

REYNA GENETICS LAB - SYNTHETIC BIOLOGY CURE



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Reyna Genetics Lab- Synthetic Biology

CURE

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TABLE OF CONTENTS

Licensing

About the Authors

About this Book

1: Inoculation

2: Lab Safety and Laboratory Notebook

3: Tips for Organizing the Lab for Large Classes

4: Visual Representation of the Protocol

5: Plasmid DNA Extraction (Mini-Prep)

6: Appendix III- Using the Nanodrop

7: DNA Double Digestion

8: Gel Loading and Running

9: Ligation of Products

10: Cell Transformation

11: Appendix I- Antibiotics and Color Coding

12: Appendix V- Common Agarose Gel Questions and Tips

13: Before you begin a semester overview

Index

Glossary

Detailed Licensing

Licensing

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About the Authors

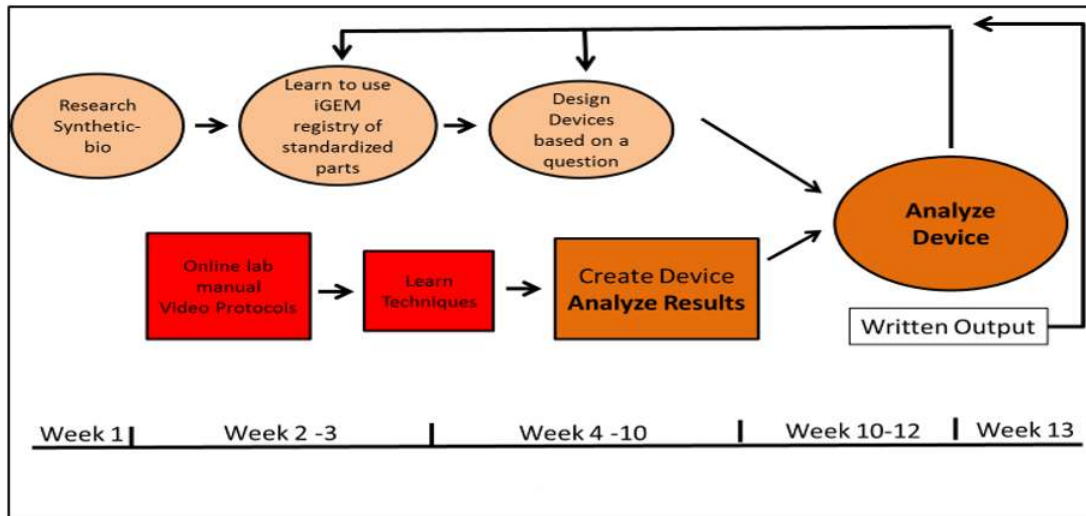
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About this Book

The protocols included in this lab manual are part of a Synthetic Biology undergraduate research course (CURE) designed by the authors. The lab presented in this manual is designed for a Genetics or Biotechnology class and has been used at both two-year and four-year institutions. The workflow for the semester long course is shown below. The lab is based off the iGEM genetic engineering competition (iGEM.org). Students are tasked with designing and then engineering a biological device in a bacterium (*E.coli*) using standardized DNA parts called Bio-Bricks from the iGEM registry. The process of engineering the device requires iteration of standard molecular biology cloning techniques which are outlined in this lab manual, some including video protocols. Additionally, provided are appendices with recipes for reagents, supply/resources list, and instructor recommendations. Instructors are encouraged to watch a series of implementation [videos](#) created for the Arkansas-CURE Projects Synthetic Biology faculty training workshop.



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1: Inoculation

Inoculation of Overnight Cultures

Small sample of bacteria is taken from a plate and placed in LB broth to be used in ZymoPure miniprep or for long term storage.

Materials	Storage Temperature
Sterile Toothpicks	Room Temp
LB Broth	Room Temp
Isolated Colony of Transformed Bacteria	Room Temp

Procedure:

Using sterile technique, pick an isolated colony from a fresh plate (less than seven days old) and inoculate LB-Miller medium.

1. You will use a **sterile toothpick** to pick the colony, and you can simply drop the entire toothpick into the liquid culture tube.
2. **Sterile 5 mL** of **LB** broth will be provided for you for this step. You may add the required antibiotic to the LB prior to inoculating with your colony (**optional**).
3. Incubate with shaking for **8–16 hours at 37°C** before harvesting. This results in maximum yields of a high-copy-number plasmid.

(Typically, after overnight incubation, the absorbance of a tenfold dilution of the culture at a wavelength of 600nm (A600) with a 1cm path length should range from 0.10–0.35.)

These cultures can now be used in the Mini-prep procedure or stored long term in glycerol. (See Appendix VI for how to make your own glycerol stock.)

Growth of Overnight Cultures – Helpful Hints if you are having difficulty.

Different culture media will also have a profound effect on the growth of different bacterial strains. Most plasmid DNA purification systems are appropriate for bacterial cultures grown in 1X Luria-Bertani (LB) medium. However, use of a LB-Miller medium containing more NaCl will produce significantly greater yields and is highly recommended. Richer media such as 2X YT, CIRCLEGROW® or Terrific Broth may be used to increase plasmid yields by increasing the biomass for a given volume of culture. Keep the biomass in a range acceptable for the plasmid isolation system used, as overloading may result in poor purity and yield of the plasmid DNA

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2: 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:

LAB NOTEBOOK

Your Name

INTRO TO BIOTECH LAB

Professor's Name

Semester/Year

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.

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?

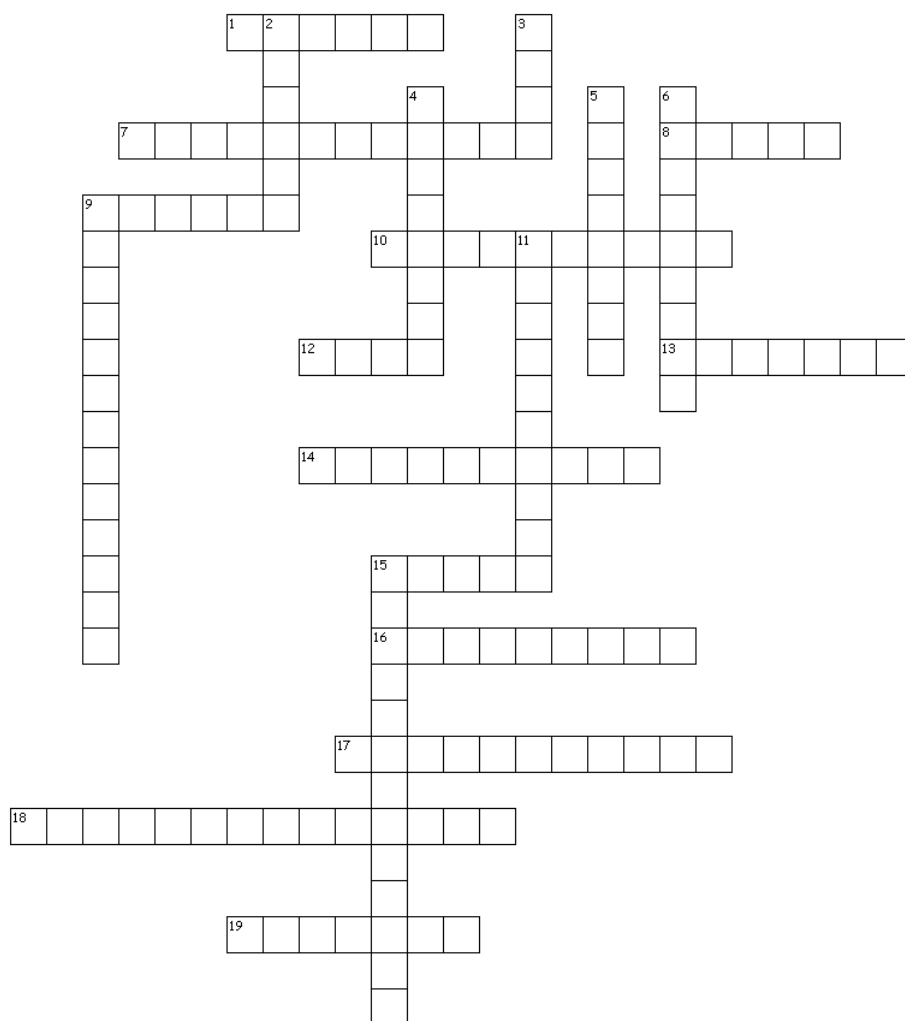
11. What are the Do's and Don'ts of keeping a legal notebook?

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

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
9. broken test tubes/beakers should be swept with a ____ and dust pan
10. COR on the NFPA sign
12. instrument used to write in a scientific lab notebook
13. used razors, scalpels and needles should be placed in the ____ waste container
14. SOP stands for Standard ____ Procedures
15. rating number for the most dangerous hazards in the NFPA sign
16. ALK on the NFPA sign
17. GLP stands for Good ____ Practices
18. GMP stands for Good ____ Practices
19. hazard that the blue color on the NFPA sign indicates

DOWN

2. the lab notebook is considered a ____ document and can be used in court
3. abbreviation for Personal Protective Equipment
4. shape of sign that lists the chemical ratings from the National Fire Protection Association
5. hazard that the white color on the NFPA sign indicates
6. table found at the front of a scientific lab notebook
9. type of waste containing recombinant DNA and tissue culture dishes
11. OX on the NFPA sign
15. hazard that the red 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
- Ionizing Radiation sign via Wikimedia Commons; public domain
- Laser Radiation sign via Wikimedia Commons; public domain
- Explosive substances sign via Wikimedia Commons; public domain
- High Voltage sign via Wikimedia Commons; public domain
- No eating or drinking sign via Wikimedia Commons; public domain
- Nonpotable water sign via Wikimedia Commons; public domain
- Do not enter sign via Wikimedia Commons; public domain
- No smoking sign via Wikimedia Commons; public domain
- Corrosive Substances sign via Wikimedia Commons; public domain
- Poisonous substances sign via Wikimedia Commons; public domain
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3: Tips for Organizing the Lab for Large Classes

Preparing for a Class

Example of a Lab Room

Pre-established Freezer Boxes:

It can be helpful to have some already stocked freezer boxes ready for your students before they get into the lab. This can work both for individual students as well as teams. These freezer boxes can be marked via color and assigned to students when the lab has started. The boxes can be pre-stocked with the various reagents your students will need so they will be able to have ready access to them.

This CryoSafe Cooler Box ([Cat# 03-410-497](#)) should consist of the following reagents:

- Restriction enzymes (EcoRI, SpeI, XbaI, PstI)
- 10x NEBuffer 2.1
- 10x DNA Ligation Buffer
- T4 DNA Ligase

It is helpful to have another color coded cardboard freezer box that is empty alongside the reagent box. This way your students will ideally have no problem storing their parts as they move through the protocol as well as ensuring fewer mixups between the various student parts as they are stored.

Pre-established Student/Team Work Stations:

Much like the freezer boxes, pre-stocked work stations allow for you to be able to provide your students with access to tools/materials such as micropipettes and microcentrifuge tubes. It is helpful to color coordinate a student/team work station with a freezer box.

This work station should consist of:

- A set of micropipettes (ranging from 1-10 uL to 100-1000 uL) per student
- At least one tube rack
- A container of microcentrifuge tubes
- A Zymo Mini-Prep kit (this can be the kit itself or pre-aliquoted amounts of the reagents)
- Sharpie
- Pipet tips (a box per type of micropipette)
- Any other room temperature reagents you would want easily accessible

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CHAPTER OVERVIEW

4: Visual Representation of the Protocol



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5: Plasmid DNA Extraction (Mini-Prep)

Zymo-Pure Plasmid DNA extraction (Micro-Centrifuge Method)

Learning Objective

- Isolation of Plasmid DNA from overnight cultures in LB.

This method is spin column-based and purifies up to 100 μg of ultra-pure endotoxin-free plasmid DNA in less than 15 minutes. The result is plasmid DNA suitable for transfection, restriction endonuclease digestion, bacterial transformation, PCR amplification, and DNA sequencing. ([ZymoPURE Plasmid Miniprep Kit](#))

Note

Watch the Youtube video for questions about the Miniprep protocol



[ZymoPURE Plasmid Miniprep video](#)

Note

The P1 reagent is temperature sensitive due to RNase being present, and should be kept in the fridge or on ice at all times. All other reagents will be stored at room temperature.

Reagent name and Code	Storage Temperature
ZymoPURE™ P1 (Red)	4°C
ZymoPURE™ P2 (Green)	Room Temp.
ZymoPURE™ P3 (Yellow)	Room Temp.
ZymoPURE™ Binding Buffer	Room Temp.
ZymoPURE™ Wash 1	Room Temp.
ZymoPURE™ Wash 2	Room Temp.
ZymoPURE™ Elution Buffer	Room Temp.
Zymo-Spin™ II-P Columns (must have purple ring)	Room Temp.
Collection Tubes	Room Temp.

Visual Procedure of Zymo Plasmid Mini-Prep



TIPS BEFORE STARTING

The first couple of times you do the Mini-Prep, on the protocol indicate/LABEL **WHERE your DNA is** for each of the steps! (e.g., DNA is in the supernatant/liquid OR DNA is in the pellet). Each step below has a “circle one” option asking where the DNA is! (pellet or supernatant).

Note

If you *understand exactly where your DNA is in each one of the steps*, you won't LOSE your DNA!!

Protocol: (make sure you have all reagents before you begin... get ice!!!)

- Using a serological pipet, pipet out **1.0 to 3 mL** (preferably 3 ml) of bacterial culture into a 1.5 ml microcentrifuge tube. Centrifuge this microcentrifuge tube at **full speed** for **20 seconds**. *You will see a small pellet form at the bottom of the tube.* (You must **repeat** steps 1-2 to get more than 1 ml worth of cells)

Where is your DNA? ☐ Pellet or ☐ Supernatant

- Remove as much media as possible by pouring it off into the Biohazard bag. **Do not disrupt the cell pellet!** (Repeat steps 1-2 to increase cell concentration: However, 1-2ml of culture should be enough plasmid.)

Where is your DNA? ☐ Pellet or ☐ Supernatant

- Add **250 µL** of **ZymoPURE™ P1 (Red)** to the bacterial cell pellet and vortex or pipet the pellet to resuspend it completely. (**P1 is stored in the fridge at 4°C. Keep on ice when not in use.**) *This step involves RNase to purify the DNA by destroying any*

RNA in the sample.

Where is your DNA? ☐ Pellet or ☐ Supernatant

4. Add **250 μ L** of **ZymoPURE™ P2 (Green)** and immediately mix by gently inverting the tube **6-8 times**. Do not vortex! Let sit at room temperature for **2-3 minutes**. Do not let sit for longer than 3 minutes. (Cells are completely lysed when the solution appears clear, purple, and viscous.)

Where is your DNA? ☐ Pellet or ☐ Supernatant

5. Add **250 μ L** of ice cold **ZymoPURE™ P3 (Yellow)** and mix thoroughly by inversion. Do not vortex! Invert the tube an **additional 3-4 times** after the sample turns completely yellow. (The sample will turn yellow when the neutralization is complete and a yellowish precipitate will form.)

Where is your DNA? ☐ Pellet or ☐ Supernatant

6. Incubate the neutralized lysate in the microcentrifuge tube on ice for **5 minutes**

Where is your DNA? ☐ Pellet or ☐ Supernatant

- Centrifuge the neutralized lysate for **5 minutes** at 16,000 xg (or **Max speed**). (Do not disrupt the white/pelleted cell debris).

Where is your DNA? ☐ Pellet or ☐ Supernatant

8. Transfer **600 μ L** of supernatant from step 7 into a clean 1.5 ml microcentrifuge tube. *Be careful not to disturb the yellow pellet and avoid transferring any white cellular debris to the new tube.*

Where is your DNA? ☐ Pellet or ☐ Supernatant

9. Add **275 μ L** of **ZymoPURE™ Binding Buffer** to the cleared lysate from step 8 and mix thoroughly by inverting the capped tube **8 times**.
10. Place a **Zymo-Spin™ II-P Column (Purple ring)** in a Collection Tube and transfer the entire mixture from step 9 by pouring or pipetting it into the Zymo-Spin™ II-P Column.
11. Incubate the **Zymo-Spin™ II-P/Collection Tube** assembly at room temperature for **2 minutes** and then centrifuge at 5,000 xg for **1 min**. Discard the flow through in the Biohazard bag.
12. Add **800 μ L** of **ZymoPURE™ Wash 1** to the Zymo-Spin™ II-P Column and centrifuge at 5,000 xg for **1 min**. Discard the flow through.
(The collection tube will hold 900ul of liquid. It is important that the flowthrough does not touch the bottom of the column!)
13. Add **800 μ L** of **ZymoPURE™ Wash 2** to the Zymo-Spin™ II-P Column and centrifuge at 5,000 xg for **1 min**. Discard the flow through.
14. Add **200 μ L** of **ZymoPURE™ Wash 2** to the Zymo-Spin™ II-P Column and centrifuge at 5,000 xg for **1 min**. Discard the flow through.
15. Centrifuge the Zymo-Spin™ II-P Column at $\geq 10,000 xg$ (**full speed**) for **1 min** in order to remove any residual wash buffer.
16. Transfer the Zymo-Spin™ II-P Column into a clean 1.5 ml microcentrifuge tube and add **25 μ L** of (warmed 50°C- optional) **ZymoPURE™ Elution Buffer** directly to the column matrix. Incubate at room temperature for **2 minutes**, and then centrifuge at $\geq 10,000 xg$ (**full speed**) for **1 minute** in a microcentrifuge. (warmed buffer and a 5 min incubation may increase plasmid concentrations)
17. Determine the concentration of your sample using a spectrophotometer (E.g. Denovix, NanoDrop, Qubit). (See appendices II, III, IV on how to use one of the aforementioned machines.)

DNA Purity: Eluted DNA is ultrapure, endotoxin-free, and well suited for transfection, transformation, sequencing, restriction endonuclease digestion, *in vitro* transcription, and other sensitive applications.

Typical: Abs 260/280 ≥ 1.8 and Abs 260/230 ≥ 2.0

18. Clearly label each plasmid tube; store it at -20 degrees and record it in the lab notebook!

What should be on your label?

Top label:

Part # (Bba_J0176)

Side label:

Team Name/Initials (SV/JH Team)

Part Name (RBS: GFP)

Concentration (ng/μL) 260/280 ratio

Plasmid Backbone (Antibiotic Resistance) / psB1C3 (Chlor)

(Date) 6/14/2021

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6: Appendix III- Using the NanoDrop

How to Use the NanoDrop



Watch the YouTube videos regarding questions for the NanoDrop.

(<https://www.youtube.com/watch?v=y3ICjycWeCA&t>)

The NanoDrop allows you to measure the concentration of DNA, RNA, and protein in a couple of microliters of the solution. It is best to use 1.5 μ L to 2 μ L for accurate measurements. Some models have the ability to measure fluorescently labeled molecules.

Procedure:

1. Wash the NanoDrop pedestal. Lift the upper arm of the NanoDrop and remove the wipe.
Add 4-5 μ L of purified water to the lower pedestal, then lower the arm.
Wait 30-60 seconds.
Lift the upper arm and use the wipe to vigorously scrub both the upper and lower pedestals.
2. Open the NanoDrop software on the computer by double-clicking the “ND-1000” icon that looks a bit like an hourglass
3. Initialize the NanoDrop:
Click on the “Nucleic Acid” button in the NanoDrop software. This brings up a dialog box; **DO NOT click “okay” until you have added water.**
Add 2 μ L of purified water to the lower pedestal, then lower the upper arm.
Click “okay” on the computer and wait about 20 seconds while the NanoDrop initializes
When it is done, lift the upper arm and dry it off with a wipe
4. Blank the NanoDrop:
Add 2 μ L of the buffer your sample is in (most likely elution buffer).
Lower the upper arm of the NanoDrop and click the “Blank” button on the software.
Wait about 20 seconds for the blank measurement to be made.
Lift the upper arm and dry the pedestal with a wipe
5. Measure your Sample:
Add 2 μ L of your sample to the lower pedestal, then lower the upper arm.
In the “Sample ID” box, type the name of your sample.
Click the “Measure” button on the software and wait about 20 seconds for the measurement to be complete.
Lift the upper arm and dry the pedestal **OR** lift the upper arm and carefully pipet up as much of the sample as you can to retain for further use.
6. Collecting Data:
Write down any measurements you are interested in.
Click the “Print Screen” button to print the complete spectrum if desired.
When finished making all measurements, click “Print Report” to get a table of all data.
7. Cleaning the Pedestal:
Add 4-5 μ L of purified water to the lower pedestal, then lower the arm.
Wait 30-60 seconds.

Lift the upper arm and use a wipe to vigorously scrub both the upper and lower pedestals.

Place a new folded lab wipe on the lower pedestal and close the upper arm.

8. Shut down and close the NanoDrop computer.

More information on what these NanoDrop values mean:

<https://tools.thermofisher.com/content/sfs/brochures/T123-NanoDrop-Lite-Interpretation-of-Nucleic-Acid-260-280-Ratios.pdf>

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7: DNA Double Digestion

DNA Double Digestion Protocol

Two restriction enzymes are used simultaneously to digest DNA in a single reaction.

Reagent name	Storage Temperature
Distilled Water	Room Temp
DNA Sample	4°C
10x NEBuffer 2.1	-20°C
Restriction Enzyme 1 (EcoRI or XbaI)	-20°C
Restriction Enzyme 2 (SpeI or PstI)	-20°C

TIPS BEFORE STARTING:

- As you add each reagent to a 1.5 ml microfuge tube, stir it in well with the pipet tip and always change your tip in between steps - **NEVER double dip your tip!**
- *VERY IMPORTANT!*** Do not dispense the liquids onto the sides of the tube. Always dispense the volumes into the very bottom of the tube!
- Enzymes are stored in glycerol and will never freeze and are ready to be used immediately. They never should be out of the table freezer block or an ice bucket. However, the buffer must be completely thawed before use. (Gently flick the tube and spin the contents to the bottom). You can store the buffers at 4°C but they will only last two weeks. *You must keep up with how long your buffer has been at this temperature!*

Procedure: (Take your time! Make sure you're doing each step correctly!)

- Pipette the following into a 1.5 ml microfuge tube: **Reagents must be added in the specified order (water is always the first reagent to add!).**

25uL final volume reaction system

If your DNA concentration is too low you can increase the reaction volume to 40-50 ul.

X μL of water to get **final reaction volume of 25 μL**

X is equal to 25 - (NEB+DNA+Enzyme 1+Enzyme 2)

2.5 μL NEB buffer 2.1

Y μL DNA

(usually ~300 ng-Use spectrophotometer to determine concentration and calculate volume needed).

Y is equal to 300 ng/DNA Concentration(ng/uL)

1 μL BioBricks enzyme 1 (either EcoRI or SpeI)

1 μL BioBricks enzyme 2 (either XbaI or PstI)

2. Mix well by pipetting slowly up and down approximately 5x. **Be gentle, and do not vortex.** Spin the samples for **5 seconds** in a balanced microcentrifuge, or flick them to collect the mixture at the bottom of the tube.

3. Incubate at 37 degrees for at least 1 hour. Samples can be stored at -20 degrees at this point, but DO NOT forget about step 4 before ligation.

4. After digestion, **incubate your samples for 80°C for 20 minutes (in heat block)**. For 3A assembly it is important you heat inactivate your samples after digestion. **THIS MUST BE DONE or NOTHING ELSE WILL WORK!**

5. Run 5 μL of your digested sample in an agarose gel in order to check that your digestion worked. (See Section 1.4 for making/pouring of agarose gel as well as running a gel electrophoresis). Make sure you run the proper controls with your samples on the gel: (1) a small volume of uncut DNA for each plasmid digested. (2) **Also, always run a lane with DNA Ladder!**

Be sure to clearly label and freeze the remainder of your digestion. This will be used for the ligation step.

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8: Gel Loading and Running

Gel Loading and Running using [MiniOne Instructions](#)

Using agarose gel electrophoresis for the visualization of digested DNA.

[Video example of pouring and running a gel](#)

Reagent name	Storage Temperature
Agarose	Room Temp
1x TBE	Room Temp
SybrGreen/SybrSafe/GelRed	Room Temp (Keep in dark place)
Digested DNA	4°C
DNA Ladder	Room Temp
Loading Dye	Room Temp

TIPS BEFORE STARTING:

Start by making a bottle of 500 ml stock of 1x TBE ($C1V1 = C2V2$) for your team's running buffer and to make agarose gels. (You can store this for future use- label the bottle clearly!) You will utilize either 10X, 25X or 50X TBE stock buffer- Pay attention to what stock solution you have been provided with!

Procedure:

1. You will make 30ml of agarose that is 1X TBE and usually 0.75-1.5% agarose. (**See Appendix V for suggestions on what percentage to use.**)
 - a. Measure ___ g of agarose (0.75-1.5%)
 - b. Add ___ mL of 1x TBE buffer.
 1. Heat gel for **1+ min** in microwave – (**Use plastic flasks!**). Swirl to make sure all crystals are in solution!
 2. Let the gel cool for 1 minute.
 3. Add **2-3 µL** of SybrGreen, SybrSafe or GelRed (*ask your instructor where this is stored; keep tube in the dark at all times*)
 4. Let the gel cool for another minute - **Do not pour HOT!!!!**
 5. Split warm agarose between the two gel containers (you will only need one). **Do not forget the combs.**
 6. Load **5 µL** of your DNA ladder per gel lane. (*Your DNA ladder should preferably be in the first lane of your gel.*)
 7. Load your samples into the wells of the gel, and mark down in your notebook the order in which you loaded the lanes. a. Loading your samples: **5 µL** of DNA mixed with **1 µL** of 6x loading dye. Load this directly in the well.
 8. Run gel for **20 min** in the Gel rig (set up according to diagrams below). While the gel is running, simulate the digest(s) in your lab (physical/digital) notebook, adding the *Quick-Load Purple 1 kb Plus DNA Ladder* as your ladder and paste the image of the expected gel in your notes. Take a picture of your actual gel results and paste them into your lab notebook.

The gel can be visualized in the gel rig (blue light) or for more sensitivity on the UV light box. WARNING: UV light is dangerous. Do not use unless you have talked to your professor!



See Appendix V for any questions you might have about the agarose gel or the electrophoresis protocol

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9: Ligation of Products

Ligation of Products

Joining of 2 DNA molecules catalyzed by DNA ligase

This process forms a phosphodiester bond between the 3' hydroxyl and 5' phosphate of adjacent DNA strands, creating recombinant DNA. ([NEB](#))

[Video example of performing a ligation](#)

Materials	Storage Temperature
Water	Room Temp
10X T4 DNA Ligation Buffer	-20°C
Linearized Plasmid Backbone (Amp,Chlor,Kan,Tet)	-20°C
Double Digested DNA insert fragments	-20°C
T4 DNA Ligase	-20°C

TIPS BEFORE STARTING:

An equal (1:1) molar quantity of each insert is best. However, 1 μL of each digested insert is commonly used for the ligation and gets consistent results. **If your parts are drastically different in size, you may want to adjust for molar quantity.** (If you are not getting good results/colonies upon transformation, start looking at changing the molar ratio of your inserts. Use [NEBioCalculator](#) to calculate molar ratios.)

Procedure

1. **Thaw 10X T4 DNA Ligation Buffer** at room temperature and then store it on ice until needed. Leave **DNA ligase (enzyme)** in the freezer block until immediately before it is needed; always keep cold!
2. **Always add water and buffer first.** Buffer must be fully thawed and mixed before use:
 - 5 μL of Water (water up to 10ul, if insert volumes are adjusted)
 - 1 μL of **10X DNA ligation buffer**
 - 1 μL (25ng) **linearized plasmid (destination) backbone**
 - 1 μL of **insert A** (digested with appropriate enzymes / denatured)
 - 1 μL of **insert B** (digested with appropriate enzymes / denatured)
 - 1 μL of **T4 DNA ligase** (actual enzyme)
3. Incubate at room temperature for **1hr to 2hr**. Ligations can go overnight or longer if stored at 4 °C (refrigerated). This works the best.
4. Heat inactivate the reaction at **65°C** (or 80°C) for **10 minutes**. (Optional but company recommended.)
5. Samples can be stored at -20°C or used immediately in the Zippy transformation protocol. It is best if you immediately transform the 5 μL of your reaction into a tube of zippy cells. (Ligation buffer can reduce transformation efficiency. Using more than 5ul may actual decrease transformation efficiency)

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10: Cell Transformation

Cell Transformation: Zippy Transformation of Z-competent Cells

Introducing foreign DNA into E. coli bacteria

Bacterial transformation is when cells take up foreign DNA from the environment. Cells may express the genetic information they received from this foreign DNA. ([ThermoFisher](#))

TIPS BEFORE STARTING:

- Depending on the situation, particularly when you get to the analysis phase and you request a different type of bacterial chassis, you will use a variety of competent cells. Be sure you know what cells you are using and if there is a specific protocol for the type of cell. (The competent bacteria described in this protocol are Zippy Competent JM109, [ZymoResearch](#))
- Most plasmids carry a marker gene for a specific antibiotic resistance. By supplementing the growth medium with the antibiotic of choice, only cells containing the plasmid of interest will propagate. **You must know the antibiotic resistance associated with your plasmid backbone!! (See Appendix I)**

All competent cells are stored in the -80 freezer. **Ask the instructor** for help when you are ready to get the competent cells. **You must have ICE, first!**

Materials	Storage Temperature
Zippy Competent Bacteria Cells	-80°C
Ligation Mixture	fresh / -20°C
LB Agar plate w/Antibiotic	Room Temp / 37°C
SOC	4°C / Room Temp
Sterile Glass Beads	Room Temp

Procedure: Read every step before you begin!

- 1) Make sure you have a warm plate.** Pre-warm by placing it in the 37°C incubator or by taking it out at room temperature before starting the procedure. *See color coding (Appendix I) to ensure you have appropriate LB+antibiotic plate type!*
- 2) Get ice, then request cells / Let the cells thaw for 1-2 minutes on ice.** – The 50 µL of cells can be split into **2 tubes of 25 µL** each for two different transformations if used immediately to transform different plasmids. *(If you don't have two plasmids to transform, consider sharing with another team.)*
- 3) Before** the cells are completely thawed add **1-5 µL** of ligation (plasmid) mixture directly to the cells (do not add to the side of the tube). For best results add the plasmid ligation to the cells while they are still slushy.
- 4) Incubate on ice for 5 minutes.**
- 5) Add 200 µL of SOC media** with no antibiotic to the cell-ligation mix.
(SOC amount = ~4X volume of cell + plasmid mix; thus only 120ul needed for 25ul transformation)
- 6). Incubate with gentle shaking (200-300 RPM) for 1-2 hrs at 37°C.** *(1 hr will work well but you get a higher transformation efficiency with 2 hr out-growths).* **Skip this step if using Ampicillin resistance and proceed directly to step 7.**
- 7) Spread transformed cells using glass beads onto a LABELED warmed (22-37°C) plate containing antibiotic.** Allow plates to dry (37°C) before flipping plates upside down. *(You do not want media/bacteria dripping onto the lid. You can flip plates early the next day, if needed.)*
- 8) Incubate overnight (18-20 hrs) at 37°C.** The next day, remove plates from the incubator, seal the lids with parafilm and store at 4°C.

The next day: All colonies should be about the same size (diameter). It is not uncommon with 3A assembly to see only 1 to 10 colonies.

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11: Appendix I- Antibiotics and Color Coding

LB Plates plus antibiotics (color coding)

Green = LB + Ampicillin^{100*}

Black = LB + Chloramphenicol^{35**}

Blue = LB + Kanamycin^{50***}

Red = LB + Tetracycline^{10****}

Antibiotic Selection

Ampicillin (Amp/A) A derivative of penicillin that kills growing cells by interfering with bacterial cell wall synthesis. The resistance gene (*bla*) specifies a periplasmic enzyme, β -lactamase, which cleaves the β -lactam ring of the antibiotic.

**Working concentration 100 μ g/mL; freezer stock 100mg/mL (in water)

Chloramphenicol (Chlor/C) A bacteriostatic agent that binds to the 50S subunit of the ribosome and blocking peptidyl transferase and inhibiting peptide bond formation. The resistance gene, chloramphenicol acetyltransferase (*cat*), specifies an enzyme that renders the antibiotic incapable of binding to the ribosome.

**Working concentration 35 μ g/mL; freezer stock 35mg/mL (in EtOH)

Kanamycin (Kan/K) A bactericidal agent that binds to 70S ribosomes and causes misreading of messenger RNA. The resistance gene (*kan*) specifies an enzyme (aminoglycoside phosphotransferase) that modifies the antibiotic and prevents its interaction with ribosomes.

***Working concentration 50 μ g/mL; freezer stock 50mg/mL (in water)

Tetracycline (Tet/T) A bacteriostatic agent that binds to the 30S ribosome and disrupts codon and anticodon interactions. The resistance gene (*tet*) specifies a multimeric transporter protein that participates in the rapid efflux of tetracycline from the bacterial cell rendering it incapable of accessing the ribosomes.

****Working concentration 10 μ g/mL; freezer stock 10mg/mL (in water)

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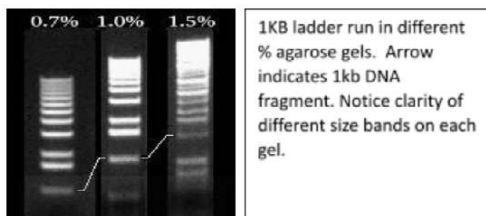
12: Appendix V- Common Agarose Gel Questions and Tips

Common Agarose Gel Related Questions and Tips

(<http://www.lifetechnologies.com/us/en/home/life-science/pcr/elevate-pcr-research/agarose-content-with-tips-and-tricks.html#2>)

1. What Percent Agarose is best?

The standard percentage of agarose used to run a DNA gel is usually around 1.0%. A higher agarose percentage enhances the resolution of smaller bands; conversely, a lower agarose percentage gives better resolution and separation of higher-molecular-weight bands. If the wrong percentage is used, it can be difficult to visualize the DNA bands reliably



% Gel	Optimum Resolution/ Linear DNA (kb)
0.5	30 to 1.0
0.75	12 to 0.8
1.0	10 to 0.5
1.2	7 to 0.4
1.5	3 to 0.2

For very small parts, 2% gel may be an option. Talk to your instructor about it!

2. How does gel casting affect the band resolution?

The recommended thickness for agarose gel is **3–4 mm**; a gel thicker than 5mm will result in fuzzy bands and higher staining background. Similarly, the amount of running buffer to cover over the gel in an electrophoresis apparatus is 3–5 mm. Too much buffer will decrease DNA mobility and cause band distortion. The thickness of the comb is also important and significantly affects resolution. A thin comb (1 mm) gives very well-defined bands, while a thick comb gives thick bands leading to reduced resolution.

3. How much DNA to run (Less is best)

The minimal quantity of DNA that may be detected is dependent on the stains used. The maximum quantity of DNA in a band that is still clear and well-defined is approximately 100 ng.

4. What is the right buffer for your agarose gel electrophoresis?

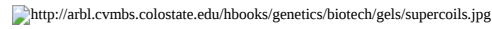
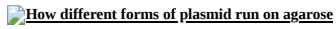
The two most popular types of buffers for running agarose gels are Tris-acetate with EDTA (TAE) and Tris-borate with EDTA (TBE). **For small DNA (<1000 bp), and if there is no plan to extract the DNA, then 1x TBE buffer is recommended.** TBE buffer has a high ionic strength and buffering capacity. TAE buffer, together with low field strength (1–2 V/cm), is preferred for separating large DNA (12–15 kb). TAE buffer interacts with agarose, resulting in lower electroendosmosis, larger apparent pore size, and lower field strength compared to agarose gels in TBE buffer. **Buffer must be added to the GEL.**

5. Why do I see so many bands?

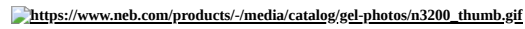
Uncut DNA will typically appear to run faster than fully linearized plasmids. Isolated, uncut plasmid should be in the supercoiled form. The use of restriction enzymes will linearize our plasmids.

(<http://bitesizebio.com/13524/how-to-identify-supercoils-nicks-and-circles-in-plasmid-preps/>)

How to identify supercoils, nicks and circles in plasmid preps

DNA Ladder (Quick-Load Purple 1kb Plus DNA Ladder, New England Biolabs)

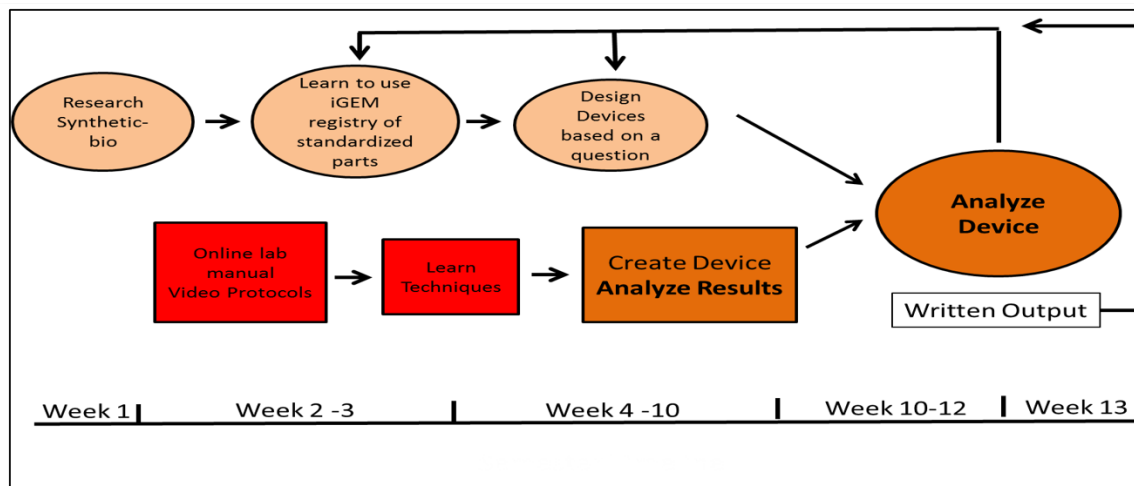


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13: Before you begin a semester overview

The protocols included in this lab manual are part of a Synthetic Biology undergraduate research course (CURE) designed by the authors. The lab presented in this manual is designed for a Genetics or Biotechnology class and has been used at both two-year and four-year institutions. The workflow for the semester long course is shown below. The lab is based off the iGEM genetic engineering competition (iGEM.org). Students are tasked with designing and then engineering a biological device in a bacterium (*E.coli*) using standardized DNA parts called Bio-Bricks from the iGEM registry. The process of engineering the device requires iteration of standard molecular biology cloning techniques which are outlined in this lab manual, some including video protocols. Additionally, provided are appendices with recipes for reagents, supply/resources list, and instructor recommendations. **Instructors are encouraged to watch a series of implementation [videos](#) created for the Arkansas-CURE Projects Synthetic Biology faculty training workshop.**



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 - 4: Visual Representation of the Protocol - *Undeclared*
 - 5: Plasmid DNA Extraction (Mini-Prep) - *Undeclared*
 - 6: Appendix III- Using the Nanodrop - *Undeclared*
 - 7: DNA Double Digestion - *Undeclared*
 - 8: Gel Loading and Running - *Undeclared*
 - 9: Ligation of Products - *Undeclared*
 - 10: Cell Transformation - *Undeclared*
 - 11: Appendix I- Antibiotics and Color Coding - *Undeclared*
 - 12: Appendix V- Common Agarose Gel Questions and Tips - *Undeclared*
 - 13: Before you begin a semester overview - *Undeclared*
 - Back Matter - *Undeclared*
 - Index - *Undeclared*
 - Glossary - *Undeclared*
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