Introduction to Biotechnology II Exercise workbook & Lab Guide

BIOL 1415 – 2018

Jack O'Grady ACC Biotechnology Program BIOL1415 2018

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INTRODUCTION TO BIOTECHNOLOGY II LAB: CLASS ORIENTATION

BIOL1414 & BIOL1415 are the key curricula for Entry Level Certificate in Biotechnology. The purpose of these two courses is to prepare students to work as entry-level biotechnology employees in potential regulatory environments. Both these courses contain not only academic knowledge in biotechnology, chemistry & biology, but provide training in other essential workforce skills; writing SOPs, filling in forms, equipment validation/calibration/verification, teamwork, and working independently.

Common Course Objectives for these courses: http://sites.austincc.edu/biology/common-course-objectives/. Biotechnicians are required to use, maintain, and troubleshoot a variety of different types of equipment, and maintain a lab notebook. As part of your lab experience, you will learn these skills during the lectures and practice them in the laboratory. Students are expected to behave professionally at all times. The lecture will provide background and relevant information about the solutions, procedure, and related techniques.

The objectives of the lab portion of the BIOL1415 course are to:

- ✓ Develop the core laboratory skills of a biosciences laboratory
- ✓ Develop critical thinking and multitasking skills
- ✓ Encourage teamwork and accountability
- ✓ Practice accuracy in calculations and writing scientifically
- ✓ Demonstrate critical skills and associated with working in a regulated company

MATERIALS:

- o No Commercial Textbook required for this course. Students will be provided an online only OER eTextbook: J. O'Grady, Introduction to Biotechnology, 2017 Edition.
- o For students wanting additional resources in laboratory math, refer to Lisa A. Seidman. "*Basic Laboratory Calculations for Biotechnology*" 2008. Pearson. ISBN: 0132238101
- o 3-ring binder, fine tip black Sharpie, earphones, and a scientific calculator (optional).
- o Safety glasses with a rating of Z87, long pants, and comfortable shoes that cover the entire foot area. A disposable lab coat is provided. However, you are welcome to bring your own clean lab coat.

LABORATORY DOCUMENTATION

Documentation in a lab notebook is an essential skill for any biotechnician. The Food and Drug Administration's (FDA) handbook states, "***if it is not written down, it was not done***." Documentation details vary from lab to lab, but it is essential to record what an individual has done and observed, helps to establish ownership for patent purposes and other legal uses, improves tractability in the manufacture of a product, and to adhere to government regulations.

The Biotechnology and Biology laboratory classes in the Biotechnology Program will have different formats and rules in regard to the notebook and lab report format. This is no different than in a typical Biotechnology workplace. Even within the same company, different

departments follow different rules and regulations regarding notebooks and reports. For example, in an R&D Department, you may use a hard-bound notebook and be required to sign it out at the beginning of the day and return it for lock up at the end of the day. In the Production Department, you may produce a 'batch record' which is a loose-leaf binder of all the SOPs, tests and other document compiled to make a particular lot batch of a product.

LAB NOTEBOOK:

Each student will maintain a 3-ring binder lab notebook to keep your workbook and laboratory exercises, notes and data throughout the semester. *Your lab notebook is graded.* In most workplaces, a hard-bound laboratory notebook is required. However, in this class, you are required to take detailed lab notes in your pre-lab and keep all materials in a binder- this is your lab notebook. Bring this binder to every class, and you will submit your lab notebook periodically during the semester for grading. Your instructor will provide you with a grading rubric for your laboratory notebook.

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

- ✓ Title Page: Course title, your name, your instructor name, and semester. Contact information such as a phone number is helpful here if it accidentally gets lost.
- ✓ Table of Contents: The notebook should be in chronological order (lab 1, 2, 3…) and tabs (or page numbers) to easily find each lab should be used. It is recommended to include lecture notes with corresponding labs.
- ✓ For each Lab Tab (in this order):
    - Relevant lecture activities
    - Lab manual instructions
    - Pre-lab exercises with experimental notes
    - Any forms generated during lab
    - Any handouts from lab
    - Data analysis (graphs, tables, charts, etc.), and Conclusion

CLASS FORMAT

BEFORE CLASS

1. Look at the schedule to see what exercise in the lab manual you will be covering in class and on which date.
2. Read assigned reading, and complete required activities before coming to class. Perform online content check to see how well you have understood the material.
3. Complete pre-lab protocol & calculations (see below). If you do not prepare before the lab, you may not have enough time to complete the exercise or may make mistakes, and the lab exercises not succeed. *Moreover, it will not be very much fun if you do not know what you will be doing! ☺*

THE PRE-LAB EXERCISE:

The purpose of the pre-lab exercise is to prepare for the Laboratory Unit. It will ensure you have read the instructions and prepared your tables and calculations for use in the lab exercise. ***The typed pre-lab exercise must be complete before beginning the lab***.

The Pre-lab must contain the following parts.

- Name, Title & Date: Create a comprehensive title of the experiment (*not "Lab unit 1"*)
- Purpose: Briefly state the purpose of all components of the experiment. Be specific.
- Hazard Communication: Summarize specific safety precautions for the lab. You may find this information in the manual or on the MSDS for that chemical.
- List of equipment and reagents: List the materials used in the lab. Be specific & include the size, and the number needed.
- Protocol: ***Write the protocol in your own words***. The protocol is numbered, and specifically directive. Do NOT copy the protocol from the manual. Include data tables.

DURING CLASS

During lab experiments, take notes in pen. WRITE EVERYTHING DOWN, including the temperature, time, observations, and any alterations to the procedure. Always show calculations. Writing down everything improves your observational skills, helps you understand the importance of each step, and provides a record of how an experiment might have gone wrong. Record you own notes, even when working in teams.

General guidelines for writing good lab notebooks:

- Write all parts of your lab notes and observations in ***ink only***. If you make an error, draw a single line through it and enter your correction in clear and legible writing. If you discard data for any reason, you must justify your decision to do so immediately and in writing.
- *Write legibly.* Remember, other people may need to read your notebook. If they cannot easily make out what you have written, they can easily misinterpret an important detail about your work, or your work may not be publishable, nor patentable.
- If you tape materials such as a gel image into your notebook or onto a form, tape all four sides. Then write **"NWUI"** ("No writing under insert") on the tape, your initials, and the date.
- Keep your records factual, concise, clear and complete in all aspects.

POST-LAB

One week after all the data has been collected you must submit a lab exercise report for grading. Make sure your report is easy to read and well organized. Include:

1. A **title page:** Descriptive title of the experiment.
2. The **pre-lab** write-up completed before class.
3. **Experimental Notes** collected during lab, this includes any **forms** filled out during the lab, and **raw data**.
4. **Results:** Report and summarize all your raw data. Results may be in a table, and graph format, depending on the lab. All tables and graphs MUST be properly labeled.
5. **Analysis:** Answer analysis questions.
6. **Conclusions:** Conclusions made must be supported by the experimental results. Report findings and compare data to the literature or class data.

__Late Lab Exercise Reports:__ Part of the skills you are learning in this class is to generate quality work while meeting expected deadlines. Late assignments are subject to the policy outlined in the syllabus. See your instructor for further information.

__Missed Classes:__ You are expected to attend every class. Since we perform graded work every class, missed classes have grade consequences. The penalty for missed labs is found in the syllabus.

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 are graded with lab exams, Employability Skills evaluations, and lab notebook grading.

Class Introduction 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 next class, set up your binder and show your instructor:

   - a lab notebook title page,
   - table of contents,
   - the lab manual

2. **Obtain a lab equipment:**
   a. **Sharpie:** a fine tip black permanent marker to use in the lab
   b. **Safety equipment:** safety glasses or goggles rated Z87 (or Z87.1) and closed-toed shoes.
   c. You are welcome to bring your own clean **lab coat** if you prefer that to the disposable one provided.

LAB UNIT 1: WORKING IN A REGULATED ENVIRONMENT

Part I: Safety & Security

Objectives: After completing this orientation exercise the student should be able to:

1. Perform site-specific bioscience safety training, and abide by the ACC lab safety policy
2. Develop site-specific security procedures. Demonstrate the difference between safety & security and discuss why security is such an important issue in a biotechnology workplace
3. Locate critical safety equipment is kept in the biotechnology lab they are working in

INTRODUCTION TO LAB SECURITY

Biotechnology laboratories are equipped with hazardous material that may pose a hazard if used carelessly. Therefore, it is important to learn how to handle hazardous material appropriately. It is often the responsibility of a biotechnician to make sure that safety rules are followed, and anyone working in a laboratory must pay attention to what they are doing and use common sense to avoid hazardous situations.

While the ACC science safety rules are designed to provide protection while working in ACC laboratories, you must become self-sufficient in protecting yourself in your future jobs in the biotechnology industry. Also, lab technicians are frequently entrusted with ensuring compliance with safety precautions in the biotechnology workplace. For this purpose, this lab exercise will introduce you to the main components of lab safety measures and security procedures that apply in a biotechnology setting. Here is a video to get started: https://www.youtube.com/watch?v=VRWRmIEHr3A

PROPER HANDLING & STORAGE OF CHEMICALS AND REAGENTS

There is no single simple formula for working safely in the laboratory since each lab facility and each experiment presents unique challenges. We will be addressing safety issues with each experiment that we do in this course and give some specific guidelines for safety throughout the semester.

A. MSDS (Material Safety Data Sheets)

While each chemical used will have unique properties, some standard practices will aid in treating them all with the level of respect that they are due. For example, labeling each chemical is required under the law and should be thorough enough so that even a person who does not work in the lab can identify any chemical. Also, every chemical in the laboratory should have a **Material Safety Data Sheet** (MSDS) or Safety Data Sheet (SDS) on file and readily available. The MSDS is a legally required technical document, provided by chemical suppliers, that describes the specific properties of a chemical. Besides the MSDS on file in the lab, several websites offer MSDS databases.

Typical information found on an MSDS:

1. **Chemical identity.** The manufacturer's contact information is here, along with contacts for emergency situations.
2. **Hazard ingredients/identity.** Some reagents have multiple components, and many single-component chemicals have alternative names. Concentration limits for airborne exposure to a chemical are listed here. Although these indices of toxicity are mainly of concern for production workers in factories, they are also useful for evaluation of short-term exposures.
3. **Physical & chemical characteristics.** This list of physical properties indicates whether the chemical is solid or liquid and how volatile it is.
4. **Fire and explosion hazard data.** Is there a flame nearby?
5. **Reactivity data.** This information is essential in determining the proper handling and storage of chemicals.
6. **Health hazards.** The best source of specific toxicology data is given here, such as symptoms of acute damage from exposure and some recommended emergency procedures. If a chemical is tested for **carcinogenicity** (cancer-causing potential), that information is listed here.
7. **Precautions for safe handling and use.** This describes how to deal with spills.
8. **Control measures.** Specific recommendations for personal protective equipment (PPE) are listed here.

B. NFPA Ratings (National Fire Protection Association)

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:

Blue for health hazard	Red for flammability	Yellow for reactivity
0 – normal material	0 – will not burn	0 – stable
1 – Slightly hazardous	1 – flash point > 200° F	1 – unstable if heated
2 – Hazardous	2 – flash point > 100° F	2 – violent chemical change
3 – Extreme Danger	3 – flash point < 100° F	3 – shock and heat may detonate
4 – Deadly	4 – flash point < 73° F	4 – may detonate

The uncolored station of the NFPA diamond is for specific hazards: **OX** – oxidizer compound, **ACID** – acidic compound, **ALK** – basic compound, **CORR**– corrosive compound, and ~~W~~– use NO WATER

C. General Safety Precautions in Handling Hazardous Chemicals in the Lab

There are four routes to exposure to hazardous chemicals that you should keep in mind while handling them:

1. **Inhalation**: avoid by the use of fume hoods and masks
2. **Skin & eye contact**: avoid by the use of lab coats, gloves, and goggles
3. **Ingestion**: Avoid eating or drinking in the lab, or leave the lab without removing gloves and washing hands
4. **Injection**: dispose of broken glass and needles properly

SAFE HANDLING PROCEDURES:

- ✓ Treat all chemicals as if they were hazardous
- ✓ Label all containers with contents, including concentrations and date
- ✓ If a hazardous material is contained, label it with a warning
- ✓ Think through the experiment 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 phones, doorknobs, light switches, etc.
- ✓ Ensure proper waste disposal and labeling.

Here are some specific tips for handling the different types of hazardous chemicals:

- **Flammable:** Do NOT heat unnecessarily, and never in the presence of a flame or source of a spark. In general, only open containers in fume hoods. When storing more than 10 gallons of flammable liquids, a special explosion proof storage cabinet is required.
- **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 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, and know the toxic properties of the chemical. When working with a dry powder, wear a mask to avoid breathing the dust. Be aware of the waste disposal procedures.

Here are some of the most common hazardous chemicals that you will encounter in the biotechnology lab:

Carcinogens	– formaldehyde	**Mutagens**	– ethidium bromide
Neurotoxins	– acrylamide	**Teratogens**	– formamide
Nephrotoxins	– acetonitrile	**Hepatotoxins**	– chloroform
Corrosives	– phenol, strong acids & bases		

D. BIOLOGICAL SAFETY: CONTAINMENT

Biohazards. You will work with live organisms in many biotechnology labs, so it is important to be able to assess any biological hazards that they may pose and to treat them accordingly. The Biology department organism use policy can be found here: http://www.austincc.edu/biology/labanimalpolicy.html

You will work with live organisms as well as recombinant DNA in many biotechnology labs, so it is important to be able to assess any biological hazards that they may pose and to treat them accordingly. In general, a live organism is a biological hazard if its release into the environment could have an effect on the health of the environment in general or humans in particular. This includes known pathogens to humans, plants, or animals, as well as benign organisms containing recombinant DNA that could render the recombinant host dangerous. In fact, the recombinant DNA itself should be treated as a biohazard, since it is usually inserted into a vector that could transform organisms in the environment if released. Similarly, tissue and cell cultures of human

or animal cells are treated as a biohazard: while they would not survive if released into the environment, most immortalized tissue culture cells contain recombinant DNA.

The routes of exposure to infectious agents are the same as those of hazardous chemicals: inhalation, contact with eyes and skin, ingestion, and injection. The same general precautions are taken in handling biological hazards as the guidelines above for handling chemical hazards, especially toxic ones.

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.
- Always remove gloves before touching phones, doorknobs, light switches, etc.
- Avoid touching your face with your hands or gloves.
- Keep personal items such as coats and book bags out of the lab, or in a designated area.
- Use mechanical pipetting devices.
- Minimize splashes and aerosol production.
- Disinfect work surfaces to decontaminate after a spill and after each lab.
- Disinfect or decontaminate glassware before washing.
- Decontaminate all regulated waste before disposal (usually by autoclaving).
- Have an insect and rodent control program in effect.
- Use a laminar flow biological safety cabinet when available.

A majority of recorded laboratory-acquired infections are due to inhalation of infectious particles. Therefore special precautions should be taken to avoid producing aerosols when working with pathogens. While performing activities that mechanically disturb a liquid or powder, the biotechnologist should make the following adjustments. Disinfectants such as bleach and detergent are used extensively to decontaminate glassware and work areas, and it is important to realize that the effectiveness of disinfectants depends on the type of living microorganisms encountered. We will discuss this more in a future module.

Activity	Adjustment
• Shaking or mixing liquids	mix only in closed containers
♦ Pouring liquids	pour liquids slowly
♦ Pipetting liquids	use only cotton plugged pipettes
♦ Removing a cap from a tube	point tubes away when opening
♦ Breaking cells by sonication in the open	sonicate in closed containers
♦ Removing a stopper or cotton plug	remove slowly
♦ Centrifuging samples	use tubes with screw cap lids
♦ Probing a culture with a hot loop	cool loop first

E. DISPOSAL OF HAZARDOUS CHEMICALS & BIOLOGICAL MATERIALS

The disposal of hazardous chemicals is subject to state and federal regulations and is ultimately overseen by the Environmental Protection Agency. Highly toxic chemicals are regulated at low levels, and less toxic chemicals are disposed of through city sewer systems at higher levels. 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. *In our laboratory, specific hazardous chemical and biological waste disposal is discussed at the start of every lab. 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.*

Safety Assignment

1. Watch safety video as directed by the Instructor. It is also available online here: https://youtu.be/uDhSSWtY3kg
2. The instructor will provide the class with a site-specific safety training sheet and will go through this as a class. *Write down all the information in the blanks and keep it available in your lab notebook while working in the ACC laboratory.*
3. You must sign an ACC Safety Contract to acknowledge that you 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.
5. The instructor may request a weekly safety check by rotating groups. Groups will give a 1 min summary to the class of any safety violations they found at the end of the week. http://www.austincc.edu/sci_safe/docs/weekly_safety_inspection_log.pdf

   Next week's safety officer(s) is: ____

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 using a deviation report.

- Aprons/lab coats clean and usable?
- Fire blankets intact and usable?
- First aid kits stocked?
- Spill kits available?
- Goggles & Safety Glasses being worn by faculty, students, and staff?
- Safety posters & signs readable?
- "Notice to Employees" present?
- Shower and eye-wash station functional and unimpeded?

Comments:

Date: ______________

Signature: _______________________ printed name: _____________________________

Signature_________________________ printed name: _____________________________

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. Following the terrorist attacks of September 2001 and the "anthrax letters" sent the same month, much attention has been directed to practical measures that will keep hazardous materials (biological and chemical) out of the hands of criminals. Many new federal laws were enacted in direct response to these terrorist attacks.

It is important that laboratory personnel take specific actions to prevent unauthorized entry to labs, secure highly hazardous materials against theft, and ensure compliance with new security regulations. It is essential to implement procedures necessary to provide security of all hazardous materials in their areas of responsibility. One objective is to minimize the risk of theft, especially during that five-minute window when the lab is left unattended. 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.

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>.

The guidelines are intended to reduce the risk of unauthorized removal of hazardous materials from your laboratory:

1. **Recognize that laboratory security is related to but different from laboratory safety and develop a site-specific security policy.** Security, as used in this discussion, refers to measures used to control access to the laboratory to prevent theft of materials or equipment from the lab.
    - Assess laboratory for hazardous materials and particular security risks.
    - Develop and implement lab security procedures for your lab group.
    - Train your 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. Consider the use of key cards or similar devices when the risk warrants.
    - Do not leave hazardous materials unattended or unsecured at any time.
    - Lock freezers, refrigerators, storage cabinets, and other equipment where biological agents, hazardous chemicals, or radioactive materials are stored when they are not in use.

3. **Know who is in your laboratory area.**
    - Consider using a logbook for staff to sign in and out of the lab each day or using carded access devices for this purpose.
    - Limit laboratory access to those individuals who need to be in the lab.
    - All lab workers (including students, visiting scientists and other short-term workers) should wear identification badges.
    - Restrict off-hours access to individuals authorized by the principal investigator.
    - Guests should be issued badges and escorted to and from the lab. Approach people you do not recognize who appear to be wandering in laboratory areas and ask if you can help direct them.
4. **Know what materials are being brought into your lab.**
    - Know what hazardous materials are being ordered and shipped to your lab.
    - Get rid of unneeded hazardous materials.
    - Use a log to sign highly hazardous materials in and out of secure storage.
    - Take periodic inventory of all highly hazardous chemicals, biological agents/toxins, radioactive materials, and controlled substances.
5. **Know what materials are being removed from your lab.**
    - Track the use and disposal of hazardous materials.
    - Require written permission before removal of highly hazardous materials from the lab.
    - Report any missing inventory.
6. **Have an emergency plan.**
    - Recognize that controlling access can make emergency response more difficult.
    - Evaluate contingency plans with administrators, safety and security officials and, if necessary, outside experts.
    - Review emergency plans with lab personnel.
    - Provide emergency responders with information on serious hazards.
7. **Have a protocol for reporting security incidents.**
    - Principal investigators, in cooperation with facility safety and security officials, should have policies and procedures in place for the reporting and investigation of incidents or possible incidents, such as undocumented visitors, missing hazardous materials, or unusual or threatening phone calls.
    - Train laboratory staff on procedures.

Security Assignment

Working with your lab partner, develop a site-specific security policy for our Biotechnology lab.

a. First, work with your lab partner to assess the laboratory for hazardous materials and security risks. What are the risks in the lab?
b. As a class, discuss ideas. Did you miss any risks?
c. As a class, develop and implement lab security procedures for the lab. How will you set up a security system in response to the risks the class has identified?

LAB ORGANIZATION

During this course, you will learn to use, calibrate and troubleshoot many pieces of equipment used in biotechnology labs, and you will be making a variety of reagents. Before you get started, it is important to learn 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 is considered practicing good lab etiquette.

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

1. Ensure that any solutions prepared are labeled properly according to SOP SOL-001 and that a solution prep form for each is created. Store them properly in the provided storage location for the class.
2. Replace any equipment, supplies, or reagents to their proper storage place.
3. Clean your personal work area; this includes removing all items from your lab bench, wiping the bench with a wet paper towel if any chemicals were used, cleaning the bench with cleaner or disinfectant and a paper towel if any microorganisms were used, and remove debris from the sink.
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 cover slips, Pasteur pipettes, broken glassware). There is a dustpan in the prep room which can be used for sweeping up broken glass. ONLY GLASS GOES IN THE GLASS WASTE.

Equipment:

- ✓ An equipment locator can is in the designated file cabinet in the lab room. Use this document to locate supplies and equipment.
- ✓ Equipment SOPs written specifically for the equipment in our department are in the SOP packet provided each semester. You will receive one 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 basic 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 can't find something, ask another group. If you still can't 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 Shutoff Valve		
Large & Small Glass Waste		
Biohazard Waste		
Liquid Chemical Waste		
General Chemicals		
Spill Kit		
Broom/dust pan		
Material Safety Data Sheets		
First Aid Kits		

Any other safety equipment of note?

Laboratory Materials/equipment	Room stored in	Location
Micropipettes		
Micropipette tips		
Microcentrifuge tubes		
Microcentrifuges		
Picofuge		
Power supplies		
Electrophoresis Chambers		
FPLC		
Scissors		
Parafilm		
Weigh boats		
Standard pH Buffers		
Test tubes		
Unopened glove boxes		
Test tube racks		
Graduated cylinders		
Low-volume spectrophotometers (NanoDrop)		
Freezers (-80 ^{o}C)		
Freezers (-20 ^{o}C)		
Refrigerator (4°C) – note the shelf!		
Shaker Incubator (37 ^{o}C)		

Part II: Basic Lab Skills in a Regulated Environment

Objectives: After completing this lab the student should be able to:

- ✓ Understand the purpose of documentation in a regulated environment.
- ✓ Validate equipment following established procedures
- ✓ Understand the importance of using validated laboratory equipment
- ✓ Properly follow SOP, and accurately fill out IQ/OQ/PQ forms
- ✓ Decide if equipment meets specification

GOOD DOCUMENTATION PRACTICES (GDPs)

Regardless of the Guidance Practices which are followed, they all exhibit the same philosophy of documentation practices, sometimes referred to as Good Documentation Practices (GDPs). The FDA uses the acronym *ALCOA (attributable, legible, contemporaneous, original and accurate)* to describe the importance of GDPs. The key to ALCOA is thorough documentation to ensure reproducibility and traceability.

The FDA's (and most quality system's) position regarding documentation is, 'if it is not written down, it was not done.' Proper documentation is essential for a regulated company from discovery all the way through to the customer's hands. It provides regulatory bodies, lawyers, patent offices, and peer review publishers the information they need to validate the product's manufacturing process. *While regulatory agencies tell you what to do, they do not tell you how.* Current good manufacturing practices (CGMP), for example, is not a prescription for production manufacturing but guidelines.

Documentation Regulations: Depending on the quality system used (and the rules surrounding that quality system) there are many different types of quality system documentation requirements. 21 CFR 211: Title 21 Chapter 1 of the CFR contains all regulations concerning the safe production of food, drug, medical device, diagnostic, and biologic products for human and animal use under FDA supervision. Documentation regulation is published under Part 211.

Regulatory submissions: Regulatory submissions are documents designed to meet the requirements of an outside regulatory agency. Pharmaceutical companies must submit an application to the FDA showing their preliminary research on a drug, their plan for clinical trials and other relevant information before they can begin field-testing a new drug in humans.

Documentation is a system of records which serves three fundamental purposes:

- A project planning tool; improves communication of project goals and priorities.
- Documentation provides a historical record of what was done, how it was done, what was changed, who did it, when it occurred, and why it was done. Accurate records are often a firm's best defense in cases of litigation.
- It is required by CGMP, ISO, QSR and GLP guidelines and regulations that clearly recognize that documentation makes good common sense.

The phrase *"documentation and traceability"* is familiar to all companies that must comply with FDA regulations. A company must be able to provide records to demonstrate traceability of all the parts of a finished product, including but not limited to, raw materials, intermediates, and final lot batches. A final product can be released if the documentation that has traced it from start to finish is complete, the product has met all required product specifications, and it has been produced in compliance with the necessary regulations. Therefore, *regulated companies have systems that ensure the work is recorded, that the appropriate documents are completed, and that the documents are stored in a secure and readily retrievable location*.

Documents that are archived must be easily retrieved in cases where customers question the quality of a product purchased, or when the company is being inspected or audited by a regulatory agency. In all cases, the consequences of missing documentation can be severe. Indeed, the company's very survival depends on these documents. Documentation is probably the first and most significant CGMP requirement that is needed in a new biotechnology company. The challenge is to establish, with limited resources and with a small staff who may have little experience in CGMPs, the same degree of CGMP compliance as larger pharmaceutical companies.

In some companies, as a new product is developed for production, the process of record keeping is often viewed as inhibiting the progress of the project. Documentation slows down and burdens day-to-day operations due to the time spent filling out and signed off on forms and then carefully archiving them. There is no shortcut, however. Although cutting corners on record-keeping may seem advantageous at first, lack of documentation can cause delays by causing primary experiments and processes to be needlessly repeated or result in faulty conclusions. *When a company manufactures a pharmaceutical, it produces two products: the drug and the accompanying documents that went into making the drug.*

TYPES OF DOCUMENTATION

Documentation is essential in all biotechnology work areas, although the specific types of documents and the systems for documentation vary according to the kind of workplace. Each company will have a set of documents to reflect their needs and requirements. There are three broad classes of documents.

- *Directive documents instruct employees how to perform a task.* Examples include standard operating procedures and protocols.
- *Data collection documents record data* to provide evidence that the directive document was performed and performed correctly.
- *Commitment documents lay out the organization's quality system;* goals and standards they commit. Mission statement, vision statement, and quality statements are all examples of commitment documents.

Laboratory notebooks: This documentation enables investigators to reconstruct their work, solve problems, detect mistakes, and prove to the scientific community that their results were properly obtained and were accurately reported. Laboratory notebooks can be used to establish a patent claim, assign credit for the discovery, document the integrity of data, and troubleshoot problems. It is essential that notes be written legibly with indelible ink. Laboratory data can be subpoenaed in litigations. It can be examined by any regulatory agency that requests it. Notebook integrity is important even in non-regulated research labs.

A lab notebook may be used to document data to support research publications that have used government funding, may support a patent application, or may support an investigational new drug application or a new drug application to the FDA. A messy lab notebook, or one not maintained with integrity, may result in losing a patent, having grant funding withdrawn, having to repay grant funding, having to pay fines, losing your job and given probation and jail time.

Standard Operating Procedures (SOPs): People in production facilities use documents other than laboratory notebooks. Standard Operating Procedures (SOPs) that describe how to perform a task are essential in production facilities. *A procedure is a written document that provides a step-by-step outline of how a task is performed*. Most production facilities (and many laboratories) use procedures to instruct personnel how to perform procedures or tasks. Everyone follows the same procedures to assure that tasks are performed consistently and correctly. SOPs must be written so that they are clear, easy to follow, and can accommodate minor changes in instrumentation. *SOPs are typically written in command sentences rather than a narrative*. The placement and distribution of SOPs are controlled and documented, and they are reviewed on a periodic basis.
Standard Operating Procedures describe what is required to perform a task, what problems may arise and how to deal with them, how to document that the task was performed correctly and, lastly, who is qualified or responsible for the work.

SOPs are important for many reasons:

- ✓ Provide consistency each time a procedure or process is performed.
- ✓ Serve as reminders to ensure that work is done properly
- ✓ Used to train new employees the correct way to perform the work
- ✓ Reduce the possibility of failure by enabling the employee to complete any task

Forms: Forms are often associated with SOPs. These forms require an individual performing the task to monitor the process or procedure as it is being performed. *Filling in blanks and initialing the steps as they go along ensures that the steps are being followed correctly*. In production, the form often has blanks to record information about ID/lot numbers of raw materials, weights, times, temperatures, and other information necessary for quality control of the product. In some production laboratories, a witness must sign key steps.

Protocols: *The term protocol may be used to refer to a procedure that will be performed one time.* A protocol is employed in some industries to refer to a task or experiment that is intended to answer a question or test a hypothesis. The protocol outlines the steps that are to be followed in performing the task/experiment. SOPs are not intended to lead to the answer of a question or test a hypothesis. Protocols in research questions are addressed continuously. In production facilities, issues relate to product performance, effects of storage (both short and long-term) on the product, quality of the product under different conditions, etc.

Reports: *A report is a formal document that describes the results of a completed task.* The report summarizes what was done, by whom, why, the data (results), and the conclusions. A report is written in a narrative addressed to a reader, with sufficient background information and technical information to achieve an appropriate amount of information. For example, reports to upper-level management may not include as many specific details as reports addressing regulators. Some reports may be published in scientific journals, such as reports of basic scientific research. Other reports, such as those of investigations performed in a company, may or may not be published, but must be made available to inspectors.

Learn about the office of research integrity (ORI). What do they do? Go to: https://ori.hhs.gov/case_summary and review the case summaries. Pick a recent case summary you find interesting and summarize the findings of the ORI and the punishment. Your instructor may have the class do this as an in-class exercise.

Lab reports and scientific papers have four common functions:

- ✓ To persuade other people to accept the hypothesis based on the data presented
- ✓ To publish data, methods, material and results for other researchers
- ✓ To make contributions to the scientific community by contributing to the body of knowledge
- ✓ To provide a record of research for documentation: legal proof, or to repeat the experiment.

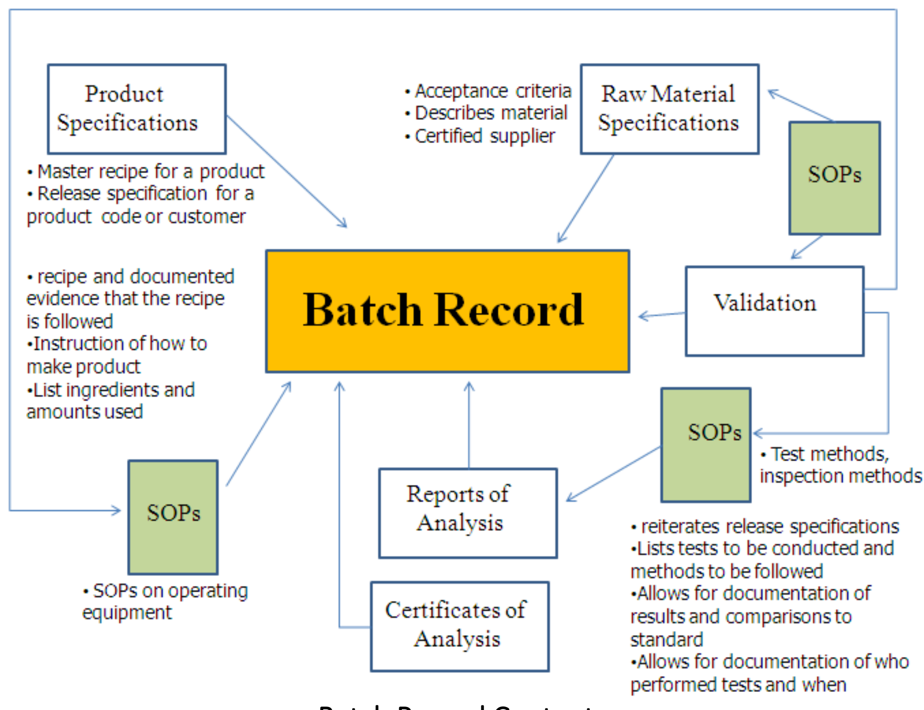
Logbooks: Logbooks are used to maintain information about the status and maintenance of equipment or instruments. Logbooks are usually bound notebooks. Whenever an instrument or piece of equipment is used, calibrated, preventative maintenance performed, and the instrument or item is repaired, that information is recorded in the notebook.

Analytical laboratory documents: Analytical laboratory documents contain data from analytical tests that measure some parameter in a sample. Clinical laboratories analyze blood for cellular components, ions, drugs and enzyme levels. The product is the test result. Documentation includes the sample being tested and the test methodology.

Labels: Labels identify instruments, raw materials, products or other items. Label format & contents are highly regulated by the FDA. **Numbering systems:** Identification numbers are used to identify items uniquely. *Identification numbers are used for traceability purposes and are used for generalized inventory; raw materials, products, equipment, and even documents!* Identification numbers should identify the item uniquely.

Chain of custody forms: Chain-of-custody is a term that refers to the maintenance of an unbroken record of possession of a sample from the time collected through delivery, receipt, storage, analysis, or disposition. Chain-of-custody documents are a method of organizing information about samples. The establishment of chain-of-custody procedures is necessary because the results of testing or analysis might be held as evidence in litigation proceedings. Each sample is assigned a unique ID number. The sample is logged in and out as it is processed. The O.J. Simpson trial illustrates the importance of chain of custody forms. The verdict was based on inadequate documentation of the chain of custody of the DNA evidence. While the DNA fingerprinting science was sound and rigorous, poor documentation of who handled the blood samples, when, where, and how, led to the acquittal.

Training records: The FDA requires a documented continuous training program for compliance with the CGMP regulations. It is the responsibility of Quality Assurance to verify that a CGMP training program is implemented and that it is an ongoing program. In addition to CGMP training, regulations require that all employees be adequately trained in their job functions whether they are new hires or existing employees who are learning new methodologies or the operation of new equipment. Training is based on the company's own written and approved SOPs. It must be well documented and provides the necessary tools and expertise needed to train the employees.



Batch Record Contents

Batch Records (Batch Production Records, or BPR) are a requirement of Good Manufacturing Practices. They are an accurate copy of the corresponding Master Production and Control Record. BPRs must be carefully designed so that all appropriate process information is documented and demonstrated in writing. The BPRs must be reviewed for accuracy and must be signed and dated by a quality group before their use in manufacturing. A BPR is a combination of

a SOP document and a form in that it directs operators in how to make the product and each step has blanks that must be filled out as the operator performs the step. For critical steps, a witness is required to watch and sign off on the BPR. Batch records are legal documents and are part of process validation compliance. The quality department issues the batch record to production. All blanks are filled in as procedures are performed. BPRs must be kept for a minimum of 1 year after the expiration date of a lot batch of the product. Most companies keep them for the predicted lifespan of the product or more.

ELECTRONIC DOCUMENTATION

The biotechnology field uses a diverse and complex mixture of both paper and electronic documentation. There are many advantages and disadvantages to both, but companies tend to choose the documentation process that best serves their needs while meeting regulatory requirements. In response to the broad use of electronic documentation and demand for systematic regulation of such documentation, in 1997 the FDA issued regulation 21 CFR Part 11 Electronic Signatures; Final Rule, to address these concerns. In 2003, the FDA released its final *"Guidance for Industry Part 11, Electronic Records; Electronic Signatures — Scope and Application".*

MANAGING CHANGE IN DOCUMENTATION

It is essential for a production facility to follow the same procedures with each batch of product produced, and it is important that all supporting laboratory analyses also follow a single set of protocols to produce a consistent result reliably. This rigid adherence to carefully described procedures helps prevent inconsistent results, but it stifles improvements that might help to improve or streamline a process. When a change needs to be made, the change must be carefully agreed upon by all parties involved, and all involved must have a procedure for enacting the change. The Quality Assurance department is usually responsible for overseeing the change process.

In summary, documentation functions to:

- ✓ Record what has been done,
- ✓ establish ownership,
- ✓ provide workers specific instructions on how to perform a task,
- ✓ establish product specifications,
- ✓ demonstrate procedure was performed correctly,
- ✓ record experimental parameters,
- ✓ provide an evidence trail, ensures traceability,
- ✓ establishes a contract between a company and a consumer and establishes a contract between a company and regulatory agencies.

VALIDATION OF PROCESSES & EQUIPMENT

PROCESS VALIDATION. Process validation is the method by which companies demonstrate that their activities, procedures, and processes consistently produce a quality result. ***METHOD VALIDATION*** refers to the testing of the raw materials, the intermediates, and the product.

EQUIPMENT VALIDATION. Different pieces of lab equipment are designed to operate within varying degrees of accuracy. For example, 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: GMP and ISO certified laboratories have written policies for equipment calibration, verification and validation for all equipment used in a cGMP lab.

- ***Calibration*** is a process that compares a known (the "standard" device) against an unknown (the target device in question). During the calibration process, the offset between these two devices is quantified, and the target device is adjusted back into tolerance (if possible). A calibration report usually contains both "as found" and "as left" data. When a micropipette is out of calibration, it is sent for recalibration.
- ***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 instrument is not within tolerance, it is sent for recalibration.
- ***Validation*** is a detailed process of confirming that the device is installed correctly, that it is operating effectively, and that it is performing without error. Validation in the pharmaceutical industry emerged from problems in the 1960s and 1970s and went together with the QA/QC philosophy that quality is built into the product not tested into the product. The FDA states that quality, safety, and effectiveness are designed and built into the product.

For example, micropipettes are validated by the manufacturer before they are sent. However, they become less accurate the more they are used. Therefore, the performance of a micropipette must be verified periodically and calibrated regularly.

Each step in the production process must be controlled to ensure that the finished product meets all quality and design specifications. The actual requirement for process validation comes from the text of the GMPs, Section 211.100 which states that ***"there shall be written procedures for production and process control designed to assure that the drug products have the identity, strength, quality, and purity they purport or are represented to possess."*** CFR - Code of Federal Regulations Title 21. (n.d.). Retrieved from http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?CFRPart=21

EQUIPMENT VALIDATION. Processes almost always involve equipment and are the focus of this laboratory exercise. For a process to proceed correctly, the equipment must be of high quality, must be properly installed, regularly maintained, and properly operated. Equipment must, therefore, be validated or qualified to ensure that it will function reliably under all the conditions that may occur during production. *Equipment qualification may be performed separately from process validation, but it is also a requirement for process validation.* Equipment 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 of equipment is a vital link in the quality chain. Equipment should be adequately inspected, cleaned, and maintained as well as, tested, calibrated, and standardized at an appropriate frequency. Laboratories usually establish schedules for such activities based on manufacturer's recommendations, and frequency of use. Equipment is one of the major areas of focus for FDA (or any other regulatory body) audits. All equipment must meet manufacturer or preset standards for operation and performance.

Every biotechnology company approaches equipment validation with a different set of evaluation tools. What remains constant is that validation of equipment occurs *before* the equipment is used to support an associated process validation. The level of detail associated with each step of equipment validation depends on the usage of equipment. It is important, though that each step of the validation, *Installation Qualification (IQ)*, *Operational Qualification (OQ)*, and *Process or Performance Qualification (PQ)* must pass before proceeding to the next. If a piece of equipment is not passing the necessary requirements written in the validation, remove the equipment from use and documentation of these deviations is included with the records.

The basic principles of equipment validation include:

1. The equipment is installed by vendor requirements, target use, and local building codes.
2. Requirements for calibration, maintenance and cleaning have been developed as SOPs.
3. Operating requirements are established, and tests are conducted to ensure equipment is functioning correctly, under ideal, and intended conditions.
4. Operator training requirements are established, completed, and recorded.

Equipment Validation can be divided into three parts:

- **Installation Qualification (IQ).** First, the equipment item is checked to be sure that it meets its design and purchase specifications and is properly installed; this is called installation qualification. Installation qualification includes, for example, checking that instruction manuals, schematic diagrams, and spare parts lists are present; checking that all parts of the device are installed; checking that the materials used in construction were those specified; and making sure that fittings, attachments, cables, plumbing, and wiring are properly connected. IQ is documented proof that the building, wiring, installation and calibration of equipment, utilities, SOPs, spare parts and specifications meet the design intention.

- **Operational Qualification (OQ).** After installation, the equipment can be tested to verify that it performs within acceptable limits. For example, an autoclave might be tested to see that it

reaches the proper temperature, plus or minus certain limits, in a set period; that it reaches the proper pressure, plus or minus certain limits, etc. The penetration of steam to all parts of the chamber, the pressure achieved at various settings, and so forth, would all be tested in the context of the operational qualification of an autoclave. OQ is documented proof that the equipment performs as specified.

- **Performance Qualification (PQ).** Once all measuring instruments are calibrated, and all equipment is validated, process validation (or qualification) can be performed. The validation of the process involves assessing the process under all the conditions that can be expected to occur during production. Testing includes checking the process endpoint(s) under these circumstances and establishing that the process consistently meets its specifications. PQ is documented proof the equipment or systems operate as intended under challenge conditions.

  PQ also involves challenging the system with unusual circumstances. FDA speaks of the "worst case" situation(s) that might be encountered during production. For example, a sterilization process might be challenged by placing large numbers of heat-resistant bacterium in the corner of the autoclave known to be least accessible to steam. The effectiveness of bacterial killing under these "worst case" conditions must meet the specifications for the process.

VALIDATION OF EQUIPMENT: CONSIDERATIONS

It is crucial when validating laboratory equipment to do so with calibrated and certified equipment and standards. Throughout the semester you will be introduced to several pieces of validation equipment. Examples of certified equipment include a certified timer, a certified thermometer and a certified tachometer which accurately measures rotor speed.

Validation of Temperature: As you will learn in this laboratory unit, the temperature is a key element in buffer preparation. Therefore, it is important to measure and record the temperature accurately. Always keep in mind that "room temperature" (ambient temperature) is a figure of speech and not a temperature. Ideal room temperature is different for everyone!

For every experiment, record the measured temperature in the lab notebook using a calibrated thermometer. Conventional laboratory glass and mercury thermometers can be highly inaccurate. Most have an error of +/- 2ºC, but some can have an error of up to +/- 4ºC! Digital thermometers (LED outputs) can be just as poor in accuracy. Some labs calibrate their conventional glass thermometers with a certified thermometer, but the best way to measure temperature accurately is to use a certified validated thermometer.

Unplanned Occurrences. After these validation activities have been performed, the collected data is analyzed as described in the validation protocol and a report is prepared. *Successful validation demonstrates that a process is efficient and reliable.* With careful validation design, planning and implementation problems are easily avoided.

Even in the most carefully designed facilities, however, unplanned occurrences do happen. These unexpected occurrences are called ***deviations***, and every company must be prepared to deal with them. Typically, the validation plan will have a form for documenting the deviation. The supervisor and the quality department will review the deviation to determine the plan of action to correct the deviation. Along with the deviation, the ***corrective action*** is also carefully documented and implemented.

Nonconformance. When a process, product or raw material is out of specification, it is called a nonconformance. Inspections, audits, and surveillance will occasionally uncover nonconformance or defects. Nonconformance problems are placed in one of three categories based on the product defect: critical, major, and minor.

- Critical defect: A defect that is likely to result in unsafe conditions for individuals using, maintaining, or depending upon the product.
- Major defect: A major defect is a non-critical defect that is likely to result in either product failure (non-life-threatening) or a significant, material reduction in the usability of the product for its intended purpose.
- Minor defect: A defect that is not likely to reduce the usability of the product for its intended purpose.

Final Thoughts: In a typical biotechnology company bound by GMP or ISO9000 regulations, ***Equipment validation*** is part of routine Quality Control procedures, usually performed in a committed period (weekly, daily, or monthly). The technician follows a ***Standard Operating Procedure*** (SOP) and fills out the ***associated form*** proving they have performed the task correctly and what the outcome of the task was. If the equipment fails the validation, the quality department must be notified through a ***deviation report***. This report outlines the parameters of the equipment that did not meet outlined specifications on the form.

References:

1. FDA. https://www.fda.gov/
2. Huxsoll, Jean F. Quality Assurance for Biopharmaceuticals. New York: John Wiley and Sons, Inc., 1994.
3. DeSain, Carol. Documentation Basics. 2nd ed. Ohio: Advanstar Communications, Inc., 2001.
4. Seidman, Lisa, and Moore, Cynthia. Basic Laboratory Methods for Biotechnology. 2nd Ed. Prentice Hall, NJ. 2009. ISBN: 321-57014-6

VALIDATION OF A pH METER

Calibration and Operation of a pH Meter: Read the SOP provided in the SOP booklet of the module of pH meter used in the lab. The definition of pH is the hydrogen ion (H+) concentration of a solution. By definition, 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. Watch this video!* https://youtu.be/UfdKhja6u2I

Pre-Lab Exercise: For *every wet-lab exercise create you will create a pre-lab exercise*, print it out, and bring to class. The pre-lab is due before you perform the lab exercise. Lab Unit 1 is completed for you on the following page. Remove this from your lab binder and use it in your lab activities in class. Include this pre-lab, with your lab notes with your lab unit 1 report.

Remember a pre-lab must include:

- ✓ A descriptive title of the entire lab unit
- ✓ The purpose of the whole lab unit
- ✓ An explicit safety statement – includes all hazards for the whole unit
- ✓ A full list of materials, with numbers, and sizes of equipment
- ✓ A numbered protocol – note the brevity, but specific direction, include A space for notes – with prompts
- ✓ Include any forms needed to complete the lab exercise (in this lab, you need IQ/OQ/PQ
- ✓ Any calculations are performed and included in the pre-lab exercise
- ✓ Data tables should be prepared ahead of time and included

Lab Unit 1B: Validation of a pH meter
(General lab manual instructions)

Materials: Retrieve the following materials before beginning the lab.

o pH meter o Instruction Manual for pH meter o SOP for pH meter from your SOP booklet o IQ/OQ/PQ Validation forms o validation equipment: certified level, certified timer, certified thermometer o pH standard buffers 4, 7, 10	o wash bottle with deionized water o Kimwipes® o 3, 25 mL beakers – for pH determination o 1, 500 mL beaker – for rinsing water waste

PART 1: Installation Qualification (IQ)

1. Retrieve all materials listed above required for this exercise. Bring the pH meter to your bench for the validation procedure.
2. Read the SOP for using the pH meter ***before*** you begin.
3. Start the pH meter validation process with the installation qualification (IQ) form.

Note: The ORDER of the forms is important! For example, in a production lab, if the equipment fails the IQ then the OQ and PQ are not performed, and the equipment is not used until the deviation is corrected.

4. Follow the instructions on the IQ Form on how to validate the pH meter.
5. Begin by assigning the IQ form number: IQ-pH-date-initials.
6. When the IQ is complete, have your instructor sign off at the bottom of the form.
7. If the pH meter passes the IQ, perform the OQ/PQ.
8. If the pH meter fails the IQ, fill out a deviation report and report the deviation to your instructor before continuing.

PART 2: Operational & Performance Qualification (OQ/PQ)

1. For the pH meter, the OQ and PQ are performed on one form. Follow the instructions on the OQ/PQ form.
2. All items used for validations must be within expiration dates for approval. Some protocols require that current copies of certificates or labels indicating expiration dates be attached to paperwork for validation approval. Verify the pH meter standards have not expired before performing this validation.
3. Perform the OQ/PQ for the pH meter according to the instructions on the form. If your pH meter fails the OQ/PQ, fill out a deviation report and discuss the deviation with your instructor.

Pre-lab Exercises for Lab Unit 1B:
Laboratory Documentation & Equipment Validation of a pH Meter

Purpose: The purpose of this exercise is to utilize regulated laboratory documentation procedures to validate a pH meter. The validated pH meter will be used in the preparation of EDTA, and Tris-Cl buffer.

Safety: It is Good Laboratory Procedures to wear appropriate Personal Protective Equipment while working in the lab. Proper PPE includes a laboratory coat, gloves, safety glasses, and close-toed shoes. For this lab unit, standard pH buffers that are acidic and alkaline may pose a risk to eyes. Wear safety glasses and wash eyes out with lots of water if any buffer splashes into the eyes.

Materials: The following items are stored in the laboratory. Retrieve them before beginning the lab.

☐ pH meter ☐ Instruction Manual for pH meter ☐ SOP for pH meter from your SOP booklet ☐ Validation forms ☐ validation equipment: certified level, certified timer, certified thermometer	☐ pH standard buffers 4, 7, 10 ☐ wash bottle with deionized water ☐ Kimwipes® ☐ 3, 25 mL beakers – for pH determination ☐ 1, 500 mL beaker – for rinsing water waste

Protocol: Work in pairs to complete the following IQ/OQ/PQ forms for the pH meter to determine if it meets the specifications required for validation. Perform the qualifications in order.

Protocol	Experimental Notes
PART 1: Installation Qualification (IQ) 1. Retrieve all materials. 2. Perform Installation Qualification 3. When the IQ is complete, have your instructor sign off at the bottom of the form. 4. If the pH meter passes the IQ, perform the OQ/PQ. 5. If the pH meter fails the IQ, fill out a deviation report and report the deviation to your instructor before continuing.	pH Meter Model: ____________ Pass IQ? _________ Was a deviation report filled out? Y / N Explanation:
PART 2: Operational & Performance Qualification 1. Verify the pH meter standards have not expired before performing this validation. 2. Perform the OQ/PQ for the pH meter according to the instructions on the form. 3. If your pH meter fails the OQ/PQ, fill out a deviation report and discuss the deviation with your instructor.	Do all reagents have valid dates? Y / N Pass OQ/PQ? _________ Was a deviation report filled out? Y / N Explanation:

Installation Qualification Record
pH meter

IQR# ____________

Instructions: Quality Technician will use this form to validate a pH meter. If at any time a "no" is checked, discontinue the validation and submit the signed form to the QA manager.

Equipment name: ____

Equipment number: _____________________ Date of installation: ___________________

Serial number: __________________________ Manufacturer: _______________________

1. Is the pH meter on a level surface? ( ) yes ( ) no

2. Is the Manufacturer's Instruction Manual available? ( ) yes ( ) no

3. Does the pH meter have the proper electrical requirements as recommended by the manufacturer? ( ) yes ( ) no

4. Is the pH electrode installed properly as recommended Manufacturer's Instruction Manual? ( ) yes ( ) no

5. Is the pH electrode stored properly as recommended by the Manufacturer's Instruction Manual? ( ) yes ( ) no

The pH meter must pass installation qualification before proceeding to operational qualification. If the above questions have all been answered "yes," then the pH meter has passed the requirements for installation qualification and is recommended for approval by Quality Assurance.

Quality Technician Signature:

Date: ______________________ Signature: ________________________________

QA Approval ☐
Date: ______________________ Signature: ________________________________

QA Fail ☐
Date: ______________________ Signature: ________________________________

Reason: ____

Operational and Performance Qualification Record
pH Meter

O/PQR# _____________

Instructions: Use this form to validate a pH meter. If at any time a "no" is checked, discontinue the validation and submit the signed form to the QA manager.

Equipment name: ________________________________ Equipment number: ________________

1. Did this pH meter pass the installation qualification? ( ) yes ( ) no

2. Turn the pH meter on. Does the LED panel light up? ( ) yes ( ) no

3. Turn the pH meter on and allow it to warm-up for at least five minutes before use.

   Start time: _____________ Date: ____________ Initials: _______
   Stop time: _____________ Date: ____________ Initials: _______

   Clock/Timer serial number: __________________ Date: ____________ Initials: _______
   Clock/Timer expiration date: _________________ Date: ____________ Initials: _______

4. Press standby to begin operation. Scroll through the mode (menu) to pH determination.
   Does the meter operate properly when scrolling through the menu? ( ) yes ( ) no

5. Press and release the MODE key until the digital display indicates pH mode. This key toggles between pH, mV, and Rel mV.

   Does the meter indicate pH mode? ( ) yes ( ) no

6. Verify the meter is working properly by verifying pH of known calibration standards. Determine and record the expiration date of the buffers used for this OQ/PQ.

   pH buffer 4.0 Manufacturer: __________________ Catalog #: _______________Exp. Date: ___________
   pH buffer 7.0 Manufacturer: __________________ Catalog #: _______________Exp. Date: ___________
   pH buffer 10 Manufacturer: __________________ Catalog #: _______________Exp. Date: ___________

   Is each buffer within its expiration date? ( ) yes ( ) no

7. Press the SETUP key twice and then the ENTER key to clear and existing standardization.

8. Rinse electrode in deionized water and blot dry. Immerse the rinsed electrode into pH four standard buffer. Stir moderately.

9. Press STD to access the Standardize mode. Press STD again to initiate standardization. The meter will automatically recognize the buffer and flash the value on the screen. When the stable icon appears the buffer value is entered and the meter returns to the Measure screen.
   Does the meter recognize the pH four buffer? ( ) yes ( ) no

10. Rinse electrode with deionized water and blot dry. Immerse the rinsed electrode into pH seven standard buffer. Stir moderately. Press STD to access the Standardize mode. Press STD again to initiate standardization.
    Does the meter recognize the pH 7 buffer? ( ) yes ( ) no

11. When the meter accepts the second buffer, it will briefly display the percent slope associated with the electrode's performance before returning to the Measure mode. If the electrode is within the range of 90-102%, the GOOD ELECTRODE message will appear.
    Does the GOOD ELECTRODE message appear? ( ) yes ( ) no

12. Rinse the electrode with deionized water and blot dry. Immerse the rinsed electrode into pH ten standard buffer. Stir moderately. Press STD to access the Standardize mode. Press STD again to initiate standardization.
    Does the meter recognize the pH 10 buffer? ( ) yes ( ) no

13. When the meter accepts the third buffer, it will briefly display the percent slope associated with the electrode's performance before returning to the Measure mode. If the electrode is within the range of 90-102%, the GOOD ELECTRODE message will appear.
    Does the GOOD ELECTRODE message appear? ( ) yes ( ) no

14. After calibration, return to Measure mode and test the pH of each buffer in duplicate.
    Actual pH: ________ Sample #1: _______ Sample #2: _____ Avg: _________
    Is the average pH within +/- 0.10 of the actual pH? ( ) yes ( ) no

    Actual pH: ________ Sample #1: _______ Sample #2: _____ Avg: _________
    Is the average pH within +/- 0.10 of the actual pH? ( ) yes ( ) no

    Actual pH: ________ Sample #1: _______ Sample #2: _____ Avg: _________
    Is the average pH within +/- 0.10 of the actual pH? ( ) yes ( ) no

14. Record the room temperature:_________ °C

Quality Technician Signature:

Date: ________________________ Signature: ___________________________________

QA Approval ☐

Date: ________________________ Signature: ___________________________________

QA Fail ☐
Date: ________________________ Signature: ___________________________________

Reason: ____

Lab Unit 1: Analysis

1. **Complete exercises in this lab unit.**

2. **Compile a post-lab report by compiling exercises in the <u>order they were completed</u>. For example, in lab unit 1, you will compile your report as follows:**
   - Title page
   - Safety training worksheet
   - Lab inspection sheet
   - Lab orientation worksheets
   - Security exercise
   - Pre-lab exercise 1B (with notes)
   - IQ/OQ/PQ forms
   - ***Analysis of results***.
     - Write a short, one paragraph summary of the topics, laboratory safety, and security. Why are both essential in a biotechnology work environment?
     - Write a short, one paragraph summary of the importance of documentation in a regulated biotechnology laboratory.
     - Write a short, one paragraph summary of equipment validation – what is it, and why is it important in a regulated biotechnology company?
     - Write a short, one paragraph summary of your results. Be specific here – equipment name, model number, and equipment number. Did the equipment pass? If not, what happened to the equipment?
   - ***Conclusions***. State the experimental conclusions of this laboratory exercise. Restate and summarize the results, and state implications for future work (if any).

LAB UNIT 2: PREPARING SOLUTIONS

Objectives:
- ✓ Prepare stock reagents commonly used in a biotechnology lab; multicomponent buffers, Tris buffer, phosphate buffer, EDTA
- ✓ Demonstrate the proper use of a pH meter to adjust the pH of a buffer
- ✓ Understand the important factors when selecting an appropriate buffer
- ✓ Create an inventory tracking form for the stock reagents that will be used the remainder of the semester

INTRODUCTION

The ability to prepare reagents is an essential skill for any biotechnician. The accuracy of calculation and measurement is critical to the outcome of any experiment, whether it will be one you do yourself or one in which you prep for someone else. There are several key aspects to making solutions that should always be followed.

- ✓ ***Check and recheck each calculation****.* It is best if two people prepare calculations independently and then cross check their answers.
- ✓ ***Read each reagent bottle twice***, once before using and once afterward. This helps ensure that the right reagent is used.
- ✓ ***Complete a Solution Prep form*** *for every solution you prepare.* This should include the formula, with the supplier and catalog number if available, as well as the concentration and the amount weighed out for each reagent, the balance or pH meter number and other pieces of relevant information.
- ✓ ***Label each bottle before filling****.* Write down the name of the solution, your initials, and the date. Some industries have special blank labels to be used for each reagent. Others use tape and a permanent marker.
- ✓ ***Record any changes observed***, no matter how trivial. This record can be used to trace back a problem to its source quickly and easily or to confirm that a problem does not lie in the reagents or their preparation.

For every solution prepared:

1. Calculations are performed *before class*, *for EVERY solution*, even the solutions you do not prepare. Include these calculations in your notebook. You will be required to have your lab partner check off your calculations.
2. You must fill out a Solution Prep Form for every solution your group prepares, and keep it in your lab notebook.
3. You must follow the SOP for correctly labeling your solutions.

Strategies for Preparing Aqueous Solutions in a Biosciences Lab

There are two main strategies when preparing solutions in the lab; **working solutions** and **concentrated stocks**. The first strategy is to make the buffer at working concentration without stock solutions. The second strategy involves making concentrated stock solutions and combining them to form the final working concentration. Both these strategies are frequently used in the lab.

Preparing Solutions—Calculations

Perform all calculations ***before*** you come to class. When you start this unit, you and your lab partner will verify each calculation ***before*** you prepare your solutions.

Briefly, *always determine what is needed and what is given, and, when performing the math, always write down the units and cancel them to ensure you end up with an answer that is in the correct units.*

- **Weight per volume** is the simplest way of expressing a concentration. This is often used for small amounts of chemicals and specialized biological reagents. For example, enzyme concentrations are often given as weight per volume--2.0 mg/ml bovine serum albumin.
- **Molarity.** Molarity is the most common unit of concentration in the biotechnology lab. The molarity of a solution is the number of moles of solute per liter of solution. The symbol for molarity is M, but it is also written as moles/Liter, or mol/L. A mole of any element always contains 6.02×10^{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.
- **Percents** may be (1) weight per volume percent, which is the grams of solute per 100 ml of solution (w/v), or (2) volume percent, the volume of solute per 100 ml solution (v/v).

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 pipet, bring the container with the liquid and the pipet to eye level. Hold the tapered end beneath the surface of the liquid. Draw the liquid up the pipet by suction until the level is just above the desired, then bring the level down to the meniscus of the volume you want. When reading the volume, ALWAYS view the pipet dead-on at eye level with the pipet held vertically, perpendicular to the ground. Pipets are operated with a hand-held electronic pump (pipet-aid) or bulb, of which there are many varieties. If you need a refresher, watch the following video on how to use a serological pipet correctly: https://www.youtube.com/watch?v=aei-tU1ZIkE&feature=youtu.be. You may also want to go to this website and test your serological pipet and micropipette skill! http://www.lsteam.org/iet/pipetting/

Operating an Electronic Balance. General guidelines to follow when using balances:

- The amount weighed determines what type of balance used in this laboratory (top-loading or analytical). Typically, a top loading balance is used for weights > 1.0 g.
- Use clean spatulas to weigh out material.
- Never put excess chemicals back into their original containers. Always ask your instructor how to discard of excess chemicals.
- Leave a CLEAN balance. Chemicals left on balance will corrode it.
- Turn off the balance when not in use.
- Analytical balances are sensitive to weight, air currents, and counter vibration. Only use them for weights < 1.0 g, and avoid bumping, banging, or moving the balance.

Buffers: Buffer solutions help maintain a biological system at its proper pH. It is essential when working with protein (and other biomolecular) solutions that you pay attention to pH! The pH determines not only the overall native conformation (and proper functioning) of your protein it can also determine how your protein interacts with other biomolecules and how your protein interacts with non-biological surfaces such as an ion exchange column. *When a solution is buffered, it **resists a change in pH**,* even when H^+ ions are added or lost from the system. Many chemicals can act as a buffer and can work at a wide range of pH.

The pKa of a buffer is the pH at which the buffer experiences little change in pH with the addition of acids and bases. When choosing a buffer, you want to choose one with a pKa one unit above or below the pH of your solution. Buffers are effective in resisting a change in pH within a range of approximately one pH unit. For example, an acetate buffer has a pKa of 4.8 and is, therefore, effective in a range of 3.8-5.8 pH units.

Not all buffering systems are compatible with all biochemistry procedures. You will need to investigate the proper buffer to use in a biochemical application. For example, although Tris is the most common buffer used in the biotechnology lab, it is an inappropriate buffer for some protein assays because it tends to react with the assay components resulting in erroneous data.

Two main factors influence the pH of a buffer: concentration and temperature. Although it is convenient to prepare a buffer as a concentrated stock, buffer pH can vary when diluted. Always check the final pH of a buffered solution if a concentrated stock was used to prepare it. Temperature also affects the pH of a buffer. Always pH a buffer at the temperature it will be used.

A ***sodium phosphate buffer*** is a useful solution in a biosciences lab because the phosphoric acid has multiple dissociation constants. This permits the preparation of phosphate buffers near any of the three pKa values (pH values); 2.15, 6.86 and 12.32. This buffer is most commonly prepared at pH 7.0 using monosodium phosphate and its conjugate base, disodium phosphate. Phosphate buffers are prepared by mixing stock solutions of desired concentration of dibasic and monobasic together in known ratios, or through titration. To prepare a sodium phosphate buffer, you need to decide the concentration and working pH of the buffer. Using Henderson-

Hassel Bach equation, calculate how much acid and base is required. Alternatively, there are many already published tables that can be used, such as the one below:

Table 1: To prepare 1 L of 0.1 M sodium phosphate buffer of the desired pH, the following mixtures should be diluted to 1 L (final volume) with H2O. Reference: Cold Springs Harbor Protocols, http://cshprotocols.cshlp.org

pH	Volume (mL) of 1 M Na_2HPO_4	Volume (mL) of 1 M NaH_2PO_4
5.8	7.9	92.1
6.0	12.0	88.0
6.2	17.8	82.2
6.4	25.5	74.5
6.6	35.2	64.8
6.8	46.3	53.7
7.0	57.7	42.3
7.2	68.4	31.6
7.4	77.4	22.6
7.6	84.5	15.5
7.8	89.6	10.4
8.0	93.2	6.8

Sodium phosphate buffers are commonly used for physiological applications since it mimics certain components of extracellular fluids, and is non-toxic to cells. Unlike Tris buffer, it is not sensitive to temperature changes but is sensitive to dilution; therefore, it is best to prepare stock solutions at or close to the final concentration. We will be preparing the stock buffer at 0.1M, and diluting it 10-fold to a 10mM working concentration in the extraction buffer. It is also highly susceptible to microorganism contamination and therefore, stored at 4°C.

EDTA: Ethylenediaminetetraacetic acid, widely abbreviated as EDTA, is an important laboratory reagent. Its usefulness in bioscience reagents is because of its role as a hexadentate ligand and chelating agent and can sequester metal ions (such as Ca2+ and Mg2+) thus making them unavailable for enzymes that may require them to function. It is commonly used in buffers such as Tris buffer to deactivate metal-dependent ions (polymerases, proteases). EDTA is an especially difficult reagent to prepare as it requires a pH of 8.0 to dissolve.

Cleaning Glassware. Properly cleaning glassware is one of the most important 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 the purpose of the clean glassware. Water source is a critical part of cleaning glassware (as we will discover in an experiment this semester!).

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!

The five standard steps to washing glassware (or plastic ware) are as follows.

1. **Pre-rinse**: Soak or pre-rinse all glassware after use to prevent contaminants from drying onto the glassware.
2. **Contaminant Removal**: Wash using approved detergents and solvents along with scrubbing will help with contaminant removal. *Use laboratory-appropriate detergent only*. Your kitchen detergent has many chemicals that interfere with biochemical reactions. Use hot water, and lab brushes 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 final rinse. This is performed 1-3 times.
5. **Drying**: This is either done in the air upside down on a rack or by heat. ***Never hand dry!*** Clean, dry glassware is stored covered in the closed cupboard to avoid contamination.

General Solution Preparation Procedure

1. Calculate the amount (g) of reagent needed to make each of the solutions in the table. Verify molecular weights on the bottles. ***Check calculation with a lab partner.***
2. Retrieve a solution preparation form and begin filling it out. Collect all the reagents, glassware needed. Record lot numbers of reagents.
3. Place a weigh boat on the top loading scale and tare. Weigh the required amount of reagent.
4. Record the exact weight (all decimal places) in your lab notebook and the Solution Prep Form. Measure approximately 50-70% of the deionized water into an appropriate sized beaker (a little larger than the solution needed) and add a stir bar.
5. Place on a stir plate and set to stir gently. Slowly, add in the dry (or liquid) reagent into the beaker while stirring. Stir to dissolve the reagent. Note: See special instructions for EDTA.
6. If preparing a buffer that requires adjustment with an acid or base, calibrate the pH meter using SOP provided.
7. Rinse the probe and put it in the beaker of reagent. Ensure not to insert the probe too deeply; it should be just below the surface. Turn on the stir plate and check the pH.
8. Add a few drops of appropriate acid or base (hydrochloric acid (HCl) or sodium hydroxide (NaOH) --CAUTION THE ACID AND BASE ARE CAUSTIC! Don't add the acid/base too fast; allow time for stabilization of the pH between drops, continue stirring. Repeat until the proper pH is obtained.
9. After all the chemicals have dissolved, and the proper pH is reached, transfer to the appropriate sized graduated cylinder and bring to volume with deionized water. Place Parafilm on top and mix by inverting.
10. Verify the pH and record final pH in solution preparation form. Pour into a bottle. Label using the label form provided and fill out the Solution Prep form.
11. If preparing a stock solution that will be used this semester, also fill in an inventory control form.

PART I: PREPARING STOCK & WORKING SOLUTIONS

A stock solution is a concentrated solution, often 10X or ten times concentrated, which is prepared in advance. When needed, a small amount of the stock solution is diluted to the desired concentration and used.

1. Ask instructor which stock solution you are responsible for preparing: 0.5M EDTA, or 1M Sodium phosphate monobasic, or 1M sodium phosphate dibasic, or 1M Tris-Cl pH 8.0.
2. Prepare stock solution, place in designated bottle with an appropriate label. Fill in an inventory control form for this solution.
3. Place bottle & inventory form in designated area.

PRE-LAB INSTRUCTIONS:

1. Prepare a pre-lab exercise for preparation of all these solutions using the template provided. *Fill in the missing areas as part of your pre-lab activities.*
2. Obtain MSDS sheets for HCl and NaOH. Read them carefully. Obtain the essential information from the MSDS about working with the chemical, and what to do for a spill. Include a summary in the pre-lab hazard section.
3. Remember to include all calculations in a calculation section (you may hand-write them). Do these calculations before class, and verify calculations with your partner.
4. Before you begin preparing solutions, ensure the molecular weight on the bottle is the same as the molecular weight you used for your calculations!

Solution	Final concentration	Final volume	Comments
EDTA Stock in purified H_2O, pH 8.0 (372.24g/mol)	0.50 M	100 mL /class	The disodium salt of EDTA is not soluble until pH is adjusted to 8.0 with NaOH. Add the correct weight of EDTA to a beaker along with half the water. Adjust pH to pH 8.0 with 6 M NaOH. Store at RT.
Sodium phosphate, monobasic stock (119.98 g/mol) NaH2PO4	1M	100 mL/ class	This solution may require being gently heated to 40°C to get into solution. Store stocks at 4°C.
Sodium phosphate, dibasic stock (141.96 g/mol) Na2HPO4	1M	100 mL/ class	This solution may require being gently heated to 40°C to get into solution. Store at 4°C.
Sodium phosphate buffer, pH 7.0	100 mM	100 mL /group	Use the table in the introduction to make the phosphate buffer. Verify pH. We will use this working solution today. Store remainder at 4°C.
Tris-Cl, pH 8.0 Stock (121.14g/mol)	1M	100 mL /class	Store at RT. Keep.
Tris-Cl, pH 8.0	10mM	100 mL /group	Prepare using 1M stock. Store remainder at ambient temperature.

PRE-LAB EXERCISE: LAB UNIT 2: PREPARING REAGENTS

TITLE:

PURPOSE: *The purpose of this lab is to prepare stock solutions that will be used throughout the semester. In this lab, the following stock solutions will be prepared:*

SAFETY: *Specific safety precautions for this lab exercise are as follows:*

MATERIALS: Create a comprehensive list of all the equipment and reagents you will need to complete this lab exercise. Include water baths, equipment, glassware, and reagents:

CC BY NC SA

PROTOCOL	EXPERIMENTAL NOTES
Part I: PREPARATION OF STOCK SOLUTIONS **Preparing Stock solutions: 100 mL, 1M Tris-Cl, pH 8.0** 1. Gather materials. 2. Place a weigh boat on a top loading scale and tare. 3. Weigh 12.11 g of Tris base, add to a 150mL beaker 4. Add a stir bar, and approximately 70mL of deionized (diH20), place on a stir plate and dissolve. 5. Calibrate a pH meter, and place probe into dissolved Tris solution. Record initial pH. 6. Add 6M HCl dropwise while mixing until pH is 7.5, then add 1M HCl dropwise until the pH reaches 8.0. 7. Pour into 100mL graduated cylinder, and bring to volume (BTV) to 100mL with diH2O. 8. Cover and mix. Transfer to a labeled storage bottle. 9. Record final pH. 10. Fill in solution preparation form, and inventory control form (if applicable).	121.14g/mol x 1 mol/L x 0.1L = 12.11g Tris base
Preparation of working stock: 100mL, 10mM Tris-Cl 1. Gather materials. 2. With a ____________, pipet _____ mL of 1M Tris stock into a 100mL Graduated cylinder. 3. BTV to 60mL with diH2O 4. Cover, and invert to mix, transfer to a labeled bottle.	Calculations: C1V1 = C2V2
Preparing Stock solutions: 100 mL 1M EDTA, pH 8.0 1. Gather materials. 2. Place a weigh boat on a top loading scale and tare. 3. Weigh ______g of EDTA base, and add to 150mL beaker 4. Add a stir bar, and approximately 60mL of deionized (diH20), place on a stir plate and dissolve. 5. Calibrate a pH meter, and place probe into solution. Record initial pH. 6. Add 6N NaOH dropwise - while dissolving keeping pH at or slightly above 8.0. 7. When the solution is close to dissolved you can switch to 1N NaOH, until pH reaches 8.0 and EDTA is completely dissolved. 8. When the solution is dissolved, pour into 100mL graduated cylinder, and BTV to 100mL with diH2O. 9. Cover and mix. Transfer to a labeled storage bottle. 10. Record final pH. 11. Fill in solution preparation form, and inventory control form (if applicable).	Calculations:

1M Sodium Phosphate monobasic (NaH_2PO_4), 100mL 1. Gather materials. 2. Place a weigh boat on a top loading scale and press tare. 3. Weigh ________g of NaH_2PO_4, and add to a 150mL beaker 4. Add a stir bar, and approximately 70mL of deionized (diH20), place on a stir plate and dissolve. 5. Pour into 100mL graduated cylinder, and BTV to 100mL with diH_2O. 6. Cover and mix. Transfer to a labeled bottle. 7. Fill in solution preparation form, and inventory control form (if applicable).	Calculations:
1M Sodium Phosphate dibasic (Na_2HPO_4), 100mL	Calculations
0.1M Sodium phosphate buffer, 100mL	Calculations

PROTOCOL	EXPERIMENTAL NOTES
PART II: TEMPERATURE AND pH OF BUFFERS 1. Aliquot each of the Tris and Phosphate *diluted working solutions* into 3, 20mL aliquots inside 50mL conical tubes. 2. Place one aliquot in a wet ice bath, one aliquot at 60°C water bath and one aliquot at ambient temperature. 3. Incubate in required temperatures for a minimum of 5 minutes. 4. Record the temperature and pH of the solution in the ice bath. Leave it in the ice bath when measuring pH. 5. Record the temperature and pH of the solution in the 60°C water bath. Set up a pH meter beside the water bath for the class to use. 6. Finally, record the accurate temperature and pH of the remaining aliquot of the buffer at ambient temperature. 7. Prepare a table summarizing temperature versus pH data. You may also want to illustrate your data in the form of a line or bar graph (put both sets of data on ONE graph). 8. KEEP stock solutions, record on inventory control sheet the volume used. 9. Discard working solutions down the drain with lots of water.	
Data Table:	

Part III: Documentation ***Each stock solution must have a corresponding solution preparation form and an inventory form. These will be kept in an inventory binder with the solutions. Each time a student removes an aliquot from the stock, it must be tracked in the inventory control binder.*** 1. For the stock solutions, your group prepared, complete the required forms. Give the completed forms to your instructor to be copied for the entire class. 2. Original forms are placed into an inventory binder and stored with the ambient temperature solutions. Ask your instructor where to put the binder and solutions. 3. As part of your lab report, AUDIT the inventory forms and include them in your report. Use a highlighter and identify areas of the form that are missing/incorrect. Write a short note beside the error to indicate what the error is. 4. KEEP the stock solutions; phosphate monobasic, dibasic, 1M Tris, 1M EDTA. Also keep remaining Tris working solutions and sodium phosphate buffer. You will use these solutions later in the semester. Ask your instructor where to store them. Part III: Clean up 1. Rinse out glassware and place dirty dishes in designated area. Remove any tape or markings you made on the glassware. You may be requested to wash the glassware today. Please ask the instructor. If you are washing glassware, please be sure to follow the SOP given for washing glassware. 2. Shut off all water baths, scales, pH meters. Return pH meter and wipe down the bench area around the pH meters.	

Lab Unit 2: Analysis

Follow the instructions in the lab manual on submitting a laboratory report.

1. For this report only, staple a ***lab grading rubric*** to the top of the lab report, and use it to *self-grade your report*.

2. ***Pre-lab exercise***. Your pre-lab exercise should have the protocol on how to prepare EACH of the solutions listed separately, including all the calculations. Include all experimental notes and calculations for every solution. Reminder, do NOT include the lab introduction.

3. ***Summary of raw data, Results.*** Summarize data in table form.
    a. Include all forms with this entire lab exercise; solution preparation and inventory control. Ensure they have been AUDITED thoroughly. You will be graded on this.
    b. Create a pH versus Temperature data table and graph and include with this report.

4. ***Analysis of results***. Research and answer the following questions. Type answers on a separate sheet.
    a. In one short paragraph, summarize the importance of accurately preparing solutions in a biotechnology lab and how solution preparation forms relate to traceability.
    b. In one short paragraph, discuss the importance of inventory control in a regulated environment.
    c. In one short paragraph, discuss advantages of preparing stock solutions.
    d. In a couple short paragraphs discuss your results. For the buffer solutions tested, does the pH change with temperature? Discuss why or why not. Research the reasons for this do not guess. ☺

5. ***Conclusions***. State the experimental conclusions of this laboratory experiment.
    - ✓ ***1-2 sentences***: Restate and summarize the results
    - ✓ ***1-2 sentences***: State implications of the work
    - ✓ ***1-2 sentences***: State future work implications

    For example:
    "In conclusion, 500 ng of genomic DNA was successfully extracted from Arabidopsis thaliana leaf and stem. Spectrophotometric and agarose gel analysis revealed the quality of this genomic DNA is satisfactory for use in PCR amplification. This Arabidopsis thaliana genomic DNA will be used in future work to amplify and isolate gapC gene using degenerate nested PCR technique."

LAB UNIT 3: WATER QUALITY IN THE BIOTECHNOLOGY LABORATORY

INTRODUCTION

Water is the primary solvent used in a biosciences lab! *Why*?! It is used to prepare reagents, clean glassware and equipment, prepare pharmaceutical products and may even be part of the final drug product. Each application requires a different quality of water, and therefore, water quality is one of the most important considerations for a biotechnology lab. It is also one of the most costly expenditures!

There are many different types of contaminants found in water. They include dissolved inorganic, dissolved organics, suspended particles, dissolved gasses, microorganisms, and most importantly for pharmaceutical companies, pyrogen, and endotoxins. There are a variety of methods used to remove these contaminants from water, and frequently many methods are used together to achieve the highest quality needed.

It is important to note here that 100% pure water does not exist. Although the techniques described below do an excellent job of removing contaminants, water is never truly free of all contaminants. Several professional societies publish standards, which contain specifications for relative water purity. One water standard is the US Pharmacopeia (USP). To learn more about what the USP does watch this video: https://youtu.be/i4Trcw0fAZY

In a pharmaceutical company, water can be used for cleaning, raw material, formulation, and a solvent. Therefore it is critical to use the highest quality water. However, what does 'high quality' mean? How do we qualify water? The USP publishes their quality standards in the USP General Chapter <1231>.

The USP: "The U.S. Pharmacopeia Convention (USP) is a scientific nonprofit organization that sets standards for the identity, strength, quality, and purity of medicines, food ingredients, and dietary supplements manufactured, distributed and consumed worldwide. USP's drug standards are enforceable in the United States by the Food and Drug Administration, and these standards are used in more than 140 countries. Since its founding in 1820, USP has helped secure the quality of the American drug supply. Building on that legacy, USP today works with scientists, practitioners, and regulators from many nations to develop and revise standards that help protect public health worldwide" (USP.org). Learn more here: http://www.usp.org/about-usp

"Pharmaceutical water systems must be appropriately designed, operated, and maintained to produce high-quality water. USP General Chapter <1231> Water for Pharmaceutical Purposes provides detailed information about nearly every aspect of maintaining, validating and monitoring a pharmaceutical water system. Validation is the process to demonstrate that the design and operation of a pharmaceutical water system consistently produce water that meets USP requirements. General Chapter <1231> provides extensive discussion of the life cycle elements to maintain a validated state of control" (USP.org).

TYPES OF WATER

The complete USP for pharmaceutical water can be found here: http://www.pharmacopeia.cn/v29240/usp29nf24s0_c1231.html
Interesting pharmaceutical facts are here: http://www.usp.org/frequently-asked-questions/water-pharmaceutical-and-analytical-purposes

The FDA & USP recognize eight types of water:

1. Non-potable
2. Potable (drinkable) water
3. USP purified water
4. USP water for injection (WFI)
5. USP sterile water for injection
6. LUSP sterile water for inhalation
7. USP bacteriostatic water for injection
8. USP sterile water for irrigation

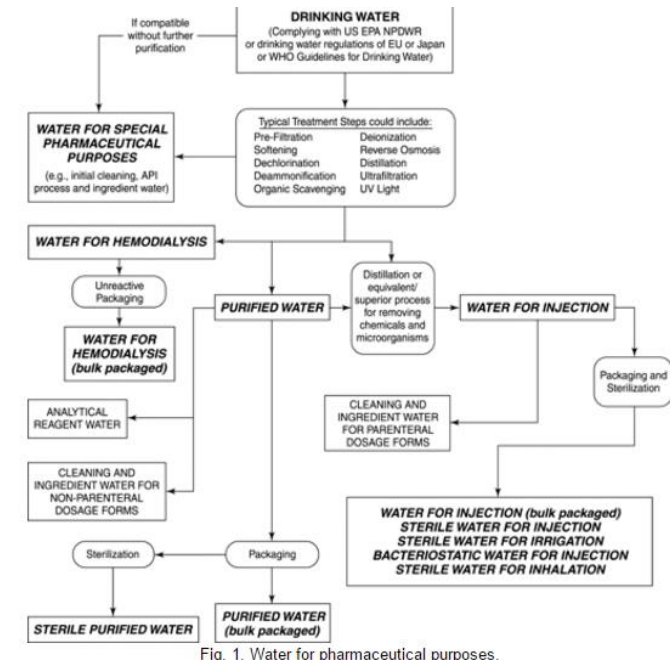


Fig. 1. Water for pharmaceutical purposes.

Source water: Source water, such as municipal water, can only be used for non-contact with product uses, such as cooling systems. Water for pharmaceutical manufacturing and testing purposes must be highly purified. Tap water can have high levels of chlorine and other ions, particulate matter and non-indicator microorganisms and viruses not tested for, by drinking quality standards.

Ultra-Pure Water: The United States Pharmacopoeia (USP) distinguishes between water for injections (WFI) and purified water. WFI water must be produced by distillation or reverse osmosis and must have extremely low endotoxin levels. It is also the water of choice for maintenance of mammalian and insect cell lines, as they are sensitive to the presence of endotoxin. Endotoxins are pyrogens, which induce fever in humans. They are part of the cell wall in gram-negative bacteria and are lipopolysaccharides—molecules containing lipids and polysaccharides. A person with a systemic gram-negative infection runs the risk of dying from septic shock when treated with an antibiotic that kills the bacterial infection. The killed bacteria release endotoxin triggering the person's immune system resulting in septic shock. Tap water is contaminated with endotoxin and therefore NOT appropriate for injection. The concentration of endotoxin is endotoxin units per milliliter (EU/mL). In a lab later this semester, we will perform a test for the presence of endotoxin known as the *Limulus* Amebocyte Lysate Test or LAL test.

Table 1: Pharmacopeia specifications for water for injection (Wikipedia.org)

Properties	European Pharmacopoeia	United States Pharmacopeia
Conductivity[B]	<1.3 µS/cm at 25 °C	<1.3 µS/cm at 25 °C
Total Organic Carbon (TOC)	<0.5 mg/L	<0.50 mg/L
Bacteria (guideline)	<10 CFU/100 mL	<10 CFU/100 mL
Endotoxin	<0.25 IU/mL	<0.25 EU/mL [C]
Nitrates	<0.2 ppm	N/A
Aluminum	<10 ppb	N/A

ISO Standards: ISO also publishes and recognizes water standards. They have three grades of water with Grade I, as the purest. Their summary guidance can be found here, and provide international harmonization of water standards:
https://www.iso.org/files/live/sites/isoorg/files/archive/pdf/en/iso_and_water.pdf

ASTM Standards: The American Society for Testing and Materials (ASTM) publishes water standards as well (D1193-06). They have four grades of water, with Type I being the purest. Type I water is typically used for cell culture, and HPLC applications and is generated using a reverse osmosis system.

- *Type I water* is the highest class of purity and is used for most analytical procedures, tissue culture and instrumentation since it has very low levels of contaminants. It is routinely prepared by reverse osmosis (RO) in combination with distillation and deionization.
- *Type II water* is suitable for most routine lab work and is prepared by distillation or RO. Although it is not as reactive as type I, it should also be stored in non-reactive containers.
- *Type III & IV water*, such as tap water, is useful for some applications such as rinsing glassware or preparing microscope slides.

ASTM further classifies water according to ***grade: A, B and C*** that are applied to these four types of water. These grades vary in the levels of bacterial and endotoxin contamination permitted. It's important to note, *water used for making pharmaceuticals requires even stricter standards than Type I water.*

PURIFICATION OF WATER

Most labs and biotechnology industries begin with partially purified tap (municipal) water, the water we drink. The quality of municipal water varies depending on geographical location and its influence on the water. You may have noticed how water tastes different in different cities. Due to the high cost of making ultrapure water, some biopharmaceutical industries choose their site depending on the quality of the local water. Although municipal water is drinkable, it needs to be further purified for lab work.

There are five major ways in which water is purified:

1. **Distillation** is often used to purify water for the lab. Water is heated to boiling; the steam travels through a cooled condenser coil, where it condenses back to liquid form and is collected in a different container. Although distillation removes many contaminants, it is not effective at removing dissolved ionized gasses. One of the main disadvantages of distillation is that it is a relatively expensive and slow process.

2. In **ion exchange,** water passes through filters with bead-shaped resins, which remove ions. Cationic resins remove positive ions, and anionic resins remove negative ions. It is essential that lab water is deionized. Ions are highly reactive and can ruin experiments, interfere in the production process and contaminate the final product. In Central Texas, we have hard water, water in which many ions, such as, calcium, are dissolved. We can see this hard water in the low sudsing of our soaps and shampoos as well as the tough-to-remove ring around the tub. Water softeners are cationic resins with Na+ loosely attached to them. As our hard water is passed over the water softener, the hard water Ca^{++} ions are exchanged for the Na^{+} ions. Many homes use these water softeners. However, because of the presence of Na+, they are not sufficient for lab purification.

   Deionization is accomplished using both a cationic exchange column with H+ (instead of Na+) ions as well as an anionic exchange column with OH-. The positive contaminants are removed by the first column and exchanged with the H+, and the negative contaminants exchanged for the OH-. The resulting ions combine to form more water molecules, and the water is purified. It is purer than tap or softened water but still contains many contaminants such as dissolved organics, bacteria, and pyrogens.

3. **Carbon Adsorption** is very effective in removing dissolved organic compounds from water. The water is passed over activated charcoal (carbon) made traditionally by burning wood. Most activated carbon is made from styrene beads today since this produces a purer carbon. The organic contaminants stick to the activated carbon and are removed. Organic removal is usually the preliminary step before deionization.

4. There are many types of **filtration methods** used in treating water:
   - The first of these are **depth filters** made of sand or matted fibers. They are often used at the beginning of the filtration system to remove large debris.

- **Microfiltration membrane filters** are filters with an appropriate pore size. Water treatment filters are usually 0.20 um which filter out bacteria, though they will not filter smaller dissolved molecules.
- Small dissolved molecules, including most organics, are filtered out of the water with an **ultrafiltration membrane** whose pore sizes are smaller than the microfilters. Molecules smaller than most proteins can be separated!
- **Reverse osmosis (RO)** filters are more restrictive than ultrafiltration membranes and with a range of 0.0001 um in diameter and smaller, removes molecules less than 300 Daltons. RO is successful at removing viruses, bacteria, and pyrogens. They also reject ions and polar molecules such as sugars. In RO, water passes through a thin membrane, which retains material based both on their size and on ionic charge. Water is usually under pressure to speed up this slow process. Reverse osmosis is often used to make Type I water.
- **Nanofiltration** is a similar process to RO and can also remove viruses and pyrogens; however, its range is a little smaller at 0.001 um.

5. There are several other methods used to purify water. Two worth mentioning are **ultraviolet (UV) oxidation** and **ozone sterilization.** In UV oxidation, water is passed for about 30 minutes over a UV lamp with a wavelength of 185 nm. The organic compounds are oxidized to simple compounds such as carbon dioxide. A wavelength of 245 nm will kill bacteria and is sometimes used to sterilize water. In ozone sterilization, ozone kills bacteria by rupturing their membranes. Ozone is highly reactive and therefore must be removed in subsequent purification steps.

WATER PURIFICATION SUMMARY

Each one of these purification methods has their advantages, limitations, and specific applications. It is important to note that a combination of these purification procedures is often employed in most labs. For example, Type I water may first be passed through activated carbon filter and then RO filter to a storage tank. Finally, a series of ion exchange columns may be used before a final ultrafiltration step. Different labs have different water needs and will choose the standard that is recommended and used in their industry based on their product. Water usage in a biotechnology company follows strict adherence to Standard Operating Procedures (SOPs). SOPs should explicitly state the source of water required, how it is purified, how the water is monitored and how the purification equipment is to be maintained.

THE MAINTENANCE OF WATER PURIFICATION SYSTEMS

Labs must monitor their water purification systems to ensure that the systems are working properly. Labs usually keep logs of the date, the monitoring system(s) used and the results. The **maintenance** of the water purification systems includes cleaning (usually daily for distillation systems), sanitation and regeneration of ion exchange systems, sanitation and checking for holes of filtration systems, and cleaning and recharging of activated carbon.

Five quality parameters are typically used to monitor the water system.

1. Resistance is a measurement of the electrical current through the water. Since ions carry the current, water without ion contaminants should have a high resistance. There is usually an attached meter to read the water resistance of water purification systems. The acceptable value for Type I water is 17.0 megohm-cm. Resistance is only useful for monitoring ions in water.
2. Bacterial counts are used to track levels of microorganisms in water. There are typically three different ways a Heterotrophic Plate Count is performed: the pour plate technique, the spread plate technique, and the membrane filtration technique. In the pour plate technique nutrient, agar is warmed to 44°C, and serial dilutions of the water sample are inoculated directly into the agar before being poured into a disposable Petri plate. In the spread plate technique, serial dilutions of the water sample are performed before being spread onto nutrient agar plates. In the membrane filter technique, a known volume of a water sample is filtered through at 0.45um filter and the filter incubated on a nutrient agar plate. In all three methods, the plates are incubated 25-35°C for 48-72 hours, depending on the method used and agar plate media. The numbers of colonies growing are counted to give the number of colony-forming-units (CFU) per mL of water. Different standards provide the maximum CFU permitted.
3. As mentioned in the introduction, pyrogens are tested with a Limulus Amebocyte Lysate (LAL) test. An extract from the blood of a horseshoe crab is mixed with different dilutions of water. Pyrogens will cause the blood extract to clot. The results are given in endotoxin units per milliliter (EU/ml).
4. Organic carbon contaminants are monitored with instruments such as the mass spectrometer, which analyze the carbon compounds.
5. The pH of ultrapure water exposed to the carbon dioxide in the air is about 5.7. In a covered container, water has a pH of about 6.0. The pH is easily monitored with a pH meter.

References:

1. Seidman & Moore, "*Basic Laboratory Methods for Biotechnology: Textbook & Laboratory Reference*," 2nd edition. 2009. Prentice Hall. ISBN: 0321570146
2. US Pharmacopeia. Retrieved on 2/24/2016 from http://www.pharmacopeia.cn/v29240/usp29nf24s0_c1231.html
3. Wikipedia.org. Ultra Pure Water. Retreived from https://en.wikipedia.org/wiki/Ultrapure_water

Lab Unit 3A: Preparation of Media & Plates

INTRODUCTION

Media is the term used for the growth solution for cells grown in the lab. Media may be either chemically defined or complex. Chemically defined media contain only substances that are clearly identified. One type of chemically defined media is ***minimal media***. Minimal media contains the minimal ingredients to support life. There are different minimal media for bacteria, yeast, and animal and plant cells. For example, the minimal media for growing human cells would have to contain vitamin C since human cells are unable to make this essential nutrient and must obtain it from their food.

Rich or complex media contain a variety of nutrients and some ingredients that are either unidentified or might be present in variable concentrations. It is not possible to define rich media. Yeast extract, the extract of all the water-soluble components of yeast, is sometimes added to media for growing a variety of microorganisms. The yeast extract cannot be chemically defined, and its composition will vary depending on the growing conditions of the source yeast.

Selective and differential media may be either chemically defined or rich. Selective media acts by killing microbes that lack a specific phenotype. For example, bacteria media containing the antibiotic penicillin will support the growth only of penicillin-resistant bacteria. Therefore the media is *selecting* for a specific trait. Differential media will distinguish between microbes that possess a given trait and those that do not, often by color or shape of the colony.

Sterilizing Media: Media is sterilized to avoid the growth of undesirable microorganisms. There are two major sterilization methods in common use in the lab: autoclaving and filter sterilization. An ***autoclave*** is in effect, a large pressure cooker, using steam and pressure to kill off any undesirable microorganisms. Typically 15 to 20 minutes autoclaving at 15 lb/in^2 pressure is sufficient to sterilize heat-resistant media and glassware or for discarding biological waste. Some media ingredients are sensitive to heat and will break down in the autoclave. These ingredients are sterilized in the lab by ***filter sterilization***. A sterile filter with holes or pores of 0.22 μM is used to filter out bacteria and other contaminating organisms or molecules. Small amounts of media are filter sterilized with a syringe filter. Larger amounts of media are filtered with a larger apparatus, which is usually a self-contained unit with chambers above and below the filter. The unsterile media is placed in the upper chamber. The lower chamber is fitted with a side arm that is attached to a vacuum so that the media is pulled through the filter by the vacuum into the lower chamber.

References:

1. Seidman & Moore, “*Basic Laboratory Methods for Biotechnology: Textbook & Laboratory Reference*,” 2nd edition. 2009. Prentice Hall. ISBN: 0321570146
2. Gabriel Bitton. “*Wastewater Microbiology*.” Chapter 8. 2005

PRE-LAB EXERCISE

Create a pre-lab exercise for all of lab unit 3 using the examples provided in lab unit 2 and the protocol below.

Preparation of Complex Media – DAY ONE

MATERIALS

○ Bacto-peptone	○ 2, 250mL graduated cylinders
○ Bacto-yeast extract	○ Aluminum foil
○ Glucose	○ Autoclave tape
○ Bacto-agar	○ Sterilmatic Autoclave
○ 1, 150 mL beaker	○ SOP AUT-002: Use and Maintenance of Sterilmatic Autoclave.
○ 1, 250 mL glass bottle	○ 60x15mm sterile Petri dishes (15)
○ 70% Ethanol	○ Clean water bath 55ºC
○ HEPA hood	○ Bottle weight (to hold bottles down in water bath)
○ Lint-free disposable cloth	

Hazard Communication

- *Steam from the autoclave and autoclaved liquid can severely burn skin. Be careful when handling molten media, and when removing liquids from the autoclave.*
- *At times pressure can unexpectedly build up in hot liquids and explode when suddenly moved or jarred. Swirl liquids gently after removing from the autoclave.*
- *When autoclaving liquids, be certain that all caps are loose, or containers can explode under high pressure!*

PROTOCOL

Part I: Preparation of Yeast Rich Media (YPD)

1. Each group of two students will need approximately 15 small agar plates for this experiment. Each dish holds approximately 10mL. Calculate the amount of each ingredient needed to prepare 150 ml of this rich media. Remember: "%" means g/100 ml

   YPD media
   1% bacto-yeast extract
   2% bacto-peptone
   2% glucose
   2% bacto-agar
2. Weigh out ingredients, recording the weights in your notebook and Solution Prep Form.
3. In a 150mL beaker, dissolve all ingredients except bacto-agar (why?) in approximately 100 ml deionized water.
4. BTV (150mL) in a graduated cylinder. Cover, and mix by inversion.
5. Pour into a 250-mL bottle, add bacto-agar (do not mix).
6. DO NOT SCREW CAP (set cap on top, do not screw). Cover cap and neck of the bottle with aluminum foil, tape foil to the bottle with autoclave tape. Label with your initials.

Part II: Operate the Autoclave

1. Carefully read the SOP on the operation of the autoclave, SOP AUT-002: Use and Maintenance of ***Sterilmatic Autoclave.***
2. Load items into the chamber. Do not crowd the items too closely, or the steam will not penetrate between them.
3. Ensure chamber drain is in the closed position. Pour 4 L of tap water into the chamber until it reaches the fill line. Do not exceed the fill line. This step may have already been done for you, ask your instructor.
4. Grasp the door handle and hold it in a vertical position while pulling down until the bottom of door rests in the door opening. Ensure the rubber seal is flush with the door. Then push door handle down to engage the lock.
5. Select the correct exhaust type: "Liquids."
6. Set the temperature to 121ºC.
7. Select the **liquid** cycle. This will heat to a higher temperature than the other cycles and exhaust more slowly to prevent evaporation and splashing.
8. Turn the timer to start operation. The timer should always be set to at least 15 minutes, as this is the minimum time for sterilization to be effective. For today's class, we will sterilize for 20 minutes.
9. When the chamber reaches the selected temperature, the timer will begin. The autoclave may take a while to begin.
10. When cycle is complete, allow the chamber pressure to drop to zero before attempting to open the door.
11. Open door completely by pulling up on the door handle. Stay to the side to avoid steam burns.
12. Remove items using insulated gloves or hot hands to avoid burns. Be very careful with molten liquid – it can store heat, and sudden movements can cause the molten liquid to explode out of the flask (and burn your skin).
13. Cool molten agar in a clean 55ºC water bath.

Part III: Clean and Prepare Equipment

This media does not contain antibiotics to kill any contaminants. Therefore, it is critical the work area and equipment, such as water baths, and bench top is clean and clutter free.

1. One group should clean a water bath: Drain water, wash gently with lab equipment detergent (ask your instructor).
2. Refill with diH2O, and set to 55ºC.
3. One group should clean a HEPA filter sterile laminar flow hood (if available): Watch this video to learn the proper procedure for cleaning a laminar flow hood (LFH): https://www.youtube.com/watch?v=XfMwduSfHN8
4. Turn on the LFH, and place sash in designated position.
5. Remove any materials from inside the hood. Always glove and cover arms with lab coat when working in a sterile hood. No bare skin – watch the wrists!
6. Use designated disinfectant to clean hood by spraying a lint-free disposable cloth (NOT inside the hood) and wiping inside each surface. Your instructor may demonstrate, there is a demonstration in the video above as well. *Never use bleach on a hood surface or*

spray caustic chemicals inside the hood because it will damage the hood and filter. If no other disinfectant is available, use 70% ethanol.

7. Wipe away any disinfectant and debris from inside the hood with 70% ethanol. Again, spray the lint-free cloth outside the hood, and wipe the hood surface, with no exposed skin, using gloves and a lab coat only.
8. The hood should remain on for at least 15 minutes before use after wiping procedure.

Part IV: Pour Plates

Pouring the plates must be done using sterile technique. If available, pour in HEPA filtered hood.

1. Prepare by first removing ***all*** extraneous materials from the work area. Wipe down the bench-top with alcohol. Set up sterile Petri dishes in a row along the bench in front of you. Keep the lids ON the Petri dishes until you need to open them.
2. Remove sterile molten agar flask from the water bath immediately before use. Wipe off the dripping water with a paper towel.
3. ***Your instructor will demonstrate this part for the class.*** Remove the foil from the molten agar flask. Quickly and carefully lift each Petri dish lid and pour the media into the bottom plate until the media is just below the line on the side of the plate (about 5 ml for the small plates). Quickly replace the Petri dish lid. ***Do not splash or create bubbles!***
4. Continue until the media in your flask has run out. You may need to gather extra plates. Rinse flask with lots of warm water and place on the cart.
5. Allow the plates to harden before moving; approximately 15 minutes.
6. After the plates have been poured, allow them to set inside hood 1-2 days. Do not turn them over while curing! Turn hood and lights off and close sash (if possible).
7. Place plates back into plastic sleeves, seal, store agar side facing down, and keep at 4°C for long-term storage.
8. Because these plates do not contain antibiotics, check plates for contamination before using.

Lab Unit 3B: Heterotrophic Plate Count of Laboratory Water

PROCEDURE HETEROTROPHIC PLATE COUNT – DAY TWO

MATERIALS (each group)

o 3, sterile 50mL beakers o pH meter and pH standards o 3, 150mL beakers o Purified KCl solution o Lab Water: Deionized, tap water, RODI (if available) o 250mL, bottled sterile water (if no RODI available	o 1, forceps in 70% ethanol o 1, vacuum flask, hose, and pump o 1, clean and sterile Buchner funnel o Filter papers o 15, YPD Agar plates o 4, Sterile Loops o 1, clean and sterile 100 mL grad. cylinder o 3, sterile grid filter paper (0.45 *u*m)

Hazard Communication

Although there is no contact hazard for this lab, you will be monitoring your water for biological contamination and utilizing media without antibiotics. Wearing clean gloves and working on clean surfaces with clean equipment is CRITICAL for this lab.

PROTOCOL

Part I: Membrane Filtration

The Membrane Filtration technique is used to enumerate heterotrophic organisms in water sources with potentially low counts. For three water sources in the lab perform the following:

1. Collect four nutrient agar plates per group (one plate for each water source and one for a negative control). Label the plate as demonstrated. Include team names, date, and the water source. *Note: Always keep the lids on the plates.*
2. Press the Buchner funnel into the vacuum flask. Attach the hose from the flask to the vacuum outlet. Ensure the vacuum source pump has been turned on. Ask the instructor where that is.
3. Find the two lab water sources (tap and deionized system). Do NOT use plastic bottles or containers to obtain water. Why? Locate the ***source tap*** for each.
4. Starting from the cleanest water, one at a time, collect 100 mL of water sample in a graduated cylinder: when collecting, *rinse out the 100-mL graduated cylinder 3X before final collection using the water you will be testing*.
5. Using clean, sterile forceps, remove the gridded filter from the wrap and backing, and place over the holes grid-side up in the Buchner funnel. Grid side up.
   NOTE: Ensure you remove the protective backing from the filter; you only want the filter paper! Also, ensure that the filter paper covers the holes in the bottom of the funnel.
6. Turn on the vacuum valve slowly and keep it at minimal suction during the filtration. Do not crank up the vacuum or you will risk rupturing any bacteria (and killing them) as they filter through the paper. Slowly pour your water sample into the Buchner funnel making sure the filter paper does not rise off the bottom of the funnel. If this happens, you must start over.
7. When the entire 100 mL has been filtered, shut the vacuum valve and remove the filter paper with sterile forceps. *Place face-up on the labeled nutrient agar plate*. Avoid air bubbles.

8. Repeat this process with the next water sample. Be sure to *rinse out the Buchner Funnel, and the 100-mL graduated cylinder with the new water source* ***before*** *you begin*.
9. Prepare your negative control plate by removing an unused filter and placing it on the plate as you did for your filtered water. What will this plate be used for?
10. When complete, turn plates over (agar and filter facing down) and tape them together. Place plates in the designated incubator. Record the incubator temperature.
11. Incubate the plates 48-72 hours at 35ºC. Record time and temperature when you remove plates from the incubator.

Part II: Spread plate technique

The spread plate technique is used when higher colony counts are expected. For each water source in the lab (sterile, deionized, tap) perform the following:

1. Collect nine nutrient agar plates per group (3 for each water source). Label the plate as demonstrated. Include your team names, date and the water source. *Note: Always keep the lids on the plates.*
2. Obtain first water sample as outlined in Part I. Pipette 10ul, 50ul & 100ul of the water sample directly onto three agar plates. *Keep plates closed throughout this procedure*. Remember, there are no antibiotics on these plates.
3. Using a sterile disposable loop quickly spread the water sample *throughout the entire surface of the plate*. Be gentle when using the loop, do not scrape the agar surface. Use the same loop for the 3, aliquots, however, change loops between water.
4. Repeat with all three water sources with a clean, disposable loop.
5. When the liquid has absorbed into the agar, invert plates and incubate for 48-72hrs at 35ºC.

Part III: pH Water Samples

For each of the three water sources perform the following:

1. Calibrate the pH meter as outlined in the pH meter SOP.
2. Collect approximately 50 mL of each experimental sample (water) into a clean 150 mL beaker. Remember to rinse the beaker 3X in the sample water before taking a final sample.
3. Add 150 μl of high purity saturated KCl to your water sample, mix well.
4. Determine the pH and record into your laboratory notebook. *Do not pH your sample while stirring the water*. Why not?
5. Remember to record ambient temperature.
6. Prepare a table summary table of results for your lab report. Do not forget to include pH data in a final comprehensive data table. Compare the results obtained for each water source to the ASTM requirements for pH.

Clean Up!

1. Rinse solutions down the sink, place glassware in designated area.
2. When plates are ready (and no longer wet!), turn plates agar side face-down, and place in designated incubator at the designated temperature. Ask your instructor and record in your notebook!

Lab Unit 3C: Microbiology Techniques

INTRODUCTION

The following excerpt is from the Water Research Center (http://www.water-research.net/) and OpenStax microbiology textbook. http://cnx.org/content/col12087/latest/.

"Bacteria are among the simplest, smallest, and most abundant organisms on earth. Most bacteria are only 1 micrometer (µm) in diameter, but they can range in size from 0.1 µm to greater than 10 µm. Bacteria are prokaryotic organisms; that is, they do not have an organized nucleus surrounded by a nuclear membrane. Prokaryotic microorganisms include bacteria and blue-green algae (cyanobacteria). Bacteria contain a single strand of DNA, and they typically reproduce by binary fission. During binary fission, a single cell divides transversely to form two new cells called daughter cells. Each daughter cell contains an exact copy of the genetic information contained in the parent cell. The process continues with each daughter cell giving rise to a generation of two new cells. The way that bacteria populations increase is an example of a geometric progression: 1 cell two cells four cells eight cells 16 cells, etc. The generation time is the time required for a given population to double in size. This time can be as short as 20 minutes for some bacteria species (e.g., Escherichia coli).

All bacteria are unicellular (single-celled) organisms. Two methods by which bacteria can be grouped are by cell shape and by differential stains. There are three key shapes: bacilli (rods), cocci (spherical or spheroid), and spirilla (spiral or corkscrew). Cells can occur either individually or as groups of cells. Grouped cells neither communicate nor cooperate with each other; however, the configurations that are observed for a particular species are fairly constant. Arrangements of spherical cells can be used in taxonomy: diplococci (paired cells), streptococci (cell chains), tetracocci (four cells arranged in a square), and staphylococci (grape-like clusters).

There are two key types of stains used on bacteria: simple stains and differential stains. Simple stains serve to increase color contrast in cells. They can react with all types of bacteria. Aniline (coaltar) dyes, such as methylene blue, crystal violet, basic fuchsin, eosin Y, and safranin O, are examples of simple stains. These are basic dyes, as opposed to acidic dyes and bind with the acidic portions of cells. Other stains do not react equally with all types of bacteria. Such stains can be used to differentiate among bacterial types; hence, the name differential stain. Differential stains are also used to detect differences among structures within cells" (cnx.org).

The Gram Stain: The Gram stain procedure is a differential staining procedure that involves multiple steps. It was developed by Danish microbiologist Hans Christian Gram in 1884 as an effective method to distinguish between bacteria with different types of cell walls, and even today it remains one of the most frequently used staining techniques. The steps of the Gram stain procedure are listed below and illustrated in Figure.

1. First, crystal violet, a primary stain, is applied to a heat-fixed smear, giving all of the cells a purple color.

2. Next, Gram's iodine, a mordant, is added. A mordant is a substance used to set or stabilize stains or dyes; in this case, Gram's iodine acts like a trapping agent that complexes with the crystal violet, making the crystal violet–iodine complex clump and stay contained in thick layers of peptidoglycan in the cell walls.
3. Next, a decolorizing agent is added, usually ethanol or an acetone/ethanol solution. Cells that have thick peptidoglycan layers in their cell walls are much less affected by the decolorizing agent; they generally retain the crystal violet dye and remain purple. However, the decolorizing agent more easily washes the dye out of cells with thinner peptidoglycan layers, making them again colorless.
4. Finally, a secondary counterstain, usually safranin, is added. This stains the decolorized cells pink and is less noticeable in the cells that still contain the crystal violet dye.

Gram stain process			
Gram staining steps	Cell effects	Gram-positive	Gram-negative
Step 1 **Crystal violet** *primary stain added to specimen smear.*	Stains cells purple or blue.		
Step 2 **Iodine** *mordant makes dye less soluble so it adheres to cell walls.*	Cells remain purple or blue.		
Step 3 **Alcohol** *decolorizer washes away stain from gram-negative cell walls.*	Gram-positive cells remain purple or blue. Gram-negative cells are colorless.		
Step 4 **Safranin** *counterstain allows dye adherance to gram-negative cells.*	Gram-positive cells remain purple or blue. Gram-negative cells appear pink or red.		

Figure 2: Gram Stain procedure.

Gram-staining is a differential staining technique that uses a primary stain and a secondary counterstain to distinguish between gram-positive and gram-negative bacteria. The purple, crystal violet stained cells are referred to as gram-positive cells, while the red, safranin-dyed cells are gram-negative (Figure). However, there are several important considerations in interpreting the results of a Gram stain. First, older bacterial cells may have damage to their cell walls that causes them to appear gram-negative even if the species is gram-positive. Thus, it is best to use fresh bacterial cultures for Gram staining. Second, errors such as leaving on decolorizer too long can affect the results. In some cases, most cells will appear gram-positive while a few appear gram-negative (as in Figure). This suggests damage to the individual cells or that decolorizer was left on for too long; the cells should still be classified as gram-positive if they are all the same species rather than a mixed culture.

Besides their differing interactions with dyes and decolorizing agents, the chemical differences between gram-positive and gram-negative cells have other implications with clinical relevance. For example, Gram staining can help clinicians classify bacterial pathogens in a sample into

categories associated with specific properties. Gram-negative bacteria tend to be more resistant to certain antibiotics than Gram-positive bacteria.

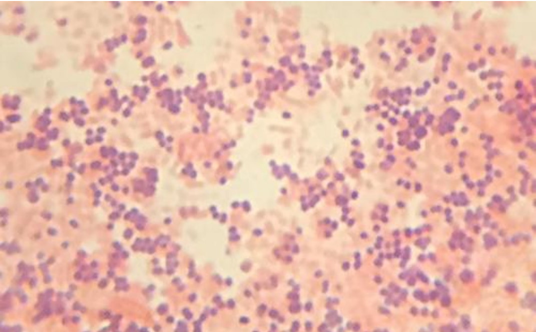


Figure 3: Gram-Stain Specimen. In this specimen, the gram-positive bacterium *Staphylococcus aureus* retains crystal violet dye even after the decolorizing agent is added. Gram-negative *Escherichia coli,* the most common Gram stain quality-control bacterium, is decolorized and is only visible after the addition of the pink counterstain safranin. (credit: modification of work by Nina Parker)

Bacteria and water:

The following excerpt is from the Water Research Center. Retrieved from http://www.water-research.net/

"Coliform bacteria have been used to evaluate the general quality of water. Testing for coliform bacteria is faster and cheaper than testing for specific organisms and pathogens. U.S. Public Health Service established a standard in 1914. Coliforms include all aerobic and facultatively anaerobic, gram-negative, non-spore forming bacilli that, when incubated at 35 C, can ferment lactose and produce gas (CO2) within 48 hrs.

Fecal coliforms are the coliform bacteria that originate specifically from the intestinal tract of warm-blooded animals (e.g., humans, beavers, raccoons, etc.). They are cultured by increasing the incubation temperature to 44.5 C and using somewhat different growth media. Two other groups of bacteria that are present in feces are fecal streptococci and Clostridium. Clostridia spores can survive a long time during adverse conditions. This genus occurs naturally in soils and polluted waters; it is not used for monitoring purposes. Fecal streptococci and enterococci are terms that have been used interchangeably; however, there are some differences between the two groups. Fecal streptococci indicate the presence of fecal contamination by warm-blooded animals. Unlike coliforms, fecal streptococcal bacteria are not known to multiply in the environment. Also, they tend to die off more quickly than coliforms.

The ratio of fecal coliforms to fecal streptococci (FC/FS) can provide information on the source of contamination (Table C); however, several precautions are in order when using these ratios:
(1) Bacterial concentrations can be greatly variable if the pH is outside of the 4.0 to 9.0 range,
(2) The faster die-off rate of fecal streptococci will change as time from contamination increases,
(3) Pollution from several sources can alter the ratio and confuse the issue,
(4) FC/FS ratios have been of limited value in identifying pollution sources in irrigation returns, bays, estuaries, and marine waters, and (5) Ratios should not be used when fecal streptococcal

counts are less than 100/100 mL.
Water quality criteria, guidelines, and standards: A health effects recreational water quality criterion is defined as a measurable relationship between the quantity of the indicator in the water and the potential risk to human health associated with using the water for recreational purposes. A water quality guideline, obtained from the criterion, is a suggested upper limit on the quantity of the indicator in the water that is associated with an unacceptable level of health risk. A water quality standard, obtained from the criterion, is a guideline set by law. The first federal water quality criteria recommendations were proposed, in 1968, by the National Technical Advisory Committee (NTAC). The criterion for bathing waters was based on studies, conducted during the late 1940's and early 1950's by the Public Health Service, of total coliform concentrations. The criterion was converted to fecal coliform concentrations using Ohio River data collected, during the original study, in 1949. The NTAC recommended that:

> *"Fecal coliforms should be used as the indicator organism for evaluating the microbiological suitability of recreation waters. As determined by multiple-tube fermentation or membrane filter procedures, and based on a minimum of not less than five samples taken over not more than a 30-day period, the fecal coliform content of primary contact recreation waters shall not exceed a log mean of 200/100 mL, nor shall more than 10% of total samples during any 30-day period exceed 400/100 mL." (NTAC, 1968)*

The standards utilized by the various states differ (USEPA, 1988a; USEPA, 1988b). The original criterion, based on fecal coliform concentrations, is still in use by many States. In many cases, water quality classifications have been developed that change the concentration of fecal coliforms required to meet the intended use of the water (e.g., primary contact, secondary contact, shellfish, etc.). Drinking water requirements: The EPA has set the following maximum contaminant levels (MCLs) on treated drinking water: "For systems that analyze at least 40 samples per month, no more than 5% of the samples may be total coliform positive. For systems analyzing fewer than 40 samples per month, no more than 1% of the samples may be total coliform positive" (AWWA, 1990b).

How to Use a Compound Microscope

"Microscopes not only magnify the object you are viewing, but they also provide increased resolution. **Resolution** is the ability to distinguish two points as separate points. For instance, if two points are very close together, they may appear to be a single spot. If magnification is increased without increasing resolution, the single spot will only look like a larger single spot, and will never resolve into two separate spots. The better the resolution, the sharper and crisper the image. The resolving power of the naked eye is approximately 0.1 mm, meaning that our eyes can distinguish two points that are 0.1 mm apart. A light microscope can improve resolution by as much as 1000-fold. Also, discernment of cellular detail can be enhanced with the use of dyes that add color and contrast to subcellular structures" (ACC, BIOL1406 lab manual).

Total magnification = Power of the Ocular lens x Power of the objective lens

As part of the pre-lab exercise, learn how to use a compound light microscope by completing this online exercise:

- Video: http://www.austincc.edu/biocr/1406/labv/microscope/index.html
- Animation Tour: http://www1.udel.edu/biology/ketcham/microscope/scope.html
- Quiz: http://www.austincc.edu/biocr/1406/laba/microscope/index.html
- Write a short protocol for focusing a gram stain sample on a glass slide.

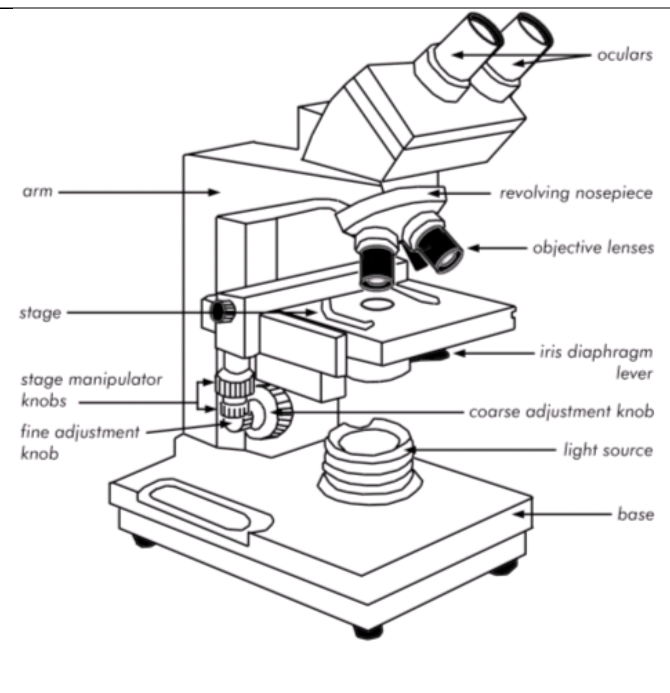
<table>
<tr>
<td>
</td>
<td>Arm - The arm is a vertical support that connects the base of the microscope to the body tube.
Ocular lenses - The oculars are the lenses you look through. Most ocular lenses have a 10X magnification.
Revolving Nosepiece and Objective Lenses - Attached to the revolving nosepiece are several lenses called objectives. Most light microscopes have objective lenses of 4 magnifications: scanning (short) -- normally 4x power, low power (medium) -- normally 10x power, high-power (long) -- normally 40 - 43x power, and oil immersion (longest) -- normally 100x power.</td>
</tr>
</table>

Figure 4: Parts of a typical light microscope

References:

1. OpenStax, Microbiology. Chapter 2. OpenStax. 01 November 2016. Retrieved from http://cnx.org/content/col12087/latest
2. Water Research Center. Retrieved from http://www.water-research.net/
3. BIOL1406 Lab Manual. Retrieved from http://www.austincc.edu/biocr/1406/labm/ex3/prelab_3_8.htm

GRAM STAIN PROCEDURE- DAY THREE

MATERIALS

Microscope Counter Biohazard bag Inoculation Loops	Gram stain reagents Glass slides 37°C heat block Water bottle Tweezers

PROTOCOL

PART I: Visual Analysis of plates

1. Collect plates from the incubator. Record the ***total*** incubation time and temperature.
2. Create a table for data in a lab notebook.
3. Start with the negative control plate. Record the number of colonies. Do you expect to find any colonies? Hint: it is the negative control!
4. Look at each of the water sample plates. Describe the colonies – shapes (round, raised), sizes, color – are these bacterial or fungal? How do they smell? Also note and count fungus, if present. Use a microscope!
5. Record the number of bacterial colonies. You may use a microscope to help count the colonies if they are too small to see by eye. It helps to tilt the plate at 45-degree angle to see the colonies. Alternatively, incubate them longer.
6. If the colonies are not separated well due to overabundance on the plate, record result as "too numerous to count" or TNTC.
7. Remember to record units! For example:
   Colony forming units ÷ 100 mL water filtered = _______ CFU/mL
   In your laboratory report, results should be described as in this example:
      "Municipal Water Heterotrophic Plate Count at 35°C/48 hours: 15 CFU/mL
      There was no fungus observed at these conditions.
      This test was performed by the membrane filter procedure."
8. Create a table summarizing results for the lab report. Hopefully, you will have one plate for each water source that led to the enumeration of the heterotrophic organisms. Compare the results obtained for each water source to the ASTM requirements – you may want to research this online.

Part II: Gram Stain SOP

1. Write a Standard Operating Procedure (SOP) for performing a gram stain. Include, how to operate a compound microscope for viewing the slide. Please use the template provided in the Appendix of the lab manual.
2. Select a few of the colonies on one of the plates to perform a Gram stain procedure.
3. Tape plates shut and dispose of in the biohazard trash (along with the inoculation loops).
4. The glass slides may go into the glass trash since they are no longer biohazardous.
5. Disinfect bench top and areas and wash hands before leaving the lab.

Laboratory Unit 3 Analysis

Remember to include the following in your lab report. Do NOT include the lab manual instructions.

1. Staple a ***lab grading rubric*** to the top of your lab report, and use it to self-grade your report.

2. ***Pre-lab exercise***, title page, laboratory notes, calculations and all forms. Include SOP for gram stain.

3. ***Summary of raw data*** in a table form.
   - ✓ Heterotrophic plate count results of each water source. Include the number of colony forming units (colonies) per mL (CFU/mL), their size, color, shape, and odor if any.
   - ✓ Report the pH results for each water source *in this table include water standards* and your conclusions as to the class of water.
   - ✓ Report gram stain results – include images if captured. Label

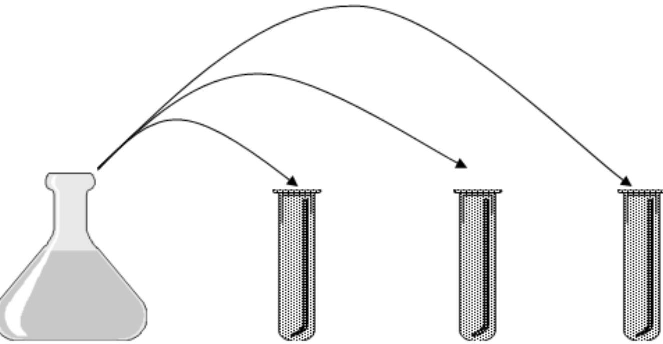
4. ***Analysis of results***.
   a. In one short paragraph, summarize the importance of water quality in a biotechnology lab.
   b. In one short paragraph, summarize the different types of water you analyzed in this experiment. Include how they are purified (For example, how is deionized water purified?).
   c. Summarize each of your results (one short paragraph each)
   d. In one short paragraph, compare the different water results to each other, and with standards.
   e. Discuss: Did you get the results you were expecting? What does this indicate about the purification systems used in our laboratory?

5. ***Conclusions***. State the experimental findings of this laboratory exercise. Restate and summarize the results, and state implications, as well as any future work recommended.

LAB UNIT 4: PYROGEN ASSAY OF LABORATORY WATER

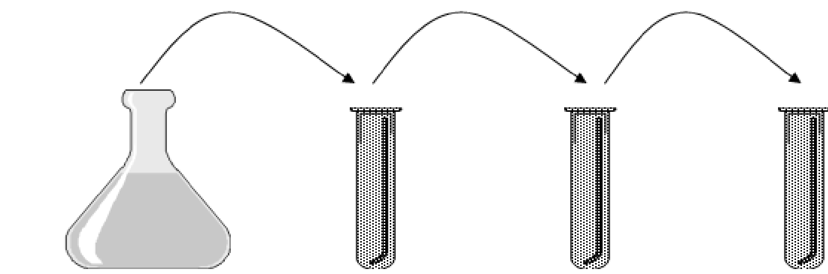
INTRODUCTION

In a previous lab, we saw how stock solutions might be diluted to prepare a buffer. There are two common types of dilutions: serial and parallel. Parallel dilutions are similar to the dilutions previously performed in lab unit 1 when 1M Tris-Cl was diluted to 10mM Tris-Cl. Say, for example, that you wanted to prepare a 1M Tris buffer pH 7.5; 0.5M Tris buffer, pH 7.5; 0.1M Tris buffer pH 7.5; and 0.25M Tris buffer pH 7.5. You could prepare four different solutions but, this would take some time. A faster method would be to make a stock solution of 2.0 M Tris buffer, pH 7.5, then remove the correct amount from the stock and bring to volume (BTV) to make the final solutions. This is a parallel dilution as illustrated below. ***The stock solution is the only source of the buffer.***



Parallel Dilutions

The other kind of dilution is the serial dilution. In this type of dilution, an aliquot is taken from the stock bottle and placed in the first tube which is then BTV to achieve the correct concentration. Then an aliquot is removed from the first tube and placed in the second tube and BTV. The second tube serves as the stock for the third tube and so on. Thus, ***a serial dilution uses the original stock solution only once.*** All other samples are taken from the previous tube, as illustrated below.



Serial Dilutions

Preparing Solutions by Parallel Dilution

When diluting a stock solution by parallel dilutions the equation to use is: $C_1 V_1 = C_2 V_2$

Where, $Concentration_{stock}$ x $volume_{stock}$ = $Concentration_{dilute}$ x $Volume_{dilute}$

Provided the concentration of the stock solution, as well as the concentration and the volume of the final diluted solution, determine the volume of the stock solution needed. For example, say you need to make 10 ml of 0.5 M solution from a stock of 2.0 M. How many ml of the stock would you use? Plug in the values into the equation: $C_1 V_1 = C_2 V_2$

$$2M \times V1 = 10\ ml \times 0.5\ M$$

$$V1 = 2.5\ ml\ of\ 2.0M\ Tris$$

Thus, we can take 2.5 ml of the stock 2.0M solution and place it in a tube, bring to 10mL with water and we will have 10.0 ml 0.5 M solution. ***Note, it is best practice to bring to volume, and not add what you believe to be the correct volume of water***. This is because some solutes and solvent chemicals do not have the same density as water and will not displace correctly.

Preparing Solutions by Serial Dilution

Serial dilutions are dilutions made from other dilutions and are prepared because many dilutions of the same dilution factor are desired and/or the final concentration desired is so small that the stock solution volume 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, and V_2 is the volume of solvent used to dilute the solution

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

Example, how will you prepare 10, 5-fold dilutions of a reagent with an end volume of 40mL in each of the 10 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 100mL beakers
2. Using a 10mL pipet, dispense 10mL of stock solution into beaker 1 and mix.
3. Using a clean pipet, transfer 10mL of the diluted mixture in beaker one 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 solution to V_2 mL of water, you remove V_1 mL of that dilution to make the next one.

METHOD VALIDATION

Method validation is the process used to determine the conditions to obtain a result reliably, and the limits of that procedure. Formal validation of test methods is required in GMP compliant laboratories. However, the aspects of validation are appropriate for any research or testing laboratory.

1. Accuracy is the closeness of a test result to the true or accepted value. Accuracy can be tested by using a reference standard value is known. If it is a method being tested, results are compared to those of a standardized assay. Tests for accuracy are also used to validate equipment. *Accuracy is calculated by determining the percent error of the mean (PEM).*

   PEM = (calculated mean – true value) ÷ true value x 100%

2. Precision is the degree of agreement between individual test results when the procedure is applied repeatedly. ***Repeatability*** is the precision of measurements made under uniform conditions and ***reproducibility*** (ruggedness) is the precision of measurements made under non-uniform conditions such as in two different laboratories. When precision is high, there is a high degree of agreement in data. ***Precision is calculated using standard deviation.***

3. Limit of Detection (LOD) is the lowest concentration of the analyte which can be detected above background by a method. There is always some error associated with any analytical measurement. An important determination that must be made is how significant a signal must be before it can be distinguishable from background. The accepted rule in analytical chemistry is that **the signal must be at least three times greater than the background noise.** It is important to note that an analytical method can never prove that a substance is not present; rather it can show only the limit which the sample cannot be detected.

   $LOD = Instrument\ Output_{blank} + 3xSD_{blank}$.

   Extrapolate from linear regression to determine concentration value of LOD.

4. Limit of Quantitation (LOQ) is the lowest concentration of the analyte that the method can quantify with acceptable accuracy and precision. Depending on the application, this is 5-10X the standard deviation of the blank.

   $LOQ = Instrument\ Output_{blank} + 10xSD_{blank}$.

   Extrapolate from linear regression to determine concentration value of LOQ.

5. Specificity is a measure of the extent to which a method can identify the presence of a compound in a sample without interference from other materials that are present. This is sometimes referred to as ***selectivity***. A very selective test will only give a positive result for the compound of interest.

6. Linearity is the ability of a method to give test results that are directly proportional to the concentration of the material of interest within a given concentration range. **The range** is defined as the limits of concentrations, from the lowest to the highest, that a method can measure with acceptable results. Tests and Assays have a range in which they exhibit linearity. For example, many assays for proteins display a standard curve that is linear at lower concentrations but flattens out at higher concentrations of the protein. Therefore, a

test for protein will be accurate only in the range of lower concentrations when the curve is linear.

7. Robustness is a measure of the capacity of a method to remain unaffected when there are small, deliberate variations in method parameters. It provides an indication of the method's reliability during normal use.

8. Ruggedness is the degree of reproducibility of the results obtained by the analysis of the same samples under a variety of conditions. Ruggedness provides a measure of reproducibility across variations of conditions normally expected, such as, from lab to lab and analyst to analyst.

STANDARD CURVES

Spectrophotometry can be used to detect the presence of compounds based upon the absorbance of light at a characteristic wavelength (e.g. p-nitroaniline absorbs light at 405 nm). The degree of absorbance is directly proportional to the concentration of the compound in solution over a linear range of concentrations. Mathematical models can be used to generate a curve fit, and this equation can then be used to determine the concentration of a test sample.

There are many methods of curve fitting that are employed in biosciences lab. The method chosen is dependent upon the assay methodology, the analyte being detected, the shape of the curve data, and current best practices to name only a few factors. Below is a brief summary of a few approaches common in a biosciences lab: linear regression, logistic regression, four and five parameter logistic regression. In this lab exercise, as well as the protein assay in the next lab, we will perform linear regression. While performing these assay, take a careful consideration as to the shape of the standard curve, and see if a different analysis may extend the range of the curve!

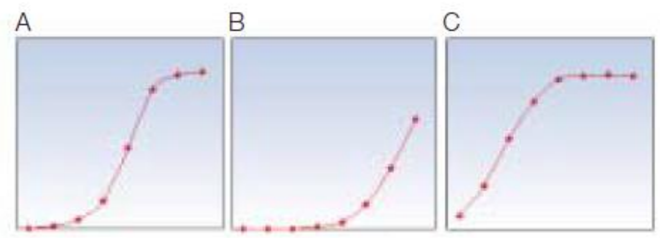


Fig. 3. Three standard curve shapes are commonly encountered when analyzing Bio-Plex cytokine assay data. A, sigmoidal or S-shaped curve; B, low-response curve; C, high-response curve.

The *linear range* is the part of the curve that is best described as a straight line. As absorption of the light increases to 100%, the instrument response becomes nonlinear. For UV-VIS spectrophotometry the limits of detection are typical ~ 2 absorbance units (99% absorbance) up to 3 absorbance units depending on the instrument. The main advantage of this method is its simplicity. A standard curve is a graphical representation of an instrument response (e.g. absorbance; *dependent variable*) to change in a parameter such as concentration (*independent variable*). It is best practice to plot the dependent variable (the measured value, such as absorbance) on the Y-axis of the graph & the independent variable (the known value that is measured, such as analyte concentration) on the X-axis. *Linear regression* may be used to find the equation of a line that 'best fits' the experimental data that falls within the linear range (the part of the curve that appears to be a straight line). This equation may be used to calculate the

concentration of a sample in solution for a given absorbance reading. The R^2 value can be utilized as an indicator of 'good fit,' meaning, how well a linear response is demonstrated by the data, and a way to validate the assay. A linear regression with an R^2 value >0.99 is excellent fit data and is a good indicator of how well the assay performed, and the confidence a technician can have using this standard curve to determine the concentration of a test sample.

A linear regression is not the only model for a standard curve, in fact, many labs use alternative models to extend the range of their curve. The most common non-linear curve used is a ***logistic regression***. A four-parameter logistic regression (***4PL***) is typically applied to a sigmoidal curve. This equation contains four parameters, hence the name, and as with the linear regression, the unknown can be determined using the 4PL equation of the line. A five-parameter logistic regression (***5PL***), as the name indicates, includes five variables, and is best used on curves that are non-symmetrical, such as with a low, or high-dose response. It is important to note that the R^2 value in logistic regression is not as appropriate as other methods for assay validation. More often a spike recovery is used, or in some situations, a back-calculation of the standards.

A ***spiked recovery*** is a simple method to assess the validity (accuracy) of the assay in determining the concentration of the test sample. Critical here for this approach is the use of a control with the same complexities as the text sample. In this respect, the spiked control can also act as an interference control, and determine if other components in a complex sample interfere with the assay, either positively or negatively. For example, in this lab exercise, we will spike a water sample with known concentrations of pyrogen. We will then determine the concentration of this spiked sample and compare it with the intended concentration. Spike recovery is generally reported in a percent, such as *"90% of the pyrogen spike into sterile water was recovered".*

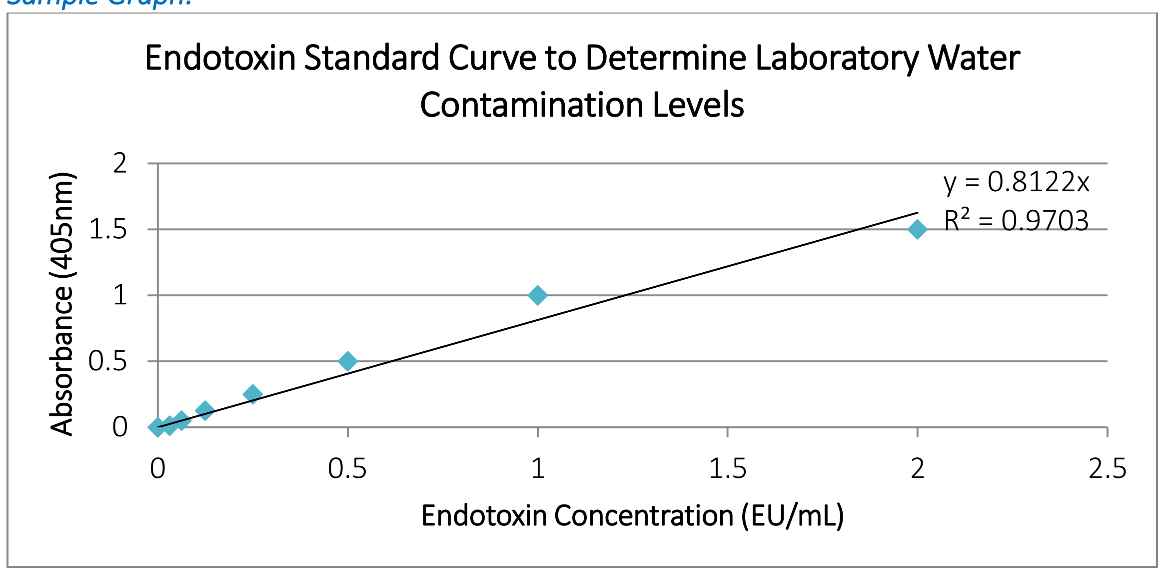
Graphing

Computer and software assistance is available to ACC students free of charge at the Learning Center on any ACC campus: http://www.austincc.edu/tutor/. Instructions on using Excel can easily be found online – try YouTube! Please refer to your BIOL1414 course notes on graphing. The instructor will provide demonstrations in class for the use of MS Excel.

In general, graphs should include the following:

1. An appropriately descriptive title.
2. Correct dependent and independent axis, with accurately labeled axes, and proper units.
3. Appropriate axis scale. Remember graph axes do not need to start at "0,0" unless you use that as a data point. Note: If you 'blank' an instrument or use a no-analyte negative control, you must use that data point in your data set, and anchor line at 0,0.
4. If you perform a linear regression, report the equation of the line and R^2 value on the graph. Remember, if you remove points from a curve to make it 'appear' more linear, you must do so with scientific and mathematical certainty. For example, provide both curves with the R^2 values to show improved linear correlation and justify your removal of those points. In general, at least 5 points in a standard curve is required for confidence in the data.

Sample Graph:



Limulus Amoebocyte Lysate Test: Pyrochrome®

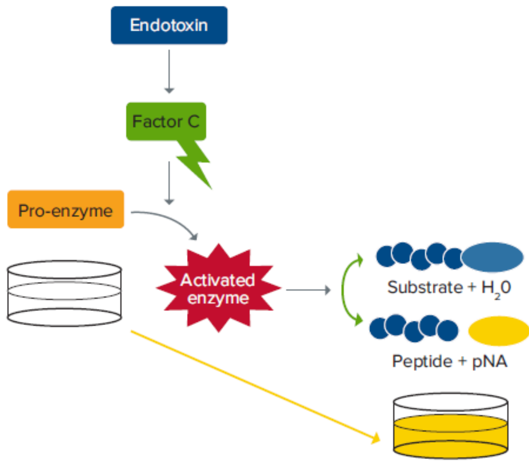
Non-sterile water that can be contaminated with microorganisms that may release toxic bacterial products, called ***Pyrogens***. *Pyrogens are derived from lipopolysaccharides that are found in the cell walls of gram-negative bacteria*. Pyrogens are not toxic directly but have an ability to induce a dangerous immune response in mammals. A person with a systemic gram-negative infection runs the risk of dying from septic shock when treated with an antibiotic that kills the bacterial infection. As the bacteria are killed, they release endotoxins which trigger the person's immune system resulting in septic shock.

The concentration of endotoxin is measured in Endotoxin Units or EU/mL. Tap water is frequently contaminated with endotoxins and should never be used in injections. According to ASTM requirement for water purity of purified water, Type I Grade A water must contain less than 0.03 EU/mL. Grade B has permissible contamination of up to 0.25 EU/mL.

In the 1970s, the FDA approved the *Limulus* Amoebocyte Lysate (LAL) test to detect endotoxin contamination in drugs, products, and medical devices. LAL is an aqueous extract from the Amoebocytes (blood cells) of the horseshoe crab *Limulus polyphemus.* Amoebocytes play a major role in pathogen defense. Amoebocytes contain a clotting factor that is released when bacterial endotoxin is encountered*. **There are three LAL current methodologies:*** gel-clot, turbidity and chromogenic. ***In this lab, we will be performing an endpoint chromogenic assay***.

Endpoint Chromogenic Assay for Endotoxin Determination For this lab, we will be using the "Pyrochrome Kit" (Cape Cod, Inc.). This kit is *a Limulus Amoebocyte Lysate (LAL) test for the detection and quantification of gram-negative bacteria endotoxin*.

The LAL reagent used in this lab is Pyrochrome®. It is a combination of a lyophilized extract of amoebocytes of *Limulus polyphemus* a colorless chromogenic substrate: Boc-leu-gly-arg-pNitroanilide. When endotoxin is present, enzymes in the LAL are activated resulting in a proteolytic cascade that cleaves the colorless artificial peptide substrate in the Pyrochrome® reagent. This proteolytic cleavage results in the liberation of the yellow product p-nitroaniline (pNA). This yellow product absorbs at 405 nm. Using the endpoint chromogenic method, the amount of pNA released is measured using a spectrophotometer, following a specific incubation time. A standard curve is generated illustrating the linear relationship between endotoxin concentration and absorption at 405 nm. Concentrations of unknown samples are interpolated from this standard curve.



Maximum sensitivity using this method is 0.005 Endotoxin Units (EU) per mL.

References:

1. Seidman & Moore, *Basic Laboratory Methods for Biotechnology: Textbook & Laboratory Reference*, 2nd edition. 2009. Prentice Hall. ISBN: 0321570146
2. VistaLab Technologies. "Performance Verification Protocol." 2010. Lit#L057-0163-001 RevC
3. Associates of Cape Cod *Limulus Amoebocyte Lysate. Pyrochrome. Instruction Manual*. 2007. PN000856 Rev005
4. Limulus Amoebocyte Lysate, Pyrochrome kit instructions. Cat# PN000856. Associates of Cape Cod Incorporated (Falmouth, MA).
5. Bio-Rad Tech Note 2861 (n.d.). *Principles of curve fitting.* Retrieved Feb 2017, from http://www.bio-rad.com/LifeScience/pdf/Bulletin_2861.pdf

UNIT 4: LAL ASSAY PROCEDURE

Materials

• Pyrogen-free serological pipettes • Micropipettes and pyrogen-free barrier tips • Multichannel pipette • Pyrogen free dilution tube • Pyrogen-free 96 well micro plate	• Pyrochrome Kit (Cat#PN000856, Assoc. of Cape Cod Inc.) • CSE (Cat EC010) • 10 ng/vial (or 20EU/mL) Endotoxin Control • LAL Reagent Water • Pyrochrome with reconstitution buffer	• Bio-Rad 96 well microplate reader • incubator at 37ºC ±1ºC • Vortex • 50% acid acetic • 3 types of lab water; tap, sterile, diH20 or list: ____________________

Hazard Communication

- ✓ ***Acetic acid can burn skin. Be careful when handling.*** *If any acid contacts skin immediately flush with lots of water. Wear protective gloves and eyewear.*
- ✓ *The toxicity of* **Pyrochrome** *has not been determined. However, prolonged or repeated contact of* **LAL** *with the skin has resulted in a Type I allergic reaction in some individuals. Use gloves and protective eyewear when handling these reagents.*

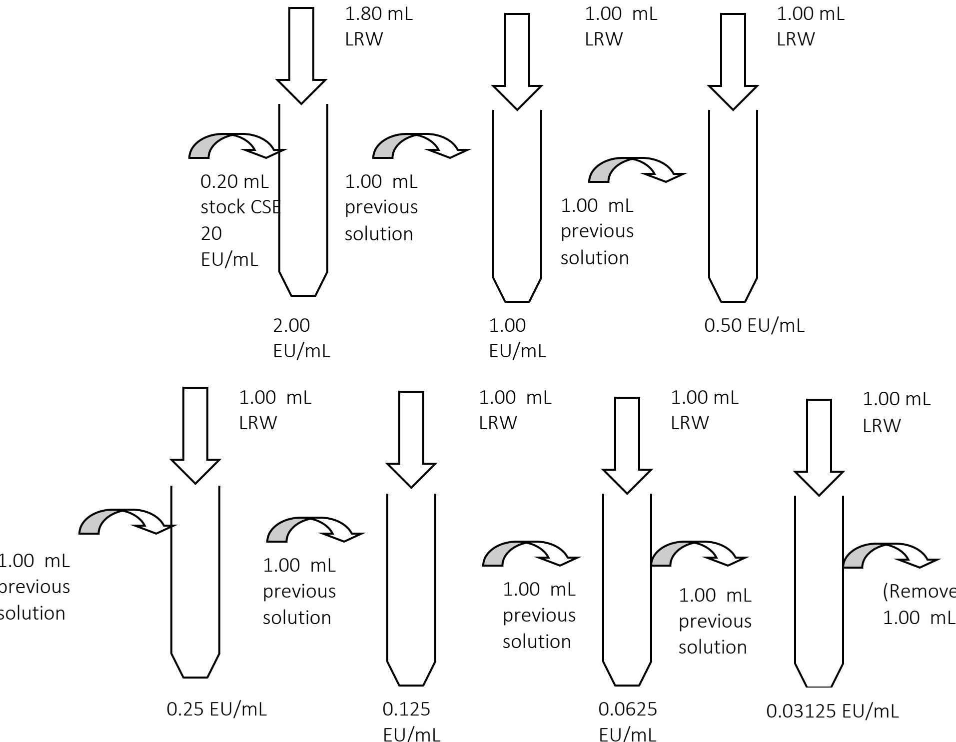
PROCEDURE

Part I: Preparing serial dilution of CSE, test reagents, and samples

1. Reconstitute the control standard endotoxin (CSE) with an appropriate volume of LAL reagent water (LRW) to obtain ***20 EU/mL.*** LRW is endotoxin-free water. This may have already been done for you, ask your instructor.
    a. The quantity of the control standard endotoxin (CSE) for each lot is different. Obtain the info for the specific lot from your instructor and reconstitute the standard with LRW to 20 EU/mL.
    b. Cover with Parafilm, vortex 30-60 seconds.
    c. Store reconstituted CSE at 2-8^{0}C, do not freeze. Vortex at least 30 seconds immediately before preparing the first dilution.
    d. Show calculations on pre-lab exercise

2. The figure below illustrates how to prepare one mL of each concentration using a 2-fold serial dilution from 2 EU/mL to 0.03125 EU/mL using a stock solution at 20 EU/mL. Show calculations on pre-lab exercise.

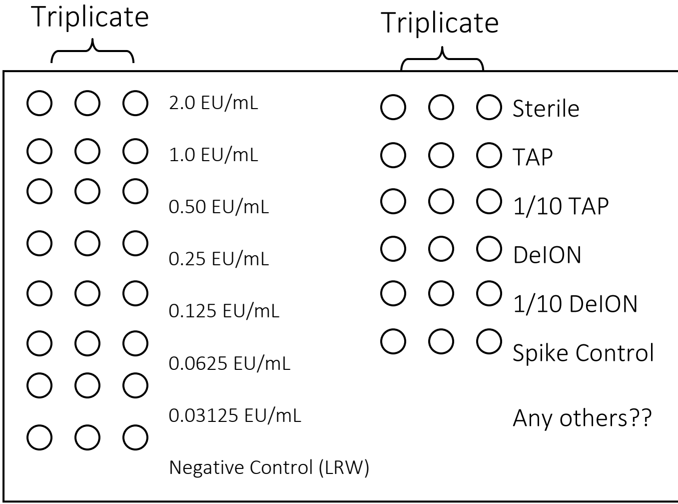
    NOTE: It is a good practice to prepare enough of each dilution, so there is an overage. You may choose to add the LRW to all the tubes first to allow for a smooth and quick dilution process, avoiding the need to change tips. PERFORM THE CALCULATIONS USING THE DILUTION EQUATIONS PROVIDED IN THE INTRODUCTION OF THIS LAB. Include them with your pre-lab.



2. Reconstitute Pyrochrome® reagent with 3.2 mL of reconstitution buffer. Invert gently, do not vortex. Let sit 5 minutes and gently remix just before use. ***Use within 8 hours.***
3. If not prepared for you, prepare 50% acetic acid (to stop the reaction) by mixing the same volume of glacial acetic acid and LRW. Prepare two mL total volume.
4. Prepare "spiked positive control" by adding 1ul of 20 EU/mL into 200 ul of sterile water. Calculate the EU/mL of your spiked sample and record in your notebook. Mix by vortexing. This is a common control used to determine if there is anything in the sample itself that may interfere with the assay performance.
5. Collect each of your water samples tested in lab unit 2 in the pyrogen-free collection tubes provided. Do NOT collect water in beakers! Turn the tap on and allow the tap to run for 5-10 seconds before collection.
6. Prepare your water samples by diluting each one 1/10 (or higher depending on your water quality results from lab 2). Use LRW for dilutions, in the pyrogen-free tubes. *WHY?!*

Part II: Endotoxin determination in Laboratory water samples

1. Dispense 50 µL of each water sample used in lab 2, into the microtiter plate wells as shown below. You may consider several dilutions based on the results obtained from water quality test results from lab 2. ***Be sure to use pyrogen-free microtiter plates!***
2. Incubate at 37°C for 10 minutes.
3. After incubation, pipette 50 µL of Pyrochrome® into each well. Utilize a multichannel pipette, if available. Gently pipet up and down once to mix reagent with the sample, avoid generating air bubbles. *Note: Since this is an enzymatic colorimetric assay, assay quality will depend on how quickly you can aliquot the reagent into every well.*



2. Return the microtiter plate to the 37°C ±1°C incubator for ***30 minutes***. Record time.
3. After the incubation time, dispense 25 µL of 50% acetic acid to each well, mix gently, do not generate bubbles. Perform these aliquots as instructed for the Pyrochrome®, above. Record the actual incubation time after the stop solution has been added to every well.
4. Ensure you have few air bubbles before reading the plate (pop with a clean micropipette tip). Place the plate in the reader and read the absorbance at 405 nm. Export data into Excel and save it to an external disk for future analysis and printing.

PART III – Data Analysis

You may need to refer to your BIOL1414 lab manual notes to generate a standard curve and to use linear regression to determine the pyrogen value of your water samples. Use MS Excel to manipulate data.

1. Using MS Excel, calculate the mean and standard deviation of each sample. Use the same significant figures as equipment: 0.000.
2. Subtract the zero (no analyte) absorbance average value from each of your standard curve, and test samples mean and report results on the same table. Mean-Blank.
3. Print a copy of your data table for your lab report.
4. Prepare a standard curve of Absorbance at 405 nm versus concentration of CSE, using all data. Do not discard any data at this point. Remember to properly label your graph! Draw a CURVE fit. Print a copy and include in your lab report.
5. Carefully look at your graph and determine the linear range of your assay. Repeat the standard curve by including *only those points in the linear range* and force the linear regression through the X/Y intercept (0,0).
6. Determine the equation of the line and the R^2 value and report these values directly on your graph. Report the linear range on your graph as well.
7. If your R^2 value is less than 0.96, you may need to remove an outlier point and re-plot. Note: A valid standard curve has at least 5 points. HINT: Evaluate your triplicate data standard deviation results.
8. Print out a copy of your final standard curve, with linear regression and R^2 value on curve. Include this second graph with your report.

9. Determine the limit of detection (LOD) and limit of quantitation (LOQ) of your assay. $LOD=Ab_{blank} + 3xSD\ Abs_{blank}$, $LOQ= Abs_{blank} + 10xSD\ Abs_{blank}$. Using the linear regression, determine concentration values. Report the values on your graph and discuss in your analysis section of your report.
10. Using your linear regression, determine the endotoxin contamination of the spiked control. Discuss result in your analysis. Report this data on your curve.
11. Using your linear regression, determine the endotoxin contamination in the water samples. Use only those values that fall within the RANGE of your standard curve. NOTE: If any samples were diluted, use ONLY the dilution that falls in the range of the curve, and multiply by the dilution factor when reporting the results. Report these results on your curve
12. Create a table with final results of all test samples and spike control. You may want to INCLUDE lab 2 water results.

NOTE: When reporting your endotoxin contamination results only report the dilutions that resulted in absorbance readings that fell into the linear range of your standard curve.

Laboratory Unit 4 Analysis & Review

1. ***Pre-lab exercise***, lab notes, forms and calculations
2. ***Summary of raw data:***
    a. Create a table and summarize your LAL data as outlined in instructions.
    a. Include the TWO graphs as outlined in the instructions. Graph 1 will have the original data. Graph 2 will have only the linear regression data with the equation of the line, R2 value, and LOD and LOQ of the assay.
    b. Report the Endotoxin contamination concentration of each of the water types tested in table form, include data from previous lab.
3. **Analysis of Results**
    a. Discuss the R^2 value, linear range, LOD and LOQ of your assay. Include Negative and positive spiked control.
    b. Discuss the results of your LAL assay. Include the pyrogen concentration of your water sample. You may want to research the expected LAL concentrations of the water you tested.
    c. Discuss how the assay results relate to your heterotrophic plate count data.
    d. The main purpose of the last two labs is to QUALIFY the water you will be using throughout the semester. Research what components are included in a Certificate of Analysis (CoA) that comes with reagents. You may ask your instructor, or lab manager for a sample CoA, or the CoA for the CSE Standard. You may work as a class, generate a CoA for EACH of the different water tested. Photocopy and share with the class, and include with your lab report. Include a copy in the Reagent Inventory Binder, and provide the Lab manager with a copy.
4. **Conclusions**

    State the experimental findings of this laboratory exercise: The Pyrogen content of each water. Restate and summarize the results, and state implications, as well as any future work recommended. For example, based on your results, what water do you recommend to use to prepare your solutions for the remainder of the semester?

LAB UNIT 5: PROTEIN CONCENTRATION

INTRODUCTION

Laboratories often use assays to quantify compounds for a number of reasons: to determine the effectiveness of each step in a purification procedure, to determine the purity of a product, and to determine the concentration of an analyte. A **protein assay** is a test that is used to detect the presence of a protein. A **qualitative assay** provides non-numerical data and simply indicates whether an analyte, or in this case a protein, or is present. A **quantitative assay** provides numerical data and is used to determine how much analyte is present.

There are several absorbance and fluorescent-based methods for determining the concentration of total protein. In this laboratory exercise, you will use a UV/VIS spectrophotometer to assay protein content in a liquid sample. A UV/VIS spectrophotometer illuminates a sample with light of a defined wavelength between 200 and 750 nm. Molecules that contain double or triple bonds can absorb light at one or more wavelengths. The wavelength(s) absorbed is characteristic of the molecular structure. The percentage of light absorbed is directly proportional to the concentration of molecules in solution. For example, pNA which contains a benzene ring absorbs light at 405 nm giving pNA its characteristic yellow color. In proteins, aromatic amino acids such as tryptophan & tyrosine absorb light at 280 nm. Absorbance at 280 nm may be used as a quick, nondestructive estimate of the presence and concentration of protein.

The colored product produced when the Bradford dye binds protein and absorbs the maximum amount of light at 595 nm, and the more protein present, more light is absorbed. Once the Absorbance or color intensity has been measured, it must be compared to a standard curve. The standard curve is created from a series of known amounts of analyte to be assayed that is treated in the same way as the sample. Ideally the standard curve is created using the same protein as your unknown. However, for most applications, due to cost and availability of a specific protein standard, the use of bovine serum albumin (BSA) is often employed.

The Absorbance units (y-axis) are plotted against the concentration (x-axis). This is typically a linear relationship with upper and lower concentration limitations. An unknown sample absorbance is compared to this standard curve so that the concentration of substrate in the sample may be determined. This may be done using an equation of the line of the linear portion of the standard protein curve. The light absorbed by a sample may be determined by monitoring the light transmitted (T). Absorbance (A) is given by: **$A = -\log T$ or $A = (EL)c$ or $A = mC$**

Beer-Lambert Law: $A = (EL)c$ or $A = mC$

Where,
A = the absorbance
E = the absorptivity for that compound at that wavelength
L = the path length
c = (or C) the concentration
(EL) = m = the slope

Absorbance increases with increasing solution concentration, path length & molar absorptivity of the solution. For low to moderate concentrations absorbance is directly proportional to concentration. Absorbance can be related to concentration by comparison to a standard curve prepared from solutions of known concentration. Ideally the standard curve is created using the same protein as your unknown. However, for many applications, due to cost and unavailability of a specific protein standard, the use of bovine serum albumin (BSA) is often employed.

The line on a standard curve has an equation Y= mX + b where m = slope and b = the Y intercept; in this case the Y intercept equals zero (subtract blank from samples so that the zero-protein control has zero absorbance). Thus, the equation for the line is simply:

A=mC or Absorbance = (slope of standard curve) x (concentration of analyte)

Two parameters are known to affect the steepness of the slope:

1) ***The chemical and physical nature of the analyte*** or material being measured, which is going to be specific for each pure analyte. This inherent tendency of a material or analyte to absorb light at a specific wavelength is called its absorptivity. The name given to this constant is absorptivity constant or extinction coefficient also known as E.
2) ***The path length of the sample*** is usually 1 cm since cuvettes are usually 1cm wide, is designated as L in the Beer's Law equation. If a different path length is used, you must apply that path length into the Beer's Law equation (divide Abs by path length in centimeters). Some spectrophotometers do this automatically.

Good laboratory practice requires that the standard curve and the sample assays are performed using use the same reagents, the same pipettes, on the same day and read within 10 min of application of the reagent. This eliminates error due to variations in the assay from day to day.

In the first part of this lab, a stock bovine serum albumin (BSA) solution is prepared and its actual concentration determined. Since it is being used for the standard curve, it is important that its actual concentration is known. In the second part of the lab, the stock solution is diluted to make the standard curve. The unknown samples will also be diluted to hopefully bring their protein concentrations within the range of the assay. The protein color reagent will be added to all samples, including a no-protein sample. This negative control will have water (or the solvent used to dilute the standard curve in) in place of sample. After an appropriate incubation time (5 min minimum to 1 hour maximum), the resulting color is read in the spectrophotometer.

Determination of Protein Concentration in a Solution

There are several absorbance and fluorescent methods routinely used to determine the concentration of protein in a solution, each has advantages and limitations such as protein-to-protein variability, interference with contaminants, accuracy, sensitivity, and reproducibility.

Absorbance methods include measurement of the protein's intrinsic UV absorbance and three such methods, generate a protein-dependent color change; the Lowry assay, the Smith copper/bicinchoninic assay and the Bradford dye assay. Most of these methods are used

routinely in almost every biochemical laboratory, depending on the application of the concentration data.

UV absorbance is based on absorption of the amino acids tyrosine and tryptophan (both have ring structures) in the protein. This method, to be accurate and quantitative, requires that a pure protein with known extinction coefficient, otherwise known as an absorptivity constant, be used, in a solution free of interfering (UV absorbing) substances. For example, a pure solution of bovine serum albumin (BSA) has an E = 0.667 $mg^{-1}cm^{-1}ml$ (43824 M-1 cm-1) so that at ***A_{280} a 1 mg/ml solution in a 1 cm path length cuvette has an absorption of 0.667***.

If the extinction coefficient is unknown, the concentration of the protein can be estimated by using either of the following equations:

A_{280} = 1 A (ml/cm.mg) x [Conc.] (mg/ml) x 1 (cm)
A_{205} = 31 A (ml/cm.mg) x [Conc.] (mg/ml) x 1 (cm)

Different proteins have widely different extinction coefficients at both 280 nm and 205 nm. Therefore, concentration estimates obtained this way should be understood as an ***estimate***. This method is used as a quick estimation of protein concentration, such as monitoring when proteins are eluting from a column (and frequently used in LC monitoring equipment). *UV absorbance is a non-destructive form of protein analysis*. It is used when the protein sample volume is too low to spare any for concentration determination (as outlined below). Many colorimetric assay methods destroy the sample.

The **Bradford dye assay** is a protein determination method that utilizes the binding of Coomassie Brilliant Blue G-250 dye to proteins. The dye exists in three forms: cationic, neutral and anionic. Under each condition, the dye absorbs at a different optimal wavelength. Under strongly acid conditions, the dye is most stable as a doubly-protonated red form. Upon binding to protein, however, it is most stable as an unprotonated, blue form.

cation (red) <=> neutral (green) <=> anion (Blue-Protein)
(470 nm) H^+ (650 nm) H^+ (595 nm)

Coomassie Brilliant Blue G-250 dye binds primarily to basic (arginine) and aromatic amino acid residues. This results in different binding capacity for different proteins. Therefore, it's best to use the protein of interest as your standard instead of BSA.

The Bradford assay is faster, involves fewer mixing steps, does not require heating, and gives a more stable colorimetric response than the assays described above. Like the other assays, however, its response is prone to influence from non-protein sources, particularly detergents, and becomes progressively more nonlinear at the high end of its useful protein concentration range. The response is also protein dependent, and varies with the composition of the protein. These limitations make protein standard solutions necessary.

In this lab exercise you will be using Bio-Rad's 1X dye reagent. There are two general protocols you can follow using this reagent: The Standard Protocol, and the Microassay Protocol. The

difference between these two protocols is the linear range each has. The Standard Protocol covers the linear range from 125-1000 ug/mL, whereas the Microassay protocol covers a much smaller linear range from 1.25-10 ug/mL. In the Microassay, a 1:1 ratio of sample:dye is used to increase sensitivity at this lower range. In the standard assay a 1:50 sample:dye ratio is used. The estimated value of your unknown will dictate which protocol you will want to follow.

Each of these assays has different formats or platforms you can use to perform the assay on. You can perform the assay in cuvettes and determine absorbance readings in a spectrophotometer or you can perform the assay in microtiter plates and determine absorbance readings on a plate reader. Note, that the method you chose may result in a different dynamic range of your assay. ***Note: *Before starting your pre-lab exercise calculations, consult with your instructor to determine which assay you will be performing****

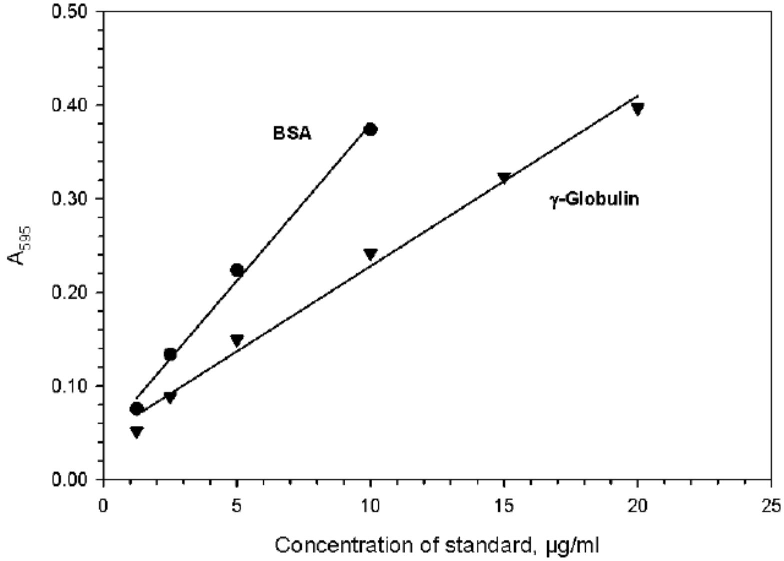


Figure from: Bio-Rad Protein Assay Reagent Instructions. (Hercules, CA). 4110065 Rev A

The Qubit Fluorometer for Protein Assays

The Qubit fluorometer for protein assays has an advantage of low protein-variability, relatively fast (15min for protein), simple to use, accurate, precise, with a high sensitivity down to 0.025 mg/mL. This curve-fit protein assay requires small sample volume (1-20ul). It is insensitive to common contaminants such as nucleic acids and has a low protein-to-protein variability. The Qubit assay is now a common methodology for protein (DNA, and RNA) quantitation in bioscience labs. To learn more: https://youtu.be/y7DLoGxg0k0

References:

1. Seidman & Moore, "*Basic Laboratory Methods for Biotechnology: Textbook & Laboratory Reference*", 2nd edition. 2009. Prentice Hall. ISBN: 0321570146
2. Bio-Rad Protein Assay Reagent Instructions. (Hercules, CA). 4110065 Rev A
3. Qubit Fluorometer Application Note (n.d.). Accurate and sensitive protein quantitation. Invitrogen. Retrieved from https://www.thermofisher.com

PART I: PROTEIN DETERMINATION USING BEER'S LAW PROCEDURE

Materials

- ☐ Unknown protein sample
- ☐ Low Volume (NanoDrop) spectrophotometer. Model: ___________
- ☐ Micropipettes and tips

Hazard Communication

Ensure the wiring for the spectrophotometer is not frayed and that the instrument is not in or near water. Wear PPE.

PROTOCOL

Estimating the concentration of unknown. The objective of this lab is to estimate the protein concentration of your unknown, and using that estimate prepare dilutions that will fit inside the Bradford standard curve you will prepare below.

1. Use the SOP for the NanoDrop low-volume spectrophotometer to determine the absorbance of your unknown at 280 nm. Unless otherwise instructed, use water to blank your unknown.
2. Print out the spectral graph from the NanoDrop and include with your report.
3. Calculate the estimated concentration of the solution using Beer's Law: A = EcL. <u>The NanoDrop does this for you.</u>
   a. When determining estimation of concentration of an unknown protein, or a protein where E is not determined, assume that E=1.
   b. If you have a spectrophotometer that does not have a 1cm path length, you MUST correct for the path length. You can correct for this easily by dividing absorbance by the path length. The NanoDrop has a program that corrects for path length – notice this on the printout.
4. From your estimation determine dilutions you will make of you unknown to ensure it falls within the linear range of the assay.

PART II: PROTEIN DETERMINATION USING BRADFORD ASSAY

MATERIALS

- ☐ Bradford reagent: 1x Dye Reagent (Bio-Rad, Cat# 500-0205) *warm to ambient temperature
- ☐ Certified 2 mg/ml BSA standard solution (Bio-Rad, Cat#500-0205)
- ☐ Unknown protein sample (same as in part I)
- ☐ Multichannel micropipette, and unopened p200 tip boxes
- ☐ Set of micropipettes and tip boxes
- ☐ Reagent reservoir (for multichannel pipet)
- ☐ UV/VIS plate reader with microtiter plates. Model: _______________
- ☐ 10, 2 mL microcentrifuge tubes

Hazard Communication

*Ensure the wiring for the spectrophotometer is not frayed and that the instrument is not in or near water. Wear gloves when handling Bradford Reagent. This reagent **will** stain your clothes and skin.*

MICROASSAY PROTOCOL:

(Bradford reagent linear range for the microassay is 1-25 µg/ml protein):

1. Remove the 1x dye reagent from the 4°C storage and let it warm to ambient temperature. Note - invert the dye reagent a few times before using.
2. Perform calculations on diluting the unknowns. Each unknown should be diluted to fit into the linear range of the standard curve. It is recommended to include an undiluted, and two dilutions of the unknown. Include dilution instructions and calculations in pre-lab.
3. Perform dilutions of the unknowns in 1.5 mL centrifuge tubes. Use water provided.
4. Leave unknown sample dilutions at ambient temperature while preparing BSA standards as outlined below.
5. Calculate the standard dilutions. ***Do this *before* class.*** Include dilution instructions and calculations in your prelab.

   Prepare 500ul-1mL aliquots of each standard using a 2 mg/ml (2 ug/ul) stock in 1.5 mL centrifuge tubes. Standards: 0, 1.625, 3.25, 7.5, 15, 30, 60, and 120 µg/ml BSA. Dilute standard BSA using water. Note: Performing the assay one time will require 500ul. If you would like to repeat the assay, prepare 1mL of each standard.
6. Fill out a plate key form to map how you will aliquot the samples into the microtiter plate. By convention, start from the top left the corner, lowest down to the highest concentration rows, in triplicate columns. Do not leave empty wells, as you will be adding reagents using a multichannel pipette.
7. Aliquot 150 ul of each BSA standard dilution series, each unknown (undiluted and diluted), and include a blank. All samples should be ***in triplicate.***
8. Quickly add 150ul of 1X dye reagent to each well using a multichannel micropipette. Mix by pipetting up and down vigorously without blowing out air from the micropipette. ***Do NOT create bubbles; they will interfere with absorbance readings!***
9. Incubate for 5 min at ambient temperature on the bench top. Remember to record the true ambient temperature in your notebook. Samples should not be incubated longer than 1 hour before reading the absorbance.
10. After incubation, record true incubation time in your notebook. Set your spectrophotometer (plate reader) to 595 nm and read samples. Export into an MS Excel spreadsheet, and save on a USB key.
11. Print out the raw data table and insert into your notebook. Remember to label your raw data very well. And include a plate key form.
12. Examine the absorbance readings of the standards and samples.
13. Ensure that at least one dilution of each of the unknowns falls in the standard curve (no higher or lower than the readings for the standard curve). If not, dilute the sample again, and repeat the assay before you leave.

Data Analysis

1. Using MS Excel, prepare a data table with all three absorbance readings for your standards as well as your samples.
2. Calculate the mean and standard deviation of each. Report results on the same table.
3. Subtract the zero (no analyte) absorbance values from each of your means and report results on the same table (Mean-Blank). Print out a copy of results table and include with your report.
4. Prepare a standard curve of Absorbance versus BSA concentration as directed in the introduction of the lab exercise – use all standard data. If the standard curve is a curve, draw a best fit <u>curve</u> (polynomial series 2 works well). Print out a copy and include with the report.
5. Carefully look at your graph and determine the linear range of your assay. Repeat the standard curve by including only those points in the linear range. Select linear regression, R2 value and force the linear regression through your X/Y intercept (0, 0).
6. If your R^2 value is less than 0.96, you may need to remove an outlier point and re-plot. Note: A valid standard curve has at least 5 points. Explain in your lab report the scientific and mathematical justification for removing points from your standard curve.
7. Determine the limit of detection (LOD) and limit of quantitation (LOQ) of your assay. LOD= Abs blank + 3xSD Abs_{blank}, LOQ= Abs blank + 10xSD Abs_{blank}. <u>Note – if you subtract your blank values the "Abs blank" is zero and you use only the SD of Blank.</u> Show your calculations in your lab report.
8. Print out a copy of your final graph. Include LOD, LOQ, linear regression, and R2 value directly on the graph.
   NOTE: LOD and LOQ are units of concentration! You must put in the absorbance value into the equation of the line to determine the concentration of the LOD and LOQ. They are not reported as absorbance units. Report the values on your graph and discuss in your analysis section of your report.
9. Using your linear regression, determine the protein contamination in the unknown sample using *only* the best dilution that fit in the middle of your standard curve. If any samples were diluted, <u>remember to multiply by the dilution factor</u> when reporting the results. Show your calculations in your lab report.
10. Discuss the validity of your assay and the confidence of your results based on the assay statistics you acquired; R2 value, LOD, LOQ, the linear range of the assay (LOQ to the highest point in the linear range).
11. Compare the result of the unknown protein sample concentrations obtained from the spectrophotometer analysis and the Bradford assay.
12. Ask your instructor for the true value of the unknown. <u>Compare your results to the true value. Which technique gave you a closer result to the true value</u>. Why?

Laboratory Unit 5 Analysis

For your Laboratory Report, remember to include the following:

1. ***Prelab Exercise***, calculations, forms and experimental observations.

2. ***Summary of raw data:***
   a. Include data table as instructed. It should include all processed data.
   b. Use Microsoft Excel to generate TWO BSA standard curves; one curve with all the points and another with only the linear portion, reporting the linear regression, LOD, LOQ and R^2 value. Remember to plot absorbance readings on the Y-axis, concentrations on the X-axis. Label graphs appropriately; include a descriptive title, axis labels with units.
   c. Include all calculations of your data in your lab report: LOD, LOQ, determination of the concentration of the unknown.

3. ***Analysis of results***
   a. Discuss the validation of your Bradford assay using the linear range of your graph and the R^2 value. Include the limit of detection (LOD) and limit of quantitation (LOQ). In your discussion refer to ***Bio-Rad's reported linear range*** of the assay performed. How do they compare to Bio-Rad's claims?
   b. Interpolate, using the Bradford BSA standard curve linear regression, to determine concentration values of ONLY the unknown dilution that fall within the curve. Discuss these values.
   c. Discuss the concentration of your unknown determined from the two methods. Are they close? Discuss your results as they relate to the true value provided to you by your instructor.
   d. What are the advantages and limitations of each method? Why would you choose one method over the other?

4. ***Conclusions***
   Restate the final results of your unknown concentration using both techniques. Restate the true value of the unknown and the implication. State any recommendations to these results – for example, after your analysis did you realize you needed another dilution of your unknown?

LAB UNIT 6: ENZYME KINETICS

INTRODUCTION

One of the more essential functions of proteins is that of ***catalysis***. Enzymes are biological molecules that help reactions in the cell to occur up to 10^{20} times faster than they normally would on their own! Without the aid of enzymes, these reactions would take place too slowly for the organism to survive! Except for catalytic RNA, ***most enzymes are globular proteins***.

Virtually all chemical reactions of living organisms are mediated by enzymes. Therefore, their characterization is crucial for the understanding of biology. The three-dimensional structure of the enzymes creates a characteristic pocket or active site into which the substrate or substrates fits. This allows for the reaction to occur without using up the enzyme—hence it is a catalyst.

The rate at which an enzyme catalyzes a reaction is characteristic for that enzyme at a given pH, temperature and ionic strength. Therefore, rate kinetic studies are often performed on enzymes. The rate, usually given as K_m, is related to the rates at which the enzyme-substrate complex is formed and the rate at which product is made. Thus

$$\text{Enzyme + Substrate} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Enzyme-Substrate complex} \xrightarrow{k_2} \text{Enzyme + Product}$$

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P$$

The ***Michaelis rate for the enzyme, K_m,*** is defined as,

$$\frac{k_{-1} + k_2}{k_1} = K_M$$

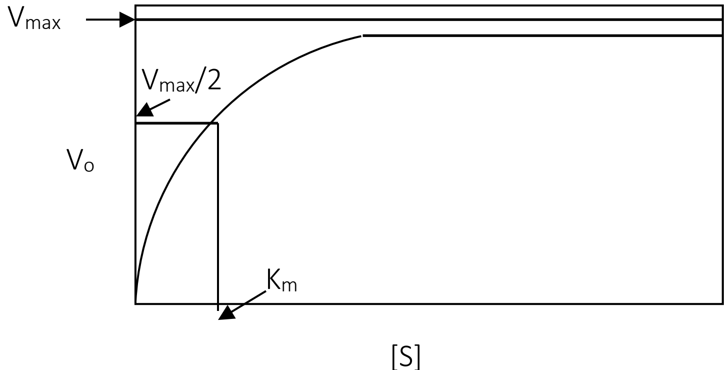
And has units of concentration. In fact, K_m ***is defined as the substrate concentration at which the enzyme catalyzes ½ the maximal velocity of the reaction.*** Note, k_{-1} is involved in dissociation of the enzyme-substrate and k_1 is involved in the formation of the complex. The question becomes how to determine this K_m.

Michaelis and Menten found that they could measure the velocity of the reaction at different concentrations of substrate. Their equation for the reaction:

$$V_o = \frac{(V_{max})\,[S]}{K_m + [S]}$$

Where, V_o = velocity
[S] = substrate concentration
V_{max} = maximum velocity of the enzyme

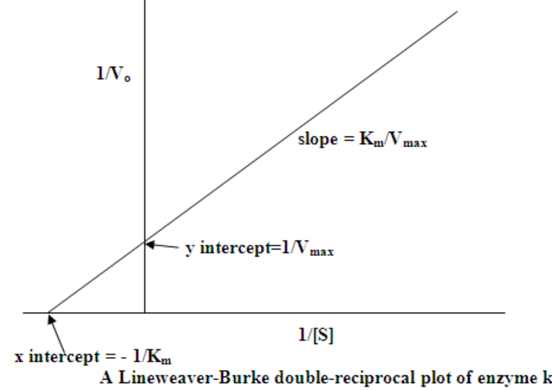
V_{max} is reached when all the enzyme is bound to substrate. Plotting V_o (y-axis) vs. [S] (x-axis) produces the following graph:



The V_{max} is hard to determine experimentally. *Lineweaver and Burke rearranged the Michaelis-Menten equation* to a more useful form:

$$\frac{1}{V_o} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

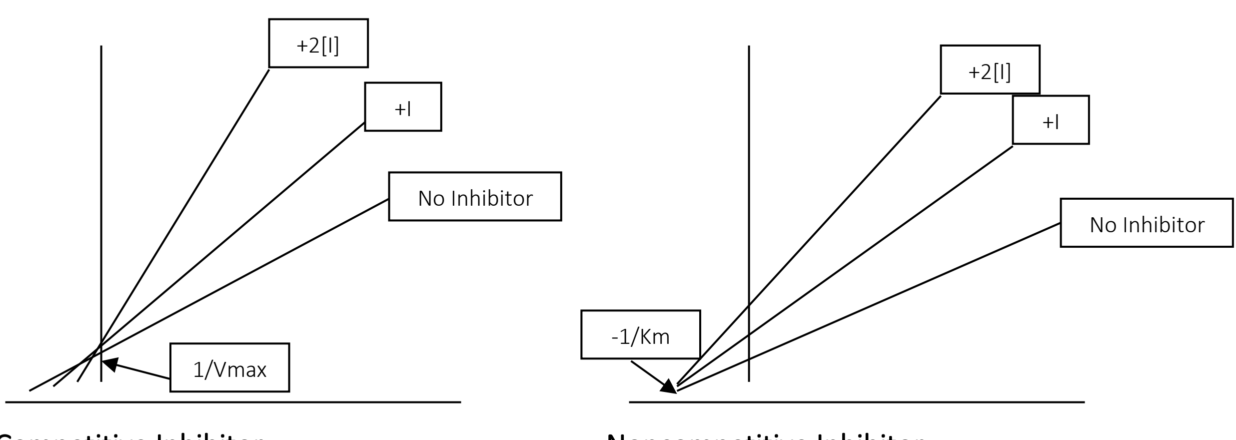
Note, this is identical to the plot of a straight line: $Y = mX + b$. In the Lineweaver-Burke equation, $Y = 1/V_o$; $X = 1/[S]$; the slope, $m = K_m/V_{max}$ and the Y-intercept, $b = 1/V_{max}$ (The X-intercept is $-1/K_m$.). Therefore, an experiment may be performed in which the velocity of a reaction (in this lab measured as the production of color which may be read in a spectrophotometer) is determined at different concentrations of substrate. By using the Lineweaver-Burke equation to plot the results, the K_m for the enzyme may be determined. A typical Lineweaver-Burke plot of an enzymatic reaction is given in the figure below.



A Lineweaver-Burke double-reciprocal plot of enzyme kinetics

Competitive or noncompetitive inhibitors may inhibit the activity of enzymes. Competitive inhibitors are molecules that sit in the active site of the enzyme and prevent the entry of the substrate. Competitive inhibitors frequently look similar to the substrate. A Lineweaver-Burke double reciprocal plot of a competitive inhibitor will have the same Y-intercept as that of the substrate. V_{max} remains the same.

Noncompetitive inhibitors do not sit in the active site but will attach to an allosteric site and change the shape of the enzyme and therefore alter the active site. The changed enzyme shape results in preventing the substrate from being converted into the product when bound to the enzyme. K_m (binding affinity) is unaffected since the inhibitor does not affect binding to the active site. In a Lineweaver-Burke double reciprocal plot of a noncompetitive inhibitor, the X-intercept, -1/Km is the same as that for the substrate. Thus, the two types of inhibitors may be distinguished from each other.



Competitive Inhibitor **Noncompetitive Inhibitor**

The value of Knowing K_m and V_{max} for an Enzyme: understanding how an inhibitor works are important, especially in the pharmaceutical industry; but what can K_m and V_{max} tell you about an enzyme? K_m is the substrate concentration that is required for the reaction to occur at half V_{max}. A high K_m means it takes a greater concentration of substrate to be half saturated. For most enzymes, K_m lies between 10^{-1} and 10^{-7} M and depends on the substrate and environmental conditions such as pH, temperature, and ionic strength. K_m is also related to the rate constants of the individual steps in the catalytic scheme (i.e. formation and dissociation of the enzyme-substrate complex). Under conditions where the dissociation of ES is much greater than the formation of P, K_m is a measure of the strength of the ES complex. ***If K_m is high, binding affinity is small***. Km increases with a competitive inhibitor because it is competing with the active site.

The maximal velocity of the enzyme, V_{max}, reveals the turnover number of an enzyme, which is the number of substrate molecules converted into a product by an enzyme molecule in a unit time when the enzyme is fully saturated with substrate. The turnover numbers of most enzymes with their physiological substrates fall in the range of 1 to 10^4 per second. ***A low V_{max} means the enzyme does not convert as much substrate to product.*** V_{max} decreases with a non-competitive inhibitor because it does not compete with the active site. V_{max} is unaffected with a competitive inhibitor because the presence of the inhibitor can be overcome by higher substrate concentrations.

The purpose of this experiment is to determine the V_{max} and K_m values for wheat germ acid phosphatase. Phosphatase is an enzyme that removes a phosphate group from a molecule. The substrate used in this experiment is *p*-nitrophenyl phosphate. The velocity of the reaction is

monitored by measuring the absorbance of the colored product at 405 nm in a spectrophotometer. *In Part II, two inhibitors of the reaction, sodium phosphate, and sodium fluoride are used;* one is a competitive inhibitor, and the other is a noncompetitive inhibitor. It is your task to determine which the competitive inhibitor is and which the non-competitive inhibitor is.

References:

1. Seidman & Moore, "*Basic Laboratory Methods for Biotechnology: Textbook & Laboratory Reference*," 2nd edition. 2009. Prentice Hall. ISBN: 0321570146
2. Campbell & Farrell, Biochemistry, 6th Ed. Thomson Brook/Cole. ISBN: 0495390410

ENZYME ASSAY PROCEDURE

Materials

o **AT Buffer:** (0.01 M sodium acetate/acetic acid buffer, pH 5 with 0.01% Triton X-100; 1 L for 4 groups) o **20 mg/mL Wheat germ acid phosphatase**: 200mg enzyme at 0.5 U/mg dissolved in 10 ml 50% Glycerol in AT buffer (keep on ice) o **100 mM p-nitrophenyl phosphate**: 250 mg/6.7 ml AT buffer; FW = 371.1 g/mole o **1 M KOH** o **100 mM stock sodium phosphate**, **dibasic** (0.355 g in 25 ml AT buffer; FW = 142.0 g/mole) o **1M stock sodium fluoride** (0.21 g in 5 mL AT buffer; FW = 42.0 g/mole)	o 1, 50-ml graduated cylinder o Set of Micropipettes and tips o 5-ml and 10-ml serological pipettes and pipet-aid o Test tubes o Multichannel pipette o 2, Multichannel buffer reservoirs o UV/VIS plate reader (model:__________________ o 1, microtiter plate for VIS o 1, timer

Hazard Communication

KOH is caustic. As part of your pre-lab exercise, research the MSDS information on the hazards of KOH. Summarize and include in your pre-lab safety section.

PROTOCOL:

Part I: Prepare the buffer with 0.5 mM sodium phosphate in AT buffer (AT+P) and 0.1 mM sodium fluoride in AT buffer (AT+F)

1. Prepare 50 mL AT+P (0.5 mM) from 100 mM sodium phosphate stock solution.

   First, calculate the amount of 100 mM stock solution needed. Then transfer correct amount of the stock solution to a 50-mL conical tube, add AT buffer to the final volume of 50 mL. Cape the tube and label it.

   $C1V1 = C2V2$ $100*V1 = 0.5*50$ $V1 = (0.5*50)/100 = 0.25$ mL

   250ul of the 100mM stock + 49.75 mL AT buffer

2. Prepare 50 mL AT+F (0.1 mM) from 1M sodium fluoride stock solution.

CC BY NC SA

First, dilute the 1M stock by 100-fold to 10 mM solution (100 ul of the 1M stock + 9.9 mL of buffer). Calculate how much of the 10 mM solution is needed to prepare 50 mL 0.1 mM solution. Transfer the correct amount to a 50-mL tube and add AT buffer to volume.

C1V1 = C2V2 10*V1 = 0.1 *50 V1 = (0.1*50)/10 = 0.5 mL

500 ul of the 10mM stock + 49.5 mL AT buffer

Part II: Prepare the substrate solutions

1. Prepare a 2-fold dilution series of substrate (from 1.6 mM to 0.025 mM) in AT buffer (sub only)
   a. Label 8 tubes from 1-8. Add 2 mL AT buffer to tube #2 to #8. Put the #8 tube on the side as the substrate-free control.
   b. In tube #1, prepare 5 mL of the1.6 mM substrate (*p*-nitrophenyl phosphate) solution from the 100 mM stock.

      C1V1=C2V2 100xV1=1.6x5 V1= (1.6x50)/100 = 0.08 mL = 80 uL
   c. Add 80 ul of the 100mM stock to 4.92 mL AT buffer
   d. Transfer 2 mL solution from the #1 tube to the #2 tube. Mix well and then transfer 2 mL of the #2 tube solution to the #3 tube, then from #3 to #4, #4 to #5, #5 to #6 and #6 to #7. The concentration of the substrate in the dilution series will be: 1.6mM, 0.8mM, 0.4mM, 0.2mM, 0.1mM, 0.05mM and 0.025mM. The #8 is substrate free blank.

Dilution	Con. (mM)
1	1.6
2	0.8
3	0.4
4	0.2
5	0.1
6	0.05
7	0.025
8	0

2. Prepare a 2 fold dilution series of substrate (from 1.6 mM to 0.025 mM) in AT +P (0.5mM) buffer
   a. Label 8 tubes from 1-8. Add 2 mL **AT+P** buffer to tube #2 to #8. Put the #8 tube on the side as the substrate-free control.
   b. In #1, prepare 5 mL of the1.6 mM substrate (*p*-nitrophenyl phosphate) solution from the100 mM stock as described above (***using AT+P buffer, not AT buffer***).
   c. Prepare the 2 fold dilution series of the substrate with the AT+P buffer as described above.

3. Prepare a 2 fold dilution series of substrate (from 1.6 mM to 0.025 mM) in AT +F (0.1mM) buffer
   a. Label 8 tubes from 1-8. Add 2 mL **AT+F** buffer to tube #2 to #8. Put the #8 tube on the side as the substrate-free control.

b. In #1, prepare 5 mL of the1.6 mM substrate (*p*-nitrophenyl phosphate) solution from the 100 mM stock with AT+F buffer as described above (***using AT+F buffer***).
c. Prepare the 2 fold dilution series of the substrate with the AT+F buffer as described above.

4. Prepare working concentration of 0.5 mg/mL of wheat germ acid phosphatase: **0.5 mg/mL working solution**: *0.5 mL of the stock + 19.5 mL AT buffer*

Part III: Set up the reactions

1. Retrieve a 96 well microtiter plate, and a plate key. Fill out the plate key with how you will aliquot your samples.
2. Aliquot 200 ul of each substrate dilution into the plate with the following layout: 3 replicates for each substrate concentration. See table below.
3. Add **20 ul** of the acid phosphatase enzyme (0.5 mg/mL) to each well using a multichannel pipette. One column a time from left to right. Start the timer right after adding an enzyme to the first column. For the enzymatic reaction, you need to work fast and in a steady pace. Mix by gently pipetting up and down 2 times.
4. At exactly 5 minutes, add 20 ul of stop solution (1 M KOH) to each well using the multichannel pipette from left to right in a same steady pace as adding the enzyme in the previous step and mix well. Incubate the plate at room temperature for 5 minutes and then read the absorbance at 405 nm in the BioTek Synergy Multimode Plate Reader.

		Column								
Dilutions	Con.(mM)	1	2	3	4	5	6	7	8	9
8	0		Sub only			sub + P			sub + F	
7	0.025		Sub only			sub + P			sub + F	
6	0.05		Sub only			sub + P			sub + F	
5	0.1		Sub only			sub + P			sub + F	
4	0.2		Sub only			sub + P			sub + F	
3	0.4		Sub only			sub + P			sub + F	
2	0.8		Sub only			sub + P			sub + F	
1	1.6		Sub only			sub + P			sub + F	

Part IV: Data Analysis

See sample data provided in Blackboard!

1. Perform all data analysis in MS Excel.
   a. Column 1-3 are three replicates of reactions with no inhibitor.
   b. Column 4-6 are three replicates of reactions with 0.5mM Sodium phosphate inhibitor.
   c. Column 7-9 are three replicates of reactions with 0.1mM Sodium Fluoride inhibitor.
2. For each data set (no inhibitor, sodium phosphate, and sodium fluoride) calculate the product concentration [P] and the reaction velocity [V_0] for each substrate concentration and fill in the following three tables.
3. Create a ***Michaelis-Menten Plot*** with a concentration of the substrate on the X-axis and the values of the reaction velocity (V_o) on the Y-axis.

CALCULATIONS:

[P]* is the product (p-nitrophenol) concentration of the enzyme reaction. It can be calculated using the Beer-Lambert Law: A=ELC.

$C = Ab_{405}/EL$, L=1 cm, E (p-nitrophenol at A405) = 17,700$M^{-1}Cm^{-1}$

Therefore,

[P] = Ab_{405}/17,700 (M)

V_0** (the reaction velocity) = d[P]/dt = [P]/ 5 (M/minutes)

Table 1: Data for inhibitor free reactions (column 1-3)

#	[S] (mM)	A_{405} R1	A_{405} R2	A_{405} R3	A_{405} Mean	Mean-blank	[P]*	V_0** (NoIn)
1	0 (blank)							
2	0.025							
3	0.05							
4	0.1							
5	0.2							
6	0.4							
7	0.8							
8	1.6							

Table 2: Data for reactions with Sodium Phosphate Inhibitor (column 4-6)

#	[S] (mM)	A_{405} R1	A_{405} R2	A_{405} R3	A_{405} Mean	Mean-blank	[P]*	V_0** (P)
1	0 (blank)							
2	0.025							
3	0.05							
4	0.1							
5	0.2							
6	0.4							
7	0.8							
8	1.6							

Table 3: Data for reactions with Sodium Fluoride Inhibitor (column 7-9)

#	[S] (mM)	A_{405} R1	A_{405} R2	A_{405} R3	A_{405} Mean	Mean-blank	[P]*	V_0** (F)
1	0 (blank)							
2	0.025							
3	0.05							
4	0.1							
5	0.2							
6	0.4							
7	0.8							
8	1.6							

Merge all three sets of data of V0 into table 4. Table 4: Combined data set

#	[S](mM)	1/[S]	*V_0 (No In)	1/V_0 (No In)	**V_0 (P)	1/V_0 (P)	***V_0 (F)	1/V_0 (F)
1	0 (blank)							
2	0.025							
3	0.05							
4	0.1							
5	0.2							
6	0.4							
7	0.8							
8	1.6							

NOTES:

*V_0 (No In): reactions without inhibitor. Data from Table 1.
**V_0 (P): Sodium Phosphate inhibitor. Data from Table 2.
***V_0 (F): Sodium Fluoride inhibitor. Data from Table 3.

GRAPHS:

1. Generate a ***Michaelis-Menten Plot*** with the data sets (include all three sets on a single graph) putting [S] on the X-axis and V_0 on the Y-axis.
2. For each set of data, calculate the inverse of the substrate concentration (1/[S]) and the inverse of the velocity (1/V_0). Record these values in table 4.
3. Create a ***Lineweaver-Burke plot*** using this data; plot values on a single graph, putting 1/[S] on the X-axis and 1/V_0 on the Y-axis. NOTE UNITS!!
4. Determine linear regression for each set of data. Report the equation of the line and the R^2 value on the graph for each set of data. You may also summarize this in a table if the graph gets too crowded.
5. Calculate the V_{max} and the K_m for the enzyme and the enzyme in the presence of each of the inhibitors. Summarize your results in a table.
6. Based on your graph and your V_{max} and K_m values, determine which of the inhibitors is a competitive inhibitor and which is a noncompetitive inhibitor.

Laboratory Unit 6 Analysis & Review

For your Laboratory Report, ensure it includes the following:

1. ***Pre-lab exercise:*** Calculations, forms, experimental notes.
2. ***Summary of raw data:***
    a. Create three tables as outlined in the lab manual to summarize your raw data and calculations. Print out and include with your lab report.
    b. For each data set, a ***Michaelis-Menten Plot*** (include all three on *a single graph*)
    c. For each set of data, a ***Lineweaver-Burke plot*** using this data (include three sets of data *on a single graph*). Report equation of the line and R^2 value on the graph for each set of data on the graph.
    d. Calculate the V_{max} and the K_m for the enzyme and the enzyme in the presence of each of the inhibitors. Summarize your results in a table.
    e. Create a CoA for Wheat Germ Acid Phosphatase. Include with your report.

3. ***Analysis of results***
    a. Discuss the validity of your assay. Evaluate the equation of the line and the R2 value.
    b. Report the assay parameters of your enzyme (Km and Vmax). How does this compare with the literature?
    c. Based on your data, which of the inhibitors is a competitive inhibitor and which is a noncompetitive inhibitor? Why? Discuss.
    d. Discuss how the values of Km and Vmax are affected by competitive and non-competitive inhibitors.

4. ***Conclusions***
    Specifically, restate final results – What are the Vmax and Km for the Enzyme? Which is the competitive and which is the non-competitive inhibitor?

LAB UNIT 7: PROTEIN EXTRACTION FROM CELLS

INTRODUCTION

Protein Production: An Industry Overview

According to an Evaluate Pharma report, the worldwide pharmaceutical industry has now topped a trillion dollars in sales, with most of this growth propelled by new FDA approvals of recombinant protein products, in particular, monoclonal antibody therapeutics, biologics, and biosimilars (generic protein products). Protein product popularity is likely a result of much of diseases come from a malfunctioning protein and the recombinant protein assists in getting the normal body functions to return. Therefore, it should be no surprise that in the U.S. alone, there were over $250 billion in sales and upwards of $150 billion annually invested in biopharmaceutical R&D in 2015 (EvaluatePharma, 2016).

What are protein products? A wide variety of proteins finds industrial applications. These include enzymes, antibodies, hormones, blood factors, growth factors and diagnostic kits. Some examples include insulin for treating diabetes, interferons for treating cancer, vaccines for diseases such as the annual flu vaccination, and the most popular upcoming drug, monoclonal antibodies for cancer treatment.

At the low-dollar end, proteins are produced in bulk quantities for the food, chemical, and pharmaceutical industries. Unlike biopharmaceutical proteins, these industrial enzymes do not require rigorous sterile purification and are produced with larger, less expensive, processes. Bulk enzymes are a billion-dollar annual market. Some examples of these types of protein products include proteases used in the brewing, cheese making, leather industry, amylases used in starch processing, alcohol production industries, and pectinases employed in the fruit juice processing industry.

Sources of Protein Products

While bulk enzymes produced for the food and chemical industries are most often isolated directly from microbial or plant sources, biopharmaceuticals are more often isolated from recombinant organisms. Although therapeutic biopharmaceutical protein drugs such as insulin were initially isolated from human and animal tissues, they are not likely to found in these natural sources in high concentrations, making the extraction and purification of these proteins prohibitively expensive. Also, contaminating residuals from natural sources can be unsafe, whether due to allergic responses in patients or due to contaminating viruses or prions.

These disadvantages are overcome by using a recombinant productions system. By isolating the gene coding for a target protein and cloning it into a high-expression vector in a recombinant host, the possibility of contaminating viruses and prions is eliminated. The higher level of expression of the protein in a recombinant host can significantly reduce purification costs, and protein engineering can be used to design improvements in stability or effectiveness of a protein product.

Expression levels of biopharmaceutical proteins in a bacterial expression host, *Escherichia coli* can range from 5 to 15% of total protein. While these proteins isolated from human and animal tissue sources might be at levels nearly undetectable, they can become the dominant protein expressed in a recombinant organism. *E. coli* was the first host used to produce recombinant proteins because it was well understood genetically and was very amenable to transformation and expression of recombinant genes. Its fermentation characteristics are well known. However, not all proteins are expressed well in *E. coli*, in part due to the bacterial host's inability to perform necessary post-translational modifications to Eukaryotic recombinant proteins. Also, *E. coli* produces an endotoxin that acts as a pyrogen when injected, and this endotoxin is tough to purify from an *E. coli* fermentation.

In the last few decades, the biotechnology industry has turned to other cell systems for recombinant hosts in recombinant protein expression systems: fungal, plant, and animal cell and tissue culture. More recently, it has created new recombinant expression system options using unconventional approaches such as farm animals and coining the terms farmaceutical and pharming. Below is a selection of unconventional expression systems currently being developed.

Table 1: Selected products using unconventional expression systems on the market or under development (Nature Biotechnology 34, pg. 117–119 (2016)).

Drug	Production process	Indication
ATryn	Purified from the milk of transgenic rabbits goats	Prevention of thromboembolic events
Ruconest	Purified from the milk of transgenic rabbits	Acute hereditary angioedema attacks
Elelyso	Produced in Transgenic carrot-based plant cell expression system	Type 1 Gaucher disease
Kanuma	Purified from the egg white of transgenic hens (*Gallus gallus*)	Lysosomal acid lipase deficiency
Pandemic flu vaccine	Virus-like particles transiently expressed in tobacco (*Nicotiana benthamiana*) leaves	Prevention of H5N1 influenza infection
VEN150	Expressed in the grain endosperm of genetically modified rice	Inflammation in HIV
SBC-103	Purified from the egg white of transgenic hens (*Gallus gallus*)	Mucopolysaccharidoses IIIB
ZMapp	Produced in transgenic tobacco plants	Ebola virus infection
PRX-106	Produced with carrot and tobacco cell culture technology	Inflammatory bowel disease
Moss-aGal	Produced in glycoengineered *Physcomitrella patens* moss cell line	Fabry disease

Downstream Processing of a Protein Product

There is no single best way to purify a given protein. The optimal protein purification strategy depends on the properties of the protein being purified, the starting concentration of the protein being purified, and the types of contaminating materials that it is being purified. Most proteins produced commercially rely on fermentation by microbial or animal cell culture. The process of harvesting and purifying a protein being produced in an industrial setting is referred

to as "downstream processing." It includes all steps of production downstream of the fermentation step. Since downstream processing of a protein can often exceed all other costs of production combined, it must be a carefully designed strategy, often requiring extensive development by scientists and engineers. An optimal purification scheme results in a maximal yield with the fewest and least expensive of steps of purification.

The following are the general steps that are part of the downstream processing of proteins. Each step will be discussed in greater detail later in this lab manual.

1. Since most proteins are not secreted from cells, the first phase of downstream processing consists of ***cell disruption***, followed by ***removal of cell debris***. Cell disruption can be performed using a relatively mild treatment with chemicals or with a more severe physical disruption by sonication. Clearing the lysate of insoluble debris can be carried out using centrifugation or by filtration.
2. Since processing of large volumes is expensive, the first purification step usually includes ***concentrating the protein extract*** to a smaller volume. Protein can be concentrated via precipitation, or by adsorbing the protein to a column such as an ion exchange, or by ultrafiltration.
3. Once the protein solution volume is reduced to a more manageable size, purification can proceed by some techniques. ***Chromatography*** offers the highest resolution, but , one ***chromatographic step is not sufficient to purify the protein to homogeneity***. Some types of chromatography include size exclusion chromatography (molecular sieving), ion-exchange chromatography, hydrophobic interaction chromatography, and affinity chromatography.
4. For biopharmaceutical products that require higher levels of purity and can command a higher price in the marketplace, some more sophisticated techniques can be used to purify a protein. These include ***immunoaffinity*** techniques and high-performance liquid chromatography (***HPLC***).
5. When the protein is purified sufficiently, it is dried by ***lyophilization***, freeze-dried, or formulated into a solution that stabilizes its activity and integrity.

Monitoring Purification Progress

During purification, it is essential to monitor how well the process is performing. There are several strategies used for this depending on the end use of the product. For example, if you are preparing and an enzyme product, you will want to develop an enzyme assay to monitor the enzyme activity as your progress through the purification scheme. Here is a list of some common strategies. A combination of any of these is used:

- Monitor absorbance at 280nm: determine estimate of total protein
- Bradford Assay: determine protein concentration
- Enzymatic Assay: to determine activity of target protein
- Denaturing Polyacrylamide gel: To determine size, modifications, purity of target protein
- Fluorescent or colorimetric tag: GFP, for example, can help locate protein in purification scheme,
- Western Blotting or ELISA: specificity of target protein

Green Fluorescent Protein

In this lab, we will purify the recombinant protein, green fluorescent protein (GFP). *GFP is a naturally occurring fluorescent protein found in the Pacific jellyfish Aequoria victoria.* Although initially chosen for its novelty of causing the transgenic organisms to glow green, GFP is successfully used as a marker for transformation. Scientists have utilized GFP as a gene fusion marker, in which the GFP gene is fused to genes of the target protein. 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. Additionally, GFP is used as a reporter of gene expression levels as well as a measure of protein-protein interactions. Therefore, GFP is a handy tool for both geneticists and cell biologists. GFP was such an important discovery, Martin Chalfie, Osamu Shimomura and Roger Tsien were awarded the 2008 Nobel Prize in Chemistry for the discovery and development of GFP.

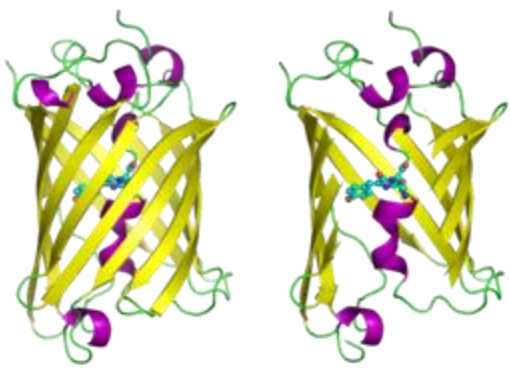


Figure 1: Cartoon GFP molecules, one complete, and one with the side of the beta barrel cut away to reveal the chromophore (highlighted as ball-and-stick). From PDB: 1GFL. (Wikipedia)

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-66, and glycine-67. These three amino acids form ***a fluorescent chromophore*** after translation by cyclization and oxidation reactions.

GFP has a beta-barrel "can" structure consisting of eleven β-strands, with an alpha helix containing the covalently bonded chromophore running through the center. Five shorter alpha helices form caps on the ends of the structure. The beta-barrel structure is a nearly perfect cylinder, 42Å long and 24Å in diameter, creating what is referred to as a "β-can" formation, which is unique to the GFP-like family. The hydrogen-bonding network and electron-stacking interactions with these side chains influence the color, intensity, and photostability of GFP and its numerous derivatives. The tightly packed nature of the barrel excludes solvent molecules, protecting the chromophore fluorescence from quenching by water.

GFP has become widely used in biotechnology as a marker for transformation because it is an easily visualized and stable protein. Furthermore, there is only one gene involved in making GFP,

and the resulting protein is not modified after translation. This means that GFP is easily translated and expressed in a wide variety of prokaryotic and eukaryotic organisms. 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°C or pH of <4.0 and >12.0. Furthermore, it can refold completely within minutes following many denaturing protocols, including sulfhydryl reagents such as 2-mercaptoethanol.

Experimental Summary

For the last half of the semester, students will *produce recombinant GFP in a bacterial system, isolate cell extract, and separate GFP using a variety of techniques: differential salt precipitation, HIC gravity chromatography, and FPLC ion exchange chromatography*. In this lab, students will extract GFP from transformed bacterial cells by freeze-thawing in extraction buffer and by sonication. Additionally, students will use several methods to *monitor the progress of our protein purification process*. We can exploit the fluorescent nature of GFP by visually observing it with a UV light (it glows green!) and quantitatively monitoring it by measuring its relative fluorescence at 509 nm in addition to determining the purity of the GFP fractions on a polyacrylamide gel.

To determine how well GFP is being purified at each step, students will create a **'Purification Table'** as a pre-lab exercise, bring it in for every lab during this GFP purification process, and add in the data for each lab. It is recommended to do this in MS Excel, so you can program the calculations and have the spreadsheet perform these calculations for you instead of doing them by hand and risking making a mistake! What are some other advantages of using a spreadsheet for this purpose?

Monitoring the Progress of Recombinant Protein Purification Scheme

A **purification table** is used during a series of protein purification steps that will assist you in organizing and presenting information about the success of your purification scheme. It will display information on yield, purity, concentration, and specific activity of your purified protein as the process progresses. You will need volume, concentration, and RFU, of your protein for each step; starting from the lysate to the final purified sample.

Figure 2 is an example of a purification table and calculations for a fluorescently tagged enzyme. In this table, you are calculating total protein, concentration, activity, specific activity, and fold/% yield purification. For your table, I would recommend you add in A280 measurements/protein concentration as well. You can use a Bradford assay to measure protein concentration, or you can use A280. How can you use the A280 and estimate the concentration of total protein using Beer's Law?

For GFP you will use fluorescence to assist you with activity calculations. A note about fluorescence: A fluorometer does not produce a particular unit, rather a relative unit (RFU; Relative Fluorescent Units). In practicality, this means, your fluorescent measurements are about EACH OTHER because each fluorometer and light source differ in their measurements. To get a true RFU value, it is best to measure all your samples together on the same machine at the same time. More on this later!

Example Purification Table

Fraction	Volume (ml)	Total Protein (mg)	Activity (units)	Total Activity (units * ml)	Specific Activity (units / mg)	Fold Purification	% Yield
Lysate	10.00	56.3	65897	658970	1170	1.0	100
DEAE	12.50	25.36	42845	535563	1689	1.4	81
Ni-Agarose	8.25	18.25	51481	424718	2820	2.4	64
S-200	4.30	3.75	48239	207428	12863	11.0	32
Reactive Blue	3.50	1.28	46578	163023	36389	31.1	25

Calculations -

1. ***Units*** **-** Normally, this is determined using an enzymatic assay. MGH has an enzyme, as one of the fusion partners, but we are determining the fluorescence of the protein as a function of the enzyme. THIS MUST BE DONE AT THE SAME TIME. You must thaw the fraction of your lysate, pooled samples and final purification sample and conduct a fluorescent assay at the same time. Different settings on the plate reader will give you a different result, thus it must be read at the same time using the same plate. Simply thaw the samples and place 100 µl into a well. Return the samples back to the tube for later assay. The units of activity will thus be measured as MGH Fluorescence (relative fluorescent units). For specifics on how to determine enzymatic activity, go to the lab website for that handout.
2. ***Total Activity*** **-** this is a measure of how many units of protein you have in a sample. Simply multiply the activity in the sample by the total volume.
3. ***Specific Activity*** **-** specific activity is a way to measure how much of a measured protein (MGH in this case) there is with all of the other contaminating proteins. Divide the Activity by the mg of protein. The higher this value, the higher purity.
4. ***Fold Purification*** **-** Divide the specific activity of each fraction by the specific activity found in the lysates. This number changes depending on the protein you are working with. There is no good or bad value. However, this number along with the percent yield, indicates if a step was worthwhile or not. A poor fold purification with a low yield is a step to avoid in the future, while a high fold purification and high yield is a great thing…
5. ***Percent Yield*** **-** Calculate the percentage of the yield for each step using the total activity from the starting step.

Try setting up an excel spreadsheet using the formulas given above.

FIGURE 2: This sample purification table comes from the National Science Foundation (2006) on the purification of a fluorescent-tagged enzyme.

Protein Extraction Methods

The first step in the purification of an intracellular protein is the disruption of the cell structure, allowing the release of proteins. There are many methods for cell disruption, listed below. When choosing a method for cell disruption, ***the goal is to find the gentlest method that gives the highest yield of protein***. Protein yield is a question of how much protein is released, or how effective the disruption technique was. It is also a question of the amount of damage the extraction method has on the proteins being released. In general, it is best to search for the mildest effective treatment to prevent damage to the proteins being isolated. Some proteins are susceptible to the shear forces required to break down cell walls, so in these cases, milder techniques should be tried. More often, proteins are sensitive to damage by exposure to air. It is best to avoid introducing air into the cell suspension and to keep the time required for cell disruption to a minimum. The cell suspension should be kept cold at all times. Although organic solvents are useful in cell disruption, most proteins are not stable using this treatment. If a protein is stable in organic solvents, this tends to be the treatment of choice. In such a case, not only is the protein efficiently released, but it is also simultaneously purified from less stable proteins.

The buffering conditions of a cell lysate are vital in the stability of proteins. The pH can change when cytoplasmic contents are released from a cell, so a buffer must be used to maintain a

constant pH. The selection of a buffer should be based on its pK_a; it should be within 1 unit of the pH that is to be maintained. The best pH to buffer at is often dependent on the protein being isolated. Most proteins are most stable around neutral pH or under slightly alkaline conditions. If the protein of choice is found to be stable at an extreme pH, buffering at this pH can help to extract the protein more selectively. The less stable proteins will denature and will be removed by the centrifugation step. This effectively purifies the protein by selective denaturation and removal of other proteins.

Most proteins are susceptible to oxidative damage, so can often be stabilized by adding a reducing agent. Dithiothreitol (DTT) and 2-mercaptoethanol are often used as reducing agents during protein isolations. These sulfhydryl reagents also protect susceptible proteins from damage by metals that may be present in buffering solutions. DDT is less volatile than is 2-mercaptoethanol, so is usually the preferred reagent used to maintain a reducing environment in a cell lysate. Since it has two thiol functional groups instead of one as in the case of 2-mercaptoethanol, DTT may be used at lower concentrations.

Table 2: Some methods of cell disruption

Method		Underlying basis
Gentle	Cell lysis	Osmotic shock: rapid immersion in hypotonic solution
	Enzyme Digest/cell lysis	Digestion of cell wall; contents release following osmotic shock
	Potter-Elvehjem homogenizer	Cells forced through a narrow gap, cell membranes disrupted by shear forces
Moderately harsh	Freeze-thaw	Slow freeze-thaw cycles break cell walls by ice crystal formation and growth
	Waring blender	Cells are broken by shear forces of rotating blades
	Organic extraction	Mixing with an immiscible organic solvent can weaken cell walls and dissolve biological membranes
	Grinding	Abrasive grinding with glass beads or a mortar and pestle, usually with frozen cells and sand or alumina
Vigorous	French press cell	Cells forced through small orifice at high pressure and disrupted by rapid pressure drop and high shear forces
	Explosive decompression	Cells equilibrated with an inert gas (e.g. N_2) at high pressure, rapidly decompressed to 1 atm
	Bead mill	Rapid vibrations with glass beads grind cell walls
(most harsh)	Ultra-sonication	High-pressure sound waves cause cell rupture by cavitation and shear forces

Protease Inhibitors: Proteins must also be protected from degradation by proteases during isolation steps. Cell disruption tends to release lytic enzymes, especially from cell rich in lysosomes. Protease inhibitors are added to a lysis buffer to reduce proteolytic damage to proteins. Many protease inhibitors can be used: PMSF, for example, is a broad inhibitor and is successfully used in animal, plant and yeast tissues. Other inhibitors include EDTA, aprotinin, chromostatin, pepstatin and benzamidine.

Protease inhibitors often have low solubility in water, and therefore stock solutions are typically made up of organic solvents. PMSF is the inhibitor most frequently used but must be prepared

fresh due to its instability in water ($t_{1/2}$ = 30 minutes at 25°C and pH 7.0). Stocks can be made up in methanol and stored in the freezer.

Clarification: Once cells have been disrupted, the cell lysate must be clarified, removing cell debris and unbroken cells either by filtration or by centrifugation. Cellular materials are highly compressible, they rapidly clog pores of filters, so centrifugation is usually the method used to remove particulate matter when the volumes are small.

Final Thoughts

It is impossible to know which extraction technique will work best for a recombinant protein. Therefore, several methods are tried and compared. In this exercise, you will examine how well the extraction techniques freeze-thaw and Sonication perform to extract GFP from your cells. ***The yield of GFP by each group will be determined by the relative fluorescence of the extraction supernatant.*** To determine the effectiveness of the extraction method, the total amount of protein will be compared to the relative fluorescence (GFP). If the extraction is too harsh, fluorescence will be low due to GFP being denatured, but the A280 will still be high (the amino acids are always present even if the protein is denatured). If the method is not effective, the fluorescence will be low due to GFP being trapped in the cells. The A280 will also be small because all the intracellular proteins will be stuck in the cells along with GFP.

References

1. Chalfie, Martin and Steven Kain, *Green Fluorescent Protein, Properties, Applications and Protocols* Wiley-Liss, 1998.
2. Seidman & Moore, "*Basic Laboratory Methods for Biotechnology: Textbook & Laboratory Reference*," 2nd edition. 2009. Prentice Hall. ISBN: 0321570146
3. World Preview 2016 Report (n.d.). EvaluatePharma. Retrieved from http://info.evaluategroup.com/rs/607-YGS-364/images/wp16.pdf
4. Green Fluorescent Protein. Wikipedia. Retrieved from https://en.wikipedia.org/wiki/Green_fluorescent_protein

LAB UNIT 7: EXTRACTION OF GFP FROM *E. coli* CULTURE

MATERIALS

☐ Stock solutions previously prepared (sodium phosphate buffer, 0.5M EDTA) ☐ 200 mM PMSF stock solution (prepared in methanol, this may have been prepared for you) ☐ 50 mL of GFP-expressing *E. coli* cell culture ☐ 15 & 50 mL conical(s) ☐ Bucket of ice	☐ Fluorometer Plate Reader ☐ Black fluorescent plates ☐ UV/VIS Spectrophotometer (low-volume, or plate reader) ☐ Sonicator and 3mm tip ☐ UV light ☐ Preparative centrifuge (to pellet your culture)

SAFETY CONSIDERATIONS

- *UV light can damage eyes, wear eye protection*
- *Use safety glasses when operating the sonicator. Prepare sure the door is closed on the sonicator before being turned on – hearing (auditory) hazard.*
- *The PMSF is hazardous. It is toxic and can burn. It is dissolved in methanol which is also toxic. Research MSDS recommendations and include them in your pre-lab exercise. Use safety glasses and gloves when working with this material!*
- *Treat GFP-transformed cells as bio-hazard materials and dispose of them accordingly.* 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:
  - ✓ Gloves and goggles should be worn at all times.
  - ✓ Wipe down the lab bench with antibacterial cleaner before starting the lab and before leaving the laboratory.
  - ✓ All materials, including plates, pipettes, loops, and tubes that encounter bacteria should be autoclaved before disposal in the garbage.
  - ✓ Wash hands thoroughly with soap and water after removing gloves.

Pre-Lab Exercise:

1. As part of your pre-lab exercises, you must perform the calculations for all the solutions below even if the class shares in the preparing of the solutions. Include these in your pre-lab where each solution is being prepared.
2. Generate a Purification Table in MS Excel – see introduction example. In MS Excel program the calculations for each column. Copy and paste this data table into your pre-lab exercise to use to collect your data. Data you will collect into this table will include volume, A280, and RFU for each sample, for labs 6, 7 & 8.

PART I: PREPARING TRANSFORMED CULTURES

This may have already been done for you. Some reagents and cultures may have been prepared in BIOL1414. It takes a few weeks to prepare the reagents and generate 50mL cultures of transformed cells for each lab group. ***Ask your instructor before you prepare a pre-lab***.

1. Transform *E. coli* with pGLO (GFP protein expression) plasmid, and plate for isolated colonies on LB-amp-ara plate. Incubate overnight at 37°C, or 2 days at ambient temperature.
2. If there are no isolated or fresh actively growing colonies, streak an isolated, pGLO transformed bacterial colony onto a fresh, warm, LB-amp-ara plate. Incubate overnight at 37°C, or 2 days at ambient temperature.
3. Prepare a starter culture by inoculating 5mL of LB-amp with one pGLO transformed colony, and incubate in a shaking incubator overnight at 37°C, or 2 days at ambient temperature.
4. Inoculate 50mL of LB-amp-ara with the 5mL of the starter culture. Clamp in tightly, and shake overnight at 37°C, or 2 days at ambient temperature.

Solution	Final concentration	Final volume	Comments
LB Medium	1% tryptone 1% NaCl 0.5% yeast extract	100ml/group	Autoclave-sterilize. Store at 4°C.
LB-amp-ara medium	LB medium 50ug/mL amp 2mg/mL ara	50mL/ group	Use sterilized LB medium, add 10mg/mL amp stock, and 200mg/mL ara stock. Store at 4°C.
LB-amp medium	LB medium 50ug/mL amp	5mL/group	Use sterilized LB medium, add 10mg/mL amp stock. Store at 4°C.
LB-amp-ara Plates	Prepared for you	1 plate/ group	These were prepared in BIOL1414.
Sodium phosphate buffer, pH 7.0	100 mM (prepared previously, check volume)	100 mL/class	Use the table to prepare the phosphate buffer as you did previously. Store at 4°C.
PMSF in methanol	0.20 M	1.0 mL/class	Prepared for you. Can be stored at -20°C.
Lysozyme in nuclease-free water	20 mg/mL	1 mL/ class	Prepared for you. Can be stored at -20°C.
Extraction Buffer	10mM sodium phosphate, 1mM EDTA, 0.5mM PMSF	20mL/ group	Prepare immediately prior to use (keep a 1mL aliquot to blank the spec).

PART II: PREPARING SOLUTIONS

1. Most of these solutions should have already been made in a previous lab. Check your inventory to ensure you have the required volume. Ask your instructor if you will be making any of the other solutions. Time permitting, you will prepare all of them, therefore please have the calculations for all solutions ready. You WILL prepare extraction buffer.
2. Before preparing the solution, check your calculations and your protocol with your partner. VERIFY the molecular weights of your dry reagents by looking on the bottle you will be using. Some reagents are hydrated (have water) and will have higher molecular weights than ones that are anhydrous.
3. Write a PROTOCOL to prepare the solutions you are assigned. Do this BEFORE you come to class.

PART III: EXTRACTION PROCEDURE

1. Ensure the preparative centrifuge has the correct rotor (for 50mL conical) and is set to 4°C pre-chilling before you begin. It can take upwards of 20min to pre-chill the centrifuge. The lid must be closed for it to chill.
2. Swirl the GFP-transformed cells before removing a 1mL aliquot into a labeled (S1-Cell) 1.5mL centrifuge tube. Place on ice.
   a. Pellet the 1.5mL culture at 13K rpm for 2 minutes.
   b. Visualize the pellet and supernatant with a UV light, and record observations.
   c. Discard the supernatant. Resuspend the transformed cells in 30ul of cell extraction buffer. Keep the tube in your sample box and store it at -20°C. You will use this for your gel and western blot analysis in a later lab.
3. Again, swirl the GFP-transformed cells before decanting approximately 45 mL into a 50 mL conical tube (do this by eye to the 45mL marking on the tube).
4. Balance your 50mL conical tube against another lab group (by weight!). You must be within 0.01g. Remember to include the lids when weighing!
5. Pellet the 45mL culture using a pre-chilled preparative centrifuge at 4000 rpm for 10 minutes at 4°C. This will need to be done together as a class. Ask for a demonstration.
6. After centrifugation, quickly remove the pelleted tubes from the centrifuge and observe with a UV light. Record whether you see fluorescence in the supernatant or the cell pellet.
7. Immediately decant the supernatant into a waste container with bleach. Be careful! Ensure your pellet does not slide out of the tube! If you do not do this immediately after centrifugation, your pellet will not stick to the side of the tube. Keep pellet on ice.
8. Add 4 mL of the extraction buffer to the cell pellet and gently pipette up and down to re-suspend the cells. Keep cell suspension on ice.
9. Save a 1mL aliquot of the Extraction Buffer in a 1.5mL tube for later sample analysis (both as a blank and to dilute samples). For convenience, keep it with your freezer box samples.

EXTRACTION PROTOCOLS:

The class will be split into different extraction groups. You will compile class data for the analysis. Prepare a pre-lab for both procedures, and make notes which groups perform which procedures.

Freeze-thaw Extraction of Cell Suspension

1. Add 200 µl of Lysozyme stock (20 mg/mL in nuclease-free water freshly made) to the cell sample and mix gently. The final concentration of lysozyme is 0.95 mg/mL.
2. Incubate the tube for five minutes at ambient temperature. If the room is cold (< 20ºC you may want to put in a 37ºC water bath).
3. Freeze lysate for 10 minutes at -80˚C and thawing in a 37˚C water bath for ~ 30 seconds (ONLY until JUST thawed). Do not warm lysate; it should stay cold. Note: It is recommended to repeat the F/T for three total cycles, time permitting.
4. Split the 4mL extraction into 2, 2mL centrifuge tubes.
5. Centrifuge at 13,000 rpm for 10 minutes (at 4˚C) in a microcentrifuge.
6. Immediately transfer the supernatants to a clean, labeled 15 mL conical. ***Keep both pellet and supernatant*** on the ice.
7. Use a UV light to examine the samples. Record observations.
8. Transfer a 100uL aliquot of supernatant to a tube and label it as S2-FT**.** Store the sample at -20˚C for later analysis.
9. The remaining 3.9 mL supernatant will be purified further in the next lab unit. Store GFP extract at -20˚C

Sonication Extraction of Cell Suspension

1. Set up the sonicator with a 3 mm micro-tip. Your instructor will demonstrate for the class.
2. Your cell suspension must be fully submerged in ice slurry (mostly ice, a little bit of water, let sit for 10 minutes to get the slurry cold). The tip must at least half/three quarter the way down in the conical without hitting bottom or sides of the tube.
3. Sonicate with a cycle of 4.0 seconds on and 2.0 seconds off over a 30-second interval. Be sure your sample stays submerged in ice water (mostly ice, less water). Repeat cycle 3 times. You must allow cooling in between each sonication procedure, or else you may accidentally denature the protein in the extract!
4. Split the 4mL extraction into 2, 2mL centrifuge tubes.
5. Centrifuge at 13,000 rpm for 10 minutes (at 4˚C) in a microcentrifuge.
6. Immediately transfer the supernatants to a clean, labeled 15 mL conical. ***Keep both pellet and supernatant*** on the ice.
7. Use a UV light to examine the sample. Record observations.
8. Transfer 100uL of the supernatant to a tube labeled S3-SON**.** Store the sample (**S3**) at -20˚C for later analysis.
9. The remaining 3.9 mL supernatant will be purified further in the next lab unit. Store GFP extract at -20˚C

PART IV: ANALYSIS

1. Examine the pellet and supernatant for both extraction methods under a UV lamp and record your qualitative observations into your data table. Where is the most GFP located (sup or pellet)?
2. Use a low volume spectrophotometer **measure the A_{280} of the S2 or S3 *supernatants*.** Record into your data table.

The total amount of protein =

measured protein concentration (mg/mL) X the total sample volume (mL)

(For example, the volume of the cell extracts via sonication is 4 mL, then the total protein of this extract sample = measured protein concentration (mg/mL) X 4mL.)

3. Use a fluorometer to **determine the RFU (the relative fluorescence unit) of the S2&S3 *supernatants.*** Record into your data table.
   a. The best way to do this is in the plate reader using a black 96-well plate. Fill in the 96-well plate-key.
   b. Aliquot 100ul of S2 & S3 into a black plate.
   c. Set the excitation and emission to 395nm and 509nm.
   NOTE: You may have to dilute the more concentrated GFP samples 1: 10 or 1:100 with the cell extraction buffer prior measuring the RFU.
   d. REMEMBER! ***Return the 100ul sample back to the correctly labeled tube to keep for future analysis***. If you prepared dilutions, keep those as well.
4. **To calculate the total amount of RFU = measured RFU/mL X dilution factor X the sample volume (mL).** For example, the volume of the cell extracts via sonication is 5 mL, if you measure RFU for a 1:10 diluted sample, then the total RFU of this extract sample = measured RFU /mL X 10 X 5mL.
5. Determine the ratio of RFU/mg protein for both extracts by divide the total RFU with the total protein of each of the extract. This value represents the specificity of the extract. Compare the values of the total protein, the total RFU and RFU/mg protein of these two samples to determine which cell lysis method works better for you. **Create a data table to summarize data. For example,**

Group	Extraction Protocol	A280 (mg/mL)	Vol extract (mL)	Total mg	RFU/100ul	Total RFU	RFU/mg
A	FT: -80/10min x 3 cycles	1.358	4mL	5.432	7862	314,480	57,894

Laboratory Unit 7 Analysis & Review

For your Laboratory Report, ensure it includes the following:

1. ***Pre-lab exercise *with notes****

2. ***Summary of raw data:***
    a. Create a summary table of the class extraction data. In your table include the qualitative analysis of all extractions; the A280, RFU, and the ratio of RFU/protein.
    b. Include in your report the Purification Table you created when starting this purification scheme.

3. ***Analysis of results***
    a. Using your table of results, what can you say about the relative effectiveness of the extraction procedure for releasing GFP? Which extraction procedure worked best?
    b. What can you say about the relative purity of the GFP released by each?

4. ***Conclusions***. Write a comprehensive conclusion of this experiment. What are the implications for future work?

LAB UNIT 8: CHROMATOGRAPHY OF GFP

Introduction

Even when produced by a recombinant host at high expression levels, proteins come in complex mixtures and frequently require multiple purification steps to remove the contaminants. The protein must be separated from hundreds of other proteins with similar properties, as well as all the lipids, nucleic acids, and carbohydrate-containing biomolecules of the cell.

One of the most effective methods of purification that can do this job is column chromatography. Like all chromatography techniques, there is a stationary phase and a mobile phase in column chromatography. The stationary phase is a fine bead particle that has defined chemical properties that proteins interact. The mobile phase is the buffer in which the protein is soluble. The separation of proteins on a column relies on their different relative affinities for the stationary, compared to the mobile, phase. Proteins with higher affinity for the stationary phase will be retained on the column while proteins with a higher affinity for the buffer will elute from the column. There are many types of column chromatography, based on the chemistry of the stationary phase. The general classifications are listed below.

Table 1: Types of chromatography commonly used for purifying proteins

Technique	Based on protein property
Gel Filtration (GFP), or Size Exclusion Chromatography (SEC)	Size, shape
Ion exchange	Net charge on protein surfaces
Hydrophobic interaction chromatography (HIC)	Hydrophobicity of amino side groups on protein surfaces
Immobilized metal affinity chromatography (IMAC)	Histidine amino acids on protein surfaces
Affinity Chromatography	Bio-recognition (ligand specific)

Gel Filtration Chromatography

In SEC, separation on the column is a function of size alone: larger molecules are excluded from the pores of the chromatography beads and elute from the column more quickly than smaller molecules. Here, the resolving power is a function of the length of the column on which the proteins are separated on, as well as the pore size of the beads, the flow rate of the elution buffer, and the relative sizes of proteins being separated. Since the permeation of the beads by smaller proteins is a diffusion dependent process, the flow rates should be slow enough to allow for this to happen. In general, the resolution of SEC on small columns is not great, but this step can also be useful for changing the buffer in which the protein is dissolved in, to the buffer used to elute the proteins from the column. Significant drawbacks to SEC is that the volume of the protein sample applied to the column must not exceed 5% of the column volume, and the protein eluted from the column is diluted by a factor of 10-fold. This means that an SEC step must usually be followed by a step that will concentrate the protein fraction.

Adsorption Chromatography

The other forms of chromatography listed in Table 8.1 are a form of "adsorption" chromatography, in that the protein that is applied to the column matrix will be adsorbed to the solid phase matrix, allowing separation of proteins that have no affinity to the stationary phase. The proteins left in the column can then be eluted by changing the buffering conditions of the mobile phase. A gradual change in buffering conditions can elute proteins one at a time, allowing their separation from each other.

Adsorption versus Absorption

Adsorption is a surface phenomenon. In the example of column chromatography, protein is adsorbed (collected) on the surface of a solid phase matrix. This is very different from ***absorption***, which describes a substance that diffuses into a liquid or solid to form a solution. For example, in spectrophotometry, we are measuring light being absorbed by a sample.

Adsorption chromatography can be used to concentrate proteins since a large volume of protein can be applied to the column without affecting the amount of protein adsorbed to the column matrix. Careful selection of elution conditions can remove the protein from the column in a much smaller volume. Since adsorption chromatography allows for both the isolation and concentration of a target protein, it is sometimes referred to as the "capture" phase of protein purification.

Stationary Phase

The stationary phase (chromatography resin) can be made from many different types of material. The resin must have many valuable qualities. It must be readily porous for large proteins, yet sufficiently rigid to sustain the hydrostatic pressure of elution buffers. At the same time, the bead material itself must not have a high affinity for proteins: the adsorption of proteins must rely solely on the ligand coating the bead, whether that is an ion exchange group, a hydrophobic group, or an affinity label. The most common chromatographic materials used to make beads are insoluble polysaccharides such as cellulose, dextran, or agarose. Sephadex is a bead-formed gel prepared by cross-linking dextran with epichlorohydrin. The dextran hydroxyl group renders the gel extremely hydrophilic, and the degree of cross-linking determines the pore size of the gel.

The lower the cross-linking, the more porous the bead is and the larger the proteins that have access to its surfaces, both interior and exterior. Unfortunately, the more porous bead have, the less mechanical strength and are compressed by high hydrostatic pressures found in large columns or when elution buffers are pumped too quickly. Cross-linked agarose-type beads, such as Sepharose, have greater mechanical stability and are preferred for larger columns and faster elution rates. Both types of beads are biodegradable, so they must be stored in a biocide such as sodium azide or high concentrations of alcohol. Although cold storage is best, the beads are damaged by freezing.

Purification Strategy

There is no single best way to purify all proteins. Finding an optimal protein purification strategy requires trial and error because the best method for purification depends on the properties of the protein being isolated as well as the proteins and other contaminants that the protein must be isolated. Different chromatography methods are tested and compared to their suitability in purifying each new protein. In choosing the best types of chromatography to use, there are at least five factors that must be evaluated:

- resolution of the method
- the capacity of the method
- the speed of the method
- % recovery of protein
- cost

In this lab exercise, you will separate your GFP protein extract from the previous lab using HIC chromatography technique.

Hydrophobic interaction chromatography (HIC)

Separation by HIC is based on the reversible interaction between a protein and the hydrophobic surface of a chromatographic medium. This interaction is enhanced in the presence of a high ionic strength buffer solution (e.g., 2M ammonium sulfate). Proteins bind to the column as they are loaded, and proteins with low affinity and other contaminants are washed from the column with high ionic strength buffer. Conditions are then altered so that the bound substances are eluted differentially, usually by decreasing the salt concentration of the elution buffer. Changes are made stepwise or with a continuously decreasing salt gradient. Target proteins are concentrated during binding and collected in a purified, concentrated form.

Some proteins with extremely high affinity to the HIC matrix must be eluted with buffers of reduced polarity (e.g. an ethylene glycol gradient up to 50%). Some proteins will elute by adding chaotropic species (urea, guanidine hydrochloride) or detergents.

Sometimes a change of pH or temperature can be used to affect the elution of proteins, as well. In general, hydrophobic interactions increase in strength with increasing temperature; there can be a 20-30% reduction in binding when the temperature is changed from 20 °C to 4°C. The interaction between proteins and hydrophobic ligands decreases with increasing pH. The effect of pH is different for different proteins, and thus the elution profiles may be improved by changing the elution pH. However, the effect of pH on elution from HIC is not that great, and it is best to work within the pH range the protein is stable.

Stationary Phase

The most popular HIC resins are cross-linked agarose gels to which hydrophobic ligands have been covalently attached. The choice of ligand will determine the degree of hydrophobicity of the HIC resin. The most popular ligands include, in the order of increasing hydrophobicity: ***methyl- < phenyl- < octyl-.*** If a protein binds too tightly, requiring a harsh elution buffer, a less hydrophobic matrix might work better.

Mobile Phase

A variety of salts may be used for loading and elution. In general, the effects of various salts on HIC mimic their effects on salting out proteins. Those salts which are most effective in salting out are most effective at binding proteins to hydrophobic matrices. Thus, ammonium sulfate is a good salt for binding most proteins to a HIC column.

After packing columns with resins in the appropriate equilibration buffer, you will apply your cell extract to the column to adsorb proteins. After non-adsorbed proteins and other contaminants are washed from the column with equilibration buffer, proteins will be eluted by gradually changing the elution buffer. You will be able to immediately tell where the GFP is at any point by directly looking for fluorescence under a black light. Fractions containing GFP will be saved for later analysis by gel electrophoresis.

Cleaning Columns

Chromatographic matrices are very expensive and with proper care, can be reused for an indefinite period. To properly care for the column matrix, you should "clean off" any tightly adsorbed proteins from the column with an extreme-pH buffer, when possible.

Many types of resin cannot withstand such harsh treatment, so ensure that you do not use this approach unless the product literature recommends it. It is critical to thoroughly remove this highly acidic or basic buffer from the column before storage. Since most chromatographic matrices used for protein purifications are made from highly biodegradable materials such as dextran, it is crucial that you store these materials in a bacteriostatic buffer.

Microbial growth can be effectively inhibited with a high concentration of ethanol or a dilute solution of sodium azide. Be sure to check the product literature for your chromatographic matrix to decide the best way to preserve your material.

References

1. Doonan, S (ed.) Protein Purification Protocols in Methods in Molecular Biology (Vol. 59) Humana Press (1996)
2. Price (Ed.) Proteins *Labfax*. Academic Press. (1996)
3. Sommer, C.A., F.H. Silva, and M.T.M. Novo. Teaching Molecular Biology to Undergraduate Biology Students. *Biochem & Mol Biol Ed.* 32(1):7-10 (2004)
4. Walsh, Gary. *Biopharmaceuticals: Biochemistry & Biotechnology*. (2nd ed). John Wiley & Sons (2003)
5. Seidman & Moore, "*Basic Laboratory Methods for Biotechnology: Textbook & Laboratory Reference*," 2nd edition. 2009. Prentice Hall. ISBN: 0321570146
6. Campbell & Farrell, Biochemistry, 6th Ed. Thomson Brook/Cole. ISBN: 0495390410

Lab Unit 8A: Column Preparation and Salt Precipitation

Material

☐ 4.1M SATURATED Ammonium sulfate ☐ 4.0M Ammonium Sulfate ☐ Previously prepared Tris and EDTA ☐ Bio-Rad Macro-prep methyl HIC resin (158-0080) ☐ Bio-Rad Poly-prep empty column (731-1550), and stopper	☐ Ice bucket ☐ UV light ☐ Preparative centrifuge – pre-chill 4ºC ☐ Stand with clamp ☐ 4, 5mL pipets & pipet-aid ☐ 1, 50mL conical ☐ 5, 1.5mL centrifuge tubes & rack ☐ 1, 2mL centrifuge tube

Safety Considerations

- ✓ *Wear gloves and safety glasses when handling and place waste in the appropriate container.*
- ✓ *UV light can damage eyes, wear eye protection*

PROTOCOL

Part I: Prepare Solutions

The following reagents will be used for purification of GFP by HIC chromatography. As part of your pre-lab exercise prepare calculations for ***every solution***. Your instructor will give you instructions on which solutions you will prepare. Check your calculations with your partner before you prepare the reagent.

Solution	Final concentration	Final volume	Comments
Saturated ammonium sulfate	4.1M	100 mL/class	54.1 g (NH4)2SO4 in 80 mL diH2O with heat and BTV to 100 mL. When solution cools down, you should see crystal formation.
Ammonium sulfate stock solution	4.0 M	150 mL/class	Store at room temperature
10X TE buffer	100 mM Tris, 10mM EDTA, pH 8.0	20 mL/group	You made the 1M Tris-HCl pH8 stock solution in lab unit 2. Use it here to make the TE stock. Store at room temperature
1XTE buffer	10mM Tris, 1mM EDTA, pH 8.0	100mL/group	Store at room temperature.
HIC equilibration buffer	2 M ammonium sulfate in 1X TE	20 mL/group	Dilute from the 4.0M stocks. Store refrigerated. Warm to room temperature before use
HIC wash buffer	1.3 M ammonium sulfate in 1X TE	10 mL/group	Dilute from the 4.0M stocks. Store refrigerated. Warm to room temperature before use

Part II: Prepare the HIC columns

1. Obtain 4 mL of 50:50 HIC resin: liquid slurry in a 15mL conical tube. The resin is supplied in an alcohol solution that must be removed by washing with water before pouring the column.
2. Allow the resin to settle and pipet off the alcohol. Avoid losing beads. There should be approximately a 2mL bed volume (1mL min).
3. Add approximately 4 mL deionized water and gently invert several times to wash the beads. Allow to settle and remove the wash with a 5mL pipet.
4. Repeat water washes for a total of 3 rinses. *NOTE: This may have been done for you already, ask your instructor.*
5. Remove all the wash water with a 5mL pipet and add approximately 4mL of equilibration buffer. Mix gently by inversion. The general rule in equilibrating columns is to add 2 column volumes of equilibration buffer and let it wash through.
6. Allow beads to settle, remove the supernatant and wash one final time with 4mL of equilibration buffer. Mix gently by inversion.
7. Snap off the end of the Poly-prep column perpendicular (don't twist) and secure to a stand using a clamp. Place a yellow cap on the end of the column. Place waste beaker under the column.
8. Add 1 mL of equilibration buffer to the column and allow soaking for 2 minutes to wet the frit.
9. Thoroughly resuspend the resin by gently inverting until you have a slurry.
10. Quickly pour the entire volume (4 mL slurry, 50:50 resin and liquid) into the column. Remove yellow stopper and allow the bead bed to pack while the equilibration buffer elutes. Allow to settle on the top of the bed looks level.
11. Ensure the bed height of the column is about 2 cm, and the top of the column bed is level. The exact height is not critical, but ensure that your beads fill the lower, more constricted part of the column. For gravity HIC chromatography, a smaller bed volume is desirable, why?
12. Replace the cap tightly just before the equilibration buffer reaches the top of the bed, leave a few mm of the buffer at the top, and *do not let the bed run dry*. It's crucial the yellow cap is placed very tight!
13. Inspect your column. Your column should have the following attributes:
    - ✓ The narrow bottom portion of the column should have 1-3 cm of resin bed volume.
    - ✓ The top of the bed should be flat.
    - ✓ There should be no air bubbles in your column, particularly in the frit.
14. If you are not continuing to the purification steps, prepare your column for temporary storage. You must use your column within one week because the equilibration buffer does not have a preservative in it.
    a. Add 1 mL of equilibration buffer GENTLY to the column, do not disturb the bed.
    b. Ensure the yellow stopper is firmly in place and wrap with a small piece of Parafilm.
    c. Cover top with Parafilm.
    d. Label your column with your names and date. Ask your instructor where to store the columns. DO NOT store at 4°C.

Part III: GFP purification via differential ammonium sulfate precipitation
Procedure

1. Combine the sonication and the freeze-thaw extract from the previous lab into a clean, unused, 50 mL conical.
2. Transfer 100uL of mixed extracts to a 1.5 mL tube and label it as the starting material (you can label it as S4-FTSON).
3. Measure the volume of the remaining extracts in the 50 mL tube using a 10 mL pipette.
4. Calculate the amount of saturated ammonium sulfate (AS) needed to give a 40% of its final concentration using the following formula.

   AS / (AS + EX) = 40/100

   Where, AS=ammonium sulfate, and EX= Extract volumes

   For example, if you had 5mL total extract:

   VolAS/(VolAS + 5mL) = 40/100
   100VolAS = 40AS + 200mL
   AS = 200/60 = 3.33 mL
5. Add the calculated volume of <u>saturated ammonium sulfate</u> to the extracts in the 50mL conical. Mix well via gently inverting the tube several times *<u>and incubate the tube on ice for 15 minutes</u>*. Balance tubes while incubating on ice (to 0.01g).
6. Centrifuge the tube at 10000 rpm for 10 minutes at 4°C. Examine the supernatant and the pellet with UV light to see where the GFP is present. Record observations.
7. Transfer the GFP supernatant into a new 50 mL conical tube by decanting.
8. Resuspend the pellet in 100 ul TE buffer and then transfer to a 1.5 mL tube and label it as the 40% ammonium sulfate precipitates (S5-40%P).
9. Add an additional amount of saturated ammonium sulfate to the supernatant to achieve a final 70% of ammonium sulfate concentration. The formula used for this calculation is:

   AS + V2 / (AS + EX + V2) = 70/100

   Where, AS is the amount of ammonium sulfate that you have added to the 40% precipitation. EX is the starting volume of your extract. *<u>V2 is the additional amount of ammonium sulfate that you need to add.</u>*
10. Add the additional volume of ammonium sulfate to make a 70% solution as calculated. Mix the solution by inversion and incubate the tube on ice for 10 minutes.
11. Centrifuge the tube at 10K for 15 min at 4°C. Examine the supernatant and the pellet with UV light to see where the GFP is present. Record observations
12. Transfer 100uL of the supernatant to a fresh labeled 1.5 mL tube (S6-70%S) and save it for later analysis.
13. Dissolve the protein pellet in 0.75 mL 1XTE buffer and transfer 100uL to a 1.5mL centrifuge tube and label it as 70% ammonium sulfate precipitates (S7-70%P). Store the samples in your sample box for later analysis. *<u>The remaining resuspended pellet (650ul) will be used for HIC chromatography</u>*.
14. If you are completing the HIC procedure today, place on ice. If completing HIC later, transfer to a 2mL centrifuge tube and label "GFP-AS."

Lab Unit 8B: HIC Purification of GFP

Material

☐ 4.0M ammonium sulfate ☐ Cellular extract labeled “GFP-AS” ☐ 1.5 mL Microcentrifuge tubes (15) & rack	☐ Previously prepared HIC column, wash buffer and 1XTE Buffer ☐ UV light ☐ 20% ethanol

Safety Considerations

Wear gloves and safety glasses when handling and place waste in the appropriate container. UV radiation can damage your eyes, wear eye protection and do not look directly at the UV light.

PROTOCOL

Part I: HIC

1. Thaw your ammonium sulfate precipitated protein labeled “GFP-AS.” Add 0.450 mL of 4.0 M ammonium sulfate to the tube and mix well by inversion. The concentration of ammonium sulfate in this solution is 1.5M with a total volume of 1.2 mL.
2. Retrieve the column prepared previously. Gently, remove the Parafilm from both ends without disturbing the resin bed. Place a waste beaker underneath the column, and carefully remove the yellow cap. Keep the yellow cap handy!
3. Allow the equilibration buffer to elute until just before it reaches the top of the resin bed. Replace yellow cap tightly. Ensure it is on tight! Do not allow the column to run dry at any time!
4. ***Add GFP extract to the column:*** Using a 1mL pipette, *slowly load all protein extract to the top of the column*. Be careful not to disturb the chromatography bed or to introduce air bubbles. Remove yellow cap and allow the cell extract to enter the column.
5. When the protein extract has entered the column bed, recap the column with the yellow cap.
6. Using the clamp height adjustment, carefully lower your column until it is just above your microcentrifuge tube rack. The most efficient way of collecting fractions is to prepare a row of pre-labeled microcentrifuge tubes. Label tubes HICF1, HICF2, etc. Take a sharpie and mark the 1mL graduation on the side of each tube, so it’s easily seen. When your fraction has reached approximately the 1 mL mark, slide the rack to the next tube. Collect 1mL fractions throughout the column chromatography procedure.
7. Carefully add 4 mL **HIC wash buffer** to the column, 1 mL at a time, being careful not to disturb the top of the bed. This is the “wash” step. Any unbound proteins will elute from the column. *GFP should remain on the column*.
8. After you have added the wash buffer, remove the yellow cap from the column and ***start collecting the column effluent*** in 1mL fractions.
9. ***Qualitative observations:*** Make qualitative notes in your notebook. Observe your column and “wash” fractions with a hand-held UV light. You should see GFP getting “trapped” in the first layers of the column by observing the column under a black light. Why? Feel free to capture images with your cell phone!
10. When the wash buffer has reached the top of the resin bed, cap with the stopper.

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11. CAREFULLY, add 4 mL of **HIC elution buffer** (1X TE) to the top of the column. Once the beads have re-settled, remove the stopper and collect 1 mL fractions. Record the volume of elution buffer required to elute GFP from the column.
12. Closely monitor the location of GFP with a UV light. Try to *collect all GFP into one fraction*. Your GFP should come out within this 4mL aliquot. If not, add 2 mL 1XTE and continue collecting.
13. *Cap all fractions and invert to mix*. This is important. Why?
14. Remove a 100ul and save a sample of each of the fractions that have a high concentration of GFP and place into clean 1.5mL tube(s), labeled S8-GFP-HIC (with the fraction #).
15. Pool the remaining volume of the GFP fraction(s) together in a 2mL tube, labeled GFP-HIC. ASK YOUR INSTRUCTOR IF YOU ARE DOING THE DIALYSIS TODAY! If not, store in your freezer box.
16. If there is not enough time in class to analyze the fractions, label fractions clearly and store in your -20ºC freezer box.

Part II: Dialysis of GFP

The GFP fraction collected from the HIC column contains a high level of ammonium sulfate. *This salt needs to be removed for the GFP to adsorb to the anion exchange column.* We will use dialysis to desalt our GFP-rich fraction(s) from the HIC column. This procedure is unique to the Slide-a-lyzer G2 Dialysis Cassette.

Material

☐ GFP-rich HIC fraction/pool GFP-HIC	☐ Paper towels
☐ Slide-A-Lyzer G2 Dialysis Cassette: 7,000 MWCO, 1-3mL capacity (Thermo #87728)	☐ 1 mL serological pipet and pipet-aid
☐ Dialysis buffer, 1L: 25 mM Tris-Cl, pH 8.16 (per class)	☐ Beaker, 1000 mL
	☐ Stir plate & stir bar
	☐ Chromatography fridge
	☐ 2mL tubes

PROTOCOL

1. If not already made for you, prepare 1 liter of 25mM Tris-Cl, pH 8.
2. USING GLOVES, remove a Slide-A-Lyzer cassette from its pouch, and label the top with your initials with an insoluble marker. Do not touch the membrane, handle by the plastic frame.
3. Immerse cassette in dialysis buffer, keeping the entire membrane portion underwater for 2 minutes. It may be necessary to hold the cassette by the plastic frame submerged in buffer.
4. Remove cassette from the buffer and tap the edge of the cassette gently on paper towels. DO NOT BLOT THE MEMBRANE.
5. Open the cassette by gently turning the cap counter-clockwise ¼ stop, gently pull out the cap. The instructor will demonstrate. Do not twist/break off the top.

6. Fill a 1 mL serological pipet with the thawed sample. Use no more than 3 mL of sample. If you have more than 3mL, use an additional cassette.
7. Insert the pipet into the cassette and inject the sample into the chamber, slowly withdrawing the pipette while dispensing.
8. Remove the excess air from the cassette by simultaneously pressing the membrane gently on both sides using gloved thumb and forefinger and inserting the cap.
9. Insert cap and lock by gently turning it clockwise.
10. Float cassette vertically in the dialysis buffer; there is an integrated float on each cassette.
11. Place the beaker with assembly on top of a stir plate in the refrigerator (4ºC) and stir gently overnight.
12. Remove cassette from the buffer and gently tap on paper towels to blot excess liquid off.
13. Open the cassette by gently turning the cap counter-clockwise until it stops and then pulling out the cap.
14. Collect the sample using a disposable 1 mL Pasteur pipet, and transfer to a clean, labeled "HIC-GFP-D," 2mL tube. Mix well by inversion.
15. Remove a 100ul aliquot and set aside for later analysis with other frozen aliquots. Label "S9-GFP-D" (desalted HIC fraction).

Part III: Clean the Bio-Rad Macro-prep methyl HIC resin (158-0080)

While one lab partner moves on to part III (analysis), the other lab partner should clean the purification reagents away, and clean the column.

1. Add 10mL of 1XTE to the column and invert to create a slurry.
2. Pour slurry into a 15mL conical. Allow beads to settle and remove supernatant by decanting.
3. Add 10mL of 1XTE to the column and repeat wash.
4. Add 10mL of the 20% ethanol to the resin and repeat wash.
5. Store with 10mL of 20% ethanol. Label the conical as follows: "Used Washed methyl HIC resin, in 20% Ethanol". Include your names and date on the label, and place in the refrigerator for long-term storage.

Part IV: Analyzing GFP Fractions and Aliquots

Depending on time this may need to be performed on a separate day. Keep this in mind as you organize your time. Do not move on to this section unless you have at least 30 min.

NOTE: Your instructor may ask you to hold off analyzing the fractions until after the FPLC purification is complete. ASK YOUR INSTRUCTOR.

Material

☐ Plate Reader	☐ UV plate
☐ All fractions and samples (labs 7-8)	☐ Fluorescent Black plate
☐ UV hand-held light	☐ Multichannel micropipette & tips

Protocol

1. Remove your samples from the freezer and allow thawing on the bench top. Include water and any other solvents in your analysis.
2. It is easiest to use a 96-well plate reader to analyze this many samples. Obtain 96-well plate key, a black plate, and a UV plate.
3. Thaw all samples thoroughly, mix by inversion, and aliquot 100ul into a 96-well black plate.
4. Use the plate reader to measure the RFU of your samples (395 nM-Ex and 509 nM –Em). Depends on the fluorescence intensity you may need to dilute your sample 1:100 or 1:200 with the TE buffer before measuring the RFU. Remember, your samples should become more concentrated with GFP.
5. Record all your data in the table previously prepared in your notebook.
6. Using a multichannel micropipette transfer your samples into a 96-well UV plate and analyze on the plate reader at 280nm. Record data in your table.
7. Return ALL aliquots to their original tubes – do not throw them out! Return all your fractions to the freezer. You will analyze several of your selected fractions using gel electrophoresis in a later lab exercise.

Laboratory Unit 8 Analysis & Review

For your Laboratory Report, ensure it includes the following:

1. ***Prelab Exercise***
2. ***Summary of raw data:***
    a. Create a summary table of your data. In your table include the qualitative analysis of your fractions, the A_{280}, RFU, and the ratio of RFU/A_{280}. Include in your table your analysis of the extract before loading on the column.
    b. Generate a graph of fraction number versus A_{280} and fraction number versus RFU (A_{509}). The usefulness of this graph is apparent if you overlay these both on one graph. Ask your instructor if you need help with this graph.
    c. Determine the quantitative yield of GFP from the column. This can be calculated by fluorescence multiplied by the total volume of GFP collected from the column. The percent yield can be calculated by comparing the total RFU of the post-column sample to the total RFU loaded onto the column.
    d. Include in your report the Purification Table you created when starting this purification scheme.

3. ***Analysis of results***
    a. Did HIC work well to purify GFP from your cellular extract? How efficient were elution conditions at releasing GFP from the column in a narrow fraction, producing a small volume of highly concentrated GFP?
    b. When did GFP elute from the column? Discuss your graph(s). Did it adsorb to the column, or come out the effluent? Did it elute with the wash buffer or with the elution buffer? Explain why GFP eluted when it did from the column?
    c. Did 100% of GFP protein come off the column where you expected it? Using your data, discuss where your GFP was 'lost' in the procedure.
4. ***Conclusions***.

LAB UNIT 9: ANION EXCHANGE FPLC

INTRODUCTION

One of the most efficient and most used methods for separating charged compounds is ion exchange chromatography. An ion exchange column is full of resin that contains charged groups on the surface. In this purification scheme of GFP, we will use a type of ion exchange resin called Q-Sepharose. The resin beads of this column contain tetra methyl amine $(CH_3)4N^+$. Electrostatic interaction binds chloride (Cl-) ions to the bead. Because there is a negative charge bound to the resin beads, this column is known as an anion exchange column.

The chloride ions loosely associated with the surface of the resin beads are exchanged for any negatively charged amino acids that are above their pI or isoelectric point. Therefore, a protein with a preponderance of negative charges will stick to the column, while other proteins with more positive charges will continue to pass on through the column and into the void volume. Since pH of the buffer system impacts how well a given protein attaches to a column, the buffer system used in separating a given protein is of paramount importance.

If a buffer can influence how a protein will attach to an ion exchange column, then changing the buffer system can, therefore, elute that protein from the column. Once the selected protein has adsorbed to the column and the undesired proteins have eluted, the buffer system is changed, allowing the selected protein to elute from the column in a more purified form.

In some more sophisticated systems, the pH of the buffer is continuously changed so allow for very precise isolation of the desired protein. This subtle change in pH is not necessary for the purification of GFP, so we will only use the 2-stage protocol of adding the GFP to the column and then changing the buffer to elute the GFP in the second step.

Note that we will also be using an anion exchange column, Q-Sepharose. Other conventional anion exchange columns are DEAE-Sephadex and DEAE-cellulose. There are also cation exchange columns such as Dowex or CM-cellulose to which a negatively charged group, such as $SO3^-$ is attached. Therefore, the exchanging (counterbalancing) ion will be a positive ion. Such a cation exchange column best separates some proteins with large numbers of basic R groups.

In this lab exercise, we will perform anion exchange chromatography to purify GFP from the HIC column further using the Bio-Rad Duo Flow FPLC system. In this automated FPLC system, the liquid is being handled by robotics. Liquid Handling Robots can be programmed to dispense liquids accurately. The automated FPLC system is different from the manual method you used in HIC separation of your GFP sample; the dispensing was performed manually, by eye. Robotics liquid handlers give the advantage of accurately dispensing volume, automation to allow the user to walk away, or perform multiple dispensing tasks, and in this case, the automation even includes analysis of the sample.

Liquid handling robotics is standard in many laboratory environments, and more frequently found in labs that are responsible for high throughput analysis, or dispensing. Robots are routinely used in pharmaceutical research labs where thousands of potential drugs can be screened efficiently.

There are various types of liquid handlers custom designed for specific applications. One commonality is a computer interface that is used to program the specific functioning of the robot, for example, dispensing volume, the number of samples to dispense, error tolerance and speed.

GFP Purification by Anion Exchange FPLC

Material

o 50 mM Tris-HCl o 50 mM Tris base o 2.0 M NaCl o UnoQ anion exchange resin cartridge (catalog #732-4102) o pH 7 and pH 10 standard buffers o Dialyzed HIC protein. HIC-GFP-D	o Duo Flow FPLC System with 1 mL sample loop o Hamilton syringe o Luer-lock syringe o Side-arm flasks, rubber tubing, and solid stoppers (for degassing) o 500 mL bottles o Fraction collection tubes, 13 x 100 mL

Safety Considerations

✓ *Wear gloves and safety glasses when handling and place waste in the appropriate container.*

✓ *UV radiation can damage your eyes, wear eye protection and do not look directly at the UV light.*

PROTOCOL

NOTES:

✓ All solutions must be degassed. Do not refrigerate solutions after degassing, because air bubbles will develop upon warming to room temperature. These solutions may have already been prepared for you. Ask your instructor.

✓ The FPLC may already be set up and ready for use. Ask your instructor.

Part I. Buffer Preparation

1. Place 500 mL of 50 mM Tris acid in a 1-L side-arm flask, add a stir bar, cap with a solid stopper, and attach the sidearm to a vacuum line. Degas for about 15 minutes with gentle stirring.
2. Repeat step 1 with 500 mL of 50 mM Tris base.
3. Repeat step 1 with 500 mL of purified water.
4. Repeat step 1 with 2 M NaCl.

Part II: Starting the System

1. Turn on the Maximizer mixer, pumps, and fraction collector. Open the Biologic software on the attached PC.
2. Check the resulting manual screen for the following system components: Maximizer and gradient pump, fraction collector, UV detector, and workstation valve AVR7-3. To view the AVR7-3 valve, click the down arrow button at the top right corner of the Maximizer Valve window (in the center of the screen). The AVR7-3 valve will appear at port 10.

Part III: Purging and Priming the Pumps

NOTE: The column should not be in-line during purging, priming, and flushing the system.

1. Immerse all four colored Maximizer inlet lines into a container of filtered, degassed purified water. Secure all three waste lines (narrow, translucent white tubing) in a large waste container.
2. From the manual screen, place the Maximizer in Local mode and use the valve port select button (under the A1/A2 Maximizer valve inlet) to select inlet port A1.
3. Connect a syringe to the priming port of pump A. Open the port by turning it counterclockwise one full turn and withdraw the syringe plunger to draw some water out. Close the priming port by turning it clockwise before removing the syringe.
4. Repeat several times until no air bubbles are visible in the red A1 inlet tubing.
5. Use the valve port select button on the Maximizer to select inlet port A2. Repeat step 3&4.
6. Repeat Steps 3 and 4 with priming ports B1 and B2.
7. From the manual screen, place the Maximizer in System mode. Locate the AVR7-3 valve on the manual screen and click “P” to place the valve in the purge position. Place the Maximizer in Local mode again.
8. Press the Purge Buttons A and B on the front of the Duo Flow pump. The indicator lights will flash green. After purging for two minutes, press the valve port select buttons on the front of the Maximizer to select the other two ports, and purge those for two minutes more. Press the purge buttons to stop the pump.
9. Place the Maximizer in System mode and place the AVR7-3 valve in the Load position by clicking “L.” Set the pump flow rate at 1.0 mL/minute and start the pump. Water will flow through the entire system and out of the fraction collector.

Part IV: pH Electrode Calibration

1. The pH electrode should be calibrated before the first run of the day. Remove the electrode from the pH monitor by unscrewing it. Place the electrode in pH 7 buffer.
2. From the Utilities menu, select pH Probe Calibration.
3. Press Set. When the pH reading has stabilized, press OK.
4. Rinse the electrode with purified water.
5. Repeat steps 2 through 5 with pH 10 buffer.
6. Replace the electrode in the pH monitor.

Part V: Equilibrating the UNO Q1 column

1. Submerge the four colored inlet tubes in the appropriate solutions and secure with tape:
    a. A1 (red): 50 mM Tris-HCl
    b. A2 (blue): 50 mM Tris base
    c. B1 (yellow): water
    d. B2 (green): 2 M NaCl
2. Prime and purge the pumps as described in Part 1.3, Steps 2-8.
3. Set the inject valve position to Load (L). Set the flow rate to 2.0 mL/minute. Set the buffer recipe by pressing the Setup button on the toolbar. Choose Tris (25 mM) and press OK. On the Manual screen, set the pH to 8.1 and Inlet B % to 0.

4. Place the UNO Q1 column in line by connecting the inlet to the inject valve and the outlet to the UV detector. Secure the column in a vertical position using the clamps.
5. Wash the column at pH 8.10, 100% B, 2 mL/minute for 3.5 minutes.
6. Equilibrate the column at pH 8.10, 0% B, 2 mL/minute for 6.5 minutes.

Part VI: Anion Exchange Chromatography

1. Open the Browser tab to locate the saved method. Locate the Method “GFP Anion Exchange” and double-click the title. The protocol for this method will open. Double check the protocol and make correction according to the following table if the saved method is different from the one posted here.

	Volume	Description	Parameters		
1	0.00	Collection Fractions of size 2.00 ml during entire run			
2	0.00	Isocratic Flow	pH: 8.10	Volume: 10.00 ml	
			0%B	Flow: 4.00 ml/min	
3	10.00	Zero Baseline	UV Detector		
4	10.00	Load/Inject Sample	Sample		Volume: 6.00 ml
			Static Loop	Auto Inject Valve	Flow: 2.00 ml/min
5	16.00	Isocratic Flow	pH: 8.10	Volume: 6.00 ml	
			0%B	Flow: 4.00 ml/min	
6	22.00	Linear Gradient	pH: 8.10	Volume: 13.00 ml	
			0%B -> 25%B	Flow: 4.00 ml/min	
7	35.00	Isocratic Flow	pH: 8.10	Volume: 6.00 ml	
			50%B	Flow: 4.00 ml/min	
8	41.00	Isocratic Flow	pH: 8.10	Volume: 10.00 ml	
			0%B	Flow: 4.00 ml/min	
	51.00	End of Protocol			

2. Click the Run button on the toolbar. The Run screen will appear. Ensure that the fraction collector rack contains at least 26 tubes starting at position 1.
3. Place the inject valve in the Load (L) position. Load the sample loop with a sample of the GFP HIC pooled fraction with a syringe and needle through port 2. Use 1 mL sample loop if your sample size is ~ 1mL. Use 5 mL sample loop for sample size larger than 1 mL. **After filling the loop, leave the syringe in the injection port.** If you remove the syringe, the sample will siphon to waste.
4. Click on the green Start button on the toolbar to launch the run. The sample will be injected automatically onto the column at the appropriate step.
5. When the run is finished, the pumps will automatically stop and a “Run Finished” message appears in the bottom right of the status bar.
6. Identify the fraction with the most GFP, mix gently by vortexing, removing a 100ul aliquot and place into clean labeled tube: S10-FPLC-GFP
7. If there is no time to continue with analysis, transfer all fractions to labeled 2mL tubes: FPLC-GFP-F1, etc.

Part VII: Analyzing GFP Fractions and Aliquots

Depending on time this may need to be performed on a separate day. Keep this in mind as you organize your time.

Material

☐ Plate Reader ☐ All fractions and samples collected, labs 7-9. ☐ UV light	☐ UV plate ☐ Fluorescent Black plate ☐ Plate reader ☐ Multichannel micropipette

Protocol

1. Remove your samples from the freezer and allow thawing on the bench top. Include water and any other solvents in your analysis.
2. It is easiest to use a 96-well plate reader to analyze this many samples. Obtain 96-well plate key, a black plate and a UV plate.
3. Thaw all samples completely, mix by inversion, and aliquot 100ul into a 96-well black plate.
4. Use the plate reader to measure the RFU of your samples (395 nM-Ex and 509 nM –Em). Depends on the fluorescence intensity you may need to dilute your sample 1:100 or 1:200 with the TE buffer before measuring the RFU.
5. Record all your data in the table previously prepared in your notebook.
6. Using a multichannel micropipette transfer your samples into a 96-well UV plate and analyze on the plate reader at 280nm. Record data into your table.
7. Return ALL aliquots to their original tubes – do not throw them out! Return all your fractions to the freezer. You will analyze several of your selected fractions using gel electrophoresis in a later lab exercise.

Laboratory Unit 9 Analysis & Review

For your Laboratory Report include the following:

1. ***Prelab Exercise***
2. ***Summary of raw data:***
    a. Create a summary table of your data, as shown in the example. In your table include the qualitative analysis of your fractions, the A_{280} total mg, RFU/mL, RFU/mg, purification fold. Include in your table your analysis of the extract PRIOR to loading on the column.
    b. Generate a graph of fraction number versus A_{280} and fraction number versus RFU (A_{509}). The usefulness of this graph is apparent if you overlay these both on one graph.
    c. Determine the quantitative yield of GFP from the column. This can be determined by the measured fluorescence multiplied by the total volume of GFP collected from the column. The percent yield can be calculated by comparing the total RFU of the post column sample to the total RFU loaded onto the column.
    d. Include in your report the Purification Table you created when starting this purification scheme.

3. ***Analysis of results***.
    a. Discuss your results. Remember that the goals of chromatography include a high overall yield as well as a high concentration of the molecule that you wish to purify. Did anion exchange work well to purify GFP from your cellular extract? How efficient were elution conditions at releasing GFP from the column in a narrow fraction, producing a small volume of highly concentrated GFP?
    b. When did GFP elute from the column? Discuss your graph(s). Did it adsorb to the column, or come out the effluent? Did it elute with the wash buffer or with the elution buffer? Explain why GFP eluted when it did from the column?
    c. Did 100% of GFP protein that you loaded onto the column come off the column where you expected it to? Using your data, discuss where your GFP was 'lost' in the procedure.
    d. Compare the anion exchange yield with the HIC yield.

4. ***Conclusions***

LAB UNIT 10: POLYACRYLAMIDE GEL ELECTROPHORESIS OF GFP

INTRODUCTION

Electrophoresis is the movement of charged particles in an electric field. Electrophoresis of protein proteins in a solid matrix and applying an electric field will separate the proteins according to their size, shape, and charge. The solid support used in the electrophoresis of proteins is typically polymerized acrylamide because of its' high resolving power while being inert.

Polyacrylamide is cross-linked with N, N'-methylene-bis-acrylamide in a polymerization reaction that is initiated by the molecule N, N, N,'N'-tetramethylethylenediamine (TEMED). If low concentrations of acrylamide and bis-acrylamide are used, the pores are larger allowing for resolution of higher molecular weight proteins. Conversely, smaller molecular weight molecules are resolved in gels with a higher concentration of acrylamide and bis-acrylamide.

PAGE gels are polymerized between two square panes of glass or plastic that are sealed around the edges and stood upright during polymerization. The resulting gels are run upright as well, giving rise to the common name of "vertical gel electrophoresis." PAGE gels may be continuous or discontinuous: continuous gels have a gradient of acrylamide concentration, whereas a discontinuous system, has two different gel concentrations forming a stacking gel and resolving gel.

In a discontinuous gels system, the lower 80 percent of the gel is the resolving gel, which contains a higher percentage of cross-links (smaller pore sizes). Above that is a stacking gel that contains fewer cross-links (larger pore size) and prepared with a buffer containing fewer mobile ions. A comb is inserted when pouring the stacking gel to produce the wells into which the samples are placed. Because the stacking gel has larger pore sizes, it provides a lower resistance than the resolving gel, so proteins will travel quickly through the stacking gel and concentrate at the interface between the stacking and resolving gels. This allows the proteins to enter the resolving gel close together and therefore increases the resolution during separation as they travel through the resolving gel.

NATIVE GELS

When an electrical field is applied to the proteins, they will travel from the negative to the positive electrode through the polyacrylamide gel matrix. Protein movement in a native gel system is influenced by many factors: size, shape, and charge. Smaller proteins will move more quickly through the gel than larger ones due to less frictional drag against the polyacrylamide matrix. For the same reason, tightly coiled globular proteins will travel more rapidly than more

loosely packed proteins or extended structure fibrous proteins in an electrophoretic gel. The ratio of charged amino acids in a protein will also influence movement through the gel; those proteins with more negatively-charge (acidic) amino acids than positively charged (basic) amino acids will travel more quickly towards to positive anode due to their large net negative charge. A protein with a net positive charge will, in fact, migrate in the wrong direction from the sample well, away from the anode. Therefore, native gel electrophoresis systems are buffered with an alkaline buffer system – to encourage an overall negative charge protein, allowing proteins to travel down through the matrix toward the positive electrode.

Native gels are used when the conformation of the protein needs to be preserved, usually because the location of the protein of interest must be identified by its structural function. For example, if an enzyme must be identified by its enzymatic activity, a native gel will be used. Also, Western blots are typically performed with native gels to ensure that the antibody detection of a protein of interest will work. In today's lab you may be asked to analyze your GFP samples using a native system. What would you expect to see on a native gel when you analyze GFP?

DENATURING GELS (SDS-PAGE)

With all these factors influencing the movement of native proteins through a PAGE gel, it is nearly impossible to predict where a given protein will migrate or analyze the results of a native gel. For proteins to behave similarly, they must be denatured into a uniformly extended shape. That way the unfolded proteins will act similarly to nucleic acids in that the short polypeptides travel further down the gel and the longer polypeptides do not go as far. Unlike DNA molecules, however, a denatured protein does not have a uniformly-charged structure. For denatured proteins to move similarly to each other in an electrophoretic gel, they must have a uniform charge-to-mass ratio.

Two protein denaturing agents are commonly used together in PAGE electrophoresis: a sulfhydryl reducing agent and an ionic detergent. Dithiothreitol and 2-mercaptoethanol are short molecules containing a sulfhydryl (-SH) group that will break up the disulfide bridges and help to disrupt any tertiary structure and quaternary structure of the proteins due to disulfide bridges. The sulfhydryl group is what gives these compounds their "rotten egg" smell. Sometimes this smell can be noxious, and it is recommended to use inside a fume hood. Additionally, DTT and B-me are hazardous chemicals and should be disposed of appropriately.

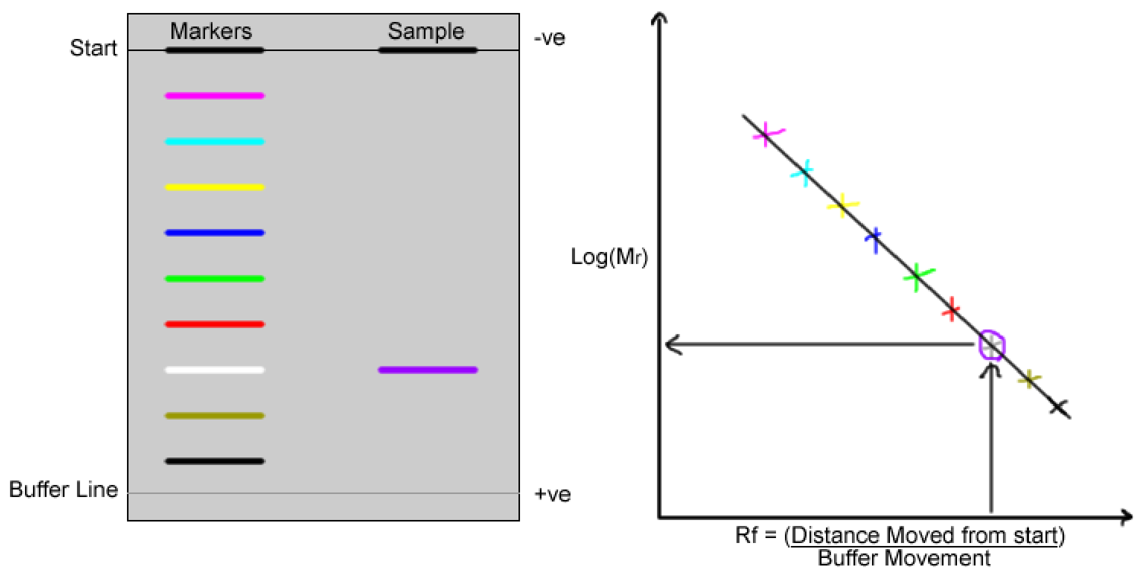
The primary denaturing agent is an ionic detergent, sodium dodecyl sulfate (SDS). SDS serves two functions: First, it disrupts the secondary, tertiary, and quaternary structures of the protein by breaking the ionic and hydrogen interactions between the amino acids of the protein, as well as interfering with the hydrophobic interactions responsible for correct folding of the protein. Second, SDS will coat the protein with negative charges from the sulfate head group of the detergent. SDS coats proteins with extensive negative charges, negating any small charge differences between proteins, permitting proteins to travel through the polyacrylamide gel and separate by size and NOT by charge.

Typically, 1% SDS and 0.1 M β-mercaptoethanol are used along with high temperatures to completely denature proteins and coat their extended structures with negative charges. This gives rise to the SDS-PAGE electrophoresis that is the most common method of identifying an unknown protein or determining its molecular weight. To determine protein molecular weight, a set of proteins markers of known molecular weight are run along with the unknown. Analysis of sizes of protein bands in the gel is then a straightforward comparison to the migration distances to the molecular weight markers.

The samples of protein are diluted with a "sample buffer." In addition to the mercaptoethanol and SDS denaturants, the sample buffer contains a tracking dye that will travel with the front and determine how far the gel has run. Glycerol is also included for increased density of the sample for it to settle into the bottom of the well and not go floating off into the electrophoresis buffer. Typically, somewhere between 10 and 40 μg protein is loaded into a well, depending on the purity of proteins in the sample. This total volume is usually about 20-40 μL, half of which is protein and half are the 2X sample buffer.

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 does not influence its migration.



BY NC SA

PRE-LAB EXERCISE

In this lab, students will use denaturing SDS-PAGE to analyze the purified GFP samples from the previous three labs. Students will capture an image of the SDS-PAGE and then transfer the protein to a nitrocellulose membrane to perform a western blot (the following lab).

1. Each student must select 1-2 samples from each of the purification steps to will demonstrate the success of the GFP purification scheme. The gels have 10 lanes; lane 1 will be a molecular weight marker, lane 2-9 will be SDS-PAGE protein samples, and lane 10 will be one GFP sample in native load buffer. Using a gel electrophoresis analysis key (see appendix), plan out your gel analysis. Bring a completed gel electrophoresis form to class with your completed pre-lab exercise.
2. Research the size you are expecting to see GFP. Bring this to class!
3. Using the catalog number of the molecular weight marker below, use the internet to retrieve a copy of the molecular weight marker product sheet. This should include an image and sizes of the protein bands that you should see in your gel. Print this out and bring it to class.
4. Complete a pre-lab exercise for SDS-PAGE analysis of GPF. Print it out and bring it to class.
5. IF you will be continuing to the Western blot lab, complete a pre-lab exercise for the Part I of the Western Blot analysis – transfer to nitrocellulose, up to and including the blocking buffer incubation. Ask your instructor!

MATERIAL

☐ GFP purification samples S1-S10. ☐ HIC and FPLC fractions ☐ 1.5 mL screw-cap tubes ☐ 1.5 mL flip-cap microcentrifuge tubes (for non-denatured samples) ☐ 10ul, Chemiluminescent marker (Thermofisher, Super Signal Protein Ladder, Cat#84785) ☐ 1mL disposable pipet ☐ 2, 10 mL serological pipets ☐ Gel-load tips ☐ Vertical Gel Running Apparatus	☐ 10X Denaturing Running Buffer (Bio-Rad, Tris/Gly/SDS, Cat #161-0732) ☐ 2X Denaturing Sample Load Buffer (126mM Tris-Cl, pH 6.8, 20% glycerol, 0.005% bromophenol blue, 4% SDS, 5% b-mercaptoethanol) ☐ 2X Non-Denaturing Sample Load Buffer (126mM Tris-Cl, pH 6.8, 20% glycerol, 0.005% bromophenol blue) ☐ Mini-Protein TGX Stain-free gel (Cat#456-8093) ☐ gel wash tray ☐ Gel cracker

SAFETY CONSIDERATIONS

- ✓ *Wear gloves when handling DTT or β-mercaptoethanol, they are highly toxic. Work in a chemical hood. Dispose of SDS-sample buffer waste in labeled fumehood solid waste container.*
- ✓ *Use only screw-cap tubes when heating samples with DTT or β-mercaptoethanol. They will pop open unexpectedly and may splash hazardous chemicals at your face.*

- ✓ 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.
- ✓ *An area of at least 6 inches around the power supply and cell should be bare of clutter and other equipment and dry.*
- ✓ *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!*

Part I: Prepare Solutions

1. Each gel cassette holder holds two gels and requires 1L of the buffer. Prepare 1L of 1X Denaturing (Tris/Glycine/SDS) Running buffer per group, using the 10X stock provided.
2. The 2X sample load buffers are already be prepared for you. Thaw them and place in a rack on your bench. Note the SDS-PAGE sample load buffer may require gentle re-heating at 37deg C to get the SDS back in solution. Warm for a few minutes at 37degC, vortex and place in a rack on your bench.

Part II: Prepare Samples

1. Before class, ***as part of your pre-lab exercise***, determine which samples to analyze on the gel and how much sample to load. If there is enough equipment and gels, each person in the class can prepare and analyze their own samples on their own gel. Each gel box can hold 2 gels.
    a. The gel has 10, 30ul wells; the first lane will have a molecular weight marker. The last lane will be purified GFP in native load buffer.
    b. Your sample selection should reflect your extraction and purification procedures – what you started with, what you didn't want, and what you purified. You have kept at least ten intentional samples, labeled S1-S10, in addition to all the fractions from the HIC and FPLC chromatography.
    c. Fill in a gel-electrophoresis form with your selection to help you label your tubes 1-10. Tube 1 is the marker; tubes 2-10 are your samples.
2. Defrost the samples and place on a rack on your bench.
3. Warm up SDS load dye at 37°C, invert gently to return SDS to solution. Do NOT put on ice; this will cause the SDS to fall back out of solution.
4. ***Denaturing Sample Preparation:*** Label SCREW-CAP microcentrifuge tubes "2-9" for denaturing samples. And add 20 μL of denaturing 2X sample load buffer to each tube. Add 20 μL of GFP sample to appropriate tube. Vortex. Boil, or heat at 94°C for 5 minutes, ONLY tubes 2-9. Flick, pop-spin and return to your microcentrifuge rack. Do NOT place samples with SDS load buffer back on the ice!
5. ***Native Sample Preparation:*** Label flip-cap microcentrifuge tubes "10". And add 20 μL of native sample load buffer to each tube. Add 20 μL of GFP sample to appropriate tube. Flick gently to mix. Pop-spin and keep on ice. Note which sample you chose to be your native GFP.
6. ***Molecular Weight Marker:*** You will be using prepared molecular weight markers. These markers already have load dye. Do NOT heat this marker; it will cause it to degrade! Thaw on ice. You will need 10 ul per gel. Label top of tube with a "1".

PART III: Analyzing GFP samples on Denaturing PAGE

1. With gloved hands, carefully unwrap Bio-Rad premade PAGE gel. Record in your notebook the percentage of the gel.
2. Pull comb straight up and out (do not wiggle), and peel off the green tape from the bottom of the gel - this is critical!
3. You will work with your lab partner to set up two gels in one gel box.
4. Your instructor will demonstrate how to set up the gels into the gel boxes, so they do not leak. Alternatively, you may watch this video: https://youtu.be/XnEdmk1Sqvg
5. Place two gels with the short plate facing inward, and close clamp. Fill up the interior of the gel clamped apparatus far above the short plate with the appropriate buffer and then check for leaks. The best way to do this is to remove the gel clamped apparatus from the gel box and place on the bench top. You will see liquid drip out of the bottom if you do not have a good seal. If this happens, pour the liquid into the gel box and repeat the clamping and leak detection procedure.
6. Insert the clamped gel apparatus back into the gel box (red to the red back electrode). Ensure it is in the correct orientation! Check with your instructor if you are unsure.
7. Fill up the gel box with the appropriate buffer to the line indicated on the gel box: 2-gels.
8. Rinse unpolymerized acrylamide from the wells by washing each well in running buffer vigorously using a 1mL pipette tip or disposable Pasteur pipette.
9. If you previously made and froze back samples, thaw them on your bench top. Pop-spin. The non-denaturing samples and molecular weight marker do not get heated. Incubate the SDS-PAGE denatured samples in a boiling water bath for 2 minutes. Vortex briefly and pop-spin, keep at room temperature. Do not place SDS samples on ice!
10. The wells are identified as #1 through #10, starting with #1 on your left as you face the front of the gel. By convention, well #1 is always the molecular weight marker lane.
11. Read the Good Laboratory Practice Tips (below) before loading samples.

GLP tips: To load a sample into a well,

a. Adjust an automatic micropipette to deliver the correct amount of the sample, and attach an ultrathin gel loading tip.
b. Withdraw the correct amount of your sample from your microcentrifuge tube slowly. Keep your tip in the sample and wait until the sample stops entering the tip. Viscous protein samples make take up to 5 seconds.
c. Insert the micropipette tip into the top of the well of the short plate. Take care not to put the tip into the bottom of the well. This takes a steady hand – it may help to support the micropipette with your other hand.
d. Ensure that 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.
e. Do not press the micropipette to the second stop – it is important to avoid blowing air bubbles into the well.
f. *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!*

12. It's important to load quickly, connect lid, and turn on power supply immediately after you have loaded your samples into the wells. If you are sharing a gel box with another group, turn the gel box sideways so that both groups can load their gels at the same time.
13. Load 10ul of molecular weight marker into Lane 1.
14. Load no more than 30μL of each sample starting from lane 2. Be sure to use a new gel loading tip for each sample loaded. Follow the order written out in your gel electrophoresis form.
15. It's important that you start running your sample immediately after both gels are loaded. It's important to work swiftly.
16. As demonstrated by your instructor, connect the electrophoresis apparatus to the power supply and plug it in. Have your instructor check your set-up and connections. When given the OK by your instructor, turn on the power supply.
17. The Bio-Rad TGX gels can run at high voltage. Set power supply to 300-250V, and run the gel for 20 minutes (until the dye front is near the end of the gel, but does not run off!). Some gels may run faster or slower, so keep an eye on your gels. Record your start time in your lab notebook.

    A few minutes after the power is applied, the blue bands should concentrate as a thin line below each well at the interface between the stacking gel and the separation gel. The blue bands should then move slowly down through the resolving gel. If this does not happen let your instructor know immediately. Did you remember to remove the green tape? Is there enough buffer in the center, far above the short plate? Is the power supply turned on?
18. When the blue dye is within 2-3 millimeters of the bottom of the gel, ***turn off the power supply and unplug it.***
19. Wearing gloves, remove the cover from the electrophoresis apparatus. With gloved hands, remove the clamped gels and pour the interior buffer back into the gel box.
20. Remove gels, rinse with diH2O. Measure the distance of the dye front in the marker lane and record in your notebook. You will need this to calculate the Rf value for your gel.
21. Using the gel cracker supplied, crack the two plates apart at the black arrows. Be careful when prying the plates apart; our gel may stick to either plate.
22. Turn the plate upside down over a Tupperware containing deionized water. Using a squirt bottle, gently pry the gel from the plate. If you use your gloved fingers, always pre-wet in water before touching the gel, or you may rip the gel. Note, if you are transferring the gel to nitrocellulose you must wash it 3x5min in diH2O.
23. Using the gel documentation system, capture a UV image of your gel to image the native GFP sample. What are you expecting to see? Print out a copy and tape it to the left in your gel documentation form. Label it "UV."
24. CHANGE the gel documentation system to "Protein," "Stain Free Gel," and capture a total protein image. Print out an image and tape it to the right, labeled "Fluorescent protein stain."
25. If you are continuing to a western blot transfer today, ensure the blot is rinsed 3x5 min in diH20, and then an additional 5 min in transfer buffer.

Optional: Stain and De-stain gels (Ask Instructor)

If you did not use "Stain-free Gels," you will need to stain your gels with Coomassie blue to see the total protein bands. Note, Coomassie blue stained protein gels cannot be used to perform Western Blots. You will need to run additional gels for Western Blot analysis. If this is the case, you may have one partner stain their gel, and the other lab partner transfer their gel to nitrocellulose. Ask your instructor for further information!

1. After removing from the plastic casing, wash the gels three times with deionized water for 5 min each using copious amounts of deionized water. Use gentle rocking and remove water completely between each wash. The SDS interferes with Coomassie Biosafe staining, it is crucial to soak your gel 3 X 5 minutes before staining!
2. Add enough Coomassie Biosafe stain just to cover your gel. Allow the gel to rock gently in stain for 1 hour (or overnight preferred).
3. Remove stain and repeatedly wash for several hours. Overnight works best. This might take a few days if you stained overnight.

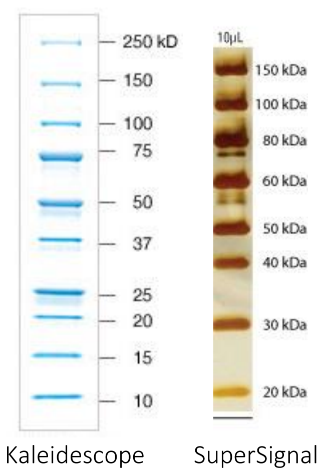
CLEAN UP!

1. Rinse apparatus with tap water and a final rinse in diH_2O. Turn upside down and leave on an absorbent towel to dry. DO NOT towel-dry; you might accidentally dislodge the electrodes!
2. Dispose of the running buffer solutions down the sink with lots of water.
3. Protein samples in native gel buffer can be discarded in the regular trash or kept to repeat analysis for Western Blot. Ask your instructor.
4. NOTE! The denaturing gel sample buffer contains either DTT or βME and therefore any tips, tubes, and excess buffers not used, must be disposed of with solid waste in the fume hood.

PART IV: Analysis of the SDS-PAGE Gel

Non-denatured GFP protein, which maintains its' fluorescence, usually shows up at around 37,000 Daltons. The fully denatured GFP can be seen at its MW of 27,000 Daltons.

1. Record the migration distance of your MW standards and your GFP in each lane for the SDS-PAGE gel only. Locate the GFP at the expected migration distance in your gel. Record your observations of the GFP band(s) and any other protein bands in each of the lanes in your gel. Distance of Dye front: ____________________ mm



Kaleidescope SuperSignal

Molecular Weight (Marker) KDa	Log MW	Migration Distance (mm)	Rf Value Migration/dye front

2. Calculate the Rf value by dividing the migration distance of the band by the migration distance of the blue dye front. Record this in the table.
3. Calculate the log of the molecular weight of each band. Record in the table.
4. Plot the log of the molecular weight (y-axis) versus the Rf value (x-axis) as outlined in the introduction of the lab manual instructions.
5. Determine the equation of the line and the R^2 value.
6. Measure the distance of the <u>predicted GFP bands</u> for each of the wells. Record this in a table in your notebook. Note, you will NOT do this for lane 10, the native GFP sample. Why not?
7. Using the equation of the line determine the molecular weight of the major protein bands in each lane (remember to anti-log!). Record this in the same table.
8. Some gel systems (such as with continuous gels) require a log/log plot to obtain a linear relationship with molecular weight and migration distance. If your results are CURVED, perform a log/log plot.
9. Determine the molecular weight of GFP in your SDS-PAGE gel using the equation of the line from your graph. <u>Is it what you expected?</u>

Laboratory Unit 10 Analysis & Review

For your Laboratory Report, include the following:

1. ***Prelab exercise –*** with experimental notes and solution calculations
2. ***Summary of raw data:***
    a. Include a labeled image of your gel, include the molecular weight marker sizes and an arrow pointing to your GFP protein on your gel electrophoresis form.
    b. Create a molecular weight graph for your SDS-PAGE analysis.
    c. Determine your GFP molecular weight. <u>Label the GFP on your labeled image with its calculated size</u>.

3. ***Analysis of results & Conclusions***.
    a. Discuss your results on your SDS-PAGE gel lane by lane. Evaluate the <u>relative abundance of total protein versus GFP</u>. How does SDS-PAGE help determine how well the purification scheme worked?
    b. In which lane(s) was the GFP band the predominant protein band? Is this expected?
    c. Discuss the native GFP sample. Does it migrate the same as the SDS-PAGE samples? Why or why not? Discuss the advantages of analyzing protein on a native gel over an SDS-PAGE gel.

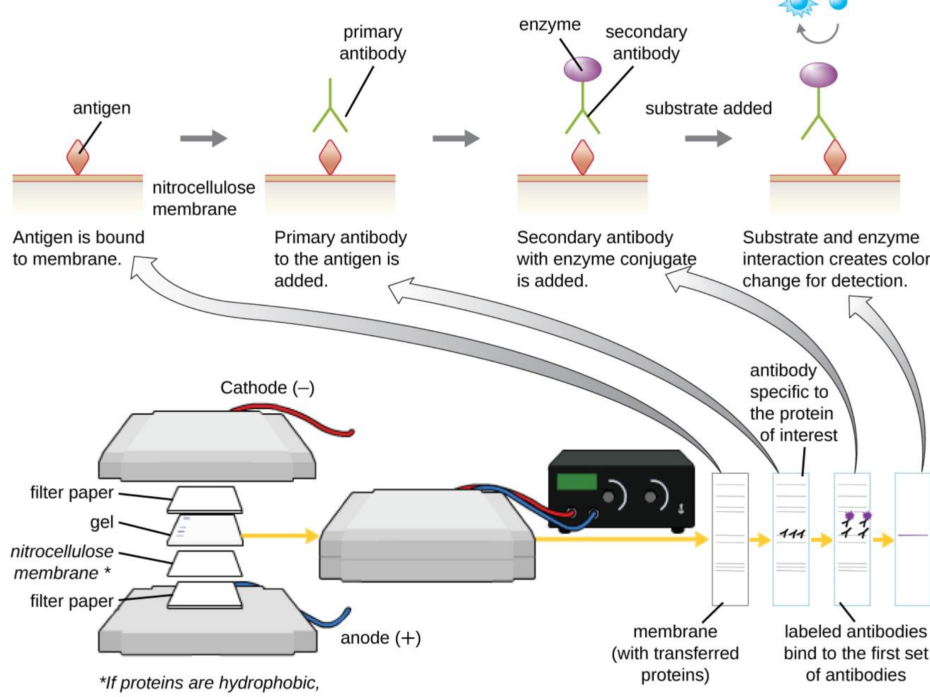
4. ***Conclusions***

LAB UNIT 11: WESTERN BLOTTING OF GFP

INTRODUCTION:

Western blotting is an important technique used in cell and molecular biology and is often utilized in research to separate and identify proteins extracted from cells. After performing protein gel electrophoresis, specific proteins can be identified in the gel using antibodies. This technique is known as the Western Blot. Following separation of proteins by PAGE, the protein antigens in the gel are transferred to and immobilized on a nitrocellulose membrane. This membrane can then be exposed to a primary antibody produced to specifically bind to the protein of interest. A second antibody equipped with a molecular beacon will then bind to the first. These secondary antibodies are coupled to another molecule such as an enzyme or a fluorophore (a molecule that fluoresces when excited by light). When using antibodies coupled to enzymes, a chromogenic substrate for the enzyme is added. This substrate is usually colorless but will develop color in the presence of the antibody. The fluorescence or substrate coloring identifies the location of the specific protein in the membrane to which the antibodies are bound

In this lab, we will use western blotting to identify recombinant GFP in samples throughout our recombinant protein purification process. Watch this video to learn more about how to set up the western blotting apparatus: https://youtu.be/VgAuZ6dBOfs



Electrophoresis: Proteins are separated by gel electrophoresis, usually using a denaturing polyacrylamide gel. In a denaturing gel, the protein mixture is separated by size alone.

Transfer: The proteins are transferred to a sheet of special membrane, usually nitrocellulose polyvinyl pyrrolidone, or nylon, though other types of membranes can be used. This transfer is done by electro-elution at 90 degrees to the gel through a buffer. The proteins retain the same pattern of separation they had on the gel. The blotting buffer is similar to the electrophoresis buffer but lacks SDS. Alcohol (methanol, ethanol, or isopropanol) is added to the blotting buffer to facilitate protein binding to the membrane.

Protein Visualization

Blocking: The membrane is incubated with a generic protein (such as nonfat milk proteins) and a nonionic detergent, such as tween-20, to bind to any remaining sticky places on the nitrocellulose. Detergents prevents nonspecific binding of the antibody to the membrane.

Direct Detection: There are two common detection systems: direct and indirect. In a direct system, the membrane is probed with a primary antibody, which binds to the target protein of interest. This primary antibody is conjugated directly with the detection system. Although this is a quick method, it does have limitations, such as; the detection system can interfere with the binding of the primary antibody. Additionally, the direct method requires a direct antibody with a detection system be chemically conjugated to it.

Indirect Western Blot: In an indirect western blot, two antibodies are used: primary antibody to the target of interest, and a secondary antibody that binds the primary antibody and has a detection system conjugated to it. The figure above depicts an indirect western blot.

Primary Antibody: An antibody (monoclonal or polyclonal) directed against the protein of interest is incubated with the membrane to allow it to bind to its specific protein. The antibody is diluted in a buffer solution, generally phosphate-buffered saline (PBS) containing a carrier protein, typically bovine serum albumin (BSA), along with some nonionic detergent such as Tween 20. The additives to the buffer help to ensure that the antibody is specific for the protein of interest, and to ensure the antibody does not bind to other proteins on the membrane.

Secondary Antibody: Since the antigen-antibody complexes are not colored, they must be treated in some way to visualize them. Usually, an enzyme such as a horseradish peroxidase or alkaline phosphatase is coupled to a secondary antibody that binds to immunoglobulin (Ig) chains of the primary antibody. Alternatives to the conjugated enzyme are fluorescent marker or dye conjugation of the secondary antibody.

Developing: The unbound secondary antibodies are washed away, and the conjugated enzyme is then presented with a colorless substrate which when reacted, will produce a colored, luminescent or fluorescent product, depending on the substrate system used. Band densities in different lanes can be compared providing information on the relative abundance of the target protein, and a molecular weight marker can provide size information.

H_2O_2 + HRP → LIGHT @ 425 nm

The enzyme/substrate system used in this lab is ***horseradish peroxidase (HRP) enzyme with luminol as the substrate***. When oxidized by HRP, in the presence of hydrogen peroxide, luminol

releases light at 425nm. The chemiluminescent western development is the most common in labs currently, however, quite a few labs are moving toward a fluorescent system. An older system, but sometimes still used, is a colorimetric 4CN substrate, which forms a purple precipitate on the nitrocellulose membrane.

References:

1. Comparative Proteomics Kit II: Western Blot Module, Biotechnology Explorer, Instruction Manual, Rev B. Bulletin #10004531
2. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3456489/
3. Microbiology. OpenStax. Rice University. Retrieved from https://d3bxy9euw4e147.cloudfront.net/oscms-prodcms/media/documents/Microbiology-OP.pdf

PART I: SEPARATING PROTEIN ON SDS-PAGE

PRE-LAB EXERCISES

- If you are not continuing on directly from lab unit 10, you will need to repeat the gel analysis from lab 10. Print out and bring a prelab exercise for analyzing your GFP samples on SDS-PAGE as outlined in lab unit 10.
- Watch this video on Western Blotting before class: https://youtu.be/VgAuZ6dBOfs

PART II: TRANSFER PROTEIN TO NITROCELLULOSE

MATERIALS – per group

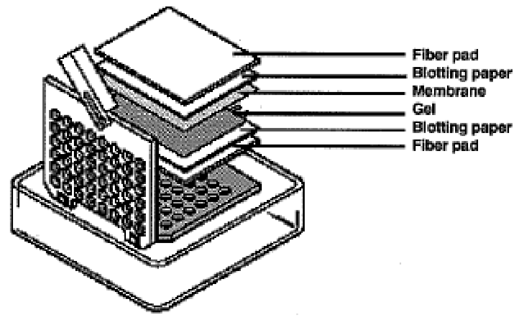
o 10X Tris/Glycine Buffer (Bio-Rad Cat#161-0734)	o 10X TBS (Bio-Rad Cat#170-6435)
o Ethanol	o BSA (non-fat milk, Sigma cat#M7409)
o Stir plate & stir bar	o Tween-20 (Sigma Cat#P3563)
o SDS-PAGE gel from lab 10	o Primary Antibody: Rabbit Anti-GFP (Sigma, Cat #G1544)
o Gel cracker & small roller	o Secondary Antibody: Goat Anti-Rabbit IgG Peroxidase (Sigma, Cat#A0545)
o Gel wash container	o Chemiluminescent developing kit
o Electro-blotting apparatus: ice pack, gel box, 1 sandwich holder, 2 western sandwich holders, 4 reusable fiber pads, transfer tray	o (optional Chloronaphthol (Thermo Cat#34012))
	o 4, snack size zip-lock bags
	o 4, 15mL conical
o Electrophoresis supply	o 1L graduated cylinder
o 2 sheets, nitrocellulose	o 100mL graduated cylinder
o 4 sheets, blotting paper	o Small blot wash container (tip box lid works)

Hazard Communication

- ✓ *The wires connecting the cell to the power supply must be in good condition, not worn or cracked. They create an electrocution hazard.*
- ✓ *Ensure the area around the power supply is dry.*
- ✓ *Wear gloves while loading and handling the gels; the* ***unpolymerized acrylamide is a neurotoxin!***

PART I: PROTEIN TRANSFER

1. Each group of two: Prepare 1.5L of 1X Tris/Glycine in 20% total volume ethanol.
2. Crack open the gel cassette as previously described. With the gel still on the plastic cassette, use a ruler to chop the top wells and a thick bottom piece off the gel. Firmly press down on the ruler's edge, do not slide the ruler across the gel or you will rip the gel. Your instructor can demonstrate.
3. Equilibrate the gel in the blotting buffer for 15 minutes, on a rocking platform.
4. Prepare for your transfer by setting the following in a small container filled with transfer buffer in the following order: Fiber pad, blotting paper, membrane, blotting paper, fiber pad.
5. Prepare your blotting sandwich as follows:



   a. Add 1 cm depth of transfer buffer to the container and insert the plastic cassette with black side down.
   b. Lay a wet fiber pad on the black side of the cassette.
   c. Lay one wet blotting paper on the fiber pad.
   d. Add a pool of blotting buffer to the top of the pad, with wet gloved hands, carefully pick up your gel and lay the gel onto the pad in a rolling motion to remove bubbles from underneath.
   e. Roll out any remaining air bubbles from under the gel with the roller provided. If you do not have a roller, you can use a test tube. Be gentle! You do not want to rip or squish and distort your gel.
   f. Add a pool of blotting buffer to the top of the gel and carefully place a wet nitrocellulose membrane on the top of the gel. Ensure it covers the gel squarely.
   g. Roll out any bubbles from underneath the membrane. There must be full contact between the gel and the membrane, or your protein will not transfer.
   h. Add a small pool of blotting buffer to the top of the membrane and add a wet blotting paper on top of the membrane. Carefully roll out any bubbles without sliding the sandwich apart.
   i. Add a wet fiber pad on top of the blotting paper.
   j. CAREFULLY close the cassette and clamp together by sliding the white tip over both cassettes. Your instructor can demonstrate if you have difficulties with this.
6. Set up the transfer tank with the black side of the cassette next to the black side of the transfer tank.
7. Insert a stir bar into the bottom of the tank and place the apparatus on a stir plate.
8. Add a frozen block and fill the tank with the white clips with blotting buffer. Turn on a stir plate.

9. Place lid on the tank, matching the power cords red-to-red and black-to-black. Allow transfer to proceed at 150V for 30 minutes (if enough time, 100V/60min works best).
10. While your gel is transferring, each group of two, prepare 100 mL of **blocking buffer**: 1XTBS, 0.1% tween-20, 1.5% BSA. Additionally, 100mL of **wash buffer**: 1XTBS, 0.1% tween-20. Both can keep for one week at 4ºC.
11. Dismantle the sandwich, rinse the membrane briefly in diH2O, and place the membrane in 20mL of blocking buffer in a small zip lock bag (keep the gel!). Remove all air bubbles from the zip lock bag.
12. The membrane can be stored in blocking buffer at 4ºC for less than one week. If you are continuing immediately to the blotting procedure, you must block for 1 hour at room temperature minimum for optimal results.
13. Carefully look at the spent transferred gel using a gel imager. Write down your observations in your notebook. Did the entire MW marker lane transfer to the gel? Did all your protein transfer to the nitrocellulose? You may capture an image and include it in your notebook – but do not attach it to your gel electrophoresis form.

PART III: IMMUNODETECTION

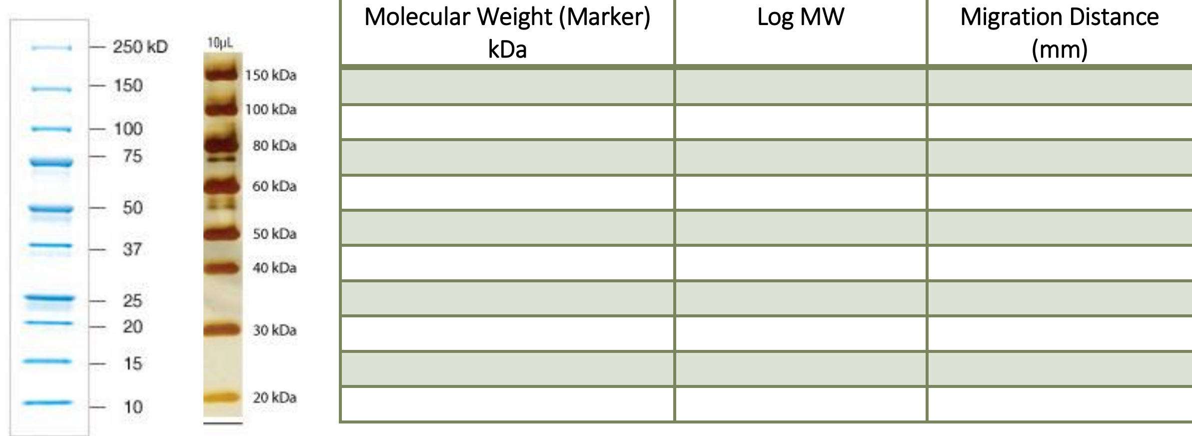
Each incubation step in this section has a time range. Depending on time restrictions in the lab you can adjust the time accordingly. For optimal results, use the maximum incubation times and do not allow the membrane to get dry at any time. ***Remember to record the actual time and temperature for your incubations in your notebook.***

1. Prepare 10mL of your primary antibody in blocking buffer, in a 1:2000 dilution.
2. Discard blocking solution down the sink with lots of water and transfer the membrane to a clean unused snack bag size Ziploc baggy. Pour in the 10mL of ***primary antibody solution*** and remove any air bubbles.
   NOTE: If you do not have Ziploc bags, you can use any small container. Find one that fits the membrane with minimal extra space (Petri dish, tip box lid, weigh boat). You do not want the membrane to dry out.
3. Incubate membrane with ***primary antibody solution*** for 15 minutes minimum (2 days maximum) with gentle rocking. Ensure the antibody solution covers the entire membrane so that it doesn't dry out during this incubation step.
4. While your membrane is incubating, prepare your secondary antibody in blocking buffer, in a 1:5000 dilution.
5. Discard antibody solution down the sink with lots of water. Transfer membrane to a small container and rinse the membrane with a small volume of wash buffer, discard in the sink. Fill the wash container half way with wash buffer and return membrane to the rocking platform. Rock for 3 minutes at a relatively brisk setting.
6. Discard the wash solution and place the membrane into a clean unused Ziploc bag. Add 10 mL of ***secondary antibody solution.*** Remove air bubbles.
7. Return to the rocking platform and incubate 15 minutes minimum (2 days maximum) with gentle rocking.
8. Discard antibody solution down the sink with lots of water. Remove membrane from the bag and place it into a wash container and rinse the membrane with a small volume of wash buffer, discard in the sink.

9. Fill the container half way with wash buffer and return membrane to the rocking platform. Rock for 3 minutes at a relatively brisk setting. Discard wash buffer and wash at least one more time.
10. Obtain the chemiluminescent reagent from your instructor. This reagent must be made up just before use and kept dark. Mix 1mL of solution A, and 1mL of solution B in a 2mL tube.
11. Place membrane on a flat surface in a small container (such as a Petri dish, weigh boat). Carefully add 2 mL of prepared reagent to the top of the membrane. The reagent will pool on the top of the membrane.
    NOTE: Alternatively, you may add 2mL of Chloronaphthol color developer and watch a purple precipitate form where your GFP protein is. The color should develop relatively quickly – within 5 min.
12. Incubate membrane 5 minutes by manual swirling.
13. While this is incubating set up the imager. There is a chemiluminescent setting on the imager – the filter must be disengaged!
14. Capture an image, print and tape it to a gel electrophoresis form. It is recommended you also capture a PDF of your image for your molecular weight standard curve analysis.

PART III: ANALYSIS

Non-denatured GFP protein, which maintains its' fluorescence, usually shows up at around 37,000 Daltons. The fully denatured GFP can be seen at its MW of 27,000 Daltons.



Molecular Weight (Marker) kDa	Log MW	Migration Distance (mm)

Kaleidoscope SuperSignal

1. Measure the migration distance of each marker and record in the table.
2. Calculate the log of the molecular weight of each band. Record in the table.
3. Plot the log of the molecular weight (y-axis) versus the migration distance.
4. Determine the equation of the line and the R^2 value.
5. Measure the distance of the GFP bands for your denatured protein: _________ mm
6. Using the equation of the line determine the molecular weight of GFP (remember to anti-log!). Is this what you predicted??
7. Some gel systems (such as with continuous gels) require a log/log plot to obtain a linear relationship with molecular weight and migration distance. If your results are CURVED, perform a log/log plot.

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Laboratory Unit 11 Analysis & Review

For your Laboratory Report include the following:

1. ***Prelab exercise*** – with notes!
2. ***Summary of raw data:***
    a. Include gel electrophoresis forms with labeled images of your protein gel and Western Blot.
    b. Table of standard curve data.
    c. Plot log molecular weight versus migration distance for your molecular weight markers. Use the equation of the line to determine the approximate molecular weight of GFP your Western Blot. <u>You must show your calculations</u>!!!

3. ***Analysis of results***.
    a. What is the molecular weight of GFP? Is this the result you obtained with your denatured samples?
    b. Look at your protein gel – do you see corresponding bands at the same migration distance as the protein detected on your western blot? How easily is it to discern your protein of interest from all the other proteins in the protein gel? What benefit does Western Blotting serve over SDS-PAGE alone?
    a. Look at your transferred protein gel – did all your proteins transfer to the membrane? Describe what you see and discuss the efficiency of transfer. How can you improve your transfer efficiency?
    b. Summarize and discuss the indirect western blot procedure. In your discussion include the species of the primary and secondary antibodies. Why is this important?
    c. Summarize and discuss the chemistry behind the detection system used.

4. ***Conclusions***

APPENDIX

Employability Skills Evaluation

AUSTIN COMMUNITY COLLEGE
BIOTECHNOLOGY PROGRAM

Student Name: ______________________________ **Score:** ______________________

Course: _____________________________________ **Semester:** ____________________

Skills	Needs Improvement		Average	Excellent	
Dependable Attendance and Punctuality. completes work promptly	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, log books)	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

Control # _______________

Name of Solution/Media: ____

Amount prepared: __________________________ **Preparation Date: ______________**

Technician(s):____________________________________ **____________________________________**

Component	Vendor/ lot #/ Control #	Date Received	Storage conditions	MW or initial concentration	Mass used	Final Concentration

Balance used	Calibration status	
pH meter used	Calibration status	
Initial pH	Final pH	Adjusted pH with
Preparation temperature	Sterilization procedure/ sterility testing	Solution storage conditions & location

Calculations/Comments:

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.

Electrophoresis Documentation Form

Date _______________ buffer ___________________ gel_____ % agarose/acrylamide (**circle one)**

Voltage _____________ start time _________ stop time _________ stain _____________

Analyst(s): ____

Experimental Summary/Description:

Lane	Sample Description	Concentration	Sample Volume Analyzed	Mass Analyzed
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				

AFFIX CLEARLY LABELED IMAGE(S) HERE

TAPE ALL 4 EDGES, AND INITIAL ACROSS THE TAPE

LABEL WELLS, INDICATE MARKER SIZE, AND SAMPLE BAND LOCATION ON THE SIDE OF THE GEL

Photographic settings (exposure time, aperture setting, etc):

Deviation Report

Date: __________________ DR#: ______________________

Detailed name & Model # of Equipment: ____

Serial Number or Equipment Number: ____

Where is the Equipment now? ____

Detailed Description of Deviation:

____

____

____

____

____

____

Recommended or Actual Correction of Deviation:

____

____

____

____

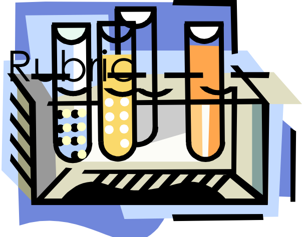
Date: _________ Name: __________________________ Signature: ____________________

<u>QA Approval (Faculty Member or Laboratory Technician)</u>

Date: ___________________ Signature: __________________________________

<u>Instructions:</u> Keep a copy for your lab notebook, keep original with the equipment and notify lab technician where the equipment is.

Lab Grading Rubric



Biotechnology Lab Report

Name ________________ Date ___________

Lab ___________________ Score __________

Formatting: A biotech lab report should be written clearly and legibly so that it can be read and understood easily by your colleagues.

Written in ink	**minus 10 pts if not done**	________
Overall neatness/legibility	**5 pts**	________
Grammar and Mechanics	**5 pts**	________

PreLab: To ensure you are properly prepared, type up a prelab before the start of the lab.

Descriptive Title	**minus 5 pts if not done**	________
Purpose - brief intro and objectives	**5 pts**	________
Materials - complete and organized	**5 pts**	________
Specific safety Precautions listed	**5 pts**	________
Procedure (-15 points if not complete on time)	**20 pts**	________

Lab Data: Record data during the lab directly in your lab notebook in ink. Write down all relevant information, including incubation times, data, calculations, measurements, readings, descriptions, and anything else that might be important.

Data is recorded during the lab	**minus 10 pts if rewritten**	________
Completely participated in data collection	**10 pts**	________
Thorough observations made	**10 pts**	________
Well organized/legible	**5 pts**	________
Experiment Success	**10 pts**	________

(Points deducted for lack of skill mastery and careless mistakes)

Analysis: The conclusion is written after the lab, but look at the questions ahead of time to know what to expect. Analyze your data as outlined in the manual. Explain your results, good or bad. The conclusion questions will be graded based on whether they are complete, correct and well written.

Data Analysis	**15 pts**	________
Conclusion	**5 pts**	________
Total:	**100 pts**	________

Inventory Control Form

Work Order #	Reagent	Quantity	Label/Lot Number #

Preparation Date: ___________________ Prepared By:___________________________________

SOP Document #: ___________________ Approved By: _________________________________

Storage Location: ____________________

Date	Purpose (Include reference number if applicable)	Volume Removed	Volume Remaining	Initials
Inventory verified by:		Date:		

Date Discarded: _________________________

SOP Template

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.	*Use this space to take notes during your experiments or other items to note during the use of this SOP*
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#."	*SOP number is inserted into the header of this template.*

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. 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, log book, 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.