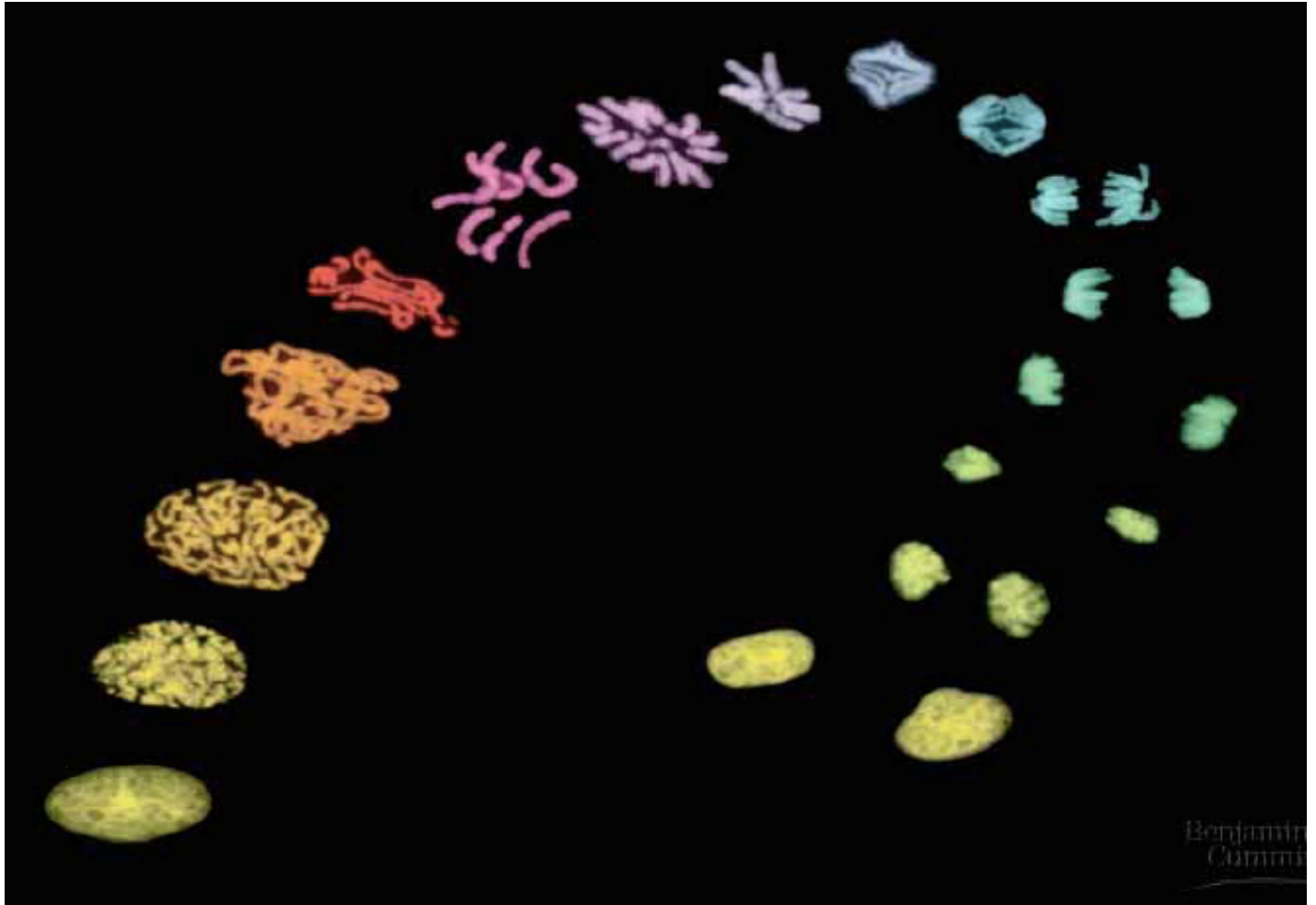# Biology 6B

Cellular & Molecular Biology

Bruce Heyer

De Anza College

Winter 2020

BIOL-6B: Cell & Molecular Biology

Course uyllabus, schedule, lecture slides, and lab supplements available from the course website:		
http://www.deanza.edu/faculty/heyerbruce/bio6b.html		
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	Office: SC 1212	Office Hours: Tue/Thu 10:30–12:20

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BIOL-6B: Cell & Molecular Biology

This course is designed to introduce you, the student of biology, to the study and understanding of the structure, genetics, biochemistry, and physiology of cells. The cell is the basic fundamental unit of life. All the processes of life, including harnessing energy, reproduction, inheritance of characteristics, and responding to the environment, can only be fully appreciated with an understanding of their cellular bases. Biol-6B will emphasize processes and structures common to most cells, and prepare you for more extensive, specialized upper-division work. The development of the field of cell biology and the focus of current innovative research in molecular biology will also be discussed. You will become more independent by learning to read, interpret, and evaluate original scientific papers.

The laboratory portion of the course provides hands-on experience using the modern instruments and methods of molecular biology. These elegant techniques provide practical experience for those pursuing careers in biological research.

COURSE OBJECTIVES

By successfully completing and passing Biol-6B, the student will demonstrate by means of objective exams, essays, oral presentations, laboratory proficiency, and written research reports, a practical competency and fluent exposition of the following topics:

♦ **Biological chemistry** — Explain the application of basic chemical principles to the complex chemistry of living systems. Understand the unique properties of water and carbon as they apply to organic chemistry. Know the classes of macromolecules and their biological significance.

♦ **Protein function** — Describe the special significance of proteins in maintaining and regulating the complexity necessary for all living systems. Define the specific actions of different functional groups of proteins. Explain how the cellular environment modifies protein activity.

♦ **Molecular genetics** — Explain how the structure of DNA relates to its function of storing and conveying information. Define a gene and describe the mechanisms for gene expression and how such expressions are regulated. Demonstrate how these genetic processes can be manipulated for the techniques of molecular biotechnology.

♦ **Cell structure** — Contrast the structure of prokaryotic and eukaryotic cells. Elaborate how the cytoskeleton sustains and transforms cellular organization and provides motility. Identify the eukaryotic organelles and their functions. Illustrate the dynamic structure of cellular membranes and their vital roles in selective permeability and compartmentalization.

♦ **Inter-cellular communication** — Describe the chemical and electrochemical mechanisms of cell-cell interaction. Contrast the actions of membrane and nuclear receptors on cellular activities.

♦ **Cell cycle** — Describe the processes of mitosis and cytokinesis in cell division. Explain the role of stem cells and regulation of the cell cycle in relation to proliferation, differentiation, apoptosis, and senescence. Postulate how aberrations of this regulation may lead to cancer.

♦ **Meiosis and sexual reproduction** — Explain the modification of cell division for meiosis and gametogenesis. Explain how recombination affects the genome. Contrast the advantages of haploid versus diploid cells, and asexual versus sexual reproduction. Distinguish the Mendelian, chromosomal, and epigenetic models of inheritance.

♦ **Bioenergetics** — Describe how photosynthetic cells harness light energy to synthesize organic molecules, and how all cells use the chemical energy in these organic molecules to power biological processes. Elucidate the chemistry of proton gradients, redox reactions, and phosphorylations as they relate to extracting and distributing energy within the cell. Explain how chloroplast structure controls the chemistry of photosynthesis, and mitochondria structure determines cellular respiration.

♦ **Laboratory research** — Perform routine procedures used in biological research laboratories, especially as related to the techniques of molecular biology. Demonstrate proficiency with standard protocols of lab etiquette, safety, hazardous materials handling, and documentation. Interpret published research articles to replicate their methodology and critique their interpretation of results.

PREREQUISITES AND ADVISORIES

Biology-6B is the second part of the three-quarter introduction to biology series for college students majoring in biology or a related science. Completion of Biol-6A (organismal biology) with a grade of C or better is a prerequisite for Biol-6B. This series is acceptable for transfer to the University of California and California State University systems and most other colleges. This course is equivalent or exceeds the rigor and depth of the corresponding introductory biology courses at these universities. Since the precise sequence of presented topics differs among institutions, it is strongly recommended that you complete the whole series at one college.

The study of cell and molecular biology requires a comfortable familiarity with chemistry. To enroll in Biol-6B, you need to have passed Chem-1A or Chem-50 with a grade of C or better, or passed the Chemistry Placement Test administered by the Testing Center. You needed to meet this chemistry prerequisite before enrolling in Biol-6A, but Biol-6B is where you'll find that you really use it.

Using equations to calculate solution concentrations, conversions, and stoichiometry in lab exercises requires above average math skills. Intermediate algebra equivalent to Math-105 or Math-114 is recommended.

Students will be writing essays and lab reports with an expected eloquence appropriate for scientific professionals. Coherent composition, accurate vocabulary, proper grammar, and correct spelling DO count! English skills equivalent to EWRT-1A or ESL-5 are highly recommended.

TIPS TO HELP YOU DO WELL IN THIS COURSE

There is no question that this class can seem intimidating with novel concepts, new vocabularies, and applied chemistry and physics. You must be prepared to invest a substantial allotment of time and effort to this endeavor. Some keys to success and satisfaction are:

- **Attend** every lecture and lab.

- **Be prepared!** Do the text reading before you come to class. If my lecture is the first you hear of a topic, you'll likely get lost. Especially with the pace we fly through topics: unprepared = frustrated. Prepare questions for unclear material — questioning is a form of *active* learning.

- Download and print out the **lecture slides**, when available, and bring them to class. But don't expect them to replace taking notes. Taking notes is another form of *active* learning.

- Develop **good study habits**. Spend time studying outside of class *every* day. Do not let yourself fall behind! Review lecture notes after each lecture. Be able to explain the concepts for each diagram presented in your own words.

- Construct **study tools**. Learning content-intensive material such as Biology often requires many steps: seeing, hearing, thinking, and doing. Create a list of terms in bold print presented in lecture. Write out flashcards and reorganize your lectures notes after each lecture as physical activities to help you process the material.
  I do not provide study guides for exams — that's *your* job! I will critique them though if you wish.

- Form a **study group!** Repeated experience has shown that those who study collectively do better. A study group will help you get to know your fellow classmates and provide intellectual reinforcement as well as moral support. Come prepared to a group study session by reviewing lecture material on your own first. Compare notes and test each other. Learn by teaching: an excellent way to learn how well you understand a matter is by explaining it to someone else.

- **Review!** The textbook supplemental CD-ROM and *Mastering Biology* website have flashcards, quizzes, games, and other tools to enhance your comprehension. They even have an online tutor to answer questions! Play the games with your study group. For access, follow the instructions on the first page of the textbook. You can go to the College Library or the Open Media Lab downstairs in Learning Center West for help with internet access.

CONDUCT

Participation in this class is expected to proceed with professionalism and mutual respect. Questions and experiences you have to clarify or enlarge on the topics being discussed are welcome. Please do not be distracting to your colleagues (including me) in class. Students are expected to be familiar with the Student Conduct Code and College Policies on academic integrity and academic freedom stated in the *De Anza College Catalogue*. Individuals found engaging in cheating, plagiarism, or disruptive behavior will be awarded a failing grade and reported to the administration for further disciplinary sanctions.

Science majors are also expected to have read the ***BHES Division Student Handbook*** for additional advice and standards. The *Handbook* may be downloaded from http://bhs.deanza.edu/StudentHandbook.pdf .

SUPPORT SERVICES

The college has a wide range of support services to provide students with assistance. These services range from tutoring and special short courses in reading and writing skills to financial aid and special programs for educational transition, reentry, and disabled students. If you would like to see if any of these programs would be of help to you, please check with the counseling office in the **Student and Community Services Center**. Consult your class schedule for a list of telephone numbers, or go to the **Student Services** website at http://www.deanza.edu/studentservices .

If you need a special accommodation for a physical or learning disability, please talk to me after the first class session so that I can make appropriate adjustments in the class to meet your needs. Visit **Disability Support Services (DSS)** and the **Educational Diagnostic Center (EDC)** in Learning Center West, room 110 for testing, advice, assistance, and special programs. Consult the **Disability Information Student Handbook (DISH)** at http://www.deanza.edu/dsps/dish .

SAFETY

The laboratory portion of Biol-6B is much more technology-oriented than was Biol-6A, requiring the use of high-voltage instruments and potentially toxic or infectious materials. All students will be required to read and sign to affirm their understanding and acceptance of the "Standard Operating Procedures" form prepared by the Biology Department. Any student who knowingly or recklessly endangers anyone's safety, or who repeatedly violates laboratory safety rules will be expelled from the class and possibly face further disciplinary actions at the instructor's discretion. If you observe any activity or situation that you think might be unsafe, please let talk to the instructor about it. Beyond this course, developing excellent lab safety habits is essential to your academic progress and scientific career.

Since De Anza College is located in a seismically active area, students should give forethought to catastrophic emergency actions. If a significant earthquake occurs during class, move away from the windows and stay indoors. If you are in lab, disconnect any gas lines or electrical devices, secure glassware, and take shelter under the lab bench.

In the event of an emergency that requires the evacuation of the room, we will exit the building and regroup outside for roll call and further instructions. Be careful to avoid traffic lanes. **Do not leave campus until you have been instructed to do so by your instructor or by emergency personnel!**

LAB OVERVIEW

Biol-6B strongly emphasizes laboratory-science skill development necessary for biology major degree programs. Therefore participation in *all* labs is expected and you *must* pass the laboratory portion to receive credit for the course. If you miss any three labs you may be dropped from the class. Non-participation is considered equivalent to non-attendance.

Read the lab experiments before you come to class and come prepared to begin work. It is next to impossible complete a lab exercise and learn anything from the process if you are reading the instructions for the first time. The safety of you and your classmates may depend on your preparedness when we are using hazardous materials.

The activities explored in lab build upon concepts presented in lecture, but they do not correlate with the sequence of topics as they are featured in lecture. The laboratory procedures used will emphasize the modern tools and techniques of molecular biology that are used to study cell biology, as well as many other aspects of life sciences.

The course lab exercises are organized around **four lab project reports**. Each report will be a group project and cover experiments conducted over several lab periods. The students at your lab table are your lab partners, and your group will turn in one report for each project.

Each project may include different kinds of experiments over several lab periods, and more than one project may overlap on the same lab period. So you will need to have very good organizational and note-keeping practices to keep track of which experiment relates to which project. The projects will become increasingly complex as the quarter progresses, and techniques that are used repeatedly will need to be accomplished with greater efficiency. At first, the instructor will give more detailed instructions on what to do and how to organize your time. But by the latter portion of the course, you will be expected to interpret the instructions and budget your time effectively within your group. It is important to finish each experiment to complete each project. The better you get at planning and time management, the more opportunities you will have to repeat experiments if needed.

The topics for the four lab project reports are:

1. **DNA restriction digest, ligation, & electrophoresis.** Use enzymes to cleave DNA at specific sites and electrophoresis to analyze the cleavage products. This project will take 2 lab periods.

2. **Bacterial conjugation.** Use direct and indirect methods to assess the transfer of genes from one kind of bacteria to another by culturing them under different conditions and observing the acquisition of heritable survival characteristics. The project will take all or part of 6–7 lab periods.

3. **pGLO.** Insert a foreign gene into bacteria, isolate the new protein gene product from the bacteria, and identify the DNA of the transferred gene in the bacterial DNA. This project will take all or part of about 7 lab periods.

4. **PV92 polymerase chain reaction (PCR).** Rapidly copy a part of your own DNA. Compare your DNA with the corresponding part of the DNA of others in the class. This project will take 4–5 lab periods.

The project instructions in the *Lab Manual* include directions for how each report should be composed.

Each report shall be graded on a scale worth 25 points. Each student's score shall be a portion of those points based upon attendance, participation, and contribution to the group effort for that project.

ONLINE LAB QUIZZES

To test your progress in the theory and practice of experimental methods, each week a quiz will be posted on the *Mastering Biology* website covering topics presented and used in the previous week's labs. Usually the quizzes will be posted Monday afternoon, and due by Wednesday morning.

LAB EXAM

The final lab class will consist of a comprehensive **lab exam** derived from all of the lab projects and methodologies. Bring a **BB-8** (large) **Examination Blue Book** for the lab exam essays and illustrations.

ONLINE HOMEWORK EXERCISES

Each lecture topic coincides with tutorials and graded textbook problem sets presented on the *Mastering Biology* website. These have been selected to enhance your comprehension of the complex concepts that may be presented too quickly in lecture. Be sure to allow sufficient time to derive the maximum benefit from these exercises. Your total score of all these graded problems will be used to calculate your percent score.

LECTURE EXAMS

There are three exams based upon material covered in lecture. (The final exam is Exam 3.) These exams are non-cumulative and will be composed of multiple choice and matching questions and diagram interpretations. A new (clean and unwrinkled) Scantron Form # 882-E (green) answer sheet and a #2 pencil will be needed for each lecture exam.
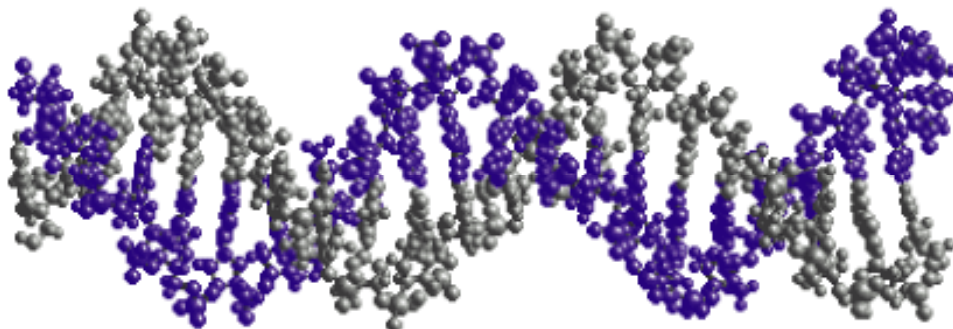
Please note the dates of all exams. If you are sick or have an emergency, contact me **BEFORE** the exam and special arrangements *might* be made in extenuating circumstances. Vacation plans are *not* extenuating circumstances! If a last-minute crisis occurred on the way to the exam, contact me before the end of the day.

GRADING

♦ **Lab Project Reports:** Four reports; each report counts 25 points. (4 x 25 = 100 points)

♦ **Online Homework & Quizzes:** Cumulative % score of all exercises and quizzes counts 100 points.

♦ **Lab Exam:** One exam; counts 100 points.

♦ **Lecture Exams:** Three exams. Each exam counts 100 points. (3 x 100 = 300 points)
The final class grade will be determined as a percentage of the maximum total 600 points:

| 92-100%= A | 89-91%= A– | 86-88%= B+ | 80-85%= B | 77-79%= B– |
| 74-76%= C+ | 65-73%= C | 53-64%= D | <53%= F

Lab Team #		
Team Label:		
Lab Partners	**Phone**	**Email**

STANDARD OPERATING PROCEDURES
DE ANZA BIOLOGICAL AND HEALTH SCIENCES DIVISION

THE FOLLOWING ARE RULES AND REGULATIONS COMPLIED FROM THE FOOTHILL/DE ANZA DISTRICT HAZARDOUS MATERIALS/CHEMICAL HYGIENE PLANS. The Environmental Protection Agency, the Water District, CAL-OSHA and the Fire Department determined these rules.

1. Absolutely no food or drinks are allowed in the lab.

2. Wear adequate foot protection. No bare feet. Do not wear open-toed shoes or sandals to lab.

3. Do not pour any fluids or chemicals down the lab sink unless specifically allowed by the instructor.

4. Students must wear eye protection, latex gloves, apron, etc., when your instructor determines that it is necessary.

5. Chemicals must be handled appropriately. Some chemicals might be corrosive, flammable, carcinogenic, volatile and/or toxic. If you have questions or concerns ask your instructor. Material Data Safety sheets (MSDS) are available for inspection upon request.

6. Chemical spills must be reported to the instructor and the lab technician. A formal Hazardous Spill Report must be completed.

7. Broken equipment can be a safety hazard. It should not be used and must be reported. Do not throw away any equipment unless allowed by the instructor.

8. Use the right container for discard items -- do NOT use these containers for trash

   - Use a Broken Glassware box for broken glass

   - Use a Sharps container for needles, razor blades, pins

   - Use a Biohazard Container for infectious waste

   - Use a Hazardous Waste collection jug for chemical waste

9. Know the location and use of all emergency equipment in each lab. Every lab has an eyewash station, a safety shower, a fire extinguisher, and a first aid kit.

10. Incase of an emergency evacuate the building calmly. Proceed to, and meet your class and instructor at the sidewalk adjacent to Parking Lot E. This allows for a head count of all students and is done for your safety.

11. At the end of every lab session, help clean up the lab. Return items back to the right location, wipe down your lab workbench, and remove labels or markings off glassware. Your help is greatly appreciated.

12. After handling chemicals and biological items, wash your hands, even if you have been wearing gloves.

I have read the above Laboratory Standard Operating Procedures. I do agree to abide by these standards, to cooperate with class instructions, and to act in accord with all safe and beneficial practices.

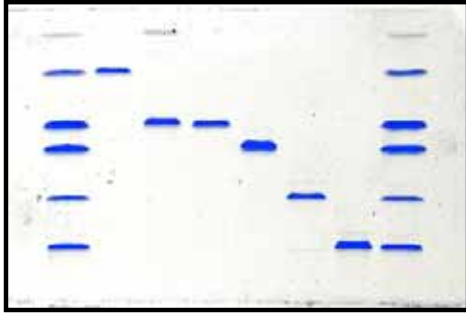**Print Name:** _____   **Class/Section:** _____

Signature: _____   **Date:** _____

x

Biology 6B
Cellular & Molecular Biology

Laboratory Manual

**Brian McCauley
& Bruce Heyer**

De Anza College 2020

P R O T E I N   E L E C T R O P H O R E S I S

In protein electrophoresis, proteins can be separated from one another by size. In the example at left, each dark line is a **band** – a bunch of identical molecules of one kind of protein. Each vertical column of bands is a **lane**. Within one lane, all the proteins started together at the top, but the smaller proteins moved faster and ended up closer to the bottom.

In today's lab you'll prepare a similar protein gel. You'll use a small meat sample as a protein source, and you'll probably see numerous bands on your gel.

By the time you complete this lab, you should understand:
- basic principles of electrophoresis
- SDS-PAGE
- what controls rate of migration in a gel
- sample buffer & running buffer
- definitions: band, lane, gel

I N T R O D U C T I O N

There are a lot of different molecules in a cell. If you want to study the function of a particular molecule – a protein, for example – you'll need to separate that molecule from all the others. Electrophoresis is a way of separating macromolecules from one another.

PRINCIPLES OF ELECTROPHORESIS

In protein electrophoresis, you force proteins to migrate through a gel. The gel can be any material that is solid, but has pore spaces large enough for the proteins to fit through. If the pores are a tight fit for the proteins, the larger proteins will move slowly, while the smaller proteins will be free to move faster. You make the molecules move through the gel by applying an electric field across the gel. Positively charged molecules will move toward the negative pole, and negtively charged molecules will move toward the positively charged pole. Uncharged molecules won't move at all.

A protein's rate of movement through the gel will be controlled by:

- **Size.** Bigger molecules move more slowly, because they don't fit through the pores easily.

- **Charge.** Strength and polarity of charge influence how fast and which direction a molecule moves. Some proteins are positively charged and some are negative; in most cases, the charge will depend on pH and other aspects of the solution.

- **Shape.** A protein that is tightly folded will seem smaller, and move through the gel faster, than a protein that is loosely folded.

- **Pore size.** Bigger pores=faster movement. Smaller pores may allow more precise separations. Pore size varies from gel to gel, but within a single gel, each lane has the same pore size.

- **Voltage.** The more voltage you apply across the gel, the faster things will move. Within a single gel, voltage is constant.

SDS-PAGE

Sometimes you just want to separate proteins by size. SDS-PAGE is a way of doing electrophoresis which ensures that a band's rate of migration is determined only by size.

SDS stands for Sodium Dodecyl Sulfate – but don't worry about remembering that. SDS is a detergent, and it accomplishes several critical things for electrophoresis:

- SDS helps proteins dissolve so you can run them on the gel (not all proteins are soluble in plain water).

- SDS denatures, or unfolds, proteins. This means that shape will not influence rate of migration in the gel.

- SDS sticks to proteins and makes them negatively charged. Since SDS sticks all over the protein, each protein ends up with the same density of charge.

[For today's lab, you may be using Lithium Dodecyl Sulfate instead of SDS; it's still called SDS-PAGE.]
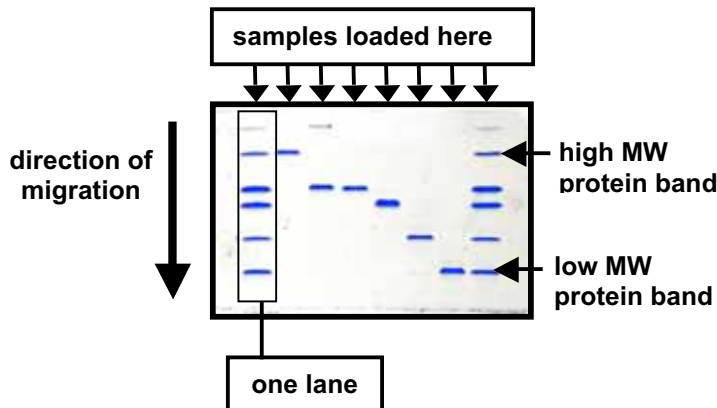
PAGE stands for Polyacrylamide Gel Electrophoresis. You should probably remember this. Polyacrylamide is used because it creates strong gels with a predictable pore size.

In SDS-PAGE, all the proteins have the same charge density and the same unfolded shape. In a single gel, the pore size and the voltage are constant. Therefore, **in SDS-PAGE, a protein's rate of migration through the gel is determined solely by size**. You get a gel that looks like the picture shown here.

SDS-PAGE is the most commonly used method of protein electrophoresis. It's the one you'll use in today's lab.

samples loaded here

direction of migration

high MW protein band

low MW protein band

one lane

THE PROTEIN SAMPLES FOR THIS LAB

Almost any biological tissue can be used for SDS-PAGE. We'll use fish, because it's mostly protein and easy to grind up.

GEL STAINING

You need to stain the proteins before you can see them. In today's lab, you'll use a stain called Coomassie. This chemical was originally developed as a dye for wool, but it happens to stain most other proteins pretty well, too. After you run your gel, you'll soak it in a Coomassie solution so you can see the bands.

ALTERNATIVE METHODS: CHROMATOGRAPHY

Electrophoresis is one way of separating proteins from one another, but there are other methods. Chromatography is similar to electrophoresis, but the key difference is that in chromatography the proteins are pulled through a gel or similar matrix by a moving solvent, rather than an electric field.

P R O C E D U R E :

SAFETY CONSIDERATIONS

✋ **Wear gloves and goggles throughout this lab.**

✋ **Sample buffer and running buffer.** The buffers you use in SDS-PAGE contain SDS, a powerful detergent. SDS can be a skin and eye irritant, so you should avoid contact by wearing gloves and safety glasses. In case of contact, wash thoroughly with water.

✋ **Electrophoresis power supply.** You'll use a power supply to apply an electric field. Be sure to keep your lab bench dry when using this electric appliance.

MATERIALS FOR PREPARING PROTEIN SAMPLE

For each lab group (usually 4 people), obtain the following:
- pipetmen and tips
- beaker for waste tips
- three 500-µl micro tubes (the small tubes) and rack
- homogenized protein samples, dissolved in LDS sample buffer
- sample reducing agent (0.5 M dithiothreitol, or DTT; this may already have been added to your sample)
- molecular weight markers (Note types)
- ice bucket with ice for protein samples

SAMPLE PREPARATION PROTOCOL

To obtain the protein samples for SDS-PAGE, we need to lyse (break open) the cells. The soluble material released from lysed cells is called the **lysate** that consists of a large variety of biological molecules including the soluble proteins. For today's lab, your sample lysates have already been prepared for you. Keep the lysates on ice so the proteins do not degrade. Next, the sample needs to be mixed with sample buffer and a reducing agent and heated to denature the proteins. For each of your lysate protein samples, prepare as follows:

1. Pipet 32.5 µl homogenized protein sample into a 500-µl micro tube.

2. Add 12.5 µl 4x LDS sample buffer (contains buffer salts, LDS, dye, & glycerol).

3. Add 5 µl sample reducing agent (dithiothreitol [DTT] solution).

4. Heat your sample at 70° C for 10 minutes.

Your "gel-ready" samples are now ready to load on the gel.

RUNNING THE GEL

Each gel has 10 wells; you won't need them all. A well can hold up to 25 µl.

1. Open the gel, take the comb out, and take the white strip of tape off. Rinse the gel with water. Assemble the gel unit. The instructor will show you how. First fill the inner buffer chamber with running buffer and make sure it doesn't leak. After a few minutes, fill the outer chamber with enough running buffer to cover the place where the tape was on the gel. Leave the lid off.

2. Load your samples into the wells. Each sample goes into its own well. Load all your samples in adjacent wells (don't skip wells between samples). Leave some empty wells on each side, so your samples are in the middle of the gel. Keep track of which sample goes in which lane. By convention, lane 1 is on the far left and lane 10 is on the right.

We measured how much protein is in your protein samples last lab. Now calculate the protein concentration in your "gel-ready" samples. The instructor will tell you what to load in each lane. You should have several lanes of your protein sample, plus a standard molecular weight marker. Write down what is on your gel:

    Lane 1:
    Lane 2:
    Lane 3:
    Lane 4:
    Lane 5:
    Lane 6:
    Lane 7:
    Lane 8:
    Lane 9:
    Lane 10:

It makes sense to load different amounts in different lanes. If there's too little protein, you won't be able to see the bands; if there's too much, the bands will be smeared together.

3. After both gels in the gel unit have been loaded, put the lid on the unit and connect the electrode cords to the power supply. Don't turn on the power yet; have the instructor check the setup.

4. Turn on the power supply and run the gel at 200 Volts. The current should be 100-115 mA (milliamps) at the beginning. (Current is expected to gradually decrease to 60-70 mA as the gel runs.) The gel should run for about 50 minutes. You should see the blue loading dye migrate to the bottom of the gel.

5. When the run is complete, turn off the power, disconnect the electrodes, and remove the gels from the gel unit.

STAINING THE GEL

1. Separate the plates of the gel cassette using the gel knife. Gently free the gel from the slotted plate by trimming away the bottom lip of the gel, then sliding the gel into a plastic staining container.

2. Cover gel with warm deionized water.

3. Let the gel sit in its container on your lab bench for 2–3 minutes with occasional agitation. Pour off the water.

4. Repeat steps 2 and 3 two more times, using enough deionized water to cover the gel.

5. After the last wash, pour 50 ml SafeStain on the gel (enough stain to just cover gel).

6. Let the gel sit in the stain for 1–3 hours with occasional agitation.
   • Alternatively, you can add 5 ml 20% NaCl solution to the SafeStain and let sit overnight.
   • Or as a quick third alternative, microwave for 45 seconds and let the gel sit on your lab bench for 5-10 minutes with occasional agitation. This quick method loses some sensitivity.

7. Pour off the used stain into a waste container. Soak the gel in 100 ml deionized water for 1 hour. Add 20 ml 20% NaCl and let soak another 2 hours or overnight.

8. Rinse the gel with 100 ml deionized water. You should now be able to see bands on your gel!

9. Transfer your gel onto a piece of plastic wrap and take a digital image of your gel. (Refer to the protocol in **Appendix 3**.) When you have a good image, throw the gel in biohazard trash.

CUTTING DNA: RESTRICTION DIGESTS & LIGATIONS

In this lab, you'll begin to work directly with DNA. This lab activity will have three parts: restriction digests and ligation (today), and gel electrophoresis (later). At the end, you should write one lab report covering all three parts.

By the time you complete this lab, you should understand:

- What restriction enzymes do
- What ligase does
- How to work with enzymes & do the appropriate calculations
- Restriction sites
- Sticky ends

INTRODUCTION

Most of the tools in a molecular biologist's toolkit were taken from cells. Cells do all sorts of tricks with DNA; learning how to manipulate DNA in a test tube usually means mimicking the way DNA is manipulated inside a cell. Cells manipulate DNA by using enzymes: proteins that catalyze specific chemical reactions. Molecular biologists get enzymes from cells, then use the enzymes in new ways.

Restriction enzymes cut DNA at specific sites. They recognize short sequences of nucleotides, called restriction sites, and cut the DNA at those sites. Restriction sites are usually four to eight base pairs long. There are many different restriction enzymes, each with its own specific restriction site. Restriction enzymes are also called endonucleases, because they cut nucleic acids somewhere in the midst of the molecule (endo- means in).

Restriction enzymes were the first DNA-altering enzymes to be isolated and used in the laboratory; in a sense, molecular biology began when a restriction enzyme was used to cut DNA in a test tube.

Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.
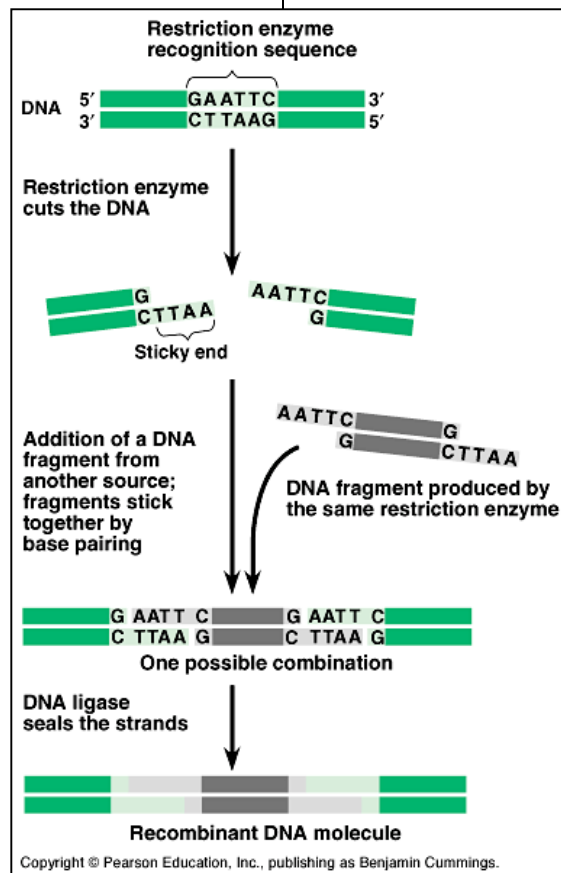
WHY DO CELLS HAVE RESTRICTION ENZYMES?

Restriction enzymes cut up DNA. Wouldn't this be a bad thing for a cell? Not if it's DNA that can harm the cell. The restriction enzymes you'll use come from bacteria, and the bacteria use the enzymes to cut up DNA from viruses or other potentially dangerous sources. So the restriction enzymes function as a self-defense weapon for the cell.

How do the enzymes avoid cutting up the cell's own DNA? A bacterial cell may lack restriction sites that can be cut by its own enzymes, or it may chemically modify the DNA (by adding methyl groups) at the restriction sites.

WHY IS CUTTING DNA USEFUL IN THE LAB?

One key reason for cutting DNA in the lab is so you can join different DNA pieces together through ligation. That's the essence of gene cloning.

For today's lab, we have a simpler purpose: you'll generate DNA fragments of known size that you can use as standards for electrophoresis.

THE ENZYMES AND DNA FOR THIS LAB

In this lab, you'll use two different restriction enzymes to cut one kind of DNA. The restriction enzymes are:

Eco R1. Isolated from *Escherichia coli*.

Hin DIII. Isolated from *Haemophilus influenzae*.

The DNA is from a virus. The virus is called lambda; it's a bacteriophage, meaning it's a virus that infects bacteria.

When you use electrophoresis to look at your results (next lab period), you'll be able to see the difference from one reaction to another, because the enzymes cut the lambda DNA into different-sized fragments. The uncut DNA should give one big band on the gel, while the cut DNAs will show various smaller bands.

P R O C E D U R E :

SAFETY CONSIDERATIONS

DNA hazards? You'll be handling some DNA, which can sometimes be subject to safety restrictions. However, the lambda DNA used in this lab is not from a human for from any organism that can infect humans. It doesn't present a hazard under normal laboratory conditions. The enzymes, Hind III and Eco RI, will be used in extremely small quantities and don't present a hazard. The other components in the reaction are simply a buffer solution, which you'll use in very small quantity.

✋ **Gloves and goggles** will still be required while performing this lab. The gloves are mainly to keep bacteria from your hands from contaminating the reaction. Bacteria have restriction enzymes of their own, and can quickly destroy foreign DNA.

PLANNING THE REACTIONS:

Working with DNA is usually pretty easy. You put some DNA and some enzymes in a little tube, and you let the enzyme do the work. The hard part is knowing how much of each ingredient to add to the tube.

The ingredients for today's reactions:

• **Lambda DNA.** This is what gets cut; you'll use electrophoresis to look at the DNA after the reaction is done. After you cut the DNA, you'll also use some of it for ligation, which is the next experiment.

• **Restriction enzyme:** Eco RI, Hin DIII, or both. Cuts the DNA.

• **Enzyme buffer.** Enzymes are proteins, and they depend on having the right 3-dimensional shape to do their job. Since the shape of a protein is controlled in part by weak interactions such as hydrogen bonds, it can be altered by changes in pH or salt concentration. When you buy an enzyme, it comes with a buffer that ensures the right environment for the enzyme. The buffer comes in a separate tube, usually as a 10x concentrate. You dilute it down to 1x before adding the enzyme.

• **Water.** You'll generally need to add a little water to make the concentrations of everything come out right.

To figure out what goes in the tube, you'll have to do a little math. You need to do all your calculations and show them to the instructor before you're ready to start doing the lab work.

How much DNA? You should use **1.0 µg of lambda DNA** for each reaction. Since you'll be using a Pipetman to pipet the solutions into your reaction tubes, you need to convert µg into µl. You'll need to know the concentration of the starting DNA solution; the instructor will give you this.

Concentration of stock lambda DNA solution: _____.

For a little guidance on how to do the math, see the notes on units and calculations in the last couple of pages of this lab manual. Write out your equation here:

You need _____µl of lambda DNA.

HOW MUCH ENZYME?

Enzyme activity is defined in terms of arbitrarily chosen units for each enzyme. For both Eco RI and Hin DIII, the unit is defined as: 1 Unit will completely cut 1.0 µg of lambda DNA in 1 hour at 37° C.

How many units of each enzyme do you need for each reaction? _____Units.

HIN DIII:

Hind III stock solution: _____ . (Get this concentration from the instructor.)

Take a look at the hints in the back pages of this book, and write out your equation for how many µl of enzyme you need:

 Minimum enzyme amount: Need at least _____µl enzyme. (Not very much, is it?)

In practice, it's a good idea to use a little extra restriction enzyme, to make sure all the DNA gets cut. For this reaction, multiply the minimum enzyme amount (which you just calculated) by 5. So you'll add _____µl Hind III.

Eco RI:

The calculations are exactly the same, but the starting concentration of the enzyme may be different.

Eco RI stock solution: _____ . (Get this concentration from the instructor.)

Again, remember to multiply your final enzyme amount by 5.

HOW MUCH BUFFER?

Eco RI and Hind III can both use the same buffer. Remember, the buffer comes as a 10x concentrate, and you need it to be diluted to 1x in your reaction tube. To figure out the amount, use $C_1V_1=C_2V_2$. In this case,

- C_1= initial concentration = 10x.

- V_1= initial volume = this is what you're trying to solve for; the answer should be in μl.

- C_2= final concentration = 1x.

- V_2= final volume = 15 μl. Generally, you just pick a final volume that will be convenient for the experiment. 15 μl will be convenient for this one. The final volume is what's in the tube after you've added everything, including water.

Now do the math! (Write out the whole equation, including the units.)

And yes, you will get to do these calculations on an exam very soon!

THE REACTIONS:

Now you know how much DNA, enzyme, and buffer to use in each reaction. You should set up four reactions, each with the same DNA but with different enzymes. The reactions will be:

- **No enzyme:** This will be just DNA, with no Eco RI or Hin DIII.

- **Eco RI:** DNA and Eco RI.

- **Hin DIII:** DNA and Hin DIII.

- **Eco/Hind:** DNA and Eco RI and Hin DIII.

✍ **Make a chart showing what goes into each reaction tube:**

	No Enzyme	Eco RI	Hin DIII	Eco/Hin
water	_____μl	_____μl	_____μl	_____μl
DNA	_____μl	_____μl	_____μl	_____μl
10x enzyme buffer	_____μl	_____μl	_____μl	_____μl
Eco RI	0.0 μl	_____μl	0.0 μl	_____μl
Hind III	0.0 μl	0.0 μl	_____μl	_____μl
total volume	**15.0μl**	**15.0μl**	**15.0μl**	**15.0μl**

You've already figured out how much enzyme, DNA, and buffer for each reaction. You know the final volume. After you've figured all this out, calculate how much water you'll need to add to bring the volume of each reaction up to 15 μl.

◈ *Be ready to do calculations like these on a quiz. You should know that you always need a buffer for the enzyme, and sometimes you aren't given the final volume.*

Check your numbers with the instructor.

MATERIALS AND SETTING UP THE REACTION:

With the calculations out of the way, the rest is easy. For each table, obtain the following:

• pipetmen, yellow tips, and a beaker for waste tips
• rack for microfuge tubes
• a small Styrofoam cooler or cup filled with ice
• one 500 μl microfuge tube for each reaction (the tiny tubes, not the larger 1.5 ml size)
• sterile water
• lambda DNA
• 10x enzyme buffer (same buffer for both enzymes)
• Eco RI enzyme (the instructor will give you the enzymes when you're ready)
• Hind III enzyme

Once you've assembled your materials, go ahead and set up your reaction according to the table you filled in above. All you need to do is pipet 4 or 5 ingredients into each tube, and you're done! As mentioned earlier, the hard thing is figuring out what goes into the tube.

TIPS FOR SETTING UP ENZYME REACTIONS:

Keep the enzyme cold. Keep everything cold. Keep the whole reaction on ice until it's completely assembled and you're ready to incubate it.

Add the ingredients in the order listed in the table above. **Add the enzyme last.** The enzyme could be destroyed if you add it before you add the buffer.

Never use a pipet tip twice –change tips every time. This prevents cross-contamination.

Keep all the ingredients in the bottom of their microfuge tubes, and mix each one with the pipet tip as you take it out. You may need to use the centrifuge to get the solution to the bottom of the tube.

After you pipet all the ingredients, incubate your reaction in the PCR machine at 37° C. We'll program the machine to run for one hour at 37° C, and then heat up to 80° C for 3 minutes to destroy (heat-kill) the enzyme. The program on the PCR machine is called Cut & Kill.

L A B   R E P O R T S

You're not done yet! The lab report for this lab will include restriction digests, ligations, and gel electrophoresis. You'll probably do both restriction digests and ligation in one day; you'll find the ligation procedure on the next page. See the end of the electrophoresis section for instructions on writing the report. (p. 22)

INTRODUCTION: LIGATION

Most DNA lab techniques fall into a few categories:

Cutting DNA with a restriction enzyme. You did this in the last lab.

Joining pieces of DNA together with ligase. You'll do this today.

Copying DNA with a polymerase. You'll do this later in the quarter, when we get to the polymerase chain reaction (PCR).

Cutting DNA into pieces, joining different pieces together in new combinations, and copying the new recombinant DNA is the essence of gene cloning. Cloning has been the foundation of much of the explosion of biological knowledge that has taken place in the last few decades. Later this quarter, in the DNA library lab, you'll have a chance to clone some viral DNA. Think of today's lab, ligation, as practice for cloning.

In the last lab, you cut some DNA with restriction enzymes. In today's procedure, you'll use the enzyme ligase to form covalent bonds joining the cut DNA fragments. The fragments could be joined exactly as they were before cutting, or they could be joined in new combinations.

LIGASE

Cells use ligase as a part of their normal DNA replication process. As you may recall, cellular DNA is synthesized in fragments, and the fragments must be joined to make complete chromosomal DNA.

Joining strands of DNA together means forming covalent bonds, and it requires energy. Some of the energy for ligation comes from breaking phosphoryl bonds on the DNA and some comes from additional ATP, which is present in the ligation buffer. The ligation buffer must always be kept cold to preserve the ATP.
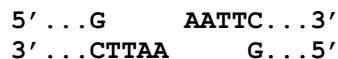
STICKY ENDS

Recall that restriction enzymes cut DNA at specific restriction sites.

You used the enzymes Eco RI and Hind III to cut your DNA. Here's The restriction site recognized by Eco RI:

```
5'...GAATTC...3'
3'...CTTAAG...5'
```

The DNA is cut like this:

```
5'...G      AATTC...3'
3'...CTTAA      G...5'
```

Note that the two strands of DNA aren't cut at the same place; each cut fragment has a single-stranded overhanging end.

Also note that the two DNA fragments can stick to each other by base pairing: the As on one strand can hydrogen bond with the Ts on the other strand. The restriction enzyme breaks the covalent bond between G and A, but the cut pieces can still stick together by hydrogen bonding. In other words, the cut DNA fragments have sticky ends. Ligase can re-form the covalent bond that was cut by the restriction enzyme.

Any two pieces of DNA that have been cut by Eco RI can stick together and be ligated. However, a piece that was cut with Eco RI won't stick to a piece that was cut by Hind III. Since Hind III's restriction site is different, it will leave different sticky ends:

```
5'...A      AGCTT...3'
3'...TTCGA      A...5'
```
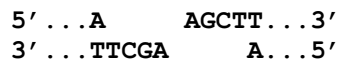
In order for two DNA fragments to be ligated together, they must have compatible sticky ends. In general, this means that they must be cut with the same restriction enzyme.

YOUR EXPERIMENT

What will happen to your cut DNA samples when you add ligase? The various pieces of Eco RI-cut DNA can be joined together in any combination. The same is true for the Hind III-cut DNA. What about the sample that was cut by both enzymes? The introduction to your lab report should include an explanation of what you expect and why. The discussion of your lab report should compare your actual results (seen on the electrophoresis gel) to your expected results.

P R O C E D U R E :   L I G A T I O N

SAFETY CONSIDERATIONS

DNA hazards? As before, you'll be handling some DNA, which doesn't present a hazard under normal laboratory conditions. The enzyme, ligase, will be used in extremely small quantities and doesn't present a hazard. The buffer and other components in the reaction pose no hazard in the quantities you'll be using.

✋ **Gloves and goggles** will still be required while performing this lab. The gloves are mainly to keep bacteria from your hands from contaminating the reaction.

PLANNING THE REACTIONS

The trickiest part is calculating how much DNA and how much enzyme (ligase) to put in your reaction.

How much DNA?

The DNA you should use for this lab is the Lambda DNA you cut with restriction enzymes in the last lab. Remember, you cut three DNA samples: one with Hind III, one with Eco RI, and one with both restriction enzymes. For this lab, you'll want a little of each of those restriction digests.

For each ligation, **use 500 ng of Lambda DNA**. You'll need to convert from ng (mass) to μl (volume) to set up the reaction. To do this, you need to know the concentration of your DNA solution. Concentration is mass/volume, so the concentration of DNA in your restriction digests is:

total amount of DNA in reaction (μg)/total reaction volume

Note that the concentration of DNA is not the same as the stock DNA solution you used in the last lab – you added some water, buffer, and enzyme to it, so the DNA concentration is different now.

✍ **What is the concentration of DNA in your restriction digests?** (Hint: the concentration in the restriction digest isn't the same as the concentration you started with, because you put other stuff in the tube.)

✍ **How many μl of each restriction digest should you use to get the 500 ng of DNA you need?**

◈ *Be ready for quiz questions involving calculations like those for this lab.*

HOW MUCH LIGASE ENZYME?

The unit definition for ligase is: 0.1 Unit is enough to ligate 1 μg Lambda/Hind III DNA. You don't have that much DNA; in practical terms, you need the smallest amount of ligase you can pipet. Using a special P-2 pipetman, you can use an enzyme volume of 0.4 μl. Add 0.4 μl to each ligation reaction.

THE REACTIONS

You should do three ligation reactions: one for Lambda DNA that was cut with Eco RI, one for the Hind III-cut DNA, and one for the Eco RI + Hind III-cut DNA. Since the three reactions will be the same except for the source of the DNA, you can make one chart showing how much of each ingredient to add to each reaction tube:

	Eco	**Hind**	**Both**	
water	_____	_____	_____	
10x ligase buffer	_____	_____	_____	= 1x final
Eco RI-cut DNA	_____	0 μl	0 μl	= 500 ng
Hind III-cut DNA	0 μl	_____	0 μl	= 500 ng
Eco+Hind-cut DNA	0 μl	0 μl	_____	= 500 ng
ligase	0.4 μl	0.4 μl	0.4 μl	
TOTAL VOLUME	**10.0 μL**	**10.0 μL**	**10.0 μL**	

✧ *Why do you think the total volume for the ligation is supposed to be so small?*

MATERIALS

For each table, obtain the following:

- pipetmen, yellow tips, and a beaker for waste tips
- rack for microfuge tubes
- a small Styrofoam cooler or cup filled with ice
- one 500 μl microfuge tube for each reaction (the tiny tubes, not the larger 1.5 ml size)
- sterile water
- your 3 tubes of restriction-digested Lambda DNA from last lab
- ligase and ligase buffer (instructor will distribute this)

SETTING UP AND INCUBATING THE REACTIONS

Set up the reactions according to your chart, exercising the usual care appropriate to working with enzymes. Keep everything on ice. **Be sure to keep the ligation buffer on ice; it contains ATP and will break down if kept warm.**

When you're done, incubate your reaction 30 minutes or longer at room temperature.

✧ *Most other enzyme reactions are incubated at warm temperatures like 37°. Why do you think this one is cool?*

When the incubation is over, keep your DNA in the freezer until next time.

WASTE DISPOSAL & CLEANUP

You may have tubes with very small amounts of leftover DNA or buffers; check with instructor before throwing any of it away.

Waste micro tubes and pipet tips go in the biohazard trash.

Dirty beakers (used for collecting waste pipet tips) go into a tub for dirty glassware. Dump the tips out first!

Dump your ice down the sink, and put everything else back where you got it.

L AB   REPORTS

You've now completed two out of the three parts for this exercise. The lab reports for this lab should include restriction digests, ligations, and gel electrophoresis. See the end of the electrophoresis section (p. 24) for instructions.

S OME   Q UESTIONS   TO   T HINK   A BOUT

◈ *Why would an organism have restriction enzymes? How might an organism protect itself from its own restriction enzymes?*

◈ *Why would an organism have ligase?*

◈ *When you're trying to ligate DNA, why does it matter which restriction enzyme you used?*

◈ *Why does each enzyme have a specific buffer?*

CUTTING DNA 2: DNA ELECTROPHORESIS
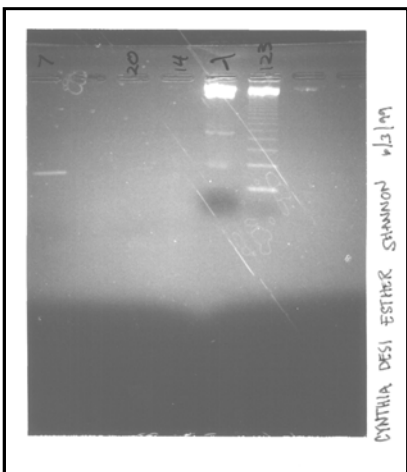
Typical electrophoresis photo from a previous Bio 6B class. Of course, *your* picture will look much better than this one.

The white horizontal bands are DNA. The DNA in this picture has migrated downward from the wells at the top. The large, bright bands at the top are large DNA molecules; the bands lower on the gel are smaller fragments.

Today you'll do electrophoresis to check the results of your restriction digests. By looking at the gel, you'll be able to see if your DNA was cut, and how many times.

After completing this lab, you should understand:

- what electrophoresis is used for

- how agarose gel electrophoresis works

- what factors control how fast and which way a piece of DNA moves in a gel

- how to determine the size of a piece of DNA from a gel

INTRODUCTION:

Electrophoresis is one of the most commonly used techniques in molecular biology. It is used to separate different macromolecules from one another so they can be analyzed. For example, if you want to study a particular protein from a cell culture, you will generally need to separate that protein from all the other proteins present in the cells. This can be tricky, since different proteins may be similar to one another; in many cases, electrophoresis is the tool you need.

For this class, we're going to focus on electrophoresis as a method of separating pieces of DNA from one another. In DNA electrophoresis, pieces of DNA are separated from one another on the basis of their size.

In today's lab, run your DNA fragments on a gel to see if your digests and ligations worked.

HOW IT WORKS:

DNA in solution is a charged molecule. Remember, DNA is deoxyribonucleic acid, and like all acids, it can give up a proton in solution. Once it gives up the proton, the DNA has a negative charge. You can use the charge to make the molecule move.

In electrophoresis, you put the DNA samples into a salty solution and apply an electric current through the solution. Electric currents have a positive end and a negative end, and salty water conducts electricity well. The electric current creates an electric field, similar to the field around a magnet. DNA, being a negatively charged molecule, migrates toward the positive pole of the electric current or electric field.

The trick with electrophoresis is to get different-sized DNA molecules to migrate toward the positive pole at different rates. You can accomplish this by putting the DNA in an agarose gel. An agarose gel is like Jell-O – it's a solid matrix, consisting largely of complex carbohydrates, and filled with water. Agarose comes from the manufacturer as a powder; when you boil it in water, it turns into a gel.

For a description and some pictures of how electrophoresis works, see fig. 20.9 in Campbell (8th ed.)

The procedure works like this:

Prepare an agarose gel by melting agarose in an electrophoresis buffer (a salty solution). The gel is prepared with wells, or small holes in which you can place your DNA samples.

Put the gel into an electrophoresis chamber, which is simply a shallow container with electrodes in it so you can run electricity through the liquid in the unit. Fill the unit with electrophoresis buffer.

Put your DNA samples in the wells.

Using a special electrophoresis power supply, apply a controlled voltage across the electrophoresis unit, so the current runs through the buffer and the gel.

Voila! The DNA starts to migrate through the gel. All the DNA molecules move in the same direction, but their rate of migration depends on how big they are. The small DNA molecules can easily fit through the pores in the agarose, so they move rapidly; the larger pieces are slowed down because they don't fit through the pores as well.

The speed at which molecules move through gels is controlled by:

The size of the molecule (smaller = faster).

The charge of the molecule (uncharged won't migrate; stronger charge = faster).

The voltage applied to the electrophoresis unit (more voltage = faster).

The density (or percent agarose) of the gel (lower % agarose = faster).

The type of agarose (some are denser than others).

In a single DNA gel, the difference in the rates at which various DNA bands move is all due to size. All the DNA in one gel has the same charge and is subject to the same gel density and voltage.

MOLECULAR WEIGHT MARKERS

In DNA electrophoresis, the relative rate of movement of different DNA molecules in the same gel is determined only by size. (All DNA is chemically the same, so it has the same charge per nucleotide.) Therefore, you can accurately determine the size of a piece of DNA by running it on a gel with some other pieces of DNA of known size.

Molecular weight markers are DNA fragments of known size that are used to determine the size of unknown DNA. You can buy DNA molecular weight markers from companies that sell molecular biology tools. Molecular weight markers are often made by taking some DNA and cutting it into specific pieces using a restriction enzyme.

For this lab, you're making your own molecular weight markers. We can find out the sizes of the DNA fragments by looking it up, since lambda is a commonly-used marker.

GEL DENSITY

DNA comes in all sizes, and you need to match the gel to the size of the DNA you want to look at. A dense gel has small pores, and large DNA molecules won't easily fit through those pores. For large DNA molecules, you need a less dense gel. Gel density is measured in terms of % agarose. A gel with 0.5 g agarose dissolved in 50 ml buffer is a 1% gel. For today's lab, you'll be looking at fairly long DNA molecules, so you'll use a 0.8% gel. See the appendix at the end of this manual for a table showing how much agarose to use for DNA molecules of various sizes.

SEEING DNA

DNA in solution is invisible. It doesn't reflect visible light. In order for you to learn anything from your gel, you need to use a trick to visualize the DNA. For this lab, and a few others this quarter, you'll use the most common reagent for visualizing DNA: ethidium bromide. Ethidium bromide is a dye that binds specifically to DNA. When the ethidium is bound to the DNA, it becomes highly fluorescent. If you shine an ultraviolet light on it, the DNA-ethidium complex absorbs the UV and emits bright orange visible light. This allows researchers to see DNA in gels, even in very small quantities. Ethidium bromide is handy, but it's also one of the

more hazardous chemicals you'll use this quarter.

◈ Why do you suppose ethidium bromide is dangerous? (Hint: it binds to DNA!)

WHAT YOU'RE LOOKING FOR

Uncut lambda DNA would be one long piece of DNA. It would run as a single large band on the gel. Lambda DNA that's been cut with a restriction enzyme should produce a number of bands, depending how many restriction sites there were. You should see different patterns of bands in your four reactions.

P R O C E D U R E

SAFETY CONSIDERATIONS

✋ **Ethidium bromide is a mutagen.** Ethidium binds to DNA, in a gel or in your cells. Like most things that bind to DNA, ethidium can alter the conformation of the DNA. This can lead to errors when the cell copies the DNA. Hence, ethidium is a **mutagen**, which means that it causes mutations, or changes in the nucleotide sequence of DNA. Your DNA is just fine the way it is. You don't want to mutate it. Mutagens are also often **carcinogens**; by causing changes in DNA, they increase the risk of cancer. Ethidium should be considered a potential carcinogen, though it has not been proven carcinogenic. **Do not microwave agarose containing ethidium!**

> ☠ **The dose makes the poison**
>
> Ethidium bromide is a dangerous chemical, but we will be using it at a very low concentration. The concentration of ethidium in the gel will be 500 ng/ml. The manufacturer (Sigma chemical) states that ethidium bromide is not known to be hazardous at the concentrations you'll be using in lab.
>
> Many chemicals are hazardous at high concentrations but much safer at low concentrations. Using the minimum amount of a chemical is half of lab safety. Safe handling, as described below, is the other half.

✋ **Gloves and goggles:** As always when handling liquids in this class, you need to use gloves and safety goggles. The ethidium bromide is the main reason for this. In this lab, the ethidium is incorporated into the gel, so you won't be handling it in concentrated liquid form. However, you should assume that everything that comes in contact with the gel is contaminated with ethidium bromide. Even though the concentration of ethidium bromide is low, you should still minimize your exposure. Also, the electrophoresis buffer is a potential eye irritant. **Wear gloves throughout this lab, even when handling the empty electrophoresis apparatus.**

✋ **High voltage:** The power supplies for electrophoresis supply a fairly high voltage to the buffer in the electrophoresis chamber (although at a fairly low current). The chamber is designed so that current can only flow through it when the lid is on. When the lid is on, you won't be able to stick your fingers in the buffer chamber. This eliminates most of the potential shock hazard. However, it is essential that you:

- Keep your lab bench dry – don't let spilled liquids come in contact with the electrophoresis apparatus.

- Make sure the voltage on the power supply is turned off before you plug the wires from the chamber lid into the power supply.

- Don't leave the power cord hanging off the edge of the bench where someone could trip on it.

✋ **Ultraviolet light:** you'll be using a high-intensity, short-wavelength UV light to illuminate your gel. UV light can be very bad for your eyes. Fortunately, we have a device that virtually eliminates the possibility of exposing yourself to this light. The device is a **UV transilluminator**, so called because it shines the light through the gel. The transilluminator has a lid on it that blocks all the UV but allows the visible light to come through. There is a magnetic switch on the lid that allows the light to be on only when the lid is closed. So if you open the lid when the light is on, the light automatically turns off. You don't need any additional protection from the UV light.

✋ **Hot agarose in the microwave:** you'll need to boil your agarose in its buffer to prepare the gel. Hot agarose can boil over very easily, and could burn you. **Only one flask should go in the microwave at a time** to reduce the risk of spills. Use a rubber flask gripper to protect your hand.

MATERIALS:

For each table, obtain the following:

- power supply
- electrophoresis chamber, lid, tray, dams, and comb
- P-20 pipetman, yellow pipet tips, and a beaker for waste tips
- DNA samples: your restriction digests & ligations
- one tube of gel loading buffer
- 50 ml graduated cylinder to measure buffer for making gel
- 125 ml flask to melt agarose in
- 250 or 500 ml beakers to carry TBE running buffer and for waste tips

PREPARE THE GEL:

You prepare the gel by pouring melted agarose into the gel tray. Here are the step-by-step instructions:

1. Seal the ends of your gel tray with buffer dams. This creates a place for you to pour the agarose. Place the comb in the slots near the end of the gel tray.

2. Using a 50 ml graduated cylinder, get 40 ml TBE (electrophoresis buffer) from the carboy near the sink. Pour the 40 ml TBE into a 125 ml flask.

3. Weigh out 0.32 g agarose, and dump the agarose into the flask that has the TBE. Swirl it around. (0.32 g agarose in 40 ml TBE makes an 0.8% gel, which is appropriate for the large size range of DNA pieces you'll be looking at. Percentage is calculated as grams agarose/100 ml buffer.)

4. Melt the agarose by heating the flask containing TBE and agarose in the microwave on high for 60 seconds. Watch for boilover, and handle with care. Use a flask gripper or a paper towel to handle the hot flask.

5. Your melted agarose is ready for the ethidium bromide to be added. Bring your flask to the station on the back bench and add the correct amount. [The correct amount is 2 μl of a 10 mg/ml stock solution. [Final concentration: $(2\ \mu l)(10\ mg/ml) = (40\ ml)(0.5\ \mu g/ml)$] Swirl the flask briefly to disperse the ethidium bromide.

6. Let the hot agarose cool for a minute or so, and pour the melted agarose into the gel tray. Make sure the comb is positioned properly, and the agarose isn't leaking through under the dams.

7. Wait until your gel hardens before you move it. This could take about 10 minutes. Tap the gel tray carefully to make sure the gel is firm.

8. Carefully remove the comb and the dams. Your gel is ready to go.

9. Add enough TBE buffer to cover the gel. Make sure that your gel is positioned so that the sample wells are nearest to the black electrode (anode).

LOAD THE GEL:

Loading the gel means placing the DNA samples in the wells.

DNA samples need to be mixed with a **loading buffer** before loading them on a gel. The loading buffer does three things: it colors the DNA sample so you can see it when you load it on the gel, it makes the sample dense enough to sink to the bottom of the well, and it contains a dye that migrates with the DNA so you can tell how far it has gone.

10. Mix the entire volume of each of your DNA samples with 10 µl loading buffer, and load each sample into a separate well. Also mix 2 µl of the uncut DNA sample with 10 µl loading buffer, and load this sample into a well. Load each sample into a separate well, and use adjacent wells (don't skip wells).

Be careful as you pipet the samples into the wells. The samples will be dense enough to sink to the bottom of the wells. Gently let the sample sink into the well. Don't poke the pipet tip into gel at the bottom of the well, or the sample may leak out through the hole you make. Don't overflow the wells.

✍ **Write down which sample you put in each lane!** (You may want to draw a picture so you remember which lane is which.)

RUN THE GEL:

11. Put the lid on the electrophoresis chamber. Make sure the red mark on the lid goes toward the red mark on the gel tray, and black toward black. Plug the wires from the lid into the power supply – black into black, and red into red. This ensures that you know which is the positive end of your gel and which is the negative end. Don't turn on the power supply yet.

12. **Have the instructor check your gel setup.** Then turn on the power supply and set the voltage to about 70 volts. Make sure you're looking at volts and not milliamps on the power supply.

13. **Run the gel** until the blue dye front reaches about halfway down the gel. Make sure it's running in the right direction!

14. **Turn off the power**, unplug the lid from the power supply, and take the lid off. Remove your gel and look at it.

OBSERVING AND PHOTOGRAPHING YOUR GEL

15. Get a piece of plastic wrap, large enough to put your gel on, and spread it out on your lab bench. Take your gel tray and gel out of the electrophoresis chamber, let the excess buffer run off into the electrophoresis chamber, and put the tray onto the plastic wrap.

16. Carry the gel (on its plastic wrap) over and set it on the UV transilluminator. Smooth out the plastic so it's not too wrinkly beneath the gel. Close the lid of the transilluminator and turn on the light. You should see bands on your gel. If you do, then proceed to photograph your gel.

17. Follow the instructions in **Appendix A3: Gel Photos — Using the Transilluminators and Digital Camera**. If all has gone well, you'll see your picture as bright bands on a dark background. See "interpreting your results," below. This photo will go in your lab report.

WASTE DISPOSAL AND CLEANUP

✋ **The gel and the gel buffer contain ethidium bromide, and must be treated as hazardous waste.** Wrap the gel up in plastic and throw it in the biohazard garbage can, but only after you're sure you have a good picture. Pour the used gel buffer into the container that is provided for it.

Clean your electrophoresis chamber and gel tray by rinsing them in the sink and drying them with a paper towel. **Be very careful of the tiny wires inside the electrophoresis chamber.** Put the chamber and tray back in the drawer.

All the used pipet tips go in the biohazard trash; put your used glassware on the cart.

I N T E R P R E T I N G   Y O U R   R E S U L T S

READING THE GEL

The DNA that you loaded in one well will run in one **lane**. Each lane may contain one or more **bands**.

You'll probably be able to see your DNA bands better in the Polaroid than in the gel itself. That's because the film is very sensitive and gives a very high-contrast image. Take a look at your picture. It's conventional to orient gel pictures with the wells at the top. That way, it's easy to compare different gels.

The picture at right shows one lane of a gel with **Lambda DNA cut with Hind III.** The numbers on the side of the picture refer to the size of the DNA fragments in numbers of nucleotides, or base pairs (bp).

Note that the largest DNA fragments are at the top, closest to the well; they migrate slowest.

Also note that the highest molecular weight bands are the brightest; you can see them better because there's more DNA there. (There are equal numbers of molecules of all the fragments, but the bigger fragments have more DNA). In this picture, you can't see the smallest bands. The same thing may be true on your gel.

Finally, note that the top band in the picture is slightly smeared. This is what happens when you try to force large DNA molecules through small pores in the gel at high voltage. Larger pieces of DNA need to be run in gels with a lower percentage of agarose and at lower voltages. The conditions for running the gel need to match the size of the DNA you're looking for.

One lane of your gel should have a molecular weight marker such as the lambda/Hind III shown above. The other lanes will have your digest and ligation samples along with uncut lambda DNA.
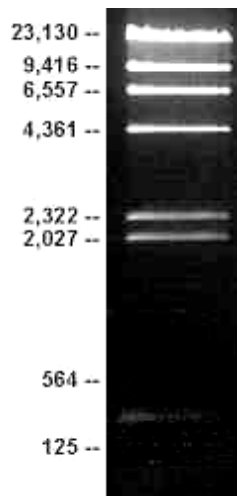
DETERMINING THE SIZE OF A BAND

If you know the sizes of the bands in one lane, you can determine the size of a band in another lane. If your unknown band ran at exactly the same speed as the 4,361-bp band of lambda/Hind III, then it's the same size. If your unknown band is in between two bands, you can interpolate.

Most people usually just guess at the sizes of their bands, after comparing with the appropriate molecular weight marker. However, you could get out a ruler and measure if you want to be more precise. If you graph the distance each band migrated, you'll find that the rate of migration is proportional to the log of the fragment size in base pairs. If you plot it out on semilog graph paper, you'll get a straight line. Then it will be easy to determine the exact size of your unknown band. However, you don't need to do that today.

SOME QUESTIONS TO THINK ABOUT

◇ Why does agarose gel electrophoresis separate DNA molecules only by size?

◇ Why is electrophoresis with DNA easier than electrophoresis with proteins? For example, with SDS-PAGE (proteins) you had to treat your sample with a special buffer and heat it before you loaded it on the gel; with DNA, you can skip that step. Why?

◇ In what situation would you want to make a gel with a higher percentage of agarose?

◇ What would happen if you reversed the leads when you plugged your gel into the power supply?

◇ What would happen if you accidentally used pure water instead of gel buffer?

L AB R EPORTS

See Appendix A4, "Writing your lab reports", pp. 121-123, for general instuctions. Your lab report should cover the restriction digests, ligations, and electrophoresis. It should include:

INTRODUCTION

Start with a *descriptive* title. The title should state clearly what the paper is about. Also include a list of authors (your lab group members) with your lab section and group number.

The Introduction section should progress from general background to specific details. Begin with an opening paragraph stating what you are doing and what is the objective of these experiments. Then proceed to describe how you intend to accomplish these objectives. For this lab, include a brief explanation of restriction enzymes, restriction sites, sticky ends, ligation, electrophoresis, and RFLP (restriction fragment length polymorphism). Conclude the Introduction with <u>specific</u> predictions of your expected results, and <u>how</u> these results will answer your questions or support/refute your hypotheses.

Keep your Introduction focused on the objectives. <u>Be concise!</u> The Introduction should only be ~1 page long.

METHODS

Don't write out the whole protocol; just refer to the lab manual. Instead, draw a flow diagram showing all three parts of the lab and how they connect with each other (e.g., the DNA goes from the restriction digest to the ligation to the gel). You don't need to show the amounts; just show what goes into each tube. Indicate how the data in the Results section were obtained. <u>The flow diagram must fit on one page.</u>

RESULTS

This section includes only what you saw, not what you think it means. Experimental results are usually presented in the form of tables or figures labeled with appropriate captions and keys.

For this lab, your data is on the gel, so your Results figure should be an image of the gel. Insert a high-resolution scan of the gel, or tape the gel photo to the middle of a page, and label what's in each lane.

DISCUSSION

This section is a discussion of what your results mean. The discussion format is typically the reverse of the introduction format: begin with specific details about your results and end with a general concluding statement.

Start with a lane-by-lane discussion of your gel. Did your DNA get cut? Did it get ligated? Finish with an overall summary. Did it all come out as you expected? If not, why?

Refer to your Introduction statements: Was your hypothesis supported or refuted? How strong is the evidence? Finish with a summary comment regarding the overall accomplishment of the stated objectives.

CONJUGATION
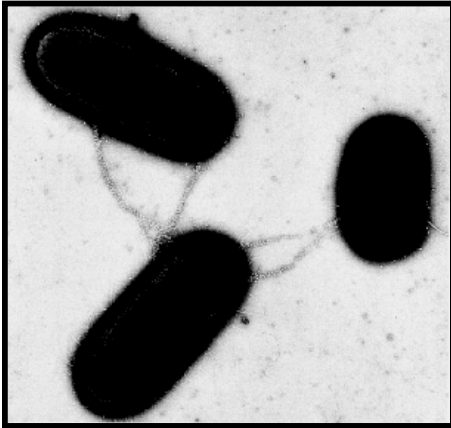
Bacteria exchanging DNA. These cells are performing **conjugation**, in which a copy of a small DNA molecule known as a **plasmid** is passed from one cell to another. The lines extending between the cells are **pili**, which are tubes that connect the cells so DNA can be moved from one cell to another.

In this lab, you'll attempt to make some bacteria become resistant to an antibiotic by allowing them to take up a plasmid containing an antibiotic resistance gene.

After completing this lab, you should understand:

- how antibiotic resistance works
- how bacteria do genetic recombination
- plasmids and their biology

INTRODUCTION:

Bacteria aren't like us. They don't have sex. Virtually all eukaryotic organisms have a sexual phase somewhere in their life cycle. This means that they have a diploid phase (two copies of each chromosome) and a haploid phase (one copy of each chromosome), and they have a mechanism for joining haploid cells (eggs and sperm) to make new diploid combinations of chromosomes. Prokaryotes usually have only one chromosome, and they have only one copy of it per cell. Therefore, they cannot perform sexual reproduction like eukaryotes do.

However, this doesn't mean that prokaryotes are stuck with forever-unchanging DNA. They have several mechanisms of mixing up their DNA with DNA from another individual (which is, after all, the most significant outcome of sex).

Conjugation: plasmid DNA in one cell is copied and a copy is transferred from one cell to another through pili, as shown in the picture above. This is the subject of today's lab.

Transduction: DNA is introduced into a cell by a bacteriophage. You'll do this in another lab this quarter.

Transformation: a cell takes up naked DNA from the environment. Under specific conditions, prokaryotic cells can take up naked DNA from a closely related cell. (The cells are able to recognize DNA from their own or a closely related species.) In the lab, it's possible to induce cells to take up all kinds of DNA from their environment. You'll also do this later in the quarter.

CHROMOSOMES AND PLASMIDS

Bacteria have a single chromosome. Like eukaryotic chromosomes, a prokaryotic chromosome consists of a DNA double helix with protein bound to it. Prokaryotic chromosomes are different from those of eukaryotes in several important ways, including:

Prokaryotic chromosomes form a large ring, or circular DNA molecule (eukaryotic chromosomes are linear).

Prokaryotic chromosomes have much less protein attached to them.

Prokaryotic chromosomes are attached to the plasma membrane, instead of floating free in a nucleus. This fact will allow you to separate the chromosomal DNA of bacteria from other DNA in the cell.

Prokaryotic chromosomes are smaller than those of eukaryotes.

Prokaryotes (and some eukaryotes) sometimes have a small "extra" DNA molecule, in addition to the chromosomal DNA. One type of extra DNA is a plasmid. Plasmids are double-stranded, circular DNA molecules, typically about 2,000-4,000 nucleotide pairs or base pairs (2-4 kilobases, or kb) long. They're just big enough to hold a few genes.

Not all bacterial cells have a plasmid, but in some cases a plasmid may be essential for a cell's survival. This is often the case when a plasmid carries a gene for antibiotic resistance.

ANTIBIOTICS AND ANTIBIOTIC RESISTANCE

Antibiotics are chemicals that kill bacteria or prevent them from growing. Most antibiotics work by blocking enzymes or biochemical processes that are specific to prokaryotes. Some bacteria are resistant to specific antibiotics because they can make a protein that destroys the antibiotic or prevents it from acting on the cell. Such proteins are encoded by antibiotic resistance genes.

Antibiotic resistance genes are often carried on plasmids, with the result that these genes can be copied from cell to cell by conjugation. In this lab, you'll indirectly observe the transfer of an antibiotic resistance gene from one bacterial strain (or type) to another.

HOW THE EXPERIMENT WORKS

You'll be presented with two strains of *Escherichia coli*, or *E. coli*:

cI – resistant to streptomycin

cII – resistant to ampicillin

In one strain, the antibiotic resistance gene is carried on the chromosome; in the other, it's on a plasmid.

The plan of the lab goes like this:

Allow the two strains to conjugate. The plasmid will be transferred, resulting in a new bacterial strain that is resistant to both ampicillin and streptomycin.

Culture the cells with both antibiotics. Cells will only grow if antibiotic resistance has been transferred from one strain to another.

Day 1:

Experiment A: Conjugate the two strains. Plate the cI, cII, & cI+II bacteria with and without different antibiotics to verify if conjugation occurred.

Experiment B: Plate serial dilutions of the cI+II culture with and without antibiotics to estimate the efficiency of conjugation.

Experiment C: Grow liquid cultures of the cI, cII, & cI+II bacteria for DNA analysis.

Day 2:

Exp. A: Observe the qualitative plates for bacterial growth.

Exp. B: Count colonies on the quantitative plates. Calculate conjugation efficiency.

Exp. C: Prepare lysates of the cI, cII, & cI+II bacteria.

Day 3:

Exp. C: Run an electrophoresis gel to look for plasmid DNA.

Safety Considerations:

✋ **Bacterial cultures.** The bacteria you'll use for this lab are *Escherichia coli*, or *E. coli*. This species is a normal resident of every human intestine. Some strains of *E. coli* can cause human disease. The *E. coli* strain used in this lab does not cause disease. However, like all bacteria, it must be handled with care. Even strains that are not pathogenic can be harmful in large quantities. Bacteria evolve rapidly, and it may be possible for harmless bacteria to become harmful. **Always use sterile technique when culturing bacteria.**

DNA hazards? You will be working with recombinant, or genetically modified, DNA (the plasmid) during this lab. In some cases, recombinant DNA is subject to federal safety restrictions, administered by the National Institutes of Health. The DNA you'll be working with is not subject to these restrictions because it contains no human or animal DNA fragments or DNA from a pathogen that can infect humans. The DNA in this lab poses no hazard.

Procedure:

✋ **Wear gloves and goggles throughout this lab.** You'll need to protect yourself from your cultures and protect your cultures from yourself.

Materials:

For each table, obtain the following:

- pipetmen, blue tips, and a beaker for waste tips
- rack for glass culture tubes
- three sterile glass culture tubes
- a Bacti-cinerator
- an inoculating loop
- one LB plate with no antibiotics (LB="Luria-Bertoni" nutrient medium)
- one LB plate with ampicillin
- one LB plate with streptomycin
- one LB plate with ampicillin and streptomycin
- tubes of cI and cII bacterial strains (the instructor will hand these out)

Conjugation protocol:

This protocol is taken from: Kreuzer, H. and A. Massey, 1996. *Recombinant DNA and Biotechnology: A Guide for Teachers*. Washington, D.C.: American Society for Microbiology.

The bacteria will do most of the work in this lab. All you need to do is put the two strains together, allow them to conjugate, and plate them out.

1. Before you start, make sure you're clear on sterile technique. Clean off your work space. Wear gloves and safety glasses.

2. Label your 3 culture tubes cI, cII, and cI+cII (use tape; don't write on tubes or caps).

3. Pipet 1 ml of cI cells into the cI tube, 1 ml of cII cells into the cII tube, and 0.5 ml of each strain into the cI+cII tube. Put the lids back on the tubes.

4. Let the tubes sit at room temperature for approximately 20 minutes. The bacteria in the cI+cII tube will conjugate.

5. While you wait, label the bottoms of all 4 of your plates like this:
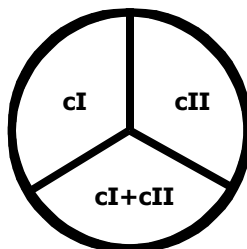
Also write your initials and the date on each plate.

When 20 minutes has passed, it's time to plate out the cells.

6. Using the inoculating loop, transfer some cells from the cI tube to the cI area of each of the four plates. Be very careful to keep each strain in its designated area of the plate. Do the same for the cII and cI+cII tubes. You should end up with four plates, each with three different strains or strain combinations.

7. Put the plates in the 37°C incubator until next lab period.

PREDICTED RESULTS

✍ You'll see your results in the next lab period. For now, write down your *expected* results (**+** for growth, **−** for no growth).

	NO ANTI	AMP	STREP	AMP+STREP
cI	_____	_____	_____	_____
cII	_____	_____	_____	_____
cI+cII	_____	_____	_____	_____

ESTIMATION OF CONJUGATION EFFICIENCY

Use the CI+CII mixture you prepared today in the previous section.

ADDITIONAL MATERIALS:

- Five sterile culture tubes
- Vortexer
- Disposable sterile spreaders
- Plate spinners
- Liquid LB medium
- Three LB plates
- Three LB plates with ampicillin and streptomycin

1. Aseptically add 0.9 ml LB medium to the five sterile culture tubes.

2. Do a 10-fold serial dilution of the CI+CII *E. coli* mixture to 10^{-5}x:

    a. Aseptically pipette 100µl of the CI+CII mixture into the first tube containing 0.9ml LB.

    b. Mix thoroughly (= 10^{-1}x dilution).

    c. Pipette 100µl of the 10^{-1}x dilution into the next tube containing 0.9ml LB.

    d. Mix thoroughly (= 10^{-2}x dilution).

    e. Continue serial dilution for the remaining three tubes (=10^{-3}x; 10^{-4}x; & 10^{-5}x).

3. Transfer & spread 100µl of the 10^{-1}x dilution onto a LB-agar plate.

    a. Aseptically pipette 100µl of the diluted culture across the plate.

    b. Use the sterile spreader or loop to spread the mixture over the surface of the agar. Try not to get too close to the edge of the plate.

4. Transfer & spread another 100µl of the 10^{-1}x dilution onto a LB/Amp/Strep-agar plate.

5. Repeat step 3 to plate 100µl of the 10^{-3}x and 10^{-5}x dilutions onto separate LB and LB/Amp/Strep-agar plates.

You should have six new plates:
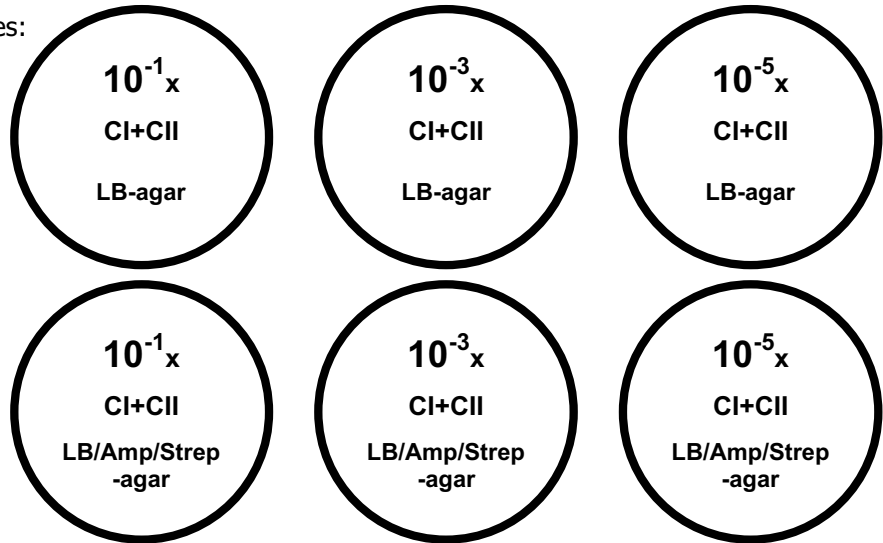
6. Incubate plates overnight in the 37°C incubator.
    Note the date & time.

S T A R T I N G   L I Q U I D   C U L T U R E S

To grow large numbers of bacteria, we need to culture them in liquid media rather than on plates. Use the tubes of cI strain, cII strain, and the cI+cII mixture you prepared today in the first section.

ADDITIONAL MATERIALS:

- Disposable sterile pipettes
- Liquid LB medium
- Stock solutions of streptomycin and ampicillin

LIQUID CULTURE PROTOCOL:

1. Aseptically add an additional 2 ml LB medium to the tube with the cI *E. coli*, 2 ml LB to the tube with the cII *E. coli*, and 2.1 ml to the tube with the cI+cII mixture.

2. Add 15 µl streptomycin to the cI culture tube. (cI should be strep-resistant.)

3. Add 15 µl ampicillin to the cII culture tube. (cII should be amp-resistant.)

4. Add 15 µl streptomycin *and* 15 µl ampicillin to the cI+cII culture tube. (Only the conjugated bacteria in this mixture should be should be resistant to both antibiotics.)

5. Cap the tubes firmly but not air-tight. Vortex gently.

6. Each tube should be clearly labeled on the tape with the contents, your lab section and group identification, and today's date. Place all three tubes in the rack in the 37°C <u>shaking</u> incubator until next class.

W A S T E   D I S P O S A L   &   C L E A N - U P

Used microfuge tubes and pipet tips go in the biohazard trash.

Gloves, paper towels, etc. go in the regular trash.

Glassware, including culture tubes and the beaker you used for waste pipet tips, goes in a cart or tub provided by the instructor; it will be autoclaved, cleaned and reused. Don't dump out any bacterial cultures in glass tubes. Please remove any tape or other markings from glassware.

SAFETY CONSIDERATIONS

✋ **Bacterial cultures. Always use sterile technique when handling bacterial cultures.**

✋ **Wear gloves and goggles throughout this lab.**

MATERIALS

- Your conjugation plates from last time

METHOD

After your plates have incubated overnight, you can see which cultures grew on each plate. That's your result from the conjugation. Count the number of colonies if you can, or otherwise note which cultures grew. Write your data in the table below. (0/+/++/+++)

	NO ANTI	AMP	STREP	AMP+STREP
cI	_____	_____	_____	_____
cII	_____	_____	_____	_____
cI+cII	_____	_____	_____	_____

1. Use sterile technique. Clean your table before beginning and use gloves and goggles.

2. Get your plates and look at them. (Save them for the liquid cultures later today.)

✍ Make sure you've looked at the colonies on your plates and recorded the results in the table above. If you can't count colonies, just describe what you see (big streak, too many to count, etc.). Do your results match your expectations? That's one of the key questions for the lab report.

C ONJUGATION E FFICIENCY R ESULTS

METHOD

1. Count the number of bacterial colonies on each "countable" plate. (Ideally with 30–300 colonies.) Record the number of colonies in the table below:

	10^{-1}x dilution	10^{-3}x dilution	10^{-5}x dilution
LB-agar			
LB/Amp/Strep-agar			

Since each colony on the plate represents one bacterial cell (colony-forming unit [cfu]) in the **100µl** of culture you placed on the plate, the concentration of bacteria in the original CI+CII tube = **(cfu/ml) = (# of colonies/0.10 ml) x (1/dilution factor)**

For example: if you count 139 colonies on the 10^{-3}x dilution plate, then the concentration of bacteria in the original culture tube was $(139/0.1)x(1/10^{-3})$= 1,390,000 cfu/ml.

The colonies on the LB plates without antibiotics represent all the bacteria in the CI+CII tube (CI cells, CII cells, and CI/CII-conjugated cells).

✍ Using the number of colonies on the LB plates, <u>calculate</u> the concentration of all bacteria in the CI+CII tube culture from last lab:

The colonies on the LB/Amp/Strep plates with represent **only** the **conjugated** bacteria in the CI+CII tube that have acquired double-antibiotic-resistance.

✍ Using the number of colonies on the LB/Amp/Strep plates, <u>calculate</u> the concentration of **conjugated** bacteria in the CI+CII tube culture:

✍ What fraction of the bacteria in the CI+CII tube culture were conjugated?

✍ How do your estimates of conjugation efficiency compare with those of the rest of the class?

✍ Under ideal culture conditions, *E. coli* can divide every twenty minutes. Assuming that our conditions are ideal, how many generations have these bacteria on your plates undergone since you plated them?

✍ Given this number of generations, about how many bacteria are in each colony on your plate descended from each original cfu? (2^n, where n=# of generations)

C E L L   L Y S I S   &   P L A S M I D   P U R I F I C A T I O N

Purifying DNA is one of the key methods of molecular biology. In this lab, you'll purify plasmid DNA from your liquid bacterial cultures. This method takes advantage of two facts about nucleic acids and bacteria:

First, bacterial chromosomal DNA is attached to the plasma membrane. If you lyse bacterial cells, the chromosomal DNA will remain attached to the insoluble complex of membrane and cell wall. Meanwhile, the plasmid DNA will be freely dissolved, because it isn't attached to anything.

Second, DNA is highly soluble in water, both plasmid and chromosomal DNA. So we should be able to extract the chromosomal DNA from the insoluble membrane complex too. You'll start with a small amount of bacterial culture broth from each of the three liquid cultures, and – with luck and good technique – end up with enough DNA that you can see it on a gel.

The first step is to wash the bacterial cells from the culture medium. Next we weaken the cell walls so the bacteria will lyse — the aqueous fluid released from the lysed cells is called the **soluble lysate**. Separate the soluble lysate containing the plasmid DNA from the insoluble membrane complex containing the chromosomal DNA by centrifugation. Pipette off the liquid as your **plasmid fraction**. Then resuspend the pellet from the bottom of the tube in warm pure water and centrifuge again. Now this liquid phase should be your **chromosomal fraction**.

MATERIALS

- Your three tubes of liquid bacterial cultures: cI, cII, & cI+cII
- 9 microfuge tubes (1.5 ml)
- Cell Resuspension Solution
- Cell Lysis Solution
- Neutralization Solution
- Alkaline protease solution
- Sterile water
- Heat block at 80°C

METHOD – PERFORM SEPARATELY FOR ALL THREE BACTERIAL CULTURES

1. Pipet 1.5 ml of bacterial culture into a microfuge tube. Pipet the thick gob of stuff at the bottom of the culture tube to get as many cells as possible.

2. Spin at 14,000 rpm (high speed) for 5 minutes to pellet cells.

3. Pour off supernatant.

4. Add 250 µl of Cell Resuspension Solution and completely resuspend the cell pellet by pipetting or vortexing. Be sure the whole pellet gets resuspended – don't leave any chunks. *(You can mix vigorously at this point because the DNA is still inside the cells.)*

5. Add 250 µl of Cell Lysis Solution and mix by inverting the tube 4 times. Incubate at room temperature until cell suspension clears somewhat (about 1-5 minutes). *(It's important to see some clearing of the solution, but don't incubate more than 5 minutes. Mix gently at this point because the cells have lysed and the DNA is free in solution. DNA molecules are so long that they can be damaged by excessive pipetting or mixing.)*

6. Add 10 µl Alkaline Protease Solution and mix by inverting the tube 4 times. *(This destroys enzymes that might damage the DNA.)*

7. Add 350 µl of Neutralization Solution and mix by inverting the tube 4 times.

8. Spin the lysate for 10 min at 14,000 rpm.

9. Get your lysate tubes from out of the centrifuge; you should now see a cleared soluble lysate (the supernatant) and some insoluble material (a white precipitate). Transfer 800 µl of the supernatant into a new, labeled microfuge tube — this is the **plasmid fraction**. Be sure not to transfer over any of the pelleted precipitate.

10. Add 500 µl sterile deionized water to the pellet in the original microfuge tube. Gently resuspend the precipitate by inverting the tube a few times.

11. Place the tubes with the precipitates in the 80°C heat block for five minutes.

12. Mix again gently. Centrifuge for 10 min at 14,000 rpm.

13. Pipette 500 µl of the supernatent into a new, labeled microfuge tube — this is your **chromosomal fraction**.

14. You should now have six clearly-labeled microcentrifuge tubes of DNA samples: plasmid fractions of cI, cII, and cI+II; and chromosomal fractions of cI, cII, and cI+II. Save these in the freezer until next lab period for electrophoresis.

WASTE DISPOSAL & CLEANUP

Used plates, microfuge tubes and pipet tips go in the biohazard trash.

Gloves, paper towels, etc. go in the regular trash.

Glassware, including the beaker you used for waste pipet tips, goes in the tub. Don't dump out any bacterial cultures that are in glass tubes. Please remove any tape or other markings from glassware.

THIRD LAB PERIOD: ELECTROPHORESIS

To determine which of our bacterial cultures have plasmids, we will perform DNA electrophoresis of the six samples prepared above.

1. Prepare a 0.8% agarose gel with 0.5 μg/ml ethidium bromide again, using the same procedure you already mastered in the "Cutting DNA" lab (pp. 18–21).

2. Remove your plasmid fraction and chromosomal fraction samples from the freezer. Thaw and mix gently. Measure the DNA content of each using the method in Appendix 2.ii.

3. This time, for each of the three plasmid fraction samples and the three chromosomal fraction samples: take 15 µl of DNA sample and add 5 µl of DNA Loading Buffer. Load all 20 µl into a sample well on the gel. [Draw the sequence of samples actually loaded below.]

4. Also take 15 µl of HinDIII-cut λ-DNA plus 5 µl DNA Loading Buffer and load it into the seventh sample well as a standard. (See Appendix 4.)

5. Run the gel electrophoresis and examine the gel results as done in the "Cutting DNA" lab.

✍ Are you able to determine if the cI+II conjugate acquired a plasmid from cI or cII?

✍ Can you discern if the streptomycin-resistance or ampicillin-resistance is carried on a plasmid or on the chromosome?

This report includes conjugation success and efficiency. The overall report format described in Appendix A4, "Writing your lab reports", should be followed (pp. 121-123).

INTRODUCTION

Include a brief explanation of what conjugation is. Define plasmid and discuss the biological significance (cost/benefit) of bacterial cells gaining or losing plasmids.

Explain why there is a significant evolutionary difference between having an antibiotic resistance gene on the chromosome and having it on a plasmid.

Describe how you "observed" conjugation with respect to the hypotheses being tested:

- H_1: If conjugation occurred and antibiotic resistance was transferred, then …

- $H_{0'}$: If conjugation did not occur, then …

- $H_{0''}$: If conjugation occurred but antibiotic resistance was not transferred, then …

- H_{2A}: If conjugation was fast/frequent, then …

- H_{2B}: If conjugation was slow/infrequent, then …

- H_3: If conjugation resulted from plasmid transfer, then, on the gel, …

METHODS

Include a flow diagram showing all the main procedures for the lab. Be sure to note anything you did that's different from what's in the lab manual. Simplify the flow diagram so it fits on one page. Make sure all links to Results are included.

RESULTS

This section should include:

- Table 1 showing the number of colonies on each section of each plate for your initial conjugation.

- Table 2 with the number of colonies on each plate for your conjugation efficiency results.

- Table 3 with the class data for the conjugation efficiency results.

- Table 4 with the "Conjugation Variants" results — cite source.

- Figures of gels or plates are optional, but must be formatted correctly

DISCUSSION

Did this experiment demonstrate that DNA was transferred from one cell to another? Did it demonstrate that the DNA conferred antibiotic resistance on the cells that received it? What other experiments might you do to be more sure of what is happening at the DNA level?

If conjugation did occur, how efficient was the process? Did your results differ significantly from the class data? Why do you think conjugation is so uncommon in bacterial populations?

Were you able to determine which antibiotic resistance gene is carried on the plasmid?

How would you design an experiment to be sure which culture was the donor, and which was the recipient? How did the "Conjugation Variants" experiment use the conclusion from the conjugation efficiency experiment to address this question?

Transformation with pGLO

plasmid from a prokaryote

gene from a eukaryote

cloned eukaryotic gene
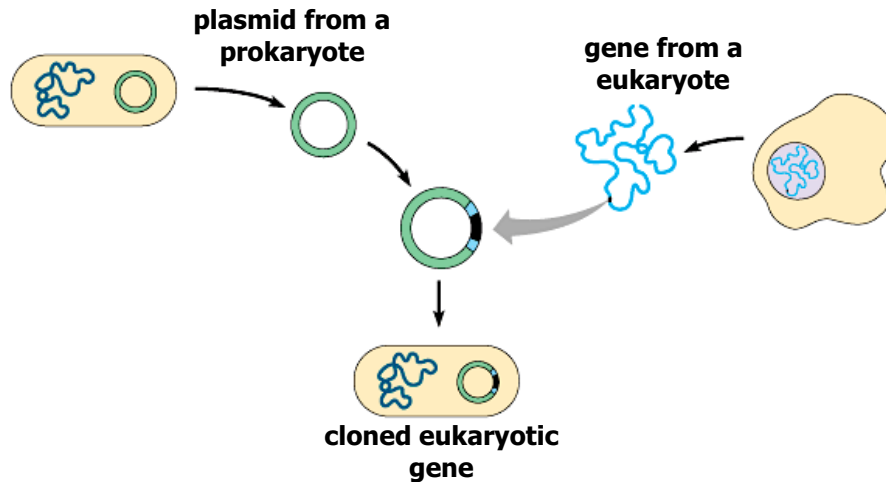
In this lab, you'll use a plasmid to insert a gene for "green fluorescent protein" into *E. coli* cells. If it works, you'll get fluorescent bacteria!

By the time you finish this lab, you should understand:

- What a plasmid is
- Transformation as a way of getting new DNA into a cell
- Using antibiotic resistance to select for transformed cells
- Operons as units of gene expression in bacteria
- What recombinant DNA is
- How to clone a gene and express and purify the protein encoded by that gene

INTRODUCTION

In the conjugation lab, you saw that bacteria can pass genes along by copying a plasmid from one cell to another. In that lab, one bacterial strain gained a gene for an antibiotic resistance protein by gaining a copy of a plasmid from another strain.

In this lab, you'll work with a plasmid again, but with a couple of key differences. First, instead of the plasmid being passed from one cell to another by conjugation, this time you'll cause the bacterial cells to take up the plasmid DNA directly from their environment, which is called **transformation**. Second, instead of seeing only the effect of the antibiotic resistance gene, in this lab you'll also see the effect of another gene carried on the plasmid – the gene for **green fluorescent protein** (**GFP**). The GFP gene codes for a protein that is green and fluorescent; if you shine an ultraviolet light on a colony of bacteria that contain this protein, you'll see the colony glowing brightly. You'll be able to see which colonies are expressing the GFP gene. This will also give you a chance to learn something about how bacterial cells regulate gene expression.

The plasmid for this lab is called **pGLO**. It's a recombinant plasmid made by the Bio-Rad Corporation. A **recombinant plasmid** is one that has a new combination of DNA pieces. For pGLO, the people at Bio-Rad inserted the GFP gene into an existing plasmid, replacing another gene.

OPERONS CONTROL GENE EXPRESSION

The pGLO plasmid was engineered to allow inducible expression of GFP. The cells don't always make GFP just because they have the gene; instead, you can induce expression of this protein by changing the cells' environment. The key to this is that the GFP gene is part of an **operon**. An operon is a bacterial gene, including the part of the gene that codes for a polypeptide and the part that helps control when this polypeptide gets made. The **arabinase operon** is one example. "Arabinase" refers to a set of enzymes that break down the sugar arabinose. Arabinose is not common, so most of the time the cells won't encounter it and don't need the enzymes to break it down. However, when arabinose is present, the cell can turn on the arabinase genes. This is the essence of gene regulation: turn on a gene when you need it, and turn it off when you don't. The function of operons is covered in detail in Chapter 18 of Campbell.

For now, you should understand several elements of the pGLO plasmid:

- The **G F P coding region** (**gfp**) – the part of the gene that actually codes for the Green Fluorescent Protein (GFP).

- The **pBAD arabinose-dependent operator/promoter**, a short stretch of DNA just before the coding region of the arabinase gene. The promoter is where RNA polymerase binds to the gene to start transcription – in other words, it's where gene expression begins. The promoter is the key spot where gene expression is turned on and off.

- The **araC gene**, which codes for a protein that acts as an arabinose receptor and a repressor transcription factor. This is how the *pBAD* promoter is arabinose-dependent.
  In the absence of arabinose, AraC binds to the operator, blocking the promoter from RNA polymerase and thus *repressing* (turning off) gene expression. If arabinose is present, it binds to AraC to prevent it from binding the operator, so RNA polymerase can get to the promoter. Thus arabinose *induces* (turns on) arabinase gene expression by removing the repressor from the operator. The *araC* gene is always on: it has its own promoter, so it doesn't depend on the arabinose-dependent promoter.

- **bla** — A gene that encodes the enzyme beta-lactamase, which breaks down the antibiotic ampicillin. Thus, a bacteria that has this gene is ampicillin-resistant.

- **ori** — the origin of replication. DNA replication starts here.

- Size: the pGLO plasmid is 5371 bp. The GFP coding region is about 800 bp, and the GFP protein is about 25 kD.
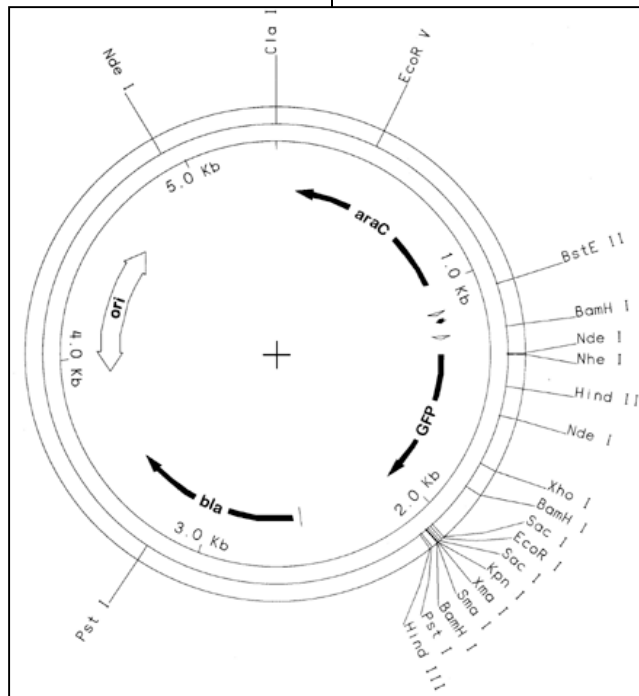
In the wild ancestor of the pGLO plasmid, the arabinase promoter controlled the expression of genes coding for arabinase proteins. In pGLO, the arabinase coding regions have been replaced with the GFP coding region. The pBAD arabinase promoter is still there, and it still turns on a gene when arabinose is present, but now it turns on a *gfp* gene instead of arabinase genes.

Add arabinose, and you get fluorescent bacteria.

WHY DID THEY CLONE GREEN FLUORESCENT PROTEIN?

The plasmid pGLO is a by-product of research on the control of gene expression. The gene for GFP originally comes from the jellyfish Aequoria victoria. This remarkable protein has turned out to be useful in research, and the people at Bio-Rad have tweaked it to make it even more fluorescent. GFP has been used in a number of studies to help pinpoint where specific genes are expressed in various organisms. The GFP gene is used to study promoter function. Suppose you find a gene, and you want to figure out what it does. One of your first questions would be, where is it expressed? If you insert the GFP gene next to the promoter for the gene you're studying, you'll see glowing cells wherever this promoter is active. It's a powerful technique –and harder than it sounds.

USING RECOMBINANT PLASMIDS TO MAKE PROTEIN

In this lab, you'll induce some bacterial cells to make green fluorescent protein. Later you'llbe able to recover this protein from the cells. This is how biotech companies manufacture cloned proteins. If you wanted a large quantity of GFP, you could either grind up a lot of jellyfish, or get *E. coli* cells to make the protein for you. If you control the operon, you can get the bacteria to make a lot of GFP, so it's a very efficient method for making protein. This approach is called **in vivo** ("in life") protein synthesis, because it happens inside live cells. The alternative would be an **in vitro** ("in glass") approach, in which you synthesize the protein in a test tube without the benefit of living cells. At this time there is no effective *in vitro* method for producing large amounts of protein.

Method

This lab has several parts; you'll complete it over <u>seven lab periods</u>.

Day 1: Transform *E. coli* cells with pGLO plasmid and plate out the cells.

Day 2: Look at the plates to see if your transformation worked (that is, are your bacteria fluorescent?). Pick some transformed colonies and transfer them to liquid cultures.

Days 3 & 4: Purify green fluorescent protein from the cells in the liquid culture. Analyze the expressed proteins by electrophoresis.

Day 5: Purify the plasmid from the transfected bacterial cells.

Days 6 & 7: Perform a restriction digest of the plasmid DNA and analyze the restriction fragments by electrophoresis.

MATERIALS

- 1 tube containing 10µl of pGLO plasmid (80 ng DNA/µl)
- 8 sterile LB Plates:
    - 1 plain LB
    - 2 ampicillin
    - 2 arabinose
    - 2 arabinose/arabinose
    - 1 ampicillin/arabinose/glucose
- 1 "starter plate" with *E. coli* colonies (this will be your source of bacteria to transform)
- 1 tube of $CaCl_2$ transformation solution
- 2 1.5-ml microfuge tubes
- 2 plastic sterile loops (not the usual metal ones)
- 2 sterile culture tubes
- LB broth liquid medium
- Plate spinner & sterile spreaders
- Pipetmen, tips, beaker for waste tips
- Make sure there is a heat block set at 42° C before you start

METHOD OVERVIEW

You'll take a colony of bacteria, add some pGLO plasmid, then heat shock the cells to make them take up the plasmid. This process is called transformation. Meanwhile, as a control, you'll do the same thing with cells but no plasmid. Then you'll spread the contents of these two transformation tubes onto plates, as shown below:

	Plates			
#	**amp**	**arabinose**	**Cells**	**expected results**
1	–	–	**pGLO–**	Lots of growth; no glow.
2	+	–	**pGLO–**	Amp but no plasmid: shouldn't grow.
3	–	+	**pGLO +**	Lots of growth. Some colonies contain plasmid; many don't. Colonies with plasmid glow.
4	+	–	**pGLO +**	Some growth, but no glow.
5	+	+	**pGLO +**	Same growth as plate 4, but all glow.
6	+	**+ + glucose**	**pGLO +**	Similar to plate 5, but less intense glow.
7	+	+	**10^{-4}x pGLO +**	"Countable" version of plate 5.
8	–	+	**10^{-4}x pGLO +**	"Countable" version of plate 3.

Using this set of treatments should allow you to find out if the cells are alive, if they get transformed, and if arabinose can induce the expression of GFP in the transformed cells. Each of the treatments is designed to answer a specific question. On an exam, you may see some hypothetical plate results and be asked to interpret them. For example, how will you know if the transformation worked? Would you be able to tell even if you didn't see fluorescent colonies?

TRANSFORMATION PROTOCOL

✋ **Read the whole method before you start.** There are critical time and temperature steps, and you must have everything ready before you start.

1. Label your 2 1.5-ml microfuge tubes + (for + pGLO) and – (for – pGLO). These are your transformation tubes.

2. Pipet 250 µl CaCl$_2$ transformation solution into each tube.

3. Use a plastic (not metal) sterile loop to transfer a single colony of bacteria from your starter plate into the (+) tube. Spin the loop in the tube until the colony is completely dispersed – no chunks. Repeat this procedure with a new sterile loop, the (–) tube, and another colony.

4. Add 10 µl pGLO plasmid solution to the (+) tube.

5. Incubate both tubes on ice for 10 minutes.

6. While your tubes are on ice, label your 5 plates according to the table above. Also make sure that you have a heat block ready at 42° C (+/-- 2° C) for the next step.

7. Place both transformation tubes in the 42°heat block for 50 seconds.

8. When your 50 seconds are up, transfer your transformation tubes immediately back to the ice. Incubate on ice for 2 minutes.

9. Add 250 µl LB broth to each transformation tube.

10. Incubate both tubes at room temperature for 30 minutes.

11. Stir the transformation mix gently with a pipet tip, then pipet 100 µl of the transformation mix onto the plates as shown in the table above.

12. Use a sterilized spreader or inoculation loop to spread the transformation mixes on the plates, then put your plates in the incubator until next time.

QUESTIONS

You might want to think about these questions to help you understand this lab better – and to prepare for a quiz. You should also address these questions in your lab report Introduction.

◇   What is the purpose of the ampicillin?   What is the purpose of the arabinose?

◇   What do these things do in this experiment: operon, promoter, coding sequence?

◇   Suppose you don't get any colonies on plates 4–7. How would you explain this? Do you think the colonies on plate 3 have pGLO?

◇   Suppose you get plenty of colonies on all eight of your plates. How would you explain this?

◇   Suppose you get colonies on all plates as expected, but none of the colonies are fluorescent. What do you think happened? How could you test your idea?

◇   Why should you expect the same number of total colonies on plates 4, 5, & 6? What would be a likely reason if the numbers are different?
    Why expect their fluorescence to be different even if their numbers are the same?

◇   What is the minimum set of plates you'd have to look at to know if the transformation worked?

◇   We are testing the hypotheses that arabinose induces *gfp* expression. What alternative hypothesis would also be consistent with the above predicted results?

pGLO 2 (Next Lab Period): record results, start liquid cultures

13. Check your plates to see if they have colonies, and if the colonies are fluorescent under UV light. Write your results in the table below.

Plates				actual results	
#	amp	arabinose	Cells	total # colonies/plate	# flourescent colonies/plate
1	–	–	pGLO–		
2	+	–	pGLO–		
3	–	+	pGLO +		
4	+	–	pGLO +		
5	+	+	pGLO +		
6	+	+ + glucose	pGLO +		
7	+	+	10^{-4}x pGLO +		
8	–	+	10^{-4}x pGLO +		

14. If you have colonies that contain pGLO, pick two pGLO+ colonies and inoculate two culture tubes containing LB broth and ampicillin – one with arabinose, one without. You can use these cultures later for protein chromatography and electrophoresis.

15. Also streak a new amp/arabinose plate with your pGLO cells. The purpose of this plate is so you can keep your cells alive for later use. Verify the fluorescence of the resulting colonies next class.

16. Calculate the total # of bacteria in the transformation reaction.
    Calculate the # of those bacteria transformed by pGLO.

17. Calculate the efficiency of your transformation reaction in two ways:

    a.   # transformed cfu / # total cfu

    b.   # transformed cfu resulting / # plasmids added

pGLO 3: PURIFYING GFP BY CHROMATOGRAPHY

In the last couple of labs, you transformed some *E. coli* cells with a plasmid containing a gene for green fluorescent protein (GFP), saw the glowing colonies caused by the expression of GFP, and started a liquid culture of cells containing the plasmid. Now you can purify GFP from those cells.

By the time you finish this lab, you should understand:

- The basic concept protein purification
- The basic concept of column chromatography
- Hydrophobic interaction chromatography (HIC), a form of column chromatography

INTRODUCTION

In the first parts of the pGLO lab, you saw that you could insert a gene (GFP) into some bacterial cells and then induce the expression of that gene by taking advantage of an arabinose-specific transcription factor (AraC) and promoter. Now you have a liquid culture of those bacteria.

What you've done so far is basically the first few steps a biotech company would use to produce a protein from a cloned gene. Now you can do the next steps: lyse the cells and purify the protein.

THE PROTEIN PURIFICATION PROBLEM

Cells make a lot of proteins. If you want a specific protein from a cell culture, you need to separate that protein from all the others.

You've already used one method for separating macromolecules: electrophoresis. Protein and DNA electrophoresis both act on the same principle: some molecules migrate faster through a gel than others. Protein electrophoresis is a very sensitive way of separating different proteins from one another, but it has some limitations. For one thing, the protein is in the gel when you finish. If you want to use that protein for something, you'd need a way to get it out of the gel. Also, you can't load very much protein on a gel. And finally, the method you used, SDS-PAGE (polyacrylamide gel electrophoresis), uses detergent (SDS) to denature the proteins. This means that you're not likely to recover functional protein.

COLUMN CHROMATOGRAPHY

Column chromatography is an alternative to electrophoresis for separating proteins or other macromolecules. In principle, it's similar to electrophoresis: you apply a sample containing a mix of solutes, and the different proteins get separated from the non-protein solutes and from each other because some travel faster than others. The key differences are what makes the proteins move, and what slows them down.

- **Electrophoresis:** proteins move because they're pulled by an electric field. They slow down because they don't easily fit through the small pores of the gel. Bigger proteins move more slowly. The gel is thin and flat.

- **Column chromatography:** proteins move through a column because they are carried by a moving solvent (the mobile phase). They slow down because some stick to the column more than others, depending on the chemical nature of the proteins and the column. The column is cylindrical and filled with some kind of matrix (the solid phase). Many kinds of column matrices are available, for separating different kinds of proteins.

In some kinds of column chromatography, the solvent is pumped through the column at a constant rate. In this experiment, we will use "spin columns" that fit on top of microfuge tubes and in the microcentrifuge rotor. You can simply apply the solvent to the top of the column and force it to flow through the column matrix by centrifugal force.

HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Hydrophobic interaction chromatography (HIC) is commonly used for separating proteins. The principle is simple. The column is filled with a "macroporous" matrix of precisely-sized, tiny plastic beads synthesized with many pores to increase its surface area. The surface has been treated to be covered with nonpolar methyl groups.

As you recall, proteins are constructed from a precise mixture of twenty different amino acids, some of which have polar side groups, and some have nonpolar groups. The Green Fluorescent Protein (GFP) is a water-soluble cytosolic protein, so normally the hydrophilic interactions of water with its polar residues far override any hydrophobic interactions from its nonpolar residues. So we would expect such water soluble proteins to migrate rapidly through the column in the aqueous buffer with little interaction with the methylated bead.

Certain salts such as **ammonium sulfate** are "chaotropic", meaning that they interfere with the ability of water to form hydrogen bonds, and therefore they reduce hydrophilic interactions between polar solutes and aqueous solvents. Thus in the presence of ammonium sulfate, the nonpolar residues of normally water-soluble proteins such as GFP will be able to stick to the hydrophobic methyl groups on the surface of the beads. To remove the proteins from the beads, simply flush the matrix with an aqueous solvent <u>without</u> ammonium sulfate.

Hydrophobic interaction chromatography takes advantage of the double-nature of proteins to purify them. Any compound that lacks nonpolar components will not stick to the matrix even in the presence of ammonium sulfate and wash right through the column. Any substance that is too hydrophobic would not be eluted by the aqueous solvent. Long analytical HIC columns can even be used to purify different proteins from each other according to their specific relative hydrophobic/hydrophilic natures by gradually decreasing the amount of ammonium sulfate

in the mobile phase. But our preparative minicolumns will just separate the mixture of soluble proteins from the non-protein components of the bacterial cell lysates for further purification and analysis — in our case, by electrophoresis.

Another advantage of HIC is that it generally does not denature proteins as they are purified. Since GFP retains its native structure, it remains fluorescent throughout the purification procedure. Hence we may check whether the protein is sticking to the column by looking for green fluorescence in either the matrix or the eluent.

LYSING THE CELLS

The first problem that you face in trying to purify pGLO is that the protein is inside the cells. To get it out, you have to lyse (split open) the cells. You can achieve this using a two-step method:

- **Lysozyme**, as the name implies, is an enzyme that causes lysis of bacterial cells. This enzyme eats away at the cells' protective cell wall, weakening them. The TE buffer not only maintains the proper pH for lysozyme activity, but is hypotonic to the cells causing them to swell by osmotic pressure.

- **Freezing** finishes the job of lysis. When ice crystals form, they break open the weakened cells.

Once you've lysed the cells, you'll have a crude mixture of cell contents (a lysate). You can pellet insoluble cell debris by centrifugation, then apply the soluble lysate to the column.

MATERIALS

- Your tubes of liquid bacterial culture containing pGLO (one with arabinose, one without)
- 8 1.5-ml microfuge tubes (4 clear, non-fluorescent tubes + 4 colored [optional] tubes)
- Lysozyme solution, on ice
- 2 chromatography columns
- HIC resin ("Bio-Rad Macro-Prep® Methyl-HIC Support" macroporous polymeric beads)
- Chromatography buffers:
    - Equilibration buffer — A high-salt buffer (2 M $(NH_4)_2SO_4$)
    - Binding buffer — A very high-salt buffer (4 M $(NH_4)_2SO_4$)
    - Wash buffer — A medium-salt buffer (1.3 M $(NH_4)_2SO_4$)
    - Elution buffer — A very low-salt buffer (10 mM Tris/EDTA; also called TE)
- Pipetmen, tips, beaker for waste tips

METHOD OVERVIEW

You should have two liquid cultures. For all the following steps in preparing a soluble lysate and hydrophobic interaction chromatography, you should process your two samples in parallel. In other words, you'll have two lysates and two chromatography columns.

You'll spin down the liquid cultures, prepare a lysate from each culture, apply the lysate to a chromatography column, then wash off the protein. When you're done, you'll have a partly purified preparation of GFP and other cytosolic proteins.

Prepare soluble lysates (*remember, you're doing two!*)

1. Remove your liquid cultures from the shaker and observe them in normal room lighting and then with the UV light. Did they grow? Are they fluorescent? Record your observations in the results table at the end of the methods section. Pipet 1.5 ml of each liquid culture into a microfuge tube (one tube for + arabinose, another for – arabinose). Use the stuff on the bottom of the tube, because that's where all the cells are.

2. Spin the tube for 5 minutes in the centrifuge at high speed (14,000 rpm). Be sure to balance the centrifuge.

3. After the spin, you should see a solid bacterial pellet. At this point, the cells contain the protein you want. Pour off the liquid supernatant into an empty tube for disposal. Observe the pellet under UV light, and record on the table on p. 46 whether you see fluorescence.
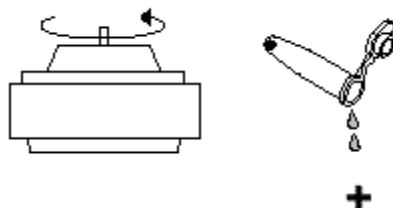
4. Add 250 µl of TE buffer to the bacterial pellet. Resuspend the bacterial pellet thoroughly by rapidly pipetting up and down.

5. Add 100 µl of lysozyme to the resuspended bacterial pellet and flick the tube to mix. Let sit at room temperature for 10 minutes. The lysozyme will start digesting the bacterial cell wall. Put the tube in the freezer until it's frozen solid. Freezing will complete the lysis of the bacteria.

6. Remove your tube from the freezer and thaw it in your hand. Vortex to mix well. Spin the tube in the centrifuge for 10 minutes at high speed. This will pellet the insoluble bacterial debris.
   * This would be a good time to start preparing your columns → Steps 9 − 11.

7. After the 10 minute centrifugation, immediately remove your lysate tube from the centrifuge. Examine the tube with the UV light. The insoluble bacterial debris should be visible as a pellet at the bottom of the tube. The supernatant contains all the soluble materials from the lysed cells – including the GFP. **The supernatant from this step is your soluble lysate.** Note whether there is fluorescence in the pellet or the supernatant in the table on the next page. If a lot of the fluorescence remains in the pellet → mix, freeze, thaw, and centrifuge again. Transfer 250 µl of the soluble lysate into the new 1.5-ml micro tube.
   * Save the unused crude fraction in the freezer.

8. Add 250 µl of binding buffer to the tube containing the soluble lysate and mix. The lysate is now ready to go on the HIC column. (The lysate could be stored in the refrigerator at this point, but you should go ahead and complete the experiment if you can. Go to Step 12.)

HYDROPHOBIC INTERACTION CHROMATOGRAPHY (one column for each sample)

9. Prepare 2 chromatography columns by pipetting 0.25 ml of HIC resin suspension into each spin column. Spin briefly (10 seconds or so on low) to pull the liquid through the column.

10. Equilibrate the columns by adding 0.5 ml of equilibration buffer to the top of the column. Spin briefly (10 seconds or so on low speed (5000 rpm)) to pull the liquid through the column.

11. Obtain 2 sets of 3 1.5-ml micro tubes and label them 1, 2, and 3. Cut off the caps, but keep them for later. (The microfuge tube won't fit in the microcentrifuge with both a spin column and a cap.)

12. Load 500 µl (all) of the lysate (from step 8) onto the top of the HIC resin in the column. Put the column into tube 1 and spin for 20 seconds on low speed (5000 rpm). The entire volume of liquid should pass through the column into the tube. Examine the column under UV light and write down your observation in the results table on the next page.

13. Transfer the column to collection tube 2. Add 250 µl of wash buffer to the column and spin for 20 seconds on low. The entire volume of liquid should pass through the column into the tube. Examine the column using the UV light and write down your observation in the results table shown below in the results section.

14. Transfer the column to tube 3. Add 400 µl of elution buffer (TE buffer) and spin for 20 seconds on low. The entire volume of liquid should pass through the column into the tube. Again, examine the column using the UV light and write down your observation in the results table below.

15. Examine all of the collection tubes using the UV lamp and write down where you see fluorescence. (See table on next page.)

16. Your proteins are in the tubes labeled 3; save these in the freezer for the protein gel in the next lab.

RESULTS & DISCUSSION

In this experiment, you're using fluorescence to detect the protein you're looking for; your results are in terms of where you see fluorescence. Record all your results below.

Sample	Fluorescence?
Raw bacterial culture [step 1]	
Bacterial pellet before lysis [step 3]	
Bacterial pellet after lysis [step 7]	
Supernatent over bacterial pellet after lysis (lysate) [step 7]	
Column, after lysate passes through [step 12]	
Chromatography Collection tube 1 (eluted lysate) [step 12]	
Column, after wash buffer passes through [step 13]	
Chromatography Collection tube 2 (wash buffer) [step 13]	
Column, after elution buffer passes through [step 14]	
Chromatography Collection tube 3 (elution) [step 14]	

QUESTIONS

You might want to think about these questions to help you understand this lab better – and to prepare for a quiz.

◇ What is the purpose of the lysozyme?

◇ Why did you put the tube in the freezer?

◇ Which buffers are more salty, and why? What would happen if you used the wrong buffer?

◇ Did you end up with GFP? How do you know?

◇ Are there other proteins still mixed in with your GFP? How could you find out for sure?

P G L O 4 : G F P E L E C T R O P H O R E S I S

In the last couple of labs, you transformed some *E. coli* cells with a plasmid containing a gene for green fluorescent protein, grew up the transformed cells, and used chromatography to partially purify GFP. Now you have your protein sample, and if it's fluorescent it contains GFP (Green Fluorescent Protein). But what other proteins are in there? How does your sample after chromatography compare to the sample before chromatography? How different is the set of proteins from the pGLO transformed cells compared to the untransformed *E. coli*? And most importantly, can you actually separate GFP from all the other proteins and identify it?

Protein electrophoresis with SDS-PAGE can answer these questions by giving you a closer look at all the proteins in your samples.

You should have two HIC-purified lysates. Both will contain a number of cytosolic proteins from your *E. coli* cultures. The key difference will be that the culture you grew with arabinose should contain GFP and the one grown without arabinose shouldn't. By comparing these two samples on a gel, you may be able to identify GFP as an "extra" band in the + arabinose lane. GFP is about 25 kD, so you'll be looking for a band around that size.

Since you already know how to do protein electrophoresis, you don't need step-by-step instructions here. You may want to refer to the earlier chapter on protein electrophoresis to refresh your memory. Ask your instructor if there are any changes. The only new thing you need to figure out is what goes on your gel, and how much.

1. Assay the [protein] in your HIC fractions according to the method in Appendix A2i.

2. Prepare "gel ready" samples of your HIC proteins according to the same as the method you used before. [See p. 5]

3. Calculate the volume of each "gel ready" sample to load 1 µg and 5 µg protein into separate wells of the gel. Remember to run a protein MW marker sample too.

protein sample	[protein] µg/ml			µl/well to load	
	Quant-iT assay	**HIC fraction**	**"gel ready" sample**	**1 µg**	**5 µg**
Ara– lysate					
Ara+ lysate					

Illustrate below how you loaded your gel.

Also today — start new liquid cultures of pGLO⁺ cells

Use a sterile inoculating loop to transfer a fluorescent colony from your pGLO⁺ culture source plate (from pGLO 2, p. 37, step 15) into a sterile culture tube with 3 ml LB/Amp/Ara medium. Place it in the shaking incubator. These cells will be used for the plasmid purification procedure in pGLO-5.

PGLO 5: PGLO PLASMID PURIFICATION

In the last couple of labs, you transformed some *E. coli* cells with a plasmid containing a gene for green fluorescent protein, grew up the transformed cells, and used chromatography and SDS-PAGE to purify GFP. You've had a good look at the protein that your transformed *E. coli* cells made. Now it's time to look at the DNA. In this lab, you'll purify plasmid DNA from your transformed cells.

DNA PURIFICATION

Purifying DNA is one of the key methods of molecular biology. In this lab, you'll purify plasmid DNA from your liquid bacterial cultures. This method takes advantage of two facts about nucleic acids and bacteria:

First, bacterial chromosomal DNA is attached to the plasma membrane. If you lyse bacterial cells, the chromosomal DNA will remain attached to the insoluble complex of membrane and cell wall. Meanwhile, the plasmid DNA will be freely dissolved, because it isn't attached to anything.

Second, DNA is highly soluble in water, but not very soluble in organic solvents, including alcohol. You can cause dissolved DNA to stick to solid glass beads by adding alcohol to the solution.

You'll start with a small amount of bacterial culture broth, and — with luck and good technique — end up with enough DNA that you can see it on a gel.

A major difference between the cell lysis method used here as opposed to the method used in the pGLO-3 experiment: In the GFP extraction, we wanted to lyse the cells while *preserving* the soluble proteins. But to extract the plasmids, we want to *destroy* the proteins before lysing the cells. Some proteins are nucleases that would damage the plasmid DNA.

PLASMID DNA PURIFICATION METHOD

Use this procedure for purifying pGLO plasmid DNA from your bacterial culture, using the Promega Wizard Miniprep kit. The method is similar to the hydrophobic interaction chromatography you did with your protein sample, but since DNA does not have hydrophobic side groups, we'll use hydrophilic interactions instead. The matrix in the "Wizard® SV" spin column consists of modified glass beads. Adding ethanol to the mobile phase decreases its polarity, so the polar DNA molecules adhere to the charged surface of the glass beads. While the DNA is retained on the matrix, other lysate compounds wash through. Then the clean DNA can be eluted from the column with pure (polar) water.

MATERIALS

- Your tube of liquid bacterial culture containing pGLO plasmid (<u>Check fluorescence</u>)
- 2 clear, non-fluorescent microfuge tubes (1.5 ml)
- Cell Resuspension Solution
- Cell Lysis (NaOH/SDS — alkaline detergent) Solution [Make sure SDS is dissolved.]
- Neutralization Solution
- Alkaline Protease (from *Bacillus licheniformes*) Solution
- Spin Column ("Wizard® SV Minicolumn")
- 2 Collection tubes
- Column Wash Solution (with ethanol)
- Sterile water

METHOD

1. Pipet 1.5 ml of the bacterial culture into a microfuge tube. Pipet the thick gob of stuff at the bottom of the culture tube to get as many cells as possible.

2. Spin at 14,000 rpm (high speed) for 5 minutes to pellet cells.
   Check for fluorescence of the pellet. Pour off supernatant.

3. Add 250 µl of Cell Resuspension Solution and completely resuspend the cell pellet by pipetting or vortexing. Be sure the whole pellet gets resuspended – don't leave any chunks. *(You can mix vigorously at this point because the DNA is still inside the cells.)*

4. Add 250 µl of Cell Lysis Solution and mix by inverting the tube 4 times. Incubate at room temperature until cell suspension clears somewhat (about 1-5 minutes). *(It's important to see some clearing of the solution, but don't incubate more than 5 minutes. Mix gently at this point because the cells have lysed and the DNA is free in solution. DNA molecules are so long that they can be damaged by excessive pipetting or mixing.)*

5. Add 10 µl Alkaline Protease Solution and mix by gently inverting the tube 4 times. *(This destroys enzymes that might damage the DNA.)*

6. Add 350 µl of Neutralization Solution and mix by gently inverting the tube 4 times. Check for fluorescence. (*What do you predict?*)

7. Spin the lysate for 10 min at 14,000 rpm. *(When the spin is done, you'll have a cleared lysate. Meanwhile, perform step 9 to get your Spin Column ready.)*

8. Insert Spin Column into Collection Tube.

9. Get your lysate from step 7 out of the centrifuge; you should now see a cleared lysate (the supernatant) and a white precipitate. Transfer 800 µl of the supernatant into the Spin Column. Be sure not to transfer any of the pelleted precipitate.

10. Centrifuge Spin Column at 14,000 rpm for 1 minute. The lysate will go through the Spin Column, but the DNA sticks to the column. Ensure all the liquid has gone through.

11. Pour off the liquid that went through the Spin Column; it is waste.

12. Add 750 µl Column Wash Solution (previously diluted with ethanol) to Spin Column.

13. Centrifuge at 14,000 rpm for 1 minute. The solution goes through, but the DNA remains stuck to the Spin Column. Pour off the wash solution from the Collection Tube. *(The wash solution contains alcohol, and the DNA is not soluble in this solution.* The DNA remains in the spin column.)

14. Add another 250µl Column Wash Solution to Spin Column and spin for 2 minutes. Throw away the wash solution that goes through the column.

15. Transfer Spin Column to a new, sterile 1.5 ml microfuge tube. Be sure there is no more wash solution in or on the spin column.

16. Add 100 µl sterile water to the spin column. *(The water will dissolve the DNA.)*

17. Spin for 1 minute at 14,000 rpm.

18. The liquid that goes through the column contains your DNA. Use 10 µl to measure the concentration of DNA in your purified sample using the protocol in Appendix A2ii.

19. If time allows, proceed directly to the pGLO-6 plasmid restriction digest.
    Otherwise save the rest in the freezer until next lab period.

PGLO 6: PGLO PLASMID RESTRICTION DIGEST

In the last lab, you purified pGLO plasmid DNA from *E. coli* cells. You could just look at this DNA on a gel, but you'll gain more information if you first cut the DNA with restriction enzymes. Go back to the first pGLO chapter, and you'll see a map of the pGLO plasmid with some restriction sites shown. If you use one or more of the restriction enzymes listed on that diagram, you should be able to predict where the DNA will be cut, and what size fragments you'll get after you cut it. If the cut DNA gives the expected fragment sizes, you can be confident that it's really pGLO and not some other DNA.

You've already done restriction digests of DNA, so you should be able to figure out how to do this lab on your own. You'll do two different digests of your plasmid DNA, using the two restriction enzymes you used before: Eco RI and Hin DIII (separately). <u>Write out all your calculations below before you do anything else.</u> You may want to refresh your memory by looking at the earlier restriction digest lab. (<u>Remember</u>: only *small* microfuge tubes fit in the thermocycler!)

You should have about 90 μl of DNA solution, which should contain plenty of DNA. Typically, your 100 μl of DNA solution contains at least 2 μg DNA (20–30 ng/μl). Plan to cut 15 μl (300ng) of it and leave the rest uncut. Later, you'll run the cut and the uncut DNA side by side on a gel.

Calculate your digest reaction with a 10-fold enzyme excess in a minimum reaction volume.

Additionally, do a third restriction digest reaction using HinDIII to cut 1 μg of lambda DNA to run on your gels as a standard.

✧   What are your predicted results? **Draw below a hypothetical gel showing how you expect uncut pGLO, HinDIII-cut pGLO, and EcoRI-cut pGLO to form bands.**

✧   Why don't we bother to cut the plasmid with a combination of HinDIII and EcoRI this time?

P G L O   7 :   P G L O   P L A S M I D   G E L

In the last lab couple of labs, you purified pGLO plasmid DNA from *E. coli* cells and cut the DNA with restriction enzymes. It's time to run a gel. Good thing you already know how to do this!

Prepare and run a DNA gel, following the procedure you used earlier. Make your gel with 0.8% agarose. The volume of the gel is the same as before. Don't forget to add the ethidium bromide.

Your samples should include uncut pGLO, HinDIII-cut pGLO, and EcoRI-cut pGLO, plus HinDIII-cut λ-DNA as a standard ladder.

WRITING THE PGLO LAB REPORT

This lab has several parts, and the report should cover all of them:

Section	Experiment	Result
Transformation	Transformation with pGLO	Plates: +/- colonies; +/- fluorescence Transformation efficiency
	Liquid cultures of transformed cells	Did the cultures grow? Were they fluorescent?
Protein expression	Preparing protein lysates	Which pellets/supernatants were fluorescent?
	Chromatography with GFP	Which fractions were fluorescent?
	Protein gel	Gel photo
Plasmid isolation	pGLO plasmid DNA purification	
	pGLO plasmid DNA restriction digest	
	pGLO plasmid DNA gel	Gel photo with cut & uncut plasmid DNA

Again, use the report format described in Appendix A4, "Writing your lab reports" (pp. 121-123). Specifically, the report should be structured as follows:

INTRODUCTION

Describe the purpose of this lab in general terms. Describe the basic characteristics of pGLO plasmid and of Green Fluorescent Protein (GFP). Describe the protein and DNA methods you used. Finish with a brief statement of the overall plan of the experiment.

METHOD

As usual, your methods section should be a flow diagram. This one is more complex than the others, but you should do one diagram that includes all the parts of the experiment, and it should fit on one page. That means that you will have to simplify a lot. Skip details of the steps; just show enough to say what the point of the technique was, rather than how you actually did it. Include both the protein gel and the DNA gel, and say what went into each lane.

RESULTS & DISCUSSION

Look at the table above for a list of the results you should include.

For this report, since you had several experiments, it makes sense discuss the results of each of the three sections separately, with the specific details and overall conclusions for that section. Then discuss the next section results and their conclusions.

After presenting and briefly discussing the individual section results, include a brief overall summary for the whole report – "In this lab, we…."

PV92 AND PCR
(POLYMERASE CHAIN REACTION)
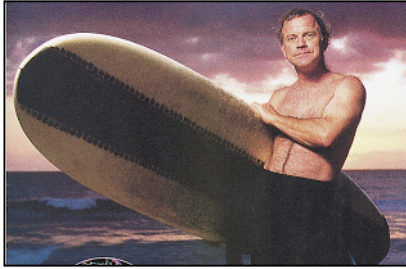
You've looked at DNA from *E. coli*. Now it's time to look at your own DNA. In this lab, you'll use the polymerase chain reaction (PCR) to copy part of your DNA so you can compare your DNA to that of other students in the class.

Kary Mullis: inventor of PCR, Nobel Prize winner, surfer, occasional crackpot, and all-around outside-the-box thinker. In this lab, you'll make use of a very good idea that Mullis had late one night while driving down the Pacific Coast Highway.

After completing this lab and the next one, you should understand:

- what the polymerase chain reaction (PCR) is used for
- how PCR works, including the function of all the ingredients in a reaction and how to figure out how much of each ingredient to use in a reaction
- how to use PCR to detect specific DNA sequences
- The nature of repetitive DNA in general and the ALU repeat in particular.
- Locus and allele; genotype; homozygous and heterozygous.
- Using Chelex resin for purifying DNA

INTRODUCTION

The polymerase chain reaction (PCR) has had a huge impact on experimental biology. PCR accomplishes one simple thing: it allows a researcher to make a large number of copies of a particular stretch of DNA. (This is known as amplifying a DNA fragment.) Because PCR allows people to accomplish this quickly and simply, it has allowed researchers in all branches of the life sciences to unlock some of the secrets hidden in the nucleotide sequences of DNA.

In this lab, you'll use PCR to amplify a particular region of your own DNA. This is one use of the technique; there are many others.

Some uses of PCR

Medicine: PCR can be used to detect specific DNA sequences. One example of this would be finding virus DNA that has been inserted into a person's cells. HIV, the virus that causes AIDS, is one example of a virus that can insert its DNA into a cell's chromosomal DNA. As a medical researcher, how would you find out if a person's DNA contains the virus? A virus

could be anywhere in the billions of nucleotides that make up the genome. PCR allows you to specifically find and copy the virus DNA sequence amidst all that excess information. If you do PCR and find HIV DNA in a cell, you've proven that the cell has been infected with the virus. In addition, you can use the virus DNA to learn more about the virus.

Population genetics: comparing DNA sequences among different individuals in a population is a good way to find out who's related to who. That's important in projects ranging from reconstructing human evolution to figuring out the breeding habits of naked mole rats. PCR allows you to start with a tiny amount of DNA and specifically amplify the region of the genome you want to study. After you've amplified the DNA, you can use DNA sequencing to find out the nucleotide sequence, which you can then compare from one individual to another.

Forensics: Because PCR can be used to amplify DNA starting from microscopic samples, it can be a good way of answering questions like "whose blood is this?" Starting from a dried blood spot, or a single hair, you could amplify enough DNA to do DNA sequencing, which could prove that the sample did or did not come from a particular individual.

Finally, PCR is widely used as an all-around molecular biology technique. Anybody who works with DNA eventually wants to copy some DNA, and PCR has become the easiest, fastest, and cheapest way to do it.

Today's lab: PV92

In this lab, you'll use PCR to copy a particular segment of your DNA, then run a gel to compare your amplified DNA to that of other students. It's a simplified DNA fingerprinting experiment.

The DNA region, or locus, that you'll copy is called PV92. The PV92 locus is part of an intron on chromosome 16. The thing that makes PV92 useful for DNA fingerprinting is that it's variable. Everybody has the PV92 locus, but some people have an extra stretch of repetitive DNA (called an Alu repeat) in it. The ALU repeat is a stretch of 300 nucleotides that occurs over and over at various locations in the human genome. Apparently, this segment of noncoding DNA has been duplicated many times during the evolution of primates, and inserted into the genome at many locations.

There are only two common PV92 versions, or alleles, in the human population – the short one, with no Alu repeat, and the long one, with the Alu repeat. The result is that for any copy of human chromosome 16, you should be able to amplify either a short or a long a PV92 PCR product. Since everybody has two copies of chromosome 16 (one from mom and one from dad), everybody has two copies of the PV92 locus. Therefore, you could possibly get both a short and a long PV92 PCR product from one person. Today you can collect DNA from each person in your lab group and test it

to find out whether that person has the short PV92, the long one, or both.

What's your genotype?

Let's call the PV92 alleles **+** (with ALU repeat) and **−** (without ALU repeat). You might have inherited the same allele from both parents, or you might have inherited a different one from each parent. If you have two copies of the same allele (+/+ or -/-), you're **homozygous**. If you have two different alleles (+/-), you're **heterozygous**.

In the PCR for this lab, the product of the + allele will be 941 base pairs long, while the product of the − allele will be 641 bp. If your genotype is +/+, you'll see one 941 bp band on the gel. If your genotype is -/-, you'll see only a 641 bp band on the gel. If your genotype is +/-, you'll see a short band and a long one.

This isn't real DNA fingerprinting

In this experiment, there are only 3 possible genotypes: +/+, +/-, and -/-. Clearly, this isn't enough information to identify DNA from a particular individual, or to say whether two individuals are related. In real DNA fingerprinting, you'd have to look at many loci together before you could definitely say that two DNA samples are the same or are closely related.

On the other hand, even today's simple experiment could be enough to prove that two DNA samples don't come from the same person. In DNA fingerprinting, it's always much easier to show that two samples are from different people than it is to show that they're from the same person.

How PCR works

The tools used for copying DNA in PCR are the same tools used by cells in copying their own DNA: a DNA polymerase, some nucleotides, primers, and the DNA to be copied (called the template). The name Polymerase Chain Reaction comes from the fact that a DNA polymerase is used to copy fragments of DNA over and over in a chain reaction.

primers

nucleotides

PCR: Basic Steps

The basic steps of PCR are similar to those of cellular DNA replication: the two strands of DNA are separated, a primer attaches to a strand of DNA, and the polymerase adds nucleotides to the 3' end of the primer, eventually creating a whole new DNA strand. The key difference between PCR and the normal DNA replication that happens in a cell is the primers. When a cell copies its DNA, it makes a large number of primers, which attach to the DNA at many sites; with many priming sites, DNA synthesis begins at many points simultaneously and proceeds until all the DNA is copied. In PCR, specific primers are used. The specific primers attach only where they can base pair with a complementary sequence on the template DNA, and only a specific section of the DNA is copied.

heat to separate template strands

anneal primers to template

CYCLE 1

extend primers

CYCLE 2

CYCLE 3

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Here's a closer look at the steps of PCR:

Denaturing, or strand separation: the two strands of the DNA double helix are separated. In living cells, the strands are separated by an enzyme (helicase). In PCR, the two strands are separated by simply heating the DNA to 90 or so. This breaks the hydrogen bonds joining the two strands, but leaves the covalent bonds of each strand intact.

Primer annealing: in this step, the primers bind to the DNA. In living cells, the primers are made of RNA are synthesized right on the DNA by an enzyme (primase). In PCR, the primers are artificial and are designed by the researcher to base pair with a specific region of DNA. For example, if you want to amplify HIV DNA, you make primers that bind to HIV

DNA but not to human DNA. Each PCR reaction normally uses two primers, one for each end of the DNA region to be amplified. Primer annealing is controlled by allowing the PCR reaction tube to cool from 90 to 45-60 . At this temperature, the hydrogen bonds that join two strands of DNA together can re-form. A large excess of primers is used to favor primer annealing rather than the re-annealing of the two original strands of DNA.

Extension: in this step, the polymerase goes to work. The polymerase used in PCR, like any DNA polymerase, can only work by adding new nucleotides to the 3' end of an existing DNA strand. The polymerase copies DNA starting at the primer. Extension happens fastest at the enzyme's optimal temperature. In PCR, the enzyme that's normally used

works at very high temperatures: 72 is its optimum, and it can survive 90 long enough to allow the denaturing step to occur. The DNA polymerase most commonly used in PCR is called Taq, which is short for *Thermus aquaticus*, the heat-loving bacterium from which the enzyme was first isolated.

Repeat: to make PCR happen, you repeat the above three steps over and over. Repeating the denaturing, annealing, and extension steps is done by controlling the temperature. The temperature can be controlled by a thermal cycler, or PCR machine, which can be programmed to put a set of PCR tubes through a programmed set of temperature cycles. (A cycle consists of denaturing, annealing, and extension. A complete reaction might require 25 cycles.) The exact temperature of each step is critical, and often determines whether the PCR is successful. PCR machines provide precise temperature control.

After the PCR is completed, you can observe the results using electrophoresis.

Why PCR is so useful

PCR has two main characteristics that make it useful:

Sensitivity: PCR can amplify DNA starting from a tiny quantity of template – as little as a single molecule. This is true because PCR is a chain reaction, making DNA copies and then making copies of the copies.

Specificity: PCR can be used to copy exactly the DNA segment you want, even if you start with a complex mixture of template DNA. This is true because each primer is complementary to only one stretch of nucleotides on the template. PCR conditions can be controlled so that the primer will bind only where it is exactly complementary to the template.

Purifying DNA for PCR

You'll need to purify some of your own DNA for this lab. Luckily, PCR is very efficient and you don't need much DNA. You can get what you need by scraping a few detached cells from the lining of your cheek.

DNA doesn't need to be very pure for PCR, but you need to get rid of two things: enzymes that might damage DNA, and cations like Mg^{++} that would interfere with the PCR enzyme. You can achieve these two goals by simply boiling your cells with a product called **Chelex**. Boiling kills the enzymes and lyses the cells. Chelex is a solid resin that is negatively charged, so it binds cations. Chelex isn't water soluble, so it will be a powder in the bottom of your micro tube, pulling away harmful ions and leaving you with ready-to-use DNA. You'll be using a version of Chelex called Insta-Gene Matrix, made by the Bio-Rad Corporation.

Questions you should answer:

✧ What does PCR do and why is it so useful?

✧ What makes PCR so sensitive (that is, why can it work with such a tiny amount of template)?

✧ What makes PCR specific about which fragment of DNA it amplifies?

✧ Be sure you can explain the purpose of each ingredient in the PCR cocktail, and each temperature step.

✧ What is the ALU repeat? How many ALU repeats are there in the human genome? How many are you trying to copy?

✧ What results do you expect to see on the gel? Why would different individuals give different results? Why would some individuals show one band, while some show two? Explain the possible results in terms of: locus and allele; genotype; homozygous and heterozygous.

M E T H O D   F O R   P R E P A R I N G   D N A   S A M P L E

Safety Considerations

✋ **Wear gloves and goggles throughout this lab.**

✋ **Personal body fluid (spit).** In general, spitting is rude. But we want the DNA from your exfoliated cheek cells in your saliva. You won't be drawing blood, but it's still possible that you could have a virus or other pathogen in your saliva. Use the same sterile technique with these samples as you would with bacterial cultures. Change your gloves after spitting back into the cup. This will reduce the chance of cross-contaminating samples.

Materials for preparing DNA sample

For each person in your lab group, obtain the following:

    a small Dixie® cup

    sterile saline solution

    large (1.5 ml) microfuge tube

    500 μl microfuge tubes containing Instagene® Chelex resin

    another 500 μl microfuge tube for each sample

    pipetmen and tips

Procedure

Note: this procedure is written as if you're going to have only one tube. However, you'll have **a tube for each person** and you can incubate them all together.

1. Put ~10 ml of sterile saline into the cup. Pour the saline into your mouth and swish it *vigorously* in your cheeks for 30–60 seconds. Vigorous swishing frees lose epithelial cells from the inside of your mouth. Expel the saline back into the cup.

2. Fill the large microfuge tube with some of the saline rinse suspension. Centrifuge at high speed for 2 minutes. You should observe a match-head size white pellet at the bottom of the tube with your exfoliated cheek cells.
   - If you don't have enough cells, carefully decant off the supernatent, add another 1 ml of oral rinse suspension, and centrifuge again. Repeat until a sufficient pellet is observed.

3. After pelleting your cells, pipatte off most of the supernatent taking care not to lose the pellet. Blot the liquid from the edge of the tube with a paper towel. ~50 μl of liquid should remain with the pellet.

4. Label 1 tube of Chelex (Instagene®) for your DNA sample.

5. Using the P-20 micropipettor, pipet up and down the liquid in with your oral pellet to evenly resuspend your cells. Transfer the entire volume to your tube with the Chelex resin. Cap *tightly* and vortex thoroughly.

6. Incubate at 56°C for 5 minutes.

7. Vortex the tube again and incubate at 56°C for another 5 minutes.

8. Vortex the tube again and incubate, this time at 100°C for 6 minutes.

9. Vortex the tube again and spin for 10 minutes with the microfuge on **low** speed.

10. Be sure that all the Chelex resin has pelleted to the bottom of the tube. Pipet 150 μl of the supernatant into a new labelled small microfuge tube. **Be sure not to transfer any of the Chelex-containing pellet!** — it will interfere with PCR.

11. Your DNA is now ready to use in PCR. Keep it on ice until you start the PCR. (If PCR cannot be run immediately, store it frozen. But we plan on running it today.)

✧ *What is Chelex? Why must we treat our DNA samples with it?*

M ETHOD   FOR   P CR

Before you get your materials together, you need to figure out your PCR recipe and program the machine. To make PCR work, you need seven basic components in your reaction tube:

Water	Added to adjust the volume and concentration of the other ingredients. Must be pure and sterile.
Reaction buffer	Controls the pH and salt concentrations to allow the enzyme to do its job. The buffer is normally included in a separate tube when you buy the enzyme. It comes as a 10x concentrate; you dilute it 10-fold to make the buffer concentration 1x in the PCR reaction tube.
MgCl$_2$	Mg^{2+} ions interact with both the nucleic acids and the enzymes, do its concentration is critical and must be optimized for each PCR experiment. Too little and no amplification, but too much and wrong products are produced. Since we chelex-treated our templates, we need to add a little Mg^{2+} back.
Nucleotides	The monomers that will be used by the polymerase to make new DNA strands.
Primers	The oligonucleotides that give the polymerase a place to start. Must be present in high concentration. In this lab, the primers base pair with the PV92 region of your DNA.
Polymerase	The enzyme that makes the new DNA. Taq polymerase is commonly used in PCR.
Template DNA	The starting DNA, part of which will be copied in the PCR reaction.

All these components must be present in the proper concentrations to allow PCR to work. The hardest part of the experiment is figuring out what goes into your reaction tube. You'll probably spend more time figuring out what to do than actually doing it. That's normal.

Recipe for a typical PCR reaction: This table shows the final concentrations for each component in your PCR reaction mix.

Component	stock	final
Reaction Buffer w/ MgCl$_2$	10x	1.0X
Nucleotide Mix	10mM	0.2 mM
Primer 1	10 μM	1.0 μM
Primer 2	10 μM	1.0 μM
Taq DNA Polymerase	5 Units/μl	1.0 Units
Template **DNA**	unknown	1 ng

When performing PCR, you normally start by preparing a **cocktail**. The cocktail is a mixture of all the ingredients except the DNA template (i.e., your sample). Make sufficient cocktail for all your samples so you only need add this one solution to each tube. Normally you would start by calculating the proper amount of each ingredient per reaction tube. "Stock" means the concentration of the stock solution – the solution you use when setting up your PCR reactions.

Then multiply the amount per tube by the total number of tubes to make enough cocktail for all your reactions. Pipet the cocktail into your reaction tubes, and then add the DNA template to each tube at the end. That way, you're sure that all the tubes get the exact same mix of primers, enzyme, etc. Making a cocktail like this also saves you a lot of pipetting and minimizes the time delay between your first and last sample getting exposed to cocktail.

For today's PCR reactions, you've got it easy: the cocktail is already made for you. All you need to do is add your DNA template to the cocktail in the reaction tube:

PV92 PCR Cocktail	**20 μl**
Template DNA [unknown]	**20 μl**
Total volume	**40 μl**

Don't start pipetting yet! Read the rest of this section first!

For this experiment, your group should have one PCR reaction for each person's DNA sample. In addition, you should have a **negative control**: a PCR reaction tube that contains everything except template DNA. This negative control tube should produce no band on your gel. You should also have a **positive control**, supplied by the instructor.

✧ *Why do you need a negative control? Why do you need a positive control?*

Program the PCR machine

Before you get your reagents out, program the PCR machine or verify that it's already programmed. The machine needs to be programmed to take your samples through the proper temperature steps. Each set of denature, anneal, and extend steps is called a cycle. For today's reactions, the machine should be set to go through 30 cycles. Before starting the first cycle, there is often a preliminary initial denaturation step; and after the last cycle, a final extension step. For PV92, the program should be:

Initial Denaturation	94	2:00
Step 1: Denature	94	1:00
Step 2: Anneal	60	1:00
Step 3: Extend	72	2:00
Step 4	GO TO step 1	29 more times
Final Extension	72	10:00
End	10°	Final hold

Set up your cocktail and individual reaction tubes

Now you're ready to start pipetting. Obtain the following materials:

- small Styrofoam cooler or cup filled with ice — Keep all the cocktail tubes and your DNA samples on ice.
- your DNA samples from members of your group
- sterile water for your negative control

- your standard positive control DNA sample: **Write down what it says on the label!** There are three *different* positive control standards and it's essential to know which one you're using. One control is the homozygous +/+, another control is the homozygous –/–, and the third positive control is the heterozygous +/–. **Predict the band pattern for each of these.**
- PCR cocktail – will be distributed by the instructor; contains the following in 20 µl:

  water

  10x rxn buffer

  nucleotide mix

  primers

  Taq polymerase

- pipetmen, tips, and a beaker for waste tips

Set up the reactions

Keep all the cocktail tubes and your DNA samples on ice. **Don't perform the following steps until the instructor tells you**; it's important that everyone gets their tubes ready at about the same time.

1. Label each PCR tube, including your group name and the template. Label by writing on the sides of the tubes; don't use tape.

2. Add 20 µl of DNA template to each tube. For the negative control, use 20 µl sterile water. The cocktail is in special, thin-walled PCR tubes, not regular microfuge tubes. So you must add the template to the cocktail, not the other way around.

After setting up your reaction tubes, make sure other groups are ready to go before you put your tubes into the machine. **All the tubes need to go in at the same time.** Your tubes need to be on ice until the reaction starts.

The machine will take more than an hour to go through all the cycles. After the PCR is complete, the DNA is fairly stable; it can sit at room temperature for a couple of days. You don't need to wait around until the PCR is done; you can leave it in the machine. In the next lab period you'll use electrophoresis to look at your PCR products.

Before you leave, you should have:
- **PCR tubes in the machine.**
- **DNA templates saved in a rack in the freezer.**

Waste Disposal & Cleanup

Dump your mouth rinse solutions into the sink; throw the cups in the regular trash.

Chelex tubes, other microfuge tubes and pipet tips go in the biohazard trash.

Beakers used for waste tips: dump the tips into the biohazard trash and put the beaker into the dirty-glassware tub on the cart (if there's not a tub, check with the instructor).

Gloves, paper towels, etc. go in the regular trash.

DNA GELS FOR PCR RESULTS

Now that your PCR is done, it's time to see if it worked. Run a gel.

Since you're looking for smaller DNA pieces than before, make your gel with 1.5% agarose. The volume of the gel should be 40 ml, the same as before.

Load 20 µl of each student DNA PCR sample, but only 10 µl of each positive control sample. Mix each sample with 4 µl DNA sample buffer.

As a marker, load 10 µl of the EZ Load molecular mass ruler, which is already mixed with sample buffer.

Run the gel at 70 volts.

DNA ASSAYS

While your gel is running, use the method in Appendix A2ii to assay the DNA concentration in your unamplified templates and in your PCR products to determine how much DNA amplification occurred.

◇ Ideally how much would you expect the DNA in your templates to be amplified after 30 cycles of PCR? How did your measured DNA concentrations match these expectations? If there was a significant discrepancy, why do you think it occurred?

WRITING YOUR REPORT

Write a report for this lab project, following the usual format (c.f., A4, pp. 121-123). Address the "Questions you should answer" in your introduction.

The results should include your ʰUⱽˑYˑcℤ8B5ˑUggUⱼmⱽXUⱨUˑUbX n℃i fˑgel photograph, and your discussion·should have a lane-by-lane·analysis of how you reached each conclusion regarding genotype·determination, as well as·comments on unpredicted bands and notes of observations you made·while doing the·experiment.

Refer to **Supplemental Exercise 4: PV92 — Analysis and Interpretation of Results.**

Add to your results the information in Tables 1–6 from that supplement. Also answer questions #5–7 within your discussion to compare your group, the whole class, and the USA population genotypic and allelic frequencies and the implication of any significant differences between observed and predicted genotypic frequencies.

Remember: if showing data not collected in this experiment, cite your source!

B ACTERIOPHAGE

In the conjugation lab, you used indirect methods to investigate the transfer of DNA from one bacterial cell to another. In this lab, you'll work directly with DNA to investigate what happens when a virus infects some bacteria.

After completing this lab, you should understand:

- what a bacteriophage is
- virus life cycles and structure
- pathogens and Koch's postulates
- how to prepare a dilution series
- how bacteriophages are used in gene cloning
- lysis, plaques, and bacterial lawns
- the application of the polymerase chain reaction (PCR) to molecular diagnostics

I NTRODUCTION:

Bacteriophages are viruses that infect bacteria. Bacteriophages (phages or Φ for short) are abundant in some natural ecosystems, and are also widely used as tools for molecular biology. A phage's ability to induce a cell to make huge numbers of copies of a specific DNA molecule can be quite useful to anyone who is trying to clone a gene.

This lab is designed to demonstrate some concepts about viruses as infectious agents and as tools for molecular biology. In addition, this lab also includes the polymerase chain reaction (PCR), a technique for copying specific DNA sequences.

Koch's postulates:

In order to prove definitively that a particular pathogen causes a disease, researchers must:

1. Find the pathogen in each individual that has the disease.

2. Isolate the pathogen from a diseased individual, and grow the pathogen in a pure culture.

3. Induce the disease in healthy individuals by infecting them with the pure pathogen.

4. Isolate the pathogen from the newly infected individuals.

It can be difficult to prove that a specific virus causes a particular disease. Simply proving that the diseased cells contain the virus isn't enough; the presence of the virus could be a coincidence. Medical researchers use Koch's postulates (see box) as a set of rules for proving that a specific virus (or other pathogen) is responsible for a particular disease.

Koch's strict set of rules was designed for working with bacterial pathogens, and sometimes it isn't possible to fulfill all four of its requirements. In this lab, you'll infect some cells with a phage, see the cells die, then try to prove that it was the virus that killed them.

VIRUSES AS PATHOGENS

Refer to Chapter 18 in Campbell et al. for a description of virus life cycles.

Many viruses are pathogens: agents that cause disease. Viruses cause a wide range of diseases in every type of organism. In this lab, you'll investigate a phage as the cause of cell death in bacteria.

VIRUS LIFE CYCLES, PLAQUES, AND LAWNS

When a virus infects a bacterial cell, the virus takes over the cell's biochemical machinery to make a large numbers of new virus particles. In many cases, the cell then bursts open, releasing the new viruses to infect other cells. This bursting is called lysis.

You'll be able to observe the effect of lysis when you grow your infected *E. coli* bacteria on a plate. If you let healthy bacteria grow all over a plate, you'll have a bacterial lawn – a faint translucent coating on the surface of the agar. If some of those bacteria are infected with a phage, you'll see a plaque – a clear spot in the lawn where the cells are dead. A plaque is formed when one infected cell lyses, releasing phage, which then infects the neighboring cells. Plaques tend to increase in size as more cells lyse and infect their neighbors.

THE POLYMERASE CHAIN REACTION (PCR)

You won't see the virus on your plates, but you can detect it using the polymerase chain reaction. As you've already learned, the polymerase chain reaction is a technique for copying a specific piece of DNA. In this lab, you'll use PCR to detect virus DNA from infected bacterial cells.

As mentioned previously, part of the point of the phage lab is to demonstrate that the phage causes lysis. You won't be able to fulfill Koch's postulates exactly; the phage only replicates inside cells, so you can't grow phage in a pure culture. You'll get an impure phage culture, containing the phage and some lysed bacterial cells. How will you recognize the virus in this culture? You can't recognize a specific phage just by looking at it; you need to examine the DNA. For that, you can use PCR.

PROCEDURE:

SAFETY CONSIDERATIONS

✋ **Bacterial cultures.** The bacteria you'll use for this lab are *Escherichia coli*, or *E. coli*. This species is a normal resident of every human intestine. Some strains of *E. coli* can cause human disease. The *E. coli* strain used in this lab does not cause disease. However, like all bacteria, it must be handled with care. Even strains that are not pathogenic can be harmful in large quantities. Bacteria evolve rapidly, and it may be possible for harmless bacteria to become harmful. **Always use sterile technique when culturing bacteria.**

Viruses. The virus you will use in this lab can only infect bacteria, and cannot cause disease in humans. However, as with all laboratory reagents and cultures, you should handle it with care.

✋ **Wear gloves and goggles throughout this lab.**

MATERIALS

For each lab group, obtain the following:

- 4 TPA or similar agar plates
- 1 tube SM liquid medium for diluting phage
- bacteriophage stock
- 4 sterile test tubes
- 3 microfuge tubes
- *E. coli* culture
- 4 melted soft agar tubes (leave these in the warm water bath until you're ready to use them)
- pipetmen, tips, and a beaker for waste tips

PLATING WITH PHAGE

The procedure for this lab is simple, but timing is important. Make sure you plan out what you're doing before you start. If you're a little too slow, your experiment won't work.

The basic procedure is to prepare a phage dilution series, add the *E. coli* cells to the diluted phage, add melted soft agar to the phage/cell suspension, and pour the whole mess onto an agar plate. The bacteria will grow embedded in the soft agar; this will create a good lawn and let you see your plaques well.

1. Before you start, the soft agar needs to be fully melted and ready at about 50 C. Before doing anything else, check to make sure that this is so.

2. Label the bottoms of your 4 plates 10^{-1}x, 10^{-2}x, 10^{-3}x, and "no phage."

3. Make a 10-fold dilution of your phage stock. Pipet 20 μl of phage stock into a microfuge tube, then pipet 180 μl of sterile SM medium into the same tube. Mix gently. The concentration of the phage stock is called 1x for convenience; your 10-fold dilution is 0.1x, or 10^{-1}x. Write "$\mathbf{10^{-1}x}$" on the tube.

4. Prepare 200 μl of 10^{-2}x phage, again using SM to dilute. Mix gently, and label the tube "10^{-2}x".

5. Prepare a tube of 10^{-3}x phage. Mix gently, and label the tube "10^{-3}x".

6. Pipet 500 μl of *E. coli* culture into each of your 4 empty test tubes. Label the tubes 10^{-1}x, 10^{-2}x, 10^{-3}x, and "no phage."

7. Add 100 μl of the 10^{-1}x phage to the cells in test tube labeled 10^{-1}x, and mix gently.

8. Take one tube of melted soft agar from the water bath and bring it back to your lab table. Pour the entire contents of the soft agar tube into the 10^{-1}x test tube. Mix gently. You are combining the phage and cells with the soft agar.

9. Immediately pour the entire contents of the soft agar tube (now containing soft agar, *E. coli* cells, and diluted phage) onto the plate labeled 10^{-1}x. Quickly spread the agar by swirling the plate once, then let this plate sit until the agar solidifies completely. If there are lumps in the agar, you should do another plate; you won't see your plaques well on a lumpy plate.

10. Repeat steps 7-9 for the 10^{-2}x and 10^{-3}x phage and the "no phage" plate. For the "no phage" plate, use 100 μl of plain SM instead of the diluted phage.

11. When you're done with all the plates, make sure the agar is all solid, then tape the plates together and put them in the 37 incubator. Put the plates upside down so condensation from the lid doesn't drip on the plates.

◈ Based on your understanding of this experiment, you should be able to answer some questions about how things might go wrong. *What if you used too little phage? What if you used too much phage? Too few or too many cells? Dead cells?*

WASTE DISPOSAL & CLEANUP

You should have four plates in the incubator.

Clean up as per usual. If you messed up any plates, put them in biohazard trash, along with your waste tips & tubes. Your microfuge tubes of diluted phage may also be discarded here.

The glass culture tubes, including those containing the phage stock and *E. coli* culture, should be placed in the rack in the contaminated-glassware tub on the cart. **Do not empty them or place them back in the original racks!** Peel the labels off all glass tubes.

Did you get a lawn? Did you get plaques? Be sure you can tell what a lawn looks like; compare a plate with a lawn to a blank plate with no bacteria.

? **Record your results for each plate**, including a good estimate of the number of plaques. Note whether you're actually counting, or just estimating.

Φ dilution	# plaques/plate	pfu/ml of stock Φ solution
10^{-1}x		
10^{-2}x		
10^{-3}x		
"No phage" control		

? **Calculate the concentration of virions (pfu/ml) in the original phage stock solution.** (Phage particles are called virions when they're not in a cell.) Each "plaque forming unit" (pfu) is assumed to represent one original virion. Use your best "most countable plate."

M A K E   A   P L A T E   L Y S A T E

If a plate has a lot of plaques, it has a huge quantity of phage particles. You can collect some of these for infecting other cells and for DNA testing by PCR in the next lab. The material that comes out of cells when they lyse is called a lysate; collect it from your plates and you have a plate lysate.

MATERIALS:

- Your plates
- SM buffer
- Pipetmen, blue tips, and a beaker for waste tips
- four 1.5-ml microfuge tubes

PREPARE THE LYSATE

1. Clean your table, use sterile technique, and wear gloves and goggles.

2. Choose the plate that has the largest number of plaques (should be your 10^{-1}x plate).

3. Pour several ml SM onto this plate. You want enough medium to completely cover the plate, but not so much that it will spill. (Later, you'll want to pipet off the SM.) **Record the amount of SM added.**

4. Swirl the plate gently and let it sit for a while. (You'll recover more virus by letting it sit longer.) The instructor will tell you how long to leave it.

5. Repeat steps 3 & 4 with your "no phage" plate.

6. Label two of your microfuge tubes "phage" and the other two "no phage".

7. Recover your plate lysates as follows: Tilt the plate slightly and pipet off the SM into a microfuge tube. Recover enough to fill the micro tube, without getting any chunks or debris. If there's not enough liquid to pipet, add some more SM to the plates. Add this amount to the total volume of SM added. The SM in your microfuge tubes is your "phage lysate."

8. Spin your "phage lysate" down for a few minutes in the microcentrifuge (high speed) to pellet any solids, including bacterial cells.

9. Transfer the supernatant to a new microfuge tube, being careful not to disturb the pelleted material. You want the phage particles, which are soluble and won't be pelleted; you don't want the bacterial debris, which forms a pellet. Don't worry about recovering all of your lysate; you'll have plenty. You can now use this lysate for infecting new cells and for PCR. Be sure to write your initials and the date on the tubes.

I N F E C T   N E W   C E L L S   W I T H   Y O U R   L Y S A T E S

Koch's postulates say that a researcher should be able to recover a pathogen from diseased individuals and infect healthy individuals with it. In this experiment, you'll find out if your phage lysate causes lysis in healthy cells.

The procedure for infecting bacterial cells with phage is the same one you used earlier.

MATERIALS

For each lab group, obtain the following:

- 3 TPA or similar agar plates (no antibiotics)
- your "phage" and "no phage" lysates
- 3 sterile test tubes
- microfuge tubes
- SM buffer for diluting phage
- *E. coli* culture
- 3 melted soft agar tubes (leave these in the warm water bath until you're ready to use them)
- pipetmen, tips, and a beaker for waste tips

PLATING WITH PHAGE

1. Before you start, the soft agar needs to be ready at about 50 C. Check to make sure that this is so.

2. Label the bottoms of your plates "10^{-3}x phage," "10^{-5}x phage," and "10^{-3}x no phage."

3. Make 10^{-3}x and a 10^{-5}x dilutions of your phage lysate. Make a 10^{-3}x dilution of your "no phage" lysate. Use SM to dilute the lysates, and do the dilutions in microfuge tubes.

4. Pipet 500 μl of *E. coli* culture into each of your 3 culture tubes. Label the tubes.

5. Add 100 μl of 10^{-3}x phage lysate to the cells in test tube labeled 10^{-3}x phage, and mix gently.

6. Take one tube of melted soft agar from the water bath and bring it back to your lab table. Pour the entire contents of the soft agar tube into the 10^{-3}x phage culture tube. Mix gently.

7. Immediately pour the entire contents of the soft agar tube (now containing soft agar, *E. coli* cells, and diluted phage) onto the plate labeled 10^{-3}x phage. Quickly spread the agar by swirling the plate once, then let this plate sit until the agar solidifies completely. If there are lumps in the agar, you should do another plate.

8. Repeat steps 4-7 for the rest of your lysates.

9. When you're done with all the plates, make sure the agar is all solid, then tape the plates together and put them in the 37 incubator.

✧ *Why are you doing 10^{-3}x and a 10^{-5}x dilutions of your phage lysate this time, when you did 10^{-1}x, 10^{-2}x and a 10^{-3}x dilutions for the first round of plates?*

WASTE DISPOSAL & CLEANUP

Save your undiluted your lysates (1x) – you still need them for PCR. Label them carefully and store them in the freezer.

You should have **three new plates** in the incubator.

You won't need your old plates any more – toss them in the biohazard trash. You can also dispose of the diluted lysates here.

Everything else, dispose of as usual: tips & tubes in biohazard trash; reusables in the tub. Remember to peel the labels off the used glass tubes and place them in the rack in the tub.

DAY 3: LOOK AT YOUR PLATES

✍ **Record your results for each plate**, including a good estimate of the number of plaques. Record what you actually see, not what you think should be there!

lysate dilution	# plaques/plate	pfu/ml of first lysate
10^{-3}x phage		
10^{-5}x phage		
10^{-3}x "no phage"		

✍ **Calculate the concentration of virions (pfu/ml) in the first lysate suspension.** Again, use your best "most countable plate."

CAN YOU FULFILL KOCH'S POSTULATES WITH YOUR RESULTS?

You should also be able to give a rough estimate of how much the phage replicated on your original plates. If your starting plate had 100 plaques, then it started with 100 phage particles infecting 100 cells. This is normally given as plaque-forming units (pfu): 100 pfu gives 100 plaques. If your phage lysate contained 1000 pfu, then the virus replicated at least 10-fold. How many pfu did your phage lysate contain? (Remember, you only put a small fraction of the lysate on each plate.)

✍ **Calculate the amount of viral replication that occurred on your first plates.**

$$= \quad \frac{\text{\# of pfu collected off of the plate}}{\text{\# of pfu put onto the plate}} \quad =$$

Assume that the total volume of first lysate equals the total volume of SM medium you added to the first lysate plate.

MAKE ANOTHER PLATE LYSATE

You still have another part of this lab to do: using PCR to see if the same virus DNA that you started with is present in your plates. To check this, you'll need the plate lysates you prepared earlier (call these the "first-round" lysates) and a plate lysate from the plates you're looking at today (call these the "second-round lysates).

Prepare plate lysates as before, from a "no phage" plate with no plaques and from a phage plate with a lot of plaques. Use the same SM volume and soak time you used to collect the first lysate.

You should now have four tubes with samples for viral identification by means of PCR:

    i. plate lysate from a first-round plate with plaques: (first phage lysate)

    ii. plate lysate from a first-round "no phage" plate (negative control)

    iii. plate lysate from a second-round phage plate with plaques (second phage lysate)

    iv. plate lysate from a second-round "no phage" plate with no plaques

You should also have a sample of the original phage stock solution from first phage lab for comparison (positive control)

YOU'RE NOT DONE YET!

Clean up your work area and get ready for PCR. Once your plates are counted and the lysates collected, you may dispose of the plates in the biohazard trash.

PCR FOR DETECTING PHAGE:

For this lab, you'll use PCR to find out whether phage DNA is present in the bacterial cultures. If the virus is present, you should get DNA produced in your reaction and see a band on your electrophoresis gel. If no phage is present, you won't get a band. In order to be sure the PCR is working, you'll need a positive control and a negative control. The positive control will be the original phage stock, and the negative control will be a "plate lysate" from the "no phage control" plate – cells, but no phage.

In this lab, you will need to prepare your own PCR cocktail. Review the ingredients and their purpose in the table in the PV92 lab.

Recipe for one PCR reaction: This table shows the final concentrations for each component in your PCR reaction mix.

Component, stock concentration	Final Concentration
5X _GoTaq_ Reaction Buffer	1.0X
MgCl$_2$, 25 mM	1.5 mM
Nucleotide Mix, 10mM	200 μM
Primer 1, "T$_2$F$_1$", 10 µM	1.0 µM
Primer 2, "T$_2$R$_1$", 10 µM	1.0 µM
GoTaq **DNA Polymerase**, 5 Units/µl	1.25 Units
Template **DNA**, concentration unknown	unknown

The final volume for each reaction will be 50 µl. The template DNA will simply be a few microliters of a plate lysate – you won't need to purify the DNA.
Fill in this chart showing what goes into a single reaction tube:

Water	_____.____ μl	
5x reaction buffer	_____.____ μl	**= 1x final concn.**
MgCl$_2$ (25 mM)	_____.____ μl	**= 1.5 mM**
Nucleotide mix (10 mM)	_____.____ μl	**= 200 μM**
Primer 1 (10 μM)	_____.____ μl	**= 1.0 μM**
Primer 2 (10 μM)	_____.____ μl	**= 1.0 μM**
Taq polymerase (5 Units/µl)	_____.____ μl	**= 1.25 Unit**
Template DNA (unknown)	5.0 μl	
Total volume	50.0 μl	

The ingredients are shown in the order in which you add them to the tube. You'll have to figure out the water last, but you should add it to the tube first.

Recipe for the PCR cocktail:

You'll be doing several reactions, which will be identical except that they will use different template DNA. When you set up your reactions, you don't need to set up each tube individually. Instead, you can make a "cocktail" containing all the ingredients except for the template DNA. Then you pipet the cocktail into the PCR tubes, and add the specific template to each tube.

You'll need enough PCR cocktail for seven reactions. In case your pipets aren't perfect (and they certainly aren't), you should always make a little extra cocktail – in this case, enough for eight reactions. Fill in your cocktail recipe:

Ingredient	1 rxn	8 rxns
Water	_____.__ μl	_____.__ μl
5x *GoTaq* reaction buffer	_____.__ μl	_____.__ μl
MgCl$_2$ (25 mM)	_____.__ μl	_____.__ μl
Nucleotide mix (10 mM)	_____.__ μl	_____.__ μl
Primer 1 (10 μM)	_____.__ μl	_____.__ μl
Primer 2 (10 μM)	_____.__ μl	_____.__ μl
GoTaq polymerase (5 Units/μl)	_____.__ μl	_____.__ μl
Template DNA	5 μl	don't add yet
Total volume	50.0 μl	360 μl

Each tube will get 45 μl of cocktail, followed by 5 μl of template. **Don't start pipetting until you read the directions on the next page.**

PROGRAM THE PCR MACHINE

Before you get your reagents out, program the PCR machine. The machine may already be programmed for you, but you should check. The machine needs to be programmed to take your samples through the proper temperature steps. Each set of denature, anneal, and extend steps is called a cycle. For today's reactions, the machine should be set to go through 25 cycles.

The program should be:

Step 1	94	30 seconds
Step 2	54	40 seconds
Step 3	72	45 seconds
Step 4	GO TO step 1	24 more times
Step 5	END	

✋ **Use gloves and goggles throughout this lab.** It's important to have clean gloves to reduce the possibility of contaminating your PCR reactions.

SET UP YOUR COCKTAIL AND INDIVIDUAL REACTION TUBES

For this experiment, your group should have seven PCR reactions:

1) plate lysate from a first-round plate with plaques: phage lysate

2) plate lysate from first-round "no phage" plate (lawn but no plaques: negative control)

3) plate lysate from second-round phage plate with plaques

4) plate lysate from second-round "no phage" plate with no plaques

5) phage stock solution from first phage lab

6) T2 phage positive control (provided by instructor)

7) SM medium with no DNA template (negative control)

Now you're ready to start pipetting. Obtain the following materials:

- small Styrofoam cup filled with ice
- pipetmen, tips, and a beaker for waste tips
- one 500-μl microfuge tube (to make the cocktail)
- seven 500-µl PCR tubes (special thin-wall tubes; <u>not</u> the microfuge tubes)
- PCR cocktail ingredients (will be distributed by the instructor):

    Sterile water

    MgCl$_2$, 25mM

    nucleotide mix, 10 mM

    T2F1 and T2R1 primers, each 10 µM

    GoTaq reaction buffer, 5x

    GoTaq polymerase, 5 U/µl

1. Keep all the cocktail ingredients and your cocktail tube on ice.

2. Label your 1 cocktail tube and 7 reaction tubes (Make sure you can distinguish your labels from the other groups' tubes — they will all be together in the thermal cycler!) Write directly on the tubes — **do not use tape!**:

    1) plate lysate from a first-round plate with plaques: phage lysate

    2) plate lysate from first-round "no phage" plate (lawn but no plaques: negative control)

    3) plate lysate from second-round phage plate with plaques

    4) plate lysate from second-round "no phage" plate with no plaques

    5) phage stock solution from first phage lab

    6) T2 positive control

    7) no template (negative control)

3. Before you pipet anything, make sure all the ingredients are thawed and mixed.

4. Prepare your cocktail on ice, according to the table you filled in on the previous page. Mix.

5. Aliquot the cocktail into the reaction tubes. After adding 45 μl cocktail to each tube, you should have about 45 μl left in the cocktail tube. Don't worry if it isn't exact, but if you're way off, you may have made a mistake.

6. Add 5 μl of the proper template to each reaction tube.

7. After setting up your reaction tubes, make sure other groups are ready to go before you put your tubes into the machine. All the tubes need to go in at the same time. Your tubes need to be on ice until the reaction starts.

The machine will take more than an hour to go through all the cycles. After the PCR is complete, the DNA is fairly stable; it can sit at room temperature for a couple of days. Later, you'll use electrophoresis to look at your PCR products.

WASTE DISPOSAL & CLEANUP

If the PCR machine is still running, leave your PCR tubes in the machine. **Save your DNA templates in the freezer in case we need to rerun them.**

Throw out the leftover cocktail along with your waste tips (biohazard).

D A Y   4 : E L E C T R O P H O R E S I S   F O R   P C R   R E S U L T S

You've done gel electrophoresis before. This time, the purpose of it is to check the results of your PCR reactions. Remember that electrophoresis separates DNA molecules by size, and allows you to see small amounts of DNA.

◈ What type of electrophoresis and what density of gel should you use?   Why?

P R O C E D U R E

The electrophoresis procedure for this lab will be the same as last time, with two small but important changes:

Agarose percentage: Since you will be looking at smaller DNA fragments this time, your gel should have a higher percentage of agarose. Prepare your gel with 2% agarose (you used 0.8% for the larger DNA molecules in the restriction digest lab). Use 40 ml TBE to make the gel, as before. Don't forget to add the ethidium bromide!

Voltage: Smaller pieces of DNA mean you can crank the voltage up a little without causing smearing in your gel. Run today's gel at 80 volts.

You should have seven PCR samples to run on the gel. You should also run a molecular weight marker, if you have it: the restriction-digested lambda DNA left over from the restriction digest lab. You should have several different samples of that DNA. You choose which one to use as a size standard, keeping in mind that the expected PCR products are around 200 bp long.

W R I T I N G   Y O U R   R E P O R T

You should do one report for your lab group, and turn it in with everyone's name on it. Your report should include the following sections.

INTRODUCTION

Explain what the lab was about and briefly outline the experiments you did and what they were intended to tell you.

METHODS

Include a flow diagram showing what you did, and note any deviations from the written protocol.

RESULTS

You should have three sections of results:

First set of plates: how many plaques on each plate?

Second set of plates: how many plaques on each plate?

PCR gel: include the gel picture. Label each lane.

DISCUSSION

In the discussion, start by discussing your specific results.

Did you get plaques as expected the first time you infected cells with phage?

I hope you did, because the rest of your experiment doesn't mean anything if you didn't.

Did you get plaques when you re-infected cells using your plate lysate?

This relates to the second and third of Koch's postulates. In other words, does the pathogen you get from the dead bacterial cells cause other bacterial cells to die the same way?

If the phage is a pathogen, you would expect to see it replicating after it enters cells. Can you tell if this has happened? How much replication?

Did you get any PCR products?

If your controls worked and you got PCR products of the expected size, you can be fairly sure that your PCR reaction contained some phage DNA to act as a template. Which reactions had products, and how do your results help to determine whether the phage is really the cause of the plaques you observed?

After discussing your results, discuss their meaning:

Can you prove that the plaques were caused by the phage?

What information do you need to do this?

What other experiments would you want to do to increase your confidence that the phage is the cause of lysis?

Finally, relate your results to the questions the experiments were intended to address. The goals of this lab include demonstrations of:

A virus life cycle

Some experiments to illustrate the experimental approach to identifying pathogens

Using PCR as a diagnostic tool

In your discussion, mention what the lab showed in terms of each of these goals.

⬧ W H A T   Y O U ' L L   B E   Q U I Z Z E D   O N :

Be sure to review the objectives at the beginning of the lab and all the sections of the lab report. Everything in the lab manual is fair game for test questions.

Some hypothetical questions might be posed, such as:

What if you had obtained a PCR product from the no phage/no plaque lysate?

What if you didn't get any PCR products at all?

What if you had obtained plaques on the "no phage" plates?

Other questions may test your understanding of the methods. For example:

When the DNA is heated in the denaturing step, why do the strands separate but not break?

In the annealing step, how can you make sure that the primers anneal to the two strands of the template instead of the two template strands re-annealing to each other?

Supplemental Exercises

# S1: SOLUTIONS & DILUTIONS

INTRODUCTION

The chemistry of life depends upon specific molecules interacting with each other. Successful interaction requires that the molecules be dissolved in water. One or more molecular species dissolved in water constitutes as solution. The molecules dissolved in water are called solutes, while the water is referred to as solvent. At this point you should review the discussion of solutions in your text book.

A key property of solutions is the concentration of solute. To a nonscientist, concentration is most often thought of in terms of percent. A "10% glucose solution" is something most of us can easily relate to and visualize (for every 100 parts of solution, 10 parts are glucose). The "parts" in a percent solutions usually refer to weight. Thus, the 10% glucose solution would consist of 10 grams of glucose for every 100 grams of solution (10 g glucose in 90 g H_2O).

Figure 1. Comparison of the composition of solutions by weight

Noting the concentration of solutions in percent by weight can lead to problems because it doesn't give any information about the concentration of molecules. For example, in comparing a 10% solution of glucose and a 10% solution of sucrose (Fig. 1), you see that each has the same weight of sugar (10g per 90g H_2O), but because a glucose molecule is about half the size of a sucrose molecule (Fig. 2), the glucose solution has about twice the concentration of dissolved solute molecules. The weight of sugar in each solution is the same, but there are twice as many sugar molecules in the glucose solution.

To get around this problem, solutions are often described by their solute concentration using the

Glucose : Molecular weigth = 180   Sucrose: Molecular weight = 342

Figure 2. Comparison of molecular sizes of glucose and sucrose
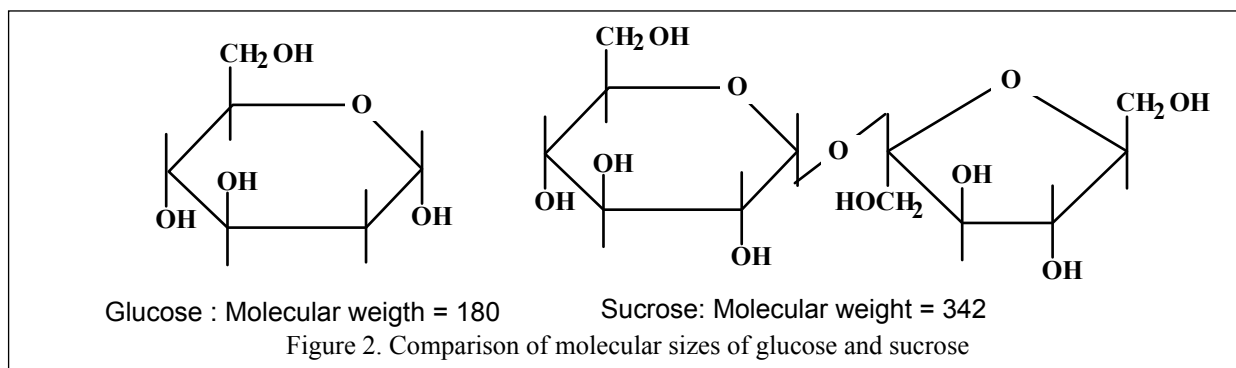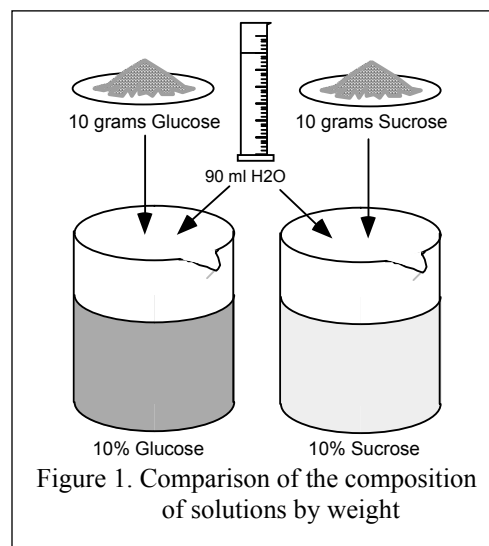
Molar designation. A Mole of a substance is equal to its molecular weight in grams. Thus for any type of substance, the number of molecules in a Mole is always the same; namely 6.022 x 10^{23} (this is an extremely large number).

Molar solutions are simply the number of moles of solute in one liter of solution. Figure 3 compares how 1 Molar glucose and sucrose solutions would be made. The concentration of solute molecules in both solutions is the same: 6.022×10^{23} molecules per liter of solution.

Figure 3. Comparison of 1 Molar solutions of glucose and sucrose
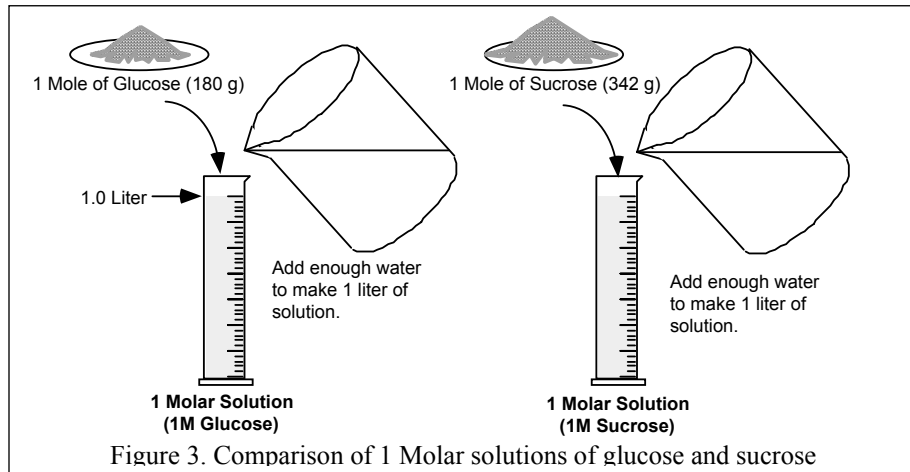
Since a great deal of experimental lab work in biology involves solutions, the ability to work with and manipulate them is very important. In this lab you will investigate several techniques for working with solutions.

In biology lab work, concentrations are often given in micro-moles or μM. A μM is one millionth of a mole per liter, or 1×10^{-6} M.

USING PIPETTES TO MEASURE SMALL VOLUMES

1. For most work in the biology laboratory, it is necessary to prepare smaller volumes of dilutions. In next series you are going to make a dilution series using and ending up with relatively small volumes.

2. There are two kinds of pipettes commonly used in laboratories (Fig. 4). The "blow out" type delivers the desired amount when completely emptied. The other type delivers the desired amount when the liquid level reaches the last calibration mark. This type is more accurate but the "blow out" type is more convenient because once it is filled to a known level, it can then be emptied completely for an accurate measurement. You will use both types at one time or another in later exercises, so always check the type your are using.

Figure 4. Comparison of "standard" and "blow out" pipettes
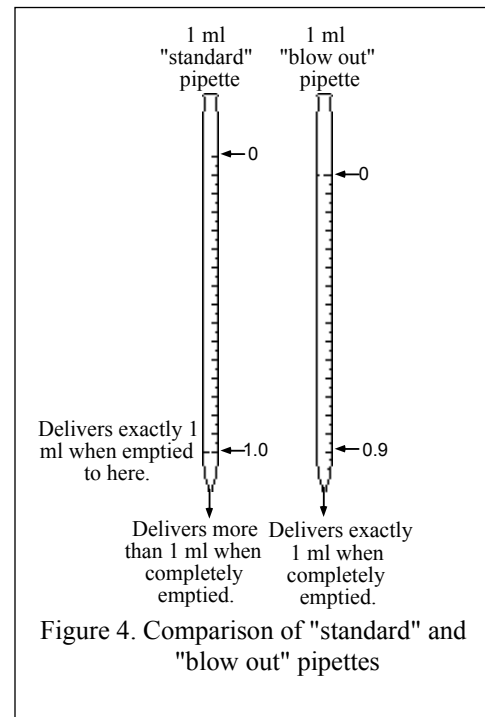
CONCENTRATION QUESTIONS

✍ How many grams of glucose would be dissolved to make 1 liter of a 0.5M glucose solution?

✍ How many molecules of glucose are in that 1 liter of 0.5M glucose solution?

✍ What is the concentration of the 0.5M glucose solution expressed in mM?

✍ What is the concentration of the 0.5M glucose solution expressed in %?

✍ How many grams of sucrose would be dissolved in 1 liter of a 0.5M sucrose solution? How does that compare to the grams of solute in the 0.5M glucose solution?

✍ How many molecules of sucrose in that 1 liter of 0.5M sucrose solution? How does that compare to the amount of solute in the 0.5M glucose solution?

✍ How much of the 0.5M glucose solution is needed to provide 100 mg of glucose?

✍ If you were to dilute 100 ml of the 0.5M glucose solution with 400 ml water, what would be the concentration of the diluted solution?

✍ If you were to dilute 10 µl of the 0.5M glucose solution with 1.99 ml water, what would be the concentration of the diluted solution?

✍ How would you prepare 10 ml of 0.1M glucose from the 0.5M glucose solution?

✍ How would you prepare 100 ml of 1% glucose from the 0.5M glucose solution?

✍ How would you prepare 20 µl of 25 mM glucose from the 0.5M glucose solution?

✍ How would you prepare 100 µl of 40 mM glucose/40 mM sucrose from the 0.5M glucose and 0.5M sucrose solutions?

WORKING WITH MICROPIPETS

INTRODUCTION

Laboratory work in molecular biology and biotechnology is usually done in minute quantities. The unit of measure used for setting up reactions is the **microliter (µl)**. One microliter is one millionth (10^{-6}) of a liter.

So: 1 L = 1,000,000 µl, and 1 ml = 1,000 µl

Practice these conversions:

1. Convert the following to ml:
   100 µl
   500 µl
   3,000 µl
   10 µl

2. Convert the following to µl:
   5 ml
   0.5 ml
   0.004 ml
   0.000001 ml
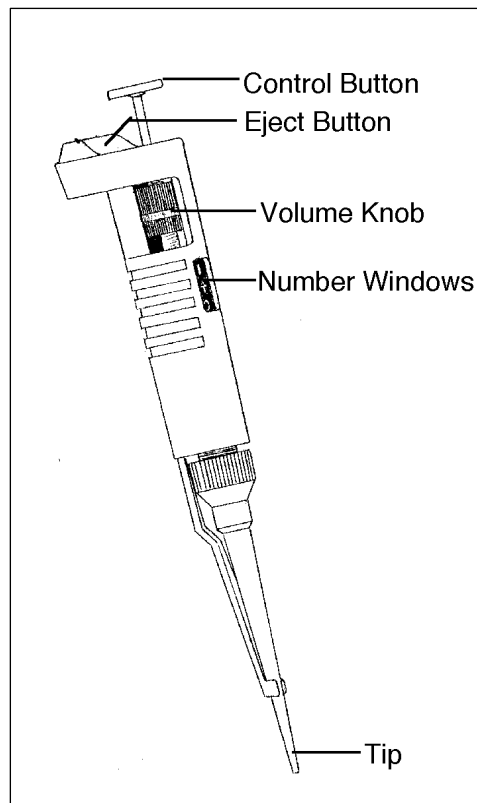
The **micropipet** is an instrument that allows us to accurately measure µl volumes of reagents. Micropipets are delicate, very expensive, and the cornerstone of our work with DNA. In this lab, you will learn to properly use and care for micropipets. A micropipet uses suction to draw up specific amounts of liquid. Its parts allow you to control how much liquid to suck up and dispense. It is essentially a hollow barrel with an adjustable plunger through it. On the left is a diagram of a micropipet and its specific parts.

The *control button*, or *plunger*, allows the user to suck up and dispense liquid.

The *eject button* allows ejection of micropipet tips after use.

The *volume knob* allows the user to dial the amount of liquid to be measured.

The *number window* shows the amount dialed.

The *tip* of the micropipet is where the micropipet tips are placed. The entire white part is called the *barrel*.

Control Button

Eject Button

Volume Knob

Number Windows

Tip

Damaging these instruments can be avoided by following a few simple rules:

> - **Never rotate the volume knob beyond the upper or lower range of the micropipet.**
> - **Never use a micropipet without a tip in place.**
> - **Never lay down a micropipet with a filled tip.**
> - **Never allow plunger to snap back after ejecting fluid.**
> - **Never immerse barrel of micropipet in fluid.**
> - **Never flame micropipet tips.**

Micropipets are designed to deliver a specified volume within a certain range, with the appropriate tip in place. You have micropipets for the following ranges:

Name of micropipet	Range of Volumes Delivered	Tip To Use
P1000	200-1,000µl	Blue
P200	20-200µl	Yellow
P20	2-20µl*	Yellow

*We use this micropipet to measure down to 1µl

Perhaps the most difficult part of using micropipets is setting them properly. On each of the micropipets, you will find 3 numbers places in the number windows. However, the numbers represent different volumes for P1000, P200, and P20:

Number	P1000	P200	P20
1st	X,000µl	X00µl	X0µl
2nd	X00µl	X0µl	Xµl
3rd	X0µl	Xµl	0.Xµl

You will notice a red line on the P1000. This represents a decimal point in ml. The red line on the P20 is the decimal point for µl.

Practice setting the following volumes:

1. P1000: 324, 1000, 546µl
2. P200: 24, 156, 87µl
3. P20: 2.4, 18.9, 6.0µl

USE OF MICROPIPETS

1. Observe the instructor's demonstration on the proper use of the micropipet before beginning this exercise.
2. Obtain two 1.5 ml microfuge tubes and fill one with distilled water. You will practice transferring liquid from one tube to the other.
3. Choose a micropipet and set the dial to a desired volume. To operate, your thumb should be at the top of the plunger, and your fingers wrapped around the body. You may have the ejector positioned under your thumb (see picture above) or facing out (I prefer it facing out).
4. Place a tip onto the micropipet by pressing the tip of the micropipet barrel firmly into a tip of the appropriate type (blue or yellow)
5. Depress the plunger to the first stop.
6. While holding the plunger down, place the tip into microfuge tube and into the liquid.
7. **Slowly** withdraw your thumb to suck liquid into tip. Watch that it goes up without air bubbles. Do not snap back plunger!
8. Place the tip into the bottom of the receiving microfuge tube.
9. Press plunger to first stop to dispense liquid. Continue to press beyond to first stop to get out all of the remaining liquid in the tip.
10. Pull tip out of liquid before relaxing the plunger back to original position.
11. Eject tip into waste container by pressing the ejector button.

USE OF MICROFUGES

1. Observe the instructor demonstrate the proper use of the microfuge and how to insert tubes in a balanced configuration. This is extremely important, because **spinning tubes in an unbalanced position will damage the microfuge!**
2. Be sure tubes you are spinning are in pairs and have approximately the same weight/volume in them.
3. Open lid and remove rotor cover. Place tubes in pairs arrange so that they are at opposite ends in the rotor.
4. Replace rotor cover and close lid.
5. Select appropriate time and push start. For short pulses, hold the pulse button for the desired time.
6. Wait for rotor to stop completely before opening lid and removing your tubes.

SMALL VOLUME PIPETTING EXERCISE

1. Obtain a P20 micropipet, yellow tips and 2 microfuge tubes.

2. Label the tubes A and B. Use the matrix below as a guide for adding appropriate volumes of the colored solutions to each tube.

3. First add the appropriate amount of water to the bottom of each tube. Be sure all of the liquid comes out and forms a small bubble of liquid at the bottom of the tube.

Tube	Red	Blue	Yellow	Water	Color Observed
A	-	2 µl	2 µl	11 µl	
B	3 µl	2 µl	-	10 µl	

4. Next add the colored solutions one at a time. Dispense the solution directly into the small bubble of liquid at the bottom of the tube.

5. After you have added all of your solutions into each tube, practice mixing the contents with a micropipet. Set your micropipet to 15 µl and slowly pipet the mixture up and down until well mixed.

6. Place all the tubes in the microfuge and apply a short (1-2 seconds) pulse. **Make sure the tubes are placed in a balanced configuration!**

7. Record the final color of the solution in each tube in the table above.

8. A total of 15 µl was pipetted into each tube. Check that your measurements were accurate by setting the P20 to 15 µl and withdrawing the contents of each tube.

LARGE VOLUME PIPETTING EXERCISE

1. Obtain a P200 and P1000 micropipet, yellow tips and blue tips and 4 microfuge tubes.

2. Label the tubes C, D, E, & F

3. Use the matrix below as a guide for adding appropriate volumes of the colored solutions to each tube.

4. Mix well (by finger vortexing or micropipetting) and place all the tubes in the microfuge and apply a short (1-2 seconds) pulse. **Make sure the tubes are placed in a balanced configuration!**

Tube	Red	Blue	Yellow	Water	Color Observed
C	40 µl	-	40 µl	920 µl	
D	-	20 µl	260 µl	720 µl	
E		120 µl	20 µl	860 µl	
F	60 µl	30 µl	30 µl	880 µl	

5. Record the final color of the solution in each tube in the table above.

6. A total of 1,000 µl (1ml) was pipetted into each tube. Check that your measurements were accurate by setting the P1000 to 1,000 µl and withdrawing the contents of each tube.

Demonstrate to yourself and to your lab partners your micropipetting skills.
Be sure you know how to:

- ❏ Convert from liters to milliliters to microliters.

- ❏ Identify micropipet parts and their purposes.

- ❏ Choose and set the correct micropipet for the job.

- ❏ Set the micropipet to the correct volume.

- ❏ Properly use the micropipet.

- ❏ Properly use a microfuge

ACTIVITY— USING MICROPIPETS TO PREPARE DILUTIONS

<u>Describe how</u> each step is to be done. Then do it.

1. Prepare 2 ml of 10% solution of blue food-color dye in a test tube.

2. Put 0.9 ml H_2O into each of ten new test tubes. Label five of the tubes 1 through 5 and the other five A through E.

3. In tubes 1–5, prepare a **two-fold serial dilution** of your 10% dye solution.
   What is the dye concentration in each tube?

4. In tubes A–E, prepare a **ten-fold serial dilution** of your 10% dye solution.
   What is the dye concentration in each of these tubes?

5. Prepare a twelfth tube containing 1 ml of 0.5% dye diluted directly from the 10% stock.
   (Describe how.)

6. Arrange the twelve tubes in order of decreasing dye concentration. Does the pattern of decreasing color match your predicted calculations?

✍ Would it be more accurate to prepare a 0.1% solution by direct or serial dilution?

MEASURING PROTEIN CONCENTRATION IN CELL LYSATES

In order to measure the concentration of a compound in a solution, we usually perform an *assay*. One type of very sensitive assay utilizes *fluorescence* — a substance absorbs light energy and re-emits that energy as a longer wavelength (lower energy) light. A device called a *fluorometer* measures the amount of the fluorescent substance in a solution by exciting the solution with the shorter wavelength light, and measuring the amount of longer wavelength light emitted.

Proteins are not usually fluorescent. But we can measure the concentration of protein in a solution by mixing it with a reagent that becomes fluorescent in the presence of protein.

To get the proteins from cells, we must first break the cells open. Bursting of cells is called *lysis*; the complex solution released from burst cells is called *soluble lysate*.

MATERIALS

- Four tubes of different tissue homogenates or bacterial culture lysates.
- 8 2-ml microfuge tubes.
- 2 ml of protein reagent "Working Solution".
- 8 fluorescence assay tubes. (These are different from microfuge tubes!)
- Qubit® fluorometer, calibrated.

METHOD

1. From each homogenate sample, pipet 1.5 ml into a labeled 2-ml microfuge tube. (Label with a marking pen directly onto the tube. Do not use tape on tubes to be centrifuged!)
2. Balance the microfuge and centrifuge at high speed for ten minutes.
3. Transfer the supernatant of each soluble lysate into new respectively labeled 2-ml microfuge tubes. Be careful not to transfer any of the pelleted cell debris.
   You will use these lysates for the protein assay today and again for the electrophoresis next lab, so <u>do not throw them away</u>!
4. Assay two volumes of each sample (**See Appendix A2i**):
   a. Plug in the Qubit® fluorometer.
   b. Pipet 198 µl of Working Solution into a fluorescence assay tube. Add 2 µl of the sample soluble lysate. Mix briefly.
   c. Pipet 190 µl of Working Solution" into another fluorescence assay tube. Add 10 µl of the sample soluble lysate. Mix briefly.
   d. Repeat both dilutions for each sample lysate.
   e. Let all eight assay samples sit at room temperature for 15 minutes.
   f. Calibrate the Qubit® fluorometer with the three protein fluorescence standards.
   g. Insert your sample tube and press GO. The fluorometer should display the protein concentration in the assay tube. Write this "[protein] in assay" number down in the table below, including the units (µg/ml). Repeat with your remaining samples.

RESULTS

Protein concentration (= [protein])

#	Sample Name	Lysate volume	[protein] in assay	[protein] in lysate	[protein] in "gel-ready" sample	volume to load 5 μg protein	μg protein in 20 μl
1		2 μl					
2		10 μl					
3		2 μl					
4		10 μl					
5		2 μl					
6		10 μl					
7		2 μl					
8		10 μl					

1. Record the reading of protein concentration from the fluorometer in "**[protein] in assay**".
2. Remember, the fluorometer reads the protein concentration in the fluorometer tube. But this assay sample was diluted with Working Solution. Use the "**lysate volume**" and "**[protein] in assay**" values to calculate the original protein concentration in the soluble lysate of each sample ("**[protein] in lysate**").
   - The "**[protein] in lysate**" calculation for the 2 μl & 10 μl "**lysate volume**" replicates of the same sample should be the same. If they are not, why not?

3. Next lab, you are going to prepare "gel-ready" samples of these four soluble lysates to load onto electrophoresis gels. Read the "Sample Preparation Protocol" on p. 5 of *Lab 1: Protein electrophoresis*. Given your "**[protein] in lysate**" concentrations you calculated today, what will the protein concentrations be in the four "gel-ready" samples you prepare next lab?
   Record these values in the "**[protein] in gel-ready sample**" column above.
4. Assuming you want to load 5 μg of protein from each sample onto your electrophoresis gel, what volume of each of the "gel-ready" samples will you load?
   Record these volumes, in μl, in the "**volume to load 5 μg protein**" column above.
5. Each sample well on the electrophoresis gel holds a maximum of 20 μl. So what is the maximum amount of protein you could load into each sample well?
   Record these amounts, in μg, in the "**μg protein in 20 μl**" column above.

S2: Virtual Molecular Biology Labs

Even as methodologies and technologies for analyzing DNA structure improved, comprehending the immense complexity of genetic information in even the simplest organism requires enormous computational power and specialized programming. It became evident that, rather than the traditional competition among research institutions, significant progress in molecular biology would only happen with collaboration and shared information. From this global cooperative have developed many online tools allowing the most modest facilities to access powerful applications. A few common ones are described at https://bitesizebio.com/19875/10-favorite-online-tools-for-molecular-biology/ .

Here we will describe how to *use a known DNA sequence* and a few of such tools to:

i. **do a virtual DNA digest. And, …**
ii. **identify probable genes by open reading frames (ORF}**

S2.i. DNA Virtual Digest

In this exercise, you'll explore the connection between nucleotide sequences, restriction enzymes, and DNA fragment sizes by using sequence analysis software to figure out your expected results from DNA digests.

Restriction map & virtual digest

DNA maps are simplified representations of nucleotide sequences, showing features of particular interest such as genes. Restriction maps are DNA maps that show restriction sites, the locations where specific restriction enzymes will cut the DNA. Here is a map of the pGLO plasmid DNA we will be using in Lab 4:

The features on this pGLO map include the locations for the *bla*, *araC*, and *gfp* genes (described in Lab 4). Also included are restriction sites for the restriction enzymes that we use in lab: PvuI, XhoI, EcoRI, and HindIII. This map will give you a general idea of what size DNA fragments to expect if you cut pGLO with each of these enzymes:

- **PvuI**: Cuts once [3054], so you get one linear piece, the size of the entire plasmid (5371 bp).
- **XhoI**: Cuts also once [1765], so you again get one linear piece, the size of the entire plasmid (5371 bp).
- **EcoRI**: Cuts once [2063], so you get one linear piece, the size of the entire plasmid (5371 bp).

NOTE: Since the plasmid is a circular DNA, cutting once gives you one linear piece. If you cut a linear DNA once, you would get two pieces. Although PvuI, XhoI, and EcoRI cut at different places, the resulting fragment will be the same size. That would not be true if cutting linear DNA.

- **HindIII**: Cuts twice, so you will get a small piece and a large piece.
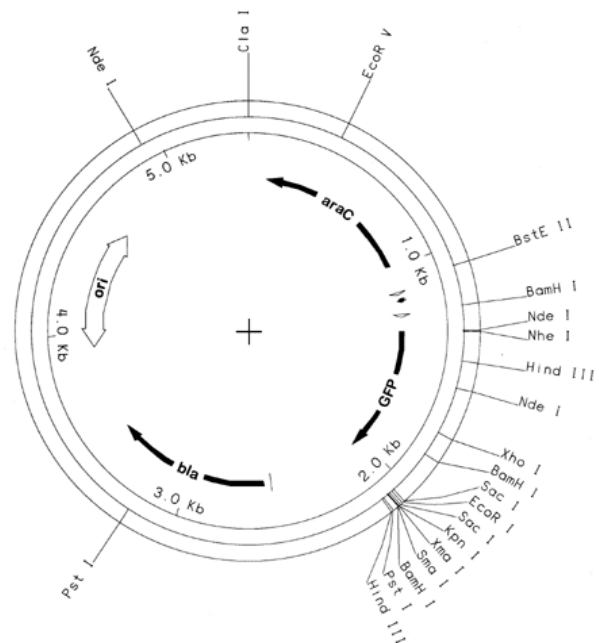
Now suppose you want to cut the plasmid with more than one enzyme at a time. Each enzyme will act on its own restriction sites, so the different enzymes won't affect each other. From the map above, you can see that if you cut with both EcoRI and HindIII, you will cut out a tiny piece between the EcoRI and HindIII sites; you probably wouldn't be able to see this small fragment on a gel, so it's not worthwhile to perform this digest. Suppose you cut with PvuI and EcoRI together.

Perform some virtual digests

To predict the results of our DNA digests in lab, we can use restriction mapping software such as this one:

http://restrictionmapper.org/

For a precise electronic restriction digest, you need to enter the nucleotide sequence of the DNA you're going to cut. To find the nucleotide sequence of some of the DNA sources we'll be using in class:

- **λ-viral** genome [linear sequence] — (Lab 2): go to
  http://www.bioinformatics.nl/molbi/SCLResources/Sequences.htm#The_sequence_of_lambda_DNA
- **pARO191** plasmid [circular sequence] — (Lab 3): go to
  http://www.bio-rad.com/webroot/web/pdf/lse/misc/pGLO_plasmid_sequence.txt
- **pGLO** plasmid [circular sequence] — (Lab 4): go to
  https://www.ncbi.nlm.nih.gov/nuccore/1027812612

Note that the sequence is shown for only one strand of DNA, but the software is smart enough to recognize that the actual DNA is double-stranded.

1. Open RestrictionMapper.org.
2. Copy that whole sequence from the above source, and paste it into the **Sequence Info** box labeled "**Paste Sequence Here**". (The software will ignore any numbers and spaces and only look at the nucleotide sequence).
3. In the **Conformation** column, Check "**Linear**" or "**Circular**" as appropriate (see above.).
4. Go to the **Include** box, "**Select Individual Enzymes**". Scroll down and click on the restriction enzyme you want to select. To do a two-enzyme digest, select the first enzyme as described; then scroll to find the second enzyme and hold down Command [Mac] or Control [PC] while selecting that enzyme. (There are a lot of restriction enzymes, so this is the hardest part).
5. Finally, in the **Menu** box, click on **Virtual Digest**.

Virtual digest will take you to a page showing the complete nucleotide sequences of the DNA fragments produced in the digest (which you probably don't care about) and also the length of each fragment (which you will be able to verify on your gel). Those fragment lengths are your expected results for this experiment.

Repeat the virtual digest procedure for the other enzymes and combinations listed below.

Make your predicted gel picture

Do a virtual digest of a specified DNA sequence with specific restriction enzymes and combinations of enzymes. For example, simulate the digestion of **λ-DNA** with **HinDIII** and **EcoRI** separately and in combination as described in Lab 2.

Make a table of the number and size of the restriction fragments produced by these different digest conditions.

Draw a diagram of the predicted gel showing the relative positions of the bands for the different digests, and label each band with its size in base pairs.

Answer the following questions:
- Why do different bands appear when the same DNA is digested with different restriction enzymes?
- Why do certain bands present when digested with HinDIII or EcoRI alone disappear when digested with both enzymes? Why do some bands *not* disappear? Why do some new bands appear that were present in neither of the single-enzyme digests?

How much DNA per band?

If you perform a restriction digest and run it on a gel, you'd like to be able to see all the bands. Your ability to see a band on the gel depends on how many nanograms of DNA are in that band, as well as how you stain the DNA. We use ethidium bromide in our gels, which allows us to visualize individual bands containing as little as 50 ng. However, the different bands from a digest won't have the same mass of DNA. If you cut some DNA into two fragments and one fragment is twice as large as the other, the larger fragment will have twice the mass of the smaller fragment.

Suppose you are doing a restriction digest of **λ-DNA** using the enzyme Hind III. If you start with 500 ng of uncut plasmid DNA, how many nanograms of DNA will be in each of the bands on your gel? **Label the bands in the Hind III lane of your gel diagram with the amount in nanograms.** Do you think you'll be able to see the smallest band?

Review

Terms and concepts

- Restriction map. Given a restriction map, you should be able to determine what the gel result would be when DNA is cut with particular restriction enzymes.
- Restriction site
- Restriction enzyme
- Restriction fragment
- Restriction fragment length polymorphism (RFLP)

Review questions

1. What is the difference between a nucleotide sequence and a DNA map?
2. On restriction mapper, why does it matter if you check the "linear" or "circular" box?
3. Suppose you have a linear piece of DNA. You cut it with Hind III, and you get one fragment of 800 bp and one of 200 bp. Draw a restriction map of this DNA.
4. When you are going to perform a restriction digest, how do you determine how much DNA you need to start with in order to see all the bands on your gel? (This is a quantitative question. On a test, I could ask you how much DNA you need to use. What information would you need to answer this question?)

S2.ii. Open Reading Frames in pGLO

This activity describes a computer-based assignment that requires you to cut and paste information among several browser windows; you'll find it easiest to complete it using a laptop or a tablet with a keyboard.

By now you've carried out numerous experiments using the pGLO plasmid. You've also examined the nucleotide sequence of pGLO to make a restriction map. In this exercise, you'll look more closely at the nucleotide sequence to identify some protein-coding genes.

Finding genes

It's not very difficult to isolate some DNA and have it sequenced. In BIOL-6B, you purify a couple of different plasmids. You could easily send these off to be sequenced commercially; you could probably get one of your plasmids sequenced for under $50. Or, if we had sequencing equipment, you could do it yourself. In the case of pGLO, though, we don't need to get it sequenced because it has already been done. We can just look it up.

However, once you have a DNA sequence, how do you figure out what it means? In the case of pGLO, you know that the plasmid contains some important sequences some are noncoding (such as the origin of replication) and some regions code for proteins. Here is a pGLO map from the virtual digest page:
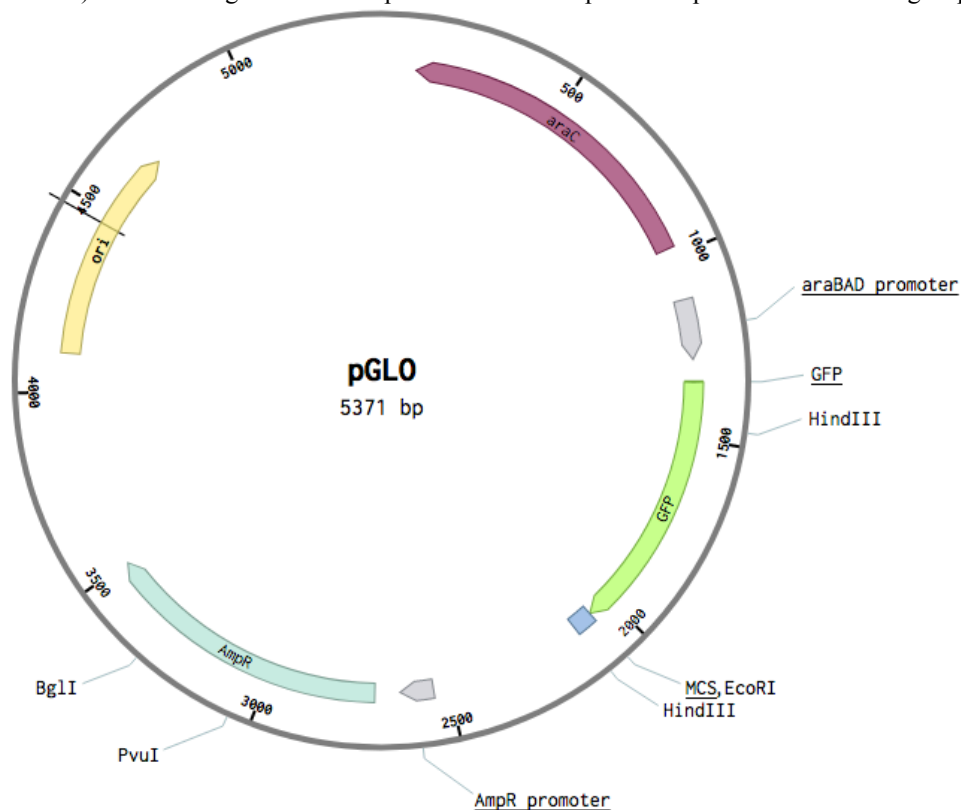
To make a map like this, you would have to start with the nucleotide sequence, analyze the sequence to find the particular regions you want to highlight, and then make a simplified map that shows the locations of important features but doesn't show the actual nucleotide sequence. There are three protein-coding genes shown in this map: *gfp* (Green Fluorescent Protein), *araC* (a protein that regulates the *gfp* operon), and *AmpR* (the ampicillin resistance gene, also known as *bla* coding for β-lactamase enzyme).

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In this assignment, you'll learn how to use online bioinformatics tools to analyze a nucleotide sequence and find the protein-coding genes. We'll use pGLO as an example, but the process is similar for any DNA sequence, whether it's a plasmid, the genome of a newly discovered bacterial species, or the human genome. Obtaining a nucleotide sequence is only the beginning; the greater challenge is to figure out the biological meaning of that sequence.

Open reading frames

To find protein-coding genes, you need to start by finding open reading frames. (In this section, I will use the word "gene" to mean a gene that codes for a protein; as you know, there are also genes that get transcribed into RNA but don't code for proteins.)

Suppose this is a double-stranded DNA nucleotide sequence:

5' ACCGCATGTCTCGGATGAAAAGCTGGGGATAGAAGCTA 3'
3' TGGCGTACAGAGCCTACTTTTCGACCCCTATCTTCGAT 5'

Remember that nucleic acid strands are always synthesized from 5' to 3', so we usually write them that way. If you want to try to find a protein-gene in this DNA sequence, you should start by finding the potential RNA sequence that could be transcribed from the DNA. In a plasmid or a chromosome, either strand could potentially be used as a template for transcription. In this example, **if the bottom strand is transcribed**, the resulting RNA sequence would be this:

5' ACCGCAUGUCUCGGAUGAAAAGCUGGGGAUAGAAGCUA 3'

Note that this is the same as the upper strand of DNA, but with U instead of T. That is why the lower DNA strand is the **template strand** and the upper strand is called the **coding strand**.

The next step is to ask whether that hypothetical messenger RNA sequence could potentially code for a protein. If the sequence is an mRNA and gets translated, the translation process will read the nucleotide sequence as codons, with **three nucleotides per codon**. There are three ways to read this above mRNA sequence into codons:

5' ACC GCA UGU CUC GGA UGA AAA GCU GGG GAU AGA AGC UA 3'

5' AC CGC AUG UCU CGG AUG AAA AGC UGG GGA UAG AAG CUA 3'

5' A CCG CAU GUC UCG GAU GAA AAG CUG GGG AUA GAA GCU A 3'

These groupings are called **reading frames**. For each mRNA, there are three possible reading frames, but only one is used for a particular protein. The correct reading frame is determined during translation by the presence of a **start codon**, which is the location where an initiator tRNA binds to the mRNA during formation of the translation initiation complex. The start codon in the mRNA is usually AUG, for both eukaryotes and bacteria. Translation begins at a start codon and continues until a **stop codon** (UAG, UGA, or UAA) is reached. A nucleotide sequence that begins with a start codon and ends with a stop codon is called an **open reading frame (ORF)**, and could potentially code for a protein. For the sequence shown above there is one open reading frame:

5' ACCGC **AUG** UCU CGG AUG AAA AGC UGG GGA **UAG** AAGCUA 3'

The start codon, AUG, is shown in green; the stop codon, UAG, is shown in red. Note that the start codon is not at the beginning of the mRNA sequence; there is a 5' untranslated region before the start codon as

well as a 3' untranslated region after the stop codon. This is always true for mRNAs. The regions before and after the ORF are not shown grouped into codons, because they are not translated.

Finding genes in a nucleotide sequence

All protein-coding genes are encoded by ORFs, but not all ORFs encode proteins. In principle, the ORF shown above could encode a polypeptide. However, most proteins are much longer so this one would probably not be a real gene. Some ORFs exist just by chance, but aren't actually used by the cell to make proteins. In general you can ignore the very short ones. It makes sense to start analyzing a nucleotide sequence by looking for the longest open reading frames, and analyze those.

Starting with a DNA nucleotide sequence, the basic procedure for finding protein-coding genes goes like this:

1. Figure out the possible RNA sequences. Since DNA is double-stranded, either strand could be used as a template, so there are two possible RNA sequences. In transcription, only a small part of the genome is copied into RNA, but you can start by pretending that the entire sequence is transcribed.
2. Search the possible RNA sequences for ORFs as described above.
3. Determine the amino acid sequences of the hypothetical polypeptides encoded by the ORFs. This is described in the next section.
4. Analyze the hypothetical amino acid sequences to see if they look like real proteins. This is described in the next section.

This is clearly a job for a computer. In this exercise, you'll explore some software that was designed to do this kind of analysis.

Keep in mind that you are looking at bacterial genes in this exercise. Analyzing eukaryotic genes could be considerably more complex due to the presence of introns.

Analyzing hypothetical polypeptide sequences

Once you've found an ORF, you can use the genetic code to translate that DNA or RNA sequence into a possible amino acid sequence:

```
5' AUG UCU CGG AUG AAA AGC UGG GGA UAG 3'
    Met  Ser  Arg  Met  Lys  Ser  Trp  Gly  Stop     {3 letter amino acid codes}
    M    S    R    M    K    S    W    G    Stop       {1 letter amino acid codes}
```

Now you have an amino acid sequence for a hypothetical protein. (Note that the codon AUG can be a start codon or a methionine within the sequence. Also keep in mind that this hypothetical amino acid sequence is much too short to be a real protein.) I am showing both the 3-letter amino acid codes and the 1-letter codes, but the 1-letter codes are always used for sequences. How can you find out if that hypothetical amino acid sequence represents a real protein, and what protein it might be? The simplest approach is to compare the hypothetical amino acid sequence to a database of known protein sequences to see if there is a match. Even if you discovered a new protein that has never been found before, there is a good chance that it is similar to some other known proteins.

This type of comparison is called an **alignment**. It's going to require a lot of computing power. Luckily, you can access some extremely powerful computers online for free through the National Center for Biotechnology Information (NCBI), which is part of the National Institutes of Health (NIH). The software you'll use is called **BLAST**, for Basic Local Alignment Search Tool.

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The assignment

The basic process will go like this:

1. Copy the pGLO DNA sequence and paste it into **ORF Finder** to identify open reading frames.
2. Determine the amino acid sequences encoded by those ORFs.
3. Use **BLAST** to compare these hypothetical proteins to a database of known proteins.
4. Figure out which what proteins are encoded by these ORFs.

While many ORFs in a sequence don't actually code for proteins, in this example we'll see a few that do.

Find open reading frames in pGLO

Get the nucleotide sequence of the pGLO plasmid from Bio-Rad (open it in a new tab).

Go to the **Open Reading Frame Finder** (**https://www.ncbi.nlm.nih.gov/orffinder/**) at NCBI and paste the pGLO sequence in the box. Under "Choose Search Parameters," **set the minimum ORF length to 300**. A minimum ORF length of 300 nucleotides corresponds to a polypeptide sequence of 99 amino acids (3 nucleotides per codon, and the last codon is the stop codon). Most proteins are bigger than that. **Check "Ignore Nested ORFs."** This will ignore any reading frame that is completely inside another. It's not impossible for proteins to be coded that way, but it's not common. Leave the other options as they are. Click **Submit** to search for ORFs within the pGLO sequence.

You should see something like this. (But this is an example using a different sequence — not pGLO!):

ORF3 (271 aa)   Display ORF as...   Mark

```
>lcl|ORF3
MSHIQRETSCSRPRLNSNMDADLYGYKWARDNVGQSGATIYRLYGKPDAP
ELFLKHGKGSVANDVTDEMVRLNWLTEFMPLPTIKHFIRTPDDAWLLTTA
IPGKTAFQVLEEYPDSGENIVDALAVFLRRLHSIPVCNCPFNSDRVFRLA
QAQSRMNNGLVDASDFDDERNGWPVEQVWKEMHKLLPFSPDSVVTHGDFS
LDNLIFDEGKLIGCIDVGRVGIADRYQDLAILWNCLGEFSPSLQKRLFQK
YGIDNPDMNKLQFHLMLDEFF
```

Label	Strand	Frame	Start	Stop	Length (nt | aa)
ORF3	-	1	5189	4374	816 | 271
ORF1	+	3	732	1088	357 | 118
ORF2	+	3	3612	3953	342 | 113
ORF4	-	2	1015	692	324 | 107

ORF3

SmartBLAST

BLAST

BLAST Database:
UniProtKB/Swiss-Prot (swissprot)

Marked set (0)

SmartBLAST best hit titles...

BLAST

At the top, there is a map of the nucleotide sequence, showing where the ORFs are located within the sequence. The map isn't very useful in this case because it displays a circular plasmid sequence as if it's linear. At the bottom right is a list of ORFs, in order from longest to shortest. In this example, ORF3 is at the top of the list because it's the longest one. The length of ORF3 is 816 nucleotides, or 271 amino acids. The amino acid sequence of the hypothetical protein encoded by ORF3 is shown in the box at left; it begins MSHIQ... et cetera.

Analyze the ORFs

Now look at the Open Reading Frame Viewer for the pGLO sequence that you just entered, and start going down the list of ORFs.

Longest ORF (ORF 4)

Click on the top ORF in the list (if you selected the options listed above, it will be called ORF4). The following questions apply to this ORF.

Question 1: How many amino acids long is the protein encoded by this ORF? (The answer is to the right of "ORF4; it says Length in aa, or amino acids).

In the left box you'll see the amino acid sequence. Is that a real protein? Click on SmartBLAST to compare this sequence to a database of all known protein sequences. That will take you to a new SmartBLAST tab in your browser. At the top of that page there is a cladogram, showing how your query sequence is related to other similar sequences. In the section called "Best hits" you'll see a short list of protein sequences that closely match the ORF sequence from pGLO. The top hit is probably exactly the same as your ORF sequence, as shown by an Ident score of 100%; this tells you that your ORF corresponds to a real protein that has been sequenced and described. Here's an example from a different plasmid sequence:

In the example above, the best hit is called "MFS transporter [Bacillus cereus]," and it is 100% identical to the query sequence that was used in the search. The ORF sequence used in that example is exactly the same as the MFS transporter.

Now answer these questions about the longest ORF from your pGLO search.

Question 2: What is the name of the best hit for this ORF? Copy the long name of the best hit (looking at your pGLO data page, not the example above).

Question 3: What is the percent identity score for the best hit? If it is 100%, then it's the exact same protein.

Question 4: What does this protein do in the pGLO lab experiments that you've done? Answer this one in your own words.

Question 5: What is the molecular mass of the protein encoded by this ORF? Go back to the Open Reading Frame Viewer page and copy the amino acid sequence and then go to the **sequence manipulation** site (http://www.bioinformatics.org/sms2/protein_mw.html) at **bioinformatics.org**. Paste the sequence into the box and hit Submit. You'll get a result in kiloDaltons (abbreviated kD or kDa).

Second-longest ORF (ORF 2)

Follow the same steps for the next ORF in the list, but you only need to answer one question for this one:

Question 6: What is the name of this protein, and what does it do in the pGLO lab?

Third-longest ORF (ORF 1)

Repeat these steps again for the next ORF in the list. Answer two questions for this one:

Question 7: What is the name of the protein encoded by this ORF? If the name of the Best hit doesn't make sense to you, look down at the long list of "Additional BLAST hits" for this one.

Question 8: What is the molecular mass of this protein? (Use the amino acid sequence from the Open Reading Frame Viewer page and the **sequence manipulation site**, as described previously). The molecular mass of the protein isn't only for theoretical interest; you might see this on your gel when you do SDS-PAGE with your pGLO cultures.

Review

Some of the concepts from this page might turn up on the lab final, even though there's no "wet" lab work for this one.

Terms and concepts

- Alignment
- Codon
- Start codon
- Stop codon
- Reading frame
- Open reading frame (ORF)

Review questions

1. What is a reading frame? How many possible reading frames are there for a given DNA nucleotide sequence?
2. What is an open reading frame? How would you recognize an open reading frame without using software?
3. What does ORF Finder do?

4. If you have already identified a particular protein by SDS-PAGE, how would you identify the ORF that encodes that protein?
5. How would you know if a particular ORF actually encodes a protein?
6. What does BLAST do?
7. In this exercise, you determined the molecular mass of two proteins encoded by ORFs on pGLO. In principle, you might see these proteins on your SDS-PAGE gel after HIC. You're culturing your pGLO-transformed cells with and without arabinose; would you expect these proteins to be the same in both +arabinose and -arabinose samples?

References

General background

Wikipedia: Genetic code; Start codon; Open reading frame.

Bioinformatics tools

NCBI Open Reading Frame Finder

Bioinformatics.org Sequence Manipulation Suite. This page hosts a variety of browser-based tools for manipulating and analyzing sequence data.

BLAST Help. You probably don't need this, but this page gives you links to detailed explanations of all the information you see on the BLAST pages.

ExPASy Bioinformatics Resource Portal. From the Swiss Institute of Bioinformatics, another site with a range of useful tools for bioinformatics.

Benchling. An suite of bioinformatics tools. Benchling requires you to make a free account, and it takes a little while to learn the interface, but it's powerful and convenient. It's what I usually use for sequence analysis.

DNA sequencing services

Just in case you're curious about how to get DNA sequencing done.

MCLab. MCLab is not affiliated with McCauley, but I like the name!

Going deeper

Why is start codon selection so precise in eukaryotes?

Start Codon Recognition in Eukaryotic and Archaeal Translation Initiation: A Common Structural Core. In case you thought the process was too simple.

S3: Restriction Digests & Restriction Mapping

1. Suppose you're doing a restriction digest of lambda DNA, similar to the way you did your previous digest. Show the calculations and amounts for everything that goes into the tube. For this experiment, you want to cut 0.8 μg of DNA. The DNA solution is at 400 ng/μl. You're going to use the restriction enzyme Hind III. The unit definition for Hind III is: 1 unit of enzyme is enough to cut 1.0 μg of lambda DNA in 1 hour at 37°. You'll do your reaction for 1 hour at 37°. The enzyme stock solution is 8,000 Units/ml. Calculate the theoretical minimum amount of enzyme needed, and multiply that amount by 5. The final reaction volume should be 15 μl.

Shown below is a restriction map. It represents a molecule of double-stranded virus DNA, 50,000 base pairs long (50 kb). Restriction sites for the enzymes DpnI and Xma1 are shown on the map.
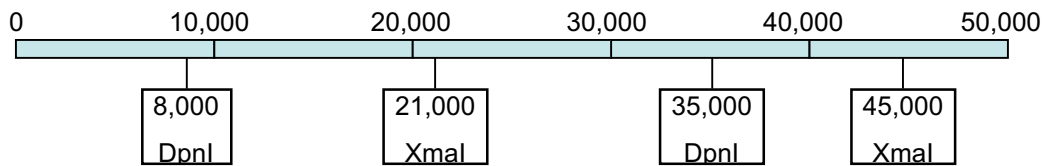
2. If you cut this piece of DNA with DpnI only, what size fragments will you get?

3. If you cut this piece of DNA with XmaI only, what size DNA fragments will you get?

4. If you cut this piece of DNA with both DpnI and XmaI together, what size DNA fragments will you get?

*Note: The following questions all refer to the DNA sequence shown in the **Table 1**, which is structured to resemble results you might see on a DNA gel.*

5. Suppose you have a 10-kb piece of DNA and you cut it with the restriction enzyme XhoI. You get two fragments: 2500 bp and 7500 bp, as shown in the table below. Draw a restriction map of this DNA showing only the XhoI restriction site (or sites).

6. Suppose you have a 10-kb piece of DNA and you cut it with the restriction enzyme HinDIII. You get two fragments: 2000 bp and 8000 bp, as shown in the table below. Draw a restriction map showing only the HinDIII site(s).

7. Referring to the table, draw a restriction map showing both the XhoI sites and the HinDIII sites.

8. Referring to the table, draw a restriction map showing all the restriction sites: XhoI, HinDIII, and EcoRI.

Table 1: Table showing restriction fragment sizes. Each column represents a lane on a gel. All lanes have the same starting DNA, cut with various restriction enzymes alone or in combination. Numbers refer to the size (bp) of the DNA fragments in that band.

| Uncut	EcoRI	HinDIII	XhoI	EcoRI + HinDIII	XhoI + HinDIII	EcoRI + XhoI
10,000						
		8,000				
			7,500			
	6,000					6,000
					5,500	
				4,000		
	3,000			3,000		
			2,500		2,500	2,500
		2,000		2,000	2,000	
	1,000			1,000		1,000
						500

9. Given the restriction map you prepared in question 8 above, draw the restriction fragment pattern you predict on the gel if you cut a piece of this DNA with all three restriction enzymes.

Cells are surrounded by a water solution that contains food molecules, gases, salts, and other substances.   This is the **external environment** for the cell.   The cell's outer surface of the plasma membrane is in contact with this external environment, while the inner surface is in contact with the cytosol.   Thus, the plasma membrane controls what enters and leaves the cell.

Small molecules may pass through the membrane.   However, the cell membrane is **selectively permeable.** That is, the membrane permits passage of some materials and does not permit passage of others.   If no additional energy is required for substances to pass through the membrane, the process is called **passive transport.**

I N T R O D U C T I O N

Time

Concentration Gradient

Dynamic Equilibrium

High   Low
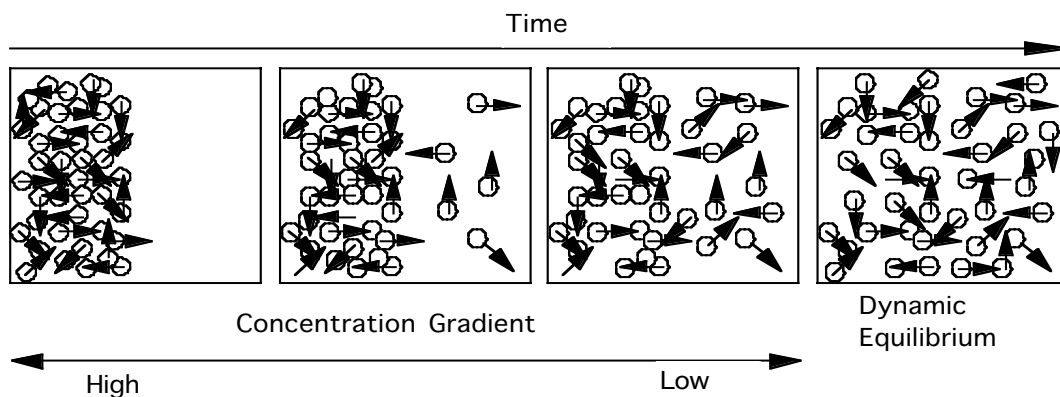
Can you remember walking into the front door of your home and smelling a pleasant aroma coming from the kitchen? It was diffusion of molecules from the kitchen through the air to the front door that allowed you to detect the odors. **Diffusion** is passive transport and is defined as the net movement of molecules from an area of greater concentration to an area of lesser concentration. To better understand how diffusion works, let us consider some information about molecular activity.

The molecules in a gas, a liquid, or a solid are in constant motion due to their **kinetic energy**. Moving molecules are constantly colliding with each other. These collisions cause the molecules to move randomly. Over time, however, more molecules will be propelled into the less concentrated area. Thus, the net movement of molecules is always from more tightly packed to less tightly packed areas.

Diffusion occurs when there is a difference in concentration from one region to another,

or from one side of a membrane to another. This unequal distribution of molecules is called a **concentration gradient**. When the molecules become uniformly distributed, **dynamic equilibrium** exists. The equilibrium is dynamic because molecules continue to move, but there is no net change in concentration over time. The process of diffusion occurs in both living and nonliving systems. In living systems, diffusion is responsible for the movement of a large number of substances, such as gases and small-uncharged molecules, into and out of living cells.

Osmosis is a specific type of diffusion; it is the movement of a solvent through a differentially permeable membrane. In biology, we are concerned with water solutions. When water is mixed with other molecules, this mixture is called an **aqueous solution**. Water is the **solvent** and the dissolved substances are the **solutes**. A solution is characterized by the solute. For example, water and sugar would be characterized as a sugar solution.

Since both the solute and solvent molecules take up space in the solution, the higher total concentration of all solutes (**osmolarity [Osm]**) corresponds with a lower concentration of water in that solution.

If a membrane separating two solutions is impermeable to all solutes, but is permeable to the water, the water will diffuse across the membrane according to its own concentration gradient. If both of these solutions have the same osmolarity (i.e., they are **isosmotic**), water will move equally in both directions across the membrane and neither will gain water from the other (i.e., they are **isotonic**).   But if one solution has a higher osmolarity (**hyperosmotic**) than the other, it has a lower water concentration and will therefore water will move faster into the hyperosmotic solution from the **hyposmotic** solution than water moving in the reverse direction.   The attraction of water by osmosis is called **osmotic pressure**. In the situation descibed, the hyperosmotic solution has a greater osmotic pressure (i.e., it is **hypertonic**) and therefore pulls water from the hyposmotic solution with a lower osmotic pressure (i.e., is **hypotonic**).
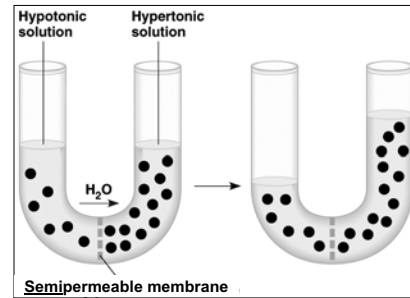
In the same way, since cell membranes are fairly permeable to water, cells placed in aqueous solution will gain and lose water with their external environment. If the solution is isotonic to the cytoplasm, the cells will gain and lose water at an equal rate (in dynamic equilibrium) and the cells with maintain their normal form.   If however, cells are placed in hypertonic solution, water will leave the cells faster than it enters and the cells will dehydrate and shrivel — a process called **crenation**.   Conversely, if cells are placed in a hypotonic solution, water will move into the cell faster than it exits and the cells will swell as the cytosol is diluted.   For cells with no cell walls, if the osmotic pressure exceeds the tensile strength of the plasma membrane, the cells will swell until they burst (**lysis**).

DETECTION OF CELL LYSIS IN ERYTHROCYTES (RED BLOOD CELLS)

Erythrocytes contain hemoglobin, a soluble protein pigment. Suspensions of intact red blood cells thus appear cloudy red. The bursting of erythrocytes is called **hemolysis**.   When the cells lyse, their ruptured membranes collapse and the hemoglobin is released into the surrounding solution.   Thus a suspension of RBCs that undergoes hemolysis appears as a clear red solution.
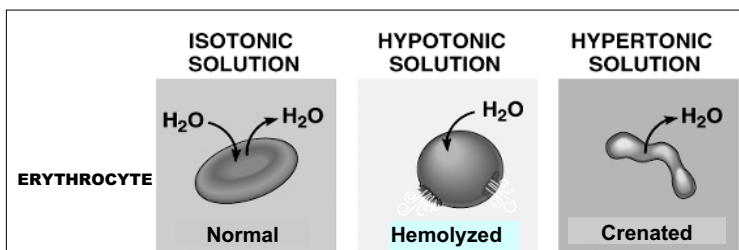
- Hold a test tube with a suspension of red blood cells in front of lined paper or printed type. If the cells are intact — normal or crenated — the cloudy solution will obscure the lines or type.   But if the cells have hemolyzed, the solution will be red, but clear enough to see the print.

SAFETY CONSIDERATIONS

Sheep blood carries a significantly lower health risk than working with human blood. But there is always the remote possibility that any blood product may contain a potential human pathogen. Therefore blood or blood-derived material should always be handled with caution as a potentially infectious agent.

✋ **Gloves and goggles** are of course required whenever working with blood or anything in contact with blood. Avoid mixing or pipetting techniques that are likely to cause spills or produce aerosols of blood-containing fluids. Avoid placing or touching blood-contaminated pipettes or tubes onto non-work areas or passing persons. Dispose of blood-contaminated disposables only in the biohazard waste container. Thoroughly disinfect your work area and all glassware and other equipment after use.

MATERIALS

- sheep whole blood or washed red blood cells
- 3.6% NaCl stock solution
- distilled/deionized water
- rack with 5 small screw cap test tubes and caps
- disposable 5-ml pipettes
- disposable droppers

PROCEDURE

1. Prepare a two-fold dilution series of the 3.6% NaCl stock solution in the five test tubes so that each tube contains 5 ml ranging from 3.6% down to 0.225% NaCl.

2. (Mix the sheep blood gently but thoroughly before taking samples.)
   To each tube add two drops of sheep blood and <u>immediately</u> cap the tube, mix gently by inversion, and record the time the blood is added in the Data Table column.

3. The tubes should be cloudy immediately after the addition of blood. As hemolysis occurs, the solutions will become transparent.

4. Record the time when the tubes become transparent in the third column of the Data Table.

5. Calculate the "Hemolysis Time" by subtracting the time the blood was added from the time the tube became transparent and record the number of minutes and seconds.

RESULTS

Tube	Solution	Molarity (M)	Osm	Time blood was added	Time solution became transparent	Hemolysis Time (min:sec)
1	3.6 % NaCl					
2	1.8 % NaCl					
3	0.9 % NaCl					
4	0.45 % NaCl					
5	0.225 % NaCl					

ANALYSIS & QUESTIONS

◈ Which solution(s) is/are definitely hypotonic to the erythrocytes?   How do you know?

◈ Which solution(s) is/are definitely hypertonic to the erythrocytes?   How do you know?

◈ Which solution(s) is/are probably isotonic to the erythrocytes?   How do you know?

◈ What is the estimated osmolarity of the normal erythrocyte cytoplasm?   How do you know?

◈ Could you discern an effect of concentration gradient upon the diffusion rate of water across the cell membrane?   Explain.

◈ The 3.6% NaCl solution is approximately the concentration of seawater. From your observations today, what would be the effect on your cells if you (or a sheep) drank seawater?

PART 2: PERMEABILITY OF CELL MEMBRANES

A **selectively permeable barrier** is one of the defining features of a living cell. The cell membrane and the associated transport proteins found in the membrane are responsible for regulating the movement of hundreds, if not thousands, of different types of molecules into and out of the cell. All molecular motion is influenced by diffusion, which is the tendency for particles to spread from higher concentrations to lower concentrations until they are evenly distributed. **Simple diffusion** refers to the passive transport of substances across the phospholipid bilayer of the cell membrane without requiring a transport protein.

In this exercise we will investigate the movement of several different types of molecules across a cell membrane and we will examine the physical properties of these different molecules to see how they relate to the relative permeability of the cell membrane to these substances.

ISOSMOTIC VERSUS ISOTONIC SOLUTIONS

Recall that we stated that if Solution A and Solution B have the same total concentration of solutes, they also have the same concentration of water and are therefore **isosmotic** (same osmolarity). We also stated that if these two isosmotic solutions are separated by a membrane *permeable to water* but *not permeable to the solutes*, water would diffuse equally to and from each solution and thus the two solutions are also **isotonic** (equal osmotic pressure).

But consider what happens if the membrane is permeable to water *and* to the solutes in Solution A, but *not* permeable to the solutes in Solution B. The Solution A solutes would diffuse across the membrane into Solution B according to their concentration gradient, thereby increasing the total amount of solute in Solution B and decreasing the amount of solute in Solution A. To maintain the isosmotic state, some water would follow the Solution A solutes into Solution B. Hence, even though Solutions A and B are isosmotic, Solution B pulls water from Solution A and therefore the two solutions are *not* isotonic.

In summary:

- All isotonic solutions must be isosmotic. But not all isosmotic solutions are isotonic.

- Isosmotic solutions are isotonic only if they are separated by a membrane that is permeable to water and is equally permeable or equally impermeable to the solutes in both solutions. (If the solutions are separated by a barrier not permeable to water, no osmosis can occur and they cannot have osmotic pressure.)

- If the two isosmotic solutions are separated by a membrane permeable to water and to the solutes of one solution but not the solutes of the other, the solution with the diffusible solutes is hypotonic to the solution with the non-diffusing solutes.

Since the water is following the diffusion of the solute, if the permeability of the membrane is greater to water than to the solutes, the rate of diffusion of the solutes determines the rate of diffusion of the water.

Recall that if we place red blood cells in a hypotonic solution, they will swell until they lyse. If the hypotonic solution is isosmotic to the cell cytoplasm, the time it takes for the cells to undergo hemolysis is proportional to the permeability of the erythrocyte membrane to the solute in the solution.

◈ From part 1 of this exercise, what did you determine to be the osmolarity of an isotonic solution with respect to erythrocytes?

Membrane Permeability: The Effect of Molecular Size

In order to study the effect of molecular size on membrane permeability, you will place red blood cells into various solutions of alcohols of increasing molecular size. If the alcohol molecules are small enough to cross the membrane, the alcohol molecules will diffuse through the membrane due to a concentration gradient, the cell will expand and eventually burst open and hemolyze. The amount of time required for hemolysis to occur is relative to the size of the alcohol molecule.

Smaller molecules will diffuse quicker and the cells will hemolyze sooner. The membrane can be permeable to larger molecules, but it will take these molecules longer to cross the membrane and cause hemolysis. Since these solutions are also isosmotic, if the alcohol molecules are not able to cross the membrane at all, the cells will not change volume and hemolysis will not occur.

We will use three different alcohols: methanol, glycerol, and mannitol (Figure 1). They differ in size, having 1, 3 and 6 carbon atoms (and -OH groups) respectively. Any difference in observed hemolysis time must be due to differences in size.

Figure 1. Alcohol molecules of similar polarity, but of increasing size.
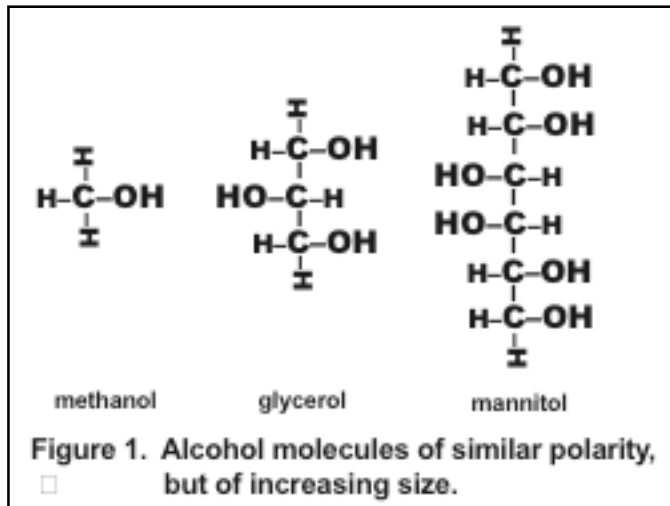
Membrane Permeability: The Effect of Molecular Polarity

Polarity is a chemical property that affects a molecule's solubility. Remember that polar molecules are more water soluble because water is itself a polar molecule. The uneven distribution of electrons create some areas on a molecule that are more negative which are balanced by other areas on a molecule that are more positive. The more negatively charged areas tend to associate with the –H side of H_2O, while the more positively charged areas tend to associate with the –OH side of H_2O.

What does water solubility have to do with membrane permeability? Remember that cell membranes are composed of a lipid bilayer. A lipid bilayer is more permeable to hydrophobic, or fat soluble molecules. Conversely, a lipid bilayer is less permeable to hydrophilic, or water soluble molecules.

Molecules with –OH groups, like alcohols, tend to be polar because oxygen is such an electronegative atom. The number of –OH groups on a molecule affects the degree of polarity a molecule will exhibit. Examine the structures of Glycerol and Propanol (iso-Propyl Alcohol) in Figure 2. Note that both have a backbone of three carbon atoms and about the same overall size. The main difference between them is the number of polar –OH groups: Propyl Alcohol has only one (C_3H_7OH), while Glycerol has three ($C_3H_5OH_3$), one for each carbon atom. This makes Glycerol a much more polar molecule than Propyl Alcohol. One would predict then, the more polar or hydrophilic a molecule, the less permeable it will be and the longer its hemolysis time.

Figure 2. Alcohol molecules of similar size, but of decreasing polarity.
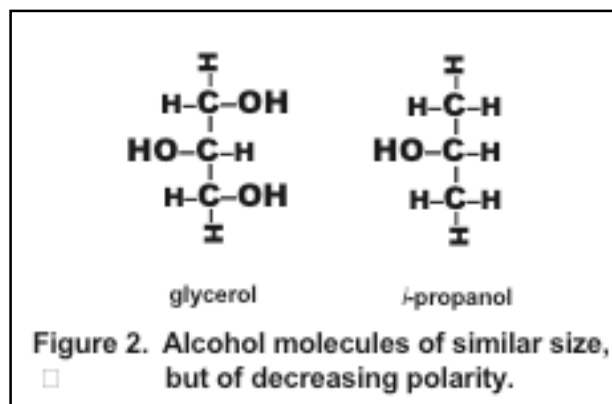
- sheep whole blood or washed red blood cells
- isosmotic Alcohol solutions (0.3M): Methanol; Glycerol; *iso*-Propanol and D-Mannitol.
- isosmotic solutions of 0.3M D-Glucose and 0.15M NaCl.
- rack with 6 small screw cap test tubes and caps
- disposable 5-ml pipettes
- disposable droppers

Reagent List						
Solute Molecule	# Carbon Atoms (size)	# Polar -OH Groups	Molecular Weight	Osmolality	Molarity (M)	g%
NaCl			58.4	300 mOsm		
Methanol	1	1	32.0	300 mOsm		
Glycerol	3	3	92.1	300 mOsm		
Propanol	3	1	60.1	300 mOsm		
Mannitol	6	6	182.2	300 mOsm		
Glucose	6	6	180.2	300 mOsm		

◇ Make a prediction, ranking the alcohol solutions in order of expected hemolysis times.

◇ Mannitol and glucose are of similar size and polarity. (Both have six carbons and six oxygens per molecule.) Why might glucose cause a more rapid hemolysis rate than mannitol?

PROCEDURE (SIMILAR TO PART 1)

1. Pipet 5 ml of each isosmotic solution into a separate tube.

2. (Mix the sheep blood gently but thoroughly before taking samples.)
   To each tube add two drops of sheep blood and immediately cap, mix by inversion, and record the time the blood is added in the Data Table column.

3. The tubes should be cloudy immediately after the addition of blood. As hemolysis occurs, the solutions will become transparent.

4. Record the time when the tubes become transparent in the third column of the Data Table.

5. Calculate the "Hemolysis Time" by subtracting the time the blood was added from the time the tube became transparent and record the number of minutes and seconds

Data Table. Hemolysis Times				
Tube #	Solute Molecule	Time blood was added	Time solution became transparent	Hemolysis Time (min:sec)
1	NaCl			
2	Methanol			
3	Glycerol			
4	Propanol			
5	Mannitol			
6	Glucose			

ANALYSIS & QUESTIONS

◈ Describe the difference between methanol, glycerol, and mannitol molecules.

◈ Describe the difference between propyl alcohol and glycerol molecules.

◈ What is the purpose of having all of these solutions isosmotic?

◈ What causes a blood solution to go from cloudy to clear?

◈ Based on your data, how does a molecule's size affect its ability to cross a cell membrane?

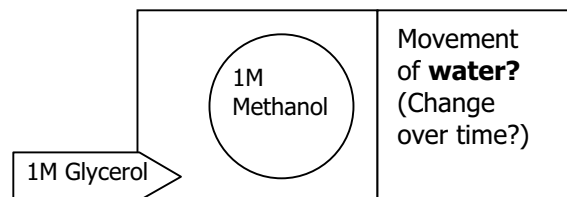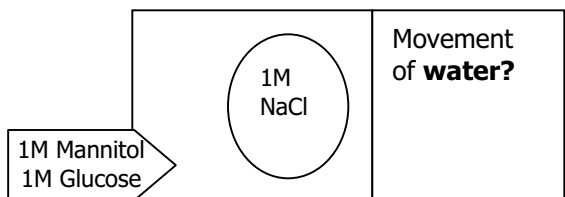◈ Based on your data, how does a molecule's polarity affect its ability to cross a cell membrane?

◈ Sodium ions and chloride ions are smaller than methanol molecules. How did the hemolysis time of isosmotic NaCl solution compare to that of methanol solution?   Explain.

◈ How did the hemolysis time of the glucose solution compare with that for the mannitol solution?   Did it agree with your prediction?   Why or why not?

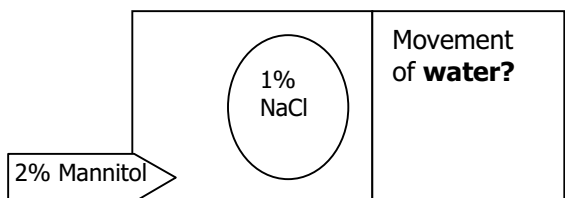1. Each of these drawings represents an artificial cell with a phospholipid bilayer membrane with permeability characteristics similar to the erythrocytes in your experiment, but filled with the solution indicated. The environment surrounding the cell is also indicated.
   - Draw arrows to indicate which way the material moves --- into the cell, or out of the cell.
   - Be sure to read the question carefully --- am I asking about the WATER or the SOLUTE.

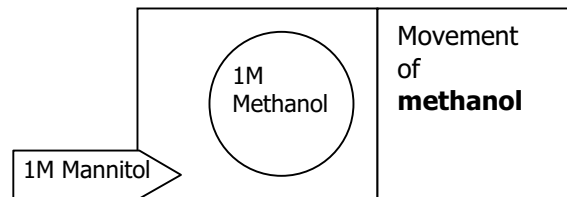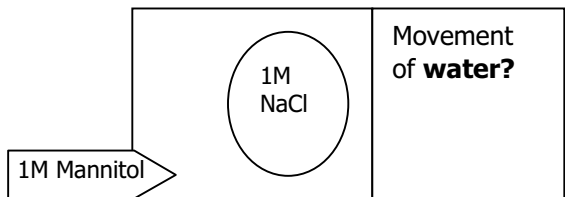|	
1% NaCl — pure water — Movement of **water?**	10% NaCl — 1% NaCl — Movement of **water?**
1M NaCl — 1M Mannitol — Movement of **water?**	1M Methanol — 1M Mannitol — Movement of **methanol**
1M NaCl — 2M Mannitol — Movement of **water?**	1M Methanol — 1M Mannitol — Movement of **water?**
1% NaCl — 2% Mannitol — Movement of **water?**	1M Methanol — 1M Glycerol — Movement of **glycerol?**
1M NaCl — 1M Mannitol 1M Glucose — Movement of **water?**	1M Methanol — 1M Glycerol — Movement of **water?** (Change over time?)
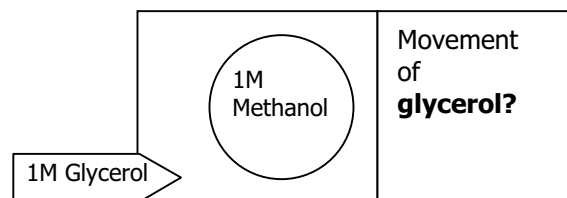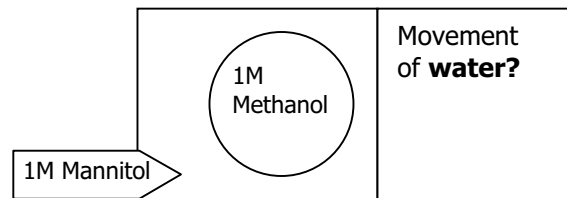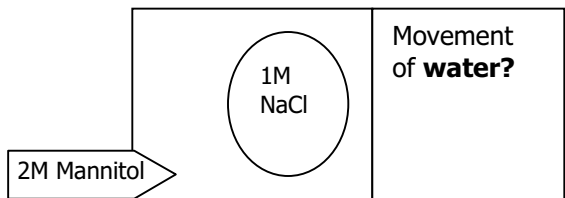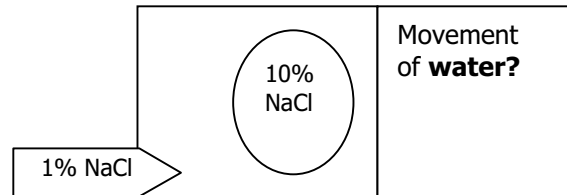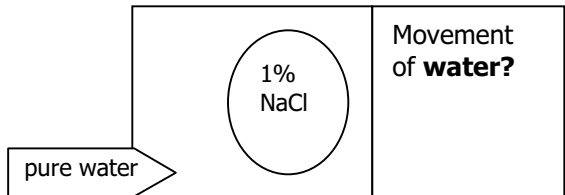
2. Indicate whether the following statements apply to **simple diffusion**, **facilitated diffusion**, **osmosis** or **active transport**. If the statement applies to more than one process, indicate which ones.

    _____ involves a pump

    _____ carbon dioxide moves out of the cell in this way

    _____ requires binding to a specific protein in the cell membrane

    _____ the terms hypotonic and hypertonic refer to this

    _____ movement "down" the concentration gradient (from high to low)

    _____ requires energy

    _____ does not require energy but does require a channel

3. When food is pickled, it is placed in a very salty solution. What does this do to the cells? Pickling is an effective way to preserve food; that is, it protects food from microbial contamination and spoiling. Why?

4. If, for some reason, you needed to analyze animal cells, why is it standard practice to suspend the cells in 0.9% saline? What would happen to the cells if you suspended them in water instead?

5. Sorbitol is used as an "osmotic laxative". It is not absorbed from the intestine. What is the effect of sorbitol and why is it effective as a laxative? (Mannitol may also be used. Does your data show why?)

6. Diarrhea is a leading cause of death worldwide in infants. It is the excessive loss of water in feces. Besides interfering with nutrition, it can cause severe dehydration and shock (Why?) Oral rehydration therapy, the administration of a solution containing glucose and NaCl, is a lifesaving treatment. Knowing what you know about the transport of glucose and salt, why does this solution promote rehydration?

7. Certain types of microbes maintain an internal pH of approximately 7, yet live in water with a pH of 3. How do they do that?

S5: PV92 — Analysis and Interpretation of Results

(modified from Technical Bulletin 4110052, Bio-Rad 2004)

Remember that this Alu sequence is inserted into a noncoding region of the PV92 locus on chromosome 16 and is not related to a particular disease, nor does it code for any protein sequence. It is simply a sequence that can be used to study human genotypic frequencies.

Because Alu repeats appear in the general population at random, the Alu insert in chromosome 16 is very useful for the study of gene frequencies in localized human populations. Theoretically, in some small, geographically isolated populations, all individuals may be homozygous +/+. In others, the individuals may all be homozygous −/−. In a "melting-pot" population, the three genotypes (+/+, +/−, −/−) may exist in equilibrium.

The frequencies of genotypes and alleles are basic characteristics that population geneticists use to describe and analyze populations. The results you obtain in this exercise provide a real-life opportunity to calculate **genotypic and allelic frequencies** of the Alu insert in your class and to use the **Hardy-Weinberg equation**.

C L A S S   D A T A   /   G E N O T Y P E   F R E Q U E N C I E S :

The results of the PCR reactions reveal your and your classmates' genotypes: +/+, +/−, and −/−. Knowing your genotypes, you can count up the alleles of your class "population" and determine their frequencies. You can then compare the allelic and genotypic frequencies of your class population to published reports of larger population sizes.

1. What is your genotype for the Alu insert in your PV92 region?

2. What are the genotypic frequencies of +/+, +/−, and −/− in your class population? Fill in the table below with your class data.

Table 1. Observed Genotypic Frequencies for the Class		
Category	**Number**	**Frequency (# of Genotypes/Total)**
Homozygous (+/+)		
Heterozygous (+/−)		
Homozygous (−/−)		
Total =		= 1.00

A L L E L I C   F R E Q U E N C I E S :

Allelic frequencies can be calculated from the numbers and frequencies of the genotypes in the population. Population geneticists use the terms p and q to represent the frequencies of the (+) and (−) alleles, respectively. Allele frequencies can be calculated from either the numbers or the frequencies of the genotypes (since they are related to each other).

$$p = \text{frequency of (+) allele} = \frac{\text{number of (+) alleles}}{\text{total number of alleles (both + and −)}}$$

$$= \frac{2(\text{\# of +/+ students}) + 1(\text{\# of +/− students})}{\text{total number of alleles (both + and −)}}$$

$$= \text{frequency of (+/+) students} + 1/2 \text{ (frequency of (+/−) students)}$$

$$q = \text{frequency of (−) allele} = \frac{\text{number of (−) alleles}}{\text{total number of alleles (both + and −)}}$$

$$= \frac{2(\text{\# of −/− students}) + 1(\text{\# of +/− students})}{\text{total number of alleles (both + and −)}}$$

$$= \text{frequency of (−/−) students} + 1/2 \text{ (frequency of (+/−) students)}$$

3. What is the frequency of each allele in your class sample? Fill in the table below with your class data. Remember, since each individual has two copies of chromosome 16, a class of 28 students (N) will have a total of 56 (2N) instances of each locus.

Table 2. Calculated <u>Allelic</u> Frequencies for the Class

Category	Number	Frequency (#/Total alleles)
(+) alleles p =		
(−) alleles q =		
Total alleles =		= 1.00

4. The following table presents data from a USA-wide random population study.

Table 3. Genotypic Frequencies for Alu in a USA Sample

Category	Number	Genotypic Frequency
Homozygous (+/+)	2,422	0.24
Heterozygous (+/−)	5,528	0.55
Homozygous (−/−)	2,050	0.21
Total =	10,000	= 1.00

Now, using the data above, calculate the allelic frequencies for the USA data as you did for your class population in Table 2.

Table 4. Calculated Allelic Frequencies for USA

Category	Number	Frequency
(+) alleles p =		
(−) alleles q =		
Total alleles =	20,000	= 1.00

5. How do your actual class data for genotypic and allelic frequencies compare with those of the random sampling of the USA population? Would you expect them to match? What reasons can you think of to explain the differences or similarities?

The **Hardy-Weinberg equation**, $p^2 + 2pq + q^2 = 1$, is one of the foundations of population genetics. It is the algebraic expansion of **$(p + q)2 = 1$**, where **$p + q = 1$**. The equation describes the frequencies of genotypes in a population that is at "**genetic equilibrium**", meaning that the frequencies are stable from generation to generation. The Hardy-Weinberg theory states that, for a population to achieve this equilibrium, the population must be quite large, the members must mate randomly and produce offspring with equal success, and there must be no migration of individuals into or out of the population, or an excessive mutation converting one allele to another. Given these conditions, and the allelic frequencies p and q, the Hardy-Weinberg equation says that

p^2 = the expected frequency of the (+/+) genotype in the population

$2pq$ = the expected frequency of the (+/−) genotype in the population

q^2 = the expected frequency of the (−/−) genotype in the population

It is important to understand that p^2, $2pq$, and q^2 are expected, theoretical genotype frequencies of a population under Hardy-Weinberg equilibrium conditions, and they may not be realized in real-life population samples if one of the conditions is not met. These theoretical frequencies are calculated using the observed values for p and q; they may or may not be the same as the observed genotypic frequencies such as those shown in Table 1. If the observed and expected genotypic frequencies are the same, this indicates that the population is in Hardy-Weinberg genetic equilibrium.

6. Using the values for p and q that you calculated in Table 2 for your class population, calculate p^2, $2pq$, and q^2. Do they come out to be the same as the genotype frequencies that you found in Table 1? If they do, your class resembles a Hardy-Weinberg genetic equilibrium. If your observed (actual) genotype frequencies are not the same as the expected values, what might be some of the reason(s) for the difference?

Table 5. <u>Predicted</u> Genotypic Frequencies for the Class

Category	Value	Frequency	Number
Homozygous (+/+)	$p^2 =$		
Heterozygous (+/−)	$2pq =$		
Homozygous (−/−)	$q^2 =$		
Total =	= 1.00	= 1.00	

7. Using the values for p and q that you calculated in Table 4 for the USA population sample, calculate p^2, $2pq$, and q^2. Do they come out to be the same as the genotype frequencies that you found in Table 3? Does this USA-wide sample suggest that the population of the USA is in Hardy-Weinberg equilibrium?

Table 6. <u>Predicted</u> Genotypic Frequencies for Alu in the USA Sample

Category	Value	Frequency	Number
Homozygous (+/+)	$p^2 =$		
Heterozygous (+/−)	$2pq =$		
Homozygous (−/−)	$q^2 =$		
Total =	= 1.00	= 1.00	= 10,000

S6: Patterns of Inheritance: Coat Color in Cats

Melanin is a dark pigment located in the skin, hair and eyes of vertebrates. Color variations arise from differences in the amount and distribution of melanin in these structures, which in turn is dependent upon a number of metabolic and developmental pathways. Cat fanciers in particular have documented a large number of genetic variations in several of the genes regulating the coloration of cat fur. Some of these genes have been subsequently localized on specific chromosomes, but many are identified only by their pattern of Mendelian inheritance. Study of the inheritance and variation of these traits has helped us understand more fully the development of the skin and associated structures in mammals.

Melanin is a water-insoluble product derived from the oxidation of the amino acid tyrosine and polymerized into irregular granules. It is synthesized inside membrane-bound vessels called melanosomes within specialized cells called melanocytes. The melanocytes may pass the melanosomes by dendritic processes to adjacent keratinocytes forming the epidermis or hair follicles. The intensity and hue of pigmentation is directly related to the number, size and distribution of melanosomes in the melanocytes or keratinocytes. Mammals lacking melanin have white, unpigmented fur and are called *albino*. Animals having an atypically increased amount of melanin are black and called *melanistic*. Malignancy of melanocytes can produce a hyperpigmented mass called a melanoma.

The following list is by no means exhaustive for genes regulating coat color in cats. But it does include many of the relevant processes in coloration. The particular mutant alleles may be specific to cats, but the wild type genes are operative for fur pigmentation in most mammals.

C (tyrosinase) gene.   The C gene codes for the enzyme tyrosinase, the enzyme catalyzing the oxidation of tyrosine to DOPA — the first step in melanin synthesis. The dominant **C** allele is the wild type, expressed as functional tyrosinase enzyme. The recessive **c** allele is a loss of function mutation expressed as a nonfunctional protein. The homozygous **cc** genotype results in an inability to produce melanin and results in albino phenotype. Hence the **cc** genotype is epistatic to the other color-related genes, since a lack of pigment precludes any variation in pigmentation. A third allele possible for this locus, c^t, is expressed as a temperature sensitive version of tyrosinase. Cats homozygous $c^t c^t$, such as Siamese as Burmese breeds, have lightly pigmented fur on their bodies close to their core, but darker pigmented fur on the cooler tips of their snout, ears, and extremities.

O (orange) gene.   Two major classes of melanins are produced in most melanocytes: the black/brown **eumelanin** and the red/orange **phaeomelanin**. The dominant wild type **O** results in predominantly black eumelanin. The recessive **o** allele causes almost entirely phaeomelanin production. The O gene is sex-linked on the X chromosome. Hence homozygous **OO** female cats and hemizygous **OY** male cats produce black fur, but homozygous **oo** female cats and hemizygous **oY** male cats produce orange fur. Because of X-inactivation, heterozygous **Oo** female cats demonstrate a pattern called tortoiseshell with a mosaic of patches of melanocytes producing either black or orange fur.

B (browning) gene.   The B gene expresses the tyrosinase-related-protein-1 (TRP-1), another protein in the metabolic pathway for variation in the structure of eumelanin. The dominant wild type **B** results in the usual black eumelanin. The recessive **b** allele results in a dark brown ("chocolate") form and another recessive b^l allele cause a light brown ("cinnamon") phenotype.

D (dense pigment) gene.   Sometimes called a diluter gene, D gene codes for melanophilin, a factor needed to transfer melanosomes from the melanocytes to the keratinocytes in the hair follicles. Recessive mutant forms result in reduced transfer and thus more muted colors. With the homozygous dd at this locus, black becomes gray, chocolate becomes lilac, cinnamon becomes fawn, and orange becomes cream colored.

S (piebald-spotting) gene.   During embryogenesis, the precursor melanocytes must migrate from their site of origin to the basement layer of the epidermis. The S gene is somehow related to this migration. The dominant **S** allele results in patches of melanocyte-free epidermis and thus spots with white fur. This gene has incomplete dominance. Homozygous **SS** cats have large white patches such as a

white blaze across the face or the tuxedo or bib pattern on the chest ("piebald"). Heterozygous **Ss** cats have fewer, smaller white patches. Wild type homozygous recessive **ss** cats lack any white spots.

Mc (tabby) gene.   In cats, when the embryonic melanocytes are migrating to the epidermis, they accumulate along the edges of the developing somites. This results in relatively darker horizontal stripes along the cat's body and face. With the dominant wild type **Mc** allele, the stripes are thin ("mackerel-striped") and often broken into rows of thin spots or bars. The recessive **mc** allele results in broader dark bands or whorls.

T (unstriped) gene.   Epistatic to the Mc gene, the dominant T^A allele results in an unstriped coloration. The wild type recessive **t** allele allows the expression of the Mc phenotype.

W (white-masking) gene.   Another epistatic gene, the dominant **W** allele triggers apoptosis in the embryonic melanocyte precursor cells before their migration. Hence the fur is almost all white despite the genotype of the other color-related genes. Since these cats can produce melanin, they are not considered true albinos. Some embryonic melanocytes often survive and may migrate to form a few isolated islands of colored fur amidst the white background. The color of the spots depends upon the C, O, B & D loci. Cats must be homozygous **ww** for the normal melanocyte migration and the uniform pigmentation phenotype to be expressed.

Problem Set

1. The ancestor of cats was presumably homozygous for wild type at all of the above loci.
    a. What was the genotype of this ancestral cat at each of these loci?

    b. What was the phenotype of this presumed ancestral cat?

2. Describe the phenotype of the cats with these genotypes (If allele not specified, assume wild type.):
    a. **CC ss OO BB dd McMc tt ww**

    b. $c^t c^t$ **ss OY bb** $T^A t$

    c. **Cc SS Oo BB** $T^A T^A$

    d. **CC Ss oY bb dd tt**

3. Describe the genotype of cats with the following phenotypes (Describe only the genes that are not homozygous for the wild type allele.):
    a. A grey cat with small white patches.

    b. A white cat with orange spots.

    c. A pale colored cat with brown tips to its tail, ears, paws, and snout.

    d. A cream-colored cat with darker cream color forming broad bands and whorls along its body.

Use Punnett squares to solve the following mating predictions.

Inheritance of a single gene:

4. A "chocolate" cat mates with a cat heterozygous at the gene for TRP-1:
   a. What is the probability of their kitten having brown coat color?
   b. What is the probability of their kitten having black coat color?
   c. What is the probability of their brown kitten being homozygous for this gene?
   d. What is the probability of their black kitten being homozygous for this gene?

5. A pure-bred uniform-color cat (homozygous for the T^A gene) mates with a mackerel-striped cat:
   a. What is the probability of their kitten having uniform coloration?
   b. What is the probability of their kitten having mackerel-striped coloration?
   c. If two of these kittens grow up and mate, what is the probability of their second-generation (F_2) kitten having uniform coloration?
   d. What is the probability of their second-generation (F_2) kitten being mackerel-striped?
   e. What is the probability of their F_2 uniform-color kitten being homozygous for this gene?
   f. What is the probability of their F_2 mackerel-striped kitten being homozygous for this gene?

6. A uniformly colored black cat mates with a black cat having small white patches:
   a. What are the possible phenotypes of their kittens?
   b. For each of these phenotypes, what is their probability and genotype?

7. A tortoiseshell cat mates with an orange cat:
   a. What is the probability of their kitten having all-orange fur?
   b. What is the probability of their kitten having tortoiseshell coloration?
   c. What is the probability of their kitten having all-black fur?
   d. What is the probability that their black kitten will be male?
   e. What is the probability that their orange kitten will be male?
   f. What is the probability that their tortoiseshell kitten will be male?

Inheritance of multiple genes:

8. A pure-bred grey cat mates with a chocolate cat:
   a. What are the possible phenotypes of their kittens?
   b. For each of these phenotypes, what is their probability and genotype?

9. An all-black cat gives birth to a mostly white kitten with black spots:
   a. If the only three male cats around were all-brown, all-black, and white with brown spots, which one sired this kitten?
   b. If this white kitten with black spots matures and mates with an all-brown cat, what are the possible phenotypes of their kittens?

10. A cat with broad pigmented bands and whorls mates with a cat heterozygous for the tabby gene. Their first kitten is albino.
    a. What is the probability that their next kitten will also be albino?
    b. What is the probability that their next kitten will be mackerel-striped?

Appendices

A1. C ALCULATIONS AND C ONVERSIONS

There are only a few kinds of calculations you'll need to do for this course, but you'll need to do them over and over. If you work in a lab, you need to pay attention to units of measurement. For the labs in this course, there are only a few kinds of units, and the prefixes that go with them.

- distance: meters (m)
- mass: grams (g)
- volume: liters (l)

Concentration can be given several ways:

- molar (M), defined as moles of solute per liter of solution
- percent (%) usually means mass/volume: 10% NaCl= 10 g NaCl in 100 ml aqueous solution.
- g/l, or μg/μl, or similar units.

The **prefixes** we normally use in this course are in multiples of 1000:

- 1 mg = 1 milligram = 10^{-3} g
- 1 μg = 1 microgram = 10^{-3} mg = 10^{-6} g
- 1 ng = 1 nanogram = 10^{-3} μg = 10^{-9} g
- 1 pg = 1 picogram = 10^{-3} ng = 10^{-12} g

Remember: m > μ > n > p. For each step, you multiply or divide by 1000. If you don't remember that a nanogram is 10^{-9} grams, just remember that there are 1000 ng in 1 μg and 1000 μg in 1 mg and 1000 mg in 1 g.

Enzyme activity units are different. It doesn't matter exactly how many molecules of an enzyme you have; it matters how much reaction they can catalyze. So each enzyme has its own unit definition. For example, a unit of a restriction enzyme might be defined as: 1 unit of enzyme is enough to completely cut 1 μg of lambda DNA in 1 hour at 37°.

The basic equation for figuring out how to make dilutions is:

$$C_1V_1 = C_2V_2$$

Where:

C_1 = the initial concentration of your stock solution

V_1 = the amount of stock solution you need to add. Usually this is what you are solving for.

C_2 = the final concentration

V_2 = the final volume – includes the volume in V_1 plus the amount of water or buffer you add.

So if you want to dilute the stock solution, just rearrange the equation to: $V_1 = C_2V_2/C_1$

Always **write down the units** when you do these calculations. A microliter is definitely not the same as a milliliter.

SERIAL DILUTIONS

Suppose I give you 1 ml of a liquid bacterial culture. The concentration of the bacteria is unknown, so we'll have to call it 1x. You need 1 ml of 10^{-6}x solution. How do you do the dilution?

Using $C_1V_1 = C_2V_2$, you could do it like this:

$$(1x)(1 \text{ ml}) = (10^{-6}x)(10^6 \text{ ml})$$

Your final volume is 1,000,000 ml, or 1,000 liters. Can you see how this would be a problem? (If not, go to the glassware cabinet and try to find a 1,000 liter graduated cylinder.)

You could also try the dilution this way:

$$(1x)(10^{-6} \text{ ml}) = (10^{-6}x)(1 \text{ ml})$$

Fine. Now get your pipetman and set it to 10^{-6} ml, or 0.001 μl (1 nl). Let me know when you're ready. Can you see why this might be difficult?

The only practical way to do large dilutions is to do serial dilutions: make a 10^{-2}x solution; then use this 10^{-2}x solution to make a 10^{-4}x solution; then dilute the 10^{-4}x solution to make 10^{-6}x. The math would be like this:

$$(\underline{1x})(10 \text{ μl}) = (\underline{10^{-2}x})(1000 \text{ μl})$$

$$\rightarrow (\underline{10^{-2}x})(10 \text{ μl}) = (\underline{10^{-4}x})(1000 \text{ μl})$$

$$\rightarrow (\underline{10^{-4}x})(10 \text{ μl}) = (\underline{10^{-6}x})(1000 \text{ μl})$$

CALCULATING HOW MUCH DNA TO USE

Suppose you're doing a restriction digest. You know you want 1μg of DNA. How many μl is that? Write down what you know:

$$1 \text{ μg} = ? \text{ μl}$$

To do the conversion, you need to know the concentration of your starting DNA solution in μg/μl. Suppose it turns out to be 0.5 μg/μl. To plug in the concentration the right way, just make sure your units come out right:

$$(1\text{μg})/(0.5 \text{ μg }/\text{μl}) = (1\text{μg})(1 \text{ μl}/0.5 \text{ μg}) = ? \text{ μl}$$

The μg cancel out, and you're left with μl on each side. Now just solve the equation, and you're done.

Key point: **write out the equation with the units**. Do this every time. It's how you check to see if you're doing it right. Don't reach for the calculator until you're sure the equation is right.

CALCULATING HOW MUCH ENZYME TO USE

Enzyme activity units are different. It doesn't matter exactly how many molecules of an enzyme you have; it matters how much reaction they can catalyze. So each enzyme has its own unit definition. For example, a unit of a restriction enzyme might be defined as: 1 unit of enzyme is enough to completely cut 1 μg of lambda DNA in 1 hour at 37°.

Again, suppose you're doing a restriction digest. If you have 1 μg of DNA, and 1 Unit of enzyme will cut 1 μg of DNA, then you need 1 Unit of enzyme. Now how much is that in μl? Suppose the enzyme concentration is 10 Units/μl. Your calculation is:

$$(1 \text{ Unit})/(10 \text{ Units}/\text{μl}) = 0.1 \text{ μl}$$

Always write down the units when you do a calculation. If the units come out right, the number generally does, too.

A2: Quant-iT Fluorescence Assays

Quant-iT protein assay

Electrophoresis works best when you load an appropriate amount of sample on the gel. If you load too much material, the bands will be smeared; if you load too little, you may not see your bands at all. In order to get the amount right, you'll use a fluorescent assay to measure, or quantitate, your concentration before loading your gel.

This assay uses a device called the Qubit fluorometer, from Invitrogen Corporation. In general, fluorometers work by illuminating a sample with ultraviolet light and then measuring the amount of visible light given off as fluorescence. You'll use the Qubit fluorometer with the Quant-iT assay kits (also from Invitrogen). You'll mix your sample with a special reagent that becomes fluorescent only when it is bound to a specific substrate (protein or DNA, depending on the reagent used). Next, you'll place your sample in the fluorometer. If there is no protein in your sample, there will be very little fluorescence, and the fluorometer will give a low reading. The higher the concentration of protein in your sample, the higher the reading will be. Since the fluorometer measures fluorescence very precisely, it will give you a precise reading of protein concentration.

The fluorometer can also be used to assay DNA concentration by using fluorescing reagents that bind DNA instead of protein. You'll learn the procedure for that in a later lab described in part ii.

i. Measuring Protein Concentration

The protein samples for Lab 1

Almost any biological tissue can be used for SDS-PAGE, because tissues always contain proteins. You don't need to start with a purified protein sample.

We'll use fish muscle tissue for today's lab, because it's mostly protein and easy to grind up. Before the lab, the lab technician will **homogenize** *15g of the sample by spinning it in a blender with 100 ml of iced buffer. This breaks down the tissue structure and* **lyses** *(or breaks open) the cells, releasing the proteins and other cell contents. The resulting suspension is called a* **homogenate** *or a* **lysate***. Then SDS is added to the lysate to help dissolve and denature the proteins. The lysate should be kept cold to keep the proteins from becoming degraded.*

Assay overview:

This procedure is simple. You mix the protein samples with the **working solution**, calibrate the fluorometer, and measure the protein concentration of your samples.

Calibrating the fluorometer

Before running your assay with a sample containing an unknown protein concentration, you'll need to calibrate the device by running three samples with known protein concentration. These samples have already been prepared for you (they come with the assay kit).

When you start up the fluorometer for the protein assay, it will first ask you to insert protein

standard #1, then #2, and then #3. After it has read the three standards, you may insert your unknown sample.

Measuring your protein concentration

After you calibrate the fluorometer, it will simply read the fluorescence of your unknown sample, compare it with its readings of the standards, and tell you the concentration of your sample in µg/ml.

The protein concentration you read on the screen is the concentration in the **assay tube** inside the fluorometer — the sample that you have diluted with working solution. In order figure out the concentration of protein in your **sample tube** (undiluted), you may need to do a little math, as described below.

Safety Considerations

- o **Wear gloves and safety glasses** throughout this lab.
- o **Quant-iT protein buffers and protein standards:** the possible hazards of these reagents have not been fully tested. Use standard laboratory procedures to **avoid eye and skin contact**.

Materials for protein assay

Note: this list assumes that we'll have three different fish protein samples to assay.

For each lab group (usually 4 people), obtain the following:

- Qubit fluorometer with power cord
- Pipetters and tips
- Beaker for waste tips
- 6 Assay tubes. You'll need three for the calibration standards and one for each of the three protein samples you're going to assay. (These are special tubes for the fluorometer; the instructor will show you the correct tubes to use.)
- 1 1.5-ml micro tube for the Quant-iT working solution
- 3 tubes homogenized fish muscle: these are your unknown protein samples
- 1 tube Quant-iT protein reagent (component A)
- 1 tube Quant-iT protein buffer (component B)
- 1 tube Quant-iT protein standard #1 (component C)
- 1 tube Quant-iT protein standard #2 (component D)
- 1 tube Quant-iT protein standard #3 (component E)
- Ice bucket with ice for protein samples

Sample preparation

Protein samples for quantitation or SDS-PAGE need to be completely homogenized – no chunks. The easiest way to do this is to throw the piece of tissue in a blender with some water or buffer. For today's lab, a large sample for the whole class will be homogenized in a blender. Keep it on ice so the proteins don't degrade. You'll take a small amount of this protein sample and mix it with other reagents to perform your assay.

Prepare the Quant-iT working solution (WS)

You'll need to prepare the appropriate amount of working solution by mixing **Quant-iT protein buffer** and **Quant-iT protein reagent** (two different things!) as follows:

995 µl	1.492 ml	4.98 ml	9.95 ml	**Quant-iT protein buffer** (component B)
5 µl	7.5 µl	25 µl	50 µl	**Quant-iT protein reagent** (component A)
1.00 ml	1.50 ml	5.00 ml	10.00 ml	**Total working solution**

You'll need approximately 200 µl of working solution for each sample. If you're going to run three standard samples and three unknown samples, you'll need about 1.2 ml working solution; make 1.5 ml to be sure you'll have enough.

1. Obtain an empty tube of the appropriate size; label it "WS" for working solution.

2. Pipet the Quant-iT protein buffer into the tube. (It's always best to start with the larger volume).

3. Add the Quant-iT protein reagent. Vortex or invert the tube to mix. The working solution is now complete.

Mix your samples with the Quant-iT working solution

Each protein sample (including the standard samples provided with the kit) needs to be mixed with the Quant-iT working solution in a transparent, thin-walled 500-µl assay tube. The final volume in each tube must be 200 µl.

4. Label your 500-µl assay tubes (1,2,3 for the standards, and a 1-letter abbreviation for each sample of fish).

5. Pipet 190 µl working solution into each tube.

6. Add 10 µl of protein standard #1 to tube 1.

7. Add 10 µl of protein standard #2 to tube 2

8. Add 10 µl of protein standard #3 to tube 3

9. Add 10 µl of your unknown protein samples to the remaining tubes.

10. Vortex all four tubes briefly. These are your assay tubes. **Allow these tubes to incubate at room temperature for 15 minutes** before using them in the fluorometer.

Calibrate the fluorometer and perform the assay

11. Plug in the Qubit fluorometer and turn it on by pressing any button. You should see the **HOME** screen.

12. On the **HOME** screen, use the up or down arrows to select the Quant-iT Protein Assay. Press GO.

13. Insert assay tube 1. Press **GO**. The reading will take about 5 seconds. Remove the tube.

14. Repeat with assay tubes 2 and 3. When all three readings are done, the calibration is complete.

15. Insert assay tube 4. The fluorometer should display the concentration of your protein sample in µg/ml. Write this number down:

_____ µg/ml **in the assay tube**

That's the concentration of the sample in the assay tube. However, what you are really trying to find out is the starting concentration of your **sample tube**, before you diluted it with working solution in the assay tube. In step 8 above, you diluted your protein sample by adding 190 µl working solution to 10 µl protein sample. You can calculate the starting concentration of your protein sample with this equation:

$$C_1V_1 = C_2V_2$$

In this case, C_2 is the number you just read from the fluorometer, V_2 is 200 µl (the volume in the assay tube), and V_1 is 10 µl the volume of protein sample that went into the assay tube). Now just solve for C_1 (initial concentration), and you have the concentration of protein in your sample tube.

Write down your calculations here:

Alternatively, if you're too lazy to do the math yourself, you can select **CALCULATE SAMPLE CONCENTRATION** on the fluorometer and follow the instructions. However, you won't be able to do that on the test!

If necessary, prepare more assay tubes

16. If your readings were out of range, perform the assay again with a larger or smaller amount of protein sample. For the first set, you used 10 µl of protein sample with 190 µl of working solution; this time, choose a different amount of protein sample and add enough working solution to make a final volume of 200 µl in the assay tube.

17. Record your assay results and calculate your starting protein concentration again.

18. Now that you have calculated the protein concentration in each of your samples, you need to calculate how much protein you loaded onto your gel well — or, what volume you need to load.

The Quant-iT DNA assay method is almost identical to the protein assay you performed earlier. You mix the DNA samples with the working solution, calibrate the fluorometer, and measure the DNA concentration of your samples.

Safety Considerations

○ **Wear gloves and safety glasses** throughout this lab.

○ **Quant-iT protein buffers and protein standards:** the possible hazards of these reagents have not been fully tested. Use standard laboratory procedures to **avoid eye and skin contact**.

Materials for DNA assay

For each lab group (usually 4 people), obtain the following:

- Qubit fluorometer with power cord
- Pipetters and tips
- beaker for waste tips
- 6 Assay tubes. You'll need three for the calibration standards and one for each of the three DNA samples you're going to assay. (These are special tubes for the fluorometer; the instructor will show you the correct tubes to use.)
- 1 1.5-ml micro tube for the Quant-iT working solution
- Your pGLO plasmid DNA sample from the previous lab
- 1 tube Quant-iT dsDNA reagent (component A)
- 1 tube Quant-iT dsDNA buffer (component B)
- 1 tube Quant-iT dsDNA standards #1 (component C)
- ice bucket with ice for DNA samples

Prepare the Quant-iT working solution (WS)

You'll need approximately 200 µl of working solution for each sample, including the two DNA standards. You may be able to share the standards and working solution with other groups.

1. Label a micro tube "WS" for working solution. Dilute the Quant-iT dsDNA reagent 1:200 in the Quant-iT dsDNA buffer (for example, 5 µl Quant-iT dsDNA reagent + 995 µl Quant-iT dsDNA buffer). Vortex briefly after pipeting. If everyone is feeling cooperative, one group could make enough working solution for the whole class.

Mix your samples with the Quant-iT working solution

Each DNA sample (including the standard samples provided with the kit) needs to be mixed with the Quant-iT working solution in a transparent, thin-walled 500-µl assay tube. **Be sure you use the special Quant-iT assay tubes**, not regular micro tubes. The final volume in each tube must be 200 µl.

2. Label your 500-µl assay tubes (1,2, and 3). Label the lids, not the sides of the tubes. Don't use tape.

3. Pipet 190 μl working solution into each tube.

4. Add 10 μl of DNA standard #1 to tube 1.

5. Add 10 μl of DNA standard #2 to tube 2

6. Add 10 μl of your plasmid DNA sample to tube 3.

7. Vortex or flick each tube briefly. These are your assay tubes. **Allow these tubes to incubate at room temperature for at least 2 minutes** before using them in the fluorometer.

Calibrate the fluorometer and perform the assay

8. Plug in the Qubit fluorometer and turn it on by pressing any button. You should see the *HOME* screen.

9. On the *HOME* screen, use the up or down arrows to select the Quant-iT dsDNA Assay (ds means double-stranded). Press GO.

10. Insert assay tube 1. Press *GO*. The reading will take about 5 seconds. Remove the tube.

11. Repeat with assay tubes 2. When two calibration readings are done, the calibration is complete.

12. Insert assay tube 3. The fluorometer should display the concentration of your DNA sample in μg/ml. Write this number down:

_____ μg/ml **in the assay tube**

Remember, that's the concentration of the sample in the assay tube. You also need to find the concentration of DNA in your sample tube using the same method you used in the protein assays. Write the sample concentration here:

_____ μg/ml **in the sample tube**

If necessary, prepare more assay tubes

13. If your readings were out of range, perform the assay again with a larger or smaller amount of DNA sample. For the first set, you used 10 μl of DNA sample with 190 μl of working solution; this time, choose a different amount of DNA sample and add enough working solution to make a final volume of 200 μl in the assay tube.

14. Record your assay results and calculate your starting DNA concentration again.

15. Now that you have calculated the DNA concentration in each of your samples, you need to calculate what volume you need to load into your gel wells.

A3: Gel Photos — Using the Transilluminators & Digital Camera

We record images of your electrophoresis gels to compare with other groups and/or other sections, and to incorporate your gel photos into your lab reports. Place your gel on the appropriate transilluminator light box. We use a digital camera equipped with a hood that fits snuggly over the illuminated plate for the best camera image. A USB cable connects the camera to the lab computer. Once the images are in the computer, they will be transferred to the class *flickr*® site where they may be viewed and downloaded online.

As described below, we will use the white-light transilluminator for our polyacrylamide gels and the ultraviolet transilluminator for our agarose gels.

You may also take photos of your gels with your cell phone. But only in addition to the images recorded on the lab computer and class *flickr*® site.
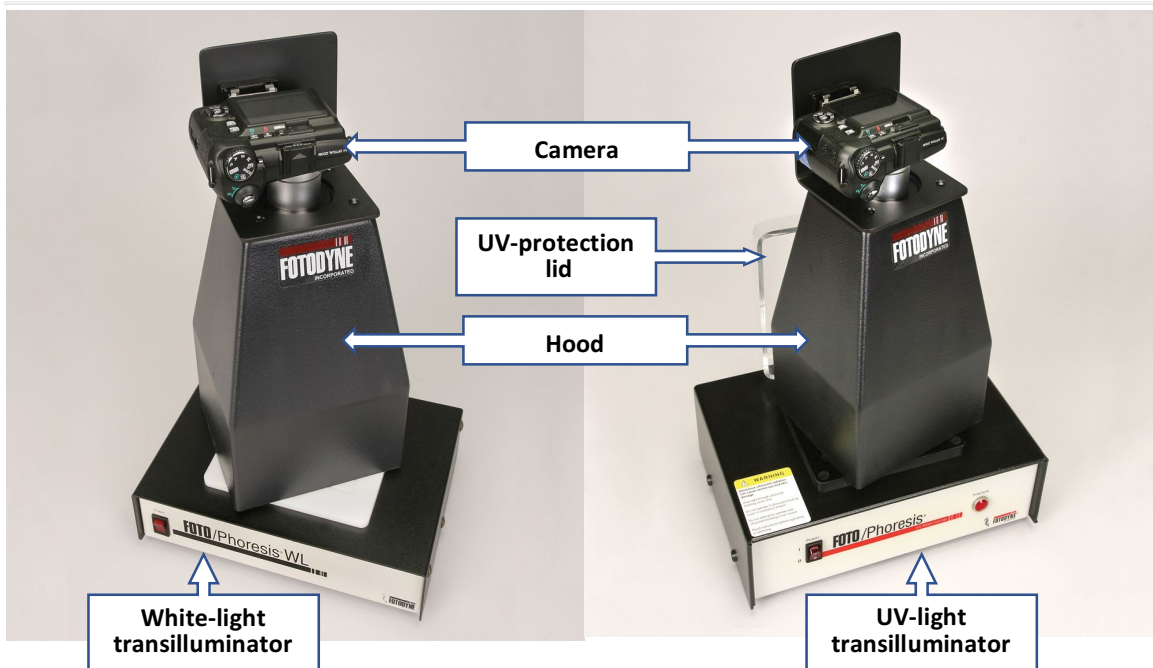

For polyacrylamide gel photos

As described in Lab 1, to analyze proteins we use polyacrylamide get electrophoresis (PAGE). To visualize the bands of separated proteins, we stain them with a blue dye, and

then destain the gel to remove the dye from the protein-free areas to produce and strong contrast between the stained bands and the gel background.
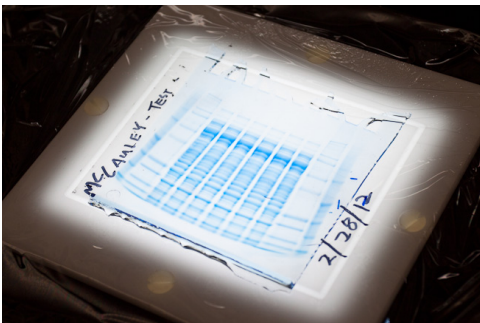
Your gel should look more or less like this picture after destaining -- strong blue bands against a clear background.

When you're ready, carry the destaining box with your gel over to the white-light transilluminator. Place a piece of plastic wrap on top of the transilluminator and write your group name, section number, and the date on the plastic wrap, leaving room in the middle for the gel.

Lift the gel out of the water using a spatula or your fingers. Slide the gel onto the plastic wrap, and you're ready to take a picture. (See "Taking the picture" section below.)
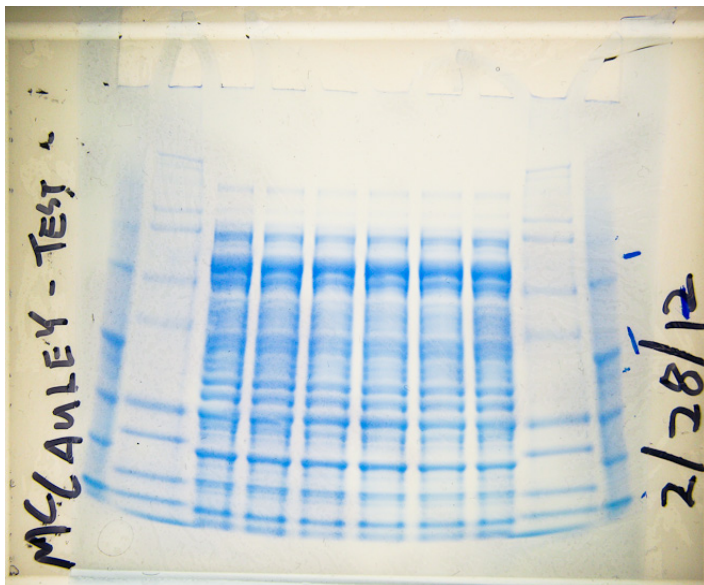
Your gel photo should look something like this. The background is nearly white, the bands are clearly visible, and the name and date are easy to read. (You'll need this information to find your gel image on the website.)

If your photo looks orange, it's because there is a UV-blocking filter on the camera, which we need for DNA gels; it will still work fine for your protein gel.

For agarose gel photos

As described in Lab 2, to analyze nucleic acids we use agarose get electrophoresis. To make the visualization of DNA bands even more sensitive, we stain it with a fluorescent dye, ethidium bromide. And since ethidium bromide only fluoresces when it is bound to the DNA, we do not need any destain steps. To stimulate the fluorescence, we must view, and photograph, the gel under ultraviolet light. The UV transilluminator emits UV light at 300 nm, optimum for fluorescing DNA stained with ethidium bromide.

Spread a piece of plastic wrap on the lab bench for the gel. Lift the gel tray out of the gel box and let the buffer drain off the gel. Then slide the gel out of its tray onto the plastic wrap.

Place the gel & plastic wrap on the UV transilluminator. Dry the edge of the plastic wrap with a paper towel and fold the bottom of the wrap over the lower region of the gel up to the sample dye line in the gel. Write your group name and the date on this area of the plastic wrap with a fluorescent highlighter pen. Make sure the plastic wrap is dry, or the highlighter ink will run into your gel and turn it bright yellow.

Put down the transparent lid on the transilluminator, turn on the UV light, and take a look at your gel. The lid is UV-absorbing acrylic to protect your eyes, and the UV light will only come on when the lid is down, but wear your safety glasses as a precaution anyway. Don't waste too much time looking at the gel; you'll be able to see it better in the image on the computer. **Don't leave the camera on the UV transilluminator longer than necessary.** The heat from the UV light will make your gel steamy, and the camera's lens will fog up.
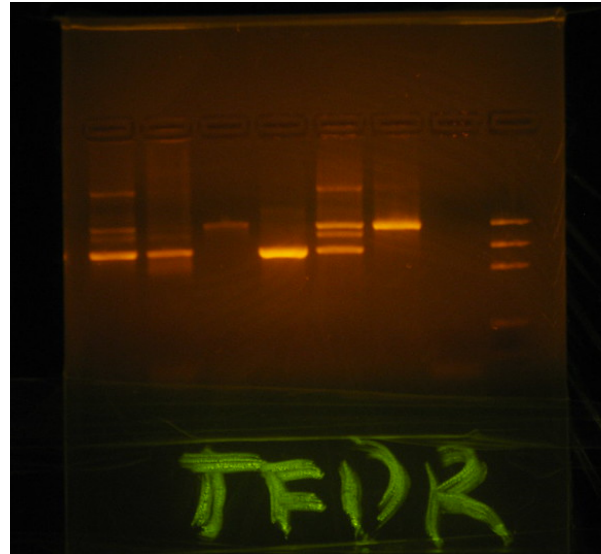
Taking the picture

Make sure the USB cable fom the computer is plugged into the USB port of the camera. If using the UV-transilluminator, there should be a UV filter on the camera lens. This filters UV light from the light box from reaching the camera so the fluorescence will appear against a dark background. If this filter is in place when using the white-light transilluminator, the images will have an orange tint.

Place the camera and hood over the gel such that the hood fits snuggly around the light plate margin. If using the UV-transilluminator, the acrylic lid must be raised and the hood positioned to engage the magnetic switch on the box. If correct, the indicator light on the front of the transilluminator will turn on to confirm the UV lamp is on.
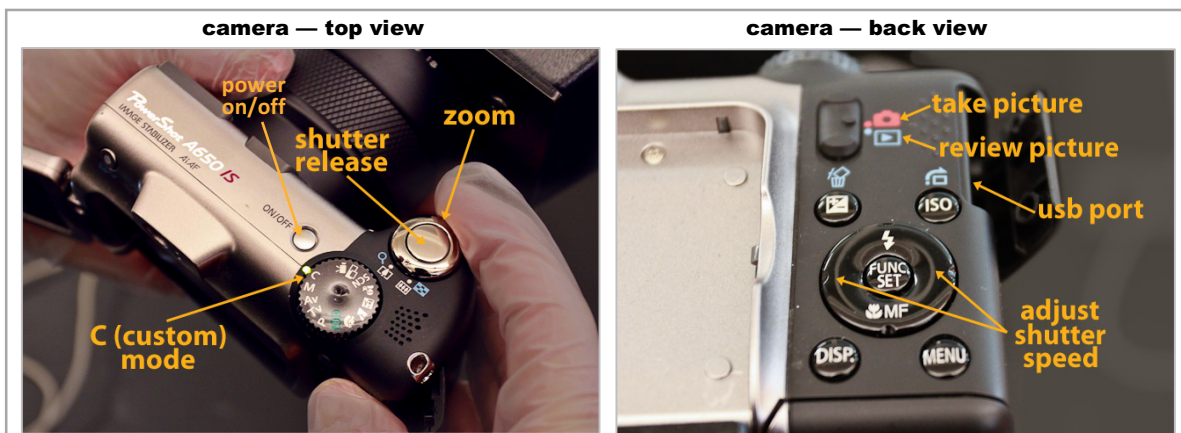
Make sure the dial on the top of the camera is set to **C (Custom) mode**, and the slide switch on the back of the camera is set to the **red camera icon** ("take picture") position. Avoid the temptation to use any mode other than "C"!

Power on the camera with the on/off button on the top of the camera. Press the shutter release button partially to focus the image in the viewfinder, and push the ring control around the shutter release button to zoom in on the desired frame. Make sure the frame includes all the bands of interest and your identifying information written on the plastic wrap. Use the right and left sides of the "adjust shutter speed" control on the camera back to brighten or darken your image to produce the best visualization of gel bands versus the gel background.

Press the shutter release button partially again to re-focus, and then press fully to take the picture while holding the camera steady. The exposure may be long, and if you move the camera, you could get a blurred picture.

When you've got your picture, move the slide switch on the back of the camera to the **blue arrow icon** ("review picture") position. The computer should already be set up to automatically copy your image to the appropriate folder on the computer and delete it from the camera. If you took more than one image, please delete all but one.

Turn off the camera when you're done. If it's left in review mode, it won't turn off by itself, and the batteries will wear down.

The instructor will upload all the images to the class *flickr*® site, so you'll be able to view it and download it later. It's important to get everybody's gel picture on flickr, so you can compare your gel to others.

A4. WRITING YOUR LAB REPORTS

The lab reports for this class should be modeled on typical scientific research papers, with a few modifications. Each lab report *must* include the following sections:

I. Title

Start with a descriptive title. Research reports are not novels with vague or cutesy titles. The title should state clearly what the paper is about.

Also include a list of authors (full names of your lab group members) with your lab section and group number, and the date of submission.

II. Introduction

The Introduction section should progress from general background to specific details.

Begin with an opening paragraph stating what you are doing and what is the objective of these experiments. Use this area to define key concepts and new terms. Then proceed to describe the particular hypothesis or hypotheses you are testing and how you intend to accomplish these objectives.

Conclude the Introduction with specific predictions of your expected results, and how these results will answer your questions or support/refute your hypotheses. Remember — these are predictions: *If* your hypothesis is correct, what specific results would you expect to see? If you are testing alternative hypotheses, how would the expected results differ depending upon which is correct?

Keep your Introduction focused on the objectives. Be concise! The Introduction should only be ~1 page long.

III. Methods

The methods sections of your lab reports will **not** resemble those of typical scientific papers. Normally, a scientific paper would include enough information to allow a reader to duplicate the experiment. In this lab, you'll be following the lab manual, so you don't need to write out the whole protocol.

Instead, draw a flow diagram showing all the parts of the lab and how they connect with each other (e.g., the DNA goes from the restriction digest to the ligation to the gel). You don't need to show the amounts; just show what goes into each tube.

In a flow diagram, all the components must connect with each other by a sequence of arrows. Even if the experiment took several lab periods, show how the components of one day lead into the next.

Indicate how the data in the Results section were obtained. For example, if a step on the methods diagram said "count the colonies on each plate", it should also say "See Table X". So Table X would be in your Results section with those counts.

The entire flow diagram must fit on one page, no matter how complex the experiment is.

IV. Results

The purpose of a scientific research paper is to present results. Everything else in the paper is there to help readers understand the results. The results section includes only what you saw, not what you think it means.

Experimental results are usually presented in the form of **tables** or **figures** labeled with appropriate captions and keys. For many labs, your results section will include a picture of a gel. You'll use digital photography in the lab; insert your gel image in the report. Label your gel photo carefully. The wells always go at the top. Generally, you should label each lane with a short descriptive label, as in the picture shown here. If you number the wells, the numbering should go from left to right. You should also include a separate table with more detail, such as the amount of DNA or protein (in μg or ng, not μl) in each lane. Don't include a description or interpretation of the gel image; that belongs in the discussion.
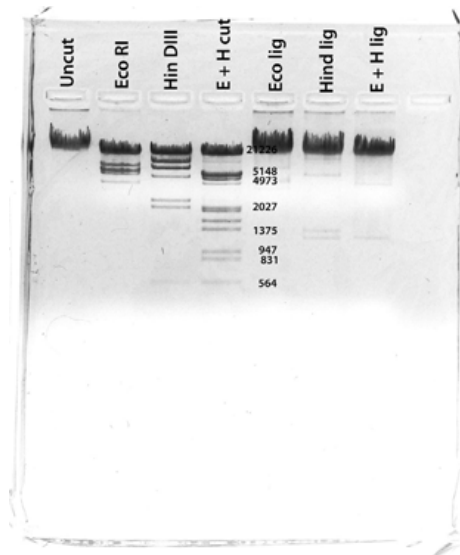
In most of your reports, you'll also need to include tables of other results, such as the number of colonies or plaques you observed on plates. Be sure to include a title for each table and units for all the numbers in the table.

To review what is required for a scientific figure or table, see the BIOL 6A *Scientific Inquiry* exercise at **http://facultyfiles.deanza.edu/gems/heyerbruce/01ScientificMethod07F.pdf**

V. Discussion

This section is a discussion of what your results mean. The discussion format is typically the reverse of the introduction format: begin with specific details about your results and end with a general concluding statement.

For example, you might start with a lane-by-lane discussion of the above gel. Did your DNA get cut? Did it get ligated? Remember that you normally have controls for each of your experiments. The control for EcoRI digestion of the DNA is the uncut DNA. Therefore, you should discuss your results in terms of comparing experimental results to control results.

Finish the discussion with an overall summary. Did it all come out as you expected? Refer to your Introduction statements: Was your hypothesis supported or refuted? How strong is the evidence? If it was not supported, why not? Come up with a hypothesis about what might have gone wrong, and state how you would test this hypothesis.

Finish with a summary comment regarding the overall accomplishment of the stated objectives.

Style Tips

• Keep your writing simple and clear. Don't write things that you don't understand.

• You'll be writing your report after you do the experiment, so use the past tense. Don't write, "we will use lambda DNA," because you already did it. It's OK to refer to yourselves as "we," because the report is written by a group of people.

• Scientific names of organisms *must* be written using correct scientific format like this: *Escherichia coli* — the name should be in italics, with the genus name capitalized and the specific epithet not capitalized. After you've used a name once, you can abbreviate the genus — *E. coli* — subsequently in that same document.

• Number the pages in your report.

• Use real greek letters where appropriate; 25 ul is not the same thing as 25 μl. In MS Word, you can find these by using Insert > Symbol. You can also find a degree symbol (e.g., 37°) this way.

• Use superscripts or subscripts where appropriate. In MS Word, you can apply superscripts with Format > Font.

Strategies for good reports

• Include all the results. This may include experiments done over several lab periods.

• Remember that your report is about your results. The most important aspect of your report is showing that you understand what your results mean. If you write an excellent discussion explaining how your results are exactly as expected, but your results show the opposite, you won't get a good grade.

• Somebody should read the whole report before you turn it in. If each person in your group writes a section of the report, and you all show up and staple it together the day it's due, you probably won't have a very good report. If your section is outstanding and someone else's is weak, then you'll all share a low score.
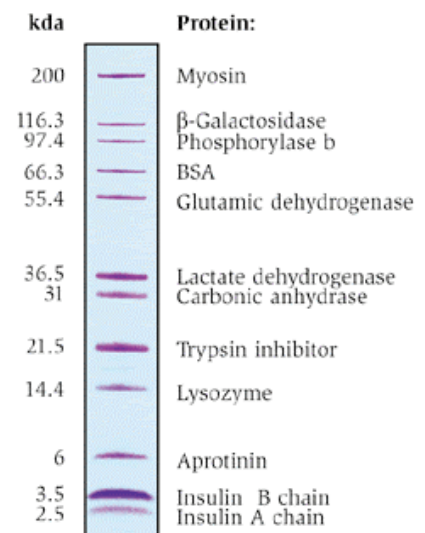
PROTEIN MOLECULAR WEIGHT STANDARD MARKERS

Sigma® Protein Markers

Protein	Subunit MW (Da)	SigmaMarker™ MW Markers			
		M 3788	M 4038	M 3913	M 3546
Urease, jack bean (hexamer)	545,000				
Urease, jack bean (trimer)	272,000				
Violet protein-dye conjugate	220,000				
Myosin, rabbit muscle	205,000	▬	▬		
α₂-Macroglobulin	180,000				
Recombinant marker protein	150,000				
β-Galactosidase, E. coli	116,000	▬	▬		
Albumin dimer, bovine serum	132,000				
Recombinant marker protein or Pink protein-dye conjugate	100,000				
Phosphorylase b, rabbit muscle	97,000	▬	▬		
Fructose-6-phosphate kinase	84,000	▬	▬		
Recombinant marker protein	75,000				
Albumin, bovine serum	66,000	▬	▬	▬	
Blue protein-dye conjugate	60,000				
IgG marker	59,500				
Catalase, bovine liver	58,100				
Glutamic dehydrogenase, bovine liver	55,000	▬	▬		
Recombinant marker protein	50,000				
Fumerase, porcine heart	48,500				
Ovalbumin, chicken egg or Pink protein-dye conjugate	45,000	▬	▬	▬	
Alcohol dehydrogenase	39,800				
Glyceraldehyde-3-phosphate dehydrogenase	36,000	▬	▬	▬	
Recombinant marker protein	35,000				
Orange protein-dye conjugate or β-Casein	30,000				
Carbonic anhydrase	29,000		▬	▬	
Triosephosphate isomerase	26,600				▬
Recombinant marker protein	25,000				
Trypsinogen, bovine pancreas	24,000		▬	▬	
Trypsin inhibitor, soybean or Blue protein-dye conjugate	20,100		▬	▬	
β-Lactoglobulin, bovine milk	18,400				
Myoglobin from horse heart or RNase B	17,000				▬
Recombinant marker protein	15,000				
Lysozyme, egg white	14,300				
α-Lactalbumin, bovine milk	14,200		▬	▬	▬
Pink protein-dye conjugate	12,000				
Blue protein-dye conjugate	8,000				
Aprotinin, bovine lung	6,500		▬	▬	▬
Insulin Chain B, oxidized, bovine	3,496				▬
Bradykinin	1,060				▬

Promega® Protein Molecular Weight Marker

kDa
— 225
— 150
— 100
— 75
— 50
— 35
— 25
— 15
— 10
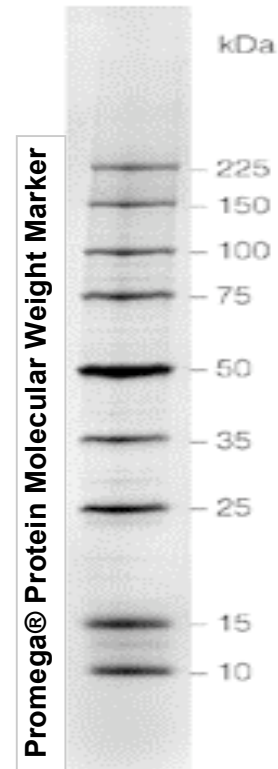
Mark 12™ Unstained Standard on a NuPAGE® Novex 4-12% Bis-Tris Gel w/MES stained with Coomassie® Blue R-250

kda	Protein:
200	Myosin
116.3	β-Galactosidase
97.4	Phosphorylase b
66.3	BSA
55.4	Glutamic dehydrogenase
36.5	Lactate dehydrogenase
31	Carbonic anhydrase
21.5	Trypsin inhibitor
14.4	Lysozyme
6	Aprotinin
3.5	Insulin B chain
2.5	Insulin A chain

DNA FRAGMENT-LENGTH STANDARD MARKER LADDERS

bp	ng/0.5µg	%
21226*	218.8	43.8
7421	76.5	15.3
5804	59.8	12.0
5643	58.2	11.6
4878	50.3	10.1
3530*	36.4	7.3

EcoRI-cut λ–DNA
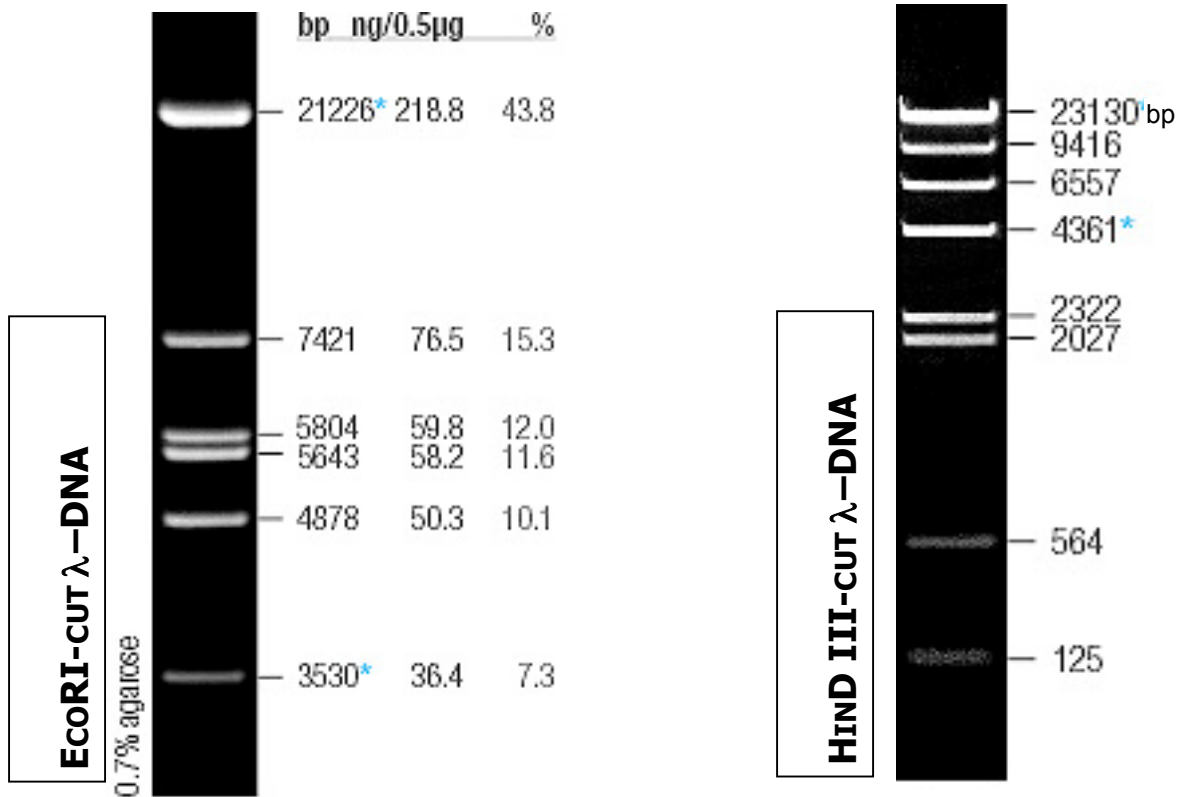
0.7% agarose

0.5µg of the Marker was run on a 20cm length of 0.7% agarose gel in 1X TAE buffer at 3V/cm 18hrs (until bromophenol blue dye reached the bottom of the gel).

HinD III-cut λ–DNA

23130 bp
9416
6557
4361*
2322
2027
564
125

EZ Load® DNA Markers

1,000 bp
700
500
200
100