INTRODUCTION TO BIOTECHNOLOGY



Orange County Biotechnology Education Collaborative

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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:

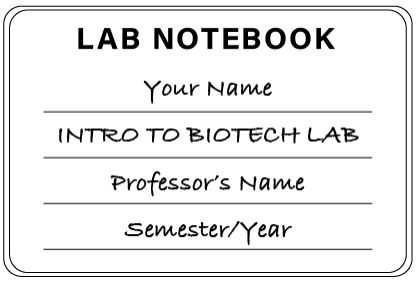


Figure 1. Lab notebook cover setup: Row 1: Name, Row 2: Intro to Biotech Lab, Row 3: Professor's Name, Row 4: Semester/Year.

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	
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)

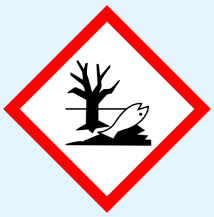
A quick assessment of a chemical's hazards is visible on its container on a Hazard Communication Standard (HCS) label. HCS labeling does not replace the more detailed Safety Data Sheet (SDS) but rather gives the following information in brief:

- 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
	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

Pictogram	Hazard Class	Hazard Type	Example Signal Word & Hazard Statement
	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
	(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

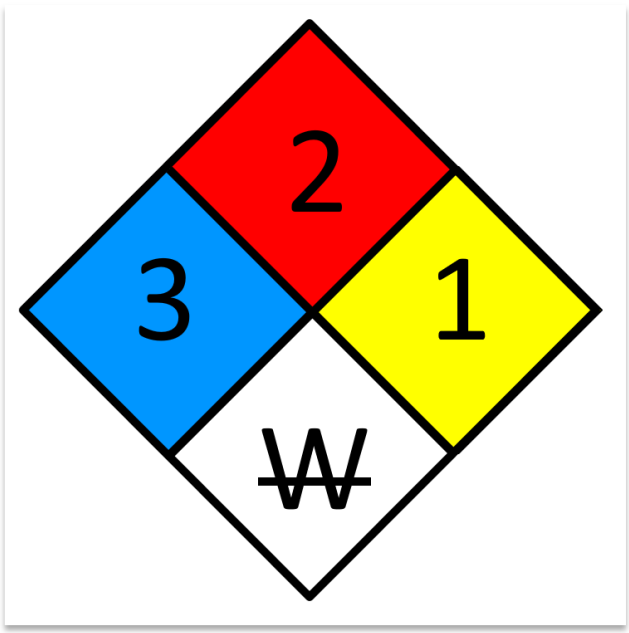
Type of Waste	Definition	Required Treatment
Chemical Waste	Any solid, liquid, or gaseous material generated in the laboratory that poses a danger to human health or the environment.	This will vary depending on the chemical. The institution you are in will have specific requirements to meet regulatory 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
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.

NFPA HAZARD RATING SYSTEM GUIDE

0	Will not typically burn	NFPA Hazard Label		0	Stable even under fire conditions.
1	Ignites only after considerable preheating			1	Normally stable except under high temperatures
2	Ignites after moderate preheating or at high ambient temperatures			2	Violent changes at high temperatures and pressures
3	Ignites at almost any temperature			3	May explode at high temperatures or with shock
4	Vaporizes at normal temperatures and pressures			4	May explode even at normal temperatures and pressure
0	No health hazards beyond normal combustible material			ACID	Acidic
1	Exposure can cause significant irritation			ALK	Alkaline
2	Intense or continued exposure can cause incapacitation or injury			COR	Corrosive
3	Short exposure can cause serious or permanent injury	BIO	Biological Hazard		Radioactive	OX	Oxidizer
4	Very short exposure can be fatal	POI	Poisonous	RAD		W	Reacts Violently or Explosively with Water

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.

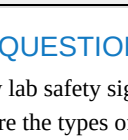
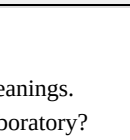
Using the SDS for sodium hydroxide provided to your group, determine what should be done in the event that the reagent: a) got on your skin b) splashed into your eyes c) was inhaled

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

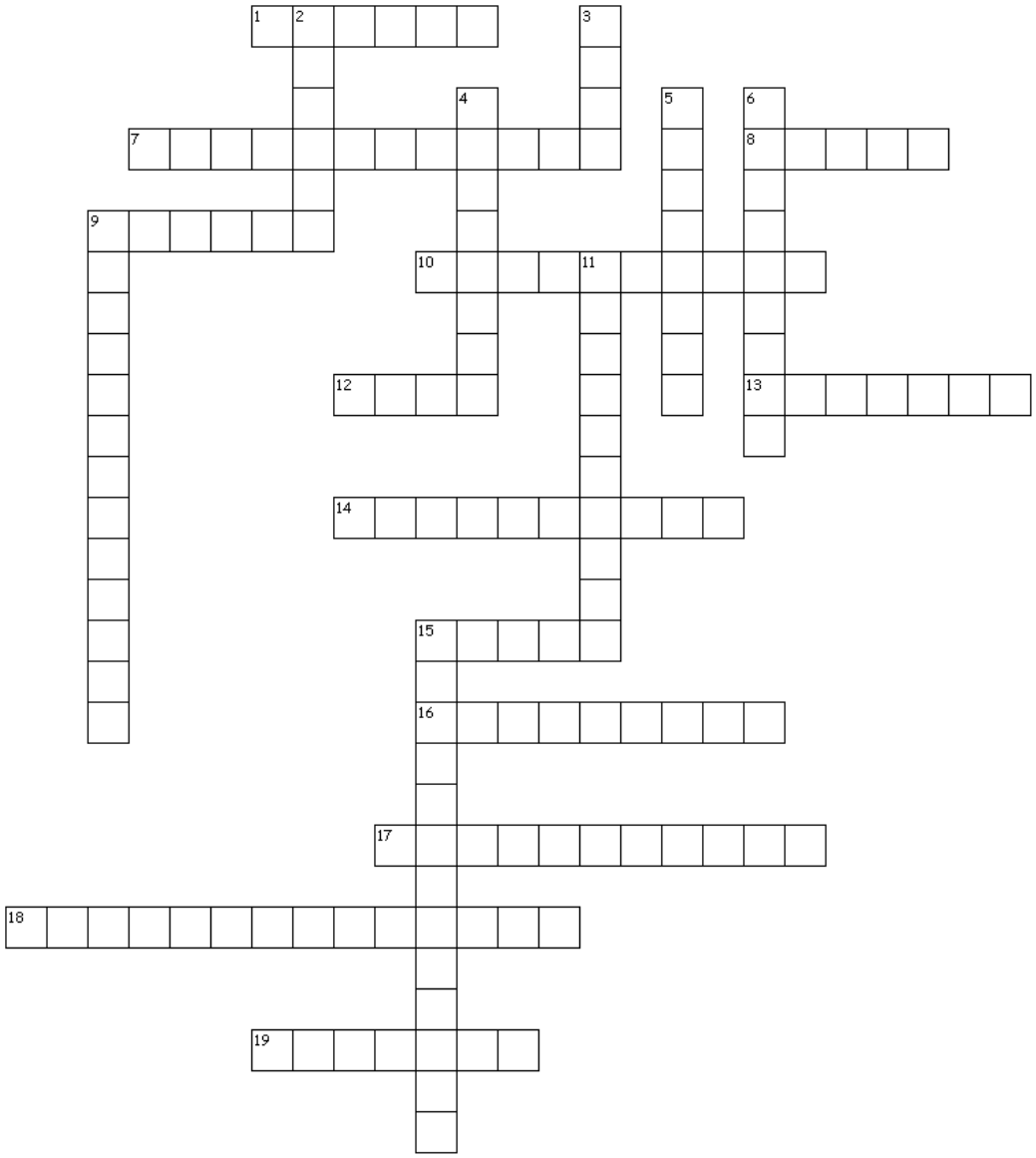
1. Review lab safety signs and know their meanings.
2. What are the types of PPE required in a laboratory?
3. How should you dispose of glass waste?
4. How should you dispose of biohazard waste?
5. What should you do if a chemical gets in eyes?
6. Given a laboratory hazard, be able to describe the correction 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	
7. hazard that the yellow color on the NFPA sign indicates	
8. governmental agency that regulates chemical safety data sheets	2. the lab notebook is considered a ___ document and can be used in court
9. broken test tubes/beakers should be swept with a ___ and dust pan	3. abbreviation for Personal Protective Equipment
10. COR on the NFPA sign	4. shape of sign that lists the chemical ratings from the National Fire Protection Association
12. instrument used to write in a scientific lab notebook	5. hazard that the white color on the NFPA sign indicates
13. used razors, scalpels and needles should be placed in the ___ waste container	6. table found at the front of a scientific lab notebook
14. SOP stands for Standard _____ Procedures	9. type of waste containing recombinant DNA and tissue culture dishes
15. rating number for the most dangerous hazards in the NFPA sign	11. OX on the NFPA sign
16. ALK on the NFPA sign	15. hazard that the red color on the NFPA sign indicates
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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- Health hazard sign via Wikimedia Commons; public domain
- Hazardous substances sign via Wikimedia Commons; public domain
- Environmental hazard sign via Wikimedia Commons; public domain
- Eyewash sign via Wikimedia Commons; public domain
- Emergency shower sign via Wikimedia Commons; public domain
- Fire blanket sign via Wikimedia Commons; public domain
- Fire extinguisher 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	**K**ing	**H**enry	**D**oes	**U**sually	**D**rink	**C**hocolate	**M**ilk	
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 2. diameter of washer, measured in a straight line across the center of the washer from outer edge to outer edge.

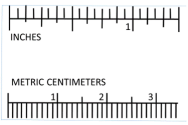


Figure 1. Ruler: centimeter scale bottom. Inch scale top.

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

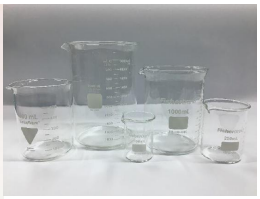


Figure 5. Beakers

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="MathJax-Element-6-Frame" role="presentation" style="position:relative;" tabindex="0">
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 container you previously attained.
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 the dial to draw 3.7 mL of deionized water into the pipet from a 50 mL graduated cylin
3. Dispense the water into the weighed beaker dialing in the opposite direction. To get the last bit out of the pipet, quickly dial one way then the other.
4. Dispense the 3.6 mL into the weighed beaker and determine its mass (g). Record this value in your table as “weight of container and water.”
5. Determine the weight of the water and record. If you accurately dispensed 3.6 mL, the weight difference should be very close to 3.6 g.
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 and water (g)	Weight of water only (g)	Actual volume dispensed (mL)	% Error
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 **closeness of repeated measurements or the reproducibility** of the measurement (how si
Precision can be affected by the measuring instrument. If a student uses a thermometer calibrated to the nearest degree to measure the temperature of a beaker of water and a second student uses a thermometer that is
Accuracy, however, is dependent upon both the **precision of the measuring instrument** and the **technical skills** of the individual taking the measurement. You will perform an experiment to determine which instrum
To calculate the “**mean” or “average”**, you will add all the measurements and then divide by the number of measurements. For example, if three groups weighed a washer and the measurements for weight were 9 gr

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 mL beaker on the scale. Record the weight in grams under the number “1” in table 1-5
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 trials using a beaker to measure 10 mL water.
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 beaker on the scale. Record the weight in grams under the number “1” (which means
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 measure 10 mL water.
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 beaker on the scale. Record the weight in grams under the number “1” (which means tha
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 measure 10 mL water.
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 accurate)?
6. What can contribute to a larger % error when making a measurement? If you had to repeat a measurement in Table 3, be sure to indicate why you think your % error was outside of the acceptable range.

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

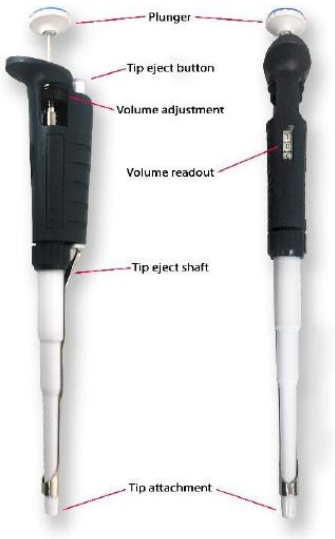


Figure 1. Labeled parts of a micropipette, front and back.

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.

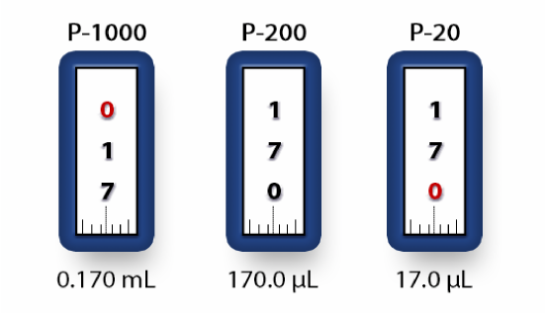


Figure 2. Reading a micropipette at various sizes.

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.

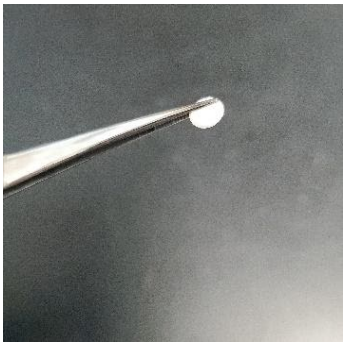


Figure 1. Forceps with sterile disk.

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.

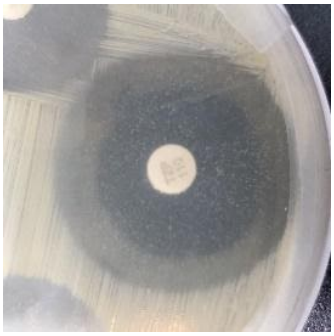


Figure 2. Zone of inhibition

5. Place plate in a 37 C incubator, with the agar side up. (note: you can place it into the same 32 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 C incubator, with the agar side up. (Some organisms in the environment do not grow well at 37 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.

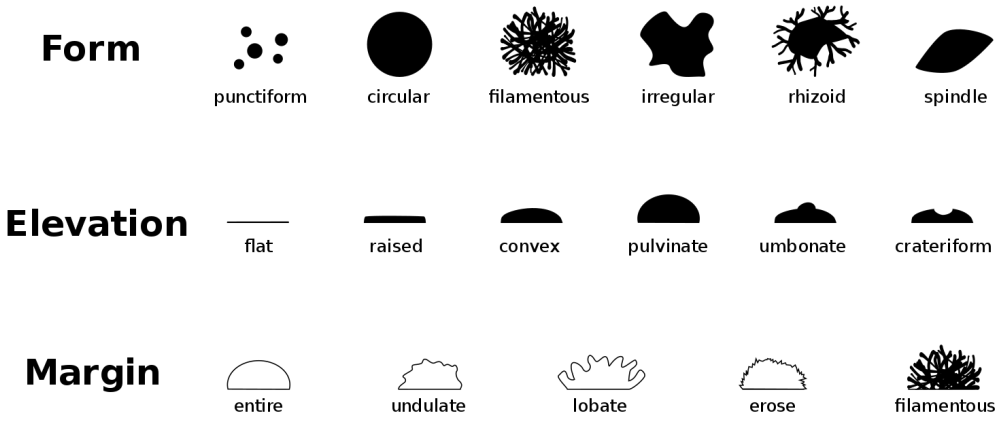


Figure 3. Morphologies of colony growth [by Madeco (CC-BY-SA) via Wikimedia Commons].

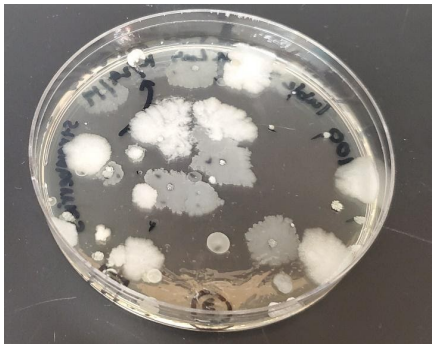


Figure 4. Nutrient agar media plate with various colony types growing on it

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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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.

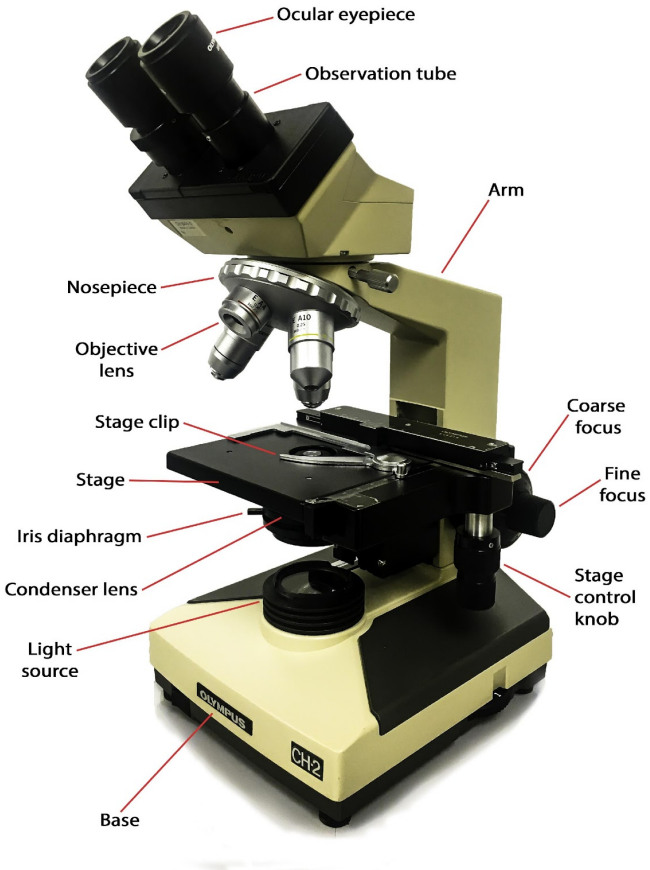


Figure 1. Labeled parts of a microscope.

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.

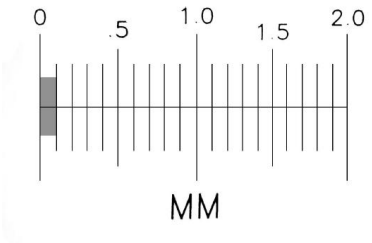


Figure 2. Stage micrometer

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

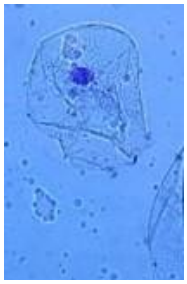


Figure 3. Human cheek cell at 400x zoom.

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.

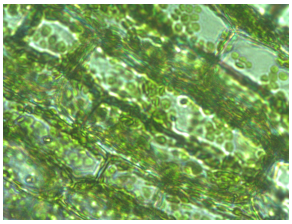


Figure 4. *Elodea* cells at 400x

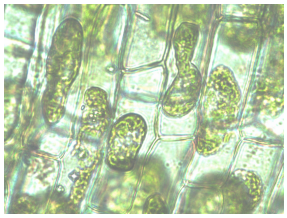


Figure 5. *Elodea* cells undergoing plasmolysis at 400x

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.

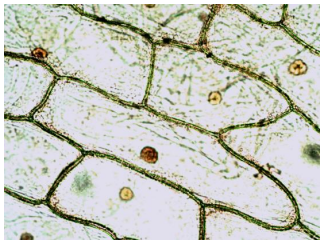


Figure 6. Onion cells at 400x

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.

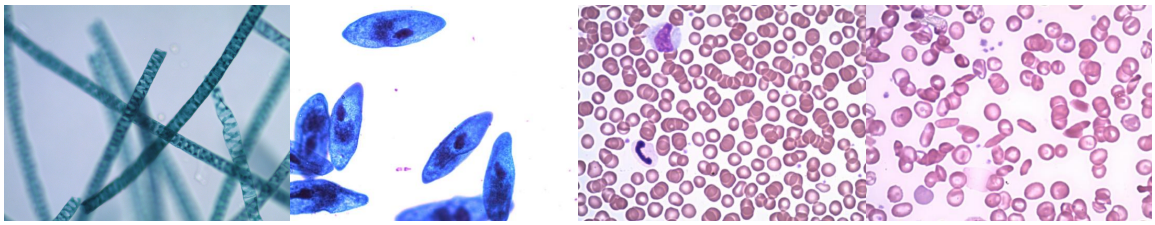


Figure 7. Specimens at 400x Magnification.From left to right: *Spirogyra*, *Paramecium*, Human Blood Smear, Human Sickle Cell.

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.

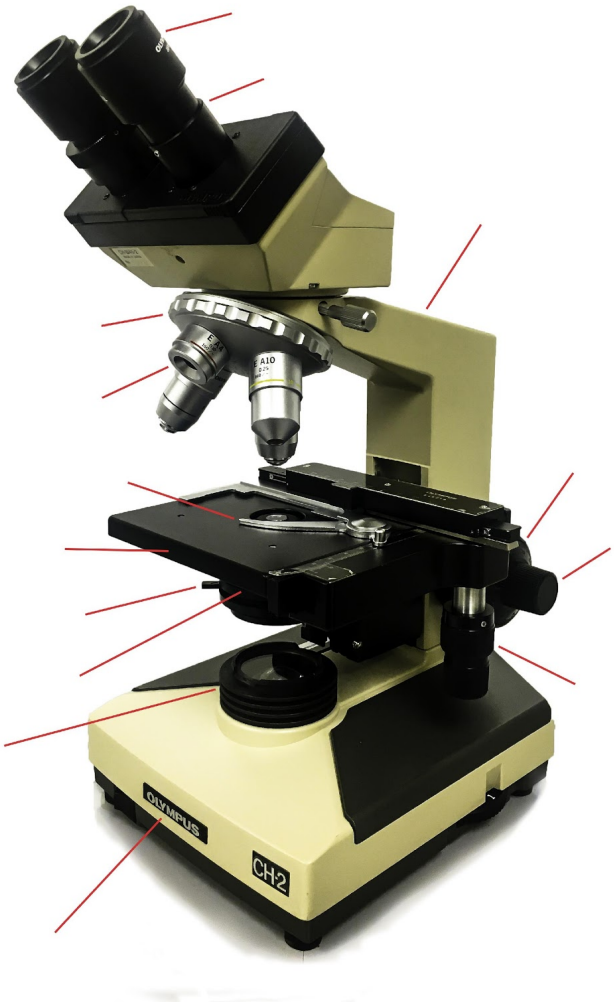


Figure 8. Blank microscope to label parts.

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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.

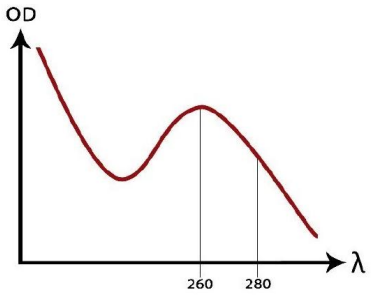


Figure 1: Curve of light absorbance and transmission

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%.

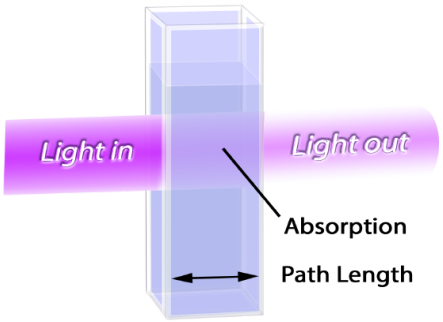


Figure 3: Light is absorbed and transmitted through the spectrophotometer cuvette and solution

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).

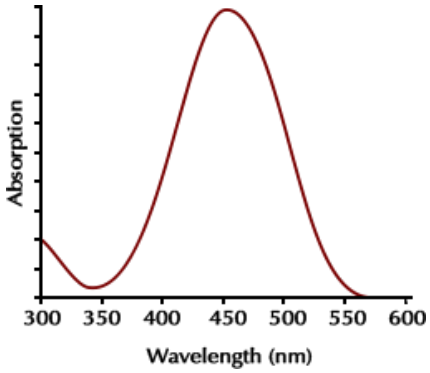


Figure 4. Curve of absorbance

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.

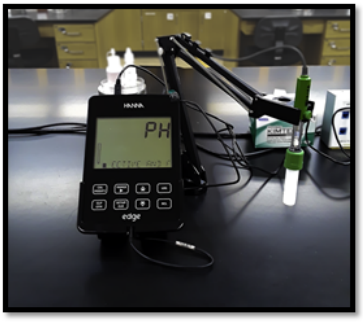


Figure 1. The pH meter

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**
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:

$$\boldsymbol{n_A} = volume\ of\ conjugate\ base\ (mL) \times \frac{1\ L}{100\ mL} \times \underset{(mol/L)}{concentration\ of\ conjugate\ base}$$

$$\boldsymbol{n_{HA}} = volume\ of\ conjugate\ acid\ (mL) \times \frac{1\ L}{100\ mL} \times \underset{(mol/L)}{concentration\ of\ conjugate\ acid}$$

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			
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 X 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.

$$concentration\,factor = \frac{volume_{initial}}{volume_{final}}$$

$$dilution\,factor = \frac{1}{concentration\,factor}$$

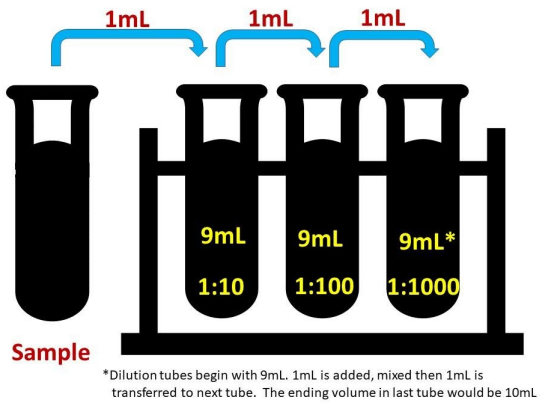


Figure 1. A ten-fold serial dilution, which can also be called a 1:10 dilution, or a series with dilution factor of 10. To determine the concentration at each step of the series, you divide the previous concentration by the dilution factor.
*Dilution tubes begin with 9-mL. 1-mL is added and mixed, then 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

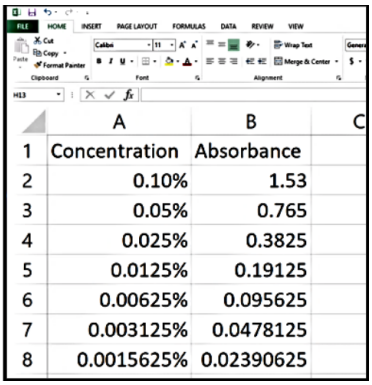
1. Pipette 2.0 mL of 5.0 µg/mL methylene blue working solution into a 15 mL conical tube.
2. Pipette 8.0 mL DI H2O into the tube to make 10.0 mL of total solution.
3. Seal the tube and invert repeatedly to mix.
4. What is the concentration of your new solution? Label the tube _____ µg/mL methylene blue.

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.

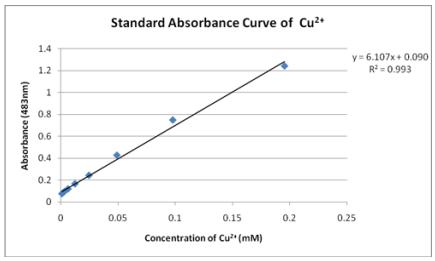


Figure 2. An example of a standard curve

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%		
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.

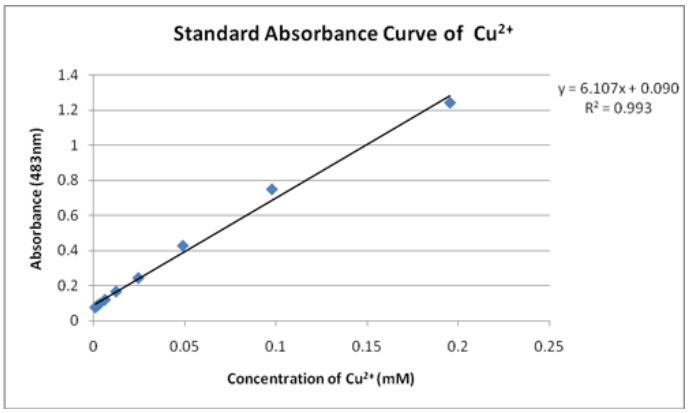


Figure 3. A standard absorbance curve of Copper II

7. Evaluate the quality of the standard curve above by using the R2 value.

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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.

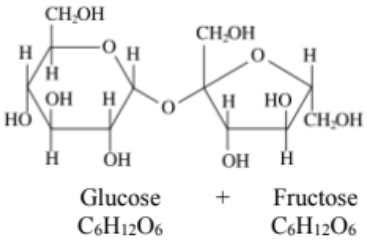
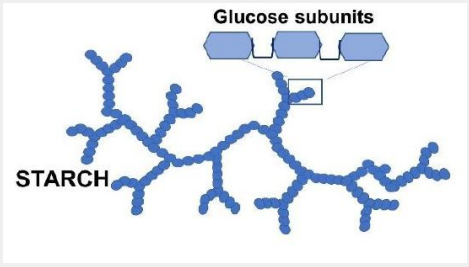
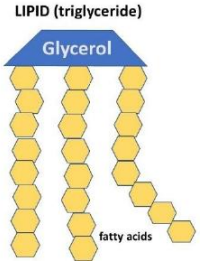
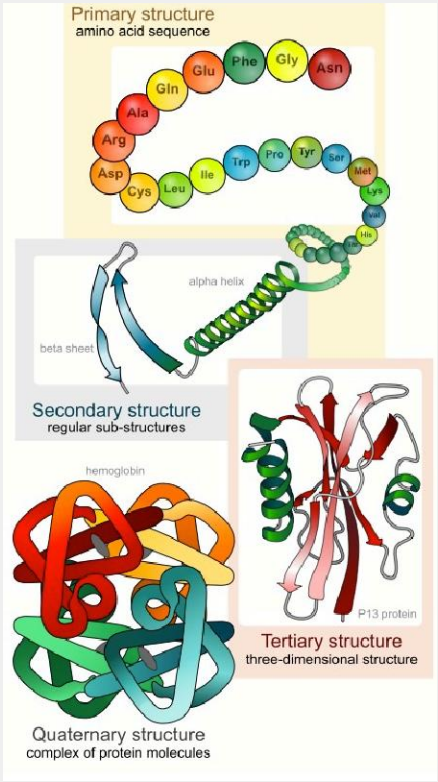
INTRODUCTION

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 Structure
Sucrose		*Common source: Table sugar* 
Starch		*Common source: Rice* 
Lipids		*Common source: Cooking oils* 
Proteins		*Common sources: cell receptors, egg, hair, feathers* 

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 C6H12O6. 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.

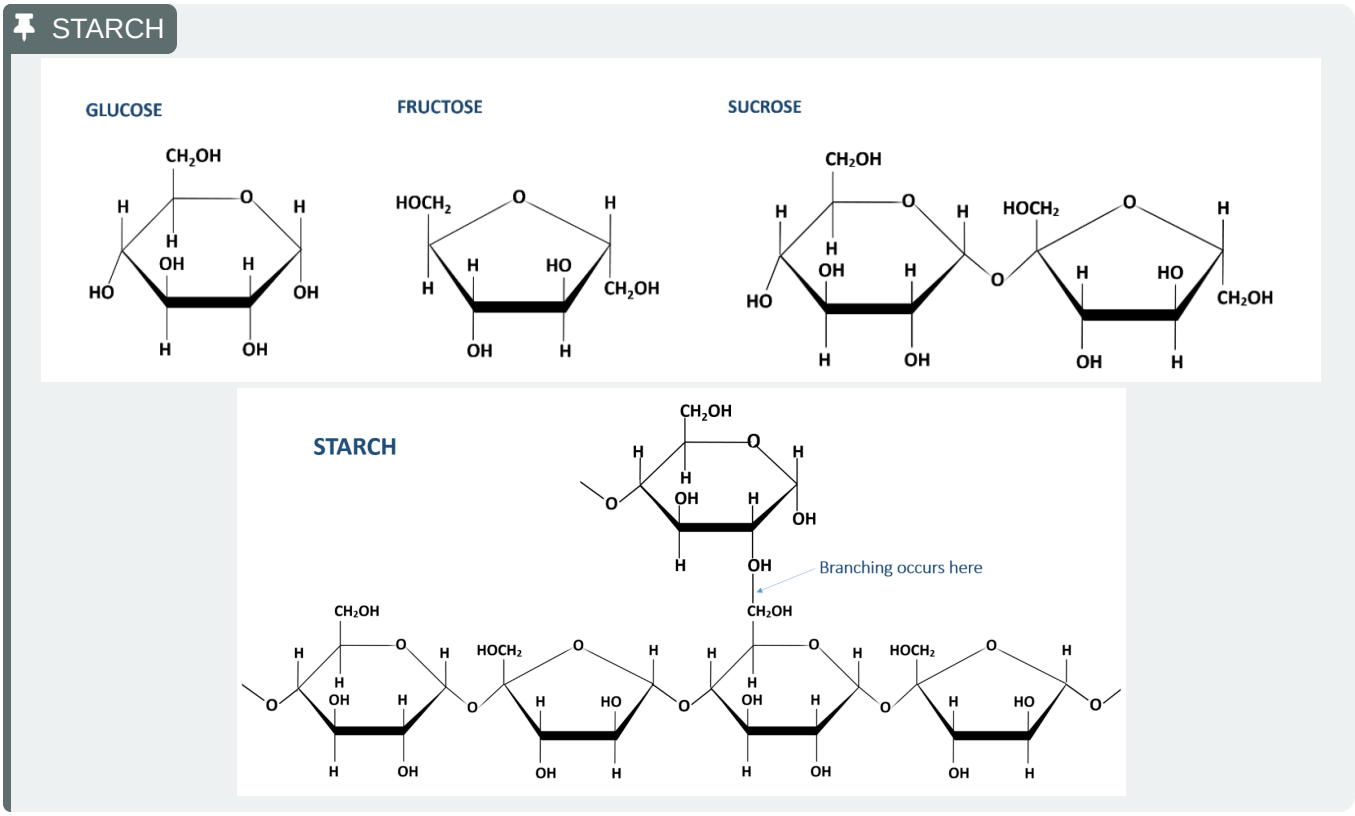


Figure 2. Monosaccharides (glucose, fructose), Disaccharide (sucrose), and Polysaccharide (starch)

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.

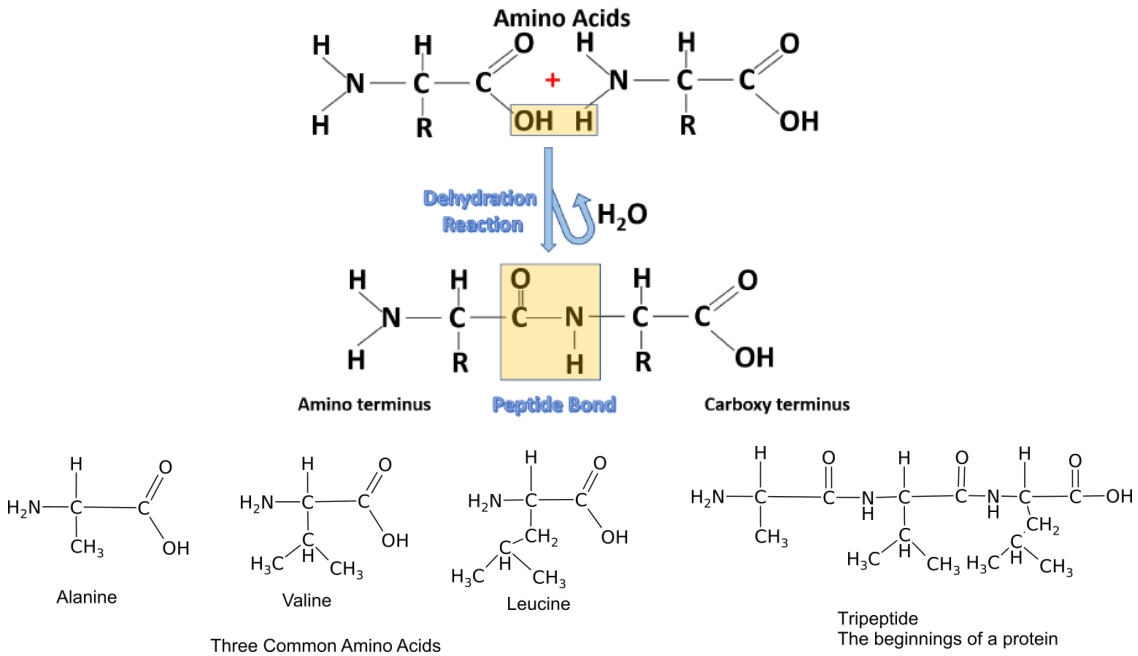


Figure 3. Amino acids forming a peptide bond.

TESTING FOR MONOSACCHARIDES WITH BENEDICT'S REAGENT

REACTION 2

Proteins + KOH + $CuSO_4$ **Purple**

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.

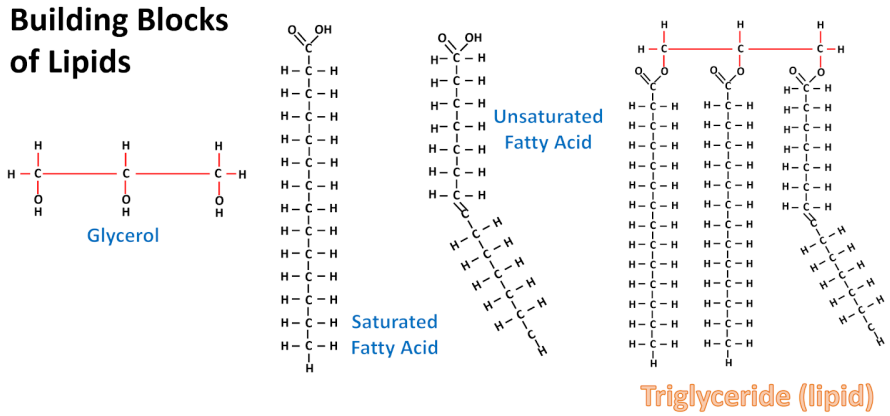


Figure 4. Saturated and unsaturated fatty acid and a lipid (triglyceride)

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

1. Sucrose Molecular Structure from LibreTexts 5.2 Carbohydrates.
2. Protein Structure diagram by Lady of Hats, Public Domain, via Wikimedia Commons.
3. Amino Acids forming a peptide bond (bottom image) by OpenLab at CitiTech CC-BY-NC-SA

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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.

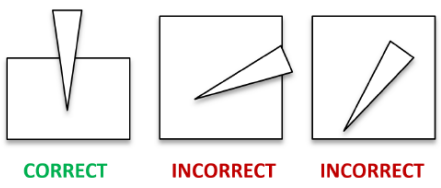


Figure 1. The correct positioning of a micropipette tip

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)	
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							
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.

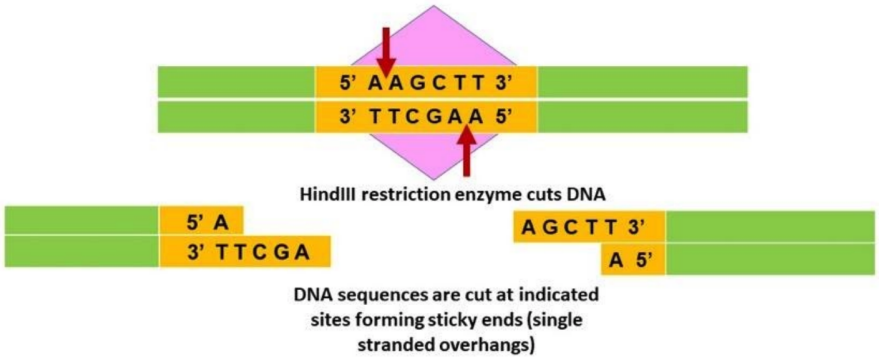


Figure 1. Recognition sequence for enzyme Hind III

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.

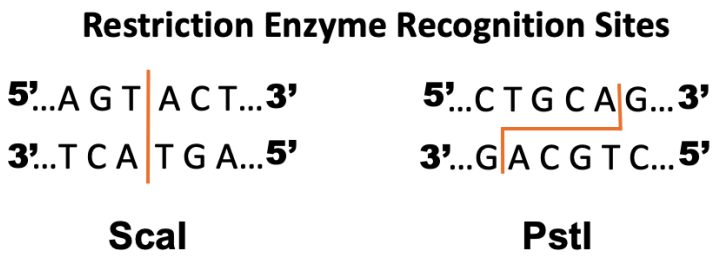


Figure 2: Restriction enzyme recognition sites.

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.

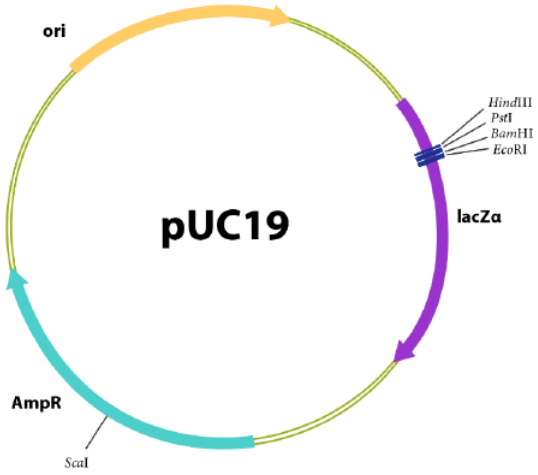


Figure 3: Plasmid map of pUC19.

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
- 37ºC 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
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. imagew228amph1amprev1ampac1ampparent1gk1-qezbOd_EasksNT7LvbYaQLJ41OZ6
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.

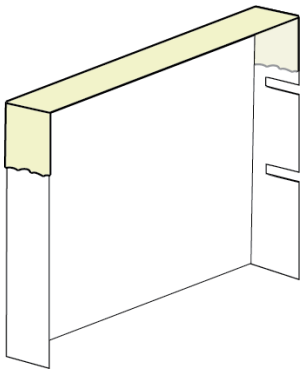


Figure 4. Casting tray with tape

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.

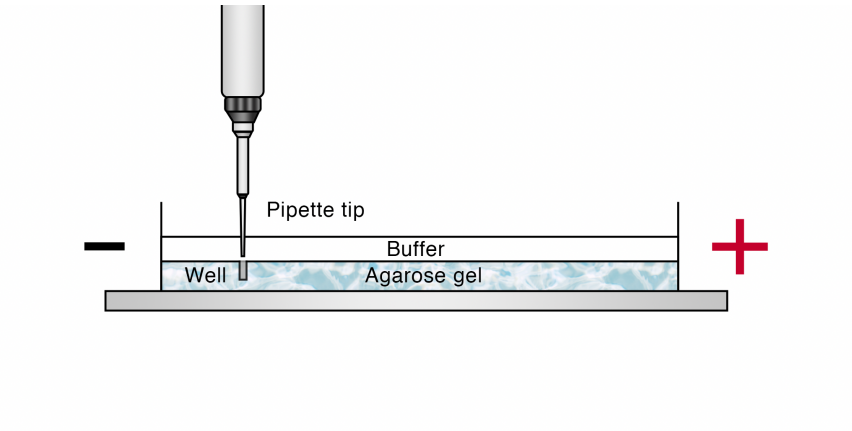


Figure 5. Pipetting a sample into the well of an agarose gel

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.

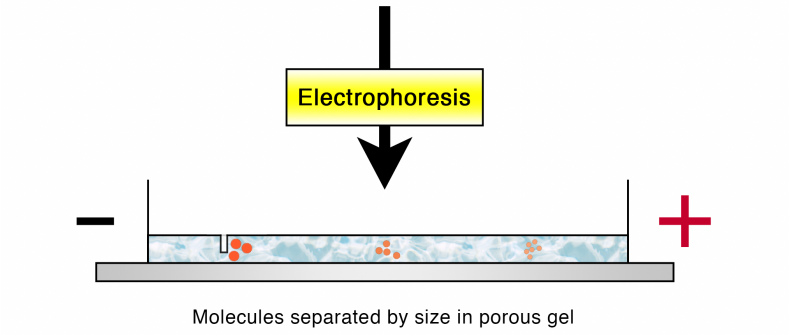


Figure 6. Electrophoresis causes the molecules to separate by size in the porous gel

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?

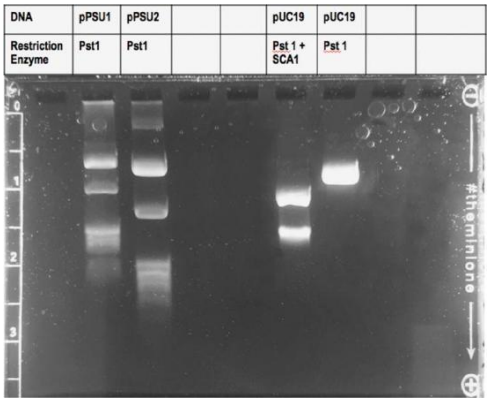


Figure 7: Restriction digests after electrophoresis

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.

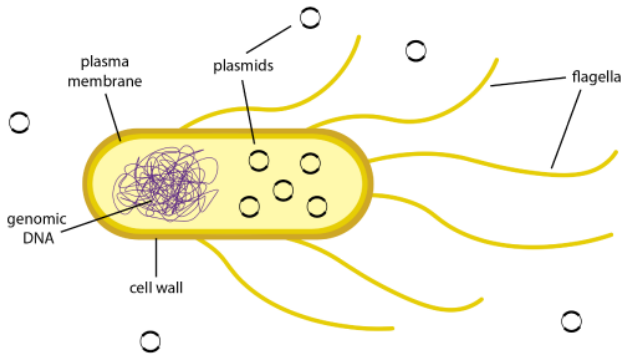


Figure 1. Bacterial transformation.

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.

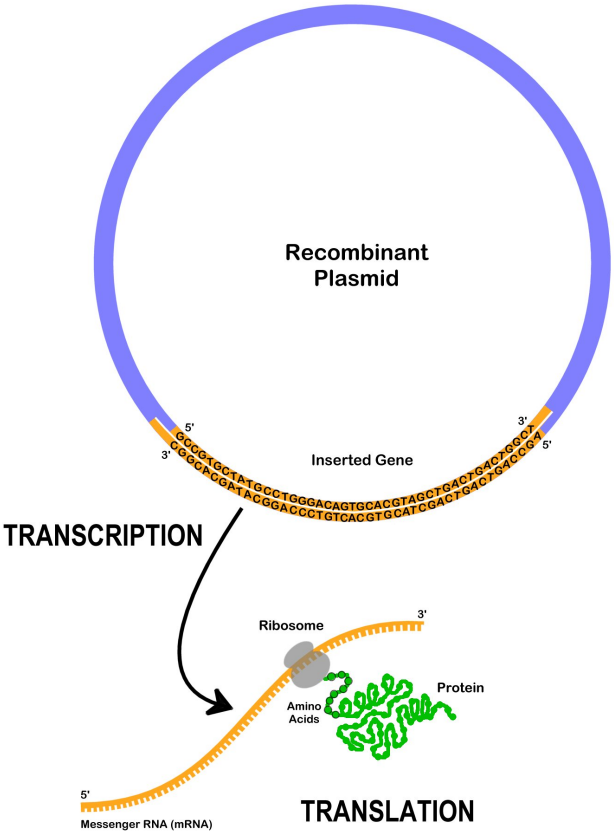


Figure 2. Gene expression from a plasmid in the bacterial cell

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+”.

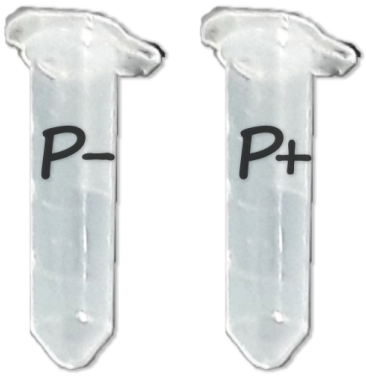


Figure 3. Labeled microfuge tubes

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

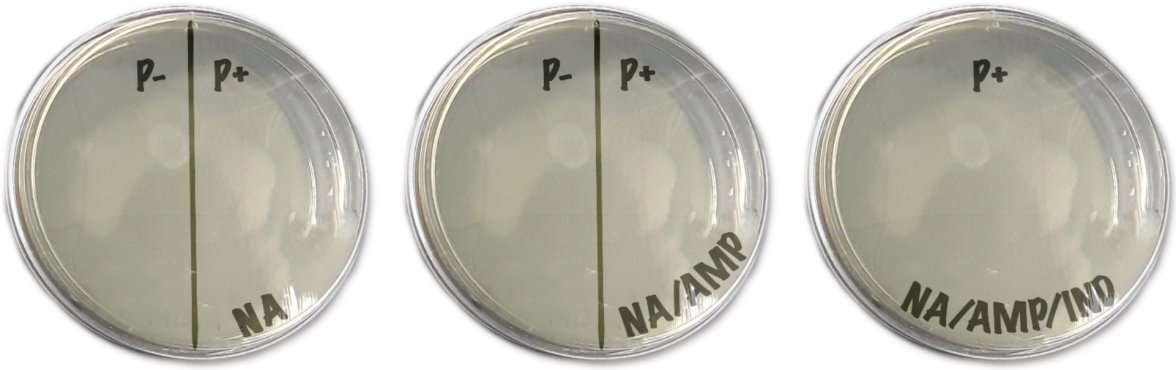


Figure 4, Labeled plates

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.

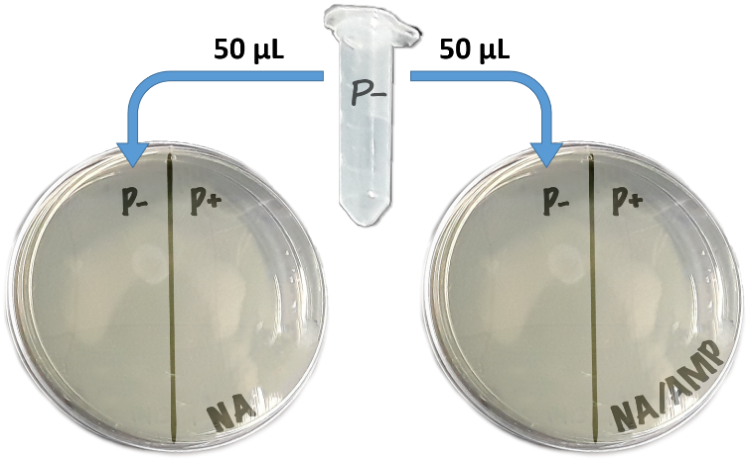


Figure 5. How to distribute the no-plasmid control treatment on the media

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.

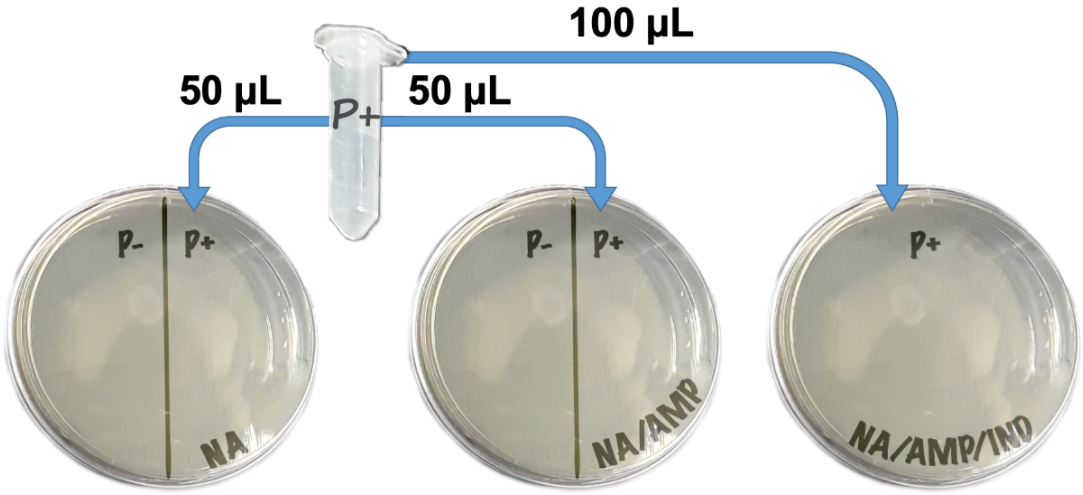


Figure 6: How to distribute the plasmid treatment onto media.

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.

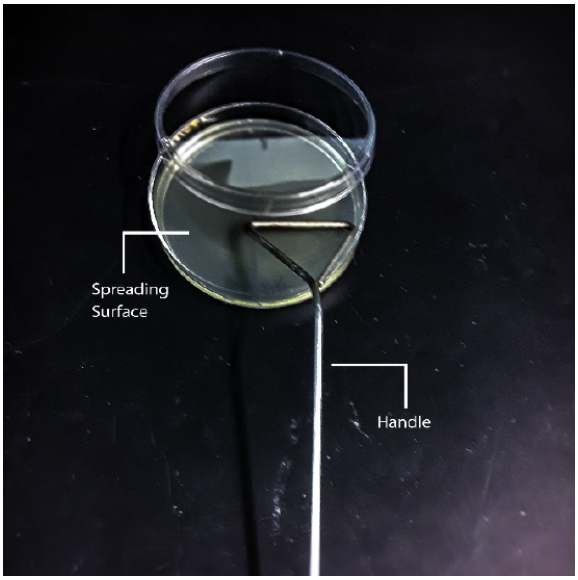


Figure 7. Spreading the *E. coli* onto the medium surface

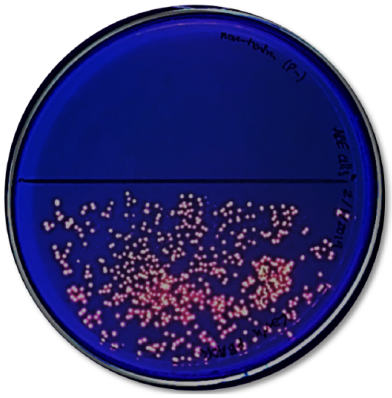


Figure 8. *E. coli* transformed with a plasmid containing a gene encoding fluorescent protein growing on NA+AMP+IND

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?

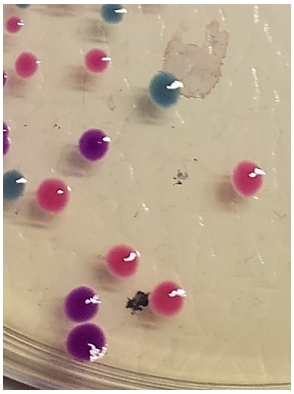


Figure 9. Transformed *E.coli* producing colored molecules on NA+AMP+IND

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)

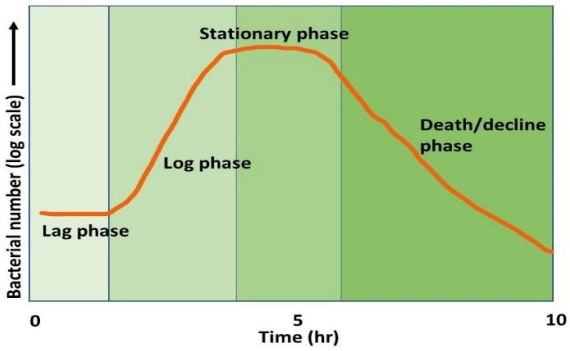


Figure 1. Bacterial growth curve outlining change in growth over time

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.

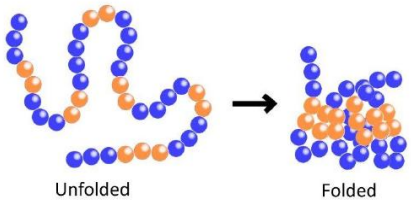


Figure 2. The folding of a protein

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.

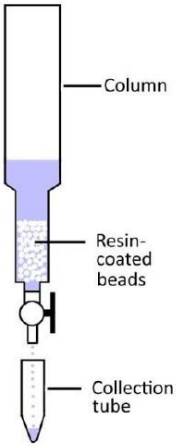


Figure 3. Liquid column chromatography

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.

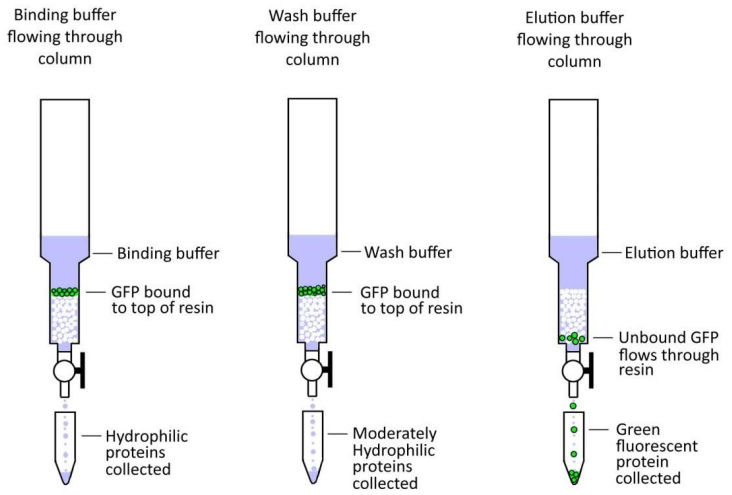


Figure 4. Separation of green fluorescent protein by hydrophobicity using column chromatography

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.

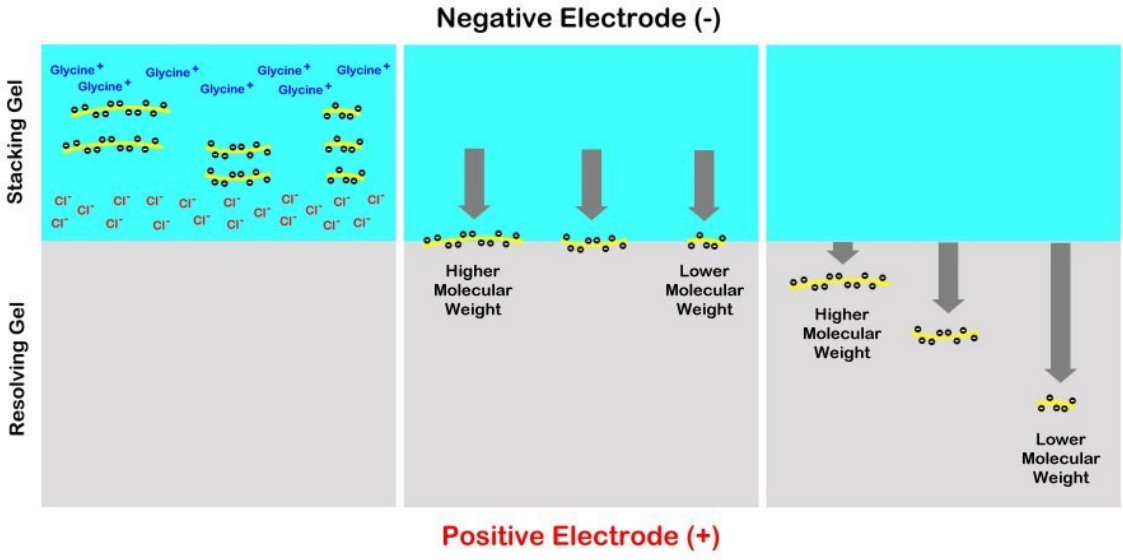


Figure 1. PAGE gel. A protein first runs through the stacking gel, where the samples spread out. Once a protein reaches the separating gel, the proteins pack together in tight bands. As they move through the resolving gel they separate by size.

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.

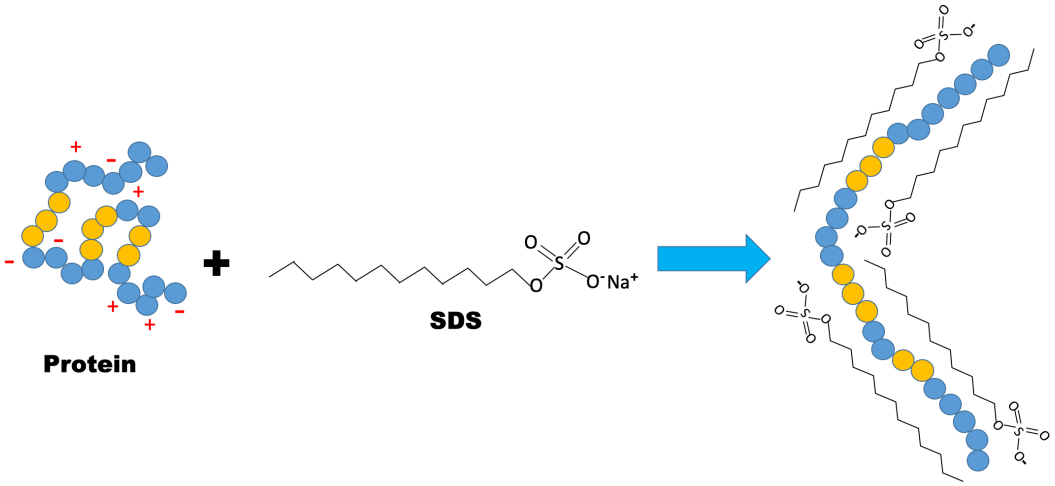


Figure 2: A protein surrounded by the SDS molecules.

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.

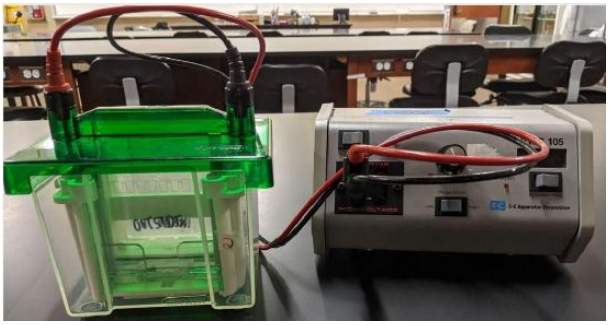


Figure 3: SDS PAGE apparatus with a power supply

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.

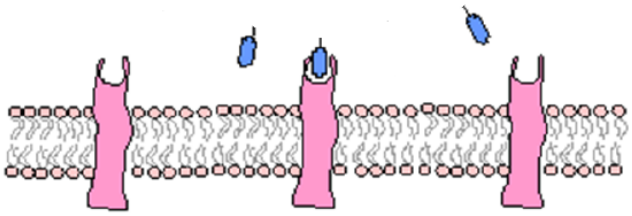


Figure 1: A chemical binding a membrane receptor

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.

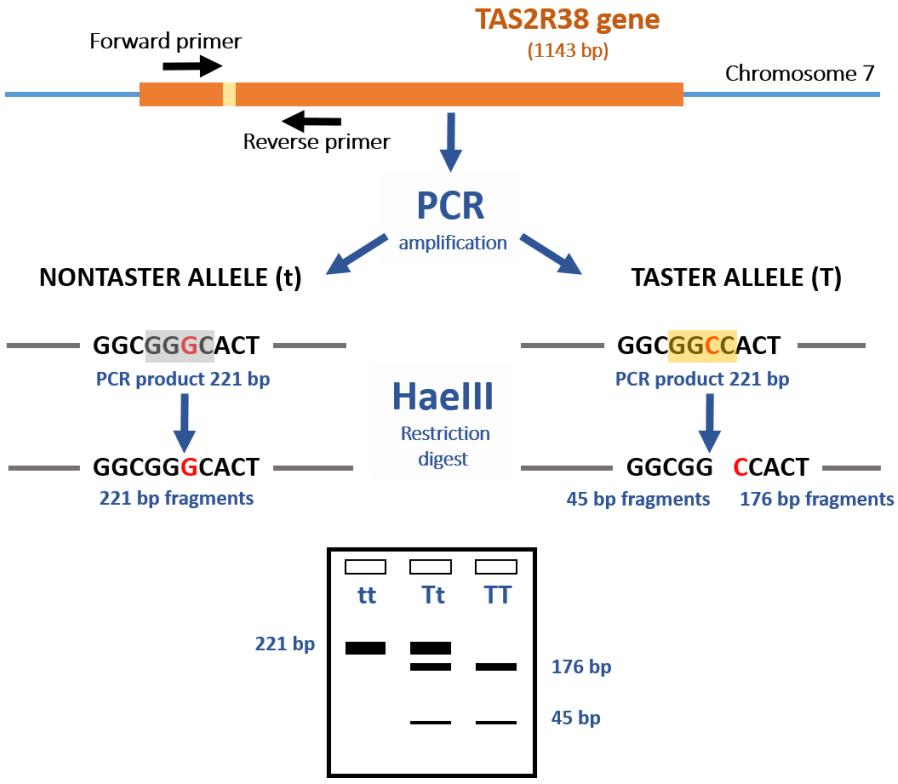


Figure 2:. Expected gel electrophoresis results post-restriction digest

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 C
Final Incubation	∞	4 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 C until next class period.

Table 5. PCR Program (optimized for MiniOne PCR System)*

Step	Duration	Temperature	Cycles
Initial Denaturation	30 sec	94 C	
Denaturation	5 sec	94 C	
Annealing	10 sec	66 C	30 Cycles
Extension	15 sec	66 C	
Final Incubation	∞	4 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 C for 15 minutes using the constant temperature mode. Enter 4 C for the final incubation. (See table 6)

Table 6. HaeIII Digest Program

Step	Duration	Temperature
HaeIII Incubation	15 mins	37 C
Final Incubation	∞	4 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	9
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.

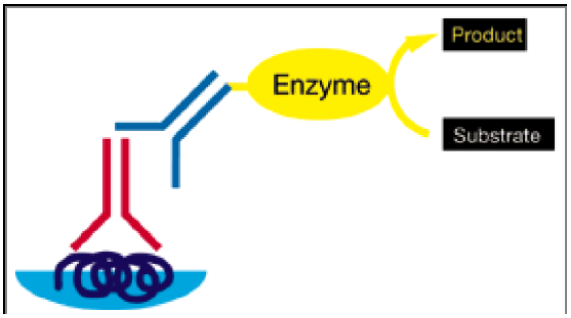


Figure 1. ELISA reactions

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

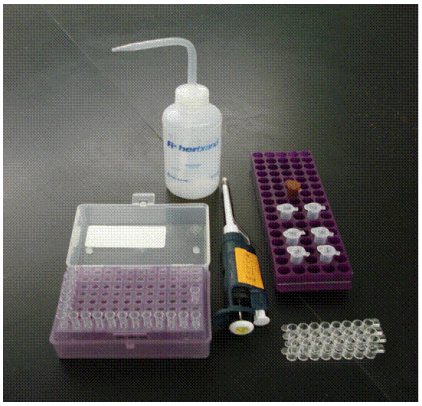


Figure 2. ELISA lab materials

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.

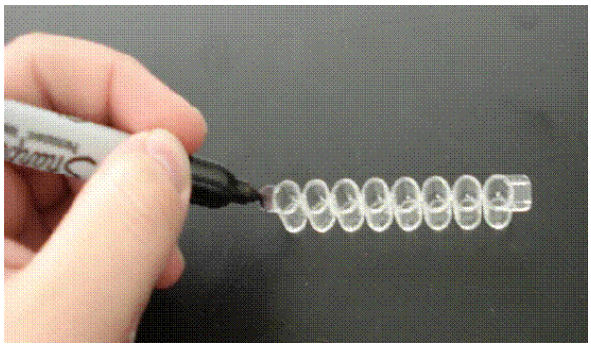


Figure 3. Marking to orient the well strip

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. imagew200amph1amprev1ampac1ampparent1zAANz2-OtJgZ9uJb3w_HiUBQblOsFrhS
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

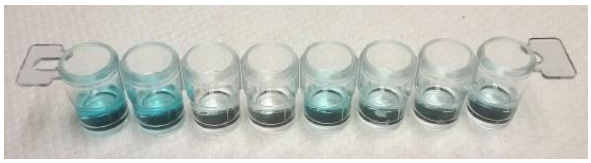


Figure 5. Positive wells (varying shades of blue) and negative wells (no color)

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.

ATTRIBUTION

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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

$$\text{concentration factor} = \frac{\text{volume}_{\text{initial}}}{\text{volume}_{\text{final}}}$$

$$\text{dilution factor} = \frac{1}{\text{concentration factor}}$$

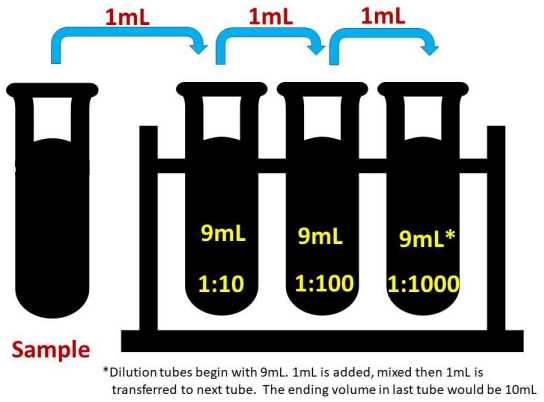


Figure 1. A ten-fold serial dilution, which can also be called a 1:10 dilution, or a series with dilution factor of 10. To determine the concentration at each step of the series, you divide the previous concentration by the dilution factor. *Dilution tubes begin with 9-mL. 1-mL is added and mixed, then 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.

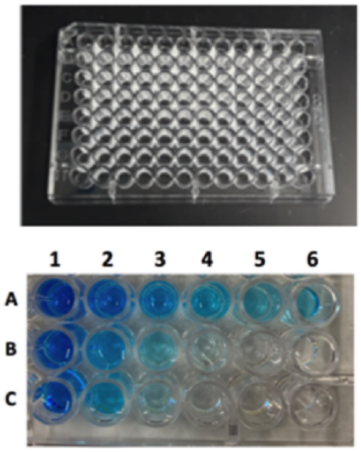


Figure 2: Microplate and serial dilutions.

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.

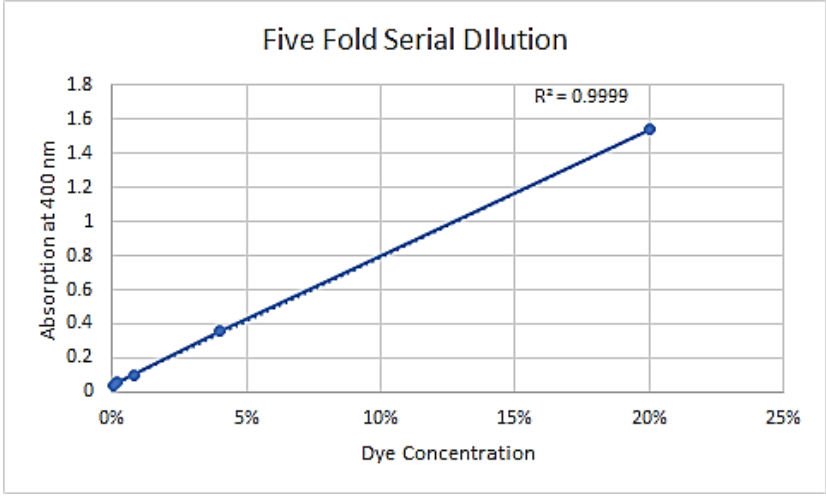


Figure 3: Standard curve of a five-fold serial dilution

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.

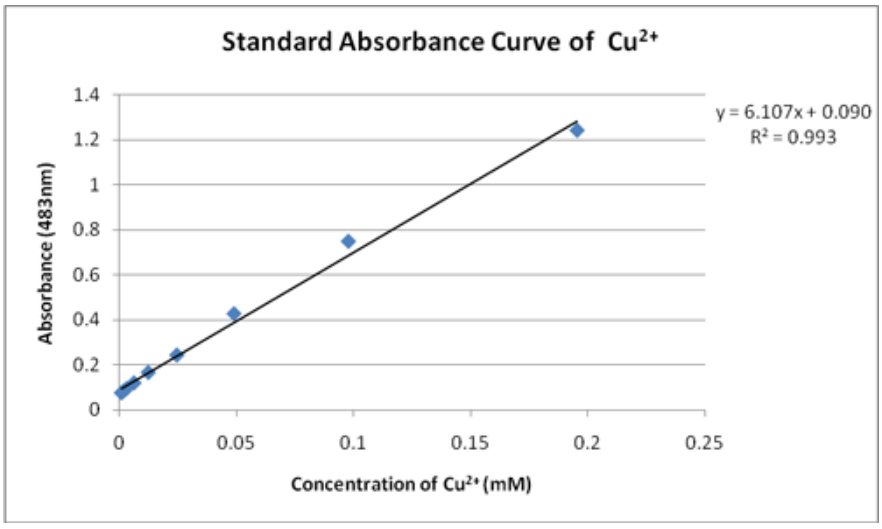


Figure 4. Standard curve of absorbance of Copper II

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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.

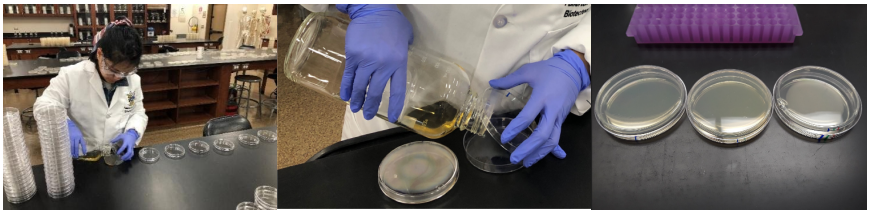


Figure 3: Pouring Plates

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.

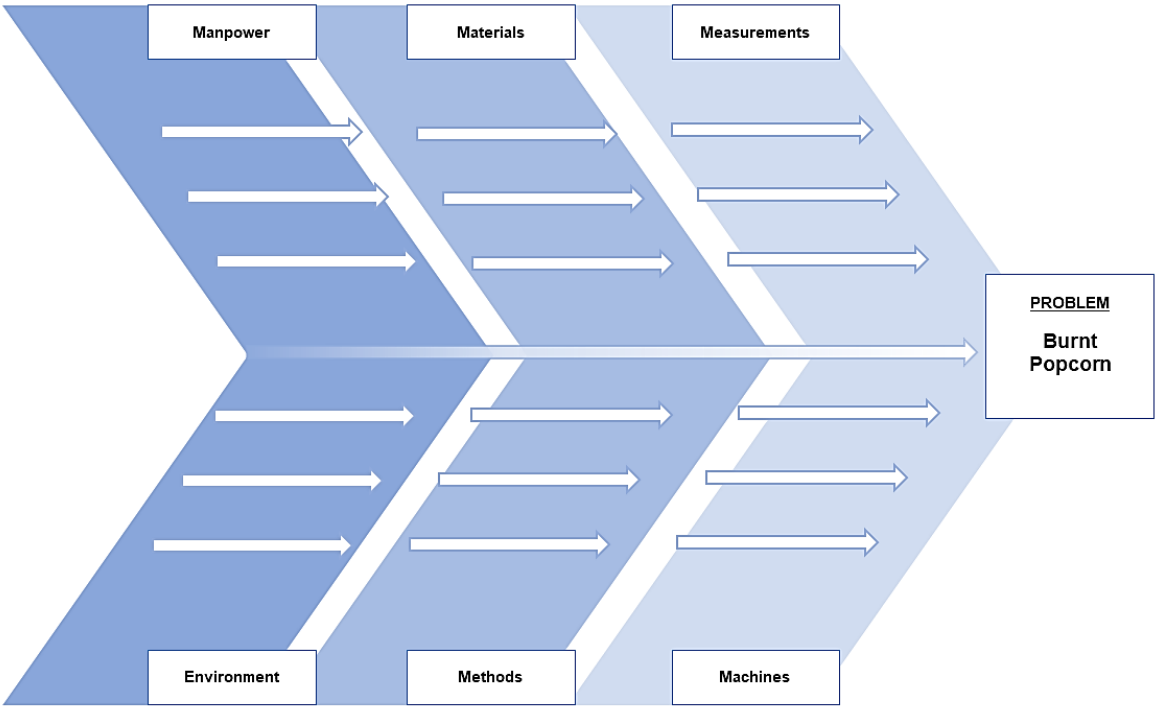


Figure 4. Blank Ishigawa fishbone diagram.

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.

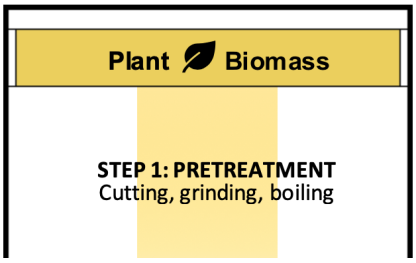
In the **pretreatment stage**, the goal is to loosen the cell wall structure so that the cellulose is exposed. Plant cell walls are made up of three primary components: **cellulose, hemicellulose**, and **lignin**. These molecules must be separated so that enzymes can reach the cellulose. Heating and 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.

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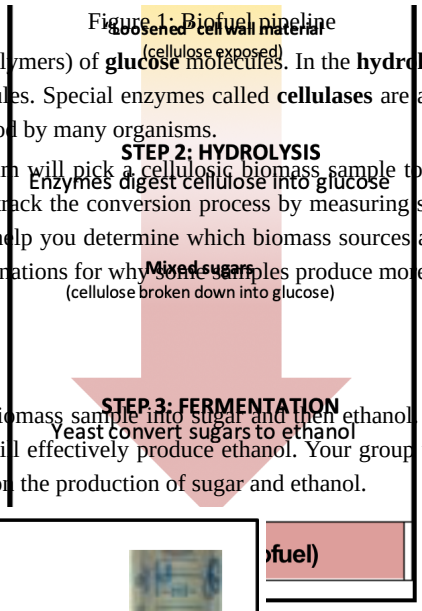


Figure 1: Biofuel pipeline

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.

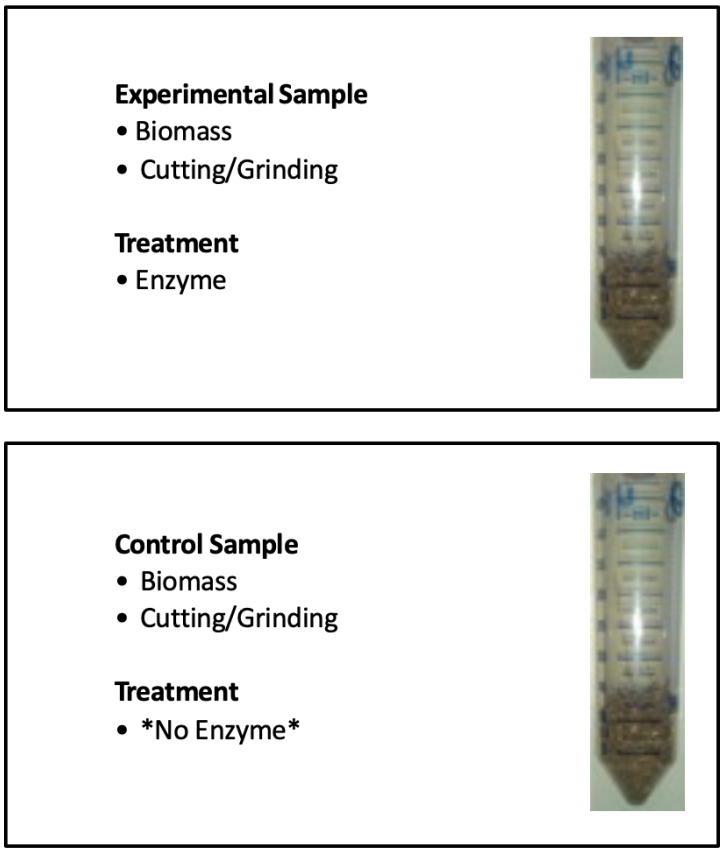


Figure 2: Experimental and control samples of biomass

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)
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).

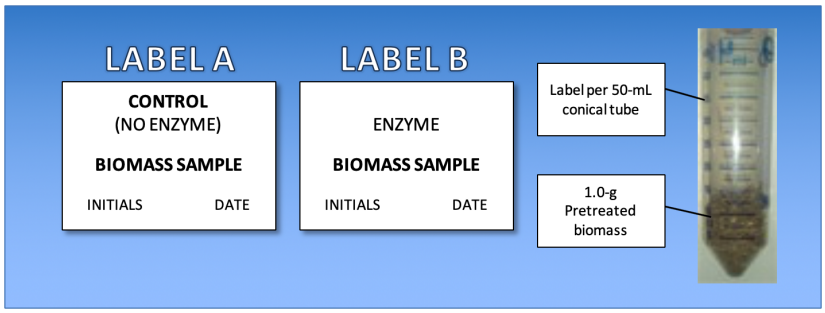


Figure 3: Example labels and diagram of sample preparation.

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.

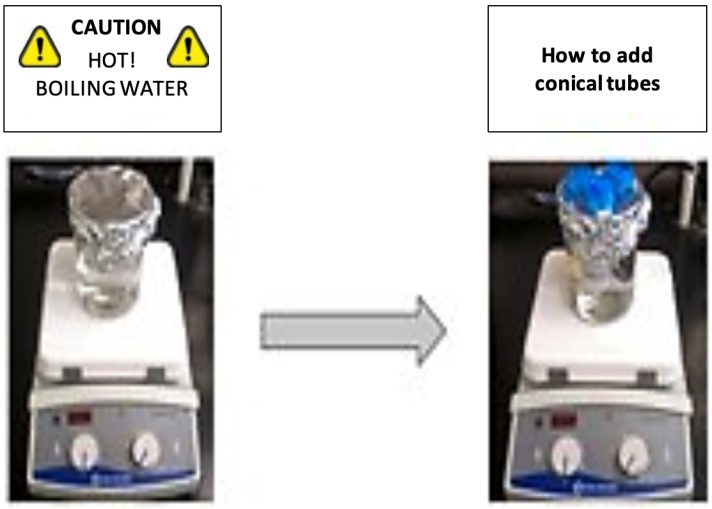


Figure 4: How to setup hotplate and tubes.

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.

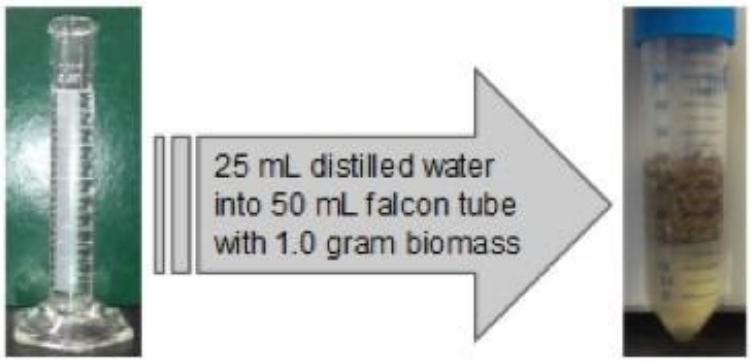


Figure 5: Contents of the falcon (conical) tube.

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?).

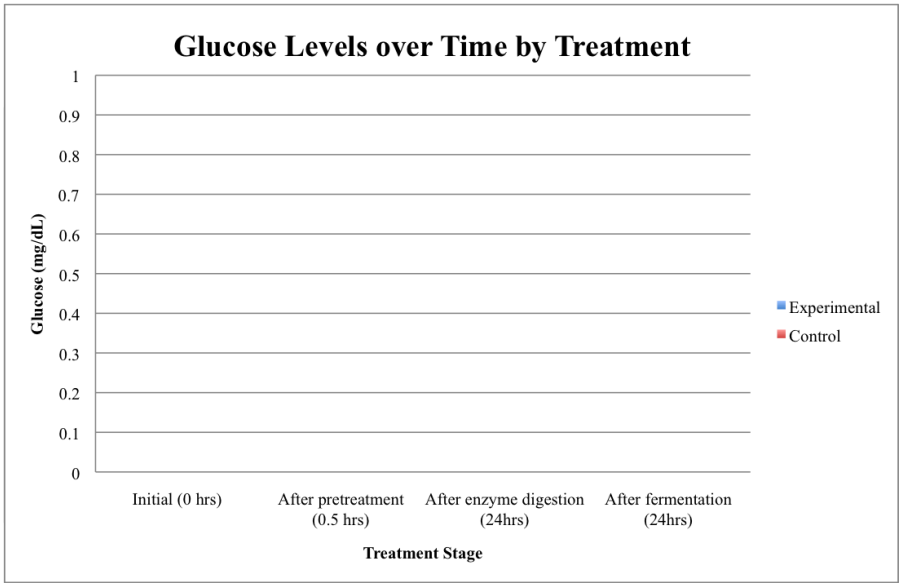
9. Take final ethanol readings. Test the ethanol concentration using the ethanol probes.
10. Record this data. Describe any detectable changes in the biomass (Appearance, odor). NOTE: For more accurate ethanol readings, allow samples to reach room temperature before taking measurements.
11. Clean tubes and lab area as instructed by your teacher.

DATA ANALYSIS

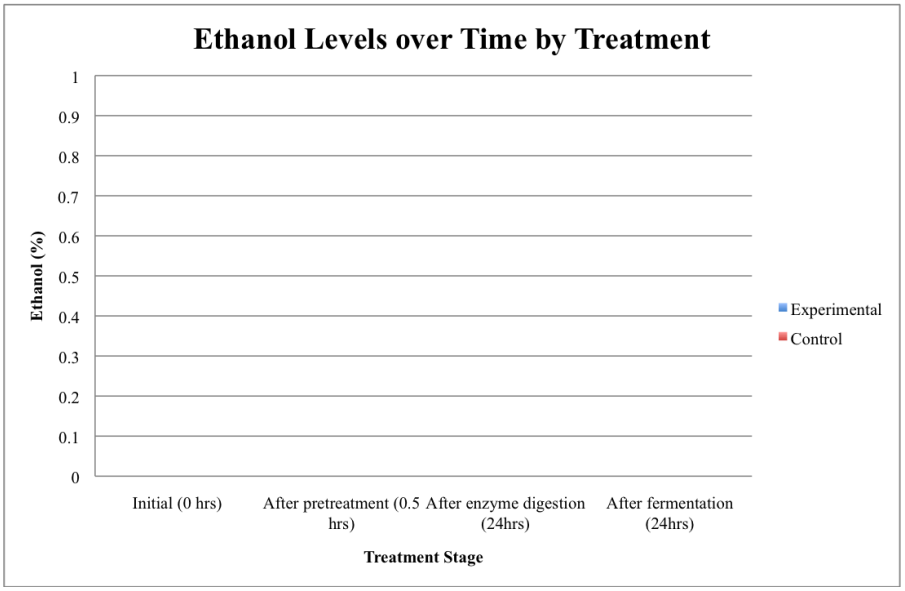
To organize and draw conclusions form your data, it is helpful to compare changes in glucose and ethanol levels over time using bar graphs. Using a computer program such as Microsoft Excel (or by hand), create two bar graphs to summarize your results (Graphs 21.1-2). The empty graphs below can serve 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.

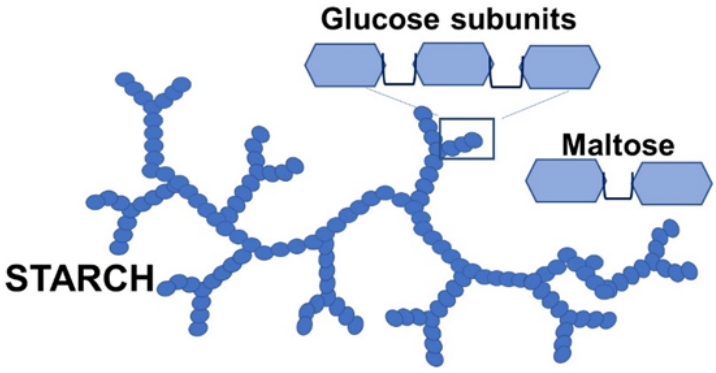


Figure 1: Branching structure of starch.

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 ^{o}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 (ºC)	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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SECTION OVERVIEW

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