Austin Community College, Biotechnology Department

BIOL 1414

Introduction to Biotechnology

Laboratory Manual

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LAB UNIT 1: INTRODUCTION TO BIOL1414 LAB

Welcome to your first course in biotechnology! This course will emphasize its laboratory component to reflect the importance of your training in biotechnology skills. Keep in mind as you work your way through this manual the specific purposes in each exercise. They will prepare you for your first job in a biotechnology laboratory, so keep a careful record of your experience. If you carefully document and archive your work, this information will be easy for you to access later and your experiences will be more valuable in your later work.

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

- Develop the basic laboratory techniques of a biotechnology or bioscience lab
- Supplement and enrich the lecture portion of the course, which deals predominantly with biotechnology techniques
- Develop critical thinking skills in the students
- Encourage teamwork and accountability among the students
- Practice accuracy in calculations and in writing scientifically
- Develop multitasking skills
- Encourage students to take charge of their learning
- Learn the responsibilities associated with working in a company

Students are expected to behave professionally at all times. Lab notebooks will be maintained and graded in the lab. The lecture will provide background and relevant information about the solutions, prep, procedure and related techniques.

Required Texts/Materials

Textbooks: Seidman & Moore, Basic Laboratory Methods for Biotechnology: Textbook & Laboratory Reference, 2nd edition. 2009. Prentice Hall. ISBN: 0321570146

Lab Manual: "BIOL1414 Introduction to Biotechnology Laboratory Manual" One copy of the lab manual will be supplied.

Supplies: 3 Ring Binder (Lab notebook), Scientific Calculator with statistics and linear regression capability, Sharpie labeling pens (fine tip).

Lab Safety: Safety glasses with a rating of Z87 and close-toed shoes

Before you can begin working in an ACC laboratory, you must first

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

We hope that you enjoy your experience in this introductory course. Following is a discussion of biotechnology, and a description of some of the activities that you will be doing in this course.

What is biotechnology?
Strictly speaking, biotechnology is the use of a living organism for one's own benefit. By this definition, biotechnology would date back to the very beginnings of civilization, when humankind first learned to cultivate crops and domesticate animals in a system of agriculture. When one thinks of modern biotechnology, however, gene splicing and recombinant organisms take center stage. Biotechnology was revolutionized when scientists first learned how to isolate and clone genes, allowing for genetic engineering.

Today, the biotechnology industry has grown and expanded to affect us on a day-to-day basis.

Below is an excerpt from Bio.org that discusses the ever expanding applications of Biotechnology in healthcare, agriculture and energy (http://www.bio.org/about_biotech/).

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

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

Heal the World
Biotech is helping to heal the world by harnessing nature's own toolbox and using our own genetic makeup to heal and guide lines of research by:

- Reducing rates of infectious disease;
- Saving millions of children's lives;
- Changing the odds of serious, life-threatening conditions affecting millions around the world;
- Tailoring treatments to individuals to minimize health risks and side effects;
- Creating more precise tools for disease detection; and
- Combating serious illnesses and everyday threats confronting the developing world.

Fuel the World
Biotech uses biological processes such as fermentation and harnesses biocatalysts such as enzymes, yeast, and other microbes to become microscopic manufacturing plants. Biotech is helping to fuel the world by:

- Streamlining the steps in chemical manufacturing processes by 80% or more;
- Lowering the temperature for cleaning clothes and potentially saving $4.1 billion annually;
- Improving manufacturing process efficiency to save 50% or more on operating costs;
- Reducing use of and reliance on petrochemicals;
- Using biofuels to cut greenhouse gas emissions by 52% or more;
- Decreasing water usage and waste generation; and
- Tapping into the full potential of traditional biomass waste products.

Feed the World
Biotech improves crop insect resistance, enhances crop herbicide tolerance and facilitates the use of more environmentally sustainable farming practices. Biotech is helping to feed the world by:

- Generating higher crop yields with fewer inputs;
- Lowering volumes of agricultural chemicals required by crops-limiting the run-off of these products into the environment;
- Using biotech crops that need fewer applications of pesticides and that allow farmers to reduce tilling farmland;
- Developing crops with enhanced nutrition profiles that solve vitamin and nutrient deficiencies;
- Producing foods free of allergens and toxins such as mycotoxin; and
- Improving food and crop oil content to help improve cardiovascular health.

Source: Healing, Fueling, Feeding: How Biotechnology is Enriching Your Life

Biotechnology Industry in Austin, Texas
The biotechnology industry has also been steadily growing in the Austin area. Today, Austin's bioscience community encompasses approximately 100 companies that employ more than 7000 people in the areas of research, diagnostics, pharmaceuticals and medical devices. (Ref: BioAustin.com). Some of these companies include Abbott Spine, Agilent, Asuragen, Bioo Scientific, Life Technologies, Luminex, Rules Based Medicine, Viagen, and Zimmer Biologics to name a few! Austin is also a major contributor to academic research in the biological sciences, both at the University of Texas and the University of Texas/M.D. Anderson Cancer Research Center in nearby Bastrop, Texas.

Watch this video to learn more about the exciting and emerging biotech industry in Austin: http://www.coolaustinjobs.com/videosBio.html

To learn more about careers in biotechnology: http://www.bio-link.org/home/sites/files/careersinbiotech20088e-j.pdf

Biotechnology Techniques and Skills Included in This Course
The State of Texas has adopted the Washington Skill Standards for Biotechnology. The Austin Community College Biotechnology Program has formally adopted and applied these standards to its program and is recognized by the Texas Skill Standards Board (www.tssb.org). Each course in the Biotechnology Program fulfills a specific set of skill standards. Those can be found here: http://www.austincc.edu/biotech/skillstandards.php

The skill standards applied to Introduction to Biotechnology (BIOL1414) are shown below.

Texas State Skills Standards (TSSB)
Key Activities for BIOL 1414 Introduction to Biotechnology

A3 Operate equipment	B2 Perform assays and experiments	B3 Troubleshoot experiments and equipment	B4 Perform data analysis	B5 Communicate results	C1 Participate in employer-sponsored safety training	C3 Identify unsafe conditions and take corrective action
C4 Suggest continuous improvements	C5 Coordinate with work team	C7 Handle and dispose of hazardous materials	C8 Maintain security	D1 Maintain lab notebook	D Create documents	

Introduction to Biotechnology – Course Competencies
The ACC Biotechnology Program has been designed to match the needs of the biotechnology job market in our immediate area. We have invited industrial partners from our community to contribute to the competency goals for each course, including this introductory course, to assure that our students are adequately prepared for positions in their companies. The following list describes the areas of expertise that you will be introduced to in this course, and may provide you with an organizational plan for the archiving of your records in your notebooks for this course. As you progress through the ACC Biotechnology Program, you can add to these archives as you build on the basics learned in this introductory course.

Basic operations in the laboratory
Purpose:
There are special approaches and precautions that must be taken in any biological laboratory. This includes procedures for safe handling and storage of hazardous chemicals and biologicals. Also, the special methods for setting up and following detailed protocols are emphasized, as well as methods for recording and archiving results properly.
Includes:
Safety in the Laboratory
Math Skills for the Laboratory
Documentation and the Lab Notebook
Molar Solutions and Dilutions
Appendices

Instruments and Equipment

Purpose:

An important part of working in any laboratory is the proper use and calibration of instruments and equipment. You will become familiar with general information about the use of lab equipment, as well as more detailed information about the step-by-step procedures for the specific instruments that you use.

Includes:

Basic Tools in the Biotechnology Laboratory	ACC Biotech Program Equipment locator
Using a Micropipette	micropipette
Calibrating Lab Instruments	balances and pH meters
Restriction Enzyme Mapping of DNA	agarose gel electrophoresis
GFP Chromatography	chromatography columns, denaturing polyacrylamide gel electrophoresis
DNA Fingerprinting: Alu PCR	thermal cycler, agarose gel electrophoresis

Working with DNA and proteins

Purpose:

It is important to be familiar with the basic techniques for purifying and analyzing biomolecules. You will learn to isolate, digest, and analyze DNA, as well as transform E. coli with a recombinant plasmid. You will also learn some basic methods to purify and analyze proteins.

Includes:

Transformation of E. coli	in vivo amplification of plasmid DNA
Plasmid Isolation	isolation of DNA
Restriction Enzyme Mapping of DNA	analysis of a restriction digest
DNA Fingerprinting: Alu PCR	isolation of genomic DNA, in vitro amplification of DNA by polymerase chain reaction
GFP Chromatography	hydrophobic interaction chromatography polyacrylamide gel electrophoresis

Immunochemistry

Purpose:

You will be introduced to basic techniques used to detect biomolecules using antibodies.

Includes:

ELISA for HIV	Enzyme-linked immunosorbent assay

Environmental microbiology

Purpose:

You will use microbes to remove environmental pollutants.

Includes:

Bioremediation: Environmental Clean-Up

Regulatory Affairs

Purpose:

You will work on writing skills and how to follow Standard Operating Procedures (SOPs) and fill out forms, in the laboratory. The regulations governing biological laboratories dictate the safety procedures and protocols for disposal of hazardous chemicals and biologicals.

Includes:

Safety in the Laboratory
Documentation and the Lab Notebook
Supplemental "SOP booklet"
Appendices: Solution Preparation & Gel Electrophoresis forms

Bioinformatics

Purpose:

Using computers to document and compile information is becoming the norm in biological laboratories. Computers are also used to access databases with genomic or statistical information.

Includes:

Bioinformatics

References

The authors would like to acknowledge the contributions of the following sources in the development of this lab manual:

1. Shoestring Biotechnology, by Kathy Frame (ed.). National Association of Biology Teachers (2002)
2. Basic Laboratory Methods for Biotechnology, by Lisa A. Seidman & Cynthia J. Moore. Prentice Hall (1999)
3. Dolan DNA Learning Center: www.dnalc.org
4. Molecular Biology Problem Solver edited by Alan S. Gerstein ISBN 0-471-37972-7
5. Geospiza web site (www.geospiza.com)
6. Bio-link web site (www.bio-link.org)
7. Seidman & Moore, Basic Laboratory Methods for Biotechnology: Textbook & Laboratory Reference, 2nd edition. 2009. Prentice Hall. ISBN: 0321570146
8. Laboratory security: http://ehs.uky.edu/ohs/labsecurity.html
9. Bio.org: http://www.bio.org/about_biotech/

Lab Unit 1-A: Documentation: The Lab Notebook & Lab Report

Introduction:

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

- to record what an individual has done and observed
- to establish ownership for patent purposes and other legal uses
- to establish criteria used to evaluate a finished product or the process to make it
- to trace the manufacture of a product
- to create a contract between a company and consumers and/or between a company and regulatory agencies
- to prove that a procedure was done correctly
- to adhere to, evaluate, and develop standard operating procedures (SOP)

Even good lab work is worthless without documentation, and careful documentation can turn an erroneous result or a failed procedure into a valuable learning experience by providing essential details needed for trouble-shooting. Furthermore, in industry, laboratory notebooks are legal documents. They are used to determine patent rights, product quality, liability, and verify the accuracy of information. Notebooks are treated as if they might be used in a court of law at any time, and you can, in fact, be called upon for questioning about your notebook in court.

An important part of this documentation process is to record what equipment and materials were used, and to show that the equipment and materials were validated and used in the correct manner. Companies must be able to produce documentation for audits by government regulatory agencies to prove that Good Manufacturing Practices (GMPs) were followed. If the material in the notebooks was not entered legibly, or information is missing, companies may be fined or the company may be held liable for damages in a product lawsuit. In research and development labs, the same careful documentation is necessary to establish rights to valuable patents. The value of a well-kept notebook cannot be overstated.

LAB NOTEBOOK:

Each student will maintain a lab notebook from which the lab reports will be derived. ***Your lab notebook will be graded***. Each of the Biotechnology and Biology laboratory classes in the Biotechnology Program will have different formats and rules in regards to your notebook and lab report format. Be sure to pay attention to your instructor, syllabus and lab manual in regards to this. 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.

General rules for writing good lab notebooks are:

- Write all parts of your lab in ink. **Writing with pencil is forbidden in the lab**. It's too easy for unscrupulous people to erase data or errors that they don't like, at which point important details about their work are lost. 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, supervisors, and possibly lawyers, will be reading your notebook, and if they cannot read your writing, your work is essentially nonexistent. If they cannot easily make out what you have written, they can easily misinterpret an important detail about your work. For example, there is a big difference between "fresh" and "frozen" even though the squiggle for each may look the same.
- Never cover information in your notebook with anything else or store information on a sheet of paper separate from your notebook. Never fold a page into your notebook. It can easily be lost.
- If you tape materials such as a graph, a manufacturer's specification sheet, or instrument readout into your notebook, 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. Write down important details that have a bearing on your results so that you can answer any questions that might be asked of you about how you did your work.

For this class, your lab notebook should include:

- A title page with the name of the course, semester and your name.
- A table of contents with page numbers
- Lab reports with notes and any appropriate results or other documentation (such as pictures of gel or manufacturers documentation about standards used) -- more information on this below
- Analysis questions for lab (at the end of each lab report)

Each lab report should include three parts:

1. The **pre-lab** write-up which is done before you begin the experiment (see below)
2. The **lab notes** which includes the standard operating procedure (SOP) used, the data and detailed observations you make while doing the lab, and any other comments you may want to remember or convey to others
3. The **analysis**, which is involves any calculations, conclusions drawn, and questions answered after the lab is completed. Most lab exercises come with a set of analysis questions to be answered.

1. Pre-lab write-up

This must be completed before coming to lab and should include the following:

- Heading – name of lab, date of lab, name of student
- A short description of the purpose of the lab
- Safety information pertaining to this lab
- A list of materials and equipment required
- Detailed list of steps, **leaving at least one space between each numbered step**

Use your own language, leaving out explanations for each step. Step numbers do not have to correspond to those on the handout but they should be in the same general order. The pre-lab can either be written into your lab notebook, using good penmanship, or typed, printed, and taped into the lab notebook as described on the previous page. Your instructor may provide you with an electronic copy of the laboratory exercise. In this case, you are required to rewrite the introduction and instructions in YOUR OWN WORDS. This action is required so that the instructor knows that you have acquainted yourself sufficiently with the lab before coming to class (i.e. so you are NOT figuring out what to do while you are trying to do the lab and therefore most likely wasting time and resources). Write only on the left half of the page, and use the right side of the page to record notes and results during lab. Use a ruler to draw a vertical line between the numbered steps and the space for notes and observations. If your pre-lab is typed, format the document to have two columns, type only in the left column, and cut or fold the page to fit into the left half of the notebook page.

The lab handouts include a lot of background material and other information in the procedural steps for your instruction in these techniques. An SOP, however should not include this type of information, and should be limited only to the actual steps taken in a procedure without explanation. You should read the instructions in your manual and extract only the action required of you during lab. This usually reduces a short paragraph to one line or less. Thus, you will create a document that is easier to follow during the lab session, and you will become adept at writing SOPs, a valuable skill in the biotechnology industry.

Composition of SOPs is an art that you must master. It is sometimes difficult to gauge the amount of detail that an SOP needs. An SOP that is too long and detailed is too cumbersome to use routinely, while an SOP lacking sufficient detail will not be lead to uniformity when different people perform the procedures. In this course, we will guide you through these decisions by providing you with a lab protocol to follow. In general, an SOP that needs the most detailed information

- is used by a large number of people
- is used infrequently so that the users will not remember exactly how it is done
- involves especially sensitive or critical steps of a process

For more information on keeping a notebook and writing SOPs, please refer to your Seidman and Moore textbook.

2. During Lab

At the beginning of the lab itself the instructor will check off your pre-lab, much as your supervisor will check off your work in industry. During lab you will take notes in pen as described above. **WRITE EVERYTHING DOWN.** Yes, we mean everything. How much did you actually weigh out? What are the supplier and the lot number of the reagent? What balance number did you use? What color was your solution? When did it start boiling? How long did each sample take to come off the column? And so on. Be sure to include any changes you made to the procedure in the lab handout, even if they were at the instructor's direction; a**lways show calculations.** In some labs, even the room temperature and humidity is recorded since that can affect the experiment. 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. Each individual should record his or her own notes, even when working in teams.

3. Post-lab Report

One week after all the data has been collected, you must submit a Lab Report for that Unit. Make sure your report format is easy to read, and well organized. Include your pre-lab, all lab experimental notes, results and analysis, any forms filled out, and post-lab question sheet. Give as much information as possible to demonstrate your understanding of the concepts.

Late Lab Reports: Part of the skills you are learning in this class is to generate quality work while meeting expected deadlines. Late lab reports will not be tolerated and will be subject to the policy outlined in the syllabus. See your instructor for further information.

Missed Lab Classes: You are expected to attend every class. Because there is no extra time in the semester, missed labs cannot be made up. Missed lab classes will have serious grade consequences. The penalty for missed labs is outlined in your syllabus. With documentation and instructor approval, the student may complete the pre-lab and as much of the post-lab analysis and questions as possible, and will receive a passing grade (70%) on the submitted lab. See your syllabus for policy on missed labs or late lab reports.

Lab Competency

Your competency in all the techniques in these lab exercises is the most important outcome of this class. Your ability to perform tasks successfully and use good lab technique will affect your grade. Your instructor will indicate on your graded report whether you have shown competency in these areas. Note that competency is not limited to lab skills, but also includes attendance, punctuality, teamwork, and tidiness. Lab competency is monitored using the lab practical exam, the workplace evaluation and the placement exam (to register for upper level Biotechnology courses).

Labeling

Labeling is very important in any lab. It is critical that you label every tube, bottle, flask, cuvette or other container you use in the lab, whatever its contents. This is especially important for any hazardous chemicals or pathogens, but be just as thorough with something as harmless as salt water. *You must label all containers with:*

- the identity of the contents and its concentration
- your initials
- the date (and time, if applicable)
- your class (for example, BIOL1414)
- OR, a number or letter corresponding to a detailed description containing the above information in your lab notebook

If the container is destined to be kept on hand for more than a day, never use a number or letter abbreviation; this will inevitably be found by someone else to whom your symbols mean nothing. Only use the abbreviated labels if you will be disposing of the contents the same day. For example, if you are doing column chromatography, you need only label the collection tubes with numbers in the order that they come off the column. However, if your instructor wants to keep one of your fractions as a control for the next semester's class, it is imperative that you label the tube with all the information above.

It is not necessary to write the lot number, manufacturer, or other details about the substance on the label, as long as you have recorded that information in your lab notebook. Only the details listed above are necessary for identification.

Lab Unit 1A – Notebook Assignment

1. **Obtain 3-ring Binder:** This will be your lab notebook and will include the lab manual, your pre-lab, and post-lab reports. We recommend a 2-3 inch binder.

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

3. **Obtain safety equipment:** You will need safety glasses or goggles rated Z87 (or Z87.1). If you wear 'flip flops' frequently, do yourself a favor and put a pair of old shoes in your car. Without closed-toed shoes you will not be permitted to participate in the lab.

Lab Unit 1-B: Biotechnology Laboratory Security & Safety

OBJECTIVES

Your performance will be satisfactory when you are able to:

- Discuss security and safety rules for the laboratory
- Recognize the correct procedure for storing and handling hazardous materials
- Find information on the classifications of chemical hazards, what types of health hazards a chemical may pose, what levels of medical attention are required following exposure to a hazardous chemical, and what personal protective equipment is required for handling a hazardous chemical
- Locate the lab safety equipment
- Locate online Material Safety Data Sheet (MSDS) databases

INTRODUCTION

Biotechnology laboratories are equipped with supplies and equipment that may pose a hazard if used carelessly and it is important that you learn how to handle them properly. 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 to you while working in ACC laboratories, you must become self-sufficient in protecting yourself in your future jobs in the biotechnology industry. In addition, 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 key components to lab safety precautions and procedures that apply in a biotechnology setting.

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 terrorists and criminals. Many new federal laws were enacted in 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 your 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 gases. The following guidelines were adapted from Appendix F of the CDC/NIH publication, Biosafety in Microbiological and Biomedical Laboratories. The guidelines are intended to reduce the risk for 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 in order to prevent theft of materials or equipment from the lab.
    - Assess your 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 card-keys 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 don't 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 prior to 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 emergency 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.

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 you some specific guidelines for safety throughout the semester.

A. MSDS (Material Safety Data Sheets)

While each chemical that you use will have its own unique properties, there are some common practices that will aid you 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) 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 web sites offer MSDS databases. They are all broken down to the same 8 sections:

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. These are all listed here. 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. The TLV (**threshold limit value**) is the maximum airborne concentration of a substance to which workers can be repeatedly exposed without adverse effects. The units used are usually **parts per million** (ppm) or mg/m^3.
3. **Physical chemical characteristics.** This list of physical properties tells you whether the chemical is solid or liquid and how volatile it is.
4. **Fire and explosion hazard data.** This is of particular interest in cases where fire-fighting methods must be selected.
5. **Reactivity data.** This information is essential in determining the proper handling and storage of chemicals. By knowing the reactivity patterns of a chemical, you know what substances or conditions from which you must isolate the chemical. For example, acids and bases react with each other rapidly, giving off large amounts of heat, so should not be stored

next to each other. Others react with water and should be stored in sealed containers with desiccants.

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 has been tested for **carcinogenicity** (cancer-causing potential) that information is listed here. In addition, levels at which a chemical has been found to be lethal (called the **LD_{50}** for lethal dose for 50% of test animals) is listed here. Since the LD_{50} is dependent on which type of animal it was tested on, as well as how the animal was exposed to the chemical, this information always requires these specifics. For example, the lethal dose for chemicals is much lower if injected than it is if ingested. The most common index reported is the LD_{50} for a rat in mg of chemical per kg of animal, administered orally (ingestion). For volatile chemicals, the toxicity of breathing it is measured as the LC_{50} (lethal concentration in air for half of the test animals), measured in ppm; in all cases, the lower the number for the LD_{50}, the more toxic the chemical.
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 given here.

B. NFPA Ratings (National Fire Protection Association)

Another quick assessment of a chemical's health hazards that is usually available on its container is a rating by the National Fire Protection Association (NFPA). A color-coded diamond shape lists numbers 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^o F	1 – unstable if heated
2 – hazardous	2 – flash point > 100^o F	2 – violent chemical change
3 – extreme danger	3 – flash point < 100^o F	3 – shock and heat may detonate
4 – deadly	4 – flash point < 73^o 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
~~**W**~~ – use NO WATER

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

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

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

Because chemicals pose so many different kinds of hazards, there are no simple rules of thumb for safe handling of them all except for some common sense measures:

- Treat all chemicals as if they were hazardous until you learn otherwise
- Label all containers with contents, including concentrations and date that they were transferred
- If a hazardous material is contained, label it with a warning
- Think through your experiment BEFORE doing it, making sure that you will not be combining incompatible chemicals
- Clean your bench top 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:

- **Flammables:** Do NOT heat these reagents 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.
- **Corrosives:** 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 gases. Strong acids and bases should never be stored together.
- **Reactive chemicals:** 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 chemicals:** 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 for unused reagents and materials that come in contact with the chemical.

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

Carcinogens – formaldehyde
Neurotoxins – acrylamide
Nephrotoxins – acetonitrile
Corrosives – phenol, strong acids & bases

Mutagens – ethidium bromide
Teratogens – formamide
Hepatotoxins – chloroform

MSDS are readily available on the internet. You can reference many chemical manufacturer websites such as Fischer Scientific and Sigma. Or try http://www.msds.com/

BIOLOGICAL SAFETY: CONTAINMENT

Official Biology & Biotechnology Department Statement on Student Use of Organisms:

"Most ACC biology classes, particularly those with laboratory components, use actual organisms during instruction in addition to images and models. ACC students generally are preparing for real-world careers requiring workers with hands-on experience. These careers include health care, veterinary work, horticultural and agricultural work. Other students plan to transfer to four-year colleges and will be participating in biological research where hands-on experience is equally important. Organisms used at ACC are fundamental in biology instruction and they are utilized to teach specific skills and knowledge. Their condition and usage varies from course to course. Students will be expected to actively participate in these activities. Students with particular concerns in this matter should consult with their instructor and/or departmental officials before enrolling in a laboratory course so that they can know what will be required of them. Some organisms are observed alive while others are dead and preserved in various ways. Student manipulation of organisms ranges from culturing living organisms to dissecting preserved ones. Some examples include, but are not limited to: bacterial culturing for microbiology courses; cat, pig or rat dissection for anatomy courses; skeleton and pelt examination for field biology; and use of frogs in physiology experiments." The web address for this policy is: http://www2.austincc.edu/biology/organismspolicy.html

You will be working 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. In general, a live organism is considered 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 cultures of human or animal cells should be 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 should be 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 at the discretion of the lab director, and adequately train all lab personnel.
- Use personal protective equipment (PPE) at all times, and keep all PPE inside the lab.
- Wash hands after handling viable materials and animals, 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 work area.

- No mouth pipetting; use mechanical pipetting devices.
- Minimize splashes and aerosol production.
- Disinfect work surfaces to decontaminate after a spill and after each work session.
- Disinfect or decontaminate glassware before washing.
- Decontaminate all regulated waste before disposal by an approved method, usually by autoclaving.
- Have an insect and rodent control program in effect.
- Use a laminar flow biological safety cabinet when available.

Seventy percent of recorded laboratory-acquired infections are due to inhalation of infectious particles, so 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.

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

Disinfectants such as bleach and ethanol 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 you are encountering:

Resistance Level	**Type of Organism**	**Examples**
Least resistant	enveloped viruses	HIV, Herpes simplex, Hepatitis B
Slightly resistant	bacteria	*E. coli, S. aureus*
Medium resistance	fungi	*Candida* species, *Cryptococcus*
Highly resistant	non-enveloped viruses	Polio virus, Mycobacteria, *M. tuberculosis*
Most resistant	spore	*B. subtilis*, *Clostridium* species

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. Extremely toxic chemicals are regulated at low levels, and less toxic chemicals can be disposed of through city sewer systems at higher levels. Biological hazards should be contained in autoclave bags made of a high melting point plastic that are sealed and autoclaved at high temperatures and pressures to completely kill any live organisms.

In our laboratory specific hazardous chemical and biological waste disposal will be discussed at the start of every lab. Chemical waste disposal containers can be found in the fume hood. Always keep the fume hood on and the lids to the chemical waste disposal containers on the containers.

Lab Unit 1B – Safety Training Assignment

1. Safety Training

a. The ACC Safety Committee has produced a video explaining safety rules and regulations. You must watch this video. We will do this in class together. It is also available online here: http://www.austincc.edu/biology/safetyvid.html

b. You must sign an ACC Safety Contract to acknowledge that you understand the safety rules and agree to abide by them before you are allowed to use the laboratory in this course.

c. The Appendix has a safety training sheet your instructor will go through with the class together. Pull this out during your safety training. ***Write down all the information in the blanks and keep it available in your lab notebook while working in the ACC laboratory.***

2. Finding MSDS and Safety Information on the Internet

Use the Internet to search for chemical company websites, university departments, or other databases containing MSDS information. Locate information for the following 3 chemicals:

a. Nicotine, an addictive substance found in tobacco.
b. Ethidium bromide, a stain commonly used for marking DNA.
c. Sodium chloride, table salt.

For each, find the LD_{50} (oral, rat, mg/kg) and whether it is a mutagen or carcinogen.

3. **Special Safety Precautions for Individual Lab Exercises.**

Find a partner to work with, and select a laboratory exercise together from this lab manual that has a list of chemicals and materials that will be used. Using information from MSDS, collect the following information and compile into one table.

- chemical name (trade name)
- Physical data (appearance, etc.)
- NFPA rating
- any health hazards/first aid measures
- LD_{50} (mg/kg, oral, rat) or LC_{50} (ppm)
- Toxicity data (carcinogen, mutagen, teratogen, neurotoxin, nephrotoxin, or hepatotoxin)
- waste disposal method/spill procedures
- any PPE needed

Lab Unit 1-C: Lab Equipment & Reagent Orientation

INTRODUCTION:
Before beginning to work in a laboratory, it is best to get an idea of where things are stored. You will be provided with a locater guide of basic lab equipment and materials in the lab room and prep room. This compiled list should be kept for later referral in the lab notebook that you bring to class each day.

OBJECTIVES
Your performance will be satisfactory when you are able to

- Identify common lab equipment pieces and describe their function
- Distinguish between glassware pieces in regard to measuring accuracy
- Understand the role of the reagents you use in the laboratory

During your training in the ACC Biotechnology program, 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. You are required to keep a list of the equipment that you learn to use and a brief description of the purpose of the machine. For example, a PCR machine is used to amplify a specific section of DNA.

LABORATORY RULES & GENERAL INFORMATION
Welcome to the ACC Biotechnology Lab. The Biotechnology Department lab techs have compiled this information to make your semester more successful and enjoyable.

Lab Courtesy
We share our main lab with the Medical Laboratory Technology Program (MLT). Please always move the supply cart into the prep room after class is over and remove any equipment or supplies from the benches so that MLT will have a clean, open space in which to work. If your lab session has been messy or you have worked with any organisms or hazardous chemicals, please clean bench tops with bleach, ethanol, and/or soapy water.

MLT has many biohazard bags in buckets on the floor around the room, as well as red sharps disposal boxes and white biohazard envelopes in green holders on the bench tops. Please never place anything into these receptacles. MLT has them incinerated at their expense. If you work with biohazards, please place contaminated tips, gloves, plates, or other items in the small biohazard bags in orange holders provided. These will be autoclaved after class.

Finally, always make sure to turn off equipment when you leave the lab. This is especially important for heated items such as water baths and optical instruments such as spectrophotometers.

Micropipettors & Tip Boxes
Each student station has a set of micropipettors. These are labeled with a number corresponding to the workstation and a letter, A or B. This way, each student can use their own set of micropipettors for the semester and they can be easily tracked and returned to their cabinet if

misplaced. Please save empty boxes by placing them on the table in the prep room. They will be refilled for you.

Glassware
During and after class, please place dirty glassware in the designated area. You are not required to wash your own glassware unless directed to do so. Do not leave dishes in sinks, on benches, or any other place other than the designated areas. If you are instructed to clean your own glassware, follow the procedure outlined in SOP-GWW-001. Please **never** use pen, wax pencil, or permanent marker to write on the white marking area of the glassware, since these residues are impossible to remove. You may use permanent marker to mark directly on the glass, or a graphite pencil to mark in the white marking area. The best method for labeling glassware is to mark it with label tape.

Broken Glass
Please use the blue broken glass boxes for broken glass disposal (used slides and cover slips, Pasteur pipettes, broken glassware). Place plastic pipettes in the regular trash, never in these boxes. There is a dustpan in the prep room which can be used for sweeping up broken glass.

Solution/Sample Storage
Please thoroughly label everything you store in a refrigerator or freezer. Items should be labeled with the following information:

- Name of substance, including concentration and/or pH if applicable
- Date stored
- Your name or initials

Store refrigerated items in the area of the fridge designated for your class as instructed. Items that are stored in other areas, or items that are unclearly or inadequately labeled, may be discarded at the discretion of the lab technician.

Storage in the -20^{o}C freezer is organized into vertical racks filled with microcentrifuge tube boxes. Your student group should obtain a box, clearly label with your group or individual names or initials, and store in the appropriate rack. Please do not start a new box for each lab activity because there is limited storage space.

Equipment Locator
An equipment locator can be found in the designated file cabinet in the lab room. Use this document to locate supplies and equipment. Most of the cabinets and drawers are labeled to help you find things. If there is an item that you cannot find or is not listed, or that you think our department needs, please contact your instructor. We cannot promise that items will be where they are listed, given the number of individuals working in this lab. Please do your part to return items to their listed location.

SOPs
SOPs written specifically for the equipment in our department are found in the top drawer of the designated file cabinet in the lab room. There is also an SOP packet that we have printed each semester. Copies are usually located on or under the table at the front of the room.

ACC BIOTECHNOLOGY STUDENT LAB CHECKOUT DUTIES

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. Instructors in our program assign courtesy points based on performance of these duties.

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

- Ensure that any solutions you have made are labeled properly according to SOP SOL-001 and that you have created a solution prep form for each. Store them properly in the provided storage location for your class.
- Replace any equipment, supplies, or reagents that you have used to their proper storage place, provided that other students are not still using them.
- 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, wiping the bench with cleaner or disinfectant and a paper towel if any microorganisms were used, and removing debris from the sink.
- Wash glassware that you have used according to SOP GWW-001, or store as directed. Never leave glassware in the basin of any sink. If left unwashed, glassware must be rinsed with tap water to remove chemical residues (not doing so is a safety violation).
- When possible, you are required to assist other students who are still working by performing some of the duties listed below.

The following should be checked for completion by the last person to leave the lab after every class:

- Replace any reagents or solutions used during the lab period to the appropriate storage place, or leave on the rolling cart. Check labels for special storage conditions (for example, some items need to be stored frozen, or wrapped in foil to block light). Take special care to ensure that hazardous substances (such as concentrated acid or flammable solvents) are stored either in the fume hood or in their designated cabinet.
- Replace equipment and consumables to the appropriate storage place (if unknown, check the equipment locator for the storage location), or leave on the rolling cart.
- Move rolling cart(s) into the prep room.
- Turn off all balances and remove any chemicals or weighing vessels from balance pans and surrounding countertop.
- Remove all items from lab benches. Wipe the benches with a wet paper towel if any chemicals were used; wipe the benches with 10% bleach and a paper towel if any microorganisms were used. Only perform this step if it has clearly not been done already.
- Place pH meters in Standby mode by pressing the Standby key make sure that pH probe tips are submerged in the provided storage solution.
- Turn hot plates off by turning heat and stir dials to “0”.
- Turn off spectrophotometers, trans-illuminators and other equipment containing lamps or bulbs to conserve lamp life.

Lab Unit 1C – Equipment Orientation Assignment

PART 1: SAFETY & LAB EQUIPMENT ORIENTATION

Explore the lab and prep room and list the locations of the following Safety-related materials as well as the basic equipment and materials that you will be using throughout the semester.

Safety Related Materials/equipment	**Room stored in**	**Location**
Eye Wash Stations (all of them!)		
Fire Extinguishers (all of them!)		
Fire Blankets		
Emergency Gas shut off Valve		
Glass Waste		
Biohazard Waste		
Liquid Chemical Waste		
Hazard Chemical Storage: flammables oxidizers		
General Chemicals		
Spill Kit		
Broom/dustpan		
MSDS		
First Aid Kits		

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

Lab Unit 1-D: Math Skills for the Laboratory

OBJECTIVES

Your performance will be satisfactory when you are able to

- Identify metric prefixes by their exponential equivalent.
- Convert metric units.
- Convert numbers to or from scientific notation.
- Multiply and divide numbers written in scientific notation.
- Distinguish significant figures.
- Set up and calculate simple dimensional analysis problems.

Exponential Numbers

The numbers that we deal with in the laboratory are often very large or very small. Consequently, these numbers are expressed in scientific notation, using exponential numbers. These rules apply to the use of exponents:

When *n* is a positive integer, the expression 10^n means "multiply 10 by itself *n* times". Thus,
$10^1 = 10$ $10^2 = 10 \text{ X } 10 = 100$ $10^3 = 10 \text{ X } 10 \text{ X } 10 = 1{,}000$

When *n* is a negative integer, the expression 10^n means "multiply 1/10 by itself *n* times". Thus,
$10^{-1} = 0.1$ $10^{-2} = 0.1 \text{ X } 0.1 = 0.01$ $10^{-3} = 0.1 \text{ X } 0.1 \text{ X } 0.1 = 0.001$

Examples: $2 \times 10^1 = 2 \text{ X } 10 = 20$
$2.62 \times 10^2 = 2.62 \text{ X } 100 = 262$
$5.30 \times 10^{-1} = 5.30 \text{ X } 0.1 = 0.530$
$8.1 \times 10^{-2} = 8.1 \text{ X } 0.01 = 0.081$

In scientific notation, all numbers are expressed as the product of a number (between 1 and 10) and a whole number power of 10. This is also called exponential notation. To express a number in scientific notation, do the following:

- First, express the numerical quantity between 1 and 10.
- Count the places that the decimal point was moved to obtain this number. If the decimal point has to be moved to the left, *n* is a positive integer; if the decimal point has to be moved to the right, *n* is a negative integer.

Examples: 8162 requires the decimal to be moved 3 places to the left
$= 8.162 \times 10^3$

0.054 requires the decimal to be moved 2 places to the right
$= 5.4 \times 10^{-2}$

Practice:

Express the following numbers in scientific notation.

20,205 = _____ 0.000192 = _____

5,800000,000 = _____ 0.0000034 = _____

40,230,000 = _____ 543.6 = _____

34.5×10^{3} = _____ 0.004×10^{-3} = _____

0.72×10^{-6} = _____ 0.029×10^{2} = _____

Addition and Subtraction of Exponential Numbers

Before numbers in scientific notation can be added or subtracted, the exponents must be equal.

Example: $(5.4 \times 10^{3}) + (6.0 \times 10^{2}) =$

$(5.4 \times 10^{3}) + (0.60 \times 10^{3}) =$

$(5.4 + 0.60) \times 10^{3} = 6.0 \times 10^{3}$

Practice:

$(5.4 \times 10^{-8}) + (6.6 \times 10^{-9}) =$ _____ $(4.4 \times 10^{5}) - (6.0 \times 10^{6}) =$ _____

$(3.24 \times 10^{4}) + (1.1 \times 10^{2}) =$ _____ $(0.434 \times 10^{-3}) - (6.0 \times 10^{-6}) =$ _____

Multiplying and Dividing Exponential Numbers

A major advantage of scientific notation is that it simplifies the process of multiplication and division. ***When numbers are multiplied, exponents are added; when numbers are divided, exponents are subtracted.***

Examples: $(3 \times 10^{4})(2 \times 10^{2}) = (3 \text{ X } 2)(10^{4+2}) = 6 \times 10^{6}$

$(3 \times 10^{4}) \div (2 \times 10^{2}) = (3 \div 2)(10^{4-2}) = 1.5 \times 10^{2}$

OR $\dfrac{(3 \times 10^{4})}{(2 \times 10^{2})} = (3/2)(10^{4-2}) = 1.5 \times 10^{2}$

Practice: All answers should be left in scientific notation.

$(3.4 \times 10^{3})(2.0 \times 10^{7})$ = _____ $(5.4 \times 10^{2)} \div (2.7 \times 10^{4)}$ =_____

$(4.6 \times 10^{1})(6.7 \times 10^{4})$ = _____ $(8.4 \times 10^{-3)} \div (4.0 \times 10^{5)}$ = _____

$(3.4 \times 10^{-3})(2.5 \times 10^{-5})$ = _____ $\frac{8.8 \times 10^{6}}{\quad} =$ _____ $\times 10^{-2}$

$(0.10 \times 10^{5})(4.9 \times 10^{-2})$ = _____ $\frac{5.2 \times 10^{-3}}{\quad} =$ _____ $\times 10^{2}$

Combine everything you have learned and perform the following calculation. Write your answer in scientific notation.

$(3.24 \times 10^{8})(14{,}000)/(3.5 \times 10^{-3})$ = _____

Metric Units

The metric system is used in the sciences to measure volumes, weights, and lengths. In the bioscience laboratory, amounts are often extremely small so it is necessary to express the values in scientific notation. You will be expected to identify the exponential number associated with each prefix.

Fill in the rest of the numbers in the table below.

Prefix	Exponential	Meaning	Symbol
Mega	10^{6}		M
Kilo-	10^{3}		k
Hecto-	10^{2}	100.0	h
Deca-	10^{1}	10.0	da
Primary unit	10^{0}	1.0	N/A
Deci-	10^{-1}	0.01	d
Centi-	10^{-2}	0.001	c
Milli-	10^{-3}		m
Micro-	10^{-6}		µ
Nano-	10^{-9}		n
Pico-	10^{-12}		p
Femto-	10^{-15}		f

Practice: Write the unit expressed by each.

0.003 g is equal to _____ g

4000 L is equal to _____ L

2×10^{6} m is equal to _____ m

5×10^{-6} L is equal to _____ L

Simple Metric Conversions: Subtracting exponentials

When measurements do not have the same units, they can be compared to each other by converting one measurement to the same unit as the other. This is simple when using the metric system, because the exponential numbers representative of each prefix differ by factors of ten. A simple way to convert decimals is to subtract the exponent of the unit you are changing to from the original unit, then move the decimal that number of spaces ---- to the right for a positive answer or to the left for a negative answer.

Example: Convert 1 kilometer into centimeters.
The exponent for kilo is 3 and that for centi is -2.

$$3 - (-2) = 5$$

This is a positive number, so move the decimal 5 places to the right.
One kilometer is equal to 100000 cm, or $1x10^5$ cm.

Likewise, changing centimeters to kilometers, one would calculate -2 – 3 = -5. The answer is a negative number, so move the decimal 5 places to the left.

Practice:

44 g = _____ kg 8.3 cm = _____ mm

2 pm = _____ fm 756 nL = _____ L

Conversion Factors and Dimensional Analysis

The use of a conversion factor is often useful in doing more complex conversions. A conversion factor is simply the ratio between the two units of measurement.

Examples: Give conversion factors for the following pairs of units.

Kilograms and grams	1000g = 1 kg	so	1000g/kg	or	1 kg/1000g
Liters and milliliters	1 L = 1000 mL	so	1 L/1000mL	or	0.001 L/mL
meters and centimeters	1 m = 100 cm	so	100 cm/m	or	0.01 m/cm

Practice the following: **Write two conversion factors for each pair of units:**

Microliters and milliliters _____ _____

Grams and milligrams _____ _____

Days and weeks _____ _____

How many days are there in 4 weeks? 28 days. How would you figure this out? You know that there are 7 days in a week, so there are 4 weeks x 7 days per week = 28 days. This problem was solved using dimensional analysis and involves a 'per expression' as a conversion factor. The per expression in this problem is 7 days/week, and you can also write it as 1 week/ 7 days, or as an equality where 7 days = 1 week. The only mathematical requirement for a PER expression or conversion factor is that the two quantities are directly proportional.

A conversion factor is used to change a quantity of either unit in the conversion factor to an equivalent amount of the other unit. The conversion follows a unit path from the given quantities (GIVEN) to the wanted quantities (WANTED). In the previous example, the one-step unit path is weeks to days, which can be written weeks→ days. Mathematically, you multiply the given quantity of 4 weeks by the conversion factor, 7 days /week, to get the number of days that has the same value as 4 weeks. The calculation setup is: 4 weeks x 7 days/week = 28 days

Notice in this unit pathway that if the units of measurement are treated algebraically, the GIVEN units of measurement cancel out (weeks divided by weeks) leaving only the WANTED units of measurement (days). When using dimensional analysis, you decide how to set up your unit pathways by analyzing the units of measurement of the given, wanted, and conversion factors. By treating the units of measurement algebraically, you determine what conversion factors are needed, and whether the conversion factors must be multiplied or divided in order to solve the problem.

When solving a problem using dimensional analysis, remember to do the following:

- Identify the GIVEN and WANTED values.
- Write down the per expressions (conversion factors) that share the units of measurement of the given and wanted values, providing a unit pathway.
- Align the given quantities and the conversion factors so that the given units of measurement cancel and the wanted units of measurement are left in the numerator.
- Write the calculation, including units.
- Calculate the numerical answer and cancel out units of measurement that disappear when divided by themselves.
- Check the answer to be sure both the number and units make sense.

Quantitative analysis is very useful when converting from one system to another or converting units.

Example: How many meters are in 2000 centimeters?
Multiply the number of centimeters given times the number of meters per centimeter.
$(2.0 \times 10^3 \text{ centimeters})\ (1 \text{ meter}/10^2 \text{ centimeter}) = 20 \text{ meters}$

Common relationships between the English and metric system are given below.

Mass	Length	Volume	
1 lb= 454g	1 in. = 2.54 cm	1.06 qt = 1 L	
1 oz = 28.3 g	1.09 yd = 1 m	1 gal = 3.785 L	
2.20 lb = 1 kg	1 mile = 1.61 km	$1 \text{ in}^3 = 6.39 \text{ cm}^3$	$1 \text{ cc}^3 = 1\text{mL}$

Practice:
How many grams are there in a 16 ounce can of soda?

Convert 555,000 meters to miles.

Convert 1 square yard to square centimeters.

Determining Significant Figures

It is important to make accurate measurements and to record them correctly so that the accuracy of the measurement is reflected in the number recorded. No physical measurement is exact; every measurement has some uncertainty. The recorded measurement should reflect that uncertainty. One way to do that is to attach an uncertainty to the recorded number. For example, if a bathroom scale weighs correctly to within one pound, and a person weighs 145 lbs, then the recorded weight should be 145 $\pm$ 1 lbs. The last digit, 5, is the uncertain digit, and is named the doubtful digit.

Another way to indicate uncertainty is the use of significant figures. The number of significant figures in a quantity is the number of digits that are known accurately plus the doubtful digit. The doubtful digit is always the last digit in the number.

Significant figures in a measurement

- apply to measurements or calculations from measurements and do not apply to exact numbers
- are independent of the location of the decimal point
- are determined by the measurement process and not the units

For example, a balance can weigh to $\pm$ 0.01 g. A sample weighs 54.69 g. The doubtful digit is 9.

When an answer given has more numbers than significant, then the last number must be rounded off. If the first digit to be dropped is <5, leave the doubtful digit before it unchanged. If the first digit to be dropped is >5, then you round upward by adding a unit to the doubtful digit left behind. For example, a student using the balance above measures 4.688 g. The correct number will be 4.69 g.

If there is only one digit beyond the doubtful digit in your number, and that digit is exactly 5, the rule is to round it down half the time and to round it up half the time so that you don't add a systematic error to your data. To keep track when to round up and when to round down, the rule of thumb is to always round to an even number in the remaining doubtful digit. For example, if a measurement on a balance with a $\pm$ 0.01 g accuracy is used to measure 4.895 g, you should record 4.90 g. If it reads 4.885 g, you should record 4.88 g as your data.

Practice:
The uncertainty of a balance measurement is ± 0.01 g. Write the numbers that should be record as data with the correct number of significant figures for the following. Some answers may already be correct.

445.81 g _____ 6.731 g _____

5872.30 g _____ 5.556 g _____

5.555 g _____ 5.565 g _____

It is sometimes confusing to determine whether a zero in a number is a significant figure or not.
Generally, a zero is a significant figure if:

- it lies between two nonzero digits in a number
- it lies to the right of a number with a decimal point
- it does not lie to the right of a number without a decimal point
- it does not lie to the left of a number

Examples: For 12.40 g, the zero is significant.
For 110 g, the zero is not significant.
For 1.004 g, the zeroes are significant
For 0.004 g, the zeroes are not significant

Practice:
Determine the correct number of significant figures in the following numbers.

10.01 g _____ 140 g _____

0.0010 g _____ 140.0 g _____

1.100 g _____ 1100 g _____

Calculations Using Significant Figures
In adding or subtracting numbers, the answer should contain only as many decimal places as the measurement having the least number of decimal places. In other words, you answer should reflect the accuracy of the measurement by correctly placing the doubtful digit. This is best done by lining up the numbers to be added or subtracted, performing the addition or subtraction, and discarding any digits to the right of the doubtful digit from the answer.

Example: For a balance that measures to ± 0.01 g, the sum of the following measurements yields:

34.60 + 24.555 g = 34.60
\+ 24.555
59.155 g = 59.16 g

Practice:
Solve the following and report your answer with the correct number of significant figures and units.

16.0 g + 3.106 g + 0.8 g (from a balance that weight to $\pm$ 0.1 g) _____

9.002 m - 3.10 m (from a meter stick that measures to the nearest cm) _____

When multiplying or dividing, the answer may have only as many significant figures as the measurement with the least number of significant figures. This is especially important to remember when using a calculator, since your calculator may give you an answer with 11 digits!

Examples: (1.13 m)(5.1261 m) = 5.79251786 m^2 = 5.79 m^2

Significant figures: 3 5 = 3

4.96001 g ÷ 4.740 cm^3 = 1.0464135 g/cm^3 = 1.046 g/cm^3

Significant figures: 6 4 = 4

Practice the following:
Solve the following and report your answer with correct number of significant figures and units.

$(4.01 \times 10^{-1}\ cm)(2.1 \times 10^{-3}\ cm)(4.97 \times 10^{-2}\ cm)$ = _____

10.96 g ÷ 12.1 cm^3 = _____

You may need to refer to the math review provided in Appendix B (such as order of operations and the manipulation of exponents when adding, subtracting, and multiplying, or dividing numbers) to solve the following.

$$\frac{1.059\ g - 0.2\ g}{0.98\ mL - 0.02\ mL} =$$ _____

$$\frac{(1.15 \times 10^{3}\ g) - (2.4 \times 10^{-1}\ g)}{(1.555 \times 10^{3}\ mL) - (6.2 \times 10^{2}\ mL)} =$$ _____

LAB UNIT 2: CURRENT GOOD MANUFACTURING PRACTICES (cGMP)

QUALITY SYSTEMS IN THE LABORATORY

There are various laboratory quality systems, all of which are intended to ensure that data from the laboratory are trustworthy and meaningful. ISO 9000 and GMP include references to quality control laboratories, which test products, raw materials, and in-process samples**.** For quality control to be successful, everyone in the company must be committed to good laboratory practices.

> ***There are three parts to good company-wide quality control:***
> 1. Trained personnel with the required skills
> 2. Clear designation of each person's responsibilities
> 3. Adequate supervision

In order to develop trained personnel, a well-run laboratory has established procedures to train new analysts and document the training. Even experienced analysts require ongoing training to refresh their skills and to learn new technologies as they are developed.

Good quality control depends on impartiality. Therefore, laboratory personnel should never be placed in a position where they have to report a certain result. A few years ago there was a court case in which a supervisor of a generic medicine company asked the QC department to report that their medicine had the correct ratio of medicine to filler, even though the test results showed that there was up to thirty percent less medicine than was approved. The QC personnel were asked to lie on documentation and cover up the manufacturing error. They responded by suing the supervisors.

Trained personnel need adequate equipment to perform all required work. A well-run facility will regularly update their QC testing equipment, training their personnel on this new equipment. Furthermore, all environmental considerations such a temperature, humidity, vibration and dust levels should be controlled and documented. The equipment should be regularly maintained and calibrated, and their maintenance and calibration must be documented to national or international standards such as National Institute for Standards and Technology (NIST).

QUALITY SYSTEMS IN THE PRODUCTION FACILITY

Facilities and Equipment

A production facility must be able to support the production of products. The following are some important consideration when designing production facilities.
If the goal is to produce drugs, the building layout should be organized so that processing steps flow in an orderly fashion from one place to another. Raw materials that have been tested and accepted for use should be stored in a space separate from unapproved materials to avoid confusing the two. The paths or flow of finished products ideally should not cross the paths of raw materials because of the possibility of confusing them or contaminating the finished product.

- The facility must have controls for environmental factors such as humidity, temperature, dust, and particulates in the air, and it should be large enough to accommodate all personnel and equipment safely.
- Besides the facility, there should be a well-thought out housekeeping program to keep the facility clean, free of rodents and other pests, and monitored environmentally in line with regulations and the appropriate standards.
- The equipment and instruments in the facility must be suitable for their purposes and properly maintained. An important aspect of equipment monitoring is that all measuring devices are operating properly.

Handling Raw Materials

Raw materials (e.g., the chemicals, bioactive entities, and inert materials such as empty vials, process containment, transport materials, and other components that go into making a product) are resources. They are defined by a unique "specification" and the "specification" document contains:

- A specific "part number"
- Chemical formula
- Description of the chemical or material
- Test methods and acceptance criteria for identity, potency, and purity.

Unlike facilities and major equipment, raw materials flow into the company, are used and/or are transformed into products, and flow out of the company in another form. Systems need to be in place to receive materials, document their arrival, and ensure that the materials are the ones that were ordered.

Once received, the materials must be quarantined until they have been tested and approved by the Quality Unit.

Principles guiding handling raw materials:

1. It is essential not to confuse or mislabel items.
2. Raw materials intended for production should not be released to production until they have been tested and found to conform to specifications.
3. Storage conditions should take into consideration hazards associated with the material, its perishability and its temperature and humidity requirements.
4. Traceability of materials must be assured.

Specifications

Specifications are descriptions that define and characterize properties that a product must possess based on its intended use. There are specifications for raw materials and for products.

The table below shows an example of specifications, in this case for three types of sodium chloride that are commercially available. Note that specifications are based on intended use.

	road salt	table salt	Analytical grade salt
Chemical purity	minimum 95%	minimum 97%	minimum 99%
Color	clear to white, yellow, red	clear to white	clear to white
Maximum allowed contan	Not specified	As 0.5 ppm Cu 2.0 ppm Pb 2.0 ppm Cd 0.5 ppm Hg 0.10 ppm	Al < 0.0005% As < 0.0001% Ba < 0.0005% Ca < 0.002% Cu < 0.0005% etc.
Physical requirements	90% of crystals between 2 and 12.5 mm	90% of crystals between 0 and 1.4 mm	95% of crystals between 0.18 and 0.3 mm
Allowed additives	Anti-caking agents of 5-10 Sodium Ferrocyanide, Fer Ferrocyanide	coating agents, hydrophob agents	not allowed
Moisture	2-3%	< 3 %	not specified

It is very important to establish specifications during the development of the product, for the product itself, all raw materials and the process as part of the application to the FDA. This is not a simple task. It requires knowledge of how the product, materials, or process will be used, the properties that will make it suitable for that use, and the ranges that are allowable. If the range for a specification is set too tightly then some adequate product or materials might be rejected. On the other hand, if the range of values for the specifications is too broad, then the quality of the product is not protected. Because the setting of specifications is both a key component of a quality program and a difficult task, the FDA scrutinizes very closely specifications for products it regulates. The FDA will not accept specifications if they are not complete, if they are unsuitable for the product, if their range is too broad, if they are unsubstantiated by testing, or if suitable analytical methods to test them are not available.

CURRENT GOOD MANUFACTURING PRACTICES (cGMPs)

The general principles of cGMP that all these regulations have in common are:

1. quality, safety, effectiveness must be designed and built into the product, not tested or inspected into the product
2. each step in the manufacturing process must be documented and controlled to ensure the finished product meets design and compendia specifications
3. process documentation provides evidence of compliance with cGMPs

Three basic criteria were used by the FDA in the design of these cGMP regulations:

1. Regulations should contain objectives and not detailed specifications; they should allow latitude for different manufacturers to find their own means of compliance.
2. Regulations should contain requirements that are considered feasible and valuable as recognized and considered by experts as assuring quality.
3. If a practice can be established to be feasible and valuable then it can be a required practice even though it does not exist in the regulations.

Considerable ambiguity exists in the acceptable cGMPs for manufacture of biological material by the biotechnology industry due to the new approaches and technology involved in this new area. Consequently, the biotechnology industry has relied heavily on regulations that have not

been finalized by the FDA. The wide range of processes that are more difficult to validate, such as fermentation production systems and chromatographic purification systems, only add to the confusion. It is difficult to set standards for quality in a field that has complex manufacturing processes. Frequently companies look to FDA Guidelines and FDA citations of lack of compliance following inspections to determine acceptable procedures in a given manufacturing process. In this regulatory environment, government has been emphasizing voluntary compliance over rulemaking, and the biotechnology industry tends to work more proactively with the FDA in establishing cGMPs for specific manufacturing processes.

DESIGNING A GMP-COMPLIANT PROCESS

The following is a brief summary on how to design a process:

1. The ***purpose*** of the process must be defined, that is, the desired output must be determined
2. An ***endpoint***(s) that demonstrates that the process has been performed satisfactorily must be defined. If appropriate, a range of accepted values for the endpoint must be established. A method to measure the desired endpoint(s) is required. In short, the specifications that define the endpoint need to be documented.
3. A method to ***measure the desired endpoint*** is required.
4. ***Raw materials*** and their specifications must be established.
5. The ***steps in the process*** must be determined, usually by experimentation.
6. The process must be ***scaled-up*** for production
7. An ***analysis*** of potential problems must be performed, noting the "critical points".
8. ***Experiments*** must be performed to determine how the process must operate at each critical point in order to make a quality product.
9. Methods to ***monitor*** the process must be developed.
10. Methods to ***control*** the process must be developed
11. Effective ***record-keeping*** procedures must be developed.
12. All ***SOPs*** required for the process must be written and approved.

References:

1. Seidman, Lisa and Moore, Cynthia. Basic Laboratory Methods for Biotechnology. 2nd Ed. Prentice Hall, NJ. 2009. ISBN: 321-57014-6
2. International Organization for Standardization: http://www.iso.org/iso/home.htm
3. FDA policies and procedures: http://www.fda.gov
4. Montgomery College: http://faculty.lonestar.edu/lloomisprice/BITC1402%20methods/1402%20homepage.htm

cGMP Production of Popcorn

GOAL:
Make a batch of GMP popcorn within a specified timeframe (1.5 hours)

OBJECTIVE:
- Understand the complexity of a GMP process.
- Gain appreciation of teamwork and cooperation of all departments.

How it mirrors industry:
- You will be frustrated at times
- You will be rushed.
- You will feel a sense of accomplishment once you made the batch!
- The actual time for the chemistry / fermentation / etc. is very small compared to the time it takes to get all GMP documentation in place.

SUPPLIES
1. Department tasks and deliverables (One copy of the lab (batch record) for the whole class, printed in color if possible)
2. Department signs: Quality Control, Quality Assurance, Material Control, Production, Supervisor.
3. Approved labels (A space for date, time, initials, lot number)
4. Quarantine labels (A space for date, time, initials, lot number)
5. 6 Boxes/Bowls for in-process material, approved material and quarantined material.
6. Microwave Popcorn (3 boxes, of 3 bags each, natural flavor, any brand)
7. Access to photocopier
8. Access to microwave
9. Water in a squirt bottle
10. 409 or other type of cleaning agent
11. Paper towels
12. Measuring cups (a graduated 2 cup works well for this)
13. Bags for the finished product (Ziplock bags for each student)
14. One binder (this will be the batch binder)

SAFETY
1. If the process is completed outside the lab (in a lecture room) with a "food only" microwave, the final product can be consumed after preparation.
2. Do not use any material from the lab, including paper towels and gloves.
3. Wash your hands before you begin!

PROCEDURE:

1. Assign job duties:
   - Supervisor: Instructor
   - Material Control: 2 people
   - QC: 2 people that like to test material
   - QA: 2 people that have an eye for written details
   - Production: 4 people that like to WORK

2. Collect the appropriate paperwork from the supervisor for your department.

3. Using your textbooks, learn about the department you have been assigned. Create a summary of responsibilities of that department. Include this in your assignment sheet at the end of the lab. How does your paperwork work into your responsibilities?

4. Each group will give an oral presentation describing an overview of their role in the company and how that role contributes to the production of the final product.

5. As a company (class), design your GMP-compliant process as outline in the introduction.

6. Design your manufacturing facility – Think about the best layout for your manufacturing facility. For example, which department do you think will be right beside the receiving door? Arrange the classroom to be your manufacturing facility. Each department will have a different area of the lab; identify that area with your signs.

7. Go to your department with your coworkers and work on your paperwork. If you're done your paperwork ahead of time feel free to observe other departments.

8. The class should create a "Batch Binder" where all in-process paperwork will be kept when it travels with the product from department to department.

9. When ready, start the production of your product! You have only 1.5 hours to complete this process, so you must hurry. Check in with your coworkers, give updates to different departments, and check-in with the supervisor if a department is delayed.

10. Remember ***"Quality is the job of everyone in the company".*** Help each other get the best quality product in the deadline given.

11. When the product has been released – enjoy!

12. The class will submit one batch binder for grading. Include in your lab report the summary of your departmental responsibilities from above and the question sheet at the end of the lab.

FLOW DIAGRAM FOR PRODUCTION:

Use this to help plan out and define your GMP process. If each horizontal box is a step, at which step does each department come in and what do they do? To get you started, the first step is done for you.

Materials Control	Quality Control	Production	Quality Assurance
Inspect incoming packages for possible signs of damage during shipping. Fill in section 1 on the "Receiving Report". Start batch binder.			

M A T E R I A L C O N T R O L

ROLE OF MATERIAL CONTROL:

- MC controls the flow of materials and limits the access of materials to prevent against "off-grade" materials being used in production.
- Material Control inspects all incoming materials/packages for integrity.
- Material control offers expertise in packaging and storing of materials.

Your Tasks are:

STORAGE AREAS:

Designate two separate areas: "**Quarantine"** and "**Approved."** Use the boxes provided and label them appropriately.

Inspect all incoming packages for possible signs of damage during shipping.

RECEIVING RAW MATERIALS:

You will receive raw materials to enter into your system.

Instructions:

1. By using information on the package - fill in **SECTION 1** on the "**Receiving Report**". Use the template provide.
2. Give a **"RECEIVING NUMBER"** to the material. Use the following as an example:
   MONTHYEAR – 001 (e.g: 0102-001 for January 2002)
3. Write this receiving number on the Receiving Report. Attach the COA to the Receiving Report.
4. Complete a **Quarantine Label** and place the completed **"Quarantine**" label on the material and transfer to the **"Quarantine" area**.
5. Give the "**Receiving Report**" and COA to QC- this is their cue to sample the material and begin testing.

LABELING RAW MATERIALS APPROVED:

1. QA will give Material Control "Approval" labels to apply to the material that is in "Quarantine".
2. Place the Approval Label to cover up the word "QUARANTINE" on the quarantine label. Move the material to the "Approved" area.
3. Production may now have the material. Give only "Approved" material to production for their use.

5. RECEIVING FINAL PRODUCTS INTO QUARANTINE:

You will need to take the final product (i.e. popcorn) and place it in quarantine.

Instructions:

1. fill in **SECTION 1** on the "**Receiving Report**". Use the template provide. Ask production team for lot number.
2. Complete a **Quarantine Label** and place the completed **"Quarantine**" label on the material and transfer to the **"Quarantine" area**.
3. Give the "**Receiving Report**" to QC- this is their cue to sample the material and begin testing.
4. QA will give Material Control the "Approval" labels to apply to the material that is placed in "Quarantine". Move the material to the "Approved" area.

RECEIVING REPORT

SECTION I : RECEIVING (Completed by Material Control)	
Material Name:	Date Received:
Supplier:	Receiving Lot No.:
Quarantine Label Applied: Yes ☐	Number of Containers:
Completed by:	Date:

SECTION II: SAMPLING and INSPECTION (Completed by QC)			
Total sample quantity (if final product otherwise N/A):	Number of Containers Sampled / Inspected:	By:	Date:

SECTION III: LABELING (Completed by QA)		
Number of labels issued: (QA)	By	Date:
Number of sample labels: (QA)	By	Date:
Attach Sample Label Below (QA)	By:	Date

Place sample label below:

P R O D U C T I O N

ROLE OF PRODUCTION:

- Execute the process according the batch record to produce a product within specifications
- Coordinate the batch record, release of raw materials, and equipment

Checklist of Items to Accomplish:

- ❑ Write Master Raw Material Specification Sheet and circulate for approvals
- ❑ Write Master Final Product Specification Sheet and circulate for approvals
- ❑ Write Master Batch Record and circulate for approvals
- ❑ Ensure Raw Materials have been tested and approved
- ❑ Clean Microwave per SOP
- ❑ MAKE GMP Popcorn! And complete batch record as you go!

HINT: PRODUCTION HAS MANY DOCUMENTS TO WRITE ….IT IS BEST IF YOU MULTI TASK AND SPREAD THE WORK AMONG THE DEPARTMENT.

Your Tasks are:

1. **WRITING MASTER SPECIFICATIONS**:

Production must write the following specifications and give to QC and QA to review and approve:

a. **RAW MATERIAL SPECIFICATION SHEET** for the KERNELS
b. **FINAL PRODUCT SPECIFICATION SHEET** for the POPCORN.

Instructions:
Use the template in your package. *Neatly complete all boxes (i.e. Vendor, storage conditions, specifications, etc.) that are* ***shaded*** *(except the signatures). All information provided should make sense and be reasonable. Try where possible to write quantitative specifications (i.e. "No more than 2 dark pieces of popcorn).*

- ❑ Circulate for signatures –

1st: Production signer in "Written By"
2nd: Production Supervisor signs
3rd: QC Supervisor
4th: QA

NOTE: QA will keep the final document.

2. **WRITING A MASTER BATCH RECORD**:

Production must write a Master Batch Record for the production of Popcorn. **Use the Template provided.**

Instructions:
Enter all the information in the boxes that are ***shaded.*** *You must describe what you think your process will be. All information provided should make sense and be reasonable.*

HOW TO WRITE A MASTER BATCH RECORD:

1. Complete all shaded areas. Sign your name on the front page
2. Give to Production supervisor for review. Sign your name on the front page.
3. Give to QA to review. Sign your name on the front page.
4. Once QA is happy with the batch record, they will issue you a copy to conduct your production.

Don't forget to check with QC regarding the testing of your **raw materials (i.e.: Kernels)**.

4. CLEANING EQUIPMENT:

Before production can use a piece of equipment it must be clean! It is a common practice in industry to have QA inspect the equipment after production cleans it.

HOW TO CLEAN EQUIPMENT:

1. Use the SOP provided to clean your equipment (i.e. Microwave).
2. Once the production operator has cleaned the Equipment, complete the documentation required on the Cleaning log and have the Supervisor inspect the equipment.
3. Request QA to **visually inspect** the microwave for cleanliness.
4. Once QA has inspected the equipment and found it acceptable, you may now use the equipment.

5. STARTING PRODUCTION!

1. Once you have the issued batch record from QA; APPROVED raw material; and CLEAN equipment, **you may start production of the POPCORN.**
2. Follow the process in your batch record and document as you go. Once the production of the popcorn is complete so should your batch record.

ENDING PRODUCTION:

1. Notify Material Control to remove your popcorn from the equipment and place it in quarantine.
2. While QC is testing the material, the Production Operator must review the record to ensure all information is complete.
3. Production supervisor must review and sign the back of the batch record.
4. Submit to QA for review.
5. Clean the equipment as documented above.
6. Wait to hear from QA if your material is approved!

Batch Production Record

Product Name: Popcorn

Effective Date:

Revision: 0

Written By:		**Date:**	
Production Supervisor Approval:		**Date:**	
Quality Assurance Approval:		**Date:**	

MANUFACTURING DIRECTIONS FOR LAB SCALE BATCH:

(completed by QA when issued)

Lot Number:

(Completed by QA on Master)

Effective Date:

Revision: 0

COMPOUND: POPCORN

INPUT MATERIALS:

INPUT MATERIALS	PRODUCTION OPERATOR	CHECKED BY

Note:

Input Materials are materials used in your process (i.e. Kernals).

Document Approval

Quality Assurance Initial:	

MANUFACTURING DIRECTIONS FOR LAB SCALE BATCH:

(completed by QA when issued)

Lot Number:

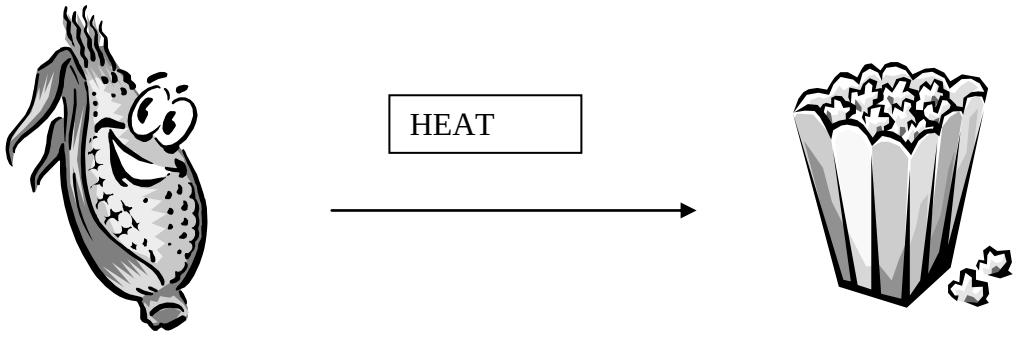
(Completed by QA on Master)

Effective Date:

Revision: 0

COMPOUND: POPCORN

REACTION SCHEME



Document Approval

Quality Assurance Initial:	

EQUIPMENT CHECKLIST

(Standard batch size)

		Inventory Number	Production Operator Initial and date
	Microwave Oven		

Document Approval

Quality Assurance Initial:	

MANUFACTURING DIRECTIONS FOR LAB SCALE BATCH:

(completed by QA when issued)

Lot Number:

(Completed by QA on Master)

Effective Date:

Revision: 0

COMPOUND: POPCORN

PROCEDURE

Date:

		Operator init./time	Coworker init./time
1.			
2.			
3.			
4.			
5.			
6.			

Document Approval

Quality Assurance Initial:	

MANUFACTURING DIRECTIONS FOR LAB SCALE BATCH:

(completed by QA when issued)

Lot Number:

(Completed by QA on Master)

Effective Date:

Revision: 0

COMPOUND: POPCORN

Date:

		Operator init./time	Coworker init./time
7.			
8.			
9.			
10.			

Comments: (initial and date any comments)

Document Approval

Quality Assurance Initial:	

MANUFACTURING DIRECTIONS FOR LAB SCALE BATCH:

(completed by QA when issued)

Lot Number:

(Completed by QA on Master)

Effective Date:

Revision: 0

COMPOUND: POPCORN

SUMMARY OF RESULTS

Actual Yield %:	%

Operator:	Supervisor:

Storage of Material

Total containers of product transferred to storage:

STORAGE CONDITIONS: Store at room temperature.

Document Approval

Quality Assurance Initial:	

CONCLUSION

Date Production Finished:		Production Operator Initials:	
Production Supervisor Approval:		Date:	
Quality Assurance Reviewer:		Date:	

DISPOSITION OF LOT # _____.___.___.

Approved ☐ **Non-Conforming** ☐

Quality Assurance Signature:		Date:

Document Approval

Quality Assurance Initial:	

STANDARD OPERATING PROCEDURE

Procedure:	Procedure No.: CLN-0001	Revision: 0
PROCEDURE FOR THE PRODUCT CHANGEOVER CLEANING OF A MICROWAVE OVEN	Effective Date: 11/15/02	
	Replaces Document: New	
Written by: Dept.: Production Date:	Dept. Approval: Dept.: Engineering Date:	
Content Review: Dept.: Engineering Date:	QA Approval: Date:	

I. PURPOSE

This procedure is to ensure proper cleaning of equipment.

III. PROCEDURE

3.1		**Product Changeover Cleaning**	**Responsibility**
	3.1.1	Enter the lot number of the batch on the "Equipment Cleaning Log".	Production
	3.1.2	Clean the equipment using water as a cleaning agent and paper towels as cleaning implements.	Production
	3.1.3	First wipe the interior top of the microwave from back to front.	Production
	3.1.4	Wipe the interior back of the microwave from top to bottom.	Production
	3.1.5	Wipe the interior sides of the microwave from back to front, then top to bottom.	Production
	3.1.6	Wipe the interior bottom of the microwave from back to front. If the microwave has a turntable inside, remove pieces and clean them using water as a cleaning agent. Visually inspect for contaminants. Replace them in the microwave when complete.	Production
	3.1.7	Complete the "Cleaning Log". Enter the SOP number used, cleaning agent, and time / date of person performing cleaning.	Production
	3.1.8	Visually inspect for contaminants. If contaminants are present, repeat steps 3.1.2 through 3.1.7. If acceptance, initial column.	Supervisor
	3.1.9	Notify QA to Inspect the equipment and verify that it is clean. Quality Assurance will sign the log in the "QA Initials Column" and check the product changeover box.	Production QA

EQUIPMENT CLEANING LOG

Equipment Name:______Microwave__________________ Make:_______________

Lot Number of batch in production	Time/Date of Cleaning	1. SOP No. 2. Solvents Used	Initials of person performing cleaning and a coworker verifying	Cleaning Status
Lot Number: ________________	**Time:** **Date:** ________	SOP # ____________ Solvent: _____________	Production Initials: ____________ Supervisor Initials: ____________	❑ Product Changeover QA Initials:______ Date:______
Lot Number: ________________	**Time:** **Date:** ________	SOP # ____________ Solvent: _____________	Production Initials: ____________ Supervisor Initials: ____________	❑ Product Changeover QA Initials:______ Date:______
Lot Number: ________________	**Time:** **Date:** ________	SOP # ____________ Solvent: _____________	Production Initials: ____________ Supervisor Initials: ____________	❑ Product Changeover QA Initials:______ Date:______

RAW MATERIAL SPECIFICATION SHEET

Receiving Number:		**Revision:** 0	**Effective Date:**

Item Description: KERNELS	
Structure: 	**Written By:** **Date:** **QC Approval:** **Date:** **Production Supervisor Approval:** **Date:** **QA Approval:** **Date:**
Hazards:	**MAY HARM TEETH!**

Storage Condition:		**Supplier:**	

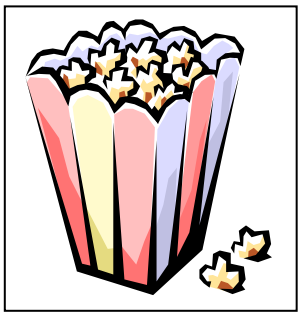
SPECIFICATIONS AND RESULTS

Test	Method	Specification	Result	QC Analyst Init. / Date
Physical Description	**Visual**			

QC Approval	Approved Non-Conforming	**Date:**
QA Approval	Approved Non-Conforming	**Date:**

FINAL PRODUCT SPECIFICATION SHEET

Lot Number:		**Revision:** 0	**Effective Date:**

Item Description: POPCORN		
Structure: 		**Written By:** **Date:** **QC Approval:** **Date:** **Project Team Approval:** **Date:** **QA Approval:** **Date:**
Hazards:	**MAY HARM TEETH!**	

Storage Condition:		**Supplier:**	

SPECIFICATIONS AND RESULTS

Test	Method	Specification	Result	QC Analyst Init./Date
Physical Description	**Visual**			

QC Approval	Approved Non-Conforming	**Date:**
QA Approval	Approved Non-Conforming	**Date:**

QUALITY ASSURANCE

ROLE OF QA:

- Review and Approval all quality related documents.
- Issue all controlled documents.
- Provide oversight on the production campaign.
- Disposition Raw materials and final products.

Checklist of Items to Accomplish:

- Review and Approve Raw Material Master Specification Sheet
- Review and Approve Final product Master Specification Sheet
- Review and Approve Master Batch Record
- Approve actual Raw Materials for use
- Inspect Microwave for Cleanliness
- Review completed Batch Record after production is complete
- Review QC data and Approve actual Popcorn

Your Tasks are:

1. APPROVING MASTER SPECIFICATIONS:

Production will be submitting for your review AND approval:

1. **RAW MATERIAL SPECIFICATION SHEET** for the KERNELS
2. **FINAL PRODUCT SPECIFICATION SHEET** for the POPCORN.

Instructions: *Review to ensure all boxes that are **shaded** have been completed. All information provided should make sense and be reasonable. Try where possible to have the team write in quantitative specifications (i.e. "No more than 2 dark pieces of popcorn). If you don't agree with the specifications or have questions feel free to go back to the Production Team for clarification.*

HOW TO APPROVE A MASTER SPECIFICATION SHEET:

To make the **specification sheet** effective complete the following:

a. Sign your name in the shaded box for "QA Approval".
b. Write in an effective date (i.e. today's date) in the upper right hand corner.
c. Make a photocopy (yes, go to the photocopier) of the original document you just signed and give a copy to QC.
d. File the original in QA.

2. APPROVING A MASTER BATCH RECORD:

Production will be submitting for your review:

1. A Master **BATCH RECORD** to make the POPCORN.

Instructions: *Review to ensure all boxes that are **shaded** have been completed. All information provided should make sense and be reasonable. HINT: The production*

team should have quantitative numbers in the process (i.e. Pop popcorn for 1.5 – 2.5 minutes). If you don't agree with the process description or have questions feel free to go back to the Production Team for clarification.

HOW TO APPROVE A MASTER BATCH RECORD:

To make the BATCH RECORD effective complete the following:

a. **Sign** your name in the shaded box for "QA Approval" on the front page and initial the bottom of all pages in the QA box designated.
b. **Write in** an effective date (i.e. today's date) in the upper right hand corner on ALL PAGES.
c. Make a **photocopy** of the Master Batch Record
d. Write in the **Lot Number** on all pages (in the box for LOT NUMBER) **ON THE PHOTOCOPY OF THE BATCH RECORD**. (See below for instructions):

INSTRUCTIONS FOR ASSIGNING A UNIQUE LOT NUMBER:

Lot number should be POP-YEAR-001. For example: POP-03-001

e. **File** the original batch record in QA.
f. **Give the photocopy** of the batch record to production. (We call this "Issuing a batch record to production")

3. APPROVING RAW MATERIALS:

Before production can use the kernels in their production run they must be approved by QC AND QA! Follow the instructions below to approve the raw materials (i.e. Kernels).

HOW TO APPROVE RAW MATERIALS:

1. Once QC finishes the "testing" on the Kernels, **review** the information written in on the specification sheet and the accompanying documentation to ensure no empty spaces.
2. If acceptable, sign your name in the box for QA at the bottom of the page on the **specification sheet** and check off "**Approved**".
3. Fill in the spots on the **Approval labels**. **Make one for each container / package PLUS 1 extra for the receiving report.**
4. Complete the remaining boxes designated "QA" on the **Receiving report**. Place your extra label on the receiving report.
5. Give **approval labels** to Material Control.

4. INSPECTING EQUIPMENT:

Before production can use a piece of equipment it must be clean! It is a common practice in industry to have QA inspect the equipment after production cleans it.

HOW TO INSPECT EQUIPMENT:

1. Production will be requesting QA to **visually inspect** the microwave for cleanliness. If it is not satisfactorily clean, have Production re-clean the microwave.
2. Once acceptable, sign/date the **Cleaning Log** in the spot for "QA initials"

5. INTERNAL AUDITING:

Feel free to audit the production area during production!

6. REVIEWING COMPLETED PRODUCTION BATCH RECORDS:

Once production of the popcorn is complete, the Production team will be submitting the completed batch record for your review. Before the popcorn is "Dispostioned" QA must review the completed batch record AND completed Quality Control testing. BOTH items must be satisfactorily before the popcorn can be approved!

HOW TO REVIEW A COMPLETED BATCH RECORD:

1. Ensure all information is recorded and completed per requirements of the batch record.
2. If any items were not completed –return to production for correction.
3. The team must not of deviated from requirements in the batch record. (i.e: if it says to "Pop the popcorn in the microwave for 2-3 minutes they must not go over 3 minutes or under 2 minutes without some justification.)
4. Once you are satisfied with the completed record, sign your name in the "Reviewed by QA" box
5. DO NOT YET APPROVE THE BATCH. YOU NEED THE QC DATA FIRST.

7. REVIEWING COMPLETED QC DATA

Once the QC data is complete – review the QC information for completeness. If both the Batch Record and QC data are acceptable you may sign both documents as "APPROVED". NOW THE POPCORN IS APPROVED.

HOW TO APPROVE QC DATA ON POPCORN

1. Once QC finishes the "testing" on the Popcorn, **review** the information written in on the specification sheet and the accompanying documentation to ensure no empty spaces.
2. If acceptable, sign your name in the box for QA at the bottom of the page on the **specification sheet** and check off "**Approved**".
3. If the batch record is complete and the QC data is complete you can now fill out the **Approval labels!**
4. Complete the remaining boxes designated "QA" on the **Receiving report**. **Make one label for each container / package PLUS 1 extra for the receiving report.**
5. Place your extra label on the receiving report.
6. Give **approval labels** to Material Control.

QUALITY CONTROL

ROLE OF QC:

- Test all materials to be used by Production.

Checklist of Items to Accomplish:

- Review Raw Material (i.e Kernels) Specification Sheet
- Test Raw Materials
- Review Final Product (i.e. Popcorn) Specification Sheet
- Test Final Product Materials

Your Tasks are:

APPROVING MASTER SPECIFICATIONS:

Production will be submitting for your review AND approval:

3. **RAW MATERIAL SPECIFICATION SHEET** for the KERNELS
4. **FINAL PRODUCT SPECIFICATION SHEET** for the POPCORN.

Instructions: *Review to ensure all boxes that are **shaded** have been completed. All information provided should make sense and be reasonable. Try where possible to have the team write in quantitative specifications (i.e. "No more than 2 dark pieces of popcorn). If you don't agree with the specifications or have questions feel free to go back to the Production Team for clarification.*

HOW TO APPROVE A MASTER SPECIFICATION SHEET:

To approve the **specification sheet** complete the following:

a. **Sign** your name in the shaded box for "QC Approval".
b. Give to QA for their review.

2. DOCUMENTS NEEDED:

NOTE: QA will give you copies of the specification sheets to document the results of the testing for BOTH the kernels and the popcorn.

3. APPROVING RAW MATERIALS:

Before production can use the kernels in their production run they must be approved by QC AND QA! Follow the instructions below to test and approve the raw materials (i.e. Kernels).

HOW TO TEST AND APPROVE RAW MATERIALS:

6. Material Control will be giving you a **RECEIVING REPORT** for both **KERNALS** and **POPCORN** (once made). This is your cue that something is in quarantine and needs to be tested by QC. Take your specification sheet to quarantine and begin to following the sampling and testing instructions.
7. Once the analyst completes the "testing" on the Kernels, **review** the information written in on the specification sheet and the accompanying documentation to ensure no empty spaces. The analyst will sign his/her initials and date in the column.
8. The QC Supervisor will review the package of data the analyst completed and If acceptable, sign your name in the box for QC approval at the bottom of the page on the **specification sheet** and check off "**Approved**".
9. Give package to QA.

4. APPROVING FINAL PRODUCT (I.E: Popcorn):

Once production has made the popcorn, you will be required to test the material. Follow the instructions below to test and approve the final product (i.e. popcorn).

HOW TO TEST AND APPROVE FINAL PRODUCTS:

1. Material Control will be giving you a **RECEIVING REPORT** for the **POPCORN** (once made). This is your cue that something is in quarantine and needs to be tested by QC. Take your specification sheet to quarantine and begin to following the sampling and testing instructions.
2. Once the analyst completes the "testing" on the Popcorn, **review** the information written in on the specification sheet and the accompanying documentation to ensure no empty spaces. The analyst will sign his/her initials and date in the column.
3. The QC Supervisor will review the package of data the analyst completed and if acceptable, sign your name in the box for QC approval at the bottom of the page on the **specification sheet** and check off "**Approved**".
4. Give package to QA.

Unit 2 - cGMP Production of Popcorn Assignment

Name: _____

Each class will submit **one** final Batch Record, which includes all the forms. You may keep a copy for your records if you wish, but only turn in one batch record for the class.

Each student must fill in this sheet and turn it in with the final Batch Record.

1) What section were you in? Provide a summary of the responsibilities of your department. And briefly describe your role in the process.

2) Who was the supervisor of the section?

3) Who were your coworkers?

4) What was the most interesting part of what you did?

5) What was the most difficult part of the process?

6) How did this exercise help you understand GMP production methods?

7) Please describe anything we could do to make this exercise more interesting or informative.

LAB UNIT 3: BASIC TOOLS IN THE BIOTECHNOLOGY LABORATORY

OBJECTIVES

Your performance will be satisfactory when you are able to

- Identify common lab equipment pieces and describe their function
- Distinguish between glassware pieces in regard to measuring accuracy
- Understand the role of the reagents you use in the laboratory

During your training in the ACC Biotechnology program, 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. You are required to keep a list of the equipment that you learn to use and a brief description of the purpose of the machine. For example, a PCR machine is used to amplify a specific section of DNA.

Concerning the equipment, to use it you **need to know its location in the laboratory. Please locate the following items in the lab.**

1. **Measurement of Volume**

Erlenmeyer flasks are used primarily to prepare solutions prior to an accurate volume adjustment. Although there are volumetric markings on these flasks, they are not calibrated and should not be relied upon for exact volume measurements.

Beakers are also used for preparing solutions, especially when a pH adjustment requires access to the solution by a pH probe. The volumetric markings on beakers are also not reliable.

Graduated cylinders are calibrated with sufficient accuracy for most volume measurements when preparing solutions. For example, the calibration of most 100 mL graduated cylinder can be relied upon to accurately measure to within +/- 0.6 mL.

Volumetric flasks are used to measure a specific volume with the highest degree of accuracy, and are used to make standard solutions for analytical assays. For example, the calibration of a 100 mL volumetric flask can have an accuracy of +/- 0.1 mL.

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

a. **Pasteur pipets** are small glass tubes used with a **bulb** to transfer volumes as small as a single drop and as large as a few milliliters. They are not graduated and are not used to measure volumes.

b. **Beral pipets (transfer pipets)** are plastic pipettes with a bulb at one end used for transfer of liquids. Sometimes they have calibration marks, which have a low level of accuracy. They are often disposable, sterile and individually wrapped.

c. **Serological, or "blowout," pipets** are graduated glass tubes used to measure anywhere from 0.1 to 50 mL. When the liquid has drained from this pipette, the final drop in the tip is transferred with a puff of air.

d. **Mohr, or "to deliver," pipets** are similar to blowout pipettes, but do not require a puff of air to accurately deliver the desired volume. They can be identified by the label "TD" on the top.

e. **Volumetric pipets** are not graduated, but are carefully calibrated to deliver a single, highly accurate volume, and are used for the transfer of exact volumes.

f. **Automatic micropipettes** are mechanical pumps calibrated to deliver highly accurate volumes generally less than 1.0 mL, and as little as 0.1 microliter. They are often adjustable for measuring different volumes and they always use dispensable plastic tips to actually transfer the liquids.

g. **Multichannel micropipettes** can deliver the same volume from as many as 12 tips simultaneously. All automatic micropipettes need regular maintenance, calibration, and validation.

2. Measurement of Weight

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

a. **Mechanical balances** weigh an object on a pan hanging from a beam that has a counterbalanced weight. We do not use mechanical balances in our lab.

b. **Electronic balances** have replaced most mechanical balances due to their greater accuracy and ease of operation. They are easier to use because they usually have a digital readout, and weighing dishes can be **tared** to read zero mass before using. Most balances used for preparation of solutions have a sensitivity of +/- 0.01 g, but **electronic analytical balances** can be sensitive to +/- 0.1 mg or less. Electronic balances require routine maintenance and recalibration.

3. Measurement of pH

Most solutions prepared in the biological laboratory must have a carefully controlled pH. Buffers are prepared by adjustment to a specific pH with strong acid and base solutions, using a meter to monitor the pH. A **pH meter** is a volt meter that measures the electrical potential between two electrodes. One electrode is in contact with your solution, and the other is in contact with a reference solution. Usually both of these electrodes are combined in a single pH probe that you place in your solution. These meters can read to the nearest 0.1 pH unit, but require frequent calibration with reference buffers of known pH.

4. **Measurement of light**

Solutions are often analyzed in the biotechnology lab by measuring how the solutes interact with light. A **spectrophotometer** measures the amount of light that is absorbed by a solution at a specific wavelength or over a range of wavelengths. If you know a wavelength at which a specific substance absorbs light, you can calculate the amount of that substance in a solution from the measured absorbance of that solution at that wavelength.

a. A **visible (VIS) spectrophotometer** measures absorbance of light in the visible region of the spectrum (wavelength of about 400-700 nm). A small vessel called a cuvette, which is generally plastic or glass and which usually has an internal diameter of 1.0 cm, is filled with the solution and placed in the spectrophotometer for measurements.

b. An **ultraviolet/visible (UV/VIS) spectrophotometer** can also measure absorbance of light in the ultraviolet region of the spectrum (about 100-400nm). These spectrophotemeters require a halogen light bulb that emits ultraviolet light and require special cuvettes that don't absorb UV light.

c. A **scanning spectrophotometer** can measure the absorbance of a solution over a range of wavelengths, creating an absorbance spectrum that can be used to identify substances in a solution.

d. A **NanoDrop spectrophotometer** is a brand of scanning UV/VIS spectrophotometer that allows the user to measure the absorbance of a very small sample of liquid (1-2 uL). This instrument makes it easy to quickly evaluate the quality and quantity of nucleic acids or proteins in a small sample prep.

e. A **microplate reader** is a spectrophotometer that can measure the absorbance in the individual wells of a plate. Usually the plates are 96 wells, but other formats are available, such as 48 wells and 384 wells. This allows the user to prepare and read many small samples at once, saving time and money. Microplate readers may also be capable of reading fluorescence and chemiluminescence, which are two types of light emission that are frequently used in biological research.

5. **Solution Preparation**

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

a. **Magnetic stirrers** come in the form of a box with a magnet inside attached to a motor that spins the magnet. When a vessel containing a magnetic stir bar is on top of the magnetic stirrer, the stir bar spins and stirs the contents of the vessel.

b. A **vortex mixer** rotates the bottom of a tube rapidly; setting up a vortex in the liquid that rapidly mixes the contents.

6. Microbiological techniques

Specialized equipment is required to isolate, transfer, and grow up cultures of microbes and tissues in the laboratory.

a. **Autoclaves** are machines that achieve a high internal temperature and pressure and are used to sterilize solutions and glassware. The kitchen pressure cooker achieves the same results and can be used instead of an autoclave.

b. A **biological safety or cell culture hood** filters small particles out of the air in order to avoid contamination of cultures or sterile media. The filters are similar to those used to decontaminate air for operating rooms in hospitals or clean rooms used in the semiconductor industry.

c. **Fermentors** are used to grow up a large quantity of cells with automatically controlled pH and levels of oxygen and other nutrients.

d. Since most cells are generally too small to be seen with the naked eye, ***microscopes*** are used to magnify their images. **Light or Brightfield microscopes** and **inverted microscopes** are the most common types found in biotechnology laboratories.

7. Preparation of biological samples for analysis

There are many pieces of equipment that are used to prepare biological samples for analysis.

a. A **Sorvall-type centrifuge, or preparative centrifuge**, has a balanced rotor that holds vessels and spins them at high speed, up to 20,000 rpm. This will cause most insoluble particles such as cells and many subcellular components to rapidly form a pellet at the bottom of the vessel. Rotors are available that hold vessels as small as a few milliliters to as large as a liter. These centrifuges are often refrigerated so that heat-sensitive compounds are not damaged during centrifugation. We do not have one of these in our lab.

b. A **tabletop, or clinical, centrifuge** is generally not refrigerated and spins at a much slower speed than a preparative centrifuge. Rotors for clinical centrifuges generally hold tubes with a capacity of 15 mL or less.

c. A **microcentrifuge** holds microcentrifuge tubes that can hold about 1.5 mL of liquid. These microcentrifuges can also spin at high speeds and are sometimes refrigerated.

d. A **sonicator** emits ultrasonic waves that can be used to disrupt cells, allowing their contents to be released into the surrounding buffer in "grind and find" strategies.

8. Separation of macromolecules

Since there are thousands of different macromolecules in each cell, purification of a specific one from all the others requires powerful separation techniques, such as chromatography and electrophoresis. Both of these approaches take advantage of physical and chemical properties that differ between the individual macromolecules.

In gel electrophoresis, the macromolecules are placed in a solid matrix, called a gel, which is under a liquid buffer. An electric field is applied to this system, and since biological macromolecules carry ionic charges, they will be attracted towards one pole of the electric field and repelled by the opposite. Thus, macromolecules characteristically migrate in either direction in the field. The migration speed is determined by the charge-to-mass ratio of the macromolecule.

a. In a **flat gel, also called a horizontal or submarine gel, electrophoresis system**, an agarose gel lies horizontally below the electrophoresis buffer. This technique is mainly used to separate large nucleic acids (DNA and RNA).

b. A **vertical electrophoresis system** holds a polyacrylamide gel in the vertical position, and is mainly used to separate proteins or small-sized nucleic acids.

Chromatography is a family of methods used to separate macromolecules through their relative affinity to a stationary phase (generally, solid chromatography beads) and a mobile phase (generally, an aqueous buffer). The chromatography beads are loaded into a tube, called a chromatography column, and buffer is dripped, or pumped, through the column to carry the macromolecules along. The macromolecules separate on their affinity for the mobile front. Some chromatography beads separate by charge (ion exchange chromatography), by hydrophobicity (hydrophobic interaction chromatography), or by a specific property of that protein (affinity chromatography). Macromolecules can also be separated by size otherwise known as size exclusion or gel filtration chromatography.

a. To overcome this limitation, **high performance (or high pressure) chromatography (HPLC)** uses high-pressure pumps and metal-jacketed columns to operate at high pressures and speed up the process.

b. A **fraction collector** collects the released mobile phase (eluent) of a chromatography column. It automatically measures a programmed volume (sometimes by the number of drops of liquid) into a line of test tubes or microcentrifuge tubes.

9. Manipulation of Nucleic Acids

You will be learning many techniques to isolate, transfer, and analyze DNA in your biotechnology training. Some of the specialized pieces of equipment used for these procedures will include:

a. **A thermal cycler** is a machine that is used for amplification of a specific section of DNA by PCR (polymerase chain reaction). The machine cycles through several temperatures, which allows an enzyme called DNA polymerase to use chemicals in solution to build DNA molecules identical to a template provided.

b. An **electroporator** is used to discharge a high-voltage, high-amperage pulse of electricity of very short duration through a cuvette containing suspended cells to disrupt their plasma membranes, allowing DNA to be introduced.

c. A **real-time PCR machine** amplifies and measures the production of amplicons in one step. It is a thermal cycler and fluorescent analyzer in one instrument and is usually computer-controlled. You do not have to load your product onto a gel to determine if it was made; the machine measures its production photometrically.

Lab Unit 3-A: Using a Micropipette

Now that you have practiced calculations and conversions, you are ready to become familiar with some of the essential tools of the biotechnician. In the next few labs, you will learn to use the micropipette, the balance and the centrifuge. These three tools are used daily in many bioscience labs around the world.

OBJECTIVES

Your performance will be satisfactory when you are able to follow these **Good Laboratory Practices (GLPs)** and make them a habit for every lab:

- Keep your work area clear of unnecessary items
- Keep everything you need within reach
- Gather all materials before you begin working
- Set up disposal areas before you begin working
- Label each container BEFORE you fill it
- Change gloves often to avoid contamination
- Never wear your gloves out of the lab
- Never do protocols from memory; always read every step every time you perform a procedure, and then check it off as it is completed
- Always cap bottles of stock solutions and chemicals when finished
- Never hold a solution in a micropipette; always eject immediately

Materials (per group)

- 20-50 mL sugar solution (dyed any color)
- 20-50 mL each of distilled water and water dyed blue
- small beakers (4)
- 1.5 mL microcentrifuge tubes (12 per group member)
- microcentrifuge tube rack
- set of 3 micropipettes
- box of 20-200 µL tips
- box of 100 – 1000 µL tips
- wash bottle with 70% ethanol
- picofuge

PROCEDURE

A. Organizing Your Work Space

When your work requires aseptic (sterile) conditions, you should wash the benchtop with 70% ethanol. Although this procedure does not need to be sterile, wash the table with ethanol to get into the habit. Collect everything (including paper towels) you will need for the lab, except things like the stock solution bottle that will be shared by the whole class. Each person in your group will do each of the measurements, so make sure you have enough containers. In order to work efficiently, you should arrange everything at your workstation so that you can reach it easily. The center of the workstation should be clear of items you are not immediately using. Always have a waste beaker for used tips when you are micropipetting; **do not use the sink for disposal of tips!**

B. Micropipetting Practice

GLP Tip: Never lay a micropipette down with a filled tip or hold it upside down or sideways. The liquid will not leak out if you hold it upright but it may enter the instrument if you hold it upside down, and contamination will result.

1. Practice setting the volume on the micropipette; each person in your group should set at least one and have it checked by other group members and/or your instructor. Look at the top of the micropipette to identify its measuring range. Remember that the highest value listed on the top is the largest volume you can measure on that pipet. On a 100 to 1000 µL micropipette, the largest measurable volume is 1000 µL; on a 20-200 micropipette, it is 200 µL. Likewise, the smaller value in the range is the smallest measurable volume; on a 2-20 µL micropipette, the smallest measurable volume is 2 µL. Set a 100-1000 µL micropipette to 0.45 mL, a 20-200 µL micropipette to 0.15 mL, and a 2-20 µL to 0.015 mL. What are these values in µL? **You should practice doing that kind of conversion in your head; it will be useful when working in a lab.**

2. Have a graduated 1.5 mL microcentrifuge tube in a rack ready to hold the liquid you measure in the next steps. Microcentrifuge tubes are often called Eppendorf tubes. Eppendorf is a popular brand of labware.

3. You will be pipetting 600µL of colored sugar solution. The color helps you see how much you are measuring. Choose the correct size micropipette and set it to 600 µL. While you are waiting to use the micropipette practice opening and closing microcentrifuge tubes with one hand or setting the other micropipettes with one hand.

4. Place a tip on the end of the micropipette. Do not touch the tip with your hands. Leave it in the box and push the end of the micropipette firmly into the tip. The smaller tips fit both the 2-20 and the 20-200 µL micropipettes. They are often yellow or clear. The larger tips are for the 200-1000 µL micropipette and are sometimes blue.

5. Using one hand, hold the micropipette and press down on the plunger with your thumb or index finger (whichever feels more comfortable). Note that there are 2 places the plunger stops. The first stop is for filling and the second stop is for delivering. Practice a few times until you can easily feel the difference between the two stops. If you are waiting to use the correct micropipette you can practice with the other micropipettes.

6. Press down to the first stop. Submerge the end of the tip just under the surface of the liquid. You may rest the tip against the side of the container just under the water line to steady it. If you submerge more than just the end of the tip, liquid will collect on the sides of the tip and drip into the collection tube when you deliver it. This will result in a larger volume of liquid than was desired.

7. Slowly release the plunger. If you release the plunger too quickly, the liquid may splash up into the micropipette and contaminate it. If you are pipetting viscous (thick) liquids, such as the sugar solution you are using, and you release too quickly, the liquid won't enter the tip fast enough and your measurement will be inaccurate. Sometimes this happens with thin liquids as well, so you should always pipette slowly. Be careful not to remove the tip from the liquid before it is filled with the desired volume or you will get an air bubble in the tip and less liquid than was desired. If you released the plunger slowly and kept the tip in the liquid but you still got a bubble, you probably pushed the plunger down to the second stop instead of the first. Practice the stops again.

8. Without removing the tip from the beaker, dispense the liquid by pushing the plunger slowly down to the first stop. Try not to make any bubbles. Repeat step 6. Drawing up the liquid twice (in labs it is called "pipetting up and down") can improve the accuracy of the measurement.

9. Dispense the liquid into 1.5 mL microcentrifuge tube and be sure it is near the 0.6 mL mark (600 µL = 0.6 mL). This is just a check to make sure you used the correct micropipette and set it correctly. Show the instructor your tube.

10. Discard the tip in a waste beaker by pressing the eject button. You may want to practice this technique a few times, as it is a very important skill to master.

C. Mixtures and Microcentrifuge Tube Labeling

1. You are going to measure different colors and amounts of water into 10 microcentrifuge tubes. Wearing gloves, choose 10 microcentrifuge tubes, close them, and label the lids 1 – 10. Always label on the top so that it can be read without removing the tube from the rack, and orient the tubes in the same direction so that you won't confuse letters like "H" and "I" and numbers like "6" and "9". Also, only use a permanent marker, such as a Sharpie, that will not erase or bleed if it gets wet. If your tubes are to be stored or mixed in a microcentrifuge etc., label your tubes with a group name, or your name, and the date of the experiment.

2. Open all of the lids of the microcentrifuge tubes so they are ready to receive the solutions. Before you begin measuring, think of what will be the most efficient way of dispensing the amount. If several of the tubes contain the same liquid, you can measure them all out before you change tips, as long as you do not touch the tip to the inside of a tube containing some other solution. Even water can be a contaminant if it changes the concentration of a given solution. You may also want to first fill all of the tubes that have the same measure of liquid so you don't have to change the setting too often. Sometimes it matters which ingredient is added first, as is the case when diluting acids and bases.

3. Measure the following amounts into the indicated tubes. Mix the contents by pipetting up and down several times. DO NOT pipet so vigorously that you make bubbles. This can degrade some sensitive solutions such as enzymes, and can also contaminate the micropipette. You may want to close the tubes as they are filled or move them back one row to avoid accidentally filling the same tube twice.

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

4. Check the accuracy of your measurements by setting a micropipette to the total volume in the tube and slowly withdrawing all of the solution from several tubes. Your pipetting was accurate if you leave no solution behind and have no air bubble in your tip. The amounts in tubes 1 and 2 are so small that if any is clinging to the side you won't be able to draw it up. If this is the case, put the tubes in a picofuge for about 10 seconds to "spin down" the liquid so it is all in the bottom of the tube. Have your instructor check your tubes before discarding them, as he or she may wish to watch you draw up the amount to check accuracy.

Lab Unit 3-A Using a Micropipette: Analysis

Part A:
Make a sketch to show how you organized your lab space.

Part B:

- If a 20 - 200 µl micropipette is set to 043 how many µl is it set to measure? _____
  How many mL is this? _____

- Why should you avoid touching the micropipette tips?

- Why should you avoid submerging the micropipette tip too deep in the liquid?

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

- What does the phrase "pipetting up and down" mean and how is this technique used?

Part C:

- On what part of a microcentrifuge tube should you write a label?

- Describe the order in which you filled the tubes in step 4 (eg. same color first, same volume first). Did this order result in maximum efficiency? If not, what order would be most efficient?

Lab Unit 3-B: Calibrating Lab Instruments

OBJECTIVES

Your performance will be satisfactory when you are able to

- Calibrate a pH meter using standard pH buffers and an SOP
- Use a pH meter to correctly determine the pH of an unknown solution
- Calibrate an electronic balance using standard weights and an SOP
- Use an electronic balance to obtain a desired mass of a substance
- Compare the measuring accuracy of glassware using an electronic balance
- LEAVE A CLEAN LAB AREA

INTRODUCTION

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

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

In this lab, you will calibrate and use pH meters and electronic balances. Both of these instruments should be calibrated each time they are turned on. If they are left on for long periods of time or used frequently during a day, they should be periodically recalibrated during that time as well.

Materials

Each group:
large waste beaker
25 mL of a solution of unknown pH
10 mL graduated cylinder
50-mL beaker with 10-mL graduations
100- or 150-mL beaker
5 mL pipette
10 mL pipette
Pipette bulb or filler
Beral (transfer) pipet

Class shares:
pH meter with SOP
electronic balance with SOP
200 g standard mass for balance calibration
pH standard buffers for meter calibration

PROCEDURE

A. Calibrating a pH meter

Several factors may affect the accuracy of a pH meter. First, most meters will only give accurate readings for solutions between -5 and 60 degrees Celsius. Second, a pH meter usually cannot measure a pH of 12 or greater accurately, and these high pH solutions can sometimes damage the electrode. Finally, solutions that have high sodium ion concentrations generally give erroneous results. While an electrode is designed to allow H^+ ions to pass through its walls, Na^+ ions may also pass through. The Na^+ ions affect the electrical potential measured by the electrode, which causes inaccurate pH readings.

1. Select a pH meter to calibrate and record its model number. Turn on and calibrate the pH meter using the SOP provided. You will be using pH 7, pH 10 and pH 4 standard buffers. The manufacturer often adds color to help identify the solutions; become familiar with these characteristic colors. Buffers are used because they have very stable pH value. Remember to always rinse the pH electrode with a wash bottle of distilled water or with the next solution before using it, catching the rinse liquid in a waste beaker labeled as such. Never rinse into the electrode storage solution or buffer.

2. After calibration is complete, use the meter to measure the pH of an unknown solution. Swirl the solution slightly to ensure that it makes good contact with the electrode before recording a reading. Record the pH in your notebook and have the instructor check your answer before going to the next section**.**

B. Calibrating and Using an Electronic Balance

The standards used to calibrate electronic balances are objects of known mass. For balances that measure to ± 0.01g, the standard is usually a 200-gram weight. These balances are used to measure amounts over 0.05 g. When you place the 200-gram weight on the balance in calibration mode, the balance recognizes the weight as 200 grams, and will then use that information to measure other masses.

1. Dispense 50 mL of orange stock solution into a 100- or 150-mL beaker.

GLP Tip: Never pipet from a stock solution. Rather, pour the approximate amount that you plan to use from a stock solution, and never return unused portions to the stock solution. This will prevent contamination. Note: This does not mean throw it out! Ask your instructor what to do with the extra if you accidentally aliquot too much.

2. Select a balance and record the model number. Follow the instructions in the SOP provided for the balance model to calibrate the balance. Have the instructor look at the live readout with the 200-gram weight on the balance before moving to the next section.

3. Place a weigh boat on the balance pan and press the tare button. This subtracts the weight of the weigh boat so that you are only weighing what is put inside it.

4. Draw up 4 mL of orange water using a 5 mL pipette and pipette filler or bulb and deliver it into the weigh boat. You might need to practice drawing up and delivering the liquid back into the flask until you can do it smoothly. If these are blowout pipets, to get the most accurate measure you must blow out the last drop (see the lab equipment exercise for ways to distinguish between blow-out and to deliver pipettes). If you have pipette filler, roll the wheel up and down quickly several times; if you have a pipette bulb, squeeze the bulb after emptying the pipette. Another option is to measure 5 mL and then deliver only down to the 1 mL mark. Remember to discard the remaining liquid in your waste beaker, not back into the stock solution.

GLP Tip: When adjusting the volume of a pipette, the bottom of the meniscus should be even with the marking to which you are adjusting. Make sure that your eye is level with the marking and meniscus in order to make sure that they are lined up correctly. Keep your pipet perpendicular to the benchtop. Make sure that there are no droplets of liquid on the outside of the pipet before you transfer it, and touch the pipet tip to the side wall of the container as you are dispensing the liquid.

5. Record the mass of the orange water in your notebook and in the analysis section. At sea level, 1.00 mL of water weighs 1.00 g. This should help you decide if your measurement was accurate.

6. Have each member of the group repeat step 4 and add 4 mL of orange water to the weigh boat (do not empty the weigh boat between measurements). Divide the total mass by the number of people in the group to find the average mass. Show calculations in your notebook.

C. Measuring Accuracy of Glassware

GLP Tip: Always inspect glassware before use. This is especially important if you are handling hazardous materials or heating the glassware. Discard chipped or cracked glassware in a specially designated glass disposal box. Never throw away glassware in the trash, and never dispose of plastic pipettes or other non-glass items in the glass disposal box.

1. Measure 10 mL of water in a 50 mL beaker using the lines on the beaker for your measurement. Tare a weigh boat on the balance. Pour the water in the weigh boat and record the mass in your lab notebook and on the analysis page. Each member of the group should repeat this step and record each measurement. Calculate the average mass of 10 mL of water measured with a beaker.

2. Repeat step 1 using a 10 mL graduated cylinder and repeat step 1 again with a 50 mL graduated Cylinder.

3. Repeat step 1 using a 10 mL pipette.

4. Repeat step 1 using a 50 mL flask.

5. Clean up your lab station. Pour the contents of the waste beaker down the sink with plenty of water, and return dirty glassware to the cart. Wipe off your lab bench with a wet paper towel. Make sure that the balances, pH meters, and the areas around them are clean and dry, and turn off the equipment.

Lab Unit 3-B: Calibrating of Instruments Analysis Assignment

Part A:
If you were given an uncalibrated thermometer, how could you use boiling water and ice to calibrate it in degrees Celsius?

Why shouldn't you measure the pH of 12 M NaOH with a pH meter (12 M is very concentrated and has a pH of about 14)? Give 2 reasons.

Based on what you know about the pH scale, why do you think the meter was calibrated with both pH 7 and pH 4 buffers?

Part B:
What does it mean to "tare" a balance?

What is a blowout, or serological pipet? How does this compare with a Mohr, or to-deliver, pipet?

The definition of a gram is the mass of 1 mL of pure water at 20˚C (about room temperature) and 1 atmosphere of pressure.

a) What should be the average mass of the water measured? Show your calculations. - _____ g

b) What was the average mass of the water your group measured? Show your calculations. _____ g

c) If there is a difference between the predicted mass (A) and the observed mass (B), how can you account for it?

Part C:
Record the average mass of the water you measured (include units):

a) 50 mL Beaker _____ ***10 mL graduated cylinder _____***

50 mL graduated cylinder _____ ***10 mL pipet _____***

50 mL Flask _____

b) Which of these measures most accurately?

c) Will it probably always be more accurate? _____ Why or why not?

LAB UNIT 4: PREPARING SOLUTIONS

OBJECTIVES

Your performance will be satisfactory when you are able to:

- Correctly prepare a solution of a given molarity leaving a CLEAN lab area
- Do parallel and serial dilutions and distinguish between the two
- Determine whether to use a parallel or serial dilution in a given situation
- Use a microcentrifuge to pellet a precipitate

INTRODUCTION

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

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

- *Check and recheck each calculation.* It is best if two people make a calculation independently and then cross check their answers.
- *Read each reagent bottle twice*, once before using and once afterwards. 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. Some Solution Prep forms will also have space to include the balance number, pH meter number and other pieces of important 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 that is prepared in the class you must do the following:

1. Calculations are performed prior to class and recorded in your pre-lab exercise. You must do this for EVERY solution, even the solutions you do not prepare.
2. You must fill out a Solution Prep Form for every solution your group prepares. Each student must turn in their Solution Prep Forms with their lab report.
3. You must follow the SOP for properly labeling your solution.

Percent concentrations may be expressed as:

1. **weight per volume** (wt/vol or w/v), which indicates the weight (in grams) of solute per 100 mL of solution (used to indicate the concentration of a solid in a liquid)
2. **volume per volume** (v/v), which indicates the volume (in mL) of solute per 100 mL of solution (used to indicate the concentration of a liquid dissolved in liquid)
3. **weight per weight** (w/w), which indicates the weight (in grams) of solute per 100 g solution (used to indicate the concentration of a solid mixed in another solid)

Note that in all cases a 100 mL (or 100 g) solution is used since *percent* means "out of 100".

Weight per volume is a common unit of concentration in the biotechnology lab. This is often used for small amounts of chemicals and specialized biological reagents. For example, enzyme and nucleic acid concentrations are often given as weight per volume (for example, 1 µg/mL DNA).

Molarity

Molarity is the most common unit of concentration in the biotechnology lab. The molarity of a solution is defined as the number of moles of solute per liter of solution. The symbol for molarity is M, but it can also be written as moles/Liter, or mol/L. A mole of any element always contains 6.02 X 10^{23} *(Avogadro's number)* atoms. Because some atoms are heavier than others, a mole of one element weighs a different amount than a mole of another element. *The weight of a mole of a given element is equal to its atomic weight in grams.* Consult a periodic table of elements to find the atomic weight of an element. For example, one mole of the element carbon weighs 12.0 g.

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

Practice:

Using a periodic table, calculate the molar mass of potassium sulfate (K_2SO_4).

PART A: MAKING MOLAR SOLUTIONS

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

Mass		= molarity		x	volume	x	molar mass	
?	g	=	moles/liter	x	L	x		g/mole

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

Example: To prepare 100 mL of 1 M NaOH (FW 40.0),
g = 40 g/mol x 1.0 mol/L x 0.1 L
g = 4 (dissolve 4 g of NaOH in 100 mL water)

Practice:
How many grams of NaCl would you need to prepare 0.5 L of a 0.10 M solution? The molar mass of sodium chloride is 58.44 g/mol.

In this exercise, you will prepare two solutions of given molarity.

PROPER USE OF SOLUTION PREPARATION EQUIPMENT

Using a Balance

Review chapter 19 in the Siedman & Moore textbook to review the proper use of a balance. In addition, read SOP for your specific pH meter supplied in your SOP booklet.
General guidelines to follow when using balances are as follows:

- The amount being weighed determines what type of balance is used in this laboratory (top-loading or analytical).
- Use clean spatulas to weigh out material.
- Never put excess chemicals back into their original containers, discard.
- Leave a CLEAN balance. Chemicals left on the balance will corrode it.
- Turn off the balance when not in use.
- You should also refer to the SOP for whatever balance is being used.

Operation of the pH Meter

Review chapter 19 in the Siedman & Moore textbook the proper use of a pH Meter. In addition, read SOP for your specific pH meter supplied in your SOP booklet.

The definition of pH is the negative log of the hydrogen ion (H+) concentration when concentration is expressed in moles per liter. For example, the H+ concentration of pure water is 1 x 10-7 moles/l, thus the pH = 7. 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. For more information on pH and conductivity meters and their use, please read chapter 22 and the appropriate Appendix in the Seidman & Moore textbook, Basic Laboratory Methods for Biotechnology, 2nd Ed. In addition, review the SOP for "Operation and Maintenance for a pH meter" ***prior to class.***

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. There are many different chemicals that can act as a buffer, and those chemicals can work at a wide range of pH. A sample table of common buffers, and the pH ranges they work in, can be found in your Seidman & Moore textbook, table 27.1.

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 what the clean glassware will be use for. Water source is a very important part of cleaning glassware!

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

1. **Pre-rinse**: Soak or pre-rinse all glassware after use. This will help prevent the contaminants from drying onto the glassware.
2. **Contaminant Removal**: Wash using approved detergents and/or solvents along with scrubbing and jets of water will help with contaminant removal. Typically, a lab glass detergent such as Alcon is used. As with all washing, hot water is preferable to cold water. Often lab brushes are used to help wash debris off glassware.
3. **Rinse**: The rinse step is essential in removing the detergent and cleaning solvents. Many SOP's specify that glassware be rinsed 3 to 5 times in tap water.
4. **Final Rinse**: Always use purified water to rinse away the final residue. This is usually done 3 times.
5. **Drying**: This is either done in the air on a rack or by heat. ***Never hand dry!*** Glassware should be dried upside down so no contaminants from the air fall in. Clean, dry glassware is often stored covered in closed cupboard to avoid contamination.

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

PART A: PREPARING A MOLAR SOLUTION

MATERIALS

Each group	***Class Shares***
50-mL beaker	Calcium chloride – $CaCl_2 \cdot 2H_20$
10 mL graduated cylinder	Magnesium sulfate – $MgSO_4 \cdot 7H_2O$
25-mL graduated cylinder or volumetric flask	balances with SOPs
masking tape or labeling tape	spatulas
permanent marker	weigh boats
PPE	stir plate or stirring hot plate
50 mL conical	stir bars
	gloves

PROCEDURE

***denotes a step that must be checked by instructor before continuing**

1. *Calculate the number of grams needed to make 25 mL of a 2.00 M solution of calcium chloride. Use the formula in the introduction. Show your calculations in the prelab notes section.

2. You will need to fill out a Solution Preparation Form for every solution you make in the lab. The blank forms are in the file cabinet and the instructions are in the Appendix.

3. Every solution you make in the lab will require a proper label. Refer to SOP booklet for information on labeling solutions.

4. Calibrate and use a balance to measure this amount of calcium chloride in a weigh boat or on weighing paper; leave the balance clean. Unless each group has its own stock of chemical, leave the container at the balance for other groups to use**.** If you are the last to use the chemical, close the container and return it to the cart.

5. Measure 15.0 mL of dH_20 (deionized water) with a 25-mL graduated cylinder and add it to a 50-mL beaker. Label the beaker "$CaCl_2$" with labeling tape and a Sharpie. Add the calcium chloride you weighed out in step 2. You will dissolve the solute (calcium chloride) in less than the final amount of solvent (water) because the solute may displace some water.

6. Add a stir bar and place the solution on a stir plate and stir until the entire solid has dissolved. If the solution you are making is nearly saturated, you may not be able to dissolve all of the solute in half the final volume of water as instructed. If this happens, you may want to add more water. Try not to add more than 80% of the final volume before the solute dissolves.

7. Bring the solution to its final volume by returning the solution to a 25-mL graduated cylinder and slowly and carefully add more water with a wash bottle, watching the meniscus (the rounded top surface of the water) until it reaches 25mL. The lowest part of the meniscus should line up with the 25-mL mark. This step is often abbreviated BTV (bring to volume).

The beaker used to dissolve the solution does not measure accurately enough to be used for bringing the solution to volume.

8. *Transfer the solution to a labeled, 50 mL conical. You can transfer the label from the graduated cylinder or volumetric flask if you wish. **Never put a solution into an unlabeled container, and always label legibly**. Cover the Erlenmeyer flask with parafilm. Cut a square of parafilm with scissors, center it over the mouth of the flask, and stretch and wrap the film around the mouth. Make sure there are no holes in the film.

9. *Repeat steps 1 – 6 to make 25 mL of a 2.00 M solution of magnesium sulfate.

PART B: PARALLEL DILUTIONS

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

1. Combining one part food coloring with 9 parts water dilutes the food coloring to 1/10. This means that there is one part food coloring in 10 parts total volume. The denominator an expression such as 1/10 is the total volume of the solution.

2. The food coloring dilution in part (a) can also be referred to as 1:9 food coloring to water. The colon (:) means "to." A 1:10 food coloring to water dilution would be 1/11, not 1/10, because the total number of parts is 11.

3. Frequently, stock solutions in biotechnology labs are concentrated and must be diluted before using. A buffer that is ten-fold more concentrated than the usable concentration is referred to as a 10X solution. One must dilute a 10X solution by a factor of 10 (by adding 1 part of the 10X stock to 9 parts of solvent) before using.

4. In dilutions, parts can be of any unit. If you combine 1 mL food coloring with 1mL water, you are using the same dilution factor (1:1, or ½) as the person who combines 1 ounce of food coloring with 1 ounce of water. If you combine one ounce of food coloring with one liter of water, the dilution factor is not 1:1, because the units are not the same.

5. To dilute a more concentrated stock solution to a less concentrated solution, the following formula is used:

$C_1V_1 = C_2V_2$
C_1 = original concentration (of stock solution)
C_2 = final concentration (of diluted solution)
V_1 = original volume (to be taken from stock solution)
V_2 = final volume (of diluted solution)

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

$C_1V_1 = C_2V_2$

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

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

$mL = 5.0$

The original volume (V_1) is almost always the quantity you must calculate, since you usually know the stock concentration and the final volume and concentration desired (if the final volume is not given, you should estimate how much you will need for the task at hand). It is not necessary for the volume to be measured in Liters in this formula, but the units for V_1 and V_2 must be the same.

In this part of the lab, you will first make dilutions of the calcium chloride solution you prepared in Part A. Then you will combine small amounts of these dilutions with the stock magnesium sulfate to precipitate calcium sulfate. The mass of precipitate formed in each case can be plotted on a graph, which, if linear, will show that your dilutions were accurately prepared.

MATERIALS

Each group

10 mL graduated cylinder
5 mL pipette
100 – 1000 µl micropipet (or 1 mL pipet)
microcentrifuge tubes (3)
microcentrifuge tube rack
masking tape or labeling tape
permanent marker
PPE
transfer pipettes

Class Shares

Magnesium sulfate – $MgSO_4 \cdot 7H_2O$
balances with SOPs
spatulas
weigh boats
vortex
gloves
15 mL conical

PROCEDURE: Parallel Dilutions

1. Calculate the volume of the calcium chloride stock solution made in Part A needed to make 10.0 mL of a 1.50 M solution. Show the calculations in your notebook.

2. In a 10 mL graduated cylinder, add the stock solution calculated above and BTV to 10mL with dH_2O. Transfer to a labeled disposable 15mL conical.

3. *Repeat steps 1 and 2 to make 10.0 mL of a 0.50 M solution of calcium chloride.

4. Label 3 microcentrifuge tubes 1, 2, and 3. Use the same labeling system as you did in the micropipetting lab.

5. Weigh each tube and record the empty weight in a data table in your notebook. This will be subtracted from the mass of the tube when it contains a pellet of calcium sulfate to obtain the mass of the pellet.

6. Pipette 500 µl of 2 M magnesium sulfate stock solution into each tube.

7. Add the following to the indicated tubes:
   Tube 1—500 µl of 2 M $CaCl_2$
   Tube 2—500 µl of 1.5 M $CaCl_2$
   Tube 3—500 µl of 0.5 M $CaCl_2$

8. Spin the tubes in a microcentrifuge for 5 minutes or until a solid pellet is formed which does not dislodge when the tube is turned upside down. Don't forget to balance the tubes by placing them across from each other and filling a tube with an equal amount of water to balance uneven numbers of tubes. If you are spinning your tubes with those of another group, be sure to mark them so you can distinguish your tubes from theirs. A simple dot or line will suffice, but your initials are better.

9. Discard the water by using a micropipette or transfer pipette. Do not disturb the pellet.

10. Weigh the tubes with pellets and subtract the weight of the empty tube to obtain the mass of precipitate formed. Record this information in your data table. The measure is not entirely accurate because there will be some water clinging to the pellet and tube even after most of it is poured off. Leaving the tubes upside down to dry overnight, or drying them in an oven, would produce more accurate results. This mass should be proportional to the concentration of calcium chloride. In the most dilute solution, there are fewest calcium ions to react with the sulfate ions in the other solution. Therefore, the smallest mass of insoluble calcium sulfate should be formed from this dilution.

11. Discard the stock solutions down the sink with plenty of water unless your instructor asks you to save them.

PART C: SERIAL DILUTIONS

Serial dilutions are dilutions made from dilutions. They are made for one of the following reasons:

1. a number of dilutions of the same dilution factor are desired

Example: You want to make a series of solutions with a repeating dilution factor of 2; in other words, the concentration of each diluted solution should be half that of the dilution before it (such as 2 M, 1 M, 0.5 M, 0.25 M).

2. the final concentration desired is so small that the original volume (C_1) cannot be accurately measured

Example: You want to prepare 1 mL of a 5 mM solution from a 10 M stock solution.

To find C_1, you would first convert millimolar to molar so that the concentration is the same on both sides of the equal sign. 5 mM = 0.005 M

Then you would use $C_1V_1 = C_2V_2$.

$$(10\ M)(\ ?\ mL) = (0.005\ M)(1\ mL)$$

$$?\ mL = \frac{(0.005\ M)(mL)}{10\ M}$$

$$mL = 0.0005\ mL \text{ (or } 0.5\ \mu l)$$

There are micropipettes that will measure 0.5 µl, but they are not available in every lab. Moreover, this small a volume is hard to measure accurately, since it is far smaller than a single drop of liquid. It is far more accurate to make a series of 1/100 or even 1/10 dilutions from a highly concentrated stock.

For serial dilutions:

$$\text{Dilution factor} = (V_1 + V_2) / V_1$$

Where V_1 is the volume of the solution being diluted

V_2 is the volume of solvent used to dilute the solution

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

Example: You want to do a series of 5-fold dilutions, each with an end volume of 40 mL.

$$\text{Dilution factor} = (V_1 + V_2) / V_1$$

$$5 = (V_1 + 40\ mL) / V_1$$

$$5V_1 = (V_1 + 40\ mL)$$

$$4V_1 = 40\ mL$$

$$V_1 = 10\ mL$$

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. Thus, you always end up with V_2 mL in each dilution but the very last one.

In this part of the lab, you will serially dilute a hydrochloric acid solution and check the accuracy of your preparations with pH measurements.

MATERIALS

Each group
Four 100-mL beakers
10- and 50-mL graduated cylinders
5- and 10-mL pipettes
100 – 1000 µl micropipette
pipette bulb or filler
glass stirring rod
labeling tape
permanent marker
goggles

Class Shares
1.00 M HCl
pH meters with SOPs
pH standard buffers and three 50-mL beakers
250-mL waste beaker
gloves

PROCEDURE: Serial Dilutions

1. Pour 20 mL of a 1 M solution of hydrochloric acid (HCl) into a labeled 50-mL beaker. Always wear goggles and gloves when working with acids. You may reuse beakers from Part A, but be sure to clean them thoroughly with soap solution rather than just rinsing.

2. *Calculate the volumes of acid and water needed to prepare 50 mL each of a 0.1 M solution, a 0.01 M solution and a 0.001 M solution through serial dilution. Show the calculations in your notebook.

3. Label 3 small beakers with the concentrations from step 2 and add the correct amount of water to each one, using the appropriate measuring device. Remember the AAA rule (always add acid) when working with acids. Never add water to acid, always add acid to water.

4. Add the correct amount of acid to each beaker and stir with a stir bar on a stir plate.

5. Measure the pH of each solution using the pH meter. Before you use the pH meter, calibrate it using the buffers and SOP provided.

6. **Pour your acid solutions into a properly labeled waste container.** Clean up your work area and the area around the pH meters.

Lab Unit 4 – Solutions Assignment

1. In part C, the last dilution made was the 0.001 M solution. How many mM is this? _____

2. If you had made this solution from the 1 M stock instead of the 0.01 M, how much stock would you have had to use?

3. Describe how to make 100 mL each of 0.4 M, 0.2 M and 0.1 M solution from a stock of 0.8 M. Would you use parallel or serial dilution for this?

4. Describe how to make 20 mL each of 1.0 M, 400 mM and 100 mM from a 2 M stock solution. Would you use parallel or serial dilution for this?

5. Draw a graph to compare molar concentration of $CaCl_2$ to amount of precipitate produced as shown by the weight of your pellet. Refer to the Appendix for graphing guidelines and how to use MS Excel to generate graphs. Draw a best-fit line and calculate the slope of the line.

6. Generate a double-log graph to compare molar concentration of HCl to pH. The log grid is on the Y-axis (pH) and the linear grid is on the X –axis (1/10 dilutions).

LAB UNIT 5: TOTAL RNA ISOLATION FROM ALFALFA SPROUTS

OBJECTIVES

Your performance will be satisfactory when you are able to:

- Understand basic structure and function of RNA
- Investigate the central role of RNA in gene expression and regulation
- Appreciate the importance of RNA in research and in the biotechnology and diagnostic/therapeutic industries
- Demonstrate safe and proper lab skills including preparation, careful attention to detail, record keeping, and teamwork
- Demonstrate safe and proper use of lab equipment including micropipettors, microcentrifuge, and electrophoresis equipment
- Isolate RNA from fresh alfalfa sprouts using Ambion's RNAqueous kit
- Analyze RNA samples prepared in the lab by comparing their appearance in a denaturing agarose gel to known RNA samples from different species and to molecular size markers

INTRODUCTION

Importance of RNA

RNA provides the link between the genetic information stored in DNA and the expression of that information through protein synthesis. It is the central piece of the central dogma. The central dogma is often written as DNA $\Rightarrow$ RNA $\Rightarrow$ protein and represents the flow of genetic information in cells. All cells store their genetic information as a sequence of nucleotides in long double strands of DNA. RNA molecules serve as intermediates to transport, regulate and translate the information from a sequence of DNA nucleotides to a sequence of amino acids. The chains of amino acids formed by the process of translation are proteins, and they are not only the building blocks of cells and organisms, but are also the regulators of almost every cellular and bodily function. In the narrowest sense, the segment of DNA that codes for a specific protein is called a gene.

Think of all the different types of cells in your body; long, branching nerve cells, bone cells in their hard matrix, muscle cells that can contract to cause movement, and many other cell types. Think of all the ways your cells have changed from the time before you were born to the present. You might not expect all these different types of cells, with all their different needs, in all their stages of development, to carry the same genetic information, but they do. All your cells have the same DNA and the same genes. The reason that cells can differentiate into various types of cells with wide ranging functions is that they can regulate the expression of their genes. Only the genes that are required by a particular cell, at a particular time, are transcribed into RNA and translated into functional proteins.

In theory, gene expression can be controlled at any stage in the path from DNA to RNA to protein. For example, there are stretches of regulatory DNA (e.g. promoters and enhancers) that act as switches to control the expression of nearby genes. The presence or absence of a

particular protein(s) will turn the switch on or off. RNA also regulates gene expression in many ways. It can be modified in ways that affect its ability to make proteins. It can be spliced, edited, or have parts added to it. Its transport and localization in the cell can be controlled. Even the rate of RNA formation and degradation can be regulated. These RNA control processes generally require regulatory proteins and/or regulatory RNA molecules, and they can be very simple or very complex.

Basic Structure and Function of RNA

All of the RNA from a cell is referred to as "total RNA". Total RNA mainly contains three types of specialized RNA molecules. They are messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). Each of these RNA types has a unique structure related to its function.

Messenger RNA is copied or transcribed as a single strand from a portion of DNA that represents a gene. It is essentially a copy of that gene, containing the same nucleotide sequence as the DNA, except that ribonucleotides (rNTPs) are used instead of deoxyribonucleotides (dNTPs). The mRNA then carries the genetic information for that particular gene to the ribosomes where the information will be translated into protein. The genetic code is made up of sets of three nucleotides called codons. Each codon corresponds to an amino acid, or to a translation start or stop signal. Messenger RNA is often modified greatly during or after transcription, especially in eukaryotic cells (e.g. splicing, capping, polyadenylation). The size of mRNA will vary depending on the size of the gene and on any modifications to the RNA that take place after transcription.

Transfer RNA is also involved in protein synthesis in the ribosomes. It takes the genetic message from mRNA (the sequence of codons) and translates it into the corresponding amino acid sequence. It is a small (65-110 nucleotides), highly structured molecule with a set of three nucleotides on one end that represent an anticodon. Each tRNA anticodon is complementary to an mRNA codon. The other end of tRNA molecules is designed to carry the amino acid that corresponds to its anticodon. A good animation of the process of translation can be found at:

www.bioweb.uwlax.edu/GenWeb/Molecular/Theory/Translation/translation.htm

Ribosomes are small organelles made of proteins and rRNA. The ribosomes are complex molecular machines that catalyze the creation of a polypeptide chain on a mRNA template, using tRNAs and various protein co-factors. The proteins they produce then carry out the actual expression of the genetic trait (the phenotype). The ribosomes of both eukaryotic and prokaryotic cells consist of two subunits. The rRNA components of these two subunits vary in number and size from species to species and thus migrate at different speeds in a denaturing agarose gel.

Table I. rNRA Sizes in Various Species

Species	rRNA	Size (kb)
Human	18S	1.9
	28S	5.0
Mouse	18S	1.9
	28S	4.7
*Drosophila melanogaster**	18S	2.2
	28S	2.8
Tobacco Leaf	16S	1.5
	18S	1.9
	23S	2.9
	25S	3.7
Yeast	18S	2.0
	26S	3.8
E. coli	16S	1.5
	23S	2.9

** D. melanogaster RNA bands tend to run at approximately 1.7 and 2.0 kb instead of at their true sizes in a denaturing gel. This is because the 28S component is split in two by the denaturing process. One of the pieces runs with the 18S component (they form one band), and the other runs by itself at a slightly faster rate.*

Applications of RNA Technology

Isolation of total RNA is typically a preliminary step in many research settings. Since each mRNA molecule carries the information from a gene, the mRNA component of total RNA is often the focus of studies involving RNA. Several techniques are commonly used to analyze mRNA. The earliest technique, which is still widely used today, is Northern blotting. It consists of separating mRNA on a denaturing agarose gel and transferring it to a membrane. Specific sequences can then be hybridized to radioactive or fluorescent probes to reveal the presence and size of specific mRNAs. A technique called reverse transcription (RT) is used to copy mRNA into DNA; DNA produced this way is known as complementary DNA (cDNA). cDNA represents all of the genes actively expressed in the sample being studied. cDNA can be amplified by Polymerase Chain Reaction (PCR) or it can be cloned into cells to create a "library" of expressed genes. This procedure is used to discover genes. Alternatively, cDNA can be labeled with fluorescent or radioactive tags, and then hybridized to an array of thousands of gene-specific DNA sequences that have been immobilized in a grid pattern (a DNA microarray). Comparison of the hybridization patterns of labeled cDNA probes from different RNA sources (for example drug-treated vs. normal tissue) can reveal which genes are expressed differently in the two samples.

Imagine the possibilities when RNA analysis techniques are applied to humans. Just as genetic screening is used to search for abnormal gene sequences, RNA analysis allows researchers to look for abnormal gene expression. These techniques are the foundation for understanding gene expression and its regulation, and lead the way towards novel diagnostic and therapeutic technologies.

Isolation of RNA: Precautions and Expected Results

The chemical structure of RNA is very similar to that of DNA, but the minor differences between the two results in a great difference in their behavior. RNA has an extra –OH group in the ring of its ribose sugar that makes it more sensitive to base catalysis (abstraction of hydroxyl proton). Because of this, RNA is less stable and more easily degraded than DNA. Also, because RNA is not double-stranded like DNA, its nucleotide bases are more exposed than in DNA.

RNase enzymes, which degrade RNA, are very common in the environment, so special care must be taken to avoid them when working with RNA. Precautions include using fresh laboratory gloves to protect the RNA from nucleases present on the skin and using RNase-free reagents. RNase*Zap*, an RNase decontamination solution, is ideal for cleaning work surfaces, micropipettes, and equipment. RNases are also found in living cells, so when working with biological samples it is important to inactivate RNases before they have a chance to degrade the RNA in the sample. To do this, samples should be either disrupted rapidly and thoroughly in a lysis solution especially formulated for RNA isolation, or they should be frozen or collected in RNA*later*, a RNA stabilization solution, as quickly as possible after they are obtained from the source.

The vast majority (~85%) of RNA in the cell is ribosomal RNA, and this is what can be seen on a gel after size separation by denaturing gel electrophoresis. The main rRNA components of the two ribosomal subunits form the bands that are usually predominant in the gel. mRNA is present, but it appears as a hazy background smear because of its variable size (0.5-10 kb) and its relatively small representation (~2%) in the total RNA. tRNA and other small RNA molecules makes up 10-15% of total RNA but are not efficiently resolved on denaturing agarose gels.

Intact total RNA run on a denaturing gel will have sharp, clear rRNA bands (e.g. 28S and 18S in mouse and rat). Although they are present in equimolar amounts, the 28S rRNA band is twice the size of the 18S rRNA band. Thus it will be approximately twice as intense as the 18S rRNA band when stained with ethidium bromide. This 2:1 ratio (28S:18S) is a good indication that the RNA sample is completely intact. Partially degraded RNA will have a smeared appearance, will lack the sharp rRNA bands, or will not exhibit the 2:1 ratio of high quality RNA. Completely degraded RNA will appear as a very low molecular weight smear. Inclusion of RNA size markers on the gel makes it possible to determine the size of any bands or smears to be determined and will also serve as a good control to ensure the gel was run properly.

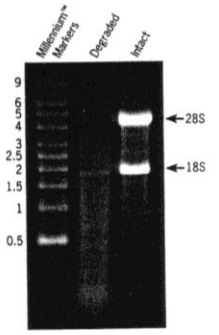


Figure 1. Intact vs. Degraded RNA.
Examples of intact and degraded RNA are shown here. Can you determine what species this RNA came from by using the table of rRNA sizes?

Reference: Ambion, Inc. http://www.ambion.com/

Extract and Purify RNA

The first step in RNA extraction is to break down cells or tissue so that the nucleic acids are released from the cells. We will use a Lysis/Binding solution that chemically disrupts cells without destroying their nucleic acids. The Lysis/Binding solution contains a detergent, a chaotrope, and a reductant. The detergent breaks down the hydrophobic membranes that

surround cells and some cellular organelles. Its action is similar to that of dishwashing detergent on greasy and oily foods. The chaotrope causes "chaos" in the cell by unfolding and deactivating proteins and other biomolecules. The reductant binds to and inactivates RNases, thus preserving RNA during the tissue disruption process. The cellular breakdown is aided by grinding or homogenizing the tissue with a small pestle that fits in a microcentrifuge tube.

The next step involves the addition of ethanol to the lysate. Nucleic acids (both DNA and RNA) are insoluble in 100% ethanol. At a concentration of 64% ethanol, RNA is most soluble and the diluted ethanol is used to maximize the ratio of RNA to DNA that will bind to the filter.

The final step in the procedure purifies the RNA away from all of the other components of the cell. This protocol uses small filter made of glass fibers to adsorb RNA molecules. The lysate/ethanol mixture is passed through the filter by centrifugation. The filter is washed with different solutions that remove unwanted components of the cell lysate from the filter. Finally, the purified RNA is eluted from the filter using a buffer solution (Elution Solution) in which the RNA is soluble. The elution solution also contains ingredients to protect and preserve the purified RNA in solution.

Agarose Gel Electrophoresis

One of the most powerful techniques for separating biomolecules is gel electrophoresis. Any separation technique that involves the migration, under the influence of an electric field, of a charged particle through a matrix is considered electrophoresis. Charged particles are attracted towards the electric pole of opposite charge. In gel electrophoresis, the matrix is a solid gel, which controls the rate of migration of particles. The rate of migration is decreases with increasing size of particles and concentration of the gel. In other words, if a particle is large and the concentration of the gel is high, its migration will be slow. Thus, one can separate uniformly charged molecules on the basis of size. RNA comes in many different three-dimensional shapes caused by intramolecular bonding interaction. Some shapes may have an advantage in moving through the agarose obstacle course. In order to accurately compare RNA sized in the gel, these intramolecular bonds must be broken (or denatured) so that the RNA takes on a random coil shape. This allows us to compare the sizes more accurately, because all the molecules now have the same shape. There are many denaturing agents, but the most common for RNA are formaldehyde and glyoxal.

The gel used to separate large molecules of nucleic acids is agarose, a complex polysaccharide isolated from seaweed. A buffer, usually consisting of an aqueous solution of organic and inorganic salts, conducts the electrical current between the positive and negative terminals.

Stains, such as methylene blue, ethidium bromide and SYBR green can be added to visualize the bands of DNA or RNA that form on the gel after electrophoresis. In this experiment, we will use SYBR green II, a stain that causes the RNA bands to fluoresce when excited with ultraviolet light.

References:

1. Adapted from Ambion's RNAqueous RNA isolation kit product literature. http://www.ambion.com/

Lab Unit 5-A: Total RNA Isolation

MATERIALS

RNaseZap
RNAqueous kit, including:
- Lysis/binding solution
- 64% ethanol
- Wash solution #1
- Wash solution #2/3
- Filter cartridges and collection tubes
- Elution solution
- Glyoxal loading dye

Plant RNA Isolation Aid (Ambion)
Alfalfa sprouts

Heat block set at 70-80°C
Ice bath
100% ethanol
RNase-free microcentrifuge with pestle
RNase-free microcentrifuge tubes
Microcentrifuge
Micropipettors and RNase-free filter tips

NOTES:
- Use RNase Zap to deactivate RNases on your lab bench, gloves, tube racks, micropipettors, and other materials.
- RNA is unstable and degrades rapidly. Work quickly.

PROCEDURE

1. Add 80 µL of Elution solution to a microcentrifuge tube and place in a heat block set at 70-80°C (to be used in a later step).

2. Obtain a sample of sprouts and determine its mass (should be approximately 20 mg).

3. Add 10-12 volumes (or at least 200 µL) of Lysis/Binding solution to the sample. For example, if the sprout sample weighs 20 mg, then use 240 µL of the solution.

4. Add a volume (in uL) of Plant RNA Isolation Aid equal to the number of milligrams of your sample. For example, if your sample weighs 20 mg, then use 20 uL of Isolation Aid.

5. Homogenize by grinding the microfuge pestle into the sample tube using a twisting motion. Process until very little particulate matter is visible.

6. Remove and discard the pestle and close the tube. Centrifuge for 5 minutes at top speed (at least 10,000 x g) to pellet cellular debris.

7. Carefully pipette the supernatant out of the tube, being sure not to pipette any solids out. Dispense the supernatant into a fresh tube and discard the pellet.

8. Add an equal volume of 64% ethanol to the lysate and mix gently by inverting several times.

9. Apply the mixture from the previous step to a filter cartridge inside a collection tube. Do not apply more than 700 µL to the filter.

10. Centrifuge at RCF 10,000 x g for one minute. Discard the flow-through but reserve the collection tube for the washing steps.

11. Apply 700 µL Wash solution #1 to the filter cartridge. Centrifuge at RCF 10,000 x g for one minute. Discard the flow-through but reserve the collection tube.

12. Apply 500 µL Wash solution #2/3 to the filter cartridge. Centrifuge at RCF 10,000 x g for one minute. Discard the flow-through but reserve the collection tube.

13. Repeat with a second 500 µL aliquot of Wash solution #2/3.

14. After discarding the flow-through, centrifuge for 10-30 seconds to remove the last traces of wash solution.

15. Place the filter cartridge into a fresh collection tube. Apply half of the preheated Elution solution (40 µL) to the center of the filter and close the cap of the tube.

16. Centifuge at RCF 10,000 x g for 30 seconds at room temperature.

17. Add another 20 µL of Elution solution and repeat the previous centrifugation step in the same tube.

Lab Unit 5-B: Denaturing Gel Electrophoresis of Total RNA

MATERIALS
Heat block set at 70-80°C
Ice bath
100% ethanol
RNase-free microcentrifuge with pestle
RNase-free microcentrifuge tubes
Microcentrifuge
Micropipettors and RNAse-free filter tips

DEPC-treated water
Horizontal electrophoresis chamber
Gel casting trays, bumpers and combs
Agarose
NorthernMax-Gly gel prep-running buffer, 10X
SYBR Green II stain
Total RNA Control
RNA size marker

NOTES:
- Use RNAse Zap to deactivate RNAses on your lab bench, gloves, tube racks, micropipettors, and other materials.
- RNA is unstable and degrades rapidly. Work quickly.

PROCEDURE
1. Prepare 1.0% agarose gels in 1X NorthernMax-Gly gel prep-running buffer. Dilute the buffer in DEPC-treated water to reduce RNases in the solution. Cool to 50°C.

2. Pour the agarose solution into a 7cmx7cm casting tray containing a comb with 25 µL wells (or larger). Allow to set at room temperature for 20 minutes.

3. Add 20 µL of RNA prep into a microcentrifuge tube and add glyoxal loading dye to a concentration of 1X. Pipette up and down to mix and pulse spin to collect liquids in the bottom of the tube.

4. Heat all samples, standards, and markers at 65-70°C for 5 minutes in a heat block. If heating in a water bath, make sure the tube caps stay above water in a floating tube holder to avoid contamination.

5. Remove comb and bumpers from the gel and place in the electrophoresis tank. Add 1X MOPS buffer to cover the gel by a few millimeters.

6. Load prepared RNA samples into the gel, noting each sample's location. Each gel must also contain at least one lane with Millenium markers and one lane with a RNA control (standard solution or previously analyzed sample).

7. Place the cover on the chamber in the correct orientation and connect leads to a power supply. Set the power supply at 125 volts and allow to run for about 25 minutes, or until the bromophenol blue tracking dye is about 2/3 of the way down the gels. Watch the gels carefully after about 10 minutes to ensure that it does not run too long.

8. Visualize RNA bands by placing gels on a UV transilluminator. Photograph gels using the gel documentation system.

9. Heat 1.0 g agarose in 72 mL DEPC-treated water until dissolved, then cool to 60°C.

10. Add 10 mL 10X MOPS running buffer and 18 mL 37% formaldehyde.

11. Pour the agarose solution into a 7cmx7cm casting tray containing a comb with 25 µL wells (or larger). Allow to set at room temperature for 20 minutes.

12. Add 20 µL of RNA prep into a microcentrifuge tube and add formaldehyde loading dye (containing 10 µg/mL ethidium bromide) to a concentration of 0.5X. Pipette up and down to mix and pulse spin to collect liquids in the bottom of the tube.

13. Heat all samples, standards, and markers at 65-70°C for 5 minutes in a heat block. If heating in a water bath, make sure the tube caps stay above water in a floating tube holder to avoid contamination.

14. Remove comb and bumpers from the gel and place in the electrophoresis tank. Add 1X MOPS buffer to cover the gel by a few millimeters.

15. Load prepared RNA samples into the gel, noting each sample's location. Each gel must also contain at least one lane with Millenium markers and one lane with a RNA control (standard solution or previously analyzed sample).

16. Place the cover on the chamber in the correct orientation and connect leads to a power supply. Set the power supply at 125 volts and allow to run for about 25 minutes, or until the bromophenol blue tracking dye is about 2/3 of the way down the gels. Watch the gels carefully after about 10 minutes to ensure that it does not run too long.

17. Visualize RNA bands by placing gels on a UV transilluminator. Photograph gels using the gel documentation system.

Lab Unit 5 - RNA Isolation Assignment

1. Describe at least two things that you did to improve your accuracy or efficiency in the lab. You may include things that you did to prepare for the lab, how you set up or handled your materials, or anything else that you can think of.

2. What is the function of the Lysis Solution?

3. What effect does ethanol have on nucleic acids? How is this helpful when we are trying to isolate them?

4. Include in your lab report you gel documentation form with a ***labeled*** picture of your gel attached. Describe your results. Your description should include how many bands you see for each sample, what are the bands you see, the relative intensity of the bands (which ones were darker or lighter), and the relative sizes of the RNA bands (using marker). Is this what you expect?

5. In this lab, you learned that RNA is a much more sensitive to degradation than DNA. What is the chemical basis for this? Why does it make sense for cells to have very stable DNA and less stable RNA?

LAB UNIT 6: TRANSFORMATION OF *E. coli* WITH A RECOMBINANT PLASMID

(adapted from Bio-Rad's pGLO Transformation Kit)

OBJECTIVES

Your performance will be satisfactory when you are able to:

- use sterile technique
- make *E. coli* cells competent for transformation
- transform *E. coli* with plasmid DNA
- select for recombinant clones on antibiotic selection plates
- analyze and troubleshoot the results of a transformation experiment

INTRODUCTION

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

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

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

The plasmids used to transform bacterial cells also have a **selectable marker** gene. This gene codes for a protein that allows the scientist to distinguish cells that have been successfully transformed by plasmid DNA from those that have not. The most common selectable markers are antibiotic resistance genes, which allow for selection of transformants by growth on media

containing the antibiotic. Non-transformed cells will die and transformed cells will survive under these conditions. In this lab exercise, a plasmid with an antibiotic resistance gene for ampicillin (amp) is used; the ampicillin resistance gene codes for an enzyme that destroys the ampicillin in the surrounding growth media.

Bioluminescence by Green Fluorescent Protein (GFP)

Bioluminescence due to microorganisms can be observed walking on the beach at night, when flashes of light originating from glowing plankton can be seen in the waves. Bioluminescent molecules are also found in some jellyfish (such as *A. victoria*) that have a specialized photogenic cell located in the base of the jellyfish umbrella. A bioluminescent protein called **green fluorescent protein** (GFP) found in the cell causes the luminescence. This protein does not require substrates, other gene products, or cofactors to produce light. When exposed to ultraviolet light, GFP will emit a bright green light.

GFP contains 238 amino acids and has a molecular weight of approximately 40,000 daltons. The chromophore, the section of the protein that actually fluoresces, is part of the primary structure of the protein. It is a tripeptide occupying positions 65 to 67 in the protein sequence, is cyclic, and is composed of the amino acids serine, tyrosine, and glycine. The importance of protein folding is clearly demonstrated with GFP, since the protein is fluorescent only when in its natural folding conformation.

Making bacterial cells competent for uptake of DNA

The efficiency of transformation can be improved by carefully managing conditions before and during the transformation. For example, the choice of bacteria and plasmid can affect the efficiency of transformation, because many plasmids have a narrow host cell range and will only transform bacterial cells of a single species. Transformation frequencies are considerably higher when using fresh bacterial cells taken from actively growing cultures. In addition, bacterial cells can be made **competent** for DNA uptake by pretreatment with chloride salts of divalent cations such as calcium, followed by a cold-shock and a heat-shock step. The metal ions and temperature changes affect the structure and permeability of the cell wall and cell membranes such that DNA molecules are able to pass through. Cells that are allowed to recover in non-selective growth media and at their optimal growth temperature following transformation also have higher transformation frequencies. The recovery time allows the transformed cell to amplify the plasmids and to express the antibiotic resistance gene required for survival on the antibiotic-containing selection medium.

LABORATORY SAFETY

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

1. Gloves and goggles should be worn at all times.
2. Wipe down the lab bench with 10% bleach before starting the lab and before leaving the laboratory.
3. All materials, including plates, pipettes, loops and tubes that come in contact with bacteria should be autoclaved or disinfected with bleach before disposal in the garbage.
4. Wash hands thoroughly with soap and water after removing gloves.

MATERIALS

Each group:
Micropipettors and sterile tips
Sterile microcentrifuge tubes
Microcentrifuge tube racks
Floating microcentrifuge racks
Gloves
Sterile inoculating loops
Ice water bath
1 LB petri plate
2 LB/amp petri plates
1 LB/amp/ara plate

Class shares:
37˚ C incubator
42˚ C water bath
Hot gloves
Long wave UV light source
pGLO plasmid solution
LB broth
Sterile 10 mM $CaCl_2$ in distilled water (on ice)
10% bleach in a bucket or beaker, or autoclave
10% bleach in a wash bottle
Biohazard bags and stands
Overnight culture of *E. coli*, grown at 34^{o}C on LB plates

PROCEDURE

Part I: Transformation of *E. coli*

1. Label one closed microcentrifuge tube "+pGLO" and another "–pGLO". Label both tubes with your group's name. Place them in a tube rack.

2. Open the tubes and add 250 µL of transformation solution ($CaCl_2$). Place the tubes on ice.

3. Use a sterile loop to pick up a single colony of bacteria from the starter plate. Pick up the +pGLO tube and immerse the loop into the transformation solution at the bottom of the tube. Spin the loop between your index finger and thumb until the entire colony is dispersed in the transformation solution. Place the tube back in the tube rack in the ice. Using a new sterile loop, repeat for the –pGLO tube.

4. Examine the pGLO plasmid DNA solution with the UV lamp. Note your observations. Immerse a new sterile loop into the plasmid DNA stock tube. Withdraw a loopful. There should be a film of plasmid solution across the ring. This is similar to seeing a soapy

film across a ring for blowing soap bubbles. Mix the loopful into the cell suspension of the +pGLO tube. Close the tube and return it to the rack on ice. Also close the –pGLO tube. Do not add plasmid DNA to the –pGLO tube because it is a negative control tube.

5. Incubate the tubes on ice for 10 minutes.

6. While the tubes are sitting on ice, label your four agar plates on the edge of the bottom (not the lid) as follows:
   - Label one LB/amp plate: +pGLO
   - Label the LB/amp/ara plate: +pGLO
   - Label the other LB/amp plate: -pGLO
   - Label the LB plate: -pGLO

7. Heat shock the sample by transferring both the +pGLO and –pGLO tubes into the 42°C water bath for exactly 50 seconds. Make sure the tubes make contact with the warm water. After 50 seconds, place both tubes back on ice. For the best transformation results, the change from the ice (0°C) to 42°C and then back to the ice must be rapid. Incubate tubes on ice for 2 minutes.

8. Remove the rack containing the tubes from the ice and place on the bench top. Open a tube and, using a new sterile pipet, add 250 µL of LB nutrient broth to the tube, pipet up and down to mix, and reclose the tube. Repeat with the second tube, using a new pipet tip. Incubate the tubes for 10 minutes at room temperature.

9. Tap the closed tubes with your finger to mix. Using a new sterile pipet for each tube, pipet 100 µL of the transformation and control suspensions onto the appropriate plates.

10. Use a new sterile loop for each plate. Spread the suspensions evenly around the surface of the agar by quickly skating the flat surface of a new sterile loop back and forth across the plate surface.

11. Stack up your plates and tape them together. Put your group name and class period on the bottom of the stack and place the stack upside down on the 37°C incubator overnight. The plates should be removed after approximately 24 hours and refrigerated until the next class period.

Part II: Analysis of transformants

1. Count the number of colonies on each plate. A convenient method to keep track of counted colonies is to mark the colony with a marking pen on the outside of the plate as you count it. Observe the plates with the UV lamp and record results in a table in the attached worksheet.

2. **Keep plates from this experiment for your next two labs!** Tape them together in a stack, label them and store them at 4^{o}C as directed by your instructor.

3. **Calculating Transformation Efficiency:**

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

You are about to calculate the transformation efficiency, which gives you an indication of how effective you were in getting DNA molecules into bacterial cells. Transformation efficiency is a number. It represents the total number of bacterial cells that express the green protein, divided by the amount of DNA used in the experiment. (It tells us the total number of bacterial cells transformed by one microgram of DNA.) The transformation efficiency is calculated using the following formula:

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

Therefore, before you can calculate the efficiency of your transformation, you will need two pieces of information:

1. The total number of green fluorescent colonies growing on your LB/amp/ara plate.
2. The total amount of pGLO plasmid DNA in the bacterial cells spread on the LB/amp/ara plate.

Transformation Efficiency Worksheet:

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

b. Total amount of pGLO plasmid DNA in the bacterial cells on the LB/amp/ara plate:

Check stock of pGLO plasmid. It's concentration is:
_____ ug/ul (0.08ug/ul)

Volume of pGLO plasmid used _____ ul

Multiply the concentration of pGLO plasmid by the amount transferred onto the LB/amp/ara plate: _____ug of pGLO

c. Total volume in the transformation solution:

CaCl2: _____ ul
pGLO plasmid: _____ ul
LB broth: _____ ul
Total Volume: _____ ul

d. Volume spread on the plate: _____ ul

e. Calculate the fraction of the total transformation solution used:

Volume spread on LB/amp/ara plate ____ = _____
Volume of total transformation solution

f. Calculate the mass of pGLO plasmid on the plate:

Fraction of total transformation x total pGLO DNA in solution
_____ x _____ ug = _____ ug

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

Number of colonies transformed = _____ transformants/ug
ug of DNA on plate

NOTE: Report transformation efficiency in scientific notation

References:

1. pGLO Bacterial Transformation Kit (Cat#166-0003EDU) Manual. 4006097 Rev E

Lab Unit 6 - Transformation of *E. coli* with pGLO Plasmid Assignment

1. Exogenous DNA does not passively enter *E. coli* cells that are not competent. What treatment do cells require to be competent?

2. The recovery broth used in this experiment does not contain ampicillin. Why?

3. Fill out the following table with observations from your experiment: Show your calculations.

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

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

5. Were there any differences between the two +pGLO plates? If so, how can you account for these differences?

6. Were there any colonies on the –pGLO on LB/amp plates? If there were any colonies, how would this change your interpretation of the results that you found on the +pGLO plates?

7. Were there any colonies on the –pGLO on LB plate? If there were no colonies, how would this change the way that you interpret the results that you found on the +pGLO plates?

LAB UNIT 7: PLASMID ISOLATION

(Adapted from Bio-Rad's Aurum Plasmid Miniprep Kit)

OBJECTIVES

Your performance will be satisfactory when you are able to:

- understand the structure and function of plasmid DNA
- demonstrate safe and proper lab skills including preparation, careful attention to detail, record keeping, and teamwork
- demonstrate safe and proper use of lab equipment including micropipettors, microcentrifuge, and electrophoresis equipment
- demonstrate knowledge of DNA isolation techniques from live organisms
- make an agarose gel of a specified concentration
- prepare and load samples onto an agarose gel
- analyze electrophoretic results

INTRODUCTION

The original alkaline lysis method for purifying plasmid DNA from bacterial cultures requires organic reagents and time-consuming steps to obtain high quality DNA. The Aurum plasmid miniprep kit has been optimized for the rapid purification of high-quality, high-yield plasmid DNA. This kit uses the silicon dioxide exoskeleton of diatoms as the DNA binding matrix. This matrix carries a partially positive charge and therefore binds negatively charged nucleic acids. The advantages of this porous substrate include ease of resuspension, high affinity for DNA, simple and efficient processing, elution in an aqueous buffer, and an inherently large surface-to-volume ratio. All of these properties contribute to the highest purity and yields of DNA. Plasmid DNA purified with the Aurum plasmid miniprep kit can be used directly for fluorescent sequencing, cell transfection, electroporation, and enzymatic restriction and modification.

Agarose Gel Electrophoresis

One of the most powerful techniques for separating biomolecules is gel electrophoresis. Any separation technique that involves the migration, under the influence of an electric field, of a charged particle through a matrix is considered electrophoresis. Charged particles are attracted towards the electric pole of opposite charge. In gel electrophoresis, the matrix is a solid gel, which controls the rate of migration of particles. The rate of migration is decreases with increasing size of particles and concentration of the gel. In other words, if a particle is large and the concentration of the gel is high, its migration will be slow. Thus, one can separate uniformly charged molecules (such as DNA) on the basis of size. The gel used to separate large molecules of nucleic acids is agarose, a complex polysaccharide isolated from seaweed. A buffer, usually consisting of an aqueous solution of organic and inorganic salts, conducts the electrical current between the positive and negative terminals. Stains, such as methylene blue, can be added to visualize the bands of DNA that form on the gel after electrophoresis. In this experiment, we will use SYBR Safe, a stain that causes the DNA bands to fluoresce when excited with ultraviolet light. A successful plasmid preparation should appear as a dense band close to the well where it was loaded; a plasmid will not migrate far in a gel due to its large size.

PROTOCOL

Part I: Isolating Plasmid DNA

Day 1: Overnight cultures
This may have been done already for you by the lab technician or instructor.

1. Obtain a plate of *E. coli* pGLO transformants and a labeled tube containing 3 mL Luria broth (LB).

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

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

4. Place in a 37°C, vigorously shaking incubator overnight.

Day 2: Plasmid Isolation & Agarose Gel Analysis of DNA

Part I: Agarose Gel

1. Prepare a 0.8% agarose gel with SYBR safe. Ensure your gel casting apparatus is on a flat surface that will be undisturbed while it solidifies. **See Appendix for instructions on preparing agarose gels.**

Part II: Isolation of plasmid DNA

1. Transfer up to 12 OD•mL of liquid culture to a labeled microcentrifuge tube. Pellet the cells by centrifuging for one minute at 12,000 rpm. Pour off all of the supernatant.

   Note: Since pGLO is a low copy plasmid, it is important to ensure you are using enough bacteria to obtain a reasonable yield of plasmid DNA to perform the restriction digest. 12 OD•mL is recommended. If you have too few bacteria, your plasmid yield will be low, if you have too many bacteria, your lysis will be inefficient and that too will result in a low yield of plasmid. To calculate the OD•mL, measure the optical density of the bacterial culture in a spectrophotometer, set to 600nm (OD_{600}), and multiply the culture volume in mL. For example, if your OD_{600} is 6, to obtain a 12 OD•mL you would need 2 mL of culture.

   Recommended: If the pellet size from step 1 is too small, repeat the centrifugation procedure in the same microcentrifuge tube with the remaining liquid culture.

2. Add 250 µL of the Resuspension Solution and vortex until the cell pellet is completely resuspended.

3. Add 250 µL of the Lysis Solution and mix by GENTLY inverting the capped tube 6-8 times. The solution should become viscous and slightly clear if the cell lysis has occurred.

4. Add 350 μL of the Neutralization Solution and mix by GENTLY inverting the capped tube 6-8 times. A visible precipitate (consisting of cellular debris) should form.

5. Pellet the cell debris for five minutes at 12,000 rpm in a microcentrifuge. A compact white pellet will form along the side or bottom of the tube. The clear supernatant in this step contains the plasmid DNA.

6. Insert a plasmid mini column into one of the 2 ml capless wash tubes supplied. Transfer the supernatant from step 5 to the column. Centrifuge at 12,000 rpm for one minute. The purpose of this step is to bind the plasmid DNA to the column.

7. Remove the spin column from the wash tube, discard the filtrate at the bottom of the wash tube, and replace the column in the same wash tube. Add 750 μL of Wash Buffer and centrifuge at 12,000 rpm for one minute. The wash buffer contains ethanol and washes away impurities from your sample.

8. Remove the spin column from the wash tube, discard the filtrate at the bottom of the wash tube and replace the column in the same wash tube. Centrifuge for an additional minute. It is important to spin twice in order to remove residual traces of ethanol. This residual ethanol will cause your samples to float out of the wells of an agarose gel and may hinder enzyme activity in future experiments.

9. Remove the spin column and discard the wash tube. Place the column in a clean wash tube. Add 50 μL of elution solution. Be sure you are covering the membrane with the solution. Let sit at room temperature for one minute to saturate the membranes on the column. Elute the plasmid DNA from the membrane by centrifuging at 12,000 rpm for one minute.

10. Discard the spin column and determine the concentration of your eluted DNA on the NanoDrop.
    a. Using the SOP provided in your SOP booklet, blank the NanoDrop with 1.5ul of elution solution. Measure 1.5ul of plasmid DNA solution
    b. Record plasmid DNA concentration in your notebook.

Part II: Running Plasmid DNA on an agarose gel

1. <u>Prepare Samples:</u> Prepare 1ug of plasmid DNA in 1X load dye in a 1.5mL microcentrifuge tube. For example:
   DNA yield is 100ng/ul = 10ul plasmid DNA
   10X load dye = 1 ul of 10X load dye
   Alternatively, prepare 20ul of the eluted DNA in 1X final concentration of load dye.

2. <u>Prepare molecular weight Marker</u>: Prepare <u>1ul</u> of 1Kb DNA Step Ladder molecular weight marker in 1x load dye in a 1.5mL microcentrifuge tube as follows:
   1ul 1Kb DNA Step Ladder
   8ul dH2O
   1ul 10X load dye

3. ***Save the remaining plasmid DNA in a labeled microcentrifuge tube for the next lab exercise. Plasmid DNA should be stored at 4^{o}C.***

4. Load sample in load dye into one well and DNA ladder in load dye into another well.

5. Run at 100 volts for approximately 30 minutes (until the dye is approximately 1cm from the bottom of the gel). Record loading position in the gel electrophoresis documentation form.

6. Visualize DNA bands by placing gels on a UV transilluminator and capture an image using the gel electrophoresis documentation system. Affix a well-labeled copy of your gel picture to the gel electrophoresis documentation form and turn this in with your lab report.

Lab Unit 7 – Plasmid Isolation Assignment

1. Define a plasmid and describe in detail how the plasmid is inserted into a bacterial host.

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

3. What is the purpose of the spin filter? How do you think DNA binds to it, and how and why does it come off of the filter?

4. Analyze your data. In your report include the gel documentation form and a ***labeled*** picture of your gel.
    a. What is the size of your plasmid? Is this the expected size for pGLO? What can account for the discrepancy (if any)?

    b. Is your yield low? What are some possible reasons for this? You can trouble shoot this by searching online for the Aurum Plasmid Mini Kit Instruction Manual.

LAB UNIT 8: RESTRICTION ENZYME MAPPING

OBJECTIVES

Your performance will be satisfactory when you are able to:

- work with enzymes without degrading them
- measure microliter amounts
- make an agarose gel of a specified concentration
- prepare and load samples onto an agarose gel
- analyze electrophoresis results to determine the size of DNA fragments

Restriction Endonuclease Mapping of DNA

Viruses form a unique group of parasitic organisms that grow only in the cells of bacteria, plants and animals. A virus that infects bacteria is called a **bacteriophage** or simply a **phage**. Viruses are valuable tools in molecular biology because they possess some essential properties of living organisms (they contain proteins and nucleic acids), yet are simpler in structure and life cycle than bacteria or eukaryotic cells.

Bacteriophage lambda, which infects *E. coli*, is probably the most studied and well understood of the double-stranded DNA phages. The protein component of this phage consists of a protective coat that forms the tail assembly and the outer shell of the head. A single molecule of double-stranded DNA is located in the core of the phage head. The DNA molecule contains 48,502 base-pairs (molecular weight $\approx 3 \times 10^7$ g/mol) that code for approximately 50 different proteins. The entire sequence of nucleotides in the lambda genome is known and the sequences that comprise the major control regions for transcription and translation have been identified. Because of the vast amount of information about the biochemistry of this phage, lambda is a popular cloning vector in genetic engineering.

In addition to agarose concentration and molecular weight, mobility of nucleic acids in agarose gels is also influenced by the molecular conformation of the nucleic acid. The 3 forms of circular DNA (plasmid) and some viral DNA are:

Form I:	Closed, circular, negatively supercoiled DNA
Form II:	"Nicked" DNA, which has been partially cut through one strand of the DNA, causing it to unwind
Form III:	Linear DNA, which has been cut by endonucleases (restriction enzymes)

Form I usually has the greatest electrophoretic mobility of all DNA forms because supercoiled DNA molecules tend to be compact. Think of it as a bullet moving through the agarose gel. Form III has decreased mobility because the linear DNA is like a long string or rod that can drag. The slowest moving of all is Form II because it is an open circle with a partially unwound strand, which causes it to drag in the gel. While the linear DNA can snake through the agarose particles, reducing drag, the circular, unwound plasmid cannot. Unlike the other forms of DNA, **linear DNA migrates through a gel at a rate that is inversely proportional to the logarithm of its molecular weight**. Therefore, the molecular weight of linear DNA can be estimated from a gel if compared to DNA fragments of known molecular weight (**markers**).

When isolating plasmid DNA, it is important to determine the relative amounts of the three forms of DNA in your sample. These can be estimated qualitatively by the relative darkness of the bands. Rough handling of the DNA or endogenous endonuclease action can result in nicking of the plasmid and **linearization**. Long-term storage of plasmids will result in increased amounts of Forms II and III due to the presence of endogenous endonucleases (see below). Uncut plasmid should be stored in the refrigerator to avoid the risk of ice crystals shearing, or damaging, the supercoiled DNA molecule. A cut plasmid can be kept in the freezer with no risk of damage.

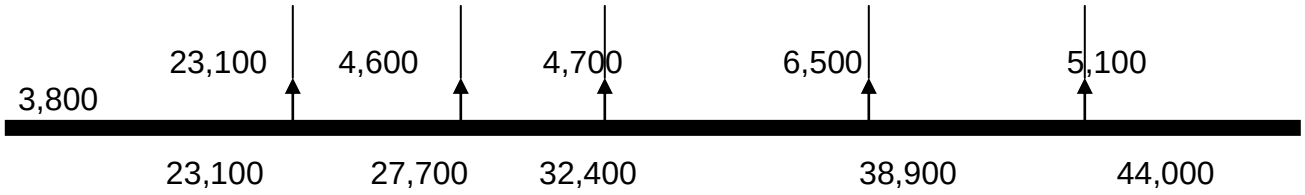
Restriction Enzymes

Restriction enzymes (**endonucleases**) are proteins found in nature that cut DNA at specific locations. They have many applications in the biotechnology lab, and you will encounter them several times in this program. A **restriction map** shows the locations at which a restriction enzyme cuts DNA (see figures 1 and 2). Purified enzymes are especially susceptible to degradation by heat, pH changes, and other factors, and must be kept under stable conditions. This instability can make it a challenge to work successfully with them. Restriction enzymes should be kept on ice at all times, and solutions containing them should be buffered at the proper pH. The concentration of a restriction enzyme is usually expressed in **enzyme units per volume**. One unit of restriction enzyme is defined as the amount of enzyme needed to digest 1 µg of DNA in 1 hour. One might purchase *Eco*RI at a concentration of 1EU/µL. Endogenous endonucleases are contaminants found in a preparation of DNA, and can degrade such samples.

Figure 1: Restriction Map of Lambda DNA – EcoR1

Arrows indicate the position of restriction sites for EcoR1. Sites are counted in number of base pairs (bp) from the left end of the phage DNA. Lambda DNA is **48,502** base-pairs long. The dark horizontal line represents lambda genomic DNA and the smaller vertical lines represent the cut sites for the respective restriction enzymes. The numbers above the line represent the size of the fragments generated by a digestion with each restriction enzyme and the numbers below represent the distance from the origin.

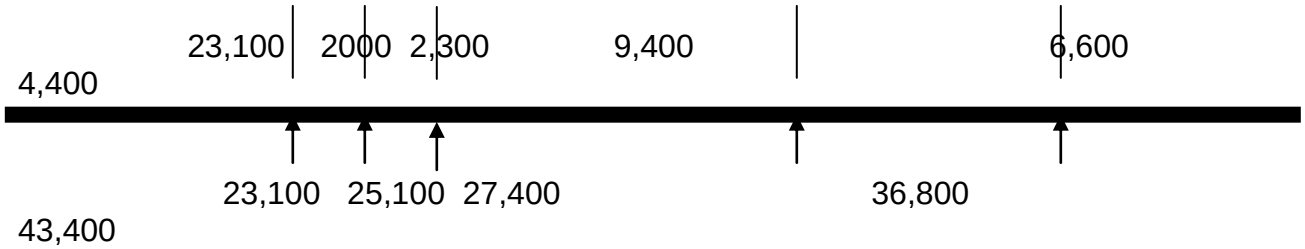
Size of EcoR1 Restriction Fragments



Nucleotide position of EcoRI Restriction Sites in the phage DNA

Figure 2: Restriction Map of Lambda DNA – HindIII
Arrows indicate the position of restriction sites for HindIII. Sites are counted in number base-pairs (bp) from the left end of the phage DNA.

Size of Hind III Restriction Fragments



Nucleotide position of HindIII Restriction Sites in the phage DNA

EcoRI recognition site = G|AATTC
C TTAA|G

HindIII recognition site = A|AGCTT
T T CGA|A

You can find this information on-line.

MATERIALS
Microcentrifuge tubes
P20 and p200 micropipets and tips
37ºC water bath
Floating tube rack

Nuclease-free water
HindIII restriction enzyme
EcoRI restriction enzyme
Universal buffer
Lambda DNA

PROCEDURE
Cut and paste or copy the following charts in your prelab.

Lambda Digest			**Volume to add**			
Component	**Concentration**	**Mass or Conc. required**	**Tube 1 (control)**	**Tube 2 (*Eco*RI, *Hind*III)**	**Tube 3 (*Eco*RI)**	**Tube 4 (*Hind*III)**
DNA	0.5 µg/µL	1 µg				
Universal buffer	10X	1X				
Water						
***Eco*RI**	0.5 units/µL	1 unit	none			
Hind III	0.5 units/µL	1 unit	none			
TOTAL			20 µL	20 µL	20 µL	20 µL

pGLO digest			**Volume to add**			
Component	**Concentration**	**Mass or Conc. required**	**Tube 1 (negative control)**	**Tube 2 (*Eco*RI, *Hind*III)**	**Tube 3 (*Eco*RI)**	**Tube 4 (*Hind*III)**
DNA	unknown	1 µg				
Universal buffer	10X	1X				
Water						
***Eco*RI**	0.5 units/µL	1 unit	none			
Hind III	0.5 units/µL	1 unit	none			
TOTAL			20 µL	20 µL	20 µL	20 µL

Part A: Restriction Digests

1. We want to digest 1 µg of DNA in these reactions. Using the concentration of the lambda DNA, calculate how many microliters need to be added to each tube to have 1 µg in each tube. The final volume in each tube after every reagent is added should be 20 µL. Using the concentration of the buffer (usually 10X), calculate how much should be added so that when the final volume is 20 µL, the buffer concentration is 1X. Using the concentrations of the two enzymes, calculate how much should be added to each to tube if you want one enzyme unit per tube. Enzyme concentrations are usually in enzyme units (U) per volume (for example, 1 U/µL). Calculate the amount of water needed in each tube, after accounting for the volumes of the other reagents, to make a final volume of 20 µL.

2. We do not know the concentration of the pGLO DNA, so instead of diluting with water, we will add it to the reaction mixture at full strength. Determine how much enzyme and buffer should be in these reaction tubes and add enough pGLO plasmid prep solution to the tubes to achieve a final volume of 20 µL.

3. Number three microcentrifuge tubes 1 – 3 and mark tube 1 with the notebook location of the charts you made in step 1. Place the tubes in ice.

4. Add the reagents to the tubes in the order listed in the chart. **Keep DNA and enzymes on ice. Use a new tip for each solution.** Tube 1 is the control, and will contain only DNA, buffer, and water. You will add *Eco*RI to tube 3, *HindIII* to tube 4, and both enzymes to tube 2. DNA should be added first to the tubes; you may keep the same tip on the micropipette without contaminating the stock solution of DNA. The buffer, which should be added second, maintains the proper pH for enzymatic activity. Water should be added next so that the buffer is the correct concentration when you add the enzyme (last). Pipet directly into the solution at the bottom of the microcentrifuge tube, and pipet up and down to mix. Avoid making bubbles, which can degrade the enzyme activity. After adding the enzymes to tubes 2 and 3, pipette up and down to mix.

5. Pop spin all the tubes in a microcentrifuge to ensure all components are condensed in the bottom of the tube and able to react.

6. Place all tubes, with lids closed, (in a floating tube rack) in a 37˚ C water bath to incubate for at least 50 minutes and up to 3 hours; the DNA should be specifically digested after 50 minutes and any longer than 3 hours may result in unspecific cutting of the DNA.

Part B: Agarose Gel Electrophoresis

7. While the tubes are incubating, the class should prepare 1.0% agarose gels in 1X TAE with 1X SYBR Safe. See Appendix for instructions on preparing agarose gels.

8. When the incubation of your reaction tubes is over, remove the tubes from the water bath, and add 10 µl of 10X gel loading solution to each. Pipette up and down to mix and spin the contents down by microcentrifuging for a few seconds.

9. Remove the bumpers and comb from a gel and place the casting tray with gel into the electrophoresis chamber. Wait until each group has put their tray in, then have one person add enough 1X TAE buffer to cover the gels. Make sure that every well is full of buffer and completely submerged. Draw a picture of the chamber and label the position of each group's gel relative to the positive and negative terminals.

10. Load 30 ul each sample into a separate well of your gel. If you have enough wells, leave the first and last empty, as they tend to be more distorted then the other wells. Record in your notebook, which sample was loaded into which well (always number from left to right).

11. Electrophorese at 80 volts for 30-60 min or until the dye has migrated to within a few mm of the end of the gel (the end nearest the positive terminal). If the loading dye has more than one color, use the aqua color as a guide. Turn off the power supply and disconnect the leads before removing the cover of the chamber. Remove your gel in its casting tray.

12. Capture image using gel documentation system. If available use orange filter on the lens (appropriate for SYBR Safe). Place a ruler beside the gel when imaging.

13. Fill out a gel documentation form and affix a well labeled copy of your gel image to it.

14. Place your gel in the regular trash. Pour the electrophoresis buffer down the sink with plenty of water, rinse the electrophoresis chamber and casting assemblies with deionized water, and place upside down over paper towels to dry. Do NOT use paper towel to dry out the electrophoresis apparatus, it may damage the electrode wires! Remember to turn off the power supply and transilluminator.

Lab Unit 8 - Restriction Enzyme Mapping of DNA Assignment

1. Maps of the restriction sites for *Eco*RI and *Hind*III in lambda DNA are given in Figures 1 and 2. Using this information, list the size (in bp) of each of the bands you should see on the gel. **Write the bands in the order you would see them on the gel (longest to shortest).**

***Eco*RI**	***Hind*III**
1. _____	1. _____
2. _____	2. _____
3. _____	3. _____
4. _____	4. _____
5. _____	5. _____
6. _____	6. _____

2. When you cut lambda DNA with both *Eco*RI and *Bam*HI, the DNA is cut into twice as many fragments. **Use the maps in Figures 1 and 2 to determine the length of the bands and write them in the order they should appear on a gel.**

DNA Fragment Length (EcoRI + Hind III)
(base-pairs)

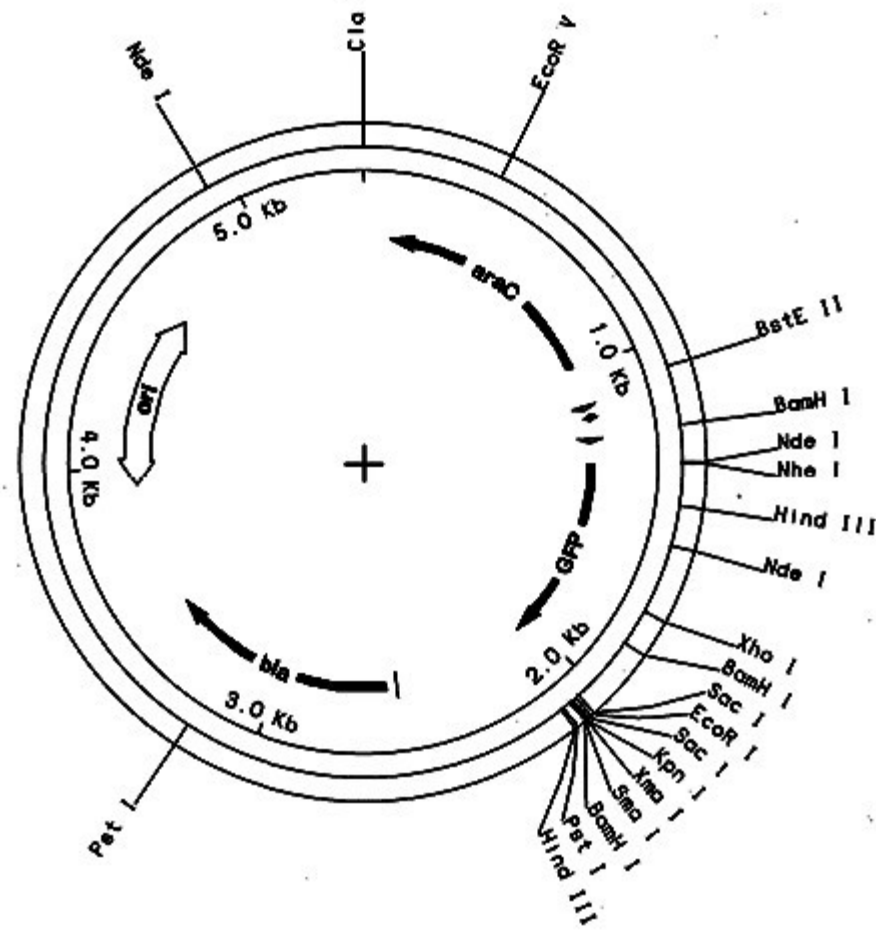
1. _____
2. _____
3. _____
4. _____
5. _____
6. _____
7. _____
8. _____
9. _____
10. _____
11. _____

3. Since there were no enzymes in tube 1, the DNA should have been uncut and appeared in one thick band. If you saw more than one band, what could it have caused this (see introduction)? What would finding more than one band say about the DNA sample that you used?

4. Plasmid DNA should be stored at 4˚ C (refrigerated) unless it has been cut, and then it can be stored at –20˚ C (freezer). Why (i.e. what might happen to it in the freezer)?

5. Did you see all 6 bands in each lane of lambda DNA cut with endonucleases? If you didn't, which bands (short or long) were not visible, and why?

6. Below is a restriction map of the pGLO plasmid:



Determine the identity of each band on the gel containing your pGLO plasmid digest. Use the molecular weight of each band and the information on this restriction map to determine your answers.

7. Using semilog paper, plot band size (kb) on the y-axis versus distance migrated on the x-axis for the ladder. If the DNA bands migrated properly, you should be able to draw a straight line through the points. Can you? Are there any points associated with bands that do not fall on the line? If so, which ones?

LAB UNIT 9: GREEN FLUORESCENT PROTEIN PURIFICATION

(adapted from BioRad's GFP Chromatography kit)

OBJECTIVES

Your performance will be satisfactory when you are able to

- extract proteins from cells
- set up a hydrophobic interaction chromatography column
- load a protein extract onto a column and collect fractions
- analyze the results of chromatographic separations
- distinguish between various chromatographic interactions and their uses
- discuss the role of green fluorescent protein

INTRODUCTION

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

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

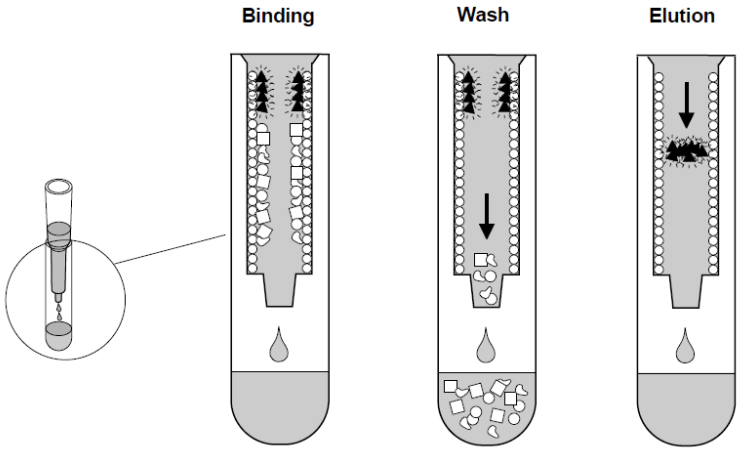
This lab exercise involves hydrophobic interaction chromatography, in which components bind, or **adsorb**, to the stationary phase due to hydrophobicity. The stationary phase is made of insoluble particles, also called a **resin**, of polysaccharide beads called Sepharose™. These beads are small (40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

Your goal in this lab is to use hydrophobic interaction chromatography to purify GFP from a bacterial cell lysate. Proteins are long chains of amino acids, some of which are very hydrophobic or "water-hating". GFP has many patches of hydrophobic amino acids, which collectively make the entire protein hydrophobic. Moreover, GFP is much more hydrophobic than most other bacterial proteins. We can take advantage of the hydrophobic properties of GFP to purify it from the other, less hydrophobic (more hydrophilic or "water-loving") proteins.

First, you will obtain a liquid bacterial culture that was prepared by isolating a single green transformant *E. coli* colony, adding it to a tube of liquid medium, and then incubating with vigorous shaking overnight. You will process this culture in order to lyse the cells and release the proteins contained therein. You will load the cell lysate onto an HIC column in a high salt buffer. The salt causes the three-dimensional structure of proteins to change so that the hydrophobic regions of the protein move to the exterior of the protein and the hydrophilic ("water-loving") regions move to the interior of the protein. This will cause most, if not all, proteins, to adsorb to the column. As the salt concentration of the buffer is decreased, the three-dimensional structure of proteins change again so that the hydrophobic regions of the proteins move back into the interior and the hydrophilic ("water-loving") regions move to the exterior.

<u>These four buffers comprise the separation scheme:</u>

Equilibration Buffer—A high salt buffer (2 M $(NH_4)_2SO_4$)
Binding Buffer—A very high salt buffer (4 M $(NH_4)_2SO_4$)
Wash Buffer—A medium salt buffer (1.3 M $(NH_4)_2SO_4$)
Elution Buffer—A very low salt buffer (10 mM Tris/EDTA)



PROCEDURE

Day One (may be performed by instructor or lab tech if time is limiting)

1. Examine your LB/AMP and LB/AMP/ARA plates from the transformation lab. First use normal room lighting, and then use an ultraviolet light in a darkened area of your laboratory. Note your observations. To prevent damage to your skin or eyes, avoid exposure to the UV light. Never look directly into the UV lamp. Wear safety glasses whenever possible.
2. Identify several green colonies that are not touching other colonies on the LB/amp/ara plate. Turn the plate over and circle several of these green colonies. On the other LB/amp plate identify and circle several white colonies that are also well isolated from other colonies on the plate.
3. Obtain two 50-mL culture tubes containing 2 mL of sterile LB/AMP/ARA medium and label one tube "+" and one tube "-". Using a sterile inoculation loop, lightly touch the "loop" end to a circled single green colony and gently scoop up the cells without gouging the agar. Immerse the loop in the "+" tube. Spin the loop between your index finger and thumb to disperse the entire colony. Using a new sterile loop, repeat for a single white colony and immerse it in the "-" tube. It is very important to pick cells from a single bacterial colony.
4. Place them in a 37° C incubator with vigorous shaking for 18-24 hours.

Day Two

1. Remove your two liquid cultures from the incubator and observe them in normal room lighting and then with the UV light. Note any color differences that you observe.
2. Transfer the entire contents of the (+) liquid culture by pipet into a 2 mL microcentrifuge tube also labeled (+). You may now set aside your (-) culture for disposal.
3. Centrifuge the (+) tube for 5 minutes at 12,000 rpm. Be sure to balance the tubes in the machine. If you do not know how to balance the tubes, ask your instructor.
4. After centrifugation, open the tube and slowly pipette off the liquid supernatant. After the supernatant has been discarded, there should be a large pellet remaining in the tube.
5. Observe the pellet under UV light. Note your observations.
6. Add 250 µl of TE Solution to the tube. Resuspend the bacterial pellet thoroughly by vortexing (don't use a micropipette, the bacteria will get stuck in the tip).
7. Add 1 drop of lysozyme by pipet to the resuspended pellet. Cap and mix the contents by flicking the tube with your index finger. The lysozyme will start digesting the bacterial cell wall. Observe the tube under the UV light. Place the microcentrifuge tube in the freezer. Freezing will cause the cells to rupture completely. Freezing slowly at -20^{o}C for

30min-1hr works efficiently at cell rupture. If time is limiting, you can use a -80 ºC freezer for 15-30 minutes.

8. Remove your tube from the freezer and thaw using hand warmth. Place the tube in the centrifuge and pellet the insoluble cell debris by spinning for 10 minutes at 12,000 rpm. Label a new microcentrifuge tube.

9. While centrifuging, prepare the chromatography column. Shake the column to resuspend the beads. If the beads have dried out, add 1mL of Equilibration Buffer and shake.

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

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

12. After the 10 minute centrifugation, immediately remove the microcentrifuge tube from the centrifuge. Examine the tube with the UV light. The cell debris should be visible as a pellet at the bottom of the tube. The liquid that is present above the pellet is called the supernatant. Note the color of the pellet and the supernatant.

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

14. Transfer the remaining 200 µl of the supernatant into a new tube labeled "supernatant".

15. Transfer 200 µl of Binding Buffer to this tube containing the supernatant. Mix by pipetting up and down a few times.

16. Save a 50ul aliquot of the supernatant in binding buffer for the SDS-PAGE analysis later.

17. Obtain 5 collection tubes and label them as shown in the table below. Include the following data table in your lab notebook:

Collection Tube Number	Prediction (do this for your prelab)	Observations Under UV Light (column and collection tube)
Tube 1 Sample in Binding Buffer		
Tube 2 Sample with Wash Buffer		
Tube 3A Sample with Elution Buffer		
Tube 3B Sample with Elution Buffer		
Tube 3C Sample with Elution Buffer		

18. When the last of the equilibration buffer has drained from the HIC column bed, gently place the column on collection tube 1. Do not force the column tightly into the collection tubes—the column will not drip.

19. Carefully load 400 µl of the supernatant (in Binding Buffer) into the top of the column by resting the pipette tip against the side of the column and letting the supernatant drip down the side of the column wall. Examine the column using the UV light. Note your observations in the data table. Let the entire volume of supernatant flow into tube 1.

20. Transfer the column to collection tube 2. Add 250 µl of Wash Buffer and let the entire volume flow into the column. As you wait, predict the results you might see with this buffer. Examine the column using the UV light and record your results.

21. Transfer the column to tube 3A. Add 250 µl of TE buffer (Elution Buffer) and let the entire volume flow into the column. Again, make a prediction and then examine the column using the UV light. List the results in the data table.

22. Transfer the column to tube 3B. Add 250 µl of TE buffer (Elution Buffer) and let the entire volume flow into the column. Again, make a prediction and then examine the column using the UV light. List the results in the data table.

23. And finally, transfer the column to tube 3A. Add 250 µl of TE buffer (Elution Buffer) and let the entire volume flow into the column. Again, make a prediction and then examine the column using the UV light. List the results in the data table.

24. Examine all of the collection tubes using the UV lamp and note any differences in color between the tubes. Cap the collection tubes and place in the refrigerator until the next laboratory period. Keep the pre-column aliquots you made as well.

Unit 9 - GFP Column Chromatography Assignment

1. In the Transformation lab, you transformed *E. coli* cells with the pGLO plasmid. The results of this procedure were colonies of cells that fluoresced when exposed to ultraviolet light. This is not a normal phenotype (characteristic) for *E.coli*. You were then asked to figure out a way to determine which molecule was becoming fluorescent under UV light. After determining that the pGLO plasmid DNA was not responsible for the fluorescence under the UV light, you concluded that it was not the plasmid DNA that was fluorescing in response to the ultraviolet light within the cells. This then led to the next hypothesis that if it is not the DNA fluorescing when exposed to the UV light, then it must be a protein that the new DNA produces within the cells.
    a. What is a protein?
    b. List three examples of proteins found in your body.
    c. Explain the relationship between genes and proteins.

2. In your own words, describe recombinant gene cloning.

3. How were these items helpful in this cloning experiment?
    a. UV light
    b. Incubator
    c. centrifuge
    d. lysozyme
    e. freezer

4. Explain how placing cloned cells in nutrient broth to multiply relates to your overall goal of purifying the fluorescent proteins.

5. Explain why the bacterial cell outer membrane ruptures when frozen (what happens to an unopened soft drink when it freezes)?

6. What was the purpose of lysing the bacteria?

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

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

9. Based on your results, explain the functions of each buffer:
    a. equilibration buffer
    b. binding buffer
    c. wash buffer
    d. TE (elution) buffer

10. Were you successful in isolating and purifying GFP from the cell lysate? Identify evidence to support your answer.

LAB UNIT 10: SDS-PAGE OF PURIFIED GFP

(adapted from BioRad's pGLO SDS-PAGE extension)

OBJECTIVES

Your performance will be satisfactory when you are able to:

- Explain how polyacrylamide gel electrophoresis can be used to analyze proteins
- Load, run and stain a polyacrylamide gel
- Analyze and interpret the results of an electrophoretic run

INTRODUCTION

General Principles of Protein Electrophoresis and SDS-PAGE

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

- What proteins are in my sample?
- What are the molecular weights of the proteins?
- What differences are there in the proteins from different sources?
- How pure is my protein of interest?
- How much protein do I have?

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

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

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

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

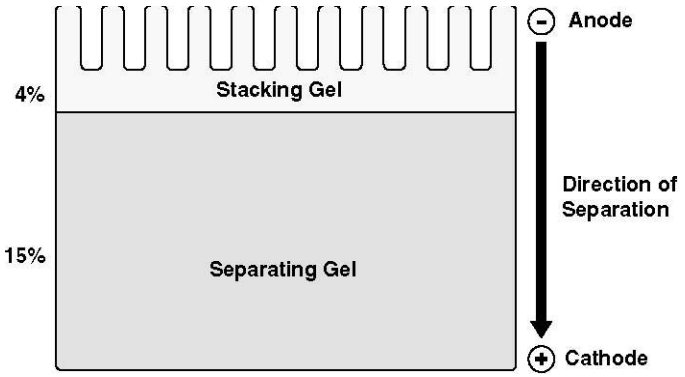


Figure 1. Precast gels are very thin polyacrylamide gels sandwiched between clear plates. Each gel has two separate zones, the stacking gel and the separating gel, which is also known as the resolving gel. In polyacrylamide gel electrophoresis, samples are loaded into wells at the top of the stacking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

The Chemistry and Physics behind Electrophoresis

The size of biomolecules is expressed in Daltons (D), a measure of molecular weight. One dalton equals the mass of a hydrogen atom, which weighs 1.66×10^{-24} gram. Most proteins have masses on the order of thousands of daltons, so the term kilodalton (kD) is used for protein molecular weights. Proteins range in size from several kilodaltons to thousands of kilodaltons. In contrast, the nucleic acids we study are often larger than 1000 base pairs, or 1 kilobase (kb), and each kilobase pair has a mass of approximately 660 kD. For example, when cloning DNA, a 2 kb fragment of DNA can be inserted into a plasmid vector of 3 kb, giving a total plasmid length of 5 kb. The mass of this 5 kb plasmid would be approximately 3.3 million daltons or 3,300 kD, much larger than the average protein!

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

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

Polyacrylamide Acts As a Molecular Sieve

The degree of sieving within a gel can be controlled by adjusting the polyacrylamide concentration. Higher concentrations of polyacrylamide resolve smaller molecular weight ranges. For example, a 5% polyacrylamide gel separates large proteins of 100 to 300 kD, while an 18% polyacrylamide gel is better for separating smaller proteins in the range of 5 to 30 kD. For this lab we will use a premade 12-15% polyacrylamide gel, which provides excellent separation for proteins in the range of 10 to 100 kD. Our attention will be focused on variations among the smaller proteins, in the range of 15 to 50 kD, since it is easier to discern differences

among these proteins. Smaller proteins migrate further through the gel and are better resolved than proteins of high molecular weights.

Running a Polyacrylamide Gel

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

Sample Preparation – Disrupting Protein Structure

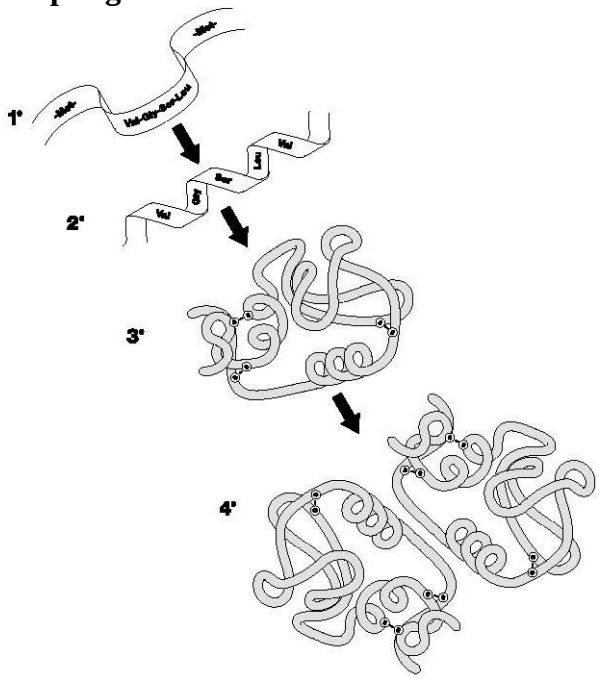


Figure 2: Secondary, tertiary and quaternary protein structure must be disrupted, or denatured to separate proteins by size.

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

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

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

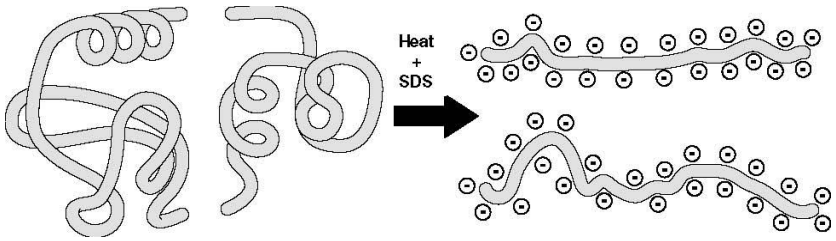


Figure 3. The combination of heat and the detergent SDS denatures proteins for SDS-PAGE analysis.

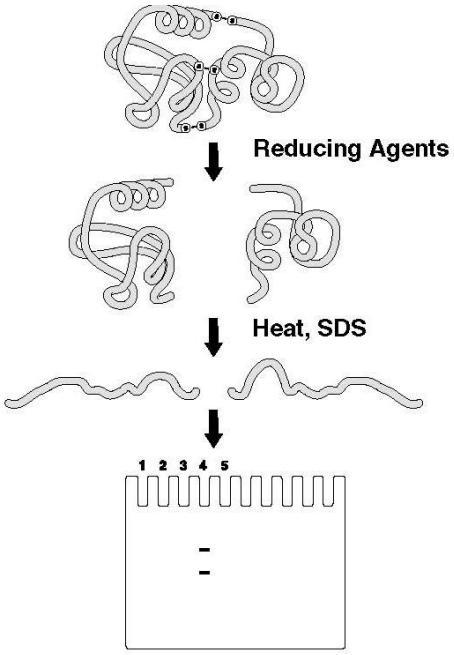


Figure 4: A quaternary protein complex denatured with reducing agents, head and SDS can be separated into individual proteins and resolved by size using SDS-PAGE.

Visualizing the Proteins

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

Molecular Weight Standards

Electrophoresis protein standards, or molecular weight markers, consist of a mixture of proteins of known molecular weight. They are available in a number of protein size ranges. The markers

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

Identifying Proteins in Polyacrylamide Gel

It is not possible to definitively identify unknown proteins in an SDS-PAGE gel without additional analysis. In an experiment like this one, each protein extract contains a complex mixture of proteins. The different proteins appear as distinct blue-stained bands on the gel. From the positions and intensities of these bands, we can determine the size and relative abundance of the proteins, but we can only make educated guesses about the identity of each protein, based on available references. Even when the molecular weight of a protein is known, and used as a criterion for identification, there are two possible sources of error. First, bands that migrate almost identically on a gel may actually be different proteins of very similar sizes. Second, proteins of very similar composition, function, and evolutionary origin may be different in molecular weight, because of variations acquired as they evolved. Definitive identification of a protein requires mass spectrometry, sequencing, or immunodetection. Immunodetection methods, such as western blotting, use antibodies that specifically recognize the proteins of interest. Such antibodies can provide positive identification when bands cannot be identified by molecular weight alone.

PROCEDURE

1. Label 7 screw-cap tubes to match the samples you collected from the column chromatography lab.
    - Protein Extract
    - Protein Extract in binding buffer
    - Column fraction 1 – proteins not bound to column
    - Column fraction 2 – proteins washed off column
    - Column fraction 3A – elution 1
    - Column fraction 3B – elution 2
    - Column fraction 3C – elution 3

2. Add 50 µl of Laemmli sample buffer to each of the tubes you just labeled. Laemmli buffer contains SDS to denature the proteins in your sample as well as tracking dye and glycerol to weigh the samples down when you load them on the gel. Your sample buffer also has reducing agent DTT, therefore you must dispose of all tips and tubes in a waste beaker.

    WARNING! DTT is harmful if absorbed through skin, causes irritation to skin, eyes, and respiratory tract. Wear gloves and wash hands, avoid from directly inhaling. Keep tubes CAPPED.

3. Add 50 µl of each sample you collected to the corresponding tube you labeled in step 1.

4. Heat all tubes for 5 minutes at 95 °C (a boiling water bath works best for this).

5. Your instructor will demonstrate how to set up your gel apparatus.

    WARNING! Acrylamide is a neurotoxin! Always handle gels with gloves.

    a. Remove the comb and tape along the bottom of the pre-made polyacrylamide gels and place your gel in the chamber with the short plates facing inside as shown by your instructor.

    b. Make sure your apparatus does not leak by pouring 1X TGS into the center and watch for leaking out the bottom. Insert into the gel box and fill to the mark "Two gels".

    c. Wash out the wells vigorously to remove unpolymerized acrylamide with a disposable transfer pipet or p1000 micropipette.

6. Load 10 µl of the Kaleidescope prestained standard. The band sizes are 250, 150, 100, 75, 50, 37, and 25, 20, 15, 10. This marker does NOT get heated!

7. Load 30 µl of each denatured protein sample into a separate well.

8. Put the lid on the tank and insert leads into the power supply, matching red to red and black to black.

9. Electophorese for 30 minutes at a constant voltage of 200 V or until the dye front reaches the bottom of the gel.

10. When the loading dye has almost reached the bottom of the gels, stop the power supply and disconnect the leads. Remove the gel cassettes. Lay a gel cassette flat on the bench with the short plate facing up. Carefully pry apart the gel plates using a spatula. The gel will adhere to one of the plates.

11. Transfer the plate with the gel adhering to it to a staining tray containing dH_2O. Rinse for 5-15 min with dH_2O to remove excess running buffer that may interfere with the Bio-Safe Coomassie blue stain. NOTE: when pouring out water washes, hold gel with your finger lightly or else it will get dumped down the sink!

12. Pour out water and add just enough Coomassie blue stain to cover the gel.

13. Stain the gels for one hour with agitation.

14. After the gels have been stained, pour off the stain down the sink with lots of water.

15. Cover the gel with dH_2O, changing several times until most of the blue background disappears and you are able to visualize discrete bands on the gel. This can take several hours to overnight.

16. The GFP protein is approximately 27 kilodaltons. Do you see a band in this range for any of your samples?

Lab Unit 10 - Protein Electrophoresis of GFP Assignment

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

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

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

4. Assume you work in a lab responsible for large scale production of GFP protein. Describe the process that we used to produce this protein. Include a discussion of the method we used to express the protein, purify the protein, and verify that the target protein is present in the sample.

5. Include your gel documentation form with a picture of the labeled gel attached. Discuss your results. What is the size of GFP? Does it match what you see in your gel? How do you know? Was the protein purification successful? Provide evidence to support your answer.

LAB UNIT 11: PCR-based VNTR Human DNA Typing

(adapted from Edvotek's PCR-based VNTR Human DNA Typing)

OBJECTIVES

Your performance will be satisfactory when you are able to:

- extract DNA from your own cells
- set up a PCR reaction tube with all required components
- operate a thermal cycler
- make and load an agarose gel
- interpret the finished gel and evaluate the quality of amplification

INTRODUCTION

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

CODIS

In 1990, the Federal Bureau of Investigation (FBI) established the Combined DNA Index System (CODIS). It is the core of the national DNA database, and developed specifically to enable forensic DNA laboratories to create searchable DNA databases of authorized DNA profiles. The CODIS software permits laboratories throughout the country to share and compare DNA data.

This system permits comparison of crime scene DNA to DNA profiles in a convicted offender and a forensic (crime scene) index. A match of crime scene DNA to a profile in the convicted offender index indicates a suspect for the crime, whereas a match of crime scene DNA to the forensic index (a different crime scene) indicates a serial offender.

As of 2011, the NDIS contained over 9,635,757 offender profiles and 370,875 forensic profiles. Ultimately, the success of the CODIS program is measured by the crimes it helps to solve. As of April 2011, CODIS had produced over 142,700 hits assisting in more than 137,100 investigations nationally and, 7500 in Texas alone!

ISOLATING DNA

The first step in forensic DNA fingerprinting is the collection of human tissue from the crime scene or victim. These tissues include blood, hair, skin, and body fluids. The sample, often present as a stain, is treated with a detergent to rupture (lyse) cell membranes and obtain DNA for further analysis. In forensics, the polymerase chain reaction (PCR) is now used to amplify and examine highly polymorphic DNA regions. These are regions that vary in length from individual to individual and fall into two categories: 1) Variable Number of Tandem Repeats (VNTR) and 2) Short Tandem Repeats (STR). A VNTR is a region that is variably composed of

a 15-70 base pair sequence, typically repeated 5-100 times. An STR is similar to a VNTR except that the repeated unit is only 2-4 nucleotides in length. By examining several different VNTRs or STRs from the same individual, investigators obtain a unique DNA fingerprint for that individual which is unlike that of any other person (except for an identical twin).

One VNTR known as D1S80, is present on chromosome 1 and contains a 16-nucleotide sequence which is variably repeated between 16 and 40 times. An individual who is homozygous for the D1S80 genotype will have equal repeat numbers on both homologues of chromosome 1, displaying a single PCR product following gel analysis. More commonly, a person will be heterozygous, with differing D1S80 repeat numbers. Amplification of DNA from heterozygous individuals will result in two distinct PCR products.

PCR: VNTR

In this experiment, polymerase chain reaction (PCR) will be used to amplify (make copies of) a short DNA sequence from human chromosome 1 at a point called the D1S80 locus that is a variable insertion. The primers used to start the amplification were designed to flank the DNA region of the D1S80 insertion site. The amplicon size (position on the gel after electrophoresis) will reveal the length of the insertion.

Set Up PCR Reactions

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

The thermal cycler must be programmed so that it is preheated and ready to run when your samples are ready. The program will begin with an initial 5 minute cycle at 94˚C, which makes sure that the DNA completely denatures (the complementary strands pull apart). Three minutes is enough time for the 3 billion base pairs in the human genome to denature. Then the machine will cycle through the steps below 32 times. Each cycle doubles the amount of DNA that was produced in the previous cycle.

94˚ C –denatures DNA enough to allow primer to attach	time at that temp: 30 seconds
65˚ C – primer anneals to complementary sequence of DNA	time at that temp: 30 seconds
72˚ C – DNA polymerase begins synthesizing new DNA	time at that temp: 30 seconds

After 32 cycles, the temperature is held at 72˚ C for 4 minutes to allow the polymerase to back-fill any amplicons that were not amplified clear to the end. Then the program is held at 4˚C until it is stopped. This allows the user to leave the thermal cycler running and take the samples out the next day if necessary. After programming, make sure the thermal cycler is preheating so that it will be warm enough to start the cycles immediately.

Lab Unit 11-A: Isolating DNA & PCR of VNTR Loci

PROCEDURE

You will isolate your own cheek cell DNA by extracting a sample and boiling it. Since DNases naturally occur in your saliva, you must protect your DNA from digestion. Addition of a chelating agent will remove divalent cations, which are necessary cofactors for DNases, and thereby inactivate these enzymes. Also, the concentration of magnesium ion must be exactly 25mM for the PCR reaction to work, so removing magnesium from the DNA in advance ensures that final concentration.

PART I: ISOLATING DNA FROM CHEEK SAMPLES

1. Label a 15 mL tube containing 2 ml of phosphate buffered saline (PBS) with your name.

2. Using a sterile cotton swab, rub the inside of your cheeks gently. Twirl the applicator while vigorously swabbing both cheeks, between the gum line and under the tongue. You are harvesting squamous cells, which are removed easily and regenerate very quickly. Submerge the swab in the saline tube and twist it vigorously for 30 seconds to dislodge the cells. Press the cotton head against the walls of the conical tube to squeeze out as much liquid as possible. Repeat with another swab. Place the used swabs in a biohazard bag or in a 15% bleach solution.

3. Transfer the cell suspension to a screw-cap microcentrifuge tube labeled "C". Centrifuge the tube at 10,000 rpm for one minute. Be sure you have a balanced configuration in the centrifuge before spinning. The cells should form a white pellet and the buffer should be clear. If the buffer is cloudy with little or no pellet, spin the tube for an additional minute. If the buffer is clear with little or no pellet, obtain another swab and repeat the above steps.

4. The pellet formed will contain cheek cells. Carefully remove the supernatant, using care not to disturb the pellet. The cheek cells in your pellet may contain some food or bacteria. This shouldn't interfere with the amplification because the primers used only recognize sites on human DNA. The DNA from food or bacteria will not be amplified.

5. Mix the tube of chelating agent by vortexing and before the agent settles, quickly transfer 100 µl of the chelating agent to the tube containing the pellet. Make sure you get some of the beads transferred. Resuspend the pellet in the solution by vortexing gently. Check to see that the pellet is fully resuspended.

6. Lyse the cells completely by placing the tube in float in a boiling water bath for 10 minutes. Do not submerge or drop the tube into the water.

7. Allow the tube to cool for 2 minutes. Vortex for 10 seconds.

8. Place tubes in a balanced configuration in a microcentrifuge and spin for 30 seconds. Transfer 50 µl of the supernatant into a clean microcentrifuge tube labeled "C", using care to avoid disturbing the pellet. Discard pellet. Hold the tube up to a light source to look for chelex beads. It is very important to make sure that no Chelex beads are transferred into the

clean tube, as they can easily chelate (trap) the Mg required by the Taq polymerase. Any carryover of the chelex to the PCR reaction will **not** yield results. If this is the case, centrifuge again and transfer 30ul of supernatant into a microcentrifuge tube labeled "C". Discard pellet.

9. Place the sample on ice while you set up your PCR reaction.

PART II: (OPTIONAL) ISOLATING DNA FROM HAIR SAMPLE

1. Label a screw cap microcentrifuge tube "H".

2. Resuspend the lysis solution by vortexing. Before the chelating agent settles, quickly add 150ul of lysis solution to the screw-cap tube. Add 1ul of proteinase K solution.

   The chelating agent removes Mg which is required by DNA degrading nucleases and DNA polymerases. The Proteinase K will digest proteins found in solution and help with cell lysis.

3. Carefully remove 3-4 hair pieces. It is important that you remove the hair so that the hair contains the sheath. The best hair to obtain is eyebrows because they are short and easily manipulated. If you use hair from other body parts, you can cut off excess hair shaft that does not contain sheath. Place hairs into bottom of screw-cap tube so that the hair is completely submerged.

4. Mix by vortexing. Ensure the hair sheaths are still submerged in the solution before transferring to a 56^{o}C water bath. Incubate tube for 15 minutes.

5. Remove from water bath and cool for 30 seconds on the bench top. Vortex for 15 seconds. Make sure the hairs are still submerged. If not, use a micropipette tip and push them back down.

6. Lyse the cells completely by placing the tube in float in a boiling water bath for 10 minutes. Do not submerge or drop the tube into the water. Be careful! The steam from the water bath is very hot!

7. Allow the tube to cool for 2 minutes. Vortex for 10 seconds.

8. Place tubes in a balanced configuration in a microcentrifuge and spin for 30 seconds. Remove 50 µl of the supernatant into a clean microcentrifuge tube labeled "H", using care to avoid disturbing the pellet. Discard pellet.
   NOTE: It is very important to make sure that no Chelex beads are transferred into the clean tube, as they can easily chelate (trap) the Mg required by the Taq polymerase. Any carryover of the chelex to the PCR reaction will **not** yield results. If your sample is cloudy, centrifuge again and remove 30uL to a clean microcentrifuge tube labeled "H". Discard pellet.

9. Place the sample on ice while you set up your PCR reaction. Alternatively, if you will not be proceeding directly to the PCR reaction, you may store your isolated DNA at -20^{o}C.

PART III: PCR OF DNA SAMPLES

For every PCR reaction you perform, it is essential to run controls to help interpret the data. For example, for this experiment, it is ideal to run a positive control with DNA that you know the primers will produce an amplicon. Also, it is ideal to run a negative control to show that the reagents are not contaminated with other non-target DNA. Depending on reagent availability, your instructor may ask you to set up positive and negative controls for your group, or the instructor may set up a positive and negative control for the class. Ask your instructor how to proceed with control set up.

1. Obtain PCR tubes containing a Ready-to-Go PCR bead. Label the sides of the tube as show in the table below.

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

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

PCR Tube	Sample	Ready-to-go PCR Bead	Volume Primer mix	Volume DNA	Volume Nuclease-free Water
C	Cheek DNA	1 bead	20ul	5 ul Cheek DNA	0 ul
H	Hair DNA	1 bead	20ul	5ul Hair DNA	0ul
+	Positive Control	1 bead	20ul	5ul Control DNA	0 ul
-	Negative Control	1 bead	20 ul	0 ul	5 ul

4. Set up the PCR machine as directed by your instructor. A 'hot start' is preferable, so wait until the PCR machine has reached 94^{o}C before loading the samples. This will help prevent any mispriming that may happen when primers prematurely anneal and DNA polymerase starts to copy the DNA.

5. When the program has completed, store tubes at -20ºC until ready to begin the next step.

Lab Unit 11-B: Analysis of VNTR loci PCR Fragments Using Agarose Gel Electrophoresis

PROCEDURE

1. Prepare a 1% agarose gel in 1X TAE buffer. See Appendix for instructions on preparing agarose gels with 1X SYBR Safe. Use a 7cm x 7cm tray which will hold 30 ml per gel.

2. Place the gel in an electrophoresis chamber and cover the gel with 1X TAE buffer. The buffer level should be about a few millimeters above the top of your gel, and it should fill all the wells.

3. Prepare PCR amplicon samples: Add 5 ul of 10x Gel Loading solution to the PCR amplicon from part B. Vortex briefly, pop-spin and store on a rack on your bench.

4. Prepare DNA marker: This is already prepared for you. Thaw, pop-spin in a picofuge and store in a rack on your bench.

5. Heat the 200 bp DNA ladder and PCR samples for two minutes at 50°C. Allow the samples to cool for a few minutes on the bench top.

6. Fill in gel electrophoresis documentation form. Load gels with 30 uL of 200bp DNA ladder and 30ul of PCR amplicons, taking note of where each sample is loaded on the form.

7. Place the cover on the chamber in the correct orientation and connect leads to a power supply. Set the power supply at approximately 80 volts and allow to electrophorese until the tracking dye is approximately 3/4th the way to the bottom of the gels (approximately 30 minutes for minigels).

8. Visualize DNA bands by placing gels on a UV transilluminator and photograph gels using the gel documentation system. Affix a copy of your gel picture to the gel electrophoresis documentation form and turn this in with your lab report.

Lab Unit 11 - Amplification of D1S80 VNTR loci by PCR Assignment

1. Include your gel documentation form in your lab report with a label picture of your gel. Analyze your data.
    - Compare the migration of your bands relative to the ladder. What are the sizes of your amplicons?
    - How many bands were present in your sample? Explain how one primer set can generate data with two bands on a gel.
    - Compare your D1S80 PCR product with those of the rest of the class. Did any students have genotypes similar to yours? How could you explain such similarities?

2. What is polymorphic DNA? How is it used for identification purposes?

3. What is CODIS? How is it used to solve crimes?

LAB UNIT 12: BIOREMEDIATION

OBJECTIVES

Your performance will be satisfactory when you are able to:

- discuss the uses of microbial ecology in bioremediation
- describe the nutritional requirements of microorganisms
- demonstrate proficiency at recording data and observations
- use sterile technique when establishing liquid cultures
- fix and stain a slide with a simple or complex stain

INTRODUCTION

Pollution comes in many forms, the most visible forms being consumer-generated trash such as paper and plastic food containers. Other pollutants that are harder to see include oil, industrial chemicals, greenhouse gases, pesticides, fertilizers, detergents and soaps. These can be found in air, water, and soil, and often have a detrimental effect on organisms and sometimes-entire ecosystems. They can also enter our bodies as we eat, drink and breathe. Thus, pollution is a problem that must be addressed if we are to maintain the quality of life we now enjoy. Over the past thirty years, the pollution remediation business has grown significantly due to increasing levels of pollution and higher demand for clean air, water, and soil. Some of these companies specialize in microbe-based environmental cleanup, also known as **bioremediation**. Certain microorganisms, including bacteria, fungi, and algae, and sometimes higher organisms such as plants, can use compounds that we consider pollutants as a food source. Thus, they consume these compounds and excrete less harmful substances.

Many bioremediation companies specialize in the cleanup of sites contaminated with a particular type of waste, such as spilled oil. If spilled in the ocean, oil clings to marine organisms, killing birds, fish, sea lions, and other species, upsetting the food chain and, therefore, the natural balance of the ecosystem. A single gallon of spilled oil can spread to cover 4 acres of water. Imagine the acreage that a damaged supertanker would cover! An oil spill of that magnitude would be an ecological disaster. However, after 3 months, 85% of the oil will be degraded by a variety of microorganisms and radiant energy from the sun (weathering). The most volatile of the components of oil will evaporate into the atmosphere, and some of the components, such as asphalt, which cannot be degraded by microorganisms, will form tar balls.

Sometimes a company will be hired to simply monitor the site of an oil spill or other polluted area. Microbes naturally present in the soil, sand or water of the site will slowly consume or absorb the pollutant, and the contamination will eventually be cleaned up. This is known as **natural attenuation**. This approach can work well in some situations, but in other cases, too much time is required for indigenous microbes to degrade the pollutant. In the interim, the pollutant is killing off other species. Thus, many bioremediation companies speed up the degradation process to minimize damage to the environment.

There are two approaches used by these companies, biostimulation and bioaugmentation. **Biostimulation** is the addition of vitamins, minerals, oxygen and other compounds to a contaminated site to speed microbial growth and enhance their activity. A simple example of biostimulation would be the addition of fertilizer to the water around an oil spill. The

concentration of nutrients, such as nitrogen and phosphorous, in the soil or water of a site limits the growth of microorganisms. With higher concentrations of these nutrients, the organisms can reproduce more quickly; building a larger population that can degrade more of the pollutant. A drawback to this approach is that a variety of organisms are stimulated, which could result in the growth of undesirable or competitive microbes. Alternatively, **bioaugmentation** is the addition of microorganisms that specifically degrade the contaminant. Companies that use this approach have collected these organisms from other sites and commercially cultivated them and one of the first examples of an organism genetically altered to benefit mankind, were bacteria specifically designed to degrade oil spills very rapidly and then expire once the oil was degraded.

Selection of Organisms
Organisms are selected or designed to withstand harsh environmental conditions such as high salt and wave action, as well as selected to thrive on the specific contaminant that must be removed. This makes them better suited to survive in the site than the indigenous microorganisms, reducing competition. Once the contaminant has been degraded, the foreign organisms have fewer advantages over the native microorganisms, so they will not survive the competition and eventually will die out. This is still being hotly debated, since many scientists think it is unwise to introduce new organisms into the environment, even if they are suppose to die once the substrate is gone, especially since there are several examples of where this action negatively impacted the environment (for example, rabbits in Australia).

Parameters for Selection
To isolate and select for these organisms, the scientist must understand their growth requirements. What are the nutritional requirements of microorganisms? Are these nutrients naturally available at the contaminated site? If cleaning up an oil spill in the ocean, media is required that duplicates the available nutrients in the ocean. Nitrogen, phosphorous, and iron are limiting nutrients in fresh and seawater environments. Bioremediation experts optimize degradation of pollution by providing elevated levels of nutrients that induce the microorganisms to degrade the pollutant or increase its metabolic rate, thereby degrading the pollutant faster. Environmental conditions such as wave action, sun and wind exposure, and temperature must also be accounted for.

Oil is a good carbon (energy) source for a variety of aerobic microorganisms. However, not all parts of crude oil are good substrates. The asphalt portion cannot be degraded and eventually forms tar balls, as mentioned above. It is important to determine what fractions of the crude oil are degraded and what parts are not degraded by microorganisms if one is going to use them to clean up oil spills.

Composition of Crude Oil
Crude oil is a complex substance made of many different components. When crude oil is refined by **fractional distillation**, these components are separated as they evaporate based on their boiling points. The table below lists all the constituents of crude oil and the temperatures at which they vaporize.

Fraction	Boiling Point (^{o}C)
Gas	<20
Petroleum ether	20-60
Naphtha	60-100
Gasoline	40-205
Kerosene	175-325
Lubricating oil	Not available (liquid)
Asphalt	Not available (solid)

Degraders of Oil in Nature

Oil is a substance found in nature, and there are microorganisms that exist already to digest it. (*Bacillus sp, Pseudomonas sp. Nocardia sp. Rhodococcus sp.* and certain fungi like *Cladosporium*) However, manmade substances, such as plastics, are not easily degraded, as nature has not had sufficient time to evolve or develop the enzyme systems to degrade them. The rule of thumb is that "what is made by nature is degraded by nature". And yet many plastics are major pollutants. The problem, then, becomes how to deal with these man-made pollutants in our environment.

Bacterial Strains Designed To "EAT" Oil

Ananda Chakrabarty and his colleagues generated the first engineered microorganisms with advanced degradative properties in the 1970s. Plasmids were transferred into a bacterial strain that could degrade several compounds in petroleum. He was also the first person to receive a U.S. patent for a genetically engineered microorganism. Although this bacterial strain never was used to clean up oil spills, the development of an "oil-eating" microbe was a significant achievement.

EXAMPLE: Method for Evaluating the Efficacy of Oil-Eating Microbes

MATERIALS
Spatula
Tap water
5 mL pipets
5 mL culture tube
Crude oil
Oil-degrading microbe culture
Paper towels
Liquid fertilizer

PROCEDURE

PART I: Experimental Design (before lab)

Lab Scenario
You work at a bioremediation company. The scientists at your company have gone to an oil-polluted site and collected soil samples. After returning to the lab, they select the desired oil-degrading bacteria. They want to develop a strain of bacteria that will degrade the oil that spills from boats at freshwater marinas. This oil pollutes both the area immediately around the marina and further out in the body of water. This affects the fish, birds and other wildlife living in the area. As a biotechnician, you have been asked to assess the ability of the bacteria to degrade motor oil spilled in fresh water. You will also need to determine if the addition of fertilizers or other nutrients (not containing a carbon source) will stimulate the degradation of the oil under the appropriate environmental conditions.

1. As part of your prelab exercise, answer the following questions:
   a. What environmental conditions are going to affect oil degradation by the bacteria, and how can you simulate those conditions in the experiment?
   b. What are the controls for the experiment and why are they important?
   c. What biostimulants do we want to try, why, and at what concentration?
   d. How are you going to determine if the oil is being degraded or not, and if the addition of biostimulants is increasing the rate of oil degradation?
   e. Can you see the oil being eaten?
   f. What compound(s) is oil converted to when it is degraded?
   g. Can you detect the metabolic products of oil degradation?
   h. Can you think of any other questions you need to answer before starting the experiment?

2. As part of the pre-lab exercise, come up with some ideas on different parameters you may want to test with your oil samples. It is recommended that each group test no more than 3 parameters. Examples of parameters you may want to consider: bioaugmentation with bacteria, and/or biostimulation with fertilizer, different temperature ranges, agitation to name a few.

3. Write up your experiment, including materials and procedure sections. An example of a possible write up is included below. You can utilize this approach but do NOT COPY it word for word.

PART II: Experimental Design (during lab)

1. The whole class will participate in a group discussion on what you have learned about bioremediation and your ideas for the experimental design.

2. As a class, design your bioremediation experiment. Create a plan for determining the optimum conditions for evaluating the efficacy of oil-eating microbes.

   Sample Procedure for Setting up the 'Oil Spill' – An Example Experimental Design
   NOTE: This is a sample procedure. You may want to set up your procedure differently.

   a. Add 5 mL of water to the required number of culture tubes. Add biostimulant to the appropriate tubes. Add oil-degrading microbe culture to the appropriate tubes. Add 3 drops of oil to each tube of water. The oil should form a thin layer on top of the water.

   b. Cap the culture tube and invert them several times to ensure good mixing and to simulate wave intermixing. Loosen the cap of the tube and incubate it vertically under predetermined conditions (i.e. room temperature, in the refrigerator, in the incubator at 37^{o}C, shaking, not shaking etc.).

   c. Illustrate and describe the original oil layers in your lab notebook.

3. To keep track of your experiment, it is recommended that you create a table similar to the one below, which gives the details of each tube. This particular table includes five variables, but you only need about three to have plenty of experimental data. Be sure to include enough negative controls to be able to determine the effects of each variable. Meaning, for every variable, or combination of variables, you will need a negative control!

   Draw a table in your notebook to outline parameters of each tube the class is preparing. A sample table is provided below.

Tube no.	Amount of Oil	Bioaugmentation Bacteria concentration	Biostimulation Fertilizer Concentration	Temperature	Agitation (yes/no)	Other

4. Set up your tubes as outlined in the class experimental design.

5. Make observations in your notebook about how the tubes looked on DAY 1. This is a qualitative assessment, so you will need to be specific and thorough in your observations.

6. In addition to your observations, feel free to take a picture of your tubes! You may use your own camera or the lab camera. Have your instructor upload the pictures to Blackboard to share with the class.

PART III: Monitor the Progress of your Remediation

1. Continue to observe the tubes every day for several weeks. If possible, invert the tubes 2 to 3 times each day to simulate wave action and promote intermixing. Compare the inoculated tubes to the non-inoculated controls under different conditions.

2. Carefully note changes in your lab notebook. How do you know when the oil is being degraded? How long does it take to degrade the oil sample?

3. Capture images of your tubes at least once a week.

4. Summarize your results in a table and hand in with your lab report.

5. Based on the data you collected during your simulated oil spill, what conditions does your lab recommend to degrade oil at a polluted site?

LAB UNIT 13 – DNA FINGERPRINTING BY SOUTHERN BLOT ANALYSIS

OBJECTIVES

Your performance will be satisfactory when you are able to:

- Understand the molecular basis of RFLP analysis using a Southern Blot
- Perform a Southern Blot

INTRODUCTION

DNA fingerprinting (also called DNA typing) is a method that allows for the identification of the source of unknown DNA samples. DNA fingerprinting involves the electrophoretic analysis of DNA fragments generated by restriction enzymes. No two individuals have exactly the same pattern of restriction enzyme recognition sites. There are several reasons for this. A large number of alleles exist in the population. Alleles are alternative forms of a gene. Alleles result in alternative expression of genetic traits which can be dominant or recessive. Chromosomes occur in matching pairs, one of maternal and the other of paternal origin. The two copies of a gene (alleles) at a given chromosomal locus constitute an individual's unique genotype. It follows that alleles have differences in their base sequences which consequently creates differences in the distribution and frequency of restriction enzyme recognition sites. Other differences in base sequences between individuals can occur because of mutations and deletions. Such changes can create or eliminate a recognition site. Variations in the lengths between these recognition sites are known as restriction fragment length polymorphism (RFLP). RFLP's are a manifestation of the unique genetic profile, or "fingerprint", of an individual's DNA.

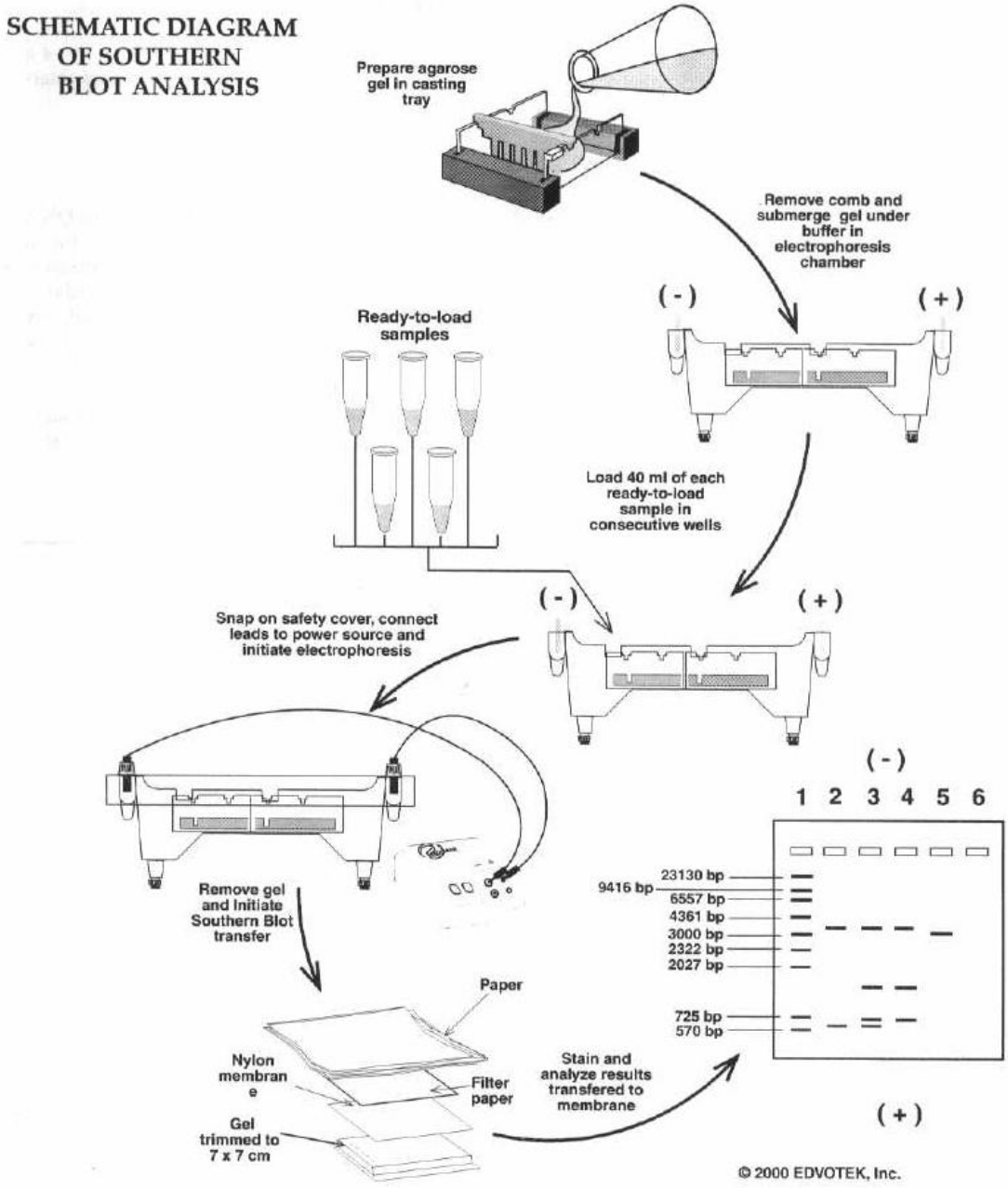
RFLP analysis of DNA is facilitated by Southern blot analysis. A replica of the electrophoretic pattern of DNA fragments in the gel is made by transferring (blotting) to nylon membrane. This is done by placing the nylon membrane on top of the gel after electrophoresis and transferring the fragments to the membrane by capillary action. The DNA becomes permanently adsorbed to the membrane, which can be manipulated much more easily than the agarose gel.

Visualization of DNA samples is accomplished through the use of well-defined probes. Probes are single stranded fragments of DNA commonly used for genetic identification at a specific chromosomal locus. Multiple probes detect multiple sequences and can yield 20 to 30 bands. Using multiple probes means the chances of two people chosen at random showing the same banding pattern is enormously remote. For example, it is calculated that two unrelated individuals having the identical DNA pattern detected by two probes is 1 in 30 billion. It should be noted that the total human population on earth is only 7 billion.

This experiment demonstrates the use of **Southern Blot Analysis** to determine paternity identification. In such a determination, DNA samples obtained from the mother, the child and possible fathers are fingerprinted. A child's DNA is a composite of its parents' DNA. Therefore, comparison of DNA fragmentation patterns obtained from the mother and child will give a partial match. Bands in the child's fingerprint that are not present in the mother's must have been contributed by the father.

In this hypothetical case, DNA was extracted from samples obtained from two possible fathers of a child. Their DNA was cleaved with the same restriction enzyme in separate reactions. The objective is to analyze and match the DNA fragmentation patterns after agarose gel electrophoresis and determine if Father 1 or Father 2 is the biological parent of the child.

This experiment employs a colorimetric detection system based on an enzymatic color change.



Lab Unit 13-A: Electrophoresis & Transfer DNA

DAY 1

PART I: SEPARATING DNA RESTRICTION FRAGEMENTS ON AGAROSE GEL
The samples are DNA that has been cut with restriction enzymes and labeled with biotin in order to aid in the detection of the DNA samples. The DNA standard is also labeled with biotin.

1. Pour a 0.8% agarose gel in 1X TAE in a 7 x 7cm tray (takes 30 ml agarose). Use a 10 well comb. Refer to Appendix for instructions on making agarose gels.

   NOTE:
   - If you do not have a 10 well comb you will need to pour two gels.
   - ***Do not add any stains*** (such as SYBR Safe or ethidium bromide) to your agarose gel. This will interfere in the procedure.

2. While the gel is cooling, heat DNA samples for two minutes at 65°C. Allow the samples to cool for a few minutes at room temperature on the bench top.

3. Load 35 µl of each sample in microcentrifuge tubes A-I into the wells in consecutive order. Record the order loaded on a gel documentation form.
   a. Standard
   b. Mother Cut with enzyme 1
   c. Mother cut with enzyme 2
   d. Child cut with enzyme 1
   e. Child cut with enzyme 2
   f. Father 1 cut with enzyme 1
   g. Father 1 cut with enzyme 2
   h. Father 2 cut with enzyme 1
   i. Father 2 cut with enzyme 2

4. Run samples for approximately 30 min at 100 volts. Time will vary depending on the gel apparatus used. Stop when the dye reaches approximately 3/4th the length of the gel. Do not run too long or the DNA fragments will run off the bottom of the gel.

PART II: TRANSFER DNA TO NITROCELLULOSE
After electrophoresis, the gel is sequentially treated in HCl and NaOH. The HCl treatment introduces apurinic sites in DNA which makes phosphodiester bonds at these sites labile and therefore introduces nicks in double stranded DNA. The NaOH treatment disrupts the interstrand hydrogen bonds between the base pairs. This procedure causes double stranded DNA restriction fragments to be converted into single stranded form.

SAFETY NOTES: During this step you must wear gloves and safety glasses when handling the gel in the HCl and NaOH solutions. Dispose of HCl and NaOH waste in a labeled waste beaker together to neutralize the pH before dumping down the sink with lots of water.

1. Depurinate the agarose gel by placing it in a small tray containing 100 ml of 0.25M HCl.
    a. Incubate at room temperature for ***NO MORE THAN 8 minutes***. Make sure the gel is immersed in the liquid and agitate periodically (or place on a shaker at a very low speed). Stop depurination if the dye becomes completely yellow.
    b. Holding onto the gel with a gloved hand, carefully discard the HCl solution in a waste beaker.
    c. Rinse the gel with several changes of 100 ml of distilled water.

2. Soak agarose gel 15 minutes in 100 ml of DNA Denaturation Solution (0.5M NaOH/0.6M NaCl). Make sure the gel is immersed in the liquid.
    a. Because of the density of the solution, the gel will float, so periodically shake the tray to immerse the gel.
    b. Discard the solution in the waste beaker.

3. Soak gel in a second 100 ml of DNA Denaturation Solution for 15 minutes.

4. While gel is incubating in the denaturation solution, prepare your membrane, filter paper and paper towel as follows. ***Wear gloves*** and keep your work area clean!
    a. Cut a piece of filter paper the size of the agarose gel.
    b. Cut a 4-5cm stack of paper towel the size of the gel as well.
    c. Wearing gloves and using forceps and scissors, trim the nylon membrane to the size of the gel. Note that the membrane is very smooth and thin and has the appearance of plastic. **DO NOT** confuse the membrane with the filter paper or with the paper separators also found in the packet of membranes. Ask your instructor if you are not sure which piece is the membrane.

5. Place a Tupperware dish on a flat level lab bench.
    a. After incubation in denaturation solution, remove the gel from the tray and place it well side DOWN into the dish. Inverting the gel places the smooth surface on top for contact with the membrane.
    b. Do not discard the DNA Denaturation Solution. Save it for pre-wetting the membrane.

6. Carefully pick up the membrane at edges with two clean forceps. Slightly bend the membrane in the middle and slowly wet (from the middle out) in the DNA Denaturation Solution you saved. Release the membrane and gently submerge it for 5 minutes until it is thoroughly saturated with DNA Denaturation Solution.
    a. Use forceps to remove saturated membrane from DNA Denaturation Solution and place it on top of the inverted agarose gel. Avoid introducing bubbles by picking up the membrane on both sides with clean forceps, slightly bend the membrane in the middle, place on gel and roll out. This will roll out any bubbles that may come in between the gel and membrane.

7. Prepare your transfer setup (ask your instructor where to set this up):
    a. Place the filter paper on top of the membrane and roll a 10 ml pipet (or mini-roller) across filter paper to remove air bubbles.

b. Carefully place the pre-cut stack of paper towels on top of the filter paper with an empty 400 ml beaker on top of paper towels.
c. Clearly label some part of this setup with your full name (or group name) and allow transfer to progress overnight.

PART III: DRYING MEMBRANE
(Instructor)
The instructor or lab tech may do this portion for you the next day.

1. Remove the tray, beaker, and all the paper towels.

2. Wearing rinsed gloves and using forceps, flip the stack (gel-nylon membrane-filter paper) over to lie on the filter paper.

3. Using a blue ink pen, draw through the six sample wells and trace their positions on the nylon membrane.

4. Using forceps, remove the gel from the membrane. Note the thickness and consistency of the now dehydrated gel. The gel can now be discarded since all further processing takes place with the nylon membrane.

   Optional: Post-transfer stain your gel in 50mL of 1XSYBR green for one hour to *observe the DNA which was not transferred to the nitrocellulose membrane*. This will give you several pieces of important information about "blotting" (transferring from an agarose gel to a membrane): Typically, there is rarely 100% transfer of all DNA from the gel to the membrane using this type of blotting method. This will allow you to see the relative efficiency of transfer of DNA using this method, and the efficiency of transfer based on size of the DNA.

5. Lay the membrane on a dry paper towel with the DNA side up (the side which was in direct contact with the gel). The bromophenol blue dye can be seen on the membrane. This is NOT the location of the DNA.

6. Using a blue ink pen, label the DNA side of the membrane with your lab group number or initials.

7. Place the membrane between two sheets of filter paper. Place in an 80°C incubator for 30 minutes. If time is an issue, this can be done until the membrane is dry.

Lab Unit 13-B: Colorimetric Detection of DNA

NOTES:

- Remember, do not handle membrane with bare hands. The oils from your hands will interfere with your detection.
- This is a 4 hr procedure if you use the maximum time for incubations. Ask your instructor for the incubation times. The minimum incubation times produce very good results.

1. Rehydrate the baked membrane for 5 minutes in 50 ml of 1X Detection Buffer. **Keep the DNA side of the membrane UP.** While the membrane is rehydrating, warm 50 ml of Membrane Shielding Buffer to 65°C. The shielding buffer (also known as blocking buffer) will block non-specific binding sites on the membrane, reducing background.

2. While holding the membrane down with forceps, pour the Detection Buffer out of the tray.

3. Add 50 ml of the warmed Membrane Shielding Buffer to the tray. The membrane should still have the DNA side facing up.

4. Cover the tray with the lid and place in a 65°C incubator for 30-60 min.

5. Turn the incubator down to 37^{o}C and carefully pour off the Shielding Buffer. Set the membrane aside (on the tray lid) and rinse out the tray with distilled water. Shake out the tray to remove excess water, but do not dry with paper towels because lint may increase the background.

6. Place the membrane back in the tray, DNA side up. Add 8 ul of SAAP to 10 ml 1X Detection Buffer. Mix well and pour over the membrane in the tray. Incubate the tray at room temperature for 10 minutes with gentle rocking to ensure that the membrane is covered with the solution. The streptavidin-alkaline phosphatase (SAAP) will bind to the biotinylated DNA during this incubation.

7. Remove the membrane from the tray and place it on the lid, as before. Wash out the tray with distilled water and shake out excess water. Place the membrane back in the tray, DNA side up. Rinse off the lid with distilled water.

8. Rinse the membrane in 100 ml of 1X Detection Buffer for 5 minutes with gentle rocking to wash off non-specifically bound SAAP.

9. Discard the solution and wash a second time in 100 ml of 1X Detection Buffer for 15 minutes with gentle rocking.

10. Discard the solution and wash a third time in 100 ml of 1X Detection Buffer for 15 mintues with gentle rocking

11. Carefully remove the membrane from the tray and place it on the tray lid (do not let dry). Add 8 ml of Color Development solution to the middle of the Tupperware. Place the membrane **DNA side DOWN** into the tray and cover with the lid. Place in a 37°C incubator for 30-60 minutes.

    NOTE:
    - The color development solution should be made fresh just prior to use!
    - The color begins to develop in 15 min and is done by 30min. If you cannot see well defined blue/purple bands allow incubation to proceed for 60 min. You can easily monitor the progression of the color development by holding up the Tupperware and looking at the membrane from underneath.
    - The color development solution contains a mixture of 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and blue tetrazolium (NBT). BCIP is a substrate for SAAP and will therefore bind to the DNA. In the presence of BCIP, NBT becomes reduced. Upon reduction NBT is converted into an insoluble blue product that precipitates on the nylon membrane indicating the position of the transferred DNA.

12. Place the membrane on the tray lid (do not let dry). Completely discard the color development solution and rinse the tray with distilled water.

13. Soak the membrane in 100 ml of Termination solution for 5 minutes. Protect the membrane from strong direct light.

14. Allow the membrane to dry in the dark between 2 sheets of filter paper. Prolonged exposure to bright light will increase the background. Alternatively, you may dry the membrane for 5 minutes at 80^{o}C in between two pieces of filter paper (if you don't do this, the membrane will curl up!).

15. Capture an image using the gel documentation system (white light), or with a camera. Good images can be obtained by taking a picture when the membrane is fully wet or fully dry.

16. Affix a well labeled picture of your gel to the gel documentation form. Submit the gel documentation form with your report.

Lab Unit 13 - DNA Fingerprinting by Southern Blot Assignment

1. Why do different individuals, even siblings, have different restriction enzyme recognition sites?

2. What is the function of probes in DNA paternity tests? Why are multiple probes used?

3. Why is a Southern Blot required for forensic and paternity DNA fingerprinting testing?

4. What was the purpose of soaking the membrane in HCl and the DNA denaturation Solution? What would you suspect might happen to your results if you left this step out?

5. Who is the father of this child? How can you tell?

LAB UNIT 14 – INDIRECT ELISA FOR HIV-1 DETECTION

(Based on Edvotec kit: Simulation of HIV-1 Detection)

OBJECTIVES

Your performance will be satisfactory when you are able to:

- Understand the molecular basis of the human immunodeficiency virus and the pathogenesis of acquired immune deficiency syndrome
- Perform an enzyme linked immunoassay (ELISA) in the context of clinical screening of serum samples for antibodies to the virus

INTRODUCTION

Mechanism of HIV Infection

An individual can be infected with HIV through an abrasion in a mucosal surface (e.g. vaginal and rectal walls), a blood transfusion or by intravenous injection with a contaminated needle. Virus or virally infected cells are found in bodily fluids such as semen and blood. During the early stages of infection in an immunocompetent person the HIV virus elicits humoral and cellular immunity responses that result in a variety of circulating IgG molecules directed at several viral epitopes. However, since the virus has a high mutation rate the variants survive and produce progeny having a similar capacity to escape immunosurveillance.

Unlike other cellular DNA polymerases, HIV DNA polymerase (reverse transcriptase) has a high error rate (1 in 10^4). These frequent mutations continually change the viral protein epitopes. This is believed to be the main mechanism of HIV immunoevasion. The most important target for the virus is hematopoietic cells such as bone marrow derived monocytes, myelocytes and immune system lymphocytes. Infection of immune system effector cells such as T cells and macrophages ultimately produce the most profound clinical consequences. gp120 binds to the CD_4 receptors on the surface of T helper (T_H) cells. These receptors are membrane bound glycoproteins involved in T cell maturation from precursor cells. T_H cells are required for the body's overall immunological responses. The viral lipid bilayer fuses with that of the cell's membranes and the viral protein capsid becomes internalized via receptor mediated endocytosis. Subsequently, the rest of the CD_4 receptors are internalized and gp120 appears on the T cell surface.

HIV Replication and Transcription

Through a complex mechanism involving several events, the reverse transcriptase synthesizes a double stranded DNA copy of the genomic RNA template. The tRNA molecule acts as the primer for the first strand synthesis. The reverse transcriptase, RNase H activity, degrades the RNA strand of the RNA-DNA duplex and the polymerase activity synthesizes a complementary DNA strand. The DNA reverse transcripts (copy DNA) migrate into the cell nucleus where they become covalently integrated into the cellular genomic DNA. The integration is catalyzed by the HIV integrase. The copy DNA integrates via specific, self-complimentary sequences at both ends called long terminal repeats (LTRs). These sequences also have important functions in viral transcription. The integrated copy DNA is called proviral DNA or the provirus. The provirus

enters a period of latency that can last for several years. The proviral DNA is replicated along with the cellular DNA and can be inherited through many generations. The HIV proviral DNA contains the major genes common to all non-transducing retroviruses. These genes are gag, pol and env. HIV also contains five or six other genes that are much smaller. Retroviral transcription is a complex process producing a variety of RNAs. Promotion of transcripts is controlled in the LTR and transcriptional termination signals are located in each major gene. Those RNA transcripts that remain unspliced become packaged in the new viral particles. The gag gene is translated into a polypeptide that is cleaved by a viral protease into four proteins that form the inner shells. Specific protease inhibitors are clinically being used to inhibit protein processing and control the further spread of the HIV virus in patients suffering from AIDS. The pol gene encodes the reverse transcriptase and the integrase which is responsible for the genomic incorporation of copy DNA. The env gene en-codes the surface glycoproteins the viral particles acquire as they bud from the cells.

Immunological Response

Macrophages are circulating monocytes and are involved in the non-specific engulfment of foreign material and normal cellular debris. These materials are degraded in the lysosomes of the cells. Peptides from foreign degraded proteins are transported to the macrophage surface where they remain bound by specialized receptors. Immunologically inactive T_H cells interact with these surface bound antigen-receptor complexes which enables them to become fully activated. HIV infects macrophages by binding to the cells' CD4 receptors. Fully activated T_H cells secrete several types of protein factors collectively known as lymphokines. Several of these factors are the interleukins which stimulate antibody secretions from B cells enable macrophage activation, stimulate general T cell growth and activate cytotoxic T cells. Cytotoxic T cells are involved in the actual destruction of foreign cells and body cells infected with different viruses. Inactive T_H cells that have been infected by HIV remain in a relatively quiescent state similar to uninfected cells. When a T_H cell containing provirus undergoes antigenic activation the integrated copy DNA becomes open to the transcription of viral RNA.

Viral replication causes the destruction of the T_H cells. Infected T_H cells also form syncytia, i.e. fused cells. Syncytia occur since the gp120 on the infected T cell surface binds to CD4 receptors on other T_H cells. Cell to cell transmission of virus can occur in syncytia without the need for a free viral intermediate. Replication of virus also proceeds in activated macrophages which causes cell death and release of infectious viral particles. These and other events ultimately cause complete immune system collapse. The long latency period after HIV infection can be understood in terms of T_H cell activation. Only certain T_H cells are capable of responding to a particular antigen. HIV infected but asymptomatic individuals will experience the usual exposure to chemicals, viruses and bacteria. Each infection activates a subpopulation T_H cells containing the provirus which eventually leads to the death of these cells. After successive waves of infections the population of T_H and macrophage cells becomes depleted and clinical AIDS develops. Thanks in large part to new antiviral treatments, there are a large number of individuals who have coexisted with HIV for over 10-20 years. Although the reason for this coexistence with HIV is not understood, the immune system appears to remain intact. Such individuals tend to eat well, exercise and practice stress reduction techniques. Genetic analysis may determine whether or not subtle genetic differences in the immune system are significant factors.

Description of the HIV Screening Simulation

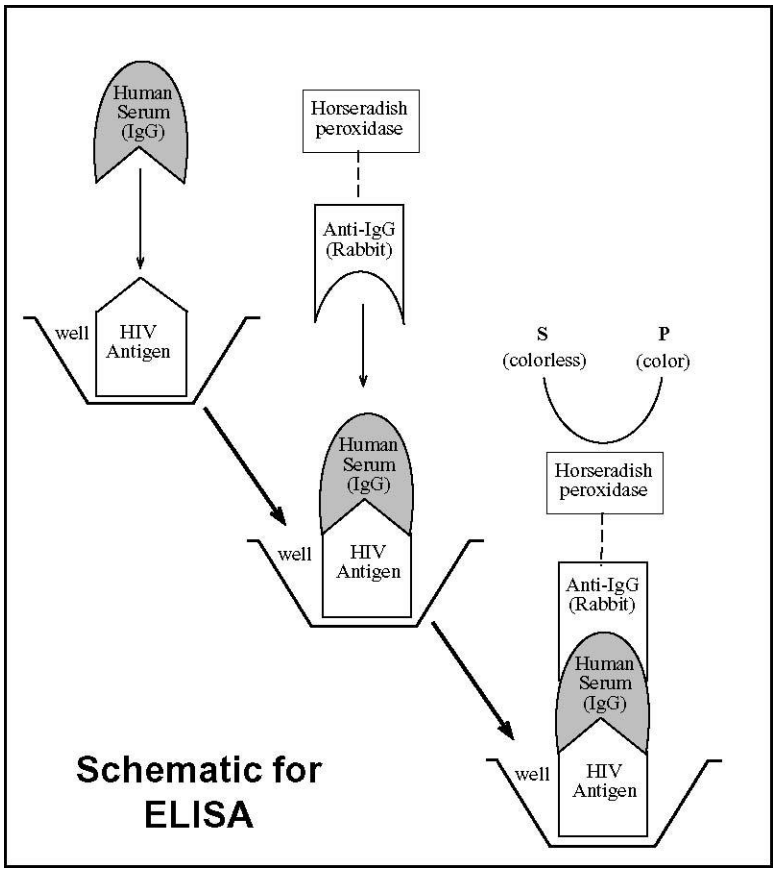
Enzyme linked immunosorbent assay (ELISA) tests were originally developed for antibody measurement. These immunoassays have also been adapted to successfully detect samples that contain antigens. This ELISA simulation experiment has been designed to detect a hypothetical patient's circulating IgG directed towards the viral (HIV) antigen.

ELISAs are done in microtiter plates which are generally made of polystyrene or polyvinyl chloride. The plates are somewhat transparent and contain many small wells, in which liquid samples are deposited. First, the antigens are added to the wells where some remain adsorbed by hydrophobic association to the walls after washing away the excess. The antigens can be the whole HIV lysate, specific HIV proteins, or a mixture of the two. There is no specificity involved with the adsorption process although some substances may exhibit low binding to the walls. In certain cases the antigens can be covalently cross-linked to the plastic using UV light. After washing away unadsorbed material, the unoccupied sites on the walls of the plastic wells are blocked with proteins, typically gelatin or bovine serum albumin.

Infection by HIV-1 causes the individual to mount an antibody response which eventually results in plasma IgG molecules that bind to different HIV proteins (and/ or different areas or the same polypeptide). In this experiment, if these antibodies are present in the plasma sample they will bind to the adsorbed antigens in the well and remain there after washing.

A solution containing the IgG antibody that binds to any kind of human IgG, is then added to the wells. If the primary antibody has remained in a well, then the secondary antibody will bind to it and also remain attached after washing. These secondary antibodies are usually raised in rabbits and goats immunized with human IgG fractions. The second IgG antibodies are purified and covalently cross linked to horseradish peroxidase. This modification does not significantly affect the binding specificity and affinity of the antibody or the enzymatic activity of the peroxidase.

After washing, a solution containing hydrogen peroxide and aminosalicylate is added to each well. Peroxidase possesses a high catalytic activity and can exceed turnover rates of 10^6 per second. Consequently, amplification of a positive sample can occur over several orders of magnitude. Many hydrogen donor co-substrates can be used by peroxidase. These co-substrates include o-diansidine, aminoantipyrine, aminosalicylic acid and numerous phenolic compounds that develop color upon oxidation. The substrate solution added is nearly colorless. Peroxidase converts the peroxide to $H_2O + O_2$ using the salicylate as the hydrogen donor. The oxidized salicylate is brown and can be easily observed in wells containing anti-HIV-1 IgG (positive plasma). It should be noted that polyclonal antibody preparations to a given antigen can have variable binding affinities due to differences in the immunological responses between animals. Different immunizations with the same antigen in the same animal can also produce variable binding affinities. The use of monoclonal antibodies directed against a single epitope eliminates this variability. Western blot analyses of positive samples are used to confirm infection by HIV.



Steps in the INDIRECT ELISA:

- Antigens are bound to ELISA plate directly or via an antibody (this has already been done in your plates).
- Serum is added to the wells. If the serum contains the antibodies for the HIV virus, they will attach to the antigens bound to the wells.
- Wells are washed thoroughly so that when a test is performed to determine the presence of antibodies, only those that attached to antigens, which were attached to the plate bottom, will be left.
- A color-changing agent is added which will react with an enzyme that was artificially attached to the antibody to produce a colored reaction.
- Wells that changed color have an antibody present attached to the antigen for which you are testing

PROCEDURE

1. Place the microtiter plate vertically and label as follows:
   Row 1 (- control)
   Row 2 (+ control)
   Row 3 (Donor Serum 1)
   Row 4 (Donor Serum 2)

2. Label 4 transfer pipets -, +, DS1, and DS2 to correspond with the wells. When instructed to remove liquids in the experimental procedure, be sure to use the appropriately labeled pipet.

3. To all 12 wells, add 100 ul of "HIV" (viral antigens).

4. Incubate for 5 minutes at room temperature (remember to record the actual temperature of the room!).

5. Remove all the liquid (viral antigens) with the appropriate transfer pipet.

6. Wash each well with Phosphate Buffered Saline solution (PBS) by adding 200 ul to each well and then removing all of the liquid from each well with the appropriately labeled transfer pipet.

   NOTE: In research labs, following this step, all sites on the microtiter plate are saturated with a blocking solution consisting of a protein mixture, such as BSA. This experiment has been designed to eliminate this step and save time.

7. Add reagents as outlined below, remembering to change tips for each reagent:
   Add 100 ul of PBS to the three wells in Row 1. (This is the negative control.)
   Add 100 ul of "+" (positive) to the three wells in Row 2. (This is the positive control.)
   Add 100 ul of Donor Serum "DS1"to the three wells in Row 3.
   Add 100 ul of Donor Serum "DS2" to the three wells in Row 4.

8. Incubate at 37°C for 15 minutes.

9. Remove all the liquid from each well with the appropriately labeled transfer pipet.

10. Wash each well with PBS by adding 200 µl to each well and then removing all of the liquid from each well with the appropriately labeled transfer pipet.

11. Add 100 µl of the anti-IgG peroxidase conjugate (2°Ab) to all 12 wells.

12. Incubate at 37°C for 15 minutes.

13. Remove all the liquid from each well with the appropriately labeled transfer pipet.

14. Wash each well with PBS by adding 200 µl to each well and then removing all of the liquid from each well with the appropriately labeled transfer pipet.

15. All 100 µl of the substrate to all 12 wells.

16. Incubate at 37°C for 5 minutes.

17. Remove the plate for analysis.

18. If color is not fully developed after 5 minutes, incubate at 37°C for a longer period of time.

19. Capture an image of your microtiter plate and affix to your notebook and label clearly.

20. Discuss the results of your experiment. Which patient(s) was HIV positive? How do you know?

Lab Unit 14 - ELISA for HIV Detection Assignment

1. What do the terms below mean?
   a. AIDS

   b. HIV

   c. ELISA

3. What does the body's immune system produce against viral infections? What does this product do in the HIV test?

4. What are the characteristic ways that a person can get HIV? How can a person avoid getting HIV?

5. Discuss your results.
   a. What does your positive control look like?

   b. What does your negative control look like?

   c. Which patient(s) was HIV positive? How do you know?

LAB UNIT 15 – MINING BIOLOGICAL DATABASES ON THE INTERNET

OBJECTIVES

Your performance will be satisfactory when you are able to:

- Locate scientific publications and biological databases of DNA and protein sequences on the Internet
- Retrieve and compare sequence information from databases
- Compare evolutionary relatedness and draw phylogenetic trees from sequence comparisons
- Study protein structure and function from database comparisons

INTRODUCTION

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

Human Genome Project goals were to:

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

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

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

Another goal of the Human Genome Project is to sequence the genomes of other species of interest, such as model organisms used by biologists, pathogens of medical importance, and crop plants of agricultural importance. This goal has also been exceeded, and today the genomes of over 900 species have been at least partially sequenced.

This large international project has not only collected enormous databanks of DNA sequence information from the genomes of dozens of species, it has also promoted the development of highly automated strategies for studying nucleic acids and proteins. Much of the reason for the

success of the Human Genome Project comes from the introduction of new **high throughput** technologies for DNA sequencing that can use automation and robotics.

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

For example of how this can work, when a scientist sequences a new stretch of DNA, this information becomes meaningful when a new gene can be discovered in the sequence. Often these genes are hard to spot, especially in eukaryotic genomes, since the majority of DNA in these organisms does not code for genes; in the human genome, genes account for less than 2% of the DNA sequence. A computer program can search the sequence for tell-tale signs of a gene from sequence data, by searching for DNA consensus sequences for translational start and stop codons, a ribosome binding site, intron splicing sites, and a promoter. In eukaryotes, a region of high guanosine and cytosine (G and C) content is frequently found near clusters of genes, so mapping GC content along a chromosome can also help to locate the presence of a gene in the DNA sequence. Since gene sequences can be highly conserved between different species, an especially powerful approach for identification of a gene in sequence data is to search databases of DNA sequences looking for **sequence similarities**. Due to these so-called ***in silico*** (in a computer program) tools and the dramatic growth of DNA sequence databases, the rate of gene discovery has increased exponentially.

The power of bioinformatics approach for the discovery of genes has been proven with the completion of the yeast genomic sequencing project in 1996. Once the genome was fully sequenced, bioinformatics approach could be used to scan for and identify genes. The genes discovered this way could be compared with the large number of genes that had already been discovered through more classical molecular and genetic techniques. The results were remarkable. Before the yeast genome was sequenced in 1996, an international collaboration of scientists studying the genetics of this model organism had identified an impressive 2,000 genes by conventional genetic analysis. When the yeast genome sequencing was completed, bioinformatics searches for similarities of DNA sequences from other organisms were able to locate an additional 2,000 genes. This means in less than one year, a single laboratory using DNA sequencing and computer searches of sequence data could both duplicate and double the gene discovery of a 20-year international effort.

Once a gene has been identified, many new questions can be asked: what kind of protein does it code for and what is its function? How does it interact with other molecules of the cell? Is it expressed at all times as a so-called "housekeeping gene", or is it a developmentally regulated gene? Is its expression tissue-specific? Is it expressed in response to an environmental factor? These questions are the same questions that have been asked by molecular and cell biologists for decades, usually by studying one gene, and its protein or proteins, at a time. With the copious amounts of information coming from the genomics project, however, biologists can ask the same questions about more complex systems. Instead of asking about one protein at a time, biologists

can now ask questions about hundreds of proteins at a time, looking for patterns of structure and patterns of expression. Looking at the proteins on a genomic scale is a new field now called **proteomics.** When a new gene has a sequence that has been found to be homologous to a gene in a database that has already been characterized, sometimes many of these questions about protein structure and function can be answered quickly by the bioinformatics approach. For example, the 2,000 new genes discovered by the yeast genomic sequencing project, discussed above, matched genes of other organisms whose function had already been determined.

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

Questions about how genes are regulated are rapidly being answered by new devices called **DNA chips** or **microarrays**. These devices are designed to allow many hybridization experiments to be performed in parallel. Oligonucleotides are synthesized on a glass surface similar to a microscope slide. Using photolithography technology used to etch semiconductor circuits into silicon for chips used in the computer industry, arrays of oligonucleotides can be laid out at a density of up to one million different oligonucleotides per square centimeter. By judicious selection of oligonucleotide sequences, complementary DNA for of all the genes expressed in an organism can be assigned at specific positions on a given microarray. A microarray can be used to easily determine which genes are being expressed in a cell. This involves harvesting the mRNA from a cell, labeling the mRNA by covalent linkage to a colored molecule, and allowing the labeled mRNA to hybridize with the oligonucleotides on the microarray. The microarray is said to be "interrogated" in this way by the labeled mRNA. The genes that are actively being transcribed into mRNA by the cell are then identified by viewing the microarray under a microscope to see which oligonucleotides were hybridized with the labeled mRNA. Although there are many variations in the design and hybridization of microarrays, they all produce massive amounts of data from single experiments, requiring computer-assisted analysis and archiving of the results.

The power of these techniques is having a major impact in medical research. For example, a biotechnology company called Sagres Discovery in Davis, California, announced in 2002 that it has identified over 1000 new oncogenes in the mouse genome after just one year of research using this approach. The practical applications of database information and new bioinformatics tools are far-reaching. For example, with the discovery of a new oncogene and its structure and function comes the possibility of a new anti-cancer drug or treatment strategy. The discovery of disease-causing genes can lead to diagnostic techniques for inherited diseases. Plant geneticists are using the detailed information coming from genomics to identify DNA markers to speed the

breeding of new traits in our crop plants. With the discovery of DNA sequence **polymorphisms** (variations in allele frequencies within populations) comes DNA fingerprinting strategies for identity testing in forensics. DNA fingerprinting is currently revolutionizing the criminal justice system.

In this exercise, you will use a computer to access GenBank, the database repository of all DNA and protein sequences housed at the National Center for Biotechnology Information (NCBI) at the National Institutes of Health (NIH).

In Exercise A you will compare amino acid sequences of proteins from different organisms to study their evolutionary relatedness. In Exercise B you will use a DNA sequence to find a protein translational sequence (**ORF** for **open reading frame**) of a plant defensin and study the function of this protein by finding homologous sequences in the protein database. In Exercise C you will use databases of the biological literature, available online through the National Library of Medicine, to discover what researchers are reporting for the structures and functions of plant defensin proteins.

Lab Unit 15-A: Hemoglobin Bioinformatics

PROCEDURE

Part A: Determine the evolutionary relatedness of species through comparisons of protein sequences

1. You will compare the sequences of the protein hemoglobin from bats, birds, and mammals. Decide whether you want to do your work with alpha-hemoglobin or with beta-hemoglobin. These are the two protein chains that carry oxygen in the circulatory systems of animals. Both of these proteins have been studied extensively in a large number of species and should work equally well. Alternatively, you may want to collaborate with a partner and do companion searches, one doing searches with α-hemoglobin, and one doing searches with β-hemoglobin. At the end of the exercise, you can compare your results with each other to determine whether your different proteins showed the same evolutionary relationships between species of bats, birds, and mammals.

2. Go to http://www.uniprot.org/uniprot. If you have problems accessing the site because the URL has changed, your instructor will help you find the new URL.

3. In the ***Protein Knowledgebase*** (UnitProtKB), under ***Query***, type "alpha hemoglobin" or "beta hemoglobin" and click ***Search***. The results of this search will come up on your screen. How many protein sequences were reported to you from this query?

   _____

4. You may scroll down and look through this long list of α-hemoglobin/β-hemoglobin sequences for one from a bat species, but it may be faster to narrow your search. Go back to ***Query*** and type "bat alpha hemoglobin" or "bat beta hemoglobin" and click ***Search.*** When you get the results of this search, how many sequences of alpha hemoglobin/ beta hemoglobin did you get for bat species?

   _____

   ⇨ NOTE: Check the species names and common names for each of the α-hemoglobin/β-hemoglobin in this sequence report to make sure that they are bat sequences. Sometimes a search won't recognize the difference, for example, between "bat" and some other word, such as "wombat"!

5. Select one bat α-hemoglobin/β-hemoglobin sequence to save to a file by clicking on the color-highlighted ***Accession*** number for that protein sequence. An **accession number** is how protein sequences are identified and archived in databases. In the case of α-hemoglobin sequences, the ***Entry name*** will start with the letters "HBA." The symbols for all α-hemoglobin will begin with "HBA."

⇨ **NOTE:** If you are working with a partner who is doing a companion study with β-hemoglobin, you will have to collaborate on your selection of which bat α-hemoglobin sequences to save.

6. The page that opens will contain information about the sequence, such as the taxonomy of the organism from which it came. Mid-way of the page, you will see a "***Sequences***" section where you will find the protein sequence written with single-letter designations of the amino acids. Here is where you will find the ***FASTA*** hyperlink. This is the best way to save sequence information to your file, because it is a sequence format that all computer search programs can understand. ***Click the FASTA link and this will bring up a page containing the sequence.***

7. Copy the amino acid sequence to a file:
   a. Highlight the amino acid sequence (the entire script on your FASTA formatted page)
   b. Right click, and select ***Copy***
   c. open Microsoft Word, right click, select ***Paste***
   d. save your file

8. Return to the web page with the list of bat alpha hemoglobin sequences//β-hemoglobin (clicking twice on the Back button on the web browser will get you there). Identify another sequence for a bat α-hemoglobin and repeat the process of highlighting the FASTA formatted amino acid sequence to your file. Save all your FASTA formatted α-hemoglobin/β-hemoglobin sequences together in one file.

9. When you have saved two α-hemoglobin/β-hemoglobin sequences from two bat species, repeat steps to get 2 sequences from bird species and 2 sequences from mammalian species. It doesn't matter which species you choose, as long as 2 are from birds and 2 are from mammals. You might want to choose species that you think are related to bats. If you are collaborating with a partner searching for β-hemoglobin sequences, your partner should search for the same species that you have chosen.

   => **IMPORTANT:** Be aware that if you are limiting your search for bird α-hemoglobin sequences with the keyword "bird," the search will only locate protein entries where the word "bird" appears. If the entry was archived under other descriptions such as "hawk" or "eagle" or "penguin," you will not find entries using the keyword "bird."

10. When you have saved six α-hemoglobin/β-hemoglobin sequences to your file (two from bats, two from birds, and two from mammals), go to http://www.genome.jp/tools/clustalw/ CLUSTALW is a computer program that you can use to search for sequence similarities between many sequences at a time and display regions of alignment.

11. Copy your entire file of sequences into the textbox. Note that the sequence descriptions proceeded by the ">" mark will be copied in with the protein sequences. This will not be a problem with your search. Without changing any of the default settings on your search, click on the blue colored ***Execute Multiple Alignment*** bar.

12. The next page will show the alignment of amino acid sequences for the 6 proteins that you have retrieved from the SWISSPROT database, using the single-letter designations for amino acids. An asterisk will appear along the bottom row of amino acid alignment at positions where there is an amino acid that is found in all 6 proteins. These amino acids are said to be **highly conserved**, since they haven't changed since these species diverged from a common ancestor.
    a. How many of the amino acids are found to be the same in all of the 6 α-hemoglobin/β-hemoglobin sequences in your alignment?

    b. What percentage of all the α-hemoglobin/β-hemoglobin amino acids are conserved in all 6 proteins?

    c. Are there any specific regions of the α-hemoglobin/β-hemoglobin sequences that are especially conserved?

       Is one end of the molecule more conserved than the other? Describe your observations.

    d. Are there any amino acids that appear more frequently in conserved regions of the protein than in the non-conserved regions? If so, which amino acids are they? Go to the table at the end of this Lab Exercise to decode the single-letter designation for amino acids.

    e. If you did find amino acids that were more frequently conserved in your alignment report, were the ones with side groups that were nonpolar, polar, or charged?

13. At the top of your CLUSTALW report, you will find the exact percentages of amino acids in the sequence alignment that are identical when comparing only two sequences at a time. For example, if your report says "Sequences (1:2) Aligned. Score: 87.2", this means that when the first two sequences saved to your file were aligned, 87.2% of the amino acids were identical in both sequences. Transfer these percentages into a table format, in which the species whose sequences you have aligned are headers for both the columns and the rows. Your table should look similar to this:

Table 1: Percent identity in amino acid alignment for α-hemoglobin

SPECIES	(bat #1 name)	(bat #2 name)	(bird #1 name)	(bird #2 name)	(mammal #1 name)	(mammal #2 name)
(bat #1 name)	100	87.2				
(bat#2 Name)		100				
(bird #1 name)			100			
(bird #2 name)				100		
(mammal #1 name)					100	
(mammal #2 name)						100

Notice that you need not fill out both halves of this table since the information is redundant.

From this table, can you see whether the α-hemoglobin/β-hemoglobin sequences are more similar for bats and birds, compared with bats and mammals? What does this suggest about the evolutionary relatedness of these species? Which species diverged from each other the most recently and have the most recent common ancestor? Which species have been divergent from each other the longest and have the most ancient common ancestor? **From the information in this you should be able to predict that bats are more closely related to either birds or mammals.**

14. A phylogenetic tree can present the relatedness of species from sequence similarity data such as your Table 1. These trees link species that are more closely related in branches, and the length of the branches is their evolutionary distance. You can draw a phylogenetic tree from your amino acid alignment report by pairing species that have the most sequence similarities to make short branches. Species who have fewer sequence similarities will branch from each other on the tree farther apart.

    The CLUSTALW on the page that your report appears on will automatically draw a phylogenetic tree for you. At the bottom of the page, click on the pull-down menu ***Dendrogram*** or ***Rooted Dendrogram***. Print out the tree that appears on the screen. Does the information on this tree agree with your analysis above of the *Percent identity in amino acid alignment for α-hemoglobins* table? Explain.

15. One way to evaluate the validity of the phylogenetic tree that you drew for bats, birds, and mammals is to compare it with trees constructed from sequences of other proteins. Compare your tree with a tree constructed by your partner searching for β-hemoglobin sequences. Does your tree derived from β-hemoglobin sequences agree with one drawn from β-hemoglobin sequences? Are the relative lengths of the branches the same?

_____

16. (OPTIONAL) Time permitting, repeat the comparisons that you made with other species such as the following:
    a. Compare whales to mammals and fish.
    b. Compare reptiles to birds and mammals.

B. Investigating proteins from DNA sequences

1. Go to http://www.ncbi.nlm.nih.gov.

   The ***proteins database*** allows you to do searches based on the type of protein that you are interested in. In the pull down menu select "***Protein***" and Type "plant defensins" in the search box, and click on ***Search*.**

2. The page that comes up will report to you how many plant defensins DNA sequences have been deposited in GenBank. How many sequences did your search report?

   _____

3. The most recently submitted sequences are reported at the top of the list. Scroll down to the end of the list to the first sequences that were submitted for plant defensin. There will be a series of sequences that were submitted at the same time that are listed separately. You can access more information about these sequences by clicking on the colored identifier for the sequence. The identifier number is called an **accession code**, and is assigned by NCBI when a DNA sequence is submitted, for the purposes of archiving the information. In the case of these plant defensin proteins, the accession codes should be something like *AX046743***.**

4. When you click on the accession code for a sequence, a page will come up that gives a more complete description of the sequence. It will contain information such as who submitted the sequence, the type of organism that it came from, whether the sequence was reported in a scientific journal or not, and some information about the sequence itself. Click on a few of the earliest plant defensins and answer the following questions.

   a. Who submitted these sequences? _____

   b. When were these sequences submitted? _____

   c. Most scientific journals and the U.S. Patent Office now required that newly sequenced DNA that is being reported must be submitted to GenBank at the time

the journal article is published or the patent is granted. Are the first plant defensins submitted to GenBank linked to a journal article or to a patent?

5. The nucleotide sequence appears at the bottom of the page that comes up when you click on the accession code. The DNA sequence is numbered from the 5' to the 3' end of the sequence. If the start codon for translation of the nucleotide sequence is known, that information will be given under the category *Features* and will be given as "CDS" for "coding sequence". When the coding sequence is known, the amino acid sequence that is translated from the nucleotide sequence will be given in the single-letter amino acid designation.

   Are any the coding sequences known for the first plant defensins that were submitted to GenBank?

6. This first submission of plant defensin sequence data included sequences from four different species: *Dimorphotheca sinuata, Picramnia pentandra, Parthenium argetatum, and Nicotiana benthamiana*. Click on the accession codes until you have located sequences for a plant defensin from each of these species.

7. Copy four nucleotide sequences from this earliest submission of plant defensins, one from each of the four species, to a floppy disk. Since you will want to use this data for searches, save the sequences in FASTA format. For each sequence, do the following:
   a. click on the colored accession code AX046743
   b. highlight the nucleotide sequence that appears at the bottom of next page
   c. use the pull-down menu at the top of the page under "Summary" to select "FASTA"
   d. copy the sequence (click on "Copy" from the "Edit" pull-down menu)
   e. open Notepad (use your mouse to go from "START" in the lower left corner of your computer screen to "PROGRAMS" to "ACCESSORIES" to "NOTEPAD")
   f. paste the sequence into Notepad, name the file, and save it to your floppy disk (Drive A)

   ⇨ NOTE: It is OK to copy and save your FASTA-formatted sequence using a word processor program rather than Notepad.

   Repeat steps a-f for three other accession codes: AX046747, AX046751, and AX046767.

8. Since there was no journal article to read about these plant defensin proteins, we don't know anything about them except their name, where they were found, and what their DNA sequence is. If someone else has sequenced a homologous gene and reported the function of the gene product, you may be able to deduce some information about the plant defensins that you have selected.

   To search GenBank for a DNA sequence that is homologous to the four sequences that you have saved, click the *Back* button until you have returned to the NCBI page that you

began with, or go to http://www.ncbi.nlm.nih.gov again. Click on *BLAST* at the top of the page and select the blue-colored *Standard nucleotide-nucleotide BLAST*. Copy one of your nucleotide sequences from your file to the text box of BLASTN by highlighting the sequence and pasting it in. Click on *BLAST!* and on the next page click on *Format.*

⇨ **NOTE:** There is an excellent and easy-to-follow tutorial on how to perform BLAST searches of GenBank at this site: http://www.geospiza.com/education/

GenBank is an enormous database, so this search may take a few minutes to complete, especially during busy times of the day. When your BLASTN report comes up, it will show a *Distribution of Hits on the Query Sequence* that displays in a figure the length of sequence that matched or **hit** the sequence that you did your search on (your **"query" sequence**). The alignment is scored by color code for how well the sequences aligned: red is the best agreement of sequences. If you roll the mouse over these color-coded lines, information about the sequence represented in the line will appear in the information box above the figure.

Below the figure, an alignment report will appear that includes some information about the species from which the sequence was taken and a score for how well the sequence aligned with your query sequence. This score is based on a statistical calculation, where a high score indicates a high level of agreement. An **E value** is also listed for each sequence in the report. This is another statistical calculation that estimates the probability that the sequences matched just due to chance. The shorter the stretch of sequence that aligned, the higher the E value, since it is more likely that a short sequence aligned due to chance.

Conduct a **BLASTN** search for each of your 4 saved plant defensin nucleotide sequences one at a time. If a search using these DNA sequences of plant defensins saved to your file does not result in a high level of sequence identity, discontinue any future searches with these. You can tell whether your search has found a good "hit" either visually by looking at the color-coded figure that shows regions of sequence homologies, or by reading the score and E values below this figure. If your search results in a long stretch of sequence homology, continue on and answer the following questions.

a. What is the name of the gene that gave you a good BLASTN hit (i.e. align well in a long stretch of sequence homology and has a Score of >100 and an E value of less than e^{-20})? What is the species and genus that this sequence came from?

_____

b. Scroll down below the color-coded alignment figure and below the blue-coded sequence alignment report. Look for a green *Alignments* header on the right side of the screen and look at the first aligned sequence. The first sequence alignment in this list will be the sequence that gave you the best hit for your query sequence. Of the 142 residues in your query sequence, how many are identical in the best hit sequence?

_____

c. Click on the color-coded accession code for this "best hitting" nucleotide sequence. The page that comes up will give you information about this DNA sequence. This sequence was published in a journal. What was the title of the journal article?

_____

If this article has been archived in the PubMed or Medline database of scientific journals, there will be a blue-colored number to the right of Medline or PubMed that will link you to these databases. If you click one of these numbers, you find more information about the journal article in which this DNA sequence was first reported. Frequently an abstract of the article is archived here. Could you find an abstract of this article in Medline or PubMed?

_____

The page with information about the best hitting nucleotide sequence will give the nucleotide sequence at the bottom of the page, and the amino acid sequence higher on the page. **Find the amino acid sequence for this protein and copy it to your file**. Follow the same procedure above for saving sequences to Notepad first in order to create this file. You will use this file to search GenBank for matching protein sequences in the next step of this exercise.

9. Our "data mining" for information about plant defensins similar to the ones patented by DuPont has not been very fruitful so far, but there are other strategies that we can try. To expand your search for homologous proteins, you can switch from nucleotide searches to a protein search, using the protein sequence that you found to be homologous to some of the DuPont plant defensins. Nucleotide sequences of homologous genes aren't as highly conserved as amino acid sequences of the proteins that they code for, so sometimes a protein sequence search yields more sequence matches than a DNA search.

   Go back to the NCBI page at http://www.ncbi.nlm.nih.gov again and select ***BLAST.*** From the BLAST page, select the blue-colored *Standard protein-protein BLAST*. Copy the amino acid sequence that you copied on your file into the query box and click on *BLAST!*. Click on *format* when the next page comes up and wait for your protein search results.

   On your BLASTP results page, you will notice a figure showing a red-colored bar designating a conserved domain. Click on *Gamma-thionin* to investigate the amino acid sequence that is conserved in a number of proteins.

   Conserved domains are amino acid sequences that have been found in a number of protein sequences that have been submitted to GenBank. A conserved domain is annotated with a **P_{FAM} number** (for "protein family") to cross reference the proteins that have this conserved domain. What is the P_{FAM} number and the name for the conserved domain of your BLASTP search?

   _____

a. Click on this P_{FAM} number to get an alignment report for this conserved domain. How many amino acid sequences are there in this alignment report, including your query sequence?

_____

b. At the top of the alignment report is a **consensus sequence** that shows the sequence of amino acids that are most often found in the alignment. How many amino acids are there in this consensus sequence?

_____

c. The alignment report is color-coded according to how well the amino acids are conserved in the consensus sequence. Red is the most highly conserved residues. To determine which positions are **absolutely conserved** (found 100% of the time in the alignment), we need to make our color code more stringent. Do this by clicking on the *View Alignment* pull-down menu and select *identity*. How many amino acids in the consensus sequence are absolutely conserved? _____

d. The conserved amino acids are cysteine (C), glutamic acid (E), and glycine (G). How often do each of these amino acids appear in the absolutely conserved consensus sequence?

Cysteine _____ glutamic acid _____ glycine _____

e. To see the 3-dimensional structure of these conserved proteins, click on *View 3D Structure* in the gray box. A colorful graphic of the 3-dimensional structure of the consensus protein will appear in the upper left side of the screen. If you can't download this graphic, you may need to download a Cn3D program from NCBI. Consult the Geospiza website at http://www.geospiza.com/education/ for help.

The sticks in the structure that will appear on your screen represent the polypeptide backbone and 4 disulfide bridges between 8 cysteines. Notice that if you click and drag on this structure, it will rotate. Click on the *Style* pull-down menu to select *Rendering Shortcuts* for *Worms*. This will change the appearance of your structure to show an alpha helix wrapped around a nail and beta-sheets represented by flat arrows. How many of each of these structures are present in these conserved proteins?

You can change the color coding on this 3-dimensional protein structure to display the degree of hydrophobicity of the amino acid residues. Click on the *Style* pull-down menu to select *Coloring shortcuts* for *Hydrophobicity*. The blue color that appears on your structure represents amino acids that are hydrophilic and the red color represents amino acids that are hydrophobic. Click on the structure and rotate it so that you can see where the hydrophilic and the hydrophobic residues lie in the 3-dimensional structure. Do you see any asymmetry in the placement of these residues, so that one side of the molecule is clustered with hydrophilic residues and the other side is clustered with hydrophobic residues? Which side of the molecule would you predict to be associated with other proteins or biological membranes?

f. To study the biological action of this gamma-thionin protein family, go back to the Conserved Domain Database page and click on the *Proteins: Click here for CDART summary of Proteins…* bar. The next page will show that there are two types of proteins that overlap the consensus sequence. What are they called?

_____ _____

g. Click on the conserved domain with the longest stretch of similar sequences. What types of proteins are there with this conserved domain?

_____

What is the mechanism of action of some of these toxins?

_____

_____

C. Searching databases for scientific literature

Although we have inferred a lot about the plant defensins by simply searching for sequence homologies, we still don't have a clear picture of what plant defensins are doing in plants, which sorts of plants make them, whether their expression is tissue-specific or developmentally regulated, etc. You can use online databases of the scientific literature to search for answers to these and other questions. We will continue our investigation of plant defensins by searching the database called PubMed maintained at NCBI by the National Library of Medicine.

1. Go to http://www.ncbi.nlm.nih.gov again, and click on the*PubMed database*. Type "plant defensins" into the query box and click *Search*. How many hits did you get for this query?

_____

2. It would probably be more convenient to narrow our search to get meaningful information more efficiently. You will notice that many of the articles listed are not in English. To limit your search to articles written in English, go back to your query page and click on *Limits* at the top of the page. On the next page, click on the *Language Types* pull-down menu to select *English*. Click on *Search* again. How many hits did you get for your query this time?

_____

3. You can peruse the abstracts of the listed articles by simply clicking on them. Select some titles that look informative and search the abstracts for general information about what plant defensins are, what they are good for, how they work, and where they are found in nature. Not all authors include this general information in their abstracts, but some do, so don't get discouraged if the articles are too detailed. You will notice that some of the articles on the

list have a colored box saying beside them saying *free in PMC* to notify you that the full length article is available for your viewing from PubMed Central without cost to you. Take advantage of the online availability of these articles by clicking on the *free in PMC* box. You will notice that most authors begin the introduction of their articles with the general information that we are interested in. General information often appears in the Conclusions section of scientific articles.

How many full-length articles were you able to find in you PubMed search for protein defensins?

_____

4. Write a short report discussing what you have learned from the literature about plant defensins about their biological role in plants. Include what you have learned in Part II (above) about the patented plant defensins that were patented by DuPont, and suggest a reason that DuPont might have gone to the expense of patenting them. Of what interest are plant defensins to the biotechnology industry?

References

1. Brown, T.A. *Genomes.* Wiley-Liss. 1999.
2. Gibas, C. & Jambeck, P. *Developing Bioinformatics Computer Skills.* O'Reilly. 2001.
3. Puterbaugh, M.N. & Burleigh, J.G. Investigating Evolutionary Questions Using Molecular Databases. *Am. Biol. Teacher.* 63:422-431. 2001.
4. Human Genome Project. 2011. http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml
5. Geospiza: http://www.geospiza.com

Lab Unit 15-B: Bioinformatics Translation Exercise

PURPOSE

The following activity is an introduction to the Biology Workbench, a site that allows users to make use of the growing number of tools for bioinformatics analysis. It is also a review of translation, mutation, and restriction analysis.

You will be working with the cDNA sequences for human hemoglobin, comparing the sequence found in normal individuals with the sequence found in those with sickle cell anemia. The software available on Biology Workbench will allow you to quickly locate the specific defect found in the sickle cell DNA by aligning the two sequences and looking for a mismatch. You will go on to use other software on Biology Workbench to discover the correct frame for the coding sequences in the cDNAs by searching for start codons and stop codons, and by aligning the two translations of amino acid sequences, you will be able to locate the exact amino acid change that appears in sickle cell β-hemoglobin. Other tools found at the Biology Workbench site will allow you to further analyze these sequences.

References

1. Biology Student Workbench (http://workbench.sdsc.edu/) Sickle Cell Anemia—Understanding the Molecular Biology
2. DNA Science, A First Course by David A. Micklos, Greg A. Freyer (second edition) CSHL Press

PROCEDURE

Using your web browser, go to the Biology Workbench page (http://workbench.sdsc.edu/) and sign in. If you have not signed in before you will have to register at the site.

A. Set up your session

NOTE: These directions may no longer be correct if the site has been modified, but your instructor will help you if that is the case. To start the exercise, choose SESSION TOOLS and input the DNA sequences that you want to work with.

1. Select START NEW SESSION and click RUN. Name the session sickle cell (or something similar). Select START NEW SESSION.

2. Go to Nucleic Tools and choose ADD NUCLEIC SEQUENCES and click RUN.

3. Open the file containing this exercise in Microsoft Word, as you will need access to the sequences.

4. Copy and paste the normal human β-hemoglobin cDNA sequence (below) into the Biology Workbench sequence box. You can browse for a file instead of cutting and pasting, but only if the document contains nothing but the sequence. Make sure the text you have copied into

the Biology Workbench contains only the DNA sequence, and delete any extraneous text. Go above the sequence box, type in a title for the sequence you have copied, and select SAVE.

Label:
>"normal β-hemoglobin"

Sequence:
ACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCAC
CTGACTCCTGAGGAGAAGTCTGCGGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATG
AAGTTGGTGGTGAGGCCCTGGGCAGGCTGCTGGTGGTCTACCCTTGGACCCAGAGGTT
CTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCAGTTATGGGCAACCCTAAGGTGA
AGGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGGACAA
CCTCAAGGGCACCTTTGCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGGAT
CCTGAGAACTTCAGGCTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGG
CAAAGAATTCACCCCACCAGTGCAGGCTGCCTATCAGAAAGTGGTGGCTGGTGTGGCT
AATGCCCTGGCCCACAAGTATCACTAAGCTCGCTTTCTTGCTGTCCAATTTCTATTAAA
GGTTCCTTTGTTCCCTAAGTCCAACTACTAAACTGGGGGATATTATGAAGGGCCTTGAG
CATCTGGATTCTGCCTAATAAAAAACATTTATTTTCATTGC

5. Repeat the process of copying, naming, and saving the sickle cell β-hemoglobin cDNA sequence, found below.

Label:
>"sickle-cell β-hemoglobin"

Sequence:
ACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCAC
CTGACTCCTGTGGAGAAGTCTGCGGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATG
AAGTTGGTGGTGAGGCCCTGGGCAGGCTGCTGGTGGTCTACCCTTGGACCCAGAGGTT
CTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCAGTTATGGGCAACCCTAAGGTGA
AGGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGGACAA
CCTCAAGGGCACCTTTGCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGGAT
CCTGAGAACTTCAGGCTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGG
CAAAGAATTCACCCCACCAGTGCAGGCTGCCTATCAGAAAGTGGTGGCTGGTGTGGCT
AATGCCCTGGCCCACAAGTATCACTAAGCTCGCTTTCTTGCTGTCCAATTTCTATTAAA
GGTTCCTTTGTTCCCTAAGTCCAACTACTAAACTGGGGGATATTATGAAGGGCCTTGAG
CATCTGGATTCTGCCTAATAAAAAACATTTATTTTCATTGC

6. Check the box beside the normal β-hemoglobin and select VIEW NUCLEIC SEQUENCE(S) in the scroll-down menu. Click RUN.

7. How large is it (how many base pairs)? _____

8. Calculate how large a protein this sequence could encode (how many amino acids). _____

B. Find the mutation

An alignment program called CLUSTALW will align the DNA sequences that you select, and locate matching and mismatching sequences.

9. Click on RETURN to go back to Nucleic Tools and check the boxes in front of both the normal β-hemoglobin and the sickle-cell β-hemoglobin.

10. CLUSTALW is a program that aligns DNA or protein sequences and compares them to find differences. We can use it to find the mutation in the sickle-cell DNA. Select CLUSTALW from the window of applications and click RUN. Leave all default settings and click SUBMIT.

11. Is this a point mutation or a frameshift mutation? Describe the exact mutation in the sickle cell DNA.

C. Translate the cDNA

A Six Frame program can quickly translate a DNA sequence into an amino acid sequence. By comparing translations for all six possible frames, you will be able to look at every possible protein sequence that can result from the DNA sequence. The most plausible amino acid sequence from all possible sequences is the longest one.

12. Click on RETURN to go back to Nucleic Tools and deselect the box next to sickle-cell β-hemoglobin cDNA. Choose the application SIXFRAME from the window of tools (you may have to scroll down). Click on RUN.

13. Leave the default settings as they are and hit SUBMIT.

14. Scroll down to analyze the results. Blue M's represent possible start codons (methionine) and red *s represent stop codons (code for no amino acid).

a. Which reading frame gave the longest protein (scroll to the bottom of the screen and look for the longest ORF (Open Reading Frame)?

b. Frame 4 is fairly long, but is not considered the longest protein. Why not?

c. About how long are the shorter proteins (look at a few sections from start codon to stop codon and estimate the average)?

d. Why should we choose the longest open reading frame as the correct translation?

e. The longest protein is not 208 amino acids long (as predicted earlier). Why not?

15. Import the longest open reading frame at the bottom of the page by checking the box in front of it and clicking the IMPORT SEQUENCES button. It will be imported into your Protein Tools section. If your Protein Tools is empty you forgot to check the box before you imported.

16. Repeat this procedure by translating all six frames with the sickle cell β-hemoglobin DNA. Find the longest ORF and click on IMPORT SEQUENCES.

D. Create a deletion mutation

To see the effect of a deletion mutation, you will edit the normal β-hemoglobin cDNA sequence by deleting one of its nucleotides.

17. Go to Protein Tools and select both the normal and sickle cell cDNA by clicking on the boxes in front of those names.

18. Select the application CLUSTALW from the tools window to align the two protein sequences. Click RUN.

19. Leave the default preferences as they are and hit the SUBMIT button.

20. Scroll down and look for the mutated amino acid(s). Describe the mutation. To find out what the single letter amino acid designation means go to the chart at the end of this activity.

21. In Nucleic Tools, select the normal β-hemoglobin sequence and select Edit Nucleic Sequences. Add or delete a nucleotide somewhere in the first half of the sequence and click on SAVE. It will be saved with "edited" after the name.

22. Select the newly edited file and translate it using SIXFRAME.

23. Import the longest open reading frame at the bottom of the page by checking the box in front of it and clicking the IMPORT SEQUENCES button.

E. Align proteins for comparison

You will find the exact amino acid that is changed in the sickle cell β-hemoglobin.

24. Go to Protein tools and check the original β-hemoglobin, the sickle cell β-hemoglobin, and the edited β-hemoglobin with the deletion, and run CLUSTALW to compare the three.

25. What amino acid change did you find in the sickle cell β-hemoglobin sequence? _____

26. What impact did the deletion have on β-hemoglobin? Is it still 147 amino acids long?

Was there only one amino acid affected as in the sickle-cell β-hemoglobin? _____

27. Did you introduce a point mutation or a frameshift mutation? _____

28. What impact might this have on the function of the protein? Would it probably have no impact on the function, change the function, or make the protein completely non-functional?

F. Simulated Restriction Analysis

You will do a restriction enzyme digest *in silico* (by computer), using a TACG program. Restriction enzymes are like spell-checking enzymes; they look for specific "words" in the DNA and then cut them. If the "word" is misspelled, then the enzyme will not cut the DNA. We will use the enzyme *Dde*I. It cuts DNA at the sequence CTGAG, but the sickle cell β-hemoglobin cDNA is missing one of these sites found in the cDNA coding for normal β-hemoglobin. This means that the *Dde*I restriction digest of sickle cell β-hemoglobin cDNA will have fewer DNA fragments than the *Dde*I restriction digest of the normal β-hemoglobin cDNA. This difference could be observed by gel electrophoresis of the two digests.

29. Go to Nucleic Tools, select the normal β-hemoglobin and then select the application TACG from the tools window. This program will allow you to digest the DNA with different enzymes.

30. Scroll down to User Specified Enzymes and type in *Dde*I. Make sure you type "I" and not "1." This enzyme will cut the normal cDNA at the sequence CTNAG (N can be any nucleotide and it will still be cut) but not the sickle cell mutant. Scroll down to Smallest Fragment Cutoff Size for Simulated Gel Map and change it to 10 (our smallest segments will be smaller than the default of 100 base pairs). Select SUBMIT.

31. What was the output of Fragment Sites by Enzyme (how many fragments at what lengths)?

32. Repeat the above steps with the sickle cell sequence. What was the output?

33. Notice that the lengths of the two missing fragments add up to the length of the new fragment. Why?

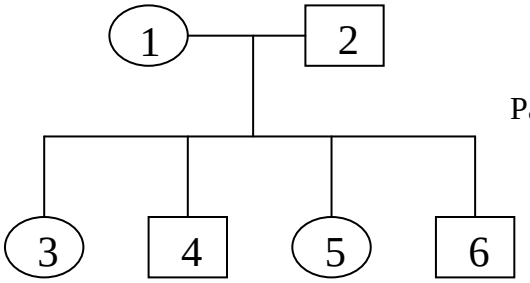
34. Use the fragment size output from steps 31 and 32 to label the bands on the gel diagram at the end of this exercise (remember that the smallest bands travel the most quickly through the gel).

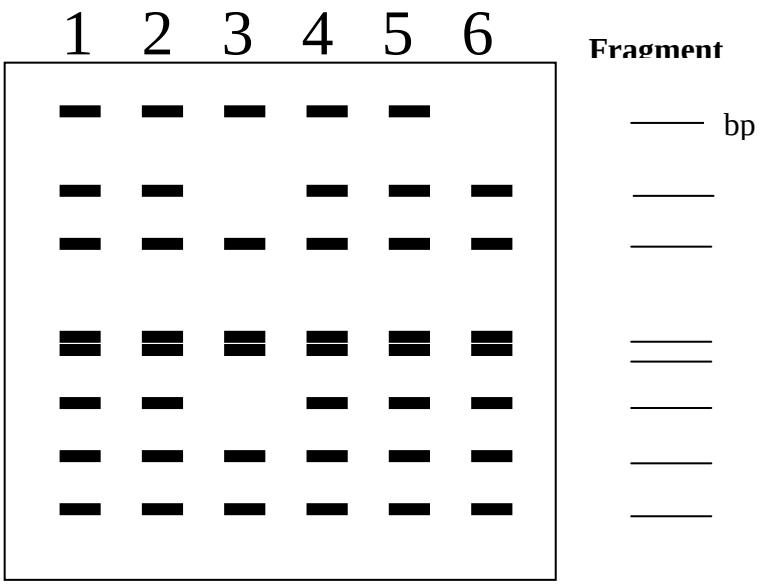
35. From banding patterns on the gels and the pedigree chart, indicate whether the person on the pedigree is **SS** (has 2 normal genes or *homozygous dominant*), **Ss** (has one normal and one sickle-cell gene or *heterozygous*), or **ss** (has two sickle-cell genes or *homozygous recessive*).

Parents	**Offspring**
1.	3.
2.	4.
	5.
	6.

36. Explain how the banding pattern helped you to determine the genotype (normal, sickle-cell anemia, or sickle-cell disease) of each individual.

PEDIGREE





Lab Unit 15-C: Finding Mutant Sequences

Objective

In this exercise, you will find DNA sequences via the database links given in Biology Workbench.

A. Using the Ndjinn database

1. Go to Nucleic Acid Tools and go to the scrollable textbox.

2. Highlight "Ndjinn- Multiple Database Search" and click RUN.

3. The next screen lists the different databases that you can search. In the search box type "beta globin". Indicate that you want to see all of the sequences, so select "SHOW ALL HITS." Since you are looking for a human gene, click on the box that is next to the GBPRI database. This is the GenBank Primate Sequence database. This database contains DNA sequences that are specific to primates only, and therefore humans are included.

4. Click on SEARCH. You will be sent to a page that contains the results of your search. From the descriptions of the search results, you need to determine which one is the wild type for beta globin. The sequence you want is "gbpri:29436- human messenger RNA for beta-globin". Select this sequence and click on the IMPORT SEQUENCE(S) button. Your sequence will be transferred to the Nucleic Tools homepage.

B. Finding the Mutant Sequence Using BLAST

1. Scroll down the text box and look for the tool called BLASTN. Compare a NS to a NS DB. This is an abbreviation for "Compare a Nucleic Acid Sequence to a Nucleic Acid Sequence Database".

2. Select this tool and ensure that your GBPRI:29436 beta globin sequence has a checkmark in the box next to it.

3. Select the GenBank primates databases to do your search in. Click on RUN.

4. Notice that the first result listed is the wt sequence. If you look over to "Score (bits)" column, you will see that the first few sequences have very high Score values. A Score above 400 means that the sequence has high homology with the sequence you are comparing it to. You can be certain of the degree of homology by looking at the "E value". The smaller the E-value, the more homologous the sequence is to the original sequence BLASTED. An E-value of zero indicates that no matches would be expected by chance- this would represent a perfect or near perfect match.

5. Since you know that the mutation is a small one (point mutation) this means the wt and mutant sequences should be nearly identical. Using this information, find sequence(s)

that matches the required parameters (low E value) and click on SHOW RECORD(s) button to get more information about the sequence(s) that you have highlighted.

6. Scroll down the "Records" window until you find the beta-globin mutant sequence. The description should have the word "sickle" in it since you are looking for sickle-cell anemia. The sequence you are looking for will be GBPRI:183944.

7. Import this sequence just as you did the normal sequence for beta-hemoglobin.

APPENDIX

Appendix A: Safety Training Sheet

Biotechnology Lab Safety Procedures and Information

Modern laboratories are equipped with supplies and equipment that may pose a hazard if used carelessly. Following safety rules, paying attention to what you are doing, and using common sense is the best way to make your experience in this course a safe one. Health and safety are paramount values in science classrooms, laboratories and field activities. You are expected to learn, understand and comply with ACC environmental, health and safety procedures and agree to follow the ACC science safety policy. You are expected to conduct yourself professionally with respect and courtesy to all. You can read the complete ACC science safety policy at: *http://www.austincc.edu/sci_safe/*

All safety policies and procedures apply to scheduled lab classes as well as open labs.

Consequences for not complying with safety procedures:

1. You will not be able to participate in a lab activity if:
   a. you are late for class and have missed safety training specific for that day's lab or field activity;
   b. you have forgotten your personal protective equipment;
   c. you refuse to wear personal protective equipment;
   d. you have not followed safety policies and procedures for that lab or field activity.
2. **You may be withdrawn from the class and not reinstated if:**
   a. you missed required safety training at the beginning of the semester;
   b. you repeatedly fail to follow lab safety policies and procedures.
3. **You may be expelled from ACC if you thoughtlessly or intentionally jeopardize the health or safety or another individual.**

Emergencies

If there is a fire, major chemical spill or other emergency:

1. Call ACC Police Dispatch as soon as possible. Tell the operator your campus _____ and exact location in the building _____.
   Location of nearest ACC phone: _____
   ACC POLICE DISPATCH: 222 (from an ACC phone)
   223-7999 (from a cell or other phone)
2. If evacuation is necessary:
   a. take your personal belongings with you if possible;
   b. on your way out, close but do not lock the classroom door;
   c. go to the designated rally point away from this building.
      - Directions to nearest exit: _____
      - Location of rally point:_____

Safety Equipment and How to Use It:

- Information about chemicals used in this laboratory can be found in Material Safety Data Sheets (MSDSs) and in a chemical inventory located _____.

- The emergency gas shut-off for this lab is located: _____. Shut off the gas immediately if gas nozzles or valves are damaged or if there is a fire.

- Fire extinguishers are located: (1) _____.
  (2) _____.

  To use a fire extinguisher:
  1) twist the pin and then pull it out of the handle
  2) hold the end of the hose and point it at the base of the fire
  3) squeeze the handle
- Fire blankets are located: (1) _____.
  (2) _____.

  If you are on fire, stop, drop and roll. Let someone else to get the fire blanket.
- A safety shower is located _____. If you spill a significant quantity of chemical, especially an acid or base on yourself immediately stand under the shower and pull the handle. Disrobe. The instructor will evacuate the room and close the doors for your privacy. Someone of your gender will stay to help you. Stand under the shower for at least 20 minutes. You will be given clothing after the shower.
- An eyewash is located _____. If a chemical is splashed or rubbed into your eyes you must use an eyewash for at least 20 minutes with your eyes held open. Someone will help you with this.
- If a person is experiencing electrical shock from touching wires or equipment, use a belt or other non-conducting material to pull them away from the electrical source.
- First aid kits are located: (1) _____.
  (2) _____.

  a. Only minor cuts and burns will be treated in the lab. Serious injuries must be treated in a medical facility. Emergency Medical Services (EMS) will be called if you are injured and are unable to take yourself to a medical facility.
  b. The instructor must fill out a report describing your injury.

Personal Protective Equipment (PPE)

1. **Safety Eyewear:**
   a. You must wear safety eyewear (safety glasses or goggles) marked Z87 when directed to do so by the lab instructor or lab safety instructions. You must bring your protective eyewear with you to every lab class. If you forget your eyewear and the lab room does not have a pair to loan to you, you will not be able to participate in the lab and may forfeit your lab grade for that day. ACC cannot guarantee that loaned safety glasses or safety goggles are uncontaminated by microbes or chemicals.
   b. People who wear contact lenses must wear goggles and may not wear safety glasses.

2. **Gloves** – You will be provided with nitrile gloves for handling hazardous chemicals and may be provided with latex gloves for handling biohazards. Please notify the instructor if you have a latex allergy. Change your gloves often, and while wearing gloves, do not touch door handles, water taps, computers, telephones or other objects that may be touched by people not wearing gloves.

3. **Shoes** – You must wear closed-toed shoes in lab.

4. **Lab Coat** – You will be required to wear a lab coat while working in the cell culture lab. This lab coat should be kept in the cell culture lab. You can also wear a lab coat if you wish during other lab courses.

5. **Other:**
   a. tie back long hair in labs involving open flames;
   b. do not wear clothing with long, loose sleeves;
   c. wear natural fiber clothing (synthetic material melts onto skin in a fire);
   d. remove watches, rings, and bracelets during lab activities involving chemicals.

Waste Disposal

You must precisely follow the waste disposal procedures. Never dispose of anything in lab without prior direction from the instructor.

- Hazardous chemical waste containers are located:
  solids _____
  liquids _____
- Biohazard bags are located: _____
- Sharps containers are located: _____
- Glass (rinsed test tubes and broken glass) disposal boxes are located: _____
- Regular trash containers are located: _____

Lab Conduct

1) Do NOT do these things:
   - come to class while intoxicated or while under the influence of drugs that impair your ability to safely perform the lab or field activity;
   - horse around or perform unauthorized experiments;
   - eat, drink, or chew (tobacco or gum);
   - bring drinks or food (even in closed containers) into the lab;
   - leave your backpacks, coats, and other personal items on the floor where they could pose a trip hazard, or on the lab bench;
   - wear loose or flowing clothing, dangling jewelry, open-toed or high-heeled shoes;
   - pipet by mouth;
   - taste chemicals or directly smell chemical fumes.

2) **Do these things:**
   - Follow all procedures in manuals, in handouts, and as given by the instructor.
   - Consult with your doctor about any special health conditions that you may have, such as asthma, allergies, or pregnancy.
   - Store backpacks, coats, and other personal items as directed.
   - Clean up your work stations, wipe your lab bench and wash your hands before leaving the lab room.
   - Report broken glass and chemical spills to your instructor immediately.

Lab Hygiene

- Clean up your individual work area/equipment and community work areas/equipment (e.g., sinks, balances).
- Put lids back on bottles and containers immediately after use.
- Do not put excess chemicals back into original containers.
- Dispose of chemicals and waste only as directed by the instructor.
- Wash your hands prior to leaving lab.
- Assume that chemicals used in lab are corrosive or irritating. If at any time chemicals come into contact with your skin wash the affected area immediately.
- Open volatile organic chemicals only inside the fume hood.

Biohazards

Diseases such as HIV and hepatitis can be transmitted from person to person through contact with human blood or other body fluids. Follow the Universal Precautions whenever exposure to human body fluids is possible:

- Consider all body fluids (saliva, blood, urine, feces, vomit) as potentially infected with a harmful pathogen.
- Do not touch or come into contact with anyone else's body fluids.
- When working with microbes, always assume they are infectious. Avoid touching contaminated objects to any other objects, even the floor and counters, and avoid direct contact, especially around broken skin.
- Always wear gloves and goggles when working with microbes or body fluids.
- Dispose of all potentially contaminated objects in a biohazard bag or a container filled with a 10% bleach solution, or follow your instructor's directions.
- Spray and wipe the work areas with 10% chlorine bleach solution before and after lab.
- Wash hands immediately after handling a biohazard.

Handling mechanical hazards

- Never touch a rapidly moving machine, such as a centrifuge, while it is moving.
- Distribute weights evenly in a centrifuge to prevent vibrations and breakage.
- Do not leave a running centrifuge unsupervised.
- If the centrifuge is vibrating excessively or "walking" across the tabletop, turn it off immediately.

Handling electrical hazards

- Do not use equipment that has any frayed or damaged wiring or plugs. Report any frayed wires to your instructor.
- Always make sure the area around all electrically powered equipment is dry before turning on the power.
- Gel electrophoresis poses a high risk for electrocution. When assembling or disassembling the gel apparatus, always be sure that it is unplugged. Connect the power supply and turn on the power supply only under the supervision of your instructor.

Handling glassware

- Dispose of disposable glass items such as capillary tubes and cover slips in a hard-sided box labeled "Glass Disposal".
- Do not use broken or cracked glassware. If you break a glass item, report the incident to your instructor and dispose of it in the "Glass Disposal" box.
- NEVER put broken glass or disposable glass items with sharp edges in the ordinary trash can. This poses a serious hazard to the person who must empty the trash.
- Avoid rapid temperature changes of any glassware, as this will often cause the glass to break. Do not place a cool glass container on a hot surface (such as a hot plate), and do not place a hot glass container on a cold surface or in a cold environment (such as a refrigerator).
- Report any cuts immediately to your instructor, and wash the wound thoroughly in running water. Check for glass in the wound, remove if necessary, dry the skin, and apply a bandage.
- Do not shake glass thermometers, and lay thermometers away from the edge of a bench on a towel or screen to avoid dropping it on the floor. If a thermometer breaks, immediately inform your instructor.

Accident procedures

- Try to contain any spills without endangering yourself and others. Spill socks and pillows, or paper towels if necessary, can be used to contain a spill and keep it from spreading. Notify the instructor immediately when a spill has occurred.
- If a caustic chemical is splashed into your eyes, notify the instructor or another student immediately so that you can be assisted to the nearest eyewash station as quickly as possible. Continue to wash your eyes for at least 20 minutes while emergency personnel are being called.
- If caustic chemicals are spilled on your skin, wash the contaminated area for at least 15 minutes. If it is a major spill, immediately remove contaminated clothing and wash for at least 15 minutes in a safety shower.
- Quickly shout an immediate warning to all your neighbors in case of a fire. It is very important that everyone in the room know as quickly as possible when there is a fire.
- All students should exit a lab in case of a fire. The lab instructor will call the Campus Police Dispatch at 222 (from any ACC phone) or 223-7999 (from an outside or mobile phone).
- Speed is the most important aspect of helping a person who is on fire. Your nearest neighbors must respond quickly by smothering the fire with a fire blanket as soon as it appears.
- Do not allow a person whose clothing or hair is on fire to move. Stop the person and quickly push them to the floor and smother the flames immediately with a fire blanket.
- The student nearest a fire blanket should bring the blanket to a person who is on fire, and once the flames are quenched, that person should be taken immediately to the safety shower.

Chemical Hazard Labels

- Label containers/test tubes if you are using more than one container per lab.
- Inform your instructor immediately if a label is damaged in any way.
- Read all labels and pay special attention to hazard information.

A typical chemical hazard label conveys two kinds of information: the category of the hazard (flammable, toxic, reactive, or corrosive) and the level of the hazard. There are two main types of labels: those shaped like diamonds and those shaped like bars. In both types the category of hazard is represented by a color and the level of the hazard is represented by a number.

1. Hazard categories are coded by color:

red	fire hazard, flammability
blue	health hazard, toxicity
yellow	reactivity
white (diamond-shaped labels)	provides more specific information about the hazard (example: acid)
white (bar-shaped labels)	tells you what kind of protective equipment (PPE) is required for handling that chemical

2. Hazard level is coded by a number:

0	minimal
1	slight
2	moderate
3	severe, serious
4	extreme

3. Refer to the training poster in your lab for examples.

4. Other types of hazard warning labels you must recognize are:
   a. biohazards
   b. radioactive materials

Appendix B: Sample Pre-Lab

Using a Micropipette

Purpose: The purpose of this lab is to become familiar with setting up a lab, to practice using a micropipette, and to learn to follow GLP's.

Safety: There are no specific safety precautions for this lab other than wearing PPE.

Materials:
20-50 ml sugar solution (dyed)
20-50 ml distilled water
20-50 ml water (dyed)
small beakers (4)
microcentrifuge tubes (12)
microcentrifuge tube rack
micropipetters (p20, p200, p1000)
box of yellow tips (1 per table)
box of blue tips (1 per table)
wash bottle with 70% ethanol
paper towels
picofuge (class shares)

Data/Notes/Observations	**Procedure**
	Part A 1. wash lab table with ethanol 2. collect and organize all materials for the lab 3. show the instructor how your group organized your space and explain why your organized it as you did.
*instructor _____	
sketch	
	Part B
0.45 ml = ____µl	1. Set the p1000 to 0.45 ml
0.15 ml = ____µl	2. Set the p200 to 0.15 ml
0.015 ml = ____µl	3. Set the p20 to 0.015 ml
	4. Set out one microcentrifuge tube in a rack

5. Set the correct micropipet to 600 µl and place a tip on the end

6. Press down to the first stop and place the tip just into the sugar solution. Slowly release the plunger and do not remove the tip before it is filled.

7. Dispense the liquid into the microcentrifuge tube by pressing to the second stop. Be sure the liquid comes to the 0.6 ml mark on the tube.

*instructor _____

8. Discard the tip in the trash beaker.

Part C

1. Label 10 microcentrifuge tubes [tube #]: [page #] on top of the lid

2. Open all of the lids and figure out the most effective way to dispense the following amounts in the indicated tubes.

*order of dispensing liquids

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

3. Dispense the liquid into each tube and mix by pipetting up and down. Do not pipet so vigorously you make bubbles.

4. Put tubes in picofuge and spin for no more than 10 seconds.

5. Have the instructor check the accuracy of your measurements.

*instructor _____

Conclusions and Analysis:

Appendix C: Forms

For every solution prepared in the laboratory, you must fill out a solution preparation form. You will find the forms in the file cabinet. A sample formed filled out is below. NEVER leave a blank space in a form – every field must be filled in, even if it says 'not performed or not applicable'.

SOLUTION PREP 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, both names are recorded here. Both students must submit a copy of the Solution Prep form in their lab report

Component	Brand/ Item #	Storage conditions/ date received	FW or initial concentration	Amount used	Final concentration

Balance used Balance number	Calibration status Did you calibrate it?	
pH meter used pH meter number	Calibration status Did you calibrate it?	
Initial pH Always record the pH the solution started at	Final pH Always record your final pH after you BTV	Adjusted pH with Chemical and concentration you used to adjusted the pH
Prep temperature Record the actual temperature the solution was prepared – NOT "RT"	Sterilization procedure Was this solution autoclaved or filter sterilized?	Storage conditions Where is this solution stored now?

Calculations/Comments:
ALL calculations used to prepare this solution are recorded here. Yes, in addition to your pre-lab, and notebook.

SOLUTION PREP FORM

Control # _____

Name of Solution/Media: _____

Amount prepared: _____ **Preparation Date: _____**

Preparer(s): _____ **_____**

Component	Brand/lot # (Vendor)	Date Received	Storage conditions	FW or initial concentration	Amount used	Final Concen-tration

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

Calculations/Comments:

Electrophoresis Documentation Form

Date _____ buffer _____ gel type _____ % agarose/acylamide-
(**circle one**)

voltage _____ start time _____ stop time _____ stain used _____

Comments on electrophoresis or staining:

Lane	**Sample description**	**Analyte loading (ug DNA or protein)**	**Sample volume/loading dye volume (Ul)**	**Analyst**
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				

Gel photo:

INSERT PHOTO HERE

Photographic settings (exposure time, aperture setting, etc)

Appendix D: Graphing Data

You will often make scatter diagrams and line graphs to illustrate the data that you collect. Scatter diagrams are often used to show the relationship between two variables. For example, in an absorbance spectrum, the variables would be the wavelength of light and the amount of light absorbed. Although this data is recorded in a table, a scatter diagram can illustrate in a more visual way the relationship between the two data sets: absorbance and wavelength.

A. Setting up a graph

To make a scatter diagram, simply plot ordered pairs of number on graph paper. The horizontal line on the graph paper is identified as the x-axis (or abscissa) and the vertical line is the y-axis (or ordinate). Each axis is labeled with an appropriate unit of measurement. Each increment of these lines represents the same amount of the measurement. For example, if you are drawing a scatter diagram of an absorbance spectrum and if one square of the x-axis represents 10 nm of wavelength of light, every other square also represents 10 nm of the wavelength of light along the x-axis. This is called a "linear scale". Similarly, if each square of the y-axis represents 0.010 absorbance units of light absorbed, each other square also represents 0.010 absorbance units along the y-axis. In other words, each axis has a consistent scale, even though the two axes may not use the same linear scale.

How do you know which variable is to be on the x-axis, and which is to be on the y-axis? The x-axis should be the **independent variable,** or the parameter that you selected to vary. In an absorbance spectrum, it would be the wavelengths across which you measured absorbance. The y-axis should be the **dependent variable,** or the data that you obtained from your measurement. In an absorbance spectrum, the dependent variable would be the absorbance that was measured over a variety of wavelengths. You can think of it as the values for dependent variables measured in your data sets depend on which independent variables you chose.

B. Approximating a "best fit" line for a scatter diagram

If you look at your scatter plot and the middle points on the graph are close to forming a straight line, it is reasonable to conclude that the relationship between the independent and dependent variables is linear. The straight line defines this linear relationship. If, for example, you have studied the effects of a particular fertilizer on fruit production in apple trees, your independent variable is the amount of fertilizer applied, while your dependent variable is the weight of apples harvested at each level of fertilizer application. If the relationship between amount of fertilizer and weight of apples harvested is linear, and you decided to plot these two sets of values, you would have a line that tells you exactly how much fruit you could expect from apple trees at a given level of fertilizer application.

It is unlikely that your data points in the apple experiment described above would all lie on a perfect line, due to variation in conditions such as sunlight and water. To get the best approximation of expected fruit yield as it relates to fertilizer application, you would draw a **best-fit line** though your scatter plot. The most valid best-fit straight lines that illustrate a linear relationship are determined using a type of statistical analysis, called linear regression analysis. However, you can approximate a best-fit line in the following way:

- If your data points seem to form a basically straight line after you have made a scatter diagram illustrating your data, place a straight edge on your graph along the data points. Move the straight edge until it is as close as possible to as many points as possible, and draw a line along the straight edge. This is easiest to do if your straight edge is transparent, such as a clear plastic ruler. There should be approximately the same number of data points on each side of your line, and the line should minimize the distance of the data points from the line as possible. Notice that your line may or may not pass through any particular plotted data point.

- If your data points seem to form a curve rather than a straight line, there is probably a nonlinear relationship between your dependent and independent variables. You will have to approximate your line by drawing the curve freehand rather than by using a straightedge. Try for the same effect, however–do not connect-the-dots. The line should be a smooth curve, which may or may not pass through any particular data point.

- Often, you will be graphing data that illustrates a relationship that is generally linear, but the linearity breaks down at the extremes of changing conditions. In this case, some of the data points on your graph will form a nearly straight line. This is an indication that at a certain point, the data that you collected no longer had a linear relationship to the independent variable. However, the information in the linear part of the graph may still be valuable. Place a straight edge so that it is as close as possible to as many points that lie along the linear part of your graph as possible.

<u>Always follow these guidelines when preparing a graph showing experimental data:</u>

1. Your graph should always be given a brief title to explain what relationship you are studying.
2. Plan how to mark off the units of measurement on each axis so that your completed graph will nearly fill the page.
3. Both axes should be clearly labeled and marked with appropriate units of measurement.
4. Both axes should have a linear scale, meaning that the same increments are consistently the same distance apart. The size of the increments on one axis does not have to be the same as that of the other, but they must both be a linear scale.
5. The x-axis should show the independent variable. This is the variable that the experimenter chooses and can change. The y-axis should show the dependent variable (the one that the experimenter observes as he or she varies the independent variable).
6. You may draw a line on your scatter plot to better illustrate any pattern that is revealed. If you find a linear relationship between your independent and dependent variable, draw a best-fit straight line through the points that are consistent with the linear relationship. If there is no linear relationship, you can leave your scatter plot as is, or draw curved lines between your data if you wish.

C. Graphing a semilog plot

Your variables may not have a linear relationship, in which case a straight line cannot represent your data. In the biological sciences, the relationship is often exponential rather than linear.

This means one value doubles for each single-unit increase in the other value. For example, each time a cell divides, the number of cells is doubled. This means that if you repeatedly count the number of cells in a culture over a given interval of time, the cell count will not rise linearly with time, but rather exponentially with time. If you graph this relationship on semilog paper, the line will be linear.

On semilog paper, the X-axis is linear (each increment is spaced equally and represents an equal unit of measurement), but the Y-axis is exponential (each increment is NOT spaced equally and does NOT equal the same unit of measurement). The hard part of using semilog paper is deciding what units belong on the Y-axis.

You will notice that there are heavier tracings and lighter tracings of the Y-axis grids on semilog paper. The heavy tracing represents a "decade," while the light tracings within the decade are assigned numbers that are equally spaced. For example, the lines within one decade might represent 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10. Depending on your data, the lines within a decade might also represent 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1,000. The important thing to notice is that the decade ABOVE the first decade represents values 10 times that of the previous decade. For example, if the first decade includes the values 1 to 10, the second decade includes the values 10 to 100, and the third decade includes the values 100 to 1,000.

Graphing with Microsoft Excel

You may find tutorials for the use of Microsoft Excel spreadsheets and graphing at the Biology Department website.
http://www.austincc.edu/~emeyerth/exceltutor1.htm
http://www.austincc.edu/biocr/1406/labm/ex2/prelab_2_10.htm
http://www.austincc.edu/biocr/1406/Excel2007tutorial/excel20073a.htm

Appendix E: Summary of Good Laboratory Practices

Ref: Molecular Biology Problem Solver, edited by Alan S. Gerstein ISBN 0-471-37972-7

To solve problems you need to understand the role of the reagent and also the physical and chemical properties of the macromolecules you are working with in your protocol.

Buffers
Role of buffers:
- Control pH
- Control Ionic Strength
- Solvating Species

Generally NOT a good idea to substitute unless the buffer is only controlling pH

Chemical Compatibility
Anticipate problems due to interaction of your buffer component with other solution components. For example, if Ca^{2+} is a necessary cofactor do not use a phosphate buffer as Ca^{2+} and phosphate form an insoluble precipitate making the Ca^{2+} unavailable. **NOTE:** Always dissolve reagent one at a time or dissolve them separately and then add together.

Buffers from Stock Solutions
Stock solutions are a quick way to store "buffer precursors." Concentrated solutions also retard bacterial growth, extending almost indefinitely the shelf stability of the solutions. Dilution of a stock solution always results in a pH change. Adjust the pH after the solution has been diluted UNLESS indicated not needed as in the case with some hybridization and electrophoresis buffers. Filtration is a good approach to sterilizing solutions except when the filter binds components of the solution (e.g. riboflavin).

Quality of the Reagents
A good rule of thumb is that it is safer to substitute a higher grade of reagent for a lower grade, rather than vice versa. If you want to apply a lower grade reagent, test the substitution against the validated grade in parallel experiments. AND it is a good idea to ALWAYS keep a small portion of a try and true reagent before switching to a new batch! (e.g. Taq, restriction enzymes, Tris).

Quality of the Water
Tap water is generally unsuitable for experiments. If you use it to wash dishes, follow with a distilled water rinse. Distillation generally eliminates much of the inorganic contamination and particularly sediments. It does not remove organics or all of the inorganic contaminants. Deionized water varies greatly in quality depending on the type and the cartridge used. Laboratory deionization cartridges are generally designed to remove both anions and cations. The highest quality water is prepared by reverse-osmosis. Reverse-osmosis is defined as

What Is the Storage Lifetime of a Buffer?
The most common causes of buffer failure are pH changes due to absorption of basic (or acidic) materials in the storage environment, and bacterial growth. For example, long-term storage of a base in glass leads to neutralization of the base and etching of the glass. Common indicators of

bacterial contamination are cloudiness of the solution. Strategies for avoiding microbial contamination includes sterilizing buffers, manipulating them using sterile technique, refrigerated storage, and maintaining stock solutions of sufficiently high ionic concentration. Always store chemicals, reagents, and solutions as indicated by the manufacturer.

⇒ ***Proper documentation concerning the preparation of the buffer cannot be overemphasized.***

DNA Isolation
Nucleases are a major problem when working with DNA and RNA. As soon as cells are broken open, nucleases are released. Samples must be lysed fast and completely and lysis buffers must inactivate nucleases to prevent nuclease degradation. If yield is important, all materials should be autoclaved or baked four hours at 300 F to inactivate the nucleases or you should use disposable materials. Where appropriate, work on ice or in the cold to slow down potential nuclease activity. Smears and failure to amplify by PCR are indicative of contamination. Large DNA molecules, such as genomic DNA, can be easily sheared during purification. Avoid vortexing, repeated pipetting, and any other form of mechanical stress.

What Practices Will Maximize the Quality of DNA Purification?

Starting Material:
The success of DNA purification depends on the initial quality of the sample and its preparation; start with a fresh sample. Process your sample as fast as possible. If you cannot process, snap freeze in liquid nitrogen or hexane on dry ice or store at –80^{o}C OR use commercial products such as those that can be purchased from Ambion, Inc.

Use only the appropriate amount of sample. Nothing will impair the quality and yield of a purification strategy more than overloading the system. Too much sample can cause an increase in the viscosity of the DNA preparation and lead to shearing of genomic DNA. Of course if you are only want to show the presence the DNA by an increase in viscous material, then overloading the system may be appropriate.

Lysis:
Procedures for lysing cells and isolating the nucleic acids from cellular components differs depending on the starting material (i.e. plants require high pressure because of the rigid cell wall, bacteria contain lipopolysaccharides that interfere with purification and cause downstream toxicity problems, fibrous tissues such as heart and skeletal muscle are tough to homogenize)

Detergents are used to solubilize the cell membranes. Enzymes that attack cellular components are added to the lysis buffers. Lysozyme digests cell walls of gram + bacteria and Proteinase K cleaves glycoproteins and inactivates to some extent, RNases/DNases. Techniques for lysing include sonication, homogenization, and grinding in liquid nitrogen.

DNA Precipitation:
To concentrate nucleic acids for resuspension in a more suitable, solvents such as ethanol (75-80%) or isopropanol (40-50%) are commonly used in the presence of salt to precipitate nucleic

acids. If volume is not an issue, ethanol is preferred because less salt will coprecipitate and the pellet is more easily dried. Salt is essential for DNA precipitation because its cations counteract the repulsion caused by the negative charges of the phosphate backbone. Temperature and time are crucial for nucleic acids at low concentrations, but above 0.25 mg/ml, precipitation may be carried out at room temperature.

Storage:
Long term storage is best in TE (10 mM Tris, 1mM EDTA pH 7-8) at low temperature (-80C). Repeated freeze/thaw will damage nucleic acids so either store in no frost free freezer or put in a storage box so that the samples are not subjected to repeated freeze/thaw. Freeze-dried DNA (but not RNA) is stable for 6 months. Commercial products, such as those from Ambion, Inc, that prevents degradation of nucleic acids, added to the product either before or after processing work very well.

Restriction enzymes
Usually class II enzymes are used. These enzymes recognize specific DNA sequences and cleave each DNA strand to generate termini with 5' phosphate and 3' hydroxyl groups. The recognition sequence is normally 4 to 8 base pairs in length and a palindrome. The point of cleavage is within the recognition sequence. A listing of restriction enzymes can be found at: http://rebase.neb.com. The role of these enzymes is to protect the bacteria against bacteriophage DNA. Nomenclature for restriction enzymes is based on a convention using the first letter of the genus and the first two letters of the species name of the bacteria of origin. For example, SacI are derived from *Streptomyces achromogenes.* (What is an isoschizomer?- restriction enzymes isolated from distinct bacterial strains having the same recognition specificity).
Enzymes of high purity are often stable for many years when stored at-20C. The greatest problem is contamination when students go into the stock tube, repeated freeze/thaw, or repeated changes of temperature. It is suggested that the stock tube be aliquoted when it is received.

For most applications 1ul is used to digest 250 ng to 1 ug of DNA. (A final dilution range of 2000 to 5000 Umits/ml is recommended when using the enzyme. The diluted enzyme is stable for long periods of time if stored at –20C). You can extend the time of incubation if you do not have enough enzyme. Always make sure that the final glycerol concentration is below 5% in the reaction otherwise you may get star activity. (Restriction enzymes are sent in glycerol –read the tube and/or C of A for this information).

You can incubate the reaction overnight (typically for 16 hours) in the recommended buffer (they usually require Mg) with 1 ug of substrate DNA in a volume of 50 ul; the only problem being contaminating endonuclease activity (you'll see this on a gel).

Electrophoresis
The choice of constant current or constant voltage depends on the buffer system and the size of the gel. Generally speaking, constant current provides better resolution because the heat in the cell can be controlled more precisely; however these runs will take longer to run. The only time constant voltage is recommended is in a native PAGE gel as there is no advantage to constant current; cooling recommended for good resolution.

Appendix F: Agarose Gel Electrophoresis with SYBR Green

SYBR GREEN:
SYBR Green is a very convenient dye for detecting nucleic acid fragments in agarose gels, as it binds nucleic acids stoichiometrically and emits fluorescent radiation on UV illumination. There is minimal background fluorescence, so destaining of gels is unnecessary. SYBR Green staining provides an extremely sensitive stain for bands that are very faint (as little as 10 ng). Although nucleic acid migration may be reduced by the presence of SYBR Green in the agarose gel (by 10-15%), it is very convenient to include the dye directly in the gel. This saves the extra time required to stain the gel in staining solution after electrophoresis.

When preparing gels with SYBR Green, use a stock solution at 10,000X. Adding 1 uL stain per 10 mL of agarose solution will create a 10,000-fold dilution from the stock. SYBR green stock should be added to the dissolved agarose after cooling, just prior to casting the gel. Because SYBR green is relatively non-toxic, it can be disposed of down the sink or in the regular trash; however, it is always a good practice to wear nitrile gloves and safety glasses when working with any chemical.

Note: There are two commercially available SYBR green stains used in the lab. SYBR Safe is used for double stranded DNA, and SYBR Green II is used for single stranded RNA.

To photograph gels stained by SYBR green, an ultraviolet transilluminator must be used. If available, use the appropriate SYBR green filter (the RRC gel doc system has both!). UV-absorbing protective eyewear is necessary when working with ultraviolet light, and exposure to this high-energy light should be kept to a minimum.

GEL RUNNING BUFFERS
Two buffers are commonly used when preparing agarose gels for separating DNA fragments:

TAE buffer:	**TBE buffer:**
40 mM Tris-base	80 mM Tris-base
5 mM Sodium acetate	80 mM boric acid
1 mM EDTA	2 mM EDTA
pH 8.0	pH 8.0

Both buffers are generally prepared from 10X or 50X stocks, which can be stored at room temperature. The 1X working solutions can be also stored at room temperature, but only for a few days. Longer storage of these solutions should be under refrigeration. Since the buffering capacity of TBE is higher, this buffer is preferred when longer electrophoresis times required. TAE is used when one wants to isolate the DNA fragments from the gel or run the electrophoresis for a short period of time. TAE buffers will electrophorese nucleic acids significantly faster.

RNA must be run on a denaturing gel. This is typically performed using a formaldehyde agarose system, or a glyoxyl denaturing agarose system. In our lab we will use the glyoxyl system for ease and safety reasons. Instructions are given in the lab manual.

GENERAL PROCEDURE FOR DNA AGAROSE GEL:

Materials required:

Agarose
1X gel running buffer
Gel-casting apparatus
Gel electrophoresis box
Power supply
Microwave oven or hot plate
Micropipettors and tips
Molecular weight standards
Balance, weigh boats
SYBR Safe stock solution, 10,000X
Ultraviolet (UV) light box (or imaging system)
Erlenmeyer flask
Saran Wrap

1. Set up a gel casting apparatus as directed by your instructor. Ensure the bumpers are snug in place to avoid leaking during casting. Insert a comb into the tray, choosing a comb by the size and number of wells it will create. Remember to include your molecular weight marker when deciding on the comb size. Set the apparatus on a flat surface that will be undisturbed while the gel is solidifying.

2. Determine the volume of agarose solution needed to accomplish your given task (approximately 30 mL are needed per gel), and calculate the mass of solid agarose needed to achieve the desired concentration.

   ⇒ NOTE: A casting tray measuring 7cm x 7 cm usually requires approximately 30 mL of agarose solution.

3. Prepare enough 1X running buffer for the gel and the electrophoresis chamber. This will depend on the specific apparatus you will use. An easy way to determine this is to fill the chamber with water and measure the volume. The mini-gel apparatus is typically less than 500mL.

4. Weigh out the required amount of agarose and add it to the appropriate amount of 1X gel running buffer in an Erlenmeyer flask. The flask should be at least twice the volume of the buffer and no more than 5X the volume. For example, to prepare 30 mL of a 1% agarose gel, add 0.3 g of agarose to 30 mL of 1X buffer in a 125-mL flask.

5. Place the flask on a level surface and carefully mark the glass at the fluid level with a permanent marker (DO NOT mark the white marking area with a permanent marker! It cannot be removed).

6. Heat the mixture until all agarose has dissolved. A hot plate or microwave oven can be used; using a hot plate will take more time. Interrupt the heating at regular intervals and swirl the container to mix the contents. The solution should be brought to a boil, but do not allow the solution to boil over. Microwave for a minimum amount of time to avoid buffer evaporation, which will cause a dramatic increase in the percentage of the gel. For example, a 30mL solution will require less than 1min total in a typical microwave.

7. When the agarose is completely dissolved, the solution will be completely clear and homogeneous; that is, you will not observe any granules or threads of non-dissolved agarose in the solution. Observe the fluid level in relation to the mark you made on the flask. If a significant amount of water has evaporated, carefully add water to return to the level of the mark and swirl the solution.

   ⇒ CAUTION: The container and contents will be hot! Use adequate precautions; handle flask with Hot Hands or an insulated glove.

8. Cool the solution to 50 – 60°C. For best results, cool the flask in a 55°C water bath. This will cool it more quickly and prevent the temperature from dropping low enough for the gel to solidify before pouring. The solution must be cooled to below 60°C to prevent damage to the plastic casting trays.

9. Add SYBR Safe stock solution (10,000X) to a final concentration of 1X. This is a 10,000-fold dilution, so for each 100 mL of agarose solution, add 10 μL of stock SYBR Safe solution (3ul for a 30mL gel).

   ⇒ NOTE: There are two SYBR stains for nucleic acids – SYBR safe for DNA and SYBR Green II for RNA

10. Pour the gel immediately into a level gel casting stand. Allow the gel to form completely; typically, 20 minutes at room temperature is sufficient. Remove the comb and bumpers from the gel, place the gel in the electrophoresis chamber, and add a sufficient volume 1X gel running buffer to just cover the surface of the gel.

    ⇒ NOTE: Do NOT pour molten agar down the sink. Left over agar can be kept in the fridge or allowed to solidify in the flask and disposed in the trash.

11. Each sample and marker should contain loading dye to a final concentration of 1X. Gel loading solution is usually supplied at a 2-10X concentration, and contains several substances. Glycerol makes loaded samples heavier than the buffer, allowing them to sink more quickly into the wells and prevents them floating away. The solution is buffered to prevent degradation of the nucleic acids it is mixed with. Finally, a visible tracking dye (usually blue, green, or orange) is present to allow the technician to monitor the progress of electrophoresis without removing the gels and observing with UV light.

12. Load samples into the wells and record the order of samples in your notebook and electrophoresis gel documentation form. You should also load an equal mass of molecular weight marker (or "ladder"). A marker is a solution consisting of predigested nucleic acids (either DNA or RNA) that appear as a series of bands. Each band corresponds to a molecular weight, and the molecular weight of unknown samples can be approximated by comparing their migration distance to that of the marker.

13. Cover the gel box with its lid, making sure to keep the black lead at the negative electrode (black) and the red lead at the positive electrode (red). Connect the gel apparatus to an

electrical power supply. Match the two leads to the two contacts on the power supply by their colors (black and red). Apply an appropriate voltage to the gel; depending on the application, this can be between 50 and 200 volts. Usually, DNA and RNA agarose mini-gels should be run at 100-125 volts. Higher voltages and shorter runs will decrease the resolution of the gel and may also cause overheating which may melt the agarose.

14. After the electrophoresis is complete (when the tracking dye is within 1 cm of the end of the gel), turn off the power supply, unplug the leads, and remove the lid from the apparatus. Wearing gloves, remove the gel. Place them in labeled weigh boats, plastic wrap, or baggies to prevent confusion about their identity.

15. Capture images using the Gel Documentation system. SYBR green works well with UV filters, but works best with the SYBR green filter if one is available.

ALTERNATIVE PROCEDURES:

1. Instead of adding SYBR Safe directly to the agarose, you may stain your gel after electrophoresis for 20 minutes on a shaker in a closed container containing 1X buffer with 1X SYBR Safe.