA of homozygous IT plants and selecting a clone of the IT gene. The gene clone was sequenced and compared to the normal susceptible ALS gene from corn. A single nucleotide substitution in the IT allele was responsible for endowing a corn plant with **resistance** to ALS herbicides. The nucleotide substitution was in the gene coding region so it changed a codon sequence in the mRNA, coding for a different amino acid in the protein. The amino acid substitution had a minor or negligible effect on the enzyme's ability to perform its normal functions. However, the amino acid substitution changed the site at which the ALS herbicide binds to the enzyme. The ALS herbicide will not interact with the ALS enzyme encoded by the IT allele, giving the plant resistance to the herbicide. This is an example of how a small change in a gene can have a big impact on a plant's phenotype in the right environment.

The IR Allele, Tissue Culture Selection for Mutations

ALS alleles that confer **resistance** to immidazolinone herbicides have also been selected from mutations that have occurred in tissue culture cells. DNA replication does not occur 100 percent without errors so it is not surprising that natural mutations occur in cells. A mutation, though, that gives a single cell in a corn plant resistance to ALS herbicides will not render the whole plant resistant. Geneticists have used the power of tissue culture to select for these mutations at the single cell level. Hundreds of thousands of corn cells can be grown in tissue culture plates that contain the herbicide. Most of the cells will quit growing and dividing when they take up the herbicide but some cells continue to grow and form clumps of undifferentiated cells called callus. Plants regenerated from these callus cells will often express the herbicide resistance as well. The IR allele of the ALS gene was selected in this way.

This allele also has an altered coding region sequence that blocks the ALS herbicides from binding. The ALS enzyme encoded by the IR allele, however, does not provide desired levels of herbicide resistance in a heterozygous plant. The molecular mechanisms causing this difference in the IR compared to IT allele are not clear. The implication of this difference in plant breeding are significant. The IT allele can be inherited from just one parent to give the 'Clearfield' or "Immi" hybrid herbicide resistance. Hybrids must be homozygous for the IR allele requiring both parents be homozygous IR inbreds (Figure 3).

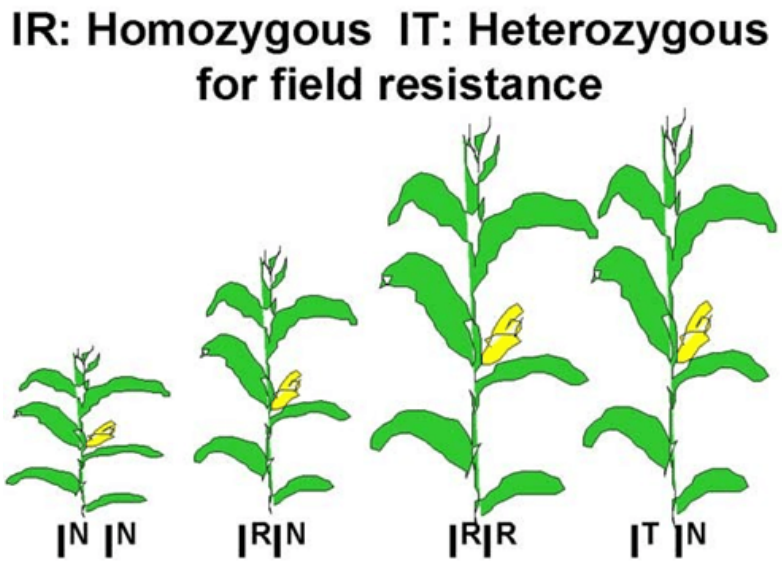


Figure 3. Corn plants sprayed with an ALS inhibitor herbicide vary in their growth. The plant can have the IR, IT or IN (normal) alleles and the genotype of the plant for the ALS genes determines its phenotype in response to these herbicides. Image by Donald Lee.

Molecular Basis of Dominance

The occurrence of the IT, IR and normal alleles of the ALS enzyme and their impact on plant phenotype provides us with an opportunity to think about dominance and lack of dominance at the molecular level. The IT allele has complete dominance to the normal allele because there is apparently no phenotype difference between homozygous dominant and heterozygous IT plants when they are sprayed with an ALS inhibitor. This suggests that in the heterozygous plants, one copy of the IT allele per cell encodes enough resistant ALS protein to drive the synthesis of sufficient quantities of Val, Leu and Ile for normal growth in the corn plant. However, the ALS enzyme encoded by the normal allele in these heterozygotes can be inhibited by the herbicide and will not contribute to amino acid synthesis.

In contrast, the IR allele has a lack of dominance to the normal allele (IN). Heterozygous plants that have one copy of the IR and one copy of the normal allele per cell do not maintain normal growth after ALS inhibitor treatment. One copy of the IR allele per cell does not encode sufficient copies of a resistant ALS enzyme to support normal growth. Instead, two copies of the IR allele per cell are needed so that all of the ALS enzyme made will not be bound by the herbicide.

The classification of the plant phenotype for characterizing possible genotypes at the ALS locus may depend on environment. In some growing environments it has been noticed that heterozygous IT hybrids will show a slight growth reduction in the sprayed plants compared to unsprayed. The difference is minor because the plants usually grow out of this difference later on and yield reduction is not a factor. This observation does emphasize that phenotype is a result of genotype and environment. Therefore, scoring traits in the proper environment will be critical to discovering the nature of gene action.

How Universal is the Genetic Code?

The table of codons used by organisms to translate mRNA into proteins is shown on the bottom of the page (Table 1). As was mentioned earlier in this lesson, the genetic code needed to be cracked one time because all organisms used the same codons to encode amino acids. As scientists began to sequence the coding regions of genes from different organisms, they discovered something called a codon preference. When you look at the codon table, you can see that the genetic code is redundant. This means that more than one codon can encode the same amino acid. This is because there are 61 codons that code for the placement of 20 different amino acids. A codon will only work in coding if a tRNA with a complementary anticodon is also found in the same cell and has the appropriate amino acid to deliver. Therefore, there could be 61 different tRNAs, one to complement each codon. Each different tRNA needs to be encoded by a different gene. If that gene is not expressed in the cell, the tRNA will not be found and a codon that needs to be complemented by that tRNA will not be complemented. In this case, the codon will act like a stop codon. The ribosome will halt **translation** and the protein made will be a shorter version of the intended protein. Organisms would not benefit from this situation so there is a tight complementation between what tRNAs genes are present and expressed in an organism's cells and what codons are used to encode a specific mRNA. In this way the genetic code will have a dialect. The language is universal but certain words are used preferentially.

Table 1. RNA Codon Table. The genetic code. The 20 common amino acids are listed in their three and one-letter formats. Stop codons are UAA, AUG and UGA

<table>
<tr><th rowspan="2">First Position</th><th colspan="4">Second Position</th><th rowspan="2">Third Position</th></tr>
<tr><th>U</th><th>C</th><th>A</th><th>G</th></tr>
<tr><td rowspan="2">U</td><td rowspan="2">Phe (F)</td><td rowspan="2">Ser (S)</td><td rowspan="2">Tyr (Y)</td><td rowspan="2">Cys (C)</td><td>U</td></tr>
<tr><td>C</td></tr>
<tr><td></td><td>Leu (L)</td><td></td><td>Stop</td><td>Stop</td><td>A</td></tr>
</table>

			Stop	Trp (W)	G
C	Leu (L)	Pro (P)	His (H)	Arg (R)	U
					C
			Gln (Q)		A
					G
A	Ile (I)	Thr (T)	Asn (N)	Ser (S)	U
					C
			Lys (K)	Arg (R)	A
	Met (M)				G
G	Val (V)	Ala (A)	Asp (D)	Gly (G)	U
					C
			Glu (E)		A
					G

Scientists are not sure why codon preferences are a part of the **gene expression** process in organisms. It may provide another level for the organism to control the amounts and kinds of proteins made in its cells. Recent experiences in genetic engineering of plants and animals, however, has made codon preference an important consideration. For example, scientists have put genes from a soil bacterium called *Bacillus thuringiensis* (Bt) into corn plant cells in order to give the corn plant the ability to make a protein that is toxic to European corn borer, a common pest to corn producers. They found that the gene would be transcribed but the mRNA would not be translated to make the desired protein. One reason was codon usage. Some of the codons the bacteria use to encode amino acids are rarely used by corn. The corn plant either lacked the tRNA to complement the codon or make the tRNA at such low levels that there were not enough copies in the cell to accommodate translation of the Bt mRNA. Therefore, the genetic engineers needed to make synthetic coding regions that substituted codons preferred by corn for those preferred by bacteria. The end result was they were able to get higher levels of the Bt protein made once these changes were made in the gene. Codon preference thus makes the genetic engineering process more challenging.

Learning Activities

Watch this animation of the transcription process.

Watch this animation of the translation process.

Watch this video overview of Transcription and Translation.

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1. Graphics used in this figure were taken from The RCSB PDB "Molecule of the Month": Inspiring a Molecular View of Biology D.S. Goodsell, S. Dutta, C. Zardecki, M. Voigt, H.M. Berman, S.K. Burley (2015) *PLoS Biol* **13**(5): e1002140. doi: 10.1371/journal.pbio.1002140 ↵

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2.4.9: Regulation of Gene Expression

Learning Objectives

- Understand the concept of gene expression.
- Understand transcriptional regulation of gene expression.
- Understand epigenetic gene regulation.4
- Understand post-transcriptional regulation of gene expression (RNA level).
- Understand post-translational protein modification and regulation.

Introduction

Every cell in a plant contains the same genetic information, the same set of genes. Yet Therefore different sets of genes are required for the various functions of different cells or tissues, as well as for plant responses to environmental stimuli or stresses. This is achieved by regulating the activity of genes according to the physiological demands of a particular cell type, developmental stage, or environmental condition. This regulation of activity is known as **gene expression**.

The term **expression** can be used in different ways that are sometimes confusing. Typically, if a gene product is produced, the gene is considered “expressed”. However, it sometimes occurs that a transcript might be produced but not a protein, or that a protein is produced but it is in an inactive state. In such cases, although a gene product is produced, the biological activity encoded by that gene is not present. For the purposes of this section, the key point is how the biological activity encoded by a gene is regulated.

The expression of genes in specific plant cells, tissues, and organs and the timing of this expression require a precise level of regulation. Expression, or genetic function, can potentially be regulated at any of the steps from transcription, RNA processing, translation, through post-translational protein modification, as discussed in lesson 1. Regulation can be qualitative (i.e. gene expression is either “on” or “off”) or quantitative (i.e. expression levels can be modulated “up” or “down”). Fluctuations in the intensities of external stimuli coupled to changes that occur at the genomic level result in different developmental outcomes or physiological states. Regulation of gene expression at the level of transcription can be brought about through chromatin and histone modifications. Also, a gene sequence can be differentially spliced to produce mRNA products of variable lengths leading to new protein products with novel functions. Some genes do not encode proteins but short forms of RNAs with regulatory functions such as induction of flowering. Finally, proteins products can be subjected to modifications such as phosphorylation or dephosphorylation to alter their functions, or can be completely degraded to turn off a gene.

Transcriptional gene regulation

Since transcription is the first step in gene expression, it makes sense in terms of cellular economy, to regulate expression at this point, and in fact this is one of the most important regulatory points. We already described the involvement of RNA polymerase in the transcription process, but in fact there are other protein factors that are required. Proteins involved in transcriptional regulation are known as transcription factors. It is the interaction of these transcription factors with specific DNA sequences that regulate the process of gene transcription.

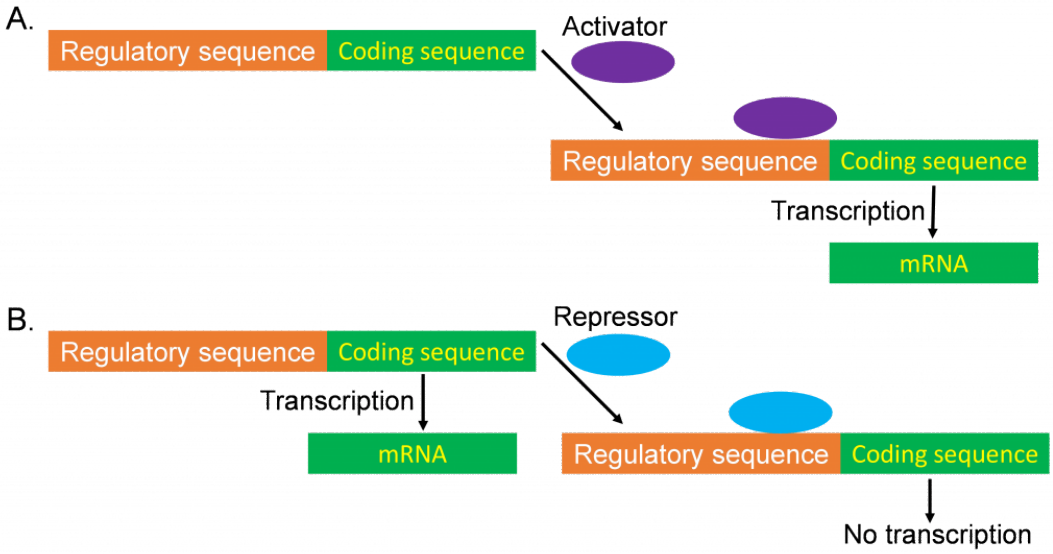


Figure 1. Positive regulation of gene transcription (A) occurs when a transcriptional factor (activator) turns on gene transcription. Negative regulation of gene transcription (B) occurs when a transcription factor (repressor) turns off gene transcription. Image by Walter Suza.

A. The concept of differential (regulated) gene expression

As just described, not every gene is expressed all the time. When a gene displays different levels of expression in different circumstances, this is known as **differential expression**. Circumstances that might apply include, but are not limited to, different plant tissues (root vs. leaf), different developmental stages (germination vs. reproductive development), or in response to different environmental stimuli (cold stress or pathogen attack).

The term differential expression can also be used to compare the expression of different genes. If two genes show different expression patterns (among plant tissues or in response to environmental stimuli), they are considered differentially expressed, whereas genes that showed very similar patterns of expression would be considered **co-expressed**.

B. Promoters

As mentioned, transcription is regulated through the interactions of proteins, transcription factors, with specific DNA sequences. Most regulatory DNA sequences governing gene transcription are located on the 5′ border of the transcribed region. This region is called the gene **promoter**.

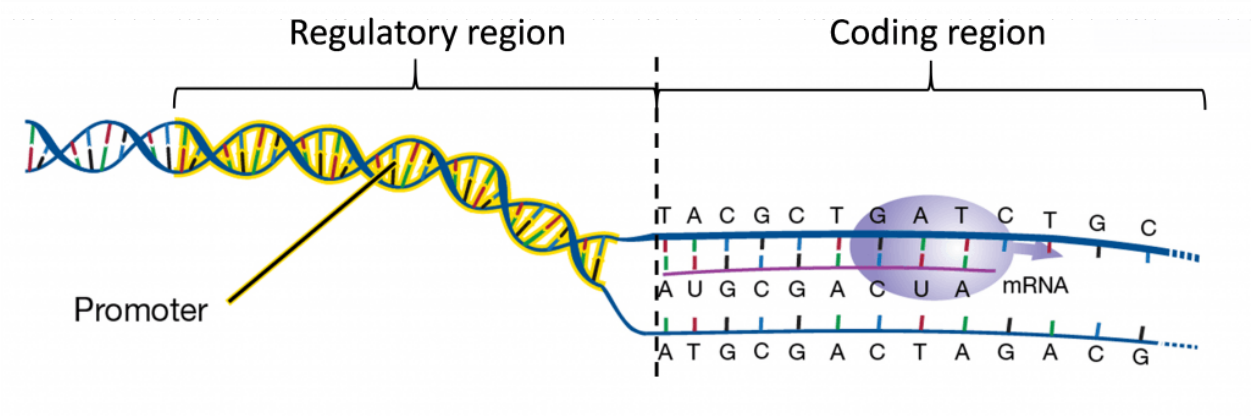


Figure 2. Promoters are part of gene regulatory sequence. Adapted from Illustration by NIH-NHGRI.

Promoters contain a core, which is required for the binding of the “basal transcriptional machinery”, including RNA polymerase. The “TATA” box, with a consensus sequence TATAA, is located within the core promoter, usually 25-30 nucleotides upstream of the transcription initiation site. Promoters also contain regulatory sequences that determine when, where, and to what level genes

are transcribed. Promoters can vary in length from a hundred to a few thousand nucleotides.

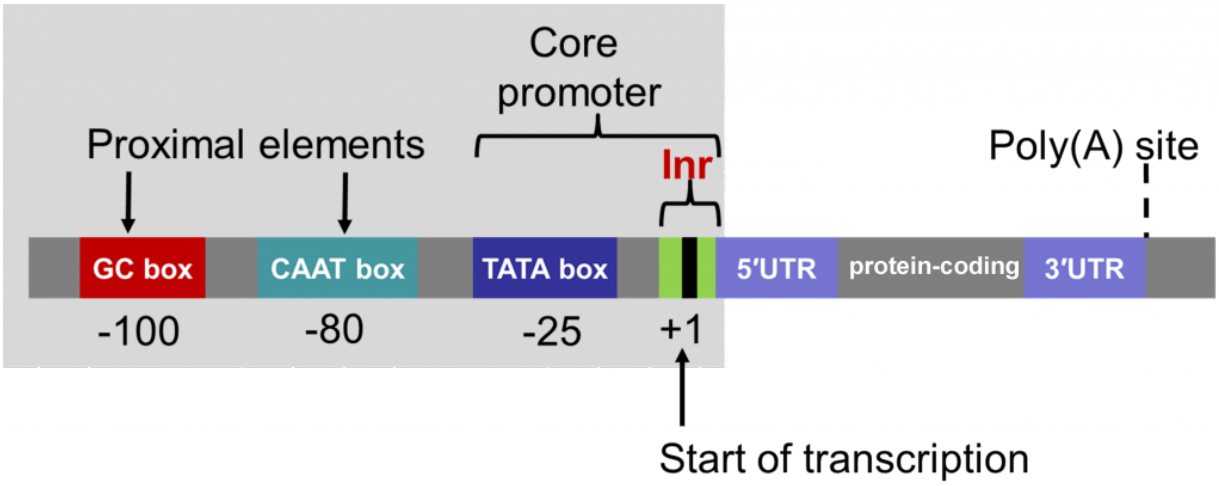


Figure 3. The promoter contains core and proximal elements. Image by Walter Suza.

The full promoter sequences of different genes that are expressed in a similar manner may be different. However, such promoters often contain short sequence "motifs" that are similar, referred to as cis elements. Early work (Benfy and Chua, 1990) to understand the function of different promoter elements in regulating gene expression in plant cells and tissues revealed that various combinations of cis elements are able to be interpreted by the cell and control gene expression. Sometimes cis elements promote gene transcription and sometimes they function to restrict transcription of genes in particular cells and tissues.

Promoter analysis is facilitated through the use of **reporter genes**. The reporter gene produces effects that are easily identifiable and quantifiable, which can be used to determine the function of a regulatory region of another gene (promoter, promoter elements, or enhancers) in cells, tissues, or organs. Such analyses are critical to crop biotechnology where targeted expression of genes in particular tissues is often desirable. To test whether a promoter is effective in conferring the expression of a gene to a particular tissue, scientists fuse the putative promoter to a reporter gene and introduce the promoter-gene fusion into plants. An example of a reporter is the GUS gene, which encodes a GUS enzyme (beta-Glucuronidase) that, when expressed, produces a blue color upon addition of a substrate. Figure 1 shows an example of GUS expression in maize seed using two promoters that act either in the endosperm or the embryo.

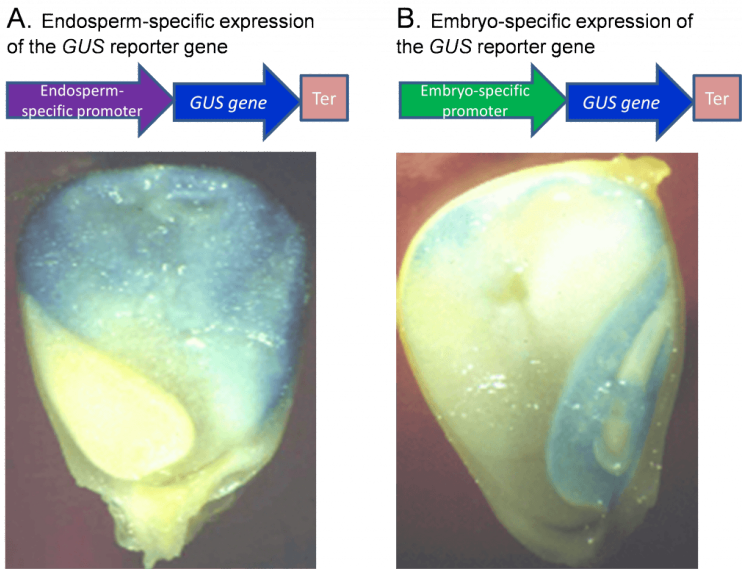


Figure 4. Promoters direct the expression of genes in plant tissues. (A) The expression of the GUS transgene is restricted to the endosperm of maize seed by an endosperm-specific promoter. (B) When the GUS transgene is fused to an embyo-specific promoter the activity of the GUS enzyme (production of the blue coloration after addition of a GUS substrate) is restricted to the embryonic tissue. Image by Shui-zhang Fei.

C. Enhancers

Enhancers are DNA sequences that increase the rate of transcription of a gene when they are present, although alone they cannot cause transcription to occur. Enhancers are usually position and orientation independent. Although they are normally located upstream of the promoter, they can also be located on the 3' region of the gene or even with the coding region. Enhancers can increase the transcription when added to genes that they are not normally associated with. This is a useful property for biotechnology, allowing promoters to be manipulated for increased levels of transcriptional regulation. Some enhancers function at all times in all cells and tissues and they are referred to as constitutive. Other enhancers function in specific tissues at specific developmental stages. Some are active only in response to environmental signals. The AACCA enhancer on the promoter of soybean β-conglycinin gene encoding a seed storage protein functions specifically in seeds. Enhancers are thought to interact with specific nuclear proteins involved in transcription. For example, enhancers might facilitate the binding of transcription factors and direct these factors along the DNA strand to the direction of the promoter. Alternatively, enhancers may facilitate changes in DNA structure such as modification of the chromatin structure.

The counterpart of an enhancer is a **silencer**. Silencers have all the properties just described for enhancers, except they function to dampen, or decrease, the levels of transcription controlled by a promoter.

D. Transcription factors

RNA polymerase binds the promoter at the TATA box and in cooperation with other proteins drives gene transcription. The proteins that interact with RNA polymerase to facilitate its binding to the promoter and to regulate its activity are known as **transcription factors**. Some transcription factors, known as "basal transcription factors" are fundamental to RNA polymerase binding and function, and are expressed in all living cells.

Other transcription factors bind to cis regulatory DNA sequences of promoters, enhancers or silencers. These proteins interact in complex ways with the basal transcription machinery, to regulate the activity of RNA polymerase, and therefore gene transcription. Thus, transcription factors regulate when or where individual genes are expressed, and to what level.

Transcription factors can function as either positive or negative regulators. That is, they can either function to induce (increase) gene transcription or to repress it. The consequence of many transcription factors depends on their interactions with other proteins. Two factors together may be required for gene activity, and exclusion of one of the factors in space and time offers a mechanism for differential gene expression. Some transcription factors might function as a positive regulator in one context but as a negative regulator in another context, depending on what other cis elements and/or transcription factors might be present.

Epigenetic regulation

A. Chromatin structure and histone modification

At the molecular level chromatin is a product of an ordered and tight packaging of the double stranded DNA around nucleosomes (a core of proteins named histones), and an association with additional proteins.

Transcriptional regulation often involves modification of chromatin structure mediated by post-translational regulation (changes in acetylation and methylation) of histones. Histone acetylation involves the addition of acetyl groups and when histones are heavily acetylated the DNA is less tightly associated with them. This often correlates with increased transcriptional activity of specific genes. The idea is that when DNA is loosely associated with histones, it is more accessible to transcription factors that require interaction with the DNA to initiate transcription. Consequently, histone deacetylation (removal of acetyl groups) by histone deacetylase enzymes (for example, FLD, p462) stabilizes nucleosomes and represses transcription. On the other hand, histone acetylation by histone ecetyltransferase destabilizes nucleosomes and promotes transcription.

As described in the text, another form of histone modification is the addition of methyl groups to histone proteins, which similarly regulates chromatin condensation or decondensation.

B. DNA methylation

The nucleotide bases of DNA can be modified by the attachment of methyl groups at various locations. The addition of these methyl groups happens after the DNA has been synthesized and is controlled by enzymes that add methyl moieties to specific

regions of DNA. The most common modified nucleotide base is C5 methylcytosine (m5C) due the activity of the enzyme DNA (cytosine-5) methyltransferase (MET). MET recognizes the unmethylated newly-replicated DNA strand and incorporates a methyl group if the template strand was methylated (hemimethylated).

Methylation status is correlated with gene expression (Figure 5) as demonstrated by the low level of methylation in regions of the genome undergoing active transcription.

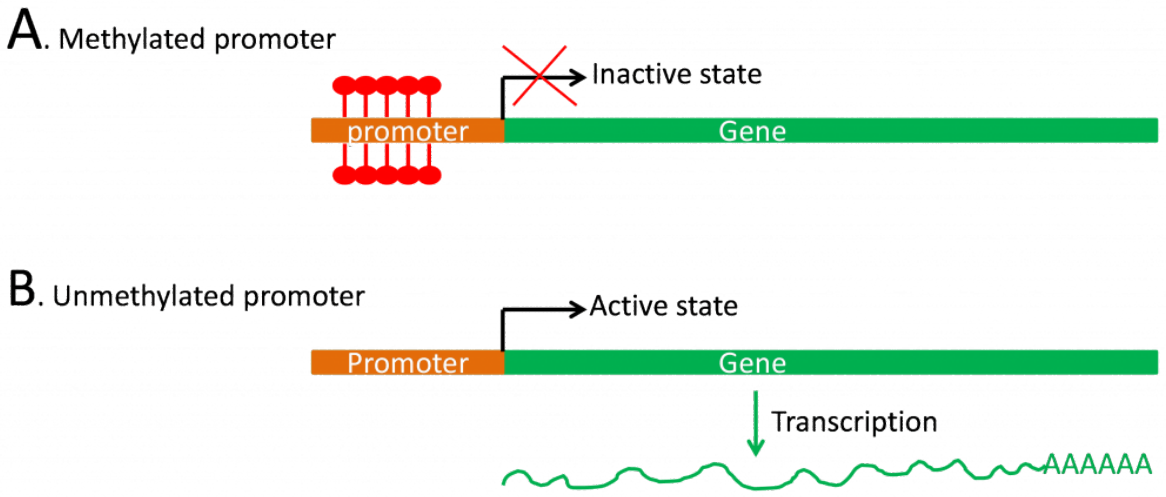


Figure 5. Absence of methylation correlates with gene expression. (A) The promoter region may be methylated during development or environmental conditions such that a gene is inactivated. (B) The same gene is activated upon the removal of the methyl groups.

Studies have shown that altering a plant's overall DNA methylation status can affect growth and development. For example, cold treatment (vernalization) induces flowering in biannuals such as Arabidopsis and winter wheat, and lowers the level of methylation of particular genes. Also, treating plants with the drug 5 azacytidine prevents methylation at the 5 position of cytosine and stimulates flowering. Recent studies also suggest a relationship between epigenetic gene regulation and heterosis, with hybrids showing higher global levels of transcription, higher histone acetylation levels and lower DNA methylation levels (reviewed in He et al., 2011).

RNA-level regulation

Regulation of gene expression occurs at many levels, including post-transcriptionally. From the standpoint of the expression level for a given gene, a critical factor is the level of fully processed, mature mRNA. When we consider the level of the mature form a particular transcript at any given time reflects the steady state balance between synthesis, processing and degradation. The Post-transcriptional regulation of RNA occurs through several mechanisms.

RNA stability and degradation

The control of mRNA degradation rate, or turnover, is an important regulatory mechanism in gene expression. As mentioned, the level of a particular transcript at any given time reflects the steady state balance between synthesis and degradation. Thus, even though a gene may be transcribed at a high rate, a high rate of RNA turnover could effectively shut off the gene. The stability of mRNA can be regulated globally, to affect all or most transcripts, or it can be very specific to a particular mRNA. Regulated mRNA turnover may be particularly important in plants, since plants cannot move to avoid harsh environmental conditions and stresses are known to induce specific changes in RNA stability. Other factors such as light and plant hormones are also known to regulate mRNA stability.

The degradation of mRNA is catalyzed by enzymes called ribonucleases. Ribonucleases include exonucleases, which degrade only from one end of the transcript, and endonucleases, which can attack the mRNA molecule internally.

Therefore, controlling ribonuclease access to the mRNA substrate is an important regulatory mechanism in gene expression. This control is achieved through various mechanisms including, controlling the amount of nucleases present, regulating the activity of the nuclease, sequestering the nuclease to particular cellular locations to restrict its access to RNA, and to control the stability of

the mRNA by decreasing accessibility to nuclease.

Four regions of an mRNA are important for its overall stability. These include, the 5′ untranslated region and the cap, coding region, 3’ untranslated region, and the poly(A) tail.

The cap at the 5′ end protects the mRNA from exonucleases that degrade RNA from the 5′ to the 3′ direction.

The 3′ untranslated region may contain short sequences that influence mRNA stability. For example, the repetition of the sequence AUUUA in the 3′ untranslated region of many animal and plant genes is associated with mRNAs with short half-lives (the time it takes for half the RNA to be degraded, after transcription is stopped).

The poly(A) tail increases mRNA stability but is not sufficient alone. It only provides stability when bound by proteins such as poly(A) binding protein (PABP). Test-tube experiments have shown that, removing PABP from a stable mRNA destabilizes it, while adding back purified PABP restores stability.

Translational regulation

As described, for most genes it is the protein product that performs the biological function. As such, regulating the amount of protein production effectively regulates gene expression. There are several known mechanisms by which the process of translation is known to be regulated. A detailed consideration of these mechanisms is beyond the scope of this course, but in general it is important to bear in mind their importance. For example, under certain stress conditions, “normal” translation is halted and only mRNAs related to stress tolerance are selectively permitted to be translated into proteins. This selective translation is important to allow plants to quickly respond to stress and to conserve energy under stress conditions.

Translation of mRNAs can play an important role in determining their overall stability. Mutations that add premature stop codons often lead to rapid degradation of the mRNA. Ribonucleases (figure 9) or other factors involved in degradation may recognize the number or spacing of ribosomes on an mRNA, and degrade those that are not produced properly. This may help prevent synthesis of proteins with incorrect functions which will negatively impact cellular processes. Errors during transcription could add or omit nucleotides which would alter the proper codon sequence creating mutant proteins.

Protein-level regulation

After translation, proteins are subject to a variety of modifications that can regulate their activity. There are many different ways by which proteins can potentially be modified and thereby regulated, and only a few of the basic mechanisms will be considered here.

Common types of post-translational modifications

The first mechanism is covalent modification by the addition of various chemical groups. A wide variety of groups can be involved, including small organic groups (methylation, acetylation) lipids (myristoylation, farnesylation, palmitylation), carbohydrates (glycosylation, glucosylation), small proteins (ubiquitination, sumoylation) and inorganic molecules (phosphorylation, sulfation). Such covalent modifications are generally accomplished by the activity of enzymes specialized to perform these modifications. Many of these modifications are also reversible; that is these groups can be added to a protein and subsequently removed. Phosphorylation is a particularly noteworthy reversible modification that is common in the regulation of many proteins. Enzymes that add phosphate groups to other proteins are called protein kinases, and those that remove phosphates are called phosphatases. As such, protein kinases and phosphatases are central to many cellular regulatory systems.

A second common mechanism of protein modification is through proteolytic cleavage. Proteolysis occurs as part of the general turnover of cellular proteins, which is required to eliminate damaged proteins and recycle amino acids. Proteolysis is important for the processing of certain proteins, for example in the removal of the signal peptide of proteins targeted to specific cellular compartments. It can also occur in a highly specific manner whereby particular proteins are targeted for degradation or for cleavage at a specific site within the protein. Proteolytic modifications are non-reversible.

Proteins can undergo modification through complex formation. Such complexes can occur among proteins or between a protein and a cofactor.

Proteins can also be modified according to conditions of the cellular environment. The redox state can result in oxidation or

reduction of proteins, particularly of sulfhydryl side groups. Cellular pH can affect the charge of ionizable side groups.

Common types of protein regulation

All of the above mentioned types of protein modification can alter protein conformations and thus have regulatory consequences on protein function or activity. Protein regulation is highly complex and there are a myriad of different ways by which this occurs. Again we will just briefly consider a few of the more common mechanisms.

One major way protein modification can regulate protein function is by **altering their activity**. This can be true for many types of proteins including, but not limited to, enzymes, transcription factors, signaling proteins, and structural proteins.

Cells are **compartmentalized** into several membrane bound organelles, including the nucleus, chloroplasts, mitochondria, peroxisomes, endoplasmic reticulum (ER), golgi, and vacuoles. Each of these compartments performs unique metabolic functions that require a set of proteins. Compartmentation is regulated for some proteins. For example, upon exposure to light the phytochrome protein moves into the nucleus where it affects the expression of light-regulated genes.

Proteolytic processing is involved in several important regulatory processes. Many proteins are synthesized in an inactive form that requires proteolytic cleavage for activation. The full-length translation product before processing is often called the precursor, or a preprotein. As mentioned, cells contain membrane-bound compartments. Since membranes are impermeable to most proteins, an active mechanism is necessary to move a protein across a membrane. For proper delivery to their organellar destinations, specific amino acid sequences, called target signals must be present in a protein (to serve as an “address”). For example, entry into the chloroplast is achieved by the presence of a target signal called the transit peptide. This is at the amino terminal end of the protein and is proteolytically cleaved during import.

Another example of proteolytic processing is seen in a plant defense response in members of the solanaceae (for example, tomato and potato). An 18-amino acid peptide hormone called systemin is secreted by plant cells that are damaged by insects or mechanical wounding. Systemin production by wounded cells is required to induce the synthesis of proteins involved in defense. Systemin induces defense responses in wounded cells, and throughout the plant. Analogous to animal peptide hormones (e.g., insulin), systemin is initially synthesized as a much larger (200 amino acids) precursor called pro-systemin. Pro-system is inactive; however, upon wounding it undergoes proteolytic cleavage to produce activated systemin.

Targeted protein degradation

The amount of protein present in a cell or tissue is determined by both its rate of synthesis and its rate of degradation. Therefore, protein degradation is an important mechanism by which the plant can regulate biological activity (i.e., a genetic function). For example, one way to shut down a metabolic pathway is by degrading one of the key enzymes controlling the rate of the entire pathway. Therefore, protein degradation is an essential component of gene regulation to meet cellular demands for growth, development, and defense.

Protein degradation must be carefully controlled to fine tune gene expression to allow plants to adapt to new environmental conditions. Often, cells will adopt several complex mechanisms for proteolytic degradation of proteins. Enzymes that cleave or degrade proteins are referred to as proteases. For example, plant vacuoles are rich in proteases that play a similar function in protein degradation as that of lysosomes in animal cells. Protease activity must be tightly regulated to prevent accidental degradation of essential proteins. Sequestering proteases in particular organelles like the vacuole separates them from other organelles and is one of to control their activity.

An important mechanism by which specific proteins are targeted for degradation is through ubiquitin-mediated proteasomal degradation. The proteasome is a large complex of multiple protein subunits that have a protease activity to degrade proteins. Proteins get marked for proteasomal degradation with a small protein called ubiquitin. Ubiquitin is covalently attached to specific proteins in response to environmental or developmental signals. This allows plants to quickly adapt to changing conditions by eliminating proteins whose functions are not advantageous under the new conditions. For example, the photoreceptor phytochrome mentioned above becomes targeted for degradation when light is no longer available. This allows plants to change their physiological functions going from daylight to night conditions. Protein degradation by the proteasome system is also an important regulatory mechanism for plant hormone signaling, for example the signaling of the defense hormone jasmonic acid, and growth hormone gibberellic acid.

Proteins have lifetimes that range from a few minutes to weeks or more. Cells continuously make proteins from and break them down to amino acids. One of the functions of protein degradation is to eliminate aberrant or damaged proteins which could harm

the cell. The second function is to facilitate the recycling of amino acids. For example, most of the amino acids required for growth of the seedling are derived from the degradation of seed storage proteins. Conversely, in annual crop plants, many of the amino acids in seed storage proteins are derived from proteins degraded in leaves and other plant parts during senescence.

Lesson Summary

The expression of genes in specific plant cells, tissues, and organs and the timing of this expression require a precise level of regulation. A single promoter may not be sufficient to regulate the expression of such gene(s) in space and time. Therefore, coding regions with the same function may have different promoters, and such genes are referred to as differentially regulated. Most regulatory sequences governing gene expression are located on the 5' border of the coding region. Transcription is often initiated between 20 and 60 nucleotides upstream of the ATG start site. Enhancers are usually position and orientation independent. Although they are normally located upstream of the promoter, they can also be located on the 3' region of the gene or even with the coding region. Enhancers can increase the transcription when added to genes that they are not associated with. RNA polymerase binds the promoter at the TATA box and in cooperation with other proteins drives gene transcription. The proteins that interact with RNA polymerase bind to regulatory sequences upstream and downstream of the transcription site.

Transcription factors can play a regulatory role by determining where individual genes are expressed. Transcriptional regulation often involves modification of chromatin by changes in acetylation and methylation of histone. The nucleotide bases of DNA can be modified by the attachment of methyl groups at various locations. Methylation status is correlated with gene expression. Alternative splicing describes an alternative mechanism of pre-mRNA processing to generate mRNAs that have different combinations of exons. The control of mRNA degradation rate, or turnover, is an important regulatory mechanism in gene expression. RNA interference (RNAi) is a post-transcriptional process involving the degradation of mRNA initiated by the formation of double-stranded RNA (dsRNA) of the target mRNA. Translation of mRNAs can play an important role in determining their overall stability. Mutations that add premature stop codons often lead to rapid degradation of the mRNA. Protein degradation is an essential component of gene regulation to meet cellular demands for growth, development, and defense. The plant can alter the activity of a metabolic pathway, by degrading one of the key enzymes controlling the rate of the entire pathway.

Exercises

1a. Which of the following could alter gene regulation

i. Deleting a promoter
ii. Altering fertilizer application rate
iii. Raising the temperature of the greenhouse
iv. Herbicide application
v. Reduce irrigation frequency

1b. The detection of a gene product, for example, RNA or protein at any one moment is a reflection of the "steady state" of the product. Describe the term "steady state" in this context.

2. You are studying expression of a gene responsible for resistance to a pathogen. You cloned the gene and made antibody to detect the protein it encodes by a procedure called Western blot analysis, and the mRNA by a procedure called reverse transcription PCR. Explain the type of regulation from the scenarios in the table.

Scenarios
Not treated with pathogen
Treated with pathogen
mRNA
Protein
mRNA

Protein

1

Absent

Absent

Present

Present

2

Present

Absent

Present

Present

3

Present

Present

Absent

Absent

4

Present

Present

Present

Absent

5

Present

Present

Present

Present

3. You are studying the expression of a gene controlling height in your crop species. You have cloned the gene and are interested in determining its expression in different parts of the plant. You use the RT-PCR procedure as in the previous problem. After the PCR part, you load products on a gel and observe the following pattern. Explain your results in the context of gene regulation.

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2.4.10: 10 - Genetic Pathways

Learning Objectives

- Understand how multiple genes often function together to control a trait
- Understand the concept of a biosynthetic pathway
- Understand the concept of a regulatory pathway
- Understand the concept of a network
- Understand how mutations in different genes of a pathway can cause the same phenotype
- Understand how mutations in different genes of a pathway might cause different phenotypes

Introduction

Genes do not function in isolation but rather suites of genes act in concert to perform biological functions. When different genes function in different sequential steps of a biological process, this is known as a **genetic pathway**. Perhaps the most conceptually intuitive type of pathway is a **biosynthetic pathway**, where a precursor molecule is chemically modified through a series of enzymatically catalyzed intermediate steps to produce a bioactive product. Since enzymes are (proteins), the genes that encode these enzymes are considered a genetic pathway. In a **regulatory pathway**, some type of stimulus leads to a change in the expression or activity of a particular gene product, which in turn acts to alter the expression or activity of another gene product or products, which in turn could regulate yet another level of activity.

Ultimately, these regulatory changes in expression or activity lead to a response to the stimulus, typically involving changes in gene expression. Again, the genes that encode the various types of regulatory molecules, mainly proteins, constitute a genetic pathway. Pathways are often targets in the biotechnological manipulation of traits, which requires an understanding of how pathways behave. Also note that many biosynthetic and regulatory systems are better described as networks because of often, pathways branch or converge and interact with other systems. However, for simplicity such complex networks will not be discussed here.

Biosynthetic pathways

Genes encode enzymes that catalyze steps in the synthesis of a compound. A **biosynthetic pathway** actually describes a process of converting a precursor substrate into a product. In addition to the precursor substrate and product, it includes the enzymes, the chemical reactions catalyzed by the enzymes and all the intermediate compounds. A pathway consists of a series of steps where a precursor molecule acts as a substrate for an enzyme, which catalyzes a chemical reaction to produce a product, which then serves as a substrate for a subsequent step.

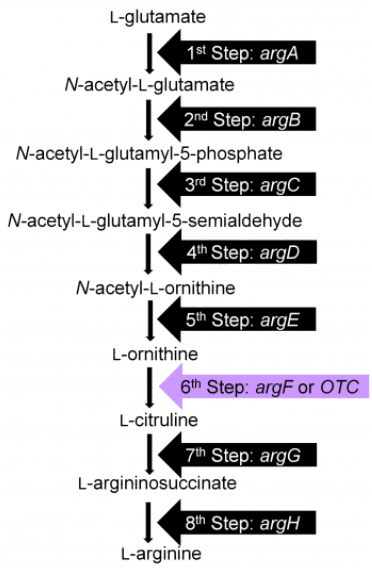


Figure 1. The arginine biosynthetic pathway. The labeled arrows represent chemical reaction steps. The italicized 4-letter symbol (e.g., argF) represents the gene that encodes the enzyme abbreviated to the right that catalyzes each step (e.g., OTC).

We have already discussed the one gene, one enzyme hypothesis which was an important advance in the discovery of how genes encode proteins. This analysis also provides a nice example of the concept of a biosynthetic pathway. This hypothesis was discovered by analyzing **auxotrophic** neurospora mutants that were defective in the production of amino acids. That is, the biosynthetic pathways producing particular amino acids were disrupted by mutations. The text discusses the analysis of the arginine pathway. Mutants that disrupt this pathway cannot grow on minimal medium lacking arginine but can grow if arginine is supplied in the medium. The same fundamental pathway also produces arginine in plants.

Mutations that disrupt the function of particular enzymes in the pathway block the progression of the pathway at that corresponding step. For example, a mutation in the argF gene would disrupt the enzyme ornithine carbamoyltransferase (OTC). This would block step 6 in the pathway, the conversion of L-ornithine to L-citruline. As a result, the intermediate compound L-Ornithine will likely accumulate, whereas all the compounds that occur later in the pathway (i.e., L-Citruline, L-argininosuccinate and L-arginine) will be deficient.

The anthocyanin pathway

The anthocyanin pathway is a slightly more complex pathway, although still simple as far as these things go. It is of significant interest to horticulturists because anthocyanin pigments are responsible for many plant colors, particularly in flowers and fruits (think red wine). Anthocyanin pigments range from orange to red, purple, and blue. In addition to the attractive colors, anthocyanins provide, there is also considerable recent interest in anthocyanins for their dietary value, acting as strong antioxidants and possibly anti-cancer agents.

Effects of mutations at different steps in anthocyanin biosynthesis.

Of course, each enzyme in the anthocyanin pathway is encoded by a gene. As such, mutations can generate variation or disrupt the pathway. The maize anthocyanin pathway and examples of mutants are shown in Figure 2. The ***a2*** mutant causes a deficiency in the anthocyanidin synthase enzyme, blocking the production of any pigmented compounds. The ***a1*** and ***c2*** mutants are similarly colorless. The ***bz2*** mutant is deficient in an enzyme related to glutathione-S-transferase, which is required for transport of anthocyanidins into the vacuole, resulting in the bronze-colored pigment due to the more alkaline environment of the cytosol. The ***bz1*** mutant (not shown) produces a similar phenotype. Mutation of the ***pr1*** gene eliminates just one class of anthocyanins. The ***pr1*** gene encodes flavanone 3′-hydroxylase, required for the production of cyanidin, which generates purple anthocyanin. In the mutant, only pelargonidin is synthesized, producing a red pigment.

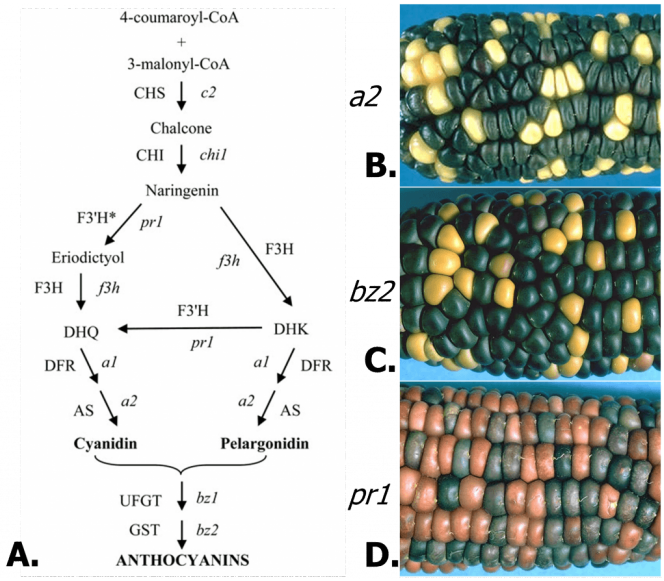


Figure 2. Maize anthocyanin mutants. A. The maize anthocyanin pathway. (Modified from Sharma et al., 2011). B-D. Ears segregating a2 (anthocyaninless2), bz2 (bronze2), and pr1 (red aleurone1) mutant kernels, respectively. Normal kernels are dark purple, nearly black, and the mutant kernels are the lighter colors. Images in B-D courtesy of M.G. Neuffer and MaizeGDB.

Regulatory pathways

A regulatory pathway refers to a situation where one gene or gene product controls the expression or activity of another gene or gene product, which may in turn also perform a regulatory function. As we discussed, gene function can be regulated at many points from transcriptional regulation to post-translational regulation. We will examine a couple of specific examples of regulatory pathways but first let's consider some of the general features of regulatory pathways. Understanding the logic of regulatory pathways will become important later when we seek to use biotechnology to manipulate traits by altering their regulation.

A simplistic but useful way to help understand the logic of regulatory pathways is to consider them as a series of switches with on and off states.

Positive vs. negative regulators

A key feature of regulatory pathways is that regulators might function either positively or negatively. A positive regulator is one that functions to activate the next component in the pathway, whereas a negative regulator would inhibit the next component. For example, a transcription factor that activated the transcription of a gene would be considered a positive regulator, while one that repressed the transcription of a gene would be a negative regulator. Another common type of regulator is a protein kinase. Protein kinases are proteins that add a phosphate group to another protein. Phosphorylation is a common type of post-translational modification that can regulate the activity of proteins; sometimes the phosphorylated protein becomes activated and sometimes it becomes repressed.

A positive regulatory step is represented with an arrow. For example, "protein A activates protein B" would be represented like:

$$\mathrm{A} \rightarrow \mathrm{B} \tag{2.4.10.1}$$

A negative regulatory step is represented with a bar. "Protein C represses protein D" would be represented like:

$$\mathrm{C} \dashv \mathrm{D} \tag{2.4.10.2}$$

Active vs. inactive states of regulators

A second point to consider in the logic of regulatory pathways is the activity state of each component. A useful way to think about this is that each component can have an "on" or an "off" state. Of course, in reality, many proteins can have intermediate levels of activity but for the sake of simplicity, we will just consider the on and off states.

The effect of a regulator being in the on or off state depends on whether it is a positive or negative regulator.

If a positive regulator is in the on state, it will function to activate the next factor. In the example of $\mathrm{A} \rightarrow \mathrm{B}$, if **A** is in the on or active state, it will function to switch **B** to the on or active state.

If a negative regulator is in the on state, it will function to switch the next factor to the off or inactive state. For $\mathrm{C} \dashv \mathrm{D}$, if **C** is in the on or active state, it will switch **D** to the off or inactive state.

Regulatory pathways sometimes contain many, sometimes few, steps. They can also contain a mixture of positive and negative regulators. Let us explore a few hypothetical examples to see how the pieces fit together to regulate plant responses to stimuli.

When a pathway is depicted, all the steps are shown. For the sake of simplicity, it is assumed that the default activity state of any given component is such that the preceding step functions to change it. We will begin with a simple example containing several positive regulatory steps that activate a cellular activity, **Activity 1**, in response to **Stimulus 1**. In the pathway shown below, the **Stimulus 1** activates **A**. Therefore, we assume that in the absence of this stimulus, **A** is in the inactive or off state. Since **A** is inactive, it is not functioning to activate **B**, which is therefore also in the inactive state, and likewise for **C**. Ultimately **Activity 1** does not occur in the absence of **Stimulus 1** but does occur in response to the stimulus.

Stimulus 1 → A → B → C → Activity 1

We can use color coding to help visualize this. Light grey represents the **OFF** state and blue represents the **ON** state.

No Stimulus 1 → A → B → C → Activity 1 (OFF)

Stimulus 1 → A → B → C → Activity 1 (ON)

Or we can make a table to help keep track of the states of each component in the presence or absence of stimulus.

Stimulus 1 →	A →	B →	C →	Activity 1
NO	OFF	OFF	OFF	NO
YES	ON	ON	ON	YES

Now let us look at another example containing both positive and negative regulators.

Stimulus 2 → D → E ⊣ F → Activity 2

In this case, components **D** and **E** would be in the inactive states in the absence of stimulus, because they would not have been activated. But **E** functions as a negative regulator of **F**. Since **E** is inactive, it is not functioning to inhibit **F**, which then remains in the active state promote cellular **Activity 2**. When **Stimulus 2** is present, **D** and **E** become activated, and **E** functions to inactivate **F**. Since **F** is off, **Activity 2** does not occur. Thus, the net response to **Stimulus 2** is the *repression* of **Activity 2**.

$$\dashv \tag{2.4.10.3}$$

$$\dashv \tag{2.4.10.4}$$

OR

Stimulus 2 →	D →	E ⊣	F →	Activity 2
NO	OFF	OFF	ON	YES
YES	ON	ON	OFF	NO

Now that we have considered regulatory pathways from a hypothetical perspective, let's consider a couple real ones. As mentioned, regulatory pathways can take on many forms. They can consist of a series of transcription factors that regulate the expression of one another's genes, ultimately resulting in the regulation of genes that effect some biological response. Here, we will look at a signal transduction pathway consisting of a series of post-translational modifications that occur response to a hormone stimulus and culminate in gene expression changes to effect a response.

Signal transduction pathway for hormone signaling

Brassinosteroids (BRs) are a class of plant steroid hormones that control many aspect of plant physiology. They are most noted for their role in promoting plant growth and mutants deficient in BR biosynthesis or signaling show dwarfism. BRs also promote a range of other responses, including yield and increased stress resistance.

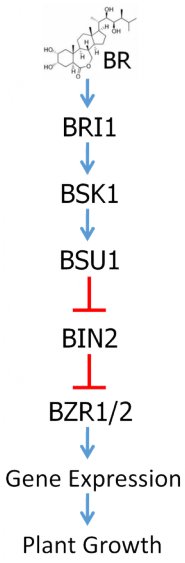


Figure 3. The brassinosteroid signaling pathway. Diagrammatic representation of the regulatory relationships among BR pathway components.

BR hormones, of which brassinolide (BL) is considered the most active, are recognized by a receptor called BRI1. BRI1 is a type of receptor known as a receptor kinase, which spans the cell membrane. On the outside of the cell is a receptor domain which specifically recognizes and binds to BR. There is also a membrane-spanning domain and then a protein kinase domain inside the cell. Binding to BR outside the cell then activates the protein kinase domain inside the cell. Protein kinases are enzymes that function to add phosphate groups to other proteins. As previously discussed, phosphorylation can alter the activity of a protein. Activation of the BRI1 receptor kinase then results in the activation of a signaling pathway that ultimately regulates the activity of transcription factors inside the nucleus, changing the expression of genes that regulate growth and stress resistance.

The BR signal transduction pathway is well studied and is summarized in Figure 3. Do not be concerned with memorizing the names of all these factors, but just focus on understanding the regulatory logic. The overall regulatory relationships of the pathway are depicted in Figure 3. The main target of regulation is a pair of closely related transcription factors called BZR1 and BZR2. BZR1/2 are negatively regulated by a protein called BIN2 in the absence of BR. BIN2 is a protein kinase that phosphorylates BZR1/2, which results in BZR1/2's exclusion from the nucleus and their proteolytic degradation. In the absence of BR, BIN2 contains a phosphate group that is required for its activity.

Binding of BR to the BRI1 receptor domain outside the cell results in the activation of the kinase domain inside the cell. Activated BRI1 then phosphorylates a protein called BSK. Phosphorylated BSK binds yet another protein called BSU1, which is a protein phosphatase. Protein phosphatases are enzymes that remove phosphate groups from other proteins. BSK binding activates BSU1 which then catalyzes the removal of the phosphate on BIN2, thereby inactivating BIN2. Thus, BSU1 is a negative regulator of BIN2. When BIN2 is inactivated, that allows BZR1/2 to accumulate, to enter the nucleus, and to regulate the expression of genes that effect the BR response, including promoting plant growth and yield.

Effects of mutation on different steps

Mutations can affect regulatory pathways in multiple ways. Most mutations are **loss-of-function** mutations where the function of the gene or gene product is partly or completely impaired. Considering the BR signaling pathway, the phenotypes of mutation will differ depending on whether the mutation affects a positive or negative regulator of the pathway. A loss-of-function mutation in the bri1 gene will block the perception of the hormone and therefore the pathway will not be activated; a dwarf phenotype will result. On the other hand, a loss-of-function in the bin2 gene will release BZR1/2 from regulation. The result will be constant BZR1/2 activity regardless of whether BR hormone is present. This might be expected to generate giant plants but in fact the effects of deregulating hormone responses are more complex—let us just call it unregulated growth.

Mutations can also be **gain-of-function**, where the gene product assumes a form that is constantly in the active state. Such mutations are typically dominant and are much less common than loss-of-function. Again, the outcome of a gain-of-function mutation on the BR signaling pathway will vary depending on whether it affects a positive or negative regulator of the pathway. A gain-of-function mutation in a positive regulator would cause deregulated hormone responses, whereas such a mutation in a negative regulator would permanently shut down the pathway and cause dwarfism.

Complementary gene action and genetic epistasis in the context of pathways

Considering that biological processes are controlled by multiple genes acting together, several genetic principles make more sense. First, it becomes clear how mutations in different genes can produce the same phenotypic effects. Since multiple genes are often required for a single biological process, disruption of different genes in the process might have the same net effect. From this follows the concept of **complementary gene action**. Mutant plants with the same phenotype are crossed together and the offspring is normal looking. The mutations in the parent plants likely affected different genes in the same pathway. Finally, the concept of **epistasis** can be best understood in the context of pathways. Note that the term epistasis is used differently by different geneticists. In quantitative genetics, epistasis refers to any gene interaction. Here, the term epistasis refers to a specific type of gene interaction where the action of one gene is masked by the action of another gene. In other words, if two mutations are combined in an individual, only one of the phenotypes is apparent. The mutant whose phenotype is apparent is said to be epistatic to the one that is masked.

Complementary gene action in a regulatory pathway

The concept of complementary gene action is no different in a regulatory pathway than in a biosynthetic pathway. Mutations in different genes that have similar roles in the pathway (e.g., are both positive regulators of the pathway) would be expected to cause similar mutant phenotypes. If such mutants in different genes were crossed together, the F1 progeny would be normal because both genes would be heterozygous and therefore a functional copy of both factors would be present.

Epistasis in a regulatory pathway

Epistasis can be quite complex, especially in regulatory pathways. Unlike complementary gene action, epistasis is not restricted to recessive loss-of-function mutations. The situation is further complicated by the presence of positive and negative regulators in a pathway. In contrast to a biosynthetic pathway, the rule of thumb for a regulatory pathway is that the more “downstream” gene is epistatic to the more “upstream” one. Let’s turn again to the BR signaling pathway and look at a couple of examples to understand why.

First consider loss-of-function mutations in the *bri1* and *bin2* genes, which in single mutants cause a dwarf phenotype and an unregulated growth phenotype, respectively. If the two mutations are combined into a double mutant plant, what do we expect? The *bri1* mutations disrupt the receptor function required to initiate signaling through the pathway. However, BIN2 functions to inhibit the activity of pathway by repressing the function of BZR1/2. If BIN2 is rendered non-functional, then BZR1/2 will be active and promote the growth response. This will be true even if previous steps are blocked by the *bri1* mutation. So the double mutant would show the *bin2* unregulated growth phenotype making *bin2* epistatic to *bri1*.

What about a gain-of-function mutation in *BZR1* or *BZR2* combined with a loss of function mutation in *bri1*? A gain-of-function mutation would render *BZR1/2* constitutively active, regardless of the activity state of BIN2, resulting in the unregulated growth phenotype. Since BIN2 activity would be irrelevant, upstream activities would also be irrelevant. Thus, the double mutant would show unregulated growth and *BZR1/2* would be epistatic to *bri1*.

Lesson Summary

We saw that genes do not act alone but function in concert with other genes to perform various biological functions such as the biosynthesis of a compound or the regulation of a response to a stimulus. When genes control a sequential series of steps, that is called a pathway. But pathways typically branch and intersect with other pathways to form networks. Mutations affect the activity of pathways in various ways, depending on the nature of the mutation (loss- vs. gain-of-function) and the function of the gene product (positive vs. negative effector).

When mutations affect different steps in a pathway, they can often have a similar overall effect on the pathway and therefore lead to similar phenotypic consequences. Such mutations will complement one another even though their phenotypes are identical. It is also possible that mutations in different steps of a pathway can cause different phenotypes. When such mutations are

combined into a double mutant individual, the phenotype of one is often masked by the phenotype of the other epistatic mutation. When pathways are linear, the “upstream” mutation is generally epistatic in biosynthetic pathways and in regulatory pathways it is generally the “downstream” mutation that is epistatic. In branched pathways or networks, it is more difficult to generalize.

Complementary gene action is important for geneticists trying to identify all the steps in a pathway. This is the basis of the classic complementation test used to determine if mutations are allelic or affect independent genes. Epistasis is also useful to geneticists who want to understand whether mutations with different phenotypes affect the same pathway, and if so, to determine the order of gene action.

When biotechnology is used to manipulate a trait, it is really the activity of a pathway or network that is being targeted. The same genetic principles outlined in this section are used to design biotechnological strategies to alter pathway activities so as to produce the desired outcome on the trait of interest.

Activity 1

To answer the following questions, refer to the pathways illustrated in Figure 3. They are labeled slightly differently but a1 encodes DFR, which catalyzes the production of leucoanthocyanidins and a2 encodes AS or LDOX/ANS, which subsequently catalyzes the production of anthocyanidins. Assume that normal, wild-type maize kernels are the deep purple/black color as in Figure 3.

1. What would you predict happens to the levels of leucoanthocyanidins in an *a2* mutant compared to normal?

a. no change
b. increase
c. decrease
d. can’t predict

Show Answer

Answer: b or d.

The expectation would be b, that leucoanthocyanidins would increase. Because the pathway is blocked at the subsequent step, leucoanthocyanidins cannot be converted to anthocyanidins. But since all the preceding enzymes remain functional, those reactions would be expected to continue resulting in the accumulation of leucoanthocyanidins.

d is also a correct response. Biosynthetic pathways do not always behave as expected. For example, there could be feedback inhibition of one or more enzymes by intermediate compounds. Bottom line, we won’t know for certain until the levels are measured experimentally.

2. What would you predict happens to the levels of pelargonidin in an *pr1* mutant compared to normal?

a. no change
b. increase
c. decrease
d. can’t predict

Show Answer

Answer: b or d.

Again, the expectation would be b, that pelargonidin would increase. Normally, some fraction of the intermediate compounds are channeled through the pathway branch leading to cyaniding. Because the pathway leading to cyanidin is blocked it might be expected that all the intermediates would be converted to pelargonidin, causing an increased accumulation.

d is also a correct response. As discussed in problem 1, biosynthetic pathways do not always behave as expected. Feedback inhibition could be a factor or it may be possible that the enzymatic capacity of the pelargonidin branch of the pathway is already saturated. To increase flux through the pathway, it could be required to increase the levels or activities of one or more rate-limiting enzymes. Again, we won’t know for certain until the levels are measured experimentally.

Activity 2

Predict the outcomes of the following mutations on the signaling output, or plant response for the BR signaling pathway.

1. A gain-of-function mutation in the BZR2 gene

a. increased output
b. no change
c. decreased output
d. can't tell

Show Answer

Answer: a. Since BZR2 is a positive regulator of BR signaling, increased output from the pathway would be expected from a gain-of-function mutation.

2. A loss-of-function mutation in the bsu1 gene. (Study the pathway and think about this one!)

a. increased output
b. no change
c. decreased output
d. can't tell

Show Answer

Answer: c. The output from the pathway would decrease causing decreased plant growth. Even though BSU1 is a negative regulator, its target is BIN2 which is another negative regulator. Without BSU1 function, BIN2 will always be in the ON state and inhibit the activities of BZR1/2, therefore decreasing the response to BRs.

Activity 3

1. Predict the phenotype of a BSK gain-of-function.

a. unregulated growth
b. no effect
c. dwarf
d. can't predict

Show Answer

Answer: a. BSK is a positive regulator of the pathway that functions to activate BSU1. Therefore a gain-of-function would cause it to positively activate the pathway, even in the absence of BR hormone, causing unregulated growth.

2. Now predict the phenotype of a double mutant between the BSK gain-of-function and a BIN2 gain-of-function.

a. unregulated growth
b. no effect
c. dwarf
d. can't predict

Show Answer

Answer: c. The gain-of-function BIN2 mutation locks BIN2 in the ON state where it would function to inhibit the activity of BZR1/2. It would be impervious to regulation by the upstream BSU1, so even if BSU1 were continually active due to the gain-of-function BSK, the BIN2 mutant would inhibit growth and therefore be epistatic.

Activity 4

Now you try a couple. Fill in each of the following tables to determine whether the hypothetical Activity will be activated or repressed in response to each Stimulus.

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2.4.11: 11 - Recombinant DNA Technology

Learning Objectives

- Understand the importance of recombinant DNA technology.
- Learn isolation of DNA and its separation on an agarose gel.
- Understand restriction and ligase enzymes and their application in gene cloning.
- Understand vectors and their application in gene cloning and expression.
- Understand polymerase chain termination reaction (PCR).

Introduction

Recombinant DNA (rDNA) technology has resulted in breakthroughs in crop and animal biotechnology. The power of rDNA technology comes from our ability to study and modify gene function by manipulating genes and transform them into cells of plant and animals. To arrive at this several tools of molecular biology are used including, DNA isolation and analysis, molecular cloning, quantification of gene expression, determination of gene copy number, transformation of the appropriate host for replication or transfer into crop plants and analyses of transgenic plants.

Definition and background

Recombinant rDNA technology involves procedures for analyzing or combining DNA fragments from one or several organisms (Figure 1) including the introduction of the rDNA molecule into a cell for its replication, or integration into the genome of the target cell.

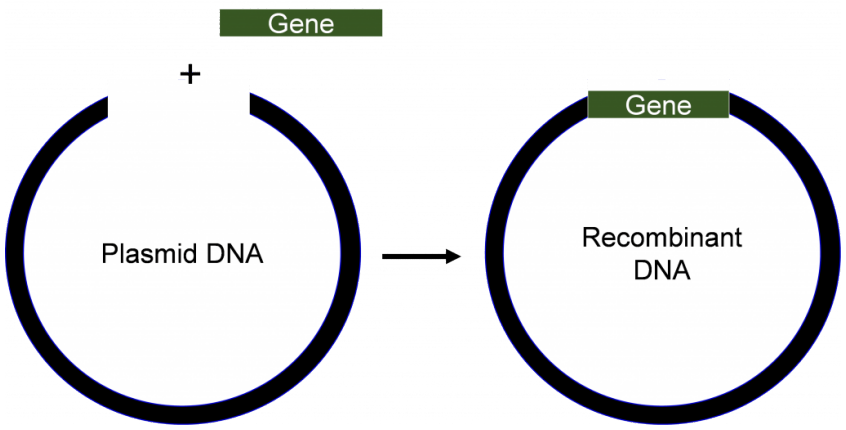


Figure 1. Recombinant DNA is made from combining DNA from different sources. Image by Walter Suza.

Advances in molecular biology in the early 1970s, including the success in creating, and transferring DNA molecules into cells, revolutionized both science and industry. The first genetically modified organisms were bacteria that made simple proteins of pharmaceutical interest, for example, insulin. As the technologies improved, other organisms including plants became amenable for improvement by rDNA technology. Table 1 provides important milestones in the development and application of rDNA technology.

Table 1.Events related to the development and application of recombinant DNA technology.

Event	Year

Mendel’s experiments published

1866

DNA discovered in cell

1869

Mutation of genes by x-rays

1927

One gene-one enzyme hypothesis

1941

DNA is identified as the genetic material

1944

Structure of DNA determined

1953

Ribosomes synthesize protein

1954

Function of mRNA proposed

1961

Genetic code determined

1961-64

Isolation of a restriction enzyme

1970

Recombinant DNA techniques developed

Early 1970s

Isolation of a single copy gene from higher eukaryote

1977

Rapid method of DNA sequencing developed

1977

Plant transformation

1983

Field testing of transformed plants

ca. 1986

Release of engineered plants to general public in the US

1995-96

Transformation of cells with rDNA produces organisms called bioengineered or genetically modified organisms (GMOs). The GMOs contain new traits from another organism. The first GMOs were *Escherichia coli* cells that were transformed with genes from human to produce various proteins for pharmaceutical purposes.

Isolation of DNA, restriction digestions and its separation on an agarose gel:

Preparation of DNA

For recombinant DNA procedures to work, a pure DNA sample must be obtained. The challenge is that plant and animal cells produce numerous other compounds that often act as contaminants and may inhibit cloning or sequencing of the DNA. Also, tissues and organs from the same plant, or different plants often contain different composition of metabolites, for example, proteins, lipids, and carbohydrates. These compounds must be separated from the DNA during isolation. To achieve this, scientists take advantage of the chemical and physical properties of different molecules inside the cell. For example, DNA is negatively charged, making it soluble in aqueous solution. However, the polar sugar phosphate groups of the DNA are repelled by non-polar solutions. Therefore, the final step in many DNA purifications protocols involves precipitation using alcohol. Other compounds, for example, proteins can also be easily separated from DNA by altering the concentration of salt in the extraction buffer.

Digestion of DNA with restriction endonucleases

Restriction endonucleases are a group of enzymes derived (primarily) from bacteria. Although there are several different types of **restriction enzymes**, those most useful for rDNA technology recognize specific short sequences in DNA and cleave the DNA at that site to produce cohesive (sticky) or blunt-ended fragments (Figure 2).

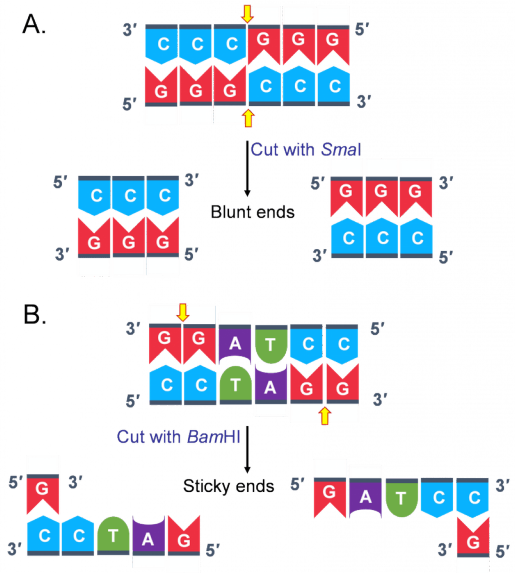


Figure 2. Example of how restriction enzymes cut DNA. (A) Treating the DNA with SmaI results in fragments with blunt ends. (B) Whereas treatment with BamHI produces fragments with “cohesive” or “sticky” ends. Image by Walter Suza.

More than 500 different restriction enzymes have been identified and can be purchased commercially. Thus, one may ask the question, how often does a restriction enzyme cut within a genomic sequence? It is not possible to give an exact answer for this question. However, let us assume that there are 4 bases on any strand of DNA. This means the probability of detecting an A (adenine) at a particular location is 1/4. Now, since most restriction enzymes recognize specific sequences of 6 bases long, the probability of finding such a site is (1/4)6 = 1 site in every 4,096 base pairs (bp). Assuming you have isolated genomic DNA from maize, and you want to digest it with a restriction enzyme that cuts every 4,096 (4,100) bp, how many fragments will you obtain? To answer this question, you need to have an idea of the size of the genome of the plant you are working with. The approximate

size of the maize genome is 2,500,000,000 bp. Thus, using an enzyme that cuts every 4,100 nucleotides one would expect to obtain 2,500,000,000 bp/4,100 bp, approximately 610, 000 fragments. Note that nucleotide distribution is not always random and thus frequency may be different for given DNA. Also, methylation of specific bases in genomic DNA can prevent cleavage at some site.

Separation of digested genomic DNA on agarose gel

The only physical features of nucleic acid fragments that are routinely used for their characterization are their size and nucleotide sequence. Molecular weight of DNA is most conveniently evaluated by electrophoresis in agarose gels. Agarose forms a gel by hydrogen bonding when cooled from the melted state. This gel, interwoven network of agarose chains, interferes with the movement of DNA through the gel (see **Lesson on PCR and Gel Electrophoresis**). Pore size, which affects rate of movement of DNA fragments of a given size, depends on the concentration of agarose. The gel is submerged in electrolyte solution; sample is loaded into wells on one end and current is applied to facilitate movement of DNA fragments. Since DNA is negatively charged it will migrate in the electrical field. Fragments separate according to size. The distance of the migration in each time is proportional to 1/log MW. Following gel electrophoresis DNA can be visualized by staining with ethidium bromide (see **Lesson on PCR and Gel Electrophoresis**) or other DNA stains.

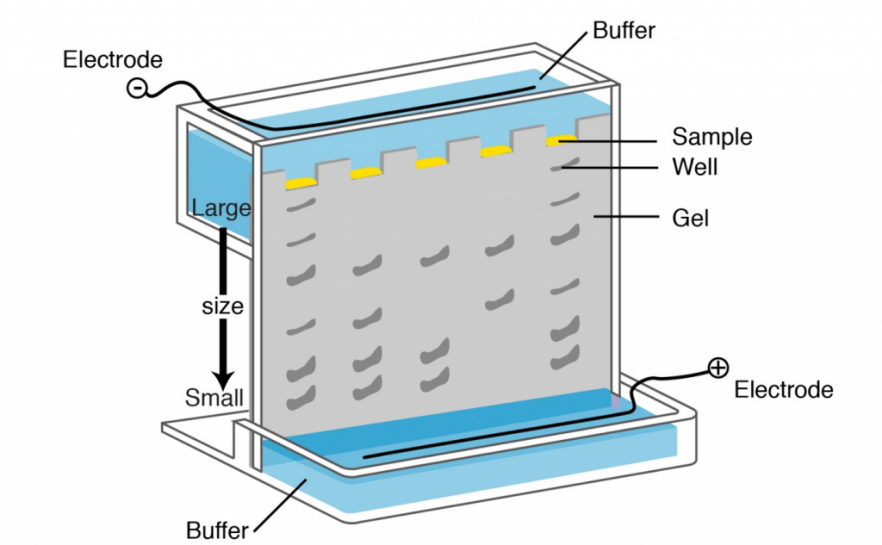


Figure 3. Gel electrophoresis is used to separate nucleic acid (DNA and RNA) or protein molecules by their size. For DNA, the sample is placed in an electric field and the negatively charged DNA will migrate to the positive electrode. Migration speed depends on the size of the DNA fragment. Use of semisolid matrix such as agarose or polyacrylamide gel facilitates separation of the DNA molecules. Image from NIH-NHGRI.

Tools for gene (DNA) cloning

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a method by which millions of copies of a DNA fragment are produced in a test tube in a matter of minutes or a few hours. The basic steps in PCR reaction were discussed in the Lesson on PCR and Gel Electrophoresis.

Once the sequence of a particular gene is known, it becomes possible to use PCR to isolate that gene from any DNA sample. Recall in **Lesson on Gene Transcription** you learned that at the genomic level a gene is made up of regulatory sequences, coding, and non-coding sequences. Thus, if the goal is to use PCR to isolate both regulatory and non-regulatory sequences of a gene, the approach would be to use DNA as the starting material.

In cloning genes by PCR, restriction enzyme sites are added at the 5′-end of the primers to facilitate cloning of PCR fragments. A few additional nucleotides (~6 nucleotides) added at the 5′-end of the restriction sites to facilitate restriction digestion of PCR products prior to cloning in a plasmid vector. Alternatively, PCR products may be cloned directly into a T-vector without restriction digestions in *E. coli* (read more about Promega cloning vector systems). After cloning into *E. coli*, the fragment is analyzed by sequencing and then sub-cloned into suitable vectors for expression studies.

Like all other biochemical processes, DNA synthesis by PCR is not a perfect process, and occasionally the polymerase enzyme will add an incorrect base to the growing DNA strand. In the context of DNA replication in a cell, the errors are corrected by the DNA polymerase, this is called “proofreading”. Commercially available polymerases may or may not have proofreading capability.

Another important consideration in PCR analysis is contamination.

Minor contamination of the starting material can have serious consequences. Recall that minute amounts of starting DNA can be amplified to millions of copies through PCR. If one inadvertently (or carelessly) mixed DNA from two different sources, the results will be confounding making it impossible to distinguish lines and may cost a laboratory time and money. Ensure that proper procedures are followed in preparing PCR assays. One common source of contamination in plant biology is the products from previous amplification processes. A completed PCR reaction will contain millions of copies of amplified fragments so that even a minute droplet or aerosol from a pipette tip will contain an enormous number of amplifiable molecules. It is always essential to run negative controls which will reveal the presence of contaminating DNA in your PCR assays.

Cloning vector definition and requirements

A cloning vector is a specialized DNA sequence that can enter a living cell and provide means for detection of its presence to a researcher by conferring a selectable property on the host cell (e.g., resistance to antibiotics), and possess means for self-replication. A vector must also possess easily distinguishable physical traits, such as size, or shape, to allow purification away from the host cell’s genome.

Ligase enzyme and gene cloning

Cutting and joining together of vector and DNA fragments from different origins results in rDNA. Recall that restriction endonucleases are used to cut DNA. To join DNA molecules together, an enzyme called DNA ligase is used. The enzyme DNA ligase is used to seal together restriction fragments by forming new phosphodiester bonds. The ligated vector and DNA fragment can now be transformed into a host cell for replication and expression.

The transformation of *E. coli* takes several steps. First, a gene of interest is inserted into a plasmid that contains a selectable marker usually encoding for resistance to an antibiotic. Second, the plasmid construct containing the gene of interest is transformed into bacterial cells by briefly exposing the mixture of ligated plasmid-DNA fragment (rDNA molecule) and bacterial cells to cold (0°C) and heat (37-42°C). The next step is to grow the transformed cells on selection media containing an antibiotic. Only the cells that have been transformed with the plasmid containing the gene of interest and the marker for resistance to the antibiotic will survive. In addition to using an antibiotic, plasmid vector systems that contain the *lacZ* gene encoding β-galactosidase allow for easier selection of positive colonies that may harbor the rDNA molecule of interest.

Types of cloning vectors

An example of a cloning vector is a **plasmid** (Figure 4), defined as an autonomously replicating extra chromosomal circular DNA which is faithfully passed on to progeny. Plasmids are double stranded circular DNA and range in size from about 1 kb – 200 kb. The most useful for cloning are 2 – 10 kb because smaller plasmids are easier to manipulate and usually produce higher copy numbers when grown in bacterial host cells.

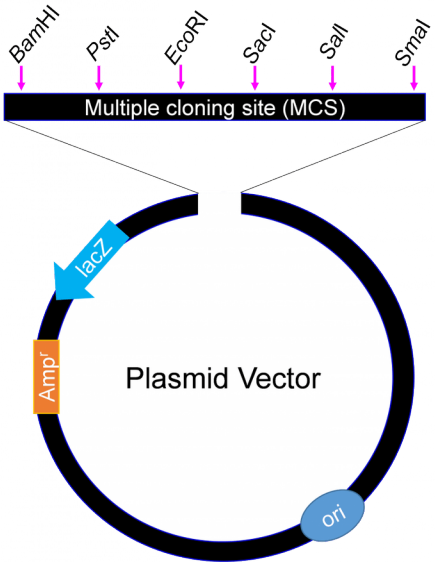


Figure 4. Basic map of a plasmid vector. The MCS contains several restriction enzymes sites and few are shown here as example. Image by Walter Suza.

Generally, no more than 10 kb is cloned into plasmids. For cloning large DNA fragments with high efficiency, a vector called **bacteriophage lambda** is used. Large chromosomal DNA fragments close to 23 kb are stable when introduced into a lambda phage vector.

mRNA as starting material for gene cloning

Recall in the **Lesson on Transcription** you learned about synthesis of RNA from DNA through the process of **transcription**. Transcription is an important step in gene expression. The mRNA produced can be isolated and "copied" back to DNA by a process called **reverse-transcription** (Figure 5). The first step of reverse transcription mimics a strategy used by retroviruses (e.g., HIV) that have RNA genomes. As part of their gene-transmission package, retroviruses also contain an enzyme called RNA-dependent DNA Polymerases, commonly referred to as **reverse transcriptase**. After infecting a host cell the retrovirus uses its reverse transcriptase to copy its single stranded RNA genome into a strand of complementary DNA (cDNA). The reverse transcriptase then synthesizes the second DNA strand from the first strand to make a double stranded-DNA copy which integrates into the host genome.

If only a gene's coding sequence is required, isolating the gene from cDNAs would be the strategy. It is important that cDNAs are synthesized from tissues expressing the gene of interest. Thus, prior knowledge of where the gene is functional is important in constructing the cDNA molecules for cloning the gene of interest.

The cDNAs produced *in vitro* (Figure 4) can be used for PCR analysis, similar to chromosomal DNA. The combination of reverse-transcription and PCR (RT-PCR) is a valuable tool in gene cloning and quantification of mRNA.

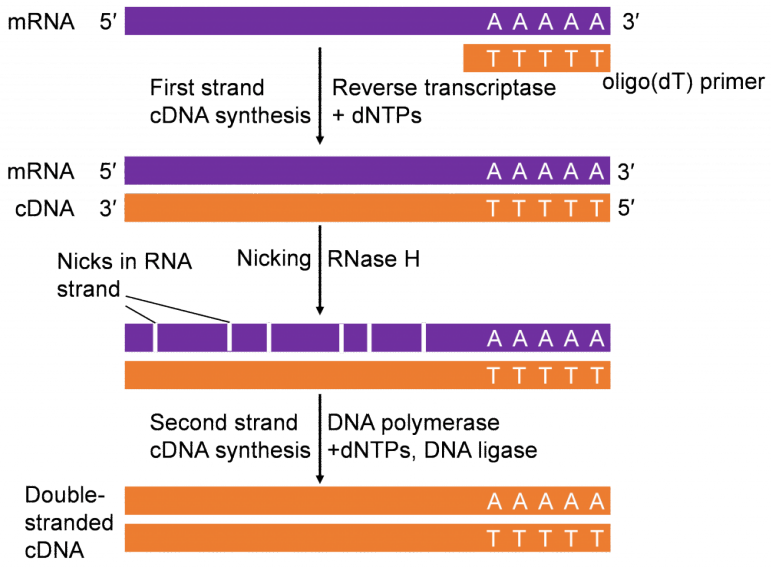


Figure 5. A population of mRNA isolated from plant tissues is combined with oligo(dT) primers that anneal to the poly(A) tail of the mRNAs to initiate the reverse transcription of cDNA from mRNA template with dNTPs. The result is hybrid molecules (mRNA-DNA). Treatment with RNAse H causes degradation of the mRNA leaving an intact single stranded DNA (first strand). DNA polymerase synthesizes the second strand by adding complementary dNTPs to the growing chain. The reagents for RT usually come in pre-mixed kits making such an assay easy to carry out routinely in most laboratories. Most mRNAs from cells in tissues analyzed are converted to cDNAs. Thus, using primers specific to a sequence, that double stranded cDNA can be amplified by PCR for various purposes for example, gene cloning. Image by Walter Suza.

Lesson Summary

Recombinant DNA technology has contributed significantly to development of agricultural biotechnology. Transformation of cells with rDNA produces organisms called bioengineered or genetically modified organisms. The tools for plant rDNA technology include, vectors, restriction enzyme, ligation enzymes, bacterial hosts, methods to isolate and multiply nucleic acids, methods to quantify nucleic acids, Agrobacterium as a vector to insert foreign DNA into plants.

Lesson Activities

Activity 1

Below is a hypothetical DNA fragment containing restriction sites for EcoRI and BamHI.

1. How many fragments will be produced after cutting the DNA with BamHI? What size will these fragments be in bp?
2. How many fragments will be produced after cutting the DNA with both BamHI and EcoRI at the same time? What size will these fragments be in bp?
3. How many fragments will be produced after cutting the DNA with EcoRI? What size will these fragments be in bp?

Activity 2

1. You need to clone an EcoRI fragment in the EcoRI site of an expression plasmid vector in E coli. The fragment is 1,809 bp long. There is a BamHI site in the fragment at 1,204 bp. In the plasmid vector, there is a single BamHI site at the promoter region for expression of the gene in E. coli. The BamHI site is 100 bp upstream of EcoRI cloning site.

Following cloning the EcoRI fragment you will get two classes of clones, only one of which will produce the protein. Describe the approach you will take to identify the correct class of clones for expression of the gene in E. coli.

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2.4.12: 12 - Genetic Engineering

Learning Objectives

- Define genetic engineering.
- List and briefly explain the five basic steps in genetic engineering. Describe why each is necessary.
- Identify the fundamental differences between genetically engineered crops and non-genetically engineered crops.
- Explain the limitations to traditional breeding that are overcome by genetic engineering.
- Identify the approximate length of time required to obtain a marketable transgenic crop line (complete the entire crop genetic engineering process).

Introduction

The production of genetically engineered plants became possible after Bob Fraley and others succeeded to use Agrobacterium tumefaciens to transform plant cells with **recombinant DNA** in the early 1980s (Vasil, 2008a). Since this breakthrough in plant biotechnology, GM crops are now routinely developed and grown in many parts of the globe. Current statistics on adoption of genetically engineered crops in the U.S. can be found on the USDA Economic Research Service's website.

Genetic engineering has been used successfully to develop novel genes of economic importance that can be used to improve the genetics of crop plants. Genetic engineering is the targeted addition of a foreign gene or genes into the genome of an organism. The genes may be isolated from one organism and transferred to another or may be genes of one species that are modified and reinserted into the same species. The new genes, commonly referred to as transgenes, are inserted into a plant by a process called transformation. The inserted gene holds information that will give the organism a trait (Figure 1).

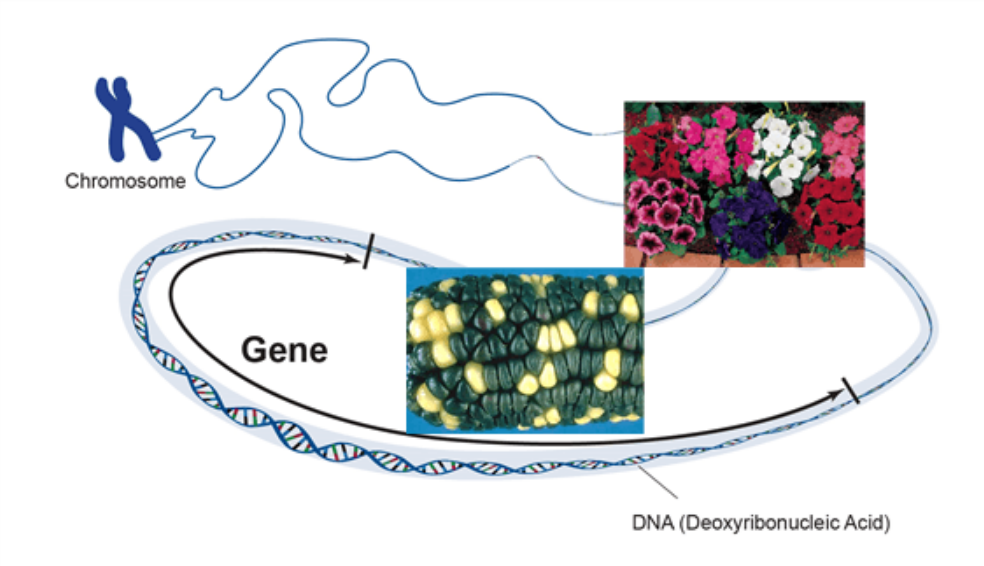


Figure 1. Traits, such as flower and seed color, are controlled by DNA. Adapted from NIH-NGHRI.

Crop genetic improvement (plant breeding) is an important tool but has limitations. First, in conventional terms, genetic improvement can only be done between two plants that can sexually mate with each other. This limits the new traits that can be added to those that already exist in that species. Second, when plants are mated, (crossed), many traits are transferred along with the trait of interest including traits with undesirable effects on yield potential.

Genetic engineering, on the other hand, is not bound by these limitations. It physically removes the DNA from one organism and transfers the gene(s) for one or a few traits into another. Since crossing is not necessary, the 'sexual' barrier between species is overcome. Therefore, traits from any living organism can be transferred into a plant. This method is more specific in that a single trait can be added to a plant.

The overall process of genetic engineering. A basic explanation of the five steps for genetically engineering a crop is provided. The five steps are:

1. Locating an organism with a specific trait and extracting its DNA.
2. Cloning a gene that controls the trait.
3. Designing a gene to express in a specific way.
4. Transformation, inserting the gene into the cells of a crop plant.
5. Cross the transgene into an elite background.

Step 1: DNA Extraction

The process of genetic engineering requires the successful completion of a series of five steps and discoveries. To better understand each of these, the development of Bt maize will be used as an example.

Before the genetic engineering process can begin, a living organism that exhibits the desired trait must be discovered. The trait for Bt maize (resistance to European corn borer) was discovered around 100 years ago. Silkworm farmers in the Orient had noticed that populations of silkworms were dying. Scientists discovered that a naturally occurring soil bacteria was causing the silkworm deaths. These soil bacteria, called Bacillus thuringiensis, or Bt for short, produced a protein that was toxic to silkworms, the Bt protein.

Although the scientists did not know it, they had made one of the first discoveries necessary in the process of making Bt corn. The same Bt protein found to be toxic to silkworms is also toxic to European corn borer because both insects belong to the Lepidoptera order. The production of the Bt protein in the bacteria is controlled by the bacteria's genes.

To be able to work with the gene responsible for making the Bt toxin, scientists must extract DNA from the Bt bacteria (Figure 2). This is accomplished by taking a sample of bacteria containing the gene of interest and taking it through a series of steps that separate the DNA from the other parts of a cell.

Step 2: Gene Cloning

The second step of the genetic engineering process is gene cloning. During DNA extraction, all the DNA from the organism is extracted at once. This means the sample of DNA extracted from the *Bacillus thuringiensis* bacteria will contain the gene for the Bt protein, but also all the other bacterium's genes. Scientists use **gene cloning** to separate the single gene of interest from the rest of the DNA extracted (Figure 2).

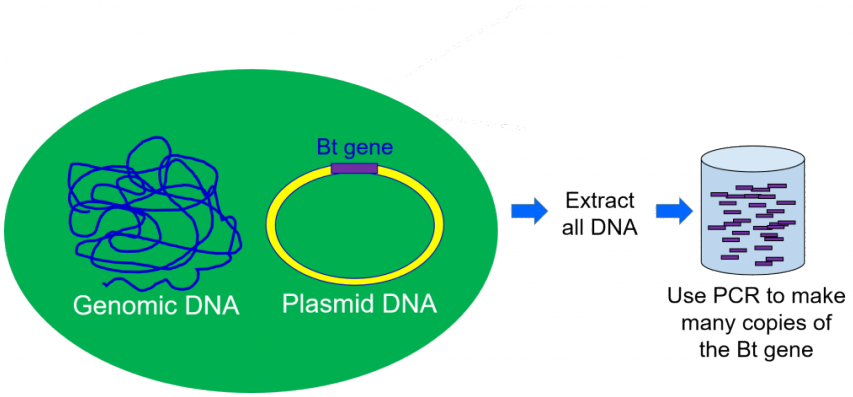


Figure 2. Using DNA from *B. thuringiensis* to clone the Bt gene. Image by Walter Suza.

The next stages of **genetic engineering** will involve further study and experimentation with this gene. To do that, a scientist needs to have thousands of exact copies of it. This copying is also done during the gene cloning step.

Step 3: Gene Design

Gene design relies upon another major discovery. This was the 'One gene One enzyme' Theory first proposed by George W. Beadle and Edward L. Tatum in the 1940's. Discoveries made during their research laid the groundwork for the theory that a single

gene stores the information that directs the cell in how to produce a single enzyme **(protein**). Therefore, there is a single gene that controls the production of the Bt protein. It is called the Bt gene.

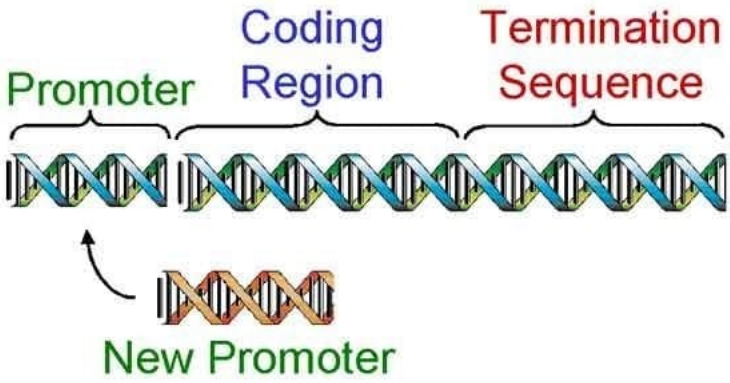


Figure 3. Replacing existing promoter with new promoter. Image by Patty Hain.

Once a gene has been cloned (Figure 2), genetic engineers begin the third step, designing the gene to work once inside a different organism. This is done in a test tube by cutting the gene apart with **restriction enzymes** and replacing certain regions (Figure 3).

Scientists replaced the bacterial gene promoter with promoters turn on the Bt gene in selected parts of the plant or promoters that can always turn on the **Bt gene** in all tissues. As a result, the first Bt gene released was designed to produce a level of **Bt protein** lethal to European corn borer and to only produce the Bt protein in green tissues of the corn plant, (stems, leaves, etc.). Later, Bt genes were designed to produce the lethal level of protein in all tissues of a corn plant, (leaves, stems, tassel, ear, roots, etc.).

Plant transformation and tissue culture

The process of transformation involves the insertion of the desired **transgene construct** (Figure 5) into cells of the recipient plant species. In this process, scientists isolate tissue or cells from the cultivar they wish to transform and use one of several methods to insert the transgene into the tissue or cells. The transgene construct contains the following key features.

a. A *promoter that acts to turn the gene on and off in the cell*. The CaMV 35s promoter from the cauliflower mosaic virus (CaMV) is commonly used in genetic engineering. Other types of promoters, such as, the nopaline synthase promoter (NOS-Pro) also may be used to express transgenes in plant tissues.
b. A *selectable marker that is used to select cells that successfully obtained the construct during the transformation process*. In figure 4, the selectable marker in the construct is NPT II (Kanr) that controls resistance to the antibiotic kanamycin. The cells of the plant used for transformation will be grown on a media containing the antibiotic. Other selectable markers that have been used successfully in plants include genes controlling herbicide resistance.
c. A *terminator sequence*, such as the nopaline synthase (NOS) is included to mark the end of the transgene sequence for proper expression in plant cells.

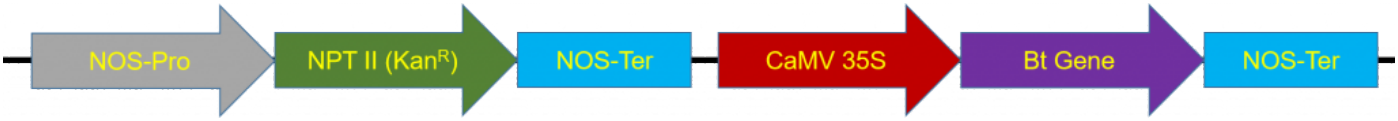


Figure 4. The basic elements of a transgene construct. Image by Walter Suza.

Two commonly used transformation methods include Agrobacterium tumefaciens-mediated transformation and biolistics transformation (aka **gene gun**), commonly referred to as particle bombardment (Figure 5). The biolistics method involves the use of high pressure to propel tungsten or gold beads coated with DNA of the gene construct into plant cells.

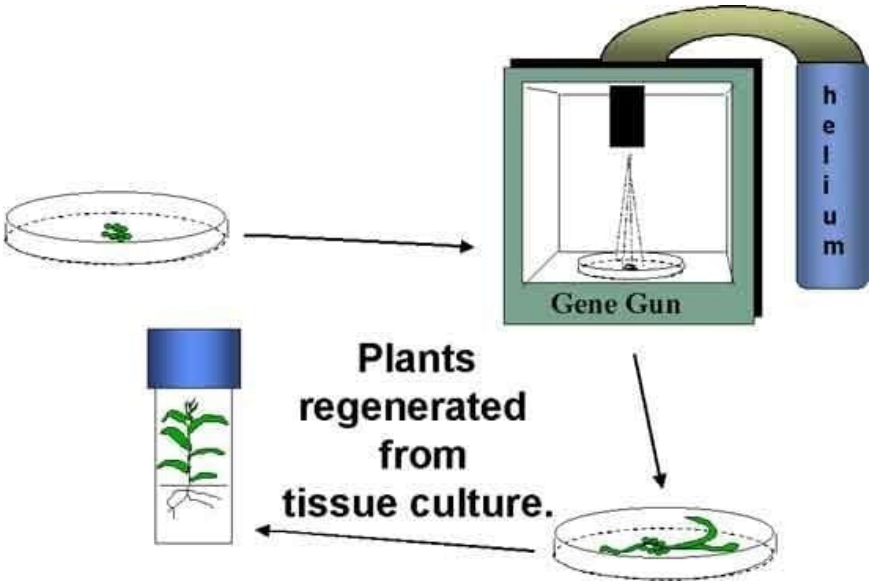


Figure 5. Using the gene gun method to transform plant cells. Image by Patty Hain.

Agrobacterium-mediated plant transformation

Crown galls are tumors of plants that arise at the site of infection by some species of the Agrobacterium. *Agrobacteria* do not enter the plant cells but transfer a DNA segment called T-DNA from their circular extra chromosomal *tumor-inducing* (Ti) plasmid into the genome of the host cells. Ti plasmids are maintained in *Agrobacteria* because a part of their T-DNA contains genes that encode unusual amino acids used by Agrobacterium. The T-DNA also encodes genes that affect host plant hormone physiology resulting in induced growth of the infected cells and tumor formation. Scientists took advantage of Agrobacterium's ability to stably integrate its T-DNA into the plant genome for introducing rDNA into plant cells. They first removed the genes that cause tumor or crown gall disease in plants from the T-DNA and engineered the plasmid for replication in both *Escherichia coli* and Agrobacterium cells. The initial replication of the construct in *E. coli* is useful for verifying the presence of the cloned gene and increasing the quantity of construct DNA for subsequent uses, including sequencing and transformation into Agrobacterium.

The steps in Agrobacterium-mediated transformation of plants are described in Figure 6.

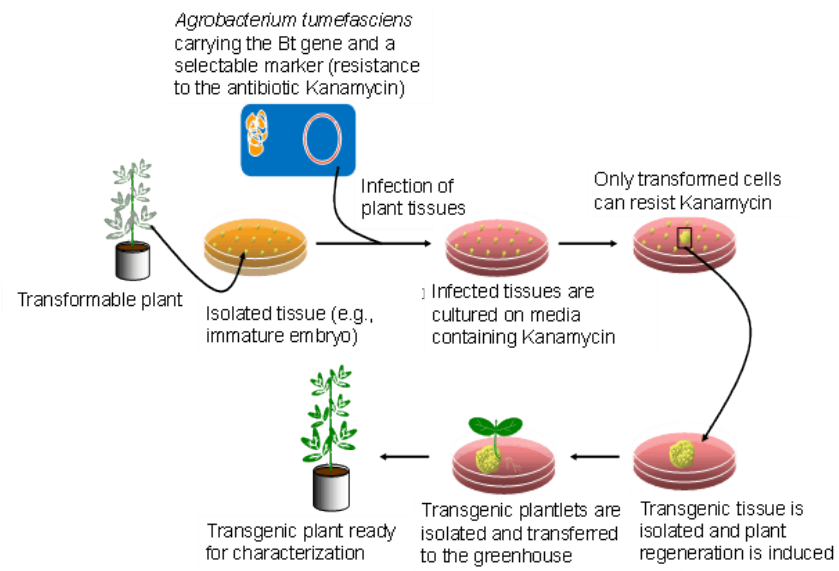


Figure 6. Transferring genes into plant cells by the Agrobacterium method. After infection of select tissue with the bacterium carrying a transgene construct with an antibiotic resistance gene as the selectable marker, the tissue is grown in a medium that contains the antibiotic that will kill all untransformed tissues or cells. Therefore, only tissues whose cells have been transformed with the transgene construct survive in the presence of the antibiotic. The surviving tissue is removed from the antibiotic and allowed to regenerate into whole plants. Normally, transgenic plants will be monitored in a controlled environment such as a growth chamber or greenhouse before they are grown in the field. Image by Kan Wang.

At present, very few host cells receive the construct during the transformation process. Each random insertion of the construct into the genome of plant cells is referred as an event. Useful events are rare because of the random nature of the transformation process. Selectable markers are very important because they allow the identification of the rare events (Figure 7). Scientists must screen many potential transformants to identify events that are useful for breeding.

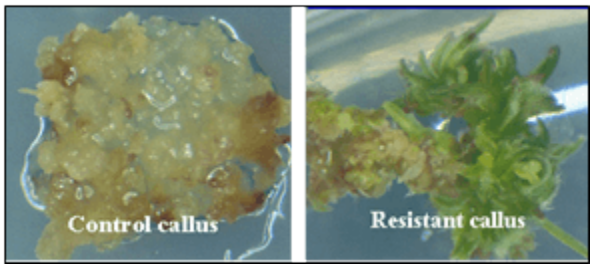


Figure 7. Selection for herbicide tolerance in buffalograss transformed with the gene that makes plants tolerant to glyphosate. The control calli lacking the glyphosate tolerance gene are killed by the herbicide that is part of the media on which the cells are grown (left panel). The calli in the right panel grew from a cell that received the glyphosate resistance gene during transformation and survived on the media. Image by Shui-Zhang Fei.

From there, the new DNA may or may not be successfully inserted into a chromosome. The cells that do receive the new gene are called **transgenic** and are selected from those that are not transgenic (Figure 7). Many types of plant cells are totipotent meaning a single plant cell can develop into an entire plant. Therefore, each transgenic cell can then develop into an entire plant which has the transgene in every cell. The transgenic plants are grown to maturity in greenhouses and the seed they produce, which has inherited the transgene, is collected. The genetic engineer's job is now complete. He/she will hand the transgenic seeds over to a plant breeder who is responsible for the final step.

Inheritance of a transgene in plants

Transformation is successful when a transgene is incorporated into one of the chromosomes. The cells that have only one copy of the transgene in their genomes are said to be hemizygous (hemi = half, zygous = zygote). Because the segregation in the progeny of a hemizygous plant is the same as for a heterozygous plant, the term heterozygous will be used in this course when referring to a plant that is not homozygous for the transgene. The trait will segregate in the progeny in the same manner as any other gene in the plant as illustrated below (Figure 8).

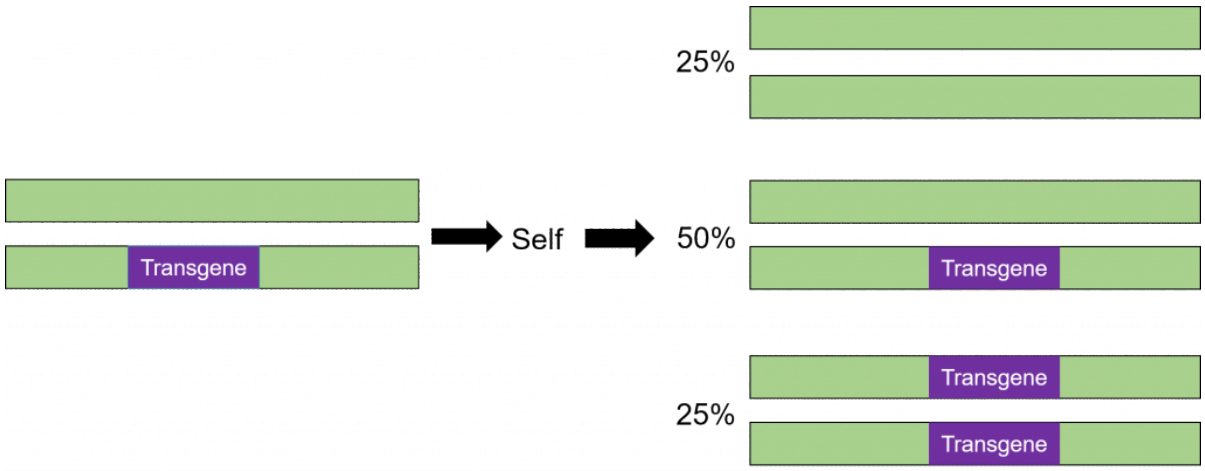


Figure 8. The ratio of possible offspring when a hemizygous diploid plant is self-pollinated. Image by Walter Suza.

Step 5: Backcross Breeding

The fifth and final part of producing a genetically engineered crop is backcross breeding (Figure 9). Transgenic plants are crossed with elite breeding lines using traditional plant breeding methods to combine the desired traits of **elite** parents and the transgene into a single line. The offspring are repeatedly crossed back to the elite **line** to obtain a high-yielding **transgenic** line. The result will be a plant with a yield potential close to current hybrids that expresses the trait encoded by the new **transgene**.



Figure 9. Using the backcross breeding method. Image by Patty Hain.

The Process of Plant Genetic Engineering

The entire **genetic engineering** process is basically the same for any plant. The length of time required to complete all five steps from start to finish varies depending upon the gene, crop species, and available resources. It can take anywhere from 6-15+ years before a new **transgenic** hybrid is ready for release to be grown in production fields.

The tissue culture process of regenerating transgenic plants from callus may result in genetic variation that is not associated with the transgene. Also, the parent line used for transformation commonly is selected for the frequency with which useful events can be obtained and not its agronomic performance. Therefore, transgenes are incorporated into commercial cultivars by conventional breeding procedures, such as backcrossing.

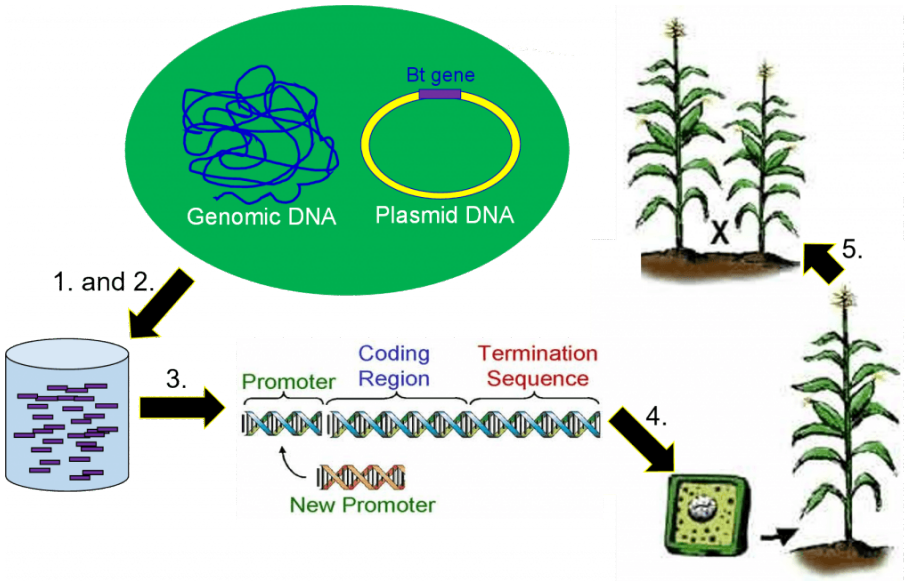


Figure 10. Crop genetic engineering includes: 1) DNA isolation 2) gene cloning 3) gene design 4) transformation, and 5) plant breeding. Image by Walter Suza and Patty Hain.

Key Takeaways

Genetic engineering is the directed addition of foreign DNA (genes) into an organism.

Five basic steps in crop genetic engineering:

1. DNA extraction – DNA is extracted from an organism known to have the desired trait.
2. Gene cloning – The gene of interest is located and copied.
3. Gene modification – The gene is modified to express in a desired way by altering and replacing gene regions.
4. Transformation – The gene(s) are delivered into tissue culture cells, using one of several methods, where hopefully they will land in the nucleus and insert into a chromosome.
5. Backcross breeding – Transgenic lines are crossed with elite lines to make highyielding transgenic lines.

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2.4.13: 13 - Introduction to Mendelian Genetics

Learning Objectives

- Outline the experimental approach Mendel used to propose the idea that genes exist, control traits, and are inherited in predictable ways.
- Compare the methods used by Mendel and Punnett to predict trait inheritance.

Introduction

In plant and animal genetics research, the decisions a scientist will make are based on a high level of confidence in the predictable inheritance of the genes that control the trait being studied. This confidence comes from a past discovery by a biologist named Gregor Mendel, who explained the inheritance of trait variation using the idea of monogenic traits.

Monogenic characters are controlled by the following biological principles:

- Living things have genes in their cells that encode the information to control a single trait. These genes are stable and passed on from cell to cell without changing.
- The genes are in pairs in somatic cells. When these cells divide to form gametes, the pair of genes is divided. One gene from the pair goes into a gamete.
- Male gametes (pollen) combine with female gametes (eggs) in the wheat flower pistil and fuse to form the next generation (zygote). Gamete union is random.
- The zygote, again, has two copies of each gene. As the zygote grows into a multicellular seed and the seed grows into a plant, the same two gene copies are found in every cell.

Let's take a short genetics history lesson to understand their confidence.

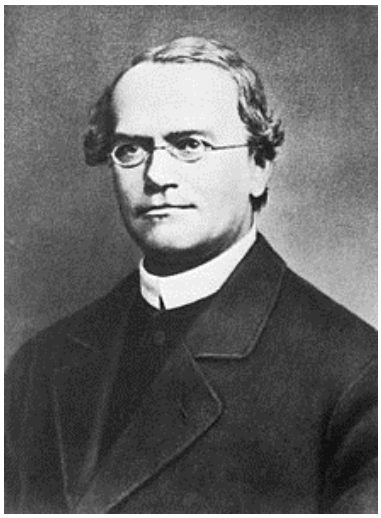


Figure 1. Gregor Mendel was born Johann Mendel.

Mendel's Peas

In the mid 1800's, an Austrian monk named Gregor Mendel (Figure 1) decided he should try to understand how inherited traits are controlled. He needed a model organism he could work with in his research facility, a small garden in the monastery, and a

research plan. His plan was designed to test a hypothesis for the inheritance of trait variation.

Since Mendel could obtain different varieties of peas that differed in easy to observe traits such as flower color, seed color and seed shape, and he could grow these peas in his garden, he chose peas as the model organism for conducting his inheritance control study. A model is easy to work with and often what you learn from the model you can apply to other organisms.

The Hypothesis

While many biologists were interested in trait inheritance, at the time Mendel conducted his experiments none of the biologists had published evidence that inheritance could be predicted. Mendel made this bold statement. His hypothesis was that he could observe "mathematical" regularities in the appearance of a trait that was passed on from parents to their offspring. Mendel had the idea that mathematical regularities could be observed and could be used to explain the biology of inheritance!

The Plan

Mendel's experimental plan was designed to test the hypothesis. He identified true breeding lines of peas by allowing them to self pollinate (which we will refer to as "selfing") and examining their offspring. Pea plants have flowers that contain both male and female reproductive parts; if a pea flower is left undisturbed, the male and female gametes from the same flower will combine to produce seeds, the next generation. If the pea always made offspring like itself, Mendel had his true breeding line. He then made planned crosses between lines that differed by just one trait (monohybrid crosses). The controlled monohybrid cross was the first step in his experiment that allowed him to look for mathematical regularities in the data for three generations. Table 1 below shows the data from a series of these monohybrid cross experiments.

The Analysis

By summarizing his data in a single table, Mendel could look for those hypothesized math regularities. A regularity is a repeated observation.

Table 1. Mendel result from crossing peas.

Character			
Cross and Phenotypes	**F_1**	**F_2**	**F_2 Ratio**
Seed form			
Round X Wrinkled	All round	5474 Round 1850 Wrinkled	2.96 to 1
Cotyledon color			
Yellow X Green	All yellow		

6022 Yellow 2001 Green

3.01 to 1

Seed coat color*

Gray X White

All gray

705 Gray 224 White

3.15 to 1

Pod form

Inflated X Constricted

All inflated

882 Inflated 299 Constricted

2.95 to 1

Pod color

Green X Yellow

All green

428 Green 152 Yellow

2.82 to 1

Flower position

Axial X Terminal

All axial

651 Axial 207 Terminal

3.14 to 1

Stem length

Tall X Short

All tall

787 Tall 277 Short

2.84 to 1

*Gray seed coat also had purple flowers; White seed coat had white flowers.

Table 1 demonstrates that Mendel was serious about the math. He generated large numbers of offspring that allowed him to observe mathematical ratios. From his table of data, we can see mathematical patterns appear with every monohybrid cross he made.

- F_1: All the plants had the same phenotype as one of the parents.

- F_2: Both phenotypes are present, the phenotype that was not expressed in the F_1 appears again in the F_2 but is always the least frequently produced. The average ratio is about **3:1** for the two phenotypes.

What was striking to Mendel was that every character in his study exhibited the same kind of mathematical pattern. This suggested that the same fundamental processes inside the plant's reproductive cells were at work controlling the inheritance of each trait.

Now Mendel had the task of providing a description of the fundamental biology process controlling each of these traits. He needed to come up with ideas that no one had yet proposed to explain biology.

New Idea #1:

The traits expressed in the pea plant were controlled by some kind of particle. These hereditary particles are stable and passed on intact from parent to offspring through the sex cells. (NOTE: Sex cells or gametes were not a new idea, Mendel was aware that biologists knew sexually reproducing plants and animals needed to make gametes.) We now call these particulate factors genes and will use that term in the rest of this reading.

New Idea #2:

Genes are stable, and genes can have alternative versions (alleles).

New Idea #3:

Genes are in pairs in somatic cells and these paired genes separate during gamete formation. Each gamete will have one gene from the pair of genes. The segregating of the paired genes from the somatic cells of the parent into gametes is random. Because segregation is random, a parent that has two different alleles for a gene pair will make two kinds of gametes and makes these gametes at equal frequencies.

From Mendel's ideas, we can see that in a situation in which there was a normal version of a gene (we can call it the R gene) and an alternate version (r), the plant could produce gametes with just the R gene or just the r gene.

New Idea #4:

Plant flowers are designed to allow male gametes (pollen) to combine randomly with the female gametes (egg). When the gametes randomly come together, they bring the genes they carry to the same zygote. This means plants could have the genotype **RR, Rr**, or **rr** in families that have both the **R** and **r** alleles.

New Idea #5:

Mendel proposed that the genes controlling a trait not only paired in somatic cells, they also interacted in controlling the traits of the plants. For the traits in his experiment, he proposed that one allele interacted with the other in a dominant fashion. That means a plant that is the genotype **RR** would have the same phenotype as an **Rr** plant. The **R** allele is dominant to the **r** allele.

Ideas and Data advance science

Those were Mendel's new ideas; he used them to make sense of his experiment data and observations. Let's think like Mendel and apply those ideas.

All the F1 were the same

Mendel's new ideas could explain this observation. Since his parents were true breeding, he was always making a cross between homozygous parents. Homo means the same, so the parents had two copies of the same version of the gene.

Crossing RR X rr plants to produce Rr

Since the **R** is dominant to **r**, then the **Rr** offspring (named the F_1) look the same (have the same phenotype) as the **RR** parent. Therefore, only one phenotype is observed in the F_1. But the F_1 genotype is different from either parent. It is heterozygous (two different alleles).

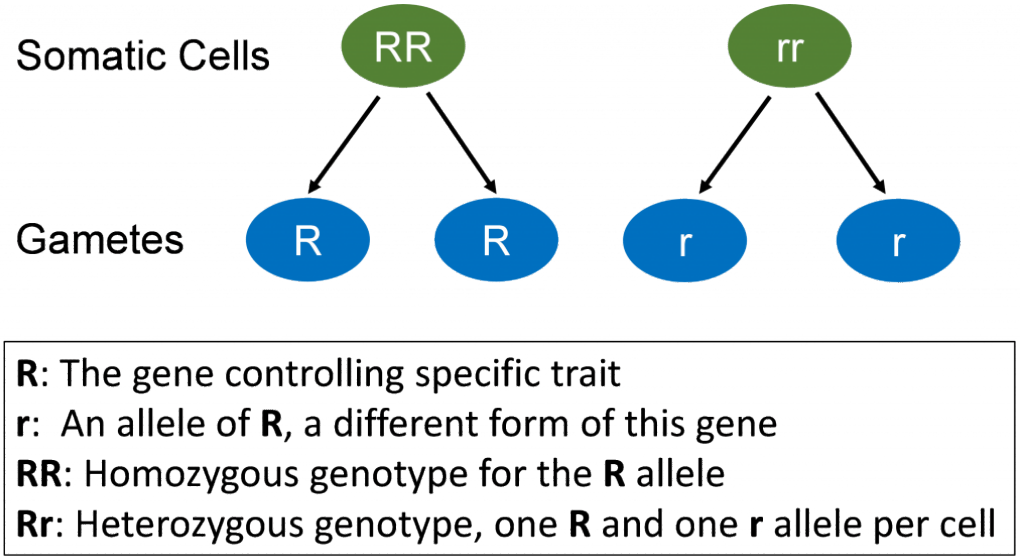


Figure 2. Mendel used letters to represent the genes (he called them particulate factors) that he proposed were found in pairs in the cells of the pea plants. These could vary in form (alleles) and segregate when gametes (sex cells) were formed as the plants prepared to sexually reproduce. Image by Walter Suza.

The F_2: both traits appear in about a 3:1 ratio

Mendel could explain the reappearance of the recessive trait and the ratio by combining the idea of genes with the idea of random segregation. Mendel used simple algebra to explain this result.

First, he wrote out a mathematical expression to account for the gametes made in the male part of the F_1 flower or in the female part.

½ R + ½ r = all the gametes made (Figure 2).

Next, he reasoned that if pollen randomly united with the egg to combine the genes in the gametes, then algebra could be used to predict the result by multiplying the gamete expressions.

(½ R + ½ r) X (½ R + ½ r) = all the F_2 offspring made.

If we do the multiplication above, we get ...

¼ RR + ¼ Rr + ¼ Rr + ¼ rr = ¼ RR + ½ Rr + ¼ rr = predicted fractions of F2 genotypes.

WAIT!

If this math is causing your brain to lose focus, you might be experiencing what Mendel's contemporaries experienced when they read his published research paper. While many biologists were motivated to understand how the variation among animals and plants was controlled and inherited, it took biologists 30 years to recognize that Mendel's new ideas to explain inheritance of traits in peas could be applied to inheritance of traits in other living organisms.

One possible explanation for this 30-year delay in appreciation is that it was difficult for biologists to understand how math could explain biology. One biologist that did understand what Mendel was describing was Punnett. Punnett decided to convert Mendel's algebra into a more graphic representation of the process of gamete segregation and random union.

The Punnett Square

Math: (¼ RR + ½ Rr + ¼ rr).

Punnett designated the gametes made in the male and female parents with single letters (Figure 3). The diagram shows that when the gametes combine, the offspring (inside the squares) again have the genes in pairs in their cells. Accounting for the random union of gametes is accomplished with the four squares in the diagram. Two squares give the same **Rr** result, one the **RR** genotype and one **rr**. Both the algebra and diagram approaches provide the same prediction. Crossing an **Rr** with an **Rr** will produce three genotypes, **RR, Rr** and **rr**. They will be produced in a ratio based on the principle of segregation.



Figure 3. The square diagram accounts for all the gene inheritance possibilities from selfing the Rr (F_1) parents. If inheritance is random, math can be applied, and the diagram used to predict both possible outcomes and the fractions expected of each.

The genes controlling the monogenic traits behaved in predictable ways

Punnett's diagram clarified for many biologists what Mendel was telling them in his published article. This was a challenging idea to understand because he was asking biologists to use something they could not see (genes) and explain something they could see (traits in peas or some other living organism).

Because Mendel recognized he was proposing a very different idea with the segregation principle, he was likely motivated to share the most convincing evidence possible. Mendel conducted additional experiments. One experiment was to test the hypothesis that there were two different kinds of F_2 which expressed the dominant trait, and these two types were being made by the F_1 in predictable fractions. How would Mendel show that F_2 which had the same phenotype did not always have the same genotype?

Mendel tested the breeding behavior of the F_2. Mendel harvested all the selfed seed produced by his F_2 and grew progeny rows of F_3. His segregation principle predicted that of the dominant F_2, there should be two that are heterozygous for every one homozygote made (on average). The results of this experiment are summarized in **Table 2**. Did Mendel's data support the hypothesis?

Table 2. Selfing dominant F_2 to produce F_3

F_2 type	**Mixed rows**	**True breeding**	**Ratio**
Round seed	372	193	1.93 to 1

F_2 type	Mixed rows	True breeding	Ratio
Yellow cotyledon	353	166	2.13 to 1
Gray seed coat	64	36	1.78 to 1
Inflated pod	71	29	2.45 to 1
Green pod	60	40	1.50 to 1
Axial flower	67	33	2.03 to 1
Tall plant	72	28	2.57 to 1

Average ratio heterozygote F_2 to homozygote F2 was 2.06 to 1.

The data show that, if we select a sample of F_2 with the dominant trait (Round seed or Yellow cotyledon), the principle of segregation predicts that there should be 2 heterozygotes for every 1 homozygotes.

Mendel's data from rows of F_3 that all came from F_2 with the dominant trait supported his hypothesis. There were always two kinds of rows (true breeding and mixed) and the rows were in a 2:1 ratio. This fits with the **principle of segregation**.

By publishing these results in a scientific journal, Mendel allowed other scientists to learn from his work. This story reveals the real power of publishing research in the "permanent" scientific literature. The power of publication does not mean you were right with your science. The real power is that other scientists can find your paper, read it, think about your ideas, and then test them. In Mendel's case, he was already dead when his fellow biologists discovered that his new ideas to explain the biology of peas were not only correct, but universal in their application.

Mendel's Dihybrid Cross Experiments

Proper credit must be given to the idea of independent assortment. Gregor Mendel was the first to put this idea down on paper based on what he observed with his pea experiments. Furthermore, Mendel performed additional experiments to back up his ideas. Let's examine his experiments with peas from the late 1800's.

The outline below describes Mendel's dihybrid cross experiments. The pattern observed in the results should look familiar!

The Experiment

- **Parents:** round seeds, yellow seeds (RRYY) x wrinkled seeds, green seeds (rryy).
- **F_1:** All round and yellow seeds (RrYy).
- **Selfing:** F_1 (RrYy x RrYy):

Mendel explained his results as follows:

The F_1 plants have the genotype RrYy and can make four kinds of gametes RY, Ry, rY and ry.

Table 3. Gametes are in equal frequencies in the male and female parts of the plant.

	Possible gametes
Male gametes	¼ RY + ¼ Ry + ¼ rY + ¼ ry
Female gametes	¼ RY + ¼ Ry + ¼ rY + ¼ ry

Table 4. Punnett square demonstrates the possible genotypes produced when the RrYy dihybrid is selfed.

	RY	**Ry**	**rY**	**ry**
RY	**RRYY**	RRYy	RyYY	**RrYy**
Ry	RRYy	RRyy	**RrYy**	Rryy
rY	RrYY	**RrYy**	RrYY	RrYy
ry	**RrYy**	Rryy	rrYy	rryy

Note that with both the Mendel algebra and Punnett square, the **RRYY** genotype occurs one time and the **RrYy** genotype occurs four times (Table 4). Mendel's algebra and Punnett's squares can be summarized to give the same results.

Selfing the F_2 to produce F_3

Table 5. Phenotype classes and their fractions in F_2.

Phenotype	**Number**	**Fraction**
Round, yellow	315	9/16
Round, green	108	3/16
Wrinkled, yellow	101	3/16
Wrinkled, green	32	1/16

The easiest experiment to perform was to let the plants self-pollinate and then keep good records. After scoring his 556 F_2 seeds (Table 5) he took the 315 that were round and yellow and planted them in one part of his garden. The plants that grew were allowed to self-pollinate. Of the 315 round and yellow seeds planted, 301 plants matured and produced seed. The seed produced was the F_3 generation. At harvest, Mendel needed to exercise the utmost care. Each F_2 plant was handled separately. The seeds from the plant were harvested and Mendel then scored the F_3 seeds that came from the same F_2 plant. This can be referred to as $F_{2:3}$ data and the table below summarizes his complete experiment using all of the F_2 phenotypes.

Mendel's F_2 data supported his principle of independent assortment. There were four different types of round yellow F_2 based on

the kinds of progeny they could produce or their breeding behaviors. Based on the F_3 progeny produced, the F_2 genotype was deduced. For example, if a round, yellow seed gave all round progeny it must have the genotype **RR**. If it gave both round and wrinkled it was **Rr**.

Furthermore, the numbers of F_2 plants with each breeding behavior were in agreement with what was expected with independent assortment. There were four times as many round and yellow F_2 that gave all four phenotypes of F_3 seeds (138) compared to the round and yellow F_2 that were true breeding (38). Overall, there were nine types of breeding behaviors demonstrated in the F_2 demonstrating that there were nine F_2 genotypes. In all cases, the fractions observed in the F_2 agreed to the principle of independent assortment. Mendel's well-planned experiment provided a convincing demonstration that genes behaved in this predictable manner.

The only thing better than performing an experiment that shows you were right about a new hypothesis is performing two experiments that show that you were right. That is what Gregor Mendel did! In his second experiment he crossed dihybrid F_1 plants with homozygous recessive plants in a test cross. This type of cross is named because the geneticist wants to perform a cross that will test or reveal the genotype of an organism. Therefore, a test cross is usually made between an organism with a dominant trait and a partner with a recessive version of this trait. Mendel performed the **RrYy x rryy** testcross and the expected progeny are shown in the Punnett square below:

RrYy gametes: **RY, Ry, rY, ry**

rryy gametes: all **ry**

	RY	**Ry**	**rY**	**ry**
ry	RrYy	Rryy	rrYy	rryy

The observed result closely matched the expected. The testcross experiment provides additional support for the principle of independent assortment.

Mendel established a rigorous precedent for using carefully planned multi-generation experiments to reveal the principles that governed trait inheritance. The beauty of Mendel's accomplishments is that both the principles and his experimental approach can be applied to understanding the genetic control and inheritance of traits in many kinds of organisms still today.

Lessson Summary

Mendel's principles of segregation and independent assortment are valid explanations for genetic variation observed in many organisms. Alleles of a gene pair may interact in a dominant vs. recessive manner or show a lack of dominance. Even so, these principles can be used to predict the future...at least the potential outcome of specific crosses.

Video Example

Watch this video about Punnett Squares for more information

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2.4.14: 14 - Deviations from Mendelian Genetics- Linkage (Part 1)

Learning Objectives

At the completion of this lesson, you will be able to:

1. Contrast the inheritance of traits that are controlled by independent genes, linked genes and pleiotropic genes.
2. Demonstrate the physical basis of linkage by drawing the key events in meiosis.
3. Calculate map distances from 2-point test cross and F2 data.
4. Assemble linkage maps from map distance information.

Maize Genetics

Maize (*Zea mays, corn*) is not only a commercially important crop; it has also been a valuable organism used in basic genetic studies to understand genetic principles. Geneticists such as Dr. John Osterman, in the School of Biological Sciences at the University of Nebraska, use corn in basic genetic studies. Let's examine one experiment.

Many seed traits in corn have been studied by geneticists. Color in the outside (aleurone) or inside (endosperm) of the seed can be important for end uses such as what color of corn chips you prefer. Starch development traits in the kernel influence what the corn seed can be used for (popcorn, sweet corn, waxy corn).

A line of corn that was true breeding for a red kernel color was crossed to a true breeding line with shrunken kernels but no red color (white or yellow). Even though the two parents differed by two traits, kernel color and kernel shape, the F_1 offspring produced from the cross were all red with plump or normal kernels.

Parents: Red, plump x white, shrunken

F_1 offspring: All red, plump

Which phenotypes would you say are dominant based upon this single result? Going a step further, what is your hypothesis for the genotypes of the parents and F_1?

The next year these red plump seeds were planted in the crossing nursery near the white shrunken line. Dr. Osterman made a **backcross** or **testcross** between the F_1 progeny and white, shrunken plants.

Thinking back on the genetics principles we have covered so far in this course, what phenotypes would you expect to observe in the testcross progeny?

If we use the principle of independent assortment and the information we already have, we would predict that there should be four types of seeds in testcross progeny.

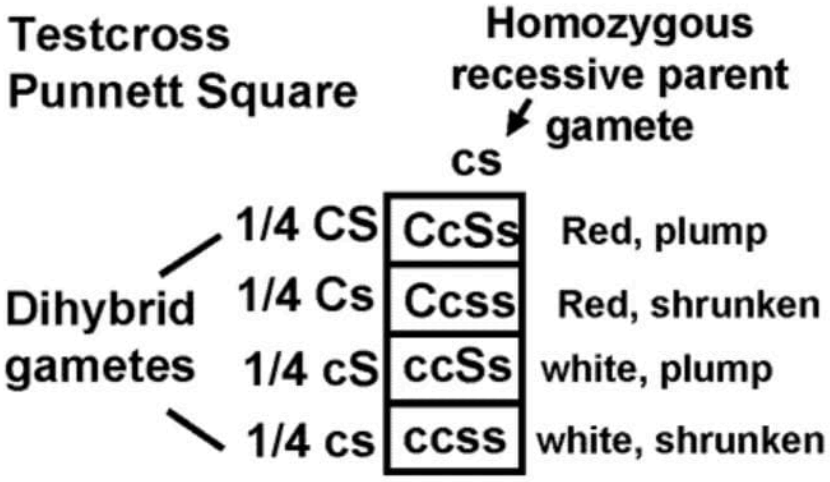


Figure 1. Seed trait inheritance in test cross progeny assuming independent assortment. Image by Donald Lee.

We should get both parental combinations of red, plump and white, shrunken. We should also get two new combinations; red, shrunken and white, plump. Furthermore, the principle of independent assortment predicts the four combinations should be in a 1:1:1:1 ratio. (Make sure you understand why!)

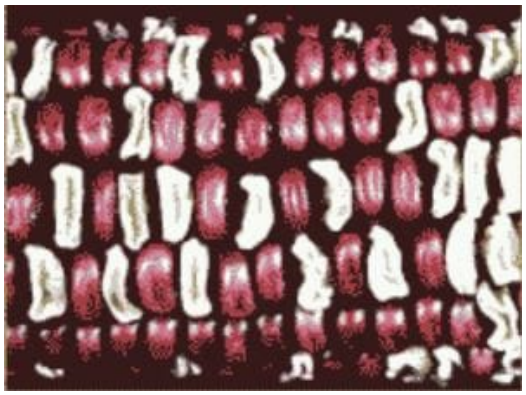


Figure 2. Testcross progeny showing red, plump and white, shrunken phenotype. Image by Donald Lee.

What did Dr. Osterman actually observe? When he harvested the ear from the first plant, peeled back the husks, and examined the kernels, all he saw were red, plump and white, shrunken seeds. Only the parental combination of traits was found among the several hundred seeds on that ear!

Half the seeds had the dominant red and plump combination while the other half had the recessive traits. While this result does not fit what we expect based on the idea of independent traits, Dr. Osterman was not surprised. Obviously, another genetic hypothesis can explain this result. Let's examine two alternative explanations.

Pleiotropy

Sometimes a single gene pair will control more than one trait. For example, barley that has good malting characteristics for brewing also tends to sprout prior to harvest. High sucrose lines of soybean have a sweeter flavor in soymilk and cause less gas production in the consumer compared to lines with normal sucrose levels. Perhaps a seed color gene in corn also controls whether the kernel is plump or shrunken. White kernels would always be shrunken, red would always be plump. When genes control more than one trait, these traits will always be inherited together. This explanation is consistent with what Dr. Osterman observed on the seeds of the testcross ear.

Linkage

The second explanation involves two genes but looks closely at their chromosome locations. Cytogeneticists have observed ten pairs of chromosomes in the somatic cells of corn. Corn has countless traits controlled by tens of thousands of genes. Since genes are on chromosomes, it makes logical sense that each chromosome is made of thousands of genes. When genes are on the same chromosome they will travel together as the chromosome is passed on to the gametes (Figure 3). If the gene for red vs. white kernel color is on the same corn chromosome as the gene for plump vs. shrunken, those genes will travel together during gamete formation. This could also explain why the red and plump gene alleles are inherited together. The same would be true of the white and shrunken alleles on the homologous chromosome in the F_1. Therefore, we could explain the result by the hypothesis of linkage; a separate gene pair controls each trait but these genes are located near each other on the same chromosome.

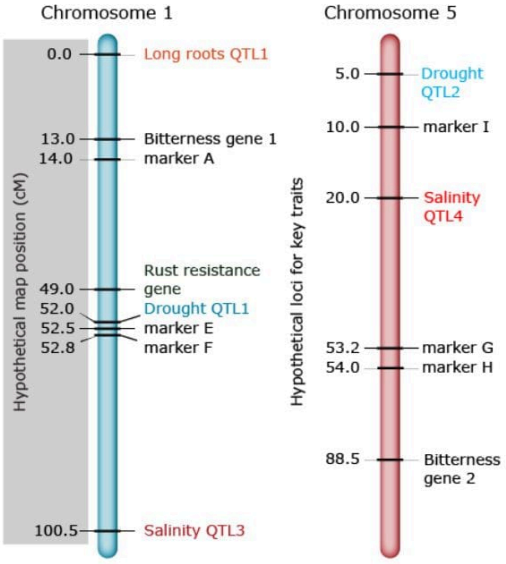


Figure 3. Genes located on the same chromosome are genetically linked. Genetic linkage analysis can be used to determine the order of genes on chromosomes. Closely linked genes are not segregating independently, like genes located on different chromosomes. Linked genes can be used as genetic markers, which have become an important tool in plant breeding. Image by Walter Suza.

More Data

Two genetic hypotheses can explain the testcross data. How can we determine which hypothesis is correct? Like all good geneticists, Dr. Osterman made an effort to generate large numbers of offspring in his studies. He had made several test crosses that summer and each cross generated ears with several hundred seeds. Upon careful examination of the seeds on all the ears, Dr. Osterman did observe a few red shrunken and white plump kernels. The new combinations were rare but their appearance in the testcross progeny allows us to eliminate one of the genetic hypotheses. Which hypothesis can be ruled out?

Linked Genes Tend to Stay Together

Because the red trait and plump trait are not always found together, one gene cannot be responsible for both traits, the occurrence of the recombinant red, shrunken and white, plump testcross offspring eliminates this as a reasonable hypothesis. Can we still use linkage as an explanation though? Yes, if we think through chromosome behavior during meiosis, we can see why genes on the chromosome tend to stay together but will not always be passed on together. Let's think about meiosis and crossing over to understand these results.

Crossing Over

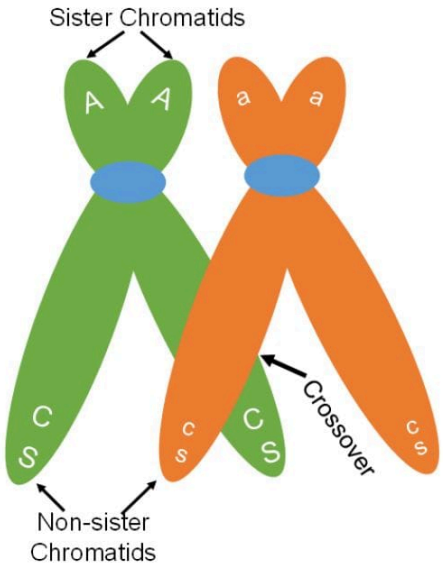


Figure 4. Crossover not between C,c and S,s. Only CS and cs. Parental gametes are made. Image by Marjorie Hanneman.

One of the key differences between meiosis and mitosis is the synapsis of homologous chromosomes during prophase I of meiosis. Synapsis is the process in which homologous chromosomes carefully pair. The pairing allows for an orderly first division to send one chromosome from each pair to separate cells. The close association of the homologous chromosomes also allows for crossing over between non-sister chromatids (Figure 4). During this process sections of the chromosomes break off and are exchanged between non-sister chromatids. When non-sister chromatids crossover, chromatids can be made that have a new combination of genes compared to the original combination on the chromosome. The original combination was inherited from the organism's parents and is called the parental combination of genes. The new combination made is called the recombinant combination. In figure 4, a crossover occurs but the original or parental combination of **CS** (red and plump) and **cs** (white and shrunken) will stay together. Crossing over can cause new gene combinations to occur on a chromosome if the crossover occurs between the linked genes.

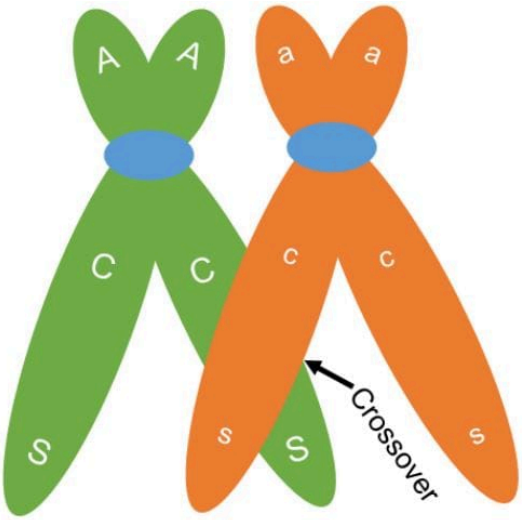


Figure 5. Crossover between the C,c and S,s gives Cs and cS recombinant plus CS and cs parental. Image by Marjorie Hanneman.

When a crossover occurs between genes, chromatids with both the parental combination and chromatids with a new combination will be made. We can see this in figure 5. Two of the chromatids are not involved in the crossing over. These chromatids will maintain the parental combination and when meiosis is complete, the two gametes made that have these chromosomes will be called parental gametes. The gametes made that have the other two chromosomes, those that went through crossing over and have the new gene combination, are called recombinant gametes (Figure 6).

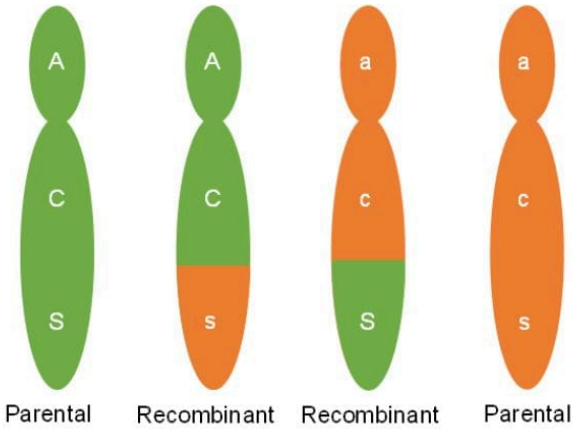


Figure 6. Parental and recombinant gametes from crossovers between C,c and S,s. Image by Marjorie Hanneman.

When crossing over occurs between two non-sister chromatids, cells will make equal numbers of recombinant and parental gametes. In looking back at Dr. Osterman's data though the number of plants that would have inherited recombinant type gametes was far below 50%. Why are parental combinations of linked genes made more frequently than new combinations? Understanding this requires us to imagine many cells going through gamete formation.

Making Lots of Gametes

Sexually reproducing organisms tend to make a large number of gametes to ensure reproductive success. Therefore, we need to think about crossing over events happening in many cells. Geneticists do not understand all the details of crossing over but in general, crossing over is a random process that occurs during **prophase I**. We also know that the occurrence of one crossover along a chromosome can reduce the chance of a second crossing over. As you look at figure 5, you can see that crossing over could occur at any point between where the **C,c** genes (the **C,c** locus) and the **S,s** locus reside on the chromosome causing recombinant gamete formation. If a crossover does not occur between the **C,c** and **S,s** loci (plural for locus), then only the parental gamete combination of **CS** and cs will be made. Considering the size of the chromosome and the relative positions of the **C,c** and **S,s** loci; how often would cells have crossovers occur between the genes compared to somewhere else along the chromosome? If crossing over is random, we would not expect a cross over to occur in very many cells between the **C,c** and **S,s** loci. Genes that are on the same chromosome tend to stay together at a rate that is proportional to how far apart they are on the chromosome. Since the **C,c** and **S,s** loci are close to each other, crossovers are rare. The **'C'** gene tends to stay with the **'S'** gene as it is passed from the red, plump line to the F1 and then to the testcross generation. The same is true of the **'c'** and **'s'** parental combination.

How does this discussion of crossing over help us understand what we see from our test cross result? The parental combinations of red, plump, and white shrunken were almost always made. The new combinations of red, shrunken and white, plump were rarely observed. It would take a recombinant gamete to make one of these rare combinations. While we never see the genes in the gametes or directly observe the genes on the chromosomes, the outcome suggests that these loci are linked close together on the chromosome. Making the connection between the results of crossing studies such as these has allowed geneticists to map genes or determine their relative positions on a chromosome. Let's go through a mapping study to see how it is done.

Two-Point Testcross Mapping

The results of a testcross study different from Dr. Osterman's is given below

- Red, Shrunken **CCss** X White, Plump **ccSS**
- F_1: all Red, Plump (**CcSs**)
- F_1 (**CcSs**) crossed with White Shrunken (**ccss**)

Table 1. Testcross progeny data

Phenotypes

of progeny
Red, plump
120
Red, shrunken
3420
White, plump
3334
White, shrunken
126
Total
7000

Obviously, the geneticist who did this experiment made a lot of test crosses to generate these numbers. How can we use the numbers to map these genes? With this information we can answer one question. 'What is the distance between the **C,c** and **S,s** loci?' We cannot get out a fancy microscopic ruler and physically measure the distance on the corn chromosome. With this information all we can do is estimate the map unit distance. Map units are a measure of the tendency for crossovers to occur between two loci. Because genes that are farther apart will have a higher likelihood of crossovers, the higher the crossover frequency, the farther apart the genes are on the chromosome. Let's apply this idea to our test cross data.

Test cross data allows us to indirectly measure the frequency of gametes made by an individual. All of the testcross progeny inherited a gamete with the recessive '**c**' and '**s**' alleles from the white, shrunken parent. Therefore, the alleles that the F_1, dihybrid parent has passed on determine the traits in the seed (Table 1). We need to be able to measure how often crossovers occurred between the **C,c** and **S,s** loci when these dihybrids made gametes. From the data we have the following:

- Gametes that were passed to the F_1 from the parent lines were **Cs** and **cS**.
- Gametes made from crossing over in the F_1 (recombinant gametes) were **CS** and **cs**.
- Gametes with the original parent combination (parental gametes) were **Cs** and **cS**.

Therefore, the frequency of recombinant gametes was 246 (120 + 126) divided by the total gametes we have information on (7000) or 246/7000 = **3.5%**

Map unit distance between the **C,c** and **S,s** loci = 3.5 Map units.

One map unit is equal to 1% recombinant gametes. Again, this is not a physical measurement. It is a relative measure of how often crossovers occurred between these loci. How reliable is this measurement? Let's look at another data set (Table 2).

- Red, plump **CCSS** X White, shrunken ccss
- F_1: all Red, Plump (**CcSs** or **CS / cs**)
- F_1 (**CS / cs**) crossed with White Shrunken (**ccss**)

Table 2. Testcross progeny data from another data set

Phenotypes
of Progeny
Red, plump

192

Red, shrunken

5

White, plump

3

White, shrunken

200

Total

400

Recombinant gamete frequency: 5 + 3 / 400 = 2%; 2 map units from **C,c** to **S,s**

Based on this result is the map unit distance measurement reliable? Yes, it is, to a certain degree. It is important to recognize the differences between the two test cross experiments.

Cis Versus Trans

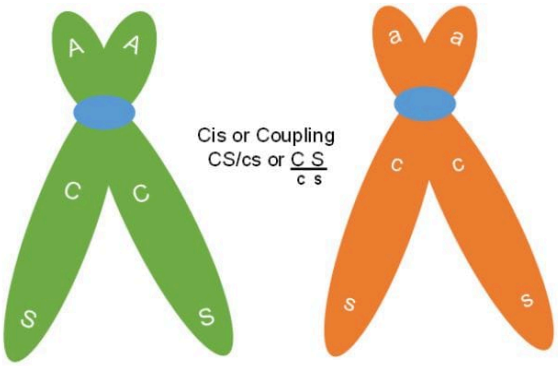


Figure 7. Dihybrid in Cis. Image by Marjorie Hanneman.

The first difference to note is that the parental gametes are not always the same allele combinations, but they are always the most frequently produced gametes. In the smaller experiment above, the parents had either both dominant or both recessive traits together. Therefore, the F_1 parent was making **Cs** and **cS** recombinant gametes. This dihybrid parent was in **cis** or **coupling phase** with respect to these genes (Figure 7).

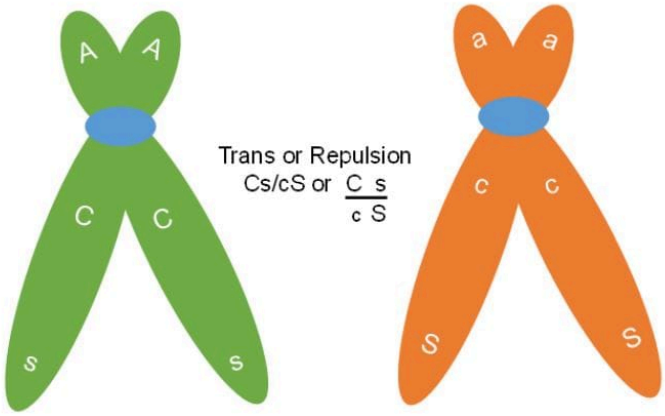


Figure 8. Dihybrid in Trans. Image by Marjorie Hanneman.

In the larger experiment with 7000 testcross progeny the dihybrid F_1 was in trans or repulsion phase for the **C,c** and **S,s** genes. In this case the **Cs** and **cS** are the parental gametes (Figure 8). Therefore, when genes are linked, they can be arranged in two ways in a dihybrid. This means that a **CcSs** plant can have its genotype in the cis arrangement with both dominant genes on one chromosome and both recessives on the other (written **CS / cs**) or can also be in trans (**Cs / cS**).

Map Distance Measurement

The first experiment gave us 3.5 map units and the latter gave 2 map units.

Why do our two experiments give us two different map unit distances? In both cases, we can see that the parental gene combinations have a strong tendency to stay together. Because the experiments were done with different population sizes, with different plants, and the difference in our map unit estimates is small, the difference could be attributed to chance. Environment may also influence the rate of crossing over to some degree. For practical purposes this gene mapping information is reliable enough to tell us that these two genes reside close to each other on the same chromosome. Once we have information on distances between other genes, we can improve our map.

Mapping Another Seed Trait Gene Pair

A third seed trait in corn is normal vs. waxy starch. Waxy starch has a different chemistry than normal starch and can be scored by staining with iodine. This gene is commercially important because some waxy corn is produced for specialty markets. Let's look at the following test cross data (Table 3):

Parent: Red, Normal (CCWW) X White, waxy (ccww)

F_1: Red, Normal (CcWw) X White, Waxy (ccww)

Table 3. Testcross data for another seed trait

Phenotypes

of testcross progeny

Red, normal

2781

Red, waxy

759

White, normal

749

White, waxy:

2711

Total

7000

- Parental gametes made by the F_1: CW and cw
- Recombinant gametes made by the F_1: Cw and cW = 759 + 749 = 1508
- **Map unit distance between the C,c and the W,w loci:** 1508/7000 = 21.5 Map Units

It is clear from the testcross data (Table 3) that the genes at the **C,c** and **W,w** loci do not have as great a tendency to be inherited together compared to the genes at the **C,c** and **S,s** loci. This would be because the loci are farther apart, and our 21.5 map units is an indicator of the relative distance.

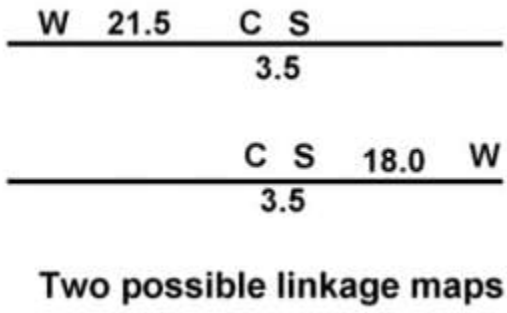


Figure 9. Two possible linkage maps from two-point data. Image by Donald Lee.

From the two distances calculated, how far apart are the **W,w** and **S,s** loci? Here is where we can try to use map unit distances to do gene mapping the same way we use miles to do road mapping. We can pencil our two possible maps (Figure 9).

In one map the **C,c** locus is in between the **W,w** and **S,s** loci. Using the 21.5 plus 3.5 map units we have calculated, the best estimate will be that 25 map units separate the **W,w** and **S,s** loci. The other map that fits our data is having the S,s locus in the middle with the **W,w** and **S,s** loci 18 map units apart (21.5 – 3.5). If the genes occupy a fixed position on the chromosome, only one map will be correct. How can we determine which is the correct map?

The Third Map Distance

A third map distance needs to be estimated in order to determine the linkage map of these three loci. We need to make a cross that gives us the opportunity to measure crossing over frequency between the **S,s** and **W,w** loci. One such cross is shown below.

- **Parent**: ssWW (shrunken, normal) X SSww (plump, waxy)
- **F_1**: sW / Sw X sW / Sw

Test cross offspring:

Plump, normal:

630

Plump, waxy:

2824

Shrunken, normal:

2900

Shrunken, waxy:

646

Total:

7000

- **Recombinant gametes**: 646 + 630 = 1276

- **Map distance S,s to W,w**: 1276/7000 = 18/2 map units

Based on the third map distance our best map for all three loci is the second alternative (Figure 9). To map all three loci using this two-point test cross data, the geneticist needed to generate three different crosses. This is how gene maps in organisms such as corn have been generated. As new genes controlling traits are discovered, crosses can be made to test if these genes are independent or linked to other genes. Once linkage is determined, additional crosses can be analyzed to position the gene on the linkage map.

Map Distances from F_2 Data

In our last test cross, the F_1 dihybrid **sW / Sw** was crossed to a **sw/sw**, shrunken waxy plant. If the shrunken waxy line was not available, the geneticist could still obtain information to determine map distance by selfing the F_1 to produce F_2 offspring. Here is the procedure.

Results of Selfing the sW/Sw F_1:

F_2 Offspring

Number

Plump, normal

254

Plump, waxy

122

Shrunken, normal

120

Shrunken, waxy

4

Total F2s:

500

Step one: Focus on the double recessive (sw/sw).

How do we obtain recombinant gamete frequencies from this F_2 phenotype information? The first thing to remember is that this is a self-cross rather than a testcross and an additional Punnett square is needed to depict how gametes produced these F_2 (Figure 10). From the Punnett square we can see that four kinds of gametes are made in both the male and female in this cross. Therefore, the plump, normal seeds can be made nine different ways in our diagram and will consist of five different genotypes (**SW/SW, SW/sW, sW/SW, sW/Sw** and **SW/sw**). We cannot tell by observing the seed phenotype though, which of the genotypes it has. The plump, waxy and shrunken, normal F_2 seeds will each consist of two different genotypes. The only F_2 phenotypes that consist of a single genotype are the shrunken, waxy. They are all **ssww** (**sw/sw**). By focusing on how this phenotype was produced in the F_2, we can estimate the frequency of recombinant gametes made by the selfed parent.

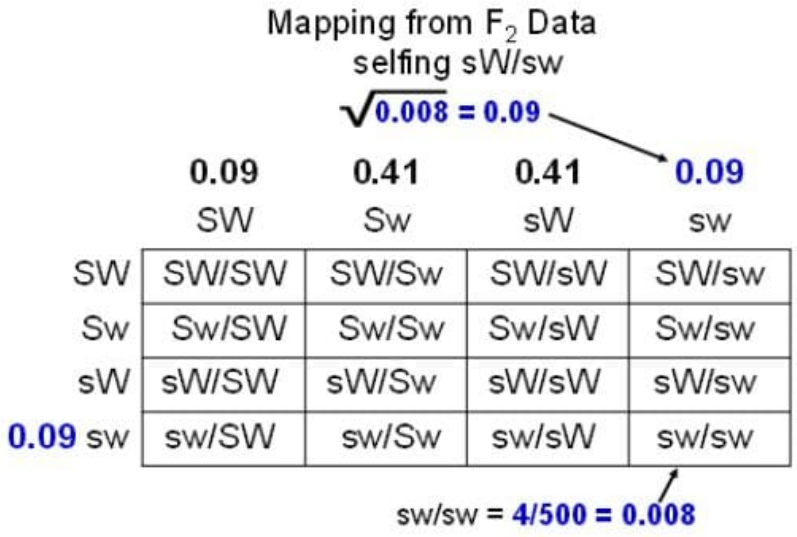


Figure 10. Mapping from F_2 data. Focus with the method described is on the double recessive, ssww or sw/sw, F_2. Image by Donald Lee.

Step two: Go from phenotype frequency to gamete frequency.

Look at the Punnett square and think about how the shrunken waxy seeds were made. First, a **sw** gamete in the male part of the plant and a **sw** gamete in the female needed to be made. The parent had the genotype **sW/Sw** that means that these would be recombinant gametes in both the male and female. Once these gametes were made, they needed to come together at random during pollination to produce the seed. Mathematically, we can say that the frequency of the shrunken, waxy seeds is the frequency of the **sw** gamete made in the male times the frequency of the **sw** gamete made in the female. If we assume that crossovers occur in the pollen forming cells at the same frequency as the egg forming cells, then the frequency of the shrunken waxy seeds is the square of the frequency of **sw**. Make sure this last paragraph makes sense to you by walking through figure 10.

Step three: Take the square root.

Now we can use our seed phenotype frequency to estimate gamete frequency in the selfed parent. Four of the 500 seeds produced were shrunken waxy. That would give our square in the lower right a frequency of 0.008 (4/500). This frequency should be the **sw** gamete frequency squared (**sw2**). If we want to know the frequency of **sw**, we can take the square root of 0.008 and get about 0.09. Now we have the frequency of one gamete estimated. This would be the same for both the male and female gametes, where 9% of all gametes produced by the **sW/Sw** parent will be **sw**. As it turns out, we can calculate the frequency of the other three gametes from this information if we just think about how the gametes were made in meiosis.

Step four: Double the frequency, decide if parental or recombinant.

In our continuing example the **sw** gamete was made when a crossover occurred in the **sW/Sw** parent. Crossovers are a reciprocal exchange between non-sister chromatids so every time a **sw** gamete was made, a **SW** gamete was also made. Therefore, if the **sw** frequency is 0.09 then the **SW** frequency is also 0.09. These are our recombinant gametes, so the frequency of recombinant gametes is 2 X 0.09 or 0.18. We can tell from this frequency that these are the recombinant gametes, they are less frequently made than the parental gametes. The parental gametes would total 82% of the gametes, each parental gamete making up half (41%) of that total. Thus, 18 map units between the **S,s** and **W,w** loci is our map distance estimate from the data. Because we use only a subset of the information in this procedure (only the double recessive numbers) this estimate is more subject to chance than the test cross data. We can use more complex math to estimate gamete frequencies from all the F_2 data but this square root method is a quick way to detect linkage and estimate map distance. In cases where geneticists cannot perform a testcross working with F_2 data is a necessary part of gene mapping. Can you think of some instances where this would be true?

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2.4.15: 15 - Deviations from Mendelian Genetics- Linkage (Part 2)

Learning Objectives

At the completion of this lesson, you will be able to:

1. Make predictions about inheritance using map unit distances and genetic markers.
2. Assemble maps from multiple-point linkage data.
3. Define the relationship between linkage maps, linkage groups and genome maps.
4. Describe how DNA or molecular markers are observed and used in gene mapping.

In this lesson you will learn to make predictions about inheritance using map unit distances and genetic markers, assemble maps from multiple-point linkage data, define the relationship between linkage maps, linkage groups and genome maps, and describe how DNA or molecular markers are observed and used in gene mapping.

Introduction

Why do geneticists map genes? This is a legitimate question after all this discussion. Gene mapping helps geneticists do two things, make better predictions and isolate genes. The following is an example of how gene mapping can help breeders make better selections.

Gene Maps and Selection

This example shows how gene mapping has a practical application. Soybeans have perfect flowers that self-pollinate. Tedious flower manipulation is required to make a hybrid seed. Soybean breeders would like to find ways to make hybrid seeds in commercial quantities. They have discovered a gene that causes a lack of pollen formation and makes plants male-sterile. Therefore, when bees visit these plants, they will make a cross-pollination and produce hybrid seed if they carry pollen from a nearby male-fertile plant. A hybrid seed production field could be set up if they could have all male-sterile plants in one row and male-fertile in the next. The genetic control of this trait is shown below:

Male-fertile plants: **MM or Mm**

Male-sterile: **mm**

Can you see the problem one would have in generating seed that is all male-sterile (genotype **mm**)? These plants will never be true breeding because they cannot self-pollinate (they have working female parts but no pollen). The only plants that can be self-pollinated to produce mm offspring are **Mm** plants. Progeny from selfed **Mm** plants will segregate 3 fertile to 1 sterile, making it impossible to obtain a pure collection of mm seeds to plant in a 'female' row for hybrid seed production. If the mm genotype could be picked out in seeds prior to planting, this would solve the problem. Unfortunately, the male sterile trait is impossible to select for in the seed but breeders discovered a trick they could use that took advantage of their knowledge of gene maps. A second trait in soybean that is easy to select for in seeds is controlled by a gene that was closely linked to the **M,m** male sterile gene. This trait is green vs. yellow seed coat. The seed coat trait is controlled as follows:

GG or Gg: green

gg: yellow

The **G,g** locus and **M,m** locus have been mapped and are about two map units apart. Therefore, we can take advantage of this linkage to select seeds from a cross that will tend to also be male sterile. This is how the process would work. The following cross is made:

GGMM (green, male-fertile) X **ggmm** (yellow, male sterile)

The cis dihybrid (**GM/gm**) will be male-fertile and can be self-pollinated. Because of linkage, the **GM** and **gm** parental gametes are made 98% of the time. Therefore, seeds that are **gg** and yellow are almost always going to be **mm** and male sterile. If the breeder sorts out the F_2 seeds based on color, about one fourth of the seeds will be yellow. Based on 2-map unit distance, about 0.24 would be the expected frequency of **ggmm** out of all the F_2 but 96% of the yellow F_2 will be male sterile (0.24 out of 0.25). Therefore 96 out of 100 seeds planted in the 'female' row of selected yellow seeds will be male-sterile (mm). Harvesting seeds from these rows will provide a high percentage of hybrid seeds. Thus, the breeder took advantage of linkage to select for a trait that was easy to detect (yellow seeds) and obtain individuals with a trait that was impossible to select (male-sterile). The power of this prediction potential has driven much of the recent efforts in gene mapping in crop plants, livestock, and humans.

Table 1. Predicting the percentage of male-sterile F_2 among yellow F_2 seeds using a chart. Chart is in text form below for additional accessibility options.

0.49 big G, big M	
0.01 big G, little m	
0.01 little g, big M	
0.49 little g, little m	
0.49 big G, big M	
0.01 big G, little M	
0.01 big G, little m	
0.01 little g, big M	
0.0001	
0.0049	
0.49 little g, little m	
0.0049	
0.2401 (selected)	

Three Point Test Cross: Multiple Point Gene Mapping

Gene mappers are motivated to map all of the tens of thousands of genes found on the chromosomes of plant or animals. Analyzing data from crosses to determine map distances for two genes at a time makes the process time-consuming and tedious. Therefore, geneticists will often attempt to map as many genes as possible from a single set of progeny. We will go through the simplest multiple point mapping example, a three-point testcross, to demonstrate this process.

We will use our three corn seed trait loci again and use data from one cross to map these three loci. The first step would be to obtain a trihybrid individual that is heterozygous at all three loci and then perform a testcross with this trihybrid.

Parents: **CCssWW (CsW/CsW) X ccSSww (cSw/cSw)**

Trihybrid: **CcSsWs (CsW/cSw) X ccssww (csw/csw)**

Table 2. Test cross offspring

Seed trait			
Gamete from trihybrid	**Number**	**Red, shrunken, normal**	**CsW**
2777	**White, plump, waxy**	**cSw**	**2708**
Red, plump, waxy	CSw	116	White, shrunken, normal
csW	123	Red, shrunken, waxy	Csw
643	White, plump, normal	cSW	626
Red, plump, normal	**CSW**	**4**	**White, shrunken, waxy**
csw	**3**	**Total number of progeny:**	**7000**

We observe eight phenotypes of seeds in the testcross progeny because the trihybrid can make eight kinds of gametes. As we knew, these three genes are linked and so the uneven ratio of phenotypes reflects the combinations of two parental and six recombinant gametes. The parental gametes are still the most frequently produced type, and the six recombinant gametes are made when crossing over occurs between the loci. We can systematically account for these crossovers if we follow the following four steps. First, we will explain the steps and then we will show how to use them to map the three genes.

Step 1: Identify the parental gametes.

These are **CsW** and **cSw**, that combination which came from the parent generation and the combination made by the trihybrid at the highest frequency.

Step 2: Classify the recombinants.

If we observe the gene combination in each of the six recombinant gametes, we can ask ourselves if the gamete has a new combination for each pair of genes. For example, **CSw** has a new combination for the **CS** and the **Cw** compared to the parental gametes but the same combination for **Sw** as the **cSw** parental gamete. Therefore, these 116 gametes represent crossovers between **C,c** and **S,s** as well as **C,c** and **W,s**. This information is recorded (see below).

Step 3: Determine recombinant gamete frequency.

Once all six recombinant gametes have been classified, the total number of crossovers between the three loci can be added up and crossover percentage determined between each pair of loci. This number will reflect gene distance but one more step is needed to complete the process.

Step 4: Add in the double crossover gametes.

Two of the six recombinant gametes were made as a result of double crossovers between the two loci that are furthest apart. These crossovers have been added to the map distances between the middle locus and the two outside loci. Now we need to add these double crossovers to the outside loci distance.

Applying these four steps to our three-point test cross data would work as follows:

1. CsW and cSw
2. **C,c** to **S,s**: 116 (CSw) + 123 (csW) + 4(CSW) + 3(csw) = 246
   C,c to **W,w**: 116 (CSw) + 123 (csW) + 643 (Csw) + 626 (cSW) = 1508
   S,s to **W,w**: 643 (Csw) + 626 (cSW) + 4 (CSW) + 3 (csw) = 1276
3. 246 / 7000 = 3.5% recombinant gametes **C,c** to **S,s** 1508 / 7000 = 21.5% recombinant gametes.
   C,c to **W,w** 1276 / 7000 = 18.2% recombinant gametes **S,s** to **W,w**.
4. From the information in step 3 we can see that the **C,c** and **W,w** loci are the farthest apart so the S,s locus must be between them. We can also see that we have underestimated the distance between the outside loci (3.5 **C,c** to **S,s** + 18.2 **S,s** to **W,w** = 21.7 map units not 21.5). While this difference is small, we can rectify this and double check our work by adding in the double crossovers. The **CSW** and **csw** gametes are made very rarely. That is because is takes two crossovers in the trihybrid's chromosome to make them, not just one.

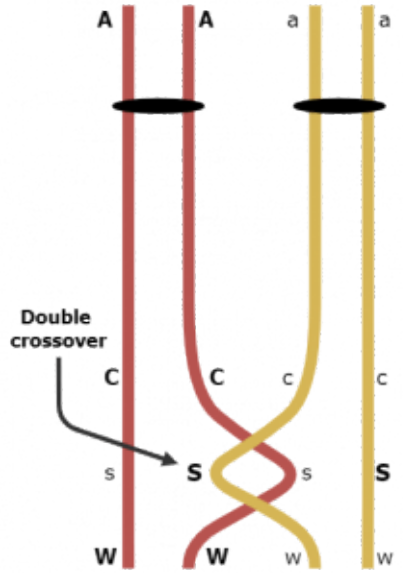


Figure 1. Double crossovers need to occur to produce all dominant allele (CSW). Image adapted from double crossover by Donald Lee, recreated in color by Abbey Elder

When two crossovers occur between the **C,c** and **W,w** loci the parental combinations of **CW** and **cw** are restored. Therefore, we did not count the **CSW** and **csw** gametes as representing crossovers when they actually represent two crossovers each (Figure 1). Thus, we should add up the double crossovers (3 + 4 = 7), multiply times two and add these 14 crossovers to the **C,c** to **W,w** distance (1508 + 14 = 1522 / 7000 = 21.7 map units). Now we have double checked our map distances and have our three-point map complete.

It can be intimidating to see all the data generated from a three-point test cross. This information, however, can be used systematically to save the geneticists time and map three genes in one experiment. The three point data also provides a more accurate measure of map distances compared to two-point data when genes are farther apart on a chromosome. This is because a third gene in between the more distant loci can account for double crossovers that would not be detectable in a two-point analysis. For this reason, map distances tend to be underestimated when genes are further apart as we saw to a modest extent in this example (21.5 two-point vs. 21.7 three-point).

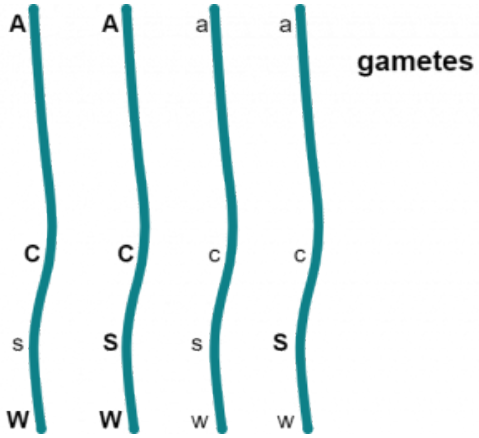


Figure 2. All recessive allele (csw) gametes. Image adapted from recessive gametes by Donald Lee, recreated in color by Abbey Elder

How would a geneticist work with a four-point test cross? There would be sixteen phenotypes in the progeny as a result of the hybrid parent making two parental gametes and fourteen recombinant gametes. These recombinant gametes would be a result of single, double, and even triple crossovers that occurred in prophase I. While the data would be tedious to work with, one data set could be analyzed to reveal the map of four genes. It is not surprising that geneticists now have written computer programs which will perform these types of calculations, save time, and reduce the chance of calculator error. Geneticist's mapping genes in economically important plants and animals will also generate mapping populations that are a result of crossing parents that have different alleles at hundreds or thousands of loci. Even though the development of computers and programs has become a part of modern gene mapping the process of indirectly measuring crossover frequency by observing the inheritance of trait

combinations is the basis of generating these gene maps.

Linkage Maps, Linkage Groups

The organism that gene mapping was first performed in was fruit flies. Working with fruit flies gave the early gene mappers many advantages. First, the fruit fly geneticists observed a great of deal of genetic variation among fruit flies for body part traits (wing size, eye shape and color, leg bristle types etc.) that were easy to see if they had a low powered microscope. The phenotype variants arose naturally or could be induced by chemical or radiation mutagenesis. Additionally, short generation time, large offspring numbers and low rearing costs allowed them to complete informative linkage experiments in a short time. Finally, cytogeneticists knew that fruit flies had four pairs of chromosomes so all the genes would map into four linkage groups. One chromosome was very small, and few genes mapped to this chromosome. The **X-chromosome** and the largest autosome have many genes and more than 100 map units separate genes on opposite ends of the chromosome.

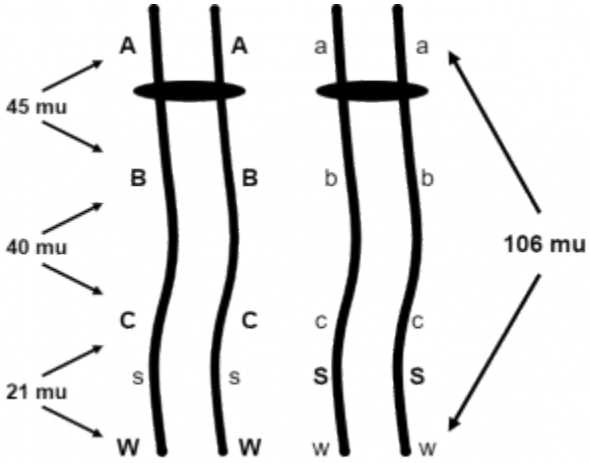


Figure 3. Genes from large linkage groups can be over 100 map units apart. Image adapted from linkage groups by Donald Lee, recreated in draw.io by Abbey Elder.

Let's stop and think about how they determined this large map unit distance. If the **A,a** and **W,w** loci are 106 map units apart, how frequently will an **AW / aw** dihybrid make the gametes **Aw** and **aW**? Since these are the recombinant gametes and map distance is the frequency of recombinant gametes, we are tempted to say 106% but that is impossible. The parental gametes for any two loci will never be made less than 50% of the time, the percentage we observe when two loci are independently assorting. In fact, 50% is the correct answer here. Anytime genes that are on the same linkage group are 50 map units or more apart, they will behave as if they are independent of one another. That is because the genes are far enough apart to always allow at least one crossover to occur between them during prophase I. So how can we ever determine that **A,a** and **W,w** are 106 map units apart? Simply by determining the distances between these genes and other genes in between them that are less than 50 map units apart (Figure 3). Figure 3 shows that distances can be found between **A,a** and **B,b**, **B,b** and **C,c**, then **C,c** and **W,w**. Each of these distances is added to arrive at the 106 map units between **A,a** and **W,w**. Therefore, if the geneticist combines the mapping information from many linkage experiments or performs a multiple point linkage analysis on a progeny group segregating for many linked genes, they can deduce the larger map unit distances.

Limited Traits, Limited Linkage Maps

Many gene mappers do not enjoy the advantages that fruit fly geneticists have in mapping genes. Cattle for example have thirty linkage groups (2n = 60), they often have one offspring per cross, and it is difficult of observe hundreds of phenotype differences controlled by single genes among cattle. Soybean have 20 linkage groups but for many decades, soybean geneticists had compiled about 30 different linkage maps and had no idea as to which maps were really part of the same linkage group. Soybean geneticists needed to discover more genes that had clear phenotype effects and then perform crosses that compared the inheritance of these new genes with the genes that had already been mapped. In the 1970's and 1980's, a new type of genetic trait, molecular markers, was discovered that greatly accelerated the gene mapping process in all organisms.

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2.5.1: GET STARTED ON YOUR RESEARCH

2.5.1.1: Narrowing a Topic
2.5.1.2: Background Reading
2.5.1.3: Developing Your Research Question

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2.5.1.1: Narrowing a Topic

Defining your research question is a process of working from the outside in: you start with the world of all possible topics (or your assigned topic) and narrow down until you have focused your interest enough to be able to state precisely what you want to find out, instead of only what you want to "write about."

Going through this process can be the hardest part of doing research, but once you have a question that is realistically scoped (not too broad, not too narrow) it will guide the rest of your work.

The Process of Narrowing a Topic

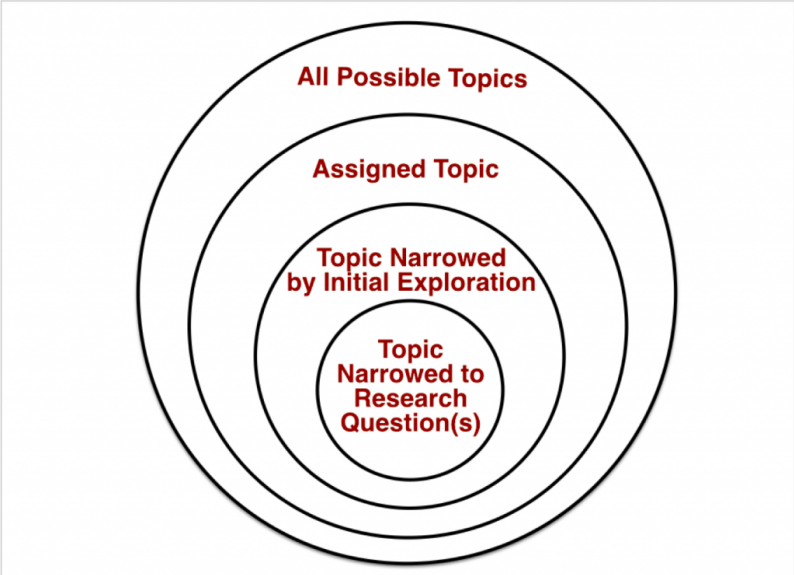


Image by Ohio State University Libraries.

ACTIVITY: Which topic is narrower?

Now it's your turn. Practice thinking about narrower topics with these 3 examples. Click the arrow to show the next question.

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TIP: Use some of the 5 Ws to help narrow your topic to a searchable question

Your assignment is to write on the topic of higher education. You decide you want to write about the high cost of tuition, but that is still too broad.

Start by asking some or all of the following questions.

Question	More specific focus
Who?	first year students, mature students, part-time students
What?	graduation rates, degree completion, attrition, dropout
When?	last 10 years
Why or how?	financial burden, employment, student debt

From asking these questions, you might come up with a research question like this:

“How does the high cost of tuition impact the degree completion of mature college students?”

But Avoid Getting too Narrow

Be careful about getting too specific with your research question. Not every question that you come up with will be searchable.

For the above question about college tuition, the important questions to ask would be who? and what? Trying to find information on the impact of rising tuition in a particular city or province will be too restrictive, and the location may, in fact, be irrelevant to the search; a large-scale study across Canada or North America would likely yield applicable information.

The bottom line is, you will be working toward a **balanced research question** that is specific enough to guide you in your research, but not too restrictive.

ACTIVITY: Find the balanced topic

Now it’s your turn. Practice thinking about balanced topics with these 3 examples. Click the arrow to show the next question.

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2.5.1.2: Background Reading

As you are exploring your topic and figuring out ways to narrow it down to a searchable question, it is a good idea to do some initial reading. For one thing, you might not know much about your topic yet. For another, such reading will help you learn the terms used by professionals and scholars who have studied your narrower topic. Those terms might become your keywords or search terms later on, so keep them in mind.

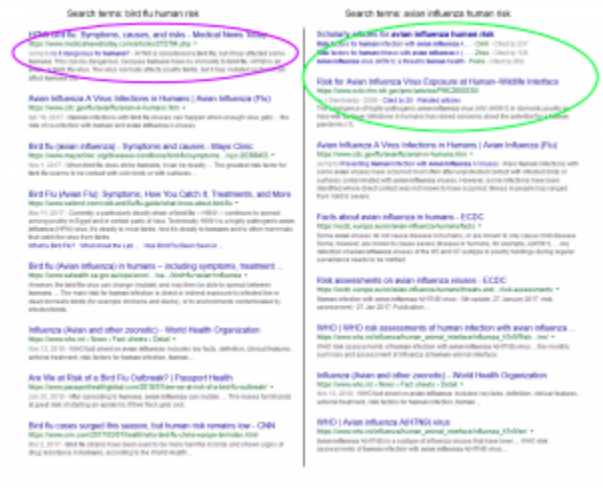
Getting your words right

It's important to understand that the search terms you use will have a direct correlation with the kinds of sources you find. And spending some time early on in your research learning relevant terms will save you time later on.

For instance, if you were going to do research about the risk of bird flu to humans, initial background reading would teach you that professionals and scholars usually use the term **avian influenza** instead of **bird flu** when they write about it. (Often, they also use H1N1 or H1N9 to identify the strain.) If you didn't learn that, you would miss the kinds of sources you will eventually need for your assignment.

Take a look at the Google search results using the terms "bird flu" and human risk vs. "avian influenza" and human risk. Compare the kinds of sources listed.

(Click on the thumbnail image for a larger view. Use your browser's back-button to return to the page.)



Comparing Google search terms

If you were to follow the linked results, you would see that the sources on the right come from government agencies and scientific journals, whereas the sources on the left come from news outlets or consumer health websites.



A note about Wikipedia

Wikipedia is a popular place to start your research and will likely be one of the top results in a Google search of your topic. While Wikipedia articles are edited and must be supported with external links and references to other, legitimate sources, it is not a good idea to rely solely on them. That's because you can't verify who has written the article, and whether the author has any credibility or expertise on the subject. Content on Wikipedia has the potential to change quickly, so your source might disappear and will be difficult for your reader to find later.

What you can do with a Wikipedia article is look at the external links, the references, and the suggestions for further reading and try to follow up with those, either in the Library's collection or elsewhere on the internet.

Try a Library encyclopedia or dictionary instead

Although you will likely start your background reading with a quick Google search, you should visit the library and its collection of reference materials early in your research. The library has access to many encyclopedias, dictionaries, and handbooks, both in print and online.

You will find a list of KPU's reference books here. You will also find links to individual reference books when you do a search using Summon. (Think of Summon as the library's search engine; it's the main search box on the homepage.)



Image by Pixabay.

Encyclopedias and **handbooks** will provide:

– a broad overview of your topic

– sub-topics and related issues

– controversies and criticisms

– key thinkers or researchers in the area

– references, recommended articles, and links to further reading

Dictionaries will offer a definition of your term and related terms that will be important as you develop your search strategy.

ACTIVITY: Use an encyclopedia article

The article below is from the Encyclopaedic Dictionary of Psychology and was found using the library's Summon search tool.

Click on the hotspots to see what information the article provides on the general topic of “eating disorders.”

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After this background work, you are now ready to start developing the research question you will try to answer for your assignment.

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2.5.1.3: Developing Your Research Question

By now, it should be clear that finding a research question is a process of exploration and refining: exploring a topic will lead to developing a question, and further refinement will help you to focus that question to something that is not too broad and not too narrow.

ACTIVITY: Watch, think, and learn

Take a minute to watch this short video on how to develop a research topic. Think about the steps the student takes starting from a wide open topic, to something too narrow, and finally, to finding a balanced topic that is searchable.



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Steps for Developing a Research Question

The steps for developing a research question, listed below, can help you organize your thoughts.

Step 1: Pick a topic (or consider the one assigned to you).

Step 2: Write a narrower topic that is related to the first.

Step 4: Do some background reading, using the Library's reference books. Do some initial research in a library database.

Step 5: Readjust your topic if you get too few, or too many, search results.

Step 6: List some potential questions that could logically be asked in relation to the narrow topic.

ACTIVITY: Summing up Module 1

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Well done! You have completed the text and activity portion of Part 1 Getting Started on your Research. You are welcome to review any part of this module at any time.

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A modules-based approach to learning research skills that emphasizes the reflective nature of information discovery, the contextual basis for evaluating that information, and a recognition that information has value.

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2.5.2: RECOGNIZE TYPES OF INFORMATION

2.5.2.1: Types of Information Sources

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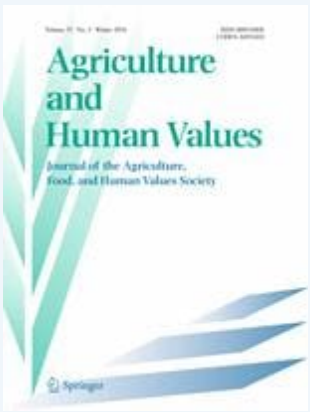
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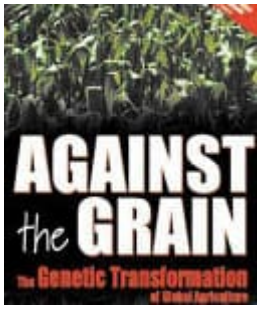
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2.5.2.1: Types of Information Sources

Consider a topic such as the **safety of genetically modified food.** Wading into this large and controversial area, you will quickly discover that information about it comes from a wide range of sources: blogs and opinion pieces, natural medicine websites, scientific research articles, government and NGO sites, as well as books, newspapers, and magazine articles.

Each of these types of sources has different content, written by people with varying levels of expertise, and written for different audiences. And each of these types of sources will have a different value for you, depending on the context and requirements of your research need. Some assignments will require that you use scholarly, academic sources that have to undergo a lengthy editorial process and therefore take longer to appear. Other assignments may allow you to use less formal, popular sources of information that may be more timely.

Newspaper		up-to-date current events, editorial opinion, commen general audience
Encyclopedia, Wikipedia		overview, background information, general audience
Website		possible commercial purposes, opinion, general audi
Magazine		current events, topics of interest to general audience
Government/NGO		reports, standards, statistics, more targeted audienc
Scholarly article		new research by scholars for other scholars, expert a

Scholarly book		in-depth coverage of a topic by expert/experts, targe audience
Social media post, blog		real-time, opinion, commentary, general audience

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2.5.2.2: What Kind of Information Do You Need?

Click the arrows below to learn about which types of information sources best meet various research needs.

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ACTIVITY: Match the resource with the research need

Think about which kind of resource would be best suited for each question.

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2.5.2.3: Popular and Scholarly Articles

For some of your research assignments, you will be expected to use “scholarly” articles. These may be called “academic” or “journal” articles, or more specifically, “peer reviewed” articles. In a later tutorial we will learn how to focus your searches in order to find these.

For now, it is useful to learn to recognize the difference between these types of publications so that whether you are searching in a library resource or on Google, you will be able to make some judgment about the kind of information source you are looking at and whether it will be appropriate for your assignment.

	Popular Articles	Scholarly Articles
Author	Journalists Professional writers	Scholars Faculty members Researchers Professionals in the field
Audience	General public	Other scholars or professionals
Visual Appearance	Often include color, photos, advertisements	Mostly text, but may include a few graphs or charts
Length	Tend to be short	Tend to be lengthy
Language	Can be understood by the average reader	Use professional jargon and academic language
Content	Gives a broad overview of issues of interest to the general public	Covers narrow topics related to specific fields
	Rarely gives full citations for sources	Includes full citations for many credible sources
Recommended For:	Enjoyable reading material	Sources for academic work
	Finding topic ideas	Professional development
	Learning basics or different perspectives for your topic	Learning about new research being conducted

Some key differences between popular and scholarly articles. Image by adstarkel.

Trade or professional articles

Depending on your area of study, you may also be asked to consider a third type of publication that is written for professionals and people within a particular field of work. These articles target a specialized audience, may report on primary research but from an applied or summary perspective, and may have advertisements of interest to people in that profession.

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2.5.2.4: Producing Information

The process of information creation follows a timeline. As soon as an event occurs, social media and online news sources are the first to provide coverage. Magazines and newspapers will follow shortly after, and journal articles and books take even longer to get published.

Knowing this will be important in your research: if you choose a very recent event to write about, you will likely not find information about it in a book or scholarly article. You may, however, need to expand your topic to look for a similar or related event, or broader treatment of the subject, which you can still use to support your writing.

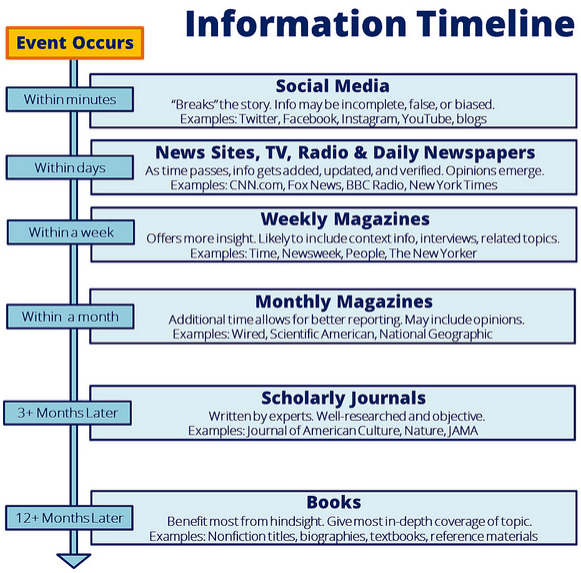


Image by adstarkel.

ACTIVITY: Explore the timeline

The following timeline details how the media covered an important news event in 2012. Maximize the screen for best viewing.

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2.5.2.5: Understanding Peer Review

Your assignment may require that you include information from “peer reviewed” articles. These articles are published in scholarly or academic journals after they have gone through a lengthy editorial process which usually involves the author making many revisions. The reviewers themselves are experts in the same field, and judge the strength of the article on the originality of the research, the methods used, and the validity of findings. The highest standard of peer review is “double-blind,” meaning that both the identity of the authors as well as the reviewers are kept anonymous in order to ensure that bias and subjectivity do not influence the process.

But be careful! Not all of the content in an academic journal is subject to peer review. There may be other content such as letters, opinion pieces, and book reviews that have been edited, but not necessarily gone through a formal peer review process.

ACTIVITY: Watch, listen, and learn

The following video describes the process of peer review.

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ACTIVITY: Summarize the peer review process

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But how can you, the researcher, recognize a peer reviewed article?

Fortunately, the library’s Summon search and most of our databases have a filter or limit which will help you find the right type of information. Various databases will use different terms: look for “academic” or “scholarly” or “peer reviewed.”

There are other clues you can look for.

Tip: Clues to help you decide if it is peer reviewed

Author’s credentials and affiliations	Look for the author’s degrees, as well as the university or research institution they are affiliated with.
References	Any peer reviewed article will have a lengthy list of sources used by the author.
Submission guidelines	Somewhere on the journal’s homepage will be a link for submitting an article for review. You may have to dig around a little!
Journal publisher	Is the journal published by a scholarly society? A university press?

ACTIVITY: Summing up Module 2

Pick the correct statement.

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Well done! You have completed the text and activity portion of Part 2 Recognizing Types of Information. You are welcome to review any part of this module at any time.

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2.5.3: DEVELOP YOUR SEARCH STRATEGY

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2.5.3.2: Check your Bias

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2.5.3.8: Library Research Tools and When to use Them

2.5.3.9: Summing up Part 3

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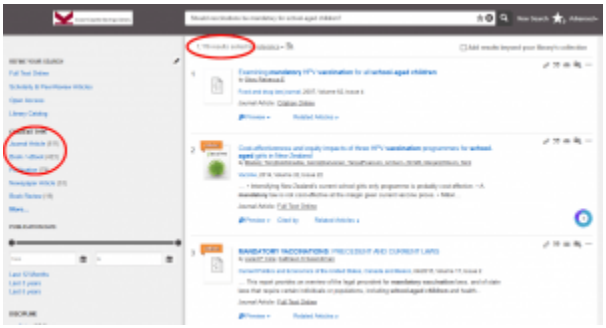
2.5.3.1: Strategy #1- Start with Just the Keywords

The first strategy in effective research is to start with a basic keyword search of your topic. Keep the focus on just the main concepts, or keywords, of your question. Typing a complete sentence or question into a search box, whether you are in Google or one of the library's research tools, will not give you as comprehensive or relevant results as just entering the two or three keywords that best reflect your question.

See what happens when you enter your search question, in natural language, directly into the library's Summon search:

The question is **"Should vaccinations be mandatory for school-aged children?"**

(Click on the thumbnail for a larger view. Use the back button in your browser to return to the page.)



Search results from natural language query

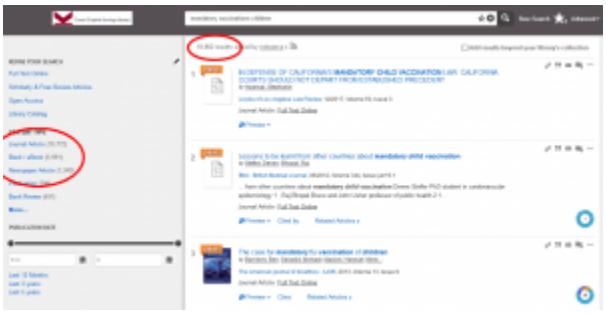
Only a little over 1,000 results come back, which is not as many as you might expect considering that Summon searches everything the library has in its collection. Furthermore, the results are rather evenly split between journal articles and books.

Now, repeat the search again, but with just the keywords that are central to the question you are researching. Removing the non-essential words in the question would leave you with something like this:

"Should vaccinations be mandatory for school-aged children?"

The search below was done using **mandatory vaccinations children**.

(Click on the thumbnail for a larger view. Use the back button in your browser to return to the page.)



Search results using keywords

This search results in over 15,000 items, with many more journal articles. That's because in the first search, Summon is looking for items in which ALL of the words of the search query are present. In the second search, only those three terms need to occur in the results. Furthermore, the words left out of the question are not essential to the overall strategy of the query.

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2.5.3.2: Check your Bias

Remove bias

Avoid any words which may imply a bias in the results: negative, positive, good, bad, benefits, harms, effects, and so on. Remember, you are searching for a balanced treatment of the question, and including biased or leading terms could skew the results of your search.

Consider what would happen if you included the term “anti-vaxxer” in your search. You would most likely end up on websites with inaccurate information.

ACTIVITY: Focus on the keywords of a research question

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2.5.3.3: Strategy #2- Examine your Results

In the previous section we looked at search results using the library's Summon search tool and found a range of items coming from scholarly journal articles, books and ebooks, newspapers, and more.

If you were to examine just a few of the top results, you would quickly see related and more specific terms that might help in subsequent searches; for example, **vaccine exemption** and **vaccine hesitancy** provide slightly different perspectives on the topic and correspond to disciplinary approaches. Articles about vaccine exemption would examine the issue from a legal perspective (an individual's right vs. population health), but articles about vaccine hesitancy might examine it from a philosophical or psychological perspective (opinions, trust in government, or misinformation). Similarly, you might find additional synonyms or alternate terms (**immunization**, **herd immunity**) that will help make your searches more complete.

As a researcher, asking yourself how these narrower and alternate terms relate to what you want to find out will be an important part of your search strategy.

Tip: Results ranked by relevance

Summon and most of the library's databases will return search results ranked in order of relevance. After performing a search, always examine closely the top few items for more precise search terms, synonyms, or other related pieces that you might add to the next search.

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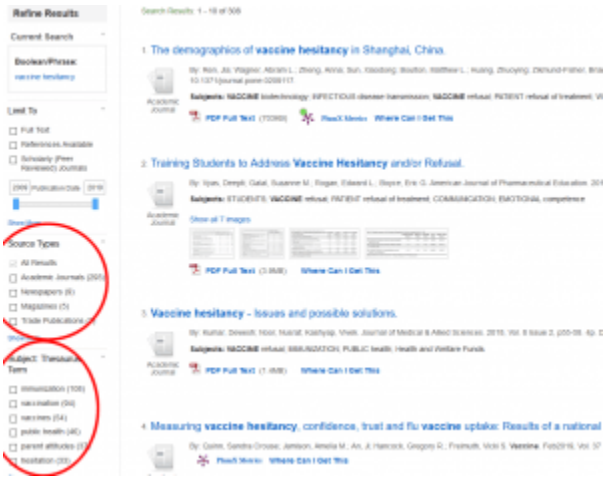
2.5.3.4: Move to a Database

Move to an article database

Moving your research over to one of the library's databases will bring a more focussed set of results.

Our earlier keyword search showed us that vaccine hesitancy might be a useful concept for finding information about what motivates some people to refuse vaccinations for their children. Doing a search with the term "**vaccine hesitancy**" in Academic Search Complete, the library's largest multi-disciplinary database, yields the following results.

(Click on the thumbnail for a larger view.)



Database search results

The majority of articles using the term "**vaccine hesitancy"** come from academic journals, indicating that it is a concept or term used by researchers or scholars in a variety of fields, but not so much in the popular or mainstream press. A close look at the subject terms also provides an indication of how the results are focussed: some will concentrate on public health, others on parental attitudes, and others on immunization more generally. This kind of strategy tells you how the issue is approached by different perspectives, and what might be most relevant for your own research.

See this page for a list of article databases the library subscribes to.

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2.5.3.5: Use the Library Catalogue

Try the library catalogue

A search of the library catalogue will yield books/ebooks, as well as videos in the collection. Again, results are ranked by relevance. Examining the first few items in the list will give you further ideas for searching. You may find books whose entire contents will be useful to your search, or you may find edited works, with a single chapter relevant for your research.

ACTIVITY: Watch, listen, and learn

The following brief video introduces you to searching the library catalogue, including using subject terms and filters.

An interactive or media element has been excluded from this version of the text. You can view it online here: https://kpu.pressbooks.pub/doingresearch/?p=352

You can access the library catalogue directly by selecting it from the library's homepage, above the Summon search box.

ACTIVITY: Analyze the details of a book

The image below was taken from the catalogue record for a book on vaccines and children. Click on the hotspots to see what kind of information about an item is available in the catalogue. Use this to further inform your search.

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2.5.3.6: Strategy #3- Add some Filters

An effective search strategy makes use of the specialized features within the library's search tools to focus on the kinds of resources you need. Unlike a Google search, Summon, library databases, and the catalogue have filters and limits that allow you to further refine your results list in a few ways.

Recall in the second module you were introduced to the idea that information comes in a variety of sources; part of your strategy should be to ensure you are getting the content you need. Does your assignment require that you use only certain kinds of sources? Is there a date restriction? Can you use a video?

Tip: Use a filter or limit

After an initial search, look for these tools to further refine your search

What do you need?	Filter/Limit
Scholarly journal? book/e-book? newspaper article? video?	Content type or source
Recent? last 10 years?	Publication date
Focussed on a specific subject	Subject headings, discipline, or topic

ACTIVITY: Selecting the best limits

Click on the arrows to answer the 4 questions below.

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2.5.3.7: Strategy #4- Citation Tracking

A final strategy is to examine the works cited by the authors of the initial sources you find.



Image by Pixabay.

Consider that scholarship is a conversation among researchers on a particular subject, with everyone offering evidence, theories, and criticism to advance what is known and what *may not* be known about a topic. Your task is to understand what connections are being made between these viewpoints, how you will integrate them in your own work, and what conclusions or advances you might be able to add.

In practical terms, as a researcher, this means paying attention to the reference lists or bibliographies of the works you find in your initial searching. This is known as **citation tracking** and is an important strategy to use to find additional resources.

There are a couple of ways to do this:

1. Follow the authors and works mentioned in the introduction or literature review section of your first article
2. Use the links that Summon, library databases, and sometimes Google Scholar provide to locate any articles that cite your article, as well as other recommended or related works. Look for: **Cited By**, **Recommended**, or **Related** articles

ACTIVITY: Click on the hotspots

The image below is taken from the introduction of an article on populism, found in one of the library's databases.

(Source: Moffitt, B., & Tormey, S. (2014). Rethinking populism: politics, mediatisation and political style. *Political Studies*, *62*(2), 381–397. https://doi.org/10.1111/1467-9248.12032)

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2.5.3.8: Library Research Tools and When to use Them

Throughout this module, we have referred to various library research tools, but we have not discussed when to use them. Learning which tools to use at the various stages of your research is also another strategy for finding the sources best suited to your assignment, and will decrease the amount of time you spend looking.

(Click the tools below for an image of their search interfaces.)

Tool	What's in it?	When should I use it?
Summon	Everything the library has in its collection: • books/ebooks • journal/magazine/newspaper articles • government documents • reference books • videos	Start here when you are new to your topic or assignment. Summon is great for seeing the breadth of what is available on your subject.
Catalogue	Almost everything the library has in its collection, except articles • books/ebooks, • government documents • videos	Use this when you know you are looking for a book or ebook, or when you are looking for a specific title. You will NOT find journal articles here. The catalogue does not have the full-text of items, but many books will have a table of contents.
Article Database	Specialized or multi-disciplinary • peer reviewed journal articles • some trade/professional publications • some newspapers	Use a database when you know you need peer reviewed journal content. Learn which databases are focussed on particular subjects.
Google Scholar	• scholarly journals articles • conference proceedings • research/government publications	Can't find what you're looking for in the article databases? Use this to go beyond the library's collection to see what else is available on Open Access websites or authors' own sites. Ask the library how to maximize access. NEVER pay for articles!

ACTIVITY: Which research tool is best?

Pick which tool would best serve the research need. Click the arrow to answer the next question.

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2.5.3.9: Summing up Part 3

ACTIVITY: Summing up Module 3

Pick the correct statements.

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Well done! You have completed the text and activity portion of Part 3 Developing your Search Strategy. You are welcome to review any part of this module at any time.

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2.5.4: EVALUATE YOUR SOURCES

2.5.4.1: Checklists

2.5.4.2: Question Authority

2.5.4.3: Who is the author?

2.5.4.4: Consider Currency

2.5.4.5: Check for Purpose and Accuracy

2.5.4.6: Explore the source

2.5.4.7: But is it Relevant?

2.5.4.8: Putting it all together

2.5.4.9: Summing up Part 4

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2.5.4.1: Checklists

There are many checklists available to help guide you through this critical process of evaluating your sources; you may have heard of the CRA(A)P test, RADAR, or something similar. All of these lists are really just devices to help novice researchers remember the criteria by which they should evaluate the information they find.

The following list is meant to be a starting point for you to develop your own internalized set of questions.

Click the arrows below.

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2.5.4.2: Question Authority

In terms of evaluating a source of information, the expertise or credibility of its author is extremely important. This is the case not only for your university assignments, but also your personal information needs; in general, we want to know that our sources are reliable and our information sound.

But this idea of authority can be complicated. Within the academic publishing world, determining someone's expertise is somewhat straightforward in that advanced degrees, a publishing record, and an affiliation with an institution of higher learning or research are the conventional indicators of authority.

Outside of the scholarly community, there are other indicators of an author's credibility; other communities may recognize authority or expertise by means of specific credentials or practical experience. For example, we generally rely on articles in the mainstream press because professional journalists are supposed to abide by a code of ethics and have a lengthy publishing record. We see a registered physical therapist to address a sports injury.

When it comes to verifying an author's credibility within the scholarly literature, library research tools can help us to make a quick determination of authority. Recall that in the last module we looked at using the scholarly or peer reviewed limits in Summon and databases to find results that are published in academic journals.

But looking a little more closely at the author and the journal, and perhaps doing some quick Google searching, can help us to make a better decision about the author's expertise in a particular area. Having an advanced degree in theoretical physics does not necessarily make someone an expert in evolutionary biology.

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2.5.4.3: Who is the author?

Activity: Check the authors

Take a look at the following record for an article from the library's Academic Search Complete database. Click on the purple hotspots.

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https://kpu.pressbooks.pub/doingresearch/?p=265

Tip: Go one step further

Going a step further to search for your author on **Google Scholar** will lead to their publication record. What else have they written?

You might also do a quick Google search for the journal's homepage. Look for author submission guidelines where a peer review process should be described, as well as the scope and aim of the journal.

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2.5.4.4: Consider Currency

To evaluate the currency of your information source, you will first need to know the requirements of your assignment. You may be able to use sources as old as 10 years, or you may need very current information. Currency is also somewhat discipline or topic dependent; research in history or literature may involve using older sources, but in the sciences and technology, currency is extremely important.

And sometimes, our research may involve examining a change in thinking or perspective over time, in which case you may need a variety of sources spanning a certain period.

Learn to ask the following kinds of questions:

- What is the publication or copyright date?
- Is it a reprint of a previous work? a new edition or revision?
- Is there newer information available on the topic?
- How might ideas and perspectives have changed since the work was published?

Activity: Decide whether the following sources are current enough for the topic

Chose the correct answer. Click the arrow to answer all 3 questions. Maximize the image by clicking the plus **+**.

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2.5.4.5: Check for Purpose and Accuracy

Making some judgment as to the purpose of your source will also help you determine whether the information it contains is accurate. Asking why something has been published, what overall purpose its author had in creating and sharing it, is part of the critical assessment you'll need to do in order to decide whether you should use it for your research.

For peer reviewed journal articles, books published by scholarly or professional publishers, government reports, and stories from mainstream news outlets, you can be fairly confident that the purpose behind such publications is to provide unbiased information, or contribute to knowledge about a certain topic. A large part of a formal review process includes careful fact-checking by the reviewers.

But evaluating sources from your Google search requires close scrutiny. Ask why a website exists. Are the authors or creators likely to be using unbiased information? Might they be motivated to spread inaccuracies or misinformation? What evidence do they use to support their claims?

Activity: Watch, think, and learn

Take a minute to watch this short video on how to evaluate sources. Think about the techniques used to determine the underlying purpose and potential bias of a website.

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2.5.4.6: Explore the source

Consider the following example.

You decide to research the topic of whether municipalities should add **fluoride** to public **drinking water**.

A Google search produces a mix of government, health, and consumer advocacy sites, including this one from a popular natural medicine website Natural News[New Tab]. (Keep this tab open to answer some of the following questions about the site.)

1. Start by examining the URL

You might begin with a quick check of the URL (the link) and the domain; this will tell you something about the overall purpose of the site. Which of the categories below does this natural medicine site fall into?

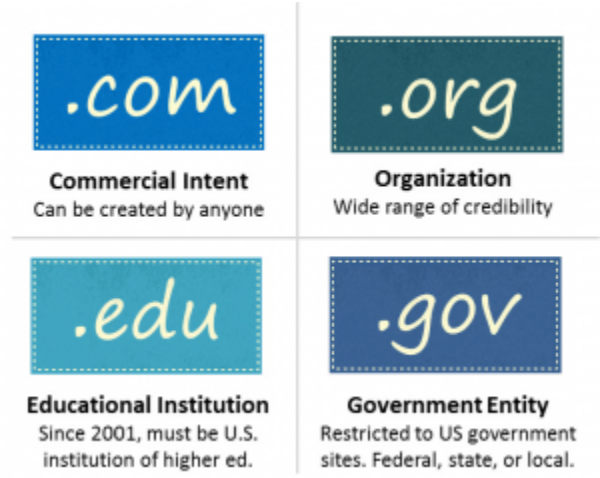


Image by adstarkel.

2. Look around the site

Further evidence of a commercial purpose is clear by the striking presence of advertisements for natural health products and apps, many of which are not related to the topic. Also note the online store. What might this tell you about the intent of the site?

Is it possible to determine the accuracy of the information? The external links provided at the bottom of the article simply refer back to **other articles on the same website**. A check of the two studies referenced **do not** conclude that fluoridated water causes the purported health problems, but conclude that further investigation is needed.

3. Leave the page

Now it's time to find something out about the website and its owner.

Open a new tab or window and do a quick Google search for the website or the owner's name (find this on the About Natural[New Tab] News page). Scan the first few results. How is the website and its owner regarded by other sources, namely the mainstream press and Wikipedia?

Notice that the Wikipedia page for **Fluoridated Drinking Water** includes a link to the controversy[New Tab] surrounding this topic. Go one step further and open the Talk[New Tab] page for this article. What do the comments from Wikipedia editors indicate?

Want further confirmation that Natural News might be less than reliable? Try searching one of these fact-checking sites:

- Snopes[New Tab]
- FactCheck.org[New Tab]

In sum, take a few extra minutes to learn something about the source you are considering.

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2.5.4.7: But is it Relevant?

You have found a source that seems current enough and the information seems accurately portrayed. You may even be able to verify that the author has some expertise or authority on the subject. It is current enough to suit the topic, and it appears to be from an academic publisher.

But there is still one more thing to consider. Is it relevant to your search? Is it written at a level you can understand? Who is the target audience? And perhaps most importantly, how will you use it in your assignment?

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2.5.4.8: Putting it all together

The following graphic illustrates the process of determining the credibility of sources you find on the internet. It is also a useful reminder of the critical questions you should be asking of all the sources you find in your research, including those you find in the library.



Image from IFLA.

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2.5.4.9: Summing up Part 4

Activity: Summing up

Your research question is: **What are the potential harms and benefits of e-cigarettes?**

Decide which of the two sources is better. Use the arrow on the right to move to the next question.

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Well done! You have completed the text and activity portion of Part 4 Evaluate your Sources. You are welcome to review any part of this module at any time.

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2.6.1: RESEARCH QUESTIONS

2.6.1.1: The Purpose of Research Questions
2.6.1.2: Narrowing a Topic
2.6.1.3: Background Reading
2.6.1.4: Regular vs. Research Questions
2.6.1.5: Influence of a Research Question
2.6.1.6: Developing Your Research Question

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2.6.1.1: The Purpose of Research Questions

The Purpose of Research Questions



Research questions are very important.

Both professional researchers and successful student researchers develop research questions. That's because research questions are more than handy tools; they are essential to the research process.

By defining exactly what the researcher is trying to find out, these questions influence most of the rest of the steps taken to conduct the research. That's true even if the research is not for academic purposes but for other areas of our lives.

For instance, if you're seeking information about a health problem in order to learn whether you have anything to worry about, research questions will make it possible for you to more effectively decide whether to seek medical help–and how quickly.

Or, if you're researching a potential employer, having developed and used research questions will mean you're able to more confidently decide whether to apply for an internship or job there.

The confidence you'll have when making such decisions will come from knowing that the information they're based on was gathered by conscious thought rather than serendipity and whim.

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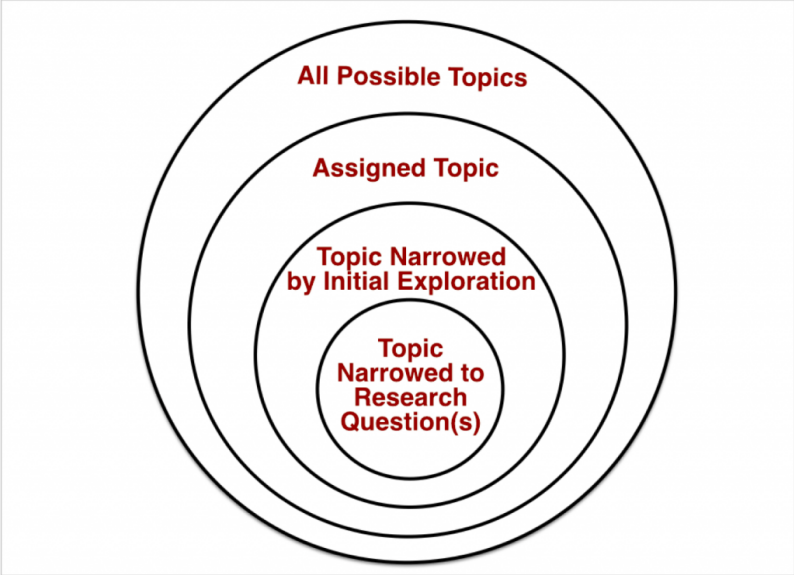
- Diablo Valley College has no license indicated.

2.6.1.2: Narrowing a Topic

Narrowing a Topic

For many students, having to start with a research question is the biggest difference between how they did research in high school and how they are required to carry out their college research projects. It's a process of working from the outside in: you start with the world of all possible topics (or your assigned topic) and narrow down until you've focused your interest enough to be able to tell precisely what you want to find out, instead of only what you want to "write about."

Process of Narrowing a Topic



Visualize narrowing a topic as starting with all possible topics and choosing narrower and narrower subsets until you have a specific enough topic to form a research question.

All Possible Topics – You'll need to narrow your topic in order to do research effectively. Without specific areas of focus, it will be hard to even know where to begin.

Assigned Topics – Ideas about a narrower topic can come from anywhere. Often, a narrower topic boils down to deciding what's interesting to you. One way to get ideas is to read background information in a source like Wikipedia.

Topic Narrowed by Initial Exploration – It's wise to do some more reading about that narrower topic to a) learn more about it and b) learn specialized terms used by professionals and scholars who study it.

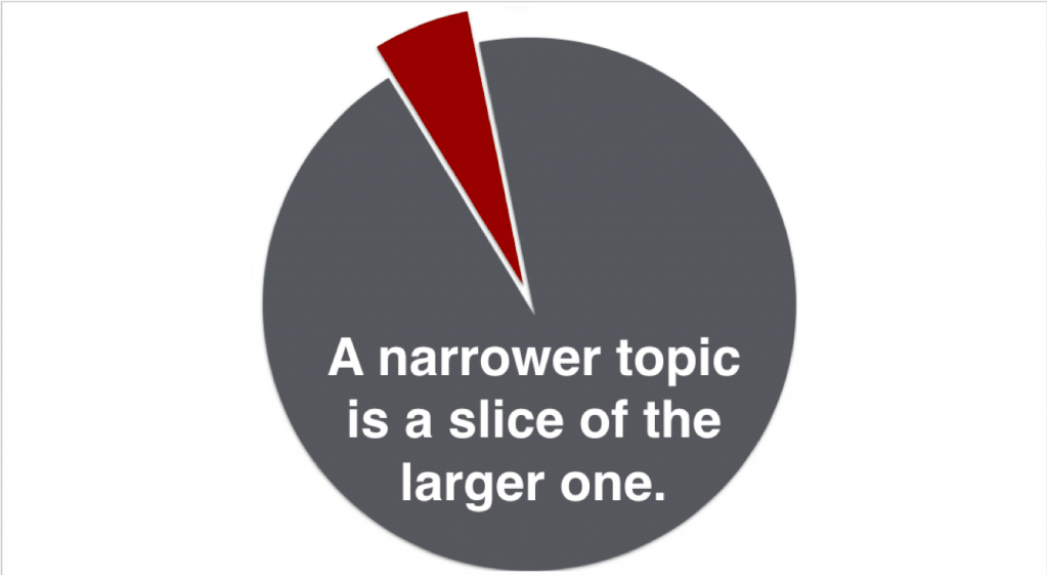
Topic Narrowed to Research Question(s) – A research question defines exactly what you are trying to find out. It will influence most of the steps you take to conduct the research.

> **ACTIVITY: Which Topic Is Narrower?**
>
> Open activity in a web browser.

Why Narrow a Topic?

Once you have a need for research—say, an assignment—you may need to prowl around a bit online to explore the topic and figure out what you actually want to find out and write about.

For instance, maybe your assignment is to develop a poster about “spring” for an introductory horticulture course. The instructor expects you to narrow that topic to something you are interested in and that is related to your class.



Another way to view a narrowed topic is as a sliver of the whole topic.

Ideas about a narrower topic can come from anywhere. In this case, a narrower topic boils down to deciding what’s interesting to you about “spring” that is related to what you’re learning in your horticulture class and small enough to manage in the time you have.

One way to get ideas would be to read about spring in Wikipedia, looking for things that seem interesting and relevant to your class, and then letting one thing lead to another as you keep reading and thinking about likely possibilities that are more narrow than the enormous “spring” topic. (Be sure to pay attention to the references at the bottom of most Wikipedia pages and pursue any that look interesting. Your instructor is not likely to let you cite Wikipedia, but those references may be citable scholarly sources that you could eventually decide to use.)

Or, instead, if it is spring at the time you could start by just looking around, admire the blooming trees on campus, and decide you’d like your poster to be about bud development on your favorites, the crabapple trees.

Anna Narrows Her Topic and Works on a Research Question

The Situation: Anna, an undergraduate, has been assigned a research paper on Antarctica. Her professor expects students to (1) narrow the topic on something more specific about Antarctica because they won’t have time to cover that whole topic. Then they are to (2) come up with a research question that their paper will answer.

The professor explained that the research question should be something they are interested in answering and that it must be more complicated than what they could answer with a quick Google search. He also said that research questions often start with either the word “how” or “why.”

What you should do:

1. Read what Anna is thinking below as she tries to do the assignment.
2. After the reading, answer the questions at the end of the monologue in your own mind.

3. Check your answers with ours at the end of Anna's interior monologue.
4. Keep this demonstration in mind the next time you are in Anna's spot, and you can mimic her actions and thinking about your own topic.

Anna's Interior Monologue

Okay, I am going to have to write something—a research paper—about Antarctica. I don't know anything about that place—I think it's a continent. I can't think of a single thing I've ever wanted to know about Antarctica. How will I come up with a research question about that place? Calls for Wikipedia, I guess.

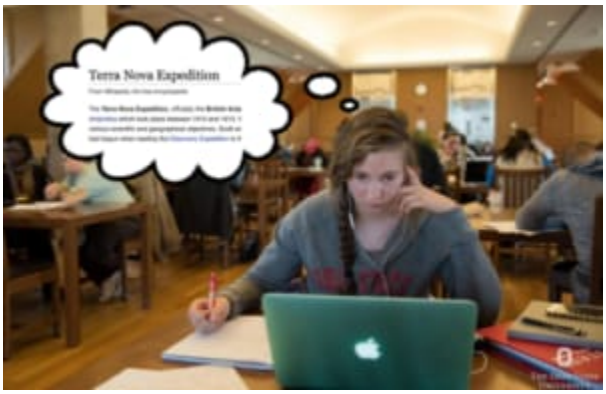


A desert?

At https://en.Wikipedia.org/wiki/Antarctica. Just skimming. Pretty boring stuff. Oh, look– Antarctica's a desert! I guess "desert" doesn't have to do with heat. That's interesting. What else could it have to do with? Maybe lack of precipitation? But there's lots of snow and ice there. Have to think about that—what makes a desert a desert.

It says one to five thousand people live there in research stations. Year round. Definitely the last thing I'd ever do. "...there is no evidence that it was seen by humans until the 19th century." I never thought about whether anybody lived in Antarctica first, before the scientists and stuff.

Lots of names—explorer, explorer... boring. It says Amundson reached the South pole first. Who's Amundson? But wait. It says, "One month later, the doomed Scott Expedition reached the pole." Doomed? Doomed is always interesting. Where's more about the Scott Expedition? I'm going to use that Control-F technique and type in Scott to see if I can find more about him on this page. Nothing beyond that one sentence shows up. Why would they have just that one sentence? I'll have to click on the Scott Expedition link.



Terra Nova...

But it gives me a page called Terra Nova Expedition. What does that have to do with Scott? And just who was Scott? And why was his expedition doomed? There he is in a photo before going to Antarctica. Guess he was English. Other photos show him and his team in the snow. Oh, the expedition was named Terra Nova after the ship they sailed this time—in 1911. Scott had been there earlier on another ship.

Lots of stuff about preparing for the trip. Then stuff about expedition journeys once they were in Antarctica. Not very exciting—nothing about being doomed. I don't want to write about this stuff.

Wait. The last paragraph of the first section says "For many years after his death, Scott's status as tragic hero was unchallenged," but then it says that in the 20th century people looked closer at the expedition's management and at whether Scott and some of his team could be personally blamed for the catastrophe. That "remains controversial," it says. Catastrophe? Personally blamed? Hmm.

Back to skimming. It all seems horrible to me. They actually *planned* to kill their ponies for meat, so when they actually did it, it was no surprise. Everything was extremely difficult. And then when they arrived at the South Pole, they found that the explorer Amundsen had beaten them. Must have been a big disappointment.

The homeward march was even worse. The weather got worse. The dog sleds that were supposed to meet them periodically with supplies didn't show up. Or maybe the Scott group was lost and didn't go to the right meeting places. Maybe that's what that earlier statement meant about whether the decisions that were made were good ones. Scott's diary said the crystallized snow made it seem like they were pushing and pulling the sledges through *dry sand*.



Rocks?!

It says that before things turned really bad (*really* bad? You've already had to eat your *horses*!), Scott allowed his men to put 30 pounds of rocks with fossils on the sledges they were pushing and dragging. Now was that sensible? The men had to push or pull those sledges themselves. What if it was those rocks that actually doomed those men?

But here it says that those rocks are the proof of continental drift. So how did they know those rocks were so important? Was that knowledge worth their lives? Could they have known?

Wow–there is drama on this page! Scott's diary is quoted about their troubles on the expedition—the relentless cold, frostbite, and the deaths of their dogs. One entry tells of a guy on Scott's team "now with hands as well as feet pretty well useless" voluntarily leaving the tent and walking to his death. The diary says that the team member's last words were "I am just going outside and may be some time." Ha!

They all seem lost and desperate but still have those sledges. Why would you keep pulling and pushing those sledges containing an extra 30 pounds of rock when you are so desperate and every step is life or death?



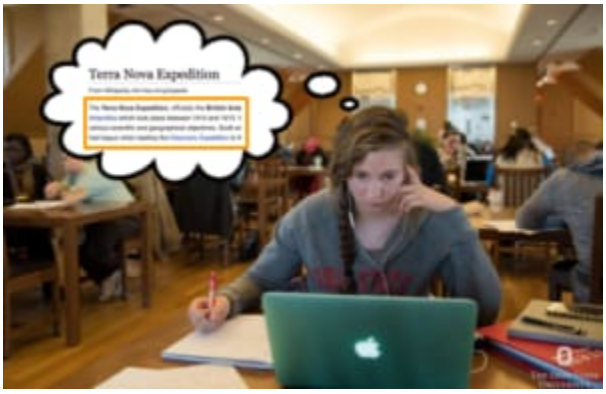
A diary…

Then there's Scott's last diary entry, on March 29, 1912. "… It seems a pity but I do not think I can write more." Well.

That diary apparently gave lots of locations of where he thought they were but maybe they were lost. It says they ended up only 11 miles from one of their supply stations. I wonder if anybody knows how close they were to where Scott *thought* they were.

I'd love to see that diary. Wouldn't that be cool? Online? I'll Google it.

Yes! At the British museum. Look at that! I can see Scott's last entry IN HIS OWN HANDWRITING!



A digital version?

Actually, if I decide to write about something that requires reading the diary, it would be easier to not have to decipher his handwriting. Wonder whether there is a typed version of it online somewhere?

Maybe I should pay attention to the early paragraph on the Terra Nova Expedition page in Wikipedia—about it being controversial whether Scott and his team made bad decisions so that they brought most of their troubles on themselves. Can I narrow my topic to just the controversy over whether bad decisions of Scott and his crew doomed them? Maybe it's too big a topic if I consider the decisions of all team members. Maybe I should just consider Scott's decisions.

So what research question could come from that? Maybe: how did Scott's decisions contribute to his team's deaths in Antarctica? But am I talking about his decisions before or after they left for Antarctica? Or the whole time they were a team? Probably too many decisions involved. More focused: How did Scott's decisions after reaching the South Pole help or hurt the chances of his team getting back safely? That's not bad—maybe. If people have written about that. There are several of his decisions discussed on the Wikipedia page, and I know there are sources at the bottom of that page.



Really, a desert?

Let me think—what else did I see that was interesting or puzzling about all this? I remember being surprised that Antarctica is a desert. So maybe I could make Antarctica as a desert my topic. My research question could be something like: Why is Antarctica considered a desert? But there has to be a definition of deserts somewhere online, so that doesn't sound complicated enough. Once you know the definition of desert, you'd know the answer to the question. Professor Sanders says research questions are more complicated than regular questions.

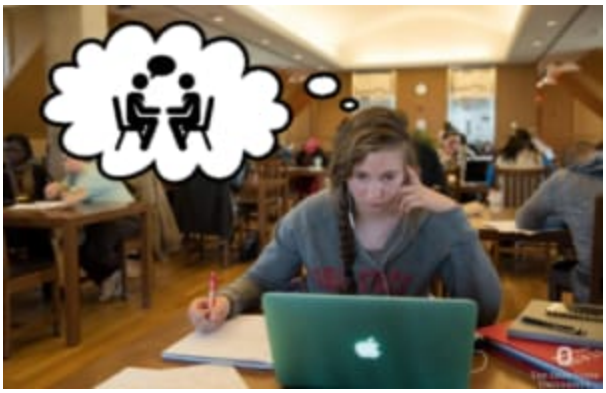
What's a topic I could care about? A question I really wonder about? Maybe those rocks with the fossils in them. It's just so hard to imagine desperate explorers continuing to push those sledges with an extra 30 pounds of rocks on them. Did they somehow know how important they would be? Or were they just curious about them? Why didn't they ditch them? Or maybe they just didn't realize how close to death they were. Maybe I could narrow my Antarctica topic to those rocks.



Why rocks?

Maybe my topic could be something like: The rocks that Scott and his crew found in Antarctica that prove continental drift. Maybe my research question could be: How did Scott's explorers choose the rocks they kept?

Well, now all I have is questions about my questions. Like, is my professor going to think the question about the rocks is still about Antarctica? Or is it all about continental drift or geology or even the psychology of desperate people? And what has been written about the finding of those rocks? Will I be able to find enough sources? I'm also wondering whether my question about Scott's decisions is too big—do I have enough time for it?



I should ask.

I think my professor is the only one who can tell me whether my question about the rocks has enough to do with Antarctica. Since he's the one who will be grading my paper. But a librarian can help me figure out the other things.

So Dr. Sanders and a librarian are next.

Questions

1. Was Anna's choice to start with Wikipedia a good choice? Why or why not?
2. Have you ever used that Control-F technique?
3. At what points does Anna think about where to look for information?
4. At the end of this session, Anna hasn't yet settled on a research question. So what did she accomplish? What good was all this searching and thinking?

Here are our answers below.

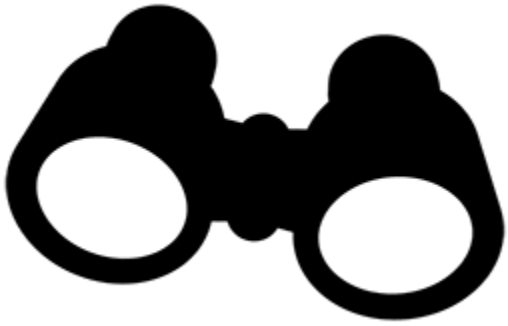
Our Answers:

1. **Was Anna's choice to start with Wikipedia a good choice? Why or why not?** Wikipedia is a great place to start a research project. Just make sure you move on from there, because it's a not a good place to end up with your project. One place to move on to is the sources at the bottom of most Wikipedia pages.
2. **Have you ever used that Control-F technique?** If you haven't used the Control-F technique, we hope you will. It can save you a lot of time and effort reading online material.
3. **At what points does Anna think about where to look for information**? When she began; when she wanted to know more about the Scott expedition; when she wonders whether she could read Scott's diary online; when she thinks about what people could answer her questions.
4. **At the end of this session, Anna hasn't yet settled on a research question. So what did she accomplish? What good was all this reading and thinking?** There are probably many answers to this question. Ours includes that Anna learned more about Antarctica, the subject of her research project. She focused her thinking (even if she doesn't end up using the possible research questions she's considering) and practiced critical thinking skills, such as when she thought about what she could be interested in, when she worked to make her potential research questions more specific, and when she figured out what questions still needed answering at the end. She also practiced her skills at making meaning from what she read, investigating a story that she didn't expect to be there and didn't know had the potential of being one that she is interested in. She also now knows what questions she needs answered and whom to ask. These thinking skills are what college is all about. Anna is way beyond where she was when she started.

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2.6.1.3: Background Reading

Background Reading



Get a good look at your topic through background reading.

It's wise to do some more reading about that narrower topic once you have it. For one reason, you probably don't know much about it yet. For another, such reading will help you learn the terms used by professionals and scholars who have studied your narrower topic. Those terms are certain to be helpful when you're looking for sources later, so jot them down or otherwise remember them.

For instance, if you were going to do research about the treatment for humans with bird flu, this background reading would teach you that professionals and scholars usually use the term avian influenza instead of bird flu when they write about it. (Often, they also use H1N1 or H1N9 to identify the strain.) If you didn't learn that, you would miss the kinds of sources you'll eventually need for your assignment.

Most sources other than journal articles are good sources for this initial reading, including the *New York Times* or other mainline American news outlets, Wikipedia, encyclopedias for the discipline your topic is in (horticulture for the crabapple bud development topic, for instance), dictionaries for the discipline, and manuals, handbooks, blogs, and web pages that could be relevant.

This initial reading could cause you to narrow your topic further, which is fine because narrower topics lead to greater specificity for what you have to find out.After this upfront work, you're ready to start developing the research question(s) you will try to answer for your assignment.

Tip: Keeping Track of Your Information

While you are in the background reading phase of your research you will come across a lot of sources and won't know yet if they will prove useful in the long run. A handy type of software to help you keep track of all your findings is called citation management software. It will also be extremely valuable when it comes to using the resources you end up needing. Three of these tools are available for free to OSU students, staff and faculty. Learn more about these tools and how to access them.



Common Citations Tools

Fuel Your Inspiration

It's worth remembering that reading, scanning, looking at, and listening to information resources is very useful during any step of the process to develop research questions. Doing so can jog our memories, give us details that will help us focus, and help us connect disparate information–all of which will help us come up with research questions that we find interesting.

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2.6.1.4: Regular vs. Research Questions

Regular vs. Research Questions

Most of us look for information to answer questions every day, and we often act on the answers to those questions. Are research questions any different from most of the questions for which we seek information? Yes.

See how they're different by looking over the examples of both kinds below and answering questions about them in the next activity.

Examples: Regular vs. Research Questions

Regular Question: What time is my movie showing at Lennox on Friday?

Research Question: How do "sleeper" films end up having outstanding attendance figures?

Regular Question: What can I do about my insomnia?

Research Question: How do flights more than 16 hours long affect the reflexes of commercial jet pilots?

Regular Question: How many children in the U.S. have allergies?

Research Question: How does his or her country of birth affect a child's chances of developing asthma?

Regular Question: What year was metformin approved by the U.S. Food and Drug administration?

Research Question: Why are nanomedicines, such as doxorubicin, worth developing?

Regular Question: Could citizens register to vote at branches of the Columbus Public Library in 2016?

Research Question: How do public libraries in the United States support democracy?

Regular Question: What is the Whorfian Hypothesis?

Research Question: Why have linguists cared about the Whorfian hypothesis?

Regular Question: Where is the Apple, Inc. home office?

Research Question: Why are Apple's marketing efforts so successful?

Regular Question: What is Mers?

Research Question: How could decision making about whether to declare a pandemic be improved?

Regular Question: Does MLA style recommend the use of generic male pronouns intended to refer to both males and females?

Research Question: How do age, gender, IQ, and socioeconomic status affect whether students interpret generic male pronouns as referring to both males and females?

Activity: Which Kind of Question

Open activity in a web browser.

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2.6.1.5: Influence of a Research Question

Influence of a Research Question

Whether you're developing research questions for your personal life, your work for an employer, or for academic purposes, the process always forces you to figure out exactly:

- What you're interested in finding out.
- What it's feasible for you to find out (given your time, money, and access to information sources).
- How you can find it out, including what research methods will be necessary and what information sources will be relevant.
- What kind of claims you'll be able to make or conclusions you'll be able to draw about what you found out.

For academic purposes, you may have to develop research questions to carry out both large and small assignments. A smaller assignment may be to do research for a class discussion or to, say, write a blog post for a class; larger assignments may have you conduct research and then report it in a lab report, poster, term paper, or article.

For large projects, the research question (or questions) you develop will define or at least heavily influence:

- Your **topic**, in that research questions effectively narrow the topic you've first chosen or been assigned by your instructor.
- What, if any, **hypotheses** you test.
- Which **information sources** are relevant to your project.
- Which **research methods** are appropriate.
- What claims you can make or **conclusions** you can come to as a result of your research, including what **thesis statement** you should write for a term paper or what **results section** you should write about the data you collected in your own science or social science study.



Your research question drives your hypothesis, research methods, sources, and your claims or conclusions.

Influence on Thesis

Within an essay, poster, or term paper, the thesis is the researcher's answer to the research question(s). So as you develop research questions, you are effectively specifying what any thesis in your project will be about. While perhaps many research

questions could have come from your original topic, your question states exactly which one(s) *your* thesis will be answering.

For example, a topic that starts out as “desert symbiosis” could eventually lead to a research question that is “how does the diversity of bacteria in the gut of the Sonoran Desert termite contribute to the termite’s survival?” In turn, the researcher’s thesis will answer that particular research question instead of the numerous other questions that could have come from the desert symbiosis topic.

Developing research questions is all part of a process that leads to greater and greater specificity for your project.

Tip: Don’t Make These Mistakes

Sometimes students inexperienced at working with research questions confuse them with the search statements they will type into the search box of a search engine or database when looking for sources for their project. Or, they confuse research questions with the thesis statement they will write when they report their research. The next activity will help you sort things out.

Activity: From Topic to Thesis Statement

Open activity in a web browser.

Influence on Hypothesis

If you’re doing a study that predicts how variables are related, you’ll have to write at least one hypothesis. The research questions you write will contain the variables that will later appear in your hypothesis(es).

Activity: Guess the Question

Despite how strong their influence is on the rest of the researcher’s tasks, research questions don’t always appear in a report of the research. Nonetheless, you can usually figure out what the researcher’s research questions were by reading the title and some of the report. Take a look at this article “Getting to the Center of a Tootsie Roll Pop®” [OSU login required] and determine what the students’ research question was.

Influence on Resources

You can’t tell whether an information source is relevant to your research until you know exactly what you’re trying to find out. Since it’s the research questions that define that, it’s they that divide all information sources into two groups: those that are relevant to your research and those that are not—all based on whether each source can help you find out what you want to find out and/or report the answer.

Influence on Research Methods

Your research question(s) will help you figure out what research methods you should use because the questions reflect what your research is intended to do. For instance, if your research question relates to describing a group, survey methods may work well. But they can’t answer cause-and-effect questions.

Influence on Claims or Conclusions

The research questions you write will reflect whether your research is intended to describe a group or situation, to explain or predict outcomes, or to demonstrate a cause-and-effect relationship(s) among variables. It’s those intentions and how well you carry out the study, including whether you used methods appropriate to the intentions, that will determine what claims or

conclusions you can make as a result of your research.

Activity: Quick Check

Open activity in a web browser.

Answer to Activity: Guess the Question

The answer to the “Guess the Question” Activity above is:

What was the students’ research question? How many licks does it take to get to the center of a Tootsie Roll Pop?

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2.6.1.6: Developing Your Research Question

Developing Your Research Question

Because of all their influence, you might worry that research questions are very difficult to develop. Sometimes it can seem that way. But we'll help you get the hang of it and, luckily, none of us has to come up with perfect ones right off. It's more like doing a rough draft and then improving it. That's why we talk about *developing* research questions instead of just writing them.

Steps for Developing a Research Question

The steps for developing a research question, listed below, can help you organize your thoughts.

Step 1: Pick a topic (or consider the one assigned to you).

Step 2: Write a narrower/smaller topic that is related to the first.

Step 3: List some potential questions that could logically be asked in relation to the narrow topic.

Step 4: Pick the question that you are most interested in.

Step 5: Change that question you're interested in so that it is more focused.

MOVIE: Developing Research Questions

As you view this short video on how to develop research questions, think about the steps. Which step do you think is easiest? Which do you think is hardest?

An interactive or media element has been excluded from this version of the text. You can view it online here: https://ohiostate.pressbooks.pub/choosingsources/?p=63

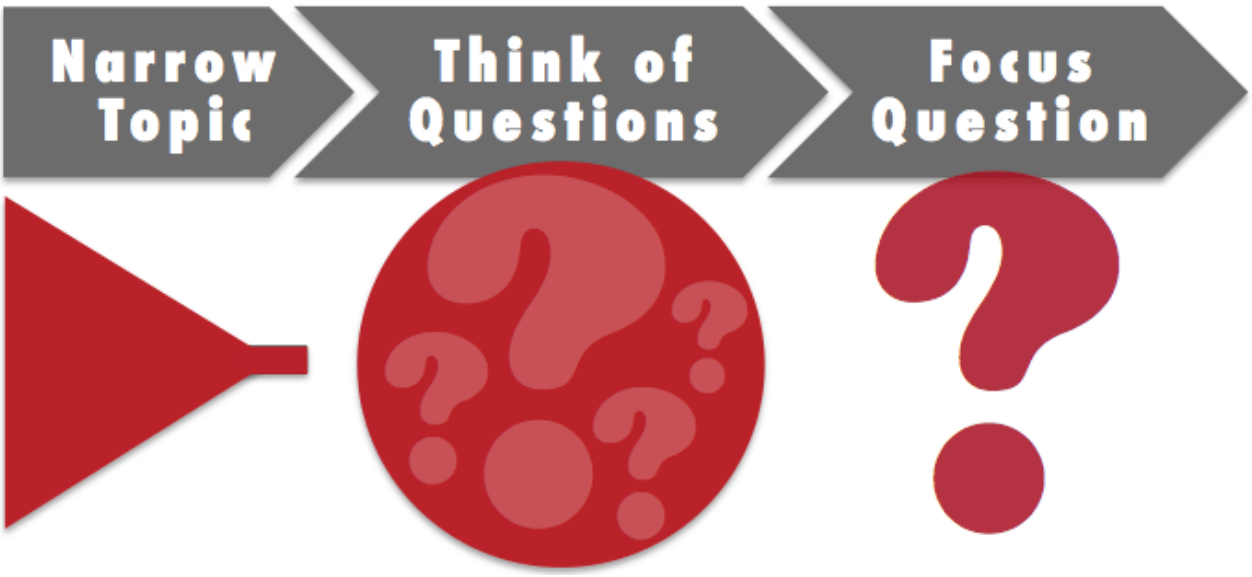
View Movie | View Text Version

Practice

Once you know the steps and their order, only three skills are involved in developing a research question:

- Imagining narrower topics about a larger one,
- Thinking of questions that stem from a narrow topic, and
- Focusing questions to eliminate their vagueness.

Every time you use these skills, it's important to evaluate what you have produced—that's just part of the process of turning rough drafts into more finished products.



Three steps for developing a research question

ACTIVITY: Developing a Research Question

Open activity in a web browser.

Maybe you have a topic in mind, but aren't sure how to form a research question around it. The trick is to think of a question related to your topic, but not answerable with a quick search. Also, try to be specific so that your research question can be fully answered in the final product for your research assignment.

ACTIVITY: Thinking of Questions

For each of the narrow topics below, think of a research question that is logically related to that topic. (Remember that good research questions often, but not always, start with "Why" or "How" because questions that begin that way usually require more analysis.)

Topics:

- U.S. investors' attitudes about sustainability
- College students' use of Snapchat
- The character Scout in *To Kill a Mockingbird*
- Nature-inspired nanotechnologies
- Marital therapy

After you think of each research question, evaluate it by asking whether it is:

- Logically related to the topic
- In question form
- Not answerable with a quick Google search
- Specific, not vague

Sometimes the first draft of a research question is still too broad, which can make your search for sources more challenging. Refining your question to remove vagueness or to target a specific aspect of the topic can help.

ACTIVITY: Focusing Questions

The first draft research questions below are not focused enough. Read them and identify at least one area of vagueness in each. Check your vagueness with what we identified. It's great if you found more than we did because that can lead to research questions of greater specificity. See the bottom of the page for the answers.

First Drafts of Research Questions:

1. Why have most electric car company start-ups failed?
2. How do crabapple trees develop buds?
3. How has NASA helped America?
4. Why do many first-time elections soon after a country overthrows a dictator result in very conservative elected leaders?
5. How is music composed and performed mostly by African-Americans connected to African-American history?

Answer TO ACTIVITY: Focusing Questions

Some answers to the "Focusing Questions" Activity above are:

Question 1: Why have most electric car company start-ups failed?
Vagueness: Which companies are we talking about? Worldwide or in a particular country?

Question 2: How do crabapple trees develop buds?
Vagueness: There are several kinds of crabapples. Should we talk only about one kind? Does it matter where the crabapple tree lives?

Question 3: How has NASA helped America?
Vagueness: NASA has had many projects. Should we should focus on one project they completed? Or projects during a particular time period?

Question 4: Why do many first-time elections soon after a country overthrows a dictator result in very conservative elected leaders?
Vagueness: What time period are we talking about? Many dictators have been overthrown and many countries have been involved. Perhaps we should focus on one country or one dictator or one time period.

Question 5: How is music composed and performed mostly by African-Americans connected to African-American history?
Vagueness: What kinds of music? Any particular performers and composers? When?

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2.6.2: TYPES OF SOURCES

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2.6.2.9: News as a Source
2.6.2.10: Data as Sources

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2.6.2.1: People as Sources

People as Sources

People don't just create the sources we use. They are actually sources them-selves. Most of us use people as sources all the time in our private lives, such when we ask a friend for a restaurant recommendation or ask whether a movie is worth watching. But you probably aren't using people as sources very often in your assignments–unless you are a journalism major, of course.

In fact, research indicates that employers such as Battelle, Nationwide Insurance, Microsoft, the FBI, the Smithsonian, the Port of Los Angeles, SS&G Financial Services, and Marriott International have been dissatisfied with their new hires' inability to gather information by talking with real people. They've found new hires unwilling or unprepared to ask the experienced employee down the hall or the expert across town for information to solve a problem. For instance, the study linked to above quotes one employer as saying about new hires:

Here's something we're targeting in interviews now—the big thing is they believe the computer is their workspace, so basic interactions between people are lost. They won't get up and walk over and ask someone a question. They are less comfortable and have some lack of willingness to use people as sources and also have a lack of awareness that people are a valid source of information...

So getting some experience using people as sources is likely to help you not just with a current research assignment but with your work in the future.

Important: Who's an "Expert"?

Experts aren't only researchers with Ph.D.s doing academic work. The question when trying to decide who can be a source is really always, who can speak with authority about any part of the subject? And the answer to that question is always contextual, a kind of "it depends."

People can speak with authority for different reasons. According to the framework for information literacy, they can have subject expertise (say, having done scholarship in the field), societal position (maybe a public office or other relevant work title), or special experience (say, living or working in a particular situation of interest or having participated in an historical event).

For instance, people who have had firsthand experience living or working with a situation (say, a survivor of school shooting if your topic is on that subject) you are studying can have a unique perspective unavailable elsewhere. And it's that up-close, firsthand view of the situation that gives them the authority that you and your audience respond to.

Of course, such sources have to be evaluated just like any other. Could they be biased? Like any source, yes. We just have to keep that possible bias in mind as we use the information from such a source. That's part of exercising the critical thinking that research assignments are famous for producing.

Potentially biased or not, sometimes a source's firsthand experience can't be beat. And recognizing what they offer can help us open up to diverse ideas and worldviews that we would otherwise miss. Don't be surprised if this kind of source takes you off in completely new directions with your assignment, ones that turn out to be much more interesting than those you were following before. For many researchers, finding sources that really open up a topic like that is one of the most rewarding—and fun—things about doing research.

Some Examples of People as Sources

Research Question	Potential Person as Source	Potential Person as Source
How are tools originally developed for medicine, geology, and manufacturing used to explore paintings and sculptures?	An art conservator who uses those tools that you read about in the newspaper or other source	The person who invented one of the tools on the floor of the factory where he works

Why do most people who qualify for food at foodbanks not ask for food?	A local food bank director	A person (perhaps a fellow student) who qualifies but does not ask for food at a food bank
How and why do city and county governments brand themselves?	An official in such a city or county who has been involved in branding decisions	The director of a company that designs branding for cities and counties

You can interview a person as a source on the phone, in email, with Skype, or face-to-face. You'll need to:

- Pay attention when reading other sources so you can identify whom to contact and know what they could have to offer.
- Prepare by learning enough about your topic so you can ask appropriate questions, know what your expert has done in relation to that topic so you don't seem ignorant of their contribution, and know how to contact them. You might also want to do a practice interview with a friend.
- Contact your source to see if they are willing to talk with you and when that would be convenient. Then follow through.

Use good interview techniques, such as trying to put them at ease, using active listening techniques to encourage them to talk, asking follow up questions, and thanking them at the end of the interview.

TIP: How to identify researchers at OSU to interview.

1. Search the database Scopus for your topic. Once you have some results, use the Affiliation option among Define Results options on the left to limit your search to Ohio State University. You may also want to limit by the Year option.
2. Pull up relevant articles' records in Scopus (you can sort by times cited) and then identify which of the authors were at the time at OSU. (Articles may have multiple authors–sometimes over a dozen or more if they are in science).
3. Go to Find People (on the OSU navigation bar on all OSU pages) and search for the OSU person's name to see if they're still at OSU and get their contact information. In many cases, they will still be at OSU, especially for fairly recent articles, because OSU updates author profiles regularly. Note that researchers may or may not be faculty–some may be staff or even students.

Citing People as Sources

Like other sources, people should be cited in your research final product, depending on the citation style you're using. For instance, in APA style, interviews, e-mail, and other personal communication should not appear in the reference list but should be in your main text only like this: (A. Authorslastname, personal communication, July 29, 2018).

See Purdue Online Writing Lab (OWL) for information on how to handle interviews and other communication with people in other styles.

ACTIVITY: People as Sources

Open activity in a Web browser

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2.6.2.2: Categorizing Sources

Categorizing Sources



Understanding types of sources helps guide your search.

Once you have your research question, you'll need information sources to answer it and meet the other information needs of your research project.

This section about categorizing sources will increase your sophistication about them and save you time in the long run because you'll understand the "big picture". That big picture will be useful as you plan your own sources for a specific research project, which we'll help you with in the next section Sources and Information Needs.

You'll usually have a lot of sources available to meet the information needs of your projects. In today's complex information landscape, just about anything that contains information can be considered a potential source.

Here are a few examples:

- Books and encyclopedias
- Websites, web pages, and blogs
- Magazine, journal, and newspaper articles
- Research reports and conference papers
- Field notes and diaries
- Photographs, paintings, cartoons, and other art works
- TV and radio programs, podcasts, movies, and videos
- Illuminated manuscripts and artifacts
- Bones, minerals, and fossils
- Preserved tissues and organs
- Architectural plans and maps
- Pamphlets and government documents
- Music scores and recorded performances
- Dance notation and theater set models

With so many sources available, the question usually is not whether sources exist for your project but which ones will best meet your information needs.

Being able to categorize a source helps you understand the kind of information it contains, which is a big clue to (1) whether might meet one or more of your information needs and (2) where to look for it and similar sources.

A source can be categorized by:

- Whether it contains quantitative or qualitative information or both
- Whether the source is objective (factual) or persuasive (opinion) and may be biased
- Whether the source is a scholarly, professional or popular publication
- Whether the material is a primary, secondary or tertiary source
- What format the source is in

As you may already be able to tell, sources can be in more than one category at the same time because the categories are not mutually exclusive.

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2.6.2.3: Quantitative or Qualitative

Quantitative or Qualitative



Information can be quantitative or qualitative.

One of the most obvious ways to categorize information is by whether it is quantitative or qualitative. Some sources contain either quantitative information or qualitative information, but sources often contain both.

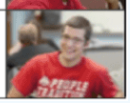
Many people first think of information as something like what's in a table or spreadsheet of numbers and words. But information can be conveyed in more ways than textually or numerically.

Quantitative Information – Involves a measurable quantity—numbers are used. Some examples are length, mass, temperature, and time. Quantitative information is often called data, but can also be things other than numbers.

Qualitative Information – Involves a descriptive judgment using concept words instead of numbers. Gender, country name, animal species, and emotional state are examples of qualitative information.

Take a quick look at the Example table below. Another way we could display the table's numerical information is in a graphic format —listing the students' ages or GPAs on a bar chart, for example, rather than in a list of numbers. Or, all the information in the table could be displayed instead as a video of each student giving those details about themselves.

Example: Data Table with Quantitative and Qualitative Data

Last Name	First Name	Age	Rank	Major	Gender	Current GPA	Photo
Adams	Grace	19	Sophomore	English	Female	3.78	
Bloomfield	Erika	21	Junior	Physics	Female	3.89	
Chow	Kimmie	20	Senior	Political Science	Female	3.77	
Crutchfield	Seth	23	Senior	Psychology	Male	3.58	
Fitch	Fredrick	18	Freshman	Art	Male	4.0	
Grover	Oscar	26	Junior	Biology	Male	3.32	

This table illustrates that information can include a range of formats, including pictures.

Increasingly, other formats (such as images, sound, and video) may be is used as information or used to convey information. Some examples:

- A video of someone watching scenes from horror movies, with information about their heart rate and blood pressure embedded in the video. Instead of getting a description of the person's reactions to the scenes, you can see their reactions.
- A database of information about birds, which includes a sound file for each bird singing. Would you prefer a verbal description of a bird's song or an audio clip?
- A list of colors, which include an image of the actual color. Such a list is extremely helpful, especially when there are A LOT of color names.
- A friend orally tells you that a new pizza place is 3 blocks away, charges $2 a slice, and that the pizza is delicious. This may never be recorded, but it may be very valuable information if you're hungry!
- A map of Ohio with counties shaded different intensities of red according to the median household income of inhabitants.

Activity: Quantitative vs. Qualitative

What quantitative and qualitative data components might you use to describe yourself? See the bottom of the page for some possible answers.

Activity: Multiple Data Displays

Take a look at the Wikipedia article about UN Secretaries-General. Scroll down and view the table of people who served as Secretary-General. In what ways is information conveyed in ways other than text or numbers? See the bottom of the page for answers.

Answer to Activity: Quantitative vs. Qualitative

The answer to the "Quantitative vs. Qualitative" Activity above is:

Quantitative: age, weight, GPA, income
Qualitative: race, gender, class (freshman, sophomore, etc.), major

Are there others?

Answer to Activity: Multiple Data Displays

The answer to the “Multiple Data Displays” Activity above is:

- A photo of each secretary general
- The flag of their country of origin
- A world map with their country of origin shaded

Are there others?

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2.6.2.4: Fact or Opinion

Fact or Opinion



An author's purpose can influence the kind of information he or she choses to include.

Thinking about the reason an author produced a source can be helpful to you because that reason was what dictated the kind of information he/she chose to include. Depending on that purpose, the author may have chosen to include factual, analytical, and objective information. Or, instead, it may have suited his/her purpose to include information that was subjective and therefore less factual and analytical. The author's reason for producing the source also determined whether he or she included more than one perspective or just his/her own.

Authors typically want to do at least one of the following:

- Inform and educate
- Persuade
- Sell services or products or
- Entertain

Combined Purposes

Sometimes authors have a combination of purposes, as when a marketer decides he can sell more smart phones with an informative sales video that also entertains us. The same is true when a singer writes and performs a song that entertains us but that she intends to make available for sale. Other examples of authors having multiple purposes occur in most scholarly writing.

In those cases, authors certainly want to inform and educate their audiences. But they also want to persuade their audiences that what they are reporting and/or postulating is a true description of a situation, event, or phenomenon or a valid argument that their audience must take a particular action. In this blend of scholarly author's purposes, the intent to educate and inform is considered to trump the intent to persuade.

Why Intent Matters

Authors' intent usually matters in how useful their information can be to your research project, depending on which information need you are trying to meet. For instance, when you're looking for sources that will help you actually decide your answer to your research question or evidence for your answer that you will share with your audience, you will want the author's main purpose to have been to inform or educate his/her audience. That's because, with that intent, he/she is likely to have used:

- Facts where possible.
- Multiple perspectives instead of just his/her own.
- Little subjective information.
- Seemingly unbiased, objective language that cites where he/she got the information.

The reason you want that kind of resource when trying to answer your research question or explaining that answer is that all of those characteristics will lend credibility to the argument you are making with your project. Both you and your audience will simply find it easier to believe—will have more confidence in the argument being made—when you include those types of sources.

Sources whose authors intend only to persuade others won't meet your information need for an answer to your research question or evidence with which to convince your audience. That's because they don't always confine themselves to facts. Instead, they tell us their opinions without backing them up with evidence. If you used those sources, your readers will notice and not believe your argument.

Fact vs. Opinion vs. Objective vs. Subjective

Need to brush up on the differences between fact, objective information, subjective information, and opinion?

Fact – Facts are useful to inform or make an argument.

Examples:

- The United States was established in 1776.
- The pH levels in acids are lower than pH levels in alkalines.
- Beethoven had a reputation as a virtuoso pianist.

Opinion – Opinions are useful to persuade, but careful readers and listeners will notice and demand evidence to back them up.

Examples:

- That was a good movie.
- Strawberries taste better blueberries.
- George Clooney is the sexiest actor alive.
- The death penalty is wrong.
- Beethoven's reputation as a virtuoso pianist is overrated.

Objective – Objective information reflects a research finding or multiple perspectives that are not biased.

Examples:

- "Several studies show that an active lifestyle reduces the risk of heart disease and diabetes."
- "Studies from the Brown University Medical School show that twenty-somethings eat 25 percent more fast-food meals at this age than they did as teenagers."

Subjective – Subjective information presents one person or organization's perspective or interpretation. Subjective information can be meant to distort, or it can reflect educated and informed thinking. All opinions are subjective, but some are backed up with facts more than others.

Examples:

- "The simple truth is this: As human beings, we were meant to move."

- “In their thirties, women should stock up on calcium to ensure strong, dense bones and to ward off osteoporosis later in life.”*

*In this quote, it’s mostly the “should” that makes it subjective. The objective version of the last quote would read: “Studies have shown that women who begin taking calcium in their 30s show stronger bone density and fewer repercussions of osteoporosis than women who did not take calcium at all.” But perhaps there are other data showing complications from taking calcium. That’s why drawing the conclusion that requires a “should” makes the statement subjective.

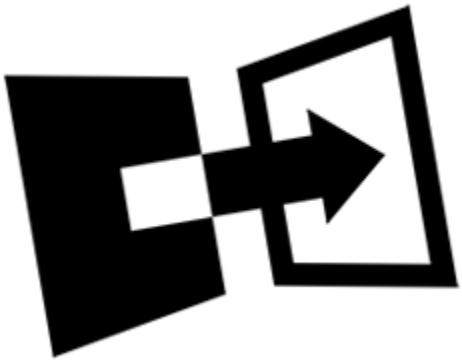
Activity: Fact, Opinion, Objective, or Subjective?

Open activity in a web browser.

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2.6.2.5: Primary, Secondary and Tertiary Sources

Primary, Secondary & Tertiary Sources



Another way to categorize information is by whether information is in its original format or has been reinterpreted.

Another information category is called publication mode and has to do with whether the information is

- Firsthand information (information in its original form, not translated or published in another form).
- Secondhand information (a restatement, analysis, or interpretation of original information).
- Third-hand information (a summary or repackaging of original information, often based on secondary information that has been published).

The three labels for information sources in this category are, respectively, primary sources, secondary sources, and tertiary sources. Here are examples to illustrate the first- handedness, second-handedness, and third-handedness of information:

Primary Source (Original, Firsthand Information)	J.D. Salinger's novel Catcher in the Rye.
Secondary Source (Secondhand Information)	A book review of Catcher in the Rye, even if the reviewer has a different opinion than anyone else has ever published about the book- he or she is still just reviewing the original work and all the information about the book here is secondary.
Tertiary Source (Third-hand Information)	Wikipedia page about J.D. Salinger.

When you make distinctions between primary, secondary, and tertiary sources, you are relating the information itself to the context in which it was created. Understanding that relationship is an important skill that you'll need in college, as well as in the workplace. Noting the relationship between creation and context helps us understand the "big picture" in which information operates and helps us figure out which information we can depend on. That's a big part of thinking critically, a major benefit of actually becoming an educated person.

Primary Sources – Because it is in its original form, the information in primary sources has reached us from its creators without going through any filter. We get it firsthand. Here are some examples that are often used as primary sources:

- Any literary work, including novels, plays, and poems.
- Breaking news.
- Diaries.
- Advertisements.
- Music and dance performances.
- Eyewitness accounts, including photographs and recorded interviews.
- Artworks.
- Data.
- Blog entries that are autobiographical.
- Scholarly blogs that provide data or are highly theoretical, even though they contain no autobiography.
- Artifacts such as tools, clothing, or other objects.
- Original documents such as tax returns, marriage licenses, and transcripts of trials.
- Websites, although many are secondary.
- Buildings.
- Correspondence, including email.
- Records of organizations and government agencies.
- Journal articles that report research for the first time (at least the parts about the new research, plus their data).

Secondary Source – These sources are translated, repackaged, restated, analyzed, or interpreted original information that is a primary source. Thus, the information comes to us secondhand, or through at least one filter. Here are some examples that are often used as secondary sources:

- All nonfiction books and magazine articles except autobiography.
- An article or website that critiques a novel, play, painting, or piece of music.
- An article or web site that synthesizes expert opinion and several eyewitness accounts for a new understanding of an event.
- The literature review portion of a journal article.

Tertiary Source – These sources *further* repackage the original information because they index, condense, or summarize the original.

Typically, by the time tertiary sources are developed, there have been many secondary sources prepared on their subjects, and you can think of tertiary sources as information that comes to us "third-hand." Tertiary sources are usually publications that you are not intended to read from cover to cover but to dip in and out of for the information you need. You can think of them as a good place for background information to start your research but a bad place to end up. Here are some examples that are often used as tertiary sources:

- Almanacs.
- Dictionaries.
- Guide books, including the one you are now reading.
- Survey articles.
- Timelines.
- Bibliographies.
- Encyclopedias, including Wikipedia.
- Most textbooks.

Tertiary sources are usually not acceptable as cited sources in college research projects because they are so far from firsthand information. That's why most professors don't want you to use Wikipedia as a citable source: the information in Wikipedia is far from original information. Other people have considered it, decided what they think about it, rearranged it, and summarized it–all of which is actually what your professors want *you*, not another author, to do with information in your research projects.

> **Activity: Which Kind of Source?**
>
> Open activity in a web browser.

The Details Are Tricky— A few things about primary or secondary sources might surprise you:

- Sources *become* primary rather than always exist as primary sources.

It's easy to think that it is the *format* of primary sources that makes them primary. But that's not all that matters. So when you see lists like the one above of sources that are often used as primary sources, it's wise to remember that the ones listed are not *automaticallyalready* primary sources. Firsthand sources get that designation only when researchers actually find their information relevant and use it.

For instance: Records that could be relevant to those studying government are created every day by federal, state, county, and city governments as they operate. But until those raw data are actually used by a researcher, they cannot be considered primary sources.

Another example: A diary about his flying missions kept by an American helicopter pilot in the Viet Nam War is not a primary source until, say, a researcher uses it in her study of how the war was carried out. But it will never be a primary source for a researcher studying the U.S. public's reaction to the war because it does not contain information relevant to that study.

- Primary sources, even eyewitness accounts, are not necessarily accurate. Their accuracy has to be evaluated, just like that of all sources.
- Something that is usually considered a secondary source can be considered a primary source, depending on the research project.

For instance, movie reviews are usually considered secondary sources. But if your research project is about the effect movie reviews have on ticket sales, the movie reviews you study would become primary sources.

- Deciding whether to consider a journal article a primary or a secondary source can be complicated for at least two reasons.

First, journal articles that report new research for the first time are usually based on data. So some disciplines consider the *data* to be the primary source, and the journal article that describes and analyzes them is considered a secondary source.

However, particularly in the sciences, the original researcher might find it difficult or impossible (he or she might not be allowed) to share the data. So sometimes you have nothing more firsthand than the journal article, which argues for calling it the relevant primary source because it's the closest thing that exists to the data.

Second, even journal articles that announce new research for the first time usually contain more than data. They also typically contain secondary source elements, such as a literature review, bibliography, and sections on data analysis and interpretation. So they can actually be a *mix* of primary and secondary elements. Even so, in some disciplines, a journal article that announces new research findings for the first time is considered to be, as a whole, a primary source for the researchers using it.

ACTIVITY: Under What Circumstances?

Instructions: Look at each of the sources listed below and think of circumstances under which each could become a primary source. (There are probably many potential circumstances for each.) So just imagine you are a researcher with projects that would make each item firsthand information that is relevant to your work. What could a project be about that would make each source relevant firsthand information? Our answers are at the bottom of the page, but remember that there are many more–including the ones you think of that we didn't!

a. Fallingwater, a Pennsylvania home designed and constructed by Frank Lloyd Wright in the 1930s.
b. Poet W.H. Auden's elegy for Y.S. Yeats.
c. An arrowhead made by (Floriday) Seminole Native Americans but found at Flint Ridge outside Columbus, Ohio.
d. E-mail between the U.S. ambassador to the United Nations, Nikki Haley, and her staff about North Korea.
e. A marriage license.

Despite their trickiness, what primary sources usually offer is too good not to consider using because:

- They are original. This unfiltered, firsthand information is not available anywhere else.
- Their creator was a type of person unlike others in your research project, and you want to include that perspective.
- Their creator was present at an event and shares an eyewitness account.
- They are objects that existed at the particular time your project is studying.

Particularly in humanities courses, your professor may require you to use a certain number of primary sources for your project. In other courses, particularly in the sciences, you may be required to use *only* primary sources.

What are considered primary and secondary sources can vary from discipline to discipline. If you are required to use primary sources for your research project, before getting too deep into your project check with your professor to make sure he or she agrees with your choices. After all, it's your professor who will be grading your project. A librarian, too, can verify your choices. Just remember to take a copy of your assignment with you when you ask, because the librarian will want to see the original assignment. After all, that's a primary source!

POSSIBLE AnswerS TO ACTIVITY: Under What Circumstances?

a. You are doing a study of the entrances Wright designed for homes, which were smaller than other architects of the time typically designed entrances.
b. Your research project is about the Auden-Yeats relationship.
c. Your research project is about trade among 19th century Native Americans east of the Mississippi River.
d. Your research project is on how Ambassador Haley conveyed a decision about North Korea to her staff.
e. You are writing about the life of a person who claimed to have married several times, and you need more than her statements about when those marriages took place and to whom.

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2.6.2.6: Popular, Professional, and Scholarly

Popular, Professional, & Scholarly



We can also categorize information by the expertise of its intended audience. Considering the intended audience—how expert one has to be to understand the information—can indicate whether the source has sufficient credibility and thoroughness to meet your need.

There are varying degrees of expertise:

Popular – Popular newspaper and magazine articles (such as *The Washington Post*, the *New Yorker*, and *Rolling Stone*) are meant for a large general audience, are generally affordable, and are easy to purchase or available for free. They are written by staff writers or reporters for the general public.

Additionally, they are:

- About news, opinions, background information, and entertainment.
- More attractive than scholarly journals, with catchy titles, attractive artwork, and many advertisements but no footnotes or references.
- Published by commercial publishers.
- Published after approval from an editor.
- For information on using news articles as sources (from newspapers in print and online, broadcast news outlets, news aggregators, news databases, news feeds, social media, blogs, and citizen journalism), see News as a Source.

Professional – Professional magazine articles (such as *Plastic Surgical Nursing* and *Music Teacher)* are meant for people in a particular profession, and are often accessible through a professional organization. Staff writers or other professionals in the targeted field write these articles at a level and with the language to be understood by everyone in the profession.

Additionally, they are:

- About trends and news from the targeted field, book reviews, and case studies.
- Often less than 10 pages, some of which may contain footnotes and references.
- Usually published by professional associations and commercial publishers.
- Published after approval from an editor.

Scholarly – Scholarly journal articles (such as *Plant Science* and *Education and Child Psychology)* are meant for scholars, students, and the general public who want a deep understanding of a problem or issue. Researchers and scholars write these articles to present new knowledge and further understanding of their field of study.

Additionally, they are:

- Where findings of research projects, data and analytics, and case studies usually appear first.
- Often long (usually over 10 pages) and always include footnotes and references.

- Usually published by universities, professional associations, and commercial publishers.
- Published after approval by peer review or from the journal's editor.

See Scholarly Articles as Sources for more detail.

Tip: Source Locator

Our Source Locator can help you see where sources of every audience expertise level (popular, professional, and scholarly) are located.

Activity: Popular, Professional, or Scholarly?

Open activity in a web browser.

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2.6.2.7: Publication Formats and the Information Lifecycle

Publication Formats and the Information Lifecycle

We can also categorize sources by publication format. That's because of the difference in time and effort sources in each format require for their production.



Sources in particular formats simply cannot exist until there has been enough time for people to create them. The result is that the sources that are created toward the end of the information lifecycle may come to very different conclusions about the event than did those sources created early on.

Sometimes the information presented in the later formats is more valid and reliable that what is in those produced earlier.

A very good example is that conclusions about the Columbine High School shooting in 1999 and the causes of that tragedy reached by books—which took years to complete after the event—were likely to be very different than the conclusions reached by news coverage created early on. For instance, many early reports concluded that the two teens responsible for the shooting had been shunned by their classmates and that it was the pain of their exclusion that had moved them to take revenge. Consequently, many K-12 schools nationwide took steps to try to ensure that all students felt included in their student bodies. But more time-consuming reportage concluded that the boys were not shunned (one had had a date for prom activities just days before) and that it was mental illness that made them kill their classmates.

Movie: Information Cycle

This video explains what kinds of information sources about an event can exist at any point in time during and after that event.

An interactive or media element has been excluded from this version of the text. You can view it online here: https://ohiostate.pressbooks.pub/choosingsources/?p=837

View Movie | View Text Version

Activity: The Information Lifecycle

Open activity in a web browser.

A Closer Look at Common Formats

Books – Usually a substantial amount of information, published at one time and requiring great effort on the part of the author and a publisher.

Magazines/Journals – Published frequently, containing lots of articles related to some general or specific professional research interest; edited.

Newspapers – Each is usually a daily publication of events of social, political and lifestyle interest.

Web sites – Digital items, each consisting of multiple pages produced by someone with technical skills or the ability to pay someone with technical skills.

Articles – Distinct, short, written pieces that might contain photos and are generally timely. Timeliness can mean that it's something that is of interest to readers at the point of publication or that is something the writer is thinking about or researching at a given point of time.

Tip: Evaluating Articles

Evaluating whether articles are **credible** enough for your information need is similar to evaluating any other source. There's more information on evaluating in Evaluating Sources.

Conference Papers – Written form of papers delivered at a professional or research-related conference. Authors are generally practicing professionals or scholars in the field.

Blogs – Frequently updated websites that do not necessarily require extensive technical skills and can be published by virtually anyone for no cost to themselves other than the time they devote to content creation. Usually marked by postings that indicate the date when each was written.

Documentaries – Works, such as a film or television program, presenting political, social, or historical subject matter in a factual and informative manner and often consisting of actual news films or interviews accompanied by narration.

Online Videos – Short videos produced by anybody, with a lot of money or a little money, about anything for the world to see. Common sites for these are YouTube and Vimeo.

Podcasts – Digital audio files, produced by anyone and about anything, that are available for downloading, often by subscription.

Activity: Best Format for Your Need

Open activity in a web browser.

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2.6.2.8: Scholarly Articles as Sources

Scholarly Articles as Sources

Articles in scholarly journals are valued for several reasons. First, they are usually trustworthy because their publication process includes a peer review that helps insure their accuracy and contribution to their disciplines. In addition, they often contain the first reports of new research, which makes their sections on methodology, data, analysis, and interpretation primary sources. Sometimes they instead consist of literature reviews summaries of multiple research studies done in the past on particular subjects of current interest. That makes those articles very helpful secondary sources.

Peer-Reviewed Sources

The most-respected scholarly journals are peer-reviewed, which means that experts in their field other than the author and editor check out each article before it can be published. It's their responsibility to help guarantee that new material is presented in the context of what is already known, that the methods the researcher used are the right ones, and that the article contributes to the field.

For those reasons, peer-reviewed articles are more likely to be credible. Peer-reviewed journal articles are the official scholarly record, which means that if it's an important development in research, it will probably turn up in a journal article eventually.

Here's a longer explanation of the peer review process, which concludes that it is good but not perfect.

Parts of a Scholarly Article

The articles you use for your assignments must also be **relevant** to your research question—not just credible. Reading specific parts of an article can help save you time as you decide whether an article is relevant.

Movie: Guided Tour of Scholarly Articles

An interactive or media element has been excluded from this version of the text. You can view it online here: https://ohiostate.pressbooks.pub/choosingsources/?p=839

View playlist

Reading a scholarly article usually takes some effort. Here's how to do it.

Activity: Parts of a Scholarly Article

Open activity in a web browser.

Finding Scholarly Articles

Most scholarly articles are housed in specialized databases. Libraries (public, school, or company) often provide access to scholarly databases by paying a subscription fee for patrons. For instance, OSU Libraries provide access to hundreds of databases via its Research Databases List that are made available free to people affiliated with the University. You can search for a journal title in these databases or view a list of databases by subject. For more information, including how to search databases, see Specialized Databases.

Databases that aren't subject-specific are called general databases. Google Scholar is a free general scholarly database available to all who have access to the Internet, and it provides some scholarly articles. For more information, see our section on using Google Scholar.

Tip: Known Article Searching

What if you have a citation for an article you need and now have to find the actual text of the article? Follow these instructions to Access to a Known Journal Article.

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2.6.2.9: News as a Source

News as a Source

News sources can provide insights that scholarly sources may not or that will take a long time to get into scholarly sources. For instance, news sources are excellent for finding out people's reactions, opinions, and prevailing attitudes around the time of an event.

So whether news sources are good for your assignment depends on what your research question is. (You'll find other relevant information at Sources and Information Needs.)

News is a strange term, because even when the information is old, it's still news. Some sources are great for breaking news, some are great for aggregated (or compiled) news, and others are great for historical news.

While news was transmitted for centuries only in newspapers, news is now transmitted in all formats: via radio, television, and the Internet, in addition to print. Even most newspapers have Internet sites today.

News must be brief because much of it gets reported only moments after an event happens. News reports occur early in the Information Lifecycle. See Publication Formats and the Information Lifecycle for more information.

When Are News Sources Helpful?

- You need breaking news or historical perspectives on a topic (what people were saying at the time).
- You need to learn more about a culture, place, or time period from its own sources.
- You want to keep up with what is going in the world today.

When Are News Sources of Limited Use?

- You need very detailed analysis by experts.
- You need sources that must be scholarly or modern views on a historical topic.

> **Activity: Using News Effectively**
>
> Open activity in a web browser.

Mainline and Non-Mainline News Sources

Mainline American news outlets stick with the tradition of trying to report the news as objectively as possibly. That doesn't mean their reports are perfectly objective, but they are more objective than the non-mainline sources. As a result, mainline news sources are more credible than non-mainline sources. Some examples of mainline American news outlets: The *New York Times*, *The Washington Post*, *The Boston Globe*, *The Chicago Tribune*, *The Los Angeles Times*; ABC News, CBS News, NBC News, PBS News, NPR News.

News from non-mainline American news outlets is often mixed with opinions. One way they frequently exhibit bias is that they leave out pertinent facts. Some examples of non-mainline American news outlets: MSNBC, Fox News, Gawker, Reddit.

Types of News Sources

Press Services—News outlets (print, broadcast, and online) get a lot of their news from these services, such as Reuters or Associated Press (AP), which make it unnecessary for individual outlets to send their own reporters everywhere. Services are so broadly used that you may have to look at several news outlets to get a different take on an event or situation.

News aggregators—Aggregators don't have reporters of their own but simply collect and transmit the news reported by others. Some sources pull news from a variety of places and provide a single place to search for and view multiple stories. You can browse stories or search for a topic. Aggregators tend to have current, but not archival news. Google news and Yahoo News are examples.

Newspaper sites – Many print newspapers also have their own websites. They vary as to how much news they provide for free. Take a look at these examples.

- The *Lantern*, Ohio State University's student newspaper
- The *Columbus Dispatch*
- *USA Today*
- *The Boston Globe*
- *The Times* of London
- *China Daily*, USA edition
- The New York Times

News Databases – Search current, recent, and historical newspaper content in databases provided free by libraries. OSU Libraries offers 69 news databases to students, staff, and faculty. They include:

- LexisNexis Academic – contains news back to 1980 from newspapers, broadcast transcripts, wire services, blogs, and more.
- Proquest Historical Newspapers – contains older content from several major U.S. newspapers.
- allAfrica – contains more than a million articles from 100 African news sources, 1996-present.
- Lantern Online – contains the archive of all of OSU's student newspaper issues, 1881-1997.

See the complete list of OSU Libraries' newspaper databases.

> **Activity: Choosing a Newspaper Database**
>
> Look at the list of OSU Libraries' newspaper databases available to OSU users. Which one would be a good place to find an article with an international left perspective on a topic? Our answer is at the end of this section.

Broadcast News Sites – Although broadcast news (from radio and television) is generally consumed in real time, such organizations also offer archives of news stories on their web sites. However, not all of their articles are provided by their own reporters: some originate from the press services, Reuters and AP. Here are some examples of broadcast new sites:

- ABC News
- BBC
- CNN
- NPR News
- NBC Learns (OSU only)

> **Activity: One-Minute World News from the BBC**
>
> Visit BBC's Video area and watch their One-minute World News to get a quick update on the world's major news stories.

Social Media – Most of the news outlets listed above contribute to Twitter and Facebook. It's customary for highly condensed announcements in this venue to lead you back to the news outlet's website for more information. However, how credible tech companies such as Facebook, Twitter, and Google are with news is in serious doubt now that their lawyers have testified to the U.S. Congress that more than 100 million users may have seen content actually created by Russian operatives on the tech companies' platforms leading up to the 2016 U.S. presidential election. Read more about their testimony at NPR and The New York Times.

Blogs – Sometimes these are good sources for breaking news, as well as commentary on current events and scholarship. Authors who write more objectively elsewhere can share more insights and opinions, more initial questions and findings about a study before they are ready to release definitive data and conclusions about their research.

Citizen Journalism – A growing number of sites cater to those members of the general public who want to report breaking news and submit their own photos and videos on a wide range of topics. The people who do this are often referred to as citizen journalists.

Examples of such sources include CNN iReport, and reddit. For more details on the history and development of citizen journalism, including addressing some of the pros and cons, read Your Guide to Citizen Journalism.

News Feeds – You can get updates on specific topics or a list of major headlines, regularly sent to you so you don't have to visit sites or hunt for new content on a topic. Look for links that contain headings such as these to sign up for news feeds:

- RSS feeds
- News Feeds
- News Alerts
- Table of Contents Alerts

Movie: What is an RSS Feed?

An interactive or media element has been excluded from this version of the text. You can view it online here: https://ohiostate.pressbooks.pub/choosingsources/?p=841

View video

Activity: RSS Feeds from Reuters

Visit Reuters News RSS Feeds to see a list of general and very specific topic areas for which you can sign up for alerts. What topic interests you? Consider signing up for one (or more).

Answer to Activity: Choosing a Newspaper Database

If you look at the database descriptions, you will notice that the one for Alternative Press Index matches the need expressed in the question.

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2.6.2.10: Data as Sources

Data as Sources

Using data as sources can help with all of your research project's information needs:

- Learn more background information.
- Answer your research question. (The evidence that data provide can help you decide on the best answer for your question.)
- Convince your audience that your answer is correct. (Data often give you ecidence that your answer that your answer is correct. (Data often give you evidence that your answer to your research question is correct or at least a reasonable answer.)
- Describe the situation surrounding your research question.
- Report what others have said about your research question.

Activity: Example of Data

Check out this very detailed data about frozen lasagna. Did you ever think this much data was available? Are there elements new to you? How might you use such data?

Movie: Reinterpreting Little Red Riding Hood

An interactive or media element has been excluded from this version of the text. You can view it online here: https://ohiostate.pressbooks.pub/choosingsources/?p=843

View video | View Text Version

What is data? The word means many things to many people. (Consider "data" as it relates to your phone contract, for instance!) **For our purposes, a definition we like is "units of information observed, collected, or created in the course of research."**

Erway, Ricky. 2013. Starting the Conversation: University-wide Research Data Management Policy. Dublin, Ohio: OCLC Research. http://www.oclc.org/content/dam/rese...13/2013-08.pdf

Data observed, collected, or cerated for research purposes can be numbers, text, images, audio clips, and video clips. But in this section on using data as sources, we're going to concentrate on *numerical* data.

TIP: From the Latin
Data is the plural of datum. (It's similar to how media is the plural of medium.)

Sometimes data is actually necessary to answer research questions, particularly in the social sciences and life and physical sciences. For instance, data would be necessary to support or rule out these hypotheses:

- More women than men voted in the last presidential election in a majority of states.
- A certain drugs shows promising results in the treatment of pancreatic cancer.
- Listening to certain genres of music lowers blood pressure.
- People of certain religious denominations are more likely to find a specific television program objectionable.
- The average weight of house cats in the United States has increased over the past 30 years.
- The average square footage of supermarkets in the United States has increased in the past 20 years.
- More tomatoes were consumed per person in the United Kingdom in 2015 than in 1962.
- Exploding volcanoes can help cool the planet by spewing sulfur dioxide, which combines with water vapor to make reflective aerosols.

So using numeric data in those portions of your final product that require evidence can really strengthen your argument for your argument for your answer to your research question. At other times, even if data is not actually necessary, numeric data can be particularly persuasive and sharpen the points you want to make in other portions of your final product devoted to, say, describing the situation surrounding your research question. (See Making an Argument)

For example, for a term paper about the research question “Why is there a gap in the number of people who qualify for food from foodbanks and the number of people who use foodbanks?,” you could find data on the website of Feeding America, the nation’s largest network of foodbanks. Some of that data may be the number of people who get food from a foodbank annually, with the number of seniors and children broken out. Those data won’t answer your research question, but they will help you describe the situation around that question and help your audience develop a fuller understanding.

Similarly, for a project with the research question “How do some birds in Australia use “smart” hunting techniques to flush out prey, including starting fires?,” you might find a journal article with data about how many people have observed these techniques and estimates of how frequently the techniques are used and by how many bird species.

Obtaining Data

There are two ways of obtaining data:

- Obtain data that already has been collected and analyzed. That’s what this section will cover.
- Collect data yourself. This can include activities such as making observations about your environment, conducting surveys or interviews, directly recording measurements in a lab or in the field, or even receiving electronic data recorded by computers/machines that gather the data. You will explore these activities in courses you take.

Finding Data in Articles, Books, Web Pages, and More

Numeric search data can be found all over the place. A lot of it can be found as part of another source- such as books; journal, newspaper, and magazine articles; and web pages. In these cases, the data do not stand alone as a distinct element, but instead are part of the larger work.

When searching for data in books and articles and on web pages, terms such as statistics or data may or may not be useful search terms. That’s because many writers don’t use those terms in their scholarly writing. They tend to use the words findings or results when talking about the data that could be useful to you. In addition, statistics is a separate discipline and using that term will turn up lots of journals in that area, which won’t be helpful to you. So use the search terms data and statistics with caution, especially when searching library catalogs. (See information on the Library Catalog. More information on searching is at Precision Searching.)

Even without using those search terms, many scholarly sources you turn up are likely to contain data. Once you find potential sources, skim them for tables, graphs, or charts. These items are displays or illustrations of data gathered by researchers. However, sometimes data and interpretations are solely in the body of the narrative text and may be included in sections called “Results” or “Findings.” (That shouldn’t keep you from displaying the data in charts, graphs, or tables as you like in your own work, though. See Data Visualization later in this section.)

If the data you find in a book, article, or web page is particularly helpful and you want more, you could contact the author to request additional numeric research data. Researchers will often discuss their data and its analysis – and sometimes provide some of it (or occasionally, all). Some may link to a larger numeric research data set. However, if a researcher shares his or her data with you, it may be in a raw form. This means that you might have to do additional analysis to make it useful in answering your question.

Depending on your research question, you may need to gather data from multiple sources to get everything you need to answer your research question and make your argument for it. (See Making an Argument.)

For instance, in our example related to foodbanks above, we suggested where you could find statistics about the number of people who get food from American foodbanks. But with that research question (“Why is there a gap in the number of people who qualify for food from foodbanks and the number of people who use foodbanks?”), you would also need to find out from another source how many people qualify for foodbanks based on their income and compare that number with how many people actually use foodbanks.

Finding Data, Data Depositories, and Directories

Sometimes the numeric research data you need may not be in the articles, books, and web sites that you’ve found. But that doesn’t mean that it hasn’t been collected and packaged in a useable format. Governments and research institutions often publish data they have collected in discipline-specific data depositories that make data available online. Here are some examples:

- United States Census Bureau
- Budget of the United States Government
- U.S. Bureau of Justice Statistics
- National Center for Education Statistics
- Daily Weather Maps NOAA)
- GeoData.gov
- The World Factbook (CIA)
- OSU Knowledge Bank

The United Nations and just about every country provide information as numeric data available online. Free and accessible data like this is called open data. The U.S. federal government, all states, and many local governments provide open data. You can find them (among other places) at site: .gov.

Other data are available through vendors who publish the data collected by researchers. Here are some examples:

- Hoover's Online (OSU Only)
- International Monetary Fund Statistical Databases
- World Health Organization Statistical Information System
- Envirofacts
- Census of Agriculture (OSU only)
- OECD Education at a Glance
- Corruption Perceptions Index

Don't know if a depository that could contain data in your discipline? Check out a data directory such as re3data.org

> **Activity: Where to Find Data**
>
> Open activity in a web browser.

Evaluating Data as Sources

Evaluating data for relevance and credibility is just as important as evaluating any other source. Another thing that is the same with data is that there is never a 100% perfect source. So just as is pointed out in Evaluating Sources, you'll have to make educated guesses (inferences) about whether the data are good enough for your purpose.

Critical thinking as you evaluate sources is something your professors will expect. But you'll benefit in other ways, too, because you'll be practicing a skill necessary for the rest of your life, both in the workplace and in your personal life. It's those skills that will keep you from being duped by fake news and taken advantage by posts that are ignorant or, sometimes, simply scams.

To evaluate data, you'll need to find out how the data were collected. If the data are in another source, such as a book; web page; or newspaper, magazine, or research journal article, evaluate *that* source in the usual way (see Evaluating Sources). If the book or newspaper, magazine, or web page got the data from somewhere else, do the same evaluation of the source from which the book or article got the data. The article, book, or web page should cite where the data came from. If it doesn't, then that is a black mark against using that data. (The data in a research journal article are often the work of the authors of the article. But you'll want to be sure they provide information about how they collected the data.)

In addition, if the data are in a **research journal article**, read the entire article, including the section called Methodology, which tells how the data were collected. Then determine the data's **relevance** to your research question by considering such questions as:

- Were the data collected recently enough?
- Is the data cross-sectional (based on information from people at any one time) or longitudinal (based on information from the same people over time)? If one is more appropriate for your research question than the other, is there information that you can still logically infer from this data?
- Were the types of people from whom the data were collected the same type of people your research question addresses? The more representative the study's sample is of the group your research question addresses, the more confident you can be in using the data to make your argument in your final product.

- Was the data analysis done at the right level for your research question? For instance, it may have been done at the individual, family, business, state, or zip code level. But if that doesn't relate to your research question, can you still logically make inferences that will help your argument? Here's an example: Imagine that your research question asks whether participation in high school sports in Columbus City Schools is positively associated with enrolling in college. But the data you are evaluating is analyzed at the state level. So you have data about the whole state of Ohio's schools and not Columbus in particular. In this case, ask yourself whether there is still any inference you can make from the data.

Research articles are sometimes difficult to read until you get used to them. Here's a helpful PDF: violentmetaphors.files.wordp...-article.pdfTo evaluate the **credibility** of the data in a research journal article you have already read, take the steps recommended in Evaluating Sources, plus consider these questions:

- Is the article in a peer reviewed journal? (Look at the journal's instructions for authors, which are often located on the journal's website, to see if it talks about peers reviewing the article and asking for changes [revisions] before publishing.) If it is a peer reviewed journal, consider that a plus for the article's credibility. Being peer reviewed doesn't mean it's perfect; just more likely to be credible.
- Do the authors discuss causation or correlation? Be wary of claims of causation; it is very difficult to determine a causal effect. While research studies often find relationships (correlation) between various variables in the data, this does not equal causation. For instance, let's return to our example above: If the study of Ohio high schools students' sports participation showed a positive correlation between sports participation and college enrollment, the researcher cannot say that participation *caused* college enrollment. If it were designed to show cause and effect, the study would not have resulted in a correlation. Instead, it would have had to have been designed as an experiment or quasi-experiment, used different statistical analyses, and would have supported or not supported its hypotheses.

ACTIVITY: Evaluating Data as Sources

Open activity in a web browser.

Data Visualization

Modern software can help you display your data in ways that are striking and often even beautiful. But the best criterion for judging whatever display you use is whether it helps you and your audience *understand* your data better than only text, maybe even noticing points that you would have otherwise missed.

Specific kinds of charts and graphs accomplish different things, which is important to keep in mind as you evaluate data and data sources. For instance:

- Line charts are usually used to show trends, comparing data over time.
- Scatter plots show the distribution of data points.
- Bar graphs usually compare categories of data.
- Pie charts show proportions of a whole.

It's important to decide what you want a display to *do* before making your final choice. Studying your data first so you know what you have will help you make that decision. Also, it may also be conventional in your discipline to display your data in certain ways. Examining the sources you were assigned to read in your course or asking your professor will help you learn what's considered conventional.

Your professors will be examining your visual display to make sure you did not misrepresent the data. For example, the proportions of slices in a pie chart all have to add up to 100%. If yours don't, you've done something wrong.

It's easy to get overwhelmed by all the choices to be made between potential displays and what each can do: Here are two sites to help you sort them out **once you know your data**:

http://datavizproject.com/

https://datavizcatalogue.com/

If you aren't ready yet to use some of the specialized tools for display, make it a point to learn how to use the data display

capabilities in Microsoft Word and/or Excel. You can find helpful tutorials on the Web. Good search statements to find those tutorials are:

- “Microsoft Word” (charts OR graphs)
- “Microsoft Excel” (charts OR graphs).

If you are OSU staff, students, or faculty, OSU Libraries’ Research Commons can help you choose a display, recommend a tool to accomplish it, and check out your finished data visualization before you have to turn it in. Contact the data visualization specialist.

If you are interested in displaying geospatial data on a map, consider how the Research Commons also helps OSU students, staff, and faculty find geospatial data and choose tools to display them.

Citing Data

Data is not copyrightable, but the expression of data is. So as with any other information source, you should cite any data you use from a source, whether it appeared in an article or you downloaded the data from a repository on the Web.

Unfortunately, data citation standards do not exist in many disciplines, although the DataCite initiative is working on them. Current workarounds include:

- Citing a “data paper,” where available.
- Citing a journal article that describes the dataset.
- Citing a book that includes the data.
- Citing the dataset as a website, where possible.

Examples: Citing Data

Data from a research database:

- APA: Department of Agriculture (USDA) (2008). “Crops Harvested”, Crop Production [data file]. Data Planet, (09/15/2009).
- MLA: “Crops Harvested”, Department of Agriculture (USDA) [data file] (2008). Data Planet, (09/15/2009).

Data from a file found on the open Web:

- APA: Center for Health Statistics, Washington State Department of Health. (2012, November). Mortality Table D1. Age-Adjusted Rates for Leading Causes of Cancer for Residents, 2002-2011. [Microsoft Excel file]. Washington State Department of Health. Retrieved from http://www.doh.wa.gov/
- MLA: Center for Health Statistics, Washington State Department of Health. Mortality Table D1. Age-Adjusted Rates for Leading Causes of Cancer for Residents, 2002-2011. Washington State Department of Health, Nov. 2012. Microsoft Excel file. Retrieved from http://www.doh.wa.gov/

Proper Use of Data

Once you have your data, you can examine them and make an interpretation. Sometimes, you can do so easily. But not always.

What if...

...you had a lot of information? Sometimes data can be very complicated and may include thousands (or millions...or billions...or more!) of data points. Suppose you only have a date and the high temperature for Columbus – but you have this for 20 years’ worth of days. Do you want to calculate the average highs for each month based upon 20 years’ worth of data by hand or even with a calculator?

...you want to be able to prove a relationship? Perhaps your theory is that social sciences students do better in a certain class than arts and humanities or life and physical science students. You may have a huge spreadsheet of data from 20 years’ worth of this course’s sections and would need to use statistical methods to see if a relationship between major and course grade exist.

You may find yourself using special software, such as Excel, SAS, and SPSS, in such situations.

Many people may have a tendency to look for data to prove their hypothesis or idea, as opposed to really answering their research questions. However, you may find that the opposite happens: the data may actually disprove your hypothesis. You should never try to manipulate data so that it gives credence to your desired outcome. While it may not be the answer you wanted to find, it is the answer that exists. You may, of course, look for other sources of data – perhaps there are multiple sources of data for the same topic with differing results. Inconclusive or conflicting findings do happen and can be the answer (even if it's not the one you wanted!).
Conflicting results on the same topic are common. This is the reality of research because, after all, the questions researchers are studying are complicated. When you have conflicting results you can't just ignore the differences—you'll have to do your best to explain why the differences occurred.

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2.6.3: SOURCES AND INFORMATION NEEDS

2.6.3.1: Sources and Information Needs
2.6.3.2: Sources to Meet Needs
2.6.3.3: Planning Your Sources

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2.6.3.1: Sources and Information Needs

Sources and Information Needs



It's easier to find appropriate sources when you start with a plan.

This section and the section on Types of Sources work together. That's because knowing the kinds of information in each category of sources will help you choose the right kind of information to meet each of your information needs. **And some of those needs are very particular.**

Information needs are why you need sources. Meeting those needs is what you're going to do with sources as you complete your research project.

Here are those needs:

- To learn more background information.
- To answer your research question(s).
- To convince your audience that your answer is correct or, at least, the most reasonable answer.
- To describe the situation surrounding your research question for your audience and explain why it's important.
- To report what others have said about your question, including any different answers to your research question.

> **Tip:**
>
> For another way to think about the work your sources do, see Roles of Research Sources.

The verbs in the list of information needs above tell you exactly how you'll use sources to carry out your research and create your final product: to learn, answer, convince, describe, and report. But you won't be doing any of that alone.

Your sources will give you information with which to reason. They'll also give you direct quotes and information to summarize and paraphrase as you create your final product. In other words, your sources will support you every step of the way during your research project.

Needs and Final Products

Background information may seldom appear directly in any final product. But meeting each of the other information needs will result in written sections of a term paper. For final products other than term papers, you'll have the same needs and will use sources to meet them. But not all needs will result in a section of your final product.

Posters & Information Needs

On a poster about your own original research, you aren't likely to have room to describe the situation surrounding your research question and why the question is important. That same lack of space may mean you do not report what others have said about your question. But that doesn't mean you didn't meet those needs and others as you carried out your research—unlike a term paper or journal article, the poster format in which you reported it just had more limited space.

For instance, in order to justify doing the research to yourself and your professor, you probably started by meeting the information need to describe the situation and why it is important. Your instructor may have you turn in that justification. And in order to do research based on what has already been found out, you will have studied what others have already reported. You also had to do that in order to make your answer to your research question more believable. But that doesn't mean you had room on your poster to say you met those needs.

Activity: Sources and Information Needs

Open activity in a web browser.

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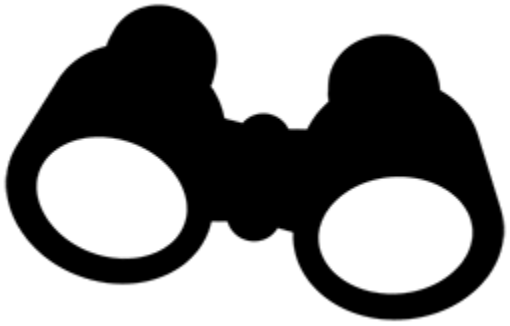
2.6.3.2: Sources to Meet Needs

Sources to Meet Needs

Because there are several categories of sources (see Types of Sources), the options you have to meet your information needs can seem complex.

Our best advice is to pay attention to when only primary and secondary sources are required to meet a need and to when only professional and scholarly sources will work. If your research project is in the arts, also pay attention to when you must use popular sources, because popular sources are often primary sources in the arts.

These descriptions and summaries of when to use what kind of source should help.



Get a good look at your topic through background reading.

To Learn Background Information

When you first get a research assignment and perhaps for a considerable time afterward, you will almost always have to learn some background information as you develop your research question and explore how to answer it.

Sources from any category and from any subgroup within a category – except journal articles – can meet students' need to learn background information and understand a variety of perspectives. Journal articles, are usually too specific to be background. From easy-to-understand to more complex sources, read and/or view those that advance your knowledge and understanding.

For instance, especially while you are getting started, secondary sources that synthesize an event or work of art and tertiary sources such as guidebooks can be a big help. Wikipedia is a good tertiary source of background information.

Sources you use for background information don't have to be sources that you cite in your final report, although some may be.

Sources to Learn Background Information

- **Quantitative or Qualitative:** Either—whatever advances your knowledge.
- **Fact or Opinion:** Any—whatever advances your knowledge.
- **Scholarly, Professional, or Popular:** Any—whatever advances your knowledge.
- **Primary, Secondary, or Tertiary:** Any—whatever advances your knowledge.
- **Publication Format:** Any—whatever advances your knowledge.

One important reason for finding background information is to learn the language that professionals and scholars have used when writing about your research question. That language will help you later, particularly when you're searching for sources to answer your research question.

To identify that language, you can always type the word glossary and then the discipline for which you're doing your assignment in the search engine search box.

Here are two examples to try:

- Glossary neuroscience
- Glossary "social media marketing"

(Putting a phrase in quotes in most search boxes insures that the phrase will be searched rather than individual words.)



Your research question may call for qualitative or quantitative sources.

To Answer Your Research Question

You have to be much pickier with sources to meet this need because only certain choices can do the job. Whether you can use quantitative or qualitative data depends on what your research question itself calls for.

Only primary and secondary sources (from the category called publication mode) can be used to answer your research question and, in addition, those need to be professional and/or scholarly sources for most disciplines (humanities, social sciences, and sciences). But the arts often require popular sources as primary or secondary sources to answer research questions. Also, the author's purpose for most disciplines should be to educate and inform or, for the arts, to entertain and perhaps even to sell. (As you may remember, primary sources are those created at the same time as an event you are researching or that offer something original, such as an original performance or a journal article reporting original research. Secondary sources analyze or otherwise react to secondary sources. Because of the information lifecycle, the latest secondary sources are often the best because their creators have had time for better analysis and more information to incorporate.)

Example: Quantitative or Qualitative Data

Suppose your research question is "How did a a particular king of Saudi Arabia, King Abdullah, work to modernize his country?"

That question may lend itself to qualitative descriptive judgments—about what are considered the components of modernization, including, for instance, what were his thoughts about the place of women in society.

But it may also be helped by some quantitative data, such as those that would let you compare the numbers of women attending higher education when Abdullah became king and those attending at the time of his death or, for instance, whether manufacturing increased while he reigned.

So looking for sources that provide both quantitative and qualitative information (not necessarily in the same resource) is usually a good idea.

If it is not clear to you from the formats of sources you are assigned to read for your course, ask your professor which formats are acceptable to your discipline for answering your research question.

Sources to Answer Your Research Question

- **Quantitative or Qualitative:** Will be determined by the question itself.
- **Fact or Opinion:** Professional and scholarly for most disciplines; the arts often use popular, as well.
- **Scholarly, Professional, or Popular:** Professional and scholarly for most disciplines; the arts often use popular, as well.
- **Primary, Secondary, or Tertiary:** Primary and secondary.
- **Publication Format:** Those acceptable to your discipline.



Sources that meet the approval of your audience will be more convincing.

To Convince Your Audience

Convincing your audience is similar to convincing yourself and takes the same kinds of sources—as long as your audience is made up of people like you and your professor, which is often true in academic writing. That means using many of those sources you used to answer your research question.

When your audience isn't very much like you and your professor, you can adjust your choice of sources to meet this need. Perhaps you will include more that are secondary sources rather than primary, some that are popular or professional rather than scholarly, and some whose author intent may not be to educate and inform.

Sources to Convince Your Audience

- **Quantitative or Qualitative Data:** Same as what you used to answer your research question if your audience is like you and your professor. (If you have a different audience, use what is convincing to them.)
- **Fact or Opinion:** Those with the purpose(s) you used to answer your research question if your audience is like you and your professor. (If you have a different audience, you may be better off including some sources intended to entertain or sell.)
- **Scholarly, Professional or Popular:** Those with the same expertise level as you used to answer the question if your audience is like you and your professor. (If you have a different audience, you may be better off including some popular.)
- **Publication Mode:** Primary and secondary sources if your audience is like you and your professor. If you have a different audience, you may be better off including more secondary sources than primary.

- **Publication Format:** Those acceptable to your discipline, if your audience is like you and your professor.



Use sources to frame the situation.

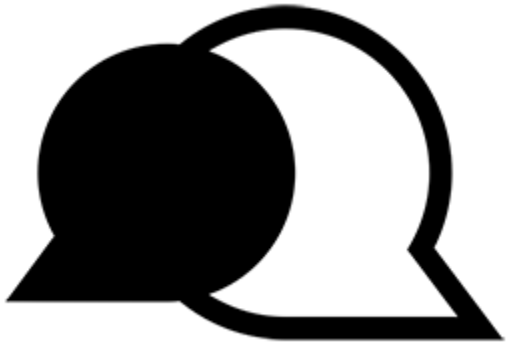
To Describe the Situation

Choosing what kinds of sources you'll need to meet this need is pretty simple—you should almost always use what's going to be clear and compelling to your audience. Nonetheless, sources intended to educate and inform may play an out-sized role here.

But even then, they don't always have to educate and inform *formally*, which opens the door to using sources such as fiction or the other arts and formats that you might not use with some other information needs.

Sources to Describe the Situation

- **Quantitative or Qualitative:** Whatever you think will make the description most clear and compelling and your question important to your audience.
- **Fact or Opinion:** Often to educate and inform, but sources don't have to do that *formally* here, so they can also be to entertain or sell.
- **Scholarly, Professional, or Popular:** Whatever you think will make the description most clear and compelling and your question important to your audience.
- **Primary, Secondary or Tertiary:** Whatever you think will make the description most clear and compelling and your question important to your audience. Some disciplines will not accept tertiary for this need.
- **Publication Format:** Whatever you think will make the description most clear and compelling and your question important to your audience. Some discipline will accept only particular formats, so check for your discipline.



Look for sources about how others have treated your research question.

To Report What Others Have Said

The choices here about kinds of sources are easy: just use the same or similar sources that you used to answer your research question that you also think will be the most convincing to your audience.

Sources to Report What Others Have Said

- **Quantitative or Qualitative:** Those sources that you used to answer your research question that you think will be most convincing to your audience.
- **Fact or Opinion:** Those sources that you used to answer your research question that you think will be most convincing to your audience.
- **Scholarly, Professional, or Popular:** Those sources that you used to answer your research question that you think will be most convincing to your audience.
- **Primary, Secondary, or Tertiary:** Those sources that you used to answer your research question that you think will be most convincing to your audience.
- **Publication Format:** Those sources that you used to answer your research question that you think will be most convincing to your audience.

Activity: Meeting Your Information Needs

Open activity in a web browser.

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2.6.3.3: Planning Your Sources

Planning Your Sources

Okay, so once you know what kinds of sources you need to meet your information needs, where should you look for them? Once more, thinking about categories can help.

Where sources are located is generally organized by audience expertise level—by whether they are popular, professional, or scholarly sources. Popular and professional are often grouped together. But scholarly sources tend to hang out by themselves. (That's why searching Google Scholar locates more of them than just plain old Google, and an academic library has more scholarly sources than a public library.) Source Locator can help you see where sources of every audience expertise level (popular, professional, and scholarly) are located. Check it out.

Even if you are not using our planning table, Bbefore you start looking, try the Plan for Sources table below along with the suggestions made in this section to think through what sources you'll need for your own research project. (There's also an example plan for sources filled in for a term paper.) Having your Plan for Sources always at your side while you search for sources will guide where you look and what you're willing to accept. It will help you keep track of whether you have found the right resources.

Also take a look at our Source Locator, whose link is below.

PLAN FOR SOURCES			
Course:	Due Date:	Type of Final Product:	
Research Question:			
Information Needs	Kinds of Sources (Popular, Professional, or Scholarly) That Should Meet Each Need	Publication Formats Likely to be Helpful in Meeting Each Need	Where to Look
To learn more background information			
To answer your research question and convince your audience			
To report what others have said			
To describe the situation and why it's important			

Thinking through the types of sources you need to meet your information needs helps you target your search. You can download the Plan for Sources table at http://go.osu.edu/planforsources.

You can download the table at http://go.osu.edu/planforsources, then fill it out with the help of our Source Locator. Using this table doesn't mean you can't change your mind if you later find another kind of source that looks too good to pass up. But making a plan first will insure that you don't just grab any source you come across. The few minutes you take to complete the table will save you time later. And it's nice to have a plan all in one place that you can put into action!

Example: Sample “Plan for Sources” Table

PLAN FOR SOURCES				
Course: ARTS & SCIENCES 3200		Due Date: 2/15/16	Type of Final Product: term paper	
Research Question: In what ways has the checklist movement affected surgery patient outcomes in U.S. hospitals?				
Information Needs		Kinds of Sources (Popular, Professional, or Scholarly) That Should Meet Each Need	Publication Formats Likely to be Helpful in Meeting Each Need	Where to Look
To learn more background information	✔	Popular Professional	Any, including magazine articles, professional blogs, and association websites and publications	Google and Bing
To answer your research question and convince your audience	✔	Professional Scholarly	Books Research journal articles Conference papers	Library catalog Library databases Google Scholar
To report what others have said	✔	Professional Scholarly	Any, including professional blogs and association websites and publications Research journal articles Conference papers	Google and Bing Library databases Google Scholar
To describe the situation and why it's important	✔	Popular Professional	Any, including magazine articles, professional blogs, and association websites and publications	Google and Bing

Completing the table puts all your planning in one place.

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2.6.4: PRECISION SEARCHING

2.6.4.1: Why Precision Searching?
2.6.4.2: Main Concepts
2.6.4.3: Related and Alternative Terms
2.6.4.4: Search Statements

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2.6.4.1: Why Precision Searching?

Why Precision Searching?



Precise searches turn up more appropriate sources.

Effective searching takes precision. This section shows you how to perform several steps to make your searching more precise—you'll turn up more sources that are useful to you and perhaps, sources that may be even crucial to your research question.

You've probably been searching in a more casual way for years and may wonder: Is going to the trouble of precision searching actually worth it?

Yes, definitely, for searches that are important to you! You're in competition with many people who are working to be as skilled as they can be. So you should use of these steps or course assignments and for information tasks you do on the job. With other tasks and searches, precision searching may be less important.

Search Strategy

This information on precision searching is based on how search tools such as Google and specialized databases operate. If you've been more casual in your searching practices, some of these steps may be new to you.

Starting with a research question helps you figure out precisely what you're looking for. Next, you'll need the most effective set of search terms – starting from main concepts and then identifying and alternate between related and terms. Those search terms need to be arranged in the most effective way as search statements, which you actually type into a search box.

An important thing to remember is that searching is an iterative process: we try search statements, take a look at what we found and, if the results weren't good enough, edit our search statements and search again—often multiple times. Most of the time, the first statements we try are not the best, even though Google or another search tool we're using may give us many results.

It pays to search further for the sources that will help you the most. Be picky.

Here are the steps for an effective search.



The steps in a precise search

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2.6.4.2: Main Concepts

Main Concepts

Identify the main concepts in your research question by selecting nouns important to the meaning of your question. Leave out words that don't help the search, such as adjectives, adverbs, prepositions and, usually, verbs. Nouns that you would use to tag your research question so you could find it later are likely to be its main concepts.

Finding the main concepts in a research question is a lot like finding the main idea in an essay or story. Often the main idea is in the first paragraph, but not always. Sometimes it's in a later paragraph or even in the conclusion. The same is true with research questions—the main concepts can be at the beginning, middle, or end. Stick to the nouns and only what's necessary, not already implied. Don't read in concepts that are not really there. Be alert to words that may have connotations other than the concept you are interested in. For instance, if you identify depression as a main idea, beware that the search engine won't automatically know whether you mean depression as a psychological state or as a condition of the economy or as a weather characteristic.

Example: How are birds affected by wind turbines?

The main concepts are birds and wind turbines. Avoid terms like affect (except the noun) and effect as search terms, even when you're looking for studies that report effects or effectiveness.

Example: What lesson plans are available for teaching fractions?

The main concepts are lesson plans and fractions. Stick to what's necessary. For instance, don't include: children—nothing in the research question suggests the lesson plans are for children; teaching—teaching isn't necessary because lesson plans imply teaching; available—available is not necessary.

Sometimes your research question itself can seem complicated. Make sure you've stated the question as precisely as possible (as you learned in Research Questions). Then apply our advice for identifying main concepts as usual.

Activity: Main Concepts

Open activity in a web browser.

Activity: More Main Concepts

Open activity in a web browser.

EXAMPLE: Does the use of mobile technologies by teachers and students in the classroom distract or enhance the educational experience?

Acceptable main concepts are teaching methods and mobile technology. Another possibility is mobile technologies and education.
Watch out for overly broad terms. For example, don't include:

- Educational experience (it misses mobile technology).
- Classroom distractions (too broad because there are distractions that have nothing to do with technology).
- Technology (too broad because the question is focused on mobile technology).

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2.6.4.3: Related and Alternative Terms

Related and Alternative Terms

For each main concept, list alternative terms, including synonyms and singular and plural forms of the words.

Sometimes synonyms, plurals, and singulars aren't enough. So also consider associations with other words and concepts. For instance, it might help, when looking for information on the common cold, to include the term virus—because a type of virus causes the common cold.

Check to make sure that your terms are not too broad or too narrow for what you want. Figuring out what's too broad or too narrow takes practice and may differ a bit with each search.

Tip: Try a Thesaurus

Have you considered using a thesaurus, such as thesaurus.com? Or adding a thesaurus to your browser search bar?

Activity: Finding Synonyms

When figuring out search terms, you can try your search terms in Visuwords <visuwords.com>, an online graphical dictionary, to see the connections visually in a diagram reminiscent of a neural net. It can help you see connections between terms that are not easy to think of.

Activity: Alternate Terms

Open activity in a web browser.

Subject Headings Instead of Keywords

All the searches we have talked about so far have been keyword searches, usually used in search engines. But sometimes it pays to use tools—such as library catalogs and journal article databases—that have subject headings that you can search. Subject headings are standardized terms that are assigned by trained experts. (Some such tools also allow keyword searching.) See the section on Specialized Databases for more detail about searching subject headings.

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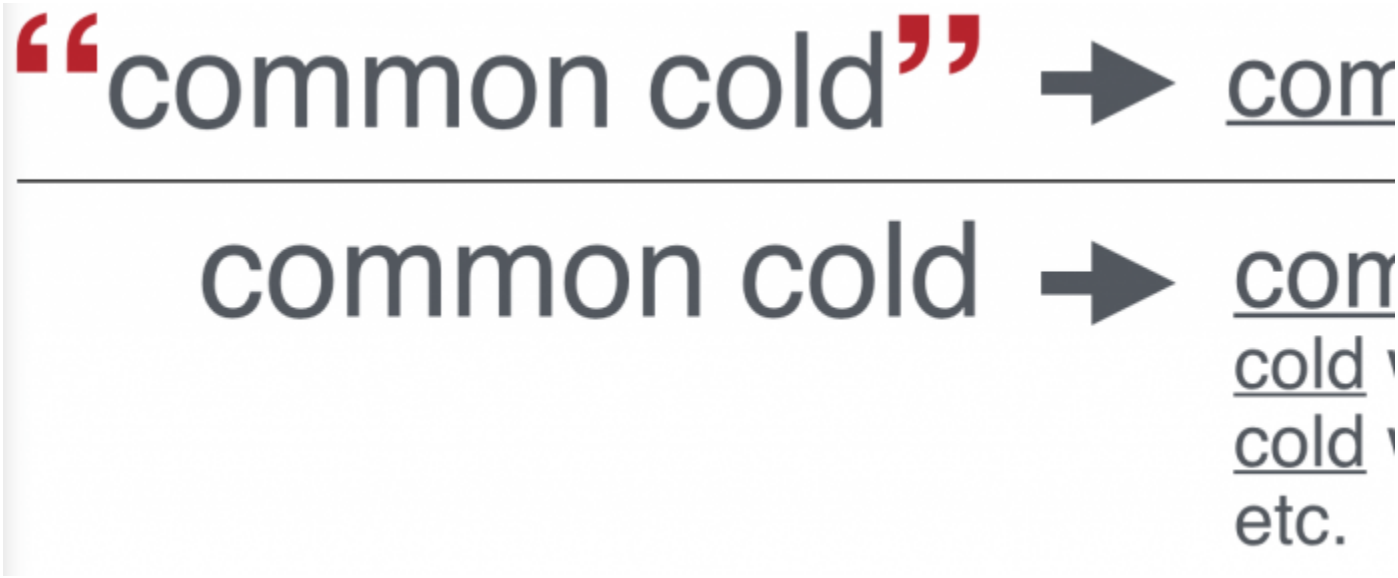
2.6.4.4: Search Statements

Search Statements

At this point in your search process, you are moving from merely identifying main concepts and similar search *terms* to developing more complicated search *statements* that can do more precise searching.

Use Quotation Marks for Phrases

Put quotation marks around any phrases among your terms so that the phrase is what's searched for, rather than the separate words. "Common cold" instead of common cold is a good example. Without those quotation marks, just think how many sources Google or other search tools would waste their/your time on things that have nothing to do with our sniffles.



Putting a phrase in quotes returns results containing that phrase,
and not the results for the individual words.

Use Wildcard and Truncation Symbols to Broaden

Consider whether using wild card or truncating symbols would help find variations of a word. For instance, the wildcard symbol in wom?n finds both woman and women, and the truncating symbol in mathematic* finds mathematics, mathematically, mathematician, etc.



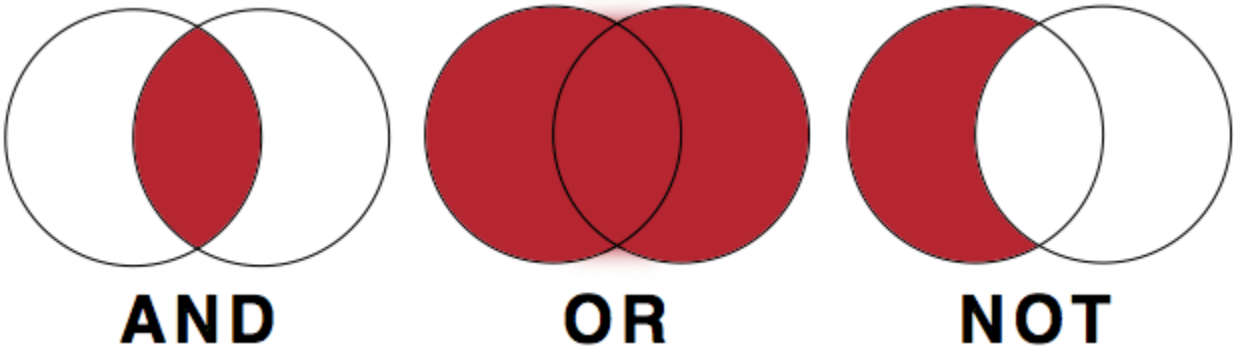
Using wildcard characters allows you to find variations of a word.

Activity: Wildcards and Truncation

Open activity in a web browser.

Consider AND, OR, NOT

You can often do more precise searching by combining search terms by using the words AND, OR, and NOT. These are known as Boolean Operators. Generally, using these operators narrows your search, making it more precise.



The Boolean operators AND, OR, and NOT exclude or include subsets of sources.

AND – If the main idea contains two or more ideas, you'll want to use AND to combine those terms in your search statement. To look for information about spiders as signs of climate change, you'll want to have both terms in the search and perform an AND search. That's what automatically happens in search engines such as Google and Bing unless you tell them to do something different by using OR or- NOT.

OR – If the main idea has several synonyms, use OR to combine them. Most search tools search for all terms (AND) by default, so you need to use the term OR between terms to let it know you want to find any of the terms not documents with all the terms. For instance, in the previous example of Latino small business growth, we would want to also use the term Hispanic.

NOT – If the main idea has a common use you want to exclude, use NOT to exclude that word. For example, if we were looking for

information about illegal drug use we would want to exclude prescription drugs from the search results. This is commonly done with NOT or the use of the minus (-) sign. In Google, to exclude a word use-word with no space between the – and the word you want to exclude. If you put a space in there, Google will not exclude the word.(When using some search tools, you have to use AND NOT before the word to exclude it.)

Using Parentheses with Multiple Operators

When a search requires multiple Boolean operators (AND, OR, NOT, or their symbols), you must use parentheses to group the appropriate terms and quotation marks with each Boolean operator. The resulting statements connect terms, remove terms, and organize search terms in ways that result in complex and precise searching.

The use of parentheses may remind you of the mathematical statements written in math courses. The reason parentheses are necessary in searching is that search tools, including Google, generally perform their operations from the left to right of a search statement. If you are using multiple Boolean operators, then the way to make sure that the search is done as a whole statement requires that you use parentheses to combine the sets in your statement.

Never use parentheses unless you are using multiple Boolean operators.



Parenthesis are used with Boolean operators to combine terms
for complex searches.

Being skillful at this task of envisioning the effects Boolean operators have on a search can help you troubleshoot your own search statements when they aren’t turning up what you expected.

Example: “United States” AND (immigration or emigration)

Can you tell that the searcher wants to find information about the United States’ immigration or emigration?

The searcher will find more with this arrangement than would turn up if the statement had been “United States” immigration emigration. That’s because the latter arrangement without parentheses would find only information that was about both United States immigration and emigration, instead of either.

Example: (cats OR dogs) AND (treatment OR therapy)

Can you tell that the searcher wants to find information about either treatment or therapy for either cats or dogs?

That’s a different search from what the searcher would have gotten if this statement had been used: cats dogs treatment therapy. Anything found with the latter statement without parentheses would have had to be about both— not just either—therapy and treatment for both—not just either—cats and dogs. So the latter statement would have turned up fewer pieces of information.

Activity: Search Analysis

Open activity in a web browser.

Practice with Search

Take some time to practice searching precisely – start by identifying main concepts, then listing related and alternative terms (with the help of wildcard and truncation symbols), and finally constructing search statements.

Activity: Search Practice

This activity focuses on the research question “How does a person’s diet affect the risk for getting cancer?” Work through the three activities below.

Search Terms – Open activity in a web browser.

Truncation – Open activity in a web browser.

Search Statements – Open activity in a web browser.

Activity: More Search Practice

This activity focuses on the research question “What is the effect of gamma radiation on crops?” Work through the three activities below.

More Search Terms – Open activity in a web browser.

More Truncation – Open activity in a web browser.

More Search Statements – Open activity in a web browser.

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2.6.5: SEARCH TOOLS

2.6.5.1: Library Catalog
2.6.5.2: WorldCat@OSU
2.6.5.3: Google Scholar
2.6.5.4: Specialized Databases
2.6.5.5: Web Search Engines
2.6.5.6: Tips for Common Search Tools

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2.6.5.1: Library Catalog

Library Catalog

The Ohio State University library catalog is searchable online and contains records for all the items owned or licensed by OSU Libraries. It also includes a circulation system that is used to check out materials. Students can use the system to have books and other materials quickly sent to their residence hall or for pick up at a library on the Columbus campus or at any of the regional campus libraries. Items in the catalog include books, journals (but not individual journal articles), documents, maps, movies, and recordings.

When to Use It

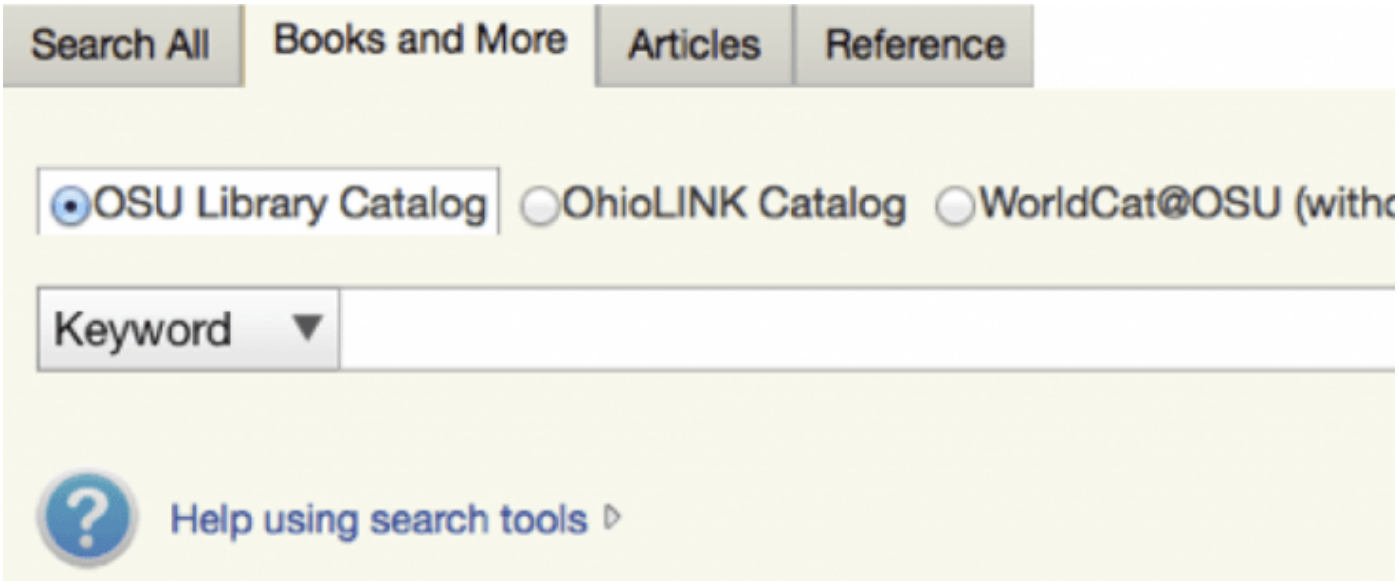
Use the library catalog to search for items that you can access because you are affiliated with Ohio State, to locate where those materials are stored, and to request them.

Note that OSU's library catalog:

- Does not contain the full-text of any materials. However, some items may include a table of contents and a link to full-text digital content.
- Does not contain specific articles. The catalog can only tell you whether a journal or other periodical title is available.

How to Use It

To access the catalog, choose the Books and More tab on the OSU Libraries' main page at http://library.osu.edu. From there, you can do a search or click on More Options to get access to other search options.



Searching Ohio State's catalog from library.osu.edu

Search Types

The catalog allows searching by author, title, journal title, subject, and keyword as well as specialty numbers such as the Library

of Congress call number and ISBN (International Standard Book Number). There is also an option for advanced search.

Additional tips:

- Keyword searches are the broadest search, as they search all information in an item record. (The search tips in Precision Searching, are based on using keywords.)
- Subjects are a very specific set of terms that are helpful for precision searches. Often, the easiest way to find subject terms is to do a keyword search first and then look at the subject terms for those that are good matches for your topic. There is more about subject heading searching in Specialized Databases later in this section.
- The Advanced Search screen allows a few additional search capabilities, such as multiple search fields to narrow the scope of a search term. You can also limit by year range, language, location, or format.

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- Compare a Human and Chimpanzee Skeleton by Shannan Muskopf is licensed CC BY-NC-SA 4.0.

2.6.5.2: WorldCat@OSU

WorldCat@OSU

WorldCat@OSU searches the holdings of libraries from all over the world (including Ohio State University Libraries and OhioLINK libraries), as well as content from thousands of journals and millions of electronic books and web-accessible documents.

When to Use It

WorldCat@OSU is good for quick searches on a topic, as a starting point, and for interdisciplinary topics. However, despite its size, it is not all-inclusive. It does not search all journals and databases, and full-text searching is limited.

How to Use It

To access WorldCat@OSU, choose the Search All tab on the OSU Libraries' main page at http://library.osu.edu. (Off-campus users will be asked to sign in with an OSU username and password or proceed as a guest for fewer options.)



Searching WorldCat@OSU from library.osu.edu

Narrowing Searches

- To specific databases – From the Search All tab, click the Advanced Search link to get access to other search options, including selecting specific databases.
- To articles – Under the Articles tab on the OSU Libraries home page, you will be using WorldCat@OSU to search only for articles.

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- A Case Study on the Endocrine System by Shannan Muskopf is licensed CC BY-NC-SA 4.0.

2.6.5.3: Google Scholar

Google Scholar

Google Scholar is a tool for finding books and journal articles that you might normally get from a library. Where possible, it provides links to online versions and to library copies to help you locate an item.

When to Use It

Use Google Scholar to find scholarly articles and books, verify citations, and explore related resources. When books are available through Google Books, some of their content may be available online.

How to Use It

Go to Google Scholar (http://scholar.google.com).

> **Movie: Using Google Scholar**
>
> Watch this tutorial on the basics of Google Scholar use.
>
> An interactive or media element has been excluded from this version of the text. You can view it online here: https://ohiostate.pressbooks.pub/choosingsources/?p=809
>
> View video

Note: Setting your school in Scholar Preferences will help you make direct connections to online sources provided by your library. If you want to locate sources in many different libraries, add WorldCat in addition to your library. (Remember to save your preferences.)

In your search results, you can connect to an online version if there is a linked option following the item's title. (If you've added Ohio State under preferences, a Find It link is shown to provide a link to full-text or to help you request the item if it's not available online. If you've added WorldCat to you preferences, the Library Search link displays the WorldCat record, which shows all of the libraries that own the item. If there are multiple references to the same item, Google Scholar groups them. You can click the versions link following a title to see a list of all versions.)

Additional Tips

- The Any Time link in the left column of results allows you to limit your search results by date.
- Find Advanced Scholar Search by clicking on the three horizontal lines icon. Advance Scholar search provides additional search fields such as author, publication, and date, as well as phrase matching and word exclusion.

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- Investigation: How Does Temperature Affect Respiration Rates of Fish? by Shannan Muskopf is licensed CC BY-NC-SA 4.0.

2.6.5.4: Specialized Databases

Specialized Databases

A specialized database—often called a research or library database—allows targeted searching on one or more specific subject areas (i.e., engineering, medicine, Latin American history, etc.), for a specific format (i.e., books, articles, conference proceedings, video, images), or for a specific date range during which the information was published. Most of what specialized databases contain can not be found by Google or Bing.

There are several types of specialized databases, including:

- Bibliographic – details about published works
- Full-text – details plus the complete text of the items
- Multimedia – various types of media, such as images, audio clips, or video excerpts
- Directory – brief, factual information
- Numeric – data sources
- Product – model numbers, descriptions, etc.
- Mixed – a combination of other types, such as multimedia and full-text

Activity: Database Types

Open activity in a web browser.

When to Use Specialized Databases

Search specialized databases to uncover scholarly information that is not available through a regular web search. Specialized databases are especially helpful if you require a specific format or up-to-date, scholarly information on a specific topic.

Many databases are available both in a free version and in a subscription version. Your affiliation with a subscribing library grants you access to member-based services at no cost to you. For example, using PubMed via OSU Libraries enables a Find It link to help you request an item.

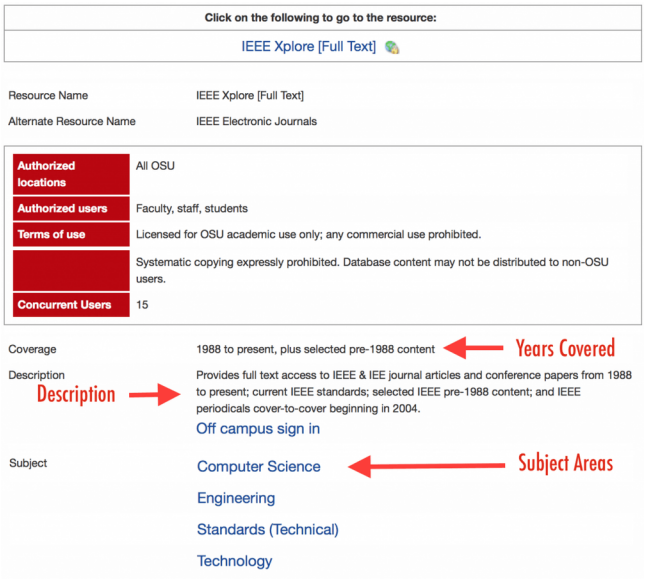
Tip: Free vs. Subscription?

In some cases, the data available in free and subscription versions are the same, but the subscription version provides some sort of added value or enhancement for searching or viewing items.

Database Scope

Information about the specific subject range, format, or date range a particular specialized database covers is called its scope. A specialized database may be narrow or broad in scope, depending on whether it, for instance, contains materials on one or many subject areas.

If you are using a database licensed by OSU Libraries and have clicked the title in the list of databases, you will see scope information at the bottom of the same page that says “Click on the following to go to the resource.”



This example shows the scope page for an OSU-licensed database.
View the live example.

Once you are aware of a database's scope, you'll be able to decide whether the database is likely to have what you want (for instance, journal articles as opposed to conference proceedings). Reading about the scope can save you time you would have otherwise wasted searching in databases that do not contain what you need.

Activity: Determining Subject Scope

Open activity in a web browser.

Activity: Years of Coverage

Instructions: In addition to subject scope, database descriptions should include years of coverage. Visit Ohio State's Research Databases List to search for the databases listed below. Which database contains the oldest information? Which covers the fewest years?

- Evidence Based Medicine Reviews
- MathSciNet
- GeoRef

Answer to Activity: Years of Coverage

The answer to the "Years of Coverage" Activity above is:

- The database containing the oldest material is GeoRef, which goes back to 1785.
- The database covering the fewest years is Evidence Based Medicine Reviews, which goes back to 1991.

How to Use Them

Use of each database varies somewhat.

See Ohio State's research database list.

Example: Academic Search Complete

Academic Search Complete (OSU only) is a general article database available through most academic and large public libraries that is often recommended for undergraduate research projects.

Movie: Academic Search Complete Database in 3 Minutes

View video

Keyword Searching

Although keyword search principles apply (as described in Precision Searching), you may want to use fewer search terms since the optimal number of terms is related to database size. Google and Bing work best with several terms since they index billions of web pages and additional terms help narrow the results. Each scholarly database indexes a fraction of that number, so you are less likely to be overwhelmed by results even with one or two keywords than you would be with a search engine.

Phrase searching (putting multiple words in quotes so Google or Bing will know to search them as a phrase) is also less helpful in specialized databases because they are smaller and more focused. Databases are better searched by beginning with only a few general search terms, reviewing your results and, if necessary, limiting them in some logical way. (See Limiting Your Search below.)

Activity: Compare Them!

Instructions:
Compare a search for items containing both phrases “United States” and “female serial killers” in the article database Academic Search Complete (OSU only) and in the web search engine Bing. (Make sure you invlude the quotation makes so they will be searched as phrases.) Notice how searching too narrowly (searching for phrases) affects results in the specialized database. How could you revise the specialized database search to get more results?

Limiting Your Search

Many databases allow you to choose which areas (also called fields) of items to search for your search term(s), based on what you think will turn up documents that are most helpful.

For instance, you may think the items most likely help to you are those whose titles contain your search term(s). In that case, your search would not show you any records for items whose titles do not have your term(s). Or maybe you would want to see only records for items whose abstracts contain the term(s).

When this feature is available, directing your search to particular parts of items, you are said to be able to “limit” your search. You are limiting your search to only item parts that you think will have the biggest pay-off at distinguishing helpful items from unhelpful items.

Searching fields such as title, abstracts, and subject classification often gives helpful items.

Tip: Full-Text Searches

Some databases allow for full-text searching, but this option includes results where a search term appears only once in dozens or more pages. Searching fields such as title, abstracts, and subject classification will often give more relevant items than full-text searching.

Subject Heading Searching

One precision searching technique may be helpful in databases that allow it, and that’s subject heading searching. Subject heading searching can be much more precise than keyword searching because you are sure to retrieve only your intended concept.

Subject searching is helpful in situations such as:

- There are multiple terms for the same topic you're interested in (example: cats and felines).
- There are multiple meanings for the same word (example: cookie the food and cookie the computer term).
- There are terms used by professionals and terms used by the general public, including slang or shortened terms (example: flu and influenza).

Here's how it works:

Database creators work with a defined list of subject headings, which is sometimes called a controlled vocabulary. That means the creators have defined which subject terms are acceptable and assigned only those words to the items it contains. The resulting list of terms is often referred to as a thesaurus. When done thoroughly, a thesaurus will not only list acceptable subject headings, but will also indicate related terms, broader terms and narrower terms for a concept.

Tip: Finding Useful Subject Headings

Try this strategy to find useful subject headings. Remember it by thinking of the letters KISS:

- **K**eyword-search your topic.
- **I**dentify a relevant item from the results.
- **S**elect subject terms relevant to your topic from that item's subject heading.
- **S**earch using these subject terms. (Some resources will allow you to simply click on those subject terms to perform a search. Others may require you to copy/paste a subject term[s] into a search box and choose a subject field.)

Activity: Searching Specialized Databases

Open activity in a web browser.

Records and Fields

The information researchers usually see first after searching a database is the "records" for items contained in the database that also match what was asked for by the search.

Each record describes an item that can be retrieved and gives you enough information so that, hopefully, you can decide whether it should meet your information need. The descriptions are in categories that provide different types of information about the item. These categories are called "fields." Some fields may be empty of information for some items, and the fields that are available depend on the type of database.

Example: Database Fields

A **bibliographic database** describes items such as articles, books, conference papers, etc. Common fields found in bibliographic database records are:

- Author.
- Title (of book, article, etc.).
- Source title (journal title, conference name, etc.).
- Date.
- Volume/issue.
- Pages.
- Abstract.
- Descriptive or subject terms.

In contrast, a **product database** record might contain the following fields:

- Product Name.
- Product Code number.

- Color.
- Price.
- Amount in Stock.

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- Comparing a Human and Avian Skeleton by Shannan Muskopf is licensed CC BY-NC-SA 4.0.

2.6.5.5: Web Search Engines

Web Search Engines

Web search engines use special software programs (called robots, spiders, or crawlers) to find Web pages and list (or index) all words within each one to make searching large quantities of page faster. Indexes capture the largest amount of information on the Web, but no index lists everything on the Internet.

Commonly used search engines include Google (https://www.google.com) and Bing (http://www.bing.com).

In addition to search engines, there are also:

- Specialized web search engines – A tool that has a specialty, usually either a subject or format focus. It ignores the rest of the information on the web. Examples include science.gov (http://www.science.gov/) and TinEye Reverse Image Search (https://www.tineye.com).
- Metasearch engines – Tools that search multiple web search engines and gives you results from all of them. Some of these return the best results from the search engines they search. Examples include Dogpile (http://www.dogpile.com) and WebCrawler (https://www.webcrawler.com).
- Web directories – Tools created by editors or trained researchers who categorize or classify web sites by subject. Directories are more selective than search engines. An examples includes Ipl2 (http://www.ipl.org).

When to Use Them

Web Search Engines and related web search tools are helpful for locating background information, news (especially if it's recent), and public opinion.

However, scholarly information is often not available through a regular web search. If you do find scholarly information through a web search engine, especially if you are off campus, you may be asked for payment to access it. Ohio State Libraries can usually get you what you need without additional payment.

Remember to follow the advice in Source Evaluation to determine whether information you locate online is suitable for your information needs.

How to Use Them

See links above. Use of each tool varies. If a search engine has an advanced search, it may include options such as specifying format, language, domain, or date range.

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2.6.5.6: Tips for Common Search Tools

Tips for Common Search Tools

Academic Search Complete

- **AND:** default (alternatively: term AND term)
- **OR:** term OR term
- **NOT:** term NOT term
- **Exact Phrase:** “exact phrase search”
- **Grouping:** term AND (term OR term)

Bing

- **AND:** default
- **OR:** term OR term
- **NOT:** term NOT term
- **Exact Phrase:** “exact phrase search”
- **Grouping:** Not available

Google

- **AND:** default
- **OR:** term OR term
- **NOT:** term -term (example: animal -cat)
- **Exact Phrase:** “exact phrase search”
- **Grouping:** term AND (term OR term)

WorldCat

- **AND:** term AND term
- **OR:** term OR term
- **NOT:** term NOT term
- **Exact Phrase:** “exact phrase search”
- **Grouping:** term AND (term OR term)

OSU Library Catalog

- **AND:** term AND term
- **OR:** term OR term
- **NOT:** term NOT term
- **Exact Phrase:** “exact phrase search”
- **Grouping:** term AND (term OR term)

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2.6.6: EVALUATING SOURCES

2.6.6.1: Thinking Critically About Sources

2.6.6.2: Evaluating for Relevancy

2.6.6.3: Evaluating for Credibility

2.6.6.4: A Source's Neighborhood

2.6.6.5: Author and Publisher

2.6.6.6: Degree of Bias

2.6.6.7: Recognition from Others

2.6.6.8: Thoroughness

2.6.6.9: Combining the Factors

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2.6.6.1: Thinking Critically About Sources

Thinking Critically About Sources



Evaluating sources often involves piecing together clues.

This section teaches how to identify relevant and credible sources that you have most likely turned up on the Web and on your results pages of the library catalog, Google Scholar, and specialized databases. Relevant, credible sources will meet the information needs of your of your research project.

In order to evaluate a source, you have to answer two questions about it:

- Is this source relevant to my research question?
- Is this a credible source– a source my audience and I should be able to believe?

It's important to determine relevance before credibility because no mater how credible a source is, if it's not relevant to your research question it's useless to you for this project.

TIP: Other Criteria from Your Professor

Don't forget that you also have to make sure your sources meet any other criteria that your professor may have given you for this assignment. For instance, professors often stipulate that, some of your sources have to be scholarly sources or articles from a particular database.

So make sure you have identified enough of the kind of sources your professor has requested.

You might already be worrying about how long evaluating sources is going to take. So let's say right off that you won't have to read all of a source to decide whether it is relevant and credible. (Later, of course, it will take a closer read to determine what direct quotes, paraphrases, and summaries you may want to use from the sources you have selected.)

Nonetheless, our advice is to not begrudge the time you spend evaluating sources. It's one of the most important things to learn at college—the opportunity to evaluate sources is one of the big reasons your professors assign research projects. And your future employers will expect you to have learned how to do it. For the rest of your professional and personal life, you will be using the critical thinking skills that make choosing the right sources possible. So learning those skills is a good investment.

Happily, you'll also get faster the more you do it.

> **ACTIVITY:** Evaluation Basics
>
> Open activity in a Web browser.

Making Inferences: Good Enough for Your Purpose?

Sources should always be evaluated relative to your purpose-why you're looking for information. But because there often aren't clear-cut answers when you evaluate sources, **most of the time it is inferences-educated guesses from available clues-**that you have to make about whether to use information from particular sources.

Your information needs will dictate:

- What kind of information will help.
- How serious you consider the consequences of making a mistake by using information that turns out to be inaccurate. When the consequences aren't very serious, it's easier to decide a source and its information are good enough for your purpose. Of course, there's a lot to be said for always having accurate information, regardless.
- How hard you're willing to work to get the credible, timely information that suits your purpose. (What you're learning here will make it easier.)

Thus, your standards for relevance and credibility may vary, depending on whether you need, say,:

- Information about a personal health problem.
- An image you can use on a poster.
- Evidence to win a bet with a rival in the dorm.
- Dates and times a movie is showing locally.
- A game to have fun with.
- Evidence for your argument in a term paper.

For your research assignments or a health problem, the consequences may be great if you use information that is not relevant or not credible.

> **Activity: Quick Check**
>
> **Instructions:** Select one answer to each question.
> Open activity in a web browser.

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2.6.6.2: Evaluating for Relevancy

Evaluating for Relevancy

Relevant sources are those that pertain to your research question. You'll be able to figure that out fairly quickly by reading or skimming particular parts of sources and maybe jotting down little tables that help you keep track. We'll show you how below, including where to look in specific kinds of sources and what questions to ask yourself as you do.

One thing to consider early on as you make inferences about relevancy is the effect that timeliness, or a source's currency, should have on deciding whether a source is relevant. **Your research question will determine that.**

For instance, if your research question is about the life sciences, you probably should consider only the most recent sources relevant because the life sciences are changing so quickly. There is a good chance that anything but the most recent sources may be out of date. So aim for sources no more than 5 years old. (An example discipline that calls for even newer sources is computer security.)

But suppose your research question is about the Edo Period in Japan (1603-1868) or about Robert Falcon Scott, who explored the Antarctic from 1901-1913. In these cases, an item from 1918 might be just as useful as an item from 2018 (although new information may have been found in the 100 year gap). But something from 1899 about Antarctica or from 1597 about Japan would NOT be current enough for these research questions.

These example research questions also give you two more clues about how to treat the timeliness or currency of sources as you consider relevance:

- Because of how long ago they lived or occurred, it would be unusual for many sources on Robert Scott or the Edo Period to have been published very recently. So, unlike sources for the biosciences, whether a source is very recent should probably not determine its relevancy to those research questions.
- Primary sources might be considered especially relevant to all three research questions. Bioscience journal articles that provide research findings for the first time count as primary sources. And primary sources (such as Scott's diaries and expedition photographs, as well as paintings, literature, clothing, and household items from the Edo Period) go a long way to explain faraway people and times. (See Primary, Secondary, & Tertiary Sources.)

EXAMPLE: Currency

Check out how currency is handled on TED. This site provides videos of speakers talking about new ideas in technology, entertainment, and design. (That's what TED stands for.) Some videos are labeled "Newest Talks," and TED tells when every video was recorded.

For your sources for which timeliness matters, see the section Where to Look, which includes where to look in websites, articles, and books for information about a source's currency.

Time-Saving Tips

Instead of thinking you have to read all of every source in order to figure out whether it is relevant, read or skim only parts of each source. If you're looking at the right parts, that should give you enough information to make an educated guess about relevancy.

But what should you be looking for as you do that reading and skimming? One way to figure that out is to first parse your research question so that you can figure out its main concepts. (This is like identifying main concepts in your research question in order to search precisely.)

For instance, suppose your research question is: How does having diverse members in a group increase the critical thinking of the group?

What are this question's main concepts? Our answer is: group diversity and critical thinking.

So when trying to judge which sources are relevant to these main concepts, you would assess whether each source you've found pertains to at least one of these concepts. We recommend you jot down a little table like the one in the example below to keep track of which sources address each main concept.

To be considered relevant to your research question, a source wouldn't necessarily have to cover all of your main concepts, but finding sources that do is the ideal. Otherwise, you just have to make do with what you've got. Don't forget that each source would have to pass the currency test, too, if currency is important to your research question. So it's wise to record your decisions about the sources' currency on your tables, too.

EXAMPLE: Sources' Main Concepts and Currency

Research question: How does having diverse members in a group increase the critical thinking of the group?

	Currency Okay	***Group Diversity***	***Critical Thinking***
Source A title	X	X	
Source B title	X		
Source C title	X	X	X

The table in this hypothetical example indicates that both Sources A and C are relevant because each pertains to at least one main concept from the research question. Currency doesn't seem to matter much to our research question, so all three sources were marked current. But since currency is all that Source B has to offer, it is not relevant for this project.

If you do make little tables for relevance, it's probably a good idea to hang on to them. You might find them helpful later in your research process.

Where to Look in Websites, Articles, and Books

The information below tells where to look and what questions to ask yourself to assess three kinds of sources' relevancy to your research question. **Whatever you do, don't stop evaluating a source after looking only a website's name or the title of another source.**

Save time by looking in particular places in sources for information that will help you figure out whether the source is relevant to your research project. Much of our advice below comes from "Speedy Reading" in *The Craft of Research*, second edition, by Wayne Booth, Gregory Colomb, and Joseph Williams, 2003, pp. 108-109.

On a **website**, check the name of the website and its articles for clues that they contain material relevant to your research question. Consider whether time should have an impact on what information can be considered relevant. If so, skim any dates, datelines, What's New pages, and press releases to see whether any website content works with the time considerations you need. Page creation or revision dates that you find can also help.

Skim any site map and index on the website for key words related to your research question. Try the key words of your research question in the search box. Do you see enough content about your keywords to make you think parts of the website could be helpful?

For an **article**, think about the title. Does it have anything to do with your research question? Consider whether time should have an impact on what sources can be considered relevant. If so, is the publication date within your parameters? Also skim the abstract to see whether the article works with the time considerations you need. For instance, if there is a time period in your research question, does the article address the same time period or was it created in that time period?

Look at the abstract and section headings in the article to locate the problem or question that the article addresses, its solution, and the outline of the article's argument for its main claim. Can those help answer your research question? Do they make it seem the article will give you information about what others have written about your research question? Do they offer a description of the situation surrounding your research question?

Do the article's introduction and conclusion sections help you answer your research question and/or offer a description of the situation surrounding your question so you can explain in your final product why the question is important? Check whether the bibliography contains keywords related to your research question. Do the sources cited by the bibliography pertain to your research question?

For a **book**, check whether the title indicates the book could be about your research question. Consider whether time should have an impact on what sources can be considered relevant. If so, is the publication date or copyright date (usually listed in the library catalog or on the back of the book's title page) too early or late for any time constraints in your research question? Maybe it is just right. Also skim some of the preface and introduction to see whether the book works with the time considerations you need.

For help answering your research question, skim the book's table of contents and any summary chapters to locate the problem or question that the book addresses, its solution, and the broad outline of the book's argument for its main claim. Do they also give you information about what others have written about your research question? Do they offer a description of the situation surrounding your research question? Look for your key words in the bibliography. Do the sources cited pertain to your research question? Skim the index for topics with the most page references. Do the topics with the most page references pertain to your research question?

ACTIVITY: Follow a Title's Clues for Relevance

Open activity in a Web browser.

ACTIVITY: Connecting the Dots beyond the Title

Open activity in a Web browser.

Connecting the Dots beyond the Title

Instructions: Now you can practice evaluating for relevance beyond the title. In the previous activity, you evaluated for currency and relevance the tittles of three sources for the research question: How does "prospect theory" in behavioral economics help explain medical doctors' decisinos to favor surgery or radiation to cure cancer in patients?

Judging by the title, the most relevant source for that research question seemed to be a journal article called **"Cancer Treatment Prescription–Advancing Prospect Theory beyond Economics," in *Journal of The American Medical Association Oncology*, June, 2016.**

Read the abstract of the article below. Then decide whether this source is relevant to your research questions above. That is, might the article help you meet any of your project's information needs about the research questions? If there is at least one need it can help meet, then you should judge the article relevant.

Answer the question below the abstract to indicate the source is relevant. Then compare your answer with our feedback.

As usual, your information needs are:

- To learn more background information.
- To answer your research question.
- To convince your audience that your answer is correct or, at least, the most reasonable answer.
- To describe the situation surrounding your research question for your audience and explain why it's important.
- To report what others have said about question, including any different answers to your research question.

Abstract

"Cancer Treatment Prescription–Advancing Prospect Theory beyond Economics," in *Journal of The American Medical Association Oncology*, June, 2016 (Note to students: This article and abstract are fictitious.)

Importance Cancer Treatment is complex. We expect oncologists to make treatment decisions according to definitive standards of care. Finding out that prospect theory demonstrates that they react very much like most other people when deciding to recommend surgery or chemotherapy for their patients indicates that more self-reflection on oncologists' part could help patients make better decisions. (Prospect theory describes how people choose between alternatives that have risk when the probability of different outcomes is unknown.)

Objective To show whether prospect theory applies to how oncologists framed their recommendations for surgery or chemotherapy for patients in good condition and bad condition.

Design, Settings, and Participants Records of 100 U.S. oncologists were examined for the years 2014 and 2015, which documented patient conditions and the way oncologists framed their recommendations regarding surgery or chemotherapy. Thus, a quasi-experimental ex post facto design was used for the study.

Main Outcomes and Measures This study explored the relationship between the way in which the oncologists "framed" the choice of surgery or chemotherapy as they made recommendations to patients, to patients' conditions, and the choice actually made. Those results were compared to what prospect theory would predict for this situation.

Results Physicians seemed to present their recommendation of surgery or chemotherapy in a loss frame (e.g., "This is likely to happen to you if you don't have this procedure") when patients' conditions were poor and in a gain frame (e.g., "By having this procedure, you can probably dramatically cut your chances of reoccurrence") when their conditions were less poor. These results are what prospect theory would have predicted.

Conclusions and Relevance This study opens up the possibility that, as described by prospect theory, a person's choice of framing behavior is not limited to how we naturally act for ourselves but includes how we act for other people, as the oncologists were acting on behalf of their patients. More research is necessary to confirm this line of evidence and determine whether oncologists' decision making and framing is the most effective and entirely according to the best standards of care.

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2.6.6.3: Evaluating for Credibility

Evaluating for Credibility

Next, you'll be evaluating each of the sources that you deemed relevant.

What are the clues for inferring a source's credibility? Let's start with evaluating websites, since we all do so much of our research online. But we'll also include where to find clues relevant to sources in other formats when they differ from what's good to use with websites. Looking at specific places in the sources will mean you don't have to read all of every resource to determine its worth to you.

And remember, the more you take these steps, the faster it goes because always examining your sources becomes second nature.

What Used to Help

It used to be easier to draw conclusions about an information source's credibility, depending on whether it was a print source or a web source. We knew we had to be more careful about information on the web–simply because all the filters that promoted accuracy involved in the print publishing process were absent from most web publishing. After all, it takes very little money, skill, and responsible intent to put content on the web, compared with what has to be done to convince print publishers that your content is accurate and that they will make money by printing it.

However, many publishers who once provided only print materials have now turned to the web and have brought along their rigorous standards for accuracy. Among them are the publishers of government, university, and scholarly (peer-reviewed) journal websites. Sites for U.S. mainline news organizations also strive for accuracy rather than persuasion–because they know their readers have traditionally expected it. All in all, more websites now take appropriate care for accuracy than what used to be true on the web.

Nonetheless, it still remains very easy and inexpensive to publish on the web without any of the filters associated with print. So we all still need the critical thinking skills you'll learn here to determine whether websites' information is credible enough to suit your purpose.

---

5 Factors to Consider

Evaluating a website for credibility means considering the five factors below in relation to your purpose for the information. These factors are what you should gather clues about and use to decide whether a site is right for your purpose.

- The source's neighborhood on the web.
- Author and/or publisher's background.
- The degree of bias.
- Recognition from others.
- Thoroughness of the content.

How many factors you consider at any one time depends on your purpose when seeking information. In other words, you'll consider all five factors when you're looking for information for a research project or other high-stakes situation where making mistakes have serious consequences. But you might consider only the first three factors at other times.

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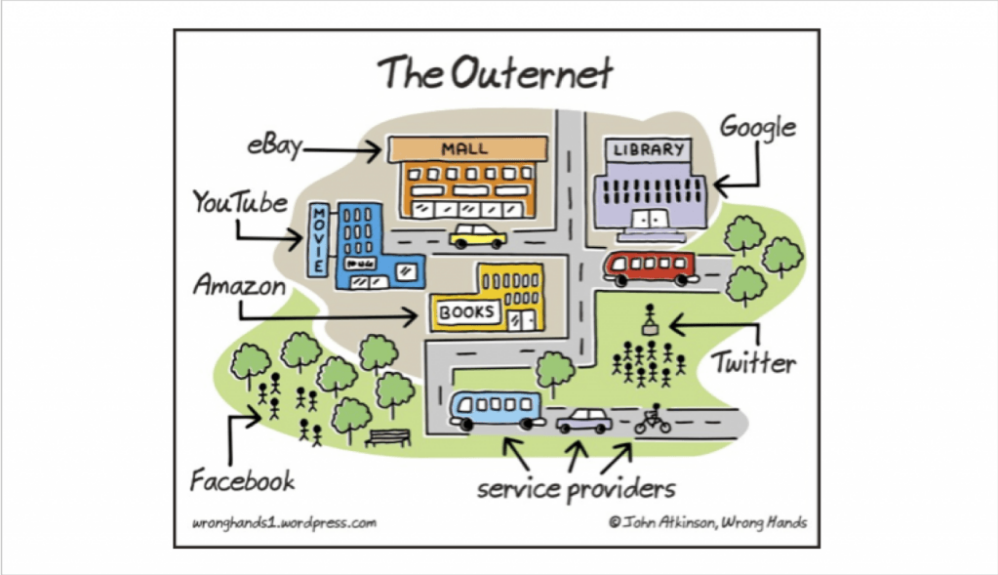
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2.6.6.4: A Source's Neighborhood

A Source's Neighborhood

To understand this concept and begin to use it, imagine that all the sites on the web constitute a community. Just like in a geographical community, there are neighborhoods in which individual sites hang out.

Thinking about what neighborhood a source is in on the web can help you decide whether the site is credible and suits your purpose.



Visualize the web as a community. (Image source: John Atkinson, Wrong Hands)

Audio: Neighborhoods on the Web

Listen to the audio clip (or read the text version) to hear how intuitive this concept is. After you listen, the next activity will show you how to apply the concept.

Listen to Audio | View Text Version

Tip: Author's Purpose for Print

Rather than examine print sources for their web neighborhood, examine them for their author's purpose. Read the introduction and conclusion and look at the table of contents to discern the author's purpose.

For instance, did the author intend to use the book or magazine article to inform/educate, persuade, sell, or entertain?

And is the author's purpose suitable for your purpose? For instance, does the fact that a resource was intended to persuade mean it can't help you answer your research question? (As you know from Sources and Information Needs, yes.)

Activity: Self-Check

Why might you want to read information on an advocacy site (from the neighborhood of sites that promote particular ideas and behavior)—even when you're writing a term paper and it's not acceptable to cite that source because it persuades instead of educates and is not objective? See the bottom of the page for the answer.

Clues About a Website's Neighborhood

Watch the Understanding Google Search Results movie to better understand how you can quickly determine what kind of information you've turned up in a Google search.

Movie: Understanding Google Search Results (no audio)

An interactive or media element has been excluded from this version of the text. You can view it online here: https://ohiostate.pressbooks.pub/choosingsources/?p=872

View Video | View Text Version

On a website, check pages labeled About Us, About This Site, Mission, Site Index, and Site Map, if available. (If such pages or similarly labeled ones don't exist, it may be a sign that the site may be less trustworthy.)

Ask yourself these questions to gather clues that will help you decide what neighborhood you're in:

- **Is the site selling products and/or services (even if there are articles and other useful information, too)?** Perhaps it's a retail, service center, or corporate site.
- **Are there membership applications and requests for contributions of money or time anywhere on the site?** They're usually a sign that you're on a site that promotes particular ideas or behavior – in other words, they're in the advocacy neighborhood.
- **Do postings, articles, reports, and/or policy papers give a one-sided view or multiple views on issues, people, and events?** If they're one-sided, the site is probably a commercial site or in the advocacy group neighborhood. If the information is even-handed and includes different sides of an issue, the site is more likely to be on the library/museum, school, or mainline U.S. news side of town. Sites there usually provide information designed to educate rather than persuade. Newspapers online or in print usually do have editorial pages, however. But labeling opinions as such helps keep mainline U.S. news sources in the newsstand neighborhood and out of the advocacy neighborhood.

Activity: Neighborhoods on the Web

Work through the three activities below to practice the concept of neighborhoods on the web.

Matching Site to Neighborhood – Open activity in a web browser.

Matching Neighborhood to Purpose – Open activity in a web browser.

Which Neighborhood? – Open activity in a web browser.

Example: Check Them Out

Think we're making a mountain out of a molehill about being careful about web sources? Please click the links below to look at three websites. Is there an inference(s) you can make that applies to all three? Perhaps that whether a website looks professionally done is not enough to insure that it is credible.

- RYT Hospital: Dwayne Medical Center – http://rythospital.com
- Dog Island – http://www.thedogisland.com
- The Manhattan Airport Foundation – http://manhattanairport.org

Making the Inference

Consider the clues. Then decide the extent that the site's neighborhood is acceptable for your purpose. It might help to grade the extent that this factor contributes to the site being suitable on a scale like this one:

- A – Very Acceptable
- B – Good, but could be better
- C – OK in a pinch
- D – Marginal
- F – Unacceptable

You'll want to make a note of the resource's grade for neighborhood so you can combine it later with the grades you give the other factors.

Answer to Activity: Self-Check

The answer to the "Self-Check" Activity above is:

Advocacy sites are useful to learn about a particular viewpoint. They may provide a wealth of information—you just have to keep in mind that it's just one side's view and then also seek out the other side's view.

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2.6.6.5: Author and Publisher

Author and Publisher



The reputation of the author and publisher influences your confidence in a source.

You'll always want to know who's providing the information for a website or other source. Do they have the education, training, or other experience that make you think they are authorities on the subject covered? Or do they just have opinions?

The more you know about the author and/or publisher, the more confidence you can have in your decision for or against using content from that source.

Authors and publishers can be individuals or organizations, including companies. (Web masters put things on the site but do not usually decide what goes on all but the smallest websites. They often just carry out others' decisions.)

Sites that do not identify an author or publisher are generally considered less credible for many purposes, including for term papers and other high-stakes projects. The same is true for sources in other formats.

Clues About an Author's and/or Publisher's Background

If they're available, first take a look at pages called such things as About This Site, About Us, or Our Team first. But you may need to browse around a site further to determine its author. Look for a link labeled with anything that seems like it would lead you to the author. Other sources, like books, usually have a few sentences about the author on the back cover or on the flap inside the back cover.

You may find the publisher's name next to the copyright symbol, ©, at the bottom of at least some pages on a site. In books the identity of the publisher is traditionally on the back of the title page.

Sometimes it helps to look for whether a site belongs to a single person or to a reputable organization. Because many colleges and universities offer blog space to their faculty, staff, and students that uses the university's web domain, this evaluation can require deeper analysis than just looking at the address. Personal blogs may not reflect the official views of an organization or meet the standards of formal publication.

In a similar manner, a tilde symbol (~) preceding a directory name in the site address indicates that the page is in a "personal" directory on the server and is not an official publication of that organization. For example, you could tell that Jones' web page was not an official publication of XYZ University if his site's address was: www.XYZuniversity.edu/~jones/page.html. The tilde indicates

it's just a personal web page—in the Residences, not Schools, neighborhood of the web.

Unless you find information about the author to the contrary, such blogs and sites should not automatically be considered to have as much authority as content that is officially part of the university's site. Or you may find that the author has a good academic reputation and is using their blog or website to share resources he or she authored and even published elsewhere. That would nudge him or her toward the Schools neighborhood.

Learning what they have published before can also help you decide whether that organization or individual should be considered credible on the topic. Listed below are sources to use to look for what the organization or individual may have published and what has been published about them.

Tip: Find Out What the Author (Person or Organization) Has Published

Library Catalogs – Search in a large library catalog to find books written by the author.

For example:

- OSU Library Catalog Link
- OhioLINK
- WorldCat@OSU

Web Article Database – Use a free web article database to search for articles by the author. Note: While you can search for free, you may not be able to retrieve articles unless searching through a library.

For example:

- Google Scholar
- MagPortal.com

Specialized Database – Locate articles written by the author by using a specialized database that covers the same topical area as information on the website. Check your library's website to find databases that you can use for this purpose. (Such databases are also called periodical indexes.)

For example:

- Use ERIC (OSU users only) to locate any articles published by the author of an education website.

Tip: Find Out What Has Been Written *About* The Author

Web Search Engine – Use a search engine to find web pages where the author's name is mentioned. (Be sure to search for the name as a phrase, as in "Jane Doe")

For example:

- Google
- Yippy

Full-Text Article Database – Use a database that searches the full-text of articles (not just descriptive information about the article) to find those that mention people and organizations.

For example:

- Academic Search Complete (OSU only)
- LexisNexis Academic (OSU only)

Specialized Biographical Sources – Use directories and indexes provided by your library to find backgrounds of people.

For example:

- Biography Reference Bank (OSU only)

Activity: Identifying Authors

Open activity in a web browser.

Making the Inference

Consider the clues. Then decide the extent that the source's author and/or publisher is acceptable for your purpose. It might help to grade the extent that this factor contributes to the site being suitable on a scale like this one:

- A – Very Acceptable
- B – Good, but could be better
- C – OK in a pinch
- D – Marginal
- F – Unacceptable

You'll want to make a note of the source's grade for author and/or publisher so you can combine it later with the grades you give the other factors.

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2.6.6.6: Degree of Bias

Degree of Bias

Most of us have biases, and we can easily fool ourselves if we don't make a conscious effort to keep our minds open to new information. Psychologists have shown over and over again that humans naturally tend to accept any information that supports what they already believe, even if the information isn't very reliable. And humans also naturally tend to reject information that conflicts with those beliefs, even if the information is solid. These predilections are powerful. Unless we make an active effort to listen to all sides we can become trapped into believing something that isn't so, and won't even know it.
— A Process for Avoiding Deception, Annenberg Classroom

Probably all sources exhibit some bias, simply because it's impossible for their authors to avoid letting their life experience and education have an effect on their decisions about what is relevant to put on the site and what to say about it.

But that kind of unavoidable bias is very different from a wholesale effort to shape the message so the site (or other source) amounts to a persuasive advertisement for something important to the author.



Look for evidence of bias in your sources.

Even if the effort is not as strong as a wholesale effort, authors can find many—sometimes subtle—ways to shape communication until it loses its integrity. Such communication is too persuasive, meaning the author has sacrificed its value as information in order to persuade.

While sifting through all the web messages for the ones that suit your purpose, you'll have to pay attention to both what's on the sites and in your own mind.

That's because one of the things that gets in the way of identifying evidence of bias on websites is our own biases. Sometimes the things that look most correct to us are the ones that play to our own biases.

Clues About Bias

Review the website or other source and look for evidence that the site exhibits more or less bias. The factors below provide some clues.

Coverage

Unbiased: This source's information is not drastically different from coverage of the topic elsewhere. Information and opinion about the topic don't seem to come out of nowhere. It doesn't seem as though information has been shaped to fit.	**Biased:** Compared to what you've found in other sources covering the same topic, this content seems to omit a lot of information about the topic, emphasize vastly different aspects of it, and/or contain stereotypes or overly simplified information. Everything seems to fit the site's theme, even though you know there are various ways to look at the issue(s).
Citing Sources	
Unbiased: The source links to any earlier news or documents it refers to.	**Biased:** The source refers to earlier news or documents, but does not link to the news report or document itself.
Evidence	
Unbiased: Statements are supported by evidence and documentation.	**Biased:** There is little evidence and documentation presented, just assertions that seem intended to persuade by themselves.
Vested Interest	
Unbiased: There is no overt evidence that the author will benefit from whichever way the topic is decided.	**Biased:** The author seems to have a "vested interest" in the topic. For instance, if the site asks for contributions, the author probably will benefit if contributions are made. Or, perhaps the author may get to continue his or her job if the topic that the website promotes gets decided in a particular way.
Imperative Language	
Unbiased: Statements are made without strong emphasis and without provocative twists. There aren't many exclamation points.	**Biased:** There are many strongly worded assertions. There are a lot of exclamation points.
Multiple Viewpoints	
Unbiased: Both pro and con viewpoints are provided about controversial issues.	**Biased:** Only one version of *the truth* is presented about controversial issues.

Examples: Bias

- The Cigarette Papers – Sources of information are documented for each chapter.
- The U.S. Immigration Debate – Shows where it gets its facts; the Council on Foreign Relations is nonpartisan.
- White Poison: The Horrors of Milk – Claims are not supported by documentation.

Making the Inference

Consider the clues. Then decide the extent that the bias you detected on the source is acceptable for your purpose. It might help to grade the extent that this factor contributes to the site being suitable on a scale like this one:

- A – Very Acceptable
- B – Good, but could be better
- C – OK in a pinch
- D – Marginal
- F – Unacceptable

You'll want to make a note of the source's grade for bias so you can combine it later with the grades you give the other factors.

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2.6.6.7: Recognition from Others

Recognition from Others

Checking to see whether others have linked to a website or tagged or cited it lets you know who else on the web recognizes the value of the site's content. Reader comments and ratings can also be informative about some sites you may be evaluating, such as blogs.

If your source isa print book, the blurbs on the front or back cover give you information from authors, experts, or other well-known people who were willing to praise the book and/or author. The same kind of "mini-reviews" may be available on the publisher's website. You can also look for reviews of the book or other source by using Google and Google Scholar.

Those links, tags, bookmarks, citations, and positive reader comments and ratings are evidence that other authors consider the site exemplary. Book reviews, of course, may be either positive or negative.



Ratings and positive comments and review are evidence that others find a source valuable.

Exactly which individuals and organizations are doing the linking, tagging, citing, rating, and commenting may also be important to you. There may be some company you'd rather your site not keep! Or, maybe the sites that link to the one you're evaluating may help solidify your positive feelings about the site.

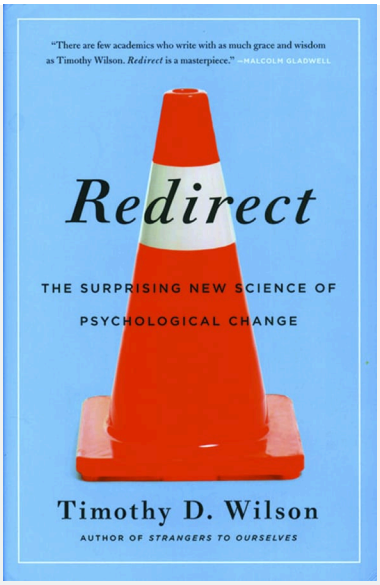
Don't let an absence of links, tags, citations, ratings, and comments damn the site in your evaluation. Perhaps it's just not well-known to other authors. The lack of them should just mean this factor can't add any positive or negative weight to your eventual decision to use the site—it's a neutral.

Tip: Peer Review and Citation as Recognition

The peer review most articles undergo before publication in a scholarly journal lets you know they're considered by other scholars to be worth publishing. You might also be interested to see to what extent other researchers have used an article after it was published. (That use is what necessitates their citation.) But keep in mind that there may not be any citations for very new popular magazines, blogs, or scholarly journal articles.

Activity: Influence You?

Would the blurb on the front cover of *Redirect* by psychologist Timothy Wilson influence you positively or negatively in your evaluation of the book?



The blurb says: "There are few academics who write with as much grace and wisdom as Timothy Wilson. Redirect is a masterpiece. – Malcolm Gladwell"

Clues about Recognition

Use Bing to find sites that link to a particular URL. (Google does not adequately support this.) Enter inbody:[URL of known site] in the Bing search box.

For example: inbody:www.deathpenaltyinfo.org

As you look over the results page, always pay attention to more than the number of sites linking to the site about which you're seeking information. Also consider the kind of sites that do that linking. Are they the kind of sites you are pleased to see associated with the site you're interested in? If you are doing academic writing, you should want them to be professional, scholarly, and/or mainline news sources—not, for instance, something from the entertainment neighborhood.

If you prefer recognition from a particular neighborhood, you can ask Bing to look specifically for links from that neighborhood. Suppose, for instance, you wonder whether scholarly sources link to the site above but don't want to look for them among all that you turned up before. You should type in the Bing search box inbody:www.deathpenaltyinfo.org site:edu in order to see whether any scholarly sources link to that site.

Find citations of an article. Although there is no simple way to find every source that cites an article in a popular magazine, a blog, or a scholarly journal, there are some ways to look for these connections.

For articles published in popular magazines or blogs, enter the title of the article in quotes in the search box of a search engine like Google. The resulting list should show you the original article you're evaluating, plus other sites that have mentioned it in some way. Click on those that you want to know more about.

Example: Finding Mentions

Here's an example using Google to find mentions for a blog article called Help Wanted: 11 million college grads by Bill Gates.

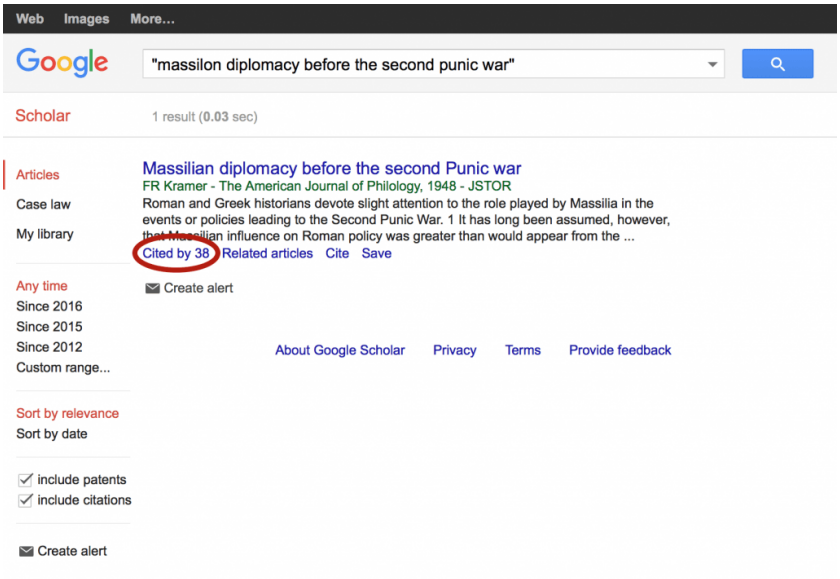
Activity: Inferences

Use Bing to determine how many scholarly sites have made links to these sites. Copy each search statement below and put it in the Bing search box to make your searches.

- inbody:www.DOAJ.org site:edu
- inbody:www.nvic.org site:edu

Sometimes you'll even have to take a look at the linking organization's document in order to figure out *why* it's linking. For instance, is it linking to a bad example that it wants to show? Like usual with source evaluation, you'll have to make inferences.

For articles published in scholarly journals, use Google Scholar to enter the title of the article in quotes. In the results list, find the article you're evaluating. (Many articles have similar titles.) Look for the number of citations in the lower left of the listing for your article. If you want more information on the authors who have done the citing, click on the citation number for a clickable list of articles or papers and get the names of authors to look up at the end of the articles or with a search engine. (This is a good way to discover more articles about your topic, too.)



Google Scholar shows how many articles have cited a given article.
View the live example.

You can also use specialized citation databases, such as Web of Science and Scopus (both OSU only), to find where an article or author has been cited.

Making the Inference

Consider the clues. Then decide the extent that the source's recognition from others is acceptable for your purpose. It might help to grade the extent that this factor contributes to the site being suitable on a scale like this one:

- A – Very Acceptable
- B – Good, but could be better
- C – OK in a pinch
- D – Marginal
- F – Unacceptable

You'll want to make a note of the source's grade for recognition so you can combine it later with the grades you give the other factors.

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2.6.6.8: Thoroughness

Thoroughness



Consider how well a source covers your topic.

Figuring out whether a website or other source is suitable for your purpose also means looking at how thoroughly it covers your topic

You can evaluate thoroughness in relation to other sources on the same topic. Compare your source to how other sources cover the material, checking for missing topics or perspectives.

Clues About Thoroughness

Click around a site to get some idea of how thoroughly it covers the topic. If the source you are evaluating is a print resource, read the introduction and conclusion and also the table of contents to get a glimpse of what it covers. Look at the index to see what subject is covered with the most pages. Is it thorough enough to meet your information need?

Tip: Related Sites

Use Google to find other sites on the same topic by entering **related:[the URL of the site you know]** in the search box.

For example: related:guides.osu.edu

Use this technique to browse other sites Google turns up. Do other sites cover aspects of the topic that are missing from the site you are evaluating? Or does your site stack up pretty well against the competition?

Activity: Comparing Websites

Open activity in a web browser.

Making the Inference

Consider the clues. Then decide the extent that the source's thoroughness is acceptable for your purpose. It might help to grade the extent that this factor contributes to the source being suitable on a scale like this one:

- A – Very Acceptable
- B – Good, but could be better
- C – OK in a pinch
- D – Marginal
- F – Unacceptable

You'll want to make a note of the source's grade for thoroughness so you can combine it later with the grades you give the other factors.

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- User:Cross-Library has no license indicated.

2.6.6.9: Combining the Factors

Combining the Factors

Once you've considered each factor used in evaluating a source, it's important to take a look at the inferences you made about them. Now is the time to look at those grades all together—to average them if you've been assigning grades—and to make one more inference.

Taking the grade on each factor into account, can you infer that the source is credible enough for your purpose? If it isn't, this is one source that can't be helpful in your project. If it is relevant and credible enough, you can use information from that resource with confidence.

Making the Final Inference

Assume you're writing a term paper and are considering using information from Site XYZ. You ran through the evaluation process as you looked over the site, and you made notes about the grades you assigned.

The grades you gave individual factors are:

- Neighborhood: A
- Author/publisher's background: B
- Degree of bias: A
- Recognition from others: No Evidence
- Thoroughness: C
- Currency of the content: A

You average the grades (A=4, B=3, C=2, D=1, F=0), remembering not to include the factor on which you gave no grade. The score was 3.4, about a B, which is a "Good, but could be better" score on the scale we used in this tutorial. You decide to use information from this site in your project.

- A – Very Acceptable
- B – Good, but could be better
- C – OK in a pinch
- D – Marginal
- F – Unacceptable

When Should You Stop?

Research shows that students often don't know when they should stop trying to find and evaluate sources for a particular project. How many sources are enough? It's hard to say, exactly. But you'll need enough to meet the information needs of your project and to meet the requirements your professor told you about.

Furthermore, you may change your mind as you continue working on your project. There is probably not a researcher alive who hasn't thought he or she had enough relevant and sources, only to change their mind later when they were actually writing the final product.

The Sources Checklist here may help you decide what you have enough of and to keep track of needs yet to be met. If you need to, you can use it multiple times as you work on the same project.

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2.6.7: ETHICAL USE OF SOURCES

2.6.7.1: Ethical Use and Citing Sources
2.6.7.2: Why Cite Sources?
2.6.7.3: Challenges in Citing Sources

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2.6.7.1: Ethical Use and Citing Sources

Ethical Use and Citing Sources



It's helpful to understand why to cite your sources.

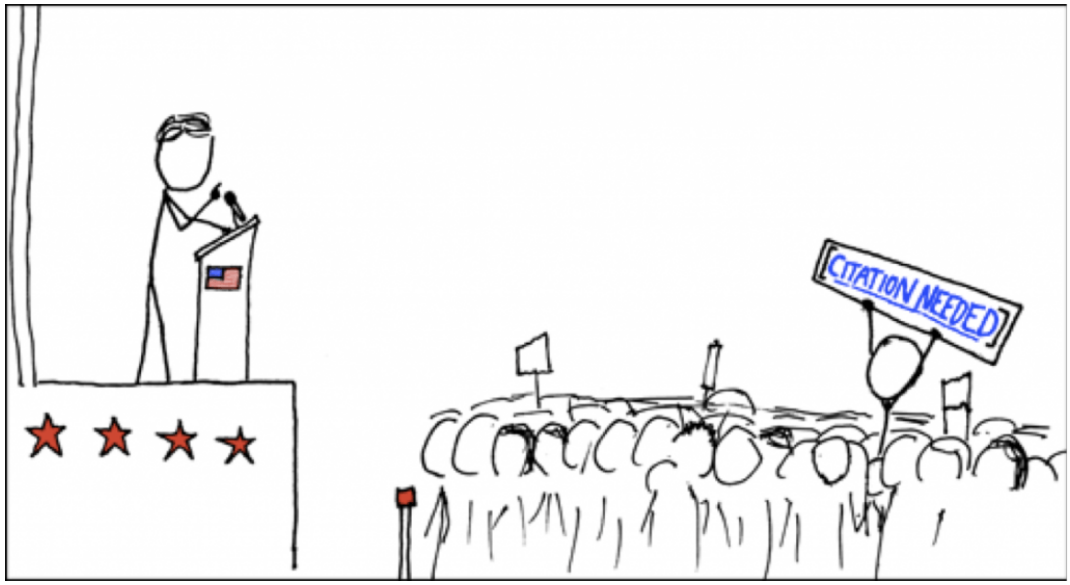
You likely know that research projects always need a reference or a works cited page (also called a bibliography). But have you ever wondered why?

There are some big picture reasons that don't often get articulated that might help you get better at meeting the citation needs of research projects. It's helpful to understand both the theory behind citing, as well as the mechanics of it, to really become a pro.

Tip: How to Cite Sources

This section introduces the concept of citing source, so you can begin your search for sources with it in mind. See the next section, How to Cite Sources for examples and the steps for citing appropriately.

In everyday life, we often have conversations where we share new insights with each other. Sometimes these are insights we've developed on our own through the course of our own everyday experiences, thinking, and reflection. Sometimes these insights come after talking to other people and learning from additional perspectives. When we relate the new things we have learned to our family, friends, or co-workers, we may or may not fill them in on how these thoughts came to us.



In everyday conversation and political speeches, evidence for arguments is often not provided. (Image source: XKDC)

Academic research leads us to the insight that comes from gaining perspectives and understandings from other people through what we read, watch, and hear. In academic work we must tell our readers who and what led us to our conclusions. Documenting our research is important because people rely on academic research to be authoritative, so it is essential for academic conversation to be as clear as possible. Documentation for clarity is a shared and respected practice, and it represents a core value of the academy called "academic integrity." It is a way to distinguish academic conversations (or discourse) from everyday conversations (or discourse).

It is hard to talk about citation practices without considering some related concepts. Here are some definitions of those concepts that are often mentioned in assignments when citation is required.

What Is Academic Integrity?

Different universities have different definitions. Ohio State University uses this definition:

Academic integrity is a commitment, even in the face of adversity, to five fundamental values: honesty, trust, fairness, respect, and responsibility. From these values flow principles of behavior that enable academic communities to translate ideals into action.

Please take a few moments to read the Office of Undergraduate Education web page that describes these values in more detail.

In other words, you must take full responsibility for your work, acknowledge your own efforts, and acknowledge the contributions of others' efforts. Working/Writing with integrity requires accurately representing what you contributed, as well as acknowledging how others have influenced your work. When you are a student, an accurate representation of your knowledge is important because it will allow both you and your professors to know the extent to which you have developed as a scholar. Part of that development is evidenced by how you apply the ruler for acknowledging the work of others.

What Is Academic Misconduct?

As you might imagine, academic misconduct is when you do not use integrity in your academic work. Academic misconduct includes many different unacceptable behaviors, but the one most relevant to what we are discussing here is submitting plagiarized work:

Submitting plagiarized work for an academic requirement. Plagiarism is the representation of another's work or ideas as one's own; it includes the unacknowledged word-for-word use and/or paraphrasing of another person's work, and/or the inappropriate

unacknowledged use of another person's ideas.

To see the full definition of academic misconduct, refer to the Ohio State University Code of Student Conduct.

Note: Check Your Syllabi

You might have noticed a reference to the Code of Student Conduct on several of your syllabi, as faculty are asked to include this statement for your benefit:

It is the responsibility of the Committee on Academic Misconduct to investigate or establish procedures for the investigation of all reported cases of student academic misconduct. The term "academic misconduct" includes all forms of student academic misconduct wherever committed; illustrated by, but not limited to, cases of plagiarism and dishonest practices in connection with examinations. Instructors shall report all instances of alleged academic misconduct to the committee (Faculty Rule 3335-5-487). For additional information, see the Code of Student Conduct.

What Is Plagiarism?

Plagiarism is defined by the OSU First Year Experience Office in this way:

At any stage of the writing process, all academic work submitted to the teacher must be a result of a student's own thought, research or self-expression. When a student submits work purporting to be his or her own, but which in any way borrows organization, ideas, wording or anything else from a source without appropriate acknowledgment of the fact, he/she is engaging in plagiarism.

Take time to look at the full definition, which also describes another form of academic misconduct called "collusion."

Plagiarism can be intentional (knowingly using someone else's work and presenting it as your own) or unintentional (inaccurately or inadequately citing ideas and words from a source). It may be impossible for your professor to determine whether plagiarized work was intentional or unintentional. But in either case, plagiarism puts both you and your professor in a compromising position.

While academic integrity calls for work resulting from your own effort, scholarship requires that you learn from others. So in the world of academic scholarship you are actually expected to learn new things from others AND come to new insights on your own. There is an implicit understanding that as a student you will be both using other's knowledge as well as your own insights to create new scholarship. To do this in a way that meets academic integrity standards you must acknowledge the part of your work that develops from others' efforts. You do this by citing the work of others. You plagiarize when you fail to acknowledge the work of others and do not follow appropriate citation guidelines.

For more information on plagiarism, see Handouts – Plagiarism from OSU's Center for the Study and Teaching of Writing.

What Is Citing?

Citing, or citation, is a practice of documenting specific influences on your academic work. See How to Cite Sources for details.

In other words, you must cite all the sources you quote directly, paraphrase, or summarize as you:

- Answer your research question
- Convince your audience
- Describe the situation around your research question and why the question is important
- Report what others have said about your question

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2.6.7.2: Why Cite Sources?

Why Cite Sources?

As a student citing is important because it shows your reader (or professor) that you have invested time in learning what has already been learned and thought about the topic before offering your own perspective. It is the practice of giving credit to the sources that inform your work.

Our definitions of academic integrity, academic misconduct and plagiarism, also give us important reasons for citing the sources we use to accomplish academic research. Here are all the good reasons for citing.

To Avoid Plagiarism & Maintain Academic Integrity

Misrepresenting your academic achievements by not giving credit to others indicates a lack of academic integrity. This is not only looked down upon by the scholarly community, but it is also punished. When you are a student this could mean a failing grade or even expulsion from the university.

To Acknowledge the Work of Others

One major purpose of citations is to simply provide credit where it is due. When you provide accurate citations, you are acknowledging both the hard work that has gone into producing research and the person(s) who performed that research.

Think about the effort you put into your work (whether essays, reports, or even non-academic jobs): if someone else took credit for your ideas or words, would that seem fair, or would you expect to have your efforts recognized?

To Provide Credibility to Your Work & to Place Your Work in Context

Providing accurate citations puts your work and ideas into an academic context. They tell your reader that you've done your research and know what others have said about your topic. Not only do citations provide context for your work but they also lend credibility and authority to your claims.

For example, if you're researching and writing about sustainability and construction, you should cite experts in sustainability, construction, and sustainable construction in order to demonstrate that you are well-versed in the most common ideas in the fields. Although you can make a claim about sustainable construction after doing research only in that particular field, your claim will carry more weight if you can demonstrate that your claim can be supported by the research of experts in closely related fields as well.

Citing sources about sustainability and construction as well as sustainable construction demonstrates the diversity of views and approaches to the topic. In addition, proper citation also demonstrates the ways in which research is social: no one researches in a vacuum—we all rely on the work of others to help us during the research process.

To Help Your Future Researching Self & Other Researchers Easily Locate Sources

Having accurate citations will help you as a researcher and writer keep track of the sources and information you find so that you can easily find the source again. Accurate citations may take some effort to produce, but they will save you time in the long run. So think of proper citation as a gift to your future researching self!

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2.6.7.3: Challenges in Citing Sources

Challenges in Citing Sources

Here are some challenges that might make knowing when and how to cite difficult for you. Our best advice for how to overcome these challenges is in the first item.

Running Out of Time

When you are a student taking many classes simultaneously and facing many deadlines, it may be hard to devote the time needed to doing good scholarship and accurately representing the sources you have used. Research takes time. The sooner you can start and the more time you can devote to it, the better your work will be. From the beginning, be sure to include in your notes where you found information you could quote, paraphrase, and summarize in your final product.

Having to Use Different Styles

Different disciplines require that your citations be in different styles: which publication information is included and in what order. So your citations for different courses could look different, particularly for courses outside your major.

Not Really Understanding the Material You're Using

If you are working in a new field or subject area, you might have difficulty understanding the information from other scholars, thus making it difficult to know how to paraphrase or summarize that work properly.

Running Out of Time

When you are a student taking many classes simultaneously and facing many deadlines, it may be hard to devote the time needed to doing good scholarship and accurately representing the sources you have used. Research takes time. The sooner you can start and the more time you can devote to it, the better your work will be. From the beginning, be sure to include in your notes where you found information you could quote, paraphrase, and summarize in your final product.

Shifting Cultural Expectations of Citation

Because of new technologies that make finding, using, and sharing information easier, many of our cultural expectations around how to do that are changing as well. For example, blog posts often "reference" other articles or works by simply linking to them. It makes it easy for the reader to see where the author's ideas have come from and to view the source very quickly. But in these more informal writings, blog authors do not have a list of citations (bibliographic entries). The links do the work for them. This is a great strategy for online digital mediums, but this method fails over time when links break and there are no hints (like an author, title and date) to know how else to find the reference, which might have moved.

This example of a cultural change of expectations in the non-academic world might make it seem that there has been a change in academic scholarship as well, or might make people new to academic scholarship even less familiar with citation. But in fact, the expectations around citing sources in academic research remain formal.

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2.6.8: HOW TO CITE SOURCES

2.6.8.1: Citation and Citation Styles
2.6.8.2: Steps for Citing
2.6.8.3: Citation Software
2.6.8.4: When to Cite

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2.6.8.1: Citation and Citation Styles

Citation and Citation Styles



Sources that influenced your thinking and research must be cited in academic writing.

Citing sources is an academic convention for keeping track of which sources influenced your own thinking and research. (See Ethical Use of Sources for many good reasons why you should cite others' work.)

Most citations require two parts:

- The full bibliographic citation on the Bibliography page or References page, or Works Cited page of your final product.
- An indication within your text (usually author and publication date and maybe the page number from which you are quoting) that tells your reader where you have used something that needs a citation.

With your in-text citation, your reader will be able to tell which full bibliographic citation you are referring to by paying attention to the author's name and publication date.

Let's look at an example.

Example: Citations in Academic Writing

Here's a citation in the text of an academic paper:

Studies have shown that compared to passive learning, which occurs when students observe a lecture, students will learn more and will retain that learning longer if more active methods of teaching and learning are used (Bonwell and Eison 1991; Fink 2003).

The information in parentheses coordinates with a list of full citations at the end of the paper.

At the end of the paper, these bibliographic entries appear in a reference list:

> Bonwell, C. G., and Eison, J. A.1991. "Active learning: Creating excitement in the classroom." ASHE-ERIC Higher Education Rep. No. 1, George Washington Univ., Washington, D.C.
>
> Fink, L. D. 2003. Creating significant learning experiences, Wiley, New York.

You can see the full article [OSU login required] from which this example was taken online.

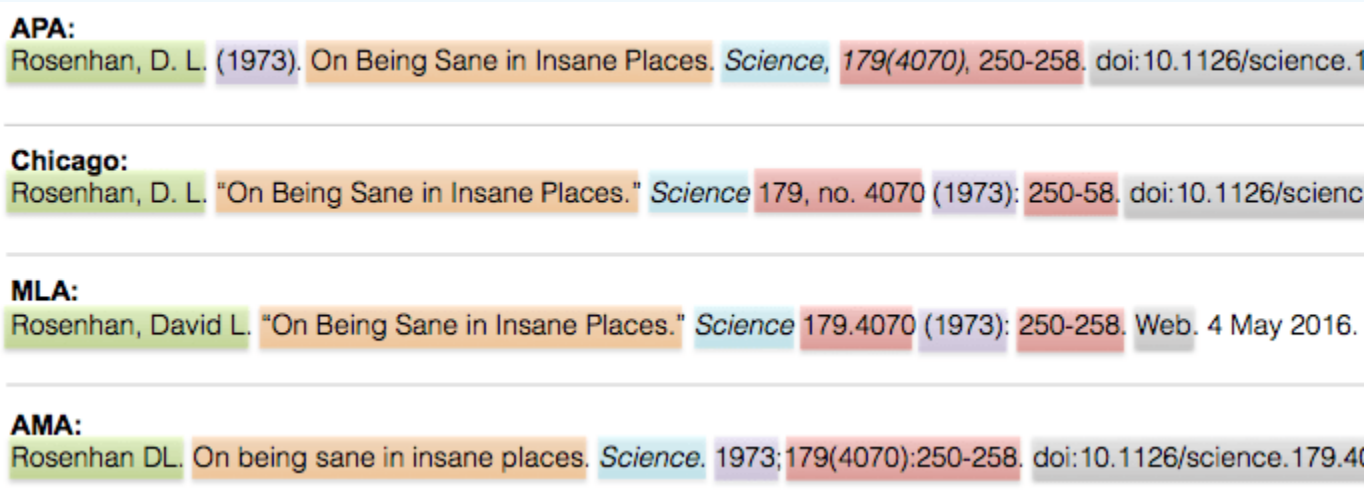
Citation Styles

Style guides set the specific rules for how to create both in-text citations and their full bibliographic citations.

There are over a dozen kinds of citation styles. While each style requires much of the same publication information to be included in a citation, the styles differ from each other in formatting details such as capitalization, punctuation, order of publication information, and whether the author's name is given in full or abbreviated.

Example: Differences in Citation Styles

The image below shows bibliographic citations in four common styles. Notice that they contain information about who the author is, article title, journal title, publication year, and information about volume, issue, and pages. Notice also the small differences in punctuation, order of the elements, and formatting that **do make a difference**.



Differences between citation practices occur mainly in formatting.

Compare citation elements (including the punctuation and spacing) in the same color to see how each style handles their information.

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2.6.8.2: Steps for Citing

Steps for Citing

To write a proper citation we recommend following these steps, which will help you maintain accuracy and clarity in acknowledging sources.

Step 1: Choose Your Citation Style

Find out the name of the citation style you must use from your instructor, the directions for an assignment, or what you know your audience or publisher expects. Then search for your style at the Purdue Online Writing Lab (OWL) or use Google or Bing to find your style's stylebook/handbook and then purchase it or ask for it at a library.

Step 2: Create In-Text Citations

Find and read your style's rules about in-text citations, which are usually very thorough. Luckily, there are usually examples provided that make it a lot easier to learn the rules.

EXAMPLE: Style Guides Are Usually Very Thorough

For instance, your style guide may have different rules for when you are citing:

- Quotations rather than summaries rather than paraphrases
- Long, as opposed to short, quotations.
- Sources with one or multiple authors.
- Books, journal articles, interviews and email, or electronic sources.

Step 3: Determine the Kind of Source

After creating your in-text citation, now begin creating the full bibliographic citation that will appear on the References or Bibliography page by deciding what kind of source you have to cite (book, film, journal article, webpage, etc.).

EXAMPLE: Using a Style Guide to Create an In-Text Citation

Imagine that you're using APA style and have the APA style guide rules for in-text citations open in OWL. In your psychogeography paper, you want to quote the authors of the book The Experience of Nature, Rachel Kaplan and Stephen Kaplan, which was published in 1989. What you want to quote is from page 38 of the book.

Here's what you want to quote:

"The way space is organized provides information about what one might want to do in that space. A relatively brief glance at a scene communicates whether there is room to roam, whether one's path is clear or blocked."

1. Skim the headings in the style guide to remind yourself of what its rules concern.
   Since it has rules about the length of quotations, you count the number of words in what you want to quote and find that your quote has 38, which is within the range for short quotations (less than 40), according to the APA style guide.According to the rule for short quotations, you see that you're supposed to introduce the quote by attributing the quote to the author (last name only) and adding the publication date in parentheses. You write:

According to the Kaplans (1989), "The way space is organized provides information about what one might want to do in that space. A relatively brief glance at a scene communicates whether there is room to roam, whether one's path is clear or blocked."

2. Then you notice that the example in the style guide includes the page number on which you found the quotation. It appears at the end of the quote (in parentheses and outside the quote marks but before the period ending the quotation). So you add that:

   According to the Kaplans (1989), "The way space is organized provides information about what one might want to do in that space. A relatively brief glance at a scene communicates whether there is room to roam, whether one's path is clear or blocked" **(p.38)**.

3. You're feeling pretty good, but then you realize that you have overlooked the rule about having multiple authors. You have two and their last names are both Kaplan. So you change your sentence to:

   According to **Kaplan and Kaplan** (1989), "The way space is organized provides information about what one might want to do in that space. A relatively brief glance at a scene communicates whether there is room to roam, whether one's path is clear or blocked" (p.38).

   So you have your first in-text citation for your final product:

   According to Kaplan and Kaplan (1989), "The way space is organized provides information about what one might want to do in that space. A relatively brief glance at a scene communicates whether there is room to roam, whether one's path is clear or blocked" (p.38).

Step 4: Study Your Style's Rules for Bibliographic Citations

Next, you'll need a full bibliographic citation for the same source. This citation will appear on the References page or Bibliography page or Works Cited page. (APA style, which we're using here, requires a page called References.) Bibliographic citations usually contain more publication facts than you used for your in-text citation, and the formatting for all of them is very specific.

EXAMPLE: Bibliographic Citation Rules Are Very Specific

- Rules vary for sources, depending, for instance, on whether they are books, journal articles, or online sources.
- Sometimes lines of the citation must be indented.
- Authors' names usually appear last name first.
- Authors' first names of authors may be initials instead.
- Names of sources may or may not have to be in full.
- Names of some kinds of sources may have to be italicized.
- Names of some sources may have to be in quotes.
- Dates of publication appear in different places, depending on the style.
- Some styles require Digital Object Identifiers (DOIs) in the citations for online sources.

Step 5: Identify Citation Elements

Figure out which bibliographic citation rules apply to the source you've just created an in-text citation for. Then apply them to create your first bibliographic citation.

Example: Using a Style Guide to Create a Bibliographic Citation

Imagine that you're using APA style and have the APA style guide rules for bibliographic citations open in OWL. Your citation will be for the book called The Experience of Nature, written by Rachel Kaplan and Stephen Kaplan and published in 1989.

1. You start by trying to apply OWL's basic rules of APA style, which tell you your citation will start with the last name of your author followed by his or her first initial, and that the second line of the citation will be indented. So you write:**Kaplan, R. and Kaplan, S**. and remind yourself to indent the second line when you get there.
2. Since you have two authors, you look for a rule regarding that situation, which requires a comma between the authors and an ampersand between the names. So you write:Kaplan, R., **&** Kaplan, S.
3. Because you know your source is a book, you look for style guide rules and examples about books. For instance, the rules for APA style say that the publication date goes in parentheses, followed by a period after the last author's name. And that the title of the book is italicized. You apply the rules and examples and write the publication information you know about your source:Kaplan, R., & Kaplan, S. **(1989). The Experience of Nature.**
4. Next, you look at the rules and examples of book citations and notice that they show the city where the book was published and the publisher. So you find that information about your source (in a book, usually on the title page or its back) and write:Kaplan, R., & Kaplan, S. (1989). The Experience of Nature. **Cambridge: Cambridge University Press.**
5. Congratulations, especially about remembering to indent that line! You have created the first bibliographic citation for your final product.

Step 6: Repeat the steps for creating an in-text citation and a bibliographic citation for each of your sources.

Create your bibliographic citation by arranging publication information to match the example you chose in Step 4. Pay particular attention to what is and is not capitalized and to what punctuation and spaces separate each part that the example illustrates.

Movie: Finding the Information You Need: PDF and HTML Journal Articles

An interactive or media element has been excluded from this version of the text. You can view it online here: https://ohiostate.pressbooks.pub/choosingsources/?p=1048

View video

Movie: Finding the Information You Need: Citing Information for Web and Online Multimedia Sources

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View video

Tip: Citation Software

If you like, you can use citation generator software to arrange the information needed for your citation according to the style guide you chose. Learn more later in this section.

ACTIVITY: Deciphering Citations

Open activity in a web browser.

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2.6.8.3: Citation Software

Citation Software

You may be familiar with the many citation generators that allow you to auto-generate reference lists from citation data. Some allow you to save and store citations to reuse them in different lists and in different work, as needed.

Such tools are worth investigating and learning about for any long-term research project. Zotero is online and available for free to anyone from anywhere. RefWorks and EndNote are available to all OSU students, faculty, and staff from anywhere because OSU Libraries subscribes to this service. For information about using any of these tools, go to software available to OSU students.



Common Citations Tools

Good reasons to use citation generation software include:

- To save time: it takes citation generation software only a few seconds to create a citation.
- To easily convert citations from one style to another.
- To have a centralized source list that is not attached to a specific project, which allows you to reuse references and their citations in various projects.

Care you must use with citation generation software includes:

- Citation generation software is only as good as the information entered into it. In other words, if you provide incorrect information or do not include some information, then your citation will be incorrect.
- Most citation generation software can create citations by searching for the information online. Sometimes software can pull the information from the wrong edition of a source, for example, or specific formatting (such as italics) might be lost. Or perhaps the generator didn't use the latest version of the style guide.
- **Always review the citations you create with this software.**

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2.6.8.4: When to Cite

When to Cite

Citing sources is often described as a straightforward, rule-based practice. But in fact, there are many gray areas around citation, and learning how to apply citation guidelines takes practice and education. If you are confused by it, you are not alone – in fact you might be doing some good thinking. Here are some guidelines to help you navigate citation practices.

Cite when you are directly quoting. This is the easiest rule to understand. If you are stating word-for-word what someone else has already written, you must put quotes around those words and you must give credit to the original author. Not doing so would mean that you are letting your reader believe these words are your own and represent your own effort.

Cite when you are summarizing and paraphrasing. This is a trickier area to understand. First of all, summarizing and paraphrasing are two related practices but they are not the same. Summarizing is when you read a text, consider the main points, and provide a shorter version of what you learned. Paraphrasing is when you restate what the original author said in your own words and in your own tone. Both summarizing and paraphrasing require good writing skills and an accurate understanding of the material you are trying to convey. Summarizing and paraphrasing are difficult to do when you are a beginning academic researcher, but these skills become easier to perform over time with practice.

Cite when you are citing something that is highly debatable. For example, if you want to claim that the Patriot Act has been an important tool for national security, you should be prepared to give examples of how it has helped and how experts have claimed that it has helped. Many U.S. citizens concerned that it violates privacy rights won't agree with you, and they will be able to find commentary that the Patriot Act has been more harmful to the nation than helpful. You need to be prepared to show such skeptics that you have experts on your side, too.

> **Tip: Why Cite Sources?**
>
> This section covers how and when to cite sources. For a discussion of *why* to cite sources, see Ethical Use of Sources.

When Don't You Cite?

Don't cite when what you are saying is your own insight. As you learned in The Purpose of Academic Argument, research involves forming opinions and insights around what you learn. You may be citing several sources that have helped you learn, but at some point you must integrate your own opinion, conclusion, or insight into the work. The fact that you are *not* citing it helps the reader understand that this portion of the work is your unique contribution developed through your own research efforts.

Don't cite when you are stating common knowledge. What is common knowledge is sometimes difficult to discern. In general, quick facts like historical dates or events are not cited because they are common knowledge.

Examples of information that would not need to be cited include:

- The Declaration of Independence was signed in 1776.
- Barack Obama became the 44th president of the United States in January, 2009.

Some quick facts, such as statistics, are trickier. For example, the number of gun-related deaths per year probably should be cited, because there are a lot of ways this number could be determined (does the number include murder only, or suicides and accidents, as well?) and there might be different numbers provided by different organizations, each with an agenda about gun laws.

A guideline that can help with deciding whether or not to cite facts is to determine whether the same data is repeated in multiple sources. If it is not, it is best to cite.

The other thing that makes this determination difficult might be that what seems new and insightful to you might be common knowledge to an expert in the field. You have to use your best judgment, and probably err on the side of over-citing, as you are learning to do academic research. You can seek the advice of your instructor, a writing tutor, or a librarian. Knowing what is and is not common knowledge is a practiced skill that gets easier with time and with your own increased knowledge.



Wikipedia, while good for early research and background information, shouldn't be cited as a source because it's already a summary.

Tip: Why You Can't Cite Wikipedia

You've likely been told at some point that you can't cite Wikipedia, or any encyclopedia for that matter, in your scholarly work.

The reason is that such entries are meant to *prepare* you to do research, not be evidence of your having done it. Wikipedia entries, which are tertiary sources, are already a summary of what is known about the topic. Someone else has already done the labor of synthesizing lots of information into a concise and quick way of learning about the topic.

So while Wikipedia is a great shortcut for getting context, background, and a quick lesson on topics that might not be familiar to you, don't quote, paraphrase, or summarize from it. Just use it to educate yourself.

Activity: To Cite or Not to Cite?

Open activity in a web browser.

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2.6.9: MAKING AN ARGUMENT

2.6.9.1: The Purpose of Academic Argument
2.6.9.2: Components of an Argument
2.6.9.3: Order of the Components
2.6.9.4: Where You Get the Components

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2.6.9.1: The Purpose of Academic Argument

The Purpose of Academic Argument



Scholarly conversation makes an argument for a given point of view.

Nearly all scholarly writing makes an argument. That's because its purpose is to create and share new knowledge so it can be debated in order to confirm, dis-confirm, or improve it. That arguing takes place mostly in journals and scholarly books and at conferences. It's called the scholarly conversation, and it's that conversation that moves forward what we humans know.

Tip: Prezi on Scholarly Publishing

View an overview of the different ways in which scholars share their work with each other and the public.

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Open in a web browser. | Alternate text version.

Your scholarly writing for classes should do the same—make an argument—just like your professors' journal article, scholarly book, and conference presentation writing does. (You may not have realized that the writing you're required to do mirrors what scholars all over the university, country, and world must do to create new knowledge and debate it. Of course, you may be a beginner at constructing arguments in writing, while most professors have been at it for some time. And your audience (for now) also may be more limited than your professor's. But the process is much the same. As you complete your research assignments, you, too, are entering the scholarly conversation.

Making an argument means trying to convince others that you are correct as you describe a thing, situation, relationship, or phenomenon and/or persuade them to take a particular action. Important not just in college, that skill will be necessary for nearly every professional job you hold after college. So learning how to make an argument is good job preparation, even if you do not choose a scholarly career.

Realizing that your final product for your research project is to make an argument gives you a big head start because right off you know the sources you're going to need are those that will let you write the components of an argument for your reader.

Happily (and not coincidentally), most of those components coincide with the information needs we've been talking about. Meeting an information need by using sources will enable you to write the corresponding argument component in your final product.

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2.6.9.2: Components of an Argument

Components of an Argument

Making an argument in an essay, term paper, blog post or other college writing task is like laying out a case in court. Just as there are conventions that attorneys must adhere to as they make their arguments in court, there are conventions in arguments made in research assignments. Among those conventions is to use the components of an argument.

Note:

This section on making an argument was developed with the help of "Making Good Arguments" in *The Craft of Research*, by Wayne Booth, Gregory Colomb, and Joseph Williams, University of Chicago Press, 2003.

The arguments you're used to hearing or participating in with friends about something that is uncertain or that needs to be decided contain the same components as the ones you'll need to use in academic writing. Arguments contain those components because those are the ones that work—used together, they stand the best chance of persuading others that you are correct.

For instance, the question gets things started off. The claim, or thesis, tells people what you consider a true way of describing a thing, situation, relationship, or phenomenon or what action you think should be taken. The reservations, alternatives, and objections that someone else brings up in your sources (or that you imagine your readers logically might have) allow you to demonstrate how your reasons and evidence (maybe) overcome that kind of thinking—and (you hope) your claim/thesis comes out stronger for having withstood that test.

Activity: Labeled Components

Read the short dialog on pages 114 and 115 in the ebook *The Craft of Research* by Wayne Booth, Gregory Colomb, and Joseph Williams. The components of an argument are labeled for you.

Example: Argument as a Dialog

Here's a dialog of an argument, with the most important components labeled.

Jerald: Where should we have my parents take us for dinner when they're here on Sunday? *[He asks the question about something that's unsettled.]*
Cathy: We should go to The Cascades! *[She makes her main claim to answer the question.]* It's the nicest place around. *[Another claim, which functions as a reason for the main claim.]*
Jerald: How so? *[He asks for a reason to believe her claims.]*
Cathy: White table cloths. *[She gives a reason.]*
Jerald: What's that have to do with how good the food is? *[He doesn't see how her reason is relevant to the claim.]*
Cathy: Table cloths make restaurants seem upscale. *[She relates her reason for the claims.]* And I've read a survey in Columbus Metro that says the Cascades is one of the most popular restaurants in town. *[She offers evidence.]*
Jerald: I never read the Metro. And Dino's has table cloths. *[He offers a point that contradicts her reason.]*
Cathy: I know, but those are checkered! I'm talking about heavy white ones. *[She acknowledges his point and responds to it.]*
Jerald: My dad loves Italian food. I guess he's kind of a checkered-table-cloth kind of guy? *[He raises another reservation or objection.]*
Cathy: Yeah, but? Well, I know The Cascades has some Italian things on the menu. I mean, it's not known for its Italian food but you can order it there. Given how nice the place is, it will probably be gourmet Italian food. *[She acknowledges his point and responds to it. There's another claim in there.]*
Jerald: Ha! My dad, the gourmet? Hey, maybe this place is too expensive. *[He raises another reservation.]*
Cathy: More than someplace like Dino's. *[She concedes his point.]*
Jerald: Yeah. *[He agrees.]*

Cathy: But everybody eats at The Cascades with their parents while they're students here, so it can't be outlandishly expensive. *[She now puts limits on how much she's conceding.]*

Activity: Components of an Argument

Open activity in a web browser.

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2.6.9.3: Order of the Components

Order of the Components

The order in which the components should appear in your argument essays, papers, and posters may depend on which discipline your course is in. So always adhere to the advice provided by your professor and what you learn in class.

One common arrangement for argument essays and term papers is to begin with an introduction that explains why the situation is important—why the reader should care about it. Your research question will probably not appear, but your answer to it (your thesis, or claim) usually appears as the last sentence or two of the introduction.

The body of your essay or paper follows and consists of:

- Your reasons the thesis is correct or at least reasonable.
- The evidence that supports each reason, often occurring right after the reason the evidence supports.
- An acknowledgement that some people have/could have objections, reservations, counterarguments, or alternative solutions to your argument and a statement of each. (Posters often don't have room for this component.)
- A response to each acknowledgement that explains why that criticism is incorrect or not very important. Sometimes you might have to concede a point you think is unimportant, if you can't really refute it.

(Again, posters often don't have much room for this part of an argument.)

After the body, the paper or essay ends with a conclusion, which states your thesis in a slightly different way than occurred in the introduction. The conclusion also may mention why research on this situation is important. (Posters often don't have much room for this component.)

A Blueprint for Argument

It's no accident that people are said to **make** arguments—they're all constructed, and these components are the building blocks. The components are important because of what they contribute. The components generally, though not always, appear in a certain order because they build on or respond to one another.

For example, the thesis or claim is derived from the initial question. The reasons are bolstered by evidence to support the claim. Objections are raised, acknowledged and subsequently responded to.



The components of argument build on each other.

ACTIVITY: Order of Argument Components

Open activity in a web browser.

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2.6.9.4: Where You Get the Components

Where You Get the Components

This section will help you figure that out which components may come from your professor, which you just have to think about, which you have to write, and which you have to find in your sources.

Here, again, are the components we'll cover:

- The research question you (or your professor) wanted answered.
- Your claim or thesis.
- One or more reasons for your thesis.
- Evidence for each reason.
- Others' objections, counterarguments, or alternative solutions.
- Your acknowledgment of others' objections, counterarguments, or alternative solutions.
- Your response to others' objections, counterarguments, or alternative solutions.

The Question You Wanted Answered

Sometimes your professor will give you the research question, but probably more often he or she will expect you to develop your own from an assigned topic. You learned how to develop research questions in another section. Though vitally important, they are often not stated in essays or term papers but are usually stated in reports of original studies, such as theses, dissertations, and journal articles.

Examples: Research Questions for Hypothetical Essays or Term Papers

- How do at least some animals' bones help control their weight?
- Did the death of his beloved daughter have any effect on the writings of Mark Twain?

Your Claim or Thesis

You write the claim or thesis–it doesn't come directly from a resource. Instead, it is the conclusion you come to in answer to your question after you've read/listened to/viewed some sources. So it is a statement, not a question or a hypothesis that you plan to prove or disprove with your research.

After you've read/listened to/viewed more sources, you may need to change your thesis. That happens all the time–not because you did anything wrong but because you learned more.

Examples: Claims (or Theses) for Hypothetical Essays or Term Papers

- Bone cells monitor whether more or less weight is pressing down on the skeleton and send biochemical signals to appetite centers in their brains to turn appetite down or up, accordingly.
- Mark Twain wrote more urgently and with less humor during the four years immediately after the death of his daughter.

One or More Reasons

You write what you believe makes your claim or thesis (the answer to your research question) true. That's your reason or reasons. Each reason is a summary statement of evidence you found in your research. The kinds of evidence considered convincing varies by discipline, so you will be looking at different sources, depending on your discipline. How many reasons you need depends on

how complex your thesis and subject matter are, what you found in your sources, and how long your essay or term paper must be. It's always a good idea to write your reasons in a way that is easy for your audience to understand and be persuaded by.

Examples: Reasons in Hypothetical Essays or Term Papers

- Animals (including humans) have a biological tendency to regain any weight that they lose and lose any weight that they gain, seemingly in an effort to maintain whatever weight they have sustained for some time. Skeletons are logical places where any gains or losses could be noted, and recent studies seem to show that osteocytes (a kind of bone cell) are involved in whether appetites go up or down after weight gain or loss.
- My content analysis and a comparison of publication rates four years before and after Mark Twain's daughter died indicate that his writing was more urgent and less humorous for four years after. It is reasonable to conclude that her death caused that change.

Evidence for Each Reason

You write this also. This is the evidence you summarized earlier as each reason your thesis is true. You will be directly quoting, paraphrasing, and summarizing your sources to make the case that your answer to your research question is correct, or at least reasonable.

Examples: Evidence for Reasons in Hypothetical Essays or Term Papers

- Report the results of studies about osteocyctes' possible effect on weight grain or loss.
- Report the results of your comparison of writing content and publication rate before and after Twain's daughter's death.

Others' Objections, Counterarguments, or Alternative Solutions

Do any of your sources not agree with your thesis? You'll have to bring those up in your term paper. In addition, put yourself in your readers' shoes. What might they not find logical in your argument? In other words, which reason(s) and corresponding evidence might they find lacking? Did you find clues to what these could be in your sources? Or maybe you can imagine them thinking some aspect of what you think is evidence doesn't make sense.

Examples: Objections, Counterarguments, or Alternative Solutions in Hypothetical Essays or Term Papers

- Imagine that some readers might think: The hormone leptin is released by fat cells when they are added to animals' bodies so it is leptin that tells appetite centers to turn down when weight is gained.
- Imagine that some readers might think: Computerized content analysis tools are sort of blunt instruments and shouldn't be used to do precise work like this.

Your Acknowledgement of Others' Objections, Counterarguments, or Alternative Solutions

So what will you write to bring up each of those objections, counterarguments, and alternative solutions? Some examples:

- I can imagine skeptics wanting to point out...
- Perhaps some readers would say...
- I think those who come from XYZ would differ with me...

It all depends on what objections, counterarguments, and alternative solutions your audience or your imagination come up with.

Examples: Acknowledgement of Others' Objections, Counterarguments, or Alternative Solutions in Hypothetical Essays or Term Papers:

- Some readers may point out that the hormone leptin, which is released by fat cells, signals appetite centers to lower the appetite when weight is gained.
- Readers may think that a computerized content analysis tool cannot do justice to the subtleties of text.

Response to Others' Objections, Counterarguments, or Alternative Solutions

You must write your response to each objection, counterargument, or alternative solution brought up or that you've thought of. (You're likely to have found clues for what to say in your sources.) The reason you have to include this is that you can't very easily convince your audience until you show them how your claim stacks up against the opinions and reasoning of other people who don't at the moment agree with you.

Examples: Response to Others' Objections, Counterarguments, or Alternative Solutions in Hypothetical Essays or Term Papers:

- But leptin must not be the entire system, since many animals do keep on the new weight.
- Unlike other content tools, the XYZ Content Analysis Measure is able to take into account an author's tone.

Activity: Quick Check

Open activity in a web browser.

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2.6.10: WRITING TIPS

2.6.10.1: When to Quote, Paraphrase, or Summarize
2.6.10.2: Helping Others Follow
2.6.10.3: Synthesis of Your Own Ideas

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2.6.10.1: When to Quote, Paraphrase, or Summarize

When to Quote, Paraphrase, or Summarize



This section features advice for using sources well in your writing projects.

If your final product is a term paper or essay, much of your writing will be devoted to:

- Reporting what others have said about your research question.
- Convincing your audience that your answer is correct or, at least, the most reasonable answer. (Giving them evidence.)
- Describing the situation surrounding your research question for your audience and explaining why it's important.

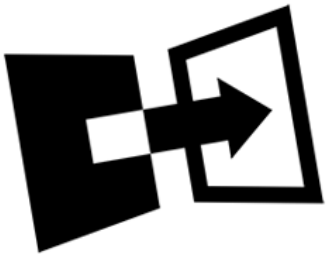
To do that writing you will often use direct quotes from your sources and will paraphrase and summarize sources. But how should you choose which technique to use when?

Tip: Citing Sources

Remember to cite your sources when quoting, paraphrasing, and summarizing. See How to Cite Sources for details.



Choose a direct quote when it is more likely to be accurate than would summarizing or paraphrasing, when what you're quoting is the text you're analyzing, when a direct quote is more concise that a summary or paraphrase would be and conciseness matters, when the author is a particular authority whose exact words would lend credence to your argument, and when the author has used particularly effective language that is just too good to pass up.



Choose to paraphrase or summarize rather than to quote directly when the meaning is more important than the particular language the author used and you don't need to use the author's preeminent authority to bolster your argument at the moment.

Choose to paraphrase instead of summarizing when you need details and specificity. Paraphrasing lets you emphasize the ideas in source materials that are most related to your term paper or essay instead of the exact language the author used. It also lets you simplify complex material, sometimes rewording to use language that is more understandable to your reader.



Choose to summarize instead of paraphrasing when you need to provide a brief overview of a larger text. Summaries let you condense the resource material to draw out particular points, omit unrelated or unimportant points, and simplify how the author conveyed his or her message.

The OSU Writing Center has more on paraphrasing and summarizing, including an example of how to do one of each. While you're at this site, you'll notice other helpful information available about essays and term paper construction.

> **Activity: Quote, Paraphrase, or Summarize?**
>
> Open activity in a web browser.

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2.6.10.2: Helping Others Follow

Helping Others Follow

As you switch from component to component in your paper, you'll be making what are called rhetorical moves—taking subsequent steps to move your argument along and be persuasive. Your readers will probably know what you're doing because the components in everyday oral argument are the same as in written argument. But *why* you're switching between components of your argument, and with these particular sources, might be less clear.

Note:

The ideas and examples in this section are informed by all three editions of Gerald Graff and Cathy Birkenstein. They Say/I Say. The third edition of the They Say/I Say provides templates of actual language to be used in written arguments. This can be extremely helpful to beginning writers because it takes some of the mystery out of what to say and when to say it. For these templates, check the book out from your library.

You can help readers follow your argument by inserting phrases that signal why you're doing what you're doing. Here are some examples:

- **To state that what you're saying in your thesis (answer to your research question) is in opposition to what others have said:**

  "Many people have believed ..., but I have a different opinion."

- **To move from a reason to a summary of a research study that supports it (evidence).** "Now let's take a look at the supporting research."

- **To introduce a summary of a resource you've just mentioned.**
  "The point they make is..."
- **If the objection is that you're not being realistic.**
  "But am I being realistic?"
- **To acknowledge an objection you believe a reader could have.**
  "At this point I should turn to an objection some are likely to be raising..."
- **To move from the body of an essay to the conclusion.**
  "So in conclusion..."

Phrases like these can grease the skids of your argument in your readers' minds, making it a lot easier for them to quickly get it instead of getting stuck on figuring out why you're bringing something up at a particular point. You will have pulled them into an argument conversation.

Examples: The Language of Arguments

The blog that accompanies the book *They Say/I Say* with Readings, by Gerald Graff, Cathy Birkenstein, and Russel Durst, contains short, elegantly constructed contemporary arguments from a variety of publications. Take a look at the They Say/I Say blog for a moment and read part of at least one of the readings to see how it can be helpful to you the next time you have to make a written argument.

Additional Advice Sources

Take a look at these sites for argument essay advice for students:

- Developing Your Thesis – Dartmouth Institute for Writing & Rhetoric

- Handouts – Ohio State Center for the Study and Teaching of Writing
- Introductions, Body Paragraphs, and Conclusions for an Argument Paper – Purdue Online Writing Lab (OWL)
- Argument Handout – University of North Carolina Writing Center
- Rewriting: how to do things with texts – Utah State University Press (Project Muse affiliates only)

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- 8: Introduction to Viruses by Linda Bruslind has no license indicated.

2.6.10.3: Synthesis of Your Own Ideas

Synthesis of Your Own Ideas

Professors want to see evidence of your own thinking in your essays and papers. Even so, it will be your thoughts in reaction to your sources:

- What was the author really trying to say?
- What parts of them do you agree with?
- What parts of them do you disagree with?
- Did they leave anything out?
- What does an author's work lead you to say?

It's wise to not only analyze—take apart for study—the sources, but also to try to combine your own ideas with ideas you found in class and in the sources.

Professors frequently expect you to interpret, make inferences, and otherwise synthesize—bring ideas together to make something new or find a new way of looking at something old. (It might help to think of synthesis as the opposite of analysis.)

Activity: Creative Thinking

Synthesis is a creative act. Are there places, things, activities, or situations that you already use to spark your creativity? Sometimes even simple things can help us be more creative. Take a look at the article 5 Ways to Spark Your Creativity for some tips.

The book *Thinker Toys*, by Michael Michalko, can help you expand your ability to think creatively. The author's web page contains fun but challenging thinking exercises, including this one that lets you practice making associations between seemingly disparate concepts.

Getting Better at Synthesis

To get an A on essays and papers in many courses, such as literature and history, what you write in reaction to others' work should use synthesis to create new meaning or show a deeper understanding of what you learned.

To do so, it helps to look for connections and patterns. One way to synthesize when writing an argument essay, paper, or other project is to look for themes among your sources. So try categorizing ideas by topic rather than by resource—making associations across sources.

Synthesis can seem difficult, particularly if you are used to analyzing others' points but not used to making your own. Like most things, however, it gets easier as you get more experienced at it. So don't be hard on yourself if it seems difficult at first.

Example: Synthesis in an Argument



Imagine that you have to write an argument essay about Woody Allen's 2011 movie *Midnight in Paris*. Your topic is "nostalgia," and the movie is the only resource you can use.In the movie, a successful young screenwriter named Gil is visiting Paris with his girlfriend and her parents, who are more politically conservative than he is. Inexplicably, every midnight he time-travels back to the 1920's Paris, a time period he's always found fascinating, especially because of the writers and painters—Hemingway, Fitzgerald, Picasso—that he's now on a first-name basis with. Gil is enchanted and always wants to stay. But every morning, he's back in real time—feeling out of sync with his girlfriend and her parents.

You've tried to come up with a narrower topic, but so far nothing seems right. Suddenly, you start paying more attention to the girlfriend's parents' dialogue about politics, which amount to such phrases as "we have to go back to...," "it was a better time," "Americans used to be able to..." and "the way it used to be."

And then it clicks with you that the girlfriend's parents are like Gil—longing for a different time, whether real or imagined. That kind of idea generation is synthesis.

You decide to write your essay to answer the research question: How is the motivation of Gil's girlfriend's parents similar to Gil's? Your thesis becomes "Despite seeming to be not very much alike, Gil and the parents are similarly motivated, and Woody Allen meant *Midnight in Paris*'s message about nostalgia to be applied to all of them."

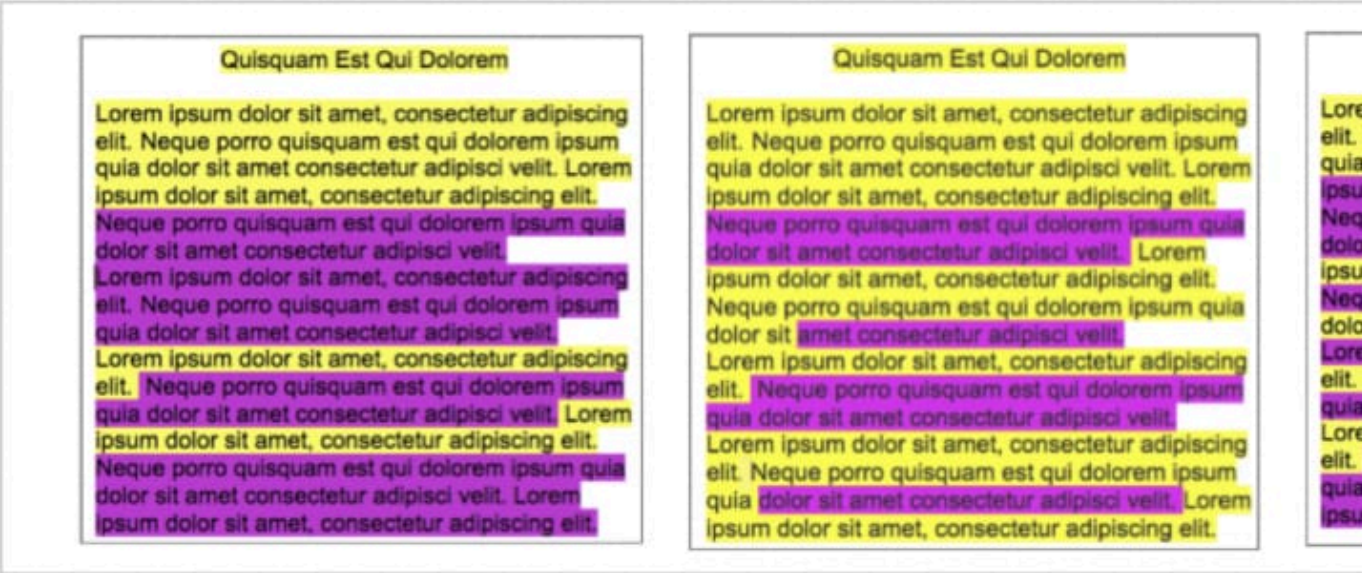
Of course, you'll have to try to convince your readers that your thesis is valid and you may or not be successful—but that's true with all theses. And your professor will be glad to see the synthesis.

There is a lot more you can learn about creating synthesis in scholarly writing. One place synthesis is usually required is in literature reviews for honors' theses, master's theses, and Ph.D. dissertations. In all those cases, literature reviews are intended to contribute more than annotated bibliographies do and to be arguments for the research conducted for the theses or dissertations. If you are writing an honors thesis, master's thesis, or Ph.D. dissertation, you will find more help with Susan Imel's Writing a Literature Review.

Activity: Balancing Sources and Synthesis

Here's a technique to quickly assess whether there is enough of your original thought in your essay or paper, as opposed to information from your sources: Highlight what you have included as quotes, paraphrases, and summaries from your sources. Next, highlight in another color what you have written yourself. Then take a look at the pages and decide whether there is enough you in them.

For the mocked-up pages below, assume that the yellow-highlighted lines were written by the writer and the pink-highlighted lines are quotes, paraphrases, and summaries she pulled from her sources. Which page most demonstrates the writer's own ideas? See the bottom of the page for the answer.



Mocked-up passages showing the division between quotes, paraphrases, and summaries and original ideas

Source: Joy McGregor. “A Visual Approach: Teaching Synthesis,” School Library Monthly, Volume XXVII, Number 8/May-June 2011.

Answer to Activity: Balancing Sources and Synthesis

The answer to the “Balancing Sources and Synthesis” Activity above is:

The Middle Sample.

The yellow-highlighted sections in The Middle Sample show more contributions from the author than from quotes, paraphrases, and summaries of other sources.

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2.6.11: COPYRIGHT BASICS

2.6.11.1: What Is Copyright?

2.6.11.2: What Copyright Covers

2.6.11.3: Rights Granted by Copyright

2.6.11.4: Respecting Copyright

2.6.11.5: Exceptions to Copyright

2.6.11.6: Creative Commons - An Alternative

2.6.11.7: Public Domain and Term of Copyright

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2.6.11.1: What Is Copyright?

What Is Copyright?



Copyright gives creators an incentive to produce and share new works by granting them exclusive rights to their work for a limited time.

Copyright is the law. While digital technology has made some aspects of copyright more complex, knowing the basics can help you to use material legally and to protect your own creative works.

You create copyrighted works regularly. When you write an original email or paper, record a song or video, or take a photograph you have created a work that is protected by copyright. It is important to know how to manage your rights as a creator.

Every day you work with copyrighted materials created by other people. Whenever you read a book, download a song, stream a video or play a video game, you are potentially dealing with copyrighted materials. It is important to understand what is and is not covered by copyright law and the ways you may use these works under the law.

Copyright Law

U.S. Copyright Law has its origin in the U.S. Constitution:

The Congress shall have the power ... to promote the Progress of Science and useful Arts, by securing for limited Times to Authors and Inventors the exclusive Right to their respective Writings and Discoveries.

– U.S. Constitution Article 1, Section 8



The U.S. Congress has the power to create laws that govern copyright. (Image Source: National Archives and Records Administration. Public Domain.)

The purpose of copyright is to encourage the creation and sharing of creative works. Copyright gives creators an incentive to produce and share new works by granting them exclusive rights to their work for a limited time. This provides an opportunity for a creator to benefit from his or her work.

Congress determines the limits of this monopoly, including the length of time that copyright coverage lasts. These limits can (and have) changed over time.

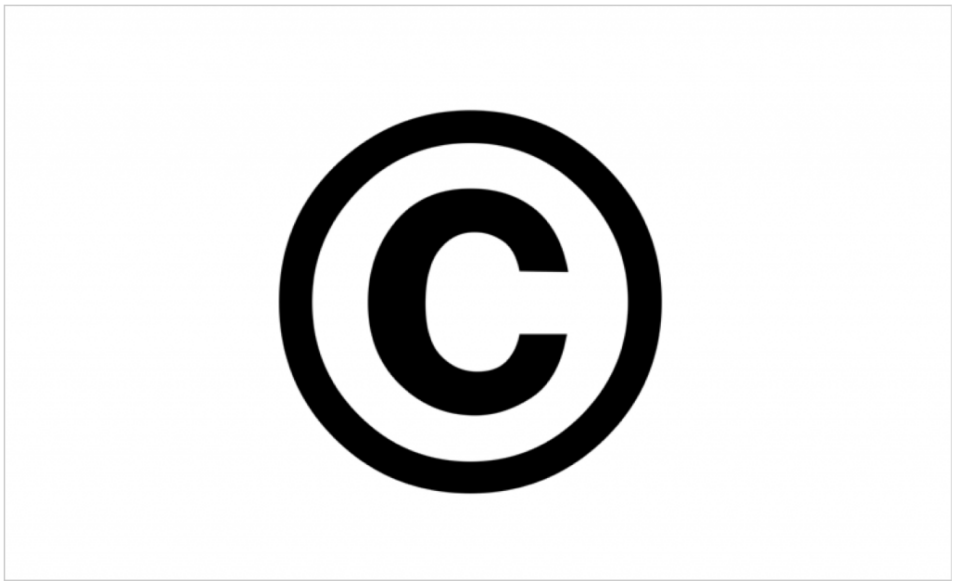
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- 10: Environmental Factors by Linda Bruslind has no license indicated.

2.6.11.2: What Copyright Covers

What Copyright Covers

The kinds of works covered by copyright are listed in Section 102 of the Copyright Act. In order for a work to be covered by copyright, it must be an original work of authorship fixed in a tangible medium of expression. (See the detailed explanations below.)



Copyright covers original work that is fixed in a tangible medium of expression.

There are several types of works that can be protected, including:

- Literary works
- Musical works, including any accompanying words
- Dramatic works, including any accompanying music
- Pantomimes and choreography
- Pictorial, graphic, and sculptural works
- Motion pictures and other audiovisual works
- Sound recordings
- Architectural works

In addition to these, new formats such as email, software, video games, and digital works including web pages and online images have all been determined to have copyright protection.

Definition: Original Work of Authorship

In copyright law, originality means that a work is independently created and possesses at least a minimum amount of creativity. For example, an alphabetized list of names and phone numbers would not receive copyright protection because it required no creativity to produce.

Definition: Tangible Medium of Expression

For a work to be "fixed in a tangible medium," it must exist in some perceptible format for more than a transitory duration. For example, a work that is fixed in a tangible medium could be written on paper, saved to a computer hard drive, or recorded on film. An improvised jazz performance that is not recorded would not have copyright protection, because the creative expression of the musician has not been saved in any tangible format.

What ISN'T Covered by Copyright?

Not all works are covered by copyright. Those not covered include:

Works already in the public domain (discussed in detail later in this book)

- *Moby Dick*
- Shakespeare's plays
- Beethoven's works

Works not fixed in a tangible medium

- A song in your head, but not recorded or written down

Ideas

- Boy meets girl, they fall in love and live happily ever after
- Hero protagonist saves the world with the help of wacky sidekick

Facts

- 1+1=2
- George IV died in 1830
- Copenhagen is the capital of Denmark

Works of the U.S. government produced by government employees

- Federal government reports
- Acts/Bills of Congress
- www.whitehouse.gov

Copyright in Cases of a Work Made for Hire

If you create something as part of your job duties, it is likely a work made for hire. In these cases, the employer is considered the author and rights holder of a work made for hire rather than the employee.

Read the United States Copyright Office's Works Made for Hire circular for a more nuanced discussion.

Activity: Copyrightable?

Open activity in a web browser.

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2.6.11.3: Rights Granted by Copyright

Rights Granted by Copyright

So, now that you know what kinds of works are covered by copyright, what exactly are the rights granted to a copyright holder?

Six exclusive rights are granted to the creator of a copyrighted work. We call these the Author's Bundle of Rights. This means the copyright holder is the only person who has the right to do these things and has the authority to grant permission for others to do these things, with some important exceptions that we will discuss later in this chapter.

If you are not the copyright holder and want to do any of the examples, you may need to get permission to do so from the holder of the copyright.

Author's Bundle of Rights

To Reproduce

- Example: Making physical and digital copies.

To Prepare Derivative Works

- Example: Creating foreign language translations, movie adaptation of a book, etc.

To Distribute

- Example: Sharing over Peer-to-Peer networks or posting online, as well as distributing physical copies.

To Perform Publicly

- Example: Performing a play, showing a movie, or reading aloud from a book to an audience outside of your normal circle of family or friends.
- Example: Playing recorded music in clubs, restaurants, stores, on the radio, etc.

To Display Publicly

- Example: Displaying in a gallery, putting posters on a noticeboard, etc.

To Perform Publicly a sound recording by means of a digital audio transmission

- Example: Streaming recording music online.

Activity: Author Rights

Open activity in a web browser.

When Does Copyright Apply?

Under current U.S. law, copyright applies as soon as an original work is fixed in a tangible medium of expression. This means that

when you save a file, take a photograph, record a song, or paint a picture your work has copyright protection.

As the creator, provided that the work is not a work made for hire, you are the owner of the copyright on your work. You do not have to register the work with the U.S. Copyright Office, publish it, or put a copyright notice on it.

If you wish to give away, sell or license any or all of the copyright on your work, you have the right to do so.

If you give away or sell your exclusive copyright to someone else, you no longer have the rights mentioned above and need to treat the work the same as any other copyrighted work created by someone else.

See Public Domain and Term of Copyright later in this section for details about the duration of copyright.

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- 12: Energetics & Redox Reactions by Linda Bruslind has no license indicated.

2.6.11.4: Respecting Copyright

Respecting Copyright

While working with other people's copyrighted works, remember that their works are under copyright protection from the moment of creation.

Additionally, U.S. Copyright Law applies to works found on the Internet. Many of the works you find online are protected by copyright, even if there is no copyright notice. Your availability to access copyrighted materials on the Internet does not necessarily mean that you have the right to use, reuse and distribute the works in any manner you wish. It is important to respect copyright, whether the works are in a physical or digital format.

Risks of Infringing Copyright

If you violate one or more of the exclusive rights of a copyright owner, the copyright owner can bring a claim against you for copyright infringement. There are a few different penalties that are possible if you are accused of copyright infringement:

- Under specific circumstances, U.S. copyright law allows criminal prosecution in cases of willful infringement.
- If the infringing work is online, such as a video posted to YouTube, the copyright owner can request the material be taken down. This may be done through a Cease-and-Desist Letter or DMCA Takedown Notice. The material will be taken down and you will be notified of the accusation of infringement. If you believe that your use of the material is legal, you can respond with your explanation of why. Some Internet Service Providers will cut off your access if you receive too many takedown notices.
- The copyright owner can sue you. They could ask for an injunction to stop your use of their work. They can also ask for either actual damages or statutory damages. Actual damages are the actual amount of money the copyright owner lost due to your activity plus any profit you made from using the work. These can be hard to determine, so the law alternatively allows for statutory damages under certain conditions. These are a set range, from $750 to $30,000 per infringed work, that the judge or jury awards to the rights holder if you are found guilty. These damages can increase to $150,000 per infringed work if your use is determined to be a "willful" infringement.
- Some rights holders may offer the option of settling out of court. This agreed settlement may be cheaper than the cost of a trial for the rights holder and you.

The accusation of infringement is not the same as a conviction. You always have the right to defend your use.

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2.6.11.5: Exceptions to Copyright

Exceptions to Copyright

U.S. Copyright Law includes exceptions that limit the rights of the copyright holder. These exceptions allow for certain uses of copyrighted material without seeking permission. Congress created these exceptions in order to balance the rights of creators and users and to enable some socially beneficial uses of copyrighted works.

Some of these exceptions are explained below.

Fair Use

Fair Use (Sec. 107) allows for various uses of copyrighted works. This is the most flexible of the exceptions in the copyright law and can apply in a wide variety of situations.

To learn more check out our section on Fair Use.

Reproduction for Libraries

Section 108 of the Copyright Act allows libraries and archives to make copies of copyrighted works under very specific conditions. For example, a patron can ask the library to make a copy of a journal article or portion of a book in the library's collection as long as it is for the patron's personal study.

First Sale Doctrine

The first sale doctrine (Sec. 109) allows you to distribute a legally acquired physical copy of a copyrighted work. This allows libraries to lend books and individuals to lend or sell used books, movies or CDs.

Classroom Display or Performance

Under Section 110(1) it is okay to display or perform copyrighted works in a face-to-face classroom setting at a non-profit educational institution. This allows a teacher to show a video or students to create and display multimedia projects in class. Section 110(2) allows for the display or performance of copyrighted works for distance learning (e.g. on a course management system), but you must fulfill many specific requirements in order to qualify for this exception.

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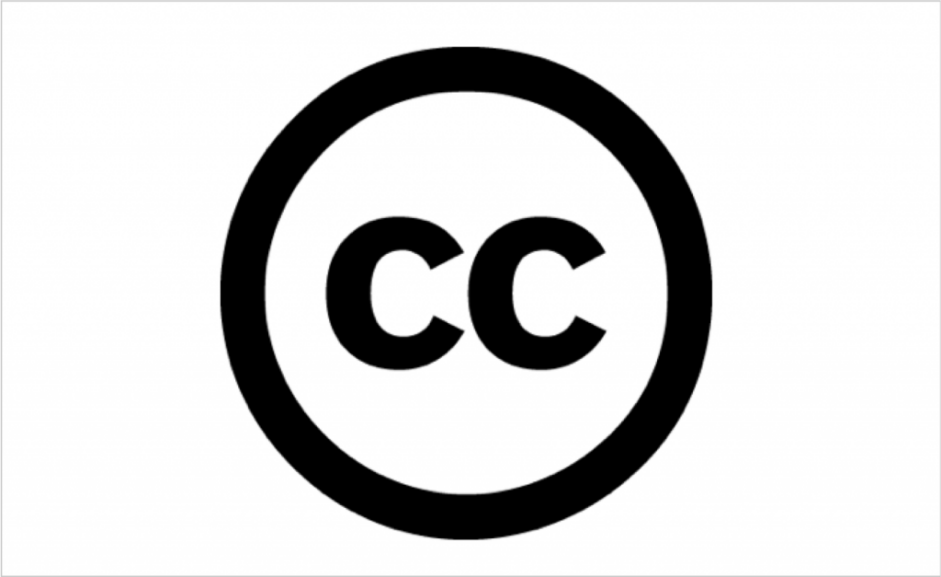
- 14: Chemolithotrophy & Nitrogen Metabolism by Linda Bruslind is licensed CC BY-NC-SA 4.0.

2.6.11.6: Creative Commons - An Alternative

Creative Commons - An Alternative

The internet has made the creation and sharing of creative works much easier than it has ever been. Most of these new works are protected by copyright as soon as they are created. But not everyone wants to lock up their creativity behind the protection of copyright. Many people want their work to be freely shared and even built upon.

Creative Commons (CC) was developed out of the desire to make it easier to share and use copyrighted works. Creative Commons allows a creator to grant licenses to their work that could include the ability to share, adapt and/or use material for commercial purposes without having to ask for permission. The creators still own the copyright, but they proactively decide to let others use their works under certain conditions.



Creative Commons allows a creator to grant licenses to their work without requiring they grant individual permission.

Movie: Get Creative

The origin and adventures of the creative commons licensing project.

An interactive or media element has been excluded from this version of the text. You can view it online here: https://ohiostate.pressbooks.pub/choosingsources/?p=604

View video

Activity: Finding Creative Commons Works

Many websites include CC licensed works. You can search them to find materials that you can freely use in creating your own work provided that you comply with the terms of the license. You can also upload your own CC licensed works to share with others.

Examples include:

- Flickr
- YouTube
- The Noun Project
- Wikipedia's Wikimedia Commons

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- 16: Taxonomy & Evolution by Linda Bruslind has no license indicated.

2.6.11.7: Public Domain and Term of Copyright

Public Domain and Term of Copyright

Copyright protection of a work doesn't last forever. Once the copyright term ends for a work, it enters the public domain. This means that no one owns the rights to the work anymore, so the work may be used by anyone, for any purpose, without permission. The public domain includes works where copyright has expired and works that were never protected by copyright in the first place (such as works of the U.S. federal government created by federal employees).



The public domain includes works where copyright has expired and works that were never protected by copyright.

Activity: Finding Works in the Public Domain

The public domain provides a great source of materials that you can use for any purpose, without requesting permission or paying a fee. The internet is full of useful sites that can help you find Public Domain materials, including:

- Columbia University list of Public Domain Sources
- HathiTrust
- Internet Archive
- Project Gutenberg

When Does a Work Enter The Public Domain?

Due to U.S. participation in international treaties and changes to U.S. copyright law, Congress has placed a limitation on the length of copyright so that works can eventually become part of the public domain and be re-used and built upon by others. Over the years the term of copyright has changed significantly.

The current term is:

- 70 years after death of author. If there are multiple authors, then it is 70 years after the death of the last author.
- If corporate, or anonymous, authorship the term is either 95 years from date of first publication, or 120 years from the date of creation, whichever comes first.

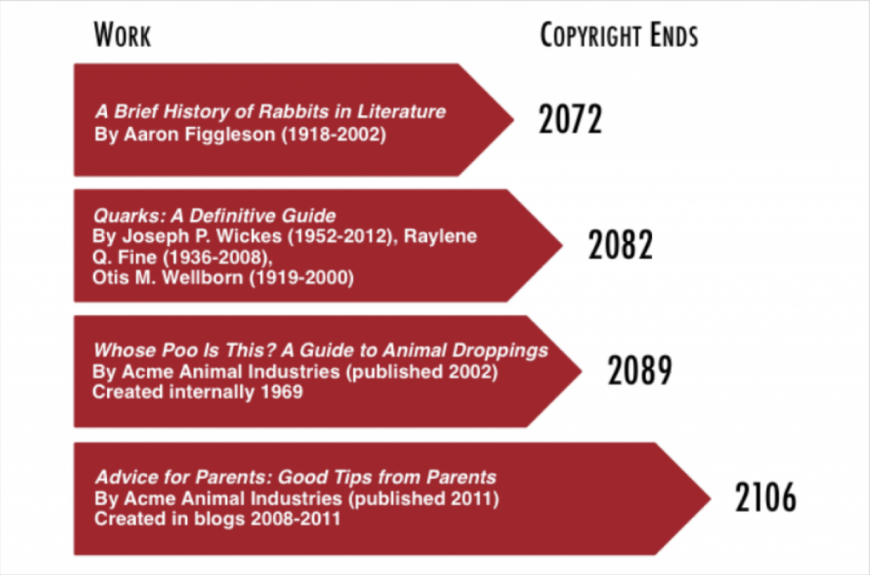
Term of Copyright

Since the duration of copyright has changed throughout the years, it can be difficult to determine when copyright expires for a particular work. Below are links to a couple of online sources to help you determine when a particular work enters the public domain.

- Copyright Term and the Public Domain in the United States
- ALA Copyright Genie

Examples: Copyright Duration

The duration of copyright depends on when the work was created and whether it was the work of a single author, multiple authors, or an anonymous or corporate author.



Copyright terms are based on factors such as the date of death of the author and on what laws were in effect when a work was created.

- *A Brief History of Rabbits in Literature* by Aaron Figgleson (1918-2002):
  Copyright ends 2072 – 70 years after the author's death
- *Quarks: A Definitive Guide* by Joseph P. Wickes (1952-2012, Raylene O. Fine (1936-2008), and Otis M. Wellborn (1919-2000):
  Copyright ends 2082 – 70 years after the death of the last author to die
- *Whose Poo Is This? A Guide to Animal Droppings* by Acme Animal Industries (published 2002) – Created internally 1969:
  Copyright ends 2089 – 120 years from the date of creation
- *Advice for Parents: Good Tips from Parents by* Acme Animal Industries (published 2011) – Created in blogs 2008-2011:
  Copyright ends 2106 – 95 years from the date of first publication

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2.6.12: FAIR USE

2.6.12.1: What Is Fair Use?

2.6.12.2: The Four Factors

2.6.12.3: Evaluating Your Case for Fair Use

2.6.12.4: Common Examples of Fair Use

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2.6.12.1: What Is Fair Use?

What Is Fair Use?



Fair Use is an exception to U.S. copyright law that allows use of copyrighted work under certain conditions.

Are you incorporating any materials in your research final product that were created by someone else, such as images or text from other works? These materials could be protected by copyright. For example, content you find online, text, books, movies, songs, email, images, and videos are most likely copyrighted. Fortunately, U.S. copyright law includes an exception that allows you to use copyrighted work in your assignments for class.

However, if you would like to share your research product outside of the classroom (such as on a webpage or blog or in your portfolio), you will need permission from the copyright owner(s) unless your use is covered under another statutory exception. Fair use is one such exception, and it can apply to a wide variety of uses.

Note: Fair Use and Educational Use

Fair Use plays an important role in education. Although educational use receives several protections in copyright law, not all educational use is automatically fair use. It's important to know that there are limits to how you can use others' creative works even as a student or teacher in the classroom.

In this section, you will learn about fair use and strategies to help determine whether or not a proposed use of someone else's copyrighted works falls under the fair use exception. Understanding how to properly perform a fair use analysis and assert your fair use rights can help you to build upon others' works with confidence.

Fair Use and Copyright – A Balance

Copyright in the U.S. is intended to promote the creation of new works by providing an incentive for creators. However, recognizing that new works often build on or incorporate existing works, the law strikes a balance between the rights of creators and the rights of users via exceptions to the exclusive rights of the creator.

The fair use exception is detailed in Section 107 of the U.S. Copyright Act. Unlike other copyright exceptions, fair use is flexible

and can apply to a broad array of uses. It is designed to be adaptable to new uses and technologies so that Congress doesn't have to create new exceptions before a new technology can be utilized.

Movie: What Is Fair Use?

Watch a short introduction to fair use from the OSU Libraries' Copyright Resources Center.

An interactive or media element has been excluded from this version of the text. You can view it online here: https://ohiostate.pressbooks.pub/choosingsources/?p=1052

View video

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- 18: Genetic Engineering by Linda Bruslind is licensed CC BY-NC-SA 4.0.

2.6.12.2: The Four Factors

The Four Factors

Most of the copyright exceptions are very specific about what kinds of uses may qualify for the exception and often include various restrictions about who can use the exception and under what precise conditions.

Fair use, on the other hand, is much more flexible and can apply to a wide variety of uses. Instead of specifying an exact type of user, type of material or amount that qualifies for this exception, the fair use statute provides a framework for the analysis and application of four factors that determine whether or not a particular use may qualify as fair use.

The four factors of fair use are:

- Purpose & character of use, including whether commercial (i.e. publishing a book) or non-commercial (i.e. using in a classroom assignment)
- Nature of the original material (i.e., is the work published or unpublished? Fact or fiction? Highly creative?)
- Amount and substantiality of the original work (are you using the entire work or just a portion?)
- Effect on the marketplace or on the work's value (will your use have a financial impact on the creator?)

When considering whether a proposed use of a copyrighted work may qualify as fair use, you must weigh all four factors together. Each factor is equally important.

Transformation

The courts have recently emphasized the concept of transformation or a transformative purpose, which falls under the first factor of fair use.

Transformation means that the way in which the work is being used is significantly different than the original use for which it was created.

In many cases a transformative use of a copyrighted work will strongly favor a determination of fair use.

There are two ways in which a use can be transformative.

First, you could actually make changes to the original work in order to use it for a new purpose. An example would be to take short clips of popular movies and remix them to create a video for the purpose of social commentary or teaching.

The second form of transformative use does not require that you alter the original work in any way. Instead, you simply use the work for a purpose that is significantly different than the use for which it was created. An example of this would be using clips from a blockbuster movie that was originally sold for mass market entertainment for the purpose of teaching and research.

> **Movie: Remix Culture**
>
> See examples of remixing that fall under fair use.
>
> An interactive or media element has been excluded from this version of the text. You can view it online here: https://ohiostate.pressbooks.pub/choosingsources/?p=633
>
> View video

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2.6.12.3: Evaluating Your Case for Fair Use

Evaluating Your Case for Fair Use

Copyright law lacks specificity, so it can be difficult to determine whether or not a particular use may qualify as fair use. Fortunately, there are a number of useful tools available online to help you consider the four fair use factors as they apply to your intended use.

A Fair Use Checklist can be very helpful for conducting a fair use analysis. The checklist indicates various criteria for each factor which have been found in a court of law to favor or oppose a finding of fair use. It is highly recommended that you use a fair use checklist to evaluate the strength of your argument for fair use.

Movie: Follow the Four Factors of Fair Use

Watch this video to see a fair use analysis using a fair use checklist.

An interactive or media element has been excluded from this version of the text. You can view it online here: https://ohiostate.pressbooks.pub/choosingsources/?p=635

View video

In a fair use analysis, you consider each of the four factors in light of your proposed use and determine whether your use is favoring or opposing fair use for that factor.

You then weigh all four factors together. You cannot rely on a numerical tallying of criteria in favor and opposing fair use in order to make a determination. You must consider all four factors holistically and determine if, taken as a whole, they favor or oppose fair use, and to what extent (e.g. strongly favoring fair use, slightly favoring, etc.).

- If, overall, your use favors fair use, then you may proceed.
- If your use instead opposes fair use, you should reassess your use and determine if you can make any changes that could strengthen your case for fair use.

There are other tools in addition to the checklist that can help you conduct a fair use analysis. The American Library Association has developed a tool called the Fair Use Evaluator.

Activity: Fair Use Criteria

Visit the Fair Use Checklist and review the criteria for each of the four factors.

Activity: Fair Use or Not Fair Use?

Open activity in a web browser.

Tips for Best Practice

While it is important to perform a fair use evaluation for each and every use of copyrighted material, there are some general rules that can often help you to strengthen a fair use claim.

Below are a few tips to consider when relying on the fair use exception in order to use copyrighted works in your endeavors.

- **Use only lawfully acquired copyrighted works** – To be able to claim fair use you must have used a legal copy of the

original work.
- **Acknowledge all of your sources with a bibliographic citation** – Giving proper credit to the original creator demonstrates good faith and may help strengthen your fair use case.
- **Use only the amount of the original work that you need to accomplish your goal** – Since the amount of the original work that is used is one of the fair use factors, it is always important to only use what you need and not add extra material.
- **Restrict the audience and/or make only the number of copies that you need** – The less you copy and share the parts of the original work, the less effect you have on the market for it.
- **Use Creative Commons licensed or public domain works** – If you use works that expressly allow you to use them or have no copyright protection, you do not need to rely on fair use and can be more confident that your use is legal.
- **Use works that you created** – If you created it, you own the copyright, with the exception of works made for hire. (When you create things for your job, typically your employer owns the copyright.)

If you are in doubt about your fair use claim, either reassess and make changes to your proposed use in order to make a stronger claim or ask for permission to use the copyrighted material – It is much easier to make changes or ask for permission before you use copyrighted material than to get hit with an infringement claim and have to make changes or face a law suit after your use.

Further Reading on Fair Use

A number of groups have developed Codes of Best Practices in Fair Use for different types of activities. These codes propose examples of fair use within specific communities of practice. Below are links to some of these Codes of Best Practices.

- Code of Best Practices in Fair Use for Online Video
- Code of Best Practices in Fair Use for Poetry
- Code of Best Practices in Fair Use for OpenCourseWare
- Documentary Filmmakers' Statement of Best Practices in Fair Use
- Association of Research Libraries Codes of Best Practices in Fair Use for Academic and Research Libraries

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2.6.12.4: Common Examples of Fair Use

Common Examples of Fair Use

Students and teachers rely on fair use in order to accomplish many of their educational goals. Below are some, but by no means all, educational activities that rely upon fair use.

Student Projects

Includes both media and text.

Your fair use analysis will change depending on how the project is presented, i.e. only the professor sees it, you present it to the whole class, you present it to a group outside of the class, or you post it online for anyone to see.

Course Reserves

Includes electronic reserves.

Instructors may copy or post small portions of books or journals for supplementary student readings, but cannot copy entire copyrighted works as a replacement for materials that students would normally be required to purchase.

Sound or Video Clips for Teaching

Students and teachers can make use of video or sound clips in creating multi-media presentations for use in the classroom.

Digitization Projects

Many university libraries rely on fair use in order to create large scale digitization projects that preserve older materials, as well as providing improved access to their collections for the purpose of research. For an example of this type of digitization project check out the HathiTrust Digital Library.

Content in Scholarly Articles

It is common to quote other researchers' writings or use others' images, graphs or charts in your own scholarly writing. These practices have long been considered acceptable under fair use.

Access for the Disabled

When specific exemptions don't fit.

While there are specific exceptions that allow for making copies of copyrighted works in order to provide access to the visually handicapped, they are sometimes too narrow to provide complete access. In these cases it is possible to rely upon fair use in order to provide access to materials.

Fair Use for Non-Educational Purposes

Fair use is not only available for educational purposes. Many other commercial and non-commercial activities depend upon fair use. Some of these common fair uses include:

- Quotes in books, news reports and blogs
- Mash-ups and remixes
- Parody, such as on television shows like South Park or Saturday Night Live
- Video or sound clips in documentary films
- Thumbnail images on search engines

Movie: Sesame Street: Gone With the Wind

Check out this parody from Sesame Street.

An interactive or media element has been excluded from this version of the text. You can view it online here: https://ohiostate.pressbooks.pub/choosingsources/?p=637

View video

Myths about Fair Use

Many people have heard of fair use and have some ideas about what it is. Unfortunately, there are many myths or misunderstandings about exactly what fair use covers, what the law states or how it can be applied. Below we dispel just a few of the most common myths about fair use.

Myth 1: All educational use is fair use.

Fact: While many educational uses are considered fair use, there are some activities that do not meet the fair use criteria. For example, a teacher can't make copies of an entire text book so that students don't have to buy it.

Myth 2: Every educational use is transformative.

Fact: Using copyrighted works for teaching can often be a transformative use, but not always. For example, using a text book created to teach Biology 101 to teach Biology 101 is not transformative.

Myth 3: All socially beneficial use is fair use.

Fact: Fair use is designed to help balance the rights of the creator and the social benefit of using copyrighted works in certain ways. Not all uses of copyrighted works that would be socially beneficial, however, qualify as fair use. For example, scanning and posting an entire medical text book online for anyone to access for free is socially beneficial but probably not fair use.

Myth 4: All commercial use precludes fair use.

Fact: Many commercial activities, such as newspapers and online news sites, rely heavily on fair use.

Myth 5: It is not possible to have a fair use when a permissions scheme exists for a work.

Fact: Just because rights holders are willing to charge you to use their copyrighted material, does not mean that fair use cannot apply. For example, the Associated Press created a licensing scheme to quote from AP stories but quoting from news stories has long been considered fair use.

Myth 6: Fair use specifies a percentage or amount of a work that is okay to use.

Fact: The law does not state that using 10% of a book or 30 seconds of a song or video clip is fair use. You can often use more than these arbitrary limits, while sometimes using even less might not be fair use. The amount of the original work used is only one of the four factors to consider.

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2.6.13: ROLES OF RESEARCH SOURCES

2.6.13.1: Thinking About Roles of Sources
2.6.13.2: BEAM- A Solution That Might Shine
2.6.13.3: Using BEAM- An Example
2.6.13.4: Practice with BEAM

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2.6.13.1: Thinking About Roles of Sources

Thinking About Roles of Sources



Knowing how to use your “players” effectively improves the outcome.
(Image source: Scott Stuart)

Does this nightmare sound like how you feel every time you have to write a term paper?

> *Your team is playing in the big game and you’re the coach. (Maybe the real coach missed the plane. Who knows–it’s a nightmare!) The stakes are high. You know your players are good athletes—you have access to the best and plenty of them. But you don’t really know good strategies of the game, so you don’t quite know how to use your players. For instance, is it better to keep your quarterback fresh by substituting often? Your kicker is not as bulky as your tackles. Is that typical of good kickers or should you find somebody else? And what about your linemen—can they tackle as well as block?*

What makes this a nightmare is *not knowing* how to use your players in a high-pressure game. Unfortunately, that situation is similar to writing a term paper if all you know are directions like these:

> Your paper must be in 12 pt. font, Times New Roman, double spaced with no more than 1″ margins, and include a minimum of 8 total articles comprised of:
>
> - At least 2 peer-review articles
> - 3 (no more than 6) popular articles (magazine or newspaper)
> - 2 (no more than 4) electronic sources (website or blog)

So you know you need sources. But directions like those aren’t much help with what to actually ***do*** with the sources in your paper. Even with credible sources, it’s very difficult to write a persuasive paper until you learn the roles that sources play—how you can use them—within your paper.

But who said anything about a ***persuasive*** paper? Perhaps one of the things you don’t know is that with most term papers and essays, the ***unstated expectation*** is that you will use your sources to make an argument. That’s because most scholarly writing makes an argument. You will be arguing that your answer to your research question (your thesis) is correct, or at least reasonable.

Obviously, it’s high time someone helped you learn all this!

For both professionals and student researchers, successful scholarly writing uses sources to fill various roles within the term paper, journal article, book, poster, essay, or other assignment.

Those roles all have to do with rhetoric—the art of making a convincing argument. Putting your sources to work for you in these roles can help you write in a more powerful, persuasive way—to, in fact, win your argument.

TIP:

For another way to think about choosing the right sources for your needs, see Sources and Information Needs.

NOTE:

This section on rhetorical roles of research sources was influenced by many sources. See the bibliography.

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2.6.13.2: BEAM- A Solution That Might Shine

BEAM: A Solution That Might Shine

This table, created from the ideas developed by Joseph Bizup, describes the roles that sources can play (some of the ways they can be used) in your finished assignment, such as a term paper. Bizup called his model BEAM, an acronym that stands for background, exhibits (or evidence), argument, and method.

Role for Sources	How to Use Them	Kinds of Sources That Can Have That Role*
Background**	Writers rely on these sources for general factual information. For instance, a writer could use background information to introduce a setting, situation, or problem in the term paper.	Usually secondary sources and tertiary sources, but, basically, just anything other than journal articles that report original research. Some examples: literature review articles, non-fiction books, and biographies (secondary) and field guides and Wikipedia (tertiary).
Exhibits or Evidence	Writers interpret and analyze sources like these in the same way they are used as exhibits and evidence in a museum or a court.	Usually primary sources. Some examples: newspaper articles from the time in question, works of literature or art, and research articles.
Argument	Writers engage with these sources that they agree with or disagree with. The sources are usually written by scholars in their field. For instance, writers often include sources that describe earlier work that is specifically relevant to their own research question and their thesis (what they consider to be the answer to that question.)	Usually primary and secondary sources. Some examples of primary sources: research articles in the sciences and humanities and recordings of performances in the arts. Some examples of secondary sources: commentaries and criticisms, such as those that appear in literature reviews, textbooks, and blogs that comment on research.
Method or Theory	Writers follow the key terms, concepts, or manner of working that are explained in these sources. That is, they pay attention to and use the relevant work of others before them to carry out their own work and then describe it in the term paper.	Often secondary sources. Some examples: literature reviews, textbooks, and blogs that comment on research.

*See Primary, Secondary, and Tertiary Sources

**See Background Reading

TIP: BEAM at a Glance

Download this BEAM Reference Chart to help you quickly determine how you might find or use a source.

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2.6.13.3: Using BEAM- An Example

Using BEAM: An Example

Using sources to function in these roles is how you enter into the scholarly conversation with all the other research and writing that has covered your topic before.

In the next few pages, you'll learn more about each role by analyzing how sources are used in the *pop culture* essay cited in the Example below. Seeing how the essay's author puts his sources to work in their various roles should help you envision how you can do the same in your own papers. The essay discusses how pop culture affects American (and global) values.

Example: Manufacturing Taste

Click on the citation below to skim through the essay. When you are finished, come back to this page and begin the next section on background sources.

Booker, M. Keith. "Manufacturing Taste: The Culture Industry, Children's Culture, and the Globalization of American Values." Pop Culture Universe: Icons, Idols, Ideas. ABC-CLIO, 2012. Web. 19 June 2012.

BEAM: Background Sources

These are sources that should be noncontroversial—the author accepts information from these sources as being authoritative (and expects readers to, as well). In other words, the sources (and the information gleaned from them) are generally trusted or undisputed. That information can serve as the incontestable foundation for your claims.

Background information is common knowledge (e.g. the sky is blue) and not necessary to be cited.

It's recommended that you cite background sources when you're unsure, but one rule of thumb suggests finding the same undocumented information in at least 5 other credible sources. It can be difficult to make this determination, so it's always a good idea to consult your professor.

Let's look at a statement in the first paragraph of the pop culture essay:

Thus, the corporate giants of the American Culture Industry (themselves now mostly multinational conglomerates) clearly must pay attention to the demands of audiences around the world in formulating, producing, and promoting the specific films, television, music, and other artifacts that are the stuff of popular culture.

How do you know that the "corporate giants are mostly multinational conglomerates" as stated in the first sentence? Or that the items listed are indeed the stuff of popular culture? These are examples of common knowledge.

Looking a little deeper...

Without context, this paragraph could also be the conclusion of a paper about what corporations should do (demonstrating the ongoing nature of knowledge itself). But the paper is not about making recommendations to the American Cultural Industry. This is an assertion that the author uses to help set up his different argument and is meant to be taken at face-value. So it's an example of how the same source can play different roles in different written assignments—all depending on how writers use them.

There is more about background sources at Getting Background Reading.

Activity: Background Sources

Which of the following would be the best example of a background source that doesn't need to be cited, according to the **BEAM** framework?

There were a total of 39 delegates who signed the U.S. Constitution; William Jackson was the 40th, but served as secretary and did not represent a state.

Thought to be limited to bat populations, the fungi responsible for the fast-spreading disease known as White-nose syndrome has been linked with similar infections affecting amphibians.

Having published over 300 reports since 2000, the Pew Internet & American Life Project has been a trusted source for research into online behavior."

Our Answer: There were a total of 39 delegates who signed the U.S. Constitution; William Jackson was the 40th, but served as secretary and did not represent a state.

BEAM: Exhibit and Evidence Sources

Generally, exhibit and evidence sources are works of literature (or other media), collected data, or some observed phenomenon, etc. that you have been asked to write about. They are what you analyze or interpret.

Looking again at the pop culture essay, the exhibits being examined are pop culture and American (as well as global) values. Specifically, the essay is examining the relationship between the two:

On the other hand, the international success of Toy Story 3, a film that deals with anthropomorphized toys and is thus essentially a consumerist fantasy of commodities come to life, also suggests that global distribution of the products of the American Culture Industry is beginning to have an impact on the tastes and values of audiences even outside the United States.

Exhibit sources are not limited to examples in the humanities; they could also be data that was collected in a scientific experiment or by a website's user survey. They can also simply serve as examples that help support a claim.

BEAM: Argument Sources

Argument sources provide you with the other voices in the academic conversation about your topic. Who else has done similar research, and how should your paper respond to what they've said? Does your paper refine or extend an existing hypothesis someone else has tested? If so, those sources belong in your paper.

Sometimes the purpose of including an argument source is to disagree with it and definitively indicate a different direction.

From our pop culture essay example:

Althusser's work remains compelling, despite the fact that theorists such as Michel de Certeau and John Fiske have argued that individuals actually have a considerable ability to resist and oppose the messages conveyed to them by official ideology, in popular culture and elsewhere.

The author is taking part and taking a stand in the ongoing scholarly discussion of culture, although this endorsement of Althusser's work could possibly be considered a method source if the argument in the article went in a different direction.

Activity: Argument Sources

Which of the follow best defines an argument source in the BEAM framework?

- It's one piece of research or scholarship that your paper is directly responding to.
- It's one of many *voices* in a larger conversation that your research paper participates in.
- It's one of several articles whose authors disagree with the premises of your paper.

Our Answer: It's one of many *voices* in a larger conversation that your research paper participates in.

BEAM: Method Sources

While argument sources help you frame your paper within the larger scholarly discussion about your topic and exhibits provide a focal point, method sources help provide underlying and sometimes implicit assumptions for your argument or analysis.

For some research, these are literally the methods you use to collect data like a focus group or a particular statistical analysis, and they provide justification for them. In other research, your paper might reveal a leaning toward a major attitude or school of thought within a discipline.

As a persuasive piece of writing, the essay has this intrinsic thread of caution and warning that is summed up in its conclusion:

"The children's film industry might not be quite as sinister as the tobacco industry, with its efforts to addict children to cigarettes. [...] Meanwhile, the lives of those audiences are now being increasingly saturated by popular culture, making it more and more difficult for individuals to form attitudes, opinions, and values that are independent of the messages promulgated by the Culture Industry."

While this is a subtle example, you would generally cite or at least credit your methods and theories that frame your analysis in your bibliography.

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2.6.13.4: Practice with BEAM

Practice with BEAM

You've just learned about the various roles sources can play in written material. In this section you'll gain practice identifying roles that other writers have used them for and then be asked to examine your own past work.

Activity: A Reading Exercise

Identifying BEAM's kinds of sources in already written materials is a good way of learning how to use them in your own writing assignments. For practice, look at an abstract of and two passages from **Lesy, M. (2007). Visual Literacy. Journal of American History, 94(1), 143-153.**

Read the abstract and passages below and identify the most likely role (background, exhibit, argument, or method) each featured source is playing in Lesy's article. See our take on each below:

ABSTRACT: The article reports on visual literacy and the psychological aspects of photography. The author offers his opinions on the complexities of photographs and reports on the various levels of meaning behind picture taking. Particular attention is given to the psychological aspects of photography and photographers. Additional article topics include the importance of historical photographs, the impact of the Internet and digital media on the profession, as well as the importance of preserving photographs.

Passage 1

Solving one scholarly problem – the need to sort out an image's multiple meanings – opens a clear view of others. No matter how mundane, utilitarian, or circumscribed a photograph's origins may be, an image is not a sentence. Images are forms of sensory data, processed by the right brain. No matter how judicious and objective a historian fancies herself, a photograph will elicit projections and associations in her, stir her imagination, before she even notices what is happening to her. A photograph "is a function, an experience, not a thing," said Minor White, a mid-twentieth-century photographer whom Walt Whitman would have recognized as a fellow poet. "Cameras are far more impartial than their owners and employers," White went on to say. "Projection and empathy [are] natural attributes in man...the photograph invariably functions as a mirror of at least some part of the viewer." SOURCE CITED: Minor White, "Equivalence: The Perennial Trend," PSA Journal, 29 July 1963), 17. 20.

Passage 2

The problem is not that there are too few images, but too many. Historical photographs exist in huge numbers, in well-ordered collections, presided over by knowledgeable curators. More and more of the collections are being digitized. Overload and saturation are only a mouse click away.

One example: in the Prints and Photographs Division of the Library of Congress there are 164,000 black-and-white photographs made between 1935 and 1945 by photographers employed by the Farm Security Administration and the Office of War Information.

"America from the Great Depression to World War 11: Photographs from the FSA-OWI, 1955-1945," Library of Congress: American Memory, http://memory.loc.gov/ammem/fsowhome.html

Our Answer

The source referred to in Passage 1 is probably a background source. The author is offering White's definition as fact. It helps support one of the author's assertions about the nature of photographs.

The source referred to in Passage 2 is an exhibit source. The author is using the Library of Congress's photographic archive as a self-evident example to support the claim that information overload is a potential problem.

Now you're ready to do role identification in a term paper you've already written yourself. (In the future, it may be helpful to do the same as a last check on term papers you are about to turn in.)

Activity: Self-Check

Directions: Re-read your term paper. (If you've already gotten feedback from your professor on this paper, also look to see whether any of that feedback applies to the roles you gave your sources.)Then consider the questions below. If you can't answer "Yes" to every question, reconsider how you have used the sources in your paper.

Do My Sources Have the Right Roles?

- Have I used background information to, for instance, introduce a setting, situation, or problem in the paper or essay?
- Did I cite/not cite the background information appropriately?
- Did I avoid using journal articles that report original research for my background information?
- Did I interpret and analyze sources as though they are exhibits or evidence in my argument?
- Were primary sources those that played the role of exhibits and evidence?
- Did I discuss and cite what others have written about my research question?
- Did I include writers who both agree and disagree with what I say is my answer to the research question?
- Did I avoid using tertiary sources to make my argument that my thesis is reasonable?
- Did I make it clear where key terms, concepts, and manner of working that I used in my research were used first by others?
- Were my sources for useful key terms, concepts, and manner of working secondary sources?

Where to Go From Here

Now that you have a better understanding of how sources are used in a paper, check out Academic Argument, which can help you plan and structure your term paper.

If you're interested in further integrating sources into your paper more effectively, check out this OSU Writing Center tutorial on summarizing, paraphrasing, and quoting.

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GLOSSARY

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Sample Word 1 | Sample Definition 1

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