BIOL1414: INTRODUCTION TO BIOTECHNOLOGY I

Exercise Workbook & Lab Guide

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2019-2020

ACC BIOTECHNOLOGY

Acknowledgments

This manual could not have been possible without the dedication of the faculty, staff, and students of Austin Community College's Biotechnology Program, in addition to our valuable educational partners, and numerous grants. Some lab material provided by the following educational partners: Bio-Link, Bio-Rad, Dolan Learning Center, and Edvotek. A special thank you to our amazing lab technicians Evelyn Apple and Jeremy Garza.

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Funding

This material was developed with the assistance of the following grants:

1. Sabbatical Funding (2018-19), Austin Community College
2. Bio-link award number 9850325 (2014-2016), 1400721 (2013), 0903317 (2010)
3. Texas Education Agency (2004-2015)
4. National Science Foundation, Award Number 0053296 (2004-2006)

Table of Contents

LAB UNIT 1: INTRODUCTION TO BIOL1414

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OBJECTIVES

In this lab activity, students will:

- ✓ Describe biotechnology
- ✓ Identify potential careers in biotechnology
- ✓ Create a lab notebook to use throughout the semester
- ✓ Learn proper PPE and lab policies expected during every lab class
- ✓ Complete lab-specific safety and security training
- ✓ Locate safety equipment & laboratory instruments that will be used throughout the semester

Welcome to your first course in biotechnology! What an exciting time to learn about biotechnology! Whether you are taking this course for general interest or you want to obtain a job working in a biotechnology lab, this course is designed to provide you with a foundation of biotechnology skills. Throughout this course, students will:

- ✓ Discover the field of biotechnology, and potential careers in the biosciences
- ✓ Learn basic laboratory techniques of a biotechnology or bioscience lab
- ✓ Develop skills in using basic biosciences laboratory equipment
- ✓ Cultivate critical thinking skills
- ✓ Encourage teamwork, accountability, and taking charge of your learning
- ✓ Practice accuracy in calculations, performing experiments, and in writing scientifically
- ✓ Learn how to work in a regulated bioscience work environment

This workbook is designed to provide students with an active learning discovery experience. Please bring it to every class. It will guide you through the course and tie in concepts between the lecture and laboratory. Before every class, students may be asked to read a chapter from the textbook, research information online, watch a video or animation, or complete quiz exercises. It is important to follow the syllabus schedule to plan and complete assigned activities. In this first lab unit, students will begin exploring the field of biotechnology and become acquainted with lab organization, policies, and safety training.

Before working in an ACC laboratory, students must first:

1. View the ACC Science Safety video.
2. Review safety policies and procedures.
3. Tour the laboratory with the instructor and locate emergency equipment.
4. Sign a safety contract, by which students agree to comply with safety regulations.

We hope that you enjoy your experience in this introductory course. The following is a discussion of biotechnology and a description of some of the activities that you may be performing this semester!

PART I: WHAT IS BIOTECHNOLOGY?

Strictly speaking, biotechnology is the use of a living organism to create a useful product. By this definition, biotechnology would date back to the very beginnings of civilization, when humankind first learned to cultivate crops and domesticate animals in a system of agriculture. When one thinks of modern biotechnology, however, DNA manipulation and pharmaceutical products take center stage. The modern biotechnology revolution came when scientists first learned how to isolate and clone genes, allowing for genetic engineering in the 1970s. However, today, the biotechnology industry has grown and expanded to improve and enrich our daily lives. Below is an excerpt from Bio.org discussing the ever-expanding applications of biotechnology in healthcare, agriculture, and energy (http://www.bio.org).

Biotechnology: Healing, Fueling, and Feeding the World (bio.org, 2014)

At its simplest, biotechnology is technology based on biology - biotechnology harnesses cellular and biomolecular processes to develop technologies and products that help improve our lives and the health of our planet. We have used the biological processes of microorganisms for more than 6,000 years to make useful food products, such as bread and cheese, and to preserve dairy products.

Modern biotechnology provides breakthrough products and technologies to combat debilitating and rare diseases, reduce our environmental footprint, feed the hungry, and use cleaner (and less!) energy, and have safer, cleaner and more efficient industrial manufacturing processes. Currently, there are more than 250 biotechnology healthcare products and vaccines available to patients, many for previously untreatable diseases. More than 13.3 million farmers around the world use agricultural biotechnology to increase yields, prevent damage from insects and pests, and reduce farming's impact on the environment. Moreover, more than 50 biorefineries are being built across North America to test and refine technologies to produce biofuels and chemicals from renewable biomass, which can help reduce greenhouse gas emissions. Recent advances in biotechnology are helping us prepare for and meet society's most pressing challenges.

Let's Explore!

Watch this! New approaches to Biotechnology: 3D Bio-Printing video

https://youtu.be/peeWHtYsmdM

In your own words, what is 3D Bioprinting?

Figure 1-1: 3-D Bio-printing a human ear

WHAT IS BIOTECHNOLOGY?

1. In your own words define Biotechnology:

2. Can you think of a biotechnology product that has improved your life? What do you think your life would be like without this product? What makes it a biotechnology product?

Modern Biotechnology

The modern definition of Biotechnology usually means the manipulation of DNA to create useful products. Deoxyribonucleic acid (DNA) is the carrier of genetic information in our cells. During this course, you will learn to isolate and manipulate DNA from several different organisms, including your own!

Let's try it out! Extract your own DNA!

You can easily isolate DNA from your cheek cell using common materials found at home. Follow the instructions here on this website (Nova). Your instructor may have these materials for you in the lab today or may ask you to try it at home!
http://www.planet-science.com/categories/experiments/biology/2012/03/extract-your-own-dna.aspx

What you need:

500ml bottled water
Dish soap
100 ml isopropyl alcohol or ice-cold Ethanol
2 x clear plastic cups or glasses
1 tbsp. table salt (3 salt packets)
Food coloring

Steps:

1. Add salt to a bottle of drinking water, recap and shake to dissolve.
2. Transfer 3 tbsp of the saltwater into a clear cup or glass.
3. Gargle the saltwater for 1 minute. Don't swallow it!
4. Spit the water back into the cup.
5. Add one drop of detergent to the saltwater. Stir gently with a spoon or swirl. Try not to create any bubbles.
6. In a separate cup, mix the alcohol and 1-3 drop(s) food coloring.
7. Gently pour the alcohol and food coloring mixture on top of the salt-water gargle. *Tilt the salt-water cup as you pour, so the alcohol mixture forms a layer on top of the saltwater.
8. Wait for 2.5 minutes. You should see white clumps and strings forming. If not, gently swirl, you should see the white strings forming. The white clumps and threads are your DNA!

Observations: Write down your observations of your DNA extraction experiment:

What's happening? When you gargle the saltwater and spit it back out, some of your cheek cells become suspended in the saltwater. The more vigorously you gargle, the more cheek cells will collect in the saltwater. The dishwashing liquid breaks down your cheek cell membranes and causes the release of DNA into the saltwater. DNA is not soluble in alcohol, so it forms an aggregate where the alcohol and salt-water layers meet. Most other substances from your cheek cells stay dissolved in the salt-water layer. The white strings and clumps you see are thousands of DNA molecules clumped together. Single DNA molecules are far too small to see with the naked eye. When you gargle the saltwater, you are also collecting some bacterial cells from the inside of your mouth, so the DNA you see is a mixture of your DNA and bacterial DNA!

CAREERS IN BIOTECHNOLOGY

Biotechnology Industry in Austin, Texas

The biotechnology industry has also been steadily growing in the Austin area. Today, Austin's bioscience community encompasses over 100 companies that employ more than 7000 people in the areas of research, diagnostics, pharmaceuticals, and medical devices (10). Some of these companies include Life Technologies, Agilent, Asuragen, PerkinElmer, XBiotech, and Luminex to name a few! Austin is also a major contributor to academic research in the biological sciences at the University of Texas and Texas State University.

Let's Explore!

1. Go to this website and explore careers in biotechnology: http://biotech-careers.org/
2. Go to https://www.indeed.com/ or https://austin.craigslist.org/d/science-biotech/search/sci and search for lab technician (biotechnology) jobs in Austin.
3. What jobs seem interesting to you?

PART II: LABORATORY SKILLS AND COMMON COURSE OBJECTIVES

The following list describes the areas of expertise that we will explore in this course. For complete Common Course Objectives refer to Biotech Program Website: http://sites.austincc.edu/biotech/common-course-objectives/

Basic Operations in the laboratory

Students will learn and practice procedures for safe handling and storage of hazardous materials, create and follow detailed protocols, and develop skills in recording data and research notes. Additionally, students will develop solution preparation skills, using basic lab equipment.

Instruments and Equipment

An important part of working in any laboratory is the proper utilization and calibration of instruments and equipment. Students will become learn how to use basic lab equipment, follow SOPs in their operation, and explore equipment validation industry standards; IQ/OQ/PQ. Students will master the use of micropipettes, top-loading and analytical balances, electronic pH meter, gel electrophoresis techniques, and a thermocycler.

Working with DNA and proteins

An important part of a lab technician job is to demonstrate basic techniques for purifying and analyzing biomolecules. Students will isolate and analyze DNA from a variety of cells, which may include plants, human cheek cells, as well as transform *E. coli* with a recombinant plasmid. Additionally, students will also learn basic methods to purify and analyze protein.

Immunochemistry

Students will be introduced to basic techniques used to detect biomolecules using antibodies and will perform the detection and quantitation of a target protein using ELISA.

Regulatory Affairs

Students will write and follow Standard Operating Procedures (SOPs) and competently complete regulatory and biomanufacturing documentation. Additionally, students will learn regulations that govern biological laboratories, demonstrate safety procedures and protocols for disposal of hazardous chemicals and biologicals, and will perform equipment validation (IQ/OQ/PQ) on a variety of basic laboratory equipment.

Bioinformatics

Students will use computers to document and compile information to analyze research data, generate graphs, and utilize protein and genomic databases, such as NCBI, BLAST, NEBcutter, and DNAsubway.

Part III: Documentation: The Lab Notebook & Lab Exercise Workbook

Documentation in a lab notebook is an essential skill for any biotechnician. The Food and Drug Administration's (FDA) philosophy is, "if it isn't written down, it wasn't done." Documentation details vary from lab to lab, but it is essential for one or more of the following:

- ✓ To establish what you will do (planning and commitment)
- ✓ To record what was done (provide traceability)
- ✓ To comply with regulations (prove what was done)
- ✓ To establish ownership

Lab Notebook:

Each student will maintain a 3-ring binder lab notebook to keep the lab manual, experimental notes and forms, and data accrued throughout the semester. The lab notebook is graded. In this course, students are required to record detailed experimental observation notes. Writing down everything improves observational skills, helps with understanding the importance of each step, and provides a record of the actual experimental procedure and if deviations occurred. Although working in groups, each student should record notes in their notebook.

For this class, the lab notebook will be a 3-ring binder, which must include:

1. Title Page: Your name, course name
2. Table of Contents: The notebook should be in chronological order (lab 1, 2, 3…) and tabs to organize each lab.
3. For each lab (in this order):
    - Completed workbook exercises for that lab
    - Any notes, and raw data recorded during the exercise
    - Any forms generated during lab
    - Any handouts from the lab
    - Any data analysis (graphs, tables, charts)
4. You are encouraged to keep any lecture notes in your binder with corresponding workbook exercise.

General guidelines for writing good lab notebooks:

- Write all lab notes and observations in ink. ***Writing with a pencil is not permitted in the lab***. If an error is made, draw a single line through it and enter the correction in clear and legible writing.
- ***Write legibly.*** Remember, coworkers, supervisors, and perhaps, the FDA may be reading these experimental notes.
- Do not re-write notes or type them. Keep all original notes generated in the lab activity.
- If you tape materials such as a graph or image into the notebook, tape all four sides, then write **"NWUI"** ("No writing under insert") across the tape, with initials, and the date.
- Keep records factual, concise, and complete.

PART IV: CLASS FORMAT

BEFORE CLASS

1. Look at the schedule to see which exercise the class will be working on that day.
2. Read assigned textbook chapters and review lecture PPT and material on Blackboard.
3. Complete assigned exercises in the workbook before coming to class. There won't be time to do this in the lab. This includes preparing calculations before class.
4. Completing prelab activities is a safety requirement. Students review potential hazards and protocols to avoid mishaps in the lab.
5. Lastly, students will have more fun if they know what to expect in the lab. Remember, lab work is a learned skill and does take thoughtful planning and practice.

DURING CLASS

1. Before beginning an experiment, students should verify with the lab partner solution calculations, protocol questions, gather reagents, and set up equipment.
2. During lab experiments, students will record experimental notes in ink. Write everything down: What mass was used? What are the supplier and the lot number of the reagent? What balance model and the number was used? Did anything unexpected occur?
3. Be sure to include any changes made to the procedure, even if they were in the instructor's direction.
4. Return reagents and equipment, dispose of waste as directed, clean lab bench and any other areas used, and report any safety hazards or broken equipment to the instructor.

POST-LAB

One week after all the data has been collected, students must submit the workbook exercise for grading. Include:

1. A typed title page with course, instructor, and student name (include lab partner name)
2. Complete *all* workbook activities and handouts.
3. All original experimental notes and data should be included. Do not re-write/type notes.
4. Include all forms completed during the exercise.
5. Analyze data using graphs and tables as directed. Include with the lab report.

Late Work & Missed Classes: Critical workforce skills developed in this course include generating quality work while meeting expected deadlines. Late assignments will be subject to the policy outlined in the syllabus. Please note, students are expected to attend every class. Since we will be performing graded work in every class that cannot be made up later, missed classes will have grade consequences. See the syllabus and notify the instructor if a class is missed.

Lab Competency: Exploring and expanding your understanding of Biotechnology is the most important outcome of this course. However, if you are interested in having a career in this field, competency in laboratory skills is even more important. Note that skill competency is not limited to lab skills, but also includes attendance, punctuality, teamwork, and tidiness. Lab skill is monitored using lab exams, Employability Skills Evaluations, and lab notebook grading.

PART V: LABORATORY SAFETY & SECURITY

LABORATORY SECURITY

Educational institutions and biotechnology companies use a wide assortment of highly hazardous materials. When working with these materials every day, it is easy to forget about the harm these materials can cause if they are stolen. It is not only important for the health and safety of the community, but it is also the law. Laboratory personnel must take specific actions to prevent unauthorized entry to labs, secure highly hazardous materials against theft, and ensure compliance with security regulations.

One easy way to increase security is to make sure that the laboratory door is locked whenever the lab is left unattended, even for a few minutes. Having multiple locked door layers, such as in our laboratory where the chemicals are locked away in a preparation room, is very practical in avoiding theft of hazardous material. Different laboratories implement various security measures, which include locking up controlled substances, balances, computers, equipment and syringes, and needles. Laboratory personnel should review and assess the security of their highly hazardous materials, such as infectious agents, toxins, radioactive materials, acutely toxic chemicals, carcinogens, explosive or reactive chemicals, and compressed gasses.

LABORATORY SECURITY PLAN:

The following guidelines are adapted from Appendix F of the CDC/NIH publication, Biosafety in Microbiological and Biomedical Laboratories, and the following source: "Laboratory Security - UK." N.p., n.d. Web. 06 Feb. 2017 <http://ehs.uky.edu/ohs/labsecurity.html>.

1. Recognize that laboratory security is related to but different from laboratory safety
    - Assess the laboratory for hazardous materials and particular security risks.
    - Develop and implement lab security procedures for a lab group.
    - Train lab group on these security procedures and assign responsibilities.
2. Control access to areas where hazardous materials are used and stored.
    - Close and lock laboratory doors when no one is present.
    - Do not leave hazardous materials unattended or unsecured at any time.
    - Lock equipment (cabinets, freezers) where hazardous material is stored.
3. Know who is in the laboratory area.
    - Limit laboratory access to those individuals who need to be in the lab.
    - Lab workers should wear badges or a means to identify them quickly visually.
    - Restrict off-hours access.
    - Guests should be issued badges and escorted to and from the lab.
4. Know what materials are brought into the lab.
    - Require approval system for materials ordering.
    - Log all hazardous materials in the lab.
    - Dispose of unneeded and aged reagents.
    - Take inventory of all highly hazardous materials regularly.
5. Know what materials are removed from the lab.
    - Track the use and disposal of hazardous materials.
    - Require written permission before the removal of highly hazardous materials.

- Report missing inventory.

6. **Have an emergency plan.**
   - Recognize that controlling access can make emergency response more difficult.
   - Post and review emergency plans with lab personnel. Practice them.
   - Provide emergency responders with information on serious hazards. Post hazards on entryway doors.
7. **Have a protocol for reporting security incidents.**
   - Create procedures for reporting security incidents such as unexpected visitors, missing materials, or unusual or threatening behavior.
   - Train laboratory staff on procedures. Practice them.

LABORATORY SAFETY

Biotechnicians frequently work with a variety of hazardous material in their jobs, which learning how to work with these materials safely is critical. Biotechnicians must employ training, experience, and common sense to stay safe. While the ACC science safety rules are designed to protect learners working in ACC laboratories, students must become self-sufficient in adopting safety concepts as they transition into the biotechnology industry.

Proper Handling & Storage of Chemicals

There is no single simple formula for working safely in the laboratory since each lab facility and situation presents a unique set of hazards. We will be addressing safety issues and provide specific guidelines with each lab unit throughout the semester.

A. SAFETY DATA SHEETS (SDS)

Every chemical in the laboratory should have a Material Safety Data Sheet (MSDS) or Safety Data Sheet (SDS) on file and readily available. Locate the Yellow SDS Binder in the lab. The SDS is a legally required technical document, provided by chemical suppliers, that provides 16 vital elements to understand the chemical hazards. This includes the identification, the hazards, first-aid, and fire-fighting and clean-up measures, to name a few. To learn more, explore the OSHA publication, https://www.osha.gov/Publications/OSHA3514.html. As part of the Pre-lab Exercises, students should look up the SDS of every chemical used to assess safe handling and disposal of reagents properly.

Sections of an SDS:

1. **Identification**: Identifies the chemical on the SDS as well as the recommended uses
2. **Hazards**: Identifies the hazards and the appropriate warning information
3. **Composition & Ingredients:** Ingredients, including impurities and stabilizing additives
4. **First-Aid:** Initial care that should be given by untrained responders
5. **Fire-Fighting Measures:** To deal with fires caused by this chemical
6. **Accidental Release Measures:** Appropriate response to accidental releases, including containment and cleanup, to minimize exposure to people, properties, or the environment.
7. **Handling & Storage:** Guidance on safe handling practices and conditions for safe storage.
8. **Exposure Control/PPE:** Recommendations for personal protective equipment (PPE)

9. **Physical & Chemical Properties:** Identifies all properties, including appearance and odor.
10. **Stability & Reactivity:** Reactivity hazards and chemical stability information.
11. **Toxicology Information:** Health effects and likely routes of exposure.
12. **Ecological Information:** Information to evaluate environmental impact.
13. **Disposal:** How to safely dispose of and what containers to use.
14. **Transport:** How to safely transport
15. **Regulatory Information:** This section identifies the safety, health, and environmental regulations specific for the product that is not indicated anywhere else on the SDS.
16. **Other Information:** This section indicates when the SDS was prepared or when the last known revision was made or other useful information.

B. NFPA (National Fire Protection Association) RATINGS

For more information on codes and standards: http://www.nfpa.org/, Another quick assessment of a chemical's health hazards that is usually available in its container, is a rating by the NFPA. A color-coded diamond shape lists numbers are rating a hazard as:

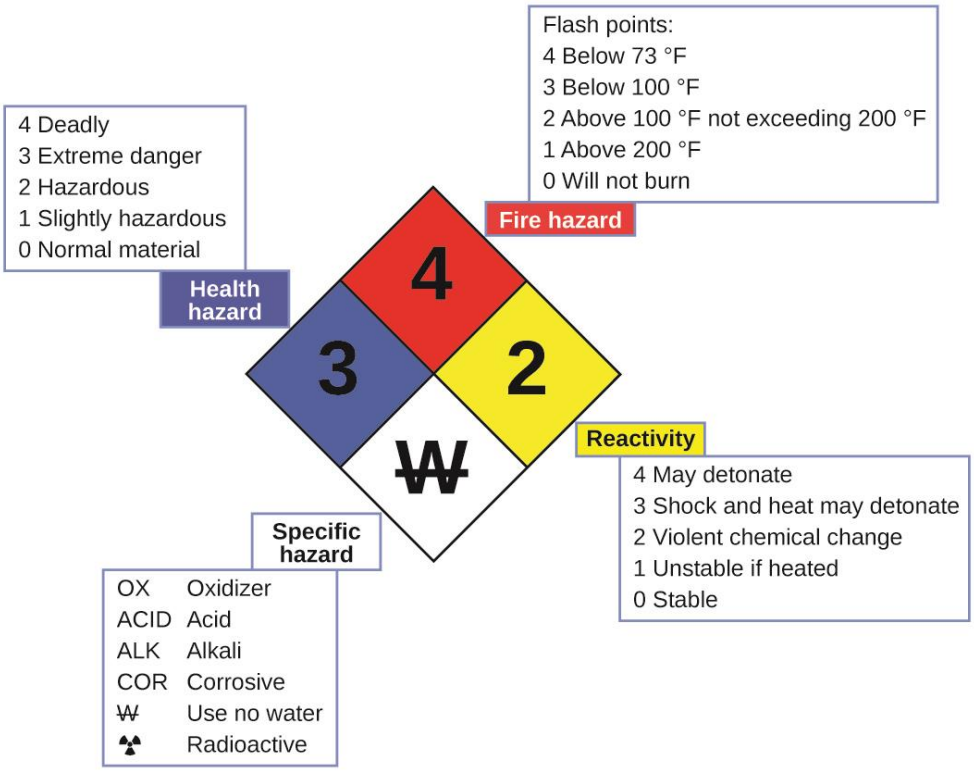


Figure 1: NFPA rating system

Image credit: https://commons.wikimedia.org/wiki/File:CNX_Chem_01_03_HazDiamond.jpg

C. GENERAL SAFETY PRECAUTIONS IN HANDLING HAZARDOUS CHEMICALS

Technicians must be mindful of four routes to hazardous chemical exposure:

1. **Inhalation:** avoid using fume hoods and masks
2. **Skin & eye contact:** avoid using lab coats, gloves, and goggles
3. **Ingestion:** Avoid eating or drinking in the lab, wear PPE, and wash hands upon leaving
4. **Injection**: dispose of broken glass and needles properly

Safe Handling Procedures

- ✓ Investigate the hazards of all reagents in the SDS
- ✓ Label all containers (even temporary) with contents, concentrations, and date
- ✓ Label hazardous material with an NFPA sticker
- ✓ Ensure incompatible chemicals are not combined
- ✓ Clean bench before and after use
- ✓ Wash hands, often and ALWAYS, before leaving the lab
- ✓ Take off lab coats and gloves before leaving the lab
- ✓ Always remove gloves before touching equipment, and change gloves often
- ✓ Ensure proper waste disposal

- **Flammable:** Do NOT heat unnecessarily, and never in the presence of a flame. When storing more than 10 gallons of flammable liquids, use a special explosion-proof storage cabinet.
- **Corrosive:** Wear **personal protective equipment** (PPE) such as lab coats, goggles, and gloves, and always add strong acids or bases to water when making solutions. Neutralize slowly to avoid the rapid generation of heat and gasses. Do not store strong acids and bases together.
- **Reactive:** Wear PPE such as lab coats, goggles, and gloves, and know the reactive properties of the chemical. Always store oxidizing chemicals away from flammable materials.
- **Toxic:** Wear PPE such as lab coats, goggles, and gloves. When working with a toxic dry powder (or irritant), weigh in a chemical hood. Be aware of waste disposal procedures.

D. BIOLOGICAL SAFETY: CONTAINMENT

Biohazards. In this course, students will work with live organisms as well as recombinant DNA and must assess biological hazards. *All live cellular material and tips, tubes, and gloves that come into contact with live organisms must be autoclave-treated before disposal. Recombinant DNA is treated as infectious material and must also be disposed of by autoclave treatment.* Students are provided with lab-specific biohazard handling training and instructions. However, there are some common practices students can use to improve safe working conditions. The routes of exposure to infectious agents are the same as those of hazardous chemicals, such as inhalation, contact with eyes and skin, ingestion, and injection. Therefore, the same general precautions are taken in handling biological hazards for handling chemical hazards.

Here are some general practices to maximize biological safety:

- Limit access to the lab and adequately train all lab personnel
- Use personal protective equipment (PPE) at all times, and keep all PPE inside the lab
- Wash hands after removing gloves and before leaving the lab
- Avoid touching face, light switches, and phones with gloved hands
- Keep personal items such as coats and book bags out of the lab, or in a designated area
- Minimize splashes and aerosol production
- Disinfect work surfaces to decontaminate before and after each lab, and during a spill
- Decontaminate all regulated waste by autoclaving before disposal
- Use a biological safety cabinet when appropriate

E. DISPOSAL OF HAZARDOUS CHEMICALS & BIOLOGICAL MATERIALS

The disposal of hazardous chemicals is subject to state and federal regulations and is overseen by the Environmental Protection Agency. Highly toxic chemicals are regulated, and less toxic chemicals are disposed of through city sewer systems. Biological hazards are contained in autoclave bags made of a high melting point plastic that is sealed and autoclaved at high temperatures and pressures to completely kill any live organisms. Chemical waste disposal containers are stored in the fume hood. Always keep the fume hood on and the lids on the chemical waste disposal containers. ***In our laboratory, specific hazardous chemical and biological waste disposal are discussed at the start of every lab.*** Therefore, students that are late to class will be unable to stay if they miss this critical safety training.

Safety Assignment

1. Watch the safety video as directed by the instructor. It is also available online here: https://youtu.be/uDhSSWtY3kg
2. The instructor will provide a safety-training sheet and will go through this with the class to provide lab-specific safety training. ***Write down all the information in the blanks and keep it available in your lab notebook while working in the ACC laboratory.***
3. Students must sign an ACC Safety Contract to acknowledge that they understand the safety rules and agree to abide by them.
4. Each lab group will perform a safety check today by filling in the Inspection sheet on the next page.
5. Each week a student will be an assigned safety officer. The safety officer will complete the following safety log form. Your instructor will assign groups weekly and provide the group's log sheets. Teams will give a 1 min summary to the class of any safety violations found and how to correct them.
   http://www.austincc.edu/sci_safe/docs/weekly_safety_inspection_log.pdf

Semester Safety Inspection Log

Campus: _____ Room number: _____

Safety Inspector(s): _____

Initial each item if acceptable or comment on necessary improvements. Immediately report problems with any of these items

- Are aprons/lab coats clean and usable?
- Are fire blankets intact and usable?
- First aid kits stocked?
- Are spill kits accessible?
- Goggles & safety glasses used in the lab?
- Safety posters & signs readable?
- Eye-wash station and shower unimpeded and functional?
- "Notice to Employees" present?

Comments:

Date: _____

Signature: _____ printed name: _____

Signature: _____ printed name: _____

PART VI: LAB ORGANIZATION

During this course, students will learn to use, calibrate, and troubleshoot many pieces of equipment commonly used in biotechnology labs. Additionally, students will prepare a variety of reagents. Before getting started, it is important to learn the basic functioning of the lab. This helps with efficiency, cleanliness, and keeping a safe work environment. The ACC Biotechnology program regards lab etiquette as an important part of the curriculum. Showing courtesy to students, staff, and instructors who share the work area by caring for equipment, leaving a clean workspace, and removing biological and chemical hazards are vital workforce skills.

Each student or group should perform the following before leaving the lab after every class:

1. Ensure that any solutions you have made are labeled appropriately according to SOP SOL-001 and that you have created a solution prep form for each. Store them properly in the provided storage location for your class.
2. Replace any equipment, supplies, or reagents that you have used to their proper storage place if other students are not still using them.
3. Clean your work area, which includes removing all items from your lab bench and wiping it with a paper towel wet with bench cleaner.
4. Rinse out dirty glassware before putting where directed. Do not use a sharpie on equipment.
5. Return equipment and reagents to cart, and move rolling cart(s) into the prep room.
6. Turn off all equipment used during the lab by the class. Make sure the scales and scale area are CLEAN.
7. You are required to assist other students who are still working before you leave the lab.

Broken Glass: Please use the blue broken glass boxes for broken glass disposal (used slides and coverslips, Pasteur pipettes, broken glassware). There are a dustpan and broom available to use for sweeping up broken glass. ONLY GLASS GOES IN THE GLASS WASTE – NO PIPETS, PAPER TOWELS, OR USED GLOVES.

Equipment:

- ✓ An equipment locator can be found in the designated file cabinet in the lab room. Use this document to locate supplies and equipment.
- ✓ SOPs written specifically for the equipment in our department are found in the SOP packet that we have provided each semester. You will receive one hard copy at the beginning of the semester for all your classes. Please KEEP this SOP booklet with you.
- ✓ Turn off all equipment before you leave
- ✓ If any equipment is not functioning properly or appears damaged, fill out a deviation report and notify your instructor. Do not return broken equipment; this is a safety hazard.

SAFETY & LAB EQUIPMENT ORIENTATION

1. With your lab partner, explore the Biotechnology Department Laboratories and Prep Room.
2. Using the Equipment Locator Key, try to find the following safety-related materials as well as the equipment and materials that you will be using throughout the semester.
3. *If you do not know what a piece of equipment looks like, it may be hard to find. Go on the internet and look it up! Try Google Images!*
4. If you still cannot find something, ask another group. If you still cannot find it, ask your instructor.
5. Turn these completed in sheets with your lab report.

Safety Related Materials/equipment	Room stored in	Location
Eye Wash Stations (all of them!)		
Fire Extinguishers (all of them!)		
Fire Blankets		
Emergency Gas shut off valve		
Large & Small Glass Waste		
Biohazard Waste		
Liquid Chemical Waste		
General Chemicals		
Spill Kit		
Broom/dustpan		
Material Safety Data Sheets		
First Aid Kits		

Laboratory Materials/equipment	Room stored in	Location
Micropipettes		
Micropipette tips		
1.5mL microcentrifuge tubes		
Picofuge		
Microcentrifuge		
Electrophoresis power supply		
Horizontal Electrophoresis chambers		
Parafilm		
Weigh boats		
Standard pH buffers		
Unopened glove boxes		
Test tube racks		
Graduated cylinders		
Erlenmeyer Flasks		
Freezers (-20^{o}C)		
Refrigerator (4^{o}C)		
Shaker Incubator (37^{o}C)		
NanoDrop Spectrophotometer		
Hotplate/stir plate		
Stir bars		

LABORATORY UNIT 1 ASSIGNMENT

1. **Obtain 3-ring Binder:** This is your lab notebook for this course and will contain the lab manual, pre-lab, and post-lab reports. We recommend a 2-3-inch binder.

   For your workbook 1 submit your binder for grading which must include:
   - a lab notebook title page,
   - table of contents,
   - the lab manual,
   - staple lab unit 1 together and submit with your binder
   - Lab Unit 1 must include:
     - ✓ title page
     - ✓ workbook
     - ✓ completed safety worksheet
     - ✓ the completed safety inspection log
     - ✓ completed equipment orientation worksheet

2. **Obtain a sharpie:** You will need a permanent marker to use in the lab.

3. **Obtain safety equipment:** You will need safety glasses, or goggles rated Z87 (or Z87.1) and closed-toed shoes.

4. **Complete all the exercises in unit 1.**

5. **Watch Safety Video:** The ACC Safety Committee has produced a video explaining safety rules and regulations. You must watch this video. We will do this in class together. It is also available online here: https://youtu.be/uDhSSWtY3kg
   a. **Sign Safety Contract:** You must sign an ACC Safety Contract to acknowledge that you understand the safety rules and agree to abide by them.
   b. **Fill-in Safety Worksheet:** Your instructor will provide you with a safety-training sheet and will go through this with you as a class. This will provide room-specific safety training. ***Write down all the information in the blanks and keep it available in your lab notebook while working in the ACC laboratory.***
   c. **Complete safety Inspection Log**: Include this in your workbook.
   d. **Fill-in Orientation Sheets**: Using the Equipment Locator Key provided, explore the laboratory with your lab partner. Fill in the attached Orientation Sheets. *If there are pieces of equipment you do not recognize, look up pictures on the internet*! Include these sheets with your report.

LAB UNIT 2: ESSENTIAL TOOLS IN THE BIOTECHNOLOGY LABORATORY

J. O'Grady, EdD, L. Fletcher, Ph.D., A. Wheeler, M.S., P. Phelps, Ph.D.

OBJECTIVES

In this lab activity, students will:

- ✓ Identify essential lab equipment
- ✓ Correctly operate a micropipette to accurately and precisely measure specified volumes
- ✓ Accurately follow an SOP to complete a specified task
- ✓ Calibrate a pH meter and operate correctly to measure the pH of a reagent
- ✓ Calibrate an electronic balance
- ✓ Select the appropriate glassware to use to measure a specified volume accurately

During lab training in the ACC Biotechnology program, students will learn to select, use, calibrate, and troubleshoot many pieces of equipment commonly employed in biotechnology labs, and use this equipment to prepare a variety of reagents. In this unit, students will learn how to follow a Standard Operating Procedure (SOP) and use it to calibrate two pieces of basic lab equipment: pH meter and electronic balance. Students will also learn how to select and use the appropriate device for measuring liquid.

Measurement of Volume

There are many different types of glassware used in a biotechnology lab. The glassware used will depend on the purpose of its usage as different glassware has various levels of accuracy of measurement! In the next few labs, students will learn how to choose the correct glassware for the appropriate situation. Learn more here: https://www.ncbionetwork.org/educational-resources/elearning/videos/measuring-volume-beakers-cylinders-erlenmeyer-flasks

- Erlenmeyer flasks are primarily for the preparation or storage of solutions, not an accurate volume adjustment. Although there are volume markings on these flasks, they are not calibrated and should not be relied upon for exact volume measurements.
- Beakers are used for preparing solutions, such as dissolving powdered reagents in water. Beakers are useful when a pH adjustment requires access to the solution by a pH probe. The volumetric markings on beakers are not reliable – and are not an accurate measuring tool.
- Graduated cylinders are calibrated with sufficient accuracy for most volume measurements when preparing solutions. For example, the calibration of most 100 mL graduated cylinder can be relied upon to measure to within +/- 0.6 mL accurately. Graduated cylinders are most frequently used to bring solutions to a final volume.
- Volumetric flasks are used to measure one particular volume with the highest degree of accuracy and are used to make standard solutions for analytical assays. For example, the calibration of a 100 mL volumetric flask can have an accuracy of +/- 0.1 mL. Note, there are no graduated markings in a volumetric flask – you can only bring solutions to one final volume in that flask.

Pipets are glass or plastic devices that are routinely used to measure and transfer liquids by drawing the liquid into the tube with a bulb or mechanical pump.

- Pasteur pipets are small glass tubes used with a bulb to transfer volumes as little as a single drop and as large as a few milliliters. They are not graduated and are not used to measure volumes.
- Beral pipets (transfer pipets) are plastic pipettes with a bulb at one end used for the transfer of liquids. Sometimes they have calibration marks, which have a low level of accuracy. They are often disposable, sterile and individually wrapped.
- Serological pipets are graduated tubes used to measure anywhere from 1 to 50 mL. When the liquid has drained from this pipet, the final drop in the tip is transferred with a puff of air. These are known as TC or to contain pipets. Serological pipets are most frequently used in bioscience labs with pipet controller (or pipet-aid), mechanical devices that aspirate liquid into and out of the pipet.
- Mohr, or "to deliver," pipets are similar to blowout pipets, but do not require a puff of air to deliver the desired volume accurately. They can be identified by the label "TD" on the top. These are sometimes called TD serological pipets.
- Volumetric pipets are not graduated but are calibrated to deliver a single, highly accurate volume.
- Micropipettes are mechanical devices with disposable plastic tips, which deliver with a high degree of accuracy adjustable microliter volumes of liquid. There are several micropipette devices available of varying sizes, such as a 0.5-10ul, 2-20ul, 20-200ul, and 100-1000ul.
- Repeater Pipettes are mechanical devices that can be set to deliver, repeatedly, a precise microliter volume. The liquid is aspirated and dispensed from a disposable tip.
- Multichannel micropipettes can deliver the same volume from as many as 12 tips simultaneously.

Measurement of Weight

Instruments for weighing materials are called balances, and most laboratories have more than one type of balance, depending on the amount of material being measured and the degree of accuracy required.

- Electronic balances usually have a digital readout and weighing dishes can be **tarred** to read zero mass before using. Most balances used for the preparation of solutions have a sensitivity of +/- 0.01 g ("top-loading balance"), but analytical balances can be sensitive to +/- 0.01mg or less. Electronic balances require routine maintenance and recalibration.

How to use an analytical balance: https://www.ncbionetwork.org/educational-resources/elearning/videos/how-use-analytical-balance

Measurement of pH.

Most solutions prepared in the biological laboratory must have a carefully controlled pH. Buffers are prepared by adjustment to a specific pH with strong acid and base solutions, using a meter to monitor the pH. A **pH meter** is a voltmeter that measures the electrical potential between two

electrodes. One electrode is in contact with your solution, and the other is in contact with a reference solution. Usually, both of these electrodes are combined in a single pH probe that you place in your solution. These meters can read to the nearest 0.1 pH unit but require frequent calibration with reference buffers of known pH. Learn more here: https://www.ncbionetwork.org/educational-resources/elearning/videos/ph-meter-calibration

Solution Preparation.

Solution preparation involves mixing liquids and dissolving solids in liquids. There are many specialized devices in addition to balances, volume-measuring devices, and pH meters engaged in these processes.

- Magnetic stirrers come in the form of a box with a magnet inside attached to a motor that spins the magnet. When a vessel containing a magnetic stir bar is on top of the magnetic stirrer, the stir bar rotates and stirs the contents of the container.
- A vortex mixer rotates the bottom of a tube rapidly, setting up a vortex in the liquid that quickly mixes the contents.

Centrifugation

Many pieces of equipment are used to centrifuge biological samples for analysis.

- A preparative centrifuge has a balanced rotor that holds vessels and spins at high speed, up to 20,000 rpm. High-speed centrifugation will cause insoluble particles such as cells, and sometimes subcellular components, to form a pellet at the bottom of the vessel. Rotors are available that hold vessels as small as a few milliliters to as large as a liter. These centrifuges are often refrigerated so that heat-sensitive material, such as cells and proteins, are not damaged due to the high heat generated during centrifugation.
- A tabletop or clinical, the centrifuge is not refrigerated and spins at a much slower speed than a preparative centrifuge. Rotors for clinical centrifuges hold tubes with a capacity of 15 mL or less and a 'swing-bucket' rotor. These are frequently found in medical laboratory testing labs.
- A microcentrifuge holds 1.5mL microcentrifuge tubes with liquid and can centrifuge at high speeds to separate liquids and particulates in solutions. There are a variety of rotor sizes available, and there are refrigerated centrifuges available as well.
- A picofuge is a fixed low-speed microcentrifuge, which spins much slower than a microcentrifuge and functions mostly to move liquid from the sides and top of a microcentrifuge tube to the bottom.

For active learning activities and videos on how to use basic biotechnology lab equipment visit this website: https://www.ncbionetwork.org/educational-resources/elearning

PART I: USING A MICROPIPETTE

Introduction to the micropipette: The micropipette is one of the biotechnician's most frequently used tools. There are different brands of micropipette, such as Brinkmann, Labsystems or Rainin. Each of the brands has its devotees, but all of them work in the same way.

All micropipettes are essentially long tubes with a handle and an adjustable piston inside. A disposable tip is placed on the bottom of the micropipette (or pipette or micropipet - you will see it written in many different ways). This tip is the only piece that is inserted into the liquid. In or near the handle is a screw/knob/button that adjusts the volume of the micropipette by moving the piston up and down. On top, there is a plunger button for filling and for dispensing the liquid. There is often a second button on top for ejecting the tip. Micropipettes come in a variety of sizes; for example, 1-20 uL, 20-200 uL, and 100-1000 uL. When choosing which micropipette to use, the rule of thumb is to ***select the smallest size pipette that can deliver the desired volume.***

A biotechnician is usually issued a personal set of the micropipette and is responsible for cleaning and verifying them regularly. During this semester, you and your lab partner will be assigned a set of pipettes. It is your responsibility to care for and maintain them.

Follow good laboratory practices and make them a habit for every lab:

1. Keep your work area clear of unnecessary items
2. Keep everything you need within reach
3. Gather all materials and set up disposal before you begin working
4. Label each container BEFORE you fill it
5. Change gloves often to avoid contamination and never wear your gloves out of the lab
6. Never perform protocols from memory; always read every step every time you perform a procedure, and then check it off as it is completed
7. Always cap bottles of stock solutions and chemicals when finished

Let's Explore!

1. *Watch this video!* https://youtu.be/uEy_NGDfo_8
2. *Describe below how to use a micropipette to measure 200ul of liquid accurately. Be specific and include tips you learned in the video.*

LEARNING TO USE A MICROPIPETTE

PURPOSE: The purpose of this exercise is to become familiar with some of the essential tools of the Biotechnician. In this lab, you will learn how to handle and operate a micropipette properly; learn to select the appropriate micropipette to measure a particular volume correctly.

SAFETY: In this laboratory exercise, we will not be using any hazardous materials, operations or dangerous equipment. However, it is part of Good Laboratory Practice to always wear close-toed shoes, lab coat, safety glasses, and gloves when working in a laboratory.

MATERIALS

Per Groups:	Per person:
☐ 50 ml conical containing sugar solution ☐ 50 ml conical containing deionized water ☐ 50 ml conical containing colored water ☐ Set of 3 micropipettes ☐ Box of 0.5-10 µL tips ☐ Box of 20-200 µL tips ☐ Box of 100 – 1000 µL tips ☐ Picofuge ☐ Plastic tip waste beaker	☐ 1.5 mL microcentrifuge tubes (13) ☐ Microcentrifuge tube rack

PROCEDURE

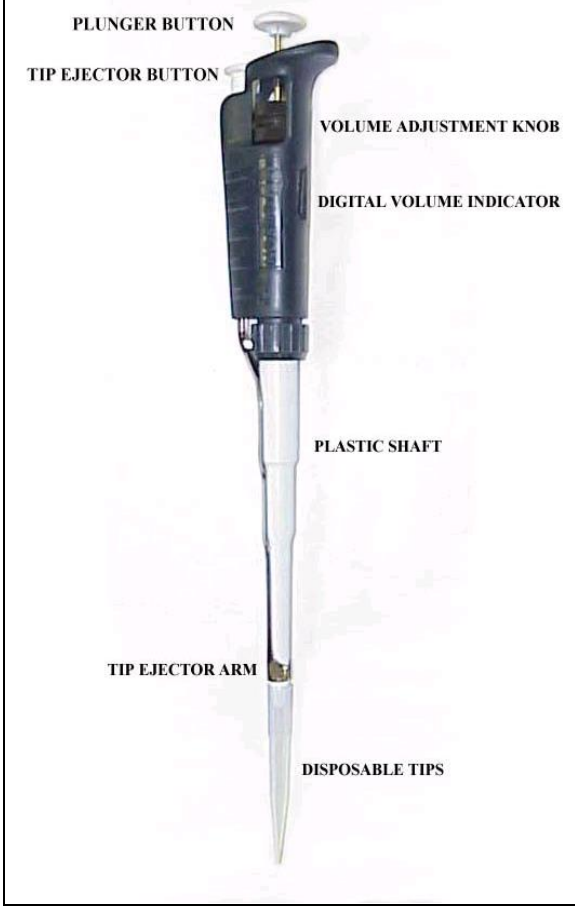
Organizing Your Work Space

1. Before you begin working, clean the benchtop with the cleaner provided by spraying a paper towel and wiping the bench with the wetted towel.
2. Collect everything you will need for the lab. Check off each material as you retrieve it and return it to your bench.
3. Note, each person in your group will perform each of the measurements. *Please obtain a microcentrifuge tube rack with microcentrifuge tubes*.
4. Locate the set of micropipettes you and your lab partner will be using. Ensure there is a complete set, with the same set numbers on them. If you do not have a complete numbered set, let your instructor know.

Draw your workspace:

Familiarizing yourself with the micropipette

Examine the set of micropipettes for your lab group. Notice the volume ranges for the set you have (top of micropipette). Familiarize yourself with the components of the micropipette.



This is a p20 micropipette. What volume is this micropipette set to dispense? _____ µl

Tips for Optimal Micropipetting Technique. Of all the factors contributing to the performance of a micropipette, ***the skill of the operator is the most critical!*** Here are some tips to help improve your micropipette technique. These tips are demonstrated in an excellent video here: http://www.artel-usa.com/tip1.aspx

a. **Pre-wet the tip.** Aspirate and entirely expel liquids three times before dispensing. This increases the humidity within the tip and reduces sample loss due to evaporation.
b. **Dispense liquids at ambient temperature.** Allow liquids to come to ambient temperature before dispensing.
c. **Examine the tip before and after dispensing.** Remove droplets from the side of the tip and ensure there are no air bubbles in the tip before or after dispensing.
d. **Use standard mode.** Depress the plunger to the first stop, immerse into liquid, aspirate by releasing the plunger slowly. Remove the tip from the liquid and depress the plunger to the second stop to dispense the entire contents.
e. **Pause consistently after aspiration.** Pause for 1 second before removing the tip from the liquid. If aspirating a viscous liquid, pause for at least 3 seconds.

f. **Pull the pipette straight out.** This is particularly important for volumes of less than 50µl. If you hold your tip at an angle, it can alter the volume aspirated.
g. **Minimize the handling of the micropipette tip.** Touching the tip with your hand will warm them up resulting in inaccurate aspiration and can lead to contamination.
h. **Immerse the tip to the proper depth.** Immerse a p1000 5-6mm, and smaller micropipettes 2-3mm only. Too little immersion will lead to aspiration of air, too much can result in liquids clinging to the side of the tip, or touching the bottom causing incorrect aspiration volumes.
i. **Use the correct pipette tip.** Use barrier tips when using biohazards or to avoid cross-contamination of your experiment is necessary (such as working with PCR).
j. **Use consistent plunger pressure and speed.** Depress and release the plunger smoothly, slowly, and consistently.
k. **Do not lay micropipettes down on the bench.** Use holders and never turn sideways when liquid is in the tip (hold straight up and down).

Practice operating a micropipette.

Follow the steps below, checking off each step as you go. Each student will perform these tasks independently.

1. Practice setting the volume on the micropipette. Look at the top of the micropipette to identify its measuring range. Remember that the highest value listed on the top is the largest volume you can measure on that pipette. On a 100 to 1000-µL micropipette, the most significant measurable amount is 1000 µL; on a 20-200 micropipette, it is 200 µL. Likewise, the smaller value in the range is the lowest measurable volume; on a 2-20 µL micropipette, the lowest measurable volume is two µL.
2. Set a 100-1000 µL micropipette to 500ul, a 20-200 µL micropipette to 150ul, and a 2-20 µL to 20ul. What are these values in mL?

   500µL _____ mL 150µL _____ mL 20µL _____ mL

3. Obtain 3, graduated 1.5 mL microcentrifuge tubes, and place them in a microcentrifuge tube rack. Close the lids, and label the tops 500ul, 150ul, and 20ul. Each student should have a rack and perform these steps. Obtain a tube of colored sugar solution – you may share with your lab partner.
4. Micropipette 500µL of ***colored sugar solution*** into a 1.5 mL centrifuge tube as follows:
    1. What micropipette will you use for this? _____
    2. Set micropipette to 500ul.
    3. Place the appropriate tip on the end of the micropipette and close tip box lid.
    4. Using one hand, hold the micropipette and press down on the plunger with your thumb to the first stop.
    5. Open sugar solution with your other hand and bring it up to eye level.
    6. Submerge the end of the tip just under the surface of the colored sugar solution and slowly release thumb aspirating up into the tip the colored solution.
    7. Notice that you have no bubbles in the tip. If you do, eject tip into the waste container and repeat.

8. Open 1.5mL centrifuge tube, bring to eye level, and dispense liquid into the tube. Close lid and eject tip into a waste container.
9. Verify the volume on the side of the tube. If it is incorrect, repeat. Keep the tube.

5. Repeat this measuring 150ul. Keep the tube.
6. Repeat this measuring 20ul. Keep the tube.
7. Have your lab partner verify your volumes.
8. Show the instructor all three tubes before you continue!

GLP TIPS!

- ✓ Always keep micropipette tip box lids and microcentrifuge tubes closed. Open only when using, then close immediately. This avoids external contamination into tubes (such as splashing, or dust, or hair or skin cells…).
- ✓ Always label the top of tubes clearly in permanent marker. If your tubes are to be stored or mixed in a microcentrifuge, label with your initials.

Testing Your micropipetting Skill

Each student will perform this task independently.

1. Retrieve a microcentrifuge rack, 10 graduated 1.5mL tubes, and colored solution.
2. Label the top of tubes with a Sharpie 1-10.
3. Using the table below, measure the following amounts into the indicated tubes. Keep lids closed!
4. Mix the contents by 'vortexing' briefly.
5. Use the pecofuge to 'pop-spin' the liquid back down into the tube.
6. Check the graduations on the side of the tube to ensure you have aliquoted the liquid correctly.
7. You can check the accuracy of your measurements by setting a micropipette to the total volume that is supposed to be in the tube and slowly withdrawing all the liquid from several tubes. If the pipetting was accurate, there will be no solution behind and have no air bubble in the tip.
8. Have your lab partner check your tubes.
9. Have your instructor check your work before discarding tubes in general trash.
10. Return all equipment and 50mL conical tubes of colored liquid to the cart.

Tube #	Contents	Tube #	Contents
1	5 µL blue	7	100 µL clear 20 µL blue
2	10 µL blue		
3	100 µL blue	8	500 µL clear 20 µL blue
4	1000 µL blue		
5	5 µL clear 20 µL blue	9	1000 µL clear 20 µL blue
6	20 µL clear 20 µL blue	10	500 µL clear 500 µL blue

Table 2-1: Testing micropipetting skill

PART 2: CALIBRATING AND USING BASIC LAB EQUIPMENT

Different pieces of lab equipment are designed to measure properties such as temperature, pH, mass, and volume to varying degrees of accuracy. If the temperature markings on the side of a thermometer are not set accurately, the instrument's measurements will not be accurate. The accuracy of these markings is due to the calibration of the thermometer.

Calibration, Verification & Validation

The manufacturer calibrates micropipettes before they are sent to you, but they do become less accurate the more they are used. Therefore, the performance of a micropipette should be verified periodically. GMP and ISO laboratories have written policies for performance evaluation for micropipettes.

- Calibration is a process that compares a known (the "standard") against an unknown. During the calibration process, the offset between these two devices is quantified, and the target device is adjusted back to tolerance (if possible). A calibration report usually contains both "as found" and "as left" data.
- Verification is simply the process of "verifying" that a device is within tolerance (within an acceptable range). Verification usually results in "as found" data. If the device is not within tolerance, it is sent for recalibration.
- Validation is a detailed process of confirming that the instrument is installed correctly, that it is operating effectively, and that it is performing without error. Validation is broken into three different tests: the installation qualification (IQ), the operational qualification (OQ), and the performance qualification (PQ).

Some equipment must be periodically calibrated because the settings are not as immovable as lines on a graduated cylinder or thermometer. The calibration of instruments such as pH meters, electronic balances, and micropipettes can be rendered inaccurate by factors such as movement, humidity, dirt, electrical field changes, and many others.

PROPER USE BASIC LAB EQUIPMENT

Use of a serological pipet and electronic pipet-aid. Serological, or "blowout," pipets are graduated tubes used to measure anywhere from 0.1 to 50 mL. They are typically made of plastic and single-use (disposable) and have the top end plugged with cotton to prevent contamination and overflow. When the liquid has drained from this pipet, the final drop in the tip is transferred (pushed out) with a puff of air. These are known as TC or to contain pipets.

When filling a serological pipet, bring the container with the liquid and the pipet to eye level. The tapered end is always held beneath the surface of the liquid. The liquid is drawn into the pipet by suction until the level is just above the volume of liquid to be delivered and then bring the level down to the meniscus of the desired volume. When reading the volume, ALWAYS view the pipet precisely at eye level with the pipet held vertically, perpendicular to the ground. To improve accuracy, pipets are designed to be used with an electronic device called pipet-aid.

Let's Explore!

1. Watch the following video on how to use a serological pipet correctly: https://youtu.be/aei-tU1ZIkE
2. Briefly describe below how to use a serological pipet to measure 5mL of liquid accurately. Be specific.

Calibrating and Operation of an Electronic Balance. The standards used to calibrate electronic balances are objects of known mass. For balances that measure to ± 0.01g, the standard is usually a 200-gram weight. These balances are used to measure amounts of over 0.05 g. When you place the 200-gram weight on balance in calibration mode, the balance recognizes the weight as 200 grams, and will then use that information to measure other masses. Refer to SOP in the SOP booklet on the calibration and operation of our specific balance model (there are several!).

General guidelines to follow when using balances are as follows:

- The amount being weighed determines what type of balance is used in this laboratory (top-loading or analytical). Typically, < 1g is best weighed on an analytical balance.
- Use clean spatulas to weigh out material.
- Never put excess chemicals back into their original containers. Always ask your instructor how to discard excess chemicals.
- Leave a CLEAN balance. Chemicals left on balance will corrode it.
- Turn off the balance when not in use.
- For more information, watch this video: https://www.ncbionetwork.org/educational-resources/elearning/videos/how-use-analytical-balance

Calibration and Operation of a pH Meter: Read the SOP provided in the SOP booklet of the module of pH meter used in your lab. The definition of pH is the hydrogen ion (H+) concentration of a solution. Any solution with a pH < 7 is acidic, and any solution with a pH > 7 is basic. In this laboratory, pH is measured by using a pH meter. The pH meter measuring system consists of a

voltmeter that measures voltage, two electrodes, and the sample that is being measured. When the two electrodes are immersed in a sample, they develop an electrical potential (voltage) that is measured by the voltmeter. ***Review the SOP for "Operation and Maintenance for a pH meter" before class.***

Let's Explore!

Watch this video on calibrating the Acument pH Meter we use in our labs. Follow along with your SOP booklet and make additional notes in your SOP booklet https://youtu.be/UfdKhja6u2I
Draw a flowchart below of how to calibrate a pH meter.

Lab Activity: Calibration of Common Laboratory Equipment

PURPOSE: The purpose of this lab is to calibrate two pieces of equipment; pH meter and a top-loading scale. The calibrated pH meter will be used to determine the pH of an unknown solution. The calibrated scale will be used to determine the most appropriate, and an accurate measuring tool to measure 10 mL.

SAFETY: In this laboratory exercise, we will not be using any hazardous materials, operations or dangerous equipment. However, it is part of good laboratory practice to wear close-toed shoes, lab coat, safety glasses, and gloves when working in a laboratory.

MATERIALS

☐ Large waste beaker	☐ 3 pH standard buffers for calibration
☐ pH meter with calibration SOP	☐ Large weigh boat
☐ A solution of unknown pH	☐ 3, 30mL beakers
☐ top loading balance with calibrated SOP	☐ Color solutions
☐ 1, 10 mL graduated cylinder	☐ Squirt bottle
☐ 200 g mass for balance calibration	☐ Kimwipes
☐ 1, 50-mL beaker	☐ 10- & 5-mL serological pipet
☐ Transfer disposable pipet	☐ Pipet-aid (automatic pipet)
☐ 1, 50mL Erlenmeyer flask	

PROCEDURE

Procedure	Experimental notes
Calibrate a pH meter. Check off each step as you move along. 1. Collect all materials and set up your workbench with your lab partner. 2. Select a pH meter to calibrate and record its model & unit number. 3. Turn on and calibrate the pH meter using the SOP booklet provided. 4. Always rinse the pH electrode with a wash bottle of distilled water, catching the rinse liquid in a labeled waste beaker. 5. Wipe the tip of the electrode with a Kimwipe before inserting into another liquid. 6. After calibration is completed – Did you receive the 'Good Electrode' message? If not, clear attempt and repeat the calibration one more time. If you did not get a 'good electrode' message, ask your instructor for assistance. 7. After calibration is complete, wash probe, tap dry and use to measure the pH of an unknown solution.	pH Meter: _____

Swirl the solution slightly to ensure that it makes good contact with the electrode before recording a reading. 8. Record the pH. 9. Check with the instructor if the pH is correct. If not, verify the pH probe by measuring the pH of buffer 7. Is it close to 7.0? If not, repeat experiment!	Experimental pH: _____ Correct pH: _____
B. Calibrating and Using an Electronic Balance. Check off each step as you move along. 1. Retrieve all materials needed and set up your workbench with your lab partner. 2. Select a balance and record the model number. 3. Follow the instructions in the SOP provided for the balance model to calibrate the balance. 4. Verify calibration by re-measuring 200g weight. 5. Place a weigh boat on the balance pan and press the tare button. 6. Place a 5 mL pipet on an automatic pipet-aid. 7. Open the lid to the colored solution and bring up to eye level. 8. Draw up 4 mL of colored water using a 5 mL pipet using an automatic pipet-aid. 9. Dispense carefully into tared weigh boat. 10. Record the mass of the orange water. Each lab partner will repeat this measurement three times, tare the weigh boat in between each measurement:	Balance: _____ Attempt 1: _____ g Attempt 2: _____ g Attempt 3: _____ g Average:_____ g
Measuring the Accuracy of Glassware. Both lab partners may complete this section together. Record data in the table below. 1. Collect and inspect glassware (ensure it is not chipped or cracked). 2. Place weigh boat on scale and tare. 3. Measure 10 mL of water in a 50 mL beaker using the lines on the beaker for your measurement. 4. Pour the water into the weigh boat and record the mass below. Don't forget your units! 5. Repeat three times. 6. Calculate the average mass of 10 mL of water measured with a beaker. 7. Repeat step 1-5 using a 10 mL graduated cylinder	Scale: _____

8. Repeat steps 1-5 using a 50 mL Graduated Cylinder. 9. Repeat steps 1-5 using a 10 mL pipet. 10. Repeat step 1-5 using a 50 mL flask. 11. Clean up your lab station. Pour the contents of the waste beaker down the sink with plenty of water and return dirty glassware to the cart. 12. Spray and wipe off your lab bench with a paper towel. 13. Return all leftover reagents and equipment where you got them. 14. Ensure the balances, pH meters, and the areas around them are clean and dry and turn off the equipment.	

RESULTS

	50 mL Beaker	10 mL Grad Cylinder	50 mL Grad Cylinder	10 mL Pipet	50 mL Flask
Measurement 1					
Measurement 2					
Measurement 3					
AVERAGE:					

Table 2-1: Measurement of 10mL of liquid using various pieces of lab equipment

Lab Unit 2: Basic Tools in the Biotechnology Laboratory

1. Why should you avoid touching the micropipette tips with your bare fingers?

2. What happens if you push the plunger to the second stop before drawing up the liquid?

3. Why should you always keep the lids on the microcentrifuge tubes closed?

4. Why is it important to verify or calibrate lab equipment before use?

5. The definition of a gram is the mass of 1 mL of pure water at 20˚C (about room temperature) and 1 atmosphere of pressure. Referring to the 4mL pipetting exercise:

What should be the average mass of the water your group measured? _____ g

What was the average mass of the water your group measured? _____ g

6. Summarize the average mass of the 10mL of water you measured (include units) below:

50 mL Beaker _____ 10 mL graduated cylinder _____

50 mL graduated cylinder _____ 10 mL pipet _____

50mL Flask _____

Circle the one you predict to measure most accurately. Put a star beside the one that did measure most accurately.

7. Was your predicted most accurate glassware the same as your measured most accurate glassware? Explain any discrepancies.

CONCLUSION: Write a short conclusion statement for this laboratory exercise.
How successful were you at using the micropipette? In what way(s) can you become more skillful? Which glassware was more accurate in your experiment? Is this an expected result?

LAB UNIT 3: PREPARING SOLUTIONS

J. O'Grady, EdD, L. Fletcher, Ph.D., A. Wheeler, M.S., P. Phelps, Ph.D.

OBJECTIVES

In this lab activity, students will:

- ✓ Accurately calculate and prepare a solution of a given molarity
- ✓ Calculate and perform parallel and serial dilutions
- ✓ Use a microcentrifuge to pellet a precipitate
- ✓ Analyze data by graphing using MS Excel; determine linear regression, R^2 value

A common task for any Biotechnician is solution preparation. This essential skill must be mastered to be a successful technician in the workplace. You will be expected to prepare many kinds of solutions correctly 100% of the time. An incorrectly prepared solution can cost a biotech company a lot of money and time. In a pharmaceutical company, an improperly prepared solution can harm someone.

Let's start with the basics - What is a solution?

It is defined as a solute (smaller amount) dissolved in a solvent (larger amount). The concentration of a solution frequently must be known to a high degree of accuracy. An incorrectly prepared solution can destroy months of hard work or cost companies thousands of dollars. Therefore, companies usually have an SOP (Standard Operating Procedure) for the preparation of each solution to minimize mistakes. All calculations are recorded in the lab notebook, even if a calculator is used. Critical calculations are double-checked by another person (and sometimes triple-checked). The exact mass and volume of reagents used are recorded in the notebook. This information, along with the date and the preparer's name or initials, is recorded on a solution preparation form and on a label on the bottle itself; these forms are provided in the Appendix.

There are several critical aspects to making solutions that should be followed at all times.

- ➢ *Check and recheck each calculation*. It is best if two people make a calculation independently and then cross-check their answers.
- ➢ *Read each reagent bottle twice*, once before using and once afterward, to ensure that the right reagent is used.
- ➢ *Complete a Solution Prep form for every solution you prepare*. Solution prep forms should include detailed and relevant information. See Appendix instructions and example.
- ➢ *Label each bottle before filling.* Use tape and a permanent marker and labeling SOP.
- ➢ *Record any changes observed*, no matter how trivial. Your notes can be used to trace back a problem to its source quickly.

Cleaning Glassware.

Properly cleaning glassware is one of the most critical aspects of the job of a Biotechnician. Improper cleaning can have disastrous and costly consequences for a company! Cleaning glassware for the lab is a lot more involved than just sticking it in a dishwasher. You will need to consider the type of glassware itself, what the dirty glassware was used for, and, most importantly, what is the purpose of the glassware. Note, the water source is a vital part of cleaning glassware!

<u>The five common steps to washing glassware (or plasticware) are as follows.</u>

1. Pre-rinse: Soak or pre-rinse all glassware after use, to prevent the contaminants from drying onto the glassware.
2. Contaminant Removal: Wash using approved detergents and solvents along with scrubbing will help with contaminant removal. Typically, a lab glass detergent, such as Alcon is used. As with all washing, hot water is preferable to cold water. Often lab brushes are used to help wash debris off glassware.
3. Rinse: The rinse step is essential in removing the detergent and cleaning solvents. Many SOP's specify that glassware is rinsed 3 to 5 times in tap water.
4. Final Rinse: Always use purified water for the last rinse and is usually performed 1-3 times.
5. Drying: This is performed by air upside down on a rack or by heat. ***Never hand dry!*** Clean, dry glassware is often stored covered in a closed cupboard to avoid contamination.

A glassware washing SOP is included in your SOP booklet. If you are required to wash glassware in lab class, you MUST follow this SOP!

Why is glassware washing a critical part of a biotech company operation?

PART I: PREPARING A MOLAR SOLUTION

Molarity

Molarity is the most common unit of concentration in the biotechnology lab. The molarity of a solution is defined as the number of moles of solute per liter of solution. The symbol for molarity is M, but it can also be written as moles/Liter, or mol/L. A mole of any element always contains 6.02 X 10^{23} *(Avogadro's number)* atoms. Because some atoms are heavier than others; a mole of one element weighs a different amount than a mole of another element. *The weight of a mole of a given element is equal to its atomic weight in grams.* Consult a periodic table of elements to find the atomic weight of an element.

Practice: Using a periodic table, calculate the molar mass of chromium oxide (CrO_2).
The atomic weight of chromium is 52.00, and that of oxygen is 16.00. Count the oxygen twice because there are two per formula unit of chromium oxide.
ANSWER: 52.00 + 2(16.00) = 84.00 g/mol

> Practice: Using a periodic table, calculate the molar mass of potassium sulfate (K_2SO_4).
>
> Molecular Mass K_2SO_4:_____ Units? _____

We can't directly measure moles, but we can measure mass. To calculate the mass of a chemical needed to prepare a given volume of a solution of desired molarity, you must convert the number of moles to mass, using the molar mass as a conversion factor.

> Mass = molarity x volume x molar mass
> __?__ g = _____ moles/liter x __L x __ g/mole

Don't forget to convert mL to L, if necessary.

Practice: How will you prepare 100 mL of 1 M NaOH (MW 40.0 g/mol)?
Calculations: g = 40 g/mol x 1.0 mol/L x 0.1 L = 4 g

Protocol:

1. Place weigh boat on an electronic balance and press tare.
2. Weight 4.0 g of NaOH and pour pellets into 100 mL beaker
3. Add 70 mL of deionized water and a stir bar.
4. Place on a stir plate and stir until dissolved.
5. Pour into 100 mL graduated cylinder and bring it to 100mL with diH2O. Cover and mix.
6. Transfer to a labeled bottle.
7. Fill in a solution preparation form.

For more practice: https://www.ncbionetwork.org/educational-resources/elearning/interactive-elearning-tools/molarity

PREPARATION OF STOCK SOLUTIONS

PURPOSE: The purpose of this lab is to prepare 2.0M CaCl2 and 2.0 M MgSO4 stock solutions.

SAFETY: Reagents used in this experiment may cause eye irritation if splashed in the eye. Use PPE, including gloves, lab coat, and safety glasses. For disposal, dump down the drain with lots of water separately. When combined, these reagents will form a precipitate and may clog the drain.

MATERIALS

☐ 2, 30 or 50-mL beakers	☐ Calcium chloride – $CaCl_2 \bullet 2H_20$
☐ 2, 25-mL graduated cylinders	☐ Magnesium sulfate – $MgSO_4 \bullet 7H_2O$
☐ labeling tape	☐ Top loading balance with SOPs
☐ permanent marker	☐ 2, spatulas
☐ 50 mL conical (2)	☐ 2, weigh boats
	☐ stir plate & stir bar

PROCEDURE

In this lab procedure, you and your lab partner will split the preparation of both solutions; one will prepare a 2.0M solution of calcium chloride, and the other will prepare a 2.0M solution of magnesium sulfate. Before you begin, perform calculations and check calculation with your lab partner!

Before class calculate the molecular weight:

a. Calcium chloride – $CaCl_2 \bullet 2H_20$ _____ g/ mol

b. Magnesium sulfate – $MgSO_4 \bullet 7H_2O$ _____ g/ mol

When you get to class, and you are collecting your reagents – VERIFY the molecular weight you calculated by verifying the molecular weight of the reagent written on the side of the bottle. Are they the same? Different?

CALCULATIONS:

25mL of a 2M $CaCl_2$ solution	25mL of a 2M $MgSO_4$ solution

Preparation of 25mL of a 2M $CaCl_2$ solution	Experimental Notes
1. Gather all materials needed & label beakers and tubes using tape. 2. Place weigh boat on the scale and press tare. 3. Weigh _____ g of $CaCl_2$ 4. Pour into a 50mL beaker 5. Add 15mL of diH2O to the beaker along with a stir bar. 6. Place on a stir plate and dissolve. 7. Transfer solution to a 25mL graduated cylinder and BTV (Bring to volume). 8. Cover with Parafilm and mix by inversion 9. Transfer to a labeled 50mL conical tube. 10. Fill in solution preparation form.	Scale Model & #: _____ Actual grams CaCl2 weighed: _____ g

Your Turn! Write a protocol below for the preparation of 25mL of 2.0 M MgSO4:

Preparation of 25mL of a 2M $MgSO_4$ solution	Experimental Notes
1. Gather all materials needed & label beakers and tubes using tape. 2. Place weigh boat on the scale and press tare. 3. Weigh _____ g of $MgSO_4$ 4.	Scale Model & #: _____ Actual grams $MgSO_4$ weighed: _____ g

PART II: PREPARING PARALLEL DILUTIONS

Parallel dilutions consist of adding additional solvent (usually water) to a solution to reduce its concentration. There are many ways of expressing dilution factors.

1. Combining one-part food coloring with nine parts of water dilutes the food coloring to 1/10. This means that there is one-part food coloring in 10-parts total volume. The denominator, an expression such as 1/10, is the total volume of the solution.
2. The food coloring dilution above can also be referred to as 1:9 food coloring to the water. The colon (:) means "to." A 1:10 food coloring to water dilution would be 1/11, not 1/10, because the total number of parts is 11.
3. Frequently, stock solutions in biotechnology labs are concentrated and must be diluted before using. A buffer that is ten-fold more concentrated than the usable concentration is referred to as a 10X solution. One must dilute a 10X solution by a factor of 10 (by adding 1 part of the 10X stock to 9 parts of the solvent) before using.
4. In dilutions, parts can be of any unit. If you combine 1 mL food coloring with 1mL water, you are using the same dilution factor (1:1 or ½) as the person who combines 1 ounce of food coloring with 1 ounce of water. If you combine one ounce of food coloring with one liter of water, the dilution factor is not 1:1, because the units are not the same.
5. To dilute a more concentrated stock solution to a less concentrated solution the following formula is used:

$$C_1V_1 = C_2V_2$$

Where,
C_1 = original concentration (of stock solution)
C_2 = final concentration (of diluted solution)
V_1 = original volume (to be taken from stock solution)
V_2 = final volume (of diluted solution)

PRACTICE: Calculate how many mL of a 1.0 M stock solution of NaCl is needed to prepare 100 mL of a 0.050 M solution (also referred to as 50 mM).

$C_1V_1 = C_2V_2$

$(1.0\ M)(?\ mL) = (0.050\ M)(100\ mL)$

$$mL = \frac{(5.0\ M)(mL)}{1.0\ M}$$

$mL = 5.0$

PREPARATION OF PARALLEL DILUTIONS

PURPOSE: The purpose of this lab is to prepare parallel dilutions using CaCl2 and MgSO4 stock solutions. These solutions will be used to determine if there is a linear relationship to the ratio of CaCl2 and MgSO4 with a mass of precipitate produced.

SAFETY: Reagents used in this experiment may cause eye irritation if splashed in the eye. Use PPE, including gloves, lab coat, and safety glasses. For disposal, dump down the drain with lots of water separately. When combined, these reagents will form a precipitate and may clog the drain.

MATERIALS

Each group	*Class Shares*
☐ One, 10 mL graduated cylinder	☐ Top loading balance
☐ 1, 5 mL serological pipet	☐ Microcentrifuge
☐ 1, 10 mL serological pipet	☐ 2, 15 mL conical tubes
☐ 100 – 1000 µl micropipette	☐ transfer pipets
☐ microcentrifuge tubes (9)	☐ $CaCl_2$ & $MgSO_4$ stocks prepared in Part A
☐ microcentrifuge tube rack	☐ permanent marker

PROCEDURE: Parallel Dilutions

*Calculate the volume of the 2M $CaCl_2$ stock solution made in Part A needed to make 10.0 mL of a 1.50 M solution. Prepare the calculations in your exercise book BEFORE you come to the lab. When you get to the lab, verify your calculations with your lab partner before you begin.

CALCULATIONS:

A: 10 mL of 1.5 M CaCl2 (using 2.0 M stock)

B: 10mL of 0.5 M CaCl2 (using 2.0 M stock)

Protocol : Preparing 10mL of 1.5M & 0.5M $CaCl_2$	Experimental Notes
Part I: Preparing solutions: 1.5M $CaCl_2$ 1. Label a 10mL graduated cylinder 1.5M $CaCl_2$ 2. Using a _____ mL serological pipet, add _____ mL of 2.0M CaCl2. 3. BTV to 10mL with dH_2O. 4. Cover and mix. 5. Transfer to a labeled disposable 15mL conical. **Part II: Preparing solutions: 0.5M $CaCl_2$** 1. Label a 10mL graduated cylinder 0.5M $CaCl_2$ 2. Using a _____ mL serological pipet, add _____ mL of 2.0M CaCl2. 3. BTV to 10mL with dH_2O. 4. Cover and mix. 5. Transfer to a labeled disposable 15mL conical. **Part III: Mixing solutions & form precipitate** 1. Label 9 microcentrifuge tubes 1-9. 2. Weigh each tube and record the empty tube weight in a data table below. 3. Pipette 500 µl of 2 M magnesium sulfate stock solution into each tube using a _____ 4. Add the following to the indicated tubes: Tube 1, 2, 3 —500 µl of 2 M $CaCl_2$ Tube 4, 5, 6 —500 µl of 1.5 M $CaCl_2$ Tube 7, 8, 9 —500 µl of 0.5 M $CaCl_2$ 5. Snap caps and invert to mix. 6. Place tubes in a microcentrifuge and ensure they are balanced. 7. Spin the tubes in a microcentrifuge for 5 minutes at 10,000rpm. 8. Gently remove as much of the water as possible using a micropipette or transfer pipet. Do not disturb the pellet. 9. Weigh the tubes with pellets and record in the table below. 10. Perform calculations as outlined in the table. 11. Discard the stock solutions down the sink with plenty of water unless your instructor asks you to save them.	

RESULTS:

Tube Number	CaCl2 Concentration (M)	Pre-weight (g)	Post weight (g)	Weight of PPT (g)
1	2			
2	2			
3	2			
Average				
4	1.5			
5	1.5			
6	1.5			
Average				
7	0.5			
8	0.5			
9	0.5			
Average				

Table 3-1: Weight of precipitate formed when various concentrations of CaCl2 are added to 2.0M MgSO4.

PART III: PREPARING SERIAL DILUTIONS

Serial dilutions are dilutions made from other dilutions, usually because:

- ✓ A series of dilutions with the same dilution factor is desired.
- ✓ The final concentration desired is so small that the original volume (C_1) cannot be accurately measured.

For serial dilutions:

Dilution factor = $(V_1 + V_2) / V_1$

Where V_1 is the volume of the solution being diluted

V_2 is the volume of the solvent used to dilute the solution

(Note: V_2 is also the ending volume of the diluted solution)

Practice: how will you prepare 10, 5-fold dilutions of a reagent with an end volume of 40mL in each of the ten tubes?

Dilution Factor = (V1 + V2) / V1

DF=5

V2 = 40 mL, Find V1

5 = (V1 + 40mL) / V1

5V1 = (V1 + 40mL)

4V1 = 40mL

V1 = 10 mL

PROTOCOL:

1. Using a 50mL graduated cylinder, dispense 40mL of diH2O into each of 10 labeled beakers.
2. Using a 10mL pipet, dispense 10mL of the stock solution into beaker 1 and mix.
3. Using a clean pipet, transfer 10mL of the diluted mixture in beaker 1 into beaker 2 and mix.
4. Continue until beaker 10 – discard 10mL out of beaker 10 to leave 40mL remaining.

IMPORTANT TO NOTE: No, really, this is important, pay attention to this!

a. **The V1 & V2 in this formula is NOT the same as the V_1 & V_2 in $C_1V_1 = C_2V_2$.**

b. **In this formula, V_2 is both the volume of solvent used in each dilution and the final volume of that dilution.**

Why? Once you have made a dilution by adding V_1 mL of the solution to V_2 mL of water, you remove V_1 mL of that dilution to make the next one. Thus, you always end up with V_2 mL in each dilution but the very last one.

PREPARATION OF SERIAL DILUTIONS

PURPOSE: The purpose of this lab is to prepare serial dilutions of HCl and determine the relationship between HCl concentration and pH. These solutions will be used to determine if there is a linear relationship between pH and HCl concentration ranging from 1M to 0.001M HCl.

SAFETY: Concentrated solutions of hydrochloric acid are extremely corrosive. The risk of damage through contact lessens as the acid is diluted; very dilute solutions are only mildly corrosive. The concentrated acid releases dangerous quantities of hydrogen chloride vapor; inhaling this can be extremely harmful. Contact with the eyes or skin can cause severe permanent damage. Always wear safety glasses and nitrile gloves when handling concentrated HCl. Do not allow the acid to contact your skin. The concentrated acid must always be used in an area equipped with proper ventilation. Dispose of waste in WASTE BEAKER IN CHEMICAL FUMEHOOD ONLY.

MATERIALS

Each group	*Class Shares*
☐ Four, 100-mL beakers	☐ 1 M HCl stock
☐ One, 50-mL graduated cylinder	☐ pH meters with SOP
☐ 3, 5- or 10-mL serological pipets	☐ pH standard buffers
☐ labeling tape	☐ 250-mL waste beaker
☐ permanent marker	☐ HCL waste beaker (Chemical hood)
☐ 3, 30-mL beakers	

PROCEDURE: Serial Dilutions

*Calculate the volume of hydrochloric acid (HCl) and water (solvent) needed to prepare a serial dilution of a 50 mL final volume, of each of 0.1 M, a 0.01 M, and 0.001 M HCl solution. Prepare the calculations below **BEFORE** you come to class. Verify your calculations with your lab partner.

CALCULATIONS:

Protocol	Experimental Notes
1. Collect all the materials before you begin. Both you and your lab partner can perform this experiment together. 2. Using tape, label 3, 100 mL beakers with the concentrations of HCl. 3. Using a 50mL graduated cylinder, add 50 mL of diH20 to each of 100mL beaker. 4. Using a _____ mL pipet, dispense _____ mL of 1M HCl to the beaker labeled 0.1M HCl. 5. Add a stir bar, place on a stir plate, and mix. 6. Using a clean _____ mL pipet, transfer _____ mL of 0.1M HCl into the 0.01M HCl beaker and mix. 7. Using a clean _____ mL pipet, transfer _____ mL of 0.01M HCl into the 0.001M HCl beaker and mix. 8. Calibrate a pH meter using calibration SOP 9. Measure the pH of each solution. 10. Create a data table below and record data. Remember to label the data table with a title. 11. Pour your acid solutions into a labeled waste container. 12. Clean up your work area and the area around the pH meters.	pH meter: _____ Temperature: _____

RESULTS:

Using a ruler, create a table below for your data. Include a descriptive title at the bottom.

Table 3-2: *(Provide a title):*

PART IV: GRAPHING DATA

As a Biotechnician, you will often make scatter diagrams and line graphs to illustrate the data that you collect. Scatter diagrams are commonly used to show the relationship between two variables. For example, in an absorbance spectrum, the variables would be the wavelength of light and the amount of light absorbed. Although this data is recorded in a table, a scatter diagram can illustrate more visually the relationship between the two datasets. Most companies use MS Excel to plot graphs; therefore, during this semester, you will prepare your graphs using this software. Your instructor will demonstrate in class.

How do you know which variable is to be on the x-axis, and which is to be on the y-axis? The x-axis should be the independent variable or the parameter that you selected to vary. The y-axis should be the dependent variable or the data that you obtained from your measurement.

Best-Fit Linear Regression.

If you look at your scatter plot and the middle points on the graph are close to forming a straight line, it is reasonable to conclude that the relationship between the independent and dependent variables may be linear. The most appropriate best-fit straight line that illustrates a linear relationship can be determined using a type of statistical analysis called linear regression.

Linear regression is generated using a computer program or a scientific calculator. A correlation coefficient (R2 value) is a mathematical relationship of linearity of the data. The closer to the value of 1 means the data is linear. The closer to the value of 0.85, indicates the data may be random. Depending on the purpose of the data analysis, an R^2 value above 0.95 may be required.

Always follow these guidelines when preparing a graph showing experimental data:

1. A graph should be given a descriptive title to explain the experimental data.
2. Adjust both axes so that the completed graph will nearly fill the page. It is not necessary to have a (0, 0) axis unless this is a data point.
3. Both axes should be labeled and marked with appropriate units of measurement.
4. The x-axis should show the independent variable, variable that the experimenter chooses, and can change. The y-axis should show the dependent variable, the one that the experimenter observes.
5. Draw a line on a scatter plot to illustrate a potential relationship. If there is a linear relationship between the independent and dependent variable (R2>0.95), draw a best-fit straight line through the points that are consistent with the linear relationship. If there is no linear relationship, leave the scatter plot as is, or draw curved lines between the data.

Graph a semi-log plot. Your variables may not have a linear relationship, in which case a straight line cannot represent your data. In the biological sciences, the relationship may be exponential rather than linear. This means one value doubles for each single-unit increase in the other value. For example, each time a cell divides, the number of cells is doubled. This means that if you repeatedly count the number of cells in culture over a given interval of time, the cell count will

not rise linearly with time, but rather exponentially with time. On a semi-log graph, the X-axis is typically linear (each increment is spaced equally and represents an equal unit of measurement), but the Y-axis is exponential (each increment is NOT spaced proportionately and does NOT equal the same unit of measure).

Graph with Microsoft Excel. In the workplace, you will never graph by hand. Using a computer-based graphing program is an essential skill of any Biotechnician. During this semester, students will have many opportunities to hone this skill by practicing graphing different types of data using various types of graphs. The most common graphing program used in this field is MS Excel and tutorials can easily be found online. A thorough tutorial on graphing data will be provided in this course.

Students may also attend Biotechnology Program Open Labs, and the Learning Lab to get individual tutoring assistance with graphing. YouTube is an excellent source of information for a specific version of MS Excel. Check it out! For Macs: https://youtu.be/ANz1TY_qg9w and for PCs: https://youtu.be/MM7dCOuhBs8

Lab Unit 3 – Preparing Solutions Assignment

1. Using MS Excel and the data generated from your experiment, create a graph to compare the molar concentration of $CaCl_2$ (x-axis) to the amount of precipitate produced, as shown by the weight of your pellet (y-axis). Include linear regression and R^2 value on the graph. Print out and attach to your report.

2. What is the equation of the line? _____ R^2 value: _____

3. <u>What does the R^2 value tell you about your data</u>?

4. Using MS Excel and the data generated in your experiment, create a graph to compare the molar concentration of HCl and pH. Graph the $-\log_{10}$ HCl concentration on the X-axis and pH on the Y-axis. Include linear regression and R^2 value on the graph. Print out and attach it to your report.

5. What is the equation of the line? _____ R^2 value: _____

6. <u>How accurate were your dilutions? How do you know?</u>

CONCLUSION: Write a conclusion statement for your experiment. Did you see a linear relationship in your MgCl2 pellets? Was there a linear relationship between the molarity of HCl and pH?

LAB UNIT 4: DNA BARCODING – ISOLATING GENOMIC DNA

J. O'Grady, EdD & Cold Spring Harbor DNA Learning Center

This lab has been modeled from the Cold Spring Harbor DNA Learning Center DNA Barcoding 101 Manual: "Using DNA Barcodes to Identify and Classify Living Things." 2012 (http://www.dnabarcoding101.org/)

OBJECTIVES

In this lab activity, students will:

- ✓ Collect and identify plants in your local environment or neighborhood
- ✓ Extract and purify genomic DNA from plant tissue
- ✓ Analyze genomic DNA preparation using agarose gel electrophoresis

INTRODUCTION

Taxonomy, the science of classifying living things according to shared features, has always been a part of human society. Carl Linnaeus formalized biological classification with his system of binomial nomenclature that assigns each organism a genus and species name.

Identifying organisms has grown in importance as we monitor the biological effects of global climate change and attempt to preserve species diversity in the face of accelerating habitat destruction. We know very little about the diversity of plants and animals – let alone microbes – living in many unique ecosystems on earth. Less than two million of the estimated 5-50 million plant and animal species have been identified. Scientists agree that the yearly rate of extinction has increased from about one species per million to 100-1,000 per million. This means that thousands of plants and animals are lost each year. Most of these have not yet been identified. Classical taxonomy falls short in this race to catalog biological diversity before it disappears. Specimens must be carefully collected and handled to preserve their distinguishing features. Differentiating subtle anatomical differences between closely related species requires the subjective judgment of a highly trained specialist – and few are being produced in colleges today.

Now, DNA barcodes allow non-experts to identify species – even from small, damaged, or industrially processed material. Just as the unique pattern of bars in a universal product code (UPC) identifies each consumer product, a "DNA barcode" is a unique pattern of DNA sequence that identifies each living thing. Short DNA barcodes, about 700 nucleotides in length, can be quickly processed from thousands of specimens and unambiguously analyzed by computer programs.

The International Barcode of Life (iBOL) organizes collaborators from more than 150 countries to participate in a variety of "campaigns" to census diversity among plant and animal groups. Including ants, bees, butterflies, fish, birds, mammals, fungi, and flowering plants and, additionally, within ecosystems – including the seas, poles, rain forests, kelp forests, and coral reefs. The 10-year Census of Marine Life, completed in 2010, provided the first comprehensive list of more than 190,000 marine species and identified 6,000 potentially new species.

There is a surprising level of biological diversity, literally in front of our eyes. For example, DNA barcodes showed that a well-known skipper butterfly (*Astraptes fulgerator*), identified in 1775, is ten distinct species. DNA barcodes have revolutionized the classification of orchids, a sophisticated and widespread plant family with an estimated 20,000 members. The urban environment is also unexpectedly diverse; DNA barcodes were used to catalog 54 species of bees and 24 species of butterflies in community gardens in New York City.

DNA barcodes are also used to detect food fraud and products taken from conserved species. Working with researchers from Rockefeller University and the American Museum of Natural History, students from Trinity High School found that 25% of 60 seafood items purchased in grocery stores and restaurants in New York City were mislabeled as more expensive species. One mislabeled fish was the endangered species, Acadian redfish. Another group identified three protected whale species as the source of sushi sold in California and Korea. However, using DNA barcodes to identify potential biological contraband among products seized by customs is still in its infancy.

Barcoding relies on short, highly variable regions of the mitochondrial and chloroplast genomes. With thousands of copies per cell, mitochondrial, and chloroplast, sequences are readily amplified by polymerase chain reaction (PCR), even from very small or degraded specimens. A region of the chloroplast gene rbcL – RuBisCo large subunit – is used for barcoding plants. The most abundant protein on earth, RuBisCo (Ribulose-1, 5-bisphosphate carboxylase oxygenase) catalyzes the first step of carbon fixation. A region of the mitochondrial gene COI (cytochrome c oxidase subunit I) is used for barcoding animals. Cytochrome c oxidase is involved in the electron transport phase of respiration. Thus, the genes used for barcoding are involved in the key reactions of life: storing energy in carbohydrates and releasing it to form ATP.

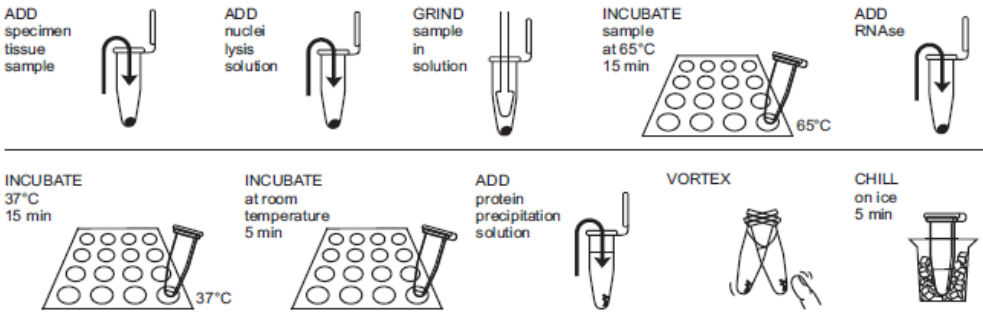
This laboratory exercise utilizes DNA barcoding to identify plants or animals – or products made from them. First, a sample of tissue is collected, preserving the specimen whenever possible, and noting its geographical location and local environment. A small leaf disc, a whole insect, or a sample of muscle tissue are suitable sources. DNA is extracted from the tissue sample, and the barcode portion of the rbcL or COI gene is amplified by PCR. The amplified sequence (amplicon) is submitted for sequencing in one or both directions.

The sequencing results are then used to search a DNA database. A close match quickly identifies a species that is already represented in the database. However, some barcodes will be entirely new, and identification may rely on placing the unknown species in a phylogenetic tree with near relatives. Novel DNA barcodes can be submitted to the database at the Barcode of Life Data System (BOLD) (www.boldsystems.org) at the University of Guelph.

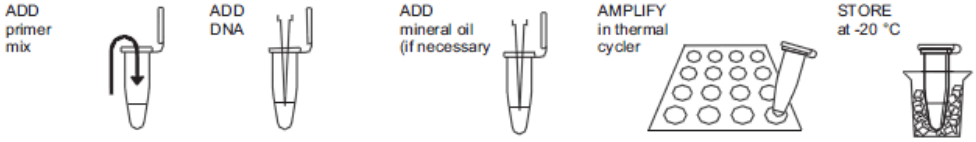
OVERVIEW OF EXPERIMENTAL METHODS

I. COLLECT, DOCUMENT, AND IDENTIFY SPECIMENS

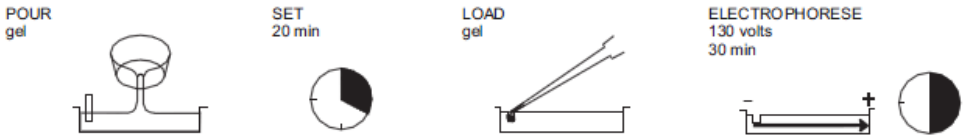
II. ISOLATE DNA FROM PLANT OR ANIMAL TISSUE



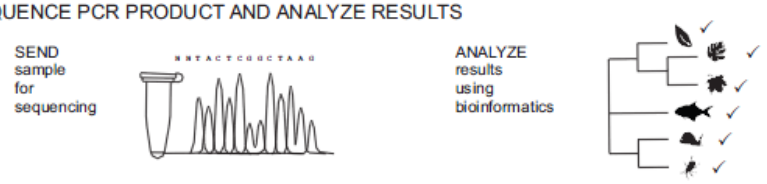
III. AMPLIFY DNA BY PCR



IV. ANALYZE PCR PRODUCTS BY GEL ELECTROPHORESIS



SEQUENCE PCR PRODUCT AND ANALYZE RESULTS



Let's Explore

1. Read the introduction to this lab and watch "The History of DNA Barcoding" video BEFORE class: https://youtu.be/Z_ClP6-E4VY
2. Write down some ideas on a class project you would like to propose.

3. Answer the following questions:
    a. What is DNA barcoding?

    b. Why is DNA barcoding important?

    c. What are a few examples of practical applications of DNA barcoding?

Bring your ideas to class! What are some ideas your classmates shared?

Write a short paragraph to describe the DNA Barcoding project you have chosen.

Lab Unit 4-A: Collect, Document, and identify Specimens

Tips on Collecting Plant Tissue Samples

1. Take only a small piece of plant leaf (1/4 inches in diameter), such as a single leaf or bud, or several needles
2. Sample young, fresh leaves or buds. ***Flexible, non-waxy leaves work best.***
3. Dormant leaf buds can often be obtained from bushes and trees that have dropped their leaves.
4. Frozen or dried leaves and herbarium samples are variable.
5. **Avoid twigs or bark. If woody material must be used, select flexible twigs with soft pith inside. As a last resort, scrape a small sample of the softer, growing cambium just beneath the bark. Roots and tubers are a poor choice because high concentrations of storage starches and other sugars can interfere with DNA extraction.
6. **Avoid poisonous or plants that can cause allergic reactions such as poison ivy

PURPOSE: The purpose of this lab is to identify and collect a sample of plant tissue to use in the DNA barcoding experiment.

SAFETY: There are no known safety issues with this lab. Note, some plants are poisonous, and we ask students not to bring those into class (such as poison ivy).

MATERIALS:

- ☐ Collection tubes, jars, or bags
- ☐ Clean and sterile tweezers, scalpel, or scissors
- ☐ Camera

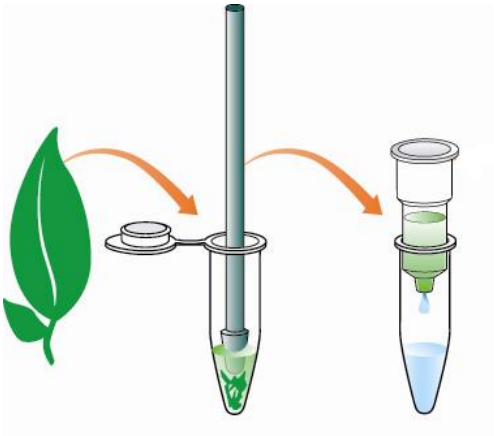
Protocol	Experimental Notes
1. Collect specimens, according to a strategy or campaign outlined by your instructor. Fresh, healthy, actively growing plant leaves work well with this protocol. 2. Capture a picture of the plant in its natural environment. Take a wide, medium, and close-up picture and include an item for scales such as a ruler, coin, or hand. 3. Use ***clean*** (wash them!) tweezers, scalpel, or scissors to collect a small sample of tissue. You should collect young leaves and flowers from plants. 4. Record where the sample was collected. The class may share their collection location on a project Google map.	Tissue Sampled: _____ Location: _____

5. Identify your specimen as precisely as possible: kingdom > phylum > class > order > family > genus > species. Taxonomic keys for local plants or animals are often available online. 6. Check to see if your specimen is represented in the Barcode of Life Database, BOLD (www.boldsystems.org). Your plant need not be novel for this project. 7. See your instructor about when to bring in your sample. ***A fresh sample is best***. However, you may freeze your sample at -20°C until you are ready to begin DNA extraction.	Known or Unknown? _____ In BOLD? _____

PASTE IMAGES OF YOUR PLANT, LOCATION, AND TISSUE SAMPLED:

Lab Unit 4-B: Isolate DNA from Plant or Animal tissue

This universal DNA extraction method uses Promega's Wizard Genomic DNA Purification Kit (Cat # A1120). The background and introduction information comes from Bio-Rad's Cloning and Sequencing Manual.



The fundamental steps of DNA purification are sample lysis and then purification of the DNA from contaminants. There is a myriad of protocols available for isolating DNA from organisms in the molecular lab. The more "classical" methods have remained virtually unchanged for decades, and the more modern methods involve commercial kits. The best method for any application depends on these fundamental considerations:

- ✓ The type of cell the DNA is isolated from will determine the cell lysis techniques used
- ✓ The purity requirements for the intended use of the DNA isolated will determine how many purification steps will be involved
- ✓ The type of DNA isolated (genomic versus plasmid DNA)

DNA extraction steps:

1. The disruption of the cell membrane and cell wall when necessary, by mechanical, chemical and enzymatic treatment.
2. Enzyme degradation is used for selective isolation of DNA (by RNase treatment) or RNA (by DNase treatment).
3. The separation of nucleic acids from other cytoplasmic components by combinations of these steps:
   a. Phenol extraction of proteins is followed by selective precipitation of nucleic acids under high salt and cold alcohol treatment.
   b. Selective precipitation of nucleic acids under high salt or cold alcohol treatment.
   c. Selective adsorption onto a chromatographic matrix in a centrifuge (a "spin column") followed by desorption by a unique buffer system.

Cell Lysis. ***The successful isolation of DNA requires methods that prevent nuclease degradation of the DNA.*** Some buffer constituents used to promote lysis and denaturation of nucleases include detergents, enzymes to inactivate DNases, denaturants such as guanidinium, and additional organism-specific components such as enzymes for cell wall, or RNase to remove contaminating RNA molecules.

In general, animal tissues are efficiently lysed, because they have no cell wall, and a gentle detergent treatment usually is enough to break open cells. Yeast and microbial cells, on the other hand, have rigid cell walls that must be weakened enzymatically before the cell will release its DNA. In the case of bacteria, lysozyme enzyme is added, while in the case of yeast a more complex mixture of enzymes must be used to degrade cell wall polymers. Plant cell walls are abraded mechanically by grinding frozen plant tissue, often with glass beads or sand and a mortar and pestle.

Remove Cellular Debris**.** This step is typically performed with centrifugation. In this protocol, the protein precipitation solution is added to aid in reducing the contaminating proteins and nucleases in the supernatant.

Isolate DNA from other cell components. The method you select for your application depends on the size and source of the DNA to be isolated. Genomic DNA can frequently be rendered insoluble and quickly precipitated by the addition of alcohol to the mixture.

Let's Explore

Watch this video on DNA extraction before coming to class: https://youtu.be/90Gsxal57-g
Draw a brief flowchart of DNA isolation from plants below:

PCR from Genomic DNA.

For PCR to be successful, the DNA extracted needs to be relatively intact. The best sources for DNA extraction include young green leaves, but fruit, roots, or germinating seeds should also suffice. ***It is better to use tissue that is still growing; as the nucleus-to-cytoplasm ratio will be more favorable, cell walls will be thinner, and the amount of potentially harmful secondary products will be less.***

There are features of plant cell structure that differ from animal cells, which make DNA extraction a challenge. First, plants have a tough cell wall made of cellulose that must be penetrated. Second, a significant part of every plant cell is a vacuole that contains acids, destructive enzymes (including nucleases), and unique secondary compounds (products produced from pathways that are not part of primary metabolism) that potentially damage DNA. To minimize contaminants from the vacuolar contents, salts and other inhibitors have been added to the lysis buffer.

Determining the Quality & Quantity of Nucleic Acids

Once the nucleic acid has been isolated, its' quality and concentration are evaluated. The amount of the isolated nucleic acid used in subsequent steps depends on its concentration. The stability of the nucleic acid and its performance in subsequent enzymatic steps is affected by its purity. Both the amount of nucleic acid isolated and its purity are affected by the type of tissue that it is isolated from, the amount of tissue used, and the isolation technique used. In this lab exercise, you will evaluate your plasmid quality and quantity using:

1. Spectrophotometric analysis of ultraviolet absorption at A_{260} will provide concentration, and a ratio of A_{260}/A_{280} (contaminating protein) and A_{260}/A_{230} (contaminating carbohydrates) will give you an idea of quality.

2. Qualitative analysis using agarose gel electrophoresis is one of the most common methods that provide valuable information about size, quality, other contaminants, and relative concentration.

The Spectrophotometer

The de facto method for quantitating nucleic acids that all other methods rely on is ultraviolet absorption. When other methods are used, a nucleic acid standard is prepared based on its absorbance at 260 nm, measured by a spectrophotometer. An advantage in the use of a spectrophotometer in nucleic acid quantitation lies in its high precision and the fact that the sample is not destroyed by the assay and can be put to further analysis after quantitation. Spectral analysis is very fast, another major reason for its routine use in a molecular lab for quantitating nucleic acids.

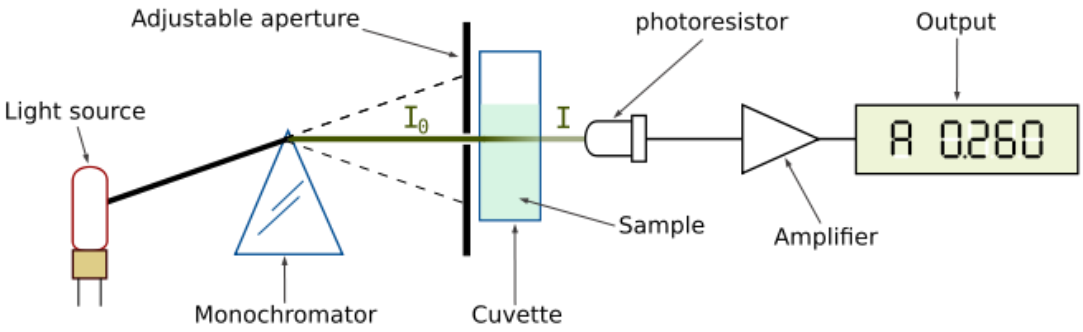


Figure: Simple diagram of a spectrophotometer. Image credit: https://commons.wikimedia.org/wiki/File:Spetrophotometer-en.svg

A spectrophotometer makes use of the transmission of light through a solution to determine the concentration of a solute within the solution. This is accomplished by placing a lamp on one side of a sample and a photocell or detector on the other side. All molecules absorb radiant energy at one wavelength or another; depending on the chemical form of their functional groups. Those that absorb energy from within the visible spectrum are known as pigments. Proteins and nucleic acids absorb light in the ultraviolet range.

The design of the single beam spectrophotometer involves a light source, a prism or grating that separates light into different colors or wavelengths, a sample holder and a photocell. Connected to each are the appropriate electrical or mechanical systems to control the illuminating intensity, the wavelength, and for conversion of energy received at the photocell into a voltage fluctuation. The voltage fluctuation is then displayed on a meter scale, is displayed digitally, or is recorded via connection to a computer for later investigation.

The concentration of colored solute in a solution is directly proportional to the intensity of its color, which in turn is proportional to the amount of absorbance of light at the wavelength that the color absorbs. The color, or absorbance, of a solution, is also proportional to the path length that the light passes. Spectrophotometers are useful for measuring concentrations of solutions because of the relation of the intensity of the color (absorbance) in a sample to the amount of solute within the sample.

This is often expressed as the **Beer-Lambert Law or Beer's Law:**

$$A = \varepsilon C l$$

Where, A is an absorbance at a given wavelength of light,
ε is the extinction coefficient (amount of color absorbance of the solute per mole),
C is the concentration of solute in the solution (doubling the concentration doubles the amount of light absorbed)
l is the path length (if you double the width of the cuvette, you double the absorbance)

Given the geometry of a spectrophotometer, what is measured at the photocell is the amount of light energy, which arrives at the photocell. The voltage meter is reading the amount of light TRANSMITTED to the photocell. Light transmission is not a linear function but is rather an exponential function. That is why the solution was APPROXIMATELY half as intense when viewed in its diluted form. Most spectrophotometers have a built-in means of direct conversion of this reading to absorbance.

Absorbance is essentially the opposite of transmittance: what light is not absorbed is transmitted. The percent transmittance is related to absorbance mathematically as:

$$A = 2 - \log (\%\ T)$$

Where A is absorbance at a given wavelength of light
% T is the percent transmittance or $\frac{\text{light transmitted through a sample}}{\text{light transmitted through a blank}} \times 100$

With the aid of spectroscopy, the quantitative analysis of nucleic acids and proteins has established itself as a routine method in many laboratories. Both nucleic acids and proteins absorb in the ultraviolet range, but while nucleic acids absorb strongly at 260 nm, proteins absorb more strongly at 280 nm.

Purity determination of DNA Interference by contaminants can be recognized by the calculation of "ratio." The ratio A_{260}/A_{280} is used to estimate the purity of nucleic acid since proteins absorb at 280 nm. Pure DNA should have a ratio of approximately 1.8, whereas pure RNA should give a value of approximately 2.1.

Absorption at 230 nm reflects biological contaminants of the sample such as carbohydrates, peptides, salts, or proteins. In addition, many chemicals commonly used in nucleic acid preparations, such as phenol, EDTA, and SDS, can be detected by their absorbance at 230 nm. In the case of pure samples, the ratio A_{260}/A_{230} should be approximately 2.2. A ratio of <2.2 indicates contaminants.

PREPARING FOR AN EXPERIMENT

Starting from this lab exercise, you will learn to create an experimental design and lab protocols. There are several important aspects of a good laboratory protocol. The first is understanding the purpose of your experiment. The purpose statement sets up the goal of the experiment, and most of the time, answers an experimental question.

The experimental question being answered by this lab is:

Writing a Title. The title offers important information about the experiment. Can you summarize the purpose or goal of the experiment in once sentence?

Title of this experiment:

Writing a Purpose Statement. For each lab, read the entire laboratory exercise and write a short, 2-3 sentence purpose statement. It should begin "The purpose of this lab…" and should include the ***experiment of the lab exercise***. For example, in one of the lab exercises, you will isolate your own genomic DNA to perform a VNTR analysis. "*The purpose of this lab is to perform a VNTR analysis of human genomic DNA."* Although you learn how to use PCR to perform this analysis, the purpose is not to learn how to perform PCR.

The purpose of this lab is to…

Writing a Hazard Communication Statement. Another important aspect of an experimental protocol is to understand any safety hazards that may arise during the experiment. This may include safe handling of a chemical such as what PPE do you wear or using a chemical fume hood. It may also include the safe disposal of reagents, such as autoclaving biohazard material. After you have read the entire laboratory exercise, provide a comprehensive list of the potential hazards that you may be exposed to during this exercise. List precautions that you will take for each hazard. For example, if you use Hydrochloric Acid, you may state the following:

> *"Hydrochloric Acid (HCl) DANGER: Corrosive. Avoid contact with skin and eyes, Avoid inhalation of fumes and mist. Do not mix with caustics or other reactive materials. Wear PPE (gloves, protective eyewear, and close-toed shoes)"*

The hazards of this lab are:

What materials do you need? Creating a comprehensive list of equipment and reagents will not only save you time but help organize your experiment. Your list should be comprehensive enough that it will take you 5 minutes to collect your items and not have to return searching for items. Being prepared for your experiment will ensure experimental success.

Writing a Procedure. The lab handouts include a lot of background material and other information in the procedural steps for your instruction in these techniques. Your procedure should not include this type of information and should be limited only to the actual steps taken in a procedure without explanation. You should read the instructions in your manual and extract just the action required of you during the lab. Your procedure should be numbered steps. Thus, you will create a document that is easier to follow during the lab session. So far, you have been writing small procedural protocols. Starting in lab 7, you will begin to write your own based on the instructions provided in the manual.

The composition of the protocol is a skill that you must master. It is sometimes difficult to gauge the amount of detail needed in a protocol. A protocol that is too long and detailed is too cumbersome to use routinely, while one lacking enough detail will not be led to uniformity when different people perform the procedures. In this course, we will guide you through these decisions by providing you with a lab protocol to follow. In general, a protocol that needs the most detailed information is used by many people, is infrequently used so that the users will not remember exactly how it is done and involves especially sensitive or critical steps of a process.

Lab Unit 4-B: Isolating DNA from Plant or Animal tissue

PURPOSE:

SAFETY:

MATERIALS:

Each Person:	Class Shares:
☐ Container with crushed ice ☐ DNA rehydration solution 100µL) ☐ 70% ethanol (600ul) ☐ Isopropanol (600ul) ☐ 2 microcentrifuge tubes (1.5 ml) ☐ Nuclei lysis solution (600ul) on ice ☐ Protein Precipitation solution (200ul) ☐ Plastic pestles ☐ Tissue specimen ☐ Microcentrifuge tube rack	☐ Microcentrifuge ☐ Picofuge ☐ Water bath or heating block at 65°C ☐ Vortex ☐ Micropipettes and barrier tips

PROTOCOL

Protocol	Experimental Notes
PART I: DNA EXTRACTION PROTOCOL 1. Obtain plant or animal tissue ~10-20 mg or ¼-inch diameter from your sample. 2. Place sample in a clean 1.5 mL tube labeled with your initials. 3. Add 100 µL of nuclei lysis solution to the tube. 4. Twist a clean plastic pestle against the inner surface of 1.5 mL tube to forcefully grind the tissue for 1 minute. Use a clean pestle for each tube if you are doing more than one sample. 5. Add 500 µL more nuclei lysis solution to the tube and mix by vortexing.	Name of Plant: _____ Where was it collected from? _____ Tissue Sampled: _____ Mass Sample: _____g

6. Incubate the tube in a water bath or heat block at 65°C for 15 minutes. Then stand tube at room temperature for 5 minutes. 7. Add 200 µL of protein precipitation solution to each tube. Vortex tubes for 5 seconds and incubate on ice for 5 minutes. 8. Place your tube and those of other groups in a balanced configuration in a microcentrifuge, with cap hinges pointing outward. Centrifuge for 4 minutes at maximum speed to pellet protein and cell debris. 9. Label a clean 1.5 mL tube with your initials. Use a fresh tip to transfer 600 µL of supernatant to the clean tube. Be careful not to disturb the pelleted debris. Discard old tube containing the pelleted debris. 10. Add 600 µL of isopropanol to the supernatant in the tube. Close cap and mix by rapidly inverting tubes several times. 11. Centrifuge for 1 minute at maximum speed to pellet the DNA. 12. Carefully pour off the supernatant from the tube and add 600 µL of 70% ethanol. Close cap, and invert tube several times to *"wash" the pellet*. 13. Centrifuge the tube for 1 minute at maximum speed. 14. Quickly, and carefully pour off the supernatant and pop-spin in Picofuge for 3 seconds. Use a 200ul micropipette with a fresh tip to remove any remaining ethanol, being careful not to disturb the pellet. 15. Air-dry the pellet for 5-10 minutes to evaporate the remaining ethanol. Do not air dry longer, or resuspension will be difficult. 16. Add 100 µL of the DNA rehydration solution to each tube and dissolve the DNA pellet by pipetting up and down several times. Incubate the DNA at 65°C for 30 minutes, or overnight at 4°C ***if*** the DNA does not easily resuspend.	Time: _____ min Temperature: _____ degC Time: _____ min

17. Store your sample on ice or at -20°C if continuing another day. PART II: SPECTROPHOTOMETRIC ANALYSIS OF DNA Determine the concentration of the DNA sample using the low-volume (NanoDrop) Spectrophotometer 1. Using the SOP provided in your SOP booklet, set the NanoDrop spectrophotometer to read DNA (nucleic acid). 2. Blank the NanoDrop with 1.5 µL of fresh rehydration solution (not your heated aliquot). Why not use water? _____ 3. Measure 1.5µL of your DNA preparation. Record data. 4. Obtain a freezer box for the whole class to store DNA. Label the box with the class name and instructor name. Ensure your tubes are clearly labeled. 5. If you do not move on to part C, store the freezer box at -20^{o}C until the next class.	Stored: _____ DNA concentration: _____ ng/µL A260/A280: _____

Lab Unit 4-C: Agarose Gel Electrophoresis of Genomic DNA

Agarose gel electrophoresis is a molecular biology technique used to separate a mixture of nucleic acid fragments according to size in an agarose matrix. DNA or RNA samples are separated by applying an electric field to move the largely negatively charged molecules toward the positive electrode. The separated nucleic acids can be visualized using a nucleic acid binding stain and exposed to UV light.

Agarose. The gel matrix is composed of agarose molecules in supercoiled bundles that are aggregated into three-dimensional structures with channels and pores through which the nucleic acid fragments move. The large pore size and gel strength make agarose suitable for nucleic acid molecules above 50 base pairs. The size of the nucleic acid fragments will dictate which pore size is most appropriate; large fragments are best separated on 0.8% gel, 1000-3000bp size fragments on 1%, and small fragments (100-500bp) on 2-3% gels. The speed of migration through the matrix is also proportional to the voltage applied, the percentage of gel, and size of the fragment. For mini-gel analysis, the voltage is typically set to 5V/cm between the electrodes. A mini-gel is set around 100V.

Because DNA molecules are double-stranded (and the same shape), they migrate through the gel matrix separating according to size alone. Smaller molecules travel faster through the gel matrix than larger molecules at a rate that is inversely proportional to the log10 of the number of base pairs. Therefore, by running a known molecular weight ladder at the same time as your nucleic acid sample, you can determine the size of the nucleic acid fragments using linear regression analysis.

SYBR Green stain. SYBR Green is a very suitable dye for detecting nucleic acid fragments in agarose gels, as it binds nucleic acids and emits fluorescent radiation on UV illumination. Because SYBR green is relatively non-toxic, it can be disposed of down the sink or in the regular trash; however, it is always a good practice to wear nitrile gloves and safety glasses when working with any chemical.

Gel Buffers. Two buffers are commonly used when preparing agarose gels for separating DNA fragment; TAE (with sodium acetate) and TBE buffers (with boric acid). Both have benefits and limitation in use. Both buffers are prepared from 50X stocks, which can be stored at room temperature.

Let's Explore

Learn more about gel electrophoresis, here!! http://learn.genetics.utah.edu/content/labs/gel/ and https://www.ncbionetwork.org/educational-resources/elearning/videos/gel-electrophoresis

PRE-LAB ACTIVITY: PREPARE AN SOP OF 50XTAE AGAROSE GEL RUNNING BUFFER:

A Standard Operating Procedure (SOP) is used for operations that are standard, meaning, laboratory tasks that you repeat the same way, and expect the same outcome no matter who is performing the procedure, when, or how many times. These are common in every workplace, but especially regulated biotechnology labs such as biomanufacturing departments. The appendix has an SOP template with instructions. Briefly:

COMPONENTS OF A STANDARD OPERATING PROCEDURE (SOP)

1. Title
2. The ID number, approval date, revision number, date of revision
3. Signatures of preparers/approvers.
4. Statement of Purpose
5. Scope describes when the procedure is relevant
6. A Statement of Responsibility describes who performs the task
7. Safety Statement. Any known hazards?
8. Materials. Lists the essential items necessary to perform the procedure.
9. Calculations required. Leave space with an example
10. Procedure. Step-wise tasks to complete the activity.
11. References to other documents/forms.
12. Instructions on how to document that the procedure was performed

PART I: PREPARE 50X TAE STOCK BUFFER

1. ***BEFORE coming to class***, use the template provided by your instructor and create an SOP for preparing 50mL of 50X TAE using the reagents listed above. You may work with a lab partner but generate an SOP to submit with your lab report. *show your calculations* (write by hand).
2. Exchange your 50X TAE SOP with your lab partner (or another lab group) for review. Check proper format and calculations. Make comments directly on the SOP. You will submit this annotated copy with your lab report as well as a final copy to reflect any corrections needed.
3. Make any corrections and print out a clean final copy of your SOP. Use this SOP to prepare a 50mL 50XTAE solution.
4. Label your reagent according to the labeling SOP and ask your instructor where it will be stored for the semester.
5. Fill in a solution preparation form to include with your report.
6. Prepare an inventory control form for your 50XTAE solution. Keep all inventory control forms with the solutions (your instructor may provide a binder for this). Every time you use an aliquot from this bottle, you must document and log it on the inventory control form.

Each group must prepare their own 50 mL bottle of the 50XTAE buffer to use throughout the semester. Perform calculations below and verify it with your lab partner. You may hand-write calculations in protocols.

The recipe for 50XTAE is as follows: **2 M Tris base** (molecular wt = 121.14 g/mol), **0.05 M EDTA** (from a 0.5 M stock provided), and **5.72% glacial acetic acid** (100% acetic acid provided in fume hood – caution!).

CALCULATIONS:

TRIS
EDTA
ACETIC ACID

ANALYZING GENOMIC DNA USING AGAROSE GEL ELECTROPHORESIS

PURPOSE: The purpose of this lab activity is to analyze the quality of plant genomic DNA using agarose gel electrophoresis that will be used in the DNA barcoding experiment.

SAFETY:

- Exposure to UV-light is a potential hazard to this lab activity. Use eye protection and do not expose eyes or skin to UV light.
- Additionally, high powered electrical equipment will be used. Do not use frayed cords or broken equipment. Wipe up any wet spills.
- Molten agarose is hot – use 'hot hands' when removing from the microwave oven and swirl carefully.

MATERIALS

☐ Agarose ☐ Electronic Balance ☐ Small weigh boat ☐ 50X gel running buffer ☐ SYBR Safe stock solution, 10,000X ☐ 125 mL Erlenmeyer flask ☐ Micropipettes and tips	☐ Gel-casting apparatus ☐ imaging system ☐ Gel electrophoresis box ☐ Power supply ☐ Microwave oven ☐ Molecular weight DNA ladder (1kB DNA ladder, Promega G7541)

CALCULATIONS

400 mL of 1X TAE buffer from 50XTAE stock
30 mL of 1% agarose gel
The volume of 10,000X SYBR safe to add to 30mL of agarose solution to make 1X final concentration

PROCEDURE: NOTE if there is enough equipment, each person should perform this experiment.

PROTOCOL	EXPERIMENTAL NOTES
1. Before starting, check calculations with a lab partner. 2. To prepare 1X TAE, measure _____mL of 50X TAE using a _____ and add to a 500mL graduated cylinder. 3. Bring to 400mL with diH2O. Cover with parafilm and mix by inversion. Set aside. 4. Set up a gel casting apparatus as directed by the instructor. 5. Weigh out _____ g of agarose and add it to a 125 mL Erlenmeyer flask. 6. Using a _____, add 30mL of 1 X TAE buffer to the 125 mL Erlenmeyer flask. 7. Place the flask on a level surface, and carefully mark the glass at the fluid level. Insert a Kimwipe in the top to prevent evaporation. 8. Heat the mixture until all agarose has dissolved using a microwave. Typically, 1 min on med-high works well. Swirl until all particles are dissolved. ***Caution! HOT!*** 9. Observe the fluid level about the mark you made on the flask. Using a squirt bottle, carefully add water back to return to the level of the mark. Swirl to mix. 10. Cool the solution to 50 – 60°C while continuing to swirl until it is cool to the touch. ***Do not cool too long, or it will solidify in the flask.*** 11. Add _____ ul of SYBR Safe stock solution (10,000X) to a final concentration of 1X. 12. Pour the gel immediately into your casting tray, and do not disturb while solidifying. Allow the gel to form completely, typically, 20min at RT or 10min at 4degC.	

13. IMPORTANT! Using a spatula, remove excess solidified agarose from the bottom of the flask and dispose of in the trash. ***Do not allow solid agarose to go down the sink.*** 14. After solidifying, break the seal between bumpers and gel using a spatula before removing. Carefully, pull the comb straight up. 15. Place the gel in the electrophoresis chamber and add the remaining 1X TAE gel-running buffer covering the surface of the gel completely. 16. Add 5ul of 5-6X load dye to a clean 1.5mL centrifuge tube. Add 10-20ul (0.5-1ug) of genomic DNA to this tube and flick to mix and pop spin in a Picofuge. 17. The molecular weight marker should already be prepared for you. Collect an aliquot (6ul) to run on the gel with your samples. 18. Load 6ul of the molecular weight marker in lane 1, and all (25ul) of the DNA sample into lane 2 (don't skip lanes). 19. Place the cover on the electrophoresis chamber and plug it into the power supply. Set to approximately 80 volts and allow electrophoresing until the blue tracking dye is about 3/4th the way to the bottom of the gel. <u>This may take almost one hour!</u> 20. Fill out a gel electrophoresis form. 21. Capture an image using the gel documentation system. Affix a copy of your gel image to the form as directed. 22. *<u>Save the remaining genomic DNA in a labeled microcentrifuge tube, return to the freezer box, and store at -20^{o}C</u>*.	

Lab Unit 4 – DNA Barcoding: Isolating Genomic DNA

**Include all forms and exercise questions answered with this workbook. This includes your research, pictures of your plants, and spectrophotometer analysis.*

1. At what wavelength does DNA absorb? How is this used to determine the concentration of your gDNA sample?

2. Prepare a table below of the genomic DNA concentration of your plant compared to your classmates. Use at least 4 student data. Are they the same? Can you explain the discrepancy?

3. Compare your gel electrophoresis results to your spectrophotometer results. What information does each provide to help you ascertain the quality of your sample?

4. **Write a conclusion statement for your experiment (NOTE – relate your conclusion statement to your experimental purpose).** *For example, "In conclusion, based on gel electrophoresis and spectrophotometry analysis, I successfully extracted genomic DNA from basil and can use this DNA for the DNA barcoding PCR reaction in the next lab"*

LAB UNIT 5: DNA BARCODING – PCR AMPLIFICATION

J. O'Grady, EdD. Adapted from Cold Spring Harbor DNA Learning Center

This lab was modeled from the Cold Spring Harbor DNA Learning Center DNA Barcoding 101 Manual: "Using DNA Barcodes to Identify and Classify Living Things." 2012
http://www.dnabarcoding101.org/

OBJECTIVES

In this lab, students will

- ✓ Use PCR to amplify genomic DNA
- ✓ Operate and program a Thermocycler
- ✓ Perform agarose gel electrophoresis on PCR samples and evaluate results
- ✓ Analyze gel data using MS Excel; use the equation of the line to determine amplicon size

INTRODUCTION

Polymerase chain reaction (PCR) is a technique for rapidly creating multiple copies of a segment of DNA utilizing repeated cycles of DNA synthesis. PCR has revolutionized molecular biology and forensics, allowing amplification of small quantities of DNA into amounts that can be used for experimentation or forensic testing. Kary Mullis, who later won a Nobel Prize for his work, developed PCR in 1983. The subsequent discovery of a DNA polymerase that is stable at high temperatures and the introduction of thermal cyclers, instruments that automate the PCR process, brought the procedure into widespread use in the late 1980s.

From trace amounts of the DNA used as starting material (template), PCR produces exponentially larger amounts of a specific piece of DNA. The template can be any form of DNA, and only a single molecule of DNA is needed to generate millions of copies. PCR makes use of two normal cellular activities: 1) binding of complementary strands of DNA, and 2) replication of DNA molecules by DNA polymerases.

PCR

The strength of PCR lies in its ability to make many copies of (amplify) a single region (target) of a longer DNA molecule. For example, a researcher wanting to study a single human gene needs to amplify only that portion from the enormous human genome of approximately 3.3 x 109 base pairs! The first step is to identify and sequence areas upstream and downstream from the DNA of interest. Once this is done, short strands of DNA that are complementary to the upstream and downstream DNA are synthesized. As in cellular DNA replication, these oligonucleotide primers are used as the starting point for copying the DNA of interest, but the primers used in PCR are DNA oligonucleotides, not RNA. PCR involves a repetitive series of cycles, each of which consists of template denaturation, primer annealing (binding to the template DNA strand), and extension of the annealed primer by a heat-stable DNA polymerase. All of the components needed for PCR are mixed in a microcentrifuge tube.

PCR Reaction Components:

1. **Template** DNA
2. *Taq* DNA **polymerase** (or another thermal stable DNA polymerase)
3. **Primers** — synthesized to complement a specific region on the template DNA. The two primers in a pair are designed to anneal to opposite ends of the region of interest. The primers are added in excess (that is, there are many more primer molecules than template molecules in the reaction tube)
4. **Nucleotides** — the four individual bases in the form of deoxynucleoside triphosphates (dNTPs), which allows them to be added to a DNA polymer. The dNTP mixture includes the same amounts of dATP, dTTP, dGTP, and dCTP.
5. Reaction **buffer** — prepared with the correct ionic strength of monovalent and divalent cations needed for the reaction and buffered to maintain the pH needed for enzyme activity

Thin-walled Tubes. The microcentrifuge tubes are specialized tubes used only for PCR. PCR tubes are plastic with very thin walls, allowing rapid transfer of heat through the plastic, and the tubes usually hold only 0.2 or 0.5 ml.

Thermocycler. The PCR reaction tubes are placed in a thermal cycler, an instrument developed in 1987 that automates the heating and cooling cycles needed during PCR. Thermal cyclers contain a metal block with holes for the PCR tubes. The metal block can be heated or cooled very rapidly. Thermal cyclers are programmable. The reaction parameters (temperatures, time at each temperature, and the number of cycles) can be stored in the thermal cycler. This means that the user can load the samples and push a button to run the reactions. Contrast this to the early researchers who had to sit by a series of water baths with a timer, switching the tubes from one temperature to another manually for hours!

Reaction Temperatures. The first step of the PCR reaction is the ***denaturation step***. Since DNA polymerase can use only single-stranded DNA as a template, the first step of PCR is uncoiling and separating the two strands of the template DNA. In cells, enzymes such as helicase and topoisomerase do this work, but in PCR, heat is used to separate the strands. When double-stranded DNA is heated to 95°C, the strands separate, or denature. Since complete denaturation of the template DNA is essential for successful PCR, the first step is frequently an extended denaturation period of 2–5 minutes. The initial denaturation is longer than subsequent denaturation steps because the template DNA molecules are longer than the PCR product molecules that must be denatured in subsequent cycles. Denaturation steps in subsequent PCR cycles are normally 30–60 seconds.

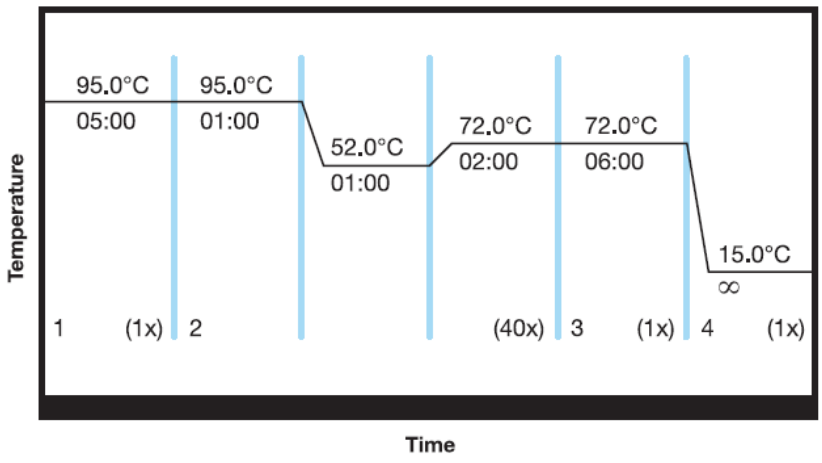
The thermal cycler then rapidly cools the reactions to 40–60°C to allow the primers to ***anneal*** to the separated template strands. The temperature at which the primers anneal to the template DNA depends on several factors, including primer length, the G–C content of the primer, and the specificity of the primer for the template DNA. If the primer sequences match the template sequences exactly, the primers will anneal to the template DNA at a higher temperature. As the annealing temperature is lowered, primers will bind to the template DNA at sites where the two

strands are not exactly complimentary. In many cases, these mismatches will cause the strands to dissociate as the temperature rises after the annealing step, but they can also result in amplification of DNA other than the target. In the annealing step, the two original strands may reanneal to each other, but the primers are in such excess that they out-compete the original DNA strands for the binding sites.

The final step is an ***extension***, in which the reaction is heated to 72°C, the optimal temperature for *Taq* DNA polymerase to extend the primers, and make complete copies of each template DNA strand.

At the end of the first PCR cycle (one round of denaturation, annealing, and extension steps define one cycle), there are two new strands for each original double-stranded template, which means there is twice as much template DNA for the second cycle of PCR. As the cycle is repeated, the number of strands doubles with each reaction. For example, after 35 cycles, there will be over 30 billion times more copies of the target sequence than at the beginning. The number of cycles needed for amplification depends on the amount of template DNA and the efficiency of the reaction, but reactions are frequently run for 30-40 cycles.

PCR generates DNA of a precise length and sequence. During the first cycle, primers anneal to the original template DNA strands at opposite ends and on opposite strands. After the first cycle, two new strands are generated that are shorter than the original template strands but still longer than the target DNA, because the original template sequence continues past the location where the other primer binds. It is not until the third PCR cycle that fragments of the precise target length are generated.



Example of thermal cycling profile. In this profile an initial denaturation step of 95°C for 5 min is followed by 40 cycles of 1-minute denaturation, 1-minute annealing, and 2-minutes extension. A final 6-minute extension time is added to ensure completion of DNA synthesis. The final hold ensures samples are kept stable until the samples are retrieved.

TEST YOUR KNOWLEDGE!

Watch PCR animation: https://www.ncbionetwork.org/educational-resources/elearning/videos/pcr-polymerase-chain-reaction

Write the exact PCR protocol the class will program into the Thermocycler. For each step, explain its purpose.

Temperature	Time	Purpose

PCR STERILE TECHNIQUE

PCR is a powerful and sensitive technique that enables researchers to produce large quantities of specific DNA from very small amounts of starting material. Because of this sensitivity, contamination of PCR reactions with unwanted DNA is always a possible problem. Therefore, utmost care must be taken to prevent cross-contamination of samples. Steps to be taken to prevent contamination and failed experiments include:

- Aerosol barrier pipet tips. The end of the barrels of micropipettes can easily become contaminated with aerosolized DNA molecules. Pipet tips that contain a filter at the end can prevent aerosol contamination from micropipettes. DNA molecules that are found within the micropipette cannot pass through the filter and contaminate PCR reactions.
- Aliquot reagents. Sharing of reagents and multiple pipetting into the same reagent tube can easily introduce contaminants into your PCR reactions. When at all possible, divide reagents into small aliquots for each team, or if possible, for each student. If only one aliquot of a reagent does become contaminated, then only a minimal number of PCR reactions will become contaminated and fail.
- Change pipet tips. Always use a new pipet tip when entering a reagent tube for the first time. If a pipet tip is repeatedly used, contaminating DNA molecules on the outside of the

tip will be transferred to other solutions, resulting in contaminated PCR reactions. If you are at all unsure if your pipet tip is clean, err on the safe side, discard the tip, and get a new one. The price of a few extra tips is a lot smaller than the price of failed reactions.

- Use good sterile technique. When opening tubes or pipetting reagents, leave the tubes open for as little time as possible. Aerosolized DNA molecules can easily contaminate tubes that are open and exposed to the air. Go into reagent tubes efficiently, and close them as soon as you are finished pipetting. Also, try not to pick tubes up by the rim or cap, as you can easily introduce contaminants from your fingertips.
- Clean Area. Bleach at a concentration of 10% destroys DNA, so wiping down surfaces with 10% bleach can get rid of any surface DNA contamination that may arise.

List and explain the components in a PCR reaction tube:

Why is a sterile technique important for PCR?

References:

1. Bio-Rad Cloning & Sequencing Manual
2. Cold Spring Harbor Laboratory's DNA Barcoding 101: http://www.dnabarcoding101.org/

Lab Unit 5-A: PCR Amplification of DNA Barcode

PURPOSE:

SAFETY:

REAGENTS, SUPPLIES, & EQUIPMENT:

○ Container with crushed ice ○ Nuclease-free water ○ P10 micropipette and barrier tips ○ Microcentrifuge tube rack ○ Permanent marker ○ 4 PCR Beads (per group of 2) ○ Thermal cycler	○ DNA from each lab partner specimen(s) ○ Positive control DNA ○ 100ul of primer/loading dye mix (25 µL each) **Primer sequences:** **rbcLa F** 5' ATGTCACCACAAACAGAGACTAAAGC-3' (forward primer) **rbcLa R** 5'- GTAAAATCAAGTCCACCRCG-3' (reverse)

PROTOCOL	EXPERIMENTAL NOTES
1. Obtain four PCR tubes containing Ready-To-Go PCR Bead. Label one tube with (+), one tube with (-), and the other two with each student initials.	
2. Use a micropipette with a barrier tip to add 23 µL of the primer/loading dye mix to each tube.	
3. Allow the beads to dissolve for 2-3 minutes at ambient temperature. Flick to mix and pop-spin to return liquid to the bottom of the tube.	
4. Use a p10 micropipette with a clean tip to add 2 µL of nuclease-free water to the (-) tube. Flick to mix.	Student DNA 1: _____
5. Use a p10 micropipette with a clean tip to add 2 µL of the first student's DNA directly into the appropriate primer/loading dye mix. Flick to mix.	Student DNA 2: _____

6. Repeat with a *clean* tip for the second student's sample DNA. 7. Use a p10 micropipette with a clean tip to add 2 µL of the positive control DNA to the (+) tube. Flick to mix. 8. Pop-spin all tubes to return the liquid to the bottom of the PCR tube and store on ice until the class is ready to begin thermal cycling. 9. The instructor will set up and demonstrate the proper use of the thermocycler. 10. Ensure the cycling is set up with the following parameters: Initial Denaturing step: 94^{o}C for 3 minutes 30 cycles of the following profile: Denaturing step: 94°C 30 seconds Annealing step: 54°C 45 seconds Elongation step: 72°C 45 seconds The final extension step 72°C 4 minutes 11. After PCR, store the amplified DNA at -20 °C until gel analysis.	

Lab Unit 5-B: Analyze PCR by Gel Electrophoresis

PRE-LAB EXERCISE: PREPARE AN SOP FOR ANALYZING YOUR DNA ON A 2% AGAROSE GEL

Use the previous SOP template and lab instructions for preparing an agarose gel and create an SOP for a 2% agarose gel. Bring in your copy, and you and your lab partner will give each other feedback. Make corrections.

PROTOCOL	EXPERIMENTAL NOTES
PART I: CASTING A 2% AGAROSE GEL 1. Before class, create a ***2% agarose gel electrophoresis SOP*** using the previous gel electrophoresis protocol. Remember to include all the sections of an SOP, paying particular attention to the SAFETY, MATERIALS, CALCULATIONS, and PROCEDURE section. 2. Check the protocol and calculations with a lab partner. 3. The DNA barcoding PCR experiment will be analyzed on one gel. Therefore, select ONE SOP (between you and your partner) that will be used to prepare the gel. 4. Use this SOP, write notes on this SOP, create a gel electrophoresis form, and make a photocopy of the originals (for the lab partner). Remember, ONE GEL, ONE SOP, ONE FORM. **PART II: ANALYZING DNA ON AN 2% AGAROSE GEL** 1. The PCR amplicon samples already contain a special load dye. ***Do not add any load dye to your sample***. 2. Load 5ul of each PCR reaction on the agarose gel. 3. Load 6ul of the DNA molecular weight ladder into the first lane. 4. Set the voltage to 80V and allow to electrophorese for approximately 1 hour. 5. Capture an image of the gel. Print out a small copy for the gel electrophoresis form, and a PDF version to analyze (see below).	

PART II: ANALYZING GEL

1. Measure the distance migrated of <u>each band that can be distinguished from each other</u> (only) in your molecular weight marker lane. Note that the 1000 & 3000 bp bands are bright. Record this data in a table below.

Molecular Weight Marker (Kb)	Log MW	Migration Distance (mm)
250		
500		
750		
Bright Band - 1000		
1500		
2000		
2500		
Bright Band - 3000		
There may be more bands that are not distinguishable from each other. Usually the largest that can be discerned from the others is 3000Kb.		

2. Using MS Excel, graph your molecular weight standard curve. ***<u>Plot Log10 molecular weight on the y-axis and migration distance on the x-axis</u>***. Label both axes and give the graph and appropriate descriptive title (NOT log MW vs. migration distance!).

3. Using MS Excel, determine the equation of the line for the linear points of the curve. The highest MW marker may not be in the linear range, so you can exclude it if it falls off the linear portion of your graph.

   <u>Equation of the Line:</u> _____

4. Determine the R^2 value for your graph. This is a correlation coefficient that will tell you how well your data correlates linearly to each other. An R^2 value >0.95 is acceptable, but >0.98 is preferred.

   <u>R^2 Value:</u> _____

5. Measure the distance of your PCR amplicon(s). Use the equation of the line to determine the molecular weight of each of your samples.

   Show Calculations:

   Size of PCR amplicon: _____ bases

Lab Unit 5 – DNA Barcoding: PCR Amplification

1. What is the name of the plant you performed the DNA barcoding experiment?

2. Was your DNA barcoding experiment successful? How do you know?

3. What is the size of your PCR amplicon? Is this what you expected? Explain.

CONCLUSION:

LAB UNIT 6: DNA BARCODING - SEQUENCING & BIOINFORMATICS

J. O'Grady, EdD, & Cold Springs Harbor

This lab has been adapted from the Cold Spring Harbor DNA Learning Center DNA Barcoding 101 Manual: "Using DNA Barcodes to Identify and Classify Living Things." 2012 (http://www.dnabarcoding101.org/). The introduction and background for this lab provided by Bio-Rad's Cloning & Sequencing Kit Instruction Manual (Cat#166-5000EDU) and exercise instructions were provided by Cold Spring Harbor's DNA Barcoding 101 Manual.

OBJECTIVES

In this lab activity, students will:

- ✓ Describe the principle behind the Sanger sequencing method
- ✓ Determine the quality of experimental sequencing data
- ✓ Locate scientific publications and biological databases of DNA and protein sequences on the Internet (DNASubway, GenBank, BLAST, NEBcutter, Clustal)
- ✓ Retrieve and compare sequence information from databases
- ✓ Compare evolutionary relatedness and draw phylogenetic trees from sequence comparisons

INTRODUCTION

Sequencing means determining the exact order of nucleotide bases (guanine (G), adenine (A), thymine (T), and cytosine (C)) in a DNA molecule. DNA sequencing began in the 1970s when two research groups developed different methods for sequencing, the Maxim-Gilbert method, and the Sanger method, at almost at the same time. Today, most researchers send their samples to core laboratory facilities where, for a nominal charge, their DNA is sequenced for them using an automated sequencer user the Sanger method. The researchers receive the sequence data the next day.

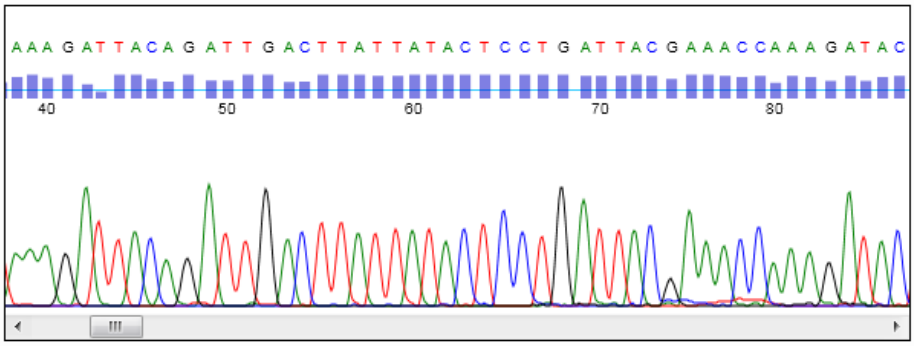
Sanger Sequencing Method. In Europe, Sanger and Coulson developed the chain termination method for DNA sequencing, or as they called it, the "plus and minus" method (Sanger et al. 1977). Since the mid-1980s, chain termination has been the predominant method used for sequencing, in large part because the technique could be automated. Frederick Sanger received a Nobel Prize for his work.

The steps in Sanger Sequencing, Chain Termination Method are:

1. Prepare a template of the DNA to be sequenced.
2. The following are added to a reaction tube in a microtiter plate well along with target DNA.
    a. Sequencing primer that will start DNA synthesis at the area to be sequenced.
    b. DNA polymerase that is heat stable
    c. Labeled nucleotides — these are deoxynucleotide triphosphates (dNTPs: dGTP; dATP; dTTP and dCTP), and they are always present in excess in the reaction.

d. Modified nucleotides called a dideoxynucleotide (ddNTP) (dye-terminator nucleotides) are added at a low concentration.

3. Allow DNA synthesis to proceed – this is similar to a PCR reaction: Cycling of denaturing DNA, annealing primer to target, elongation from primer by DNA polymerase. During synthesis, almost all of the nucleotides that are incorporated into the new DNA strand are labeled dNTPs, because the dNTPs are in excess. However, when a ddNTP is incorporated, DNA synthesis will stop on that strand, as there is no 3'-hydroxyl to form the next phosphodiester bond.
4. Use capillary gel electrophoresis and fluorometry to separate the labeled DNA fragments by size, and a computer reads the fluorescent tag, processes the data, and provides a sequencing chromatogram.
5. The result is a graph called a chromatogram or electropherogram, on which the bases are represented by a sequence of colored peaks. The peak height indicates the intensity of the fluorescent signal. The automated sequencer interprets the results, assigning G, A, T, or C to each peak. If the software cannot determine which nucleotide is in a position, it will assign the letter N to the unknown base.



Example of a sequencing chromatogram

To learn more about DNA Sequencing watch this animation: https://youtu.be/ONGdehkB8jU

Whole Genome Sequencing. *From Wikipedia (https://en.wikipedia.org/wiki/Whole_genome_sequencing)*
The DNA sequencing methods used in the 1970s and 1980s were manual, for example, Maxim-Gilbert sequencing and Sanger sequencing. The shift to more rapid, automated sequencing methods in the 1990s finally allowed the sequence of whole genomes. The first organism to have its entire genome sequenced was *Haemophilus influenzae* in 1995. After it, the genomes of other bacteria and some archaea were first sequenced, largely due to their small genome size. *H. influenzae* has a genome of 1,830,140 base pairs of DNA.

In 1999, the entire DNA sequence of human chromosome 22, the shortest human autosome, was published. The first plant genome - that of the model organism *Arabidopsis thaliana* - was also fully sequenced by 2000. By 2001, a draft of the entire human genome sequence was published. In 2004, the Human Genome Project published an incomplete version of the human genome. To date, researchers have sequenced the complete genomes of thousands of organisms. For a complete list: https://en.wikipedia.org/wiki/Lists_of_sequenced_genomes

Analysis of DNA Sequences Using Bioinformatics Tools

The ability to determine the exact DNA sequence of genes that emerged in the late 1970s and a technique to synthesize large quantities of target regions of DNA using polymerase chain reaction (PCR) was developed in the early 1980s. An electronic repository for the many genes being discovered was created in the late 1980s. This database, GenBank, is operated by the National Center for Biotechnology Information (NCBI) and funded by the U.S. National Institutes of Health (NIH).

GenBank is accessible via the Internet to scientists, teachers, and students worldwide free of charge. Major efforts to completely sequence entire genomes were initiated in the 1990s and have now been completed for humans as well as for numerous model organisms studied by scientists. The capacity for isolating and sequencing genes has grown so quickly that the number of submissions to GenBank has doubled every two years since 1993 (NCBI, 2005). The challenge of analyzing all of the DNA sequences deposited in GenBank spurred the development of numerous computer programs for interpreting DNA and protein sequence data. This computer-aided analytical approach is called bioinformatics. In addition to GenBank, other databases housing sequence information is available, as are a wide range of software programs and tools designed to obtain, analyze, and organize this information.

DNA Sequencing Data

Once the sequencing reaction has been performed, and the samples have been analyzed on a sequencing instrument, the result is a data file that contains a chromatogram. A chromatogram is a representation of the DNA molecules generated from the Sanger chain-termination sequencing protocol, where the sequence of peaks represents the sequence of bases. A chromatogram provides information on the peak intensities, the time course in which they eluted, and the base calls that the instrument made for these peaks. The data can be analyzed manually by opening the data file in a reader-style program such as DNA Subway, which can be found free on the Internet.

In this laboratory exercise, we will analyze the DNA Barcoding sequences using a software program called "DNA Subway" found here: https://dnasubway.cyverse.org/ Using this software program, students will:

1. Evaluate the quality of the raw data (DNA barcoding sequences)
2. Trim off any unusable portion
3. Pair up sequences (forward and reverse)
4. Establish a consensus
5. Use BLAST to identify plant by comparison to other sequences in the NCBI database (or determine if you have a novel sequence)
6. Evaluate the relatedness of your DNA Barcode to other published sequences or classmate sequences

REFERENCES

1. Bio-Rad Cloning & Sequencing Kit Instruction Manual (Cat#166-5000EDU)
2. Cold Spring Harbor DNA Barcoding 101 laboratory: http://www.dnabarcoding101.org/

Lab Unit 6-A: Preparing Plates for Sequencing

PURPOSE: The purpose of this lab is to prepare PCR amplicon samples from the previous lab for sequencing.

SAFETY: There are no hazardous materials used in this lab. However, it is critical to use gloves, clean tips, and careful pipetting skills to avoid contaminating samples when Micropipetting into the microtiter plate.

MATERIALS

Each group	*Class Shares*
☐ Plant amplicon samples that showed a clear single amplicon ☐ p10 and p20 Micropipettes ☐ Plugged tips	☐ Bottle sterile water ☐ Sequencing plate ☐ Plate key (identifying sample placement)

PROCEDURE: Serial Dilutions

Note:

- We will send your plant DNA PCR sample for sequencing if you observed only a SINGLE amplicon on your agarose gel.
- You will not need to add any sequencing reagents or primers. The sequencing reagents and primers used (M13 forward and reverse) will be added by the sequencing company.
- Typically, the sequencing plate is set up the same day PCR samples are analyzing. Ask your instructor.

1. Thaw plant DNA PCR amplicon at room temperature, pop spin.

2. The sequencing plate requires 20 ul. Using a p20 micropipette, measure and ensure there is 20ul. If you are short, add the difference with the sterile water provided.

3. The class must create a plate key (diagram) and assign a place where students will aliquot their PCR reactions. Each student must prepare two aliquots; 10ul for the forward sequencing reaction, 10ul for the reverse reaction.

4. Aliquot 10ul of the PCR reaction into each of two wells in the plate strip as instructed.

5. The instructor will cap the strips and give the sequencing strips and the plate key to the lab technician. Keep a copy of the key for your records.

Lab Unit 6-B: Bioinformatics

DNA Subway is a bioinformatics workspace that wraps high-level analysis tools in an intuitive and appealing interface. By "riding" different lines (workflows) you can predict and annotate genes in up to 150,000 base pairs of DNA sequence (Red Line), prospect entire plant genomes for related genes and sequences (Yellow Line), determine sequence relationships (Blue Line) and analyze RNA-Seq reads to measure differential expression (Green Line). For today's bioinformatics exercise, you will ride the blue line to view, compile and compare your DNA sequences to published sequences at the NCBI database.

PART I: CREATING A DNA SUBWAY ACCOUNT

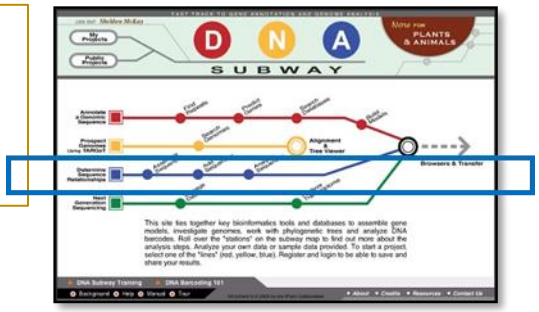
Pre-lab activity: **It is important you create a DNA subway account BEFORE you come to class.

1. Go to DNA Subway at https://dnasubway.cyverse.org/
2. Click "Register" in the top left corner to create an account.
3. Complete the demographic information with your name, our institution, and complete the remainder with the drop-down menu. You can select anything or "does not apply" or "prefer not to say."
4. ***Note, it may take up to 24hrs before it approves your account.*** Do not try to create another account.
5. Ask your instructor for assistance if you have any difficulty. Remember to ***write down your username and password!*** ☺

USERNAME: _____ PASSWORD: _____

PART II: BIOINFORMATICS ANALYSIS OF PLANT DNA BARCODE

For the activity below, create an MS-Word document and add a screen capture at each of the steps below as appropriate. Print and submit with your lab report. The results should be approximately 3-5 pages long.



The following directions explain how to use the ***Blue Line of the DNA Subway***.

1. Obtain instructional manual
   a. Download and read the blue line instructions. https://dnabarcoding101.org/files/DNA_Subway_Guide.pdf
   b. Watch the video on how to use the Blue Line: https://youtu.be/7WF--Ba2P10

2. Upload DNA Sequences
    a. Log into DNA Subway https://dnasubway.cyverse.org/.
    b. You may use a "Guest" account. However, it will not save your work.
    c. Select "Determine Sequence Relationships" (Blue Line) to begin a project.
    d. Select "rbcL" from the "Select Project Type" section.
    e. "Select Sequence Source" to upload sequence(s) in ab1 (files ending with .ab1) or FASTA format.
    f. Click "Browse" to navigate to a folder on your desktop or drive containing your sequence(s).
    g. You must select more than one sequence by holding down the Ctrl key while clicking on both Forward and Reverse file names.
    h. Once you have selected the sequences you want, click "Open."
    i. Provide a title in the Name Your Project section.
    j. Write a short description of your project in the Description section.
    k. Click "Continue."

3. Process Sequences
    a. Upload sequences
    b. View sequences – screen capture both good and poor DNA sequences
    c. Trim off poor sequences from end
    d. Use pair builder to pair the forward and reverse sequences
    e. Use consensus builder to create consensus
    f. Address any mismatched sequences and trim off ends that don't have two sequences contributing to the consensus

4. BLAST
    a. Use BLAST to search NCBI for matches to your sequence
    b. View top search results – is this what you expected? Include with report
    c. Add at least three top searches to your Project
    d. Add sequence data
    e. Add reference data

5. Align Sequences using MUSCLE
    a. Print out and include with your report

6. Generate Phylogenic Tree
    a. Include with your report
    b. Stop analysis here (do not attempt to publish to GenBank).

Lab Unit 6 – DNA Barcoding: Bioinformatics

Capture each of the steps above and paste into an MS word document. This should be about 3-5 pages long – NO LONGER. Print out and include with your report. In the minimum, you should have the top example of good sequence, poor sequence, consensus, BLAST result, MUSCLE alignment, and Phylogenetic tree.

1 What is DNA sequencing?

2 List all the components of a Sanger Sequencing reaction. Describe what each component is for in the reaction.

3 List the steps for the Bioinformatics pipeline – how did you analyze and compile the raw sequence data to determine what plant you had?

4 Describe the quality of <u>your</u> DNA sequences.

5 Did you have to trim Ns from your sequence? What are Ns? Why did you trim them before aligning?

6 Did your bioinformatics activity correctly identify your target plant? How do you know? Discuss.

CONCLUSIONS:

LAB UNIT 7: PCR-BASED VNTR ANALYSIS OF HUMAN DNA

J. O'Grady, EdD, L. Fletcher, Ph.D., A. Wheeler, M.S., P. Phelps, Ph.D.

Adapted from Edvotek's PCR-based VNTR Human DNA Typing (Cat#334)

OBJECTIVES

In this lab activity, students will:

- ✓ Demonstrate safe handling of biohazard material
- ✓ Explain how PCR can be used in identifying individuals or alleles
- ✓ Extract DNA from human cheek cells
- ✓ Operate a thermocycler to perform PCR reaction
- ✓ Analyze amplicon using agarose gel electrophoresis
- ✓ Interpret and evaluate the quality of amplification
- ✓ Employ MS Excel graphing software to determine amplicon size

INTRODUCTION

Although human DNA from separate individuals is identical in more places than it is unique, many regions of the human genome exhibit a great deal of diversity. Such sequences are termed **polymorphic** (having many forms) and are used for diagnosis of genetic disease, forensic identification, and paternity testing. Many polymorphisms are located in the estimated 98% of the human genome that does not code for proteins. Since no genes that encode proteins are found in these regions, changes, or **mutations**, in these regions do not have an effect on the individual and are more likely to be passed on to offspring. Mutations in protein-coding regions are far more likely to be detrimental to the health and longevity of the individual.

CODIS: In 1990, the Federal Bureau of Investigation (FBI) established the Combined DNA Index System (CODIS). It is the core of the national DNA database and developed specifically to enable forensic DNA laboratories to create searchable DNA databases of authorized DNA profiles. The CODIS software permits laboratories throughout the country to share and compare DNA data. This system permits comparison of crime scene DNA to DNA profiles in a convicted offender and a forensic (crime scene) index. The **NDIS** (National DNA Index System) is the national level of CODIS containing the DNA profiles contributed by federal, state, and local participating forensic laboratories. The NDIS contains over 13,041,408 offender profiles, 2,860,423 arrestee profiles, and 804,902 forensic profiles as of September 2017. As of September 2017, CODIS has produced over 392,684 hits assisting in more than 377,507 investigations and over 66,000 arrests just in Texas! Learn more here, at the CODIS fact sheet:
https://www.fbi.gov/services/laboratory/biometric-analysis/codis/codis-and-ndis-fact-sheet

ISOLATING DNA: The first step in forensic DNA fingerprinting is the collection of human tissue from the crime scene or victim. These tissues include blood, hair, skin, and body fluids. The sample is treated with a detergent to lyse cell membranes and obtain DNA for further analysis.

In forensics, the polymerase chain reaction (PCR) is now used to amplify and examine highly polymorphic DNA regions. These are regions that vary in length from individual to individual and

fall into two categories: 1) Variable Number of Tandem Repeats (VNTR) and 2) Short Tandem Repeats (STR). A VNTR is a region that is variably composed of a 15-70 base pair sequence, typically repeated 5-100 times. An STR is similar to a VNTR except that the repeated unit is only 2-4 nucleotides in length. By examining several different VNTRs or STRs from the same individual, investigators obtain a unique DNA fingerprint for that individual, which is unlike that of any other person (except for an identical twin).

D1S80 is a VNTR region present on chromosome 1 and contains a 16-nucleotide sequence that is variably repeated between 16 and 40 times. An individual who is homozygous for the D1S80 genotype will have equal repeat numbers on both homologs of chromosome 1, displaying a single PCR product following gel analysis. More commonly, a person will be heterozygous, with differing D1S80 repeat numbers. The amplification of DNA from heterozygous individuals will result in two distinct PCR products. In this experiment, PCR will be used to amplify a short DNA sequence from human chromosome 1 at a point called the D1S80 locus that is a variable insertion (one of the CODIS loci). The primers used to start the amplification were designed to flank the DNA region of the D1S80 insertion site. The amplicon size (position on the gel after electrophoresis) will reveal the length of the insertion.

PCR: The primer mixture you will use contains a 25 bp forward primer that starts copying one strand and a 26 bp reverse primer that starts copying the complementary strand. These primers match only one site on human DNA, so only the DNA fragment between the two primers is copied. It would help if you also had *Taq* DNA polymerase, buffer, KCl, $MgCl_2$, and dNTP (nucleotides with each of the four bases – A, T, C, and G) in your reaction mixture to achieve amplification. All of these are supplied in a single-use, solid bead or pellet. These components, your DNA, and the primers are all of the ingredients needed to perform the amplification. PCR beads must be stored desiccated at room temperature, or they will absorb water from the air, and the enzyme will be degraded.

The thermal cycler must be programmed so that it is preheated and ready to run when your samples are ready. The program will begin with an initial 5-minute cycle at 94˚C, which makes sure that the DNA completely denatures (the complementary strands pull apart). Five minutes is enough time for the 3 billion base pairs in the human genome to denaturing. Then the machine will cycle through the steps below 35 times. Each cycle doubles the amount of DNA that was produced in the previous cycle.

94˚C /30 seconds: denatures amplicon DNA
65˚C /30 seconds: primer anneals to a complementary sequence of DNA
72˚C /30 seconds: DNA polymerase begins synthesizing new DNA

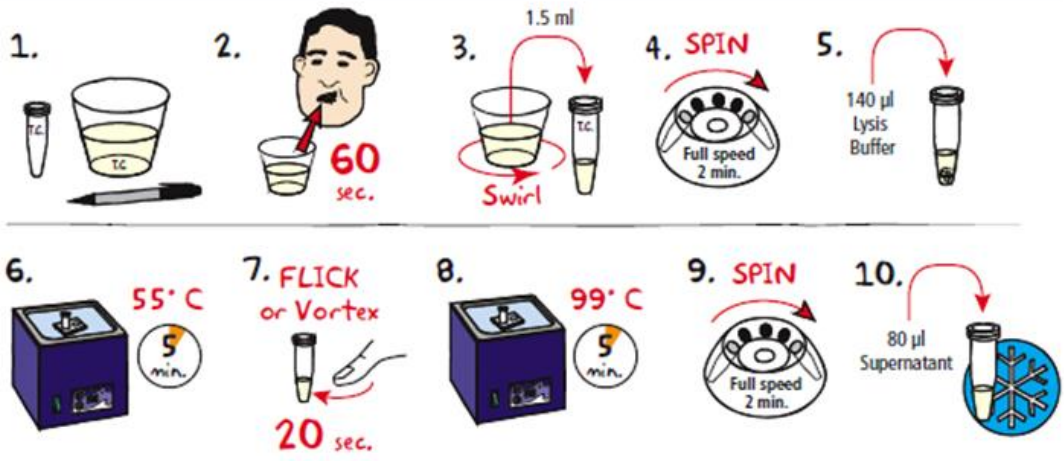
After 35 cycles, the temperature is held at 72˚ C for 4 minutes to allow the polymerase to back-fill any amplicons that were not amplified clear to the end. After programming, make sure the thermal cycler is preheating so that it will be warm enough to start the cycles immediately.

Lab Unit 7-A: Isolating DNA & PCR of VNTR Loci

PRE-LAB ASSIGNMENT! Create a protocol for Lab 7 (A&B). Include:

a. Title page with title, name, date
b. Purpose
c. Checklist of materials
d. Safety Considerations
e. Protocol (use format in previous labs)

PROCEDURE: PART I: ISOLATING DNA FROM CHEEK SAMPLES



1. **LABEL** a 1.5 ml screw top microcentrifuge tube and a cup with your lab group and/or initials.
   NOTE: Saline solution MUST be used for cheek cell wash. Sports drinks will inhibit amplification of DNA by Polymerase Chain Reaction in Module II.
2. **RINSE** your mouth vigorously for 60 seconds using 10 ml saline solution. **EXPEL** the solution into cup.
3. **SWIRL** the cup gently to resuspend the cells. **TRANSFER** 1.5 ml of solution into the labeled tube.
4. **CENTRIFUGE** the cell suspension for 2 minutes at full speed to pellet the cells. **POUR** off the supernatant, but **DO NOT DISTURB THE CELL PELLET! REPEAT** steps 3 and 4 once more.
5. **RESUSPEND** the cheek cells in 140 µl lysis buffer by pipetting up and down or by vortexing vigorously.
6. **CAP** the tube and **PLACE** in a waterbath float. **INCUBATE** the sample in a 55° C waterbath for 5 minutes.
7. **MIX** the sample by vortexing or by flicking the tube vigorously for 20 seconds.
8. **INCUBATE** the sample in a 99° C waterbath for 5 minutes. *NOTE: Students MUST use screw-cap tubes when boiling DNA isolation samples.*
9. **CENTRIFUGE** the cellular lysate for 2 minutes at full speed.
10. **TRANSFER** 80 µl of the supernatant to a clean, labeled microcentrifuge tube. **PLACE** tube in ice.
11. **PROCEED** to Module II: Amplification of the D1S80 Locus.

STEP 4:
If cell pellet size is not large enough, REPEAT steps 3 - 4 until students can easily see the pellet.

STOP **OPTIONAL STOPPING POINT:**
The extracted DNA may be stored at -20°C for amplification at a later time.

PART II: PCR OF DNA SAMPLES

CONTROLS: For every PCR reaction you perform, it is essential to include controls to help interpret the data. More than just a "negative" or "positive" control, you want to find ways that controls can help you understand the results you obtain. For example, you may want to include:

- ✓ Positive control DNA that you know the primers will produce an amplicon
- ✓ A no-template control (negative control) to show that the reagents are not contaminated with DNA
- ✓ Negative control DNA, so you know the primers are specific to only the target DNA. This is also a type of negative control.
- ✓ Sometimes you may want to include internal positive control, so you can demonstrate there is DNA in your sample. When you get a negative result, is it because you had no DNA or the primers did not pick up a target in your sample?

1. Each group (pair) will need four Ready-to-Go PCR beads. Label the sides of the tube as shown in the table below (not the lids!).

2. Add 20 uL D1S80 primer mix to each PCR tube and flick to dissolve. Pop-spin and place on ice. Once the bead is dissolved, it is very important to keep on ice until the samples are transferred to the PCR machine.

3. Add to each PCR tube the DNA or Nuclease-free water, as outlined in the table below. Mix gently by pipetting up and down or flick and pop-spin in the Picofuge and return to the ice. Make sure the bead is completely dissolved.

PCR Tube	Sample	PCR Bead	Volume Primer mix	Volume DNA	Nuclease-free Water
S1	Student 1 gDNA	1 bead	20ul	5 ul DNA	0 ul
S2	Student 2 gDNA	1 bead	20ul	5ul DNA	0ul
+	+ Control DNA	1 bead	20ul	5ul Control DNA	0 ul
-	No (DNA) template	1 bead	20 ul	NO DNA added!	5 ul

4. Set up the PCR machine as directed by your instructor. The PCR program for this lab is as follows:
   94°C for 5 min
   30 cycles of: 94°C for 30sec, 65°C for 30sec, 72°C for 30sec
   72°C for 4 min

5. Keep PCR reactions on ice and wait until the Thermocycler has reached 94°C. Press pause and carefully load the samples ensuring the lids are snapped in all the way, and the tubes are in the plate holes — press resume.

6. When the program has completed, store tubes at -20°C until ready to begin the next step.

Lab Unit 7-B: Analysis of VNTR Loci PCR Amplicons

PART I: ANALYZING VNTR PCR AMPLICONS ON A 2.5% AGAROSE GEL

1. Prepare a 2.5% agarose gel in 1X TAE buffer. We will use a high percentage of agarose gels to ensure proper resolution of multiple alleles (amplicons) close in size that are relatively small.
2. The molecular weight marker is already prepared — load 6ul in the first lane.
3. Add 5ul of 6X load dye to PCR samples, mix, pop-spin before loading all 25ul into the gel.
4. Set voltage to 80V and electrophorese for approximately 1 hour.

PART II: ANALYZING GEL

1. Measure the distance migrated of **ONLY** **each band you can resolve** in the molecular weight marker lane. Note that the 1000 & 3000 bp bands are bright. Record this data in a table below.

Molecular Weight Marker (Kb)	Log MW	Migration Distance (mm)
250		
500		
750		
Bright Band - 1000		
1500		
2000		
2500		
Bright Band - 3000		
There may be additional larger bands that are unresolved. Exclude them.		

2. Using MS Excel, graph your molecular weight standard curve. ***Plot Log10 molecular weight on the y-axis and migration distance on the x-axis***. Label axis and give the graph and appropriate descriptive title (NOT log MW vs. migration distance!).

3. Using MS Excel, determine the equation of the line for the linear points of the curve. The highest MW marker may not be in the linear range, so you can exclude it if it falls off the linear portion of your graph.

4. Determine the R^2 value for your graph. This correlation coefficient will tell you how well your data correlates linearly to each other. An R^2 value >0.95 is acceptable, but >0.98 is preferred.

5. Measure the distance of your PCR amplicon(s). Use the equation of the line to determine the molecular weight of each of your samples. **Show Calculations**

6. Generate a table with the class data. In the table, including the researcher's name and the size of the PCR amplicon(s).

7. Include your tables and graph in your lab report.

Lab Unit 7 - Amplification of D1S80 VNTR loci by PCR

1 What is VNTR? How can it be used to identify people?

2 What is CODIS? How is it used to solve crimes?

3 Include your gel documentation form in your lab report with a labeled image of your gel.

4 Include a well-labeled graph with equation of the line and R^2 value.

5 Analyze your data.

a. Determine the size of the amplicon(s). Show calculations here.

b. Some students had two bands in their PCR reactions. Explain how one primer set can generate data with two bands on a gel.

c. Compare your D1S80 PCR product with those of the rest of the class using a table below.

d. Did any students have genotypes similar to yours? How could you explain such similarities?

CONCLUSION: In conclusion,

LAB UNIT 8: RECOMBINANT DNA TECHNOLOGY

J. O'Grady, EdD, L. Fletcher, Ph.D., A. Wheeler, M.S., P. Phelps, Ph.D.
Adapted from Bio-Rad's pGLO Transformation Kit (Cat#166-0003EDU)

OBJECTIVES

In this lab activity, students will:

- ✓ Retrieve and compare sequence information using bioinformatics databases
- ✓ Employ bioinformatics tools: NEBcutter, BLAST, Jmol, and Clustal
- ✓ Use a sterile technique to prepare media and starter cultures
- ✓ Transform *E. coli* with plasmid DNA
- ✓ Select for recombinant clones on antibiotic selection plates
- ✓ Calculate transformation efficiency

Molecular cloning is a set of methods used to construct recombinant DNA and incorporate it into a host organism; it makes use of several molecular tools that are derived from microorganisms. Cloning is frequently the first step of a research project to produce a recombinant DNA molecule. In this lab, the protein Green Fluorescent Protein has been inserted into a plasmid vector. This vector has then been inserted into *E. coli* bacteria. As *E. coli* replicates, it makes a copy of this foreign DNA as well.

Recombinant Protein. Additionally, the bacteria can be induced to make protein from this foreign DNA strand. In the figure below, human insulin gene is inserted into bacteria on a foreign plasmid vector. Then insulin is made inside the bacteria. The bacteria are lysed, and the insulin is purified from the extract. Insulin was the first FDA-approved recombinant protein.

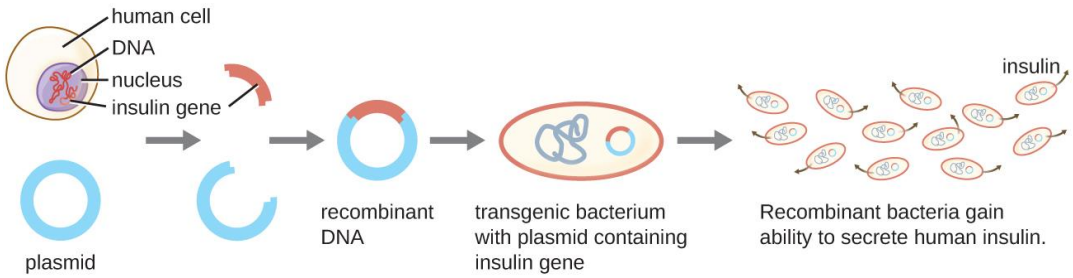


Figure 1: Recombinant DNA Technology. Image Credit OpenStax.

Recombinant DNA technology has dramatically influenced and advanced numerous fields – from medicine to agriculture. Recombinant DNA technologies have helped create genetically altered plants, which can withstand different environmental conditions and reduce the use of pesticides to yield more abundant crops. They have also helped produce advances in medicine, such as treatments for cancer, recombinant therapeutic protein drugs, and vaccines. One interesting use of recombinant DNA technology is in the field of animal husbandry. Recombinant DNA technologies about animal husbandry have led to the development of transgenic animals and clones, including the first successfully cloned animal, Dolly, the sheep. To learn more, visit Khan Academy: https://www.khanacademy.org/science/biology/biotech-dna-technology

Lab Unit 8-A: Bioinformatics of Green Fluorescent Protein

The Human Genome Project (HGP), completed in 2003, was a 13-year project coordinated by the U.S. Department of Energy and the National Institutes of Health. During the early years of the HGP, the Welcome Trust (U.K.) became a primary partner; additional contributions came from Japan, France, Germany, China, and others. For more information:
http://www.ornl.gov/sci/techresources/Human_Genome/project/hgp.shtml

Human Genome Project goals were to:

1. identify all the approximately 20,000-25,000 genes in human DNA,
2. determine the sequences of the 3 billion chemical base pairs that make up human DNA,
3. store this information in databases,
4. improve tools for data analysis,
5. transfer related technologies to the private sector, and
6. address the ethical, legal, and social issues that may arise from the project

Though the HGP is now complete, analyses of the data will continue for many years. Follow this ongoing research on the HGP page:
http://www.ornl.gov/sci/techresources/Human_Genome/project/timeline.shtml

An essential feature of the HGP project was the federal government's long-standing dedication to the transfer of technology to the private sector. By licensing technologies to private companies and awarding grants for innovative research, the project catalyzed the multibillion-dollar U.S. biotechnology industry and fostered the development of new medical applications.

Another goal of the Human Genome Project is to sequence the genomes of other species of interest, such as model organisms used by biologists, pathogens of medical importance, and crop plants of agricultural importance. This goal has also been exceeded, and today the genomes of tens of thousands of species have been sequenced. Check out the list here, at the NCBI website:
https://www.ncbi.nlm.nih.gov/genome/browse/#!/overview/

The Human Genome Project has been a catalyst for change in the way biologists approach the study of living things. Biologists today using the sophisticated laboratory technology for sequencing DNA are collecting data faster than they can interpret it. A new field called bioinformatics is developing for the storage and management of the data stored in these rapidly growing databases, as well as for the use of a computer as a general tool for discovering how living things work.

The power of bioinformatics approach for the discovery of genes has been proven with the completion of the yeast genomic sequencing project in 1996. Once the genome was fully sequenced, bioinformatics approach could be used to scan for and identify genes. The genes discovered this way could be compared to a large number of genes that had already been discovered through more classical molecular and genetic techniques. The results were remarkable. Before the yeast genome was sequenced in 1996, an international collaboration of

scientists studying the genetics of this model organism had identified an impressive 2,000 genes by conventional genetic analysis. When the yeast genome sequencing was completed, bioinformatics searches for similarities of DNA sequences from other organisms were able to locate an additional 2,000 genes. Meaning, in less than one year, a single laboratory using DNA sequencing and computer searches of sequence data could both duplicate and double the gene discovery of a 20-year international effort.

Once a gene has been identified, many new questions can be asked: what kind of protein does it code for, and what is its function? How does it interact with other molecules of the cell? Is always it expressed as a so-called "housekeeping gene," or is it a developmentally regulated gene? Is the expression tissue-specific? Is it expressed in response to an environmental factor? These questions are the same questions that have been asked by molecular and cell biologists for decades, usually by studying one gene, and its protein or proteins, at a time.

With the copious amounts of information coming from the genomics project, however, biologists can ask the same questions about systems that are more complex. Instead of asking about one protein at a time, biologists can now ask questions about hundreds of proteins at a time, looking for patterns of structure and patterns of expression. Looking at the proteins on a genomic scale is a new field now called proteomics.

When a new gene has a sequence that has been found to be homologous to a gene in a database that has already been characterized, sometimes many of these questions about protein structure and function can be answered quickly by the bioinformatics approach. For example, the 2,000 new genes discovered by the yeast genomic sequencing project, discussed above, matched genes of other organisms whose function had already been determined.

A bioinformatics approach is playing an increasingly important role in protein structure studies. Although the final structure of a protein is determined by the amino acid sequence of that protein, we have yet to model the correct folding of a protein by its amino acid sequence by computer. There is progress, however, in achieving this so-called "holy grail" of proteomics. As our database of protein structures grows, it is easier to predict protein structures based on similarities in amino acid sequence. Also, we have discovered by analysis of sequence databases that there are certain conserved protein families with high sequence homology in part, if not all, of the amino acid sequence. Computer programs can currently predict protein structures by homology modeling when the sequence homology is as low as 25%. Therefore, if the amino acid sequences agree by more than 25%, the computer program can accurately predict the secondary and tertiary structure of the amino acid sequences.

GenBank. In this exercise, you will use a computer to access GenBank, the database repository of all DNA and protein sequences housed at the National Center for Biotechnology Information (NCBI) at the National Institutes of Health (NIH). Researchers and scientists submitted the sequences in GenBank. A unique number identifies each submission and is called an accession number. In the first part of this bioinformatics activity, you will use the accession number of the

pGLO plasmid (also known as pBAD-GFPuv) to search GenBank and retrieve the plasmid sequence for pGLO.

Bioinformatics and Gene Structure. Once a sequence is determined, scientists can use computer programs to search the DNA for genes. Genes have a start codon (ATG) that signals the start of the protein and a stop codon, which signals the end of the protein and for the ribosome to release the mRNA. These two codons must be 'in the frame.' This means that each codon (every three nucleotides) must code for a specific amino acid, from the start codon until the stop codon is reached. This is referred to as an open reading frame or ORF. Since not all ORFs encode a protein, you will use a common research tool called BLAST. This putative protein sequence for each ORF will be queried against the protein database in GenBank using BLAST. You will determine which sequence most closely matches the ORF.

Once the gene and ORF have been identified, an essential piece of information to clone or manipulate the gene using recombinant DNA technology is restriction mapping. In this lab, you will use the free software NEBcutter to determine the restriction map of the pGLO plasmid. The Protein Data Bank (PDB) is a valuable research database, which hosts 3-D protein structures identified by a PDB-ID number. This software program also hosts Jmol, which you will use to view the 3-D structure of the GFP protein.

References

1. Human Genome Project. 2011. http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml
2. Geospiza: http://www.geospiza.com
3. Biotechnology: A Laboratory Skills Course. Bio-Rad, pp 264-269
4. Khan Academy: https://www.khanacademy.org/science/biology/biotech-dna-technology

BIOINFORMATICS TIPS

1. When opening the new software, open in a new tab; keep the previous software open as well.
2. At each step of the exercise, copy and paste your progress into an MS word document. SAVE the file as you go through the exercise.
3. Most software programs will let you right-click on figures to allow you to copy then paste the figure into your MS word document. ☺
4. Browsers require updates and plug-ins. Before you begin, UPDATE your browser software and install any additional plug-ins required. This may take some time, so prepare ahead.
5. All of the software and tools are available free online so that you can perform this exercise at home or in the computer center or learning lab.

PRE-LAB QUESTIONS

1. What is Bioinformatics?

2. What is GenBank?

3. What is an accession number? What is the accession number for pGLO plasmid?

Part I: Using GenBank to search for and download the pGLO plasmid sequence

1. Open MS Word and save the open document with the lab name. Create a title at the top of the document. Remember to save your work as you go!

2. Open a web browser and go to the National Center for Biotechnology Information (NCBI) website. (www.ncbi.nlm.nih.gov/)

3. Click the analyze button and then nucleotide on the pull-down menu "All Databases."

4. The accession number for pGLO plasmid is U62637. Type this accession number in the search window at the top (with the nucleotide dropdown) — press Search.

5. Click on the result. Find the FEATURES section of the accession (left-hand side). How many coding sequences (genes) are reported for this plasmid sequence? _____

6. Find the ORIGINS section of the accession. Report the first 15 bases of the plasmid sequence.

Part II: Using NEBcutter to determine the restriction site map and ORFs of pGLO Plasmid

1. NEB has an excellent array of recombinant DNA, cloning online tools. Go to http://nebcloner.neb.com/ and explore. Click on the traditional cloning workflow and watch the video on molecular cloning. Draw a flowchart of cloning workflow below:

2. In a new tab, go to NEB website: www.neb.com and click on NEBcutter under Tools & Resources (http://nc2.neb.com/NEBcutter2/)

3. Type in the Accession number for pGLO plasmid (U62637) into the "GenBank Number" box. Click on the Circular button at **The Sequence is** section. This is important! Leave the other settings at default and click **submit**.

4. Once you press submit, the window will reload and show an image of the plasmid with restriction sites and with the ORFs identified. Right-click on the restriction map, select copy **and paste it into your MS Word document** with an appropriate heading.

5. Click on one of the ORFs, a, b, or c. This ORF will open in a new window with the amino acid sequence of this gene. Copy and paste this gene sequence into your MS Word document with an appropriate heading.

6. To identify this gene, copy the sequence starting at ">" and paste it into the NCBI Protein BLAST database here: https://blast.ncbi.nlm.nih.gov/Blast.cgi Click on Protein Blast, then copy sequence into "Enter Accession Number."

7. Scroll down and click BLAST at the bottom of the page. Note that this may take quite a few minutes, depending on how busy the server is. Be patient. The window will refresh multiple times before it is complete.

8. When results are loaded, copy the conserved domain image "superfamily" at the top of the page (move cursor over top image, right-click, and copy image) and paste into your MS word document with an appropriate heading.

9. Scroll down to Sequences producing significant alignments (under the multicolored chart). The sequences at the top of the table are the results, which are the closest match to the query sequence.

10. Click on the accession number for the TOP RESULT only (on the right). Copy and paste this amino acid sequence (ONLY!) into your result MS word document. DO NOT copy the entire document – this is hundreds of pages long!

11. Go back to NEBcutter and repeat with the remaining ORFs.

12. Fill in the following table:

ORF in Plasmid	Top Search Result (protein name & organism)

Part III: Using JSmol to view a 3-D model of GFP

1. Go to the Protein Database website https://www.rcsb.org/

2. The PDB ID# for GFP is 3i19. Enter this in the search field and click on Go.

3. Right-click on the 3D image of GFP protein structure, press copy, and paste into your MS Word document with appropriate heading.

4. Click on "3D View Structure" and explore the various 3D protein features of GFP. Note: As a reminder, you should be doing this work in an updated browser, such as Chrome. NOT MS explorer.

5. Observe the protein from all sides and answer the following questions:

a. GFP is composed of several structures that comprise a barrel – what is the name of this secondary structure? How many are needed to make the barrel?

b. Do you see any alpha-helices? How many are there?

c. Describe the 'active site' for fluorescence.

Part IV: Using Clustal Omega to compare GFP sequences from different species of jellyfish

GFP is a protein found in many species of jellyfish. It was originally isolated in *Aequorea victoria*. ClustalQ is an alignment tool that can be used to compare sequences of similar proteins.

1. Return to the NCBI website (www.ncbi.nlm.nih.gov/)

2. Copy and paste the FASTA format of each of the following GFP sequences into your MS Word document, and press save! Searching the accession numbers below, click on FASTA format, and copy and paste the entire file from ">" onward.
    a. AAC53663.1: pGLO plasmid
    b. AAA27722: GFP from *Aequorea victoria*
    c. AAN41637: GFP from *Aequorea coerulescens*
    d. AAK02062: GFP from *Aequorea macrodactyla*

3. Copy and paste each FASTA file one after another. Start each on a new line, but do NOT insert a paragraph spacing or line between sequences.

4. Go to the Clustal website: http://www.ebi.ac.uk/Tools/msa/clustalo/

5. Copy and paste all four FASTA files and paste them into the search window provided. Ensure Protein and Clustal with numbers is selected. Click submit. It may take a few minutes to complete the request.

6. Click on Show Colors. Copy and paste your aligned sequences into your MS word results document. The colors represent different chemical properties of the amino acids:
    - Red: small hydrophobic
    - Blue: Acidic
    - Magenta: Basic
    - Green: Hydroxy, sulfhydryl, amine, and G
    - Gray: Unusual amino acids

The GFP plasmid was generated by genetically modifying the native form of GFP to obtain a brighter and more stable fluorescent molecule. Looking at the consensus sequence and the different colored amino acid identifies the differences between the native GFP from *Aequorea victoria* and the GFP that has been genetically modified in the pGLO plasmid. What amino acids were changed? How do you think those changes modified the structure?

7. Click on the Phylogenic Tree to observe the relationship between these species. Copy and paste it into your MS Word document (you may need to do a print screen for this tree).

8. Using your phylogenic tree results, what two GFP proteins are most alike? What two are most different? Discuss what this means – evolutionarily speaking?

9. Save and print out a copy of your results from your MS Word file and include with your report.

Unit 8-A: BIOINFORMATICS

For this portion of the lab report, print out your well-labeled and well-organized results with your exercise answers and include this with your lab report. Answer the following questions and attach to the back of your lab report:

1. What is the NCBI? Why was it used in this exercise?

2. How many amino acids are in GFP protein? _____

3. List the genes in the pGLO plasmid. What is the purpose of each of these genes in this plasmid?

4. Describe the 3-dimensional structure of GFP.

CONCLUSIONS:

Lab Unit 8-B: Transformation of E. coli with a Recombinant GFP

DNA recombination or molecular cloning consists of the insertion of DNA fragments from one type of cell or organism into replicating DNA of another type of cell. The cell is said to be transformed, and many copies of the inserted DNA can be made in the cell. If the inserted fragment is a functional gene coding for a specific protein, many copies of that gene and translated protein could be produced in the host cell if there is a promoter preceding the site of insertion. This process has become necessary for the large-scale production of proteins (*Bacillus thurengiensis* toxin, insulin, human growth hormone, Factor VIII, etc.) that are of value in agriculture, medicine, and other sciences.

While transformation is a relatively rare event under natural conditions, it is possible to manipulate conditions to make transformation frequencies higher in the laboratory. For example, plasmids can be used as vectors to carry fragments of DNA into bacterial cells. *Plasmids are closed, circular DNA molecules that are capable of autonomous replication within a host cell.* There are many naturally occurring plasmids, but the plasmids used in the biotechnology laboratory are those that have a high copy number in host cells. After the host cell has been transformed with a high copy number plasmid, the plasmid will multiply and be maintained at levels of hundreds to thousands of copies within each cell.

Plasmids have been genetically engineered to contain a cluster of restriction enzyme sites within a short region of the plasmid called a multiple cloning site, or MCS. This allows for the insertion of DNA fragments produced from a restriction digest to be incorporated into plasmid DNA at its multiple cloning sites after digestion with the same restriction enzyme. After allowing the sticky ends of fragments of target DNA to anneal to the complementary sticky ends of the plasmid, the DNA insert is fixed in place with covalent bonds by DNA ligase, forming a recombinant DNA (rDNA) plasmid. In this experiment, the plasmid contains a gene coding for a green fluorescent protein (GFP) isolated from a jellyfish (*Aequorea victoria*).

The plasmids used to transform bacterial cells also have a selectable marker gene. This gene codes for a protein that allows the scientist to distinguish cells that have been successfully transformed by plasmid DNA from those that have not. The most common selectable markers are antibiotic resistance genes, which allow for the selection of transformants by growth on media containing the antibiotic. Non-transformed cells will die, and transformed cells will survive under these conditions. In this lab exercise, a plasmid with an antibiotic resistance gene for ampicillin (amp) is used; the ampicillin resistance gene codes for an enzyme that destroys the ampicillin in the surrounding growth media.

Making bacterial cells competent for the uptake of DNA

The efficiency of transformation can be improved by carefully managing conditions before and during the transformation. For example, the choice of bacteria and plasmid can affect the efficiency of transformation, because many plasmids have a narrow host cell range and will only transform bacterial cells of a single species. Transformation frequencies are considerably higher when using fresh bacterial cells taken from actively growing cultures. Also, bacterial cells can be

made competent for DNA uptake by pretreatment with chloride salts of divalent cations such as calcium, followed by a cold-shock and a heat-shock step. The metal ions and temperature changes affect the structure and permeability of the cell wall and cell membranes such that DNA molecules can pass through. Cells that are allowed to recover in non-selective growth media and at their optimal growth temperature following transformation also have higher transformation frequencies. The recovery time allows the transformed cell to amplify the plasmids and to express the antibiotic resistance gene required for survival on the antibiotic-containing selection medium.

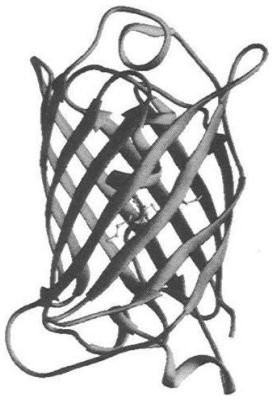
Green Fluorescent Protein (GFP)

The green fluorescent protein (GFP) is a fluorescent protein naturally occurring in the Pacific jellyfish *Aequoria victoria* that has been successfully cloned into some organisms from bacteria to mice. Although originally chosen for its novelty of causing the transgenic organisms to glow green, GFP has been successfully used as a marker for transformation. Recent studies have created gene fusion in which the GFP gene is fused to genes of target markers on either the N- or C-terminus of the protein that they encode. The GFP becomes a marker for the intracellular location of the target gene product, tracking its migration by fluorescence microscopy into the nucleus, mitochondria, secretory pathway, plasma membrane or cytoskeleton. GFP can also be used as a reporter of gene expression levels as well as a measure of protein-protein interactions. Therefore, GFP is a very useful tool for both geneticists and cell biologists.

To date, the GFP gene has been introduced and expressed in many biological systems including yeast, bacteria, fish, plant, fruit fly, and many types of mammalian cells including human cells. This was such an important scientific discovery that Martin Chalfie, Osamu Shimomura, and Roger Y Tsien were awarded the 2008 Nobel Prize in Chemistry in 2008 for their roles in the discovery and development of GFP.

The green fluorescent protein is a medium-sized protein of 238 amino acids and a molar mass of 27,000 Daltons. In spectrophotometry, it shows a major absorption peak at 395 nm and a minor absorption peak at 475 nm. The characterizing molar extinction coefficients are 30,000 and 7,000 $M^{-1}cm^{-1}$ respectfully. ***Fluorescence at 509 nm*** is not energy-requiring and depends on the amino acids serine-65, tyrosine-77, and glycine-67. This trimer forms a fluorescent chromophore after translation by cyclization and oxidation reactions.

Once isolated, the GFP is stable across a wide range of temperatures and pH. It is very resistant to denaturation, requiring treatment with 6 M guanidine hydrochloride at 90^{o}C or pH of <4.0 and >12.0. Furthermore, it can renature completely within minutes following many denaturing protocols, including sulfhydryl reagents such as 2-mercaptoethanol.



Overall Shape of GFP Monomer (*Carson, M, 1987. J. Mol. Graphics **5**:103-106.)*

GFP consists of a dimer, each made of a barrel-shaped cylinder made primarily of β pleated sheets on the outside and α-helices on the inside, a structure that is unique among proteins. This structure produces a compact domain that surrounds and protects the fluorophore located at the center of each cylinder as shown in the figure above. The N-terminal region of the protein acts as a "cap" on the end of the protein, further protecting the core fluorophore. When this cap is disrupted, the fluorescence may be easily quenched. The dimers are probably held together with the hydrophilic interactions of the pleated sheets on the outside of the cylinders.

GFP PLASMID – pGLO

The pGLO plasmid contains several genes that enable replication of the plasmid DNA and expression of the fluorescent trait (phenotype) in bacteria following transformation. Some of the essential genes include:

Figure 2: Plasmid Map: pGLO

- GFP — the jellyfish gene that codes for the production of Green Fluorescent Protein.
- amp^r— A gene that encodes the enzyme beta-lactamase, which breaks down the antibiotic ampicillin. Bacteria containing the amp^r gene can be selected by placing ampicillin in the growth medium.
- Ori — The origin of pGLO plasmid DNA replication.
- araC — A gene that encodes the regulatory protein that binds to the pBAD promoter. Only when arabinose binds to the araC protein is the production of GFP switched on.
- pBAD Promoter — A specific DNA sequence upstream of the GFP gene, which binds araC-arabinose and promotes RNA polymerase binding and transcription of the GFP gene.
- Multiple Cloning Sites — Regions of known restriction (*Nde*I, *Hin*dIII, *Eco*RI, etc.) sites that permit insertion or deletion of the gene of interest.

In this lab, you will transform *E. coli* bacteria with a plasmid that has the GFP gene inserted into it. To learn more about Bio-Rad's pGLO transformation kit lab exercise, watch the following video: https://youtu.be/c40UudFIlGw Summarize the procedure in a flow chart below:

PREDICTION: Predict the results you expect with this transformation experiment:

1. Label each of the following four plates with the agar (LB, LB-amp etc.)
2. Label each experimental condition (+pGLO, -pGLO)
3. Draw what you predict and explain why (right)

DRAW PREDICTION	EXPERIMENTAL CONDITION	EXPLAIN

Lab Unit 8: pGLO Transformation

PURPOSE: The purpose of this experiment is to transform competent E. coli bacteria with a pGLO plasmid. These transformed cells will be used in a later experiment to produce recombinant GFP.

SAFETY:

The *Escherichia coli* strain used in this experiment is not considered a pathogen, but *E. coli* bacteria colonize the intestinal tracts of animals. Although it is rarely associated with any illness in healthy individuals, it is good practice to follow simple safety guidelines in handling and disposal:

1. Gloves and goggles must be worn at all times.
2. Wipe down the lab bench with antibacterial cleaner before starting the lab and before leaving the laboratory.
3. All materials, including plates, pipettes, loops, and tubes that come in contact with bacteria should be autoclaved before disposal in the garbage.
4. Wash hands thoroughly with soap and water after removing gloves.

MATERIALS

Each group:	Class shares:
☐ Micropipettes and plugged tips	☐ Benchtop cleaner
☐ Sterile microcentrifuge tubes	☐ 37˚ C incubator
☐ Microcentrifuge tube racks	☐ 42˚ C water bath & floats
☐ Floating microcentrifuge racks	☐ Hot gloves
☐ Gloves	☐ Longwave UV light source
☐ Sterile inoculating loops	☐ Fresh starter culture of *E. coli*
☐ Ice bath	☐ Petri Dishes
☐ 2 LB Petri plates	☐ (Lyophilized *E. coli)*
☐ 2 LB/amp Petri plates	☐ pGLO plasmid solution (0.08ug/ul)
☐ 1 LB/amp/ara plate	☐ Petri dishes for preparing agar plates
☐ Biohazard bags and stands	☐ Agar packet
☐ LB broth	☐ Transformation solution
☐ Cold 50 mM $CaCl_2$	☐ Ampicillin vial
	☐ Arabinose vial

PROCEDURE	EXPERIMENTAL NOTES
Part I: Preparing Plates (day 1) Note: Part of this part may have already been completed. ***Ask your instructor.*** 1. Label Petri plates with a sharpie, as demonstrated by your instructor. There will be 16 plates LB, 16 plates LB/amp, and eight plates LB/amp/ara. a. One stripe = LB (only) b. Two stripes = LB-amp c. Three stripes = LB-amp-ara 2. Clean the area that will be used to pour plates. 3. Prepare nutrient agar a. The agar plates should be prepared at least three days before the experiment, left out at room temperature for two days. b. To prepare the agar, add 500 ml of purified water to a 1L or larger clean Erlenmeyer flask or glass bottle. c. Add the entire contents of the LB nutrient agar packet. Swirl the flask to mix the agar, and autoclave-sterilize 20min, 121^{o}C. d. Cool in a clean 50°C water bath. 4. Prepare arabinose and ampicillin a. ***Note***: Arabinose requires at least 10 minutes to dissolve - be patient. With a new sterile pipet, add 3 ml of transformation solution directly to the vial to rehydrate the sugar. Mix the vial; a vortex helps. b. With a new sterile pipet, add 3 ml of transformation solution directly to the vial to rehydrate the antibiotic. 5. Pour LB nutrient agar plates (LB, LB/amp, LB/amp/ara) a. Aliquot, by pouring, LB nutrient agar into the 16 plates that are labeled LB. b. Stack the empty plates 4 to 8 high and starting with the bottom plate lift the lid and the upper plate straight up and to the side with one hand and pour the LB nutrient agar with the other. c. Fill the plate about one-third to one-half (~12 ml) with agar, replace the lid and continue up the stack. Pour 16 plates in this fashion and label them as LB.	

6. Now, add the hydrated ampicillin to the remaining LB nutrient agar. Swirl briefly to mix. Pour into the 16 plates that are labeled as LB/amp as above.

7. Last, add the hydrated arabinose to the remaining LB nutrient agar containing ampicillin. Swirl briefly to mix and pour into the eight plates labeled as LB/amp/ara using the technique utilized above.

8. Allow plates to set at ambient temperature for the next class. Keep plastic sleeves for plates! You may leave at ambient temperature for two days — store at 4°C.

9. Next class leave out one LB (only) plate per group and put the rest back into the sleeves — store upside down (agar side up) in the fridge.

Part II: Starter Culture (day 2)

1. Rehydrate lyophilized *E. coli* by directly adding 250ul of transformation solution directly into the vial.

2. Recap, swirl to mix, and allow to stand for 5 min at ambient temperature.

3. Each group prepares a started culture plate by streaking plate for isolated colonies.
   a. Label the top of an LB (only plate) with your group initials, the date, LB, and *E. coli* starter culture, as demonstrated by your instructor. ***Keep a lid on the plate at all times.*** Remember, this plate has no antibiotics! Anything from the air that lands on the plate will grow in this nutrient-rich environment.
   b. Shake culture to mix just before use.
   c. See streaking demonstration by instructor and as shown in the figure below.
   d. Using a 10ul loop, insert the loop into bacteria vial and remove a loop full of rehydrated E. coli. The loop will have a shine to it (like when you are blowing bubbles).
   e. Dispense the 10ul loop of culture on a labeled LB only plate, spread as on a single line on the plate (see figure below). Dispose of the loop.
   f. Obtain a clean loop and repeat streak, dispose of the loop. Repeat two more times. See the diagram below and the demonstration by your instructor.

g. Return lid, turn upside down and incubate 24-48hrs at 25-37°C. The colonies should be 1mm wide. *h. NOTE: You must use a fresh starter plate. Prepare 1-2days before the transformation section.*	

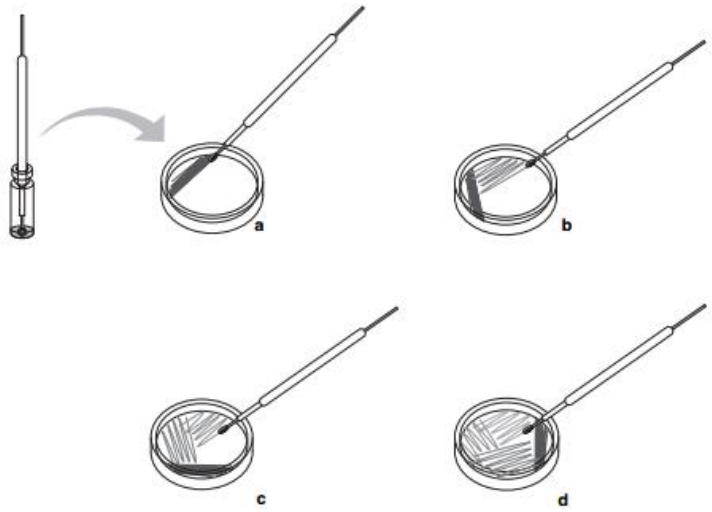


Figure 3: Streaking for isolated colonies. This is your starter plate.
Watch this video for a detailed explanation: https://youtu.be/0heifCiMbfY

PROTOCOL	EXPERIMENTAL NOTES
Part III: Transformation of *E. coli* (day 3) *BIOHAZARD! Note, all tips, tubes, and gloves must be disposed of in the biohazard trash. Clean benchtop before and after working. Discard gloves in biohazard trash and wash hands before leaving the lab.* 1. Label the top of one closed microcentrifuge tube "+pGLO" and another "–pGLO." Label both tubes with your initials along the side. 2. Add 250 µL of transformation solution ($CaCl_2$) into both tubes. Place the tubes on ice. 3. Use a sterile loop to pick up a single colony of bacteria from the starter plate. Pick up the +pGLO tube and immerse the loop into the transformation solution at the bottom of the tube.	

4. Spin the loop between your index finger and thumb until the entire colony is dispersed in the transformation solution. Place the tube back on the ice. 5. Using a new sterile loop, repeat for the –pGLO tube. 6. Examine the pGLO plasmid DNA solution with the UV lamp. Does it glow? 7. Add 10 ul of the pGLO plasmid into the cell suspension of the +pGLO tube. Close the tube and return it to the ice. ***Do not add plasmid DNA to the –pGLO tube.*** Why, not? 8. Incubate the tubes on ice for 10 minutes. 9. While the tubes are incubating on the ice, label your four agar plates around the bottom of the plate with the agar (not the lid). See the instructor for a demonstration. Ensure you are labeling the correct plate! ○ Label one LB/amp plate: +pGLO ○ Label the LB/amp/ara plate: +pGLO ○ Label the other LB/amp plate: -pGLO ○ Label the LB plate: -pGLO 10. Heat shock the sample by transferring both tubes into the 42°C water bath for exactly 50 seconds (use a timer!). 11. Immediately after 50 seconds, place both tubes back on the ice. For the best transformation results, the change from the ice (0°C) to 42°C and then back to the ice must be immediate! 12. Incubate tubes on ice for 2 minutes. 13. Remove the tubes from the ice and place them on the benchtop. 14. Open each tube, add 250 µL of LB broth to the tube, pipet up and down to mix, and reclose the tube. 15. Repeat with the second tube, using a clean pipet tip.	

16. Incubate the tubes for 10 minutes at room temperature.

17. Flick the closed tubes with your finger to mix. Using a clean pipet tip for each sample, transfer 100 µL of the appropriate reagents onto the plates as follows:
    - LB/amp plate: +pGLO tube
    - LB/amp/ara plate: +pGLO tube
    - LB/amp plate: -pGLO tube
    - LB plate: -pGLO tube

18. Use a new sterile loop for each plate. Spread the liquid evenly around the surface of the agar by quickly skating the flat surface of a new sterile loop back and forth across the entire plate surface. Return the lid and allow liquid to absorb into the plate on the benchtop. This may take 5-10 minutes.

19. Once the liquid had fully absorbed into the surface of the plate, invert, and stack your plates together. You can tape them together and label them with group initials.

20. Place the inverted stack in the 37°C incubator overnight. *Why do you incubate plates with the agar side facing down?* The plates should be removed after approximately 18-24 hours and refrigerated until the next class period.

21. If there are extra LB (only) plates, use them to practice streaking for isolated colonies. Use your *E. coli* starter culture LB plate. Put these into the 37°C incubator overnight with your other plates.

Part IV: Analysis of transformants (day 4)

PROTOCOL	EXPERIMENTAL NOTES
1. Observe the plates with the UV lamp. Do any plates have glowing green colonies? Why? 2. Count the number of colonies on each plate and record it in a table below. A convenient method to keep track of counted colonies is to mark the colony with a marker on the outside of the plate as you count it or use a clicker counter. 3. Record results in a table in the attached worksheet. 4. <u>Keep plates from this experiment for your next two labs!</u> Seal plates closed with Parafilm, or place them in a ziplock baggy, so they do not dry out. 5. Label and store plates at 4^{o}C as directed by your instructor. 6. Calculate transformation efficiency by completing the worksheet on the following page.	

RESULTS TABLE:

Experimental Conditions	# of colonies/plate	The appearance of colonies under UV light
+pGLO on LB/amp		
+pGLO on LB/amp/ara		
-pGLO on LB/amp		
-pGLO on LB		

Transformation Efficiency Worksheet

In many experiments, it is important to transform as many cells as possible genetically. For example, in some types of gene therapy, cells are collected from the patient, transformed in the laboratory, and then put back into the patient. The more cells that are transformed to produce the desired protein, the more likely, that the therapy will work. The transformation efficiency is calculated to help scientists determine how well the transformation is working.

$$\text{Transformation efficiency} = \frac{\text{Total number of cells growing on the agar plate}}{\text{Amount of DNA spread on the agar plate (in µg)}}$$

1. Total number of transformed colonies on the LB/amp/ara plate: _____

2. The total amount of pGLO plasmid DNA in the bacterial cells on the LB/amp/ara plate:
   Check stock of pGLO plasmid. Its concentration is: _____ ug/ul (0.08ug/ul)

   Volume of pGLO plasmid used _____ ul

   Multiply concentration of pGLO plasmid by the volume transferred to tube: _____ug pGLO

3. Total volume in the transformation solution:
   CaCl2: _____ ul
   pGLO plasmid: _____ ul
   LB broth: _____ ul
   Total Volume: _____ ul

4. Volume spread on the plate: _____ ul

5. Calculate the fraction of the total transformation solution used:
   $\frac{\text{Volume spread on LB/amp/ara plate}}{\text{Volume of total transformation solution}}$ = _____

6. Calculate the mass of pGLO plasmid on the plate:
   Fraction of total transformation x total pGLO DNA in solution
   _____ x _____ ug = _____ ug

7. Calculate the number of colony forming units (CFUs) per ug of DNA transformed
   Transformation Efficiency:

   $\frac{\text{Number of colonies transformed}}{\text{ug of DNA on the plate}}$ = _____ = _____* transformants/ug

   *NOTE: Report transformation efficiency in a whole number

Lab Unit 8-B: Transformation Assignment

1. What is a bacteria colony? Is a single colony one bacteria cell?

2. Describe how you would properly streak for a single, isolated colony. Why is this important?

3. Exogenous DNA (such as a plasmid) does not passively enter *E. coli* cells that are not competent. What treatment do cells require to be competent?

4. Discuss Results.
    a. Were there any differences between the two +pGLO plates? Explain.

b. Were there any colonies on the –pGLO on LB/amp plates? Why or why not?

c. Were there any colonies on the –pGLO on LB plate? Why or why not?

d. What was the Transformation Efficiency you calculated for your group? What does this say about the efficiency of the transformation in your experiment?

CONCLUSION:

LAB UNIT 9: PLASMID DNA ISOLATION

J. O'Grady, EdD. Adapted from Bio-Rad's Aurum Plasmid Mini-prep Kit (Cat# 732-6460)

OBJECTIVES

In this lab activity, students will:

- ✓ Describe the structure and function of plasmid DNA
- ✓ Distinguish plasmid DNA isolation from previous genomic DNA isolation approaches
- ✓ Demonstrate safe and proper lab skills of working with biohazard material
- ✓ Isolate plasmid DNA from bacteria
- ✓ Use spectrophotometry to analyze the quality and quantity of a nucleic acid preparation

INTRODUCTION

As you explored in the previous lab, biotechnicians frequently genetically recombine foreign DNA into a bacterial plasmid. *Plasmids are closed, circular DNA molecules that are capable of autonomous replication within a host cell.* There are many naturally occurring plasmids, but the plasmids used in the biotechnology laboratory are those that have a high copy number in host cells. After the host bacterial cell has been transformed with a high copy number plasmid, the plasmid will multiply and be maintained at levels of hundreds to thousands of copies within each cell. Once you get the plasmid into the host, how then do you get it back out?

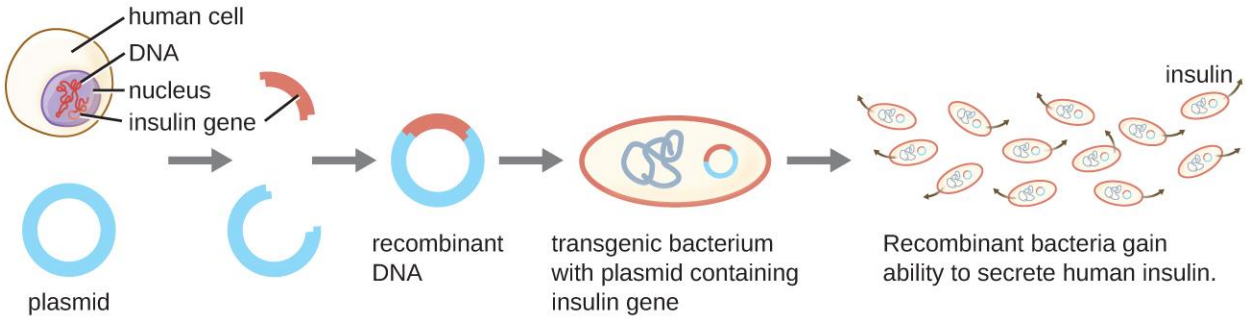


Figure 9-1: Recombinant DNA technology is the artificial recombination of DNA from two organisms. In this example, the human insulin gene is inserted into a bacterial plasmid. This recombinant plasmid can then be used to transform bacteria, which gain the ability

The isolation of nucleic acids is a common practice in the molecular lab. Generally, nucleic acid isolation first requires the disruption of the cell membrane (and cell wall when present) using a mechanical, chemical, or enzymatic treatment. After the disruption and centrifugation of the cellular debris, the supernatant can be selectively separated by a variety of techniques. Genomic DNA extraction typically involves the extraction of protein (using phenol, enzymes, or binding reagent), followed by alcohol precipitation of the DNA. But when you have both genomic DNA and plasmid DNA present, how can you selectively target plasmid DNA alone?

ALKALINE LYSIS PLASMID PURIFICATION

The *alkaline lysis method for purifying plasmid DNA* from bacterial cultures can selectively isolate plasmid DNA from a complex molecular mixture.

1. Transformed bacteria are grown overnight, and approximately 1-5mL of culture is centrifuged to collect bacteria.
2. The bacterial pellet is resuspended completely in a buffer.
3. The resuspended bacteria are gently incubated with alkaline detergent and enzymatic lysis solution that will disrupt the cell wall and plasma membrane releasing the cellular components. The alkaline pH will denature linear (genomic) DNA molecules, while the supercoiled circular plasmid DNA will be unaffected.
4. A neutralization solution (acetic acid) is added, which will cause sudden renaturation the larger gDNA causing it to aggregate. Since the plasmid DNA is supercoiled it is not impacted by the sudden pH change.
5. After centrifugation, the genomic DNA is pelleted with the cellular debris and the plasmid DNA remains in the supernatant.
6. The supernatant is passed through a slightly positive silica membrane column, which has a high affinity for negatively charged DNA molecules. Other molecules easily pass through this porous membrane.
7. The DNA can be eluted from the silica membrane using a Tris-EDTA buffer.

Watch this video on alkaline lysis plasmid purification. Read the protocol below. Draw a flowchart summarizing this lab procedure.
https://youtu.be/O4oLyd2mZv8

MONITORING BACTERIAL GROWTH USING OPTICAL DENSITY

Optical density is a common microbiology technique used to monitor the growth of microorganisms. Although a spectrophotometer is used in this measurement (600nm), it is not the amount of light absorbed, but rather, *light scattered by the microorganisms*. A typical microbial growth curve can be plotted by monitoring the OD600 by time. The OD600 indicates indirectly through light scattering an approximate number of live cells. See figure below.

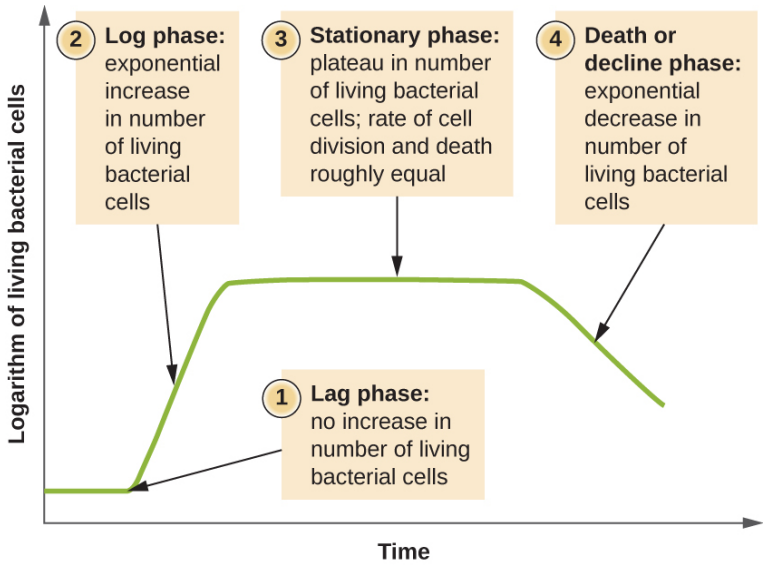


Figure 2: A typical microbial growth curve. Image credit: OpenStax

During the lag phase, the cells are adjusting to their new environment and may not reproduce immediately. Depending on the species this lag phase can last a few hours (typically) up to a few days as it adjusts to its new growth conditions. In the log phase of growth, the cells reproduce logarithmically. This is an important stage for researchers as the metabolic rate of individual cells is at its maximum, the cell wall is intact, and growth is stable and healthy. As nutrients become depleted, bacterial growth slows and enters the stationary phase. During this phase nutrients are depleted, waste is built up, and the rate of reproduction stops (the number of dying and produced cells is equal). It's important to note that during this phase the metabolic rate of live cells slows. During the death phase, the number of dying cells outnumbers the live cells substantially. There may still be some live cells in the culture. For some organisms, during this phase, they may develop resting structures such as endospores.

Determining the Optical Density of the bacterial culture

Twelve OD•mL is recommended to obtain a reasonable yield of plasmid DNA to perform any downstream application such as transformation, sequencing, or restriction digest. If there are too few bacteria, plasmid yield will be low, if there are too many bacteria, the lysis will be inefficient, the genomic DNA may be too goopy to centrifuge, and that too will result in a low yield of the plasmid. To calculate the OD•mL, measure the optical density of the bacterial culture in a spectrophotometer, set to 600nm (OD600), and multiply the culture volume in mL.

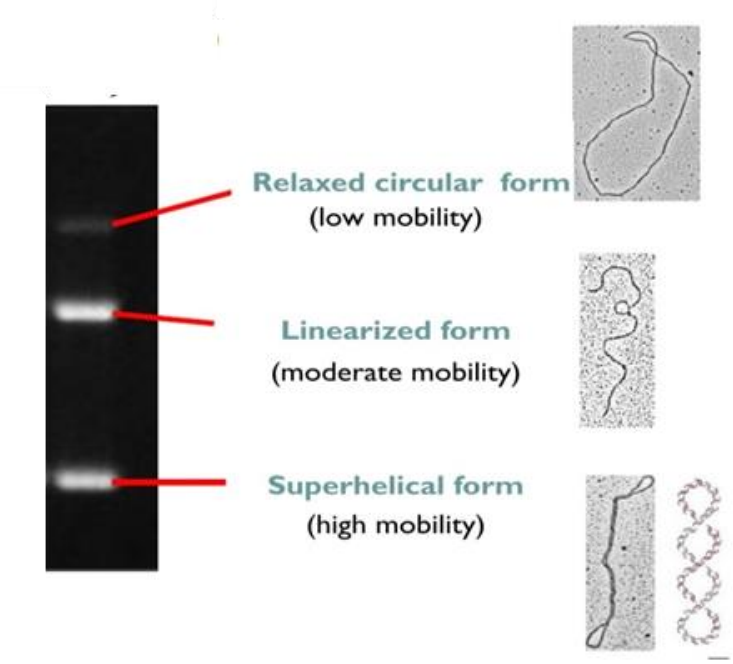
AGAROSE GEL ELECTROPHORESIS TO ANALYZE NUCLEIC ACID

As discussed in previous labs, agarose gel electrophoresis is a molecular biology technique used to separate a mixture of nucleic acid fragments according to size in an agarose matrix. DNA or RNA samples are separated by applying an electric field to move the largely negatively charged molecules toward the positive electrode. In this lab, you will analyze your plasmid DNA quality using agarose gel electrophoresis.

Agarose Separation of Plasmids. Since DNA molecules are double-stranded (and the same shape), they migrate through the gel matrix separating according to size alone. Smaller molecules travel faster through the gel matrix than larger molecules at a rate that is inversely proportional to the log10 of the number of base pairs. Therefore, by running a known molecular weight ladder at the same time as your nucleic acid sample, you can determine the size of the nucleic acid fragments using linear regression analysis. In addition to agarose concentration and molecular weight, the mobility of nucleic acids in agarose gels is also influenced by the molecular conformation of the nucleic acid.

The three forms of circular plasmid DNA are:

1. Form I: Closed, circular, negatively supercoiled DNA
2. Form II: "Nicked or relaxed" DNA, which has been partially cut through one strand of the DNA, causing it to unwind
3. Form III: Linear DNA, which has been cut by restriction enzymes



Form I usually have the greatest electrophoretic mobility of all DNA forms because supercoiled DNA molecules tend to be compact. Think of it as a bullet moving through the agarose gel. The size of closed circular plasmid DNA cannot be determined on an agarose gel relative to the bands of a linear marker. The slowest moving DNA of all is Form II because it is an open circle with a partially unwound strand, which causes it to drag in the gel. While the linear DNA can snake through the agarose particles, reducing drag, the circular, unwound plasmid cannot. Form III, or plasmid DNA that has been cut with restriction enzymes, has decreased mobility because the linear DNA is like a long string or rod that can drag.

Unlike the other forms of DNA, *linear DNA migrates through a gel at a rate that is inversely proportional to the logarithm of its molecular weight.* Therefore, the molecular weight of linear DNA can be estimated from a gel if compared to DNA fragments of known molecular weight (markers).

Lab Unit 9A: Plasmid DNA Isolation

PURPOSE: The purpose of this lab is to isolate plasmid DNA from transformed cells.

SAFETY: What are safety considerations when working with *E. coli* and plasmids? Think about both handling these materials AND waste disposal. Refer to previous lab activities.

MATERIALS:

PROTOCOL

Day 1: Overnight cultures

This may have been done for you. Ask your instructor.

1. Obtain your plate of *E. coli* pGLO transformants on LB/amp/ara and a labeled tube containing 5 mL Luria broth with ampicillin (LB/amp).

2. Use a sterile inoculating loop to pick up a single transformant colony glowing green.

3. Place the loop into the LB and swirl to completely transfer the bacteria into the broth.

4. Place in a 37°C, vigorously shaking incubator 18-24hrs. It is important that this culture be fresh – alternative procedures include 37°C /48hrs or 25°C/48hrs (highest yield). It is not recommended to store cultures at 4°C before extracting this may result in a low yield.

Day 2: Plasmid Isolation & NanoDrop Analysis of DNA

PROTOCOL	EXPERIMENTAL NOTES
Calculate the volume of culture needed to prepare 12 OD•mL of liquid culture: 1. Plug-in, and warm-up spectrophotometer and collect materials, including SOP for operating spectrophotometer. 2. **Dilute culture 10-fold:** Completely resuspend bacteria and remove a 50ul aliquot to a clean 1.5mL centrifuge tube. Add 450mL of warm LB. Gently invert to mix. 3. Set the spectrophotometer to 600nm. 4. Blank (zero) the spectrophotometer with LB broth. 5. Measure the diluted sample.	Spectrophotometer model and unit: _____ OD600: _____ OD•mL = _____ (OD) x dilution factor 10 x (X mL culture) Solve for X: _____

Plasmid Isolation

PROTOCOL	EXPERIMENTAL NOTES
The volume of culture needed for 12 OD•mL of culture is typically between 4 and 6mL. Therefore, we will be centrifuging the remaining culture into one 2 mL tube. 1. *Pellet 4mL of the 5mL culture as follows:* a. Using a p1000 plugged micropipette tip, aliquot 2mL of culture directly into a 2 mL centrifuge tube. b. Centrifuge 2mL of culture at 12,000rpm for one minute. c. Decant supernatant directly into biohazard waste container as directed by your instructor. d. Using a clean tip, add an additional 2mL of culture directly into same tube and repeat centrifugation. e. Decant all the supernatant into the waste beaker. f. Change gloves. 2. Add 250 µL of the Resuspension Solution and vortex until the cell pellet is completely suspended. Do not use a micropipette as the pellet is sticky and will get lodged and clog the tip. 3. Add 250 µL of the Lysis Solution and mix by GENTLY inverting the capped tube 6-8 times. The solution should become viscous and slightly clear if the cell lysis has occurred. 4. Add 350 µL of the Neutralization Solution and mix by GENTLY inverting the capped tube 6-8 times. A visible precipitate (consisting of cellular debris) should form.	

5. Pellet the cell debris for five minutes at 12,000 rpm in a microcentrifuge. A compact white pellet will form along the side or bottom of the tube. *The clear supernatant in this step contains the plasmid DNA.* 6. Insert a plasmid mini-column into one of the 2 ml cap-less wash tubes supplied. 7. Transfer the supernatant to the column. Centrifuge at 12,000 rpm for one minute. The purpose of this step is to bind the plasmid DNA to the column. 8. Remove the spin column from the wash tube, discard the filtrate at the bottom of the wash tube, and return the column into the same wash tube. 9. Add 750 µL of Wash Buffer and centrifuge at 12,000 rpm for one minute. The wash buffer contains ethanol and washes away impurities from your sample. 10. Remove the spin column from the wash tube, discard the filtrate at the bottom of the wash tube and replace the column in the same wash tube. 11. Centrifuge for an additional minute to dry membrane and remove residual traces of ethanol. 12. Remove the spin column and discard the wash tube. *Place the column in a clean elution tube*. 13. Add 50 µL of elution solution directly on top of the white membrane in the middle of the column. Let sit for one minute to saturate the membranes on the column. 14. Elute the plasmid DNA from the membrane by centrifuging at 12,000 rpm for one minute. 15. Transfer your plasmid DNA preparation to a clean, labeled 1.5mL microcentrifuge tube. If you are not analyzing your plasmid preparation today, store your plasmid at 4°C in a storage box provided.	

Lab Unit 9-B: Spectrophotometric Analysis of Plasmid DNA

Determine the concentration of your eluted DNA on the low-volume (NanoDrop) Spectrophotometer. Include this worksheet in your lab report!

1. Using the SOP provided in your SOP booklet set the NanoDrop spectrophotometer to DNA (nucleic acid).
2. Blank the NanoDrop with 1.5ul of elution solution.
3. Measure 1.5ul of plasmid DNA solution. If possible, print out the spectral graph from the NanoDrop and include it with your report.
4. Record plasmid DNA concentration as well as the A260/A280 ratio:

   Concentration: _____ ng/µL

   A260/A280: _____ A260/A230: _____

5. What does the A260/A280 ratio tell you? What is the optimal ratio for plasmid DNA? How does your ratio compare?

6. Obtain a storage box for the whole class to use. Label the box with the class name and instructor name. Ensure your tubes are labeled, so they are not mixed up with your classmate's tubes. Store the box at -20^{o}C until the next lab exercise.

****OPTIONAL*** ANALYZE YOUR PLASMID ON AN AGAROSE GEL- Use previously prepared SOP.**

Frequently researchers will analyze their plasmid DNA isolations on an agarose gel to determine the quality of the plasmid preparation. Intact supercoiled DNA migrates quickly as one band on an agarose gel – but not according to size – why? When there are small nicks in the plasmid, you may get several different populations that migrate differently.

Lab Unit 9 – Plasmid Isolation

1. The transformed cells (with plasmid DNA) were grown in an LB-ampicillin culture. Why was ampicillin used? How did the cells survive when exposed to this antibiotic?

2. In this lab, you added Cell Lysis solution to your bacterial pellet. What is the purpose of this solution, and what do you think would happen to the results of your experiment if you left out this step?

3. What is the purpose of the spin filter? How does DNA bind to the filter? How do you get DNA to elute from the filter?

CONCLUSION:

LAB UNIT 10: RESTRICTION ENZYME DIGEST

J. O'Grady, EdD, L. Fletcher, Ph.D., A. Wheeler, M.S., P. Phelps, Ph.D.

OBJECTIVES

In this lab activity, students will:

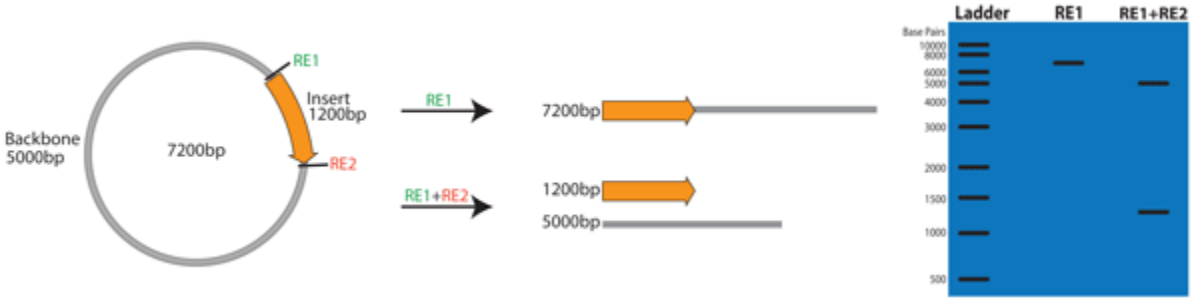
- ✓ Describe how restriction enzymes can be used in recombinant DNA technology
- ✓ Demonstrate safe and proper lab skills of working with recombinant DNA material
- ✓ Analyze DNA fragments on an agarose gel
- ✓ Analyze the size of DNA fragments using graphing techniques; determine linear regression, R2 value and determine the size of DNA bands using the equation of the line

INTRODUCTION

Restriction enzyme digests are a tool used almost daily by molecular biologists. Recall that restriction digests can be used to cut DNA at specific nucleotide sequences, allowing for subsequent manipulation of the DNA, and often to generate recombinant DNA molecules. Restriction digests are also important as a diagnostic tool. Creating recombinant DNA molecules requires numerous steps involving different DNA molecules.

In this lab, you are continuing to work with the plasmid pGLO DNA you just isolated. Recall that in the previous lab you transformed bacterial cells with the pGLO plasmid. Bacterial colonies from this transformation were grown up in LB nutrient broth containing ampicillin as a selective agent. In this lab, you isolated and purified plasmid DNA from these bacterial cells. At this point, it is wise to confirm that the plasmid DNA isolated from your bacterial "overnight" is indeed the pGLO plasmid, and not chromosomal DNA or foreign DNA. To do this, we will first presume that we have in fact isolated the correct DNA from the transformed bacteria.

Verifying Plasmid Insert. Most of the time researchers know the sequence and map of their plasmid DNA. Using this map, you can select the appropriate restriction enzyme to determine if you, in fact, have the plasmid with the insert. Restriction analysis can even be used to determine the orientation of the insert in the plasmid. In the example below, digestion with enzyme RE1 will linearize the 7200bp plasmid into one single 7200bp fragment. Alternatively, digestion with both RE1 and RE2 will result in two bands; the 1200bp insert and the 5000bp backbone.



pGLO Plasmid. The pGLO contains several genes that enable replication of the plasmid DNA and expression of the fluorescent trait (phenotype) in bacteria following transformation. Some of the essential genes and other elements include:

- **GFP:** The jellyfish gene that codes for the production of Green Fluorescent Protein
- **amp^r**: A gene that encodes the enzyme beta-lactamase, which breaks down the antibiotic ampicillin.
- **Ori:** The origin of pGLO plasmid DNA replication
- **araC:** A gene that encodes the regulatory protein that binds to the pBAD promoter. Only when arabinose binds to the araC protein is the production of GFP switched on
- **pBAD Promoter**: A specific DNA sequence upstream of the GFP gene, which binds araC-arabinose and promotes RNA polymerase binding and transcription of the GFP gene
- **Multiple Cloning Sites:** Regions of known restriction (*Nde*I, *Hin*dIII, *Eco*RI, etc.) sites that permit insertion or deletion of the gene of interest.

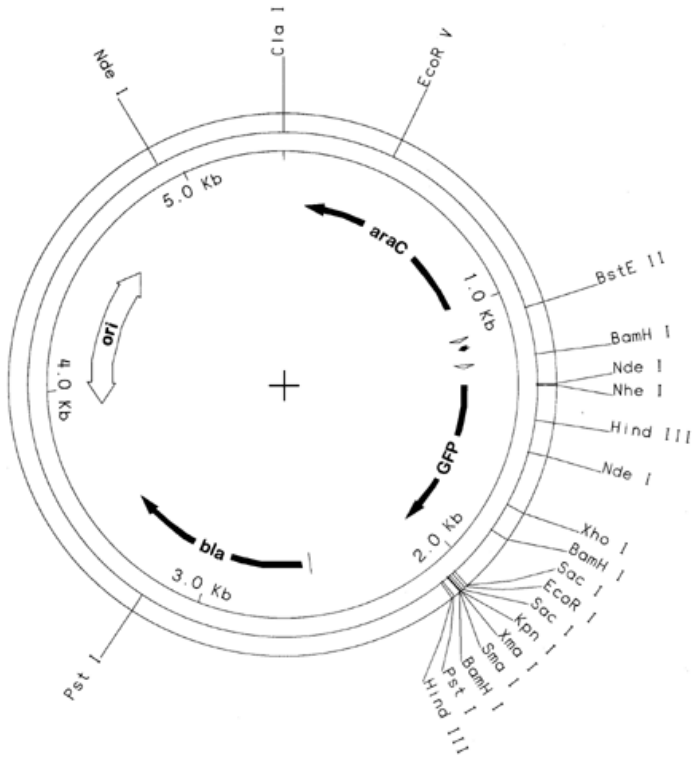


Figure 0-1:Restriction Map of pGLO plasmid DNA

How to choose percentage of Agarose Gel. When performing restriction analysis, you will obtain varying sizes of DNA. Depending on the size of DNA fragments you will choose a concentration of agarose gel that will help resolve those fragments. The following table is a recommended agarose percentage for size separation.

Other factors affecting the resolution of bands include voltage, using a wider comb, and loading less DNA. Note, you would also choose an appropriate marker (DNA ladder) to correspond to the size fragments you will obtain. In our lab, we use a broad ladder and only consider the linear portion of that ladder to determine the size of the DNA fragment.

% Agarose	Size Fragments (bp)	Recommended Voltage
0.8	2,000 to 30,000 (Genomic DNA and large plasmids)	80-125V or lower for gDNA
1.0	500 to 10,000 (most general applications)	100-125V or lower
1.5	200 to 3,000 (better resolution for smaller fragments)	100V or lower
2	50 to 1,000 (better resolution for small fragments)	80V or lower
2.5	50 to 800 *better resolution of small fragments from each other in the same lane	80V or lower

Lab Unit 10-A: Bioinformatics of Plasmid DNA

Part I: NEB Cutter: As part of your pre-lab exercise, complete the following exercise.

1. Open up NEB cutter: http://tools.neb.com/NEBcutter2/
2. Insert the Accession number for this plasmid (Accession #: U62637).
3. Click on "All commercially available" and "circular" DNA. Press submit.
4. The default is single cutters, but you can toggle between single cutters and multiple cutters by pressing the button at the bottom of the screen "Display."
5. Copy and paste the single cutter map into an MS word document. Submit this with your report.
6. Based on this map, predict the size of fragments if you cut this plasmid with the following enzymes:
    - EcoRI: _____
    - Double digest: EcoRI & EcoRV: _____
7. From this map, can you see one or two restriction enzymes that will cut out GFP? What are they? _____
8. Confer with your classmates. As a class, decide the best enzyme(s) to use to determine if you have GPF in your plasmid: _____
9. Ask your instructor if you have these enzymes available in the lab.
10. Predict the size of restriction fragments you expect to see on the agarose gel after restriction digestion with these enzymes. You may use NEB cutter!
    _____
11. What percentage of agarose gel do you recommend to resolve these fragments from each other? See introduction!
    _____

Lab Unit 10-B: Restriction Enzyme Digest of Plasmid DNA

MATERIALS

- Microcentrifuge tubes
- p20, p200 micropipette tips
- 37°C water bath
- Floating tube rack
- Nuclease-free water
- Restriction enzymes as determined above
- Universal Buffer
- Isolated pGLO Plasmid DNA
- Control pGLO plasmid DNA
- Prepared molecular weight marker

SAFETY

It is important to note that recombinant DNA must not be disposed of in regular trash. Dispose of any tips and tubes that have been exposed to recombinant DNA in biohazard trash provided.

PROCEDURE SUMMARY CHART

Component	Concentration	Mass or Conc. required	Tube 1 Undigested pGLO	Tube 2 Digested pGLO
pGLO Plasmid DNA	_____ng/ul	1 µg		
Universal buffer	10X	1X		
Nuclease-free Water				
_____ *Enzyme 1*	_____ units/µL	1 unit (minimum volume is 1ul)	none	
_____ *Enzyme 2*	_____units/µL	1 unit (minimum volume is 1ul)	none	
TOTAL			20 µL	20 µL

PROCEDURE:

1. It is ideal to digest a maximum of 1 µg of DNA in these reactions to see DNA fragments on an agarose gel. Using the concentration of the isolated plasmid DNA, calculate the volume needed to have 1 µg in each tube. The final volume in each tube after every reagent is added should be 20 µL.

NOTE: If your concentration is below 100ng/ul, you will need to use the maximum volume of your plasmid.

Calculate actual mass used in each reaction:
Concentration DNA:_____ng/ul x _____ul = _____ng

2. Using the concentration of the buffer (10X), calculate how much should be added so that when the final volume is 20 µL, the buffer concentration is 1X.

Calculations:

3. Calculate the amount of water needed in each tube, after accounting for the volumes of the other reagents, to make a final volume of 20 µL. Record in the table.

4. Add the reagents to the tubes in the order listed below. **Keep DNA and enzymes on ice. Use a new tip for each solution.** Tube 1 is the control and will contain only DNA, buffer, and water, *NO* enzyme! Why not?
   a. Add water first to each tube (if needed).
   b. The buffer, which should be added second, maintains the proper pH for enzymatic activity. This must be thawed and vortexed to resuspend buffer components.
   c. Then DNA should be added. Make sure you use a clean tip for each DNA sample.
   d. The enzyme should always be added LAST. Pipette the enzyme directly into the solution at the bottom of the microcentrifuge tube, and pipet up and down to mix. The enzyme should be retrieved directly from the freezer and returned immediately to the freezer. It is in glycerol and does not need to be thawed. Pop-spin before opening, KEEP ON ICE, DO NOT VORTEX. Return to freezer immediately.
5. Gently flick to mix, pop spin all the tubes in a Picofuge to ensure all components are in the bottom of the tube.
6. Place all tubes, with lids closed, (in a floating tube rack) in a 37˚ C water bath to incubate for at least 60 minutes and up to 3 hours. However, for this procedure, *it may be beneficial to incubate overnight*.

Lab Unit 10-C: Agarose Gel Electrophoresis of Restriction Digest DNA Fragments

Part I: Prepare Agarose Gel with SYBRsafe

1. Use previous labs to create a protocol to analyze the restriction digests on a 2% agarose gel.
2. The molecular weight marker should already be prepared for you. Load 6ul in the first lane.
3. Add 5ul of 5-6X load dye to each of the restriction digests. Flick to mix and pop spin in a Picofuge, load all the sample into the well.
4. Set the power supply to approximately 80 volts and allow to electrophorese until the tracking dye is approximately 3/4th the way to the bottom of the gels (approximately 1 hour).

PART II: Data Analysis

Include this worksheet with your lab report!

1. Using the large printed out copy of your images, measure the distance migrated by each band in your molecular weight marker (in mm!). Record this data in a table below. It is recommended you look up an image of the marker used online (Promega G7541). The 1000, 3000, 8000, and 10,000 bp bands are brighter intensity making them easier to identify.

Molecular Weight Marker (Kilobases)	Log MW	Migration Distance (mm)
250		
500		
750		
1000		
1500		
2000		
2500		
3000		
Larger bands may not be resolved well. Do not include them.		

2. Using MS Excel, graph your molecular weight standard curve. Plot log molecular weight on the y-axis and migration distance on the x-axis. Label axis and give the graph and appropriate descriptive title (NOT log MW vs. migration distance!).

3. Using MS Excel, determine the equation of the line for the linear points of the curve. The highest MW marker may not be in the linear range, so you can exclude it if it falls off the linear portion of your graph.

4. Equation of the Line: _____

5. Determine the R^2 value for your graph. This correlation coefficient will tell you how well your data correlates linearly to each other. An R^2 value >0.95 is acceptable, but >0.98 is preferred.

6. R^2 Value: _____

7. Measure the distance of each of the DNA bands in your lanes. Use the equation of the line to determine the molecular weight of each of your samples. Record in the table below.

Experimental Samples	Migration Distance (mm) of each band	Log MW of each band	MW (kilobases) of each band
Tube 1: Undigested control plasmid			
Tube 2: Restriction digested sample pGLO plasmid			

8. Turn a copy of your graph with your completed lab report.

9. Did your plasmid migrate at the expected size? Why or why not? See your NEB cutter predictions.

Lab Unit 10 – Restriction Digest Assignment

1. Include your NEB restriction digest map with your report.

2. Based on this map, predict the size of fragments if you cut this plasmid with the following enzymes:
   - EcoRI: _____
   - Double digest: EcoRI & EcoRV: _____

3. Which enzyme did the class choose to cut out GFP from this plasmid? Why?

4. Predict the size of restriction fragments you expect to see on the agarose gel after restriction digestion with these enzymes. You may use NEB cutter to help you out!

5. Analyze data. Determine the size of the insert and vector.

   MW Size of Insert: _____

   MW Size of Vector: _____

6. Did you obtain the predicted size of your insert and vector? Why or why not?

CONCLUSION:

LAB UNIT 11: COLUMN CHROMATOGRAPHY

J. O'Grady, EdD, L. Fletcher, Ph.D., A. Wheeler, M.S., P. Phelps, Ph.D.,
Adapted from Bio-Rad's GFP Chromatography kit (Cat # 1660005EDU)

OBJECTIVES

In this lab activity, students will:

- ✓ Demonstrate the safe handling of the biohazardous material
- ✓ Extract proteins from cells
- ✓ Separate a complex protein mixture using hydrophobic interaction chromatography
- ✓ Discuss the role of green fluorescent protein in Biotechnology

INTRODUCTION

Chromatography is a very powerful method for separating complex mixtures of biomolecules into separate components. There are many types of chromatography, but in each case, the separation of components of a mixture is based on differences in the chemical and physical properties of the components.

In all types of chromatography, the separation takes place between two different phases: the stationary phase that does not move and the mobile phase that moves steadily past the stationary phase. Different components of a solution will separate due to their differential affinity for the stationary, compared with the mobile phase. The stationary phase can be a flat sheet (as in paper or thin layer chromatography) or a column of material (as in liquid or gas chromatography). The separations can be based on molecule size (as in size exclusion or gel permeation chromatography), by charge and polarity (as in ion-exchange chromatography), or by specific binding (as in affinity chromatography).

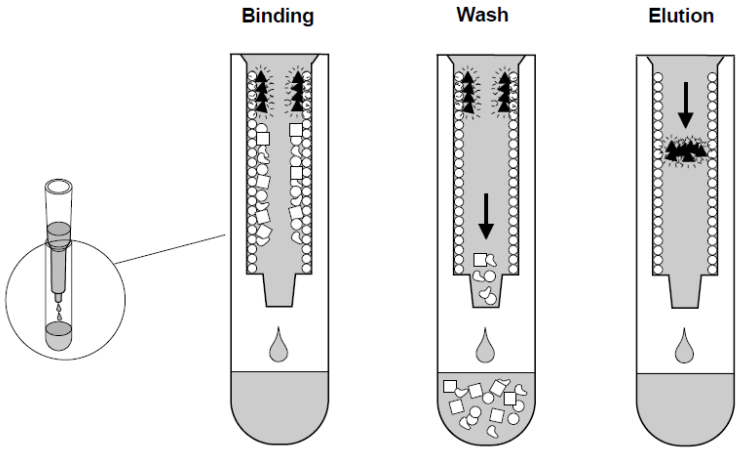
This lab exercise involves hydrophobic interaction chromatography, in which components bind, or adsorb, to the stationary phase due to hydrophobicity. The stationary phase is made of insoluble particles, also called a resin, of polysaccharide beads called Sepharose™. These beads are small (40 to 165 μm diameter), and made of agarose that has been chemically cross-linked to make the beads less likely to be crushed in a large column. While sepharose is hydrophilic, cross-linking phenyl groups make it into the hydrophobic phenyl sepharose. Proteins with patches of hydrophobic (literally, "water-fearing") amino acids on their surfaces will be attracted to the phenyl groups on the resin. Higher salt concentrations and higher temperatures strengthen these hydrophobic attractions. Under high salt conditions, even the least hydrophobic proteins will bind to the phenyl sepharose beads, but at low salt conditions, only the most highly hydrophobic proteins will remain bound to the phenyl sepharose beads. You may use an equilibration buffer with high salt concentration to bind the proteins in a mixture to phenyl sepharose and use elution buffers with successively lower concentrations of salt to separate them from each other according to their relative hydrophobicity.

Your goal in this lab is to use hydrophobic interaction chromatography to purify GFP from a bacterial cell lysate. Proteins are long chains of amino acids, some of which are very

hydrophobic. GFP has many patches of hydrophobic amino acids, which collectively make the entire protein hydrophobic. Moreover, GFP is much more hydrophobic than most other bacterial proteins. We can take advantage of the hydrophobic properties of GFP to purify it from the other, less hydrophobic (more hydrophilic or "water-loving") proteins.

First, you will obtain a liquid bacterial culture that was prepared by isolating a single green transformant E. coli colony, adding it to a tube of the liquid medium, and then incubating with vigorous shaking overnight. You will process this culture to lyse the cells and release the proteins contained therein. You will load the cell lysate onto a HIC column in a high salt buffer. The salt causes the three-dimensional structure of proteins to change so that the hydrophobic regions of the protein move to the exterior of the protein, and the hydrophilic ("water-loving") regions move to the interior of the protein. This will cause most proteins to adsorb to the column. As the salt concentration of the buffer is decreased, the three-dimensional structure of proteins changes again so that the hydrophobic regions of the proteins move back into the interior and the hydrophilic ("water-loving") regions move to the exterior.

<u>These four buffers comprise the separation scheme:</u>
Equilibration Buffer—A high salt buffer (2 M (NH4)2SO4)
Binding Buffer—A very high salt buffer (4 M (NH4)2SO4)
Wash Buffer—A medium salt buffer (1.3 M (NH4)2SO4)
Elution Buffer—A very low salt buffer (10 mM Tris/EDTA)



******ASSIGNMENT**** Before coming to class, read the entire detailed protocol and complete the missing pieces to the short protocol you will use in class to perform the experiment.*

TITLE:

PURPOSE: The purpose of this experiment is to:

MATERIALS: Thoroughly evaluate this procedure and come up with a detailed list of materials (reagents & equipment) you will need to complete this exercise. Be specific! If you need micropipette tips, what size? If you need microcentrifuge tubes, how many? What equipment do you need?

Materials & Reagents needed per Person	Equipment & Materials to share with the class

SAFETY PRECAUTIONS

UV radiation is harmful to your eyes. Be very careful when handling the hand-held UV light and always point down or away from you or your lab partner (not up towards your face and eyes). *Always use eye protection when using UV light.*

What other safety precautions do you need to consider in this lab?

PROCEDURE DAY 1: INDUCTION OF TRANSFORMED BACTERIA

PROCEDURE	EXPERIMENTAL NOTES
1. Examine your LB/AMP and LB/AMP/ARA plates from the transformation lab with and without UV light. To prevent damage to your skin or eyes, avoid exposure to the UV light. Never look directly into the UV lamp. Wear safety glasses whenever possible. 2. Obtain a culture tube containing 5 mL of sterile LB/AMP/ARA medium and one culture tube containing 5mL of LB/AMP. Using a sterile inoculation loop, lightly touch the "loop" end to a single green colony and gently scoop up the cells without gouging the agar. Immerse the loop in the LB/AMP/ARA culture tube. Spin the loop between your index finger and thumb to disperse the entire colony. 3. Using a new sterile loop, repeat and immerse it in the LB/AMP culture tube. What do you predict will be the result tomorrow when you look at both tubes with a UV light? 4. Place them in a 37° C incubator with vigorous shaking for 18-24 hours. After incubation store at 4°C until extraction.	

PROCEDURE DAY TWO – ISOLATING GFP USING HIC CHROMATOGRAPHY

The detailed procedure for part II is on the next few pages. Using this procedure, create a quick guide protocol for part II on the next page. Part I is already done for you as an example.

PROTOCOL	EXPERIMENTAL NOTES
PART I: CELL LYSIS 1. Collect culture tubes and invert to mix. 2. Observe tubes in normal room lighting and then with the UV light. Record 3. Pour a 2mL aliquot of the LB-amp-ara culture into a clear 2mL microcentrifuge tube. 4. Centrifuge the tube for 1 minute at 12,000-14,000 rpm. Discard the supernatant and pour an additional	Culture incubation conditions: _____ LB-amp-ara: _____ LB-amp: _____ Pellet observations: _____

2mL aliquot to the same tube and centrifuge as above. Discard supernatant in liquid biohazard waste. 5. Add 250 µl of TE Solution and resuspend pellet thoroughly by vortexing. 6. Add one drop of lysozyme by pipet to the resuspended pellet. Cap and mix by inversion. 7. The lysozyme will start digesting the bacterial cell wall. Observe the tube under the UV light. 8. Place the microcentrifuge tube in a -80 ºC freezer for 5 min. 9. Thaw for 1 min at 37ºC. 10. Repeat freezing two more times. 11. Centrifuge for 5 minutes at 12,000-14,000 rpm. 12. Transfer 50 µl of the supernatant into a new tube labeled "Protein Extract." 13. Transfer the remaining 200 µl of the supernatant into a new tube labeled "supernatant."	Lysis Observations: _____ Time 1: Time 2: Time 3: *prepare column while waiting Observations with UV light:

PART II: CHROMATOGRAPHY

PROTOCOL	EXPERIMENTAL NOTES
1. Remove the two liquid cultures from the incubator (or fridge). If they have been sitting invert to mix. 2. Observe tubes in normal room lighting and then with the UV light. Note any color differences that you observe. 3. Transfer the entire contents of the GFP glowing liquid culture by pipet into a clear, 2 mL microcentrifuge tube labeled GFP as follows: a. Mix bacterial culture tube by inversion a few times and pour a 2mL aliquot into a clear 2mL microcentrifuge tube.	

b. Centrifuge the tube for 1 minute at 12,000-14,000 rpm. Be sure to balance the tubes in the centrifuge.
c. After centrifugation, observes the pellet and supernatant under UV light. Note your observations in your notebook.
d. Discard the supernatant and pour an additional 2mL aliquot to the same tube and centrifuge as above.

4. After centrifugation, open the tube and slowly pour off the liquid supernatant into a liquid biohazard waste container.

5. Pour more of the liquid bacterial culture into the SAME tube and centrifuge as above (it is recommended to use all 4mL of culture).

6. After the supernatant is discarded, add 250 µl of TE Solution to the pellet. Resuspend the bacterial pellet thoroughly by vortexing (don't use a micropipette, the bacteria will get stuck in the tip).

7. Add 1 drop of lysozyme by pipet to the resuspended pellet. Cap and mix the contents by flicking the tube with your index finger.

8. Place the microcentrifuge tube in the freezer. Freezing will cause the cells to rupture completely. Freezing *slowly* at -20^{o}C for 30 min-1 hr works efficiently at cell rupture. If time is limiting, you can use a -80 ºC freezer for 15-30 minutes. When placing in freezer, do not put in a rack. It will prevent freezing.

   Recommended: *2x20min freeze-thaw cycles at -20^{o}C.*
   If there is not enough time for this, 3x5min freeze-thaw cycles at -80^{o}C also work very well.

9. Remove the tube from the freezer and thaw quickly.

10. While the sample is freeze-thawing, prepare the chromatography column. Shake the column to resuspend the beads. If the beads have dried out, add 1mL of Equilibration Buffer and shake.

11. Shake the column down to consolidate the beads at the bottom. Remove the top cap and snap off the tab bottom of the chromatography column. Allow the liquid buffer to drain from the column into a beaker (this will take 3–5 minutes).

12. Equilibrate the column by adding 2 mL of Equilibration Buffer to the top of the column, 1 mL at a time, being careful not to disturb the bead bed at the top. Drain the buffer.

    NOTE: If the column is not dripping, notify your instructor. Sometimes the tip (2mm) may need to be cut off with a pair of scissors to open up the hole at the bottom of the column.

13. After the centrifugation, immediately remove the microcentrifuge tube from the centrifuge. Examine the tube with the UV light. The cell debris should be visible as a pellet at the bottom of the tube. The liquid that is present above the pellet is called the supernatant. Note the color of the pellet and the supernatant.

14. Transfer 50 µl of the supernatant into a new tube labeled "Protein Extract." We will analyze this aliquot on the SDS-PAGE gel to determine how well our purification worked to separate GFP from the other proteins in the extract.

15. Transfer the remaining 200 µl of the supernatant into a new tube labeled "supernatant." Add 200 µl of Binding Buffer to this tube containing the supernatant. Mix by pipetting up and down a few times.

16. Obtain five 1.5mL collection tubes and label them, as shown in the table below. When the last of the equilibration buffer has drained from the HIC column bed, move the column to collection tube 1.

17. Carefully load all the GFP extract in Binding Buffer into the top of the column. Examine the column using the UV light. Note your observations in the data table. Let

the entire volume of supernatant flow into the column and tube 1. 18. Move the column to collection tube 2. CAREFULLY add 250 µl of Wash Buffer and let the entire volume flow into the column. As you wait, predict the results you might see with this buffer. Examine the column using the UV light and record your results. 19. Move the column to tube 3A. CAREFULLY add 250 µl of TE buffer and let the entire volume flow into the column. 20. Move the column to tube 3B. VERY CAREFULLY add 250 µl of TE buffer and let the entire volume flow into the column. 21. Finally, move the column to tube 3C. CAREFULLY add 250 µl of TE buffer (Elution Buffer) and let the entire volume flow into the column. 22. Examine all the collection tubes using the UV lamp and note any differences in color between the tubes. Record in your notebook. 23. Cap the collection tubes and label with your initials. Place tubes (and pre-column aliquot) in a labeled freezer box. Store at -20^{o}C until next lab.	

DATA TABLE:

Collection Tube Number	Prediction under UV light	Observations Under UV Light
Tube 1 Sample in Binding Buffer		
Tube 2 Sample with Wash Buffer		
Tube 3A Sample with TE Buffer		
Tube 3B Sample with TE Buffer		
Tube 3C Sample with TE Buffer		

Unit 11 - GFP Column Chromatography

1. How were these items helpful in this recombinant protein experiment?
    a. UV light

    b. Incubator

    c. Centrifuge

    d. lysozyme

2. What was added to the nutrient broth to trigger the expression of the GFP? Briefly describe how this works.

3. What was the purpose of lysing the bacteria after pelleting?

4. Briefly describe hydrophobic interaction chromatography and identify its purpose in this lab.

5. Based on your results, explain the essential components and functions of each buffer:
    a. equilibration buffer

    b. binding buffer

    c. wash buffer

    d. TE (elution) buffer

6. Using your data table, discuss your experimental results. Which fraction contained your GFP protein?

CONCLUSION: Were you successful in isolating and purifying GFP from the cell lysate? Identify evidence to support your answer. Feel free to include your photos with your report!

LAB UNIT 12: POLYACRYLAMIDE GEL ELECTROPHORESIS

J. O'Grady, EdD, L. Fletcher, Ph.D., A. Wheeler, M.S., P. Phelps, Ph.D.
Adapted from Bio-Rad's pGLO SDS-PAGE extension (Cat# 1660013EDU)

OBJECTIVES

In this lab activity, students will:

- ✓ Explain how polyacrylamide gel electrophoresis can be used to analyze proteins
- ✓ Analyze protein samples using PAGE
- ✓ Plot molecular weight marker with MS Excel and using linear regression to determine the size of GFP in chromatography fractions

INTRODUCTION

General Principles of Protein Electrophoresis and SDS-PAGE

Electrophoresis ("to carry with electricity") is the migration of charged molecules in an electric field toward the electrode with the opposite charge. This technique is widely used in molecular biology research to answer a variety of questions by examining proteins. For example:

- ✓ What proteins are in my sample?
- ✓ What are the molecular weights of the proteins?
- ✓ How pure is my protein of interest?
- ✓ How much protein do I have?

Ulrich Laemmli developed his system of polyacrylamide gel electrophoresis with two gel phases so that all of the proteins in a gel begin separating or resolving, at the same time. Since sample volumes can vary from lane to lane, forming vertically narrow or broad bands in the wells, not all of the proteins in a sample enter the stacking gel zone simultaneously. However, the low percentage (4%) of the stacking gel allows the proteins to migrate rapidly and accumulate at the edge of the denser resolving gel, regardless of their sizes. The samples of mixed proteins are thus concentrated into uniformly thin bands in each lane before they move into the denser (5-20%) resolving gel and begin to separate according to their weights.

There is no obvious visual border between the stacking and resolving zones of a commercially prepared gel, but if you watch your samples immediately after turning on the power supply, you will see the protein samples being focused into a narrow band at the interface. Prestained protein markers first stack into a tight band, and then the individual prestained proteins become distinct as the proteins begin to separate according to their molecular weights.

Why are we using polyacrylamide gels, and not agarose gels, to analyze proteins?

The gel matrix formed by polyacrylamide is much tighter and able to resolve much smaller molecules than agarose gels. Polyacrylamide gels have pore sizes similar to the sizes of proteins. Nucleic acids are orders of magnitude larger than proteins, and agarose is usually the preferred medium for these molecules. However, when separating very small fragments of DNA, for example, during DNA sequencing, polyacrylamide is the matrix of choice.

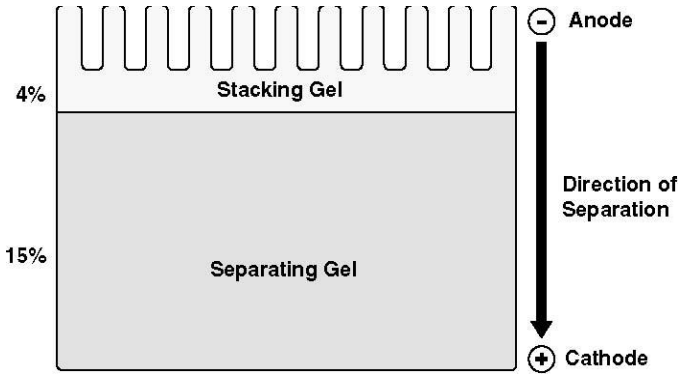


Figure 1. Precast gels are very thin polyacrylamide gels sandwiched between clear plates. Each gel has two separate zones, the stacking gel and the separating gel, which is also known as the resolving gel. In polyacrylamide gel electrophoresis, samples are loaded into wells at the top of the stacking gel, and the proteins move downward toward the positively charged electrode.

The Chemistry and Physics behind Electrophoresis

The size of biomolecules is expressed in Daltons (D), a measure of molecular weight. One Dalton equals the mass of a hydrogen atom, which weighs 1.66 x $10^{-}24$ g. Most proteins have masses about thousands of Daltons, so the term kilo-Dalton (kDa) is used for molecular weight. Proteins range in size from several kD to thousands of kD.

In contrast, the nucleic acids we study are often larger than 1000 base pairs, or 1 kilobase (kb), and each kilobase pair has a mass of approximately 660 kD. For example, when cloning DNA, a 2 kb fragment of DNA can be inserted into a plasmid vector of 3 kb, giving a total plasmid length of 5 kb. The mass of this 5-kb plasmid would be approximately 3.3 million Daltons or 3,300 kD, much larger than the average protein!

A molecule's electrical charge and its mass affect its mobility through a gel during electrophoresis. The ratio of charge to mass is called charge density. Since every protein is made of a unique combination of amino acids, each of which may have a positive, negative, or neutral charge, the net charge of each protein is naturally different. The inherent charges of proteins must be removed as a factor affecting migration for polyacrylamide electrophoresis to be effective as a method of protein molecular weight determination.

The intrinsic charges of proteins are obscured by placing a strongly anionic (negatively charged) detergent, SDS, in both the sample buffer and the gel-running buffer. SDS coats the proteins with negative charges and keeps them denatured as linear chains. In this form, proteins migrate in a polyacrylamide gel as if they have equivalent negative charge densities, and mass becomes the only variable affecting the migration rate of each protein. This technique is called SDS-PAGE.

Polyacrylamide Acts As a Molecular Sieve: The degree of sieving within a gel can be controlled by adjusting the polyacrylamide concentration. Higher concentrations of polyacrylamide resolve smaller molecular weight ranges. For example, a 5% polyacrylamide gel separates large proteins of 100 to 300 kD, while an 18% polyacrylamide gel is better for separating smaller proteins in the

range of 5 to 30 kD. For this lab, we will use a premade 12-15% polyacrylamide gel, which provides excellent separation of proteins in the range of 10 to 100 kD. Our attention will be focused on variations among the smaller proteins, in the range of 15 to 50 kD, since it is easier to discern differences among these proteins. Smaller proteins migrate further through the gel and are better resolved than proteins of high molecular weights.

Polyacrylamide Gel: Polyacrylamide gels are pre-cast in a plastic cassette. The gel cassette is inserted into a vertical electrophoresis apparatus, and the running buffer is added until each well is covered with buffer. Samples, controls, and molecular weight markers are loaded into the wells. A lid is placed on the apparatus, and leads are plugged into a power supply. A current is applied at a constant voltage, bubbles rise from the electrodes, and the loading dye and proteins in the samples begin to enter the gel.

Sample Preparation – Disrupting Protein Structure

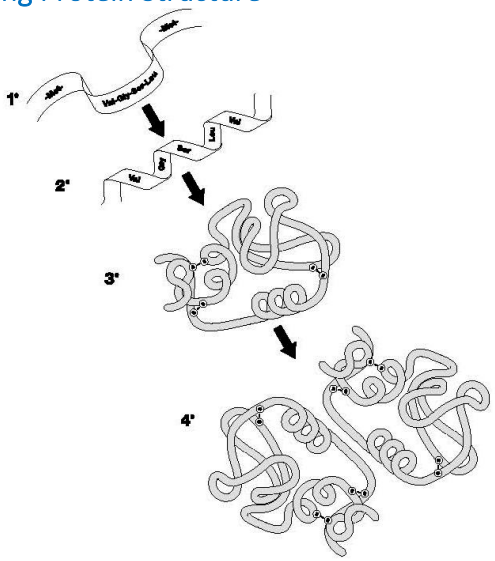


Figure 2: Secondary, tertiary, and quaternary protein structure must be disrupted or denatured to separate proteins by size.

To determine the molecular weights of proteins, the secondary (2°), tertiary (3°), and quaternary (4°) structures of the protein complexes within a protein extract are disrupted before electrophoresis. This process of structural disruption is called denaturation.

1. Primary structure = order of amino acids
2. Secondary structure = domains of repeating structures, such as β-pleated sheets and α-helices as a result of H bonding between peptide backbone
3. Tertiary structure = 3-dimensional shape of a folded polypeptide, maintained by disulfide bonds, electrostatic interactions, hydrophobic effects, H bonding of the R groups on the amino acids
4. Quaternary structure = several polypeptide chains associated together to form a functional protein

Secondary, tertiary, and quaternary structures are disrupted by the combination of heat and SDS. A reducing agent, such as β-mercaptoethanol (BME) or dithiothreitol (DTT), may be added to ensure complete breakage of disulfide bonds. These three factors – heat, ionic detergent, and reducing agent – completely disrupt the 2°, 3°, and 4° structures of proteins and protein complexes, resulting in linear chains of amino acids which allow the molecules to snake through the gel at rates proportional to their molecular masses.

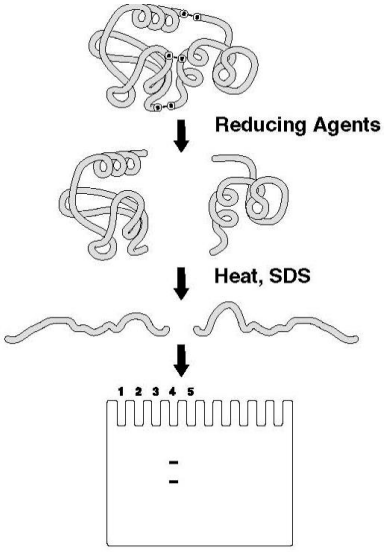


Figure 3: A quaternary protein complex denatured with reducing agents, head, and SDS can be separated into individual proteins and resolved by size using SDS-PAGE.

Visualizing the Proteins: After the electrophoresis is complete, the gel is stained so that blue-colored protein bands appear against a clear background.

Molecular Weight Standards: Electrophoresis protein standards, or molecular weight markers, consist of a mixture of proteins of known molecular weight. They are available in some protein size ranges. The markers to be used should correspond to the sizes of the proteins of interest. Molecular weight standards are available either prestained or unstained. Unstained markers are not visible until the gel is stained with a protein stain, such as Bio-Safe™ Coomassie stain. The prestained Kaleidoscope standards used in this lab are visible as they separate on the gel. The dyes bound to the Kaleidoscope marker proteins affect the migrations of the proteins, and the actual sizes of the dyed molecules differ slightly from batch to batch. Please refer to the size chart that comes with each vial of Kaleidoscope prestained standards for the calibrated molecular weights of each of the dyed proteins.

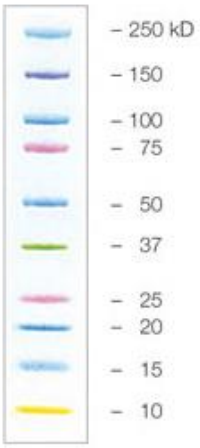


Figure 4: Kaleidoscope prestained standards

Gel Loading Tips

1. Using a thin gel-loading tip, aliquot the correct amount of your sample from your microcentrifuge tube *slowly.*
2. Insert the micropipette tip into the top of the well to at least four mm of the bottom of the well in between the two plates. It helps to lean the tip on the tall plate and using both hands to steady tip.
3. Ensure the pipette tip is between the two plates and v*ery slowly and gently* expel the solution from the micropipette tip into the well while holding the micropipette steady. The blue solution should fall to the bottom of the well, gradually filling it.
4. Do not press the micropipette to the second stop – it is important to avoid blowing air bubbles into the well.
5. Do not release your thumb until you have slowly withdrawn the micropipette tip from the well so that you avoid removing the sample that you have so carefully loaded!

Using Molecular Weight Marker to Determine MW of Unknown

There is a linear relationship between the log of the molecular weight of the protein and migration distance (Rf) when proteins are separated on a denaturing polyacrylamide gel. This relationship can be exploited to determine the molecular weight of a target protein. Using the equation of the line and the migration distance of the target protein, you can determine its approximate molecular weight. This relationship works best with denaturing gels because the three-dimensional shape and charge of the protein do not influence its migration – it migrates by size alone.

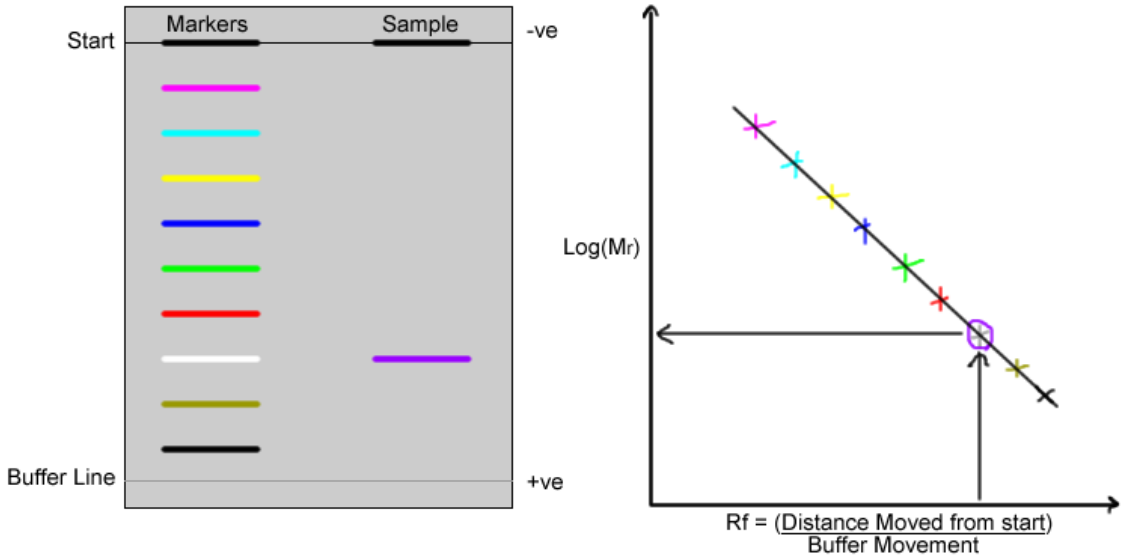


Figure 5: Using a Standard Curve to determine the MW of an unknown

PROTEIN ANALYSIS OF GFP HIC SAMPLES USING SDS-PAGE

PURPOSE: The purpose of this lab activity is to analyze GFP samples from the chromatography procedures.

MATERIALS

☐ 1, polyacrylamide gel per person	☐ 95°C water bath and floater
☐ Chromatography samples	☐ Vertical Gel Apparatus (one per lab group)
☐ Kaleidoscope molecular weight marker	☐ 1000mL, 1X Denaturing Gel Running Buffer per vertical gel apparatus
☐ Sample load buffer	☐ Coomassie Safe stain
☐ Gel load tips & micropipettes	☐ Ruler
☐ Screw cap tubes	☐ Cracker to open gel
☐ Hand-held UV light	

SAFETY

1. The wires connecting the cell to the power supply must be in good condition, not worn or cracked. Broken or worn wires not only cause rapid changes in resistance that adversely affects electrophoresis, but they also create an electrocution hazard.
2. An area of at least 6 inches around the power supply and cell should be bare of clutter and other equipment and dry.
3. *Acrylamide is a neurotoxin!* Wear gloves while loading and handling the gels; the unpolymerized acrylamide is a neurotoxin! Most prepared gels are preserved using sodium azide, which is very toxic!
4. *The sample load dye has either DTT or beta-mercaptoethanol, which are hazardous.* Handle with gloves and avoid breathing in vapors and dispose of tips and tubes in hazardous waste containers. Keep tubes closed when aliquoting.
5. Coomassie blue will stain clothing and hands. Wear gloves when handling..

CALCULATIONS:

1L of 1X Running Buffer (from 10X stock):

PROCEDURE	EXPERIMENTAL NOTES
Part I: Preparing Protein Samples 1. Label 7 screw cap tubes A-G. Prepare protein samples collected from the column chromatography lab: A. Protein Extract undiluted B. Column fraction 1 – proteins not bound to column C. Column fraction 2 – proteins washed off column D. Column fraction 3A – elution 1 E. Column fraction 3B – elution 2 F. Column fraction 3C – elution 3 G. *Column fraction with the most GFP* (***this tube will not be heated***) 2. Add 50 ul of Laemmli sample load buffer to each of these tubes. 3. Add 50 µl of each sample you collected to the corresponding tube you labeled in step 1. 4. Heat tubes A-F ONLY (***NOT tube G***) for 5 minutes at 95°C (a boiling water bath works best for this). 5. Add _____ mL of 10X running buffer to a 1L graduated cylinder. BTV with di H2O, cover, mix. 6. Your instructor will demonstrate how to set up your gel apparatus. a. Remove the comb and tape along the bottom of the pre-made polyacrylamide gels and place your gel in the chamber with the short plates facing inside, as shown by your instructor. b. Make sure your apparatus does not leak by pouring 1X TGS into the center and watch for leaking out the bottom. Insert into the gel box and fill to the mark "Two gels." c. Wash out the wells vigorously to remove unpolymerized acrylamide with a disposable transfer pipet or p1000 micropipette. **Part II: Separating Protein Samples on the SDS-PAGE gel:** 1. Load 10 µl of the Kaleidoscope prestained standard in the far left lane. The MW band sizes are 250, 150, 100, 75, 50, 37, and 25, 20, 15, 10 kDa. This marker does NOT get heated!	

2. Load 30 µl of each protein sample (A-G) into a separate well. Remember to fill in the gel-documentation form! 3. Put the lid on the tank and insert leads into the power supply, matching red to red and black to black. 4. Electrophorese for 30 minutes at a constant voltage of 200 V or until the dye front reaches the bottom of the gel. Alternatively, you can use 250V for 20 min with special fast gels from Bio-Rad (ask your instructor). 5. When the loading dye has almost reached the bottom of the gels, stop the power supply, and disconnect the leads. 6. Remove the gel cassettes. Rinse with water and wipe with a paper towel. 7. Using a hand-held UV light, look to see if you can see your GFP protein glowing. If you can, take a sharpie and mark on the plate where you see it. 8. Measure with a ruler the migration distance of the glowing GFP and note what lane it is in. 9. Also, Measure the blue dye front. You will need this for the graphing procedure in part IV. 10. Lay a gel cassette flat on the bench with the short plate facing up. Carefully pry apart the gel plates using a spatula. The gel will adhere to one of the plates. 11. Transfer the plate with the gel adhering to it to a tray containing dH2O. Rinse for 15 min with dH2O to remove excess running buffer that may interfere with the Bio-Safe Coomassie blue stain. This is best done as three 5-minute washes.	VOLTAGE: _____ TIME: _____ DISTANCE GFP: _____ DISTANCE DYE FRONT: _____

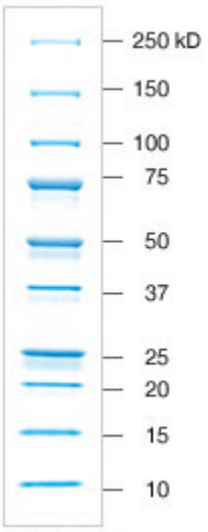
12. Using the UV lightbox, capture an image of your gel BEFORE staining. Do you see the GFP glowing in any of your lanes? Which lane(s)?

Part III: Staining SDS-PAGE Gel with Coomassie Safe Stain

1. Pour out the water and add just enough Coomassie safe stain to cover the gel. Stain the gels for one hour (to overnight) with gentle agitation.
2. After staining the gels, rinse the stain down the sink with lots of water.
3. Cover the gel with large volumes of dH_2O, changing several times until most of the blue background disappears, and you can visualize discrete bands on the gel. This can take several hours to overnight.
4. The GFP protein is approximately 27 kDa. Do you see a band in this range for any of your samples?

Part IV: Gel Analysis

Measure the migration distance (in mm!) of each band in the molecular weight marker lane. Record this in the data table below. Complete the table below.



Molecular Weight (Marker) KDa	Log MW	Migration Distance (mm)	Rf Value Migration/dye front
250			
150			
100			
75			
50			
37			
25			
20			
15			
10			

1. Plot the log of the molecular weight (y-axis) versus the Rf value (x-axis).
2. Determine the equation of the line and the R^2 value.
3. Measure the distance of the predicted GFP in each well. Create a table to record your sample data.
4. Using the equation of the line determine the molecular weight of the major protein bands in each lane. Record this in the same table.

Lab Unit 12 – SDS-PAGE Assignment

1. Why did you use polyacrylamide gels to analyze your protein fractions rather than agarose gels?

2. Explain the purpose of heating the samples with a buffer containing SDS and DTT.

3. Distinguish between the primary, secondary, tertiary and quaternary structure of the protein.

4. Include your gel documentation form with a picture of the labeled gel attached. Discuss your results.
    a. What is the size of GFP according to the literature?

    b. What is the size of GFP in your gel?

    c. Does it match the theoretical size of GFP? Why or why not? Discuss.

    d. Did you have any lanes where GFP glowed with UV light? Why or why not?

CONCLUSION: Was the protein purification successful? Provide evidence to support your answer.

LAB UNIT 13: ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Jack O'Grady, EdD.

This lab is based on Bio-Rad's ELISA ImmunoExplorer Kit (Cat#166-2400EDU). The introduction, instructions, and analysis is copied in part or completely from the instruction manual.

OBJECTIVES

In this lab unit, students will:

- ✓ Describe the mechanism for an ELISA assay, including what components are needed
- ✓ Perform an ELISA to determine the concentration of an unknown protein
- ✓ Graph data using MS Excel, perform linear regression and utilize an ELISA standard curve to determine the concentration of an unknown protein sample

INTRODUCTION

Immunology is the study of the immune system and how the body protects itself against disease. Over 100 years ago, biologists found that animals' internal immune systems respond to invasion by "foreign entities" or antigens. When an invader enters the body, it provokes an immune response that begins with the production of proteins called antibodies.

Like magic bullets, antibodies seek out and attach themselves to invading entities (antigens), flagging the invaders for destruction by other cells of the immune system. The antigenic invaders may be any molecules foreign to the body, including components of infectious agents like bacteria, viruses, and fungi. Today, antibodies have become vital scientific tools used in biotechnology research and to diagnose and treat disease. The number of different antibodies circulating in the blood has been estimated to be between 106 and 1011, so there is usually an antibody ready to deal with any antigen. In fact, antibodies make up to 15% of your total blood serum protein. Antibodies are very specific; each antibody recognizes only a single antigen.

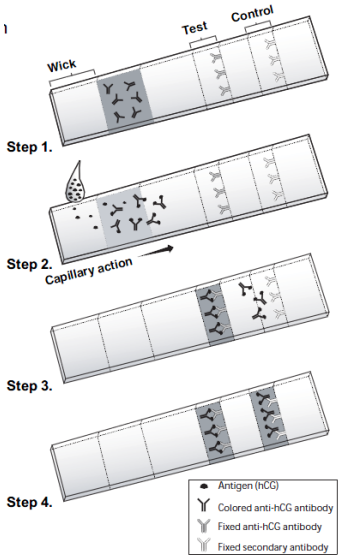
You are about to perform an ELISA (enzyme-linked immunosorbent assay). The ELISA relies on antibodies to detect the presence of antigens in liquid samples. Because they are antibody-based, ELISAs are called immunoassays. ELISAs can detect minute amounts of disease agents in samples, such as body fluids (before the body has had a chance to mount an immune response). Smallpox virus is an example of a disease agent that can now be detected using an ELISA. If exposure is detected and treated with a vaccine within 2–3 days, patients do not develop smallpox. Other applications for ELISA include testing for West Nile virus, HIV coat protein p24, SARS virus, anthrax spores, hormones such as hCG in pregnancy tests, illegal steroids in drug tests, bacteria in food safety tests, and the presence of genetically modified organisms contaminating non-GMO food.

Where Is ELISA Used in the Real World?

With its rapid test results, the ELISA has had a significant impact on many aspects of medicine and agriculture. ELISA is used for such diverse purposes as home pregnancy tests, disease detection in people, animals, and plants, detecting illegal drug use, testing indoor air quality, and determining if food is labeled accurately. For new and emerging diseases like severe acute

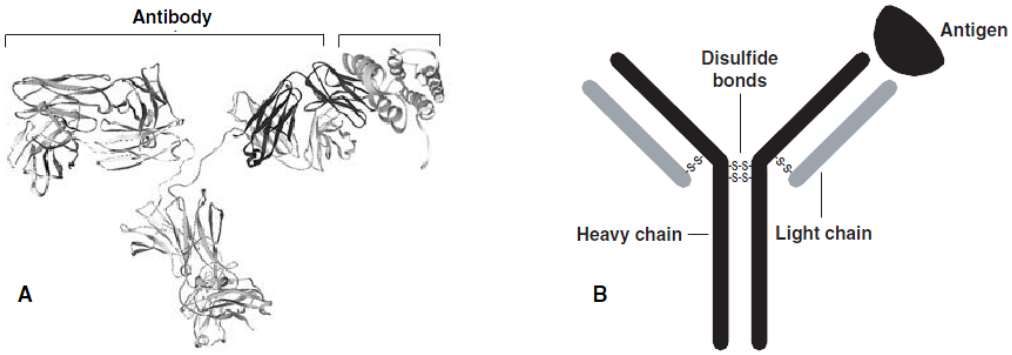
respiratory syndrome (SARS), one of the highest priorities of the US Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) has been to develop an ELISA that can quickly and easily verify whether patients have been exposed to the virus.
Over-the-counter kits that are based on the same principles as this ELISA activity include home pregnancy and ovulation tests, and tests for the presence of illegal drugs like marijuana and cocaine.

Some tests give positive or negative results in a matter of minutes. For example, home pregnancy dipstick tests detect levels of human chorionic gonadotropin (hCG), a hormone that appears in the blood and urine of pregnant women within days of fertilization. The wick area of the dipstick is coated with an anti-hCG antibody labeled with a pink compound (step 1). When the strip is dipped in urine, if hCG is present it will bind to the pink antibody, and the pink hCG-antibody complex will migrate up the strip via capillary action (step 2). When the pink complex reaches the first test zone, a narrow strip containing an unlabeled fixed anti-hCG antibody, the complex will bind and concentrate there, making a pink stripe (step 3). The dipsticks have a built-in control zone containing an unlabeled secondary antibody that binds unbound pink complex (present in both positive and negative results) in the second stripe (step 4). Thus, every valid test will give a second pink stripe, but only a positive pregnancy test will give two pink stripes.



How Are Antibodies Made?

When exposed to antigens, all mammals generate an immune response and produce antibodies, proteins that recognize and bind tightly to the specific antigens. Each antibody recognizes only a single antigen. Animals such as goats, rabbits, and mice can be injected with an antigen and, after a period, their serum will contain antibodies that specifically recognize that antigen. If the antigen was a disease-causing agent, the antibodies could be used to develop diagnostic tests for the disease. In an immunoassay, the antibodies used to recognize antigens like disease agents are called primary antibodies.



A) Structure of IgG bound to the HIV capsid protein p24 as determined by X-ray crystallography (Harris et al. 1998, Momany et al. 1996). These structures can be downloaded from the Protein Data Bank (www.pdb.ufmg.br, (Berman et al. 2000) using the PDB identification codes 1IGY and 1AFV and manipulated using free online software such as Rasmol and Protein Explorer. **B)** A commonly used representation of an antibody bound to an antigen.

Secondary antibodies recognize and bind to primary antibodies in an immunoassay. They are prepared by injecting antibodies produced by one species of animal into another species. This works because the antibodies produced by different species are different enough from each other that they will provoke an immune response. For example, if you want a secondary antibody that will recognize a primary human antibody, inject human antibodies into an animal like a rabbit. After the rabbit immune response, the rabbit serum will contain antibodies that recognize and bind to human antibodies. Secondary antibodies are frequently labeled to make them visible.

In this experiment, the secondary antibodies you will be working with are conjugated to an enzyme named horseradish peroxidase (HRP); HRP in the presence of its substrate, TMB, produces a blue color.

Controls in Immunoassays

For an immunoassay to be valid, it must include both positive and negative controls, i.e., samples that will give known results. Controls are always run side by side with experimental samples. If you do not run a positive control and the experiment provides negative results, how can you be sure the results are truly negative? What if the assay did not work? If a positive sample gives a negative assay result, it is called a false negative.

Conversely, if you do not run a negative control and the experiment gives all positive results, how can you be sure the results are truly positive? What if the assay was contaminated with antigen? If a negative sample gives a positive assay result, it is called a false positive.

Controls are also needed to guard against experimental error and to ensure that the assay is working correctly. There can be problems with reagents, which can degrade due to age or poor storage conditions. Operators can make mistakes by choosing the wrong reagents, causing errors in dilutions, or in pipetting or failing to remove unbound reagents. Poor record-keeping is another source of false assay results. Most of these possibilities can be checked within the assay with the appropriate controls. Now let's put this all together.

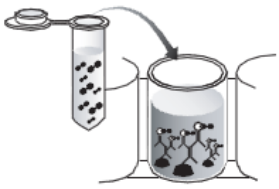
The main steps in this antigen detection ELISA are:

1. Add your sample and control samples to the wells in a microplate strip. Your samples contain many proteins and may or may not contain the antigen. Incubate for 5 minutes to allow all the proteins in the samples to bind to the plastic wells via hydrophobic interaction. This is called an immunosorbent assay because proteins adsorb (bind) to the plastic wells.

2. Add primary antibody to the wells and incubate. The antibodies will seek out the antigen from the many proteins bound to the well. If your sample contains the antigen, the antibodies will bind it tightly and remain in the well.

3. Detect the bound antibodies with HRP-labeled secondary antibody. If the primary antibodies have bound to the antigen, the secondary antibodies will bind tightly to the primary antibodies.

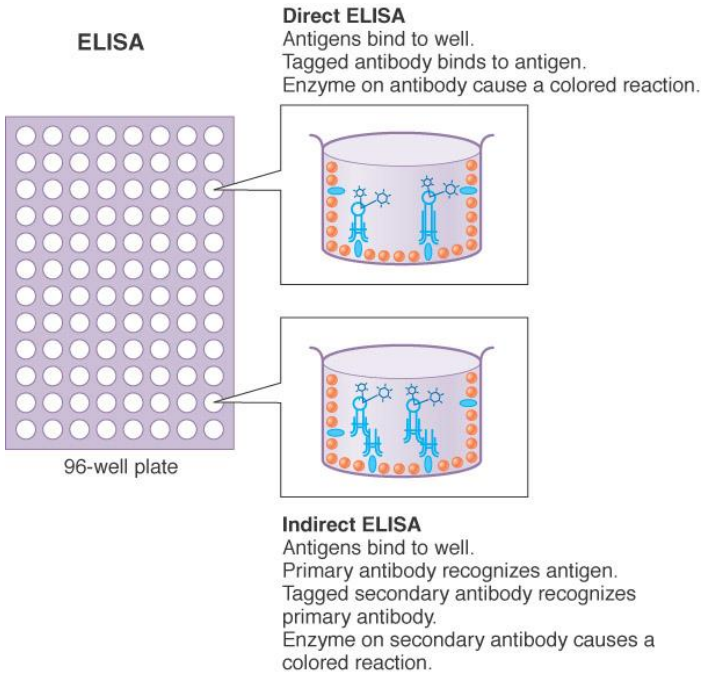
4. Add enzyme substrate to the wells, wait 5 minutes, and evaluate the assay results. If the antigen was present in your sample, the wells will turn blue. This is a positive diagnosis. If the wells remain colorless, the antigen was not present in your sample and the diagnosis is negative.



Like most ELISA assays, this kit relies on a Horseradish Peroxidase (HRP) conjugated antibody and the TMB (3, 3´, 5, 5´-tetramethylbenzidine) substrate. TMB is a chromogen that yields a blue color when oxidized with hydrogen peroxide (catalyzed by HRP) that has significant absorbance peaks at 370 nm and 652 nm. The color then changes to yellow with the addition of acid with maximum absorbance at 450 nm. The relative amount of protein in the well will be directly proportional to the amount of signal that is obtained at 450 nm.

This kit uses an indirect ELISA format, in which a sample is added to a microtiter plate well. After coating the well with the sample, the unbound sample is removed, the wells are washed, and a primary antibody to the target protein is added. Unbound primary antibody is washed away, and a secondary antibody, which recognizes this primary antibody, binds. The secondary antibody is conjugated to HRP, is added (the “detection antibody”). The signal is generated by reaction with the TMB substrate as described above. The intensity of the signal (measured at 450 nm) is directly proportional to the amount of target protein in the sample. Dilutions of a standard are

used to construct a standard curve, from which the concentration of target protein in the samples are determined by interpolation.



ELISA antibodies can recognize antigens directly or by recognizing another antibody that recognizes the antigen

QUANTITATIVE ELISA

A set of known standards is analyzed along with the samples at the same time using the same reagents. The range of known standard concentrations is determined by both target antigen concentration as well as dynamic range of ELISA kit used.

A standard curve is constructed by plotting the absorbance obtained from each reference standard against its concentration in ng/mL, as shown below. The following figure is a typical antigen standard curve. Using the equation generated by the standard curve, the target antigen concentration (ng/mL) of an unknown sample can be determined.

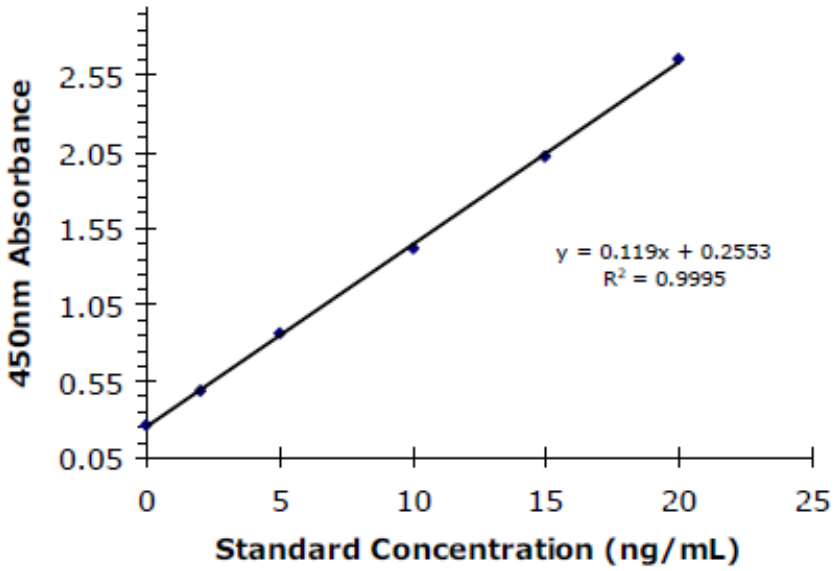
To determine the total amount of target antigen in the sample, use the following equation and ***solve for the x value***:

$$y = mx + b, \text{ rearrange: } x = (y-b)/m$$

Where:

b=the y-axis intercept of your standard curve
m=the slope of your standard curve
x=the antigen concentration
y=the 450 nm Absorbance of the sample

For example: If the OD reading of the sample is: 1.248, using the above equation and the Standard Curve shown above the antigen concentration would be: 8.342 ng/mL

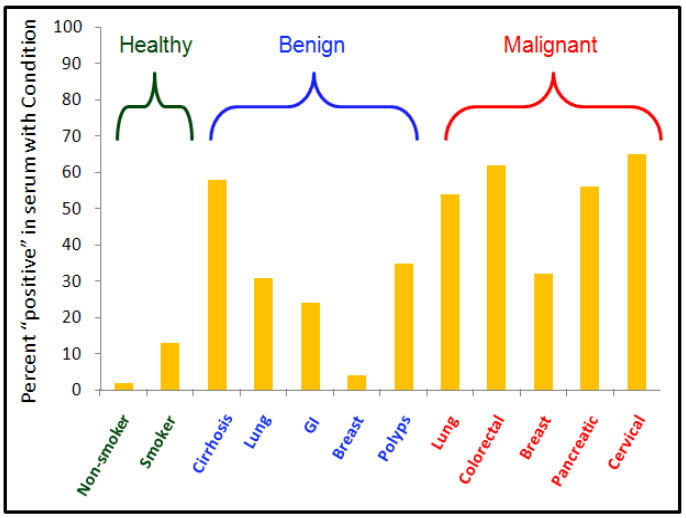


Sample Standard curve for ELISA

ELISA DIAGNOSTIC TEST FOR CANCER DETECTION

Despite advances in detection and treatment, cancer remains a deadly disease with more than 1.63 million new cases diagnosed each year in the United States (American Cancer Society, 2012), and approximately 7.6 million people die worldwide (Center for Disease Control, 2013)

Early cancer detection is key to decreased total numbers of death from cancer. Protein biomarkers are showing promise in aiding early detection system. Carcinoembryonic Antigen (CEA) is one such antigen that has shown promise in assisting early detection, disease progression monitoring as well as determining response to pharmacologic intervention. The figure below is data reproduced from Bayer Diagnostics/Siemens white paper.



Any number of conditions may cause an increase in CEA antigen levels in serum. The upregulation of CEA may be an indication of many potential problems. Additional diagnostic testing helps pinpoint the actual problem. ***CEA serum levels for healthy nonsmokers are less than***

3ng/mL. The CEA level for smokers is elevated and puts them at risk of cancer at up to 15 ng/mL. With cancer patients, CEA levels are significantly elevated in serum and usually, exceed 20 ng/mL.

In this simulation serum ELISA lab, we will determine the CEA levels of two patients ("A" and "B"). You will run a set of CEA antigen standards and determine the concentration of your patients from a standard curve.

References:

1. MaxDiscovery™ GAPDH ELISA Kit Manual – 3401-01 *from* ©BIOO Scientific Corp
2. Ellyn Daugherty, Biotechnology: Science for the New Millennium. 2012. EMC Paradigm Publications. ISBN: 978-0-76384-284-0
3. *Bio-Rad's ELISA Immuno Explorer Kit (Cat#166-2400EDU*)

Let's Explore

Watch the following video on using ELISA to detect and quantitate specific antigens. Draw a single well to show how Indirect ELISA works. https://youtu.be/RRbuz3VQ100

TITLE: *(write a fully descriptive title to your experiment – Write this here and on your title page!*

PURPOSE: *(write a purpose statement to your experiment)*

SAFETY: *(review the procedure below. Write a safety statement your experiment)*

MATERIALS: Review the protocol below and devise a detailed list of reagents and equipment you will need to complete this ELISA assay. Verify your list with your lab partner!

PROCEDURAL FLOWCHART: Watch this video https://youtu.be/849HN1ueUhs and draw a simple flowchart below summarizing the ELISA procedure you will be performing:

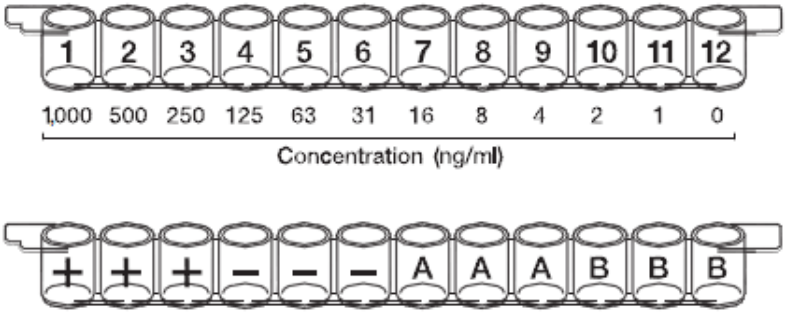
PROTOCOL

NOTE: *Check off each step as you complete them! Write down observations as you move through the experiment.*

PART I: ELISA ASSAY:

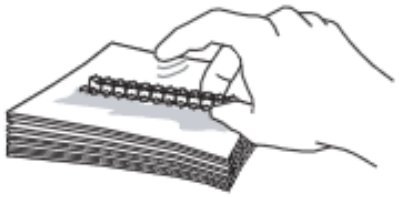
<u>You will perform the ELISA ASSAY on BOTH strip plates at the same time! Why?</u>

1. Label the outside wall of each well on one 12-well strip with the numbers 1–12. Label the first three wells of a second 12-well strip with a "+" for the positive controls, the next three wells with a "–" for the negative controls, the next three wells with the initials of one of your sample tubes, and the last three wells with the initials of the second sample tube.



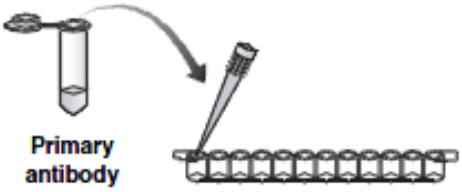
2. Use a pipet to add 50 µl of PBS from the yellow tube labeled "PBS" to wells labeled #2 through #12.
3. Add 100 µl from the yellow tube labeled "1,000 ng/ml AG" to the well labeled #1.
4. Perform serial dilution from well #1 through well #11 in the following manner:
   a. Pipet 50 µl out of well #1 and add it to well # 2. Pipet up and down gently three times to mix the sample in well #2.
   b. Using the same pipet tip, transfer 50 µl from well # 2 to well # 3 and mix the sample in well # 3.
   c. Using the same pipet tip, transfer 50 µl from well # 3 to well # 4 and mix the sample in well # 4.
   d. Repeat this transfer and mixing step, moving to the next well each time. STOP when you reach well # 11; discard the 50 µl of solution from well #11 into a waste container.
5. In the second microplate strip, use a fresh pipet tip to transfer 50 µl of the positive control (+) from the violet tube into the three "+" wells.
6. Use a fresh pipet tip to transfer 50 µl of the negative control (–) from the blue tube into the three "–" wells.
7. Use a fresh pipet tip to transfer 50 µl of each of your team's samples into the appropriately initialed three wells.

4. Wait 5 minutes while all the proteins in the samples bind to the plastic wells.
5. Wash the unbound sample out of the wells:
    a. Tip the microplate strip upside down onto the paper towels so that the samples drain out, then gently tap the strip a few times upside down on the paper towels. Make sure to avoid splashing sample back into wells.



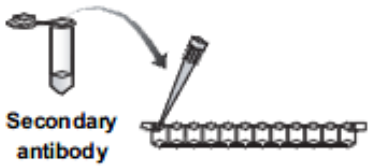
    b. Discard the top paper towel.
    c. Use a transfer pipet filled with wash buffer from the beaker to fill each well with wash buffer taking care not to spill over into neighboring wells. The same transfer pipet will be used for all washing steps..

    d. Tip the microplate strip upside down onto the paper towels so that the wash buffer drains out, then gently tap the strip a few times upside down on the paper towels.
    e. Discard the top 2–3 paper towels.
6. Repeat wash step 5.
7. Use a <u>fresh</u> pipet tip to transfer 50 µl of primary antibody (PA) from the green tube into all 12 wells of the microplate strip.

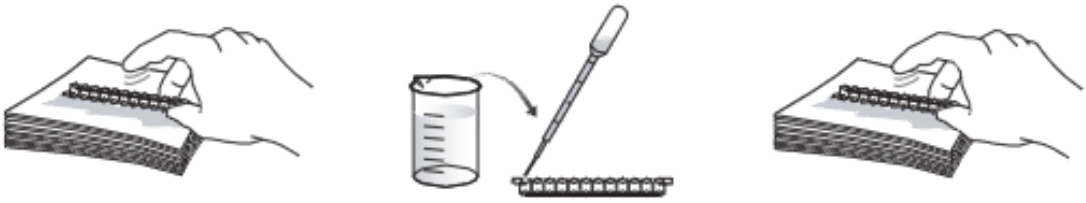


8. Wait 5 minutes for the primary antibody to bind.
9. Wash the unbound primary antibody out of the wells by repeating wash step 5 **two** times.

10. Use a fresh pipet tip to transfer 50 µl of secondary antibody (SA) from the orange tube into all 12 wells of the microplate strip.



11. Wait 5 minutes for the secondary antibody to bind.
12. Wash the unbound secondary antibody out of the wells by repeating wash step 4 **three** times.



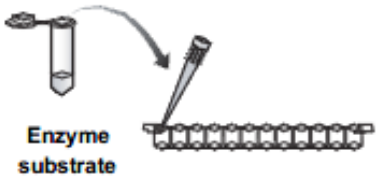
The secondary antibody is attached to an enzyme (HRP) that chemically changes TMB (the enzyme substrate), turning it from a colorless solution to a blue solution. Predict which wells of your experiment should turn blue and which should remain colorless and which wells you are not sure about.

_____

_____

_____

13. Use a fresh pipet tip to transfer 50 µl of enzyme substrate (SUB) from the brown tube into all 12 wells of the microplate strip.



14. After 5 min, add 50 ul of 0.18M sulfuric acid. This will turn the blue wells to a bright yellow color.
15. Analyze in a plate reader set to 450nm.
16. Using the Excel spreadsheet of your data, determine the CEA concentration of your patient samples A & B and assess the risk of cancer for each patient by comparing your results to the literature provided in the introduction.

Part II: Quantitative ELISA Analysis

Standard Concentration (ng/mL)	Absorbance (450nm)	Absorbance – Blank Absorbance
0		
1		
2		
4		
8		
16		
31		
63		
125		
250		
500		
1000		
Negative control		
Patient A		
Patient B		

1. Using MS Excel, graph your standard curve. Plot (absorbance-blank) on the y-axis and CEA concentration on the x-axis. Label both axes (with units) and give the graph and appropriate descriptive title.

2. Using MS Excel, determine the equation of the line ***for the linear points of the curve*** as follows. Note the graph may have a sigmoidal ("S") shaped curve. Notice and high and low points on the graph that may not be in the linear range and remove them. Keep the "no analyte" (0, 0) point.
    a. Input data into MS Excel in two columns, with the CEA concentration on the left column, and the corresponding absorbance (-blank) on the right column.
    b. Select data, click on insert, click on scatter plot
    c. Right-click on one of the points, select, add a trend line
    d. Click on Linear, set intercept at 0,0, click on display equation on chart and display R2 value on the chart
3. The R^2 value is a correlation coefficient that will tell you how well your data correlates linearly to each other. An R^2 value >0.95 is acceptable, but >0.98 is preferred.
4. Click on the chart elements (Right corner of graph box, "+" and select axis, axis title, and chart title. Also, complete the corresponding titles. Please print out a copy of your graph and include it with your report.
5. Using the equation of the line, determine the CEA concentration of your sample.

Lab Unit 13 – ELISA Assignment

1. Why do you need a positive control for every ELISA? Why do you need a negative control for every ELISA?

2. What is the equation of the line for your graph? _____
3. Use this equation of the line to determine the level of CEA in the serum of patient A and patient B. Show your work:

Patient A: _____ *Patient B:* _____

4. Is patient A or patient B at-risk for cancer? How do you know? (hint see CEA levels figure description)

5. Write a conclusion statement for your experiment:

LAB UNIT 14: PREPARING FOR A CAREER IN BIOTECHNOLOGY

J. O'Grady, EdD, & L. Fletcher, Ph.D.

OBJECTIVES

In this activity, students will:

- ✓ Identify career interests
- ✓ Find potential jobs in the biosciences
- ✓ Create a basic resume

Exciting Biotechnology Careers

Biotechnology is a diverse career requiring a full range of academic and workforce skills. Biotechnology offers career opportunities in Bioscience: Medical, Agricultural, Environmental; Applied chemistry: Testing; Physics & Engineering: Biomedical devices; and Computer Science: Bioinformatics.

Biotechnology Industry in Austin, Texas

The biotechnology industry has also been steadily growing in the Austin area. Today, Austin's bioscience community encompasses over 100 companies that employ more than 7000 people in the areas of research, diagnostics, pharmaceuticals, and medical devices. Some of these businesses include Xbiotech, Agilent, Asuragen, Perkin Elmer, Fischer Scientific, Pfizer, CPL, and PPD to name a few!

Austin is also a major contributor to academic research in the biological sciences at the University of Texas, Texas State University, and the M.D. Anderson Cancer Research Center in nearby Bastrop. Additionally, many students obtain jobs at the Texas Department of State Health Services where they test seasonal flu samples! To find out more biotech opportunities in Texas, explore here: https://texaswideopenforbusiness.com/industries/biotechnology-life-sciences Explore Biotech jobs in Austin: https://www.indeed.com/q-Biotech-l-Austin,-TX-jobs.html. Even Craig's List has a biotech section! https://austin.craigslist.org/d/science-biotech/search/sci

Biotechnology is an international collaborative movement, and students should consider expanding their career search across the globe! Many companies have research and development, manufacturing, or administrative facilities in several different countries. For example, Bioo Scientific a local startup company was recently acquired by the world-wide company Perkin Elmer. You can learn more about Bioo Scientific here! http://www.biooscientific.com/

Bio-Link is a national biotechnology career and education organization that has an excellent biotechnology career exploration tool. Go here to learn about potential careers in Biotechnology across the nation: http://biotech-careers.org/

PART I: Careers in Biotechnology Assignment: Perform this activity before class! You will share it with the class today!

1. Take a moment to reflect on the various techniques you've explored over the semester. And write down a favorite lab activity or topic explored this semester. Why was this your favorite?

2. Reflect on your answer to the previous question. What area of the Biotechnology Industry (as a career) interests you the most? Why?

3. Using the resources provided, or search on your own, list three Biotechnology companies, State or academic labs, in Austin, you may want to work. Include their website.

4. Find one posting online for a job you would like to have when you graduate from the biotechnology program. Write a short three-sentence biography of the company and a 3-sentence summary of the job - including the title and description of duties.

PART II: Writing a Resume for a Job in the Biotechnology Industry

The purpose of a resume is to get an interview! Your resume will open doors for you in your career, so it is important that you take time to create a memorable resume. It is a marketing brochure, and you are the one that you are marketing. While writing your resume, remember that you intend to summarize the major highlights of your career in a way that will emphasize your professional accomplishments that are appropriate for the specific job you are applying.

It is required that you prepare a resume in advance of the internship course you will take at the end of the program. That is why you are being provided an opportunity to work on your resume in this course. You will continue to update your resume during every course with the new techniques you have learned. When you are ready to register for the internship program, you will have an accurate and professional resume ready to go! After instructor approval, this resume will be given to prospective mentors and employers.

CAREER WORKSHOPS:

There are many free and excellent workshops available to you. Workforce Solutions has an office in the Eastview Campus (EVC), building 1000, and they have weekly workshops ranging from job hunting, resume building to mock interviews. It is recommended you visit their offices and see what it is they do there. You will be visiting them when you graduate to help you find a job. If you have never prepared a resume, the '***Basic Resume Lab***' workshop is recommended. If you already have a working resume, the '***Advanced Resume Workshop***' is recommended. You can find a calendar of their workshops at their website: http://www.wfscapitalarea.com/

RESUME CHECKLIST

- ✓ Make sure that your **name, one email, and one phone number** is prominently displayed at the top. No need to put your home address. Call yourself and ensure the voice mail is accessible and has a personal and professional message.
- ✓ **Summary.** It is preferable to provide a summary section (rather than an objective). A summary tells the company what you offer them. Tailor the summary to the job posting. The summary should include information that sets you apart from other applicants.
- ✓ **Your educational background.** List where you have attended schools and what degrees you have earned, and year awarded. If you are currently working on a degree, post-date your graduation. Bold the degree, not the university. No need to list your GPA.
- ✓ **List employers chronologically.** Unless there is a work history that relates to the job you are applying for, list the most recent first. Each entry should include the position title, organization's name, and years you were employed.
- ✓ **Use other categories if appropriate:** Specific job skills and training, Awards, Scholarships, Publications & patents, Presentations & posters, Professional Associations, and Community Service.
- ✓ The program was composed of several courses. Think of each course as a company in terms of what you mastered. Also consider the wording used in the skill standards. The standards were approved by industry and therefore use that language.

Anita J. Ob

Anita.job@gmail.com • 512.555.5555

Summary

Highly motivated and experienced biotechnician with a Bachelor of Science in Biochemistry and intensive training in a biotechnology certification program seeks employment in the biotechnology industry. Experience in experimental protocol planning, execution, and documentation with attention to detail. Confident leadership and teamwork skills developed in the military.

Technical Skills Summary

- Protien: Protein purification using column chromatography (FPLC, HPLC); protein analysis using SDS-PAGE, western blot, ELISA, gas chromatography, and protein quantification with Bradford Assay and spectrophotometry.
- Nucleic acid technology: Rna isolation, genomic and plasmid Dan purification, pcr (end-point and real-time), agarose gel electrophoresis, mammalian cell culture, recombinant Dna technology, and VNTR analysis.
- Extensive solution preparation, including media, multi-component solutions, and buffers.
- QA/QC: Equipment qualification and process validation, composing SOPs, domentation
- cGMP

Education

Advanced Technical Certificate, Biotechnology, Austin Community College **Bachelor of Science, Biochemistry,** Texas A&M, Corpus Christi (2009)

Work Experience

X Biotech, Research Intern *May– Sept*

- Performed ELISA, FPLC, SDS-PAGE, and western blotting to determine purity, identity, and activity of recombinant antibodies.
- Performed PCR, plasmid preparation, and dna agarose gel analysis.
- Experience in ELISA and FPLC protocol development.

Austin Organics, Greenhouse Manager *2010 –2016*

- Managed the construction and day-to-day operations of the first organic certified hydroponic lettuce greenhouse in Texas.
- Handled direct customer sales, ordering materials, planting, and harvesting schedules.
- Worked with Microbial Earth to culture aerobic microorganisms for composting.

United States Navy, Aviation Electronics Technician *1999 –2003*

- Worked as an electronics technician repairing and maintaining mission-critical avionics and automated test equipment.
- Instrumental in instituting new repair protocols.
- Responsible for maintaining documentation in accordance with a quality system.

RESUME WORKSHEET

To create your resume, it is helpful to first reflect upon your past and current experiences. When you begin, you will quickly realize there is a large amount of information you will need to research to create a resume. Do not leave this to the last minute before class! You will need access to personal records: email, pay stubs, job descriptions, transcripts, etc…

1. In the worksheet below list, all work experiences, training, and achievements. Try to identify what you did in that experience (see example).
2. After making your list, identify those experiences that you have enjoyed and learned something that you would like to use in the future. Circle or highlight those.
3. Highlight in a different color (or circle in red pen) those items that will require further research.
4. Include this worksheet in your report.

Work	Volunteer	Education	Other
Example: *A shift supervisor at McDonald's* *-helped customers* *-handled $$$* *-supervised staff*	*Example:* *Habitat for Humanity* - *30 hours of service in 2012* - *Recruited 3 new volunteers*	*Example:* *Biotechnology certificate* - *Complete in 2014* - *Favorite classes: BITC1240, BITC2441*	*Example:* *-SSTEM Scholarship* *-Biotechnology club officer*

RESUME CRITIQUE

A great way to really learn about good resume preparation is reading other resumes. Swap your final resume with a classmate and critique according to the table below. Include your resume and the critique sheet from your lab partner in your final lab report.

RESUME CRITIQUE SHEET

Name: _____ Score: _____

CHARACTERISTICS	SCORE 1……5……10 Developing Strong	FEEDBACK
Overall appearance ✓ Easy to read & inviting ✓ Professional appearance		
Format ✓ Contains contact information ✓ Complete ✓ Appropriate length ✓ Consistent layout/format		
Organization ✓ Strongest qualities first ✓ Emphasizes important skills, credentials & accomplishments		
Content ✓ Stresses skills, education, & accomplishments ✓ Targeted for the job – supports the summary ✓ Appropriate content for a resume		
Language ✓ Appropriate tense ✓ Action verb-accomplishments ✓ Spelling ✓ Grammar		

ADDITIONAL FEEDBACK:

LAB UNIT 14: ASSIGNMENT

1. PRE-CLASS EXERCISE – Before you come to class, perform the activities in the introduction and the following resume
    - Career activity in Part I
    - Find your current resume and bring it to class. If you only have a paper copy, bring this to class. If you have an electronic copy, bring that as well, as you will use it to update your resume. If you do not have one, not to worry, you will create one in class!
    - Fill in the "Resume Worksheet" with new experiences to add to your resume, or if you do not have a working resume, use it to collect all the information you will need to put on your resume. Bring this to class.
    - Using the "Resume Critique Sheet," critique your current resume (if you have one). Bring this to class.

2. As a team, evaluate Anita J. Ob's resume. What are some things you like about it? What are some issues? How would you fix them?

3. Create a basic resume or update a current working resume. Print out your draft resume and write DRAFT on the top.

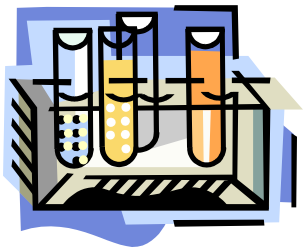
4. Swap resumes with your classmate and 'edit' them using a red pen. Fill in the "Resume Critique Sheet" for your classmate. Return the edited resume and critique sheet to your classmate. Include both the resume with the markups and the critique sheet with your report.

5. Make corrections based on your classmate's comments if you see fit. Remember, your resume is your own 'marketing brochure'. Print out a final resume to include with this assignment.

6. Staple your final resume to the top of your assignment as your title page. Include all activities, rubrics, versions of your resume, and worksheets with your assignment.

APPENDIX

BIOL1414: Laboratory Report Rubric



Name _____ **Date** _____

Lab _____ **Score** _____

Formatting:

Title Page	**1 pts** _____
Overall neatness/legibility	**2 pts** _____
Grammar and Mechanics	**2 pts** _____

Workbook Exercises: Complete all exercises in the Workbook.

Complete all exercises in the workbook	**6 pts** _____

Lab Work:

Teamwork: Active participation in lab exercise	**2 pts** _____
Thorough observations made in (written in ink)	**3 pts** _____
Safety: Wear PPE, lab clean up, operate equipment	**2 pts** _____

Results & Analysis:

Tables, Graphs, Calculations, Forms	**5 pts** _____
Lab Analysis Questions	**5 pts** _____
Conclusion	**2 pts** _____

Total: **30 pts** _____

Feedback:

Employability Skills Evaluation Form

AUSTIN COMMUNITY COLLEGE, BIOTECHNOLOGY PROGRAM

Student Name: _____ Score: _____

Course: _____ Semester: _____

Skills	Needs Attention		Developing	Exceptional	
Dependable Attendance and Punctuality, completes work in a timely manner	1	2	3	4	5
Organizational skills	1	2	3	4	5
Working with others, good team worker	1	2	3	4	5
Safe & secure work habits in a regulated environment	1	2	3	4	5
Communication skills	1	2	3	4	5
Resourcefulness, able to work independently	1	2	3	4	5
Documentation in a regulated environment (lab notebook, SOP, forms, batch binders, LOGBOOKS)	1	2	3	4	5
Keeps work area clean and orderly	1	2	3	4	5
Produces quality work	1	2	3	4	5
Problem solving skills	1	2	3	4	5

EVALUATOR: _____ DATE: _____

Additional Comments:

Solution Preparation Form

For every solution prepared in the laboratory, fill out a solution preparation form. Foms are in the file cabinet. A sample formed filled out is below. Never leave a blank space in a form – fill in every field, even if it was 'not performed' state so.

SOLUTION PREPARATION FORM Control # Use label SOP

Name of Solution/Media: Complete name, include concentration, pH

Amount prepared: _____ Date: _____

Preparers(s): If two students prepared the solution, record both names here. Both students must submit a copy of this (same) Solution Prep form in their lab report

Component	Vendor/ lot #/ Control #	Date Received	Storage conditions	MW or initial concentration	Mass used	Final Concentration
Water is not a listed component						

Balance used Balance Model and Number	Calibration status Did you calibrate it? Alternatively, did you verify it?	
pH meter used pH meter Model and number	Calibration status Did you calibrate it? Alternatively, did you verify it?	
Initial pH Always record the pH the solution started at	Final pH Always record the final pH after BTV	Adjusted pH with Chemical and concentration used to adjust the pH
Prep temperature Record the accurate temperature – NOT "RT."	Sterilization procedure Was this solution autoclaved or filter sterilized?	Storage conditions Where is this solution stored now?

Calculations/Comments:

Record ALL calculations used to prepare this solution.

SOP Template

Evelyn Goss & Jeremy Garza

Title: How to Write a Standard Operating Procedure (SOP)	
Institution: Austin Community College	Department: Biotechnology Program
Approved By:	Date of Approval: 10/07/2017
Prepared By: Jack O'Grady	Revision Number: 002

1.0 SCOPE AND APPLICATION

The purpose of this Standard Operating Procedure (SOP) is to train users to write an SOP for use in ACC Biotechnology labs and teaching curriculum. SOPs provide consistency each time a procedure or process is performed, serve as reminders to ensure that work is completed correctly, can be used to train employees the correct way to complete a new task, and also reduce the possibility of failure by enabling the employee to complete any function described in the SOP.

2.0 SUMMARY OF METHOD

Briefly, summarize the procedure.

3.0 WORKFLOW

An overview of the workflow. Create a flowchart of the procedure.

4.0 RESPONSIBILITIES

Include qualification user should have to complete task satisfactorily. A technician or student is responsible for adhering to all applicable duties outlined in this SOP. The instructor is responsible for overseeing all activities, ensuring all work satisfies the specific tasks described in this SOP.

5.0 DEFINITIONS

Identify specialized terms, abbreviations, or acronyms used in this SOP.

6.0 SAFETY GUIDELINES

6.1 Health and Safety Warnings

Indicate operations that could result in injury. Explain what will happen if the procedure is not followed correctly. List warnings here, and at critical steps, in the procedure.

6.2 Precautions

Indicate activities that may result in equipment damage, sample degradation, or possible invalidation of results. List here and at critical steps in the procedure.

7.0 WASTE MANAGEMENT

Hazardous materials disposal guidelines.

8.0 INTERFERENCES

Describe process components that may interfere with accuracy of final product

9.0 QUALITY CONTROL

This section dedicated to allowing self-verification of the quality and consistency of the work. Describes preparation of appropriate QC procedures and QC material for calibration, performance evaluations, standard preparation, and the frequency at which they should occur. Also, details limits/criteria for QC results and actions required when data exceed limitations.

10.0 REAGENTS, MATERIALS, AND EQUIPMENT

10.1 List reagents: name, manufacturer, CAS, catalog number (if known).

10.2 List specific instruments/software used in this SOP: vendor and model number. Include SOPs to use and calibrate this equipment.

11.0 CALCULATIONS

Show mathematical steps to be followed for preparation of procedure.

12.0 PROCEDURE

Use this space for detailing the experiment procedure of each step listed in the workflow. Must be numbered steps. 1. Assign a title for the SOP that briefly and clearly states what it will describe. a. The title reflects a full description of the purpose of the procedure. b. The title is written directly into Title Box at the top of an SOP. 2. Assign an ID number and revision number to the SOP. a. The ID number reflects the type of procedure. Example: SOP-HPLC-xxx refers to SOPs for HPLC instruments. SOP-HPLC-001 refers to a specific SOP written for an HPLC procedure. This section will also list the date when the document was approved. b. Revision numbers indicate how many times the document has been revised. The writer begins with the letter A and proceeds alphabetically each time a new version is approved. This section will also list the date when the document was revised and approved again. c. SOP ID number is placed at the top right-hand corner of the SOP labeled "SOP#." 3. In Section **1. 0 Scope and Application**, write a statement of the purpose and scope of the procedure. Scope should be brief but descriptive and specific, include situations when this SOP is used. 4. In Section **2.0 Summary of Method**, briefly, summarize the procedure.	*Use this space to take notes during your experiments or other items to note during the use of this SOP* *SOP number is inserted into the header of this template.*

5. In Section **3.0 Workflow**, briefly summarize the workflow, using a list of steps or a graphical representation such as a flowchart.
6. In Section **4.0 Responsibilities**, define the responsibility of the procedure. Include qualifications and credentials user should have to complete task satisfactorily.
7. In Section **5.0 Definitions**, include any specialized terms that will be needed to follow the SOP.
8. In Section **6.0 Safety Guidelines**, define as follows:
    a. Identify any hazardous chemicals and how they might enter the body or otherwise cause danger. Example: "Concentrated sulfuric acid released strong fumes that cause lung, eye, and skin irritation and can result in serious burns if spilled on the body. Only work with concentrated sulfuric in a fume hood while wearing nitrile gloves, lab coat, and safety glasses."
    b. Indicate what personal protective equipment (PPE) is required for performing the procedure. Examples: "Sodium dodecyl sulfate causes lung, eye, and skin irritation. Wear a respirator while weighing the dry chemical."
    c. Identify other physical hazards (flammability, electrical, mechanical) and how to work safely to reduce the risk of injury. Example: "Hydrogen gas is extremely flammable if concentrated. Avoid any open flame around hydrogen tank."
    d. Indicate proper disposal of biohazards, chemicals, and contaminated items. Example: "Place used agar plates in a red biohazard bag, tie and deposit in large cardboard biohazard collection area. Place pipet tips contaminated with 2-mercaptoethanol in the designated collection bag inside the fume hood."
9. In Section **7.0 Waste Management**, include instructions for how to safely dispose of any hazardous materials used in, or generated by, the procedure.
10. In Section **8.0 Interferences**, describe any known processes that may interfere with the accuracy of the final product.
11. In section **9.0 Quality Control**, list instructions related to quality control of the procedure, instrument, or materials used for the SOP.
12. In Section **10.0 Reagents, Materials, and Equipment**, list materials are needed to perform the procedure.
    a. These can include equipment, supplies, chemicals, and facilities.
    b. Be specific about the manufacturer and model of products if a specific model is required.
    c. Specify temperatures for water baths and incubators.

d. Include SOP of associated equipment use and calibration. 13. In Section **11.0 Calculation,** include an example of the calculation needed to perform this task and provide space for the user to write any calculations needed. 14. In section **12.0 Procedure**, write a procedure in numbered steps that provides instructions to the user exactly how to perform the operation. a. Steps should be written as commands in the present tense. b. Steps should be placed in chronological order, particularly when an advanced preparation is required. Example: "One hour before starting procedure, place solution A at room temperature to equilibrate." c. Each step should provide only the details required for that step, minimizing background information or explanation. d. If a common problem arises during the procedure, include a step for how to prevent or correct the problem. Example: "The pellet may become dislodged from the tube if it was not dense enough. Centrifuge again for two minutes, increasing speed to 10,000rpm." 15. In Section **13.0 Data and Records Management**, list instructions for how to manage data from, or records related to, the procedure being followed. Provide guidelines for how to document the procedure was performed; this may be a form that was filled out, logbook, or recording the action in a lab notebook. 16. In Section **14.0 References**, cite sources you used to write the SOP; this is often the user manual for a piece of equipment, a product insert, or another SOP or procedure. 17. Submit the written SOP to the supervisor for approval. 18. Destroy or limit access to previous versions of the SOP. Always keep a copy of the old versions for historical reference.	

13.0 DATA AND RECORDS MANAGEMENT

The records section should include post-data calculations or presentation to be performed, forms to be used, and include data and record storage information.

14.0 REFERENCES

List any references, associated documents, and forms.

Seidman LA, Moore CJ. 2009. *Basic laboratory methods for biotechnology: textbook and laboratory reference.* Upper Saddle River, NJ: Prentice Hall.