Austin Community College, Biotechnology Department

BITC 2441

Molecular Biology Techniques

Laboratory Manual

Spring 2011

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ISBN: BITC2441F2011

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LAB UNIT 1: INTRODUCTION TO MOLECULAR TECHNIQUES LABORATORY

The Biotechnology 2441 course at Austin Community College is one of the advanced courses in the Biotechnology program. It is designed for degree students who have completed the first year of biotechnology or its equivalent or those who are already employed in the field and are interested in increasing their skills in DNA and RNA technology.

In Molecular Biology Techniques you will be learning many of the common techniques used to work with both DNA and RNA. This will include isolation techniques, enzymatic DNA amplifications, sequencing, bacterial transformation with plasmid vectors, and quantitative-PCR.

This class is designed as an advanced course for those who have already completed at least one year of the biotechnology program or who are currently employed in the field. The techniques learned here will build on those introduced in BIOL1414 Introduction to Biotechnology and BITC 1402 Biotechnology Laboratory Methods. Unless you are already working in the field or have written permission of one of the full-time biotechnology faculty, you must have had these courses and passed a basic competencies exam with a grade of 70%, prior to enrolling in Molecular Biology Techniques. Registration will be revoked by the instructor for those students who have not fulfilled these requirements.

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

- Develop the advanced laboratory techniques of nucleic acid technology
- Supplement and enrich the lecture portion of the course
- Enhance skills to identify the important parameters in the design of a laboratory to conduct the most commonly-used molecular diagnostics protocols
- Enhance skills to identify the important parameters in the design of a quality system for molecular analyses
- Develop proficiency in the techniques required in order to perform the most commonly-used molecular biology techniques
- Develop critical thinking skills
- Enhance skills to design a well-controlled assay
- Develop proficiency in troubleshooting when molecular experiments fail
- Learn about the important design parameters and optimization of a molecular technique
- Practice accuracy in calculations and in writing scientifically
- Reinforce multitasking skills and the ability to work in a timely manner
- Encourage teamwork and accountability among students

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 Biotechnology Molecular Techniques are below.

Texas State Skills Standards (TSSB)

Key Activities for BITC2441 Biotechnology Molecular Techniques

A1 Maintain laboratory and equipment	A3 Operate equipment	A5 Clean and prepare items for lab	A6 Prepare biological and/or chemical materials	B1 Assist in designing experiments	B2 Perform assays and experiments	B3 Troubleshoot experiments and equipment
B4 Perform data analysis	B5 Communicate results	B6 Investigate new technologies and methodologies	C1 Participate in employer-sponsored safety training	C2 Participate in emergency drills and emergency response teams	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	D2 Create documents	D3 Enter and manage laboratory information electronically	

Course Policies:
Students are expected to behave professionally at all times and follow the ACC laboratory safety rules at all times. The lab exercises are project-based and are performed as close to industry situations as is reasonable and possible. Lab notebooks will be maintained and lab reports are required for all lab units on the week following the completion of that unit in the lab.

There will be lecture every class followed by the lab unless specifically stated otherwise in the lab manual. Thus there will be two labs on most weeks. The lecture is intended to give background and relevant conceptual information about the solution prep, procedures and related techniques encountered during the lab exercise and will also include material from the lab manual and textbook. You are required to pre-read both the manual and textbook and come to class prepared to fully participate. If you have questions about the material, this is the time to ask!

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

LAB NOTEBOOK

Each student will maintain a lab notebook in a three ring binder. The lab notebook will contain both experimental methods and observations along with the organized lab reports. The binder will be turned in at the end of the semester for grading, and each lab report will be tuned in individually for grading weekly.

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.
- 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 <u>lab notebook</u> must include:

- <u>A title page</u> with the name of the course, semester and your name.
- <u>A table of contents</u> with page numbers: Organize chronologically by LAB
- <u>Each Lab</u>
  - <u>Lab Manual Instruction</u>
  - <u>Pre-lab</u> as outlined below

- Experimental Results: This includes any forms filled out during the lab, raw data images from instrument printouts, raw data recorded into a data table, and any observations made during the lab.
- Lab report 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

PRE-LAB REPORT

The Pre-lab must be prepared before beginning the lab. The lab instructor will sign off on the pre-labs at the beginning of class to ensure that the students are prepared for the lab. The Pre-lab must be typed and printed out before the lab.

Your pre-lab must consist of the following:

- A Title: the title from the lab manual is sufficient.
- Date: when you turned your report in for grading.
- Student's name and names of any partners: those individuals directly responsible for the data and lab exercise.
- Introduction: An extensive background *IN YOUR OWN WORDS* of the purpose of the experiment and the approach taken to accomplish these goals should appear at the beginning of your report.
  - What questions do you want to answer with your experiments, or what exactly do you wish to accomplish? This will be similar to the introduction in the lab manual, but it will be in your own words. Feel free to use other sources. Do NOT plagiarize. This should be one page or more depending on the lab.
  - Remember to include any equations, chemical formulas as appropriate.
- Table of materials: Read through the protocol carefully and list all the equipment and materials needed. You should have an idea of where these materials will be in the lab. This list should be complete enough that your lab setup for the day should not require more than 5 minutes.
- Table of reagents: Read through the protocol carefully and create a reagent table that includes the following information:
  - the name of the chemical in the reagent used or the solution prepared
  - an explanation of its purpose in the lab procedure
  - any safety precautions that should be taken when handling the chemical
- Protocol: In your own words, write a brief description of the steps of the protocol. Any calculations such as for preparations of solutions that can be done prior to performing the exercise must be written out in the protocol. Data tables to be filled out during the lab exercise should be prepared beforehand in this section.

You must present your pre-lab work to your instructor to have it approved and signed off on prior to performing any experiments. You will not be permitted to participate in the lab without your pre-lab. This means you will receive a zero grade for this lab!

Although collaboration with lab partners is okay, each student must prepare a pre-lab and lab report individually. You are not permitted to plagiarize (intentionally or not!) any portion of the pre-lab.

EXPERIMENTAL OBSERVATIONS DURING LAB

During Lab, you must take complete notes as you perform the each step. WRITE DOWN EVERYTHING! How much exactly was weighed for each reagent? How much solution exactly was used? What mistakes were made? What did you observe?

Without adequate information, you cannot troubleshoot an experiment that turns up inexplicable results – or no results at all! Even for experiments that go well, you jeopardize your ability to reproduce these results in the future if it isn't exactly clear how you performed the experiment. Biological experiments are always complex, subject to subtle effects and unknown contributors.

Your results and observations must be recorded in pen, only. Remember, if you make a mistake cross out that mistake and initial the cross out. Never use White Out and never rewrite the lab. In industry, and even in some academic labs, your notebook will serve as a legal document in a patent court.

FILLING OUT FORMS:

Whenever a solution is prepared for use in the lab exercise, a **Solution Prep Form** must be filled out and a Control Number assigned for that solution. The Control Number for every solution used during the lab exercise must be written in your lab notebook in order to cross-reference it to the Solution Prep Form describing how it was prepared. This careful annotation of solutions used plays an important role in troubleshooting failed experiments. All Solution Prep Forms for solutions used in an experiment should be included in your notebook, not your lab report. If you are using a solution prepared by another student in the class, you will be responsible for obtaining the appropriate Solution Prep Form from them.

Similarly, whenever an electrophoresis gel is run, the details about what was loaded in each lane and the running conditions of the gel must be recorded in a Gel Electrophoresis Documentation Form. When sharing gels with other students, you will be responsible for obtaining all the necessary information from our classmates.

THE LABORATORY REPORT

After completing the lab exercise, you will write a formal report that summarizes your lab in a professional and scientific format. In the workplace your supervisor may ask you to summarize your experiments in a report form. A report is professional, it includes a scientific perspective of your experiment and it should stand alone. Meaning, your supervisor should not have to reference your notebook to gain further insight into the report's results.

Your report will be typed, be written with scientific language, have a professional format, contain no spelling and grammar mistakes, and will include the following:

- Title page, with name, date, and appropriate descriptive title for the lab.

- Introduction: You may copy this directly from your pre-lab. It should be one page long minimum, depending on the lab.

- Materials & Methods: Summarize the general procedure in your own words. This should be about ½ page long. Do NOT copy your detailed procedure from your pre-lab.

- Results: Do NOT include your raw data, notebook pages, or forms in the report!
  - You need to consider ways of compiling your results into tables and graphs here. Ask your instructor for guidance if you are unsure of appropriate ways to present your data in your report.
  - These tables and graphs should be titled and annotated with enough detail that they can stand alone in summarizing your results quickly. This may mean that raw data has been averaged, graphed, or manipulated mathematically, but in every case, it should be obvious to the reader where the numbers came from and what they represent.

- Analysis & Conclusions: Your analysis should be approximately one page, depending on the lab. Summarize your results and any conclusions that can be drawn from them. ***Use scientific language and mathematical concepts to defend your conclusions***. Avoid 'feeling' words and descriptions.
  - This is the most important section of your write-up, because it answers the questions, "Did you achieve your proposed goals and objectives?" And "What is the significance of the data?" Any conclusion that you make must be supported by the experimental results. If it is possible to compare your data to controls or known values obtained from reliable sources, then calculate the percentage error and why your results differed.
  - This is also the place where you discuss the success of the experiment. When you encounter unexpected or disappointing results, you should provide a likely explanation for them here. If the results cannot be interpreted, you should reason on how to troubleshoot the experiment and how to improve your approach or technique in order to make it more successful.
  - This is also a good place to compare your results with those of your classmates in order to interpret your results.

- Analysis Questions from the Lab Manual: Some units have additional questions at the end. Answer the questions using explicit language and complete sentences.

- References: Learning is not an island but a road. You learned everything from somewhere, so list all books, journal articles, and websites that were used to write up the experiment. This includes this lab manual and your textbooks.

The entire unit is due the week after the data has been collected. Late lab policy is outlined in the syllabus.

Missed Lab Policy

Students are allowed to miss only one lab during the semester and only with instructor approval. Approval will be based on illness or how unavoidable the absence was. For example, a death in the family is unavoidable, but leaving early for spring break is avoidable and won't be approved. There will be no make-up lab but students who complete the pre-lab and the post lab report using data from a teammate, may receive a passing grade (as much as a 70%) from submission of the lab report.

Lab Unit 1-B: Lab Safety & Security

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 $\mathbf{LD_{50}}$ 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

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

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
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, they 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 1-B PROTOCOL:

PART 1: Safety Training

1. 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 available online here: http://www.austincc.edu/biology/safetyvid.html

2. 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.

3. 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.***

Lab Unit 1-C: Lab Room 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. A current copy is typically kept in the lab for your reference.

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.

Micropipettes
Two sets of four micropipettes are stored in the cabinet below each workstation. 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 micropipettes for the semester and they can be easily tracked and returned to their cabinet if misplaced.

Pipette Tips
The tips intended for regular, daily use are located in cabinet 1B, and are restocked frequently. Take a pack of each size tip needed and store them in the cabinet or drawer at your workstation; do not stockpile more than one box per size. Filter (aerosol-resistant) tips for special use in PCR and RNA work are kept in the prep room in cabinet 9B. Please avoid commingling these tips with the regular tips in cabinet 1B because they are more expensive and not necessary for everyday use. One exception is the p10 size tips which we will provide the filter tips only. 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 either on the bottom shelf of the cart or next to the sink in the prep room. 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°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 10% bleach 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 it to the right side of the sink in the prep room. 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 encouraged 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 (LCD will read “STANDBY”), and 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 1-C PROTOCOL:
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		
Gloves		
Test tube racks		
Graduated cylinders		
Spectrophotometers		
Erlenmeyer Flasks		
Freezers (-20°C)		
Refrigerator (4°C)		
Shaker Incubator (37°C)		
NanoDrop		
Hotplate/stir plate		
Stir bars		

Lab Unit 1-D: Competencies Check

Introduction
The biotechnology industry in this area has determined a number of competencies or skills that are required for an entry-level biotechnician. The complete listing of these competencies is provided on our web site (http://www.austincc.edu/biotech/). In order to succeed in BITC 2441, Molecular Biology Techniques, you will need to readily perform some basic calculations such as those used in making solutions. You will also need to use certain basic techniques such as using a balance, and operating a micropipette, pH meter and spectrophotometer. This paper and pencil exercise reviews the starting competencies for BITC 2441 in order for you to determine your readiness for this class.

Safety Precautions: *None needed for this laboratory.*

Protocol:
1. Working in groups of 2-4, answer the following questions on the attached sheets. Work with the others in your group to formulate one answer. Make sure you write your names at the top under "Answerers"

2. Pass your answered questions to another group and receive their set of answered questions. Write your names at the top under "Questions Graded".

3. Correct each of the questions you have received, checking with each other within your group to make sure your corrections are correct.

4. Hand in your graded questions. You will receive credit for both answering questions correctly and grading questions correctly.

COMPETENCY CHECK QUESTIONS

Question Answerers: ______________________________ date ____________________

______________________________ date ____________________

Question Graders: ______________________________ date ____________________

______________________________ date ____________________

QUESTIONS TO ANSWER	**GRADER'S COMMENTS**
1. Describe how you would make a 1.00 M Tris-Cl pH 8.0 stock solution (50 mLs). The molar mass of Trizma base is 121.1 g/mol. Include in your answer how exactly you would adjust the pH and how you would store this stock solution.	
2. Describe how you would make a 50 mL solution of 50mM pH 8.0 Tris-Cl and 0.9% NaCl from these stock solutions: 1.00 M Tris-Cl pH 8.0 & and 20% NaCl. Include in your calculations how much water must be added.	

3. Describe how you would make 3 serial dilutions of 5-fold dilutions of your 1.00 M stock Tris-Cl pH 8.0, in which you are left with 4.0 mL of diluted solutions. Include in your calculations how much water must be added. What are the final concentrations of each dilution?	
4. What are the maximum and minimum volumes of the following micropipettors? What do the settings on the micropipettors look like when adjusted for these minimum and maximum settings?	

P1000 micropipettor		P200 micropipettor	
Minimum setting _______ uL	Maximum setting _______ uL	Minimu m setting _______ uL	Maximu m setting _______ uL

P20 micropipettor		P10 micropipettor	
Minimum setting ______ uL	Maximum setting ______ uL	Minimu m setting ______ uL	Maximu m setting ______ uL

5. Which micropipettor would best be used to measure each of the following volume measurements? Draw the appearance of the numbers on the micropipettor after adjusting for each measurement.

2.5 uL micropipettor used _________
volume setting

45 uL micropipettor used _________
volume setting

720 uL micropipetteter used _________
volume setting

Analysis & Questions for Lab Unit 1

For this Unit One lab exercise, there will be no formal lab report. Instead, complete the following and turn in for grading one week from today.

1. Obtain materials for lab: safety glasses, closed-toed shoes, a sharpie, scientific calculator and a 3-ring binder to use in this lab class.
2. Submit the following filled-in sheets:
   a. Safety Forms
   b. Lab Equipment sheet
   c. Competency check sheets (you may have done this in class already).
3. Answer questions below and hand in with competency check sheets.

Analysis Questions to include in your report:
You may have already read Chapters 9, 10, & 11 in Basic Laboratory Methods for Biotechnology, 2nd Ed. Seidman & Moore (2009), in your previous Biotechnology courses. If you have not, or it's been a while, please review them. Answer the following questions:

A. Lab Emergency Scenarios
You may find the ACC Environmental Health website useful for answering the following questions: www,austincc.edu/ehs/HAZCOM.html. This site provides links to information about emergency procedures and chemical reactivity's.

1. Below are some common lab emergencies. Briefly describe what to do in the following situations for each of these chemicals that are commonly used in the biotechnology laboratory**:** hydrochloric acid, acetone, sodium hypochlorite, and ammonium sulfate.
   a. The chemical spills on your hand
   b. The chemical splashes into your eye
   c. A 250 mL beaker of the chemical drops and breaks on the floor

2. What are the NFPA ratings for the above 4 chemicals, and what do these ratings signify?

3. What would you do if there is a small fire in the lab? Describe the steps in the order that they should be taken.

B. Toxicity
Molecular biology laboratories must sometimes work with hazardous chemicals. Toxicity information can be found in MSDS reports, which are shipped with the chemicals sent out from vendors. MSDS reports are also readily available online by searching for "MSDS" in a search engine such as www.google.com. An MSDS list is also linked to the ACC Environmental Health website at www.austincc.edu/ehs/HAZCOM.html . A glossary of MSDS terms is available at www.ilpi.com/msds/ref/index.html.

Below are 5 chemicals commonly used in molecular biology labs. Using MSDS information, prepare a table of the following chemicals that lists the LD_{50} (oral rat, g/kg), carcinogenicity, NFPA ratings, the target organs that the chemical affects, and your description of what

precautions must be taken when using them.   Also, calculate the dose that a 150# (70 kg) person would have to swallow for a 50:50 risk of death, based on rat toxicity data.

Chemical	***Use***
Acrylamide	Subunit for PAGE (polyacrylamide gel electrophoresis)
Ethidium bromide	Dye that detects DNA
Diethylpyrocarbonate (DEPC)	Inhibitor of RNases
Phenol	Protein denaturant used to purify nucleic acids by extraction
Chloroform	Protein denaturant used to purify nucleic acids by extraction

To put the toxicity of these chemicals into perspective, prepare a similar table for:

Chemical	**Use**
Ethanol	Party aid
Aspirin	Post-party aid
Caffeine	Study aid for college students

Unit 1 References:

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

LAB UNIT 2: QUALITY SYSTEMS IN THE MOLECULAR LAB

Introduction
All laboratories are in the business of making measurements and reporting results of such measurements. The reliability of such measurements play an important role in the conclusions that can be safely drawn from them. ***Quality cannot be automatically assumed, and must be demonstrated and monitored by the laboratory***.

The need for validation of results has been recognized by government agencies such as the Food and Drug Administration (FDA) and the Environmental Protection Agency through the institution of Good Laboratory Practices, which govern regulatory compliant laboratories. These regulations provide a quality system for good recording of data, plans, and procedures in the laboratory, helping them to ensure against everything from sloppiness to dishonesty. While adherence to such guidelines is clearly more critical in laboratories that have a big effect on public health and safety, all laboratories can benefit from basic quality guidelines.

Some benefits of adherence to a quality system include:

- ✓ more reliable data and reproducible results allows a steady progress in a research lab, since misleading results are avoided
- ✓ protection of one's reputation—nothing is more damaging than the release of anomalous data and information
- ✓ protection of patent rights to intellectual property—a patent court will assume that if it wasn't written down, signed and dated, it wasn't done at all
- ✓ protection from litigation

So, while attention to quality while operating in a laboratory requires additional time, effort and expense, it is a worthwhile investment and a good habit to keep.

Every aspect of lab operations has an impact on quality:

- ♦ the ordering of the proper reagents and equipment to adequately perform the needed measurement
- ♦ the correct maintenance and use of materials and equipment to perform experiments
- ♦ careful adherence to established protocols or standard operating procedures (SOPs)
- ♦ careful lab technique in performing protocols
- ♦ careful documentation of observations and raw data
- ♦ complete reporting of results

The following discussion will present some important approaches for maintaining high quality of results in a laboratory.

Reagent Quality
Chemicals exist in varying grades of purity:

- ♦ ultrapure, chemically pure (CP) - Ultrapure chemicals have been put through additional purification steps for use in specific procedures such as chromatography, atomic

absorption, immunoassays, molecular diagnostics, standardization, or other techniques that require extremely pure chemicals. These reagents may carry designations such as "HPLC" or "molecular" grade on their labels

- Analytic reagent grade (AR) - The American Chemical Society (ACS) has established specifications for analytic reagent grade chemicals, and chemical manufacturers will either meet or exceed these requirements. Labels on these reagents state the actual impurities and their levels.
- United States Pharmacopoeia (USP) or National Formulary (NF) - USP and NF grade chemicals are used to manufacture drugs, and the limitations established for this group of chemicals are based only on the criterion of not being injurious to individuals. Chemicals in these groups may be pure enough for use in most chemical procedures; however, it should be recognized that their purity standards are not based on the needs of the laboratory
- Technical or commercial grade - This is the lowest purity, to be used primarily in manufacturing and should never be used in an analytical or research lab.

Organic solvents also have varying grades of purity:

- reagent grade (ACS) - certified to contain impurities below certain levels established by the ACS
- chromatographic grade - minimum purity of 99% determined by gas chromatography
- spectroscopic - purity determined by spectroscopy (less sensitive and reliable means of testing)
- chemically pure - approaches the purity level of analytical or reagent grade (AR)
- practical grade - the lowest grade

Although purity of reagents generally corresponds with cost, most molecular techniques are sensitive to trace amounts of contaminants, and the highest purity should be used.

Water is the most frequently used reagent in the laboratory. Purification of water includes distillation, ion exchange, reverse osmosis, or any combination of the above. There can be carryover of organic materials in both distillation and ion exchange processes. The quality of distilled water is highly dependent on how well the equipment is maintained. Deionized water can vary greatly in quality depending on the type and efficiency of the deionizing cartridges used. The deionizing resins can themselves increase the organic contaminant level in the water by leaching of resin contaminants, and should always be followed by a bed of activated carbon to eliminate the organics so introduced. Ion exchange and carbon columns are notorious for harboring microbes and should be carefully monitored for contamination. Reverse osmosis generally provides the highest quality of water, but does not remove dissolved gases. Generally, reverse osmosis in combination with another purification step works well for a molecular lab.

Water can be further sanitized by ultrafiltration, ultraviolet light, or ozone treatment and autoclaving. Sanitization helps to reduce the threat of nuclease contaminants, and is generally required for quality results in the molecular lab. Ultrafiltration works well to remove microorganisms and any pyrogens or endotoxins. Ultraviolet or ozone sterilization tends to leave behind residual products, and these techniques are most often used only on highly purified water. Commercial systems that produce the highest quality of purified water usually apply a multiple-

step cleanup process including reverse osmosis, mixed-bed ion exchangers, carbon beds, and filter disks. In-line conductivity meters indicate when the cartridges have been exhausted and the system is failing. Since water devoid of ions is a poor conductor of electricity, a high resistance is an indicator of a well-functioning water purification system.

Laboratory Supplies

Several types of supplies are common to most of today's laboratories and care should be taken in their selection to assure that they are well-suited.

1. **Thermometers.** Some lab procedures, especially enzyme reactions, are sensitive to temperature. In addition, many reagents used in the molecular lab must be stored at a proper temperature. Liquid-in-glass thermometers, using a colored liquid, are replacing the more traditional mercury-in-glass devices. They usually measure temperatures between -20 to 400°C. Partial immersion thermometers must be immersed to the proper height as indicated by the continuous line etched on the thermometer stem. Total immersion thermometers are used for refrigeration or incubator applications. An electronic thermometer is often incorporated in instruments, refrigeration units, and incubators. All thermometers must be calibrated before use, and for critical applications they should be calibrated against a NIST-certified or NIST-traceable thermometer.

   It is important to accurately measure and record the temperatures in which buffers are prepared, and enzymatic reactions are performed. Always keep in mind that "room temperature" is a figure of speech and not an actual temperature. For every lab you are performing an experiment (at room temperature, in a 37°C incubator, in a 95°C heat block etc…) <u>you will be required to record in your notebook the actual temperature the experiment took place</u>. Common laboratory glass and mercury thermometers can be highly inaccurate. Most have an error of +/- 2°C, but some can have an error of up to +/- 4°C! Digital thermometers (LED outputs) can be just as poor in accuracy. Just because you set the water bath to 37°C, doesn't mean the actual temperature of the water is 37°C. Some labs calibrate their common glass thermometers with a certified thermometer, but <u>the best way to measure temperature accurately is to use a certified validated thermometer</u>.

2. **Glassware.** Glass used in the laboratory differs with respect to temperature and chemical stability. In general, disposable glassware is made of flint (soda lime) glass, which has a relatively low melting point and is more susceptible to attack by strong acids and bases. While borosilicate glass (Pyrex or Kimax) suffices for most laboratory applications, aluminosilicate glass (Corex or Vycor) should be used to store strong acids and bases. Chemical instability, in which glass is etched by solutions, results in contamination of the solution, and should be avoided. Glassware that is to be washed should be rinsed immediately, and washed glassware should be rinsed several times with distilled or deionized water to ensure that the soaps and tap water salts have been thoroughly removed. Many molecular techniques are extremely sensitive towards detergents and salts, so caution must be exercised with non-disposable glassware.

   <u>NOTE: Amber glass is not recommended for storage of molecular biology solutions that are light sensitive. Small amounts of metals in the glass can catalyze unwanted oxidation in aqueous solutions, leading to the possible degradation of key components in complex</u>

solutions. In a lab, most will wrap bottles with foil, or store in a dark cupboard to protect solution from light.

3. **Plasticware.** The unique high resistance to corrosion and breakage has made plasticware a popular choice in the laboratory. Its low expense makes it a preferred choice for disposable materials. Four types of resins are most often used in the laboratory: polystyrene, polyethylene, polycarbonate, and polypropylene. Of these, polypropylene withstands autoclaving and high temperatures the best, while the strength of polycarbonate is preferred in high-speed centrifugations. Polystyrene has the advantage of being transparent, but is chemically and thermally instable. Polyethylene is the most flexible and has good chemical stability towards inorganic chemicals. None of these plastics are resistant to nonpolar organic solvents; for this Teflon plastics are generally the preferred plastic and glass is a more reliable storage material to be used. See the appendix for a more thorough description of the chemical and physical properties of plastics.

4. **Pipets.** The most common operation in a molecular lab is the measurement and transfer of specific volumes, so this central activity must be impeccable. All types of devices that measure volume are calibrated to different degrees of accuracy; it is important to know what accuracy your device has been certified at before using it for a critical measurement. Generally, volumes less than 20 mLs are best measured by pipets. The table below lists the classification of different types of pipets.

   Since pipets come in different forms and widely different degrees of accuracy in volumetric measurement, it is important that you be aware of the type of pipet that you are using. This is especially important for the blow-out versus self-draining types of pipets. All blow-out pipets are identified by either (a) an opaque band one-quarter of an inch wide or (b) two bands one-quarter inch apart at the top end of the pipet. There are pros and cons to both kinds of pipets. All self -draining pipets are calibrated with water, so if the liquid has a different surface tension or viscosity from water, your measurements will not be accurate. On the other hand, not everyone will exert an equal amount of blow-out force, so measurements with a blow-out pipet are often not as reliable. Since most solution of critical volumes measured in the molecular lab is aqueous, the self-draining pipets work best.

   Pipets can be purchased with cotton plugs which allow gases past and filters out any aerosols that might be formed during the pipetting procedure. Since cross contamination is a major concern, cotton plugged pipets are preferred in the molecular lab, even if that means that you are using a disposable pipet of low volumetric accuracy.

   Washing of pipets for reuse requires precautions to assure adequate cleaning and rinsing of possible contaminants. Pipets should be rinsed and cleaned immediately after use or soaked in a bleach solution until cleaned by batch. The key to adequate cleaning is number rinses, especially following any use of detergents. A final rinse with deionized or distilled water is critical to ensure that no tap water salts remain in the pipets.

   Micropipettes must be validated at least once a year by measuring the weight of measured volumes of a liquid of known density (such as water). The standard deviation of a series of

replicates and the percent coefficient of variation [%CV = (standard deviation / mean) X 100] is measured, and micropipettes are recalibrated whenever the error exceeds the limits specified by the vendor or the laboratory management.

Category of pipet	**Type of pipet**	**Description**
By design	To contain (TC)	Holds or contains a particular volume, but does not dispense that exact volume
	To deliver (TD)	Will deliver the volume indicated
Drainage characteristics	Blowout	The last drop of liquid should be expelled into the receiving vessel
	Self-draining	Contents of the pipet drains by gravity only; the pipet tip should not be in contact with the accumulating fluid being delivered
Type by purpose	Serologic	Has calibration marks to the tip and is generally a blowout pipet
	Mohr	Has calibration marks that do not go all the way to the tip and specific volumes must be measured by the difference between two calibration marks
	Volumetric	Does not have graduation marks to the tip and is a self-draining pipet; measures one volume only with a high degree of accuracy
	Transfer	May or may not have calibration marks; not intended for accurate volumetric measurements
	Disposable	All types of pipets described can be purchased in a disposable glass or plastic form; generally not as accurate in volumetric measurements
	Micropipettes	Mechanical pumps calibrated to deliver highly accurate volumes generally less than 1.0 mL, and as little as 0.1 microliters. They can be fixed or adjustable volumes; always use disposable plastic tips to actually transfer the liquids. Multichannel micropipettes can deliver the same volume from as many as 12 tips simultaneously. All automatic micropipettes need regular maintenance, recalibration, and validation.

5. **Balances.** Electronic balances use an electromagnetic force to counterbalance the weighed sample’s mass. Analytical balances are more accurate and must be used to weigh analytic standards and very small masses. Balances must be calibrated with standard weights that span the range of the instrument. The frequency of calibration is a function of the manufacturer’s specifications and the requirements of the lab management. Balances must

be kept scrupulously clean and must be located away from traffic and air currents in the lab. Placement on a slab of marble helps to ensure that vibration interference is minimized.

6. **pH meters.** Careful control of pH by buffer solutions is critical to the success of molecular biology experiments. All pH measurements should be preceded by calibration to certified standard buffers, as these instruments quickly lose their calibration. The first calibration buffer should be at or near pH of 7.0, and the second calibration buffer should bracket the pH that you need to measure. Unfortunately, tris(hydroxymethyl) amino methane ("Tris") buffers and many other biological samples are known to damage AgCl electrodes by causing precipitates with silver ions, so calomel electrode is a better electrode for the molecular lab. The mercury in a calomel electrode makes their use a drawback, and some manufacturers now offer a nonmetallic, redox couple reference system which is not hazardous to the biological samples or lab personnel. It contains no ions to cause precipitates with proteins as well. New electrodes are also available with isolated Ag wire references and AgCl inner billing solutions that do not come in contact with the sample. These electrodes use a polymer gel to keep the sample and the silver separated. Electrode manufacturers are the best source of information on tris-insensitive electrodes. Electrodes need to be conditioned prior to use for reliable operation. Electrodes must be stored between uses in a storage solution specified by the manufacturer.

Standard Operating Procedures (SOPs)

Many of the quality issues in laboratories can be addressed by formally written standard operating procedures (SOPs) of routinely-performed lab procedures. SOPs can be written to describe such things as:

- procedures for reagent receipt, storage, and preparation
- equipment operation
- equipment maintenance and repair
- methods for taking and recording data

These SOPs should have sufficient detail to promote consistency in performing the procedures, something that is especially important and challenging in the larger labs. Having SOPs and insisting that they are followed provides a lab director with a measure of control over potential variables in experiments. If SOPs are strictly followed and documented in lab notebooks, anomalous results or data that fall outside of an acceptable range can be more easily evaluated. Careful documentation of SOPs used during an experiment allows for the reconstruction and evaluation of the experiment. Any changes to an SOP must be formally agreed upon by all involved. All expired SOPs must be removed from the lab operations, but archived for any future reference for historical data.

While different laboratories develop their own SOPs, many protocols and assay procedures in the regulated lab setting, such as the clinical or diagnostic lab, are determined by regulatory agencies. The FDA and EPA have quality systems and procedures outlined in their Good Laboratory Practices (GLP) guidelines. Many assay procedures are specified by the FDA and EPA as acceptable. Often, assays adopted by the FDA come from by the United States Pharmacopoeia (USP). The FDA, USP, or EPA approved methods often set the standard for unregulated laboratories striving to establish the quality of their work.

Documentation of Data

There are at least four characteristics of quality in lab measurements and data:

- completeness – the information is totally there, self-explanatory, and whole
- consistency – there is reasonable agreement between different replicates within an experiment, replication of results of the same experiment from day to day and between different laboratories using the same information
- accuracy – the agreement between what is observed and what is recorded
- reconstructability – the recorded data and results should be able to guide any person through the all relevant events of the study

Raw data must be recorded properly to preserve and adequately archive them. The FDA defines raw data and its proper recording in its GLPs:

All data generated during the conduct of a study, except those that are generated by automated data collection systems, shall be recorded directly, promptly, and legibly in ink. All data entries shall be dated on the date of entry and signed or initialed by the person entering the data. Raw data includes the worksheets, calibration information, records and notes of original observations, and activities of a study that are necessary for the reconstruction and evaluation of the study. May include photographic materials, computer printouts, automated and hand recorded datasheets.

Those who carelessly or sloppily record their data eventually suffer the consequences when data are lost or their records are unintelligible. Such data are not acceptable to the patent office or a regulatory agency, and can even lead to a lawsuit if data appears to be incomplete or obscured. Legibility is another absolute requirement of data records. Data must be readable and understandable. If questions arise later, the individual responsible may be sought out and asked to clarify an entry. Most lab work is time dependent, so the time and date must be recorded on each page of recorded data, along with signatures of the individual responsible for collecting the data.

If data requires corrections or additions at a later date, the FDA and EPA GLPs address this:

Any changes to entries shall be made so as not to obscure the original entry, shall indicate the reason for such change, and shall be dated and signed at the time of the change.

A single line drawn through an entry requiring correction will allow the reconstruction of the mistake. White out is never allowed.

Most laboratories expect data collected or generated by instruments and computer programs to be treated in the same way as hand-generated data. The GLPs state:

In automated data collection systems, the individual responsible for direct data input shall be identified at the time of the data input. Any changes to automated data entries shall be made so as not to obscure the original entry, shall indicate the reason for the change, shall be dated and the responsible individual shall be identified.

The name of the individual collecting data, along with the date of data collection, should appear on any automated data collection. A paper printout should be signed and dated by the operator and taped or glued into the lab notebook with the rest of the experimental information. If the printout is too large for the page, it should be shrunk to fit the page. No writing should be covered by the paper taped or glued into the notebook.

The exact description of an experiment must precede the data that is collected and recorded in the lab notebook. If an SOP is in place that describes the experiment in sufficient detail, it can be referenced. Any information that is unique to the experiment or not specifically discussed in the SOP should be added. It should include any deviation from established methods or SOPs. Failed experiments must be reported even though the procedure was successfully repeated, and a description of what may have gone wrong should be included. Information pertinent to reconstructing and evaluating an experiment includes careful documentation of reagents used. A system must be in place to record and archive details about each solution that is prepared or purchased for use. This is especially crucial in a molecular lab where such solutions are commonly complex and have a relatively short shelf life that is subject to the methods used for storage.

Documentation Forms. Computer-generated forms are often used to standardize data collection. They offer advantages of an easy format to fill data into, and generally can ensure that the data collection and descriptions are more complete. For example, SOPs followed can be included in the forms, and header information can ensure that the sample numbers, solutions used, etc are included. This works well only if all blanks are filled out on the form, and any blanks that do not apply to the experiment at hand are justified and marked as such. Some information that forms for experimental results should include are:

- the instrument used to collect the data
- the person operating the instrument
- the date and time of the operation
- all conditions of the experiment and settings of the instrument
- cross-reference to a protocol or SOP
- the units of measurement associated with the data
- all system-calculated or hand-calculated results

A quality system should be in place to monitor the pre-experimental operations of the laboratory. Information must be recorded and archived to track materials and reagents as they are received, maintained, and used in the laboratory. In addition, equipment calibrations and maintenance logs should be maintained in order to document their status. Some forms that are valuable in such a quality system may include:

1. **Reagents Received Forms:** includes vendors and lot numbers, when received, expirations dates, how it is stored and where. NOTE: That date that reagent bottles are opened should be added to this log, and the bottle label should be also marked with the date that the bottle is first opened. If reagents are tested for potency or purity, that information is recorded here, and any observed loss of potency or purity is observed here once the reagent is put into use in the laboratory. Historical information, such as any loss of refrigeration for heat-sensitive reagents is recorded on this form, as well.

2. **Solution Prep Forms:** should include details about the reagents used, calculations for amounts used, equipment used to make measurements, the individual who prepared the solution, the date that the solution was prepared, how and where the solution is stored, and a control number to identify the solution. The solution label should reflect details on the data of preparation, the solution contents and concentrations, and the control number. These forms are archived for easy access by lab personnel. (See appendix for forms.)

3. **Instrument Calibration Logs.** Any calibration measurement that is made on an instrument must be documented for recording any trends or defects. Corrective measures made for an instrument that is found out of calibration should be included.

4. **Instrument Maintenance Logs.** This records the proper care of instruments, insuring their correct operation and safety in the laboratory.

5. **Culture Logs.** Many molecular labs maintain cryopreserved cultures. A record of t he cultures must include the cell type, any recombinant ore genetic constructs of the cell line, the culturing history of the preserved specimen, the cell density and crypreservative of the specimen, and the location of the cells.

6. **Freezer Logs.** Many solutions, cell cultures, and reagents of a molecular lab need to be preserved in an ultralow freezer (-70 to –80°C). Any deviation from this low temperature can be harmful to the contents of these freezers, so it is important to not allow them to be opened for any long periods of time. To prevent long-term rummaging through such freezers, logs are maintained for rapid location of its contents.

7. **Inventory Logs.** It is especially useful to larger laboratories to keep a log of supplies and equipment to monitor the inventory and to be able to locate equipment.

In this lab exercise, we will address three important concerns of a molecular lab:

Part A. The Design of a Molecular Lab
The physical layout of a molecular lab plays in important role in contamination problems. In this exercise, students will design an ideal layout for a lab that routinely performs PCR amplifications.

Part B. The Verification of a Micropipette
The correct use and verification of micropipettes has an important impact on the precision and accuracy of results in a molecular lab, and will be tested in this exercise.

Part C. Preparation of Reagents
Every experiment requires exacting solutions often made by the researcher. These solutions must be made exactly the same every time to prevent variance in experimental results. In this exercise, students will make common laboratory solutions.

Lab Unit 2-A: Design of a Molecular Lab

Introduction:
One strategy used to avoid the cross-contamination of DNA in a molecular lab that routinely amplifies DNA, lies in the original design of the lab itself. Ideally, separation of different stages of amplification tests will be carried out in separate rooms. It is especially important that any analytical work that requires the opening or transfer of amplicons to be separated from all other areas of a molecular diagnostic lab, and that strong measures are taken to avoid any carryover of amplicon DNA through the air, through supplies or materials, or on personnel. A ventilation system that creates negative air flow through HEPA filtration or directly to the outside of the building will help to "quarantine" a PCR detection room. Disposable personal protective equipment (PPE) such as lab coats should be used by lab personnel and should not leave a PCR detection room. Any supplies, equipment, or materials should likewise not leave a PCR detection room without first being treated and/or contained. Chemical and physical decontamination measures should also be used: work areas can be treated with a 10% bleach solution followed by wipe down with deionized water, and ultraviolet lights to irradiate rooms overnight can also degrade amplicon DNA contamination.

In reality, many if not most molecular labs do not have the space required to separate the different stages of amplification reactions, and rely instead on laminar flow hoods equipped with air filters and ultraviolet decontamination lights. Nevertheless, in this situation it is still wise to maintain a traffic pattern of personnel and materials that prevents the carryover of amplicons from PCR reactions into PCR prep areas.

Scope of the Laboratory Exercise:

In this lab exercise, you will work with a lab partner to design your ideal of a molecular lab to prevent amplicon cross-contamination. You will also outfit your lab with **necessary materials and equipment** required in each of the rooms that you design, based on a general description of the steps that might be involved in conducting a PCR experiment that follows. Indicate in your lab lay-out drawing exactly where you would place these instruments and supplies to make them convenient for day-to-day operations and to avoid cross-contamination issues. You may want to make a story about what your lab is named and what work it carries out. And you are the Chief Scientist in charge!

Many of our biotech interns are, in fact, given responsibility for the design and commissioning of a new molecular lab, so you may in fact be called upon to do so yourself in the future. This exercise will help you to prepare for that day!

General Description of a PCR Experiment

Stage 1. Specimen preparation.
Specimens are received and archived for analysis, including the tests to be run. DNA is extracted from samples for PCR amplification.

Stage 2. Master Mix preparation.
All the reagents common to all DNA samples to be amplified are prepared in the proper concentrations and volumes.

Stage 3. Amplification.
Master Mix, primers, and DNA preps from clinical samples are combined in PCR tubes and placed in a thermal cycler.

Stage 4. Post-amplification analysis.
For end-point analysis, amplicons are analyzed, usually by gel electrophoresis.

Lab Unit 2-B: Verification of a Micropipette

Introduction:
All critical measurements of volume are done in the molecular lab with a set of micropipettes. A good maintenance and calibration schedule is the key to ensuring that your micropipette is working properly. A few maintenance suggestions include:

- Always store micropipettes in an upright position to prevent the nose cones or pistons from being bent.
- Keep the micropipette clean: dust or smears of materials on a nose cone can prevent proper sealing of micropipette tips and a clogged nose cone will affect the volume that is aspirated. Unscrew the nose cone to check for debris in the piston, O-ring, and seal. Check the orifice at the tip of the nose cone to make sure that it is free of debris. Follow manufacturers' direction for cleaning of micropipette parts. Validate measurements (test for accuracy) of a micropipette following a thorough cleaning procedure.
- Make sure that the nose cone is fitting securely on the micropipette and has not undergone any twisting.
- Watch for leaks and replace an O-ring to get a tight seal when necessary. Validate measurements (test for accuracy) of a micropipette following an O-ring replacement.

The protocols and schedules for micropipette validations (tests for accuracy) of micropipettes may be subject to guidelines provided the American Society for Testing Materials (ASTM), International Standardization Organization (ISO), FDA Good Manufacturing Practices (GMP), or FDA and EPA Good Laboratory Practices (GLP) standards. Clinical laboratories may follow specific guidelines provided by their certifying agencies and regulations: National Committee for Clinical Laboratory Standards (NCCLS), College of American Pathologists (CAP), Clinical Laboratory Improvement Amendments (CLIA), and Joint Commission on Accreditation of Healthcare Organizations (JCAHO). A minimum of a quarterly evaluation is generally recommended.

Most micropipette manufacturers advise users to check the calibration of their micropipettes by simply weighing a measured amount of water repeatedly to calculate the mean, standard deviation, and percent coefficient of variation [%CV = (standard deviation / mean) X 100]. Some parameters that must be addressed in this approach include:

1. The water must be highly pure and at the same temperature as the equipment in use.
2. The balance must be a highly sensitive analytical balance that is properly calibrated.
3. The procedure must be in a timely manner to avoid evaporative loss of water, and careful technique must avoid leaving contaminating masses on the weigh container, such as fingerprints.
4. The number of replicates must be greater than 4.
5. The test volumes should be at the largest volume of the micropipette and at half the largest volume of the micropipette.
6. Your micropipetting technique must be impeccable.

After taking all the measurements and making all the needed calculations, the results should be compared to the manufacturer's stated specifications. If the micropipette does not meet the

specifications, the calibration of the micropipette must be changed. This can be accomplished in different ways depending on the brand and style of the micropipette. In some, you adjust the piston stroke length, which changes the amount of movement that the piston has during an aspiration/dispensing step. Others may require the volume display to match the volume that was actually dispensed. Once the micropipette has been adjusted, it should be retested to ensure that the new calibration is correct.

TECHNIQUES TIPS: Micropipetting small volumes

1. As the volume being measured and transferred goes down, the care that must be taken to assure accuracy goes up exponentially! It is very important that you develop a careful technique and good habits in order to get good results in a molecular lab. The SOP for micropipetting gives some tips for good technique, and here are some more suggestions for especially small volumes:

2. You should submerge the micropipette tip only 2 mm or so into the solution to be measured. The depth should be slightly greater when measuring larger volumes. This shallow depth ensures that the pipet tip does not fill up through hydrostatic pressure in addition to the desired aspirated volume. Maintain the same submersion depth during the aspiration of liquid. DO NOT pre-wet your micropipette tip.

3. While aspirating liquids into the micropipette tip, the micropipette should be in a completely vertical position.

4. All movements of the piston should be smooth and not abrupt. Aspirating a sample too quickly can cause the sample to vortex, possibly over aspirating the sample. When aspirating viscous liquids (i.e., serum, blood, enzymes), leave the tip in the liquid for 1-2 seconds after aspiration before withdrawing it. After you withdraw the tip, make sure that an air bubble does not appear in the tip.

5. Once there is liquid in the tip, NEVER lay pipettes on their sides! It is very important that the pipettes themselves never have direct contact with liquids. Doing so may allow water or vapor to touch the metal shaft inside the micropipettor, rusting it and reducing accuracy. It costs over $100 to fix these problems.

6. Before dispensing the liquid, check the pipet tip for any extra liquid adhered to the outside of the tip. Do NOT remove extraneous liquid with a tissue, as this can contaminate the tip and remove some liquid from the inside of the tip by capillary action. If the excess liquid cannot be removed by touching it off on the sides of the container, dispense the liquid back into the container, discard the tip, and try again—this time without submersing the tip in the liquid as much.

7. When dispensing the sample, hold the pipet at a slight angle, with the tip touching the side of the receiving vessel if possible. Depress the control button slowly and smoothly to the first stop to dispense the liquid from the tip.

8. If a droplet remains inside the tip by capillary action, the volume dispensed is not correct. To prevent this from happening, you should touch off the wall of the receiving vessel as you blow out the droplet with a final puff of air (depressing the thumb to the second stop of the micropipette).

9. If transferring a small volume into another solution, it is helpful to deliver the sample in the micropipette tip directly into the solution and to rinse out the micropipette tip by pumping solution up and down several times. The action of pipetting repeated up and down also helps to get a good mixing of small volumes together. (Some scientists have reported to me that this should be done as many as 7 times to ensure complete rinsing and transfer of sample from a micropipette tip when the volumes are on the order of a microliter!

10. NOTE: genomic DNA (gDNA) solutions should be exempted from this rule—excessive shear forces will degrade its integrity. Pipetting of gDNA should be done very slowly to avoid shearing the molecules into fragments. Vortexing is also damaging to gDNA molecules, and should be avoided. The best way to mix gDNA is by gentle inversion of the solution, or by pipetting up and down slowly with a wide-bore pipet tip.

11. Eject the tip using the second control button. It is preferable to eject tips into a waste container that is conveniently place at your workbench, for later disposal. Tips that have contacted a biohazardous waste should be ejected directly into a biohazard waste receptacle.

12. ALWAYS change tips before pipetting a different solution or after a tip has touched another solution in order to avoid cross-contamination!

Calibration, Verification & Validation

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

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

Verification of a Micropipette

In this lab you will verify the performance of your pipette. Read Chapter 20 in your Seidman & Moore textbook for more information on micropipetting techniques. Gilson has prepared a Verification Procedure for their pipettes. You can retrieve this on-line from their website. Some of our pipettes are Gilson, but not all of them.

The following are important factors affecting pipette verification. Ideally you would take all of these into account, however, for our lab today we will only be able to perform some of these.

1. Atmospheric Conditions: All equipment for this procedure should be placed in the same room together and allowed to stabilize at the same atmospheric conditions (temperature, barometric pressure and humidity) at least 2 hours prior to starting this procedure. For accurate results the procedure should be performed when all equipment is under stable and homogenous atmospheric conditions.

2. Barometric pressure, temperature and humidity: Ideally, you would record the ambient temperature, barometric pressure and humidity prior to the verification. Recommended temperature should be stable and between 20-23°C. Recommended relatively humidity should be greater than 50% but stable. Barometric pressure should be at 1013+/-25hPa.

   Water density correction would take all of these conditions into consideration by extrapolating from a Z factor chart. We will convert the mean water weight to the mean volume measured assuming the density of water is 0.9982 g/ml at 20°C.

3. Evaporation rate: A low humidity environment may have adverse effect on the performance data. Pipettes may appear to be reading lower than specifications if you do not account for evaporation rate of the water during the micropipetting procedure. This is particularly important when measuring volumes less than 50ul.

4. Pipette Operation: Consistency of micropipetting technique contributes significantly to the reproducibility of results. Maintain a steady rhythm when aspirating and dispensing samples and recording results. If you are interrupted during the data collection you must start over. One test cycle should take less than 1 minute.

5. Weighing Containers: Special containers are typically used for a pipette verification procedure. These containers control evaporation during the gravimetric data collection. A different sized container is used depending on the pipette used and volume dispensed. Small volume containers contain lids to help with evaporation.

6. Water: Deioinized or distilled water should be used for this procedure. All the water needed should be collected in one vessel and the water and vessel allowed to come to ambient temperature at least two hours before use.

7. Immersion depth: Immersing a tip too deeply in a sample forces additional liquid into the pipette tip. Immersing a tip too shallow may cause air bubbles to be aspirated into the pipette tip. For a p100/p200 immersion depth should be 2-3mm. For a p1000 immersion depth should be 2-4mm.

Scope of the Laboratory Exercise:

In this lab exercise, you will select a set of micropipettes that you will use for the rest of the semester and evaluate its calibration.

> ***Safety Precautions:***
> *Wear gloves throughout this lab.*
> *Avoid touching your face and especially your mouth.*
> *Check for hazard ratings on each chemical.*

Materials:

Carefully read through the following protocol and make a list of specialized equipment that you foresee as necessary for performing the protocol   These are the things that you must line up in your work area in order to complete the work described, excluding some common lab equipment that is shared with the rest of the class such as pH meters, pH standards, and balances.

Make a similar list of chemicals and reagents that are required to complete the protocol for this day. As described in your Introduction chapter, you must make a table for these chemicals and reagents and include the following information:

a. the name of the chemical(s) in the reagent used or the solution prepared (include concentrations)
b. a brief explanation of its purpose in the lab procedure
c. any safety precautions that should be taken when handling the chemical(s) or solution
d. how the chemical(s) or solution should be stored properly

(You may need to leave space for any information that is unavailable before the lab exercise, such as the lot number and/or expiration date of the materials.)

PROTOCOL

A. Gravimetric Procedure. (Based on an ASTM Method E 1154-89 “Standard Specification for Piston or Plunger Operated Volumetric Apparatus”, taken from Seidman, L.S. & C.J. Moore. *Basic Laboratory Methods for Biotechnology.* Prentice Hall. (2009))

Summary of Method: The correct functioning of a micropipette is determined by checking the accuracy and precision of the volumes it delivers. Weights of water samples delivered by the instrument is converted to a volume based on the density of water (~1g/ml). Whereas many micropipetting calibration companies will check at three, but for this exercise, you will perform calibrations at two volumes: one at the maximum and minimum setting and another at half the maximum setting.

1. You will find a collection of micropipettes in the drawer at your bench. You will use this set through the semester so write down the number set. If the micropipettes have been scrambled, ask around for the micropipettes to make a matched set.

2. The analytical balance used to check the micropipette must be well-maintained and properly calibrated. It must also be in a draft-free, vibration-free environment.

3. The water used should be purified and degassed. Discard water after one use.

4. Document all relevant information including date, micropipette serial number, temperature and humidity when the check was performed, name of the person performing the evaluation, etc.

5. If the water and micropipette are not stored at the same temperature, allow at least 2 hours for temperature equilibration. If the relative humidity of the room is less than 50%, place a small beaker of water inside the closed weighing chamber of the balance for at least 30 minutes to raise the relative humidity in the chamber.

6. Set the micropipette volume, and tare the balance to a vial.

7. Use your best micropipetting technique (see Techniques Tips above) to measure and deliver a volume of water into the vial. Immediately record the weight measurement. Working quickly, repeat steps 6 & 7 ten times. Avoid prolonging this process, as you want to avoid evaporative losses, and be sure to tare between each measurement.

8. Repeat the 10 measurements at the second volume setting for your micropipette.

9. For each volume setting, calculate the mean weight of the water for the 10 measurements. Convert the mean water weight to the mean volume measured as follows: the density of water is 0.9982 g/mL (its density at 20°C and normal pressure). Divide the mean water weight (g) by this density conversion factor (g/ml) to get a value in ml.

10. Determine the % accuracy or the "inaccuracy" (also called the percent error) of the micropipette at each volume setting as follows:

$$\%\ \text{accuracy} = \frac{\text{mean volume observed}}{\text{volume setting}} \times 100\%$$

$$\%\ \text{error} = \frac{\text{mean volume observed} - \text{volume setting}}{\text{volume setting}} \times 100\%$$

11. Determine the precision (repeatability) of the micropipette at each volume setting by calculating the standard deviation (SD) or coefficient of variation (CV) as follows:

$$SD = [\Sigma (X_i - X_m)^2 / n-1]^{1/2}$$

where X_i is each volume measurement,
X_m is the mean volume measurement, and
n is the number of measurements (10)

$$CV = \frac{SD \times 100\%}{X_m}$$

12. Check with the manufacturer's specifications for the micropipette to see if your measurements are within the expected range. If out of calibration, according to the manufacturer's specifications, fill in a deviation report to notify the lab technician that the micropipette needs to be recalibrated. Include a copy of the deviation report in your lab notebook.

B. Colorimetric Procedure.

Summary of Method: When measuring volumes less that 10uL, evaporative losses and imprecision in the analytical balance create proportionately large sources of error in gravimetric measurements. An additional approach is to pipet a colored solution into a volume of water, followed by measuring the absorbance of the solution that you have created. This procedure will be used to check the precision of a P10 and P20 micropipette.

1. Turn on a spectrophotometer to allow it to warm up for at least 10 minutes before use.

2. Place 20 test tubes in a rack and locate a 5 mL volumetric pipet. Pipet 5 mL of diH_2O into each test tube.

3. Set the micropipette to its maximum setting, and use your best micropipetting technique (see Techniques Tips above) to measure and deliver a volume of food coloring into each test tube of water. Use the dark, undiluted food coloring for this procedure.

4. Consult the Operating Manual to your spectrophotometer and blank the spectrophotometer against water in a cuvette. (If you don't know the wavelength to use for your food coloring, you will need to scan the absorbance spectrum to locate the wavelength that has the maximum absorbance.)

5. Repeat the 10 measurements at the half maximal volume setting for your micropipette.

6. For each volume setting, calculate the mean absorbance of the water for the 10 measurements.

7. Note that in the absence of an external standard, it is not possible to determine the accuracy of your measurements (explain why in your notebook). Determine the precision (repeatability) of the micropipette at each volume setting by calculating the standard deviation (SD) or coefficient of variation (CV) as follows:

$$SD = [\Sigma (X_i - X_m)^2 / n-1]^{1/2}$$

where X_i is each absorbance measurement, X_m is the mean absorbance measurement,

and

n is the number of measurements (10)

$$CV = \frac{SD \times 100\%}{X_m}$$

8. Check with the manufacturer's specifications for the micropipette to see if your measurements are within the expected range. If out of calibration, according to the manufacturer's specifications, fill in a deviation report to notify the lab technician that the micropipette needs to be recalibrated. Include a copy of the deviation report in your lab notebook.

9. Report your results in your note book.

TROUBLESHOOTING GUIDE: Micropipetting

Problem	Possible cause	Solution
Micropipette drips or leaks	Tip is loose or does not fit correctly	Use manufacturer recommended tips Use more force when putting the tip on the micropipette
	Nose cone is scratched	Replace the nose cone
	Seal of the nose cone leaks	Replace the nose cone
	Piston is contaminated by reagent deposits	Clean and lubricate the piston (as per the manufacturer recommendations)
	Piston seal is damaged	Replace the piston seal and lubricate the piston (as per the manufacturer recommendations)
	Nose cone has been loosened	Retighten the piston cone (usually by twisting)
Push button does not move smoothly	Piston is scraping due to contamination	Clean and lubricate the piston (as per the manufacturer recommendations)
	Seal is swollen due to reagent vapors	Open micropipette and allow it to dry; lubricate the piston if necessary (as per the manufacturer recommendations)
Inaccurate volumes	Micropipette is leaking	(see above)
	Micropipette calibration has changed	recalibrate according to manufacturer specification
	Poor Micropipetting technique	check Micropipetting SOP and Technique Tip (above) for guidance, and practice

Lab Unit 2-C: Preparation of Reagents

Introduction

The ability to make reagents is an essential skill for any biotechnicians. The accuracy of calculation and of measurement is critical to the outcome of any experiment, whether it be one you do yourself or one in which you prep for someone else. 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, the supplier and catalog number if available, as well as the concentration, the expiration date of the chemical, when it was received, how it was stored upon receipt, and the amount weighed out for each reagent. If the pH is adjusted or the solution is sterilized, information about these procedures should be documented. Some solution prep forms will also have space to include the balance number, pH meter number and other pieces of important information. The storage conditions for the solution that was prepared should also be recorded here.
- ***Assign a Control Number for each solution prepared.*** The solution prepared should be assigned a unique identifier in order to cross-reference the solution to the Solution Prep Form filled out while it was being made. The control number will be archived in alphabetical order, so it is important to choose a relevant letter to begin the control number that you designate for your solution. For example, if you are making a SDS solution, you may want to have your control number begin with "SDS". The rest of the control number should also be descriptive. For example, you might want to identify the solution with your initials. Since you may make up the same solution sometime later on, you might also add the date to the control number in order to give it a unique identify, starting with the year. (see specific example below)
- ***Label each solution bottle before filling***. Write down the name of the solution, its concentration, its pH if it is a buffer, your initials, the control # assigned to the solution, and the date. Some industries have special blank labels to be used for each reagent. Others use tape and a permanent marker. There are labeling software programs and systems for labeling and making electronic records for solutions prepared in laboratories.
- ***Record any changes observed in materials during solution preparations*,** no matter how trivial they might seem. This includes the formation of gas bubbles and any change in color. 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.

TECHNIQUES TIP: Using the pH Meter
Small errors in the pH adjustment of a buffer can have large effects on sensitive enzymes used in the molecular lab. There are many things that you can do to improve the performance of a pH meter.

1. Never assume that a pH meter is in calibration. Even when properly maintained and cared for, a pH meter undergoes considerable drift in a matter of hours after calibration. Follow the appropriate SOP to determine whether your pH meter is in calibration and to bring it into calibration. The standard buffers used to calibrate a meter should bracket the pH of the sample to be measured. The first buffer should be pH 7.0.

2. Verify that the standard buffers that you are using have not expired. This is especially important for pH 10 buffer, where CO_2 dissolved from the air will cause the pH to go down over time.

3. Avoid direct contact of solids or surfaces on the bulb of the pH electrode as it has a very fragile membrane. The electrode should not be wiped dry because static discharge can build up on the electrode.

4. Follow the manufacturer instructions for the proper care and use of an electrode. Some electrodes, such as gel-filled electrodes, should be stored in pH storage solution, and might be ruined if stored dry.

5. The best indicators of the electrode condition are the slope of the calibration curve and response time required to obtain a stable pH reading. As any electrode ages, the slope decreases from 100%. The recommended operating range varies by manufacturer but is usually 92 – 100%. The response time will become longer as the sample components coat the sensing glass bulb with continued usage. This can often be remedied with cleaning and/or replacing the filling solution, following the manufacturer directions.

Review of solution concentration calculations:

A. Making Molar Solutions
In this unit we will be making solutions that will be used for the rest of the semester. In this and in most molecular biology labs, you will use water that has been purified by reverse osmosis/deionizing cartridges/carbon filter/ultrafiltration to make all solutions. Never use tap water unless specifically indicated

The formula for making molar solutions from dry chemicals is:

g needed	=	formula weight	x	molarity	x	volume
g needed	=	g/mole	x	mole / liter	x	liter

where, the formula weight, also called the molecular weight is given as gram/mole. The formula weight is usually listed as F.W. on the reagent bottle. The molarity is the number of moles/liter and is abbreviated as M. The volume of the solution is listed in liters. Make sure that you are always using compatible units of measurement—don't switch from liters to milliliters, for example, without making to proper conversions.

Example 1: Make 1 liter of 0.5 M solution of NaCl (F.W. = 58)

a) To get the grams of NaCl needed, first convert each of the values to the standard. That is, 58 becomes 58 g/mole, 0.5 M becomes 0.5 mole/L and 1 liter stays at 1L. By doing this step first, you will be able to cancel units and make sure that your answer is correct.
b) Then, plug the values into the equation and solve:
   g needed = 58 g/mole x 0.5 mole/L x 1 L
   The moles and the liters cancel out, leaving the g needed = 29 g
c) You would weigh out 29 g NaCl and place it in a beaker with something less than 1 liter of purified water. It is important to dissolve the solute (NaCl) in less than the required solvent (water) because the beaker is not an accurate measurement container.
d) When the NaCl is dissolved, you do a quantitative transfer of the solution into a 1 liter graduated cylinder, making sure that all the solution transfers by washing the beaker out a few times with a wash bottle of purified water. Then bring it to volume (BTV) of 1 liter with purified water. By dissolving the reagent in less than the final volume and THEN BTV, you make sure that you do not overshoot the volume and make a solution that is too dilute. If you have a volumetric flask, they are much more accurate in measuring volumes.

Example 2: Make 100 mL 25 mM Tris (FW 121.1), pH 7.5

a) As in example 1, first convert each of the values to the standard. Therefore the formula weight becomes 121.1 g/mole, 25 mM becomes 0.025 mole/liter (to go from mM to M divide by 1000) and 100 mL becomes 0.1 L.
b) Plug these values into the formula:
   <u>g needed = 121.1 g/mole x 0.025 mole/L x 0.1 L</u>
   The moles and the liters cancel out (but only if you have made the conversions beforehand) and g needed = 0.30275 g
   This needs to be rounded off to 0.30 g since balances will not measure more precisely than this.
c) You would weigh out 0.30 g Tris and place it in about 80 mL distilled or deionized water in a beaker along with a magnetic stir bar.
d) Then adjust the pH to 7.5 with acid, usually HCl. Monitor the pH on a pH meter, making sure that the pH probe is submerged but not getting tapped by the moving stir bar.
e) Quantitatively transfer your buffer to a 100 mL graduated cylinder, washing the buffer across several times with a wash bottle of purified water. Lastly, BTV at 100 mL with purified water. Note that pH is adjusted before BTV, and then quickly checked afterwards to confirm it has not changed with the addition of the slight amount of water.

B. Making Percent Solutions

The sales tax in this state is 8.25%. That means that we pay $8.25 for every $100 worth of merchandize. Percent solutions work the same way, except that instead of dollars, grams and mL are used instead. Thus, a 5% solution means 5 g solid dissolved in 100 mL water or 5 mL liquid dissolved to 100 mL water.

Example 3: Make 100 mL 2% (w/v) tryptone.

In this simple solution, you would place 2 g tryptone into about 80 mL water in a beaker. Use a magnetic stir bar and stir plate to dissolve the tryptone completely. Once the tryptone is dissolved, quantitatively transfer it to a graduated cylinder and BTV at 100 mL.

Note that moles and molarity are never needed in making percent solutions.
Sometimes the percent solution will be designated as (w/v) or (v/v) as in the protocol below. (W/v) means weight to volume so in a 2% (w/v) you would weigh out 2 g reagent per 100 mL water. The term (v/v) refers to a liquid reagent. For a 100 mL of 5% (v/v) glycerol you would measure out 5 mL glycerol to be added to 95 mL water. Note that you need to subtract the volume of the glycerol from the water in order to get the correct final volume.

Example 4: Make 500 mL of 50% (v/v) glycerol.

For 100 mL 50% (v/v) glycerol you would combine 50 mL glycerol and 50 mL water. Since you need five times that amount you would combine 250 mL glycerol with 250 mL water.

C. Combined Molarity and Percent Solutions

Several of the solutions used in the molecular lab are a complex combination of solutes. Often, some of the reagents are given as molar solutions and some given as percents. This is sometimes done when making media for bacteria and other cells. Treat each ingredient individually, added them to the water and allowing them to dissolve before transferring to a graduated cylinder and bringing to volume.

<u>**Review of Dilutions from Stocks:**</u>

A. From a concentrated stock

We frequently make up a stock solution that is more concentrated than the working solution. That way we can keep the stock on our bench and dilute it easily and quickly when necessary. The formula for diluting from a stock solution is:

For a parallel dilution: $C_1V_1 = C_2V_2$

Where C_1 is the concentration of the stock solution,
V_1 is the volume of the stock solution needed (this is usually the unknown),
C_2 is the final concentration of the solution and
V_2 is the final volume of the solution.

Example 5: Make 10 mL of 20 mM solution from a stock of 100 mM.

The most difficult part of these problems is deciding what value is what. One way to solve this is to write over each value C_1, V_2, etc. Thus, the problem would look like this:

V_2 (10 mL), C_2 (20 mM), C_1 (100 mM)

Make 10 mL of a 20 mM solution from a stock of 100 mM

Plug these values into the formula and solve for V1.

$$100 \text{ mM} \times ?\text{ mL} = 20 \text{ mM} \times 10 \text{ mL}$$

$$?\text{ mL} = \frac{20 \text{ mM} \times 10 \text{ mL}}{100 \text{ mM}}$$

$$= 2 \text{ mL}$$

Therefore you would take 2 mL of the stock solution and add to 8 mL (10 mL – 2 mL) water to get the desired final concentration.

Note that although molarity is used, you do not need to know the formula weight of the reagent in the solution. Furthermore, you do not need to covert to liters and moles/liter as you had to do when dealing with molar solutions. The only caveat is to make sure that the units on each side of the equation are the same. In this case we have mM and mL on both sides of the equation and so are all set. However, it you had mM and □M, then you would have to convert one to the other.

B. Dilution from a "times" stock

Sometimes stock solutions are given as a "times" stock such as 10X. (A 10X stock is usually read as "ten X".) This means that the stock is ten times as concentration as the final solution. In order to dilute a "times" stock, follow the same dilution formula as above.

Example 6: Make 50 mL working solution from a 10X stock solution.

In this case, we can do the same as we did above:

V_2 (50 mL), C_2 (1X), C_1 (10X)

Make 50 mL working solution (1X) from a 10X stock.

The implication is that the working solution, C_2, is 1X. Therefore when we plug in the values

$$10\text{X} \times ?\text{ mL} = 1\text{X} \times 50 \text{ mL}$$

Solving as above gives us 5 mL of the 10X stock solution added to 45 mL (50 mL – 5 mL) water to make the 1X working solution.

Scope of the Laboratory Exercise:

In this lab exercise, you will prepare stock solutions that you will use in other lab exercises throughout the semester.

> ***Safety Precautions:***
> 2. *Wear gloves throughout this lab.*
> 3. *Avoid touching your face and especially your mouth.*
> 4. *Check for hazard ratings on each chemical.*

Materials:

Carefully read through the following protocol and make a list of specialized equipment that you foresee as necessary for performing the protocol. These are the things that you must line up in your work area in order to complete the work described, excluding some common lab equipment that is shared with the rest of the class such as pH meters, pH standards, and balances.

Make a similar list of chemicals and reagents that are required to complete the protocol for this day. As described in your Introduction chapter, you must make a table for these chemicals and reagents and include the following information:

1. the name of the chemical(s) in the reagent used or the solution prepared (include concentrations)
2. a control number or lot number & vendor for the solution or reagent
3. a brief explanation of its purpose in the lab procedure
4. any safety precautions that should be taken when handling the chemical(s) or solution
5. how the chemical(s) or solution should be stored properly

(You may need to leave space for any information that is unavailable before the lab exercise, such as the lot number and/or expiration date of the materials.)

Protocol

Be sure to fill out solution prep forms for each of the solutions that you prepare. Assign to each form a Control Number, using a numbering system that reflects each reagent identity uniquely. Identification numbers should identify what the item is and which solution prep that it is. The system ensures that no two raw materials will have the same number.

For example: TE–PP-050906 would correspond to an item prepared on September 6, 2005

05	is the year
09	is the month
06	is the day of the month
TE	Letters refer to a specific item, usually an abbreviation of the solution name, such as “Tris/EDTA buffer” in this case. The letters should be as

	informative as possible, and may include the initials of the person who prepared the reagent.
PP	These are the initials of the person responsible for preparing this solution.

Label all reagent containers made with the following information:

a. Solution Name
b. Who prepared the solution
c. Date Prepared
d. Control # from the Solution Prep Form
e. Concentration, and pH if it is buffered

PROCEDURE

Your instructor will give you specific instructions on preparing these solutions below. Regardless of which solution you make, you MUST prepare calculations for all of the solutions for your pre-lab exercise.

<u>0.5 M EDTA pH 8.0 (50 mL)</u>

NOTE: EDTA is not very soluble in water until titrated to an alkaline pH with a large amount of NaOH. Be sure to allow for this by starting out your titration with a minimal volume of water. The pH of the solution will drop following each addition of NaOH as more EDTA dissolves into the solution, so be patient between each addition to allow it to equilibrate.

1. Calculate the amount of ethylenediaminetetraacetic acid, disodium salt (EDTA), sodium salt, required to make 50 mL of a 0.50 M solution.

2. Weigh out the EDTA and add approximately 30 mL deionized water into a 50 mL beaker. Add a stir bar, and begin stirring on a stir plate. You will notice the EDTA will NOT go into solution! Why not?

3. Standardize a pH meter and adjust the EDTA slowly by adding 6 M NaOH one drop at a time and stirring on a magnetic stirrer until you have adjusted the pH to 8.0. Make sure that the pH probe is submerged but that the moving stir bar is not tapping the pH probe (The EDTA is not highly soluble, but will readily dissolve at this pH).

4. Transfer to a graduated cylinder and wash residual solution from beaker with small amount of deionized water. BTV (Bring to Volume) with deionized water to 50 mL.

5. Parafilm, mix, and transfer to a labeled 50 mL plastic conical centrifuge tube.

6. Store at room temperature.

1.0 M Tris-Cl pH 8.0 (50 mL)

NOTE: The pH of Tris-Cl is temperature dependent and changes drastically at 0.03 pH units per degree fall in temperature. For instance, if you want pH 7.6 at 4C, prepare the buffer at pH 7 at room temperature.

1. Calculate the amount of tris(hydroxymethyl)amino methane (Tris or Trizma base) required to make 50 mL of
   a 1.0 M solution.

2. Weigh out the tris and dissolve in approximately 35 mL deionized water into a 50 mL beaker.

3. Standardize a pH meter and adjust the solution pH slowly by adding 6 M HCl one drop at a time and stirring on a magnetic stirrer until you have adjusted the pH to 8.5. Make sure that the pH probe is submerged but that the moving stir bar is not tapping the pH probe.

4. Further adjust the pH to 8.0 with an HCl solution of lower concentration. Be careful not to overshoot the endpoint pH. If you overshoot, throw the solution away and start again.

5. Transfer to a graduated cylinder and wash residual solution from beaker with small amount of deionized water. BTV (Bring to Volume) with deionized water to 50 mL. Record a final pH after BTV. Is your pH the same? If not, talk with your instructor.

6. Parafilm, mix, and transfer to a labeled 50 mL plastic conical centrifuge tube.

7. Store at room temperature.

1.0 M Tris-Cl pH 7.5 (50 mL)

Repeat the procedure 1-7 (above) with Tris to make a 10 mL 1.0 M stock solution at pH 7.5. You will need to adjust the pH with 6M and 1M HCl. (Begin with the higher concentration of acid and switch to the lower concentration as you approach the final pH. Don't overshoot the pH! Store solution at room temperature.

10% SDS (20 mL)

NOTE: SDS is potentially hazardous if inhaled. Please don't shake the powder before opening.
1. Calculate the amount of SDS required to prepare a 20 mL solution of 10% (w/v).
2. Weigh the SDS and dissolve in approximately 8 mL of water in a beaker.
3. Quantitatively transfer to a graduated cylinder and BTV with deionized water to 10 mL.
4. Parafilm, mix, and transfer to a labeled 15 mL conical centrifuge tube.
5. Store at room temperature – the SDS will come out of solution at lowered temperatures.

Sterile LB broth (80 mL)
1. Measure 0.8 g tryptone, 0.4 g yeast extract, and 0.8 g NaCl and add to approximately 50 mL purified water.
2. Stir to dissolve and quantitatively transfer to a 100 mL graduated cylinder. BTV.

3. Mix and pour into a 125 mL autoclavable bottle. Leave the cap partially screwed down but not tight. Cover the cap with aluminum foil.
4. Label and add autoclave tape.
5. Autoclave 20 minutes at 121°C, slow exhaust, following the SOP for the use of the autoclave.
6. Cool and store at room temperature. Store refrigerated or use immediately after adding antibiotics.

References:

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3. Gad, S.C. & S.M. Taulbee. *Handbook of Data Recording, Maintenance and Management for the Biomedical Sciences.* CRC Press. (1996)
4. Gerstein, A.S. *Molecular Biology Problem Solver: A Laboratory Guide.* Wiley-Liss. (2001)
5. GLP Standards FDA (HFE-88) Office of Consumer Affairs, 5600 Fisher's Lane Rockville, MD 20857
6. Good Laboratory Practice (GLP) Regulations, 21 CFR 58, 1979. www.fda.gov
7. ISO Guide 25, The International Organization for Standardization., 1, Rue de Varembe, Case Postak 56, CH-1211 Geneve 20, Switzerland
8. Seidman, L.S. & C.J. Moore. *Basic Laboratory Methods for Biotechnology.* Prentice Hall. (1999)
9. US Pharmacopeia, USP645

Analysis & Questions for Lab Unit 2

Answers to the following questions should be added to the end of your lab report:

1. What would you do to make sure that a light-sensitive liquid reagent is stored properly?

2. What types of reagents would you NOT recommend for storage in glass?

3. Why is it an advantage to cotton-plug your pipets in a molecular lab?

4. What are some advantages that you can think of for the use of forms for recording data in a molecular lab?

5. Where is the documentation forms stored for use in the molecular lab that you are using this semester?

6. Frequently buffer solutions used in the molecular lab are complex, containing multiple solutes. The following is a typical "Master Mix" for a polymerase chain reaction (PCR). Using the stock solutions provided below whenever possible, describe how you would make 100 uL of 5X Master Mix. Include in your description all the calculations, as well as the volume of water added to your final 5X solution.

5X Master Mix for PCR: 0.5 mM of each dNTP, 7.5 mM $MgCl_2$, 5X PCR buffer, 5 uM forward primer, 5 uM reverse primer

Stock solutions	Calculations	Volume added (uL)
20 mM ATP		
20 mM GTP		
20 mM CTP		
20 mM TTP		
25 mM $MgCl_2$		
10X PCR buffer		
10 mM forward primer		
10 mM reverse primer		
Purified water		
	(total volume)	

LAB UNIT 3: NUCLEIC ACID ISOLATIONS

Introduction:

An efficient method of nucleic acid extraction that produces purified, high-quality nucleic acid product is crucial to the success of PCR, sequencing, and many other molecular techniques involving enzymatic transformations. Many methods of nucleic acid extraction have been developed, and the specific method of choice depends on the type of tissue source, as well as the intended subsequent use of the purified nucleic acid. Although many vendors offer kits for nucleic acid extraction, it is important to understand the principle of the extraction techniques in order to evaluate the best one to use for any particular application. Each extraction strategy comes with its own advantages and drawbacks, possible problems associated with them that you need to watch out for.

Some specific parameters that should be evaluated when trying out a new kit or strategy for preparing nucleic acids for a test application include:

1. **Simplicity.** The greater the number of steps, the greater the time required and the more possibilities of a failure. Multiple steps also tend to reduce yields.
2. **Efficiency.** The greater the yield of nucleic acid from the tissue, the less sample and reagents required. Different tissue types will extract with different efficiencies, depending on the exact extraction strategy selected. In general, the more efforts to purify DNA, the low the expected yields.
3. **Purity of nucleic acids extracted.** Some molecular assays require highly pure sample, while others are more robust at lower degrees of purification. Some extraction techniques can address the specific impurities commonly contaminating cellular extracts better than do others.
4. **Specificity.** The type of nucleic acid being extracted (DNA vs RNA) will guide your selection of extraction method. In extracting pathogenic nucleic acids from clients' tissues, the extraction method should be chosen that can selectively purify from the more abundant gDNA of the client.
5. **Sensitivity.** Some types of tissue extract easily, while others require much harsher treatments in order to get nucleic acids out of cells. The harsher treatments are more laborious and pose a greater risk of carryover of reagents or nucleic acid degradation that can interfere with subsequent analysis.
6. **Integrity of the nucleic acids.** Harsh treatments, especially those that introduce shear forces, can greatly decrease the average size of genomic DNA prepared.
7. **Adaptation to high-throughput techniques.** As the demand for molecular analyses increases, the ability to automate extraction techniques becomes more important.
8. **Reliability.** Since extraction methods deal with a range of tissue sources and tissue storage conditions, an extraction technique that is the most fool-proof is always preferred. The method that has the least fail-rate will save lab time and reduce the number of specimens that require retesting.
9. **Expense.** A great deal of both time and expense can be saved by determining exactly what cellular contaminants actually need to be removed. For example, some PCR applications might only require cellular lysis and no subsequent purification steps, depending on the tissue being tested and how quickly the extract can be processed.

The major factors that interfere with nucleic acid purification are:

1. **Nucleases.** The isolation of nucleic acid is a race against internal degradation from nucleases. Lysis buffers are the key to avoiding this problem, by promoting a rapid and thorough lysis of cells, and by including inhibitors of nucleases. Most lysis buffers either contain protein-denaturing reagents such as detergents and enzyme-inhibiting components such as EDTA. These components must be carefully selected in order to avoid inhibition of enzymes used later in testing. Keeping samples cold prior to nuclease inactivation is also key to winning the race against nucleases, as the enzyme activity is slowed at the lower temperatures. To avoid reintroduction of nucleases later on during sample processing, reagent purity must be guarded, and materials must be kept nuclease-free. The use of disposable materials or the use of heat and chemical treatments of non-disposable materials can effectively prevent nuclease problems. Signs of nuclease contaminant are apparent in smears or lack of signal in electrophoresis gels, or failure to amplify by PCR.

2. **Shearing.** The quality of genomic DNA can be improved by avoiding vortexing, repeated pipetting through narrow tips, and freeze-thawing of sample. Mechanical techniques for disrupting cell wall materials found in bacteria, plants, and fungi contribute to shearing of gDNA. Sonication, grinding in liquid nitrogen, and shredding devices such as rigid spheres or beads are used to lyse difficult samples, but lead to low quality gDNA. Enzymatic cell wall disruption, such as the use of lysozyme to break down bacterial cell walls, can improve the size of gDNA isolated.

3. **Chemical contaminants.** Cells can be sources of contaminants that interfere with nucleic acid isolations and down-stream applications. Polysaccharides and cell wall materials can react irreversibly to create an unusable final product, so CTAB (cetyltrimethyl ammonium bromide) is often used to precipitate gDNA and separate it from these interfering contaminants. Low levels of certain purification reagents can lead to failures in subsequent enzymatic treatments, and must be removed. Phenol-based purifications leads not only to phenol interferences with many enzymes, but oxidation products of phenol can also damage nucleic acids. Freshly-prepared phenol solutions must be used, and traces of phenol must be removed by chloroform extraction, followed by precipitation of nucleic acids in ethanol. Many impurities, such as guanidinium isothiocyanate, can be removed by ethanol precipitation of nucleic acids, followed by ethanol washes of the nucleic acid precipitate. Ethanol itself, however, can interfere with subsequent enzymatic treatments, and must be carefully removed. Drying in the open air is commonly used to remove traces of ethanol, but overdried nucleic acids can be difficult to redissolve. Phosphate buffers inhibit many polymerases, including *Taq* polymerase, and should be avoided. High levels of EDTA should be avoided, as it can inhibit many enzymes that act on nucleic acids. Heparin should be avoided, because it inhibits *Taq* polymerase and has been shown to lead to nicked DNA molecules.

There are many general protocols for isolating nucleic acids from a variety of cells available on-line. Detailed technical manuals are available on the kits at the vendors' websites and on blackboard. In this laboratory exercise, nucleic acids will be isolated from different sources by a variety of methods. Some of the methods include:

1. Promega's Wizard Genomic DNA Purification Kit (Cat# A1120), salt precipitation step to purify gDNA from fresh tissue. www.promega.com/tbs
2. Promega's Wizard Genomic DNA Purification Kit, salt precipitation step to purify gDNA from whole tissue.
3. Quiagen Spin MiniPrep (Cat# 27104) of plasmid DNA from *E. coli* www.qiagen.com
4. Ambion's RNAqueous Kit (Cat# AM1912), a phenol-free kit which uses a silica spin column to purify RNA from fresh tissue. www.ambion.com
5. BiooScientific's MaxRecovery BiooPure Kit (Cat# 5301-01), a phenol based kit using 1-bromo-3-chloropropane (BCP) to purify RNA from fresh tissue.www.biooscientific.com

Experimental Outline:
Your instructor will have details on which procedure you will perform. A week before this lab, you will need to consult your instructor so you have the correct pre-lab prepared.

Once the nucleic acids have been isolated by these 5 methods, they will be stored under different conditions to evaluate the effects of storage on their quality over time. Quality of nucleic acids will be evaluated in Unit 4 of this lab manual.

Lab Unit 3-A: DNA Isolations

Introduction:

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

a. Where the DNA is isolated from will determine the cell lysis techniques used.
b. The purity requirements of the intended use of the DNA being isolated will determine how many purification steps will be involved.
c. The type of DNA being isolated: genomic DNA has different physical properties from those of plasmid DNA.

The successful isolation of DNA requires methods that prevent nuclease degradation of the DNA. Some buffer constituents used to promote lysis and denaturation of nucleases include:

- Detergents - used to solubilize cell membranes: Popular choices are SDS (sodium dodecyl sulfate, aka SLS, sodium lauryl sulfate), Triton X-100, and CTAB (cetyltrimethyl ammonium bromide)
- Proteinase K - sometimes added to cleave glycoproteins and to help the detergents to inactivate DNases.
- Denaturants such as urea, guanidinium salts, and other chaotropes are sometimes applied to inactivate enzymes. Heat is often applied to enhance the lysis of cells and the denaturation of proteins.
- For microbial sources of DNA, enzymes must be added to break down cell walls in order to make the cells susceptible to lysis.
- RNases are often added to a lysis buffer to remove contaminating RNAs, which can interfere with the intended use of the DNA being isolated.

In selecting a lysis method for a particular application, top priority should be given to choosing a method that has simplicity and speed (least number of steps and solutions required). Remember that every constituent added to cells during the lysis procedure could become a culprit by sabotaging the activity of an enzyme later on. You will want to remove anything added to your DNA at the beginning of DNA isolation sometime later, so try to keep the number of constituents in your lysis buffer to those that are absolutely needed. The number of steps in a cell lysis protocol should also be kept to a minimum, since any delays during this part of the DNA isolation procedure runs the risk of DNA degradation by nucleases in the cells. DNA will not be safely stabilized until it has been purified from all protein contaminants. In general, animal tissues are easily lysed, due to the fact that they have no cell wall, and a gently detergent treatment usually is sufficient to break open cells. Yeast and microbial cells, on the other hand, have rigid cell walls that must be weakened enzymatically before the cell will release its DNA. In the case of bacteria, lysozyme enzyme is added, while in the case of yeast a more complex mixture of enzymes must be used to degrade cell wall polymers. Plant cell walls are generally

abraded mechanically by grinding frozen plant tissue, often with glass beads or sand and a mortar and pestle.

The second phase of DNA isolation protocols is the purification of the DNA released from the cell from other components of the cell and the lysis buffer. The method you select for your application depends on the size and source of the DNA to be isolated. When plasmid DNA is being isolated from bacteria such as *Escherichia coli (E. coli),* an alkaline solution of SDS is sufficient to release plasmid DNA, leaving behind the genomic DNA still associated with the cellular debris. The genomic DNA is then conveniently removed from the plasmid DNA by a quick centrifugation step. Genomic DNA can frequently be rendered insoluble and quickly "spooled" from the lysed cells by addition of alcohol to the mixture. The spooled DNA can be transferred to a fresh buffer to redissolve the genomic DNA.

For some applications, this low level of DNA purity will suffice. Often, though, there are proteins or polysaccharides (especially in plant sources of DNA) that coprecipitate with the DNA and interfere with subsequent enzymatic treatments. Classically, the further purification of DNA involves the removal of proteins by aqueous phenol solutions, followed by numerous alcoholic precipitation steps to remove traces of phenol from the isolated DNA. Alcohol precipitations of DNA also serve to concentrate the DNA into a smaller volume, and to purify the DNA from any water-soluble contaminants. The phenol extraction is an inefficient method of purification and suffers from a poor yield of DNA. Also, phenol reagents are unstable, and fresh solutions must be used or the quality of the reagent must be monitored, generally by observed changes in pH. This, along with safety concerns in the use of phenolic solutions, is a serious drawback in this method of DNA purification.

An alternative procedure is the use of so-called "spin columns", which are small chromatography columns that purify the DNA from other solutes. While this procedure is more expensive than phenol extractions and alcohol precipitations, the purification and yields of product by spin columns are improved. Also, the reagents used are more stable, so provide a more reliable, or "robust", method. The final DNA prepared with spin columns is free of protein and salt contaminants and can be used directly in restriction digests, Southern blotting, and PCR applications. All components of this system are stable at room temperature for one year. Another advantage of purchased kits for plasmid preparation is that the quality of reagents can be tested and assured the venders. For these reasons, many biotechnology labs routinely use kits for their plasmid preparations.

Binding and elution from silica beads has become the method of choice for isolation of genomic DNA from animal tissues. A high concentration of chaotropes serves to bind nucleic acids to silica surfaces. The adsorption step to bind DNA to the silica particles is followed by wash steps, usually with salt/ethanol solutions which will not interfere with the strong binding of nucleic acids but will wash away remaining impurities and excess chaotrope. Elution of DNA from silica columns requires the use of nuclease-free water or low ionic strength buffers such as TE. This is an advantage since it means that the isolated DNA can be used directly in further manipulations without further cleanup. a spin-column method that does not require the use of time-consuming and toxic phenol/chloroform extractions or ethanol precipitations. The final

genomic DNA prep is free of protein and salt contaminants and can be used directly in restriction digests, Southern blotting, and PCR applications.

A potential problem with the use of silica columns for the binding of DNA is the possibility of overloading the column with DNA, resulting in a wash-through of non-adsorbed DNA and reducing the overall yield of DNA. There is also some loss of material that does not elute from the silica resin. The smaller the DNA size is, the tighter is its interaction with silica surfaces. Although size is not a problem with isolations of genomic DNA, loading the silica resin with too little DNA can also lead to a low overall yield of DNA eluted from a silica column.

Protocols for the use of spin columns are unique to each vendor, and so the vendor's protocol should be followed when they are used. Some examples of some more general protocols are found below, along with a discussion of how they work.

Method 1: Genomic DNA Extraction by Salt Precipitation

The principle of the method:
The *Promega Wizard Genomic DNA Purification Kit* can be used to purify DNA from many tissue types including whole blood, animal and plant tissue, culture cells and bacteria in a five-step process:

1. Lysis of the cells in the Nuclei Lysis Solution with mechanical homogenization (tissue) or after removing red blood cells in Cell Lysis Solution (Blood).
2. (optional) An RNase digestion step removes RNA molecules from the solution.
3. Cellular proteins are removed by a salt precipitation step which precipitates the proteins but leaves the high molecular weight genomic DNA in solution.
4. Genomic DNA is concentrated and desalted by isopropanol precipitation.
5. DNA purified using this is up to 50 kb in size, with fragments of approximately 20-30 kb predominating. DNA of this length denatures completely during thermal cycling and can be amplified with high efficiency.

The purification by this kit is suitable for subsequent amplification by *Taq* polymerase, digestion with restriction endonucleases, and membrane hybridizations such as Southern and dot/slot blots.

EXPECTED YIELD: from 10-20 mg of animal tissue, 15-20 ug of gDNA

Tasks to perform prior to the lab

1. Set waterbath or incubator to 37°C.
2. Set waterbath or incubator to 65°C for rehydration of purified DNA.

NOTE: All centrifugation steps are carried out at room temperature.

For Tissue samples only –

1. Add 300 uL of Nuclei Lysis Solution to a 1.5 mL microcentrifuge tube and chill on ice.

2. Add 10-20 mg of fresh or thawed tissue to the chilled Nuclei Lysis Solution and homogenize for 60 seconds using a small homogenizer. Ensure the tissue is fully homogenized. If it is not homogenized properly your yield will be low.

3. Add an additional 300 uL of Nuclei Lysis Solution and vortex to mix.

4. Incubate the lysate at 65°C for 15-30 minutes.

5. Optional: Add RNase Solution (1.5 uL) to the nuclear lysate and mix the sample by inverting the tube 2-5 times. Incubate the mixture at 37°C for 15 minutes, and then cool to room temperature.

6. Allow the sample to cool to room temperature for 5 minutes.

7. To the room temperature sample, add 200 uL of Protein Precipitation Solution and vortex vigorously at high speed for 20 seconds. Chill sample on ice for 5 minutes.

8. Centrifuge at 16,000 x *g* for 3 minutes at room temperature. The precipitated protein will form a tight white pellet.

    a. NOTE: If no pellet is observed, you may have not cooled the sample to room temperature before adding the Protein Precipitation Solution. In that case, cool the sample to room temperature at least 5 minutes, or chill on ice for 5 minutes, vortex for 20 seconds, and centrifuge for 3 minutes at 16,000 x *g* and proceed with the protocol. Alternatively, perhaps the Protein Precipitation Solution was not thoroughly mixed with the nuclear lysate. Always mix the nuclear lysate and Protein Precipitation Solution completely.

9. Carefully remove the supernatant containing the DNA (leaving the protein pellet behind) and transfer it to a clean 1.5 mL microcentrifuge tube containing 700 uL of room temperature isopropanol.

    b. NOTE: Some supernatant may remain in the original tube containing the protein pellet. Leave this residual liquid in the tube to avoid contaminating the DNA solution with the precipitated protein.

10. Gently mix the solution by inversion until the white thread-like strands of DNA form a visible mass.

11. Centrifuge at 16,000 x *g* for 1 minute at room temperature. The DNA will be visible as a small white pellet.

12. Carefully decant (typically pour off, but it may be better to aspirate or remove by pipetting off with a micropipettor) and discard the supernatant.

13. Add 600 uL of room temperature 70% ethanol to the DNA pellet. Gently invert the tube several times to wash the DNA pellet and the sides of the centrifuge tube.

14. Centrifuge at 16,000 x *g* for 1 minute at room temperature.

15. Carefully aspirate (remove by pipetting off) the supernatant using either a drawn Pasteur pipette or a gel loading pipette tip. The DNA pellet is very loose at this point and care must be used to avoid aspirating the pellet into the pipette.

16. Invert the tube on clean absorbent paper and air-dry the pellet for 5 minutes at room temperature. Do not let air-dry too long or it won't go back into solution.

17. Add 100 uL of DNA Rehydration Solution (10 mM Tris-HCl & 1 mM EDTA pH 7.5) to the tube, and rehydrate the DNA by incubating at 65°C for 1 hour. Periodically mix

the solution by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature or at 4°C.

18. For a stability test, aliquot into 4 separate tubes 25 uL of purified DNA to be analyzed by gel electrophoresis at a later date. Carefully label tubes with the date, your name, and the source of DNA, the method of extraction, and the storage temperature. Store one tube at room temperature (25°C), one tube in the refrigerator (4°C), and the rest of the tubes in the –20°C freezer in the assigned freezer rack.

TROUBLESHOOTING GUIDE

Symptoms	***Comments***
Poor DNA yield	The sample was too old. Best yields are obtained with fresh tissue. Samples that have been stored at 2-5°C for more than 5 days may give reduced yields. The DNA pellet was lost during isopropanol precipitation. Use extreme care when removing the isopropanol to avoid losing the pellet.
Degraded DNA (<50kb is size)	Improper collection or storage of the sample. Obtain a new sample under the proper conditions.
DNA pellet difficult to dissolve	Samples may have been over dried. Rehydrate DNA by incubating 1 hour at 65°C, and then leave the sample at room temperature or at 4°C overnight. Caution: Do not leave the DNA at 65°C overnight! Samples were not mixed during the rehydration step. Remember to mix the samples periodically during the rehydration step.

Method 2: Plasmid DNA Extraction by Silica Spin Column

Isolation of plasmid DNA from Escherichia coli

Introduction:
It is essential that the bacterial cells be harvested during the early log phase of the culture in order to be able to lyse the cells effectively and get a high yield of DNA. The most reliable way to ensure that your cells are at the right culture phase is to grow a liquid culture overnight, inoculate from the overnight culture into fresh medium, and monitor the optical density of the culture over the next 2-3 hours.

The plasmid DNA is then extracted by an alkaline lysis method in which the cell membrane and wall are disrupted and proteins are denatured by SDS (sodium dodecyl sulfate) in a highly alkaline solution. SDS is a detergent consisting of a 14-carbon hydrocarbon attached to a sulfate head group. Sodium is the counterbalancing salt to the sulfate.

The chromosomal DNA is separated from the much smaller plasmid DNA by differential centrifugation in the presence of DNase-free RNase that digests the RNA. This procedure has many variations although most involve the same steps of extraction of DNA, differential centrifugation and incubation with RNase. If it involves small culture volumes such as those performed in the lab, this procedure is usually termed "miniprep". A culture of greater than 5 mL is generally referred to as a "midiprep" and a "maxiprep" refers to the isolation of plasmid from volumes of cultured cells greater than 100 mL and as much as liters of culture medium.

Addition of acidified potassium acetate to the cell lysate lowers the pH and precipitates the SDS-membrane-protein complexes. Since chromosomal DNA is attached to the membrane fraction, it will precipitate as well. After centrifugation to remove this precipitate, the supernatant is treated with RNase to remove any residual RNA. At this stage, many plasmid prep protocols also use phenol or chloroform to remove residual proteins. Phenol must then be carefully removed from the DNA because residual phenol will interfere with subsequent enzymatic procedures. Alternatively, you may use a spin-column method.

The principle of the spin-column method:
The Qiagen Spin MiniPrep plasmid DNA Kit can be used to purify plasmid DNA absorbed on silica spin columns in the presence of high salt from bacteria after alkaline lysis in four-step process:

1. Preparation and clearning of the bacterial lysates. Lysis of the cells in the lysis Buffer P2. P2 contains the strong base sodium hydroxide (alkaline conditions). Addition of N3 to cell lysate to precipitates cell debris, proteins and genomic DNA leaving the plasmid DNA in solution.
2. Addition of supernatant to silica gel spin column for adsorption of nucleic acids. A component of the N3 (and PB) Buffer is the chaotropic salt guanidine hydrochloride for inactivation of nucleases. It is a harmful irritant.
3. Removal of loosely bound contaminants from the spin column with washes in Buffer PB and PE.

4. Elution of nucleic acids from the spin column in EB Buffer (10 mM Tris, 0.5 mM EDTA pH 9.0) or distilled water.

EXPECTED YIELD: from 1 ml culture of bacteria, ~8-10ug of plasmid DNA.

The purification by this kit is suitable for subsequent amplification by *Taq* polymerase and restriction enzyme digest. Co-purification of RNA may interfere with many enzymatic reactions, but will not interfere with amplification by *Taq* polymerase.

SAFETY TIPS:
- *Wear gloves throughout this lab.*
- *Avoid touching your face and especially your mouth.*
- *Check for hazard ratings on each chemical.*

PROCEDURE

Part I: Culture of the Transformants
The lab technician may have done this for you.

1. Obtain a culture tube of 2.5 mL sterile liquid LB+amp and label it with your initials.

2. Using a sterile inoculating loop, pick a single, well isolated colony from freshly-streaked LB+Amp plate.

3. Inoculate the media.

4. Incubate the tubes at 37°C with vigorous shaking (approximately 300 rpm) for 8 hours.

5. Inoculate a flask of 80 mL of sterile liquid LB+Amp medium in a 500 mL culture flask with 0.1 mL of overnight culture and shake at 300 rpm at 37°C for 1-2 hours until the culture has an absorbance of 0.5-0.6 at 600 nm.

Part II: Plasmid DNA Isolation
Tasks to perform prior to the lab
- Set water baths or incubators to 65°C.
- Equilibrate all solutions except TE at room temperature prior to beginning the procedure.
- Verify ethanol (96-100%) was added to Buffer PE as directed on the label, and mark the label as having had ethanol added. Buffer PE is stable for 1 year when stored closed at room temperature.
- Verify RNase A has been added to Buffer P1. P1 should be stored at 4°C.
- Verify no salt precipitate has formed in buffers P2 and N3. Redissolve by warming to 37°C; do not shake P2 vigorously due to presence of SDS detergent.

NOTE: All centrifugation steps are carried out at room temperature.

1. Harvest 1 mL bacterial culture in a 1.5 mL microcentrifuge tube by centrifugation at 16,000 x *g* for 3 minutes at room temperature.

2. Aspirate and discard the supernatant (spent media) into a biohazard waste container. Although the media should not contain live bacteria, it should be treated as potentially infectious.
3. Resuspend the pelleted bacterial cells in 250 uL **Buffer P1**. No cell clumps should be visible after resuspension.

4. Add 250 uL **Buffer P2** and mix gently but thoroughly by inverting 4-6 times. DO NOT VORTEX as this will shear the genomic DNA and it will be captured with plasmids on the silica membrane.

    a. NOTE: Close Buffer P2 immediately after use to avoid acidification of Buffer P2 from CO_2 in the air.

5. Add 350 uL **Buffer N3** and mix immediate and thoroughly by inverting the tube 4-6 times. The solution should become cloudy and viscous (snotty). DO NOT VORTEX as excessive bubbles will form.

6. Centrifuge for 10 minutes at 16,000 x *g* to pellet the cell debris. A compact white pellet will form with some additional floating precipitates.

7. Apply the supernatant to the **QIAprep spin column** by aspirating.

NOTE: Special precautions are necessary when handling spin columns in order to avoid cross-contamination, especially when planning to subsequently perform PCR analysis on the isolated nucleic acids.

- Carefully apply the sample to the column, pipetting the sample into the center of the column without actually touching the column or wetting the rim of the column.
- Change pipet tips between all liquid transfers. The use of aerosol-barrier pipet tips is recommended.
- Avoid touching the column membrane itself with the pipet tips. A puncture of the column surface will cause the solution to rapidly channel the column through instead of percolating through the silica resin.
- Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.
- Close the columns before placing them in the microcentrifuge.
- Open only one column at a time following centrifugation, taking care to avoid generating aerosols.
- Label both the column and the collection tube to avoid cross mixing samples

8. Place the column in a collection tube and centrifuge 1 minute at 16,000 x *g*.

9. Decant (pour off) the flow through contained in the collection tube down the sink and wash the column by adding 500 uL **Buffer PB**. Incubate for 5 min at the bench top.

10. Return the column to the collection tube and centrifuge for 1 minute at 16,000 x *g*.

11. Decant (pour off) the flow through contained in the collection tube down the sink and wash the column by adding 750 uL **Buffer PE** (note the change in buffer).

12. Return the column to the collection tube and centrifuge for 1 minute at 16,000 x *g*.

13. Decant (pour off) the flow through contained in the collection tube down the sink.

14. Return the column to the collection tube and centrifuge for 1 minute at 16,000 x *g*. This removes residual wash on the bottom of the spin column.

15. Place the QIAprep column in a clean 1.5 mL microcentrifuge tube.

16. Elute the DNA by adding 70ul Buffer EB (10mM Tris Cl, pH 8.5) or water to the center of column.
    a. Ensure that the elution buffer is dispensed directly onto the center of the column for optimal elution. If water is used for elution, verify the pH is between 7.0 and 8.5 for maximum elution efficiency.
    b. Allow the EB to contact the membrane for 1 minute prior to centrifugation.

17. Centrifuge 1 minute at 16,000 x *g*.

18. Discard the QIAprep column and keep the flow through containing your purified plasmid DNA.

19. For a stability test, aliquot into 4 separate 1.5 mL tubes of 15 uL of purified DNA to be analyzed by gel electrophoresis at a later date. Carefully label tubes with the date, your name, and the source of DNA, the method of extraction, and the storage temperature. Store one tube at room temperature, one tube in the refrigerator, and the rest of the tubes in the –20°C freezer.

TROUBLESHOOTING GUIDE

Symptom	Comments and Suggestions
No DNA in the cleared lysates before loading	• Check the storage conditions and age of the buffers. • Pelleted cells should be completely resuspended in Buffer P1 before Buffer P2 is added. Verify an even suspension was obtained.
DNA is found in the flow through of cleared lysate	• The column is overloaded. If rich culture media such as TB or 2x YT are used, culture volumes should be reduced. Also, if high copy number plasmids are used with LB, culture volumes may need adjusted. • Also ensure that RNA is not clogging up the column and that RNase A was added to Buffer P1
Clogged spin column membrane	• Centrifuge for 1 minute at full speed or until all the lysate has passed through the column. In some samples, cellular debris may clog the membrane completely. In this case, repeat procedure with new sample diluted in PBS. In plasma samples, cryoprecipitates may form due to repeated freezing and thawing. Do not use plasma that has been frozen and thawed more than once.
Little or no DNA in the eluate.	• Low concentration of DNA in the sample • Inefficient cell lysis. (See above) • Isopropanol used instead of ethanol. The substitution of isopropanol for ethanol decreases yields. • Insufficient incubation of spin column with elution buffer or water. To ensure elution efficiency, pipet Buffer EB or water onto the center of the spin column without disturbing the column. Incubate the column for 5 minutes at room temperature before centrifugation. • Incorrect pH of the elution water. Low pH will reduce DNA yield. Ensure that the pH of the water is at least 7.0 or use Buffer EB for elution.
Poor purification; high levels of proteins	• Inefficient cell lysis—see above. • No ethanol added to the lysate before loading onto the spin column. Repeat procedure with new sample
Nuclease Contamination	• When using endA+ host strains such as HB101 and its derivatives, the JM series, or any wild-type strain, ensure that the wash step with Buffer PB is performed.
Genomic DNA in the eluate	• The lysis step should not exceed 5 minutes. • The culture could have been overgrown and thus dead cells and degraded genomic DNA was present. • The lysates was not handled correctly and shearing of genomic DNA occurred.

Lab Unit 3-B: RNA Isolations

Introduction:

RNA may be used in the molecular lab to make cDNA in order to clone genes, to study gene regulation, to determine the size and structure of specific messages, the identify gene products, to determine rates of specific mRNA synthesis or degradation, to name a few. There are many procedures for isolating RNA, depending on the type of RNA to be isolated, the type of tissue that the RNA is being isolated from, and the intended use of the isolated RNA. The successful isolation of intact RNA by any procedure requires that four important steps be performed:

1. effective disruption of cells or tissue
2. denaturation of nucleoprotein complexes
3. inactivation of endogenous ribonuclease (RNase) activity
4. purification of RNA away from contaminating DNA and protein

The most important of these is the immediate inactivation of endogenous RNase activity which is released from membrane-bound organelles upon cell disruption. RNases are very stable and generally require no cofactors such as magnesium and other divalent cations, so they cannot be inhibited or inactivated by adding chelators such as EDTA.

Potent RNase inactivating ingredients that denature proteins (including RNases) are added to the lysing buffer. The most effective and most commonly used denaturant is guanidinium isothiocyanate (usually abbreviated as GITC or GuSCN). Another strong protein denaturant is phenol (which is also very caustic!), and historically, phenol or mixtures of phenol and chloroform were used in RNA isolation. Detergents are also able to denature proteins and most lysis solutions contain detergents. These denaturing agents also disrupt cell membranes and aid in solubilization of the tissue.

Once RNA has been isolated, it is usually resuspended in water or water with trace amount of salts or EDTA. At this point the RNA is unprotected and highly vulnerable to degradation. Therefore it is extremely important that surfaces and solutions, which come into contact with the RNA preparation, are completely free of active RNase.

The choice of an RNA isolation method depends upon a number of factors, such as:

1. the source of RNA
2. the type of RNA to be purified
3. the relative abundance of the RNA
4. the sample size

Most RNA isolation methods recover total RNA, not mRNA. Since the mRNA fraction is only about three to five percent of the total RNA, it is sometimes an advantage to purify mRNA from the rRNA and tRNA fractions. This is generally accomplished by taking advantage of the poly A tail found at the 3' ends of most mRNAs; the total RNA is mixed with a solid matrix, usually cellulose, which is attached to a homopolymeric tract of T residues. The poly-dT on the cellulose hybridizes to the poly A at the end of the mRNA, and then the cellulose (with the captured mRNA) can be recovered by centrifugation or filtration. Preparations of mRNA are often referred to as "poly A-selected RNA", and this term is generally synonymous with mRNA.

Some assays can tolerate partial degradation of the mRNA. For example, the reverse transcription-polymerase chain reaction (RT-PCR) assay amplifies only a small portion of the RNA and does not need the entire length of the nucleic acid. Other assays and applications, like Northern blots and construction of full-length cDNA libraries, do require that the mRNA is intact.

Because of the stringent requirements for high-quality reagents, many if not most labs prefer to purchase RNA isolation kits from commercial sources where the quality of the materials have been validated and spin columns help to speed the extraction process.

RNA will be isolated using Ambion's RNAqueous Kit similar in methodology to the QIAprep spin columns for plasmid DNA. In addition, we will try a newly developed phenol-based reagent from another local company called Bioo Scientific. Detailed technical bulletins about this and other RNA isolation kits are available at each company's website. Other RNA isolation protocols are described in the Appendix of this manual.

TECHNIQUE TIPS:

1. Observe all the guidelines for creating an RNase-free working environment in your workspace and lab (see in the box below).
2. Keep reagent bottles closed if not in use.
3. Keep tissues, reagents, and RNA samples on ice to prevent degradation by RNases.
4. Avoid aerosols: Pulse spin microfuge tubes on a picofuge to force solutions to the bottom of the tube before opening.
5. Use glycogen as an RNA carrier for samples with low cell numbers (fewer than 200,000 cells per 300 mL RNA Lysis Solution) or non-cellular body fluids. Add glycogen (1 mL of 20 mg/mL glycogen per 600 mL isopropanol) to the isopropanol prior to or following the addition of the RNA-containing supernatant from the protein-DNA precipitation step.

CREATING A RNase-FREE ENVIRONMENT

RNases are ubiquitous and notoriously difficult to inactivate. The following notes are good things to consider in setting up for an RNA isolation:

1. Two of the most common sources of RNase contamination are the researcher's hands and bacteria or molds that may be present on airborne dust particles or laboratory equipment and supplies. To prevent contamination from these sources, sterile technique should be employed when handling any of the reagents used for RNA isolation or analysis. ***Gloves should be worn at all times.***

2. To avoid RNA degradation, all solutions, glassware, and plasticware that come into contact with "unprotected" RNA should be treated to remove RNases. Diethylpyrocarbonate (DEPC) inactivates RNases by reacting with histidine residues, found at the active site and so is the usual method for producing RNase-free equipment. To render water RNase-free, it is treated as follows:
   a. Measure water into RNase-free glass bottles.
   b. Add diethylpyrocarbonate (DEPC) to make a 0.1% (v/v) solution. (ie add 0.1 mL

DEPC for each 100 mL of water).

c. Shake vigorously to mix
d. Autoclave for 15 minutes at 121° C on liquid cycle, OR incubate at least 12 hours at 37° C and then heat to 100° C for 15 minutes.

NOTE:
Tris buffers cannot be treated with DEPC.
<u>DEPC is a suspected carcinogen and should be handled with care</u>. Always use gloves and open under a fume hood.

3. Disposable plasticware such as pipette tips and microcentrifuge tubes is generally RNase free if used straight out of the package and the package is kept closed and gloves are worn when items are touched and removed. It is best to pour tubes from an unopened bag onto an RNase-free environment (such as plastic wrap).

4. Nondisposable glassware and plasticware should be treated before use to ensure that it is RNase-free. Glassware can be made RNase-free by baking it at 300°C for 4 hours or by baking it at 200° C overnight. (NOTE: Corex centrifuge tubes should be rendered RNase-free by treatment with 0.05% DEPC overnight at room temperature and not by baking. This will reduce the failure rate of this type of class during centrifugation.)

5. Nondisposable plasticware should be thoroughly rinsed before use with 0.1 M NaOH/1 mM EDTA and then with DEPC-treated water.

6. Deionized water sources can be a potential contributor of RNase activity. If degradation of RNA occurs, it may be necessary to test the lab's water source for RNase activity.

7. Chemicals and equipment for use in RNA isolation and analysis would be best reserved separately from other uses. Wear gloves when handling this equipment, and use only baked spatulas and untouched weigh boats or weigh paper.

8. Use filter-tips for micropipetting when isolated RNA is to be used in amplification procedures.

9. Use heat-blocks and not water baths. Dirty water baths are a source of RNases.

Method 1 - RNAqueous Protocol

The principle of the method:
The Ambion RNAqueous Kit can be used to purify all but small RNAs from cells from virtually any source (except whole blood) in a three-step process:

1. Lysis of the cells in a guanidinium-based lysis solution.
2. Adsorption of nucleic acids to a silica gel spin column, followed by washes and elution.
3. An optional DNase treatment required for sensitive amplification such as RT-PCR.

RNA purified using this method is total RNA containing mRNA, rRNA, tRNA and ncRNA. Specific RNA transcripts can be amplified out using gene specific primers.

EXPECTED YIELD: varies widely with the transcriptional activity of the cell

Tasks to perform prior to the lab:
It is critical that this protocol be performed quickly and efficiently to avoid degradation of RNA by RNases. Careful preparation will be essential to the yield of RNA as well as the quality of the RNA that you isolate.

1. If you cannot isolate RNA from fresh tissue immediately, weigh 40-50 mg of tissue and transfer it into 600 uL of RNA*later* solution in a 1.5 mL microcentrifuge tube. Store frozen at –10°C until the next lab period.
2. Heat an aliquot of the Elution Solution to ~75°C in an RNase free tube. Typically each sample is eluted in ~100 uL, but additional solution may be heated to allow for evaporation.
3. Equilibrate all solutions at room temperature prior to beginning the procedure.
4. Prepare a 64% Ethanol Solution. Add 38.4 mL of ACS grade (or better) 100% ethanol to the bottle labeled Water for 64% Ethanol, which contains 21.6 mL of RNase-free water. Mix well and place a check mark in the empty box on the label to indicate that the ethanol has been added.
5. Prepare Wash Solution #2/3. Add ethanol (100%) to Wash Solution 2/3 as directed on the label, mix well, and mark the label as having had ethanol added. Store at room temperature if the Wash Solution is to be used within 1 month. Store at 4°C for longer times, but warm to room temperature before use.

All centrifugations in this protocol are at full-speed: a RCF of ~16,000 x *g* – which corresponds to 13,200 rpm in an Eppendorf Model 5415D microcentrifuge.

PROTOCOL

Sample preparation: This may be done for you by the instructor or the lab tech.

1. Weigh 40-50 mg of tissue and transfer it into 600 uL RNA*later* solution in a 1.5 mL microcentrifuge tube. This could be stored frozen at –10°C for an extended period of time until you are ready to process your sample.

2. If your tissue was frozen in RNA*later*, it should be fully thawed and the preserved tissue removed. Blot the excess RNA*later* from the sample.

Cell lysis

1. Transfer your 40-50 mg of tissue to 300 uL of Lysis/Binding Solution. Homogenize the tissue with a pestle that fits into the microcentrifuge tube. After completely homogenized add an addition 300ul of Lysis/Binding solution.

2. Vortex vigorously to lyse the cells. Invert the tube to be sure the solution is homogenous. Samples that were stabilized in RNA*later* will require more vigorous vortexing to resuspend and lyse cells. *Lysate should be somewhat viscous, but if it is so viscous that it is difficult to pipet, then add more Lysis/Binding Solution and homogenize again to reduce the viscosity.*

3. Centrifuge 3 minutes at 16,000 x *g* in a microcentrifuge to <u>pellet cell debris</u>.

RNA purification

1. Heat an aliquot of the Elution Solution to ~75°C in an RNase free tube. Typically each sample is eluted in ~100 uL, but additional solution may be heated to allow for evaporation.

2. For each sample, place a Filter Cartridge into one of the Collection Tubes supplied in the kit. Label the lid of the resulting Filter Cartridge assembly and the ring of the cartridge. Label a second Collection Tube for each sample; these will be used to elute the RNA from the spin column filter.

3. Remove supernatant into a clean centrifuge tube and add equal volume of 64% ethanol to lysate and vortex briefly to mix.

4. Apply no more than 700uL of the sample mixture from the previous purification step (aqueous phase with added ethanol) to a filter cartridge inside a collection tube at a time.

5. Centrifuge for 15-60 seconds at 12,000 RPM to pass the sample through the filter. Do not exceed 15,000 x *g* (~13,000 RPM), as this may result in disintegration of the spin column. Discard the flow-through and reuse the collection tube for the washing steps.

6. Apply the remaining sample mixture and centrifuge as described.

7. Apply 700 µL Wash Solution #1 to the filter cartridge. Centrifuge for 15-60 seconds. Discard the flow-through and reuse the collection tube for the next washing step.

8. Apply 500 µL Wash Solution 2/3 to the filter cartridge. Centrifuge for 15-60 seconds. Discard the flow-through but reserve the collection tube and reuse the collection tube for the next washing step.

9. Apply 500 µL Wash Solution 2/3 to the filter cartridge. Centrifuge for 15-60 seconds. Discard the flow-through and reuse the collection tube for the next washing step.

10. After discarding the flow-through, centrifuge for 1 minute to remove the last traces of wash solution.

11. Place the filter cartridge into a freshly labeled collection tube. Apply half of the preheated Elution solution (65 µL) to the center of the filter and close the cap of the tube. Leave the assembly at room temperature for 30 seconds. Then centrifuge 30-60 seconds to recover the RNA from the filter.

12. Perform a second elution with an additional preheated 65 µL of Elution solution. Repeat the previous centrifugation step, collecting the Elution Solution into the same Collection Tube.

OPTIONAL (Time Permitting)
DNase I Treatment: to remove any contaminating gDNA from the eluted RNA

1. Add 1/20th volume of 20X DNase Buffer and 1 uL DNase I (8 units/uL) to the eluted RNA and mix gently but thoroughly.

2. Incubate 30 minutes at 37°C.

3. Add 20% volume of DNase Inactivation Reagent, mix thoroughly, and leave at room temperature for 2 minutes.

    a. NOTE: The DNase Inactivation Reagent is supplied as a slurry. Before removing it, vortex the tube vigorously to ensure that it is completely resuspended. To pipet the reagent, insert the pipet tip well below the surface and observe the aliquot withdrawn to ensure that it is mostly white, without a significant amount of clear fluid. If treating multiple samples, revortex the reagent as needed to resuspend the particles. The DNase Inactivation Reagent may become difficult to pipet after multiple uses due to depletion of fluid from the interstitial spaces. If this happens, add a volume of Elution Solution equal to approximately 10-20% of the bed volume of the remaining reagent and vortex thoroughly to recreate a pipettable slurry.

4. Pellet the DNase Inactivation Reagent by centrifugation of the sample for 1 minute, and then transfer the RNA solution to a new RNase-free tube.

Stability Test Setup
For a stability test, aliquot into 4 separate tubes 25 uL of purified RNA to be analyzed by gel electrophoresis at a later date. Carefully label tubes with the date, your name, the source of

RNA, the method of extraction, and the storage temperature. Store one tube at room temperature, one tube in the refrigerator, and the rest of the tubes in the –20°C freezer.

TROUBLESHOOTING GUIDE

Symptom	***Comments and suggestions***
RNA contains residual gDNA, causing amplification of gDNA in a reverse transcriptase PCR (RT-PCR) experiment	Some samples have an unusually high white blood cell (WBC) count. This may result in a higher DNA concentration in the processed sample. Retreat the eluted RNA with DNase I. RT-PCR samples that are subjected to >30 PCR cycles, and/or when an unusually large amount of RNA (>1ug) is used, will amplify residual gDNA. Reduce the number of PCR cycles and/or reduce the amount of template used.
Difficulty recovering aqueous phase after organic extraction	A suboptimal mixing of the sample during the phenol/chloroform extraction step cause by a tube that is too full can reduce the amount of aqueous phase that can be recovered to less than 0.8 mL. Samples with an unusually high WBC count may also lead to this problem. To increase the amount of aqueous phase, ♦ After the centrifugation step of the phenol/chloroform extraction, remove as much of the aqueous phase as possible, without carryover of the organic phase, and transfer to a new tube. Determine the volume recovered. ♦ Add additional Lysis Solution to the organic phase to bring the total aqueous phase to 1.2 mL. For example, if 800 uL of aqueous phase was initially recovered in the extraction, add 400 uL additional Lysis Solution to the tube with the remaining organic phase. ♦ Mix the Lysis Solution + organic phase by brief vigorous vortexing, then centrifuge for 1 minute at maximum speed to separate the phases. ♦ Remove the aqueous phase and pool it with the aqueous phase recovered initially. ♦ Add one-half volume of 100% ethanol, mix well, and continue the purification procedure.
RNA is partially degraded	An overload of sample, such in the case of blood with an unusually high WBC, can exceed the capacity of the reagents to inactivate endogenous RNases. Blood with high WBC counts stored in RNA*later* for prolonged periods and/or at elevated temperatures (>30°C) will also be more susceptible to RNase degradation. A suboptimal amount of RNA*later* added may likewise be inadequate to stabilize RNA in blood samples. To improve RNA quality ♦ Use a smaller volume of blood (300-400 uL instead of 400 uL) in the purification procedure. ♦ Store samples stabilized in RNA*later* at –20°C instead of

	at room temperature, and/or isolate the RNA after a shorter time following stabilization in RNA*later*. ♦ For RT-PCR assays, some degradation of RNA can be tolerated, especially for the small amplicons typically used in real-time RT-PCR (qRT-PCR). Therefore, it may be possible to use partially degraded RNA for qRT-PCR experiments; for such experiments we recommend using PCR primers that amplify an amplicon of 100-300 bp. ♦ The optimal level of RNA*later* is at least two volumes for each volume of sample that is to be stabilized. Aspirate up and down several times in the RNA*later* to rinse the pipet and to ensure adequate mixing. Close the tube and mix the cell suspension thoroughly by vortexing or inversion.
Low RNA yields	RNase degradation can lead to low RNA yields (see suggestions above). You can also expect sample-to-sample variation in the endogenous levels of RNA.

Method 2 – Bioo Scientific MaxRecovery BiooPure Reagent

The principle of the method:
The Bioo Scientific MaxRecovery BiooPure RNA isolation reagent from Bioo Scientific is a single-phase reagent for extraction of total RNA or enriched small RNA from solid tissues, cultured cells, and cell-free fluids such as serum and plasma. It contains guanidinium, a chaotrope that inactivates nucleases, and phenol, which denatures and separates proteins and DNA from RNA.

This protocol describes how BiooPure can be used to isolate total RNA from animal tissue in a 3-step process:

1. Lysis of the cells in a guanidinium-based lysis solution.
2. Adsorption of nucleic acids to a silica gel spin column, followed by washes and elution.
3. An optional DNase treatment required for sensitive amplification such as RT-PCR.

RNA purified using this method is total RNA containing mRNA, rRNA, tRNA and ncRNA. Specific RNA transcripts can be amplified out using gene specific primers.

Whole blood is made of blood cells and yellowish liquid plasma containing mostly water and dissolved proteins, glucose, clotting factors, mineral ions, hormones and carbon dioxide. Blood serum is plasma without fibrinogen and clotting factors.

EXPECTED YIELD: varies widely with the transcriptional activity of the cell

Tasks to perform prior to the lab

1. It is common to use RNase Zap to deactivate RNases on your lab bench, gloves, tube racks, micropipettes, and other materials when working with RNA.

2. Collect the fish tissue stored in RNA*later*.
3. Collect the BiooPure reagent stored at 4°C, 1-bromo-3-chloropropane (BCP) an alternative to chloroform.
4. You will also use Isopropanol, 75% ethanol (made with nuclease-free water), nuclease free water
5. Set a heat block at 65°C

PROTOCOL

Extract the RNA

1. Blot tissues with a Kimwipe to remove excess RNA*later*.

2. Prepare a sample lysate by grinding tissue in a homogenizer. Use approximately 50 mg of tissue and 1 mL BiooPure reagent. Grind the tissue until homogeneous (no visible chunks remain). Not all tissues will homogenize completely.

   NOTE: Lysates in BiooPure reagent may be stored at -20°C until further processing. In general RNA is stable in lysates for at least several months.

3. Decant the lysate into a 1.5 mL microcentrifuge tube if not already in one.

4. Add 100 uL BCP to the lysate. The ratio is 100 uL BCP (or 200 uL chloroform) to 1 mL lysate.

5. Pulse-vortex the mixture for 20 seconds, 1 second at a time.

6. Centrifuge for 15 minutes at 12,000 x *g*, cooled to 4ºC.

7. Remove the aqueous top phase containing your RNA (which should be colorless) to a fresh tube. Avoid removing material from the interface of the two phases to prevent transfer of DNA and protein. Note the volume of the aqueous layer, which is typically 60% of the volume of BiooPure reagent used.

Precipitate the RNA

8. Add an equal volume of isopropanol to the extract from the previous step (if you have 600 uL, add 600 uL isopropanol) and mix by vortexing. This will precipitate all RNA.

9. Incubate at room temperature for 10 minutes. Record actual ambient temperature.

10. Centrifuge for 15 minutes at 12,000 x *g*, cooled to 4ºC if possible.

11. Remove the supernatant, leaving behind 50-75 uL of fluid behind to avoid disturbing the pellet. **Save the supernatant in a spare 1.5 mL tube**, as the RNA pellet may be invisible and can easily be lost when pouring or pipetting off the supernatant.

12. Wash the pellet by adding 500 uL of 75% ethanol and vortexing briefly but vigorously to dislodge the pellet.

13. Centrifuge for 5-10 minutes at 10,000 x *g*, cooled to 4ºC if possible.
 NOTE: a slower speed is generally used in this step compared to earlier steps to avoid re-pelleting any impurities.

14. Remove the supernatant, leaving behind 20-50 uL of fluid. **Save the supernatant**, as the RNA pellet may be invisible and can easily be lost when pouring or pipetting off the supernatant.

15. Re-spin the tube for about 10 seconds to recover fluid from the sides of the tube, and then remove the remaining liquid with a small bore (10 or 20 uL) pipet tip. A pellet should be visible unless using cell free serum. It is recommended that a co-precipitant such as nuclease free glycogen or linear polyacrylamide be used for the precipitation from cell free extracts such as serum.

16. Resuspend the RNA pellet in 50 uL of nuclease-free water or 0.1 mM EDTA. Vortex vigorously and spin down briefly.

17. Heat at 65ºC for 5 minutes to solublize the pellet completely, then vortex and spin down again.

Stability Test Setup
For a stability test, aliquot into 4 separate tubes 25 uL of purified RNA to be analyzed by gel electrophoresis at a later date. Carefully label tubes with the date, your name, and the source of RNA, the method of extraction, and the storage temperature. Store one tube at room temperature, one tube in the refrigerator, and 2 tubes in the –20°C freezer. Any remaining RNA store at –80°C

TROUBLESHOOTING GUIDE

Symptom	*Comments and suggestions*
Contamination of the aqueous phase with DNA and/or protein	It is imperative you do not transfer too much of the aqueous phase. To maximize recovery of aqueous phase without introducing DNA and protein contamination, first remove ~ 80-90% of the aqueous phase into a clean 1.5 mL microcentrifuge tube, the transfer the remaining ~ 10-20% into a second 0.5 mL microcentrifuge tube. Centrifuge the second aliquot to cause contaminating material from the interface of the two phases to separate at the bottom of the tube, and then remove the aqueous phase into the initial larger aliquot.

REFERENCES:

1. Ausubel, F.M. et al. *Short Protocols in Molecular Biology.* 5th Ed. Wiley. (2002)
2. Berger, S.L. & A.R. Kimmel, eds. *Guide to Molecular Cloning. Methods in Enzymology*. Vol. 152. Academic Press. (1987)
3. Gerstein, A.S. *Molecular Biology Problem Solver: A Laboratory Guide.* Wiley-Liss (2001)
4. Protocols and Handbooks from Ambion, Promega, Bioo Scientific and Quiagen as found on blackboard or at the corporate websites.

Analysis and Questions for Lab Unit 3:

Your lab report should include:

1. Introduction to nucleic acid extraction (1 page)
2. *Results discussion – procedural problems (see below) (1/2 page- 1 page)
3. *Conclusions discussion – compare and contrast the 2 methods of each DNA/RNA extraction (1/2 page – 1 page). Can you make any conclusions yet?
4. Answer questions below.

Please keep all notebook pages and experimental notes in your lab notebook.

*Because you will not be visualizing your results from these extraction procedures until Lab Unit 4, your Lab Unit 3 report should contain a lengthy discussion comparing and contrasting the two methods. Be sure to include any procedural problems which occurred during your extraction procedures. For example, did you lose your pellet? Did you readily see your pellet? Was there no 4deg centrifuge to use? Did you miscalculate g's versus RPM??

Compare and contrast your two methods. Which procedure did you like best and why (Salt precip vs Silica spin column vs Phenol and precip)? Which method was easiest to perform?

Additional Analysis Questions:

Answers to the following questions should be added to the end of your lab report. If your answer has appeared previously in your lab report, indicate the location in your answer.

1. What purpose does the ethanol addition prior to spin column purification of nucleic acids?
    a. What types of contaminants can block the spin columns used to purify the nucleic acids of microbes and plants?
    b. What is the purpose of the high temperature incubation elution solutions prior to eluting nucleic acids from the spin columns?

2. Compare and contrast the lysis buffers used in all procedures.
    a. What are some important components to the lysis buffer? Why?
    b. How do the DNA lysis buffers differ from the RNA lysis buffers? How are they the same?

3. In table form, predict the expected results of the stability assay.

LABORATORY UNIT 4: MEASURING NUCLEIC ACID QUALITY & QUANTITY

Introduction and Background:

The isolation of nucleic acids is a common practice in the molecular lab. You previously performed a combination of the steps listed below to isolate your nucleic acid:

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

Once the nucleic acid has been isolated, it can go on to the next step in its use only after its quantity and quality have been evaluated. The amount of the isolated nucleic acid used in subsequent steps depends on its concentration. The stability of the nucleic acid and its performance in subsequent enzymatic steps is affected by its purity. Both the amount of nucleic acid isolated and its purity are affected by the type of tissue that it is isolated from, the amount of tissue used, and the isolation technique used.

There are three general techniques used to evaluate the concentration and purity of an isolated nucleic acid:

1. Spectroscopic analysis of ultraviolet absorption will provide concentration and a ratio of A_{260}/A_{280} (contaminating protein) and A_{260}/A_{230} (contaminating carbohydrates) will give you an idea of quality. The NanoDrop is most commonly used as it requires microliters of sample volume.
2. Qualitative analysis using agarose gel electrophoresis is one of the most common methods that provide valuable information of size, quality and relative concentration.
3. A bioanalzyer, such as the Agilent Bioanalyzer, is a chip-based nucleic acid analysis system. This combines both systems above to produce highly accurate and reproducible information on both quality and quantity of the nucleic acid.

In this lab exercise, you will evaluate the purity and concentration of DNA and RNA solutions isolated in Unit 3 by all three of these methods. You will discover their relative advantages and drawbacks by comparing your results. You will begin your stability study of DNA and RNA isolated in Unit 3 and stored at different temperatures by evaluating the quality of the DNA and RNA after initial storage. Quantity measurements of these solutions will also allow you to evaluate the yields of nucleic acids isolated by the different methods used in Unit 3 as compared to the other groups.

Lab Unit 4-A: Nucleic Acid Analysis by Spectrophotometry

Introduction:

The de facto method for quantitating nucleic acids that all other methods rely on is ultraviolet absorption. When other methods are used, a nucleic acid standard is prepared based on its absorbance at 260 nm, measured by a spectrophotemeter. An advantage in the use of a spectrophotometer in nucleic acid quantitation lies in its high precision and the fact that sample is not destroyed by the assay and can be put to further analysis after quantitation. A word of caution, however, is needed: ultraviolet light damages DNA, causing mutations in the nucleotide sequences, so the duration of exposure to ultraviolet light should kept to a minimum. A spectroscopic analysis is very fast, another major reason for its routine use in a molecular lab for quantitating nucleic acids. A drawback to the use of a spectrophotometer lies in its low sensitivity and the large volumes that cuvettes come in (generally >0.5 mL). This means that small volumes of sample or low concentrations of nucleic acids are not easily assayed with a standard spectrophotometer. Another drawback to ultraviolet absorbance in nucleic acid quantitation lies in its interference by contaminating proteins.

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

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

The concentration of colored solute in a solution is directly proportional to the intensity of its color, which in turn is proportional to the amount of absorbance of light at the wavelength that the color absorbs. The color, or absorbance, of a solution is also proportional to the path length that the light passes through. This is often expressed as the **Beer-Lambert Law, or Beer's Law:**

$$A = \varepsilon C l$$

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

Spectrophotometers are useful for measuring concentrations of solutions because of the relation of intensity of color (absorbance) in a sample to the amount of solute within the sample. For example, if you use a solution of red food coloring in water, and measure the amount of blue light absorbed when it passes through the solution, a measurable voltage fluctuation can be induced in a photocell on the opposite side. If now the solution of red dye is diluted in half by the addition of water, the color will be approximately 1/2 as intense and the voltage generated on the photocell will be approximately half as great. Thus, there is a relationship between the voltage and the amount of dye in the sample. Nucleic acid solutions are uncolored because the wavelengths that they absorb are outside the visible spectrum of light.

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

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

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

Where A is absorbance at a given wavelength of light

% T is the percent transmittance, or $\frac{\text{light transmitted through a sample}}{\text{light transmitted through a blank}} \times 100$

The following table demonstrates how absorbance and % transmittance are related:

Absorbance	% T
∞ (infinity)	0
3	0.1
2	1.0
1	10.0
0	100

Most spectrophotometers have a built in means of direct conversion of this reading to absorbance.

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

Converting OD into concentrations:

The absorption of 1 OD (Optical Density or Absorbance Unit, a dimensionless quantity) is equivalent to approximately 50 µg/ml dsDNA, approximately 33 µg/ml ssDNA, 40 µg/ml RNA or approximately 30 µg/ml for oligonucleotides. Purity determination of DNA Interference by contaminants can be recognized by the calculation of "ratio". The ratio A_{260}/A_{280} is used to estimate the purity of nucleic acid, since proteins absorb at 280 nm. Pure DNA should have a ratio of approximately 1.8, whereas pure RNA should give a value of approximately 2.0. Absorption at 230 nm reflects biological contaminants of the sample such as carbohydrates, peptides, salts, or proteins. Also, many chemicals commonly used in nucleic acid preparations, such as phenol, EDTA, and SDS, can be detected by their absorbance at 230 nm. Enzymes such as restriction endonucleases and polymerases are inhibited by low levels of phenol and SDS. DNA polymerases and DNase I are notably sensitive to the presence of EDTA in nucleic acid samples. In the case of pure samples, the ratio A_{260}/A_{230} should be approximately 2.2.

Glass absorbs UV light and thus is inappropriate for use in a UV spectrophotometer cuvette. Quartz and UV-transparent plastic cuvettes are available for measuring absorbance in the UV range.

High throughput spectroscopy

Laboratories requiring a high throughput can adapt spectrophotometric procedures to a microplate reader format. The fundamental differences between conventional cuvette-based spectrophotometer instruments and microplate readers must be taken into account when converting assays to a microplate reader. The most important difference has to do with path length of the solution being assayed. Spectrophotometers use horizontal photometry, where a beam of light passes horizontally through a cuvette of defined width. The light path length has been standardized to 1.00 cm, allowing for easy comparison of data. The vertical photometry of a microplate reader has a path length that depends on the amount of fluid added to each well. In order to be comparable, from one well to the next, all volumes must be identical, and in order for microplate readings to be comparable to a spectrophotometer, the absorbance measurement must be corrected to 1.0 cm.

Historically, microplate readers have been used for the determination of colorimetric ELISA assays. These readers use a tungsten-halogen light source and band-pass filters to provide a wavelength range for the measurement of different colors of light. Recent improvements in microplate technology allow for selection of specific wavelengths for absorbance reading through the use of diffraction-grating monochrometers to separate different wavelengths from the light source. The use of monochrometers allows spectral scans to be performed on samples. Further advances in microplate reader technology allows for ultraviolet wavelengths, by substituting the tungsten-halogen light source with a xenon-flash lamp. While a tungsten-halogen lamp can be used effectively for wavelengths ranging from 340 nm to 800 nm, the xenon-flash lamps produce light over a much broader spectrum, allowing measurements from

200 nm to 1000 nm. Microplates that are UV-transparent must be used for wavelengths less than 300 nm.

A nucleic acid standard is the most convenient way for path length corrections in a microplate reader. For example, if a nucleic acid standard has an absorbance of 0.500 in a standard 1.0 cm cuvette, and an absorbance of 0.100 in a microplate assay, all values from that microplate assay will have a path length 0.1/0.5, or 0.20 cm. A correction factor of 5 (multiplication of all microplate readings by 5) will allow all measurement to be compared to standard spectrophotometer readings.

Since path lengths in a microplate are much smaller than 1.0 cm, all absorbance reading by microplate readers suffer from a lower sensitivity. The need for improved sensitivity has driven the use of fluorescence assays for microplate assays. The detection limit for nucleic acids by microplate ultraviolet absorbance readings is about 200 ng. The use of the following fluorescent stains can increase this sensitivity limit considerably.

Nucleic acid stain	**Specificity**	**bias**	**Sensitivity (per well)**	**Excitation wavelength (nm)**	**Fluorescence wavelength (nm)**	**Vendor**
SYBR green II	RNA or ssDNA		100 pg RNA or single-stranded DNA per band	254nm	530	Invitrogen
SYBR safe	dsDNA	Minor groove	500 pg/band in a minigel	280nm	530	Invitrogen
PicoGreen	dsDNA		1.2 pg	485	530	Molecular Probes
Hoechst dye 33258	dsDNA		5 ng	360	460	Hoechst
OliGreen	ssDNA, RNA, dsDNA	thymine bias, ssDNA & RNA	150 pg	485	530	Molecular Probes
RiboGreen	RNA, DNA	RNA	100 pg	485	530	Molecular Probes

Note that OliGreen can be used to detect all types of nucleic acids, double-stranded DNA fluoresces to a lesser extent than do single-stranded DNA or RNA. Also, thymine fluoresces much more than does cytosine. This means that the selection of a standard for this assay must carefully match the type of nucleic acid and the thymine-to-cytosine ratio of the nucleic acid samples being assayed. Similarly, although RiboGreen stain binds preferentially to RNA, DNA will interfere and should be either eliminated prior to the RNA assay, or the RNA standard must have a corresponding amount of DNA added to it.

Ultralow-volume spectroscopy
NanoDrop Technologies has developed an instrument that can measure absorbance and fluorescence with minimal consumption of sample. The NanoDrop Spectrophotometer and the NanoDrop Fluorospectrometer can accurately measure a wide range of biomolecules in volumes as small as 1 microliter. Rather than using cuvettes for sample retention, these systems use fiber optic technology and inherent surface tension of liquid samples to hold a one microliter sample in place between two surfaces during the measurement.

In order to make a measurement, 1 uL of sample is placed directly onto instrument measurement surface and an upper surface is engaged to sandwich the sample, forming a path length between a light source and a detector. Once the measurement is complete, the user simply blots both surfaces with a standard lab wipe to prepare for the next sample.

The NanoDrop Spectrophotometer has a UV-Vis wavelength range of 220 to 750 nm, and the path length can be mechanically adjusted for ultrahigh concentrations of analyte. The detection limit for dsDNA by absorbance at 260 nm is 2 ng/uL (2 ug/mL), and concentrations as high as 3600 ng/uL can be obtained without the need to perform dilutions. Spectral analysis may also be done on this instrument, for a quality measure of the sample.

Scope of the Laboratory Exercise:

In this lab exercise, you will compare the absorption spectra of protein and DNA solutions. Also, you will perform some serial dilutions on your samples to discover over what range of absorbances Beer's Law applies, where the relationship between absorbance and concentration is a linear one.

Safety Precautions:

5. *Wear gloves throughout this lab.*
6. *Avoid touching your face and especially your mouth.*
7. *Check for hazard ratings on each chemical.*

Materials (As part of your pre-lab exercise):
Carefully read through the following protocol and make a list of specialized equipment that you foresee as necessary for performing the protocol These are the things that you must line up in your work area in order to complete the work described, excluding some common lab equipment that is shared with the rest of the class such as pH meters, pH standards, and balances.

Make a similar list of chemicals and reagents that are required to complete the protocol for this day. As described in your Introduction chapter, you must make a table for these chemicals.

(You may need to leave space for any information that is unavailable before the lab exercise, such as the lot number and/or expiration date of the materials.)

Protocol

PART I: Absorbance spectra of RNA solutions

1. Retrieve one tube of each RNA sample from each storage condition and thaw/store on ice.

2. **NanoDrop:** Using the SOP for operating the Thermo Scientific NanoDrop spectrophotometer, scan the UV absorption spectrum of a 1.5ul sample of your RNA isolations. Prior to measuring the absorbance, set the nucleic acid type to RNA. Enter the A_{230}, A_{260}, A_{280} and the ratios A_{230}/A_{260} and A_{260}/A_{280} ratios for each of your nucleic acid samples into your data table in your notebook.

3. **Save your samples,** including diluted samples, on ice for further analysis.

PART II: Absorbance spectra of DNA solutions

1. Retrieve one tube of each DNA sample from each storage condition and thaw/store on ice.

2. **NanoDrop:** Using the SOP for operating the Thermo Scientific NanoDrop spectrophotometer, scan the UV absorption spectrum of a 1.5ul sample of your DNA isolations. Prior to measuring the absorbance, set the nucleic acid type to DNA. Enter the A_{230}, A_{260}, A_{280} and the ratios A_{230}/A_{260} and A_{260}/A_{280} ratios for each of your nucleic acid samples into your data table in your notebook.

3. **Save your samples,** including diluted samples, on ice for further analysis.

Lab Unit 4-B: Nucleic Acid Analysis using Agilent Bioanalzyer

Introduction

From Agilent Technologies' website:
About Lab-on-a-Chip Technology
Agilent Technologies is the leader in commercial microfluidic Lab-on-a-Chip technology. This technology utilizes a network of channels and wells that are etched onto glass or polymer chips to build mini-labs. Pressure or electrokinetic forces move pico liter volumes in finely controlled manner through the channels. Lab-on-a-Chip enables sample handling, mixing, dilution, electrophoresis and chromatographic separation, staining and detection on single integrated systems. The main advantages of Lab-on-a-Chip are ease-of-use, speed of analysis, low sample and reagent consumption and high reproducibility due to standardization and automation.

About The RNA Integrity Number
The RNA integrity number (RIN) is a software tool designed to help scientists estimate the integrity of total RNA samples. The expert software automatically assigns an integrity number to a eukaryote total RNA sample. Using this tool, sample integrity is no longer determined by the ratio of the ribosomal bands, but by the entire electrophoretic trace of the RNA sample. This includes the presence or absence of degradation products. In this way, interpretation of an electropherogram is facilitated, comparison of samples is enabled and repeatability of experiments is ensured. The assigned RIN is independent of sample concentration, instrument and analyst therefore becoming a de facto standard for RNA integrity.

The RNA Integrity Database (RINdb) is a freely accessible repository holding hundreds of user submitted total RNA traces. By searching the database scientists can now see what is a "normal" profile for different tissue types as well as the effects of using different RNA extraction methods and kits.

It is important to understand what the RIN can and cannot do.
What the RIN can do:

- Obtain a numerical assessment of the integrity of RNA.
- Directly compare RNA samples, e.g. before and after archival, compare integrity of same tissue across different labs.
- Ensure repeatability of experiments, e.g. if RIN shows a given value and is suitable for microarray experiments, then the RIN of the same value can always be used for similar experiments given that the same organism/tissue/extraction method is used.

What the RIN cannot do:

- Tell a scientist ahead of time whether an experiment will work or not if no prior validation was done (e.g. RIN of 5 might not work for microarray experiments, but might work well for an appropriate RT-PCR experiment. Also, a RIN that might be good for a 3' amplification might not work for a 5' amplification).

PROTOCOL

1. Before class, as part of your pre-lab exercise, download and read the Agilent Bioanalyzer manual from Blackboard or the Agilent website and write a brief summary of the protocol.

2. After determining your DNA and RNA concentrations with the NanoDrop, dilute your stocks to 200ng/ul, place on ice, and provide the Biotech Dept. lab technician with the solutions so they can prepare and run your sample on the Agilent BioAnalyzer.

3. Write down additional notes on your protocol when watching the Agilent BioAnalyzer demonstration.

4. Retrieve data from lab technician. Include raw data in your notebook.

5. Summarize data with your final report.

Lab Unit 4-C: RNA Analysis by Denaturing Agarose Gel Electrophoresis

Introduction:

Isolated RNA can be analyzed by electrophoresis on an agarose gel. Care is taken during the electrophoresis to ensure that the single-stranded RNA does not form secondary structures through intramolecular base pairing. This can be done by running a denaturing gel of glyoxal (or formaldehyde) and DMSO in MOPS buffer. The RNA fraction is visualized by adding ethidium bromide or SYBR green to the sample loading solution. The loading buffer also contains a tracking dye to determine how far the smallest RNA molecules have migrated while the gel is run.

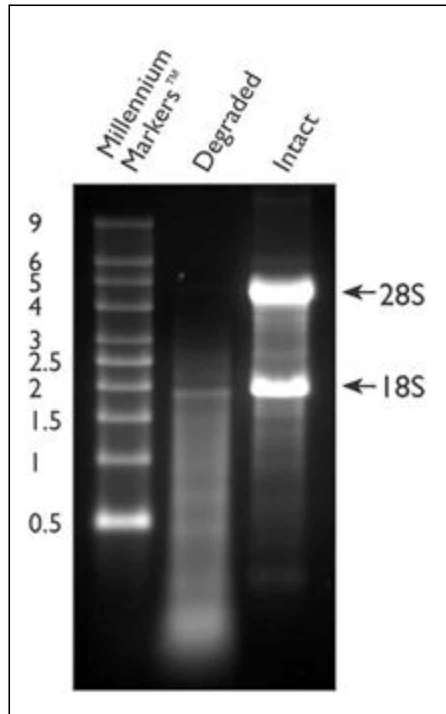
As with all agarose gels, the smaller fragments run faster and therefore further into the gel than the larger fragments. Plant RNAs consist of a heterogeneous population of molecules that can be separated into three classes by zone sedimentation:

23-25S	rRNAs	~55% by weight
16-17S	rRNAs	~20%
4S	tRNAs and other small RNA molecules	~25%

When running RNA gels, we will expect to see the different sized bands. The two major rRNA molecules called 28S and 18S are about 5 kilobases (kb) and about 2 kb in size, respectively. These show on the agarose gel as two sharp, intense bands. Smearing of these bands indicate degradation of the RNA. Contaminating DNA will show up as bands larger than the 28S rRNA migrating the least distance. Checking for degraded 28S RNA and DNA bands will indicate the quality of the RNA preparation. If the 28S RNA is not degraded and there is no contaminating DNA, you can be confident you have a good preparation of RNA.

There is usually a smaller, more diffuse band representing low molecular weight tRNAs and 5S rRNA, though are sometimes lost in RNA preps.

Due to the hardiness and ubiquitous presence of RNases, as well as the inherent chemical instability of RNA molecules, RNA quickly degrades with storage and poor lab technique. As the RNA degrades, the bands on a gel become smaller in size (migrate faster through the agarose) and diffuse in appearance (see figure below).



Intact vs. Degraded RNA. Two µg of degraded total RNA and intact total RNA were run beside Ambion's RNA Millennium Markers™ on a 1.5% denaturing agarose gel. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample. The degraded RNA appears as a lower molecular weight smear.
http://www.ambion.com/techlib/append/supp/rna_gel.html

Scope of the Laboratory Exercise:

In this lab exercise, you will prepare and run a denatured agarose gel to indicate the quality and quantity of RNA prep.

Safety Precautions:
- Wear gloves. Buffers in this system contain irritants.
- Keep hands away from face and especially the mouth.
- Be sure the electrophoresis equipment is not near water or that the cords are not frayed.
- When viewing the gels on the gel box, always wear goggles to protect the eyes from UV light.

Materials (As part of your pre-lab exercise):

Carefully read through the following protocol and make a list of specialized equipment that you foresee as necessary for performing the protocol. These are the things that you must line up in your work area in order to complete the work described, excluding some common lab equipment that is shared with the rest of the class such as pH meters, pH standards, and balances.

Make a similar list of chemicals and reagents that are required to complete the protocol for this day. As described in your Introduction chapter, you must make a table for these chemicals.

(You may need to leave space for any information that is unavailable before the lab exercise, such as the lot number and/or expiration date of the materials.)

Protocol

Running the RNA Denaturing Gel
Read the product literature on NorthernMax (http://www.ambion.com/catalog/CatNum.php?AM8671) and Millennium Markers (http://www.ambion.com/catalog/ProdGrp.html?fkProdGrp=153) at the Ambion website. See appendix for detailed explanation of preparing agarose gels.

1. Assemble gel casting trays and combs. You will run all of your RNA samples stored at different temperatures (thawed previously). Prepare enough gels for your experiment, and remember to account for a well for the molecular weight ladder.

2. Prepare 1.0% agarose gel (RNase-free) in 1X NorthernMax solution in an Erlenmeyer flask that is at least 5X larger volume than the agarose gel solution that you are preparing. Remember that 1% = 1g/100ml. If you do not know the volume requirements for your gel, you can measure it as follows: assemble the casting tray for your gel, fill the tray to the height that you want your gel to be (0.6cm), and pour this volume into graduated cylinder. Write this volume down for future agarose gels you may run.

3. Make a mark on the flask where the liquid line is and place the flask of 1.0% agarose suspension into the microwave until the buffer just begins to boil. The immersion in water ensures that any specs of agarose adhered on the wall of the flask will melt. Overheating can cause the agarose to bubble over and out of the flask.

4. Swirl to make sure that the heated agarose is completely dissolved and that no particles are adhering to the walls or floating in the solution. If the gel level has dropped from the mark made previously, add ddH_2O to replace the volume lost by evaporation.

5. Be careful and use orange HotHands to touch the glass as it will be hot. To avoid unequal cooling and gelling in any part of the agarose solution, swirl gently until cool enough to pour. Alternatively, you can stir it on a stir plate with a stir bar.

6. When the gel flask is tolerable to touch (approximately 55 - 65°C) add 10,000X SYBR green II stain for RNA (3ul/30mL). Ask your instructor to check your calculations. Make sure that the stain has mixed completely into solution.

7. Pour the agarose solution into the gel casting tray and pop any bubbles with the large end of a micropipette tip. Leave the gel to solidify undisturbed.

8. Tansfer 3 μg of your RNA preps into RNase-free tubes. Place the rest of the RNA preps on ice.

9. Add an equal volume of gel-loading solution to the RNA, vortex briefly, and incubate in a 50^oC heat block for 30 minutes. This step denatures any RNA secondary structure.

10. Prepare the RNA Millennium Markers for the gel electrophoresis in the same way that you are preparing your samples.

11. Remove combs and bumpers and position the gel in the electrophoresis chamber with the wells next to the cathode (negative/black) lead. Cover the gel with about 0.5-1 cm 1X NorthernMax running buffer.

12. Picofuge your RNA markers and samples to make sure all the solutions are at the bottom of the tube. Using RNase-free tips, load RNA markers in one of the wells of each gel that is run. Load your samples of RNA in the other wells, recording which well was used for each one in a gel documentation form.

13. Run the gel at 5-10 volts per centimeter measured between the electrodes. This is typically 80 volts for short gels. Run until the tracking dye is most of the way down the gel. Approximately 30 minutes.

14. Capture the images using the gel documentation system and affix a well-labeled copy of your gel to your gel documentation form.

Analysis of the RNA Gel

1. The markers are 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 9 kb respectively. Identify the size for each of the bands of marker RNA.

2. Identify the 5 kb 28S rRNA band and the 2 kb 18S rRNA band. Are they distinct or diffuse? Also identify the small diffuse tRNA bands.

3. Was there any degradation in the samples stored at room temperature? What about at 4°C? Check for DNA contamination by looking for a band near the top of the gel.

4. In your analysis compare and contrast the RNA isolation methods in terms of quantity, quality, and stability and DNA contamination.

Lab Unit 4-D: DNA Analysis by Agarose Gel Electrophoresis

Introduction:

While spectrophotometry of a DNA or RNA solution can be used to measure the concentration of nucleic acid, it cannot give any indication of the purity of the nucleic acid in the solution. Both DNA and RNA will give rise to absorbance, so the amount of DNA contamination in an RNA prep and the amount of RNA in a DNA prep is unknown by this method.

Electrophoretic separation of DNA and/or RNA bands by size can indicate quality of nucleic acids. By running standards of nucleic acids of known concentration in the same gel, the quantity of nucleic acids found in the sample loaded on the gel can be estimated. DNA size markers should bracket the size of DNA in your sample. For gDNA or large plasmids, lambda DNA fragmented by *Eco*RI restriction enzyme or PhiX174 DNA cut with *Hae*III restriction enzyme produces markers from 70 bp to 20,000 bp in length. Restriction digests of plasmids produce DNA markers of smaller sizes. Ladders of synthetic DNA are also available commercially.

The maximum loading capacity for a well is 200 ng per band, and as little as 5 ng of dsDNA may be detected by ethidium bromide, or even lower with SYBR green (500pg).

To assist in loading of samples to an electrophoresis gel a sample buffer, sometimes referred to as a loading buffer, is added to each sample. This buffer contains a colored dye that helps to visualize accurate load of wells. The dye also acts as a tracking dye on the gel. Highly colored and negatively charged, the dye molecule travels at the same rate as the smallest-sized DNA fragments. When the dye molecules have migrated near the bottom of the gel, you will know that it is time to stop the electrophoresis. The sample buffer also contains a ~20% glycerol solution to give your sample sufficient density to drop into the well and not escape the well while loading sample.

Agarose gels have larger pore sizes than do polyacrylamide gels, so agarose gels are used for separating most DNA molecules, while polyacrylamide gels are used only when small fragments need to be resolved. Pore sizes can be varied in different gels by varying the concentrations of the gel materials, as shown in the tables below.

Range of separation of acrylamide gels

Acrylamide (%w/v) (acrylamide:bisacrylamide 29:1)	***Optimal range of DNA separation (bp)***	***Comigration sizes (bp) Bromophenol Blue***	***Xylene cyanole***
3.5	100 – 1000	100	460
5.0	80 – 500	65	260
8.0	60 – 400	45	160
12.0	40 – 200	20	70
20	6 – 100	12	45

Range of separation of agarose gels

Agarose (%w/v)	*Optimal range of separation of linear (kbp)*
0.3	50 – 5.0
0.6	20 – 1.0
0.7	10 – 0.8
0.9	7 – 0.5
1.2	6 – 0.4
1.5	4 – 0.2
2.0	3 – 0.1

Agarose powder comes in many grades. Higher grades are used in preparative work. Low-melting agarose is more expensive, and is used if DNA bands are to be cut out of the gel and DNA extracted by melting the agarose. For general analytical purposes, most electrophoresis-grade agarose preparations suffice.

Scope of the Laboratory Exercise:

In this lab exercise, you will prepare and run a DNA gel, loading sample lanes and lanes with DNA of known concentrations in order to determine the quality and concentration of the sample DNA.

Safety Precautions:

Wear gloves and keep hands away from face, especially the mouth.

- *Be sure the electrophoresis equipment is not near water or that the cords are not frayed.*
- *When viewing the gels on the gel box, always wear goggles to protect the eyes from UV light.*

Materials (As part of your pre-lab exercise):

Carefully read through the following protocol and make a list of specialized equipment that you foresee as necessary for performing the protocol These are the things that you must line up in your work area in order to complete the work described, excluding some common lab equipment that is shared with the rest of the class such as pH meters, pH standards, and balances.

Make a similar list of chemicals and reagents that are required to complete the protocol for this day. As described in your Introduction chapter, you must make a table for these chemicals.

(You may need to leave space for any information that is unavailable before the lab exercise, such as the lot number and/or expiration date of the materials.)

Protocol

Note: plasmid DNA will run as many diffuse bands depending of the degree of supercoiling. In order to use electrophoresis to evaluate its purity, it must first be linearized with a restriction enzyme. The pBR322 family of plasmids, which includes pUC18, pUC19, and pGEM, has a single EcoRI recognition site that may be used for a restriction digest that will create a single unique size band on an electrophoresis gel if the plasmid DNA is pure.

1. Depending on available apparatus, you may wish to run your plasmid DNA on a 1% agarose gel and your genomic DNA on a 0.8% agarose gel. If apparatus is limiting, prepare a 0.8% agarose gel, with SYBR safe, in 1X TAE buffer as described in the appendix. Remember to make sure you make enough gels for your samples, and remember to include one well for the molecular weight ladder.

2. **Prepare Markers**: While the gels are cooling, prepare a molecular weight standard (DNA markers) for a lane in your gel. If not already prepared in sample buffer, add 10ul of nuclease free water to a nuclease-free 1.5mL tube. Add 3uL of 6X sample buffer (also known as “loading buffer”) and 5 uL of DNA markers. Mix by vortexing briefly and pop-spin. NOTE: You will use a different marker for your pDNA and your gDNA!

3. **Prepare Samples**: add 1 uL of 6X loading dye to 5 uL of your unknown DNA sample. Your unknown should be diluted into an approximate concentration range of 0.1-0.5 ug/uL. For convenience, 1 uL loading buffer can be spotted onto a sheet of Parafilm, and 5 uL of sample can be pipetted directly into a spot. Load the resulting 6 uL into an empty lane in the gel.

4. When the gels are ready, remove the combs and dams, fill electrophoresis chamber to just above the gel.

5. Load samples and markers into the wells. Be sure to record what is loaded in your gel electrophoresis documentation form.

6. Electrophorese at 80-100 volts for approximately 30 minutes or until the tracking dye approaches the bottom of the gel. If your tracking dye leaves the gel, you have run it too long!

7. Capture image and affix a well-labeled copy of your gel to your gel documentation form.

8. Observe the number of bands of DNA in your sample DNA lanes.

9. Was there any degradation in the samples stored at room temperature? What about at 4°C? Check for RNA contamination by looking for two bands near the center of the gel.

10. In your analysis compare and contrast the DNA isolation methods in terms of quantity, quality, and stability and RNA contamination.

References:
1. Ambion Technotes: "Denaturing Agarose Gel Electrophoresis of RNA"
2. http://www.ambion.com/techlib/append/supp/rna_gel.html
3. Ausubel, F.M. et al. *Short Protocols in Molecular Biology.* 5th Ed. Wiley. (2002)
4. Berger, S.L. & A.R. Kimmel, eds. *Guide to Molecular Cloning. Methods in Enzymology*. Vol. 152. Academic Press. (1987)
5. Gerstein, A.S. *Molecular Biology Problem Solver: A Laboratory Guide.* Wiley-Liss. (2001)
6. Seidman, L.S. & C.J. Moore. *Basic Laboratory Methods for Biotechnology.* Prentice Hall. (1999)

Analysis and Questions for Unit 4:

Results:
1. Compile a table with the class NanoDrop data for all DNA and RNA extractions. Include the ratios as well as the concentration.
2. Compile a table with all the class data for the stability data for all the DNA and RNA extraction methods at each of the storage conditions.
3. Compile a table with the class chip analysis (Agilent) for all DNA and RNA extractions.
4. Include a well-labeled figure of your DNA and RNA extractions.

Discussion:
1. Compare and contrast the DNA and RNA extraction methods – successes and failures. Why do you think one method was successful over another? If your extraction failed give some plausible explanations.
2. Compare and contrast the storage and stability data obtained. How does your stability table of predicted (lab report unit 3) and experimental results match up?
3. Compare and contrast the different methods of analyzing the quality and quantity of your nucleic acids samples.

Analysis Questions:
1. Discuss the principle behind using spectrophotometry of nucleic acids to determine quality and quantity by answering the following questions:
   a. Discuss why you performed a scan from 230nm to 280nm. What is absorbing at 230nm, 260nm and 280nm? Be specific.
   b. What is the A260/A280 ratio supposed to be for pure DNA and RNA? Why?
   c. Is it possible to reliably detect small amounts of RNA contamination in a DNA prep by comparison of the A_{230}/A_{260} ratio? Explain your reasoning.
   d. Is it possible to reliably detect small amounts of protein contamination in a DNA prep by comparison of the A_{260}/A_{280} ratio? Explain your reasoning.

2. Create a table and compare the methods of qualitative and quantitative analysis of nucleic acid using spectrophotometry (Spec20 and the NanoDrop), the Agilent bioanalyzer and an agarose gel. What are the advantages and limitations of each?

3. Why is RNA run on a denaturing gel and DNA is is not?

LABORATORY UNIT 5: CLONING GAPDH FROM PLANTS

The information provided in laboratory unit 5 is in part or in whole from the Bio-Rad Sequencing & Cloning Kit (Cat #166-5000EDU) Manual.

Introduction and Background

The aim of this lab is to clone portions of the glyceraldehyde phosphate dehydrogenase (GAPDH) *GapC* gene from plants, insert these gene fragments into a plasmid vector, and analyze the sequence of resulting clones using bioinformatics. It is hoped that the results of this lab will generate entirely novel sequence from plants that have not been characterized yet.

Genomics can be expensive and unless a researcher or company is interested in the specific gene product, then its code may be unknown. Thus for some of these plant genomes, the *GapC* gene sequence is unknown. Some of the GAPDH gene sequences have been previously described and can be found on the NCBI Genebank database. You have the option once performing an initial search of nucleotide databases (such as Genbank) to ascertain whether a particular plant species has had its *GapC* gene sequenced, you have the option to choose a plant species where the *GapC* sequence is known or unknown. If you succeed in your project, and the GapC gene is novel, you can publish your research!

GAPDH Cloning Project Overview

For the entire Lab Unit 5 research project you will be using the Bio-Rad Cloning Kit (Cat #166-5000EDU). The Manual has an extensive description of the theory and the protocols involved in this project, and only a brief summary of each step is presented here (below).

A word of caution: this project has many different phases, requiring perhaps 8 weeks of continuous effort. A mistake at any step of this process can prevent you from reaching your ultimate goal of sequence information from the *gapC* gene that you are studying. Your success will rely heavily on your lab preparation PRIOR to coming in to work on this project. Not only should you make yourself familiar with each step of the project, you should plan ahead to make sure that the timing of your protocol is streamlined. For example, here are some pitfalls that you should avoid:

- It takes a long time for incubators to come up to temperature – make sure that you set these up well before they are required. These incubators and temperatures should be listed in your pre-lab equipment list.
- Proper labeling of microcentrifuge tubes and reagent containers is also very time-consuming. Arrive at a shorthand notation system that not only works for you, but also ensures that if your samples should be scrambled or lost in a freezer that they can be identified and returned to you. THEN, make sure that you plan ahead to make sure that tubes are properly labeled ahead of time, BEFORE you need the tubes.
- There will be a large number of samples stored by each student in the freezer during the course of the semester. Make sure that you store your samples so that they are not likely to become scrambled or lost by accidental upturning in the freezer. For example, the worst method of freezer storage would be in an open beaker. The best method of freezer

storage would be in a carefully labeled container with a rack to keep your samples in order and a lid to ensure that they cannot spill out if upturned.

- Take care of your reagents: make sure that there is no cross contamination (fresh pipet tips, only!) and that all enzymes are kept VERY cold prior to use. Once you have thawed enzymes, they should be mixed and picofuged to make sure that all the liquid is a uniform solution and located at the bottom of the tube. Once you and your classmates have taken as much enzyme as needed from the stock, it should be immediately returned to the freezer for storage.

Keep track of your outcomes as you work your way through this project, so that you can avoid problems as you continue. For example, if you know that a previous step has yielded a very low level of PCR template for the next step, you can add a higher amount of template to that PCR reaction mix. Conversely, if you know that you have a high level of template going into a PCR reaction, you would best cut back on the prescribed level of template recommended by the Bio-Rad protocol.

Cloning & Sequencing Project Outline:

Choosing a Plant

The first step of this project, you and your lab partner will choose two plants. One that the GapC gene sequence is known, the other the sequence is unknown. You will prepare an oral presentation for the class outlining your preliminary research for your project and project outline. You will be expected to defend why you chose the plants you picked.

DNA Extraction

This stage extracts all nucleic acids from the plant cells using tissue grinding followed by DNA purification using column chromatography. The final eluate contains genomic DNA and RNA from the cells.

Optional analysis of the eluate may be performed using standard techniques. DNA can be viewed using agarose gel electrophoresis. Loading ~10 µl of gDNA 0.8-1% gel electrophoresed at 75 v for one hour is recommended. Genomic DNA will be seen as a faint band at the top of the gel. RNA will be viewed in 2 major bands much further down the gel. Note: some plant extractions may have yielded too little DNA to be visible on a gel- however this will likely amplify in the next PCR step. Spectroscopy or fluorometry that specifically quantifies double stranded nucleic acids can be used to quantify the DNA. Reading the absorbance of the DNA at 260 nm using spectrometry will have reduced accuracy due to the presence of RNA in the prep. RNA can be removed using RNase I and precipitating the DNA using standard techniques to remove nucleotides.

GAPDH PCR

In this stage students will perform two round of PCR to amplify a portion of the GAPDH gene from their genomic DNA. The initial PCR uses degenerate primers to amplify a pool of DNA fragments. In this case, this means that one or more positions in the nucleotide sequence have more than one nucleotide synthesized at that position. Whereas nucleotides are usually synthesized as having one nucleotide at one position, degenerate nucleotides have one or more

positions that can have more than one nucleotide synthesized at a single position. This idea can be demonstrated in the example below, where the Ns stand for any deoxynucleotide.

5' – AGNTTTGCNTGTGAAC – 3'

The second round of nested PCR uses more specific primers to amplify a specific GapC gene from the pool. Different plants will have different affinities for the primers and thus have different efficiencies in the PCR reaction. Students may well obtain a clone-able fragment after the first round of PCR; in this case, this would mean that a PCR product of sufficient quantity (generally visible on an agarose gel is sufficient) and quality (means few or single bands), and of the expected size. However, the PCR could also be so inefficient that students cannot detect PCR product after the first round. In order to obtain target DNA of sufficient quantity and purity to clone, we highly recommend performing both rounds of PCR. However even then it is possible that no band may be visible following nested PCR either because the DNA extraction was unsuccessful, or the primers had insufficient affinity for the target DNA- see notes above on which plants to choose above.

Controls in the lab include genomic DNA from *Arabidopsis thaliana* which should be treated as an additional genomic DNA sample and amplified in the initial round of PCR and the product amplified in the second round of nested PCR. In addition, plasmid DNA encoding the targeted GAPDH fragment from *Arabidopsis* is also included. This should be used as a template in each round of PCR to ensure the PCR reaction is working. By performing these controls students will not only understand the use of controls in experiments, but control reactions can also serve as a source of clonable PCR product should their own samples not amplify. A negative (no template) control is also recommended.

Agarose Gel Electrophoresis of PCR products

Time constraints may determine when students analyze their PCR reactions. Experiments to be analyzed prior to ligation:

1. Initial PCR (5 samples)
2. Nested PCR (5 samples)
3. Optional) Purified PCR products (2 samples)

It is recommended that students analyze their initial PCR and nested PCR on a single gel to allow direct comparison of PCR products. After choosing the plant *gapC* that will be cloned, the PCR product needs to be purified (Stage 4). If time permits it would be valuable for students to compare the PCR reaction prior to purification and the product after purification by electrophoresis to see the removal of small products. This would require the running of an additional agarose gel once the purification of PCR products is complete.

Purification of PCR products

In this stage students will purify their PCR reactions using size exclusion chromatography to remove excess primers, nucleotides and enzymes. Columns are first prepared by resuspending the resin and then centrifuged to create the column bed. Samples are then applied to the column bed and centrifuged to spin out the purified PCR product. It is important that the speed of the centrifuge for this stage is adjusted according to the protocol. Details are in the student manual,

but in brief, the speed required is 735 g which is much slower than most benchtop microcentrifuges. Note- as an alternative to the calculation stated in the student manual, many conversion tables and calculators are available on the web.

Following the PCR purification an option to analyze the PCR reaction prior to and after purification is available. This will depend on the time constraints.

Cloning: Ligation and transformation

In this stage the purified PCR products are ligated into a plasmid vector and then transformed into bacteria. The vector is pJet1.2 blunt. This vector is already digested with blunt ends and ready for ligation of PCR products. Rather than the traditional lacZ gene to assist with colony selection using blue white cloning on x-gal/IPTG plates, pJet1.2 selects successful ligations through the disruption of an otherwise lethal gene, *Eco47IR*, which enables positive selection of the recombinants. PCR fragments are blunt-end cloned into pJet1.2, which first requires that the 3' dA overhangs are removed from the PCR products in a blunting reaction prior to ligation. The ligation reaction is a fast ligation reaction, complete in 5-10 min. A very minimal increase in transformants benefit is gained, by extending the ligation time beyond 10 min.

The transformation protocol involves creating competent cells and immediately performing the transformation. This method permits relatively high transformation efficiency (10^7 per µg DNA) without the requirement of a refrigerated centrifuge (although useful if available), commercial competent cells or a -70°C freezer to store competent cells. Once made, these cells must be used immediately or discarded; they cannot be stored at -70°C for later use.

Isolation of Plasmid DNA

In this stage the students isolate plasmid DNA from transformed bacteria. Prior to this lab, students (or instructor) need to inoculate four LB Amp broth mini-preps per team from the transformed colonies grown after the transformation. The mini-prep method is an alkaline lysis protocol, with the purification step performed on a purification column. After plasmids are isolated, they are analyzed by restriction enzyme digestion and agarose gel electrophoresis to determine whether they contain the PCR products.

DNA Sequencing

In this stage students combine plasmid and sequencing primers ready to mail to a sequencing service. The Dept of Energy Joint Genome Institute (JGI) values the skills being taught through this laboratory and is providing a free sequencing service for US educators using this laboratory. Plates will be sequenced on a 2 week basis, thus results may take 2 weeks to arrive. Results will be posted as zip files on an FTP server ready for download. If results are desired more promptly, local sequencing services may be utilized. Most major universities have core labs that will sequence for a fee; alternatively there are many commercial sequencing services.

Lab Unit 5-A: Choosing a Plant Species

Introduction:

There are many methods of DNA extraction for plants, many specific to each plant type. Although every attempt has been made to make this lab as universal as possible to all plants, the fact that this lab uses a single DNA extraction method means there will be some plants for which the DNA extraction method does not succeed. These may be plants with very tough extracellular matrix or cell walls or some other reason that makes it difficult to extract DNA. Moreover, different plants will yield different quantities of DNA and the ability of that DNA to amplify may vary, for many unpredictable reasons. For example, plants that yield very little gDNA, may well amplify easily, while other plants that yielded a lot of gDNA may amplify poorly.

Likewise, PCR primers have been designed in this kit to amplify GAPDH from the majority of plants; however, although extremely conserved on the protein level, there is a good deal of variation between GAPDH coding DNA sequences of different plant species. Thus there may be some plants whose DNA amplifies poorly or not at all with the primers provided. Alternatively, a particular plant may contain a metabolite that interferes with PCR, preventing amplification.

Students will choose two plant species for this set of protocols. The first is a plant known to be successful with this protocol (see table 1) in order to ensure success and comfort with the techniques taught. Although not a true positive control, use of this plant will allow the student to compare data and results with a less well-characterized plant species that may not work. The second plant should be less well characterized, with the risk that it may be unsuccessful. By encouraging independent research, there exists great opportunities to contribute to table 1, future years of this course, and to publish data in scientific databases of gene sequence. The student will have the opportunity to troubleshoot and choose to extract plant DNA from different parts of the plants, using alternative extraction methods. Once DNA has been successfully extracted, students may also explore optimizing PCR reactions and redesigning primers that fail.

To increase success and to demonstrate additional techniques, the PCR protocol has two stages. First DNA is amplified from genomic DNA using degenerate primers. Then a further round of nested PCR is performed to both increase specificity and quantity of PCR product. Depending on the plant chosen, it may be that no PCR product is visible on an agarose gel after the first round of PCR, or you may choose to directly clone a well amplified fragment after the first round, to reduce the time spent on the course. However, it is recommended that you perform the cloning protocol with the 2 stage (nested) PCR to have the best chance of student success.

With this in mind you will choose two plants at the start of the experiment. To increase the chance of success, you could choose a plant known to work well, and a less well characterized plant. You could also choose two different tissues (roots or leaves for instance) of a plant known to work well. If your goal is to obtain well characterized sequence to be uploaded into GenBank, multiple groups should plan to research the same plant species, so that multiple redundantly verified sequences are created. This ensures proper depth of coverage for the sequence to be acceptable to GenBank.

In addition the lab contains controls to assay the validity of the results: genomic DNA from *Arabidopsis thaliana* and an *Arabidopsis thaliana* GAPDH gene fragment cloned into a plasmid vector previously. *Arabidopsis thaliana* (thale cress) is native to Europe, Asia and Africa, but more importantly, it is a model organism due to its small genome (fully sequenced), small size and rapid life cycle. Using these true positive controls ensure that at each step, the products from the controls will allow continuation of the experiment in the event that the students experiments are not successful, thus allowing the techniques to be learned and performed in a timely manner, even if novel data is not achieved. Student troubleshooting and repetition is greatly encouraged.

Figure 1 *Arabidopsis thaliana*

Scope of the Laboratory Exercise:

In this lab exercise, you will select a plant known to work from Table 1 that you wish to work with. You must go online to check for sequence data of the *gapC* gene for the plant that you choose. At the same time, you will select a plant that is not listed in the Table 1, nor listed in the plants known to fail using the Bio-Rad. ***Keep in mind that whatever plant tissue you choose must be available to you to bring to class.***

Part of your grade for this report will be to prepare a presentation for the rest of the class of your findings.

PROCEDURE:

1. Before class, you and your lab partner should choose 2 plant species that interest you and that you know you can locate easily to bring into the lab for this experiment. One of these two species should be selected from the list in the Bio-Rad manual that has been shown to work well with the PCR primers designed for this kit. This information is located in Table 1 of the Bio-Rad manual.

2. Make sure that your second plant species selection is not listed in the Bio-Rad manual table of plant species so that it has NOT been shown (yet!) to be a species that works well, and should also NOT be found in the list of species that have been shown to fail to amplify with the PCR primers designed for this kit. This second species, then, will be your unknown and will allow you to expand our knowledge of *gapC* sequence information. This selection should be one that you would like to investigate for some reason. Perhaps you would like to see your favorite Texas wildflower get more attention at GenBank, or perhaps you are interested in an herbal remedy that you use. Make sure that you are selecting something that you can easily locate and bring into the lab. As a reminder, the other species should be one NOT found in this table.

3. When you get to class, share your choices with the instructor. Ensure each group has a different set of plants.

4. GenBank data can be accessed at the National Center for Biotechnology Information website hosted by the National Library of Medicine at the National Institutes of Health (www.ncbi.nlm.nih.gov). You may search for the gene "gapC" and limit your search by adding the species that you are interested in, or just "in plants". You may select from many different databases, including the publications available in PubMed, the protein sequence, and the nucleotide sequence database. Look for information about the *gapC* gene in the "nucleotide" drop down menu and answer the following questions:
   a. What protein does the *gapC* gene code for, and what major metabolic pathway does it participate in?
   b. What cellular compartment is the *gapC* protein found in?
   c. What cellular compartment is the *gapC* DNA sequence found in (cytoplast, nucleus, mitochondria, etc)?
   d. How many plant *gapC* nucleotide and protein sequences can you find deposited in GenBank?
   e. How many plant *gapC* genes have been completely sequenced?

5. Select the accession that has the protein sequence of *gapC* gene found in *Arabidopsis thaliana*.
   a. How many amino acids are there in this protein? How many different domains are there in this protein, and what is the exact function of each domain?
   b. From the search report page, select "conserved domains" for the *gapC* gene found in *Arabidopsis thaliana*. Are there any other proteins that have sequence homology to *gapC* gene? If so, what are these genes and how are their functions in the cell similar and how are they different in from *gapC* gene?
   c. Click on the *gapC* conserved domain (red) and evaluate the amino acid sequence similarities to other proteins. Click on the proteins accession numbers and evaluate how related these organisms are.

6. Research the plant species that you have selected, answering the following questions.
   a. How closely related are the two species that you have selected?
   b. What is the scientific (Latin) name and common name of your plant species?
   c. Has the *gapC* gene found in these two species been sequenced?
   d. Are any of the species that you have selected closely related to one of the plants whose *gapC* genes have been sequenced?

7. Prepare an informal presentation of what you and your lab partner have discovered about the two species that you have selected and their gapC genes to present to the class.
   a. Generate a 5 slide (or less) PowerPoint to use with your presentation. Your presentation should be less than 10 minutes in length.
   b. Your presentation should NOT include general information about GAPDH, it should only have information on the two species you chose, why you chose them and why believe you will succeed.
   c. Try to persuade your classmates that your selections are interesting enough to merit study in some way, such as in their usefulness to mankind, or their intrinsic beauty, or maybe just because of their close relatedness to a species whose gapC gene has already been sequenced (hence, you expect a good chance of success in your project).

Analysis and Questions for Unit 5-A:

Your report for this lab will be a different format previously. Details are listed below. Part of your grade will be for your report; the other part of your grade will be for your presentation.

a. **Introduction:** Your introduction should include background information on the gene you will be cloning. Use questions 4a-e to help guide your introduction. Do NOT write this as a Q&A – write a one page introduction on GAPDH and the plants you chose.

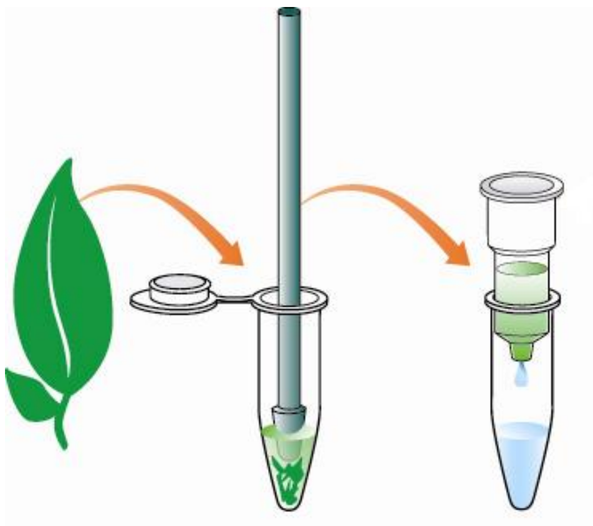
b. **Results:** Include all the data collected on the two plant species chosen and **summarize** appropriately in your report.

c. **Discussion & Analysis:** In your discussion and analysis section include a discussion that covers *all* of the questions listed in the lab instructions. DO NOT list the questions and answers underneath.

   Include in your discussion why your selections are interesting enough to merit study in some way, such as in their usefulness to mankind, or their intrinsic beauty, or maybe just because of their close relatedness to a species whose *gapC* gene has already been sequenced (hence, you expect a good chance of success in your project).

d. **Presentation:** Prepare an informal presentation of what you have discovered about the two species that you have selected and their gapC genes to present to the class. Generate a 5-10 slide PowerPoint to use with your presentation. Your presentation should be less than 10 minutes in length.
   a. Try to persuade your classmates that your selections are interesting enough to merit study in some way, such as in their usefulness to mankind, or their intrinsic beauty, or maybe just because of their close relatedness to a species whose gapC gene has already been sequenced (hence, you expect a good chance of success in your project).
   b. Upload your PPT presentation with your lab report to Blackboard.

Unit 5 – B: DNA Extraction



Introduction:

This project is an opportunity to perform novel research – to clone and sequence a gene that has not yet been analyzed and to add to the body of scientific knowledge. The first step in this exercise is to choose an interesting plant species to work with. Some model species that plant biologists study, for example *Arabidopsis thaliana, Chlamydomonas,* or crop plants like rice and wheat, have already had their genomes sequenced. You may choose to reproduce and confirm this sequence data. Alternatively, you may choose to select a species that is less studied. There are over 250,000 plant species known to exist on the planet, providing plenty of options. Also, you could choose a variety or cultivar (within a species) that no one has examined yet.

In order to clone a gene from an organism, DNA must first be isolated from that organism. This genomic DNA is isolated from one or two plants using column chromatography. Plant material is weighed, and then the material is ground in lysis buffer with high salt and protein inhibitors using a micropestle. The solid plant material is removed by centrifugation, then ethanol is added to the lysate and lysate is applied to the column. The ethanol and salt encourage DNA to bind to the silica in the chromatography column. The column is then washed and the
DNA is eluted using sterile water at 70oC.

For PCR to be successful, the DNA extracted needs to be relatively intact. The best sources for DNA extraction includes young green leaves, but fruit, roots, or germinating seeds should also suffice. It is better to use tissue that is still growing, as the nucleus:cytoplasm ratio will be more favorable, cells walls will be thinner, and the amount of potentially harmful secondary products will be less. There are two features of plants that make DNA extraction different from animals. First, plants have a tough cell wall made of cellulose that has to be penetrated. Second, a major part of every plant cell is a vacuole that contains acids, destructive enzymes (including nucleases), and unique secondary compounds (products produced from pathways that are not part of primary metabolism) that potentially damage DNA. To minimize contaminants from the vacuolar contents, salts and other inhibitors have been added to the lysis buffer.

Although every attempt has been made to make this lab as universal as possible to all plants, the fact that this lab uses a single DNA extraction method means there will be some plants for which the DNA extraction method does not succeed. These may be plants with very tough extracellular matrices or cell walls, or plants with some other characteristic that makes it difficult to extract DNA. Moreover, different plants will yield different quantities of DNA and the ability of that DNA to amplify may vary. For example, plants that yielded very little genomic DNA (gDNA) 9 may amplify easily, while other plants that yielded a lot of gDNA may amplify poorly. Likewise, PCR primers have been designed to amplify *GAPDH* from the majority of plants. However, although *GAPDH* is extremely conserved on the protein level, there is a good deal of variation among *GAPDH* DNA sequences of different plant species, so there may be some plants whose DNA amplifies poorly, or not at all, with the primers provided. Alternatively, a particular plant may contain a metabolite that interferes with PCR, preventing amplification.

Materials:

1. Biotechnology Explorer, Nucleic Acid Extraction Module (Catalog #166-5005EDU)
2. Two plants chosen in Lab Unit 5A

Protocol:

1. Download the Biotechnology Explorer ***Nucleic Acid Extraction Module*** from Blackboard or the Bio-Rad website. Read in its entirety very carefully before coming to class. This is what you will prepare your pre-lab exercise from.
2. Perform DNA extraction as outlined in the Nucleic Acid Extraction module from both your chosen plant species. We will not be performing the RNase treatment nor concentration step.
3. If you will not be determining the quantity and quality of your DNA on the same day as the extraction, store at -20°C.
4. Run ~10 µl of your extracted gDNA on a 0.8-1% agarose gel.
   - You may work with another group (or two) in running one gel since you will only have 2 samples each.
   - Note: Be sure to run an appropriate <u>genomic</u> marker.
   - Include a copy of your gel in your notebook and also prepare a well-labeled figure for your Lab Unit 5-B report.
5. Using the NanoDrop, run a spectrophotometric analysis of your DNA sample as previously performed in lab 4.
   - Share your spectrophotometer results with the class.
   - Collect spectrophotometer data from the class.
   - Prepare ONE table of the class results for your lab report.

Analysis and Questions for Unit 5-B:

Results:

1. Summarize the class NanoDrop results in tabular form.
2. Include a well-labeled and titled figure of your gel electrophoresis of your plant gDNA.

Analysis Discussion & Conclusion

Be sure to include in your discussion of your results and the class results the following. Do not answer these as a Q&A; include them in the body of your discussion.

- What was the lowest concentration level of gDNA (in ug/ul) that was detectable from your DNA isolation agarose gel based on the detection limits of dsDNA by staining in gel electrophoresis? You may need to consult your textbook/the SYBR green handout in order to answer this question.
- Is there a trend in the types of plants that resulted in high yields and low yields of gDNA?
- Did you have difficulty extracting DNA from your plant? If you did, discuss why you think so. If your extraction was successful, discuss why you think your extraction was successful and other groups were not as successful.
- Is there any evidence of RNA contamination in your gDNA isolates? If so, how might you eliminate this contaminant?

Analysis Questions:

1. What purposes does the ethanol addition prior to column purification of your gDNA?
    a. What types of contaminants are purified from gDNA by the column purification method? What kinds of contaminants would you expect to be poorly resolved from gDNA by this method?
    b. What types of contaminants can block the spin columns used to purify the gDNA?
    c. What is the purpose of the high temperature incubation prior to extracting gDNA from the spin columns?

2. Why is it more difficult to extract DNA from plants?

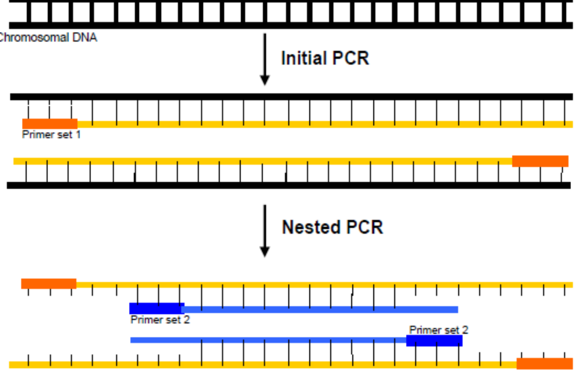
3. Why is it recommended to extract DNA from young plants?

Unit 5-C: Polymerase Chain Reaction

Introduction:

The overall purpose of this experiment is to clone a portion of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH). Because it is a vital metabolic enzyme involved in one of the most basic of biological processes — glycolysis in respiration — the GAPDH protein is highly conserved between organisms, especially vital domains of the enzyme, such as the active site. However, this does not mean that the gene and DNA sequence are identical in different organisms. Much of a gene does not code for protein; this “intronic” DNA is not subject to the same selective pressures as DNA that codes for protein. In addition, for the gene exons — gene sections that do encode for proteins — there is degeneracy of the genetic code such that different DNA triplet codons encode the same amino acid. Also some regions of the enzyme less vital to function (other than an active site) do not have the same degree of selective pressure and although there is conservation of protein sequence, it may not be as stringent in some areas as others.

To clone a known gene from an uncharacterized organism, PCR primers (short synthetically synthesized, single-stranded oligonucleotides often 17–25 bases) must be designed that are complementary to conserved regions of *GAPDH* genes. However, even conserved regions are not identical between organisms. A best guess of the gene sequence is made using a comparison alignment from the sequences of *GAPDH* genes from different but related organisms, with the understanding that the primers will not be an exact match to the sequence and may amplify nonspecific sections of DNA in addition to the target sequence. A second set of primers is then designed (interior to the first set of primers) and used to amplify the PCR products from the first round of PCR. This technique is called "nested PCR" and is based on the extremely slim chance of nonspecifically amplified DNA also encoding these interior sequences, whereas the target sequence should contain these sequences, even if the match is not perfect. In other words, if the wrong fragment was amplified with the first primers, the probability is quite low that the wrong fragment will be amplified during the second round of PCR. As a result, the PCR products generated from nested PCR are very specific. Additionally, since the nested PCR primers are in the interior of the first fragment, the PCR products generated during the second round of PCR are shorter than the first one. See figure below for an illustration of nested PCR.



The products of the initial PCR reaction will be diluted and used as the templates for the nested PCR reaction. *Arabidopsis* genomic DNA has been included as a control for these PCR reactions. In addition, a plasmid encoding the targeted region of *GAPDH* from *Arabidopsis* will be used as a further control.

As each PCR reaction takes approximately 3–4 hours to run, it is most practical to run the PCR reactions on separate days. Since the reagents used in these experiments function optimally when prepared fresh, just prior to running the PCR reactions, it is highly recommended that the reagents be prepared just prior to setting up the PCR reactions.

Important note: PCR is extremely sensitive to contamination by DNA from many sources. All manipulations involving reagents to be used for PCR should be handled with care so that contamination is minimized. To avoid contamination, it is recommended that PCR reactions are set up in an area of the laboratory or classroom that is separate from the DNA extraction area, and/or that the lab benches are thoroughly swabbed down with a commercial cleaner or 10% bleach (ethanol does not destroy DNA). In addition, aerosol barrier pipet tips should always be used to set up PCR reactions (and for preparation of template DNA). It is recommended that micropipettes be carefully cleaned with a 10% solution of bleach before performing PCR. In research labs, PCR flow hoods are frequently utilized to prevent contamination.

Controls in the lab include genomic DNA from *Arabidopsis thaliana* which should be treated as an additional genomic DNA sample and amplified in the initial round of PCR and the product amplified in the second round of nested PCR. In addition, plasmid DNA encoding the targeted GAPDH fragment from *Arabidopsis* is also included. This should be used as a template in each round of PCR to ensure the PCR reaction is working. By performing these controls students will not only understand the use of controls in experiments, but control reactions can also serve as a source of clonable PCR product should their own samples not amplify. A negative (no template) control is also recommended.

Lab Unit 5C Protocol:

The following protocols are available on Blackboard. You must read the entire instructions and prepare your pre-lab accordingly. You don't have to print out the entire instructional manual, but make sure you have detailed instructions to follow in your pre-lab! Your instructor will go through the schedule in more detail with you. This section will take approximately 2 weeks.

- Biotechnology Explorer GAPDH PCR Module (Catalog #166-5010EDU)
- Cloning & Sequencing Explorer Series, PCR Purification
- Cloning & Sequencing Explorer Series, Agarose Gel Electrophoresis
- PCR Kleen™ Spin Purification Module (catalog #732-6300EDU)

Procedural Outline:

1. Day 1: Long PCR
2. Day 2: a. Exonuclease treat long PCR
   b. Nested PCR
3. Day 3: a. Purify nested PCR
   b. Agarose Gel of PCR amplicons

***GAPDH* PCR (Day 1)**

1. Plan the initial PCR experiment: one initial PCR reaction will be performed for each of the genomic DNA samples extracted.
2. Prepare DNA samples for PCR
3. Set up PCR reactions
4. Run PCR

Optional: Although this protocol recommends analyzing the PCR products after both rounds of PCR have been completed, PCR results can be assessed using electrophoresis directly after this reaction is complete. Positive controls should yield visible bands. It is possible that some plant genomic DNA will not yield a visible band during the initial round of PCR and yet still be amplified after the second round of nested PCR.

Note: If this is done — DO NOT add loading dye directly to the PCR reactions as loading dye may interfere with the subsequent round of PCR.

Nested PCR (Day 2)

PCR products generated in the previous step will be further amplified (i.e., serve as the template) in a second round of PCR. However, before performing the nested PCR, the primers that were not incorporated into PCR product must be removed so that they do not amplify target DNA in the second round of PCR. To do this, an enzyme that specifically digests single-stranded DNA, exonuclease I, will be added to the PCR reactions. After the initial PCR primers have been digested, exonuclease I also needs to be inactivated before it is introduced into fresh PCR reactions to prevent it digesting the nested PCR primers. In nature, this enzyme is involved with proofreading and editing newly synthesized DNA.

Following exonuclease I treatment, diluted PCR products from genomic templates generated in the first round of PCR will be amplified using the nested primers. Plasmid DNA will also be

amplified in this step to serve as a positive control for PCR. A no-template negative control will also be run.

The fragment of GAPDH that has been targeted varies in size between plant species. The expected size of the fragment from the first round of initial PCR is expected to be 0.5–2.5 kb. The expected size of the fragment from the second round of nested PCR is expected to be slightly smaller than the product from the initial round of PCR. It is likely that some plants may amplify multiple bands — which correspond to multiple GAPDH genes within the plant's genome. The nested PCR should result in a single band or doublet — corresponding to the GAPC and/or GAPC2 gene of the organism. This is probably due to amplification of two GAPDH genes that are very homologous (genes that share similar structures and functions that were separated by a duplication event).

Nested GAPDH PCR (Day 2)

1. Treat PCR reactions from initial PCR with exonuclease I
2. Heat-inactivate exonuclease I
3. Prepare PCR reactions for 2nd round
4. Run PCR

Purify & Analyze GAPDH PCR (Day 3)

Set aside 5ul aliquot of your nested PCR BEFORE purification. Electrophorese 5 µl of the long PCR, 5ul of the unpurified nested PCR and 5 µl of the purified PCR on an agarose gel. Compare amplification success, amplification sizes, and relative quantity of each.

Choose a plant GAPDH to clone

Once the class has their results, it is time to pick a plant to clone. Although two plants were chosen to investigate, ***only a single plant's GAPDH gene will be cloned***. It is recommended that the plant chosen be ***the one that generated the cleanest PCR product*** (fewest background bands), ***with good band intensity of an appropriate size***. It is acceptable to clone doublets since each plasmid is expected to ligate a single DNA fragment. Be aware that two different gene sequences may be obtained from different minipreps.

It is highly recommended that the entire class clone GAPDH from the same plant, so that the data obtained will be more reliable. Cloning the same gene multiple times will provide significant coverage, which will help to resolve any ambiguous base pairs when the gene is sequenced. Remember, the ultimate goal of this laboratory is to provide new data for the scientific community at large, thus it is vital the data provided be as correct as possible. It is recommended that one or two groups perform an additional PCR purification, ligation, and transformation of the control Arabidopsis GAPDH PCR fragment, as a positive control for the class.

Purification of PCR Products

The next step after generating DNA fragments is to find a way to maintain and sequence these products. This is done by ligating (inserting) the fragments into a plasmid vector (small circular pieces of double-stranded DNA found naturally occurring in bacteria) that can be propagated in

bacteria. To increase the success of ligation, it is necessary to remove unincorporated primers, nucleotides, and enzymes from the PCR reaction.

This is done by using size exclusion column chromatography. In size exclusion chromatography small molecules like proteins, primers, and nucleotides, get trapped inside the chromatography beads while large molecules, like DNA fragments, are too large to enter the beads and pass through the column into the microcentrifuge tube. Without this cleaning step, we would be unsuccessful in the next steps of the cloning process: blunt-ending our PCR product with a proofreading polymerase, and ligating it into a vector. The opportunity also exists here to run gel electrophoresis of PCR product samples before and after cleaning to demonstrate the efficacy of the spin-column cleaning. The NanoDrop may not yield accurate DNA quantitation results from the Kleen Spin columns, therefore a gel analysis is the most appropriate method to determine recovery from the column.

Important note: PCR Kleen spin columns are designed to be used in variable-speed bench top microcentrifuges capable of generating a force 735 x g.

Additional tasks to perform prior to next stage:
Starter cultures must be inoculated one day prior to the transformation with a starter colony from the HB101 LB agar starter plate. Incubate cultures with shaking overnight at 37°C. This may have already been done for you.

DAY 3: Analyze PCR reaction
1. Set aside 5ul aliquot of unpurified nested PCR product.
2. Purify PCR amplicon for cloning.
3. Agarose Gel analysis: aliquots from long PCR, nested PCR, purified PCR
4. Choose which PCR amplicon one you will clone.

Analysis and Questions for Unit 5-Part C:

Results:

1. Create a table compiling the results of all of your classmates, showing the yields of nucleic acid from the gDNA isolation, the long PCR, and the nested PCR results. Include the plant source of gDNA, and the approximate size of nucleic acids in your table.
2. Include a well-labeled figure of your own group results.

Analysis:

- Looking at the combined results of your class, what is the range of template concentration in the long PCR and the nested PCR reactions?
- Do the gDNA template concentrations in the long PCR reaction mixture fall within the concentration range recommended by your textbook?
- A poor yield of amplicon can result from a template concentration that is too low and one that is too high in the PCR reaction mixture. Do you see any evidence of this in your class results?
- What is a plausible reason that a template concentration that is too high can result in a low amplicon yield?
- What is a plausible reason that the amplicon sizes differ widely?
- Indicate if primer-dimers were the only amplicon detected for your PCR reactions. What are some possible reasons that these amplifications failed?

Additional Analysis Questions:

1. Controls for a step of a procedure helps with troubleshooting that step should you obtain disappointing results.
   a. What controls were run in both your PCR reactions? Thoroughly explain why each was used.

2. What are some advantages of using two PCR amplifications (long and nested PCR) in isolating the *GapC* gene?
   a. What are some reasons that the long PCR reactions might have no amplicon yield?
   b. What are some reasons that the nested PCR reactions might have no amplicon yield?
   c. What is different in the two primer sets used for the two PCR amplification steps? Explain thoroughly.

3. What is the purpose of the exonuclease I treatment of the long PCR amplicons?

4. In what ways is the purification procedure for amplicons different than that of the gDNA purification? Why?

5. Why is it important to purify the nested amplicon prior to ligation?

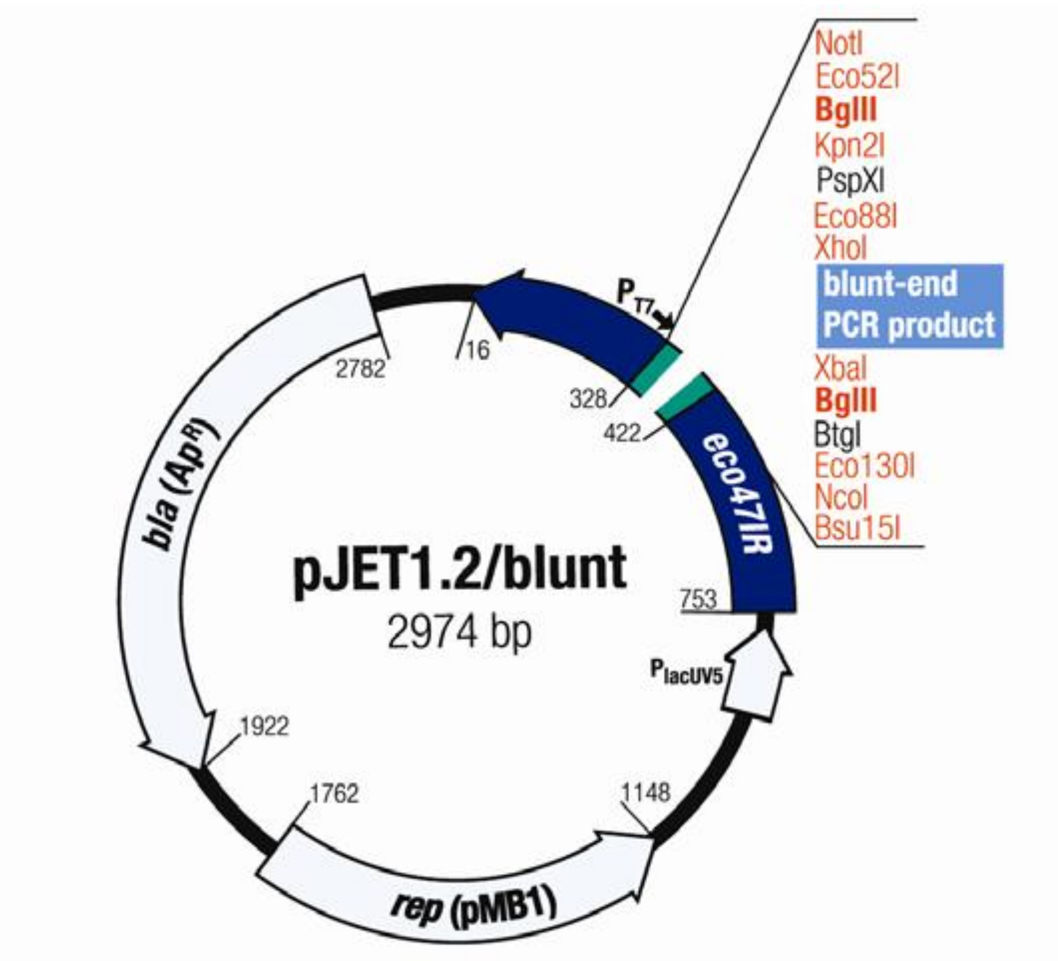
Unit 5 D: Ligation & Transformation of GAPDH PCR Products

Background
At this stage, the PCR product will be inserted (ligated) into a plasmid vector. The plasmid is supplied ready to use and has already been opened ready to receive the fragment. However, prior to ligating the fragment into the plasmid, the PCR fragment must first be treated to remove a single adenosine nucleotide that is left on the 3′ ends of the PCR fragment by Taq DNA polymerase. This is performed by a proofreading DNA polymerase (enzymes with a 3′ proofreading exonuclease domain that allows the polymerase to remove mistakes in the DNA strands). This polymerase functions at 70oC but not at lower temperatures, so it is not necessary to inactivate this enzyme after use.

Once blunted, the PCR fragment is combined with the plasmid. A T4 DNA ligase (an enzyme that catalyzes the formation of phosphodiester bonds between the 5′-phosphorylated PCR fragment and the 3′-hydroxylated blunt plasmid) is added and the ligation reaction is completed in 5–10 minutes.

During ligation, many different products are produced. In addition to the desired ligation product where the PCR fragment has inserted itself into the plasmid vector, the vector may religate, or the PCR product may ligate with itself. Relatively few of the DNA molecules formed during ligation are the desired combination of the insert and plasmid vector. To separate the desired plasmid from other ligation products and also to have a way to propagate the plasmid, bacteria are transformed with the ligation reaction. Bacteria naturally contain plasmids, and plasmid vectors are natural bacterial plasmids that have been genetically modified to make them useful for molecular biologists. In order to get a plasmid into bacteria, the bacteria must be made "competent". The bacteria must be actively growing, ice-cold, and suspended in transformation buffer that makes them porous and more likely to allow entry of plasmids.

Bacteria are then actively grown in culture media and pelleted, cooled, and resuspended in transformation buffer two times to ensure they are competent. It is vital to keep bacteria on ice at all times. Bacteria are subsequently mixed with the ligation reaction and plated on warm LB ampicillin IPTG agar plates that will only permit bacteria expressing ampicillin resistance genes (encoded by the pJet1.2 plasmid) to grow. These plates also contain isopropyl β-D-1-thiogalactopyranoside (IPTG), which induces expression of the ampicillin resistance gene. Plates are then incubated at 37°C overnight. To ensure the cells were made competent by this procedure, a control plasmid will also be transformed.



Lab Unit 5D Protocol:

The following protocols are available on Blackboard. You must read the entire instructions and prepare your pre-lab accordingly. You don't have to print out the entire instructional manual, but make sure you have detailed instructions to follow in your pre-lab! Your instructor will go through the schedule in more detail with you. This section will take approximately 2 weeks.

- Biotechnology Explorer, Ligation & Transformation Module (Catalog #166-5010EDU)

Procedural Outline:

1. Prepare Agar Plates (this may have already been done for you).
2. Prepare bacterial cells and solutions for transformation (this may have already been done for you).
3. Perform blunting reaction on clean nested PCR product.
4. Set up ligation reaction.
5. Transform & plate transformed cells onto LB IPTG Amp agar plates.

Analysis and Questions for Unit 5-D:

Results:

1. Report the number of transformants present on each plate (if any). Compile class data into one table. Include controls. When you have more than 300 colonies, you write “TNTC”, which means “Too Numerous to Count”.

Analysis:

- Describe why each of these plates was necessary (controls) and, if necessary, the troubleshooting information that you got from the results in each of the control plates.
- Assuming a 100% success in the ligation reaction, what amount of plasmid DNA was added to your competent *E. coli* cells in your transformation reactions? Report in your table.
- Calculate the transformation efficiency of your reactions based on the calculation above. Report your answer in transformants per ug recombinant plasmid in your table
- Discuss the success and failures of the ligation and transformation experiment. Include in your discussion both your experimental GAPDH amplicon transformants as well as ALL of the controls used in this experiment. Discuss the importance of each of these controls.

Additional Analysis Questions:

1. Describe how the pJET1.2 plasmid vector DNA was prepared prior to the ligation reaction.
   a. Why must the plasmid be prepared by a proofreading polymerase prior to ligation?
   b. Explain why the concentrations of the plasmid and the amplicon must be controlled in order for ligation of amplicon to plasmid to succeed.

2. What is the selectable marker for transformants? How was the plasmid DNA prepared for you prior to the ligation reaction?
   a. What are the products of the *bla* and the *eco471R* genes?
   b. What is the positive selectable marker for recombinant transformants?

3. What type of *E. coli* strain did you use in your transformation experiment, and how was it prepared for transformation?
   a. Why is it necessary to use a culture in early- log phase for transformation?

4. Which generally yields the highest transformation efficiency: electroporation or cold calcium chloride/heat shock preparation of competent cells?

Unit 5 E: Plasmid DNA Mini-Prep & Genomic Sequencing of Cloned GAPDH

PART I: PLASMID DNA MINI-PREP OF CLONED GAPDH

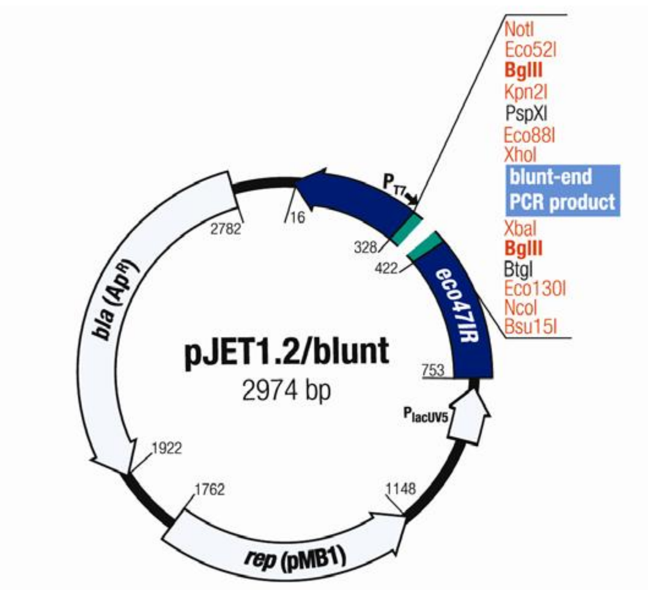
Introduction:

In this stage the students isolate plasmid DNA from transformed bacteria. Prior to this lab, students (or instructor) need to inoculate four LB Amp broth mini-preps per team from the transformed colonies grown after the transformation. The mini-prep method is an alkaline lysis protocol, with the purification step performed on a purification column. After plasmids are isolated, they are analyzed by restriction enzyme digestion and agarose gel electrophoresis to determine whether they contain the PCR products.

It is necessary to analyze the plasmids that have been successfully transformed to verify that they have the PCR fragment inserted. To do so, a sufficient amount of plasmid DNA is obtained by growing a small culture of bacteria, purifying the plasmid from the bacteria, and performing restriction digestion (using an enzyme that cuts double-stranded DNA at specific recognition sequences) on the plasmids. This allows for assessment of the PCR fragment size that was inserted and comparison to the size of the PCR fragment ligated. The plasmid used to ligate the PCR products is pJet1.2 (see figure).

The blunted PCR product was inserted into the vector. pJet1.2 contains a BglII restriction enzyme recognition site on either side of the insertion site. Thus, once the plasmid DNA has been isolated, a restriction digestion reaction will be performed to determine the size of the insert.

Transformed bacteria containing plasmids should have been grown to saturation in LB ampicillin medium prior to this lab. In addition, an agarose gel is needed for the analysis of the restriction digest.



Protocol:

The following protocols are available on Blackboard. You must read the entire instructions and prepare your pre-lab accordingly. You don't have to print out the entire instructional manual, but make sure you have detailed instructions to follow in your pre-lab! Your instructor will go through the schedule in more detail with you. This section will take approximately 2 weeks.

1. Cloning & Sequencing Explorer Series
   Plasmid Purification
2. Aurum Plasmid Mini-prep Kit
   Bio-Rad, Cat#732-6460

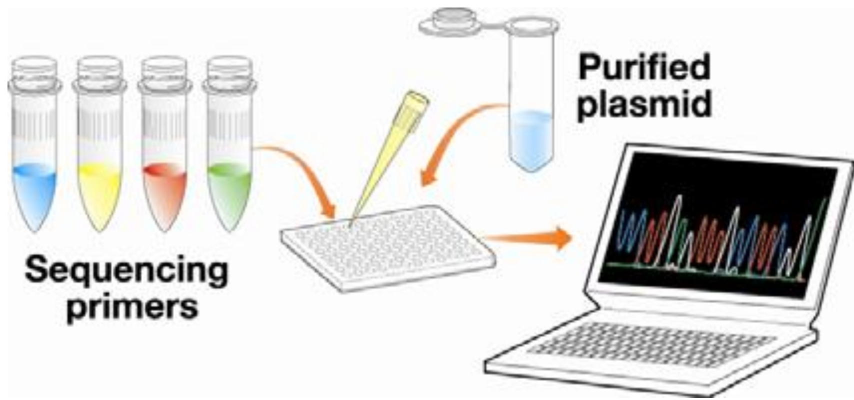
Procedural Outline:

1. Inoculate mini-prep cultures
2. Plasmid DNA Mini-prep
3. Restriction Digest
4. Run on agarose Gel
   Optional: It is recommended that 5 µl of undigested DNA also be run next to your digested samples. Prepare these samples by combining 5 µl of miniprep DNA with 5 µl of sterile water. In addition, you may also want to run 5ul of the original PCR fragment beside the digested DNA to compare sizes.

PART II: PREPARING SEQUENCING REACTIONS OF GAPDH CLONE

Introduction:

The aim of this laboratory is to obtain sequence of a GAPDH gene from an unstudied organism. In addition, confirmation that the PCR product is GAPDH is required. To verify the PCR and to obtain novel sequence, the plasmids containing fragments suspected to be GAPDH genes need to be sequenced.



Sequencing reactions rely on the basic principles of DNA replication like PCR and as such require primers to initiate the replication. However, sequencing is performed in just one direction and so instead of a primer pair, sequencing makes use of single oligonucleotides. Since a single sequencing run generates a read length of 500–700 base pairs (bp), multiple primers for sequencing in both directions are required to provide full coverage of the gene and to provide increased depth of coverage; thus, the same region is read in multiple reactions.

The primers used for DNA sequencing are different from the primers used to amplify the GAPDH gene via PCR. Sequencing primers are designed to complement the DNA sequence of the cloning vector, rather than the insert DNA. If the primers used for PCR were also used for sequencing, then part of the clone's sequence would be missing, because sequencing starts about 20–50 bases away from the primer itself. This is a function of the size of DNA polymerase. Most commercially available cloning vectors are designed to have sites that are relatively far from the cloning region and that will bind to widely available sequencing primers. These universal sequencing primers allow researchers to work with different cloning vectors at the same time. However, the current method used in DNA sequencing only generates ~500 bp of usable DNA bases, whereas the GAPDH gene in some species is much larger. Therefore, internal sequencing primers are used to primer-walk (the primers are designed to obtain a contiguous sequence from an internal region of the gene of interest). In our case, double stranded primer walking (providing contiguous sequence information from both DNA strands) will be performed.

In this lab, four sequencing reactions for each plasmid will be run — two in the forward direction and two in reverse. Two primers are to the vector sequence on either side of the PCR product and two primers have been designed to anneal to conserved regions within the GAPDH PCR product. The plasmid DNA will be combined with the sequencing primers and then mailed to a sequencing laboratory, which will perform the sequencing reactions, analyze the results, and end the DNA sequence back for analysis.

The technique for determining the exact order of As, Ts, Cs, and Gs in cloned DNA is called the dideoxy or Sanger method, named for Dr. Fred Sanger of Cambridge, England, who invented it in the mid-1970s. In this approach, the plasmid clones are heat-denatured (to separate the complementary DNA strands using high temperatures) and used as template to synthesize new strands of DNA. To do this, the template is incubated with DNA polymerase, sequencing primers, 21 deoxynucleotide triphosphates (dNTPs: dATP, dTTP, dCTP, dGTP) and relatively small amounts of dideoxynucleotide triphosphates (ddNTPs: ddATP, ddTTP, ddCTP, ddGTP). The difference between the deoxy- form and the dideoxy- form of a nucleotide triphosphate is a missing -OH group on the 3′ carbon of the deoxyribose. This missing -OH (hydroxyl) group is necessary for normal DNA synthesis, so if a growing chain of DNA happens to utilize a ddNTP, instead of a dNTP, the DNA synthesis reaction is stopped. This termination event occurs rarely enough that all possible lengths of DNA get synthesized during the process.

For instance, a sequencing reaction that provides the DNA sequence for 700 bases would essentially have involved synthesizing 700 different strands of DNA, covering the entire range of possible lengths. These different lengths of DNA are resolved by electrophoresis and visualized. A common visualization method is to 'end-label' each of the four types of ddNTPs with a different label, originally a radioisotope that can be distinguished after electrophoresis.

The Sanger method is now routinely modified to use a fluorescent dye to end-label the ddNTP, followed by dye detection with a digital camera after capillary electrophoresis. This approach detects and records the dye fluorescence and shows the output as fluorescent peaks on a chromatograph.

DNA sequence output is based on the fact that longer strands of DNA move more slowly during electrophoresis than shorter lengths, and that the digital camera can detect the color of the fluorescent dye that labels each of the bands. Since the specific color of the dye attached to each of the different ddNTPs is known, and since that specific ddNTP will end-label the growing DNA strand on the plasmid template, the task of correlating the order of colors with a specific sequence of DNA is relatively straightforward.

After the sequencing reactions are set up, they are mailed to a sequencing service. The Dept of Energy Joint Genome Institute (JGI) values the skills being taught through this laboratory and is providing a free sequencing service for US educators using this laboratory. Plates will be sequenced on a 2 week basis, thus results may take 2 weeks to arrive. Results will be posted as zip files on an FTP server ready for download. If results are desired more promptly, local sequencing services may be utilized. Most major universities have core labs that will sequence for a fee; alternatively there are many commercial sequencing services.

Tips for this stage
It is vital that the number identifying the plate next to the barcode on the label on the sequencing plate is recorded in a secure place. This is the information that will be needed to obtain the sequence information from JGI.

It is highly recommended that students mix their plasmids and sequencing primers in micro-tubes, prior to pipetting these into the 96 well plate. This should prevent (or reduce) the likelihood of students pipetting their samples into the wrong well. Using lab tape to cover up completed wells also may help prevent mis-pipetting.

A 96 well plate plan has been provided for the instructor to plan the location of the student's samples. Each student team should be assigned a numbered column and this information needs to be recorded. It may also be a good idea to dedicate rows A thru D to the forward sequencing primers and rows E thru H to reverse sequencing primers. This may simplify analysis of the 96 sequence files.

Protocol

1. Biotechnology Explorer
   Sequencing & Bioinformatics Module
   Catalog #166-5025EDU

NOTE: Record your plate number in your notebook! You will need it to retrieve your sequencing data from the database!

Analysis and Questions for Unit 5-E:

Analysis:

Include in your data analysis and conclusion section the following:

- What is the size of the pJET1.2 plasmid DNA? Does this size correspond to what you observed in your agarose gel?
- What were the sizes of the *GapC* insert generated from your BglII digestions? How did this size compare with the nested amplicon size? What are some plausible reasons for any discrepancy?
- Which plasmids contain fragments that you suspect are *gapC* gene fragments that are good candidates for sequencing? Explain your reasoning.
- Do you have any digests with more than two bands? What could cause this? What do the sizes of the bands add up to?
- Do you have any digests with no insert or with an insert that does not correspond to your PCR product? What could cause this?

Additional Questions:

1. In a table, compare the purification of plasmid DNA with the procedure that you used to purify gDNA. In what ways were the two procedures the same, and in what ways did they differ? How did they differ in terms of yield and contamination?

2. Why do undigested plasmids appear smaller than the expected sizes in agarose gels?

3. If every colony should have an insert, why perform more than one miniprep?

4. Why chose only one miniprep to use for sequencing reactions?

5. What are all the components of the Sanger sequencing reaction and what role does each of these components play in the sequencing reaction?

6. Why is your insert sequenced from both a forward and a reverse primer? Why are more than one forward and more than one reverse primer used? To what sequence are these primers directed? Why is only one primer included in a sequencing reaction?

7. How does automated sequencing that uses Sanger principles differ from traditional Sanger sequencing?

Unit 5-F: Bioinformatics

INTRODUCTION

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

GenBank is accessible via the Internet to scientists, teachers, and students worldwide free of charge. Major efforts to completely sequence entire genomes were initiated in the 1990s and have now been completed for humans as well as for numerous model organisms studied by scientists, like the bacterium *Escherichia coli*, the common yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, the plant *Arabidopsis thaliana*, mice, and rats. The capacity for isolating and sequencing genes has grown so quickly that the number of submissions to GenBank has doubled every two years since 1993 (NCBI, 2005).

The challenge of analyzing all of the DNA sequences deposited in GenBank spurred the development of numerous computer programs for interpreting DNA and protein sequence data. This computer-aided analytical approach is called bioinformatics. In addition to GenBank other databases housing sequence information are available, as are a wide range of software programs and tools designed to obtain, analyze, and organize this information.

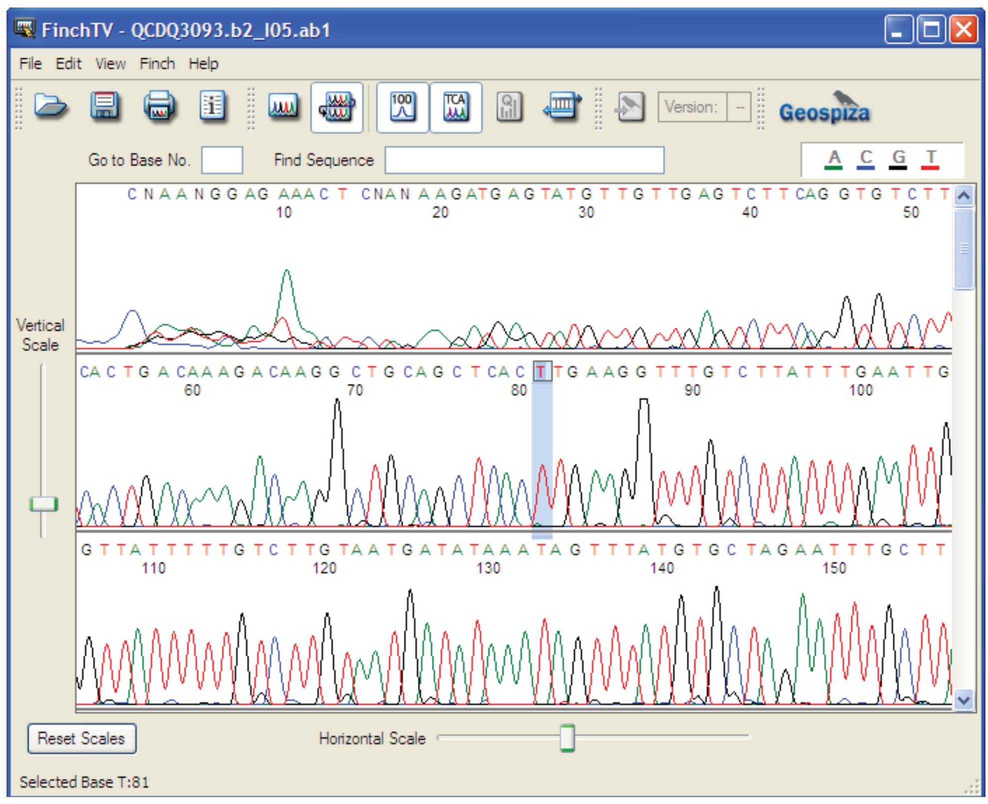
The primary tools that will be used in this module to analyze sequence information are:

- iFinch for Educators: a web tool from Geospiza, Inc., that offers data management, analysis and viewing of DNA sequencing data
- BLAST (Basic Local Alignment Search Tool): an online tool from NCBI for comparing primary sequence data
- CAP3 Sequence assembly program: an online tool that allows assembly of a set of contiguous sequences (contigs) from sequence data

DNA Sequencing Data
Once the sequencing reaction has been performed and the samples have been run on a sequencing instrument, the end result is a data file that contains a chromatogram. A chromatogram is a representation of the DNA molecules generated from the Sanger chain termination sequencing protocol, where the sequence of peaks represents the sequence of bases.

A chromatogram provides information on the peak intensities, the time course in which they eluted, and the base calls that the instrument made for these peaks. The data can be analyzed manually by opening the data file in a reader-style program such as FinchTV that can be found for free on the Internet. An example chromatogram is shown below. The trace shows the peaks for each base in the order they eluted off the sequencing instrument. Above each peak is the

letter code for the base that the sequencing instrument “called” for each peak (hence the term base call).



BLAST Searches
One of the initial steps in analyzing a novel sequence is to determine if the sequence is like any others that have been sequenced before. To do this the user-entered (query) sequence is compared to a database containing other sequences and the best match is determined. The most commonly used tools for this analysis are the BLAST (Basic Local Alignment Search Tool) family of search tools that are designed to find short (local) regions where pairs of sequences match. The BLAST family of programs and information and these programs can be found on the NCBI webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

CAP3 Sequence Assembly Program
Currently, the average length of sequence generated by a Sanger sequencing reaction is around 700 bases. Since most genes are kilobases in length, many overlapping sequences must be assembled together to provide the sequence of a single gene. To do this manually would be laborious and time consuming, and therefore, the ability to computationally assemble multiple sequences is critical in determining entire gene sequences.

CAP3 is a piece of software that can assemble a series of sequence fragments and generate the most likely full length sequence from all the fragments (consensus sequence). This allows the construction of one long sequence for a gene that has been generated from overlapping individual sequencing reactions. Programs such as CAP3 can also use the quality of the data from each sequence to aid in decisions on a consensus sequence.

Protocol

1. Biotechnology Explorer
   Sequencing & Bioinformatics Module
   Catalog #166-5025EDU

In this part of the laboratory, DNA sequences will be run through a series of analyses. This portion of the laboratory is quite open-ended: the level of complexity and the depth of the analyses are entirely up to the instructor, class and time allotted.

Time constraints may prevent following all steps in the process, but the following types of analyses are suggested:

1. Obtain DNA sequences and upload them to the iFinch web site (Geospiza, Inc.). Tutorials and other resources are available at *http://www.geospiza.com/education*
2. Review the quality of the data and view sequence traces using Geospiza's iFinch programs.
3. Use iFinch and SQL programs to search for cloned sequences and vector sequence.
4. Identify the cloned GAPDH sequences.
5. Use the sequences to perform a BLAST search on the NCBI GenBank nucleotide database.
6. Assemble sequences into a contig using the CAP3 program.
7. Identify introns and exons (including addition of annotations).
8. Predict mRNA sequence and check with BLAST.
9. Translate the mRNA sequence to predict the sequence of the proteins.
10. Publish sequence.

Analysis and Questions for Unit 5-F:

1. Did you get data from the primers that anneal to the plasmid (pJET SEQ F and pJET SEQ R) and from the primers that anneal to the cloned GAPDH insert (GAP SEQ F and GAP SEQ R)?
2. If you did not get any good data from the primers that anneal to the cloned GAPDH insert (GAP SEQ F and GAP SEQ R), what might be the cause?
3. If other classmates also sequenced the same plant as your group, did they get good data using the GAP SEQ F and GAP SEQ R primers?
4. If a base has a quality value of 20, what is the chance that the base has been mistakenly identified?
5. What are the characteristics of a high quality base?
6. Record which GAPDH gene you predict has been cloned for each of your minipreps.
7. Do all the sequences for each individual miniprep have homology to the same GAPDH gene, or do the different sequences have homology to different GAPDH genes?

Unit 5-G: Research Paper & Class Presentation

Refer to Appendix D of the Bio-Rad "Cloning & Sequencing Explorer Series" Manual for an excellent detailed overview of "Preparation of Research Papers and Presentations". A summary is provided below.

PART I: Research Paper

Research papers are an important means of communication in science. In this assignment you will write a formal scientific paper based on your GAPDH gene research project. You and your lab partner may work together on the paper and submit one paper together. The format of this paper will be in the form of a scientific journal, such as Science or Nature. This paper (and presentation) is the capstone of this course and is worth 10% of your final grade.

Your paper should include the following:

1. Title: Your paper should have an appropriate descriptive title which should be descriptive, yet succinct.

2. Abstract: Your abstract should be a ***one paragraph summary of your paper***. The first sentence should be a topic sentence, briefly describing the project. The next few sentences provide background about the project as well as stating the overall research objective. The next few sentences can be devoted to briefly describing the methodology. The most important results should be summarized next with a brief conclusion. The final sentence of the abstract summarizes the project as a whole.

3. Introduction: This is a one page introduction should contain ***background information that is pertinent to all of the different aspects of the project***. In the case of this research project, there are four essential topics that should be discussed in the Introduction section. First, there should be a description of the gene being isolated (GAPDH) and its biological function. Second, there should be information about the plant species whose DNA is being studied. Third, there should be a short summary about the basic strategy was used to determine this GAPDH sequence. Fourth, finish the Introduction with a sentence or two stating the specific objectives for the study.

4. Materials & Methods: In this 1-2 page section, you will ***summarize all the materials and methods used to isolate the GAPDH gene***. It should be specific enough that the reader could replicate your work! There should be a description of how the genomic DNA was extracted from the plant, how it was purified, amplified, ligated in to the vector, and used to transform bacterial cells. Remember to include the DNA sequence of the PCR primers used. A brief description of how the resulting DNA sequence was analyzed should also be included. Be sure to outline the experimental controls that were used in your experiment.

5. Results: ***The Results section of the research paper is a presentation of the outcome of the experiment*** with both figures and tables showing data and a thorough description of the results in the text. Each figure and table should have a short legend, and should be as informative as possible. The Results section should also include the accession number of the DNA sequence in the GenBank. This could be the one you submitted or the one you

compared your results to. The writer should also include a legend or caption, a verbal text that corresponds to each of the tables and figures included in the report. The body text of the results section should help the reader by summarizing the important observations about the data. Set aside speculations or narratives about the implications of the data for the Discussion section of the paper. ***The Results section should be largely factual descriptions of observations.***

6. Discussion: The Discussion section of the paper is where the writer can ***speculate about the implications of the research*** and how the results coincide with or differ from the research of others. Technical problems that may have occurred during the experiment can be described in this section, as well as recommendations for future research. The final few sentences of the discussion should summarize the project as a whole.

7. References: At the end of the paper is a list of references. This section is a complete list of references that were ***actually used and cited in the paper***. Remember to reference all citations throughout the paper in the specific area it was cited.

PART II: Oral Presentation of Research Paper

Another important way of relaying research to others is through oral communications. This might involve speaking to fellow students in a classroom setting, but could also be in your workplace, presenting your research at the weekly R&D meeting!

For this assignment you (and your partner) will present your paper to the class in a 15 min oral PowerPoint presentation. You will briefly summarize the success of your project and, if possible, detail a justification for submission of your sequence information to GenBank.

Format Overview:

Most research talks are organized the way written reports are arranged, with a Title, Introduction, Materials and Methods, and Results and Discussion. Since audience members cannot go back and review the material (like they can with a written report), ***it is important to be succinct and to arrange the talk in a logical fashion.***

There are other important differences between oral and written presentations. It is impractical to distribute copies of the written paper during the presentation, so instead speakers will usually project their presentation on a large screen using Microsoft PowerPoint. It is important that your slides be clearly presented to the audience. Here are a few tips:

- ✓ It is important to display the text in as large a font as possible so the audience can read it.
- ✓ Instead of showing long paragraphs, encapsulate the salient points in bulleted lists.
- ✓ Be concise and accurate, and give priority to the most important features of the research.
- ✓ Do not read directly from the slides. Use the points on the slides to help guide you.
- ✓ At the end of the talk, the speaker should ask the audience for questions or comments. This is a good opportunity to get perspectives from other people about the research and presentation, as well as to reiterate important features of the experiment.

UNIT 6: REAL-TIME POLYMERASE CHAIN REACTION - GMO APPLICATION

The information provided in this lab is, in part or in whole, directly from the Bio-Rad GMO Real Time PCR Educational Kit Manual (Cat#166-2500EDU and Cat#166-2500EDU)

Introduction

"With the world population exploding and farmable land disappearing, agricultural specialists are concerned about the world's ability to produce enough food to feed the growing population. Environmentalists are concerned about the overuse of pesticides and herbicides and the long-term effects of these chemicals on the environment and human health.

Might there be a solution to both of these problems? The biotechnology industry thinks so. Its proponents believe genetically modified organisms (GMOs), particularly genetically modified (GM) crop plants, can solve both problems. This proposed solution, however, has met with great opposition throughout the world. Dubbed "frankenfoods" by opponents and restricted in most European countries, GMOs are widely produced and sold in the United States. Currently in the US, foods that contain GMOs do not have to be labeled as such.

Genetic manipulation of crop plants is not new. Farmers have been genetically modifying crops for centuries. Crop breeding to encourage specific traits, such as high yield, is still an important part of agriculture today. However, there is now the option to place genes for selected traits directly into crop plants. These genes do not have to originate from the same plant species—in fact, they do not have to come from plants at all.

One popular class of GM crops has a gene from the soil bacterium Bacillus thuringiensis (Bt) inserted into their genomes. Bt crops produce a protein called delta-endotoxin that is lethal to European corn borers, a common pest on corn plants. Farmers who plant Bt crops do not have to apply pesticide because the plants produce the toxic protein inside their cells. When the corn borers feed on the genetically modified plant, they die. Other GMOs include those that are herbicide-resistant delayed for fruit ripening, are resistant to fungi or drought, have increased crop yield, or bear improved fruits.

Many people object to the use of GM crop plants. They argue that there is a potential to create super-weeds through cross-pollination with herbicide-resistant crops or that superbugs will evolve that are no longer resistant to the toxins in pest-resistant crops. Many are concerned with potential allergic reactions to the novel proteins or antibiotic resistance arising from the selectable markers used to develop the crops or other unforeseen effects on public health.

Proponents of GM foods argue these crops are actually better for the environment. Fewer toxic chemicals are put into the environment and thus fewer toxic chemicals can harm the environment and human health. In addition, these crops can preserve arable land by reducing stresses on the land, improve the nutritional value of food in developing countries, and allow crops to be grown on previously unfarmable land. Whatever position one takes in the GMO debate, it would be

beneficial to be able to test foods found in the grocery store for the presence of GMO-derived products. This can be done in several ways. One would be to use an antibody-based test such as the enzyme-linked immunosorbent assay (ELISA), which can detect the proteins that are produced specifically by GM crops. However, the ELISA is not useful for testing foods that have been highly processed, because the proteins have most likely been destroyed and different GM foods produce different proteins. Another method is to use the polymerase chain reaction (PCR) to look for a DNA sequence common to GM foods. DNA is more resistant than proteins to processing and can be extracted from even highly processed foods. It is these GMO DNA sequences that we will be testing for in this laboratory. In the first lesson you will extract genomic DNA from food samples, in the second lab you will run PCR reactions to amplify GMO and natural plant sequences from the DNA, and in the third lab you will electrophorese the amplified samples to visualize the DNA.

Let's see if your favorite food contains GMOs!" (1)

Applications of Real-Time PCR

"PCR has found so many applications in the biotechnology lab that it has been said: "PCR is to biology what petroleum is to transportation" (Pray, 2004). Although there have been many ways that PCR has been adapted for the detection of specific nucleic acids in cells, real-time PCR is becoming the most widely used application of PCR in the research lab for genomic and gene expression analysis, and is rapidly establishing itself as a technique in the clinical diagnostic lab (Bustin et al, 2005; Kubista, 2006; Leutenegger, 2001; Mackay, 2004; Stevens, 2003).

The need for faster, more accurate, and more economical systems with a high throughput has fueled the popularity of real-time PCR. Using genomic DNA as the template for amplification, real-time PCR can be used in infectious disease diagnostics to rapidly determine levels of specific pathogens in various tissues (Mackay, 2004; Leutenegger, 2001; Stevens, 2003; Templeton, 2003). The molecular diagnostic lab also relies heavily on real-time PCR for detection of aneuploidies and the diagnosis of other genetic diseases (Gibson, 2006; Jiang, 2004; Stevens, 2003; Watson, 2005). In microbiology labs, real-time PCR can be used to detect and quantitate various microbial contaminants in environmental samples (Mackay, 2004). This approach is especially invaluable in the analysis of microbes that are difficult to grow in culture.

In food testing labs, real-time PCR is used to test for food integrity, food contamination, and GMO content of food.

Alternatively, using RNA as the template, reverse transcriptase can be used to generate DNA template for real-time PCR reactions, a strategy referred to as quantitative reverse transcriptase PCR (qRT-PCR), or as transcription-mediated amplification (TMA). This approach has become a valuable tool in the study of gene expression, where changes in transcription levels of various mRNAs can be compared with those of a gene that does not undergo changes in transcription, most often these tend to be the so-called "housekeeping genes". This technique has also become an important assay in the molecular diagnostic lab, where it can be used to determine the viral loading by retroviruses, or to diagnose disease by expression profiles (Bernard, 2002; Bustin & Mueller, 2005; Kubista, 2006; Saleh-Lakha, 2005; Wong, 2005)." (2)

Theory of Real-Time PCR

"Conventional PCR does well to detect the presence of the DNA that the primer pair targets. Conventional PCR detects the amplified product (amplicon) by an end-point analysis — running the DNA on an agarose gel after the reactions are completed. If the target DNA sequence is not there, no amplicon will appear on the agarose gel. If as little as a single DNA molecule that contains the target DNA sequence is in the sample, the amplification by 25-30 cycles is sufficient to generate detectable amplicons via electrophoresis. Thus, conventional PCR makes a highly sensitive assay for specific DNA sequence, which is useful for the diagnosis of diseases, especially viral types.

It is also a rapid, highly sensitive and specific assay for microbes in environmental samples. Through the use of reverse transcriptase, conventional PCR has also become the standard for the detection of RNA targets, useful for analysis of gene expression in research and medical diagnosis. In this case, reverse transcriptase generates DNA from an RNA template, forming a template for the PCR polymerase amplification.

Real-time PCR is based on the same principles as conventional PCR. The reaction requires both forward and reverse primers bracketing the target region (amplicon), nucleotides, and a DNA polymerase such as Taq. However, real-time PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses — in "real time". The difference is the addition of a fluorescence chemistry, which enables product amplification to be monitored throughout the entire real-time reaction using specialized Bio-Rad thermal cyclers equipped with fluorescence detection modules. The measured fluorescence reflects the amount of amplified product in each cycle.

Real-time PCR results can either be qualitative (presence or absence of a sequence) or quantitative (number of copies of DNA). Real-time PCR that is quantitative is also known as qPCR. The main advantage of using real-time PCR over conventional PCR is that real-time PCR allows you to determine the starting template copy number with accuracy and high sensitivity over a wide dynamic range. Conventional PCR can at best be semi-quantitative and the methods to obtain quantitative data can be quite complicated. One advantage of conventional PCR is better determination of the sizes of the amplified PCR products using conventional gel electrophoresis. Therefore, separating the real-time PCR products on a gel following amplification allows the visualization and determination of the size of the amplified products." (2)

References:

1. Biotechnology Explorer GMO Investigator Kit. (Cat #166-2500EDU). Kit Instructions. 10002155 Rev A.
2. Application Note: Biotechnology Explorer GMO Investigator Kit: A Quantitative Real-Time PCR Extension (Cat #166-2500EDU). Kit Instructions. Rev 07-0164 407 1205

Unit 6-A: Real Time PCR - Introductory Procedure

Procedure

<u>BEFORE CLASS:</u>
Read GMO kit instructions posted to Blackboard.

1. Biotechnology Explorer GMO Investigator Kit. (Cat #166-2500EDU). Kit Instructions. 10002155 Rev A.
2. Application Note: Biotechnology Explorer GMO Investigator Kit: A Quantitative Real-Time PCR Extension (Cat #166-2500EDU). Kit Instructions. Rev 07-0164 407 1205

<u>For Week One we will be following Application Note</u>: Biotechnology Explorer GMO Investigator Kit: A Quantitative Real-Time PCR Extension.

<u>DAY 1:</u>
Run DNA standard curve on Real Time PCR machine (see application note)

1. Each student will prepare one dilution set. For lab partners, one partner will prepare the PMM dilutions, the other will prepare the GMM dilutions.

<u>DAY 2:</u>

1. Run PCR amplions on an agarose gel
2. Analyze Real Time PCR results (see application note). Although each student prepared one primer set, you will individually analyze both primer sets for their lab report.

RESULTS

1. Prepare standard curves for PMM and GMM dilution series. Do this with the Real Time PCR software. Include the equation of the line and R^2 value.
2. Prepare a table with class results comparing slopes, y-intercepts, and correlation coefficients (R^2 –value), and E value with those of the rest of your class.
3. Prepare a melt curve analysis for your PMM and GMM dilution series.
4. Include a well-labeled gel figure.

Analysis

1. Which dilutions were the first to cross the threshold line, and which were the last? Why?\

2. Did you observe any amplification in your NTC (no template control) tube? Can you explain the results that you got in this NTC reaction tube?

3. Did you obtain primer dimers on your reactions? Explain the effect amplification of primer dimers have on your fluorescence progress curves of your qPCR reactions.

4. When a PCR reaction is running at 100% efficiency, in other words is doubling amplicon products perfectly with each thermal cycle, it will take 3.3 cycles to multiply the product 10-fold. Therefore, the 10-fold dilution series in this experiment should have the fluorescence

progress curves spaced 3.3 cycles apart. Is this what you observe in your progress curves? Explain your observations.

5. Do any of your reactions have more than one amplicon? If so, which reactions had more than one amplicon? What could be the cause of the second amplicon?

6. What is the range of melting temperatures of each type of amplicon that the PCR reaction generated? Why do they differ? What effect would the amplification of primer-dimers have on your melting curve analysis of your qPCR?

7. Using linear regression, the correlation coefficient (r^2) of your standard curve is acceptable for qRT-PCR at a value of 0.98, and is perfect at a value of 1.00. Is the quality of your standard curve data in the acceptable range?

8. Using linear regression, the slope of your standard curve line should be -3.322. If the slope is less than this value, the efficiency of the amplification is low. Calculate the efficiency of the standard curves that you prepared from your average Ct values. The x-axis should be log template concentration (copy number) and the y-axis should be the corresponding Ct. The formula for efficiency (E) is:

   $E = 10^{(-1/S)} - 1$ where S = slope of the graph

   a. If your reactions were running with 100% efficiency, the slope of your standard curve would be –3.3. Is this what you observed?
   b. Calculate the efficiencies of the standard curves of your serial dilutions.
   c. What factors could give rise to efficiencies that are less than 100% or more than 100%?

9. <u>Comparing class data:</u> Compare the correlation coefficients with the slopes of each of the lines. How do your correlation coefficients compare with the class averages? Compare the R^2 values of all the standard curves. What is the range of values among the 12 standard curves? What can you conclude about your pipetting technique from your standard curves? What does this range reveal about the reproducibility of pipetting technique between classmates?

Unit 6-B: Determining GMO Content in Food

Procedure

BEFORE CLASS:

Read GMO kit instructions posted to Blackboard.

1. Application Note: Biotechnology Explorer GMO Investigator Kit: A Quantitative Real-Time PCR Extension.
2. Biotechnology Explorer GMO Investigator Kit. (Cat #166-2500EDU). Kit Instructions. 10002155 Rev A.

Each student must choose a food to test and bring to class. You must do this BEFORE you come to class! You will be extracting DNA on day 1 of this project.

DAY 1:

1. Extract DNA from food (see GMO investigator kit instructions).
2. Real Time PCR extracted DNA (see application note)

DAY 2:

1. Run PCR reactions on Gel
2. Real-Time Analysis – Advanced (see application note)

Analysis Questions

1. Use your melt-curve analysis to verify that the Ct values of your amplicons are genuine PCR amplicons and not primer dimers. Report the melting temperatures of your amplicons, and explain how you can exclude primer dimers from this data.

2. Compile the Ct results of your qRT-PCR results, using the following as a template.

Template DNA	***Master mix***	***Ct values***	***Average Ct value***	***Description of the test food***
Test food DNA	PMM primers			
Test food DNA	GMM primers			
Positive control DNA	PMM primers			
Positive control DNA	GMM primers			
NTC	PMM primers			
NTC	GMM primers			

a. Were you able to verify that there is no GMO contamination of your reagents or facility that could lead to false-positive results?
b. Which of the test food samples had successfully amplified the plant DNA using plant control primers, and which of the test food samples had successfully amplified the plant DNA using GMO primers? What are the most likely explanation for any amplifications using PMM primers?
c. Were able to find GMO DNA in your test food? If so, what is the ratio of GMO DNA copy number, compared to the plant reference gene DNA? Compare the Ct values for your test food DNA in plant master mix and the Ct values for your test food DNA in GMO master mix. What can you say about the relative abundance of chloroplast DNA compared with GMO DNA in your extract?

3. Which foods did others in your class find GMO DNA in? Which foods were found to not have any GMO DNA?

4. Compare qRT-PCR results of your positive control DNA here with those of your Laboratory 1 results.
   a. Using the standard curve that you made comparing the Ct values with copy number of plant reference gene DNA find the copy number of plant reference gene DNA in your test food extract. Show your work for full credit.
   b. Using the standard curve that you made comparing the Ct values with copy number of GMO DNA find the copy number of GMO DNA in your test food extract. Show your work for full credit.

LABORATORY UNIT 7: PRIMER DESIGN

Introduction and Background

As you have discovered in the previous unit, PCR can be a valuable technique for quickly isolating a gene for sequence analysis when there is enough sequence information to allow for the design of primers for the amplification of the target gene from gDNA. A problem in trying to design primers off of DNA sequences from a different species is when sequence differences between the two species can result in primers that inadequately complement the target sequence, ending with a PCR failure. In an attempt to find "universal primers" that will work well for a given gene in all species, it is best to first locate regions of the gene that has the greatest sequence similarity. Sequence information can be downloaded from GenBank, and multiple sequence alignment programs can be used to look for regions of highly conserved DNA sequences. Alignment programs are freely available to the public on the internet such as Clustal (www.ebi.ac.ukclustalw or http://pir.georgetown.edu) or Geneious (free trial - http://www.geneious.com/).

Once some conserved regions have been found, a consensus primer sequence can be chosen by the most commonly occurring nucleotide at each position of the conserved sequence. These primers should be analyzed for structure and predicted mispriming. Alternatively, degenerate primers can be prepared, in which alternative nucleotides are included in the sequence. Degeneracy can be tolerated at the 5' end of the primer, but mismatches at the 3' end of the primer reduce both annealing specificity and PCR yield.

An additional problem in the design of universal primers for eukaryotic genes lies in the variability of intron sequences from one species to another. Since changes in intron sequences generally have loss-of-function effect on the protein that the gene encodes, introns have less similarity among different species. The nucleotide sequences differ more in introns than in exons, and intron position and length differs between different species as well. Taking this variability into consideration, introns should be avoided when designing universal primers and exons should be the focus of a search for highly conserved DNA sequences.

GENERAL CONSIDERATIONS IN PRIMER DESIGN

The most important step in a successful PCR reaction lies in the careful design of the primers used. A poorly designed primer can result in little or no product due to nonspecific amplification and/or primer-dimer formation leading to reaction failure. The major aspects of primer properties that affect their performance include specificity, melting temperature (T_m), and intra-primer and inter-primer complementarity. Several parameters must be taken into account when designing primers for PCR, including the length of the amplicon. You should avoid amplicons that exceed 1600 bp. Specific primer design parameters are discussed in detail below.

1. Primer Length

Primer length critically affects PCR success by influencing specificity, melting temperature, and time required for annealing. Generally, the sequence must be complex enough so that the likelihood of annealing to sequences other than the chosen target is low. A primer length of 18-30 bases has sufficient specificity for most PCR applications. Shorter primers are more likely to lead to nonspecific annealing, while longer primers have a higher likelihood of containing

secondary structures such as hairpin loops. Longer primers also have a lower annealing efficiency, leading to lower yields because the annealing time is too short for a high level of primer annealing.

2. Primer Melting Temperature

For most PCR applications, the optimum melting temperature of primers ranges from 55°C to 60°C. Under PCR reaction conditions, the T_m of a primer depends on its length and sequence composition. Generally speaking, the higher the GC content of a primer, the higher its T_m. **It is very important that both of the primer pairs have very similar T_m** since they will be annealing at the same temperature during the PCR reaction.

3. Secondary Structures in Primers

A tendency for a primer to fold back on itself to form a double-helical hairpin structure spells disaster for a PCR reaction. This is because the *Taq* polymerase will treat the hairpin as a primed template and extend the sequence, thus creating a new primer sequence that no longer is complementary to the target DNA sequence. To avoid hairpin secondary structures, you should avoid primers that have >3 bp complementarity within its sequence.

4. Avoid Primer Dimers

Primer sequences should be examined for their ability to base pair into a double helix, forming a "homodimer" by intra-primer complementarity. Similarly, primer pairs should be examined for inter-primer complementarity, which can also lead to the formation of "heterodimers". Because high concentrations of primers relative to template, primers may anneal to each other much more readily than they anneal to the template, even if they are only partially complementary to each other. A small amount of sequence complementarity is tolerated, because the annealing temperature should be well above the melting temperature of a short stretch of base-pairing. To avoid interference with annealing of primer to the DNA template, you should avoid >3 bp complementarity between primers.

5. GC Clamp

Primers with a G or C residue at its 3' end have better priming efficiencies. The so-called "GC clamp" helps to ensure correct binding at the 3' end of the primer due to the stronger hydrogen bonding of GC residues. Primers that have a T residue at the 3' end tend to have reduced specificity.

6. Avoid Repeats and Runs

A sequence of 4 or more nucleotides which is repeated can generate secondary binding sites, or "false priming sites", for primers. A primer sequence with an internal repeat can also lead to inefficient annealing. For example, this sequence with an internal repeat (underlined), <u>AATCGA</u>CGATTAT<u>AATCGA</u>, can anneal inaccurately to its complementary sequence, leading to a shift in the annealing site. An internal repeat and a sequence of 4 or more identical nucleotides in a homopolymeric run can cause ambiguous binding of primers to their target site ("slippage effect"). Poly (A) and poly (T) stretches should be also avoided as these regions will have especially low T_m, and may open up stretches of primer-template complex at the temperatures of the PCR reaction.

7. Primer Specificity
It is important to avoid more than 4 G's or C's in a row at the 3'-end of a primer, because this can lead to mispriming. If you keep the G/C content of a primer between 40 and 60%, this will also help to avoid mispriming. Also, before you settle on a set of PCR primers sequences, you should check their specificity for the target DNA sequence by doing a BLAST search to rule out other homologous sequences in nontarget DNA in the GenBank database.

SPECIFIC CONSIDERATIONS IN DESIGN OF DEGENERATE PRIMERS

When designing "universal primers" for a particular target sequence that will work for all species, decisions must be made about how to deal with sequence mismatches from one species to the next. Basically, you must first search for regions of the target sequence that are highly conserved in order to avoid mismatches. When mismatches are not avoidable, as is usually the case, you can order "degenerate primers" that include all possible sequence variations. You should take these parameters into consideration when deciding where to tolerate mismatches and degeneracy:

1. Avoid introns
Since exons are more highly conserved, you are more likely to find consensus sequences in exon sequences.

2. Avoid intron-exon boundaries
Since you are targeting DNA, a primer sequence that spans two exons will not work. Therefore, before deciding on your primer sequences, check for intron positions. Be aware that this can vary from one species to the next. An intron found in one species might be displaced in the gene of another species, or it can be absent altogether.

3. Avoid 3' mismatches
Primer specificity is mostly determined by the 3'-end sequences, so when mismatches in this region should be avoided. It is critical that a universal primer includes 8 – 10 unique bases at the 3'-end of the primer sequence. A mismatch of the 3'-terminal nucleotide reduces priming efficiency the most, and should be avoided altogether.

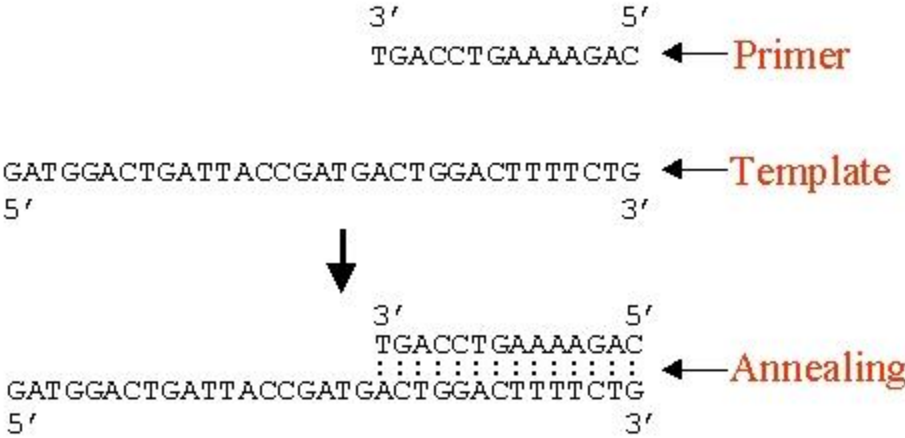
4. Minimize degeneracy
A PCR primer sequence is called degenerate if some of its positions have several possible bases. The degeneracy of the primer is the number of unique sequence combinations it contains. Introduction of degeneracy in primer sequences decrease their specificity. Chances are that apart from the common targets, the primer can now bind to altogether different targets causing mispriming and background noise. It also has another disadvantage in lowering of the primer concentration. This lowered PCR primer concentration means that more than 512 fold degeneracy should be avoided. To calculate degeneracy, simply multiply the number of alternative nucleotides at each position. For example, the degeneracy of the following oligonucleotide:

CG<u>N</u>TA<u>N</u>GCTAAT<u>N</u>CGTA (where <u>N</u> is A or T or C or G) would be 4 x 4 x 4 = 64.

Lab Unit 7A: Introduction to Primer Design

What is a primer?

A primer is a short synthetic oligonucleotide which is used in many molecular techniques from PCR to DNA sequencing. These primers are designed to have a sequence which is the reverse complement of a region of template or target DNA to which we wish the primer to anneal.



Analysis of primer sequences

When designing primers for PCR, sequencing or mutagenesis it is often necessary to make predictions about these primers, for example melting temperature (Tm) and propensity to form dimers with itself or other primers in the reaction. The following program will perform these calculations on any primer sequence or pair.

Go to: IDT DNA and Select Oligo Analyzer.
http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/

The programs will calculate both the Tm of the primers, as well as any undesirable pairings of primers. When primers form hairpin loops or dimers less primer is available for the desired reaction. For example Hairpin and Self dimers:

Hairpin

```
Oligo, 3 bp (Loop=4), delta G = -0.1 kc/m

        5'  GGGAAA┐
             |||   |
3'  TATCTAGGACCTTA┘

Oligo, 2 bp (Loop=3), delta G = 2.1 kc/m

         5'  GGGAA┐
             || |  A
3'  TATCTAGGACCTTA┘
```

Self-Dimer

```
4 bp, delta G = -6.6 kc/m (bad!) (worst= -36.6)
        5' GGGAAAATTCCAGGATCTAT 3'
               ||||  ||||
3' TATCTAGGACCTTAAAAGGG 5'

4 bp, delta G = -5.4 kc/m (bad!) (worst= -36.6)
      5' GGGAAAATTCCAGGATCTAT 3'
               ||||
3' TATCTAGGACCTTAAAAGGG 5'
```

Some thoughts on designing primers:

1. Primers should be 17-28 bases in length
2. Base composition should be 50-60% (G+C)
3. Primers should end (3') in a G or C, or CG or GC: this prevents "breathing" of ends and increases efficiency of priming
4. Tms (melting temperature) between 55-80°C are preferred
5. 3'-ends of primers should not be complementary (ie. base pair), as otherwise primer dimers will be synthesised preferentially to any other product
6. Primer self-complementarity (ability to form 2° structures such as hairpins) should be avoided
7. Runs of three or more Cs or Gs at the 3'-ends of primers may promote mispriming at G or C-rich sequences (because of stability of annealing), and should be avoided

Also keep in mind that most oligonucleotide synthesis reactions are only 98% efficient. This means that each time a base is added, only 98% of the oligos will receive the base. This is not often critical with shorter oligos, but as length increases, so does the probability that a primer will be missing a base. This is very important in mutagenesis or cloning reactions. Purification by HPLC or PAGE is recommended in some cases.

Oligonucleotide length	Percent with correct sequence
10 bases	$(0.98)^{10} = 81.7\%$
20 bases	$(0.98)^{20} = 66.7\%$
30 bases	$(0.98)^{30} = 54.6\%$
40 bases	$(0.98)^{40} = 44.6\%$

Designing Degenerate Oligonucleotides.

A group of degenerate oligonucleotides contain related sequences with differences at specific locations. These are used simultaneously in the hope that one of the sequences of the oligonucleotides will be perfectly complementary to a target DNA sequence.

One common use of degenerate oligonucleotides is when the amino acid sequence of a protein is known. One can reverse translate this sequence to determine all of the possible nucleotide sequences that could encode that amino acid sequence. A set of degenerate oligonucleotides would then be produced matching those DNA sequences. The following link will take you to a program that will perform a reverse translation. http://arbl.cvmbs.colostate.edu/molkit/rtranslate/

For example, the amino acid sequence shown in purple below could be encoded by the following codons.

```
AspGluGlyPheLeuSerTyrCysTrpLeuProHisGln
GATGAAGGTTTTCTTTCTTATTGTTGGCTTCCTCATCAA
  C  G  C  CT CAGC  C  C   T C  C  C  G
        A     A  A           A  A
        G     G  G           G  G
```

One could then select the 14 base sequence (in blue) to generate a smaller set of degenerate oligonucleotides. Each oligonucleotide in the set would have one base changed at a time (shown in purple below). A total of 32 unique oligonucleotides would be generated.

```
TATTGTTGGCTTCC

TACTGTTGGCTTCC

TATTGCTGGCTTCC

TACTGCTGGCTTCC
```

When ordering degenerate oligonucleotides, you just let the company know that you want a mixture of nucleotides added at a specific position using the code below. By adding the mixture, oligos will incorporate one of the bases, leading to a mixture of oligonucleotides.

Standard Mix Base Definitions	
R	A, G
Y	C, T
M	A, C
K	G, T
S	C, G
W	A, T
H	A, C, T
B	C, G, T
V	A, C, G
D	A, G, T
N	A, C, G, T

PROCEDURE

PART I: DESIGNING PRIMERS

```
>Paddlefish
CCTTGGCCTCTGCCTAATCACACAGATTCTAACAGGATTATTTCTCGCAATACACTACACAGCTGACA
TCTCAACAGCCTTCTCCTCCGTCGCCCACATCTGTCGAGATGTTAACTACGGATGACTAATTCGAAAC
ATTCATGCAAACGGAGCCTCCTTTTTCTTCATCTGCCTCTACCTTCACGTAGCCCGAGGCATATACTA
TGGCTCATACCTCTACAAAGAAACCTGAAACATCGGAGTAGTTCTCCTACTCCTAACTATAATAACCG
CCTTCGTAGGATATGTGCTCCCATGAGGACAGATATCCTTCTGAGGAGCCACCGTAATTACCAACCTT
CTTTCCGCCTTCCCCTACATCGGGGACACCCTAGTACAATGAATCTGAGGTGGTTTCTCAGTAGACAA
CGCCACCCTAACC

>Shovenose Sturgeon
CCTAGGCCTCTGCCTTATTACACAAATCTTAACAGGACTATTTCTTGCAATACACTACACAGCTGACA
TTTCAACAGCCTTCTCCTCCGTCGCCCACATCTGCCGAGACGTAAACTACGGGTGACTAATCCGAAAC
GTCCACGCAAATGGCGCCTCCTTCTTCTTTATCTGCTTGTACCTTCACGTCGCACGAGGTATATACTA
CGGCTCCTACCTCCAAAAAGAAACCTGAAACATCGGAGTAGTCCTCTTACTCCTCACCATAATAACCG
CCTTCGTAGGCTATGTACTGCCCTGAGGACAAATATCATTTTGAGGGGCAACCGTAATCACTAACCTC
CTTTCCGCCTTCCCGTACATCGGCGACACATTAGTGCAATGAATCTGAGGCGGCTTTTCAGTC
```

1. Open up your pre-lab exercise Microsoft word document.

2. Use an **[Alignment](http://bioweb.uwlax.edu/GenWeb/Molecular/Seq_Anal/Alignment/alignment.htm)** program to identify conserved regions in both sequences.
   http://bioweb.uwlax.edu/GenWeb/Molecular/Seq_Anal/Alignment/alignment.htm

3. Record your alignment results in your document. Remember to periodically save your work as you work on this lab exercise.

4. Design PCR primers 15-25 bp long that will amplify both of these two sequences. ***Recall that the two primers need to bind to the target DNA such that the free 3´ ends of each primer point towards each other.*** You may wish to review the rules used design primers.

5. Record the sequence of your two primers. Be sure to write them in the correct orientation!

PART II: ANALYZE PRIMERS

1. After you have identified the sequence of your primers, check the primers with the oligo analyzer program used to calculate melting temperature (T_m) and the formation of primer dimers. Record the Tm of your primers. http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/

2. If the T_m is less than 55°C or bad hairpins or dimers form, try another region of sequence. And redesign your primers. Recheck the melting temperature.

3. Do your primer pairs meet the requirement of similar Tm? What will happen if your primer pairs have different melting temperatures?

4. On your alignment sequence, highlight and record your two primers.

5. What is the size of the PCR products from your primers when used on each of the two species?

LAB REPORT:
1. Introduction: One page summary on primer design
2. Results: Summarize results of primer design
3. Analysis: Analyze the primers you have designed. Base your analysis on scientific evidence and recommended characteristics of a good primer set.
4. Conclusion Statement

Lab Unit 7B: Degenerate Primer Design of GapC gene

In this exercise, you will

1. Download complete plant *GapC* sequences from GenBank.
2. Save the sequences in a FASTA format for import into a multiple sequence alignment program called CLUSTAL W. This format is one of the more common one used in bioinformatics, and is easily identified by a > symbol that precedes an identification line for the sequence. This line is terminated by a hard line bread or return character followed by the actual sequence data. The sequence is unnumbered and may consist of one long line without line breaks, or it may include line breaks. The software programs using this sequence will treat everything as sequence data until the next > character, so management of the > character is critical to the use of FASTA formats.
3. Align your sequences in CLUSTAL W using software and a server freely available to the general public online.
4. Search for regions of sequence similarity in your alignments in order to design “universal primer sets” for *GapC* genes in plant genomes.
5. Design primers from consensus sequences discovered in your alignments. These primers must be spaced 1000-1600 base pairs from each other, and must pass the quality tests described above for PCR primers.

Safety Precautions: *Don't stare at the computer screen for too long ;)*

Materials:

This exercise will require a computer with an internet connection along with some media for storing program files

Protocol:

There are some general file-keeping considerations that must be kept in mind during the database searches and manipulations that will be done in this exercise, listed below:

- Although you can work with your lab partner, you must generate your own work for this lab!
- You will be generating many files, so it is important that your file names be very descriptive. This means that your names must describe the species included and that updated versions of files must be easily distinguished from each other.
- Many of the files that you generate will be very large and you may be working much of the time in an ACC computer lab. Although you may download data into the hard drive of the computer that you are using, it is important that you have sufficient portable storage media such as flash drives in order for you to be able to take your work home with you. If you lack sufficient portable storage space, don't forget that you can email files to yourself to download onto your home computer later.
- Don't forget to save your work as you progress through the lab! It is a good idea to both email your work to yourself as well as store files on portable media in case of a memory drive or email failure.

Protocol steps	**OBSERVATIONS, COMMENTS, & CALCULATIONS**
DOWNLOADING DNA SEQEUNCES 1. Go GenBank (www.ncbi.nlm.nih.gov) to retrieve the sequences of *GapC* genes from plant species. Search in the “nucleotides” database for “GapC in plants, complete sequence”. This will give you all the full-length GapC sequence information that has been submitted to GenBank to date.	How many “core nucleotide records” are there for the GapC gene in plants? How many different species are represented in these records?
FASTA format 4 nucleotide sequences of the complete GapC gene	
2. Click on the *Arabidopsis thaliana* mRNA sequence hotlink to go to this record. You will want to download the sequence in a FASTA format. Select FASTA from the Format bar. Highlight everything text starting with “>gi… followed by accession number, genus, species, etc, and through nucleotide sequence.) Copy this to a Microsoft Word file, saving it as a sequence file named for the gene and the genus. It is best to save this file as a Rich Text File (.rtf) since this format is used by all word processing software and is compatible across programs as a way of importing text. 3. Repeat this process to download the nucleotide sequences of the *GapC* gene for at least three other species in FASTA format. Try to find sequences from representative species to help ensure that your primers will in fact be “universal”. For example, you might include an herbaceous plant, a tree, a cereal, and a cactus in order to get a good cross-section of different types of plants.	Record the species and the length of the nucleotide sequences for GapC. *NCBI now has a tool to design primers. You are free to use this and compare the primers you design by hand with the ones the NCBI tool provides.* The GenGank accession number often begins with two letters such as “NC” followed by 6-10 numbers. The GenBank sequence file includes a lot of information about the sequence such as who submitted it, where it is published in the scientific literature, where the genes are found in the sequence. The nucleotide sequence itself is an easy-to-read numbered format. The FASTA format has considerably less information, but can be exported into CLUSTAL programs for alignments.

4. Put all of the sequences that you will align into a single document. CLUSTAL W will identify sequences in its alignment by the first 10 characters following the > character in your FASTA files. To make your identifiers more informative, add a 10-character name immediately following the > sign in all of your FASTA formatted sequences. Avoid leaving any spaces or using any hyphens in your names.

5. Save this document of combined gene sequences with an appropriately named Word file.

CLUSTAL W multiple sequence alignment

6. Now you are ready to do a multiple sequence alignment. There are a number of CLUSAL W programs that you can access for free online. For example, go to http://www.ebi.ac.uk/Tools/clustalw2/index.html and copy your entire sequence file into the sequence box. Make sure that the “>” signal gets copied with your sequences into the box. (An alternative site is: http://pir.georgetown.edu.

7. When your multiple alignment comes up, the aligned sequences will read from left to right, with 60 bases per line.

Finding prospective primer sequences

8. Locate the conserved nucleotide sequences. Below the alignment, you will find an asterisk at the bases that are conserved in all of your sequences. Locate all regions that have a long run of conserved bases, and mark those regions with a number. The best size for primers is between 18 and 30 nucleotides long. If you cannot find consensus sequences that long, locate sequences that have the smallest number of nucleotides that are

It is easier to evaluate your results if you print them out so that you can see more at once. You may need to adjust the margins and font size to keep the sequences in alignment with each other. If they won’t align, check to make sure that they are in “Courier New” font.

If you are **not** finding many conserved regions, look for a single species that may be causing the most trouble with finding

not homologous. Another approach to avoiding excessive degeneracy is to focus on sequences that vary between one or two nucleotides at a position, as opposed to a position that can be any of the 4 nucleotides.

9. Cut and paste the conserved sequences into a table along with the number that you have assigned to it. Here are the annotation system that we will use:

Annotation, by order	***Example***
Position of primer in the gene sequence	Use the number of the Arabidopsis nucleotide as your reference for this numbering system.
Direction of primer	"F" for forward "R" for reverse

homologous sequences. A clue as to which sequence might be a problem can be found in the phylogenetic tree at the top of the alignment page. If your troublesome species is being placed as a highly distant relative to the other species, you might consider lowering your goals from "universal primers" to primers that work well with a certain family of plants.

10. For sequences that are not completely homologous, assign a letter to signify the type of dissimilarity according to the following table of the IUB universal nomenclature for mixed bases.

Letter designation	***Stands for***
R	A, G
Y	C, T
M	A, C
K	G, T
S	C, G
W	A, T
H	A, C, T
B	C, G, T
V	A, C, G
D	A, G, T
N	A, C, G, T

Since these sequences are the entire top strand, add "F" to the numbers that you have assignment to them for "Forward".

11. Add the following columns to your primer table: Tm, hairpins, and homodimer. 12. Search for possible primer pairs by looking for an appropriate distance separating them. The ideal distance should be between 800 and 1600 bases apart. 13. Create a second table, identical to the first one, with the complementary sequences to the forward primers. Don't forget to add "R" to your numbering system for these reverse primers. 14. Create a third table for primer pairs, entering the numbering designation, the approximate size of amplicon they would make, and the forward and reverse primer sequences into columns of this table. Leave separate columns headed "Heterodimer" and "BLASTn" **Primer design** 15. Now you are ready to analyze the quality of your prospective primer sequences. Go to www.idtdna.com and select "SciTools" and then "Oligo Analyzer". 16. Cut and paste your first primer sequence into the text box and select "analyze". Enter the Tm for this primer into your primer table. **If the Tm does not fall between 50- 65°C, go back to the text box and trim your primer to reduce the Tm or add nucleotides to increase the Tm.** To decide which end to take off or add nucleotide sequences, refer to the introduction guidelines for primer design. 17. Repeat the Tm step until you get a primer that has a Tm between 55 - 65°C. 18. Select "Hairpin" and enter this information into your primer table.	You will be evaluating these primer sequence prospects for their melting temperature, as well as their likelihood for forming hairpin secondary structures and for forming complementary base pairs between strands.

19. Select "Self dimer" and enter the Gibb's Free Energy for the formation of a double helix between primer molecules into your primer table.

20. Repeat these steps for all of your forward and reverse primers. Make sure that your reverse primers can be paired with a forward primer that will yield an amplicon of acceptable length (1000 to 1600bp).

21. Eliminate any primers from consideration that have a high propensity to form hairpins or homodimers ($\Delta G < |-8$ kcal/mol$|$).

22. For all primers that have acceptable parameters so far, create a "Primer Pair" table and cut and paste all possible forward and reverse primer combinations into the textboxes. Make sure that the primers that you pair are a good match: they should yield an amplicon of acceptable length and the Tm of primer pairs must fall within 1°C of each other.

23. Using OligoAnalyzer, check the pair for heterodimers.

24. Make a "Hetero-dimer" column and record the Gibb's Free Energy value for the formation of a primer dimer by each of the primer pairs in your primer pair table.

25. There is one more step to evaluate your primers: they must be unique to plants (Viridiplantae). Go to www.ncbi.nlm.nih.gov and select "BLAST" from the Resources menu in order to do a nucleotide BLASTn search of GenBank of your prospective primers. http://blast.ncbi.nlm.nih.gov/Blast.cgi

26. Optimize for “Highly similar sequences (megablast)”. Enter a prospective primer into the text box, and select “BLAST”. When the next window comes up, select “Format” and wait for the results of your database search for similar sequences. 27. The blast should reveal VERY small E-values (less than 0.0001) for the GapC gene of various plant species. Print out the first page of your BLASTn results, and indicated in your primer pairs table whether you have done a BLASTn search and whether there were any exact matches to nontarget sequences (other genes). 28. Turn in your tables summarizing your results and your BLASTn printouts in your report. Indicate the gene that the primer appears in, and whether it is a “Forward” primer or whether it is a “Reverse” primer (oriented in a direction of decreasing numbering of the nucleotides on a gene map). Write a paragraph indicating which of your primer pairs would make the best choice and give the reasoning for your choices.	

LAB REPORT:

1. Introduction: One page summary on primer design
2. Results: Summarize results of primer design
3. Analysis: Analyze the primers you have designed. Base your analysis on scientific evidence and recommended characteristics of a good primer set.
4. Conclusion Statement

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/*

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: Forms

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 Gel 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 C: Agarose Gel Electrophoresis using SYBR green

SYBR GREEN:
SYBR Green is a very convenient dye for detecting nucleic acid fragments in agarose gels, as it binds nucleic acids stoichometrically 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 on 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. Place a black rubber bumper on a lab bench with the slit facing upwards. Press the clear plastic casting tray into the slit, pushing down towards the bench with the palm of your hand. Repeat with a second bumper. Other gel casting assemblies may have a different design; if so ask your instructor how to assemble the unit.

2. Insert a comb into the tray, choosing a comb by the size and number of wells it will create.

3. 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.

4. Weigh out the required amount of agarose and add it to the appropriate amount of 1X gel running buffer in an Erlenmeyer flask that is 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 buffer in a 125-mL flask.

   ⇒ NOTE: A casting tray measuring 7cm x 7 cm usually requires approximately 30 mL of agarose solution.

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 in step 5. 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 loaded into the gel 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. 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 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. If you have run more than one gel, it is very important to label the gels because they are identical. 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.

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.

A. 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.

B. 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