## Table of Contents

<table>
<thead>
<tr>
<th>Lab</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Lab Safety Rules</strong></td>
<td>i</td>
</tr>
<tr>
<td></td>
<td><strong>Lab Equipment</strong></td>
<td>v</td>
</tr>
<tr>
<td>LAB 1</td>
<td>Worksheet for “Scientific Investigation” (Morgan/Carter #1 – 7e)</td>
<td>1</td>
</tr>
<tr>
<td>LAB 2</td>
<td>Measurement in the Metric System</td>
<td>7</td>
</tr>
<tr>
<td>LAB 3</td>
<td>Gel Filtration Chromatography</td>
<td>31</td>
</tr>
<tr>
<td>LAB 4</td>
<td>Macromolecules</td>
<td>43</td>
</tr>
<tr>
<td>LAB 5</td>
<td>Microscopes and Cells</td>
<td>57</td>
</tr>
<tr>
<td>LAB 6</td>
<td>SDS-PAGE</td>
<td>77</td>
</tr>
<tr>
<td>LAB 7</td>
<td>Diffusion &amp; Osmosis</td>
<td>89</td>
</tr>
<tr>
<td>LAB 8</td>
<td>Enzymes: β-Galactosidase</td>
<td>99</td>
</tr>
<tr>
<td>LAB 9</td>
<td>Restriction Enzyme Digestion of DNA</td>
<td>121</td>
</tr>
<tr>
<td>LAB 10</td>
<td>DNA &amp; Gene Expression</td>
<td>135</td>
</tr>
<tr>
<td>LAB 11</td>
<td>Restriction Enzyme Mapping</td>
<td>153</td>
</tr>
<tr>
<td>LAB 12</td>
<td>Genetic Transformation</td>
<td>161</td>
</tr>
<tr>
<td>LAB 13</td>
<td>Sickle Cell Genotype by Southern Blot (Edvotek #315)</td>
<td>169</td>
</tr>
<tr>
<td>LAB 14</td>
<td>PCR Amplification of DNA</td>
<td>189</td>
</tr>
<tr>
<td>LAB 15</td>
<td>Mitosis &amp; Meiosis</td>
<td>201</td>
</tr>
<tr>
<td>LAB 16</td>
<td>DNA Cloning</td>
<td>215</td>
</tr>
<tr>
<td>LAB 17</td>
<td>Reading Primary Research Articles</td>
<td>225</td>
</tr>
<tr>
<td>LAB 18</td>
<td>Fermentation &amp; Respiration</td>
<td>229</td>
</tr>
<tr>
<td>LAB 19</td>
<td>Photosynthesis</td>
<td>239</td>
</tr>
<tr>
<td>LAB 20</td>
<td>ELISA</td>
<td>249</td>
</tr>
<tr>
<td>LAB 21</td>
<td>Principles of Genetic Inheritance</td>
<td>259</td>
</tr>
<tr>
<td>LAB 22</td>
<td>Drosophila Genetics</td>
<td>285</td>
</tr>
</tbody>
</table>
The following rules should be observed at all times to ensure safety in the laboratory:

1. Do NOT eat, drink or bring any food or beverage (including water) into the laboratory. In addition, never put anything (e.g., pencils, pens) into your mouth while in the laboratory. This is for your own protection. Potentially harmful chemicals may be used in this room.

2. Keep your table top clear of any unnecessary materials. Textbooks, backpacks, purses and all other materials not in use should be removed from your lab table.

3. Wear enclosed shoes in the laboratory, and pull long hair back. Do not wear sandals or open-toed shoes to avoid chemical or physical injury due to spills, broken glass, etc. Long hair should be pulled back to avoid contact with any lab materials and loose, hanging clothing should be avoided.

4. Begin AND end each laboratory session by cleaning your work area. Spray your table top with the disinfectant provided, wipe with a paper towel and allow to air dry to ensure the surface is clean before you and your belongings come in contact with it. Repeat at the end of the laboratory session to leave a clean surface for the next group to use your table.

5. Immediately report all accidents, spills or injuries to your instructor. Your instructor will indicate how best to deal with the situation.

6. NEVER pipette materials by mouth. Only use the pipette pumps provided in the lab, this way there is no chance of your ingesting the substance being measured.

7. Exercise care with hot plates. Hot plates should be OFF when not in use and positioned so they outside your work space away from the edge of your table. Flammable materials should be kept a safe distance from the hot plate.

8. Inform your instructor of any missing lab materials or equipment. Do NOT attempt to find what you need by searching the laboratory, simply ask the instructor to help locate what you need.

9. Discard waste material from your experiments as indicated by your instructor or the lab manual. Certain lab materials should never be disposed of in the sink or garbage. Be sure to ask your instructor if you uncertain how to handle any waste material.

10. Wash your hands each time you leave the laboratory and at the end of each laboratory session. This is important to protect you and any one you contact from materials you may have handled in the lab.

11. Be sure your lab table is clean before you leave, and clean any labware you have used. This is important since we do not have the staff to clean up after everyone, so please do your part to maintain a clean lab that is ready for the next lab session.

12. Push your lab chair under the table before you leave so it will not be an obstruction.
STATEMENT OF COMPLIANCE WITH BIOLOGY 6 LABORATORY SAFETY RULES

I, ________________________________, have read, understood, and will comply
(please print your name)
with the Biology 6 Laboratory Safety Rules, and have a copy of these rules and guidelines to keep in
my possession.

Student Signature: ________________________________

Date: _________________
EQUIPMENT USE IN BIOLOGY 6

In this course you will use a variety of equipment, much of which is stored in the cabinets and drawers at each lab bench. Great care has been taken to organize equipment so that each group has the necessary type and number of each. You will often be using many or all of these on a single day. It is imperative that you know where your group’s equipment is stored, how to properly store it and the numbers of each you should have. At the end of each lab period it is your responsibility to ensure that all of your equipment is accounted for and properly stored. Failure to do so will result in deducted lab participation points. Note that each cabinet is labeled to clearly indicate which equipment it holds and how they should be organized. You can always reference the table below if you are unsure where your equipment is located.

Biology 6 Common Equipment Storage. FR: Front Right, FL: Front Left, US: Upper Shelf, BS: Bottom Shelf, T:Table.

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Number of pieces of equipment per table</th>
<th>Equipment Location</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(T1)</td>
<td>(T2)</td>
</tr>
<tr>
<td>P10</td>
<td>*Only two in the entire lab.</td>
<td>In a box on the cart at the FR.</td>
</tr>
<tr>
<td>P100</td>
<td>1</td>
<td>BS, FL cabinet at T3.</td>
</tr>
<tr>
<td>Heat block</td>
<td>1</td>
<td>BS, FL cabinet at T3.</td>
</tr>
<tr>
<td>Microcentrifuge</td>
<td>1</td>
<td>BS, FL cabinet at T3.</td>
</tr>
<tr>
<td>Power supply</td>
<td>1</td>
<td>BS, FL cabinet at T3.</td>
</tr>
</tbody>
</table>

The equipment you will be using is also expensive! For example, the list price for a single pipetman is $367 and the microcentrifuges you use cost $900 each. This equipment is also delicate. Incorrect handling of a pipetman will ruin its calibration making it useless. Recalibrating a single pipetman can cost from $30 to $60 dollars. It can cost significantly more to repair a microcentrifuge. Take care to handle the equipment as indicated by your instructor and/or detailed in the laboratory manual.
Ex. 1-1: QUESTIONS AND HYPOTHESES

Lab Study A: Asking Questions

On what basis should you decide which questions could be answered scientifically?

Indicate whether or not each question below can be answered scientifically by circling “yes” or “no”:

1. Do kids who play violent video games commit more violence? (yes or no)
2. Did the consumption of seven cans of “energy drink” cause the heart attack of a motorcyclist in Australia? (yes or no)
3. Will increased levels of CO₂ in the atmosphere stimulate the growth of woody vines such as poison ivy and kudzu? (yes or no)
4. How effective are extracts of marigold and rosemary as insect repellents? (yes or no)
5. Should it be illegal to sell organs, such as a kidney, for transplant purposes? (yes or no)

Lab Study B: Developing Hypotheses

Write an explanatory hypothesis for each of the following questions:

1. Does supplemental feeding of birds at backyard feeders affect their reproductive success?

2. Do preschool boys in coed classes develop better verbal skills than boys in all-male classes?

Indicate which of the following statements would be useful as a scientific hypothesis investigated using scientific procedures. Remember to consider if the hypothesis could be falsified and if a measurable and controllable experiment could be carried out to test it.

1. The use of pesticides in farming causes deformities in nearby frog populations.

2. Sea turtles are more likely to hatch during a new moon.

3. Drinking two or three cups of coffee a day reduces the risk of heart disease in women.

4. Manatees and elephants share a recent common ancestor.

5. Organic food is healthier than conventionally produced food.
Ex. 1-2: DESIGNING EXPERIMENTS TO TEST HYPOTHESES

Lab Study A: Determining the Variables
For the soybeans, several dependent variables are measured, all of which provide information about reproduction. What are they?

What was the independent variable in the investigation of the effect of sulfur dioxide on soybean reproduction?

Can you suggest other variables that the investigator might have changed that would have had an effect on the dependent variables?

Why is it important to have only one independent variable?

Why is it acceptable to have more than one dependent variable?

What are the controlled/standardized variables in this experiment?

Lab Study B: Choosing or Designing the Procedure
What was the level of treatment in the soybean experiment?

Describe replication in the soybean experiment.

What was the control treatment in the soybean experiment?

What is the difference between “the control” and “controlled variables”?

Lab Study C: Making Predictions
State a prediction based on the following hypothesis: “Exposure to sulfur dioxide reduces soybean reproduction.”
List the components of a scientific investigation from asking a question to carrying out an experiment.

List the variables that must be identified in designing an experiment.

What are the other components of an experimental procedure?

**Ex. 1-3: DESIGNING AN EXPERIMENT**

Record the body parts and the corresponding questions chosen by the class below.

Record the hypotheses for each of the questions below.

Outline your experimental plans for testing each hypothesis.

What is the dependent variable in each of your experiments?

What is the independent variable in each of your experiments?

What are the controlled variables?

What is the control?

Are there any levels of treatment?

Describe the replication in these experiments.

Predict the results of each experiment (in general) based on your hypotheses (if/then).
### Ratios of Height to Two Body Parts for Each Student

<table>
<thead>
<tr>
<th>Student</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Part #1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Part #2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Part #1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Part #2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Ex. 1-4: PRESENTING AND ANALYZING RESULTS

**Lab Study A: Tables**

Using the student average data from above, design a table to present the results for students in your class for H/BP for each body part measured. Include in your table ratios for newborns. Compose a title for your table.
Lab Study B: Figures

Using the data you’ve gathered, draw a **bar graph** in the grid below that shows the relationship between the dependent and independent variables for both body parts. Be sure to label each axis clearly and indicate the units, if applicable, for each axis. Also, include a legend to distinguish newborn and student ratios, and give your graph an appropriate title.

a) What is the *independent* variable for each experiment, and on which axis would you graph this?

b) What is the *dependent* variable, and on which axis would this variable go?

Ex. 1-5: INTERPRETING AND COMMUNICATING RESULTS

Using your tables and figures, analyze your results looking for relationships between the variables and for general trends. Write a summary statement for your results *being sure to also address whether the data support or falsify each hypothesis.*

Critique your experiment using the table below to indicate weaknesses and suggest improvements.

<table>
<thead>
<tr>
<th>Weaknesses in Experiment</th>
<th>Improvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td></td>
</tr>
</tbody>
</table>
REVIEWING YOUR KNOWLEDGE

1. Match each description on the right with the corresponding term on the left:

   ____ control                      A. variables kept constant during experiment (not manipulated)
   ____ controlled variables        B. tentative explanation for an observation
   ____ level of treatment          C. what the investigator varies in the experiment
   ____ dependent variable          D. process used to measure the dependent variable
   ____ replication                 E. appropriate values to use for the independent variable
   ____ procedure                   F. treatment eliminating or standardizing independent variable
   ____ prediction                  G. what investigator measures or records, i.e. what is affected
   ____ hypothesis                  H. number of times the experiment is repeated
   ____ independent variable        I. statement of expected results based on the hypothesis

2. Circle the dependent variable and underline the independent variable for each experiment below:

   Scientists investigating the effects of increased temperatures on plants in urban environments measured the size of ragweed flowers in Baltimore city lots and rural fields.
   
   Scientists determined the abundance of apple aphids on leaves of 120 apple trees on 5 days in October. The autumn leaf color of the apple trees varied with 40 red trees, 40 yellow trees, and 40 green trees.
   
   Synapse number and level of synaptic proteins in the brains of fruit flies are measured during wakeful periods and periods of sleep.

3. Suggest a control treatment for each of the following experiments:

   Geneticists are studying the inheritance of “wolfman syndrome”, where the body and face are covered with dark hair. They studied three families in which 16 individuals had the syndrome. They discovered DNA deletions in four genes in each of the 16 persons.
   
   Nutrition experts investigate if eating yogurt every day will reduce gum disease.
   
   The number of T-lymphocytes are counted in the blood of vultures that feed on carcasses of livestock treated with antibiotics.

4. In a recent study of 10,000 women, scientists reported that women who had breast cancer had a history of heavier antibiotic use than women who did not have breast cancer. What possible explanations for this correlation can you suggest?

5. What is the essential feature of science that makes it different from other ways of understanding the natural world?
Bio 6 – Metric System Lab

Overview

In this laboratory you will make a variety of measurements in metric units, and practice converting units within the metric system. You will also practice graphing data sets and making aqueous solutions.

The Metric System of Measurement

How many teaspoons are in a cup? How many inches are in a mile? How many ounces are in a pound? If you know the answers to all of these questions, you are one of the few people in the world who can completely understand the English System of Measurement. The United States is one of the few countries that still uses the English System of Measurement (not even England uses it!).

The English System was developed over many centuries by the kings and noblemen of the Roman and British empires. In fact, the foot was literally the length of the actual foot of an English king, which happens to be 12 inches (each inch being the length of three seeds of barley). The following are units used in the English System today:

**LENGTH**
- 1 mile = 8 furlongs = 1,760 yards = 5,280 feet = 63,360 inches

**VOLUME**
- 1 gallon = 4 quarts = 8 pints = 16 cups = 128 ounces = 256 tablespoons

**MASS**
- 1 ton = 2,000 pounds = 32,000 ounces = 2.24 x 10^8 grains

**TEMPERATURE**
- Fahrenheit - Water freezes at 32°F and boils at 212°F

Did you know how to do these conversions already? Probably not. Very few scientists, much less everyday citizens, can remember how to convert units within this system, even though they’ve used it their entire lives. Basically, the English system is so difficult to work with that most countries in the world, and all scientists, have adopted a much easier system called the Metric System of Measurement.

The metric system of measurement has been adopted by most countries in the world and all scientists for two primary reasons: 1) there is a single, basic unit for each type of measurement (meter, liter, gram, °C) and 2) each basic unit can have prefixes that are based on powers of 10 making conversions much easier. Once you learn the basic units and the multiples of 10 associated with each prefix, you will have the entire system mastered.
Basic Units of the Metric System

**LENGTH** – The basic unit of length in the metric system is the **meter**, abbreviated by the single letter **m**. A meter was originally calculated to be one ten-millionth of the distance from the north pole to the equator, and is ~3 inches longer than a yard.

**VOLUME** – The basic unit of volume in the metric system is the **liter**, abbreviated by the single letter **l** or **L**. A liter is defined as the volume of a box that is 1/10 of a meter on each side. A liter is just a little bit larger than a quart (1 liter = 1.057 quarts).

**MASS** – The basic unit of mass in the metric system is the **gram**, abbreviated by the single letter **g**. A gram is defined as the mass of a volume of pure water that is 1/1000th of a liter. 

[NOTE: 1/1000th of a liter = 1 milliliter = 1 cubic centimeter = 1 cm³ = 1 cc].

**TEMPERATURE** – The basic unit of temperature in the metric system is a degree Celsius (°C). Water freezes at 0°C and boils at 100°C.

Prefixes used in the Metric System

Unlike the English System, the metric system is based on the meter (**m**), liter (**L** or **l**) and gram (**g**), and several prefixes that denote various multiples of these units. Specifically, each basic unit can be modified with a prefix indicating a particular “multiple of 10” of that unit. Here are the more commonly used prefixes and what they mean:

<table>
<thead>
<tr>
<th>Prefix</th>
<th>Symbol</th>
<th>Multiplier</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mega</td>
<td>M</td>
<td>10⁶</td>
<td>1,000,000</td>
</tr>
<tr>
<td>Kilo</td>
<td>k</td>
<td>10³</td>
<td>1,000</td>
</tr>
<tr>
<td>No prefix</td>
<td></td>
<td>10⁰</td>
<td>1</td>
</tr>
<tr>
<td>Deci</td>
<td>d</td>
<td>10⁻¹</td>
<td>0.1 or 1/10 (i.e., tenths of a unit)</td>
</tr>
<tr>
<td>Centi</td>
<td>c</td>
<td>10⁻²</td>
<td>0.01 or 1/100 (i.e., hundredths of a unit)</td>
</tr>
<tr>
<td>Milli</td>
<td>m</td>
<td>10⁻³</td>
<td>0.001 or 1/1,000 (i.e., thousandths of a unit)</td>
</tr>
<tr>
<td>Micro</td>
<td>µ</td>
<td>10⁻⁶</td>
<td>0.000001 or 1/1,000,000 (i.e., millionths of a unit)</td>
</tr>
<tr>
<td>Nano</td>
<td>n</td>
<td>10⁻⁹</td>
<td>0.000000001 or 1/1,000,000,000 (i.e., billionths of a unit)</td>
</tr>
</tbody>
</table>

Here is how simple the metric system is using the basic units and the prefixes:

What is one thousandth of a meter? **a millimeter (mm)**
What is one millionth of a liter? **a microliter (µl)**
What is 1,000 grams? **a kilogram (kg)**
Let us now examine these units more closely by using them to make actual measurements and converting from one metric unit to another.

**DISTANCE in the Metric System**

In the United States, when we travel by car, distances are measured in miles. For example, from the Mission College to downtown Los Angeles is about 20 miles. In almost every other country, such distances are measured in kilometers. One kilometer is exactly 1,000 meters and approximately 0.6 miles. Thus, 1 km is equal to 0.6 miles.

What are some other real-world examples of metric units of length? One **micrometer (µm)** is $1/1,000$th the size of a millimeter or $1/1,000,000$th of a meter. When you observe a cheek cell under the microscope in a future lab, it is about 40 µm in diameter. Typical bacteria are about 5-10 µm in diameter.

One **nanometer (nm)** is $1/1,000$th the size of a micrometer or $1/1,000,000,000$th of a meter. Objects this small are far too tiny to observe even in a light microscope. If you line up five water molecules side-by-side, the length would be about 1 nanometer.

**Exercise 1 – Measuring distance**

1. Obtain a wooden meter stick. If you look on the back of the meter stick, one meter is approximately 39 inches or about 3 inches longer than one yard (36 inches). Using the meter stick, estimate the size of the laboratory by measuring its width and length to the nearest **meter**.

2. Observe that the meter is divided into 100 equal units called centimeters. A centimeter is about the width of a small finger. Using the meter stick, estimate the dimensions of a regular piece of notebook paper to the nearest **centimeter**.

3. How tall are you? Go over to the medical weight and height scale to measure how tall you are to the nearest **centimeter**.

4. Next, obtain a small plastic metric rule. Observe that each centimeter is divided into 10 small units called millimeters. A millimeter is about the thickness of a fingernail. Using the small plastic ruler, estimate the diameter of a hole on a regular piece of notebook paper to the nearest **millimeter**.

**VOLUME in the Metric System**

A variety of devices are used to measure volume in the metric system. In the next exercise you will familiarize yourself with measuring volumes greater than 1 ml using beakers, graduated cylinders and pipettes.
Exercise 2A – Measuring volumes larger than 1 ml

1. Obtain a one liter (L or l) beaker. One liter is equal to 1,000 cubic centimeters (cc = cm³ = milliliter = ml). Fill the beaker with one liter of water. To do this, add water until the meniscus (top level of the water) reaches the 1 liter marker on the beaker. Pour the water into a 2 liter soda bottle. Repeat.

   Once again, fill the beaker with one liter of water by adding water until the meniscus reaches the 1 liter mark. Over the sink, add the 1 liter of water to the 1 quart container provided. Notice that 1 liter is just a little bit more than 1 quart. In fact, 1 liter = 1.057 quarts.

2. One way to measure the volume of a fluid in a laboratory is to use a graduated cylinder. Whereas beakers are generally used to hold fluids, graduated cylinders are used to accurately measure volumes. Obtain a 50 milliliter (ml) graduated cylinder. Fill the graduated cylinder with water until the meniscus reaches the 50 ml mark. Add the water to a 1 liter (1,000 ml) beaker. Notice that 50 ml is equal to 1/20th of a liter. Next, use the graduated cylinder to measure the fluid in the flask labeled “A” to the nearest 0.5 ml. Record this volume on your worksheet.

3. Pipettes are used to measure smaller liquid volumes whereas graduated cylinders are used to measure larger volumes. Obtain a 10 ml glass pipette and attach it snugly to a pipette pump. Notice whether or not the pipette is a delivery or blowout pipette. Blowout pipettes are designed for measuring fluids all the way to the end of the pipette so that the liquid measured can be completely “blown out” of the pipette. Delivery pipettes have a gap at the end of the pipette and are designed to “deliver” the liquid down to the desired marking only. The remainder is discarded or returned to the original container. (NOTE: blowing out a delivery pipette will yield the wrong volume)

   Using the roller on the pipette pump, gradually suck up some water until the meniscus reaches the 0 ml mark. Measure 10 ml of the water into the sink by moving the roller in the opposite direction. Next, measure the amount of fluid in the test tube labeled B to the nearest 0.1 ml using the 10 ml pipette. Record this volume on your worksheet.

To measure volumes less than 1 ml you will need a micropipettor like the one shown on the next page. Micropipettors will allow you to accurately measure volumes at the microliter (µl) level. While we don’t deal with microliter volumes in everyday life, in the laboratory measurements as little as 1 µl are used routinely. For this reason micropipettors are essential in any laboratory environment.
You should have at least four types of micropipettor at your table, each capable of measuring volumes in the indicated ranges:

- **P1000**: 100–1000 µl
- **P200**: 50–200 µl
- **P100**: 20–100 µl
- **P20**: 50–200 µl
- **P10**: 1–10 µl (not at all tables)

Let's first get acquainted with each part of a micropipettor and its corresponding function. Be sure to look at one of the micropipettors at your table as you read the next three paragraphs:

The end of the micropipettor labeled “tip attachment” is where you will attach a disposable plastic tip. The liquid you measure will be contained within the tip and thus will not make contact with the micropipettor itself. When you are finished measuring your liquid, you will discard the tip and use a new tip for the next measurement. This avoids contamination of your sample as well as the micropipettor. The **tip eject button** will move the **tip eject shaft** down to eject the disposable tip.

The **volume readout** shows the number of microliters (µl) the micropipettor is set to measure. The volume readout is adjusted by turning the **volume adjustment** knob. Each micropipettor has a range of volumes it is designed to measure which is indicated on the end of the plunger. **If you set the micropipettor to a volume outside this range you can damage the instrument’s internal mechanism which may destroy its accuracy.** Remember, you should **never** adjust the volume readout outside the range the micropipettor is designed to measure.
The plunger is pressed downward with your thumb and then released to draw liquid into the disposable tip. The measured liquid can then be expelled from the tip by depressing the plunger. If you press down on the plunger you will reach a point of resistance called the “first stop” as shown in the illustration. When depressed to the first stop, the plunger will draw in the volume indicated on the volume readout as it is released. To measure 20 µl for example, you would set the volume readout to “200” for the P20 or “020” for the P100, submerge the tip on the end of the micropipettor into the liquid to be measured, and slowly release the plunger to its rest position. The liquid can then be expelled from the tip by depressing the plunger to the first stop. If any liquid remains in the tip at this point, the plunger can be depressed beyond the first stop toward the “second stop” (see illustration). This will force any remaining liquid out of the tip. The only time you will concern yourself with the second stop is for this purpose.

Exercise 2B – Measuring volumes less than 1 ml

In addition to the micropipettors, you should have 3 racks of disposable micropipettor tips – large tips for the P1000, medium tips for the P200, P100 & P20, and small tips for the P10. You should also have a small microcentrifuge tube of blue liquid, five empty microcentrifuge tubes and a microcentrifuge tube rack. Everyone at your table should practice measuring and transferring the volumes indicated in the exercises below. Before you begin, be sure you are in a comfortable position with everything you will need in front of you or within comfortable reach.

Follow the instructions below when using each micropipettor to measure the desired volumes:

- adjust the volume readout of the micropipettor to the desired volume
- place the appropriate disposable tip snugly on the “tip attachment” end of the micropipettor by firmly inserting it into a tip in the rack
- open the small tube of blue liquid, depress the plunger to the first stop, immerse the end of the tip into the liquid, and slowly release the plunger (you can hold the tube in your opposite hand while doing this)
- hold the tube you want to transfer to in your opposite hand and insert the tip with your sample
- slowly depress the plunger to the first stop to expel the liquid into the tube, being sure to NOT release the plunger just yet!

NOTE: if necessary you can push the plunger toward the second stop to expel any remaining liquid

- move the tip out of the liquid, and then release the plunger (this is extremely important, you do NOT want to release the plunger before removing the tip from the liquid or else you will pull most of the sample back into the tip!)
- dispose of your used tip into the biohazard waste container at your table

1. Use the P1000 to transfer 500 µl of blue liquid to a tube labeled “500” (volume readout = 050).
2. Use the P200 to transfer 100 µl of blue liquid to a tube labeled “100” (volume readout = 100).
3. Using the P100, transfer 25 µl of blue liquid to a tube labeled “25” (volume readout = 025).
4. Using the P20, transfer 5 µl of blue liquid to a tube labeled “5” (volume readout = 050).
5. Using the P10, transfer 1 µl of blue liquid to a tube labeled “1” (volume readout = 010).
**MASS in the Metric System**

Mass is an inherent property of matter whereas weight refers to the mass of an object and the force of gravity acting upon it. For example, your mass is the same whether you are on earth or in outer space. Your weight, however, will be different on the earth vs outer space (where you can be essentially “weightless”). So technically you will be measuring the weight of various objects in the following exercise to illustrate the property of mass. To do so you will use a metric balance, much like the one shown on the left, to determine the mass/weight in grams.

**Exercise 3 – Measuring mass**

1. Place an empty 50 ml graduated cylinder on the balance and determine its mass in grams.

2. Next, fill the graduated cylinder with 50 ml of water and measure the mass of both the cylinder and the water. From this value subtract the mass of the cylinder to get the mass of the water.

   **NOTE:** By definition, one gram is the mass of exactly 1.0 ml of pure water, thus 50 ml of water has a mass of 50.0 grams. How far off was your measured mass from the true mass of 50 ml of water?

3. Next, take a large paper clip and place it on the balance and determine its mass in grams.

**TEMPERATURE in the Metric System**

Temperature is a measure of the amount of heat a substance contains and can be measured in degrees Fahrenheit or degrees Celsius (also known as degrees Centigrade). The metric unit for temperature is °Celsius (°C), which is based on water freezing at 0 °C and boiling at 100 °C. Notice that this range of temperature is conveniently divided into 100 units. In the Fahrenheit system water freezes at 32 °F and boils at 212 °F, thus dividing the same range of temperature into 180 units.

Here in the United States we are more familiar with temperatures expressed in degrees Fahrenheit, however the scientific community and much of the rest of the world measures temperatures in degrees Celsius. Thus it is especially important here in the United States to be able to convert from one temperature system to the other. There are two simple formulas to do so which are shown on the following page. Instead of simply taking for granted that the formulas will convert temperatures correctly, let’s take moment to see how they are derived. In so doing you will not only learn the basis of each formula, but you will never have to remember the formulas since you can derive them yourself!
Recall that for every 100 degrees Celsius there are 180 degrees Fahrenheit. Thus a degree Fahrenheit is smaller than a degree Celsius by a factor of 100/180 or \( \frac{5}{9} \). Conversely, a degree Celsius is larger than a degree Fahrenheit by a factor of 180/100 or \( \frac{9}{5} \). When converting from Fahrenheit to Celsius or vice versa you must also consider that 0°C is the same temperature as 32°F. Thus the Fahrenheit system has an extra 32 degrees which must be taken into account. Keeping these facts in mind, let’s see how to convert temperatures between the two systems.

**Converting Celsius to Fahrenheit**

Since each degree Celsius is equal to 9/5 degrees Fahrenheit, simply multiply the degrees Celsius by 9/5 and then add 32. Note that the extra 32 degrees are added only after you have converted to degrees Fahrenheit, producing the following formula:

\[
\left(\frac{9}{5} \times \degree C\right) + 32 = \degree F
\]

**Converting Fahrenheit to Celsius**

Since each degree Fahrenheit is equal to 5/9 degrees Celsius, simply subtract 32 from the degrees Fahrenheit and then multiply by 5/9. Note that the extra 32 degrees are subtracted while still in degrees Fahrenheit, i.e., before you convert to degrees Celsius. This produces the following formula:

\[
\left(\degree F - 32\right) \times \frac{5}{9} = \degree C
\]

**Exercise 4 – Measuring and converting temperature**

1. Use the thermometer at your table to measure the following in degrees Celsius:
   - the ambient temperature of the lab
   - a bucket of ice water
   - a beaker of boiling water

2. Convert the temperatures on your worksheet from Celsius to Fahrenheit or vice versa.

**Converting Units in the Metric System**

In science, numerical values are commonly represented using **scientific notation**. Scientific notation is a standardized form of **exponential notation** in which all values are represented by a number between 1 and 10 times 10 to some power. For example, 3500 in scientific notation would be 3.5 \( \times \) 10\(^3\), and 0.0035 would be 3.5 \( \times \) 10\(^{-3}\). Scientific notation is much more practical when dealing with extremely large or small values. For example, consider the masses of the earth and a hydrogen atom:

- **mass of the earth**: \( 5.97 \times 10^{27} \) grammes = \( 5,970,000,000,000,000,000,000,000,000,000,000 \) grammes
- **mass of a hydrogen atom**: \( 1.66 \times 10^{-24} \) grammes = \( 0.00000000000000000000000166 \) grammes
In these examples scientific notation is clearly much more practical and also easier to comprehend. Instead of counting all those zeroes or decimal places, the factors of 10 associated with each value are clearly indicated in the exponent. Scientific notation is especially useful in the metric system since the various metric units represent different factors of 10. Therefore it is important to be able to convert between scientific and decimal notation as outlined below:

**Converting from decimal notation to scientific notation**

**STEP 1** – Convert the number to a value between 1 and 10 by moving the decimal point to the right of the 1st non-zero digit:

- e.g. 0.00105 OR 1,050
- \( 10.5 \)

**STEP 2** – Multiply by a power of 10 (i.e., \( 10^n \)) to compensate for moving the decimal:

- the power will equal the number of places you moved the decimal
- the exponent is negative (-) if the original number is less than 1, and positive (+) if the original number is greater than 1

- e.g. 0.00105 = 1.05 \( \times 10^{-3} \)
- 1,050 = 1.05 \( \times 10^3 \)

**Some things to remember about the conventions of writing numbers in decimal notation:**

- if there is no decimal in the number, it is after the last digit (e.g., 1,050 = 1050.0)
- zeroes after the last non-zero digit to the right of the decimal can be dropped (e.g., 1.050 = 1.05)
- all simple numbers less than 1 are written with a zero to the left of the decimal (e.g., .105 = 0.105)

**Exercise 5 – Converting decimal notation to exponential notation**

1. Complete the conversions of simple numbers to exponential numbers on your worksheet.

**Converting from exponential notation to decimal notation**

You simply move the decimal a number of places equal to the exponent. If the exponent is negative, the number is less than one and the decimal is moved to the left:

\[ 1.05 \times 10^{-3} = 0.00105 \]

If the exponent is positive, the number is greater than one and the decimal is moved to the right:

\[ 1.05 \times 10^3 = 1,050 \]
Exercise 6 – Converting exponential notation to decimal notation

1. Complete the conversions of exponential numbers to simple numbers on your worksheet.

Converting Units within the Metric System

Converting from one metric unit to another is simply a matter of changing a value by the appropriate factor of 10. With decimal numbers that means simply moving the decimal one place for every factor of 10 – to the right to increase the value or to the left to decrease. With exponential numbers you simply increase or decrease the exponent by 1 for every factor of 10. In other words, all you really need to figure out is A) whether the value should increase or decrease and B) by what factor of 10 to do so.

Although this seems fairly straightforward, many students struggle with such conversions and rely on various formulas to do them. This will work just fine, but oftentimes students end up with an answer that is wildly off the mark due to moving a decimal in the wrong direction or changing the exponent in the wrong way. One should immediately realize there is a problem with such errors, however when the process is divorced from intuition and common sense it is easy to make such a mistake and think nothing of it. So instead of focusing on a formula to follow, let’s approach this by appealing to common sense and common experience. Once your common sense has been engaged, converting from one metric unit to another should be easier than it seems.

When dealing with the metric system we usually talk about grams, meters and liters, however the metric system can be just as easily applied to something we are all familiar with and hold dear – money. The basic unit of money in this country is the dollar, with the smallest unit being the penny. We all know that 100 pennies equals a dollar, so a penny is clearly 1/100 th of a dollar, or in metric terms, a centidollar \((10^{-2} \text{ dollars})\). We also know that 10 dimes equals 1 dollar, so a dime is actually a decidollar \((10^{-1} \text{ dollars})\). And if you’re fortunate enough to have 1000 dollars in the bank, you are the proud owner of a kilodollar \((10^{3} \text{ dollars})\). With this in mind, let’s do some conversions within this system.

If asked, “How many pennies is equal to ten dollars?” you should have little problem figuring this out. Since one dollar is equal to 100 pennies and there are 10 dollars total, it should be clear that 10 dollars is equal to 100 x 10 or 1000 pennies. Now let’s consider the same problem in metric terms:

\[
10 \text{ dollars} = _______ \text{ centidollars}
\]

Seeing the problem in this form may make the answer less intuitive. Nevertheless it is the same problem to which you can apply the same common sense – there should be 100 times more centidollars (pennies) than dollars. You should also realize that this is true for not only this problem, but for any problem involving dollars and centidollars. Let’s now consider the reverse in which you convert pennies (centidollars) to dollars:

\[
1483 \text{ centidollars} = _______ \text{ dollars}
\]

You know the answer immediately, right? The answer is 14.83 dollars since every 100 pennies (centidollars) is equal to one dollar. You intuitively knew to decrease 1483 by a factor of 100 to arrive
at the correct answer since there should be more of the smaller unit (pennies or centidollars) and less of the larger unit (dollars). This is all there is to metric conversions, realizing if the value should increase or decrease and by what factor of 10 to do so. Hopefully this has shown you that you already know how to do metric conversions intuitively, you just need to think of them in terms that are familiar to you. Let’s now try one that’s a bit more challenging:

500 decidollars = _______ kilodollars

In this problem we are actually converting dimes to thousands of dollars. This may take a little more thinking, however you can probably figure out that a decidollar (dime) is 10,000 times smaller than a kilodollar (10 dimes per dollar x 1000 dollars per kilodollar). It should be clear now that 500 decidollars is equal to 10,000 times less kilodollars, thus you simply move the decimal 4 places to the left to arrive at the correct answer of 0.05 kilodollars. You can use a calculator if you like, but it really is not necessary since there is essentially no calculating involved.

Next we will do several sample problems involving more traditional metric units to show you how simple and straightforward this can be as long as you are familiar with the metric prefixes which are reproduced for you in the box below. Refer to this as needed and you should have little trouble.

<table>
<thead>
<tr>
<th>BASIC UNIT</th>
<th>Mega (M)</th>
<th>Kilo (k)</th>
<th>No prefix</th>
<th>Deci (d)</th>
<th>Centi (c)</th>
<th>Milli (m)</th>
<th>Micro (µ)</th>
<th>Nano (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^6$</td>
<td>$10^3$</td>
<td>$10^0$</td>
<td>$10^{-1}$</td>
<td>$10^{-2}$</td>
<td>$10^{-3}$</td>
<td>$10^{-6}$</td>
<td>$10^{-9}$</td>
</tr>
</tbody>
</table>

Before we do a few more sample problems, it is important to realize that this is really a two step process in which is outlined on the next page.

1. **Identify whether your value should increase or decrease.**

2. **Determine the factor of 10 by which the value should change.**

Once you have completed these two steps it’s simply a matter of adjusting your decimal or exponent accordingly.

As you realize from doing the problems involving money, there should always be a larger number of smaller units (e.g., pennies) and a smaller number of larger units (e.g., dollars). So the first step is easy. Let’s now do a problem similar to the ones you’ll find on your worksheet:
28 mg = _____ µg

You are converting larger mg (0.001 g) to smaller µg (0.000001 g), so clearly the value of 28 should increase. Since a µg is 1000 times smaller than a mg (0.001/0.000001 = 1000), the value of 28 should increase by a factor of 1000. Therefore:

\[ 28 \text{ mg} = 28 \times 1000 \text{ µg} = 28,000 \text{ µg} \]

If you prefer working with exponential numbers then the problem works out this way:

\[ 28 \text{ mg} = 28 \times 10^3 \text{ µg} = 2.8 \times 10^4 \text{ µg} \]

Let’s try one more problem which is presented in a slightly different way:

How many kilometers (km) is 3.7 centimeters (cm)?

This question can be expressed in the following problem:

\[ 3.7 \text{ cm} = _____ \text{ km} \]

In this problem, km (10³ m) are clearly larger than cm (10⁻² m) so the value of 3.7 should decrease. A cm is \(10^5\) times larger than a cm \((10^3/10^{-2} = 10^5)\), so the value of 3.7 should decrease by a factor of \(10^5\). Therefore:

\[ 3.7 \text{ cm} = 3.7 \times 10^0 \text{ cm} = 3.7 \times 10^{-5} \text{ km} \]

In this problem it is more practical to use exponential notation, however the original value was in decimal form. Converting 3.7 to an exponential number \((3.7 \times 10^0)\) makes it easy to reduce the value by a factor of \(10^5\) by subtracting 5 from the exponent.

While this approach to making metric conversions is meant to engage your common sense, you may prefer a more traditional method. This involves multiplying the original metric value by a ratio of metric units equal to one that, due to cancellation, leaves you with an equivalent value in the desired units. To illustrate how this works, let’s consider the first problem on the previous page:

\[ 28 \text{ mg} = _____ \text{ µg} \]

In this problem you want to convert the units from mg to µg, so multiply 28 mg by a ratio equal to one that will cancel the mg and leave you with µg:

\[ 28 \text{ mg} \times 1000 \text{ µg/mg} = 28,000 \text{ µg} \]
Since 1000 µg equals 1 mg, a ratio of 1000 µg/mg is equal to one. The key to this method is determining how many of the new units (µg) equal one of the original units (mg). Once this is determined simply put the original unit on the bottom (to allow its cancellation) and the equivalent value in the desired units on top.

Let’s do one more example to make sure this is clear:

\[
3.7 \text{ cm} = \text{ _____ km} \\
3.7 \text{ cm} \times 10^{-5} \text{ km/cm} = 3.7 \times 10^{-5} \text{ km}
\]

Now that you see how to solve these conversion problems you are ready to complete the final exercise for this lab.

**Exercise 7 – Metric conversions**

1. Complete the metric conversions on your worksheet.

---

**Working with Concentration**

**Units of Concentration**

It is essential in biology to understand the concept of concentration – the amount of a substance per unit volume. The concentration of a substance can be expressed with a variety of units:

- Molarity (moles per liter)
- Mass concentrations (e.g., mg/ml)
- Percent solutions (g/100 ml)
- “X” concentrations (relative to the desired final concentration – e.g. 10X)

You will use all these units of concentration throughout the course so it is important that you are familiar with them and can use them in calculations.

**Calculations Involving Concentration**

Many biological experiments involve the use of concentrated stock solutions that need to be diluted to the correct concentration in a solution or biological reaction. A simple way to calculate the amount of concentrated stock solution to be added to a solution or reaction is to use the following formula:

\[
\text{volume needed} = \left(\frac{\text{final concentration}}{\text{stock concentration}}\right) \times \text{total volume}
\]
For example, if you have a 10X stock solution of something you want to add to a 50 µl total reaction volume such that its final concentration is 1X, you would need to add 5 µl of the stock solution to the reaction:

\[
\frac{1X}{10X} \times 50 \mu l = 5 \mu l
\]

In the next example the units are different, however the calculation is much the same. Assume you have a 500 mM stock solution of NaCl which you will use to make a 1 liter solution containing 10 mM NaCl. The amount of stock NaCl solution you will need to add is:

\[
\frac{10 \text{ mM}}{500 \text{ mM}} \times 1 \text{ liter} = 0.02 \text{ liters or 20 ml}
\]

In the following exercise you will practice making such calculations...

**Exercise 8 – Calculations involving concentration**

1. Complete the concentration problems on your worksheet.

**Making Solutions**

Another essential skill in the lab is making solutions. You should have already learned how to do this in your chemistry courses, nevertheless it will be good practice to make some solutions in this lab.

**Exercise 9 – Making solutions**

1. Make 1 liter of 1X TAE using a 50X TAE stock solution. The materials you will need can be found at the side of the lab near the window.

2. Make 50 ml of physiological saline solution which is 154 mM or 0.9%. The materials you will need can be found at the side of the lab near the window.

**Graphing**

The previous lab (Morgan/Carter #1) addressed the graphing of data, however students typically need additional practice. Refer to the graphing guidelines in the previous lab to ensure you graph the data below properly.

**Exercise 10 – Graphing practice**

1. On your worksheet, use the grids provided to graph the three sets of data provided.
Metric System Lab Worksheet

**Exercise 1 – Measurement of distance**

Laboratory width: ________ m  
Laboratory length: ________ m  
Calculate approximate area: width _____ m  \( \times \)  length _____ m = ________ m\(^2\)  

Paper width: ________ cm  
Paper length: ________ cm  
Calculate approximate area: width _____ cm  \( \times \)  length _____ cm = ________ cm\(^2\)  

Paper hole diameter: ________ mm  
Your height: ________ cm, which is equal to ________ m

*Indicate which metric unit of length you would use to measure the following:*

- length of a fork: ________
- width of a plant cell: ________
- size of a small pea: ________
- length of your car: ________
- height of a refrigerator: ________
- distance to the beach: ________
- diameter of an apple: ________

**Exercise 2A – Measurement of volume**

Volume in Flask A = ________ ml  
Volume in Test Tube B = ________ ml

**Exercise 3 – Measurement of mass**

Mass of Graduated Cylinder = ________ g  
Mass of Graduated Cylinder with 50 ml of water = ________ g  

Mass of 50 ml of water *(difference of the two numbers above)*: ________ g  

Difference between your measurement and actual weight of 50 ml of water (50 g): ________  

Mass of Large Paper Clip = ________ g
Exercise 4 – Measurement of temperature

Ambient temperature in lab _____ °C

ice water _____ °C

boiling water _____ °C

Convert the following temperatures using the formulas on page 10 of the lab exercises:

Mild temperature: 68°F = _______ °C

Body temperature 98.6°F = _______ °C

Cold day 8°C = _______ °F

Very hot day 40°C = _______ °F

Exercise 5 – Converting from decimal notation to exponential notation

Convert the following decimal numbers to exponential numbers:

243,000 = ____________  ____________ = 0.096

68.3 = ____________  ____________ = 0.00055

803.05 = ____________  ____________ = 0.0000019

Exercise 6 – Converting from exponential notation to decimal notation

Convert the following exponential numbers to decimal numbers:

2.7 x 10⁴ = ____________  ____________ = 10⁷

1.08 x 10⁶ = ____________  ____________ = 4.562 x 10⁻³

4.0103 x 10⁻² = ____________  ____________ = 3 x 10⁵

Exercise 7 – Metric conversions

Convert the following measurements to the indicated unit:

335.9 g = _________________ mg

_______________________ m = 0.00886 km

0.0939 μl = _________________ ml

_______________________ kg = 894 mg

45.82 ng = _________________ μg

_______________________ dl = 90.5 cl

20 kilotons = __________ megatons

_______________________ μm = 0.037 mm

12 megabase pairs (mbp) = __________ kbp

_______________________ mm = 110.5 nm

2.5 mg = _________________ g

_______________________ μl = 0.0046 L
Exercise 8 – Calculations involving concentration

You are putting together a reaction in a total volume of 30 µl and stock solution A is 5 mM. What volume of stock solution do you need to add so that the final concentration of A in the reaction is 200 µM?

How many microliters of stock solution B do you need to add to a reaction if the stock concentration is 1 µM, the total volume of the reaction is 25 µl, and the final concentration of B needs to be 0.2 µM?

You are planning an enzymatic reaction and the buffer solution supplied with the enzyme is 5X.

a. How much of this 5X buffer will you add to the reaction if the total volume is 30 µl?

b. After adding what you thought was 5X buffer, you discover that it is actually a 10X buffer and there is none left so you can’t start over. How do you fix the problem so that you have a final concentration of 1X buffer in the reaction?

The enzyme you plan to use in a reaction is at a stock concentration of 10 units/µl. What volume of enzyme do you add if you want a total of 3 units of enzyme in the reaction?

You are responsible for preparing the 1X TAE needed for a Biology 6 laboratory session. The stock TAE solution is 50X and the class will be running a total of 8 samples, each requiring 500 ml of 1X TAE.

a. What total volume of 1X TAE will you need to make?

b. How much 50X TAE and how much water will you combine to make this volume of 1X TAE?
Exercise 9 – Making solutions

Have your instructor initial below to verify that your group has made the indicated solutions:

1X TAE _________    Physiological Saline _________

Exercise 10 – Graphing practice

Identify the independent and dependent variables for each data set, and graph each data set on the grids provided at the end of your worksheet:

1. **Average monthly high and low temperatures in downtown Los Angeles from 1981 to 2010**

<table>
<thead>
<tr>
<th></th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sep</th>
<th>Oct</th>
<th>Nov</th>
<th>Dec</th>
</tr>
</thead>
<tbody>
<tr>
<td>average high (°F)</td>
<td>69</td>
<td>69</td>
<td>71</td>
<td>73</td>
<td>75</td>
<td>79</td>
<td>84</td>
<td>85</td>
<td>84</td>
<td>79</td>
<td>73</td>
<td>68</td>
</tr>
<tr>
<td>average low (°F)</td>
<td>50</td>
<td>51</td>
<td>53</td>
<td>55</td>
<td>59</td>
<td>62</td>
<td>65</td>
<td>66</td>
<td>65</td>
<td>60</td>
<td>54</td>
<td>49</td>
</tr>
</tbody>
</table>

Independent variable:_________________ Dependent variable:_________________

2. **Average distance from sun and temperature data for the planets of the solar system**

<table>
<thead>
<tr>
<th></th>
<th>Mercury</th>
<th>Venus</th>
<th>Earth</th>
<th>Mars</th>
<th>Jupiter</th>
<th>Saturn</th>
<th>Uranus</th>
<th>Neptune</th>
</tr>
</thead>
<tbody>
<tr>
<td>average distance from sun (millions of miles)</td>
<td>36</td>
<td>67</td>
<td>93</td>
<td>142</td>
<td>484</td>
<td>887</td>
<td>1784</td>
<td>2794</td>
</tr>
<tr>
<td>average surface temperature (°C)</td>
<td>179</td>
<td>453</td>
<td>12</td>
<td>-43</td>
<td>-153</td>
<td>-185</td>
<td>-214</td>
<td>-225</td>
</tr>
</tbody>
</table>

Independent variable:_________________ Dependent variable:_________________

3. **National per capita CO₂ emissions for 2010**

<table>
<thead>
<tr>
<th></th>
<th>Brazil</th>
<th>Canada</th>
<th>China</th>
<th>France</th>
<th>India</th>
<th>Japan</th>
<th>Mexico</th>
<th>Iran</th>
<th>Russia</th>
<th>U.S.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂ emissions per person (metric tons)</td>
<td>2.2</td>
<td>14.9</td>
<td>6.2</td>
<td>5.5</td>
<td>1.7</td>
<td>8.9</td>
<td>4.1</td>
<td>7.6</td>
<td>11.8</td>
<td>17.6</td>
</tr>
</tbody>
</table>

Independent variable:_________________ Dependent variable:_________________
Bio 6 – Gel Filtration Chromatography Lab

Adapted from the Bio-Rad Biotechnology Explorer Size Exclusion Chromatography Instruction Manual

Introduction to Chromatography

Chromatography is commonly used in biotechnology for purifying biological molecules, like proteins, or medicine or other uses. Chromatography allows the separation of individual components from complex mixtures. Chromatography consists of a mobile phase (solvent and the molecules to be separated) and a stationary phase either paper (in paper chromatography), or glass beads, called resin (in column chromatography), through which the mobile phase travels. Molecules travel through the stationary phase at different rates because of their chemistry.

Some Common Types of Chromatography

In gel filtration chromatography, commonly referred to as size exclusion chromatography (SEC), microscopic beads which contain tiny holes are packed into a column. When a mixture of molecules is dissolved in a liquid and then applied to a chromatography column that contains porous beads, large molecules pass quickly around the beads, whereas smaller molecules enter the tiny holes in the beads and pass through the column more slowly. Depending on the molecules, proteins may be separated, based on their size alone, and fractions containing the isolated proteins can be collected.

In affinity chromatography, a biomolecule (often an antibody) that will bind to the protein to be purified is attached to the beads. A mixture of proteins is added to the column and everything passes through except the protein of interest, which binds to the antibody and is retained on the solid support. To get the protein to elute from the column, another buffer is used to disrupt the bond between the protein of interest and the antibody. Often this elution buffer contains high concentrations of salt or acid.

In ion exchange chromatography, the glass beads of the column have a charge on them (either + or -). A mixture of protein is added to the column and everything passes through except the protein of interest. This is because the beads are picked to have the opposite charge of the protein of interest. If the charge of the beads is positive, it will bind negatively charged molecules. This technique is called anion exchange. If the beads are negatively charged, they bind positively charged molecules (cation exchange). Thus, a scientist picks the resin to be used based on the properties of the protein of interest. During the chromatography, the protein binds to the oppositely charged beads. When the contaminant is separated from the protein of interest, a high salt buffer is used to get the desired protein to elute from the column.

Principles of Gel Filtration Chromatography

In this laboratory you will investigate the principles of gel filtration chromatography. Gel filtration chromatography is a very powerful technique for the physical separation of molecules on the basis of size. This technique can be used to estimate the molecular weight of a molecule or to purify a molecule of interest. In this procedure, a mixture of molecules dissolved in liquid (the mobile phase) is applied to a chromatography column which contains a solid support responsible for separation (the matrix). This is the stationary phase. In this case, the matrix consists of microscopic porous spheres, or “beads” that rest on a porous disk (the frit) that sits at the bottom of the column. The mass of beads within the column is often referred to as the column bed. The beads act as “traps” or “sieves” and function to filter small molecules which become temporarily trapped within the pores. Larger molecules pass around or are “excluded” from the beads. There are a variety of different types of matrices used in gel filtration chromatography, each capable of separating a range of molecular weights, its fractionation range. The column you will
use is prefilled with beads that effectively separate or “fractionate” molecules between 3,000 and 60,000 daltons. As the liquid flows through the column, molecules below 60,000 daltons enter the beads and pass through the column more slowly. The smaller the molecule, the more time they spend in the beads and the slower they move through the column. Molecules less than 3,000 daltons will not separate effectively since they will all equally penetrate the beads. The shape of a molecule also influences how quickly it moves through the column. Molecules with a more compact shape (like a sphere) will enter the beads more effectively than molecules that are elongated. Thus, more compact molecules will pass through the column more slowly than elongated ones. In the column you will use today, molecules greater than 60,000 pass around the beads and are excluded from the column. Thus, 60,000 daltons is the exclusion limit of this column. The total volume between the beads is known as the void volume.

The liquid used to dissolve the biomolecules to make the mobile phase is called a buffer. The mixture of biomolecules dissolved in the buffer is called the sample. The sample is placed on the column bed and the biomolecules within the buffer enter the top of the column bed, filter through and around the porous beads, and ultimately pass through a small opening at the bottom of the column. For this process to be completed, additional buffer (elution buffer) is placed on the column bed after the sample has entered the bed. The mobile phase (liquid) is collected as drops into a series of collection tubes. A set number of drops is collected into each tube. The larger molecules which pass quickly through the column will end up in the early tubes or “fractions”. The smaller molecules which penetrate the pores of the stationary phase end up in the later fractions. The schematic below illustrates the differential fractionation of large and small molecules on a gel filtration column.
The Sample

Hemoglobin and Vitamin B12 are the two biomolecules of differing sizes in your sample. You will use gel chromatography to separate these two molecules determine which is larger. You will be able to trace these two molecules throughout the separation process based on their color; hemoglobin is brown whereas Vitamin B12 is pink.

Hemoglobin

Hemoglobin, a protein found in red blood cells, functions to transport oxygen to the tissues of the body. The hemoglobin used in this experiment is bovine hemoglobin. The use of bovine hemoglobin (rather than the human counterpart) avoids the potential health hazard presented when using human blood products. Hemoglobin is made up of four polypeptides (small proteins) which associate to form a large, globular protein. Hemoglobin gets its name from the heme group, the iron-containing component of hemoglobin which physically binds oxygen. The iron-containing heme group is responsible for the red-brown color of hemoglobin. The closely related protein, myoglobin, is found in muscle and is responsible for delivering oxygen to muscle tissue. Muscles which are very active and require a lot of oxygen are dark in color because of a high myoglobin content. An example would be the red-brown color of the dark meat of chicken.

Hemoglobin is the main component of red blood cells (RBCs), the oxygen carrying cells of the body. Again, it is the heme group of hemoglobin which gives RBCs their distinctive red of hemoglobin which gives RBCs their distinctive red color. Different forms of hemoglobin are produced during different stages of development. Fetuses produce a form of hemoglobin which has a higher affinity (tighter binding) for oxygen than does adult hemoglobin. Because fetuses depend upon their mothers for their oxygen supply, it is important that maternal hemoglobin can easily give up its oxygen to the fetal hemoglobin. For this reason, obstetricians advise their patients to avoid vigorous exercise during pregnancy. Vigorous exercise depletes the tissues of oxygen, which sets up a competition between the transfer of oxygen to maternal tissues or to fetal hemoglobin.

In addition to oxygen, hemoglobin can also bind carbon monoxide. Hemoglobin actually has a higher affinity for carbon monoxide than for oxygen. Suffocation from carbon monoxide occurs when oxygen bound to hemoglobin is displaced by carbon monoxide, which in turn deprives body tissues of oxygen.

The body can adapt to environmental changes which require increased amounts of oxygen delivery to tissues. At high altitudes, where the amount of oxygen in air is decreased, the body responds by increasing the number of red blood cells produced. This effectively increases the number of molecules of hemoglobin in the blood supply, which has the effect of increasing the oxygen supply to the tissues. For this reason, athletes will train at high elevation to increase the amount of RBC’s, and thus increase their oxygen capacity, which is needed for rigorous exercise.

Sickle cell anemia is a molecular disease of hemoglobin. A single change or mutation in the gene which encodes hemoglobin results in a mutation in the amino acid sequence. This mutation changes the three-dimensional structure of the polypeptides of hemoglobin, causing them to “stick” together as rod-like structures. The abnormal rod-like hemoglobin molecules distort the structure of red blood cells, causing them to have a sickle shape. Unlike their round counterparts, the sickle-shaped RBC cannot freely pass through capillary beds, and thus the capillary beds become blocked. The blocked capillary beds of organs and tissues make delivery of oxygen difficult, resulting in extreme fatigue and even death. Because sickle-cell anemia is a genetic disorder which results from a mutated genetic sequence, at this time there is no cure. However, the side effects of sickle cell anemia can be alleviated by frequent blood transfusions from
people who have normal hemoglobin and red blood cells. Sickle cell anemia is a genetic disease in which
the individual has inherited a defective mutant hemoglobin gene from both parents. Individuals with the
sickle cell trait have received an abnormal gene from only one parent, and the single defect actually
confers an evolutionary advantage. In Africa, expression of the sickle cell gene positively correlates with
malaria infections. Malaria is a deadly disease caused by a mosquito-borne parasite. The parasite infects
and ultimately kills RBCs. The parasite can infect normal RBCs, but cannot infect sickle cell RBCs. Thus, the
sickle cell trait helps confer resistance to malaria and results in a positive evolutionary adaptation.
Unfortunately, expression of two copies of the gene is deleterious.

Vitamin B12

Vitamin B12 is a vitamin that is essential to humans and other vertebrates. Vitamin B12 is an essential
cofactor of several biochemical reactions which occur in the human body. One function of vitamin B12 is the
breakdown of fats. Sources rich in vitamin B12 include eggs, dairy products, and meats. Vitamin B12 is not
found in plants and vegetable foods. Thus, people who have strict vegetarian diets are often deficient in
vitamin B12, unless they take some supplementary vitamins.

Pure molecules of vitamin B12 cannot be absorbed by the intestines. Vitamin B12 must bind to a carrier
protein in the intestinal tract. When vitamin B12 binds to this carrier protein, the complex is able to pass
through the intestine and into the blood stream, where it is eventually taken up by the liver.

Because vitamin B12 is only required in minute quantities (humans require ~3 µg/day), vitamin B12
deficiencies are extremely rare. However, some individuals have a genetic disorder in which the gene that
codes for the carrier protein is mutated. Individuals with this mutation do not synthesize the carrier
protein necessary for absorption into the blood stream. Thus, even though these people have adequate
intakes of vitamin B12, they still show signs of deficiency because they lack the required carrier protein.

Workstation Check (✔️) List

Your workstation. Materials and supplies that should be present at
your workstation prior to beginning this lab are listed below.

<table>
<thead>
<tr>
<th>Student workstation items</th>
<th>Number required</th>
<th>(✔️)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collection tubes</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Sizing chromatography columns</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Column end-caps</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pipette</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Lab marker</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Test tube rack</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Instructor workstation items</th>
<th>Number required</th>
<th>(✔️)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein mixture</td>
<td>1 vial</td>
<td></td>
</tr>
<tr>
<td>Column buffer</td>
<td>1 bottle</td>
<td></td>
</tr>
</tbody>
</table>
Laboratory Protocol

1. Place the 12 collection tubes in your test tube rack. Label 10 collection tubes sequentially from 1 to 10. Label the last two tubes “waste” and “column buffer”. Label either the tubes or the rack with your name and laboratory period.

2. Pipet 4 ml of Column Buffer into the tube labeled column buffer.

3. Remove the cap and snap off (do not twist) the tab at the end of the Poly-Prep sizing column. Drain all of the buffer into the “waste” collection tube. Cap the bottom of the column with the column end cap.

4. Gently place the column onto collection tube 1 (do not jam the column tightly into the collection tubes—the column will not flow). You are now ready to load the protein sample onto the column.

5. Remove the end cap from the column. Observe the top of the column bed; all of the buffer should have drained from the column. This is best observed by looking directly over the column—the “grainy” appearance of the column beads should be visible. Carefully load one drop of protein mix onto the top of the column bed. The pipette should be inserted into the column and the drop should be loaded just above the top of the column so that it minimally disturbs the column bed.
6. Allow the protein mix to enter the column bed. This is best observed by looking directly over the column. Carefully add 250 µl of column buffer to the top of the column. This is best done by inserting the pipet tip into the column so that it rests just above the column bed. Carefully let the buffer run down the side of the tube and onto the top of the bed. Begin to collect drops into tube 1.

![Column buffer](image1)

7. When all of the liquid has drained from the column, add another 250 µl of column buffer to the top of the column. Add the buffer as before, by placing the pipette just above the top of the column and letting the buffer run down the side of the tube. Continue to collect drops into tube 1.

![Column buffer](image2)

8. When all of the excess liquid has drained from the column, add 3 ml of column buffer to the top of the column. This can be done by adding 1 ml from the pipette three times. At this time the protein mix has entered the column far enough so that slight disturbances to the column bed will not affect the separation. Transfer the column to tube 2 and begin to count the drops that enter into each tube. Collect 5 drops of buffer into tube 2.

![Column buffer](image3)
9. When 5 drops have been collected into tube 2, transfer the column onto tube 3. Collect 5 drops of buffer into each collection tube. When 5 drops have been collected into a tube, lift it off and transfer it to the next tube.

10. Continue collecting 5 drops into each tube. When you reach tube 10, collect a final 10 drops. Cap the column when finished collecting drops. Store your samples and column according to your teachers’ instructions.

11. **Results** - In your lab notebook, create a table listing the color and relative intensity of each fraction, *for each color, brown and pink*. To do so, score the intensity of the darkest faction of a particular color as a “5” and a completely colorless faction as “0”. Estimate the relative intensity of the remaining fractions relative to these two samples.

12. **Graph your data** – In the results section of your lab notebook, create a graph of the relative color intensity (dependent variable) vs fraction number for both hemoglobin and Vitamin B12.
Gel Chromatography Study Questions

1. Examine the 10 fractions that you collected. Which tube contains the “peak” fraction for hemoglobin and vitamin B12? The peak fractions contain the highest concentration of protein/vitamin and will be the most intense in color.

2. Which molecule, hemoglobin or vitamin B12, exited the column first? Would this molecule be the larger or smaller of the two?

3. In one sentence, describe how gel filtration chromatography works to separate molecules.

4. Outline the basic steps involved in setting up and running a gel filtration column.

5. Define the following terms:
   - Stationary phase (matrix)
   - Frit
   - Elution buffer
   - “packing the column”
   - Void volume
6. Why do larger molecular weight molecules elute from the column faster than smaller molecular weight molecules?

7. What is meant by the “stationary phase” and the “mobile phase” when discussing chromatography?

8. How does the shape of a molecule influence how quickly it will elute?

9. What is the fractionation range of a particular gel?

10. Think about the color of the protein mix that was applied to the column—why was the mixture a reddish-brown color?

11. Why do you think the column needed to be “dry” (the absence of buffer on the top of the column bed) when the protein mix was loaded?

12. Why did you need to add more buffer after the protein mixture was loaded onto the column (at step 7 and 8 of the protocol)?
13. If the following mix of molecules were purified using size exclusion chromatography, what would be the order in which the molecules pass through the opening in the bottom of the column? Mixture containing: hemoglobin, 65,000 daltons; myoglobin, 17,000 daltons; myosin, 180,000 daltons.

First molecule to appear:

Second molecule to appear:

Third molecule to appear:
Bio 6 – Macromolecules Lab

Overview

In addition to water and minerals, living things contain a variety of organic molecules. Most of the organic molecules in living organisms are of 4 basic types: carbohydrate, protein, lipid and nucleic acid. Many of these molecules are long polymers, and thus collectively referred to as macromolecules. In this laboratory you will learn how organic molecules are put together, with an emphasis on proteins and carbohydrates. In addition, you will learn several methods of detecting carbohydrates and proteins in complex samples such as foods.

Part 1: BUILDING MACROMOLECULES

The Structure of Organic Molecules

All living things consist of both organic and inorganic molecules. Organic molecules contain the elements carbon (C) and hydrogen (H), and more specifically, carbon–hydrogen bonds. Molecules lacking C–H bonds are considered to be inorganic. For example, oxygen gas (O₂), water (H₂O) and carbon dioxide (CO₂), despite their obvious importance for life, are inorganic molecules. Methane (CH₄), ethanol (C₂H₆O) and glucose (C₆H₁₂O₆) on the other hand, are all organic. In general, organic molecules are derived from living organisms, hence the association of the word organic with natural, living things.

It is helpful to think of organic molecules as skeletal carbon structures (carbon skeletons) to which various chemical groups are attached. To illustrate this, let’s take a look at the structure of a simple organic molecule that we are all familiar with, ethanol:

\[
\begin{align*}
H & \quad H \\
H-C-C-O-H & \quad H \quad H \\
\text{ethanol} & 
\end{align*}
\]

Notice that a molecule of ethanol contains a core carbon structure or skeleton consisting of 2 carbon atoms connected to each other by a single covalent bond. The remaining unpaired electrons in the carbon atoms are involved in covalent bonds with individual hydrogen atoms or a hydrogen-oxygen combination known as a hydroxyl group. The single hydrogen atoms and the hydroxyl group are examples of common functional groups (though a hydrogen atom technically is not a “group”) that are attached via covalent bonds to carbon skeletons. Let’s look at another slightly larger organic molecule, the simple sugar glucose:
If you look carefully you’ll notice that the glucose molecule above (shown in both its linear and ring forms) is simply a 6-carbon skeleton to which numerous hydrogens and hydroxyl groups are attached (as well as a double-bonded oxygen).

Hydrogen atoms and hydroxyl groups are by no means the only functional groups found in organic molecules, so let’s get acquainted some other common functional groups in addition to these two and take note of their chemical properties when bound to a carbon framework:

<table>
<thead>
<tr>
<th>functional group</th>
<th>molecular formula</th>
<th>property (at pH 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hydrogen</td>
<td>*R–H</td>
<td>non-polar</td>
</tr>
<tr>
<td>hydroxyl</td>
<td>R–OH</td>
<td>polar</td>
</tr>
<tr>
<td>methyl</td>
<td>R–CH₃</td>
<td>non-polar</td>
</tr>
<tr>
<td>amino</td>
<td>R–NH₂</td>
<td>basic (binds H⁺)</td>
</tr>
<tr>
<td>carboxyl</td>
<td>R–COOH</td>
<td>acidic (releases H⁺)</td>
</tr>
<tr>
<td>aldehyde</td>
<td>R–CHO</td>
<td>slightly polar</td>
</tr>
<tr>
<td>ketone</td>
<td>R–C–R</td>
<td>slightly polar</td>
</tr>
</tbody>
</table>

*R = unspecified organic side chain

Exercise 1A – Constructing functional groups

Diagram the structural formulas for the functional groups shown above on your worksheet and then build them with your molecular model kit. In your structural formulas, represent the bond that will connect to an R-group as a line sticking out from your function group. In your models, each functional group should have a covalent bond connector that is not connected to anything on one side to indicate where the R-group is attached. Keep in mind that if your structure is correct there should be no atoms with unfilled holes. Once all structures are complete, show them to your instructor and save the hydrogen, amino and carboxyl groups for the next exercise. Below is a key to the components of your kit:

- WHITE = hydrogen atom
- BLACK = carbon atom
- RED = oxygen atom
- BLUE = nitrogen atom

*short stiff connectors (use for single covalent bonds)*
*long flexible connectors (use for each bond in double & triple covalent bonds)*
Monomers and Polymers

The organic molecules we classify as carbohydrates, proteins, lipids and nucleic acids include single unit monomers (one unit molecules) as well as chains of monomers called polymers (many unit molecules). Terms like dimer (two unit molecule) and trimer (three unit molecule) are also used. For example, carbohydrates can be monomers (such as glucose and fructose), dimers (such as sucrose and lactose), and polymers (such as starch and glycogen). For carbohydrates, such molecules are more specifically referred to as monosaccharides, disaccharides, and polysaccharides (saccharide is Greek for “sugar”).

![Sucrose](image1.png)

**sucrose**  
(a disaccharide)

![Starch](image2.png)

**starch**  
(a polysaccharide)

Proteins are another important type of biological polymer. The monomers from which proteins are assembled are amino acids. In a moment you will use some of the functional groups you just made to construct an amino acid. The general structure of an amino acid is shown below:

![Amino Acid](image3.png)

Notice that there is a central carbon atom (essentially a carbon skeleton consisting of one carbon atom) connected via covalent bonds to the following:

- a hydrogen atom
- an amino group (–NH₂)
- a carboxyl group (–COOH)
- a variable “R-group”
All amino acids have in common the first 3 functional groups: the hydrogen, amino and carboxyl groups. Proteins are constructed from up to 20 different amino acids, and the R-group is different for each giving each amino acid its unique properties. Let’s examine the R-groups (highlighted in green) of ten different amino acids, after which you will assemble one of these amino acid using a molecular model kit:

**glycine**  
\[
\begin{align*}
\text{H}_2\text{N} &- \text{C} - \text{COOH} \\
\text{H} &
\end{align*}
\]

**serine**  
\[
\begin{align*}
\text{H}_2\text{N} &- \text{C} - \text{COOH} \\
\text{CH}_2 &- \text{OH}
\end{align*}
\]

**alanine**  
\[
\begin{align*}
\text{H}_2\text{N} &- \text{C} - \text{COOH} \\
\text{CH}_3 &
\end{align*}
\]

**cysteine**  
\[
\begin{align*}
\text{H}_2\text{N} &- \text{C} - \text{COOH} \\
\text{CH}_2 &- \text{SH}
\end{align*}
\]

**proline**  
\[
\begin{align*}
\text{H}_2\text{N} &- \text{C} - \text{COOH} \\
\text{H} &- \text{C} - \text{C} - \text{CH}_2
\end{align*}
\]

**aspartic acid**  
\[
\begin{align*}
\text{H}_2\text{N} &- \text{C} - \text{COOH} \\
\text{CH}_2 &- \text{COOH}
\end{align*}
\]

**valine**  
\[
\begin{align*}
\text{H}_2\text{N} &- \text{C} - \text{COOH} \\
\text{H}_3\text{C} &- \text{CH}_3
\end{align*}
\]

**threonine**  
\[
\begin{align*}
\text{H}_2\text{N} &- \text{C} - \text{COOH} \\
\text{HC} &- \text{OH}
\end{align*}
\]

**methionine**  
\[
\begin{align*}
\text{H}_2\text{N} &- \text{C} - \text{COOH} \\
\text{CH}_2 &- \text{CH}_2
\end{align*}
\]

**asparagine**  
\[
\begin{align*}
\text{H}_2\text{N} &- \text{C} - \text{COOH} \\
\text{CH}_2 &- \text{CO} \text{NH}_2
\end{align*}
\]

**NOTE:** The structures above are partial structural formulas. You will need to determine the complete structural formula for each amino acid you will build.
Exercise 1B – Building an amino acid

1. On your worksheet, diagram the complete structural formula for the amino acid your instructor has assigned you to build.

   NOTE: The diagrams shown on the previous page are partial structural formulas to help guide you in determining the complete structural formula (with all covalent bonds shown).

2. Identify and label the indicated components of your structural formula (see worksheet).

3. Build the amino acid with your molecular model kit as follows:
   a) to a single carbon atom, attach the following functional groups you’ve already made:
      - a hydrogen atom
      - an amino group
      - a carboxyl group
   b) construct the “R” group for your amino acid separately
   c) attach your “R” group to the remaining bond in your central carbon atom

4. Show your amino acid model to your instructor to verify its structure.

5. Find someone at another table who has built the same amino acid as you and superimpose them to see if you have built the same enantiomer.

Assembling and Breaking Down Polymers

Living organisms such as yourself are continuously building polymers and breaking them down into monomers. For example, when you eat a meal you ingest large amounts of polymers (proteins, starch, triglycerides) which are subsequently broken down into monomers (amino acids, glucose, fatty acids) within your digestive system. Within your cells there is a continuous cycle of building new protein, carbohydrate, lipid and nucleic acid polymers, and breaking down “old” polymers into their respective monomers (amino acids, sugars, fatty acids, nucleotides).

There is a common theme to the building and breaking down of biological polymers. Whenever a monomer is added to a growing polymer, a molecule of water (H₂O) is released in a process called condensation or dehydration. For example, when two amino acids are joined in a growing polypeptide, the –OH of the carboxyl group of the first amino acid will combine with an H from the amino group of the second amino acid. This results in the simultaneous formation of a covalent bond between the two amino acids, a peptide bond, and the joining of –OH and H to form a water molecule as shown on the next page.
**Exercise 1C – Assembling a polypeptide**

You will work with other members of your group to assemble a small polypeptide containing each of the amino acids your group has just built. Assemble the polypeptide in alphabetical order (e.g., alanine before glycine), and the amino acid you have built will be added at the appropriate position. To reproduce how polypeptides are actually assembled in living cells, the polypeptide should be assembled as follows (AA = amino acid):

1. Position AA1 and AA2 so that the –COOH of AA1 is next to the –NH₂ of AA2.
2. Remove the –OH from the –COOH of AA1, and an H from the –NH₂ of AA2.
3. Combine the –OH and H to form H₂O and set it aside (you will use it in Exercise 1D).
4. Connect the carbon from the original –COOH in AA1 to the amino group of AA2.
5. Repeat steps 1 to 4 with each additional amino acid until the polypeptide is assembled.

- Show the polypeptide you have built to your instructor to verify its structure.
- Diagram the structural formula of your polypeptide on your worksheet.

*NOTE: Be sure to save your water molecules for the next exercise.*
The breakdown of a polymer into monomers essentially reverses the process of its assembly. Whenever a monomer is removed from a polymer, a water molecule must be inserted in a process called **hydrolysis**. This is illustrated below for the breakdown of a dipeptide into individual amino acids:

**Exercise 1D – Breaking down a polypeptide**

*Break down the polypeptide your group has just assembled as follows:*

1. Break the peptide bond between the last 2 AAs in your polypeptide.

2. Use a water molecule (from exercise 1C) to restore the H on the amino group of AA just removed and the –OH on carboxyl group of the other AA.

3. Repeat steps 1 and 2 for each successive peptide bond until the polypeptide is completely broken down into its original amino acid monomers.
Part 2: DETECTING MACROMOLECULES

In the exercises to follow, you will test various food items for the presence of simple sugars, starch and protein using chemical reagents specific for each. When doing such tests it is always important to include control reactions. As you learned in the first lab, a control experiment is one in which the independent variable (e.g., the source of sugar, starch or protein in test samples) is “zero” or some background level. For example, if you are testing for starch you want to be sure to include a sample that you know does NOT contain starch. The perfect negative control for this and other such experiments is plain water since it does not contain starch or anything else. This sort of control is referred to as a negative control since it is negative for what you are trying to detect. The importance of performing a negative control is two-fold:

1) To verify that a negative sample actually gives a negative result with the reagents you are using.

2) To allow you to see what a negative result looks like for the sake of comparing with your other test samples.

You will also want to include a positive control for each of your experiments, i.e., a sample that DOES contain the substance you are testing. For example, when you test various foods for the presence of starch you will want to include a sample that you know contains starch. The ideal positive control in this case would be simply a starch solution (water with starch and nothing else). The importance of performing a positive control is also two-fold:

1) To verify that a positive sample actually gives a positive result with the reagents you are using.

2) To allow you to see what a positive result looks like for the sake of comparing with your other test samples.

If either control fails to give the predicted outcome in a given experiment, then the results for all of your test samples are suspect. If your controls give the expected outcomes, then you can be confident that the results for your test samples are reliable.

Now that you understand the importance of performing positive and negative controls, you are ready to test the following foods for the presence of simple sugars, starch and protein:

cow's milk  
rice milk  
almond milk  
chicken broth  
white grape juice  
coconut water  
soda  
diet soda
**Exercise 2A – Detection of simple sugars**

*Benedict’s reagent is a chemical reagent that will reveal the presence of any monosaccharide as well as the disaccharides lactose, maltose or mannose (not sucrose). The reagent itself is blue, however when it reacts with monosaccharides (or the disaccharides indicated) it will change to a green, yellow, orange or reddish brown color depending on how much sugar is present (green to yellow if low levels, orange to reddish brown if high levels).*  

*Materials you will need include:*

- Benedict’s reagent
- 10 test tubes and a rack
- 8 food samples to test
- Deionized water (negative control)
- Glucose solution (positive control)
- Boiling water

NOTE: Before you start, remove the hot plate from your drawer and plug it in. Half fill a large beaker with water, place it on the hot plate and turn on the heat dial ~halfway.

1. On your worksheet, hypothesize which foods will test positive for sugar.

2. Plan and carry out Benedict’s tests of all 8 liquid foods plus the positive and negative controls (i.e., 10 samples total) based on the following:
   - each Benedict’s test should have 2 volumes of Benedict’s reagent plus 1 volume of test sample
   - tests must be mixed and placed in boiling water for 5 minutes before observing the result

**Exercise 2B – Detection of starch**

*Iodine solution will reveal the presence of starch. The reagent itself is a clear light brown or orange color, however when it reacts with starch it will change to an opaque dark blue or black color. Materials you will need include:*

- Iodine solution
- 10 test tubes and a rack
- 8 food samples to test (use solid pieces of potato, banana, coconut, peanut)
- Deionized water (negative control)
- Starch solution (positive control)

1. On your worksheet, hypothesize which foods will test positive for starch.

2. Plan and carry out iodine tests of all 8 liquid foods plus the positive and negative controls (i.e., 10 samples total) keeping based on the following:
   - each iodine test should have 3 drops of iodine solution plus 2 ml of test sample
   - each reaction should be mixed and observed immediately at room temperature
Exercise 2C – Detection of protein

Biuret reagent will reveal the presence of protein. The reagent itself is blue, however when it reacts with protein it will change to a purple or lavender color. Materials you will need include:

Biuret reagent
10 test tubes and a rack
8 food samples to test
deionized water (negative control)
albumin solution (positive control)

1. On your worksheet, hypothesize which foods will test positive for protein.

2. Plan and carry out Biuret tests of all 8 liquid foods plus the positive and negative controls (i.e., 10 samples total) based on the following:
   - each Biuret test should have 1 volume of Biuret reagent plus 2 volumes of test sample
   - each reaction should be mixed and observed immediately at room temperature

Exercise 2D – Analysis of an unknown sample

The unknown sample you’ve been given may contain any combination of sugar, starch, protein or just plain water. You are to test the sample for all three macromolecules as you did in Exercises 2A, 2B and 2C. Record the results and your conclusions on your worksheet.

**PLEASE be sure to dispose of all tube contents in the chemical disposal jug in the flow hood and wash all of your tubes with soap and hot water leaving them upside in your test tube rack in the sink.**

**We don’t have the staff to wash so many tubes and your cooperation will be greatly appreciated. **

**THANK YOU!**
Macromolecules Lab Worksheet

Exercise 1A – Constructing functional groups

Draw the complete structural formulas (indicating all covalent bonds) for the following functional groups using an “R” to indicate organic structures the group is attached to (e.g., R–S–H):

–OH     –CH₃     –NH₂

–COOH    –CHO     –C–

Match each functional group below with the correct chemical property on the right (choices may be used more than once).

  _____ amino group
  _____ carboxyl group
  _____ hydrogen
  _____ hydroxyl group
  _____ methyl group
  _____ aldehyde group
  _____ ketone group

A. acidic
B. basic
C. polar
D. slightly polar
E. non-polar

Exercise 1B – Building an amino acid

a) Draw the complete structural formula for the amino acid you built with your molecular model kit.
b) Circle and label the amino, carboxyl and R groups, and mark the central carbon with an asterisk (*).

  amino acid name: _______________
**Exercises 1C & 1D – Assembling and hydrolyzing a polypeptide**

*Draw the complete structural formula for the polypeptide your group assembled, and circle the peptide bonds. Be sure to show your instructor your polypeptide and demonstrate its hydrolysis.*

**Exercises 2A, 2B & 2C – Detecting macromolecules**

- For each test below, place a checkmark (✓) next to the foods you predict will be positive for the test. This is your hypothesis.
- For each test below, circle the positive control and underline the negative control.

Record your data below indicating the color of each test and the amount of the substance (−, +, ++, +++):

<table>
<thead>
<tr>
<th><strong>Benedict’s reagent</strong></th>
<th><strong>Iodine solution</strong></th>
<th><strong>Biuret reagent</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>sample</td>
<td>color</td>
<td>sugars?</td>
</tr>
<tr>
<td>cow’s milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rice milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>almond milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chicken broth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>white grape juice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>coconut water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>soda</td>
<td></td>
<td></td>
</tr>
<tr>
<td>diet soda</td>
<td></td>
<td></td>
</tr>
<tr>
<td>glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sample</td>
<td>color</td>
<td>starch?</td>
</tr>
<tr>
<td>cow’s milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rice milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>almond milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chicken broth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>white grape juice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>coconut water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>soda</td>
<td></td>
<td></td>
</tr>
<tr>
<td>diet soda</td>
<td></td>
<td></td>
</tr>
<tr>
<td>starch</td>
<td></td>
<td></td>
</tr>
<tr>
<td>water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sample</td>
<td>color</td>
<td>protein?</td>
</tr>
<tr>
<td>cow’s milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rice milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>almond milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chicken broth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>white grape juice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>coconut water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>soda</td>
<td></td>
<td></td>
</tr>
<tr>
<td>diet soda</td>
<td></td>
<td></td>
</tr>
<tr>
<td>albumin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>water</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Indicate below the foods that have simple sugars, starch, and protein based on your test results:

<table>
<thead>
<tr>
<th>Foods with sugars</th>
<th>Foods with starch</th>
<th>Foods with protein</th>
</tr>
</thead>
</table>

➢ Which foods were not consistent with your hypothesis? Explain.

Exercise 2D – Unknown # ______

<table>
<thead>
<tr>
<th>test</th>
<th>color</th>
<th>substance detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benedict’s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biuret</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Review questions:

1. List the 4 major groups of macromolecules.

2. Referring to your list above, what types of macromolecules might be present in your unknown sample that would not be detected by the tests you performed?

3. What is an enantiomer?

4. Which of the 20 amino acids is not an enantiomer and why?
Bio 6 – Microscopy & Cells

Objectives

1. Explain each part of the compound microscope and its proper use.
2. Examine a variety of cells with the compound microscope and estimate cell size.
3. Examine larger specimens with the stereoscopic dissecting microscope.

Introduction

In this laboratory you will be learning how to use one of the most important tools in biology – the compound light microscope – to view a variety of specimens. You will also use a slightly different type of light microscope called a stereoscopic dissecting microscope. The first lens used to magnify things was developed in the first century A.D. These were pieces of glass shaped in a convex form – thicker in the middle and tapering off to the sides – and were the first magnifying glasses that could increase the image of an object about 10 to 20X. The creation of glass lenses improved dramatically at the end of the 16th century, vastly improving the magnifying power. By 1609, Galileo Galilei refined the methods of lens making in an effort to view objects in the sky.

About half a century later, the Dutchman Anton van Leeuwenhoek further improved the art of lens making, allowing him to view objects in pond water that had never been viewed by humans – microorganisms – life at a tiny level. At the same time, an English physicist named Robert Hooke improved the technology of van Leeuwenhoek and confirmed the existence of tiny organisms in pond water. He also famously examined a piece of cork and observed tiny boxes arranged in such a way that they looked like the “cells” (rooms) in a monastery if you removed the roof and looked in from above. Today the best compound light microscopes are able to magnify objects up to 2,500X without losing their resolution – the sharpness of the image itself.

Part 1: THE COMPOUND LIGHT MICROSCOPE

The Parts of the Compound Light Microscope

Exercise 1A – Getting familiar with the microscope

You will first get acquainted with the major parts of the compound light microscope before learning the proper way to use it. Get a microscope from the cabinet below your lab bench, being sure to handle it by the arm and base (refer to image on page 2), and place it on the bench in front of you. Remove the cover and place it below, out of the way, and then plug in the microscope. The ocular lens (eyepiece) and stage should be facing you. Read the description of each part of your microscope on the next two pages being sure to follow all instructions, and then complete the matching exercise on your worksheet.
OCULAR LENS (eyepiece) – Your microscope will have either one (monocular) or two (binocular) ocular lenses. These are the lenses you will look through when examining a specimen with the microscope. Take a look at the side of your ocular lens and you will notice a label of “10X”. This indicates that each ocular lens magnifies the image by a factor of 10 or 10X.

OBJECTIVE LENSES – Notice the set of objective lenses on the revolving nosepiece. These lenses allow you to change the degree of magnification. Some of our microscopes have four objective lenses while others have only three. The degree of magnification for each objective lens is indicated on its side. Let’s take a look at each progressing from the shortest to longest objective lenses, being sure to rotate the revolving nosepiece to click each objective lens into position above the stage before examining it:

4X – This objective magnifies the image by a factor of 4. It is referred to as the “scanning objective” since it is used to scan the slide to locate the specimen before viewing it at higher magnification. Your microscope may not have this objective lens, in which case you can begin with the 10X objective.

10X – This objective magnifies the image by a factor of 10 and is referred to as the “low power” objective.

40X – This objective magnifies the image by a factor of 40 and is referred to as the “high power” objective.

100X – This objective magnifies the image by a factor of 100. It is referred to as the “oil immersion objective” since it requires a drop of immersion oil on the slide to provide good resolution. You will not be using this objective lens.

For now, make sure that the low power objective is clicked into position above the stage, and keep in mind that you will only be using the low power and high power objectives. Also keep in mind that the total magnification of any image you see through the ocular lens is the product of the objective and ocular lens magnifications (for example, when using the lower power lens the total magnification is: 10X ocular lens x 10X low power objective lens = 100X).
STAGE and STAGE CLIP — The stage is the flat surface upon which you will place each slide you will examine. Notice that there is a moveable stage clip that can be used to secure the slide on the stage. Open and close the stage clip to see how it will snugly hold your slide in position.

MECHANICAL STAGE KNOBS — To move the slide on the stage when it is secured in the stage clip, you will use the mechanical stage knobs on the underside of the stage to move the slide backward/forward and right/left. Adjust each knob to see how one knob controls backward/forward movement and the other knob controls right/left movement.

COARSE FOCUS and FINE FOCUS KNOBS — In order for a specimen on a slide to be in focus, the distance between the specimen and the objective lens must be just right. The coarse focus knob, the larger of the two, will move the stage or objective lens (depending on the microscope) up and down quickly and quite visibly, altering the distance between them. It is very important that the coarse focus knob is only used with the low power or scanning objective lenses, otherwise the microscope or objective lenses could be damaged. Adjust the coarse focus knob to observe how quickly the focal distance changes. In contrast, the fine focus knob will move the stage or objective lens such a small amount that it is hardly noticeable to the naked eye. This is the knob you will use to get the perfect focal distance so the image will be crystal clear.

CONDENSER LENS — Just underneath the stage is the condenser lens. This lens serves to capture and focus light from the lamp below onto the slide mounted on the stage. On many microscopes the condenser lens can be adjusted up or down with a knob beneath the stage. Examine the condenser on your microscope to see if it is adjustable. If so, be sure to adjust it as high (close to the stage) as possible since, for our purposes, this is where it should be set.

DIAPHRAGM — The diaphragm is located within the condenser and is one of the most important pieces of the microscope, though it is often neglected by many students. The diaphragm allows you to adjust the amount of light passing through the slide by adjusting the diaphragm lever. Most of the time the diaphragm will be all the way open to allow the maximum passage of light. However it is important to adjust the diaphragm at times to reduce the amount of light passing through your specimen should the image be too bright or dim, and also to increase the contrast to allow you to see the specimen more easily against the background. For now, open the diaphragm all the way, and when using the microscope, do not forget to use the diaphragm.

LAMP — The lamp emits light to illuminate the specimen so that you can actually see something.

BASE and ARM — The base is the bottom of the microscope that sits on the table, and the arm is the vertical framework ascending from the base along the back of the microscope. When handling the microscope always hold the arm while supporting the base with your other hand.

**Proper Use of the Compound Light Microscope**

**Exercise 1B — Steps to follow when using the microscope**

If you really want to be able to see a specimen on a slide, you must follow the steps on the next page *every time you look at a new slide*. The microscope will be your friend if you always use the following steps in their proper order. Before you begin, be sure your microscope is plugged in and the power is “on”.

59
**Step 1.** Get a slide of the letter “e” from the tray on the side counter. This an example of a prepared slide, a slide that is already made for you and meant to be reused. (i.e., don’t dispose of it, please return it to the tray when you are finished!)

**Step 2.** Use a piece of lens paper to clean any smudges (fingerprints, grease, etc.) off the slide. Place the slide on a white piece of paper find the specimen (the letter “e”) on the slide with your naked eye, noticing its location and orientation.

**Step 3.** Lock the scanning objective lens into place (it should “snap” into place) if you have not already done so. You will always, always, always start with either the low power or scanning objective when you want to view a slide since these lenses provide the largest field of view.

**Step 4.** Use the coarse focus knob to move the stage (or objective lens) so that they are as far apart from each other as possible. Open the stage clip and place the slide snugly in the corner of the stage clip (make sure the slide is completely flat) before releasing the clip to hold the slide firmly in place. Then use the mechanical stage knobs to position the slide so that the specimen (i.e., letter “e”) is centered over the condenser and the light that passes through it.

**Step 5.** Next, using the coarse focus knob once again, move the slide and objective lens as close together as the knob will allow.

*(NOTE: To this point, you have not yet looked into the oculars. This may be surprising, but this is the proper way to use a microscope so that you will actually see something!)*

**Step 6.** Now, look into the ocular lenses. Using the coarse focus knob, SLOWLY increase the distance between the slide and objective until the specimen is in focus.

*If the light is too intense, adjust the diaphragm lever (or dial near the lamp if present) until the light level is comfortable before trying to locate the specimen.*

If you have difficulty locating and focusing on your specimen (the letter “e”), make sure that it is properly centered and you may need to adjust the course focus more slowly. If you still can’t locate it, ask your instructor for assistance.

**Step 7.** Adjust the diaphragm lever so there is sufficient contrast between the specimen and the background, closing it no more than is necessary. *This step is especially important for live specimens since you may not be able to see them otherwise.*

**Step 8.** Now use the fine focus knob to get the specimen in proper focus. *You should now be able to see the object clearly.* Before going to the next step (increasing the magnification), be sure to center your specimen in the field of view as best you can.

**Step 9.** Now that you have centered and focused the object as best you can using the scanning objective, rotate the low power objective into place over the slide being sure it “clicks” into position. Use the fine focus knob (NOT the coarse focus) to bring the object into perfect focus. Repeat with the high power objective if you want a more magnified image.

*(REMINDER: Only use the coarse adjustment knob with the low power and scanning objectives)*

FOLLOW THESE STEPS EVERY TIME YOU WANT TO VIEW A NEW SLIDE AND YOU WILL BECOME A GOOD MICROSCOPIST!
Part 2: PROPERTIES OF LIGHT MICROSCOPY

In this section we will focus on some of the key properties relating to light microscopy. To help you understand each property you will first read an explanation and then do an exercise to illustrate that particular property. Let us begin with the property of magnification...

**Total Magnification**

The total magnification of an image is quite simple – it is the product of the ocular lens magnification times the magnification of the objective lens you are using:

\[
magnification \text{ of ocular} \times magnification \text{ of objective} = \text{ total magnification}
\]

For example, if the ocular lens magnifies the image by a factor of 10 (10X), and the objective lens magnifies the image by a factor of 50 (50X), the total magnification of the image is 500X:

\[
10X \times 50X = 500X
\]

Many students make the mistake of adding the two magnifications, so remember that total magnification is the product (multiplication) of the ocular and objective lens magnifications.

**Exercise 2A – Determining total magnification**

On your worksheet, calculate the total magnifications for the examples given, then calculate the total magnification when using each of the objective lenses on your own microscope.

**Field of View**

The field of view (FOV) is the actual “circle” you see when looking in the microscope. Although this circular field of view appears to be the same no matter which objective lens you are using, this is not the case. The circular area you are actually viewing will decrease as you increase the magnification:

<table>
<thead>
<tr>
<th>total magnification</th>
<th>field of view</th>
</tr>
</thead>
<tbody>
<tr>
<td>40X</td>
<td>⬤</td>
</tr>
<tr>
<td>100X</td>
<td>⬤</td>
</tr>
<tr>
<td>450X</td>
<td>⬤</td>
</tr>
<tr>
<td>1000X</td>
<td>⬤</td>
</tr>
</tbody>
</table>
A good analogy is to imagine yourself viewing the Earth from space as you gradually move closer and closer to Mission College. Initially your field of view is the entire western hemisphere, but as you approach the Earth’s surface your field of view will progressively shrink to encompass the western United States, Southern California, the San Fernando Valley, Sylmar, etc. Although your field of view is shrinking, the image in your field of view is becoming increasingly magnified. This is really no different than looking into your microscope at increasing levels of magnification.

It is also useful to know the diameter of the field of view (FOV diameter) at a particular magnification, since you can use this information to estimate the size of the specimen you are viewing. The FOV diameter at low power for your microscope (100X) is ~1.8 mm. Using this FOV diameter, you can calculate the FOV diameter at other magnifications. This is done by multiplying by the ratio of the magnifications:

\[
\text{known FOV diameter} \times \frac{\text{total mag. (known FOV)}}{\text{total mag. (unknown FOV)}} = \text{unknown FOV diameter}
\]

If you want to know the FOV diameter at 500X, you could calculate it as follows:

\[
1.8 \text{ mm} \times \frac{100X}{500X} = 1.8 \text{ mm} \times \frac{1}{5} = 0.36 \text{ mm} = 360 \mu\text{m}
\]

Once you know the FOV diameter, you can estimate the dimensions of your specimen. For example, assume you are viewing the specimen below at 500X total magnification and, based on your calculation above, you know FOV diameter to be 360 µm. It appears that ~4 of your specimens would fit across the FOV end to end (i.e., length = 1/4 of FOV), and ~10 side to side (i.e., width = 1/10 of FOV). Thus you would estimate the dimensions of your specimen to be:

\[
\text{LENGTH} = \frac{1}{4} \times 360 \mu\text{m} = 90 \mu\text{m} \\
\text{WIDTH} = \frac{1}{10} \times 360 \mu\text{m} = 36 \mu\text{m}
\]
Exercise 2B – Field of view and estimating size

Before you can estimate the size of a microscopic specimen, you must first determine the diameter of the field of view at the magnification you are using. Once you have that information you are prepared to estimate the size of any specimen you observe at that magnification:

1) Calculate the FOV diameter for each possible total magnification on your microscope given the FOV diameter at low power (100X) is 1.8 mm.

2) Examine a prepared slide of Paramecium at low power and estimate the length and width of a single Paramecium.

3) Examine a prepared slide of Euglena at high power and estimate the length of a single Euglena.

Part 3: A MICROSCOPIC VIEW OF CELLS

Review of Cell Structure

All living organisms consist of one or more cells and come in a tremendous variety. There are single-celled prokaryotic organisms such as the bacteria, single-celled eukaryotic organisms such as the protozoa (e.g., Paramecium) and yeasts (a type of fungus), and multicellular eukaryotes such as most fungi (e.g., molds, mushrooms) and all members of the plant and animal kingdoms. Before you examine cells from some of these organisms, let’s review some of the general features of our three basic cell types:

Prokaryotic Cell

Notice that a prokaryotic cell does not have any distinct internal compartments. This does not mean that prokaryotic cells have no internal organization, they simply do not have any structures we refer to as organelles. In contrast, animal and plant cells contain a variety of organelles. Take
Now that you are well acquainted with the structures and organelles found in cells, you will use your microscope to observe cells from most of the major groupings. The most comprehensive groupings are the **domains**, of which there are three: the **Bacteria, Archaea** and **Eukarya**.

All species in the domains Bacteria and Archaea are single-celled prokaryotes. Until recently, the bacteria and archaea were grouped into a single kingdom called Monera. However, research in the last few decades has revealed that these organisms, though similar in microscopic appearance, are vastly different in DNA sequence and physiology. As a result they are now placed into entirely different domains. The domain Eukarya consists of all eukaryotes — i.e., organisms made of cells with a nucleus and other organelles. Within the domain Eukarya are the three traditional kingdoms still in use — **Fungi, Plantae** and **Animalia** — with all remaining eukaryotes lumped into a complex group called the **Protista**. The Protista used to constitute a single kingdom, however their diversity has led to a complex reclassification that is still in progress. For simplicity we will refer to all of these remaining eukaryotes as Protista or **protists**, keeping in mind that they are no longer comprise a single kingdom.
Let us now take a look at cells from organisms in these major groups.

**Bacteria**

By far the most abundant organisms on our planet are the single-celled prokaryotes known as the **bacteria** and **archaea**. Since archaea are very difficult to culture in a laboratory setting, the only prokaryotes you will observe are bacteria. You will observe two species of bacteria, one having a round or **coccus** shape and the other having a rod or **bacillus** shape. Since bacteria are much smaller than eukaryotic cells, you will need to observe them at 1000X under the oil immersion lens in order to see them clearly.

**Exercise 3B – Examining prokaryotes**

*Slides showing two distinct types of bacteria have been prepared for you. These cells are not alive, and the bacteria are stained to add contrast and help you distinguish between the two types. You will view these cells at 1000X under “oil immersion” as demonstrated by your instructor:*

1) Find a region of the slide where there is empty space around the bacteria and draw a sample of each type of bacteria on your worksheet.

2) Describe the morphology (shape of individual cells) and arrangement (how multiple cells in contact with each other) for each type of bacteria.

**Protozoa**

The **Protista** consist of two general types of eukaryotic organisms: the **protozoa** and the **algae**. The protozoa (literally “before animals”) are single-celled eukaryotes that have “animal-like” qualities, whereas the algae are photosynthetic organisms that do not have the features of true plants. In the next exercise, you will observe a variety of live protozoa as you learn how to prepare your own wet mounts of these organisms:
Exercise 3C – Examining protozoa

You will prepare three different wet mounts of live protozoa as outlined below: Paramecium (view at low power), Euglena (view at high power) and a sample of pond water (low or high power). The protozoa you will see can move quite fast under the microscope, so prepare each wet mount as follows to ensure that they are slowed enough for you to view them:

1) Place one drop of “protoslo” on a clean glass slide (this will help slow the critters down!)
2) Using a transfer pipet, add one drop of sample (from the bottom of the container) to the protoslo and slowly add a cover slip over the sample, laying it down gently at an angle.
3) Mount the slide on your microscope and prepare to view the slide at low power.
4) To help you find the level of focus for the protozoa you want to examine, focus on either a bubble or the edge of the cover slip.
5) Close the diaphragm lever almost all the way to increase the contrast, and locate a specimen. Unless you do this, there will not be enough contrast to see any specimens.

Yeast

The kingdom Fungi includes multicellular fungi such as molds and mushrooms, as well as single-celled fungi which are collectively known as the yeasts. Yeasts are immensely important to humanity. They are essential for producing certain foods and beverages (e.g., bread, beer, wine, chocolate), and have allowed scientists to effectively study the nature of eukaryotic cells and to produce commercial medicines such as insulin for diabetics. In the next exercise you will look at the species of yeast commonly referred to as “baker’s yeast” or “brewer’s yeast”: Saccharomyces cerevisiae.

Exercise 3D – Examining yeast (Saccharomyces cerevisiae, aka “brewer’s yeast”)

Prepare and observe live yeast cells as instructed below. You will observe these cells at 1000X under “oil immersion” which will be demonstrated by your instructor:

1) At the front of the lab, obtain a clean glass slide and place a drop of methylene blue dye on it.
2) Add one drop of yeast suspension.
3) Obtain a single glass cover slip and placing it at angle, gently and slowly lay it down over the leaf at an angle until it lies flat on top of it.
4) Find the yeast at low power (100X), then place a drop of immersion oil on the cover slip observe under oil immersion at 1000X as demonstrated by your instructor.
5) Find and draw an example of a single live yeast cell (they will be gold, dead cells will be blue) and identify its nucleus.
6) Find and draw an example of a “budding” yeast which is in the process of cell division by mitosis.
Plant Cells

The kingdom **Plantae** includes organisms such as mosses and ferns as well as the familiar cone-bearing plants (**Gymnosperms**) and flowering plants (**Angiosperms**). All plants are multicellular and sustain themselves by the process of photosynthesis. Most plants have distinct organs and tissues consisting of different cell types. For the next exercise, you will observe live plant cells in a leaf from the aquatic plant **Elodea**:

**Exercise 3E – Examining plant cells**

*Prepare and observe your own wet mount of a leaf from the aquatic plant Elodea as instructed below, and answering the corresponding questions on your worksheet:*

1. At the front of the lab, obtain a clean glass slide and place a drop of water on it.
2. Using a pair of tweezers, break off a single **Elodea** leaf and place it in the drop of water on the slide, making sure it is as flattened out as possible.
3. Obtain a single glass cover slip and placing it at angle next to the leaf, *gently* and *slowly* lay it down over the leaf at an angle until it lies flat on top of it.
4. Observe the leaf at low power and draw a sample of what you see on your worksheet.
5. Observe the leaf at high power and draw several cells including whatever organelles you can see. Be sure the label the nucleus, chloroplasts, central vacuole, and cell wall.

Animal Cells

All species in the kingdom **Animalia** are multicellular, consisting of a wide variety of organs, tissues and cell types. Like the protists, fungi and plants, animals belong to the domain Eukarya since their cells have a nucleus and other organelles as shown in the diagram on page 9. The animal cells you will observe today will actually be your own (*Yes, you are a member of the kingdom Animalia!*).

**Exercise 3F – Examining animal cells**

*Prepare a wet mount of your own cheek cells as described below, and answer the corresponding questions on your worksheet. Since your cheek cells are transparent, you will add the dye methylene blue to provide contrast between your cells and the background.*

1. At the front of the lab, obtain a clean glass slide and place one drop of water and one drop of the dye methylene blue on the slide.
2. Obtain a toothpick and *gently* rub one end of it on its side along the inside of your cheek.
3. Place the end of the toothpick that made contact with your cheek in the mixture of methylene blue and water on the slide, and move it around to transfer some cheek cells.
4. Discard the toothpick in the biohazard bag, and gently place a cover slip over the slide as you did before.
5. Observe your cheek cells at low and high power and draw samples of cheek cells you see at each magnification, being sure to identify the cell nucleus.
Part 4: THE STEREOSCOPIC DISSECTING MICROSCOPE

Up until now you have been exclusively using a compound light microscope. While it is ideal for viewing tiny microbes that can be mounted on a slide, there are biological specimens that are too large and/or thick to be mounted on a slide and viewed with the compound microscope (yet too small for the naked eye). In this case you will want to use the **stereoscopic dissecting microscope** or “dissecting microscope” for short. Two advantages of this microscope are 1) you can manipulate your specimen (turn, flip, dissect) using your hands or tools while viewing it under magnification (hence term “dissecting”), and 2) by looking through both oculars you can see the image in three dimensions (“stereoscopic”).

The dissecting microscope is a *simple* light microscope since the image you see is magnified through a single magnification lens. The magnification on your microscope adjustable allowing you to view your specimen at 8X through 35X (10X oculars x 0.8-3.5X adjustable objective). While the total magnifications possible on this microscope are low, they provide the advantages of a very large field of view and a very thick depth of focus. This will allow you to see most, if not all, of your specimen clearly and in three dimensions.

Your dissecting microscope contains a single focus knob and two different light sources controlled by knobs on either side of the arm of your microscope. Turn them on and you will notice that one light source is below the stage and the other is above the stage. The light below the stage produces light that will pass through a transparent specimen, what we call **transmitted light**. The light above the stage produces light that will bounce or reflect off the specimen. We refer to this as **reflected light**, which is used to illuminate a non-transparent specimen from above.

You will first examine the letter “e” slide to see how the image created compares to the compound microscope and then examine various 3-dimensional biological objects as well as your own hand and other items of interest.
Exercise 4 – Using the stereoscopic dissecting microscope

Examine the samples indicated below, and as you do so, adjust the lighting to give you the best image, and answer the corresponding questions on your worksheet.

1) Examine the letter “e” slide at 35X, noting its orientation viewed with the microscope relative to your naked eye.

2) To become more familiar with viewing specimens under the dissecting microscope, examine the biological items provided on your bench at various magnifications using transmitted and/or reflected light.

3) Examine one of your fingers at various magnifications using reflected light and feel free to examine anything else of interest.

Before you leave, please make sure your table is clean, organized, and contains all supplies listed below so that the next lab will be ready to begin. Thank you!
Exercise 1A – Parts of the compound microscope

Write the correct label for each part of the microscope shown below:

Exercise 1B – Using the compound microscope

Match each part of the compound microscope on the left with its function on the right:

___ base and arm
___ coarse focus knob
___ condenser lens
___ diaphragm
___ fine focus knob
___ high power objective
___ lamp
___ scanning or low power objective
___ mechanical stage knobs
___ ocular lens
___ stage
___ stage clip

A. eyepiece, what you look in to see an image
B. adjusts position of slide left/right, front/back
C. used to bring the image into sharp focus
D. flat surface on which slide is placed
E. secures slide in place before viewing
F. focuses light from the lamp on the slide
G. used only with the low power objective
H. used to handle the microscope properly
I. adjusts the amount of light passing through slide
J. used when you first examine a slide
K. light source used to illuminate specimen
L. used to produce a more magnified image after the specimen has been located on the slide
Exercise 1B – Using the microscope

Answer the following questions as you work through this exercise:

- How is the letter “e” on the slide oriented when you see it with the naked eye as you mount it on the stage (i.e., is it right side up or upside down)?

- How is the letter “e” on the slide oriented when you see it under low or high power magnification?

- What effect, if any, does the compound light microscope have on the orientation of the image?

Exercise 2A – Total Magnification

Fill in the charts below. For your actual microscope, you will find the magnifications of the ocular and objective lenses printed on the side of each lens.

<table>
<thead>
<tr>
<th>SAMPLES</th>
<th>YOUR MICROSCOPE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ocular lens magnification</td>
</tr>
<tr>
<td>5X</td>
<td>50X</td>
</tr>
<tr>
<td>5X</td>
<td>100X</td>
</tr>
<tr>
<td>10X</td>
<td>50X</td>
</tr>
<tr>
<td>20X</td>
<td>20X</td>
</tr>
</tbody>
</table>

Exercise 2B – Field of View and Estimation of Size

Calculate the diameter of the field of view for each total magnification on your microscope in millimeters (mm) and then convert to micrometers (µm). NOTE: You will use this to estimate sizes below:

Scanning (40X): 1.8 mm x 100X/40X = _____ mm = _______ µm

Low power (100X): FOV diameter = 1.8 mm = _______ µm

High power (400X): 1.8 mm x 100X/400X = _____ mm = _______ µm

Oil immersion (1000X): 1.8 mm x 100X/1000X = _____ mm = _______ µm

Draw and estimate the length of a single Paramecium (low power) and Euglena (high power):
Exercise 3A – Cell Structure

Label each structure for the plant and animal cell diagrams below:

Match each cell structure/organelle on the left with its function on the right:

<table>
<thead>
<tr>
<th>Structure</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>nucleus</td>
<td>A. modification, sorting of proteins</td>
</tr>
<tr>
<td>endoplasmic reticulum</td>
<td>B. where cellular respiration occurs</td>
</tr>
<tr>
<td>Golgi apparatus</td>
<td>C. gelatinous outer layer of prokaryotic cells</td>
</tr>
<tr>
<td>mitochondrion</td>
<td>D. small structure that carries out protein synthesis</td>
</tr>
<tr>
<td>chloroplast</td>
<td>E. cellular projection used for motility</td>
</tr>
<tr>
<td>plasma membrane</td>
<td>F. synthesis of lipids, secretory pathway proteins</td>
</tr>
<tr>
<td>cell wall</td>
<td>G. stores water and other materials in plant cells</td>
</tr>
<tr>
<td>capsule</td>
<td>H. contains the chromosomes (DNA)</td>
</tr>
<tr>
<td>flagellum</td>
<td>I. where photosynthesis occurs</td>
</tr>
<tr>
<td>ribosome</td>
<td>J. provides external support/structure in some cells</td>
</tr>
<tr>
<td>centriole</td>
<td>K. barrier between inside/outside of cell</td>
</tr>
<tr>
<td>central vacuole</td>
<td>L. plays an important role in cell division</td>
</tr>
</tbody>
</table>

Place a check mark or “X” indicating a structure/organelle is present in the indicated cell type:

<table>
<thead>
<tr>
<th>CELL TYPE</th>
<th>nucleus</th>
<th>endoplasmic reticulum</th>
<th>Golgi apparatus</th>
<th>mitochondrion</th>
<th>chloroplast</th>
<th>central vacuole</th>
<th>plasma membrane</th>
<th>cell wall</th>
<th>capsule</th>
</tr>
</thead>
<tbody>
<tr>
<td>bacteria</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>animal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Exercise 3B – Prokaryotes: Bacteria and Archaea

- Describe or draw the appearance of each of the 2 bacterial species addressing both the shape of cells (e.g., coccus or bacillus) and their arrangement (e.g., chains, clusters, single cells, pairs, etc.).

  Morphology:
  Arrangement:
  total magnification _________

Exercise 3C – Protozoa

Draw samples of live Paramecium and Euglena as seen in the microscope.

  Paramecium
  Euglena
  total magnification _________
  total magnification _________

Exercise 3D – Yeast

Draw samples of yeast cells seen in the microscope, and be sure to identify and label the cell nucleus.

  total magnification _________
Exercise 3E – Plant Cells

Draw Elodea cells at low and high power:

Elodea (low power)  Elodea (high power)

total magnification ______  total magnification ______

Exercise 3F – Animal Cells

Draw several cheek cells at low and high power:

Cheek cells (low power)  Cheek cells (high power)

total magnification ______  total magnification ______

Exercise 4 – Dissecting Microscope

Answer the following questions:

➢ Notice the orientation of the letter “e” on the slide with your naked eye and then again when viewed through the dissecting microscope. Is the orientation the same or different?

➢ How does this compare to the compound microscope?

➢ What type of illumination was best for viewing the letter “e” slide, transmitted or reflected?

➢ What type of illumination was best for viewing your finger, transmitted or reflected?
Bio 6 – SDS-PAGE Lab

Objectives

Upon completion of this laboratory you will understand how to load and run protein samples on an SDS-polyacrylamide gel, stain the gel, and analyze the resulting bands of protein on the gel to estimate the molecular weight of each protein.

Introduction

SDS-PAGE is a very common laboratory technique used to analyze proteins. The acronym SDS-PAGE stands for sodium dodecyl sulfate – polyacrylamide gel electrophoresis. Sodium dodecyl sulfate or SDS is a detergent commonly used in biology laboratories to denature proteins, i.e., disrupt the 3-dimensional structure of proteins without breaking the polypeptide backbone. Polyacrylamide is a polymer prepared as a gelatinous medium or “gel” through which proteins can be resolved based on molecular weight (MW). Electrophoresis refers to the movement of charged soluble particles such as proteins through a medium during exposure to an electric field. SDS-PAGE is therefore a technique by which proteins move through a polyacrylamide gel that is subjected to electric current.

The rate at which a protein moves through the microscopic pores of a polyacrylamide gel during electrophoresis is dependent on three physical properties – molecular weight, 3-dimensional shape, and net charge. Separation of proteins based solely on the property of MW is possible only if the variables of 3-dimensional shape and net charge are eliminated. This is accomplished by the detergent SDS, which due to its amphiphilic properties of being hydrophobic on one end and charged on the other is able to disrupt all non-covalent interactions in a protein – i.e., denature the protein. This results in unfolded or linear polypeptides that have a net negatively charge as shown below:

SDS thus eliminates any differences in shape and overall charge among proteins, leaving one variable physical property – molecular weight – to influence the rate at which a protein moves through the gel.
As shown below, SDS-PAGE is run vertically and the gel consists of a “stacking gel” on top of a “resolving gel” (aka “running gel”). Both ends of the gel are in contact with an electrolyte buffer solution containing the positive and negative electrodes which generate electric current through the gel.

The stacking gel contains cavities called **wells** into which you will load protein samples. When electric current is applied, negatively charged solutes such as SDS-treated proteins will experience electromotive force toward the oppositely charged positive electrode and thus move into the stacking gel. The stacking gel has an acrylamide percentage and pH that ensures all proteins enter the resolving gel at the same time. Once in the resolving gel, proteins will migrate at a rate inversely proportional to MW. When the gel is finished running, it is stained to reveal the position of each protein on the gel as shown below:
Each stained band on the gel corresponds to a one or more types of protein with the same molecular weight (keep in mind that each band contains billions of proteins of that MW). The larger the protein, the slower it moves through the gel and thus the higher its position. Smaller proteins move at faster rate and are seen as bands further down the gel. A protein MW standard (a collection of proteins of known size) is always run on the gel and used to estimate the sizes of proteins in the other lanes.

**Part 1: Running an SDS-PAGE gel**

To begin this lab you will denature and load several different protein samples on a polyacrylamide gel after which you will run the gel as described. Preparing an acrylamide gel for SDS-PAGE is a bit tricky, so the polyacrylamide gels have been prepared for you. To denature your proteins, it is essential that you add **sample loading buffer** and then boil the sample. The sample loading buffer contains several key components needed to fully denature the proteins and have them run through the gel properly:

- SDS to denature the polypeptides
- Dithiothreitol (DTT) to break disulfide bonds (covalent bonds between cysteines)
- Glycerol to make the sample dense enough to sink into the well
- Bromophenol Blue, a negatively charged dye to monitor gel progress

The loading dye is prepared at a 2X concentration so that it can be diluted to 1X when mixed with the protein sample. Once loading dye has been added, the heat from boiling facilitates denaturation of the proteins and breaking of disulfide bonds. Disulfide bonds (refer to chapter 5 of your textbook) are the only covalent interactions formed between amino acid R groups and are **not** disrupted by SDS, thus the need for DTT. Only in this fully denatured state will each protein sample move through the gel at a rate dependent on molecular weight only.

Loading samples into the wells of the gel can be challenging since the gel is very thin, too thin to insert the pipet tip directly into the well. As shown in the picture, you will need to gently rest the pipet tip on top of the inner plate at an angle so that the pipet tip is resting just above the well you want to load. It is especially important that you do not press the pipet tip down between the plates, otherwise you may pry them apart. If this happens, even just a little bit, your samples will leak down between the gel and the plate and be lost. Before you load your protein samples you will load a practice sample so you will have some experience before loading your real samples.
Exercise 1 – Loading and running your gel

All of the materials you will need can be found on carts at the side of the lab where you will obtain an ice bucket and a tray of materials. Once you have all your materials, be sure to half fill the beaker with hot tap water and put it on a hot plate set to the maximum so boiling water will be ready when you need it:

1. Each protein sample is 5 \( \mu \text{l} \). Add 2X sample loading buffer to each protein sample so the final concentration is 1X (i.e., the 2X sample loading buffer should be half of the total volume).

2. Tap each tube gently to mix and boil the samples for 5 minutes (the 5 \( \mu \text{l} \) protein molecular weight marker should not be boiled, it already contains 1X sample loading buffer and is ready to load).

3. Identify the wells you plan to use for your protein samples (avoid using deformed wells), and indicate in your lab notebook how you will load your gel. Each person should practice loading 10 \( \mu \text{l} \) of 1X sample loading buffer in the unused lanes. Your instructor will demonstrate how to do this.

4. Load all of the protein molecular weight marker and each protein into their designated lanes.

5. Place the cover on the gel apparatus and connect the red and black plugs to the corresponding colors in the power supply. **NOTE:** If the buffer level in the upper chamber is not above the inner plate of the gel, there will be no flow of electric current.

6. Be sure the power supply is plugged in and set the toggle switch below the digital display to “Volts”, the Range Select switch to “High” and the Voltage Select knob to “Min.”

7. Turn on the power (toggle switch on upper left) and adjust the Voltage Select knob until the digital display reads just over 200 volts.  
   **NOTE:** If the gel is running properly you will see a curtain of tiny bubbles rising from the wire in the upper buffer chamber. You should also see a much lower amount of “bubbling” from the wire in the lower buffer chamber. If you don’t see this, be sure to consult your instructor.

8. Let the gel run until the bromophenol blue dye is ~1 cm from the bottom of the gel (~30-40 minutes).

9. When the run is complete, turn the Voltage Select know to “Min.” and turn off the power.
Part 2: STAINING PROTEINS IN A POLYACRYLAMIDE GEL

To visualize the proteins in your polyacrylamide gel you will use a stain called Coomassie Brilliant Blue. This is not the only protein stain one could use, though it is a very commonly used stain to view proteins on polyacrylamide gels. The staining process may require an overnight incubation so you may not see the protein bands on your gel until the next laboratory session.

Exercise 2 – Staining a polyacrylamide gel

Be sure to wear gloves when you handle the gel and staining solutions, and to clean up as indicated by your instructor:

1. When the gel run is complete be sure the Voltage Select knob is set to “Min.” and the power is off, then remove the cover of the gel apparatus.
2. Carry the gel apparatus to the sink and discard the running buffer from both chambers into the sink.
3. Loosen the clamps holding the gel in place, carefully remove the plates containing your gel and lay it on a flat surface with the taller outer glass plate facing up.
4. Use the plastic spatula supplied as a wedge to carefully pry apart the 2 plates. The gel should stick to one plate or the other as you do so. If it seems to stick to both, ask for assistance from your instructor.
5. Place ~100 ml of fixative solution in the staining tray provided.
6. Gently separate a bottom corner of the gel from the glass plate using the spatula, carefully peel the gel off the plate (it’s OK if the stacking gel tears off, you won’t need it) and place it in the fixative solution. If the gel curls up, carefully unravel it with your gloved hand until it flattens out.
7. Place a Protein InstaStain card (which contains Coomassie Brilliant Blue stain) face down in the fixative solution over the gel, place the lid on the container (be sure to label the lid with your group number) and place the container on the slow rocker at the side of the lab. Leave for 1 to 3 hours or overnight.
8. When the protein bands are clearly visible, photograph your gel as indicated by your instructor being sure to include a ruler aligned with the top of the resolving gel. Store an image of the gel in your lab notebook.

Part 3: ESTIMATING THE MOLECULAR WEIGHT OF PROTEINS ON A POLYACRYLAMIDE GEL

You will use the protein molecular weight standard you ran on your gel to estimate the molecular weights of the other proteins on your gel. As mentioned earlier, the protein molecular weight standard is a set of proteins of known molecular weight. Since the rate at which a denatured protein moves through a polyacrylamide gel during electrophoresis depends only in its molecular weight, you can estimate the molecular weight of a protein based on its position on the gel in relation to the molecular weight standard proteins. In theory, the most accurate way to do this is to create a standard curve on semi-log graph paper by plotting molecular weight in daltons (Da) or kilodaltons (kDa) vs distance traveled on the gel in cm. The distance traveled refers to the distance from the middle of the protein band to the top of the resolving gel (not the bottom of the well in the stacking gel).
The graph above shows a sample standard curve consisting of 4 known proteins (NOTE – the standard proteins on your gel are different, this is an example). Once the standard curve is drawn, the molecular weight of any protein on the gel can be estimated using the distance it traveled on gel. For example, if a protein on the gel pertaining to this standard curve traveled 7.4 cm, identify 7.4 cm on the X-axis and move vertically to find the corresponding position on the standard curve. The molecular weight on the Y-axis that corresponds to this point on the standard curve will be your estimate. In this example the molecular weight estimate would be ~45,000 daltons or 45 kDa. While this method can be very precise, its accuracy depends on the quality of the distance measurements and the precision of the best fit curve.

A simpler method to estimate molecular weight is to compare the protein band of interest to the protein molecular weight standard and visually estimate its molecular weight. For example, if your protein of interest is positioned approximately halfway between standard proteins that are 40 kDa and 50 kDa, then you would estimate 45 kDa. If the band is just above and very close to the 40 kDa standard protein then you might estimate 41 or 42 kDa. While this may not seem very precise, it can give you a value very close to that obtained using a standard curve and for most purposes this should be sufficient. In the next exercise you will estimate the MW of proteins using both methods described above.
Exercise 3 – Estimating protein molecular weight

NOTE: A 12% polyacrylamide gel can effectively resolve or separate proteins between 20 kDa and 100 kDa, so be sure NOT to include molecular weight standard proteins outside this range in your standard curve.

1. Place your stained gel on a white transilluminator and use a small ruler to measure in cm the distance from the middle of the band to the top of the resolving gel for each molecular weight standard protein* between 20 kDa and 100 kDa. Record these values in your notebook.

2. Plot these values on the semi-log graph paper found at the end of this lab and draw the best fit line through the points. A copy of this standard curve should be placed in your lab notebook.

3. For each protein sample loaded on your gel, identify the most prominent band (this will be the protein of interest, the other bands are contaminants), measure the distance from the middle of the band to the top of the resolving gel. Record these values in your notebook.

4. Estimate the molecular weight of each protein using the standard curve as shown on the previous page and record in your notebook.

5. Estimate the molecular weight of each protein by visual comparison with the protein molecular weight standards and record in your notebook.

6. Answer the study questions associated with this lab.

NOTE: For this laboratory you will turn in a formal lab report.

*The protein molecular weight standard most likely used on your gel is shown below. If a different protein molecular weight standard was used your instructor will provide the details.
1. SDS-PAGE is an acronym referring to 3 specific components (SDS, PA and GE). Indicate what SDS-PAGE stands for and briefly describe each of these 3 components.

2. What does it mean to “denature” a protein and why is this important for SDS-PAGE?

3. Describe how you denatured the proteins you used for SDS-PAGE.

4. What substance in sample loading buffer is responsible for breaking disulfide bonds?

5. Diagram a disulfide bond under “BEFORE” and also diagram what it should look like after boiling in the presence of the substance from the previous question (“AFTER”).

BEFORE

AFTER
6. What is the purpose of loading “protein molecular weight standards” on the gel?

7. List the molecular weights of the standard proteins run on your gel and check the ones that you used to make your standard curve.

8. Describe each of the two methods you used to estimate protein molecular weight.
Bio 6 – Diffusion & Osmosis Lab

INTRODUCTION

Diffusion is a phenomenon we are all familiar with. If there is an odor in the room, you know it will gradually spread, i.e., diffuse, from its source and eventually dissipate. If you put a drop of food coloring in a glass of water, you know it will gradually spread until it is uniformly distributed. What you may not realize is the underlying basis of this movement. While in each case the diffusing substance seems to move in a directional manner – away from its source, in reality this movement is due to random motion of the molecules involved. How can this be?

In any liquid or gas, the molecules are moving very rapidly and randomly, and they only change direction when they experience a collision or some other force. There is nothing directional about the movement of any single molecule. However, the collective movement of many molecules can be directional provided there is a difference in concentration – the amount of a substance per unit volume. This is because the collective movement of molecules will always be from a region of higher concentration to a region of lower concentration.

This overall or net movement of molecules from higher to lower concentration is due to a simple fact – random molecular motion will always result in more molecules moving from higher to lower concentration than the reverse. To illustrate this, imagine a closed box of flies is brought into a room that already contains a few flies buzzing around in the room. Clearly the flies in the box are at a higher concentration than in the room outside the box. If the box is opened – i.e., the barrier is removed – it should be obvious that more flies will fly out of the box than into it. Even though each fly moves in a random fashion, the collective or net movement of flies will be out of the box – from higher to lower concentration. Once the concentrations of flies in and out of the box are equal, the system will be in equilibrium – the rate of flies entering the box will be the same as flies leaving it.

So how does diffusion relate to biology and cells? Cells take advantage of diffusion whenever the direction of diffusion – high to low concentration – is in the cell’s interest. For example, cells in your body continually consume oxygen gas (O₂) and thus, as long as O₂ is continually provided by the bloodstream, oxygen will diffuse into cells from high to low concentration. Cells also take advantage of diffusion to get rid of carbon dioxide (CO₂) waste which is higher in cells than in the bloodstream. Diffusion in the opposite direction occurs in your lungs in relation to the air you breathe – O₂ at higher concentration in the air diffuses into your blood, and CO₂ at higher concentration in your blood diffuses out into the air you exhale. As long as there is no barrier to movement, a substance will always undergo net movement from high to low concentration. In addition, cells do not need to exert any energy to allow diffusion to occur, they simply need to let it happen.

It is important to realize that cells also need to move substances from low to high concentration, a process called active transport, which requires the exertion of energy by the cell. This phenomenon will not be addressed in this lab, nevertheless it is extremely important and is a major use of energy in cells.

The last phenomenon we need to address before you begin this lab is osmosis. Osmosis refers to the special case of the diffusion of water in relation to a semi-permeable barrier such as a cell membrane. Water is the solvent in which all biological processes occur, and like any other substance, water will always
diffuse from higher to lower concentration, even across a membrane. Where things get interesting is
when water contains solutes that cannot pass through a membrane or any other barrier. This is the case
in cells where most solutes, unlike $O_2$ and $CO_2$, are unable to pass through the membrane barrier. In this
scenario, water will continue undergo a net movement from higher to lower concentration whereas the
solutes are trapped on one side of the membrane. This results in a net loss or gain of water, and a
corresponding change in osmotic pressure, depending on which side of the membrane has a higher water
concentration as shown in the example below.

Also note the terminology regarding tonicity – the relative solute concentrations. If a cell is in hypotonic
solution (lower solute concentration), there will be a net movement of water into cells from higher to
lower concentration and a corresponding increase in osmotic pressure inside the cell. In animal cells this
leads to swelling and even cell lysis. Plant cells, having a cell wall, are protected from lysis and will have
a turgid appearance. In hypertonic solution (higher solute concentration), there will be a net movement
of water out of cells from higher to lower concentration. This results in reduced osmotic pressure inside
of cells and a shriveled or crenate appearance in animal cells, and plasmolysis in plant cells where the cell
membrane pulls away from the cell wall. In isotonic solution (equal solute concentration), there will be
no net movement of water since solute concentrations inside and outside the cell are equal (water still
moves in and out of the cells, but at equal rates so there is no net change).

**Part 1: Osmotic Pressure**

To illustrate the principle of osmotic pressure, your instructor will submerge dialysis tubing containing a
hypertonic solution into a hypotonic solution – pure water. The dialysis tubing is permeable to water but
not the solute. Over time, osmosis will result in a change in osmotic pressure that affects the level of
solution visible in a pipet allowing you to measure the change of that level over time.
**Exercise 1 – Osmotic Pressure Demo**

This exercise will be set up for the whole class at the front of the lab. All you need to do is he following:

1. Record the liquid level in the pipet at the beginning of the experiment (your instructor will clarify).
2. At the end of the lab, measure the new position of the liquid level in the pipet and record the amount of time that has passed between your initial and final measurement.
3. Answer the associated questions on your worksheet.

**Part 2: Osmolarity**

The term **osmolarity** refers to the concentration in moles per liter of all solutes in a solution, including living cells. This includes the sum of all ions, sugars, proteins, etc. Your goal in the next exercise will be to determine the osmolarity of potato tissue. As you will see, this can be done by placing pieces of potato into solutions of various osmolarities, graphing the % change in weight of each potato piece, and determining at what osmolarity a potato piece would experience no change in weight. This would only occur in isotonic solution that, by definition, is the same osmolarity as the potato.

**Exercise 2 – Determining the Osmolarity of a Potato**

All materials you will need for this experiment can be found on carts at the side of the lab by the window:

1. Place 100 ml of the following sucrose solutions into the appropriately labeled styrofoam cups: pure water (0.0 M sucrose), 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M and 0.6 M sucrose.
2. Use the coring tool to produce several potato cores, cut off the ends (to remove skin), and use a razor blade to cut 7 one inch pieces of potato.
3. Weigh each one inch potato piece to the nearest 0.01 g, and as you do so, record this weight on your worksheet and the sucrose solution in which it was placed.
4. Let the potato pieces soak in their sucrose solutions for 1 ½ hours (at this point, move on to the next exercise).
5. Reweigh each potato piece after removing it from the sucrose solution and dabbing off any excess liquid with a paper towel. Record each weight on your worksheet and calculate the net change in weight (final wt – initial wt; note that a loss of weight will result in a negative number) and percent change in weight \((\text{final wt} – \text{initial wt}) / \text{initial wt}\).
6. Graph molarity of sucrose solution (independent variable) vs % change in weight (dependent variable) and draw the best fit line through your points.
7. The molarity at which your line crosses 0% change in weight is your estimate of the potato osmolarity.
**Part 3: Semipermeable Membranes**

Dialysis tubing is similar to a cell membrane in that water and some other small molecules can pass through it, but many larger molecules cannot. In the next exercise, you will determine which of 3 solutes can pass through the microscopic pores of the dialysis tubing. The solutes you will be testing are listed below in increasing order of molecular size:

- **iodine solution** (MW = 127)
- glucose (MW = 180)
- starch (MW = >693)

Based on these sizes, see if you can predict which solutes will pass through the dialysis tubing.

**Exercise 3 – Testing the Permeability of Dialysis Tubing**

*All the materials you will need can be found on carts on the side of the lab near the window. The dialysis tubing can be found submerged in a beaker of water. Before you begin, predict which of the solutes above will pass through the dialysis tubing:*

1. Tie one end of the dialysis tubing (your instructor will demonstrate).
2. Into the open end of the dialysis tubing add two transfer pipet volumes (5-6 ml) each of glucose and starch and solutions.
3. Add several droppers full of iodine solution to the beaker of water until it is visible yellow in color.
4. Submerge the dialysis tubing in the beaker of iodine water and drape the open end over the lip of the beaker. Leave for 30 minutes.
5. Visually analyze the contents of the beaker and the dialysis tubing to determine if starch and/or iodine solution passed through the dialysis tubing (starch and iodine produce a dark blue color).
6. Test the contents of the dialysis tubing and the beaker for the presence of sugar using Benedict’s solution just as you did in the “Macromolecules” lab to determine if the dialysis tubing is permeable to glucose.
Part 4: Osmosis in Cells

Cells are similar to dialysis tubing in being semipermeable – permeable to water and some solutes, but not others – and thus subject to changes in osmotic pressure when placed in a hypotonic or hypertonic environment. In the next two exercises, you will examine the appearance of plant cells (with a cell wall) and animal cells (no cell wall) in hypotonic, isotonic and hypertonic solutions.

Exercise 4A – Osmosis in Plant Cells

All the materials you will need can be found on carts at the side of the lab by the window. Before you examine each slide, predict the net movement of water in each case – into or out of the cell – and how this would affect the cells:

1. Working with a partner, make two wet mounts each with a single Elodea leaf, one in a drop of pure water and the other in a drop of hypertonic salt solution.

2. Examine each at 400X under the compound microscope record the appearance of each on your worksheet.

Exercise 4B – Osmosis in Animal Cells

1. Observe the images of red blood cells on the next page and determine which is in hypotonic solution, which is in isotonic solution, and which is in hypertonic solution.
**Diffusion & Osmosis Lab Worksheet**

**NAME: __________________**

### Exercise 1 – Osmotic Pressure

Refer to the pipet graduations on the osmotic pressure demo to record the solution levels before and after the experiment as well as the elapsed time:

Before: _____________  After: ______________   Total Time: __________

Did the osmotic pressure inside the dialysis tubing increase or decrease?

Explain the reason for the change in osmotic pressure in terms of solute and water concentrations:

### Exercise 2 – Osmolarity of Potato

Record your potato weight measurements in the table below:

<table>
<thead>
<tr>
<th>[sucrose]</th>
<th>0.0 M</th>
<th>0.1 M</th>
<th>0.2 M</th>
<th>0.3 M</th>
<th>0.4 M</th>
<th>0.5 M</th>
<th>0.6 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>initial wt</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>final wt</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt change</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Graph sucrose concentration vs percent weight change in the table below:
Based on your graph, what is the osmolarity of the potato?

Exercise 3 – Testing the Permeability of Dialysis Tubing
Indicate the solutes you think will pass through the dialysis tubing:

Record below the color of the solutions inside the dialysis tubing and inside the beaker before and after the experiment:

Inside the dialysis tubing:
Before ________________________________  After _________________________________

In the beaker:
Before ________________________________  After _________________________________

Based on these results, what can you conclude about the permeability of the dialysis tubing to iodine and to starch?

Indicate below the color of each of your Benedict’s tests:

Dialysis tubing ___________________________  Beaker _____________________________

Based on these results, what can you conclude about the permeability of the dialysis tubing to glucose?

Exercise 4A – Osmosis in Plant Cells

Draw examples of how Elodea cells appear in hypotonic and hypertonic solution:

<table>
<thead>
<tr>
<th>Pure water (hypotonic)</th>
<th>Salt solution (hypertonic)</th>
</tr>
</thead>
</table>

total magnification ______  total magnification ______
Indicate the terms that apply to the appearance of plant cells in each solution:

Hypertonic - Hypotonic -

**Exercise 4B – Osmosis in Animal Cells**

*Indicate which images – A, B or C – represent red blood cells in the solutions below:*

hypotonic -

isotonic -

hypertonic -
Bio 6 – Enzymes: \( \beta \)-Galactosidase

Overview

Upon completion of this lab you will:

1) Be able to describe an enzyme and describe enzymatic activity.
2) Be able to use a spectrophotometer, understand the principles of absorbance and transmittance, be able describe how this information can be used to determine the amount of a substance, and relate this to enzyme activity.
3) Understand how changes in environmental conditions, such as pH and temperature, influence the rate of enzyme activity.
4) Be able to describe how changes in enzyme or substrate concentration influence the rate of enzyme activity.
5) Be able to distinguish between competitive and non-competitive inhibition.
6) Effectively graph and interpret graphs of enzyme activity.

Introduction

Enzymes, i.e. protein catalysts, are of particular importance to cells. Enzymes speed up chemical reactions without being used up or altered in the process, thereby permitting the chemical reactions necessary for life to take place in a reasonable time frame. During an enzymatic reaction, the substrate, the molecule which the enzyme acts upon, is converted into a new form, the product. The substrate binds to the active site of the enzyme, forming an enzyme-substrate complex. This interaction promotes catalysis and, once completed, the product is released, and the enzyme is ready to bind another substrate molecule. Thus, a single enzyme is capable of processing a great number of substrate molecules. When we study enzymes, we are generally then, most interested in how much work an enzyme can do, i.e. its activity. In today’s lab you will assay (measure) the activity of one particular enzyme, \( \beta \)-galactosidase.

Enzyme activity is expressed units. The units for each enzyme is specifically defined as a given amount of change in a given amount of time under a specific set of conditions. For example, a unit of restriction enzyme is the amount of restriction enzyme capable of digesting 1 µg of DNA in 1 hour at 37°C.

The enzyme \( \beta \)-galactosidase cleaves \( \beta \)-D-galactosides (something hooked to galactose by a \( \beta \) linkage) into galactose and an alcohol. For example, it cleaves lactose into galactose and glucose. In the \( \beta \)-galactosidase assay we will use the artificial galactoside o-nitrophenyl-\( \beta \)-D-galactopyranoside (ONPG) as a substrate. \( \beta \)-galactosidase cleaves ONPG into galactose and o-nitrophenol.

\[
\text{ONPG (colorless)} \quad \xrightarrow{\beta \text{-galactosidase}} \quad \text{Galactose (colorless)} + \quad \text{O-Nitrophenol (yellow)}
\]

99
Note that the substrate is colorless but one of the products of the reaction, o-nitrophenol, is yellow. Thus, we can visualize the progress of this enzymatic reaction by the change in color; the more intense the color change, the more product has formed. Using a spectrophotometer, we can quantitate the intensity of the reaction’s color by measuring how much light is absorbed by the reaction. O-nitrophenol specifically absorbs light at the 420 nm wavelength (i.e. violet light). Thus, the spectrophotometer will emit light at 420 nm and measure how much of it is absorbed; the more o-nitrophenol there is, the more light will be absorbed at this wavelength.

We will define the units of β-galactosidase as the amount of ONPG converted to o-nitrophenol and galactose (as measured by a change in absorbance at 420 nm) per minute at 28°C.

\[
\text{Units} = \frac{(\text{change in } \text{ABS} @ 420 \text{ nm})}{(\text{minute}) (\text{ml of enzyme used})}
\]

NOTE – o-nitrophenol is toxic and care should be taken when handling all solutions. Wear gloves at all times and properly dispose of all liquid waste in the designated waste containers. No waste should be disposed of down the drain.
IN THE FIRST EXPERIMENT, YOU WILL BE SETTING UP A TIME COURSE. A TIME COURSE IS A SET OF ASSAYS IN WHICH THE REACTION TIME IS VARIED. THIS WILL ALLOW YOU TO SEE IF THE AMOUNT OF REACTION PRODUCTS CHANGES OVER TIME. BELOW IS A FLOW CHART OF THE OVERALL PROCEDURE THAT SHOULD HELP YOU SEE WHAT YOU ARE DOING IN THIS EXPERIMENT. MAKE SURE YOU CAREFULLY READ THE FULL DIRECTIONS, THOUGH, TO SEE EXACTLY HOW EACH STEP IS DONE.

AFTER COMPLETING THE TIME COURSE EXPERIMENT (PART I) YOUR GROUP WILL EXAMINE THE INFLUENCE OF A PARTICULAR FACTOR ON THE ACTIVITY OF β-GALACTORSIDEASE BY CHOOSING TO COMPLETE ONE OF THE ADDITIONAL EXPERIMENTS (PART II, PART III, PART IV OR PART V). WHILE THE REACTION CONDITIONS WILL VARY, YOU WILL RUN THE ASSAYS IN THE SAME MANNER AS THE TIME COURSE.

---

Z buffer and β-galactosidase enzyme are mixed in a tube to place the β-gal enzyme in a suitable salt/pH environment

**INITIATION**: ONPG, a substrate for the β-galactosidase enzyme, is added, and the β-gal enzyme begins to cleave the ONPG into colorless galactose and yellow o-nitrophenol

Reaction is placed at 28°C (a suitable temperature for β-gal) so the ONPG cleavage can continue to occur for the prescribed time

**TERMINATION**: At the specified time, Na₂CO₃, which dramatically shifts the pH so the β-gal enzyme cannot function, is added to terminate the reaction

The amount of reaction is measured by measuring the yellow product, o-nitrophenol, in a spectrophotometer.

---

In the blank “0” reaction, the termination step is done early to prevent reaction.

Start timing here

End timing here
Materials:

- Z Buffer, pH 7.0, Bottle with 45 mL
- β-galactosidase stock, Conical Tube with 5 mL
- ONPG, 2 mg/ml, Conical Tube with 15 mL
- 1 M Na₂CO₃, Bottle with 40 mL
- Plastic cuvette
- P1000s, p200s and appropriate tips
- Test tubes (NOT the screw cap type – make sure they are clean!)
- Aliquot precisely (no more, no less) into 15 ml conical tubes each of the following:
  - 4 mL of each “Compound X” stock (1, 5, 15, 25, 50, 100 mM)
  - 4 mL of each pH (4, 5 6, 8, 9, 10)

---

NOTE – unless otherwise noted in the instructions or, by reason of your experimental design, each reaction should be composed of the following:

1.68 mL Z Buffer, pH 7.0*
0.12 mL β-galactosidase
0.36 mL ONPG

* This volume will generally stay the same but you will use other versions of this buffer depending in two experiments as follows:

Part II. INHIBITION OF β-GALACTOSIDASE – use the 0.5 mM Compound X (prepared in Z Buffer pH 7.0)

Part IV. INFLUENCE OF pH ON β-GALACTOSIDASE ACTIVITY – use the Z Buffer with the appropriate pH for each sample condition.

NOTE: Due to time constraints, your instructor may delegate different groups to do different experiments and share the resulting data with the rest of the class.
Part I. TIME COURSE

Make all subsequent measurements carefully. This is a timed assay. Before you begin the assay, read through ALL the steps and know exactly what you are going to do. Make a table of what time each reaction will be initiated and terminated to help you plan.

Preparation of the time course tubes:

1) Label two clean test tubes as A and B. Into Tube A, add 2 ml Z Buffer pH 7 and 0.12 ml β-galactosidase. Into Tube B, add 1.8 ml Z Buffer pH 7 and 0.36 ml 2 mg/ml ONPG. Set these aside for now.

2) Label six clean test tubes with the following times and your initials: 0, 5, 10, 15, 20, 25 min.

3) Add 1.68 ml of Z buffer (pH 7) to each tube (0, 5, 10, 15, 20, 25 min). Z buffer contains a buffering agent to keep the pH constant at pH 7 during the reaction, as well as salts, Mg2+, and other agents that stabilize the enzyme.

4) Add 0.12 ml of β-galactosidase enzyme to each tube.

5) Set aside the tubes that are labeled 5, 10, 15, 20, and 25. For the next few steps you will work ONLY with the “0” tube.

Setting up the “blank” (no reaction) tube:

6) Take ONLY the tube labeled “0” and add 0.9 ml of 1M Na2CO3 to this tube (the Na2CO3 will dramatically shift the pH so no reaction can occur). Mix the tube’s contents. This “0” tube will serve as your blank. A blank is a mock assay in which no reaction will take place. The blank will be used by the spectrophotometer as a comparison to the reaction tubes. DO NOT do this step with the other tubes!

7) Take ONLY the tube labeled “0” and add 0.36 ml of 2 mg/ml ONPG. Mix the tube contents. The ONPG would ordinarily start the reaction, but because Na2CO3 is present no reaction will occur. DO NOT do this step with the other tubes!

8) Put the “0” tube in the 28˚ waterbath. It should not have any reaction, so in principle the temperature does not really matter, but you are trying to make the tube as similar as possible to the tubes you will process in the steps below.

Running the time course -- Here comes the timed part!!!

9) Now you will work with the tubes labeled 5, 10, 15, 20, 25 that you previously set aside. In principle you can work with the tubes in any order, but it is most time-efficient to do the next step starting with the 25 minute tube, then the 20 minute tube, then the 15 minute tube, then the 10 minute tube, then the 5 minute tube. This way the tubes that need to react the longest are started first. Have a timer or clock handy.

10) Starting with your 25 minute tube, initiate the reaction by adding 0.36 ml of 2 mg/ml ONPG to a tube. Mix thoroughly for a couple of seconds, then let the sample incubate at 28˚ in a waterbath. This temperature is a bit warmer than room temperature and is suitable for β-galactosidase to do its job. Record the time you added the ONPG. Figure out when the reaction will need to be terminated (e.g., since you are working with the 25 minute tube, you will need to terminate it 25 minutes after the time you added the ONPG) and write that down for future reference. Now initiate the reaction in the 20 minute tube by adding 0.36 ml ONPG, recording the start time, mixing, placing the sample at 28˚, and calculating the termination time. Repeat with the 15 minute, 10 minute and 5 minute tubes. Incubate Tube A and Tube B for 5 minutes.
11) As you have calculated, you will terminate the reactions at various times after initiation. Terminate the “5” tube, Tube A and Tube B reactions after 5 minutes, the “10” tube after 10 min., the “15” tube after 15 min., the “20” tube after 20 min., and the “25” tube after 25 min. of incubation with ONPG. Terminate each reaction by adding 0.9 ml of 1M Na₂CO₃. As you terminate each tube, mix the contents and place the tube on the bench.

Sodium carbonate (Na₂CO₃) overwhelms the buffering capacity of Z buffer and shifts the pH of the reaction to 11. β-galactosidase is not active at pH 11.

12) The blank “0” tube does not need any additional Na₂CO₃ as you have already added some. This tube may be taken from the waterbath at any time because its reaction has already been terminated.

13) Turn on the GENESYS 10S UV-Vis spectrophotometer. Note that you will be using the rectangular cuvettes that should always be filled with 2 ml of sample to make a measurement.

14) The instrument should be set in Basic ATC mode to take readings. You can toggle between Absorbance, %Transmittance, and Concentration modes by pressing the “Change Mode” button. Set the mode to Absorbance. Press “Set nm” to set the wavelength. Enter 420 and press “Set nm” again to set the wavelength of the spectrophotometer to 420 nm.

15) Once you have done this, carefully pipet 2 ml of your blank sample into a cuvette. Gently wipe the transparent sides of the cuvette with a Kimwipe to remove any debris and smudges. Then place the cuvette with the blank into the B position of the cell holder. Be sure to align the transparent sides with the direction of the beam (forward and back; the opaque sides will face left and right). You want to ensure that you always insert the cuvette in the same orientation to minimize the effects of any imperfections in the cuvette. It may be helpful to make a small mark at the top of one side with a Sharpie marker as a reference. Whenever inserting the cuvette, make sure the mark faces you.

16) Close the lid to the sample compartment. Press “Measure Blank” to set the absorbance of the spectrophotometer to zero using this cuvette. The blank corrects for any faint yellowness in the sample that is not due to enzyme activity. The display should now read “0.000A”.

17) Remove the cuvette and pipet the blank solution back into its test tube.

18) Next, carefully pipet 2 ml of the least yellow reaction into the cuvette. Place the cuvette into the B position of the cell holder and close the lid. Read the absorbance at 420nm for the assay off the display and record this number in your notebook (make a table!). Repeat for the other samples, including Tube A and Tube B. You do not need a fresh cuvette for every reaction tube; you can simply pipet the cuvette’s contents back into the reaction tube and replace them with that of the next reaction. You can rinse and blot the cuvette between samples to avoid carry-over but in practice this is usually not necessary. When you measure from the least yellow to the most yellow reaction there is only a slight error introduced from the few drops that cling to the cuvette because they are more weakly colored than the new sample and therefore can’t shift the color much. If you do rinse, be sure to dispose of the liquid into the waste beaker at your bench.

19) Once completed, dispose of all your liquid waste into the waste beaker at your bench. Rinse the test tubes with a couple of milliliters of water and dispose of the rinse waste into the same waster beaker.
Part II. INHIBITION OF β-GALACTOSIDASE

Some compounds act as inhibitors, blocking the activity of an enzyme. Some inhibitors mimic the substrate and thereby compete with the substrate for the active site. These are known as competitive inhibitors. Other inhibitors bind to the enzyme at a location other than the active site. In so doing, they cause a change in the conformation (i.e. shape) of the enzyme, preventing it from interacting properly with the substrate. These are known as non-competitive inhibitors. Both types of inhibition are reversible. The removal of a non-competitive inhibitor will restore the enzyme to an active conformation and normal rates of enzymatic activity. In the case of competitive inhibitors, increasing the concentration of substrate will increase the probability that the substrate fills the active site instead of the inhibitor. Thus, competitive inhibitors can be out competed by adding in additional substrate to a reaction.

In this experiment you will determine whether compound X is a competitive or non-competitive inhibitor for β-galactosidase. Propose a hypothesis about the nature of inhibition of β-galactosidase by Compound X (i.e., competitive or non-competitive). To test your hypothesis, you will need to:

1. Aliquot 4 mL 0.5 mM Compound X in Z Buffer pH 7 into a conical tube.
2. Make an appropriate blank solution (as you did in the time course).
3. Prepare a dilution series of your ONPG substrate:
   a. Number three microcentrifuge tubes 1 through 3.
   b. Pipet 500 μl of Z Buffer pH 7 into each tube.
   c. Make serial dilutions as follows:
      i. Tube 1: Add 500 μl of 2 mg/ml ONPG and gently pipet to mix. The concentration of ONPG in Tube 1 is now 1 mg/ml.
      ii. Tube 2: Add 500 μl of ONPG from Tube 1 and gently pipet to mix. The concentration of Tube 2 is now 0.5 mg/ml.
      iii. Tube 3: Add 500 μl of ONPG solution from Tube 2 and gently pipet to mix. The concentration of Tube 3 is now 0.25 mg/ml.
4. Run 5-minute enzyme assays (@ 28°C) using 1.68 ml 0.5 mM Compound X with each different concentration of ONPG (Tube 1, Tube 2 and Tube 3 that you just made). Make a table showing the contents and conditions of each reaction. Write a prediction for your experiment in the proper “if, then” format. Confirm your experimental design and prediction with your instructor before continuing. Make sure to keep proper records in your notebook.

Setup four reactions as follows:
1.68 ml of 0.5 mM Compound X (in Z Buffer, pH 7)
0.12 ml of β-galactosidase (1x)
0.36 ml of 2 mg/ml, OR 1 mg/ml, OR 0.5 mg/ml, OR 0.25 mg/ml ONPG

Remember, as soon as the substrate and enzyme come in contact, the reaction has started!

5. Don’t forget to include the control reaction (no Compound X)!
   1.68 ml of Z Buffer, pH 7
   0.12 ml of β-galactosidase (1x)
   0.36 ml of 0.25 mg/ml ONPG

6. Incubate the samples for 5 minutes at 28°C
7. Stop the reactions with 0.9 ml Na₂CO₃ after 5 minutes.
8. Measure the absorbance for each sample and record your results in your notebook.
Part III. INFLUENCE OF ENZYME CONCENTRATION ON β-GALACTOSIDASE ACTIVITY

In this experiment you will determine the influence of enzyme concentration on the rate of the reaction. To do so, you will make serial dilutions of your β-galactosidase stock to generate a range of enzyme concentrations which you can then assay. Propose a hypothesis about how changing the enzyme concentration will influence the rate of the enzymatic reaction. Write a prediction for your experiment in the proper “if, then” format. To test your hypothesis, you will need to:

1. Prepare the enzyme dilution:
   a. Number five microfuge tubes 1 through 5.
   b. Pipet 250 μl of Z Buffer pH 7 into each tube.
   c. Make serial dilutions as follows:
      i. **Tube 1 (1:1 dilution):** Add 250 μl of β-galactosidase stock (1x) and gently pipet to mix.
      ii. **Tube 2 (1:3 dilution):** Add 250 μl of β-galactosidase solution from Tube 1 and gently pipet to mix.
      iii. **Tube 3 (1:7 dilution):** Add 250 μl of β-galactosidase solution from Tube 2 and gently pipet to mix.
      iv. **Tube 4 (1:15 dilution):** Add 250 μl of β-galactosidase solution from Tube 3 and gently pipet to mix.
      v. **Tube 5 (1:31 dilution):** Add 250 μl of β-galactosidase solution from Tube 4 and gently pipet to mix.

2. Make an appropriate blank solution (as you did in the time course).

3. Run 5-minute enzyme assays (@ 28 oC) with each of the enzyme concentrations (including a no enzyme reaction). Make a table showing the contents and conditions of each reaction. Confirm your experimental design and prediction with your instructor before continuing. **Make sure to keep proper records in your notebook.**
   Your six reactions should each be set up as follows:
   1.68 ml of Z Buffer, pH 7
   0.12 ml of 1x, OR 0.5x, OR 0.25x, OR 0.125x, OR 0.0635x, OR 0.0312x, β-galactosidase
   0.36 ml of 2 mg/ml ONPG
   **Remember, as soon as the substrate and enzyme come in contact, the reaction has started!**

4. Incubate the samples for 5 minutes at 28 oC

5. **Remember to stop the reactions with 0.9 ml Na₂CO₃ after 5 minutes.**

6. Measure the absorbance for each sample and record your results in your notebook.
Part IV. INFLUENCE OF pH ON β-GALACTOSIDASE ACTIVITY

The pH of the environment can impact the 3D shape of the protein. Each enzyme has an optimal pH at which it works best. For example, the enzyme pepsin contributes to digestion, working effectively in the highly acidic stomach juices near pH 2. On the other hand, arginase has an optimal pH of about 9. In this experiment, you will determine the optimal pH for β-galactosidase. Deviation from the optimal pH range for an enzyme can lead to loss of proper 3D shape, i.e. denaturation.

Propose a hypothesis describing the rate of activity of β-galactosidase in various pH conditions.

Write a prediction for your experiment in the proper “if, then” format. To test your hypothesis, you will need to:

1. Make an appropriate blank solution (as you did in the time course).
2. Run 5-minute enzyme assays (@28°C) with Z Buffer at pH 4, 5, 6, 7, 8, 9, & 10. Make a table showing the contents and conditions of each reaction. Write a prediction for your experiment in the proper “if, then” format. Confirm your experimental design and prediction with your instructor before continuing. Make sure to keep proper records in your notebook.
   Your seven reactions should each be set up as follows:
   - 1.68 ml of Z Buffer, pH 4 OR pH 5, OR pH 6, OR pH 7, OR pH 8, OR pH 9, OR pH 10
   - 0.12 ml of β-galactosidase (1x)
   - 0.36 ml of 2 mg/ml ONPG
   Remember, as soon as the substrate and enzyme come in contact, the reaction has started!
   - Incubate the samples for 5 minutes at 28°C
3. Remember to stop the reactions with 0.9 ml Na₂CO₃ after 5 minutes.
4. Measure the absorbance for each sample and record your results in your notebook.
Part V. INFLUENCE OF TEMPERATURE ON β-GALACTOSIDASE ACTIVITY

Temperature is another environmental factor that can influence the activity of an enzyme. As temperature rises, the speed of molecules increases leading to more frequent collisions between molecules. In an enzymatic reaction, this means an increase in the collisions between the substrate and the active site, and hence, an increase in the speed at which product can be formed. Typically, a rise of 10°C results in a 2x or 3x increase in the rate of a reaction. However, the 3D shape of the protein can be lost if temperatures increase too high. In this case, the protein is said to be denatured. Because the 3D shape of the protein is critical to its ability to interact with and convert substrate to product, high temperatures can abrogate enzymatic activity. Just as with pH, each enzyme has an optimal temperature range. Some enzymes work best near body temperature, 37°C. Others, like Taq DNA polymerase, an enzyme from the thermophilic bacterium *T. aquaticus*, can operate at much higher temperatures (75-80°C).

Propose a hypothesis describing the rate of activity of β-galactosidase at various temperatures. Write a prediction for your experiment in the proper “if, then” format. To test your hypothesis, you will need to:

1. Make an appropriate blank solution (as you did in the time course).
2. Run 5-minute enzyme assays using Z Buffer pH 7 at 0°C (ice bath), 22°C (room temp), 28°C, 37°C and 50°C. Make a table showing the contents and conditions of each reaction. Write a prediction for your experiment in the proper “if, then” format. Confirm your experimental design and prediction with your instructor before continuing. Make sure to keep proper records in your notebook.
   Your five reactions should each be set up as follows:
   - 1.68 ml of Z Buffer pH 7
   - 0.12 ml of β-galactosidase (1x)
   - 0.36 ml of 2 mg/ml ONPG
   Remember, as soon as the substrate and enzyme come in contact, the reaction has started!

3. Incubate each reaction for 5 minutes in the waterbath with the appropriate temperature.
4. **Remember to stop the reactions with 0.9 ml Na2CO₃ after 5 minutes.**
5. **Measure the absorbance for each sample and record your results in your notebook.**
Part I. β-GALACTOSIDASE TIME COURSE

Identify the dependent and dependent variables in this experiment.

What is the purpose of Test Tube A? Of test Tube B? (i.e. what information do they provide?)

Why did you add Na₂CO₃ only to the 0 time point tube? What was the purpose of this tube?

Record the time course results in the table below:
Create a figure for the time course data, plotting your data on graph below. Give your figure a proper number, title and legend information.

Explain your results.

It is critical not to use the same pipet tip to measure out your enzyme and substrates. Why? What would happen if you did?
Part II. INHIBITION OF β-GALACTOSIDASE

Hypothesize about the nature of inhibition by Compound X (i.e., competitive or non-competitive).

Predict the results of the experiment based on your hypothesis (if/then).

Which test tube is the control?

Why was the concentration of the ONPG increased?

Record the results in the table below. If the data is not your own, indicate the Bio-6 section (AM/PM) and individuals responsible for generating the data in the table legend.
The independent variable is __________________________ and should be plotted on the ______ axis.

The dependent variable is __________________________ and should be plotted on the ______ axis.

Create a figure, plotting the data on graph below. Give your figure a proper number, title and legend information. If the data is not your own, indicate the Bio-6 section (AM/PM) and individuals responsible for generating the data in the figure legend.

Explain the results in terms of your hypothesis.
Part III. INFLUENCE OF ENZYME CONCENTRATION ON β-GALACTOSIDASE ACTIVITY

Formulate a question about enzyme concentration and reaction rate.

Hypothesize about the effect of changing enzyme concentration on the formation of o-nitrophenol.

Predict the results of the experiment based on your hypothesis (if/then).

Record your results in the table below. If the data is not your own, indicate the Bio-6 section (AM/PM) and individuals responsible for generating the data in the table legend.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The independent variable is __________________________ and should be plotted on the _______ axis.

The dependent variable is __________________________ and should be plotted on the _______ axis.
Create a figure, plotting the data on graph below. Give your figure a proper number, title and legend information. If the data is not your own, indicate the Bio-6 section (AM/PM) and individuals responsible for generating the data in the figure legend.

Explain the results in terms of your hypothesis.
Part IV. INFLUENCE OF pH ON β-GALACTOSIDASE ACTIVITY

Formulate a question about pH and reaction rate.

Hypothesize about the activity of β-galactosidase in various pH conditions.

Predict the results of the experiment based on your hypothesis (if/then).

Record the results in the table below. If the data is not your own, indicate the Bio-6 section (AM/PM) and individuals responsible for generating the data in the table legend.

<table>
<thead>
<tr>
<th>pH</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The independent variable is __________________________ and should be plotted on the _______ axis.

The dependent variable is __________________________ and should be plotted on the _______ axis.
Create a figure, plotting the data on graph below. Give your figure a proper number, title and legend information. If the data is not your own, indicate the Bio-6 section (AM/PM) and individuals responsible for generating the data in the figure legend.

Explain the results in terms of your hypothesis.
Part V. INFLUENCE OF TEMPERATURE ON β-GALACTOSIDASE ACTIVITY

Formulate a question about temperature and reaction rate.

Hypothesize about the activity of β-galactosidase at various temperatures.

Predict the results of the experiment based on your hypothesis (if/then).

Record the results in the table below. If the data is not your own, indicate the Bio-6 section (AM/PM) and individuals responsible for generating the data in the table legend.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The independent variable is __________________________ and should be plotted on the _______ axis.

The dependent variable is __________________________ and should be plotted on the _______ axis.
Create a figure, plotting the data on graph below. Give your figure a proper number, title and legend information. If the data is not your own, indicate the Bio-6 section (AM/PM) and individuals responsible for generating the data in the figure legend.

Explain the results in terms of your hypothesis.

What do you think would happen to reaction rate in the tube incubated in ice if this tube, with enzyme and substrate already mixed, were placed in the 37°C water bath? (explain your answer in terms of the effect of various temperatures on enzyme structure and the rate of enzyme activity)
REVIEWING YOUR KNOWLEDGE:

catalyst          enzyme

substrate        active site

cofactor         coenzyme

competitive inhibitor         non-competitive inhibitor

Compare and contrast competitive and noncompetitive inhibition.

Why does adding additional substrate overcome competitive but not noncompetitive inhibition?
Bio 6 – Restriction Enzyme Digestion Lab

Objectives

Upon completion of this laboratory you will understand how to: 1) set up and carry out a restriction enzyme digest of DNA, 2) carry out agarose gel electrophoresis of DNA, 3) stain DNA in an agarose gel and determine the approximate length of each DNA fragment, and 4) identify undigested or partially digested DNA fragments on the gel.

Introduction

A restriction endonuclease is an enzyme that catalyzes the hydrolysis or “cutting” of the DNA sugar-phosphate backbone at very specific sequences. Most people refer to restriction endonucleases as “restriction enzymes”, so from this point on the term restriction enzyme will be used. To understand what a restriction enzyme does, let’s consider three different restriction enzymes:

<table>
<thead>
<tr>
<th>restriction enzyme</th>
<th>restriction site</th>
<th>cut DNA product</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>5’…GAATTC…3’</td>
<td>5’…G AATTC…3’</td>
</tr>
<tr>
<td></td>
<td>3’…CTTAAG…5’</td>
<td>3’…CTTAA G…5’</td>
</tr>
<tr>
<td>SmaI</td>
<td>5’…CCCGGG…3’</td>
<td>5’…CCC GGG…3’</td>
</tr>
<tr>
<td></td>
<td>3’…GGGCC…5’</td>
<td>3’…GGG CCC…5’</td>
</tr>
<tr>
<td>PstI</td>
<td>5’…CAGCTG…3’</td>
<td>5’…CAGCT G…3’</td>
</tr>
<tr>
<td></td>
<td>3’…GTGAC...5’</td>
<td>3’…G TCGAC…5’</td>
</tr>
</tbody>
</table>

Each of the restriction enzymes shown above recognizes and cuts a very specific double-stranded DNA sequence referred to as a restriction site. There are literally hundreds of known restriction enzymes, each cutting a specific restriction site which is typically 4 to 8 base pairs. If you look carefully at each restriction site above you will notice that they are palindromic, i.e., the sequence on both strands is the same when read 5’ to 3’. This feature is characteristic of most restriction sites.

Another important feature of restriction enzymes is how they cut their corresponding restriction sites. Some enzymes such as SmaI cut the restriction site exactly in the middle on both strands producing cut DNA products with blunt ends. Most restriction enzymes cut their corresponding restriction sites in a staggered fashion leaving single-stranded overhangs. In the examples above, EcoRI produces ends with single-stranded 5’ overhangs and PstI produces ends with 3’ overhangs.

The majority of restriction enzymes cut 6 base pair palindromes as seen with the examples above, however not all restriction sites fit this pattern as shown in the examples on the next page:
<table>
<thead>
<tr>
<th>restriction enzyme</th>
<th>restriction site</th>
<th>cut DNA product</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AluI</strong></td>
<td>5’...AGCT...3’</td>
<td>5’...AG</td>
</tr>
<tr>
<td></td>
<td>3’...TCGA...5’</td>
<td>3’...TC</td>
</tr>
<tr>
<td><strong>NotI</strong></td>
<td>5’...GCGGCCGC...3’</td>
<td>5’...GC</td>
</tr>
<tr>
<td></td>
<td>3’...CGCCGG...5’</td>
<td>3’...CGCCG</td>
</tr>
<tr>
<td><strong>HaeII</strong></td>
<td>5’...RGCGCY...3’</td>
<td>5’...RGCGC</td>
</tr>
<tr>
<td></td>
<td>3’...YCGCGR...5’</td>
<td>3’...YRGCGR</td>
</tr>
<tr>
<td><strong>ScrFI</strong></td>
<td>5’...CCNGG...3’</td>
<td>5’...CC</td>
</tr>
<tr>
<td></td>
<td>3’...GGNCC...5’</td>
<td>3’...GGN</td>
</tr>
</tbody>
</table>

N = any base,  R = purine,  Y = pyrimidine

Notice that AluI, ScrFI and NotI have 4, 5 and 8 base pair restriction sites, respectively. HaeII and ScrFI are also atypical in that they have restriction sites that accommodate more than one base at select positions. The first and sixth positions in the HaeII restriction site can be any purine (A or G) or pyrimidine (C or T), respectively, whereas the third position of the ScrFI restriction site can be any of the four bases (A, C, G or T).

It should be no surprise that the number of times a DNA sample is cut by a given restriction enzyme depends on how often its restriction site appears in the sequence. This of course will vary from one DNA sample to another since every DNA sequence is unique. Nevertheless you can still calculate the probability of a given restriction site appearing in a DNA sample by random chance. This will allow you to estimate the average distance between each copy of a given restriction site, and thus the approximate number of times your DNA will be cut (assuming you know the total number of base pairs in your sample) as well as the average size the resulting DNA fragments. To illustrate this let’s look at the EcoRI restriction site:

**EcoRI**

<table>
<thead>
<tr>
<th>5’...GAATTC...3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>3’...CTTAAG...5’</td>
</tr>
</tbody>
</table>

Since there are four possible bases at any given position in a DNA sequence, there is a 1 in 4 chance that position will contain a particular base. For example the probability of any position in a DNA molecule containing the base G is \( \frac{1}{4} \). Since the EcoRI restriction site requires a G at position one, an A at position two, and so forth, the probability of any particular six base stretch of DNA being 5’-GAATTC-3’ is:

\[
\frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} = 1/4096
\]

The nature of probability dictates that the probability of multiple events occurring together is the product of the probabilities of each individual event. This applies to flipping coins, rolling dice, or the identity of nucleotides in DNA. Thus the probability of any six base sequence in a DNA molecule being 5’-GAATTC-3’ is \( (\frac{1}{4})^6 \) or \( 1/4096 \). What this means is that, simply due to random chance, the EcoRI restriction site should appear on average once every 4096 base pairs. Looking at this another way, EcoRI should cut DNA on average once every 4096 base pairs and thus the resulting DNA fragments should average ~4096 base pairs in length.
The calculation is slightly different when a restriction site contains positions with some flexibility as indicated by R, Y or N. The probability of a position containing any of the four possible bases is of course 4 out of 4 or 1, and the probability of a position containing a purine (A or G) or a pyrimidine (C or T) is 2 out of 4 or ½. With this in mind, let’s look at a couple more examples:

<table>
<thead>
<tr>
<th>restriction enzyme</th>
<th>restriction site</th>
<th>probability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HaeIII</strong></td>
<td>5’…RGCGCY…3’</td>
<td>½ x ¼ x ¼ x ¼ x ¼ x ½ = 1/1024</td>
</tr>
<tr>
<td>3’…YCGCGR…5’</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ScrFI</strong></td>
<td>5’…CCNGG…3’</td>
<td>¼ x ¼ x 1 x ¼ x ¼ = 1/256</td>
</tr>
<tr>
<td>3’…GGNCC…5’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DNA can also exist in a linear form or a circular form, something you will need to consider when cutting a DNA sample with a restriction enzyme. To see why this is important, let’s consider two hypothetical DNA molecules, one linear and one circular, each with a single EcoRI site:

As you can see, the DNA fragments resulting from restriction enzyme digestion of linear vs circular DNA will differ. With circular DNA you will get as many linear fragments as there are restriction sites in the molecule, whereas a linear molecule will yield one more fragment than the number of restriction sites. This is no different than cutting a piece of string with a pair of scissors. If a linear piece of string is cut 3 times you will end up with 4 pieces, whereas a circular piece of string cut 3 times will yield 3 pieces.

**Partial restriction enzyme digestion of DNA**

Any DNA sample used in a restriction enzyme digestion contains huge numbers identical molecules of DNA. For example, 1 µg of a 3000 bp plasmid contains over 300 billion (3 x 10¹¹) copies of the plasmid! So even if there is only one restriction site per plasmid for the enzyme being used, that’s over 300 billion sites that need to be cut. To cut them all and thus achieve “complete digestion” requires a sufficient amount enzyme and a sufficient amount of time. If complete digestion is not accomplished for any reason then you would have what is called “partial digestion”. This simply means that a significant proportion of the target restriction sites were not cut. You will be able to view the difference between a complete and partial digestion of a DNA sample at the end of this laboratory exercise.
Part 1: RESTRICTION ENZYME DIGESTION OF A DNA SAMPLE

Planning your reactions

Before you begin to put together a restriction enzyme digestion you need to have a plan. This is important to make efficient use of your time and materials. The materials you will use are expensive and you don’t want to make mistakes that force you to discard anything and start over. Before making your plan you need to consider each component of a restriction enzyme digest:

**DNA**
- What DNA sample do you want to cut and how much will you need per reaction?
- How many total reactions do you plan to carry out?

**Restriction Enzyme**
- What restriction enzyme will you use to cut your DNA sample?
- How many units* of restriction enzyme will you need in a single reaction?

* By definition, one unit of a restriction enzyme is enough to completely cut 1 µg of DNA in 1 hour, though in practice this is not always the case.

**Reaction Buffer**
- What reaction buffer is best for the restriction enzyme you plan to use?

**Ultrapure Water** (contaminants in impure water can interfere with the reaction)
- How much water will you need per reaction?

**Temperature**
- Every restriction enzyme works best at a particular temperature, so you need to determine the temperature at which you will incubate your reactions.

Once it is clear what components you will need per reaction and how many reactions you need to carry out, you can then make your plan. The most efficient way to put together your reactions is to pool together all components that are common to each reaction. This saves time and effort, is much more accurate, and minimizes mistakes. To illustrate this assume you want to cut 1 µg of 10 different DNA samples with 5 units of the restriction enzyme BamHI, and the available components are as follows:

- 10 different DNA samples (each at a concentration of 0.5 µg/µl)
- BamHI restriction enzyme (20 units/µl)
- reaction buffer supplied with the enzyme (10X concentration)
- ultrapure water

The reaction buffer required for a given restriction enzyme will be specified by the manufacturer of the enzyme. This information is available in the manufacturer’s catalog such as the one found at your table,
or online. For now, let’s assume we have all necessary components and are ready to make our plan. First we need to determine the total volume of each reaction, and the volume needed of each component per reaction. Let’s plan on a total reaction volume of 20 µl which will dictate the volume we need of each component:

**Components needed for a single reaction**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA sample</td>
<td>2 µl</td>
</tr>
<tr>
<td>BamHI</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>10X buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>water</td>
<td>15.75 µl</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>20 µl</strong></td>
</tr>
</tbody>
</table>

Let’s review the volume added for each component to clarify how we arrived at these numbers. The original DNA sample is at a concentration of 0.5 µg/µl so we will need 2 µl to get 1 µg total (2 µl x 0.5 µg/µl = 1 µg). The BamHI enzyme is at a concentration of 20 units/µl so we will only need 0.25 µl to get 5 units total (0.25 µl x 20 units/µl = 5 units). The reaction buffer and BSA are both at a 10X concentration (10 times the working concentration), so we will need 1/10th of the total volume of each (1/10 x 20 µl = 2 µl) to make the final concentration 1X. Last and certainly not least, we need to add enough ultrapure water so that the total volume is 20 µl. This is extremely important. If you don’t add the correct amount of water, the final concentrations of all other components will be off which can adversely affect your reaction.

If you plan to carry out a single restriction enzyme reaction then you are ready to proceed, however in this scenario we plan to carry out 10 reactions that differ only in the DNA sample being digested. As mentioned earlier, the best way to do this is to pool together all common components which are: the restriction enzyme BamHI, 10X buffer, and ultrapure water. It is always a good idea to make enough of your “pool” to accommodate one extra reaction to ensure that you don’t come up short. So in this example we will make a pool to accommodate 11 reactions as shown below:

**Pooled components for 11 reactions**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PstI</td>
<td>2.75 µl</td>
</tr>
<tr>
<td>10X buffer</td>
<td>22 µl</td>
</tr>
<tr>
<td>water</td>
<td>173.25 µl</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>198 µl</strong> (divided by 11 = 18 µl ea)</td>
</tr>
</tbody>
</table>

As you can see, each component common to all reactions was multiplied by 11. To ensure the amounts are correct, the total is divided by 11 resulting in 18 µl of the pool per reaction. This is exactly what we expect since the pool contains all components but the DNA which will account for 2 µl of each reaction (20 µl total – 2 µl DNA = 18 µl for everything else). It is extremely important to verify your calculations in this way to avoid a mistake that will affect every reaction.
Putting your reactions together

Now that you understand how to plan a set of restriction enzyme digests, you are ready to plan and carry out your own reactions. All of your reaction components except the water are very temperature sensitive, the enzyme in particular. Thus, it is important to keep them as cool as possible which means keeping them on ice until needed. Before you begin, first get an ice bucket filled with crushed ice. You can then acquire all needed components, thaw them if necessary, and store them on ice.

Once you have made your plan and have all necessary components on ice, keep in mind that the order in which components are added to your pool is very important. You want to avoid exposing the enzyme to an environment drastically different from the reaction itself. For example, you don’t want to add the enzyme to the 10X buffer and then add the water last. If you were to do it this way, the enzyme will be exposed to a much higher concentration of salts and other components than in the final reaction. As a rule of thumb, it is always best to add the water first and the enzyme last. For this reason, your pool should be put together in the order shown below:

1) ultrapure water
2) 10X buffer
3) DNA (only if the same for all reactions)
4) restriction enzyme

Now you are ready to carry out your restriction enzyme digest of DNA. The DNA you will be cutting is a small circular piece of DNA called a plasmid. Plasmids are commonly found in bacteria and contain additional genes not found in the bacterial chromosome. Plasmids are also very useful in the process of gene cloning as you will see later in the course. The plasmid DNA you will be cutting is 3342 base pairs (bp) in length, and the enzyme you will be cutting with is EcoRI.

Exercise 1 – EcoRI digestion of plasmid DNA

Each reaction should contain 1 µg of plasmid DNA (0.5 µg/µl) with 5 units of the restriction enzyme EcoRI (20 units/µl) in a total of 20 µl. Each student should prepare at least one reaction as described below:

1. Look up the restriction enzyme EcoRI in the New England Biolabs catalog at your desk and determine a) what 10X buffer should be used with this enzyme, and b) at what temperature the digestion should be carried out. Set a heat block (dry bath) or a water bath to the appropriate temperature.
2. Devise a plan (refer to previous two pages) to pool together all common ingredients so that there will be enough for 4 reactions plus 1 extra reaction.
3. Combine the components for your pool in a microcentrifuge tube in the order shown above, mixing gently by tapping the bottom of the tube after each addition. If necessary, do a quick spin at the end to bring all liquid to the bottom of the tube.
4. Label four microcentrifuge tubes “1, 2, 3 and 4” aliquot the calculated amount of pool into each tube.
5. Place each tube in the heat block at your table set to the appropriate temperature.
6. Remove tube 1 after 2 minutes and place on ice, remove tube 2 after 5 minutes and place on ice, remove tube 3 after 10 minutes and place on ice for 5 minutes, then place all 3 tubes in the freezer.
7. Leave tube 4 to digest for at least one hour (or overnight), then place in the freezer.
Part 2: AGAROSE GEL ELECTROPHORESIS

To visualize the products of your restriction digest you will need to carry out a very common laboratory technique called agarose gel electrophoresis. Agarose is a polysaccharide derived from red algae that is used to form a horizontal gel slab. When a DNA sample is loaded on an agarose gel and subjected to electric current, the DNA fragments in the sample move through the gel at a rate inversely proportional to DNA fragment length. Once sufficient separation has been accomplished the gel can be stained and the DNA fragment sizes visualized.

The process of agarose gel electrophoresis involves 3 general steps which we will address in order:

1) **Preparing** the gel
2) **Running** the gel
3) **Staining** the gel

**Preparing an agarose gel**

An agarose gel is a combination of agarose powder and an electrolyte solution called 1X TAE. "1X" refers to “one times the working concentration” (a stock solution of 50X TAE is diluted to make 1X TAE). “TAE” refers to tris-acetate (pH buffer system, electrolytes) plus EDTA (prevents DNA degradation). To prepare an agarose gel you need to know two things: 1) the percentage of agarose required in the gel and 2) the total volume of the gel. You are actually making a percent solution (grams/100 ml) in which the percent agarose is simply the weight of agarose powder in grams divided by the total volume in ml, multiplied by 100. The calculation to determine how much agarose powder you will need is simple. Once you know the volume you want, the amount of agarose is simply the desired agarose concentration (% agarose) multiplied by the desired total volume:

\[
0.8\% \times 50 \text{ ml} = 0.4 \text{ g}
\]

Notice that we ignore the volume of the agarose itself, which means that the actual percentage of agarose is slightly lower. The difference, however, is insignificant and the convention in laboratories is to assume that the total volume of your gel is determined by the electrolyte solution alone.

Once the agarose powder and 1X TAE have been measured, they are combined and boiled in a microwave oven after which the agarose solution is cooled to 50°C in a water bath. This is important since the agarose solution must be warm enough to remain liquid but not too hot to pour into a gel casting tray (if the gel mixture is too hot it can warp the casting tray). A comb suspended over the casting tray will result in the formation of cavities in the gel called wells as the gel cools. The solidified agarose gel slab will be placed in an electrophoresis chamber and completely submerged in 0.25X TAE **running buffer** (the lower concentration will allow you to run your gel faster). DNA samples can then be loaded into the wells and subjected to electric current, i.e., electrophoresis.
Exercise 2A – Prepare an agarose gel

The DNA sample you are currently subjecting to restriction enzyme digestion will be run on a 1% agarose gel. The gel casting apparatus you will be using requires a total volume of 60 ml. Calculate the amount of agarose you will need and follow the instructions below as you prepare your gel:

1. Weigh the necessary amount of agarose powder and add it to a 250 ml flask.
2. Add 60 ml of 1X TAE to the agarose in the flask.
3. Place a folded paper towel on the scale and weigh the flask. Record this weight and zero the scale.
4. Heat the mixture uncovered for 1 minute 30 seconds in a microwave. This will boil the mixture long enough to completely dissolve the agarose.
5. Using the rubber “hot hand” protector, remove the flask from the microwave and swirl to ensure the agarose is completely dissolved. If not, then boil in the microwave for an additional 30 seconds.
6. Place the flask on the scale and, using a water bottle, add deionized water until the weight is ~3 ml above the original weight of the flask/agarose mixture which you recorded in step 3. Swirl to mix.
7. Place the flask in a 50°C water bath for ~15 minutes to allow the gel mixture to reach 50°C. The gel mixture is cool enough when the base of the flask is comfortably warm to the touch.
8. While the gel mixture is cooling, set up the gel casting tray:
   • ensure the rubber seals are firmly in the grooves along each end of the casting tray
   • insert the tray into the electrophoresis chamber so that the ends of the tray are pressed snugly against the sides
   • insert the comb (use side with thinner teeth) into the notches at one end of the casting tray
9. Pour the 50°C gel mixture into the casting tray and allow the gel to cool for ~15 or 20 minutes.
10. Remove the casting tray containing the gel and turn it 90 degrees so that it rests parallel in the chamber with the wells on the left and the plugs facing away from you.
11. Add just enough 0.25X TAE to completely submerge the gel.
12. Carefully remove the comb from the gel. The gel is now ready for your DNA samples.

Loading and running an agarose gel

“Loading a gel” refers to adding DNA samples to the wells of an agarose gel. To ensure that the DNA samples remain in the wells prior to electrophoresis, a gel loading dye solution is added to each sample. Gel loading dye contains two key components:

- glycerol – makes the solution denser than water
- bromophenol blue – a negatively charged dye

The glycerol ensures that the DNA sample will sink to the bottom of the well and stay there when loaded. If not for the glycerol, the DNA sample would rapidly diffuse out of the well and be lost. The bromophenol blue dye, like DNA, is negatively charged and will migrate through the gel in the same direction as DNA. The migration of this dye thus allows one to monitor the progress of electrophoresis.
The actual loading of DNA samples into a well requires careful technique as shown below to ensure that the sample ends up in the well with minimal loss and without damaging the surrounding gel.

The keys to successfully loading an agarose gel are listed below:

- position the electrophoresis chamber so that you can comfortably load your samples without moving the chamber when you are finished (“sloshing” can result in the loss of your samples)
- get in a comfortable position (sitting is recommended unless it is easier to stand)
- steady your arms by leaning your elbows on the bench top
- use your opposite hand to steady the micropipettor as shown in the picture
- position the micropipettor tip just above, but not inside, the well to avoid puncturing the gel
- load the sample slowly
- remove the tip from the liquid before releasing the plunger

If you follow these suggestions, you will be sure have minimal sample loss when your load your gel.

Running a gel is fairly straightforward. The electrophoresis chamber is connected to a power supply (without disturbing the gel now loaded with DNA samples) such that the cathode is opposite the DNA samples. The power supply is then turned on, the voltage set, and the gel should be allowed to run until the bromophenol blue dye is approximately halfway across the gel.
As electrophoresis progresses, the DNA fragments migrate toward the cathode (due to the negative charge of DNA molecules) at a rate inversely proportional to the length of the DNA fragment. In other words, **shorter DNA fragments will migrate faster and longer DNA fragments will migrate more slowly.** The net result is a separation of DNA fragments based on length which is revealed upon staining.

While the relative length of DNA fragments is easy to see upon staining a gel, the *actual* length of a DNA fragment cannot be determined without running a sample of DNA fragments with known lengths on the same gel. Such a sample is called a **DNA ladder.** An example of a stained gel containing a lane with a DNA ladder (*) is shown below:

![DNA ladder image]

Relative to the known sizes of the ladder (k = kilobase pair), the length of the DNA fragments in each lane can be estimated. For example, the fragments in lane 2 are ~3000 base pairs (bp) and ~400 bp.

**Exercise 2B – Load and run an agarose gel**

1. Consult your lab mates to plan what samples will be loaded into what lane on your gel. Be sure to include one lane for the DNA ladder, ideally in the middle of the gel, and one lane for uncut plasmid DNA, both of which will provided by your instructor.

2. Add 2 µl of gel loading dye to each restriction digest. Mix by tapping and then quick spin your sample to bring all liquid to the bottom of the tube.

3. Make sure your gel is oriented so that the negatively charged DNA samples will run towards the positively charged anode, then load 10 µl of the DNA ladder and 10 µl of the uncut plasmid DNA.

4. Load all 22 µl of each restriction digest into the appropriate lanes.

5. Connect the electrophoresis chamber to the power supply, set the toggle switches to “low” and “volts”, and turn the knob to “min”. Turn on the power and adjust the knob until the readout just over 250 volts **(NOTE: if the gel is set up correctly a large amount of bubbles should rise from the anode)**

6. Run the gel until the bromophenol blue dye has moved a little more than halfway across the gel (about 15 minutes), then turn off the power.
Staining an agarose gel

Visualizing DNA fragments as bands on a gel requires the addition of a dye that is attracted to DNA. Two common dyes used for this purpose are ethidium bromide and methylene blue. Ethidium bromide is a more sensitive stain used routinely in research labs despite its classification as a mutagen (chemical that can cause mutations in DNA). Ethidium bromide is perfectly safe as long as it is handled properly. Methylene blue is a less sensitive dye that takes more time to stain and is commonly used in educational labs. To ensure that all bands on your gel are clearly visible and to save time, you will stain your gels with ethidium bromide.

Exercise 2C – Stain your agarose gel

1. Put on a pair of gloves and keep them on throughout the staining procedure.
2. Remove the casting tray with the gel from the electrophoresis chamber and carefully slide the gel off the tray into the plastic container at your table.
3. Drip a few drops of running buffer (0.25X TAE) on top of the gel to moisten it.
4. Peel the plastic film off an InstaStain ethidium bromide card and place it face down on top of the region of the gel you want to stain.
5. Place the gel casting tray on top of the staining card and a beaker on top of the casting tray. Leave for 5 minutes. (NOTE: the extra weight will ensure good contact between the staining card and the gel)
6. Remove the staining card from the gel and discard as biohazard waste.
7. Photograph your gel as indicated by your instructor and store a copy in your notebook.

Estimating DNA fragment sizes

Once your agarose gel is stained and bands of DNA are visible on the gel, you can estimate the approximate length of various DNA fragments by comparison with the DNA ladder. The DNA ladder you have used is called “1 kb-Plus Ladder” since it is a collection of DNA fragments that are in multiples of 1000 bp (1 kb) or 100 bp. The specific lengths of each DNA fragment in the ladder are shown on the right.

DNA fragment length can be estimated by plotting the distance each band travelled on semilog graph paper, however this is tedious and really no more accurate than doing a visual estimate as shown on the previous page.
Before you examine your gel you need to be aware that a circular piece of DNA (i.e., uncut plasmid) can exist in two different forms as illustrated below: relaxed and supercoiled.

During agarose gel electrophoresis, circular DNA in its relaxed form migrates at a different rate than supercoiled circular DNA. For this reason uncut plasmid DNA run on a gel usually results in two bands – a lower, faster migrating band of supercoiled DNA and higher, slower moving band of relaxed circular DNA. A linearized plasmid of the same size will migrate on the gel at a rate different from either circular form. Thus the same DNA molecule can migrate at 3 different rates and therefore end up in 3 different positions on a gel depending on whether it is linear, supercoiled or a relaxed circle. Since circular DNA runs at a different rate on a gel than linear DNA, a DNA ladder (which consists entirely of linear DNA fragments) can only be used to estimate the size of linear DNA fragments. Keep this in mind as you analyze your stained gel.

Exercise 2D – Estimate the length of each DNA fragment in your restriction digests

1. Compare each of your restriction digests with the uncut plasmid DNA on your gel and identify any bands that match and thus are uncut supercoiled or relaxed circular DNA. The lengths of DNA molecules in these bands cannot be estimated in relation to the DNA ladder, so don’t bother to do so.

2. The remaining bands on your gel should contain linear DNA fragments. Compare each of the remaining bands for each restriction digest to the 1 kb-Plus ladder and estimate the length of each fragment to the nearest 50 or 100 base pairs.

3. Examine the bands in the lane containing the DNA sample digested for the longest period of time (at least one hour) and:
   - determine how many EcoRI sites are in the plasmid
   - estimate the distance in base pairs (bp) between each EcoRI site
Restriction Enzyme Digestion Lab – Study Questions

1. What is a restriction enzyme? Give an example of one and its restriction sequence.

2. Indicate how often the following restriction sites should appear (on average) in a DNA molecule.

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlwNI</td>
<td>5’ - CAGNNNCTG - 3’</td>
<td>3’ - GTCNNNGAC - 5’</td>
</tr>
<tr>
<td>AvaI</td>
<td>5’ - CYCGRG - 3’</td>
<td>3’ - GRGCYC - 5’</td>
</tr>
</tbody>
</table>

3. What do you notice about each sequence above that is characteristic of most restriction sites?

4. Describe what is meant by the terms “sticky ends” and “blunt ends” with regard to linear DNA.

5. Why should you include a DNA ladder every time you run DNA samples on an agarose gel?

6. What is the relationship between DNA fragment length and the distance it will run in a gel?

7. A plasmid and a linear DNA molecule are each cut 7 times by a restriction enzyme. How many DNA fragments will be produced in each case?
8. You want to cut 1 µg of a plasmid with 4 units of the restriction enzyme HindIII for two hours at each of five different temperatures: 10°C, 20°C, 30°C, 40°C & 50°C. The plasmid stock is 250 ng/µl, the enzyme is 10 units/µl, the reaction buffer is 5X, and the total volume of each reaction is 20 µl. Using the New England Biolabs catalog, indicate your plan for setting up these reactions below.

9. What is a partial restriction enzyme digestion? How would you recognize partial digestion of a DNA sample on a stained agarose gel?

10. Describe how the rate of migration and thus the position on a gel of a DNA fragment will differ if the fragment is linear, supercoiled or a relaxed circle.
Overview

In this laboratory you will investigate three aspects of genes: 1) the structure of DNA - the molecule of which genes are made; 2) the copying or replication of DNA as it occurs during S phase of the cell cycle, and 3) the expression of genes into the protein products they encode. In addition, you will learn how to isolate DNA from cells.

Introduction

Deoxyribonucleic acid, or DNA for short, is a nucleic acid commonly referred to as “genetic material”. As you learned in a previous laboratory, genes are actually specific segments of chromosomes which are simply really long pieces of DNA! So given the obvious importance of DNA we want to make sure you understand DNA structure, how DNA is copied in cells, and how individual genes are expressed into “gene products”, which for most genes is a specific protein.

Part 1: DNA Isolation

DNA is surprisingly easy to purify from plant and animal tissues. You may recall from an earlier lab that chromosomes, which are made of DNA, are stored in the nuclei of eukaryotic cells. So to purify chromosomal DNA you need to somehow release it from the cell nuclei and then separate it from all other cellular materials. To do this will require three general steps:

1) breaking open cells in the tissue to release the DNA from cell nuclei
   - this is done by mashing the tissue in a solution with detergent and salt to break up cell membranes

2) removing the insoluble material (the DNA remains dissolved in liquid since it is soluble)
   - this is done by filtering the tissue “mash” and to isolate the liquid portion

3) precipitating the DNA in the liquid collected (making the DNA insoluble)
   - this is done by adding the right amounts of salt and alcohol to make the DNA insoluble

Now that you have a basic understanding of the principles involved, let’s purify some DNA from strawberry tissue...
Exercise 1 – Purification of DNA from strawberry tissue

Obtain your materials from the front of the lab, carefully follow the steps below, and in about 5 minutes you will have isolated strawberry DNA!

1. Add 4 ml of detergent/salt solution to the strawberry slice in a Ziploc bag.
2. Remove most of the air from the Ziploc bag, seal it, and thoroughly mash the strawberry slice in the detergent/salt solution (squeeze with hands or press on table top).
3. Set up a funnel over a glass beaker and line the funnel with 2 or more layers of cheese cloth (fold the cheese cloth into 2 or more layers).
4. Transfer as much of the strawberry mash as you can from the Ziploc bag to the cheese cloth in the funnel (if you like you can cut a corner of the bag and squeeze out its contents).
5. Allow the liquid from the strawberry mash to pass through the cheesecloth and collect in the beaker (you can squeeze the cheesecloth to help as much liquid as possible pass through).
6. Add 10 ml of ice cold ethanol to the liquid collected in the beaker and mix thoroughly by swirling the beaker.
7. Use a hooked glass rod to stir & collect the precipitated DNA.

Part 2: DNA STRUCTURE & DNA REPLICATION

DNA Structure

Like the proteins and polysaccharides you learned about earlier in the course, DNA is a polymer. Recall that a polymer is a chain of smaller molecules or monomers. For example, proteins are polymers of amino acids (i.e., amino acids are the monomers in proteins). So if DNA is a polymer of smaller monomers, what are the monomers in DNA? The answer is nucleotides.

All nucleotides in DNA have the same basic structure: the sugar deoxyribose connected to a phosphate group on one side and to one of four possible nitrogenous bases (“bases” for short) on the other. The phosphate group is acidic and thus negatively charged. This is why DNA has a net negative charge. Because all nucleotides in DNA contain deoxyribose they are called deoxyribonucleotides, though for simplicity we will just call them “nucleotides”. As shown below, the four different nucleotides in DNA are each referred to by their base: adenine (A), cytosine (C), guanine (G) and thymine (T).
These four nucleotides shown above can be linked together in any order to form a nucleotide polymer that is referred to as a **single strand** of DNA like the example shown below.

Notice that each nucleotide is connected to the next via the sugar (deoxyribose) and phosphate, thus forming what is called the "**sugar-phosphate backbone**" of a DNA strand. Notice also that each end of the sugar-phosphate backbone is different: what we call the **5' end** has a free phosphate group, while the other end has a free hydroxyl (–OH) group on the sugar, what we call the **3' end**. The 5' to 3' order of the bases in each nucleotide of a DNA strand constitutes its "**DNA sequence**".

The base of each nucleotide in a strand of DNA sticks out perpendicular to the sugar-phosphate backbone. This is important because DNA is actually a **double-stranded** molecule. The two strands of a DNA molecule are held together by interactions between the bases on each strand, a phenomenon known as **base pairing**.

On the next page are three different illustrations, each representing a double-stranded DNA molecule. Notice the base pairing between the two strands and that they are **anti-parallel** (the 5' and 3' ends are
oriented in the opposite direction). The two strands also form a coil or helix, hence the reference to DNA as a double-stranded helix or simply a “double helix”.

The base-pairing in a DNA molecule involves chemical attractions between certain bases due to hydrogen bonding. For base pairing to occur, the bases must have complementary chemical groups (e.g., partial negative matched with partial positive or vice versa) and complementary sizes (one larger purine – A or G – and one smaller pyrimidine – C or T). For these two reasons, the base pairing in DNA is limited to adenine with thymine (A:T base pairs) and guanine with cytosine (G:C base pairs):

The fact that each base can pair with only one partner gives DNA a remarkable property: the sequence of one DNA strand determines the sequence of the other. For example, if the sequence of one DNA strand is all A’s, the other strand must be all T’s for base pairing to occur between the strands. Let’s consider another example starting with the sequence of one strand:

5’ – C A T G C A A C G T C C A A A T T A G T – 3’
Since each strand of DNA molecule must be **complementary**, the two strands must be in an **anti-parallel** orientation and have nucleotides that will **base pair** with **all** the nucleotides on the opposite strand. Therefore, the double stranded sequence of this short DNA molecule must be:

\[
5' - C\ A\ T\ G\ C\ A\ A\ C\ G\ T\ C\ C\ A\ A\ T\ T\ A\ G\ T - 3' \\
3' - G\ T\ A\ C\ G\ T\ T\ G\ C\ A\ G\ G\ T\ T\ T\ A\ A\ T\ C\ A - 5' 
\]

Look carefully at this sequence and you will see that each strand is, in fact, anti-parallel to the other, and all of the nucleotides positioned across from each other can form a base pair (A:T or G:C). These two criteria are **essential** for the strands to interact with each other via base pairing to form a double helix. So if you know the sequence of one DNA strand you can easily determine the complementary sequence of the other strand since there is only one possibility.

At this point you know enough about DNA structure to do the next exercise in which you will build a model of double-stranded DNA after being given the sequence of a single DNA strand...

**Exercise 2A – Building a double-stranded DNA molecule**

*To build your DNA molecule use the model kit and key to the parts at your table. Your instructor will assign your group a single-strand DNA sequence after which you will follow the instructions below:*

1. On your worksheet, write the single-strand DNA sequence you’ve been given, indicating the 5’ and 3’ ends, and refer to the handout to familiarize yourself with the components of your DNA model kit.
2. Use the DNA model kit to build each nucleotide in your single strand sequence separately.
3. Assemble the nucleotides in the order of your sequence, beginning at the 5’ end.
4. Write the **complementary** sequence to your original single-strand sequence on your worksheet.
5. Build the complementary sequence as you did in steps 2 and 3 above.
6. Put the two DNA strands that you’ve just built together to form a double-stranded DNA molecule, being sure they are anti-parallel and that the base pairs match up properly.

*NOTE: Save the DNA molecule you have just built for use in Exercise 2B.*

**DNA Replication**

In a previous lab you learned that when a cell enters the cell cycle, in order to divide it must first copy all of its chromosomes – all of its DNA. This, as you should recall, will occur during **S** phase of the cell cycle (S for DNA **Synthesis**). The process of copying DNA, DNA synthesis, is formally known as **DNA replication**. DNA replication is an extremely complex process involving many different enzymes playing specific roles in the process. In the lecture part of this course you will learn about some of these enzymes, however for the purpose of this laboratory we will not be concerned with these details. We simply want you to learn how the process unfolds, knowing that many different enzymes are required.
The process of DNA replication depends on base pairing between nucleotides. As it turns out, each original DNA strand is used as a **template** or guide to produce a complementary DNA strand. This requires that the base pairs between the two original strands be disrupted, thus “unzipping” the DNA. A new strand with complementary nucleotides is then produced for each original strand. The net result is two identical copies of the original DNA molecule! Let’s revisit the DNA sequence in the example from the previous section to see how this would work:

```
5’ – C A T G C A A C G T C C A A A T T A G T – 3’
3’ – G T A C G T T G C A G G T T T A A T C A – 5’
```

For the above DNA molecule to be copied by DNA replication, the two strands must first be separated:

```
5’ – C A T G C A A C G T C C A A A T T A G T – 3’
3’ – G T A C G T T G C A G G T T T A A T C A – 5’
```

Once separated, each strand can be used as a template to produce a complementary strand. Each new complementary strand must, of course, be anti-parallel, and the enzymes that synthesize the new strands can only do so in a 5’ to 3’ direction as indicated below:

```
5’ – C A T G C A A C G T C C A A A T T A G T – 3’
3’ – G G T T T A A T C A – 5’
```

```
5’ – C A T G C A A – 3’
3’ – G T A C G T T G C A G G T T T A A T C A – 5’
```

Each new complementary nucleotide can only be added to the 3’ end of the growing new strand, one nucleotide at a time. Once the process is complete, you can see that the original DNA molecule has been accurately replicated in a 5’ to 3’ direction:

```
5’ – C A T G C A A C G T C C A A A T T A G T – 3’
3’ – G T A C G T T G C A G G T T T A A T C A – 5’
```

```
5’ – C A T G C A A C G T C C A A A T T A G T – 3’
3’ – G T A C G T T G C A G G T T T A A T C A – 5’
```

Now that you have a basic understanding of DNA replication you are ready to complete the next exercise...
**Exercise 2B – Replication of a DNA molecule**

Replicate the DNA molecule you built in Exercise 2A by following the instructions below. Although you may intuitively see how it all fits together, please DO NOT deviate from the instructions. It is important that you follow the instructions exactly to reinforce how this process works in cells. This is the whole point of the exercise! You’re basically playing the role of DNA polymerase which can only replicate DNA, with the help of several other enzymes, in the manner outlined below:

1. Completely “unzip” your DNA molecule by separating the two strands.
2. Working with one strand at a time, identify the nucleotide at the 3’ end of the molecule.
3. Build a nucleotide *complementary* to this nucleotide and base pair the new nucleotide with the nucleotide at the 3’ end of the original template strand.
4. Build a nucleotide complementary to the next nucleotide in the template strand and add it to the 3’ end of the preceding nucleotide, base pairing it with the template strand.
5. Repeat step 4 until the complementary strand is complete.
6. Repeat steps 2 through 5 with the other original strand to complete the DNA replication process.

**NOTE:** *Save the DNA molecules you have just produced for use in Exercise 3A.*
A gene is much like a recipe in a cookbook, with a chromosome being like the cookbook itself. Gene expression in this analogy would be equivalent to using a recipe in the cookbook to make a food item. However instead of instructing how to prepare a food item, a gene contains instructions on how to construct a protein (or in some cases an RNA molecule).

Like DNA replication, gene expression is very complicated and involves many different enzymes. Thus, we will leave most of these details to the lecture portion of the course and simply address how the process unfolds in general. The process of gene expression requires two distinct cellular processes: 1) the transcription of DNA encoding the protein into a very similar nucleic acid polymer called RNA (ribonucleic acid), and 2) the translation of the RNA sequence into a protein – a polymer of amino acids. How a DNA sequence encodes the amino acids in a protein involves the “genetic code”, something we will also address. Before we look into these concepts, however, we first need to become familiar with RNA.

RNA

RNA, like DNA, is a polymer of nucleotides. The nucleotides from which RNA is made, however, contain the sugar ribose which has one more –OH group than deoxyribose. The nucleotides in RNA are thus called ribonucleotides, though for simplicity we may also refer to them as “nucleotides” as we have done regarding DNA. The bases in ribonucleotides are the same as in deoxyribonucleotides with one exception: the base uracil (U) is used in place of thymine (T). Below is a diagram illustrating these differences:

One other key difference between RNA and DNA is that RNA exists as a single-stranded molecule. Even though RNA is single-stranded, its nucleotides still participate in base-pairing as you will see, with uracil (U) forming base pairs with adenine (A).
Transcription

Returning to the cookbook analogy, transcription is essentially making a photocopy of a recipe. The recipe is the DNA sequence encoding the instructions to build a protein, and RNA serves as a simple photocopy of the original recipe. This is a pretty good analogy, however it does not take into account the two strands of a DNA molecule, each having a different sequence. In fact, only one DNA strand of a gene actually contains the instructions for building a protein, the strand we call the coding strand. The complementary strand, called the template strand, does not contain any instructions yet it is extremely important in the process of transcription.

Transcription is actually very similar to DNA replication in that DNA is “unzipped” and used as a template to make a complementary strand of RNA instead of DNA. Unlike DNA replication, this occurs only within a single gene at a time (DNA replication results in the copying of entire chromosomes), and only one DNA strand, the template strand, is used to make RNA. The resulting RNA is complementary to the DNA template strand and thus a copy of the DNA coding strand sequence, with uracil (U) in place of thymine (T). To illustrate this, let’s pretend the DNA sequence we used earlier is to be transcribed:

\[
\begin{align*}
5' - &\text{C A T G C A A C G T C C A A A T T A G T} - 3' \quad \text{coding} \\
3' - &\text{G T A C G T T G C A G G T T T A A T C A} - 5' \quad \text{template} \\
5' - &\text{C A T G C A A C G T C C A A A T T A G T} - 3' \quad \text{coding} \\
5' - &\text{C A U G C A A} - 3' \\
3' - &\text{G T A C G T T G C A G G T T T A A T C A} - 5' \quad \text{template} \\
5' - &\text{C A T G C A A C G T C C A A A T T A G T} - 3' \quad \text{coding} \\
3' - &\text{G T A C G T T G C A G G T T T A A T C A} - 5' \quad \text{template} \\
5' - &\text{C A U G C A A C G U C C A A A U U A G U} - 3' \quad \text{RNA}
\end{align*}
\]

As you can see, the RNA produced is complementary to the template strand and identical in sequence to the coding strand, with U’s in place of T’s. In our cookbook analogy, we have just created a photocopy of the recipe! The RNA molecule is now ready to be used in the process of translation to make the protein encoded by its sequence. It is important to realize that, even though all RNA molecules are made by the process of transcription, they can be used in a variety of ways. At the moment we are only considering one role for RNA, serving as a copy of the coding strand in a gene. RNA used for this purpose is called messenger RNA or mRNA for short. Another role for RNA will be addressed when we look at the process of translation.
Let’s move on to the next exercise reinforcing the concept of transcription...

**Exercise 3A – Transcription of DNA into RNA**

Your group will transcribe the DNA molecules you produced in Exercise 2B. To do so you will consider the strand with the original sequence given by your instructor as the **coding strand**, and the other strand as the **template strand**. Once you have identified your coding and template strands, proceed as described below keeping in mind that this is how transcription occurs in cells:

1. Completely “unzip” your DNA molecule by separating the two strands.
2. Identify the nucleotide at the 3’ end of the **template strand**.
3. Build a **ribonucleotide** complementary to this nucleotide and base pair it with the nucleotide at the 3’ end of the DNA template strand.
4. Build a ribonucleotide complementary to the next nucleotide in the template strand and add it to the 3’ end of the preceding ribonucleotide, base pairing it with the DNA template strand.
5. Repeat step 4 until the complementary mRNA is complete.
6. Unzip the mRNA from the DNA template strand, and restore the DNA to its original double-stranded state.

*NOTE: Save the RNA molecule you have just produced for use in Exercise 3C.*

---

**The Genetic Code**

Before we look at translation, you need to understand the **genetic code**. While this may seem like yet another thing you need to learn in biology class, having knowledge of the genetic code is truly a remarkable privilege. Scientists and thinkers from the past would have given anything to know what you are about to learn, arguably the most fundamental biological process there is: how genes store information, information in the form of a DNA sequence that can be expressed into proteins and passed on to the next generation. In other words, you are about to learn what genetic information actually **means**, not just for human beings, but for **all** life on earth.

Once it was known that genes are made of DNA which somehow codes for proteins, a number of scientists set out to uncover the underlying genetic code. Since proteins are polymers of amino acids, they reasoned correctly that the sequence of nucleotides in DNA (or more specifically the sequence of bases) must somehow encode specific amino acids and their order in a protein. Recall from Lab 4 that there are 20 amino acids used to make proteins. This fact was well known in the early 1960s when these investigations began, thus the genetic code was assumed to consist of at least 20 different short nucleotide sequences, presumably of the same length.

Given that there are only 4 different one nucleotide sequences (A, C, G, T), 16 different two nucleotide sequences (AA, AC, AG, AT...), and 64 different three nucleotide sequences (AAA, AAC, AAG, AAT...), a code consisting of three nucleotide sequences was clearly the best candidate. Through some very clever experimentation the genetic code was soon discovered to be just that, a 3-nucleotide code which is read in a 5’ to 3’ direction. We now refer to each 3-nucleotide combination in the genetic code as a **codon**. The meanings of all 64 codons have been determined and are represented in the following chart:
This chart uses uracil (U) instead of thymine (T) since RNA, not DNA, is used to make proteins during the process of translation (though both nucleotides have the same meaning in the genetic code). The chart is organized to make it easy to find any particular codon. Codons in each row begin with the same base, codons in each column have the same second base, and codons in each box differ only in the third base.

If you look carefully you will notice that all 20 amino acids are represented in this code, with some being represented by only one codon (e.g., UGG for tryptophan), and others being represented by as many as six codons (e.g., leucine). You will also notice that there is a single start codon (AUG, which also codes for methionine), as well as three stop codons (UAA, UAG, UGA). The importance of these codons in the production of a polypeptide will be addressed as we move on to the final step of gene expression, translation, but not before you complete the next exercise.

**Exercise 3B – Understanding the genetic code**

Use the chart of the genetic code on this page to complete the corresponding exercises on your worksheet.

**Translation**

A messenger RNA molecule (mRNA) produced by transcription in the nucleus of a cell is transferred to the cell cytoplasm where its sequence is translated into proteins by **ribosomes**. This process, known as translation or protein synthesis, also involves another functional type of RNA, **transfer RNA (tRNA for short)**. Each tRNA is attached one of the 20 different amino acids, ready to deliver it to a ribosome when needed, and also contains a 3-nucleotide **anticodon**. The role of ribosomes is to: 1) facilitate base-pairing of mRNA codons with tRNA anticodons, and 2) catalyze the formation of peptide bonds between amino acids delivered by consecutive tRNAs. This basic process is illustrated in the diagram below:
The process begins when a ribosome locates the start codon – AUG. This is extremely important to ensure that the ribosome not only begins where it is supposed to, but also uses the correct reading frame. From this point the ribosome, working with two codons and two tRNAs at a time, will move in a 5’ to 3’ direction down the mRNA until it reaches a stop codon and ends the process. To see how important start codons and reading frames are, let’s consider the mRNA sequence you produced earlier:

5’ – CAUGCAACGUCCAAUUAUGU – 3

Beginning with the AUG start codon and moving in the 5’ to 3’ direction, a ribosome would translate this mRNA sequence into the following polypeptide (see your genetic code chart):

met – gln – arg – pro – asn (stop)

If translation was to begin at CAU instead of the start codon AUG, the ribosome would be using the wrong reading frame and would produce an entirely different polypeptide:

5’ – CAUGCAACGUCCAAUUAUGU – 3

his – ala – thr – ser – lys – leu

There is also a third reading frame which would yield a completely different polypeptide as well:

5’ – CAUGCAACGUCCAAUUAUGU – 3

cys – asn – val – gln – ile – ser

As you can see, beginning translation at the start codon ensures that the codons are read correctly and the polypeptide is made correctly. In this way, a DNA sequence transcribed into an identical RNA sequence can be used to consistently produce many copies of the same polypeptide.
Now that you understand the process, let’s put gene expression into context. Pretend you just ate a starchy meal, say a mashed potato. As a consequence, your blood sugar (glucose) will go up and special cells in your pancreas will produce more insulin to help restore your blood sugar to normal levels. Insulin is a protein consisting of a single polypeptide. To make more insulin, these pancreatic cells will have to **express the insulin gene**. This means transcribing the insulin gene into mRNA followed by ribosomes translating insulin mRNA into insulin polypeptides. A single insulin gene (you actually have two, one from mom and one from dad) can yield many mRNA copies by transcription, and each mRNA can yield many insulin polypeptides. Thus huge amounts of the gene product, in this case the protein hormone insulin, can be expressed from a single gene.

**Exercise 3C – Translation of mRNA into a polypeptide**

*In this exercise you will function as a ribosome and translate the mRNA molecules you produced in Exercise 3A into a short polypeptide. Before you do so, be sure that all your tRNAs are connected to the correct amino acid and then proceed exactly as described below:*

1. Identify the AUG start codon in your mRNA sequence.
2. Create a tRNA using your model kit that has an anticodon complementary to AUG and an attached amino acid.
3. Base pair the anticodon of this tRNA with the AUG start codon in the mRNA.
4. Create a tRNA using your model kit that has an anticodon complementary to the next codon in your mRNA molecule and an attached amino acid (be sure you are going in a 5’ to 3’ direction).
5. Base pair the anticodon of this tRNA with the mRNA codon.
6. Form a peptide bond between the amino acids of the adjacent tRNAs and remove the first tRNA (the dipeptide just formed should now be attached only to the second tRNA).
7. Repeat steps 4 through 6 based on the 3rd codon in your mRNA after which you should release your polypeptide from the last tRNA which should then be detached from the mRNA.

*NOTE: Although not shown in this exercise, in a cell a ribosome would facilitate this process.*
Ex. 1 – Purification of DNA from strawberry tissue

➢ How did you release the DNA from cells in the strawberry slice?

➢ What did you add to the liquid filtrate to make the strawberry DNA insoluble?

➢ Describe the appearance of your strawberry DNA after it was precipitated.

➢ What property of DNA would make it water soluble? Did any of your precipitated DNA appear to dissolve in water?

Ex. 2A – Building a double-stranded DNA molecule

➢ Write the DNA sequence your group was given below, then write the complementary strand just below it (be sure to also indicate the 5’ and 3’ ends of each DNA strand).

➢ What does it mean to say that the strands in a DNA molecule are anti-parallel?

➢ What chemical groups identify the 3’ and 5’ ends of a DNA strand?

Ex. 2B – Replication of a DNA molecule

➢ Using different colors for “old” and “new” DNA strands, write the DNA sequences of both DNA molecules resulting from your completion of the DNA replication exercise.
Based on your model of DNA replication, does the original DNA molecule stay intact?

What is meant by the term DNA template?

Ex. 3A – Transcription of DNA into RNA

Using different colors for RNA and DNA strands, write the DNA sequence of your original DNA molecule as well as the RNA molecule produced by transcription. As always, be sure to label the 5’ and 3’ ends of each strand, and correctly label the DNA template and coding strands.

DNA and RNA differ in three basic ways. What are these three differences?

Ex. 3B – Understanding the genetic code

Indicate all codons that specify the amino acid serine.

If the genetic code consisted of codons with 4 nucleotides, how many different codons would there be?

Determine the amino acid sequence encoded by the following mRNA sequence, and don’t forget to begin with the start codon:

5’ – GCGUAUGACCGUUAUAGAUGGGCGUCUCCACACUGAAUACUAACGAAU – 3’
What is the reading frame of a DNA sequence? Why is this so important?

Ex. 3C – Translation of an mRNA into a polypeptide

Write out the mRNA sequence you generated by transcription in Exercise 3A, and below the sequence indicate the amino acid sequence your mRNA encodes:

The amino acids used during translation are attached to what kind of molecule?

Describe the two basic roles of ribosomes during the process of translation.

Questions for Review:

a) Briefly describe the roles of the following enzymes in DNA replication (in the bacterium *E. coli*):

- DNA polymerase I
- DNA polymerase III
- Helicase
- Topoisomerase (Gyrase)
- Primase
- DNA ligase

b) Describe the role of RNA polymerase in transcription.
Bio 6 – Restriction Enzyme Mapping Lab

Objectives

In this laboratory you will cut plasmid DNA with various combinations of restriction enzymes to map the relative positions of the corresponding restriction sites on the plasmid. In the process you will learn how to plan and carry out the simultaneous digestion of DNA with multiple restriction enzymes, and how to examine the resulting DNA fragments to deduce the relative position of each restriction site on the plasmid.

Introduction

In the previous lab you learned how to digest a DNA sample with a restriction enzyme and examine the resulting restriction fragments by agarose gel electrophoresis. You will use the same techniques in this laboratory for the purpose of creating a restriction map – a map of the relative positions of multiple restriction sites. The DNA molecule for which you will create the map is a circular plasmid that contains a total of 3342 base pairs (bp) of DNA:

Before you begin it is important to practice creating a restriction map to see how this is done. Let’s consider a hypothetical 3000 bp plasmid and create a restriction map involving three restriction enzymes: EcoRI, HindIII and XbaI. In order to create the map you will need first of all to know how many times each enzyme cuts the plasmid. This can be determined by cutting samples of the plasmid with each restriction enzyme alone. You will also need to cut samples of the plasmid with combinations of these enzymes in order to deduce the relative positions of their restriction sites. This will involve cutting samples of the plasmid with all possible combinations of two enzymes as well as all three enzymes together. Let’s assume you have done this and got the following restriction fragments (in bp) for each digestion:

- **EcoRI (alone)**: 3000 bp
- **HindIII (alone)**: 3000 bp
- **XbaI (alone)**: 3000 bp
- **EcoRI + HindIII**: 2200, 800 bp
- **EcoRI + XbaI**: 1700, 1300 bp
- **HindIII + XbaI**: 2500, 500 bp
- **EcoRI + HindIII + XbaI**: 1700, 800, 500 bp
Recall from the previous lab that a circular DNA molecule such as a plasmid will yield as many fragments as there are cuts in the molecule. Based on this it is clear that each restriction enzyme cuts the plasmid only one time, so the map will contain only three restriction sites, one for each enzyme. The double digests allow you to come to the following conclusions and ultimately the positions the restriction sites relative to each other:

- the EcoRI and HindIII sites are 800 bp apart
- the EcoRI and XbaI sites are 1300 bp apart
- the HindIII and XbaI sites are 500 bp apart

Based on this the only map that works is the following:

![Diagram of restriction site positions](image)

Drawing the map is simple. Diagram a circle representing the plasmid and arbitrarily place one restriction site at the top (EcoRI in this example). Choose one of the remaining restriction sites (e.g., HindIII) and position it relative to the first based on the double digest involving both enzymes. All that’s left is to position the third restriction site (XbaI in this example) for which there are two possibilities. Relative to EcoRI, XbaI can only be positioned 1300 bp on one side or 1300 bp on the other. In one case, that puts XbaI 500 bp away from the HindIII site which is consistent with the HindIII + XbaI double digest. In the other case, XbaI is positioned 900 bp from the HindIII site which is not consistent with the data. The correct position for the XbaI site can therefore only be 1300 bp from EcoRI and 500 bp from HindIII. The triple digest was not essential to deduce the map, however it is important to confirm the map you deduced. In this case the map you deduced is consistent with the triple digest and you can be confident it is correct. If they did not match, you would know you made a mistake somewhere and would have to reexamine your data.

**Part 1: RESTRICTION ENZYME DIGESTS**

**Planning your restriction digests**

Digesting a DNA sample with multiple restriction enzymes simultaneously is not as straightforward as it may seem. Each restriction enzyme functions optimally under very specific conditions of temperature, pH, salt concentration, etc. In a simultaneous double or triple digest, the enzymes involved must work in the same environment and therefore must have compatible buffer systems. You may remember from
the previous lab that you added **10X reaction buffer** when preparing your reaction. The 10X reaction buffer is supplied by the manufacturer of the enzyme and contains all necessary components for the enzyme at 10X the normal concentration. When diluted to 1X it will provide the optimum chemical environment for the enzyme to function. In reality, the optimum chemical, as well as temperature, environments for two different restriction enzymes is frequently different. It is up to you to determine if the two (or three) restriction enzymes you plan to use can function well in the same environment. If not, the DNA sample will have to be digested sequentially with each enzyme (i.e, each enzyme alone).

To illustrate this, let’s go back to the example in the introduction involving the restriction enzymes *EcoRI*, *HindIII* and *XbaI*. The enzymes you will use are purchased from the biotech company **New England Biolabs**. Like any other biotech company that produces enzymes, New England Biolabs produces a variety of 10X buffers that can be used with its enzymes. The ideal 10X buffer for each enzyme they produce is indicated in the New England Biolabs catalog, a book that contains a wealth of information about each enzyme in addition to pricing. There should be several New England Biolabs catalogs at each table, so be sure to locate one as we are about to use it (this information can also be obtained online at [www.neb.com](http://www.neb.com)).

Find the section on restriction enzymes and look up *EcoRI* (they will be listed alphabetically). The information we are most interested in are the:

- recommended temperature
- recommended 10X buffer
- activity with the other 10X buffers

According to the manufacturer, *EcoRI* digestion should be carried out at 37°C using a unique *EcoRI* 10X buffer which is supplied with the enzyme. Lastly, and most importantly, we want to see *EcoRI* activity in other 10X buffers. New England Biolabs has a system of four different 10X buffers (NEBuffer 1.1, NEBuffer 2.1, NEBuffer 3.1 and CutSmart) that cover the needs of most restriction enzymes (*EcoRI* is unusual in being provided with its own unique 10X buffer). The activity of a restriction enzyme in each of the four standard buffers will always be indicated since it is essential for planning multiple digests (which is what we are doing!). Notice that *EcoRI* has 100% activity only in buffer NEBuffer 2.1.

Let’s now look at *HindIII*. Notice that *HindIII* digestion should be carried out at 37°C using 10X NEBuffer 2.1, and it has 100% activity **only** in NEBuffer 2.1. Looking at *XbaI*, digestion should be carried out at 37°C using CutSmart buffer, and the enzyme is also 100% active in NEBuffer 2.1. Based on the information you just obtained for all three enzymes, we should be able to carry out successful double and triple digests involving these three enzymes at **37°C in 1X NEBuffer 2.1**.

Now that you understand what you need to consider when planning a restriction digest involving multiple enzymes simultaneously, you can plan the restriction digests you will carry out in order to map the following restriction sites on your plasmid DNA:

- **BamHI**
- **NdeI**
- **ScaI-HF**
Exercise 1 – Restriction enzyme digestions of plasmid DNA

The class will work together to plan the 7 different restriction enzyme digestions needed for this exercise – each enzyme alone, each enzyme paired with each other enzyme, and all three enzymes together. Each group will then be responsible for preparing enough of one reaction to be distributed among all groups in the class.

1. Look up the restriction enzymes BamHI, NdeI and Scal-HF in the New England Biolabs catalog at your table (or online) and identify the a) reaction temperature, b) recommended 10X buffer, and c) other 10X buffers that will give 100% activity for each enzyme. As a class you will determine a set of reaction conditions that will work for all 7 restriction enzyme digestions.

2. Set a heating block (dry bath) or water bath to the desired reaction temperature.

3. As a class, determine the components needed for each reaction which should contain 1 µg of plasmid DNA (0.5 µg/µl stock) and 5 units of each restriction enzyme (20 units/µl stock) in a total volume of 20 µl. Your group will then be assigned a specific reaction to prepare for all groups in the class.

4. Plan and put together your group’s assigned reaction so there will be enough for each group in the class plus one extra (e.g., for 8 groups, pool together the equivalent of 9 reactions). Label enough tubes for each group in the class, transfer 20 µl of your reaction to each tube, and bring to your instructor.

5. Acquire one tube for each different restriction enzyme reaction at the direction of your instructor.

6. Place all tubes in a heat block (or water bath) at the appropriate temperature and incubate overnight.

Part 2: AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis of your restriction digestes will be identical to Lab 9, so refer to the Restriction Enzyme Digestion Lab to load, run and stain your gel (NOTE: You should also be able to prepare and run your gel by following your procedure for this lab in your lab notebook).

Exercise 2 – Agarose gel electrophoresis of restriction digestes

1. Pour a 60 ml 1% agarose/1X TAE gel keeping in mind that you will need at least 9 lanes. Be sure to use the side of the comb with thinner teeth to ensure sharper bands when you stain your gel.

2. Plan how your group will load the gel and record in your lab notebook. Keep in mind you will also be loading 1 kb-Plus DNA ladder and uncut plasmid DNA (10 µl each). NOTE: It is a good idea to place the DNA ladder in the middle so that it will be no further than 4 lanes from any sample.

3. Add 1/10 volume of gel loading dye to each restriction digest, tap to mix and quick spin.

4. Once your gel is submerged in 0.25X TAE, load all of each sample into the appropriate lane.

5. Run the gel at just over 250 volts for ~15 minutes (until bromophenol blue is a little beyond halfway).

6. Stain your gel with an InstaStain ethidium bromide card as in the previous lab. If necessary you can use more than one stain card to ensure all DNA samples in your gel are completely stained.

7. Photograph your gel as indicated by your instructor and store a copy in your notebook.
Part 3: CREATING A RESTRICTION MAP

Now that you can see the restriction fragments for each of your digests, you can estimate the length of each fragment by comparison with the 1 kb-Plus DNA ladder. The lengths of each fragment in the 1 kb-Plus DNA ladder are shown on the right.

It should be clear that the sum of all fragments in a given digest should add up to the total size of the plasmid which is 3342 base pairs. Since you can only estimate fragment size to the nearest 50 bp, it would be appropriate to round off the total plasmid size to 3350 bp. Since smaller fragment sizes can be estimated more accurately than larger fragments, you should first estimate the sizes of the smaller fragments and then subtract their sizes from the total of 3350 to determine the size of the largest fragment.

Exercise 3 – Construct a restriction map

1. Estimate the length of each DNA fragment in each restriction digest and record in your notebook.

2. Draw a circle in your notebook to represent the plasmid and construct your map by following the approach described on pages 1 and 2.

NOTE: For this laboratory you will turn in a formal lab report.
1. An unknown DNA molecule was cleaved using several restriction enzymes individually and in various combinations. The DNA fragment sizes were determined by agarose gel electrophoresis and the restriction enzyme recognition sites were mapped. Subsequently, the DNA was sequenced and an extra recognition site was found for one of the enzymes. All the other mapping data was consistent with the sequence data. How could you explain this extra site that was missed, assuming the DNA sequence had no errors.

2. A plasmid was cleaved with several restriction enzymes, individually and in various combinations. The following fragment sizes (base pairs) were determined by agarose gel electrophoresis.

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Fragment Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>4364</td>
</tr>
<tr>
<td>AvaI</td>
<td>4364</td>
</tr>
<tr>
<td>PvuII</td>
<td>4364</td>
</tr>
<tr>
<td>PstI</td>
<td>4364</td>
</tr>
<tr>
<td>EcoRI + AvaI</td>
<td>2938, 1426</td>
</tr>
<tr>
<td>EcoRI + PstI</td>
<td>3608, 756</td>
</tr>
<tr>
<td>AvaI + PvuII</td>
<td>3722, 642</td>
</tr>
<tr>
<td>AvaI + PstI</td>
<td>2182</td>
</tr>
<tr>
<td>PvuII + PstI</td>
<td>2824, 1540</td>
</tr>
<tr>
<td>EcoRI + PvuII</td>
<td>2296, 2068</td>
</tr>
</tbody>
</table>

Create a restriction map based on this data, indicating the distances between each restriction site.

Why was only one size band detected in the AvaI + PstI double digest?
3. Consult the New England Biolabs catalog (or website) to plan a triple digestion (one reaction containing 3 different restriction enzymes) of 1 µg of plasmid DNA in a 30 µl total volume using 5 units of each of the following restriction enzymes: \textit{BglII}, \textit{NcoI} and \textit{NotI}. Assume the plasmid DNA stock is 400 ng/µl and each restriction enzyme is 20 units/µl.
Introduction
This lab will illustrate the central dogma, the sequential flow of information from DNA to RNA to protein:

![Diagram showing DNA to RNA to Protein]

To illustrate this principle, you will perform a procedure known as genetic transformation. In doing so, you will alter the genotype, the collection of genes, of an organism. Remember that a gene is a piece of DNA which provides the instructions for making (i.e. it “codes for”) a protein. The activity of a protein contributes to one or more traits (the observed phenotype). Genetic transformation literally means “change caused by genes,” and involves the insertion of a gene into an organism in order to change an organism’s trait(s). This technique is used in many areas of biotechnology. In agriculture, genes coding for traits such as frost, pest, or spoilage resistance can be genetically transformed into plants. In bioremediation, bacteria can be genetically transformed with genes enabling them to digest oil spills. In medicine, diseases caused by defective genes are beginning to be treated by gene therapy; that is, by genetically transforming a sick person’s cells with healthy copies of the defective gene that causes the disease.

You will use a procedure to transform bacteria with a gene that codes for Green Fluorescent Protein (GFP). The source of this gene is the bioluminescent jellyfish *Aequorea victoria*. GFP causes the jellyfish to fluoresce and glow in the dark. Following the transformation procedure, the bacteria express their newly acquired jellyfish gene and produce the fluorescent protein, which causes them to glow a brilliant green color under ultraviolet light.

Moving genes from one organism to another is typically accomplished with the aid of a plasmid. In addition to one large chromosome, bacteria naturally contain one or more small circular pieces of DNA called plasmids. Plasmid DNA usually contains genes for one or more traits that may be beneficial to bacterial survival. In nature, bacteria can transfer plasmids back and forth allowing them to share these beneficial genes. This natural mechanism allows bacteria to adapt to new environments. The recent occurrence of bacterial resistance to antibiotics is due to the transmission of plasmids.

The pGLO plasmid carries several genes. One gene, *bla*, codes for the protein β-lactamase, which degrades the antibiotic ampicillin. A bacterial cell carrying the *bla* gene would thus be resistant to the ampicillin antibiotic. This gene is constitutively expressed in cells meaning that it is constantly expressed (transcribed and translated). The pGLO plasmid also carries the *GFP* gene which codes for the Green Fluorescent Protein. Expression of the *GFP* gene in pGLO is controlled by the pBAD promoter. A promoter is the region of DNA where RNA polymerase binds to begin transcription of a gene. A third gene, *araC*, codes for the protein AraC and is also constitutive. In the absence of the sugar arabinose, the AraC protein binds to the
pBAD promoter, blocking RNA polymerase and thereby preventing the GFP gene from being transcribed. However, in the presence of arabinose, AraC binds to arabinose directly, resulting in a conformational change in the protein. This change in shape promotes the binding of RNA polymerase to the promoter, which in turn causes the transcription of the GFP gene into messenger RNA (mRNA), followed by the translation of this mRNA into GFP.

The incidence of bacterial transformation in nature is very low. However, cells can be chemically treated with CaCl₂ to make them competent to pick up DNA from the environment. Chemically competent cells are incubated with plasmid DNA and then briefly heat shocked to induce them to pick up foreign DNA. Only a very small number of cells will successfully take up the plasmid. However, the bla gene serves as a selectable marker. Selection for cells that have been transformed with pGLO DNA is accomplished by growth on ampicillin plates. Only cells that have successfully been transformed will be capable of growth in the presence of ampicillin.
The transformation procedure involves three main steps. These steps are intended to introduce the plasmid DNA into the *E. coli* cells and provide an environment for the cells to express their newly acquired genes.

To move the pGLO plasmid DNA through the cell membrane you will:
1. Use a transformation solution containing CaCl₂ (calcium chloride).
2. Carry out a procedure referred to as heat shock.

For transformed cells to grow in the presence of ampicillin you must:
3. Provide them with nutrients and a short incubation period to begin expressing their newly acquired genes.

---

**Day 1 – Bacterial Transformation by Heat-shock**

**Responsible Handling of *E. coli***

*E. coli* is an organism which is a normal part of the bacterial flora of the gut. It is rarely associated with illness, and adherence to simple handling and disposal techniques makes *E. coli* a non-threatening experience for students. Specifically, when handling *E. coli*, don’t allow any utensil in direct contact with the organism to touch the skin, clothing or working surface. Dispose of all items in contact with *E. coli* in the biohazard bag. Wipe the lab bench with disinfectant at the beginning and end of the lab. Lastly, don’t forget to wash your hands before leaving the lab!

1. Obtain a sterile Eppendorf tube. Close the cap and mark it “+”. Place this tube on ice. (pGLO will be added to the “+” tube; none will be added to the “−” tube which you will label shortly.)

2. Obtain the tube of competent *E. coli* cells from your instructor. These cells are suspended in a solution of calcium chloride to render them competent. Completely thaw the cells on ice. Keep this tube on ice and do not allow it to warm.

3. Aliquot 25 µl of competent cells into the Eppendorf tube labeled “+” from step 1.

4. Label the tube containing the remaining competent cells with “−” (this is the tube of cells that will not be given any pGLO plasmid). Keep both tubes on ice.

5. Add 20 ng of plasmid DNA to the “+” sample. *Gently tap to mix and keep the tubes on ice after adding the DNA.* Add an equivalent volume of ultrapure water to the “−” sample.

6. Incubate both tubes on ice for 10 minutes.

7. While the tubes are incubating, label the **bottom** of the media plates with your lab group name and date. Then labels as follows:
   - Label one LB/amp plate: + pGLO
   - Label the LB/amp/ara plate: + pGLO
   - Label the other LB/amp plate: - pGLO
   - Label the LB plate: - pGLO
8. Following the 10-minute incubation on ice, “heat shock” the cells. To do this, remove both tubes directly from ice and immediately immerse them in the 42°C water bath for **exactly 90 seconds**. Immediately return both the tubes directly to ice for 2 minutes.

9. Add 1 mL sterile Luria Broth (LB) medium to each tube. Gently tap the tubes with your finger to mix the LB with the cell suspension. Incubate the tubes at 37°C for 30 minutes.
   - At this point, the bacteria are recovering from the heat shock. During this time they will begin to express any genes they have taken up during the procedure.
   - While you wait for the cells to recover, label and draw your predicted results on the worksheet.

10. Once the recovery is complete, gently mix the cells and then plate 100 µl of each transformation reaction on the appropriate plates. Your instructor will demonstrate the proper method for plating your samples. Complete one plate at a time, from start to finish. Cells from the “−” tube should be spread on the –pGLO plates, and cells from the “+” tube should be spread on the +pGLO plates.

11. Wrap your 4 plates together with tape and place the plates **upside down** in the incubator. Incubate them for approximately 18–24 hours at 37°C.

---

**Day 2 - Data Analysis**

1. Observe the colonies through the petri plate lids. **Do not open the plates.** Record your observed results in the table below.
   a. Note whether there is bacterial growth on each plate. If the cell growth is too dense to distinguish individual colonies, record “lawn”.
   b. Note the color of the colonies under normal visible light.
   c. Using the UV pen, examine the cells. Make a note of any that fluoresce green.
pGLO Transformation Lab Worksheet

In the circles below, label and draw your prediction for each plate:

Record your observations in the table below:

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No Growth or Colonies or Lawn; color(s)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Different from predictions? If so, suggest why.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The goal of data analysis for this investigation is to determine if genetic transformation has occurred.

2. Which plate(s) are controls? Which are the experimental group(s)?

3. Which of the traits that you originally observed for *E. coli* did not seem to become altered? In the space below list these untransformed traits and how you arrived at this analysis for each trait listed.

<table>
<thead>
<tr>
<th>Original trait</th>
<th>Analysis of observations</th>
</tr>
</thead>
</table>

4. Of the *E. coli* traits you originally noted, which seem now to be significantly different after performing the transformation procedure? List those traits below and describe the changes that you observed.

<table>
<thead>
<tr>
<th>New trait</th>
<th>Observed change</th>
</tr>
</thead>
</table>

5. If the genetically transformed cells have acquired the ability to live in the presence of the antibiotic ampicillin, then what might be inferred about the other genes on the plasmid that you used in your transformation procedure?

6. From the results that you obtained, how could you prove that the changes that occurred were due to the procedure that you performed?
7. If a fluorescent green color is observed in the *E. coli* colonies then a new question might well be raised, “What are the two possible sources of fluorescence within the colonies when exposed to UV light?”

Explain:

a. Recall what you observed when you shined the UV light onto a sample of original pGLO plasmid DNA and describe your observations.

b. Which of the two possible sources of the fluorescence can now be eliminated?

c. What does this observation indicate about the source of the fluorescence?

8. Summarize the evidence that indicates whether your attempt at performing a genetic transformation was successful or not successful.
EXPERIMENT OBJECTIVE:

In this experiment, you will learn about an important application of biotechnology to biomedical diagnosis, as it related to sickle cell anemia.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment Components</td>
<td>3</td>
</tr>
<tr>
<td>Experiment Requirements</td>
<td>4</td>
</tr>
<tr>
<td>Background Information</td>
<td>5</td>
</tr>
<tr>
<td>Experiment Procedures</td>
<td></td>
</tr>
<tr>
<td>Experiment Overview and General Instructions</td>
<td>11</td>
</tr>
<tr>
<td>Module One: Agarose Gel Electrophoresis</td>
<td>12</td>
</tr>
<tr>
<td>Module Two: Southern Blot Transfer</td>
<td>14</td>
</tr>
<tr>
<td>Study Questions</td>
<td>18</td>
</tr>
<tr>
<td>Instructor's Guidelines</td>
<td></td>
</tr>
<tr>
<td>Notes to the Instructor</td>
<td>20</td>
</tr>
<tr>
<td>Pre-Lab Preparations</td>
<td>22</td>
</tr>
<tr>
<td>Experiment Results and Analysis</td>
<td>23</td>
</tr>
<tr>
<td>Study Questions and Answers</td>
<td>24</td>
</tr>
<tr>
<td>Appendices</td>
<td></td>
</tr>
<tr>
<td>A  Agarose Gel Preparation For Southern Blot Analysis</td>
<td>26</td>
</tr>
<tr>
<td>B  Quantity Preparations for Agarose Gel Electrophoresis</td>
<td>27</td>
</tr>
<tr>
<td>Material Safety Data Sheets</td>
<td>28</td>
</tr>
</tbody>
</table>
Components & Requirements

This experiment contains enough reagents for 5 groups.

Experiment Components

DNA Samples for Electrophoresis
- A Sickle cell gene sample
- B Sickle cell trait (carrier) sample
- C Normal gene sample
- D Mother’s DNA sample
- E Child’s DNA sample
- F Father’s DNA sample

Components for Membrane Transfer
- 5 Pre-cut Southern Blot Nylon Membrane (7 x 7 cm)
- 5 Pre-cut Blotting Filter Paper (7 x 7 cm)
- Blue-Blot DNA Stain™ Solution (10x)
- NaCl
- NaOH

Other Reagents & Supplies
- UltraSpec-Agarose™ powder
- Concentrated electrophoresis buffer

Before use, check all volumes of Standard DNA fragments and DNA samples for electrophoresis. Evaporation may have caused samples to become more concentrated.

If needed, tap tubes or centrifuge, then add distilled water to slightly above the 1.0 cm level and mix.

All components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

This experiment is a simulation. THIS EXPERIMENT DOES NOT CONTAIN HUMAN DNA. None of the experiment components are derived from human sources.
Requirements

- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Automatic micropipets with tips
- Balance
- UV Transilluminator
- Waterbath (65°C)
- 80°C incubation oven
- Microcentrifuge (optional)
- Microwave, hot plate or burner
- Assorted glassware (beakers, flasks and graduated cylinders)
- 250 ml flasks or beakers
- Hot gloves or beaker tongs
- Safety goggles and disposable laboratory gloves
- Plastic wrap
- Paper towels
- Forceps
- Small plastic trays for soaking gels
- Distilled or deionized water
- Concentrated HCl

Online Ordering now available  
www.edvotek.com

Visit our web site for information about EDVOTEK’s complete line of “hands-on” experiments for biotechnology and biology education.

Technical Service Department

1-800-EDVOTEK  
Mon - Fri  
9:00 am to 6:00 pm ET

FAX: (301) 340-0582
Web: www.edvotek.com
email: info@edvotek.com

Please have the following information ready:

- Experiment number and title
- Kit lot number on box or tube
- Literature version number  
  (in lower right corner)
- Approximate purchase date
The Sickle Cell Gene

A single nucleotide change in the DNA sequence of an important gene can affect health and disease. A large number of genetic diseases are identified where such changes have been correlated to the changes in a single nucleotide. More recently, mutations in oncogenes and tumor suppressor genes such as p53, have been associated with lung, colon and breast cancer. Other mutations in genes such as the BRCA 1 and II genes have been identified as markers with potential as diagnostic tools for breast cancer.

Human genetics follows the basic findings of the Augustine monk, Gregor Mendel, who studied plant genetics in the mid-1800’s. Mendelian genetics, which predicts traits inherited by offspring, is based on the inheritance of two alleles, or forms of the gene. These two alleles are inherited one from each parent. Alleles, and corresponding traits, can be either dominant or recessive. When a dominant allele is inherited, the trait coded by that allele will be apparent in the offspring. The presence of a dominant allele will, in effect, mask the trait coded by the recessive allele. To observe a recessive trait, it is required that both parental alleles be the recessive type. If both alleles are the same type, either both recessive or both dominant, the individual is homozygous with respect to that trait. If an individual has one dominant and one recessive, the individual is heterozygous for the particular trait.

Mendelian inheritance can be demonstrated with a 2 x 2 matrix, as shown in Figure 1. Parental alleles are placed on the sides of the matrix, and the genotype (what is genetically inherited) and phenotype (the way we look) of the offspring can be predicted. By convention, the dominant allele is denoted by an uppercase letter and the recessive allele by a lowercase letter. For example, assuming both parents each carry one dominant allele and one recessive allele, we can predict that 3/4 of their children will have the dominant phenotype and 1/4 of their children will have the recessive phenotype. Genotypically, 1/4 of the children will carry two dominant alleles; 1/2 of the children will carry one dominant and one recessive allele, and 1/4 will carry two recessive alleles. These estimates would be observed if there are a large number of offspring from two parents, as in the case of insects or plants.

Hemoglobin, which is present in red blood cells, is the carrier of oxygen to cells in the body. In capillaries carbon dioxide, which is a by product of metabolism, enters red cells and is converted to carbonic acid. The acidic pH reduces the affinity of oxygen binding to hemoglobin resulting in the release of oxygen in cells. Likewise when the bound carbon dioxide is released from red cells in the lungs there is an increase in pH which favors the binding of oxygen to hemoglobin. In individuals who suffer from certain blood diseases such as sickle cell anemia, the binding and subsequent transport of oxygen is compromised due to the mutation of a single nucleotide. This results in a
The Sickle Cell Gene

deficiency of oxygen and carbon dioxide exchange in the patient. In sickle cell anemia patients, the substitution of the polar side chain (Glu) with a nonpolar hydrophobic side chain (Val) results in the polymerization of the unoxygenated form of hemoglobin and subsequent precipitation of such polymers in red blood cells. The precipitation gives red blood cells a sickle shape due to the lack of diffusion through capillaries.

In the United States, sickle cell anemia is of special interest since it is estimated that 8% of African Americans are carriers of the sickle trait. Therefore, pregnancies at risk of an offspring suffering from sickle cell anemia is 8% x 8%, which equals 0.64%. It is of interest to note that heterozygous individuals for Hb S have a high resistance to the malaria parasite, part of whose life cycle is spent in red blood cells. Historically, sickle cell anemia provided a selective advantage in some regions of the world such as parts of Africa. This can also explain the reason for the high frequency of this homozygous gene amongst African Americans.

Hemoglobin is made up of two α chains and two β chains. The gene where the α is located is on the short arm of chromosome 16, while the β-globin gene cluster is on the short arm of chromosome 11. In addition to the adult form of Hb encoded within the β Hb cluster are the Hb forms that substitute for the adult β Hb during the various stages of development. Hemoglobin S (Hb S) is the variant form of adult hemoglobin A (Hb A) in which an amino acid substitution occurs in the β globin chain. The amino acid substitution is that of Valine (Val) in Hb S for the glutamic acid (Glu) normal Hb A hemoglobin (Figure 2). This significant finding was reported in 1957 by Vernon Ingram who was able to determine this structural change using peptide mapping analysis which ushered molecular medicine. These procedures are tedious and difficult. It should be noted that this predates Polymerase Chain Reaction (PCR) and DNA Sequencing.

The single base mutation is an A to T in the triplet codon of the amino acid residue number 6 from the amino acid end in the beta chain of hemoglobin. This change introduces an amino acid with a polar (neutral) side chain valine instead of the acidic glutamic acid (negative) residue and changes the property of the hemoglobin molecule. This substitution also changes
The Sickle Cell Gene

the electrophoretic mobility of Hb S compared to Hb A. At slightly basic pH, such as 8.4, Hb S will be relatively more positive than Hb A and therefore will travel slower towards the positive (anode) electrode. This change in mobility is used as a diagnostic test of the presence of Hb S.

With the advent of biotechnology, fetal DNA from cells can be obtained by amniocentesis and analyzed with a high degree of accuracy. DNA from a few cells can provide sufficient DNA to amplify using Polymerase Chain Reaction (PCR). Alternative methods can include growing cells in culture to yield sufficient DNA for analysis. The basis of the test is the recognition by restriction enzymes of specific palindromic sequences in DNA. In the normal β globin gene, the sequence of nucleotides that specifies amino acids 5, 6 and 7 (Pro-Glu-Glu) are CCT-GAG-GAG. The point mutation in codon 6 converts the A to T changing the sequence CCT-GTG-GAG. The palindrome recognition site of the restriction enzyme Mst II is CCTNAGG, where N can be any of the four nucleotides. Close examination of the sequence shows that Mst II will recognize the normal β globin CCT-GAG-G where N is a G, but not the mutated form. The restriction enzyme digests can then be analyzed by electrophoresis and Southern blotting using the appropriate probe for the β globin gene. Alternatively, DNA Sequencing can be used to determine the conversion of A -> T that is the basis of the sickle cell trait and sickle cell anemia.

Other blood disease such as β-thalassemias are attributed to various point mutations or other translational product aberrations. Almost 400 different hemoglobin (Hb) variants of known structure have been identified. The early recognized variants were historically assigned alphabetical initials based sequence of discovery or hematologic features.

ABOUT POLYMERASE CHAIN REACTION

PCR has two important advantages. The first is sensitivity, which allows for DNA fingerprinting identification using much smaller amounts of DNA since PCR amplifies DNA. The second advantage is the speed of PCR analysis, which allows critical questions to be answered more quickly as compared to Southern Blot analysis.

PCR amplification requires the use of a thermostable DNA polymerase, such as Taq polymerase. Purified from a bacterium known as Thermus Aquaticus that inhabits hot springs, Taq polymerase is commonly used in PCR because it remains stable at near-boiling temperatures. Also included in the PCR reaction are the four deoxynucleotides (dATP, dCTP, dGTP, and dTTP) and two synthetic oligonucleotides, typically 15-30 base pairs in length, known as “primers”. These components, together with the DNA to be amplified, are incubated in an appropriate buffer that contains Mg2+. The primers are designed to correspond to the start and end of the DNA to be amplified, known as the “target”.

Duplication of this document, in conjunction with use of accompanying reagents, is permitted for classroom/laboratory use only. This document, or any part, may not be reproduced or distributed for any other purpose without the written consent of EDVOTEK, Inc. Copyright © 1997, 1998, 1999, 2001, 2006 EDVOTEK, Inc., all rights reserved
In Search of the Sickle Cell Gene

The Sickle Cell Gene

Figure 3: Polymerase Chain Reaction
The Sickle Cell Gene

The PCR reaction mixture (which contains the DNA polymerase, buffer, deoxynucleotides, primers, and template) is subjected to sequential heating/cooling cycles at three different temperatures (Figure 3).

- In the first step, the enzyme reaction is heated to near boiling (92° - 96°C.) to denature or "melt" the DNA. This step, known as "denaturation" disrupts the hydrogen bonds between the two complimentary DNA strands and causes their separation.

- In the second PCR step, the mixture is cooled to a temperature that is typically in the range of 45° - 65°. In this step, known as "annealing", the primers, present in great excess to the template, bind to the separated DNA strands.

- In the third PCR step, known as "extension", the temperature is raised to an intermediate value, usually 72°C. At this temperature the Taq polymerase is maximally active and adds nucleotides to the primers to synthesize the new complimentary strands.

ABOUT SOUTHERN BLOTS

Analysis of complex DNA is facilitated by Southern blot analysis. After electrophoresis, the gel is sequentially treated in HCl and NaOH. The HCl treatment introduces apurinic sites in DNA which makes phosphodiester bonds at these sites labile and introduces nicks in double-stranded DNA. These Apurinic sites result when the purine base is removed, as in adenine residue from the A=T base pair. The NaOH treatment disrupts the the interstrand hydrogen bonds between the base pairs. The sequential acid and base treatments therefore result in the formation of small fragments from large DNA fragments. This facilitates the transfer of DNA fragments onto the nylon membrane. This procedure causes the double stranded restriction fragments to be converted into single stranded form. A replica of the electrophoretic pattern of DNA fragments in the gel is made by transferring (blotting) to a membrane of treated nylon. This is done by placing the nylon membrane on the gel after electrophoresis and transferring the fragments to the membrane by capillary action or by electrotransfer. The DNA becomes permanently adsorbed to the membrane, which can be manipulated much more easily than the gel. At this point the DNA is not visible on the nylon membrane.

Analysis of the transferred DNA is often done by hybridization with a DNA probe. Currently, non-isotopic detection systems are employed to detect DNA bound to the membrane. These probes are chemically synthesized and can be easily labelled by fluorescent tags or by radioisotopes.
In Search of the Sickle Cell Gene

The Sickle Cell Gene

A solution containing the single-stranded probe is incubated with the membrane containing the transferred, single-stranded DNA fragments. Under the proper conditions, the probe will only base pair (hybridize) to those fragments containing the complementary repeated sequences. The membrane is then washed to remove excess probe and is exposed to a sheet of x-ray film. Only those DNA fragments that have hybridized to the probe will reveal their positions on the film because the localized areas of radioactivity cause exposure of the x-ray film. This process is known as autoradiography. The hybridized fragments appear as discrete bands (fingerprint) on the film and are in the same relative positions as they were in the agarose gel after electrophoresis. The reason that well-defined bands can be visualized is because only a small fraction of the hundreds of thousands of fragments present contain sequences complementary to the probe.

In this experiment, two parents will be tested to determine if they are carriers of the sickle cell trait. In this hypothetical case, the parents have concerns about the possibility that their child is a carrier of the sickle cell gene. They have decided to determine the hemoglobin status of their child. In this experiment, simulated DNA digests will be separated by gel electrophoresis, followed by a Southern Blot, and then analyzed.
**EXPERIMENT OBJECTIVE:**

In this experiment, you will learn about an important application of biotechnology to biomedical diagnosis, as it related to sickle cell anemia.

**BRIEF DESCRIPTION OF THIS EXPERIMENT:**

Mutations in DNA can be inherited from one or both parents. Many germline genetic diseases are passed on from one generation to the next in Mendelian genetics. Thus if one parent is a carrier of a gene mutation that causes a genetic disease while the other does not, the offspring could be a carrier of the gene in one chromosome while the other chromosome will carry the normal gene. Such individuals could be carriers of the trait for the disease but usually do not manifest clinical traits.

In this experiment, you will separate DNA samples by electrophoresis, after which you will perform a Southern blot. You will then analyze the results of simulated DNA from hypothetical parents and offspring.

**LABORATORY SAFETY**

1. Wear gloves and goggles routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. **DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.**
4. Exercise caution when using any electrical equipment in the laboratory.
5. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.

**LABORATORY NOTEBOOK RECORDINGS:**

Record experimental results in your laboratory notebook or on a separate worksheet. Before starting the Experiment, write a hypothesis that reflects the experiment and predict experimental outcomes. During the Experiment, record (draw) your observations, or photograph the results. Following the Experiment, formulate an explanation from the results and determine what could be changed in the experiment if the experiment were repeated. Write a hypothesis that would reflect this change.
Module One: Agarose Gel Electrophoresis

AGAROSE GEL REQUIREMENTS FOR THIS EXPERIMENT

- Recommended gel size: 7 x 7 cm
- Number of sample wells required: 6
- Agarose gel concentration: 0.8%

PREPARING THE AGAROSE GEL

1. Close off the open ends of a clean and dry gel bed (casting tray) by using rubber dams or tape.

2. Place a well-former template (comb) in the first set of notches at the end of the bed. Make sure the comb sits firmly and evenly across the bed.

3. To a 250 ml flask or beaker, add agarose powder and buffer as indicated in the Reference Tables (Appendix A) provided by your instructor. Swirl the mixture to disperse clumps of agarose powder.

4. With a marking pen, indicate the level of the solution volume on the outside of the flask.

5. Heat the mixture using a microwave oven or burner to dissolve the agarose powder.

6. Cool the agarose solution to 60°C with careful swirling to promote even dissipation of heat. If detectable evaporation has occurred, add distilled water to bring the solution up to the original volume marked in step 4.

After the gel is cooled to 60°C:

7. Place the bed on a level surface and pour the cooled agarose solution into the bed.

8. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes.

9. After the gel is solidified, be careful not to damage or tear the wells while removing the rubber dams or tape and comb(s) from the gel bed.

10. Place the gel (on its bed) into the electrophoresis chamber, properly oriented, centered and level on the platform.

11. Fill the electrophoresis apparatus chamber with the appropriate amount of diluted (1x) electrophoresis buffer (refer to Table B on the instruction sheet from the Appendix provided by your instructor).
Module One: Agarose Gel Electrophoresis

LOADING THE SAMPLES

12. Make sure the gel is completely submerged under buffer before loading the samples and conducting electrophoresis. Load 18-20 µl of each DNA sample.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Tube</th>
<th>Sample Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>Sickle cell gene sample</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>Sickle cell trait (carrier) sample</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>Normal gene sample</td>
</tr>
<tr>
<td>4</td>
<td>D</td>
<td>Mother’s DNA sample</td>
</tr>
<tr>
<td>5</td>
<td>E</td>
<td>Child’s DNA sample</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>Father’s DNA sample</td>
</tr>
</tbody>
</table>

Reminder:
Before loading the samples, make sure the gel is properly oriented in the apparatus chamber.

RUNNING THE GEL

13. After the DNA samples are loaded, properly orient the cover and carefully snap it onto the electrode terminals.

14. Insert the plugs of the black and red wires into the corresponding inputs of the power source.

15. Set the power source at the required voltage and conduct electrophoresis for the length of time determined by your instructor.

16. Check to see that current is flowing properly - you should see bubbles forming on the two platinum electrodes.

17. After the electrophoresis is completed, disconnect the power and remove the gel from the bed for Southern blot analysis.
Module Two: Southern Blot Analysis

Quick Reference:
The depurination procedure must be brief (no longer than 8 minutes). Prolonged exposure to HCl completely depurinates DNA strands. Subsequent treatment with a denaturation solution would fragment the depurinated DNA molecules into very short oligonucleotides, which are poor targets for probe-based detection.

During this procedure, the bromophenol blue tracking dye in the gel will change color. After 8 minutes, the dye will be greenish to slightly yellow in color.

OVERVIEW

In this module, you will transfer the DNA fragments from the agarose gel, to a nylon membrane. After the transfer, the membrane will be baked for a short time to fix the DNA to the membrane.

DEPURINATION AND DENATURATION
(Approximately 1 hour)

The HCl treatment introduces apurinic sites in DNA which makes phosphodiester bonds at these sites (obtained upon hydrolysis of purine bases) labile and introduces nicks in double-stranded DNA. Apurinic sites result when the purine base is removed, such as an adenine residue from the A/T base pair. The NaOH treatment disrupts the the interstrand hydrogen bonds between the base pairs. The sequential acid and base treatments result in the formation of small fragments from larger DNA fragments. This procedure causes the double stranded restriction fragments to be converted (melt) into single stranded form and that facilitates the transfer of DNA fragments onto the nylon membrane.

1. After electrophoresis, depurinate the agarose gel by placing it in a tray containing 200 ml of 0.25 M HCl. Leave at room temperature for 8 minutes. Make sure gel is immersed in the liquid. Stop depurination if the dye becomes completely yellow before 8 minutes.

2. Carefully discard the HCl solution; do not reuse the solution.

3. Rinse the agarose gel with several changes of 200 ml distilled water.

4. Soak the agarose gel for 15 minutes in 200 ml of DNA Denaturation Solution (0.5 M NaOH/0.6 M NaCl). Make sure gel is immersed in the liquid.

5. Periodically shake the tray to immerse the gel, which will float because of the density of the solution.

6. Discard the solution.

7. In a second 200 ml of DNA Denaturation Solution (0.5 M NaOH/0.6 M NaCl), continue soaking the agarose gel for 15 minutes. Do not discard the DNA Denaturation Solution. Save the solution to wet the nylon membrane in Step 12.
Module Two: Southern Blot Analysis

SETTING UP THE SOUTHERN BLOT TRANSFER
(Aproximately 30 minutes)

After the second denaturation of the gel, set up the Southern Transfer:

8. Place a sheet of plastic wrap (such as Saran Wrap) on a flat level lab bench.

9. Remove the gel from the tray and place it (well side down) directly onto the plastic wrap. Inverting the gel places the smooth surface on top for contact with the membrane.

10. Wearing gloves and using forceps and scissors, trim the nylon membrane to the size of the gel.

11. Carefully pick up a membrane at the edges with clean forceps.

12. Slightly bend the membrane in the middle and slowly wet the membrane (from the middle out) in the DNA Denaturation Solution contained in the tray from step 9.

13. Release the membrane and gently submerge it for 5 minutes until it is thoroughly saturated with DNA Denaturation Solution.

14. Use forceps to remove the saturated membrane from the DNA Denaturation Solution and place it on top of the inverted agarose gel.

15. Trim the white blotting filter paper to the same size as the gel and the membrane.

16. Place the white filter paper on top of the membrane.

17. Roll a 5 or 10 ml pipet across the filter paper to remove air bubbles.

18. Carefully place a stack of paper towels approximately 4 - 5 cm thick on top of the filter paper.

19. Place an empty tray on top of the paper towels. Put a small object, such as an empty 400 ml beaker, inside the tray for weight.

20. Allow the blot transfer to progress 3-4 hours or overnight.
Module Two: Southern Blot Analysis

After incubating for 3-4 hours (or overnight):

21. Gently remove the tray, beaker, and all the paper towels.

22. Wearing rinsed gloves and using forceps, flip the stack (gel - nylon membrane - filter paper) over to lie on the filter paper.

23. Using a blue ink pen, draw through the six sample wells and trace their positions on the nylon membrane.

24. Using forceps, remove the gel from the membrane.

Note the thickness and consistency of the now dehydrated gel. The gel can be discarded since all further processing takes place with the nylon membrane.

25. Lay the membrane on a dry paper towel with the DNA side up (the side which was in direct contact with the gel). The bromophenol blue tracking dye will be visible on the membrane.

26. Using a blue ink pen, label the DNA side of the membrane with your lab group number or initials.

27. For optimal results, completely dry and fix the DNA to the membrane:
   - place the membrane between two small sheets of filter paper
   - place into an 80°C oven for 30 minutes

Optional Stopping Point

The dried membrane can be stored at room temperature, away from moisture, and between two sheets of Whatman filter paper until you are ready to continue with the non-isotopic detection procedure.
Module Two: Southern Blot Analysis

NON-ISOTOPIC DETECTION OF DNA

During this procedure you will visualize the DNA on the membrane. Blue Blot DNA Stain™ is a non-isotopic reagent, developed by EDVOTEK for classroom use, that eliminates all the associated hazards of working with radioactive isotopes or chemicals used in non-isotopic labeling.

28. Place the membrane with the DNA side up in 100 ml of dilute Blue-Blot™ solution.

29. Soak the membrane at room temperature for 10 to 15 minutes.

30. Remove the membrane with forceps and rinse in 200 ml of distilled water.

31. Replenish the distilled water once, or until the membrane is destained and DNA bands are clearly visible.
1. Sickle cell trait and sickle cell anemia are due to a mutation in what gene? How do the genotypes differ between the two conditions?

2. Describe how the sickle cell gene and protein differ from the wild-type versions.

3. What is run on a gel when performing a Southern Blot? What about a Northern Blot?

4. What is run on a gel when performing a western blot? What is the probe for a western blot?

5. What are the roles of HCl and NaOH when treating the gel after electrophoresis?

6. Describe what it means to carry out “hybridization with a DNA probe”.
Bio 6 – Polymerase Chain Reaction (PCR) Lab

Objectives

In this laboratory you will plan and carry out the Polymerase Chain Reaction (PCR) technique to amplify a specific DNA sequence within a larger DNA molecule. You will then analyze the resulting PCR products by agarose gel electrophoresis to determine if a) amplification was successful, b) if there is any evidence of DNA template contamination, and c) the least amount of template that resulted in amplification.

Introduction

The Polymerase Chain Reaction (PCR) technique is essentially DNA replication *in vitro* targeted to a very specific region of a DNA sample. As a result, the DNA in the target region is amplified exponentially due to repeated rounds of DNA replication. For example, consider that the human genome consists of ~3 billion base pairs of DNA. PCR makes it possible to take a sample of human DNA and selectively amplify any desired portion of it provided it is no larger than several thousand base pairs. The remaining DNA is more or less ignored by the replication machinery. The importance of PCR cannot be overstated. It has *completely revolutionized* biological research, forensics, diagnostic testing, and any other field that involves DNA analysis.

So how does PCR accomplish the selective amplification of a relatively small portion a complex DNA sample? To answer this question you need to understand how DNA replication works. Recall that DNA replication in bacteria requires the following components:

- DNA template*
- deoxyribonucleotide triphosphates (dNTPs)*
- origin of replication
- helicase
- DNA gyrase (topoisomerase)
- RNA primase
- DNA polymerase III*
- DNA polymerase I
- DNA ligase

* all that is required for PCR

All of these components and more are required for a bacterial cell to completely copy a very large piece of DNA, the bacterial chromosome which in *E. coli* is ~4 million base pairs. The replication of a relatively small region of DNA *in vitro* via PCR, however, requires only three of these components: *template DNA, dNTPs* and a *DNA polymerase*. An origin of replication and RNA primase are not necessary since a sequence-specific pair of *DNA primers* produced synthetically are added to the reaction. The sequence specificity of the primers is what limits DNA replication to the desired region of DNA and nowhere else. Helicase and topoisomerase are not needed to unwind and release tension in DNA, their jobs are not necessary due to a combination of high temperature and the short length of the DNA being amplified. The equivalent of DNA polymerase I and DNA ligase are also unnecessary due to the absence of RNA primers and Okazaki fragments during the process of PCR.
Since PCR requires very high temperatures as you will see, a typical DNA polymerase cannot be used since it will be denatured by the intense heat. A DNA polymerase that can function at very high temperatures is essential, and lucky for us, there are organisms that have just such a polymerase: hyperthermophilic bacteria. One such bacterial species is *Thermus aquaticus*, discovered around 1970 in the hot springs of Yellowstone National Park. *Thermus aquaticus* thrives at 70°C and can survive temperatures as high as 80°C. This means that its version of DNA polymerase III, an enzyme called *Taq* polymerase, can remain functional up to at least 80°C. As it turns out, *Taq* polymerase retains its enzyme activity even after almost one hour at 95°C! Thus, *Taq* polymerase would prove ideal for the PCR technique. DNA polymerases from other hyperthermophilic microbes have since been discovered and are used in PCR, however *Taq* polymerase is still used routinely and is the enzyme you will use in your PCR reactions.

In addition to the components already identified, DNA replication in a PCR reaction also requires specific pH conditions and concentrations of chloride, potassium and magnesium ions. These components are contained in a 10X buffer supplied by the manufacturer of the *Taq* polymerase. The components you will need to assemble a PCR reaction are listed below:

- DNA template
- dNTPs
- Primer 1
- Primer 2
- 10X buffer
- H2O
- *Taq* polymerase

Once assembled, a PCR reaction must then be placed in a device called an automated thermocycler (aka “PCR machine”). An automated thermocycler is a machine that is programmed to cycle through various temperatures for specific periods of time to allow the PCR amplification of DNA to occur. Let’s look at a typical “PCR program” below and then consider the purpose for each step:

- 5’ @ 95°C
- 30” @ 95°C
- 30” @ 60°C  x 30
- 60” @ 68°C
- 5’ @ 68°C

The initial 5 minute treatment at 95°C will completely denature the DNA template, i.e., separate complementary DNA strands from each other. What follows is 30 cycles of: 30 seconds at 95°C to denature DNA, 30 seconds at 60°C to allow the primers to hybridize (i.e., form base pairs) with complementary sequences in the DNA template, and 1 minute at 68°C to allow *Taq* polymerase to carry out DNA replication at its optimal temperature. DNA replication will occur because the synthetic DNA primers base-pair to complementary sequences in the DNA template. This provides 3’ ends for the *Taq* polymerase to add to and thus synthesize a new complementary strand of DNA. Each time this cycle is repeated, copies of the desired DNA sequence increase by a factor of two. The final 5 minutes at 68°C allows any unfinished DNA strands to be synthesized to completion. After the program is complete (about 2 hours), the desired DNA sequence will have been amplified by a factor of $2^{30}$, or over 1 billion times!
To help visualize what is happening in during repeated cycles of PCR, let’s look at the diagram below:

As this illustration shows, it is only the region of DNA between the two primers that gets amplified. The key to targeting PCR amplification to your desired DNA sequence is to design a pair of primers that flank the region you want to amplify and direct DNA replication in converging directions. As long as you know the DNA sequence where you want primers to hybridize, you can simply submit an order to a biotech company for complementary primers and they will synthesize them for you for a relatively inexpensive fee (less than $20 a piece!).
**Part 1: PCR AMPLIFICATION OF A DNA SAMPLE**

**Planning your PCR reactions**

Just like the restriction enzyme digests you did previously, it is essential that you plan your PCR reactions on paper and pool together all common ingredients before you begin to actually put the reactions together. If you do not do this, you will be prone to making mistakes and wasting expensive materials. Since there are quite a few components that go into a PCR reaction, it is useful to construct a table such as the one below to account for all components in each reaction. Once the table is completed, it is easy to identify and add up the common ingredients to include in your pool:

<table>
<thead>
<tr>
<th>tube #</th>
<th>DNA template</th>
<th>vol/ ng</th>
<th>forward primer</th>
<th>vol/conc</th>
<th>reverse primer</th>
<th>vol/conc</th>
<th>10X buffer</th>
<th>10 mM dNTPs</th>
<th>Taq units</th>
<th>H₂O</th>
<th>total vol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>plasmid DNA</td>
<td>1 µl</td>
<td>primer 1</td>
<td>10 µM</td>
<td>primer 2</td>
<td>10 µM</td>
<td>5 µl</td>
<td>1 µl</td>
<td>0.4 µl</td>
<td>40.6 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>1 µl</td>
<td></td>
<td>0.1 µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1 µl</td>
<td></td>
<td>10³ µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1 µl</td>
<td></td>
<td>10⁴ µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>1 µl</td>
<td></td>
<td>10⁻⁴</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>none</td>
<td>--------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>41.6 µl</td>
</tr>
</tbody>
</table>

This table illustrates the plan for 6 PCR reactions that vary only in the amount of template DNA, much like the PCR reactions you will carry out. Tube #6 will serve as a negative control. This is very important to confirm that no contaminating DNA ended up in your reactions. Notice that, despite differences in the amount of template DNA in each reaction, the total volume of template DNA added to all but the control is still 1 µl. This is easily accomplished by creating a 10-fold dilution series (serial dilution) of the template DNA, something you will also do. All other components are the same for each reaction and thus can be included in your pool (for the control reaction, the extra 1 µl of water can be added later).

Keep in mind that the final concentrations of the primers and dNTPs are very important. This is why both the concentrations and volumes of these components are indicated. Do the math and you will see that the final concentration of each primer is 0.2 µM, and the final concentration of dNTPs is 0.2 mM. If the final concentrations of these components are significantly off, your PCR reactions will not work. Now that we have identified all the common components in this set of reactions, let’s plan a pool for 6 reactions plus one extra:

- ultrapure H₂O 284.2 µl
- 10X buffer 35 µl
- primer 1 7 µl
- primer 2 7 µl
- dNTPs 7 µl
- *Taq pol.* 2.8 µl
- **TOTAL** 343 µl (divided by 7 = 49 µl ea)
As you can see, the amounts of each component in the pool have been verified since it will result in 49 µl of the pool per PCR reaction – 50 µl minus the 1 µl of template DNA (or water) which will be added separately.

Before you move on and prepare your own PCR reactions, let’s take a look at how you will prepare a serial dilution of the template DNA. Stock DNA solutions are typically 1 µg/µl, so let’s assume this is what you start with. What is needed for the PCR reactions are samples of the plasmid DNA at 1ng/µl, 0.1 ng/µl and so forth down to 10⁻⁴ ng/µl. This can be accomplished by diluting some of the stock DNA sample by a factor of 1000 and then carrying out a 10-fold serial dilution from there as shown below:

![Diagram of dilution process]

By adding the appropriate amount of diluent (what you are diluting with, ultrapure water in this case) to each tube first, the dilution series can be accomplished very quickly and easily. When setting up serial dilutions, keep in mind that the dilution factor is the volume added/total volume. This is why a 10-fold dilution for example requires 1 part sample and 9 parts diluent (i.e., 1 divided by 9 + 1 = 1/10). Once the appropriate amount of diluent has been added to each tube, you simply measure 1 µl from the original stock DNA solution, transfer it to a tube with 999 µl of water and mix to create a 1/1000 dilution (1 ng/µl). The next step would be to transfer 100 µl of this dilution to a tube with 900 µl of water and mix to dilute the sample 10-fold more (0.1 ng/µl), and so on down the line. If you measure accurately and mix thoroughly before each transfer (e.g., by vortexing), you will end up with a very accurate dilution series derived from a single µl of original stock DNA solution.

You are now ready to plan your PCR reactions and prepare a serial dilution of your template DNA...
Exercise 1A – Planning a set of PCR reactions

For the remainder of this lab you will work in groups of 3 or 4, though all planning on paper should be recorded individually and included in your notebook:

1. Use the table at the end of this lab to plan a set of 5 PCR reactions based on the following:
   - each reaction should have one of the following amounts of plasmid DNA as template: 1 ng, 10^{-3} ng (1 picogram), 10^{-6} ng (1 femtogram), 10^{-9} ng (1 attogram), no template DNA (negative control)
   - M13 forward and M13 reverse primers are at a stock concentration of 10 \mu M and should be used at a final concentration of 0.2 \mu M
   - each reaction should contain 0.2 mM dNTPs (from 10 mM stock) and 2 units of Taq polymerase (5 units/\mu l stock) in a total of 25 \mu l

2. Plan a “pool” of all components that will be common to each reaction, making sure you will have enough for one extra reaction.

3. Gather all necessary components for your PCR reactions and place them on ice.

4. Prepare your template DNA by serial dilution as described on the previous page.

Running your PCR reactions

Now that you have your plan in order, all you need to do is assemble your PCR reactions and run them in the automated thermocycler at your table. Before you can run your reactions, though, you need to know what parameters to use (i.e., temperatures, times, number of cycles, etc) which are indicated below.

Exercise 1B – Assembling and running PCR reactions

1. Use your plan from the previous exercise to put together your pool and assemble your PCR reactions into labeled PCR tubes.

2. Place your tubes in the thermocycler at your table and close the lid. Program the thermocycler with the following parameters with the aid of your instructor:

   \[
   \begin{align*}
   5’ @ 95^\circ C \\
   30” @ 95^\circ C \\
   30” @ 60^\circ C \quad \text{x 30} \\
   60” @ 68^\circ C \\
   5’ @ 68^\circ C \\
   \text{hold @ 4^\circ C}
   \end{align*}
   \]

3. Start the thermocycler and the program should be complete in \sim 2 hours.
### Part 2: ANALYSIS OF PCR PRODUCTS

The standard way to analyze PCR products is by agarose gel electrophoresis which is no different than what you have done in previous laboratories.

#### Exercise 2 – Agarose gel electrophoresis of PCR reactions

1. Prepare and pour 60 ml of a 1% agarose gel in 1X TAE as described in previous labs (be sure to submerge the gel in 0.25X TAE running buffer).

2. Plan how you will load your gel and record in your lab notebook.

3. Add 1/10 volume of gel loading dye to each of your PCR reactions, mix and quick spin.

4. Load all of each PCR reaction on your gel along with 10 µl of 1 kb-Plus ladder.

5. Run the gel at just over 250 volts for ~15 minutes.

6. Stain the gel with an InstaStain Ethidium Bromide card as described in previous labs.

7. Photographic your gel and place a copy in your notebook.

8. Determine the length of each PCR product and compare the amount of PCR product in each reaction. You should also identify what concentrations of DNA template, if any, resulted in no observable PCR product.

**NOTE: For this laboratory you will turn in a formal lab report.**
<table>
<thead>
<tr>
<th>tube #</th>
<th>DNA template vol</th>
<th>forward primer vol</th>
<th>reverse primer vol</th>
<th>10X buffer vol</th>
<th>10 mM dNTPs vol</th>
<th>Taq units</th>
<th>H₂O vol</th>
<th>total vol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
PCR Lab – Study Questions

1. You plan to test PCR amplification using 1 ng (10^{-9} g), 0.01 ng (10^{-11} g), 0.1 picograms (10^{-13} g) and 1 femtogram (10^{-15} g) of template DNA. Diagram how you would create a serial dilution of the DNA template to produce these amounts assuming the stock DNA concentration is 1 µg/µl.

2. Indicate your plan for a set of PCR reactions in which you will amplify 1 ng of human DNA template obtained from 3 different suspects in a crime and a sample from the crime scene. Assume each DNA sample is at a stock concentration of 1 ng/µl, each primer is 10 µM, the Taq polymerase is 5 units/µl and you want to use 2 units of enzyme in a total volume of 50 µl per reaction.

<table>
<thead>
<tr>
<th>tube #</th>
<th>DNA template</th>
<th>vol/ ng</th>
<th>forward primer</th>
<th>vol/ conc</th>
<th>reverse primer</th>
<th>vol/ conc</th>
<th>10X buffer</th>
<th>10 mM dNTPs</th>
<th>Taq/ units</th>
<th>H₂O</th>
<th>total vol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Indicate your pool below:
3. Explain what happens in a PCR reaction at 95°C, 55°C and 68°C, and why the process is repeated 30 times.

4. PCR amplification of human DNA samples (such as those collected at a crime scene) is vulnerable to contamination. Considering how PCR works, contamination from what source is the main concern and why would this be a problem?

5. You want to PCR amplify the highlighted region in the DNA sequence shown below. Design a pair of primers that are each 15 nucleotides long which will target PCR amplification to the desired sequence. Be sure to indicate the 5’ and 3’ ends of each primer sequence.

5’ – GCTCGATTCGAACTGTCGACTTCGGAATCGGGTTGAACCCACATCGCGACTGCATCGACTAC – 3’
3’ – CGAGCTAAGCTTAGCTAAGCTTAGCCCCTTTGGAACCGAGGATCCATCGCTGTGTAATGAGCTTGATG – 5’

6. One of your PCR reactions contained 1 femtogram (10^-15 g) of plasmid DNA template. Given the plasmid is 3342 bp and the average molecular weight of a base pair is 660 g/mol, calculate how many molecules of plasmid DNA were in that PCR reaction. Also calculate how many molecules are in 1 attogram (10^-18 g) of plasmid DNA. (NOTE: 1 mole = 6.02 x 10^23)
Bio 6 – Mitosis and Meiosis Lab

*Adapted from the University of Redlands

It is critical to understand the position and movement of chromosomes during mitosis and meiosis in order to understand the role of these processes in inheritance. In the first part of today’s lab you will use socks as models for chromosomes and then use these chromosomes to model the number of chromosomes, the nature of those chromosomes (are they made of sister chromatids or not? Is there a homologous chromosome or not?), and the dynamics of these chromosomes during mitosis and meiosis.

You will work with your lab group; each lab group will receive a bag of socks. The objects of mitosis and meiosis will be defined as follows:

| One sock = a chromosome prior to S phase |
| Letters = allele of a certain gene that is present on the chromosome |
| Socks of the same type= homologous chromosomes. These may differ from each other in the alleles that are present. |
| Magnets = cohesin (the protein that keeps sister chromatids together) |

For each of the phases below, after modeling with the socks, draw a diagram of the chromosome arrangement. Represent your chromosomes as follows:

- **Homologous chromosomes** can be indicated by depicting genes, usually indicated by letters, on the pair of chromosomes. To show that these chromosomes have the same genes, the same letters are used for each chromosome - both chromosomes might have the letter "A", for example. However, homologous chromosomes can have different versions, or alleles, of their genes, so a gene on one chromosome is often drawn with capital letter and while on the other a lower-case letter is used - A vs. a. Alternatively, homologous chromosomes can also be shown by making the lines different colors or textures. These slight lettering or drawing differences all are ways to indicate that while these chromosomes have the same genes, the alleles on these chromosomes can differ. Because these chromosomes are homologous, any genes shown should be shown on both chromosomes, chromosomes should be drawn as the same length, and the centromere, if shown, should be at the same position.

- **Different chromosomes** should be drawn as different lengths and their centromeres can be at different positions along the strand.
EXERCISE 1. MODELING MITOSIS

Don’t forget to draw each stage! Include the plasma membrane, nuclear membrane, centrosomes, mitotic spindle, and chromosomes when appropriate.

INTERPHASE

1. G1 (a stage of interphase):
   Start with the following assumptions. The cell that is undergoing mitosis is from an organism that is diploid and has a total of 4 chromosomes (another way of describing this is that n=2 where n is the number of chromosomes in a gamete from that species). Thus, you need to select the right number and the right types of socks to model interphase for a cell. Check your starting arrangement with your instructor.

2. G2 (a stage of interphase):
   Now assume that this cell is going to undergo cell division and has thus gone through S phase and has reached G2. Model how the chromosomes will look. Make it as representative as possible. How many socks should there be? How should these socks be arranged? Have the instructor check your solution. If approved, go ahead and draw your model.

MITOSIS

3. Prophase (a stage of mitosis):
   Now assume that this cell has proceeded to prophase. Model how the chromosomes and cell will look. Has the number or arrangement of the chromosomes changed? Some sorts of changes may not be possible to depict with this model.

4. Prometaphase (a stage of mitosis):
   Model the chromosomes and cell will look in prometaphase. Has the number or arrangement of the chromosomes changed?

5. Metaphase (a stage of mitosis):
   Model the chromosomes and cell as they would look during metaphase. What would the distinction be between prometaphase and metaphase? Are the numbers of chromosomes the same as in prophase? Is the arrangement of these chromosomes the same as in prophase or different? Show the lab instructor your solution.

6. Anaphase (a stage of mitosis):
   Model the difference between metaphase and anaphase. What aspect of the model should change compared to your solution for metaphase? Have the group nearest to you check your model—do they agree that you have modeled this appropriately?

7. Telophase (a stage of mitosis) and Cytokinesis:
   Show the outcome of mitosis by showing the two groups of chromosomes that will exist at the end of telophase. How do these grouping compare to each other? How does each of the groupings compare to the starting point of this exercise (G1 stage of interphase)? Is this consistent with what your understanding of mitosis?
   Before proceeding to the next section check with the instructor.
EXERCISE II. MODELING MEIOSIS

Start the modeling as you did for mitosis: The cell that is undergoing meiosis is from an organism that is diploid and has a total of 4 chromosomes (another way of describing this is that n=2 where n is the number of chromosomes in a gamete from that species). Thus, you need to select the appropriate number and the appropriate types of socks to model interphase for a cell.

An instructor should look at your simulation at each of the following steps. As before, draw each step.

INTERPHASE

1. G1, S, and G2 (stages of interphase)
   a. Using the socks before you, model how the cell will look during interphase prior to the S phase. Is there any difference between this and the equivalent step in mitosis?
   b. Now model the cell after S phase is complete. What is the difference prior to S phase and after S phase is complete? Are there differences after S phase in meiosis compared to after S phase in mitosis?

MEIOSIS - FIRST DIVISION (Meiosis I)

2. Prophase I
   In prophase I an interesting phenomenon occurs that makes the arrangement of chromosomes of prophase I of meiosis distinct from prophase of mitosis. Make sure your model of prophase I accounts for this distinction. Show your model of prophase I to the lab instructor.

3. Prometaphase I
   Show the arrangement of chromosomes in prometaphase I. How does your solution differ from your modeling of prophase of mitosis?

4. Metaphase I
   Show the arrangement of chromosomes in metaphase I. How does your solution differ from your modeling of metaphase of mitosis? This solution should imply how the separation that occurs in anaphase I is distinct from the separation that occurs in anaphase of mitosis. Have the instructor examine your model at this step.

5. Anaphase I and Telophase I
   Now show what happens during anaphase I, and telophase I. How many chromosomes are left in each nucleus by the end of telophase I? How many cells have been made from a single cell at the end of meiosis I? How do the outcomes of meiosis I differ from the outcomes of mitosis?

Make sure to have your instructor check your solution at this stage. You will go on to model meiosis II. If your solution is confused at this point, further work will only add to the confusion!
**MEIOSIS – SECOND DIVISION (Meiosis II)**

6. **Prophase II**
Show how the chromosomes of the organism exist in prophase II in each of the two cell products of meiosis I. What is the difference between this and prophase I?

7. **Prometaphase II**
Show how the chromosomes of the organism exist in prometaphase II. How does this differ from prometaphase I?

8. **Metaphase II**
Show how the chromosomes of the organism exist in metaphase II. How does this differ from metaphase I?

7. **Anaphase II**
Show how the chromosomes exist in anaphase II. What structures will be pulling the chromatids apart?

8. **Telophase II and Cytokinesis**
Show how the chromosomes exist in telophase II. How many chromosomes will be in each of the four meiotic products? How does this compare to the number of chromosomes in the original cell prior to meiosis? How many of each type of chromosome exist? If you merged two meiotic products (egg and sperm) how many chromosomes would exist? How would this compare to the starting number?

**Exercise III. EXAMINING CELLS IN GARLIC AND ONION ROOT TIPS UNDERGOING MITOSIS**

**Introduction**
During the process of germination plant tissues, particularly the roots, grow rapidly. This rapid growth is the result of frequent cell division; that is, cells in the root are proliferating. The rapidly proliferating cells are located in a specialized region at the tip of the root, just behind the root cap. This region is known as the root apical meristem and it produces the cells that contribute to the growth of the root.

Like all cell division, the cell division that occurs in the root apical meristem can be described as occurring in phases; the summation of these phases is known as the cell cycle. During the cell cycle, the chromosomes must be duplicated and partitioned equally between the resulting “daughter” cells. Failure to faithfully partition the chromosomal material will most likely result in the death of one or both of the daughter cells. Mitosis (or M phase) is the part of the cell cycle where the chromosomes become readily visible under the microscope. In class, we have discussed interphase and the various phases of mitosis: prophase (the chromatin begins to coil), metaphase (the paired chromatids become aligned at the metaphase plate), anaphase (the chromosomes begin to move toward the poles, and telophase (the chromosomes have reached the poles). In today’s laboratory, you will use the tips of garlic or onion roots as samples to observe the various phases of mitosis.
Method (adapted from a protocol by Ken Miller, Brown University)

1) Each group will receive a garlic root bulb that has been germinating for a few days. These should have young roots with cells that are rapidly dividing. It is important that during the following procedure the root tip does not dry out.

2) Examine the roots and note that the very tip of the root, about the last 2mm or so, is slightly more cream-colored than the rest. This section is the part you want. Remove several of the root tips with a pair of scissors or razor blade. Use the smaller roots and take only the last 2 mm (less than an 1/10 of an inch) of the tip.

3) Use a dissecting needle or forceps to place the roots in an eppendorf tube with 0.5 ml of 1M hydrochloric acid (HCl). This is fairly concentrated strong acid, so take care opening and closing the tube (you may wish to leave the tube open so that it will not splash in your eyes upon opening. Caution: Wear safety goggles.

4) Incubate the tube at 60˚C for 3 minutes. After the incubation is over, pour the mixture out of the tube and into a watch glass. (Hold tube at the bottom when you pour to avoid getting HCl on your fingers).

5) Select a root tip from the HCl-treated batch and transfer it to a glass microscope slide. Add a small drop (just enough to cover the tissue) of Toluidine Blue stain. Incubate for 2 -3 minutes at room temperature. Alternatively, your instructor may describe how to use acetocarmine to stain the sample.

6) Place a cover slip on top of the root tip on the microscope slide and place the slide on top of a paper towel. Place the cover slip at a slight angle so a small bit hangs off the edge of the slide. Using a pencil eraser or your thumb on the cover slip, use direct vertical pressure to push and squash the root tip. Some suggest dropping a pencil, eraser side down, about 10 cm (about the length of the palm of your hand) onto the cover slip to crush the tip. Because the tip is a 3D object, it must be crushed to make it only one or two cell layers thick, allowing for microscopic observation. Use the small overhanging bit of the cover slip to lift it up, then put it back down to help distribute the stain to the newly exposed cells from the inside of the root.

7) Now observe your specimen under the microscope. Remember, start with the lowest power objective (4X) to find your specimen, focus and then rotate the next highest power objective into place. The best objectives to use to examine the cells are the 10X and 40X objectives. The 100X objective is an oil-immersion lens and will not work for this purpose. The 10X objective is best for the purpose of surveying your sample, looking for any unusual features. Note that you should be able to make out individual cells. The chromosomes and the nuclei will stain blue if you have used Toluidine Blue. The vast majority of cells will have a circular staining pattern in which the entire nucleus is stained. Look for exceptions to this staining pattern. These may be cells in mitosis. In areas where cells are dividing the cells are less elongated and more boxy in shape. Once you find such an exceptional cell try using the 40X objective to get a closer look. Look for cells in the various stages of mitosis. If your sample is under- or over-stained or not crushed adequately, try staining and crushing another tip, modifying the procedure to correct the problem.

8) Attempt to find cells that are in each of the stages of mitosis. For each stage of mitosis, sketch the cell.

9) Next, look at a previously prepared slide of stained onion root tips. These have been sliced, rather than crushed, making it easier to tell which section of the root is being examined. These will show...
the mitotic figures with more clarity. At low magnification, look up and down the root tip length. Unlike your sample, this sample has cells that were fixed in place. Notice that the root tip has the shape that you would expect.

Consider the following questions: Are there particular areas where there is little or no mitosis? Are there areas where many of the cells are in mitosis? Based on examining the cells in the microscope, is it possible to determine whether the cells are in mitosis or meiosis?

10) **Draw a sketch of a portion of the root tip.** Use the 40X objective and find a place where there are at least several cells in mitosis.

11) **Show the instructor cells at each of the stages of mitosis.** While waiting for the instructor, peruse the specimen to see if you can determine which stage(s) of mitosis are most common and which stage(s) of mitosis are least common. **What might differences in the frequency of appearance of the different stages of mitosis mean?** That there are differences in frequency may seem puzzling at first, because every cell that goes through mitosis goes through each stage. Think about how the slice of the root is a sample at one particular time and how the various events each happen at their own pace. **What aspect of a commonly seen stage might make it easier to “catch” a cell while it is in that stage?**

**Exercise IV. EXAMINING CELLS IN WHITEFISH BLASTULA CELLS UNDERGOING MITOSIS**

Examine the images of whitefish blastula cells undergoing mitosis given by your instructor. Match each image to the proper stage: interphase, prophase, metaphase, anaphase, early telophase, late telophase/cytokinesis.
Ex. 1: MODELING MITOSIS

G1

Prophase

G2

Prometaphase
Telophase and Cytokinesis:
Ex. 2: MODELING MEIOSIS

Prophase I

Prometaphase I

Metaphase I

Anaphase I
Telophase I and Cytokinesis:

Prophase II:

Metaphase II:
Anaphase II:

Telophase II and Cytokinesis:
Ex. III: EXAMINING CELLS IN GARLIC AND ONION ROOT TIPS UNDERGOING MITOSIS

Ex. IV EXAMINING CELLS IN WHITEFISH BLASTULA CELLS UNDERGOING MITOSIS

A _________________________________
B _________________________________
C _________________________________
D _________________________________
E _________________________________
F _________________________________

Summarize the major differences between mitosis and meiosis in the table below:

<table>
<thead>
<tr>
<th></th>
<th>Mitosis</th>
<th>Meiosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crossing over</td>
<td></td>
<td></td>
</tr>
<tr>
<td>When chromosomes split</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of divisions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of cells resulting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of chromosomes in daughter cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetic similarity of daughter cells to parent cells</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Reviewing Your Knowledge

1. Define the following terms:

   centromere:
   centriole:
   centrosome:
   kinetochore:
   sister chromatid:
   cell plate:
   cleavage furrow:
   diploid/haploid:

2. In the photomicrograph of dividing ONION root cells below, identify INTERPHASE and the following phases of mitosis: PROPHASE, PROMETAPHASE, METAPHASE, ANAPHASE, TELOPHASE, CYTOKINESIS

3. Mitosis is important as organisms, both animals and plants, increase in size and grow new tissues and organs. Unlike animals, plants continue to grow throughout their lives. Where would you expect mitosis to be most common in the body of a mature plant?

4. What role would mitosis play in the body of an adult animal?
5. What advantage does the process of crossing over bring to reproduction?

6. Why would the method of cytokinesis in animal cells not work in plant cells?
Bio 6 – DNA Cloning Lab

Objectives

In the next four laboratory sessions you will attempt to “clone” a fragment of DNA by ligating the DNA fragment with a plasmid vector, transforming bacterial cells with the recombinant plasmid, isolating plasmid DNA from potential bacterial clones, and analyzing each plasmid clone for the DNA fragment by restriction enzyme digestion and agarose gel electrophoresis.

Introduction

The analysis of a gene, or any DNA fragment, requires that one clone the DNA sample. The term DNA cloning refers to the process of recombining a DNA fragment of interest with a DNA vector carried in a host microbe such as a bacterium or virus. In bacteria, the DNA cloning vector is typically a plasmid, a nonessential piece of DNA that is extrachromosomal and circular. In viruses the vector is the viral DNA itself. The cloning of relatively small DNA fragments (less than several thousand base pairs) is typically accomplished using plasmid vectors in bacteria. Larger DNA fragments require viral vectors or vectors called artificial chromosomes that are used in yeast or bacterial hosts. Regardless of the vector, once the process is complete a clone of the host containing the recombinant vector can be cultured to produce essentially unlimited amounts of DNA containing the fragment of interest.

You will be cloning a relatively small DNA fragment in a plasmid vector using a bacterial host, so we will focus on this particular method which involves three general steps:

1) LIGATION – combining the DNA fragment of interest with the plasmid vector

2) TRANSFORMATION – introduction of the recombinant vector into a bacterial host

3) SCREENING – identifying bacterial clones carrying the DNA of interest

The first two steps will be accomplished in a single lab session. The process of screening for desired bacterial clones will involve three separate lab sessions.

Part 1: LIGATION

Before you carry out a ligation of the plasmid vector with the DNA fragment to be inserted, it is important to review the properties of a plasmid vector, and how both the plasmid and DNA to be inserted must be treated before ligation can occur. Let’s begin with the plasmid vector...
The Plasmid Vector

The plasmid vector you will use is called pUC19, a very common cloning vector used in labs throughout the world. This plasmid has four important characteristics that make it useful as a cloning vector:

1) origin of replication
2) ampicillin resistance gene
3)  \textit{LacZ} gene encoding the enzyme $\beta$-galactosidase
4) multiple cloning site (MCS) within the $\beta$-galactosidase the gene

An \textit{origin of replication} ("rep" in the map above) is a DNA sequence that is essential for any plasmid. Without it the plasmid would never be copied by DNA replication in the bacterial host and thus never passed on to daughter cells. The \textit{ampicillin resistance gene} ($Ap^R$) encodes an enzyme that degrades the antibiotic \textit{ampicillin}. This ensures that only bacteria containing the plasmid will survive exposure to ampicillin which is extremely useful in the screening process. The \textit{multiple cloning site} (MCS) consists of a short stretch of DNA containing a variety of unique restriction sites. This is where a DNA fragment of interest will be inserted into the plasmid. The \textit{LacZ} gene produces the enzyme $\beta$-galactosidase which is involved in the catabolism of the sugar lactose. $\beta$-galactosidase can also catalyze the hydrolysis of an artificial substrate called \textit{X-gal}, a molecule that resembles lactose and yields a blue product when hydrolyzed. Colonies of bacteria containing the plasmid will therefore be blue if X-gal is in the medium. The location of the MCS within the \textit{LacZ} gene is extremely useful for the screening process since a DNA fragment inserted into the MCS will disrupt the \textit{LacZ} coding region and thus no functional enzyme is produced. As a result, bacteria containing the plasmid with DNA inserted into the MCS should have a white color. This is the basis of what is commonly called "blue/white selection" in the cloning process.
Preparing the insert DNA and plasmid vector

Ligation of a plasmid vector with a DNA fragment is accomplished using the enzyme DNA ligase. Simply mixing the DNA molecules to be joined with DNA ligase, however, is not sufficient to get the job done. The two DNA molecules to be joined must be cut with restriction enzymes to create compatible DNA ends. This will also direct ligation to the desired location in the vector. For most ligations this means cutting the plasmid vector and the DNA insert with the same restriction enzyme as illustrated below:

If the plasmid and insert DNA are cut with the same restriction enzyme, the ends of each fragment will be the same and thus compatible. This is true whether the enzyme produces 5’ overhangs, 3’ overhangs or blunt ends. Compatible 5’ or 3’ overhangs, though, provide an extra advantage in that the overhangs are complementary creating “sticky ends” which greatly enhance the efficiency of ligation.

When the plasmid vector is cut or linearized with a single restriction enzyme, it can easily be ligated with itself to restore the original plasmid without any inserted DNA. This is a ligation product you clearly want to avoid and can do so by treating the cut plasmid DNA with a type of enzyme called a phosphatase. Phosphatases catalyze the removal of phosphate groups from a substrate, and treating the cut plasmid vector with a phosphatase will remove the 5’ phosphates from each end of the DNA molecule. Without the 5’ phosphates, the linearized plasmid cannot be ligated to itself. The DNA insert, however, is not treated with phosphatase and can still be ligated with dephosphorylated plasmid vector.
This issue can be avoided by cutting the plasmid with 2 different restriction enzymes and cutting the ends of the insert with the same 2 enzymes (this is the case for the vector and inert you will be ligating). With 2 different ends the plasmid can’t ligate with itself so dephosphorylation is not necessary.

**Planning a ligation reaction**

As with any other enzymatic reaction, it is essential that you plan a ligation reaction first to ensure that you assemble the correct amounts of each component. First of all, it is important to identify the components that go into a ligation reaction:

- prepared plasmid DNA
- prepared insert DNA
- 10X DNA ligase buffer
- H₂O (ultrapure)
- DNA ligase

A typical ligation reaction will be carried out in a total of 20 µl with ~2 units of DNA ligase and 50 ng of prepared plasmid DNA. The molar ratio of insert to plasmid DNA is typically 3:1. This ratio maximizes the yield of ligation products with only one copy of the insert DNA. Assuming a reaction will have 50 ng of plasmid DNA, a 3-fold molar excess of insert DNA can be calculated as follows:

\[ 50 \text{ ng of plasmid} \times \frac{\text{length of insert (bp)}}{\text{length of plasmid}} \times 3 \]

For example, if the plasmid is 3000 bp and the insert is 600 bp, the calculation would be:

\[ 50 \text{ ng} \times \frac{600}{3000} \times 3 = 30 \text{ ng of insert DNA} \]

Once assembled, the reaction is incubated at room temperature for 1 ½ hours. Although the ligase works best at 37 °C, this is too warm for the sticky ends to effectively base pair. Room temperature is cool enough for the base pairing of complementary sticky ends and results in a more efficient ligation.

**Exercise 1 – Ligation of plasmid and insert DNA**

You will ligate 50 ng of pUC19 (2686 bp) with a 3-fold molar excess of a 650 bp DNA insert, both of which have been cut with the restriction enzyme EcoRI. Working with the other students at your table, plan and carry out the ligation of the two fragments as described below:

1. Refer to the guidelines above and plan a ligation using 2 units of DNA ligase and a total reaction volume of 20 µl. The plasmid and insert DNA are both at a stock concentration of 10 ng/µl, and the DNA ligase stock is 5 units/µl.

2. Assemble your reaction, being sure to add the enzyme last, mix gently by tapping and quick spin.

3. Incubate the reaction at room temperature for 10 minutes.
Part 2: TRANSFORMATION

Once your ligation reaction is complete, you are ready to induce bacterial host cells to internalize the ligation products, a process called transformation. This is not a trivial process since the bacteria you will be transforming have to transfer the DNA across a rigid cell wall sandwiched between two membranes. However when chemically pretreated to make them competent and exposed to a brief heat shock, the bacteria readily internalize DNA from their immediate surroundings. If the internalized DNA is a circularized plasmid, it will be copied and transmitted to all daughter cells (bacteria can complete DNA replication only if the molecule is circular).

Your ligation reaction will produce a very heterogeneous collection of DNA molecules. Most molecules will be linear plasmid and insert DNA along with various ligation products that did not circularize. If internalized by bacteria, none of these DNA fragments will be copied or passed on to daughter cells. The only ligation products that can successfully transform bacteria are recircularized plasmids, regardless of the presence of the DNA insert.

Exercise 2 – Transformation of bacteria with ligation products

1. Completely thaw a tube of competent bacterial cells on ice.

2. Add 1 µl of your ligation reaction to the thawed competent cells, tap gently to mix, and place on ice for 30 minutes.

3. Place the tube in a 42°C water bath for 30 seconds, then immediately place on ice without mixing and leave for 5 minutes.

4. Transfer all 50 µl of the heat-shocked bacteria to tube containing 1 ml of sterile LB medium and place the tube in a 37°C shaker for up to 1 hour.

5. Use a P200 with a sterile tip to drip 100 µl of the transformed bacteria on the surface of an LB agar plate containing ampicillin and X-gal. Sterilize a plate spreader with alcohol and a brief flaming and spread the transformed bacteria on the plate as demonstrated by your instructor.

6. Label the plate and place lid down in a 37°C incubator overnight.

Part 3: SCREENING FOR POSITIVE BACTERIAL CLONES

Inoculating bacterial clones

At this point in the DNA cloning process you should have an agar plate containing both blue and white colonies. It is important to realize that each colony is derived from a single bacterial cell and thus all the bacteria in a given colony are clones of the original cell. In addition, the only bacterial cells capable of growing into a colony are those that acquired a plasmid with ampicillin resistance during the
transformation process. Recall that blue colonies should consist of bacterial clones transformed with a plasmid that does not contain a DNA insert in the MCS. Conversely, each white colony should consist of bacterial clones that do have a DNA fragment inserted in the MCS.

The first step in the screening process is the inoculation of randomly selected colonies from which you will purify plasmid DNA to test by restriction enzyme digestion...

**Exercise 3A – Inoculation of selected bacterial clones**

*For this exercise you will randomly choose 3 white colonies and 1 blue colony for inoculation. The white colonies are clones that potentially contain your 650 bp DNA insert. The blue colony is a negative control.*

1. Obtain 4 sterile plastic culture tubes and label them 1 to 4.
2. Use a sterile pipette to add 3 ml of sterile LB medium with ampicillin to each tube.
3. Inoculate 3 random white colonies into tubes 1 to 3 as follows:
   - sterilize a pair of metal forceps by dipping in alcohol and briefly flaming (*be careful not to drip flaming alcohol!*)
   - grab a sterile toothpick with the forceps and touch a white colony with one end of the toothpick
   - place the entire toothpick, inoculated end down, into the corresponding labeled tube
4. Inoculate a single blue colony into tube 4 in the same manner.
5. Make sure the cap of each tube is loose, and incubate the tubes in 37°C shaker overnight.

**Purifying plasmid DNA**

You are now ready to purify the plasmid DNA from each of the six bacterial cultures you inoculated in the previous laboratory session. In most laboratories this is referred to as doing “plasmid mini-preps” since you are isolating plasmid DNA on a small scale. There are many different approaches to doing plasmid mini-preps, many of which involve commercially produced kits for this purpose. Commercial kits are very useful and reliable, though they can be expensive. Luckily there are several very effective plasmid mini-prep protocols that require relatively inexpensive materials common to most laboratories. You will be carrying out one such protocol which is referred to as the “boiling method”.

In principle, the process is rather straightforward. The bacterial cells are lysed in a solution containing a detergent plus *lysozyme*, an enzyme that degrades the bacterial cell walls. The lysate is then briefly boiled to coagulate and remove the proteins and chromosomal DNA from solution. The much smaller plasmid DNA remains in solution and can be easily purified by *alcohol precipitation*, a process by which DNA is made insoluble by adding the right amount of alcohol.
Exercise 3B – Plasmid purification (boiling method)

1. Label four microcentrifuge tubes 1 to 4 and set a beaker of water to boil on your hot plate.

2. Pour 1.5 ml of the corresponding bacterial culture into each tube, being careful not to spill or over fill.

3. Spin the tubes at full speed in a microcentrifuge for 30 seconds. This will pull the bacterial cells to the bottom of the tube.

4. Use a small fine-tipped transfer pipet to remove and dispose of the supernatant into a small beaker (this is biohazard waste which will be handled by the lab technician).

5. Repeat steps 3 and 4 to get rid of any remaining liquid.

6. Use a P1000 to completely resuspend each bacterial pellet in 300 µl of STET/lysozyme solution, and leave at room temperature for at least 2 minutes. This will disrupt the bacterial membranes and cell wall releasing the cellular contents. STET/lysozyme solution contains the following:
   - 8% sucrose (S)
   - 5% Triton X-100 detergent (T)
   - 50 mM EDTA (E)
   - 50 mM Tris-HCl pH 8.0 (T)
   - 2 mg/ml lysozyme

7. Place all six tubes in a boiling rack and boil for exactly 50 seconds, then spin the tubes at maximum speed in your microcentrifuge for 10 minutes.

8. Use a sterile toothpick to remove the “goop” from the bottom of each tube and dispose of the goop in the biohazard waste.

9. Estimate the approximate volume of liquid remaining in each tube (refer to the markings on the tube) and add an equal volume of isopropanol to the remaining liquid in each tube, vortex to mix, and place on ice for 10 minutes to precipitate the plasmid DNA.

10. Spin the tubes at maximum speed in your microcentrifuge for 10 minutes to pellet the precipitated plasmid DNA.

11. Remove the liquid supernatant in each tube using a small fine-tipped transfer pipet and discard into the small beaker of biohazard waste. Quick spin and remove any remaining liquid using a P200. Air dry the DNA pellet (leave lid open) for ~5 minutes.

12. Resuspend each DNA pellet in 50 µl of TE with 10 µg/ml RNase (to degrade any RNA in the samples).

Analyzing plasmids by restriction enzyme digestion

To see if any of your bacterial clones contain a plasmid with a DNA insert of the desired length, all you need to do is cut a sample of the plasmid DNA with an appropriate restriction enzyme and subject the resulting DNA fragments to agarose gel electrophoresis. If the restriction site used to ligate the insert DNA with the plasmid vector was recreated when the fragments were
joined together, then you can simply cut the DNA with the same restriction enzyme. On occasion the cloning restriction site is not recreated and therefore you would need to examine the MCS and cut with restriction enzymes that are on either side of the original cloning site.

Exercise 3C – Restriction enzyme digestion and agarose gel electrophoresis

Any plasmid clones containing the desired DNA insert should have recreated EcoRI restriction sites on each side of the DNA insert. Digestion of a positive plasmid with EcoRI should therefore release the 650 bp DNA insert which will be detected by agarose gel electrophoresis:

1. Plan EcoRI digests of your four plasmid samples such that each reaction contains 5 µl of plasmid DNA and 5 units of each enzyme in a total volume of 20 µl (refer to Lab 11 if necessary).
2. Pool all common components (everything but the plasmid DNA) such that you have enough for one extra reaction and assemble your digests (refer to Lab 9 if necessary).
3. Incubate each reaction at 37°C overnight.
4. Prepare and pour 60 ml of a 1% agarose gel with 1X TAE (use 0.25X TAE as the running buffer).
5. Add 2 µl of gel loading dye to each reaction, mix and quick spin.
6. Load all 22 µl of each sample on the agarose gel along with 10 µl of 1 kb-Plus DNA ladder and 10 µl of uncut plasmid DNA and run at just over 250 volts for ~15 minutes.
7. Stain the gel with an InstaStain ethidium bromide card as described in Lab 9.
8. Produce a photographic record of your gel and place in your notebook.
9. Examine the length of each DNA fragment in each lane. A positive clone should produce a 2686 bp band corresponding to the linearized plasmid, and a 650 bp band corresponding to the DNA insert you attempted to clone.

NOTE: Positive clones on your gel should look just like the digests you did in the Restriction Enzyme Digestion lab.

Once a positive clone has been identified, the next step would normally be to sequence the DNA insert in the plasmid to confirm its identity. Once confirmed, a culture sample of the positive clone is preserved and can be used to grow as much of the plasmid as needed.

NOTE: For this laboratory you will turn in a formal lab report.
DNA Cloning Lab– Study Questions

1. What is a plasmid?

2. What is a vector? What vector was used in this experiment?

3. Explain how having a MCS (Multiple Cloning Site) in the middle of the Lac Z gene of pUC19 allows for the selection of bacteria containing plasmids with or without an insert.

4. What are the roles of X-Gal and ampicillin in the cloning process?

5. Define the terms ligation and transformation.

6. What enzyme is required to covalently bond a linear DNA fragment into a plasmid?
7. Explain what is meant by the term “competent cell” as it relates to transformation.

8. Diagram what you would expect to see on your agarose gel for EcoRI digests of plasmid DNA from white colonies vs blue colonies in your cloning project.

9. Pretend you cloned a DNA insert cut with SpeI into the XbaI site of pUC19 resulting in the destruction of the XbaI site (SpeI and XbaI have slightly different restriction sites that produce the same sticky ends which when joined produce a sequence that can’t be cut by either enzyme). What restriction enzymes would you cut the plasmid samples with to release the insert?

   HINT: Refer to the pUC19 MCS sequence on the second page of this lab (page 158) and the New England Biolabs catalog to find compatible enzymes for a double digest.

10. What are the roles of lysozyme and phosphatase in the cloning process?
Reading a Primary Research Paper

Overview

In this laboratory you will learn how to approach reading a primary research paper. To do so you will 1) become familiar with the components of a primary research paper, 2) review the process of reading a primary research paper, 3) analyze as a class a primary research paper selected by your instructor, and 4) independently analyze a primary research paper of your own choosing.

Introduction

A primary research paper is a formal publication of original research in an academic research journal which presents experimental results generated by the authors along with materials and methods used in generating the results. The paper will also provide relevant background information and address the significance of the results. In contrast, a textbook, newspaper article, or any article in a research journal referred to as a “review” or “synopsis” for example will not present any original research, only a summary of research already public in a given area.

Reading a primary research paper is not like reading a newspaper or a book. They are densely packed with important information that can be technically challenging. Students tend to think they have to start at the beginning and understand every word in every sentence, but this can be quite tedious and is not always necessary. You simply need to understand the parts of the article that are relevant to your interests. There is no right or wrong way to read research papers, and over time you will develop your own approach. Before attempting to read a primary research paper it is important to first understand how a research paper is organized as shown below.

I. The Components of a Primary Research Paper

All primary research papers have a similar format which includes the following components:

1. Title of the Article

   - Every primary research paper is proposing to add something new to a particular field of study. In one phrase, the title tells you what the authors claim this to be.
   - The authors are typically listed below the title, with the order reflecting the level of credit each deserves for the work being presented. The principle investigator(s) who secures the funding and oversees the project is listed last.
2. **Abstract**
   - This a very brief, to the point summary of the findings presented in the paper and their significance. The abstract is prominently placed at the top of the first page and is generally the equivalent of a paragraph.

3. **Introduction**
   - Following the abstract is the introduction which is generally 1 to 2 full pages. This is where the authors provide background information relevant to the findings of the paper. Specifically, the authors will address the “big picture” pertaining to the topic of the paper, clarifying what is already known, what is not known, what is controversial or unclear, what needs to improve, etc. The authors will finish the introduction by stating very specifically what their findings contribute to the body of knowledge in this area.

4. **Results**
   - The results section is the most important part of the paper since this is where the authors present all the evidence for their claims. Each experiment or data set is presented in a logical order which, in a sense, tells a story leading to the major claim(s) of the paper. For each experiment or data set, the authors will:
     - clarify its purpose or importance
     - indicate the basic method or technique used
     - present the results in a figure, table or graph
     - interpret the results

5. **Discussion**
   - In the discussion the authors will tie together all the evidence presented in the results section to make a case for what they claimed in the title, abstract and end of the introduction. Any uncertainties or alternative interpretations will also be addressed. In some journals the discussion is included in the results section.

6. **Materials and Methods**
   - This is where the authors provide sufficient details for each experimental method so that anyone in the field can scrutinize the methods and, most importantly, repeat any aspect of their work.

7. **References**
   - This section will contain complete references for all referenced material in the paper. This provides the reader the opportunity to examine, verify or simply refer to the work of other researchers that the authors have deemed relevant to their study.
All primary research papers will contain the components above, however each journal may organize, present and even name some of these components differently. For example, in some journals the Materials and Methods section is located after the Introduction whereas in others it is located after the Discussion. Some journals simply call this section “Methods”. Some journals have separate “Results” and “Discussion” sections while others have a single “Results and Discussion” section. Each journal has its own format, so don’t be surprised to see a lot of variety in formatting.

II. A Step by Step Approach to Reading a Primary Research Paper

The following guidelines are by no means the only way to read a research paper, however they should be a useful guide for understanding a paper well enough to come to your own conclusions and to present an oral and/or written review of the paper. To illustrate each step your instructor will provide a primary research article to be reviewed as a class using the 3-step approach outlined below.

1. **Identify the main claim(s) of the paper.**
   - this can be found in the title, abstract and end of the introduction
   - keep this in mind as you read the rest of the paper
   - once you finish analyzing the paper you can decide for yourself whether or not their claims are supported

2. **Analyze each experiment or data set independently.**
   - this is the most important thing you will do and is best done by outlining each experiment or data set as follows:
     a) in your own words, write the question being asked or purpose of the experiment (i.e., what are they trying to show?)
     b) identify the experimental method or approach (you should refer to the Materials and Methods or other resources as needed since you can’t interpret their results if you don’t understand what they did)
     c) independently interpret the results presented in the corresponding figure, table or graph in light of the purpose or question addressed by the experiment
       o this is where you will identify any shortcomings in the experimental design such as insufficient controls or sample sizes
     d) determine whether or not the authors’ interpretation is well supported

3. **Conclude whether or not the main claim(s) of the authors are supported by the evidence presented in the results section**
• first read the discussion to make sure you fully understand what the authors are claiming to contribute to this field of study
• determine whether or not you agree with the authors’ claims
• consider what, if anything, you think the authors should have done differently

By following these steps you should be able to understand and critique any primary research paper. This will take time and frequently requires additional research into the methods used (or consulting someone who is familiar with a particular method), however this will allow you to come to your own conclusions about a research paper and not simply assume the authors’ claims are well supported.

Exercise 1 – Analyze a research paper

Do the following for the primary research paper designated or this lab by your instructor:

1. Consult with members of your group to identify the main claim or claims of the paper.
2. Your group will analyze one experimental result in the paper as indicated by your instructor.
3. Each group will present their conclusions to the class after which the authors’ claims will be addressed.

Now that you have a good idea how to go about analyzing a primary research paper, you should be prepared to do this on your own. You will need to first identify the paper that you will work with which can be time consuming, so be sure to start your search without delay. A good place to start is PubMed Central, an online source for freely available research and review articles in the biomedical sciences:

http://www.ncbi.nlm.nih.gov/pmc/

As you look for a primary research paper in a field that interests you, be sure:

• the paper is actually a primary research paper
• the paper is not too long, otherwise you will have too many experiments to analyze
• you are familiar with the experimental methods (look at the Materials and Methods)

Once you have identified one or more papers of interest, consult your instructor to be sure you have an appropriate paper for this assignment.

Exercise 2 – Analyzing your own research paper

To complete this lab you will analyze the primary research paper you have chosen for the “Article Review” assignment and produce an outline addressing each aspect of the paper indicated in the guidelines. This will help you write your formal article review and prepare your oral presentation. The outline will be worth 10 points and your instructor will clarify when it is due.
INTRODUCTION

The cells of all living organisms require energy to keep themselves alive and fulfilling their roles. Where does this energy come from? The answer is energy released from molecules of the nucleotide adenosine triphosphate or ATP.

As you can see from the diagram above, the hydrolysis of ATP to ADP (adenosine diphosphate) and inorganic phosphate (P) is exergonic and thus releases energy which cells can use to do any number of things. Once hydrolyzed, ATP can be regenerated from ADP and P, though this is endergonic and thus requires energy. The energy needed to regenerate ATP is obtained from “food”, whatever that may be.

The food we eat is first digested by enzymes as you learned in the previous lab. Once the polymers in your food (e.g., polysaccharides, triglycerides, protein) have been broken down by enzymes into monomers (e.g., monosaccharides such as glucose, fatty acids, amino acids), they enter the blood circulation and are delivered to the cells of the body. Within cells, the processes of fermentation and cellular respiration will further catabolize (break down) these molecules, harvesting the energy they contain for the synthesis of ATP.

Let us now take a brief look at fermentation and cellular respiration to see how each process produces ATP using energy released from molecules of glucose. Keep in mind that, although we are focusing on glucose, other molecules such as fatty acids can be used for the same purpose, though in slightly different ways.
Part 1: FERMENTATION

To produce ATP from glucose, whether by fermentation or cellular respiration, cells must first partially break it down by glycolysis ("sugar" "separation"). The enzymes involved in glycolysis are located in the cell cytoplasm and sequentially break down each 6-carbon molecule of glucose to two 3-carbon molecules of pyruvate. In the process, enough energy is extracted to produce 2 molecules of ATP.

\[
\text{glucose (6-carbons)} \rightarrow \rightarrow \rightarrow \text{2 pyruvate (3-carbons)}
\]

\[
2 \text{ADP} + 2 \text{Pi} \rightarrow 2 \text{ATP}
\]

In conjunction with glycolysis, cells will carry out fermentation if there is no oxygen (O₂) available. When you overexert yourself for example, your muscles do not receive enough oxygen and temporarily ferment glucose. In another familiar example, yeast will ferment when placed in an enclosed environment with a source of carbohydrate such as grapes (for making wine) or hops and barley (for making beer).

Interestingly, fermentation does not produce any additional ATP. What it does do is regenerate an important molecule needed for a particular step in glycolysis. This molecule is the electron carrier NAD⁺, which if depleted will bring a halt to glycolysis and ATP production, resulting in cell death. Fermentation therefore contributes to ATP production indirectly by allowing glycolysis, and the production of 2 ATP per glucose, to continue unhindered.

\[
2 \text{NAD}^+ \rightarrow 2 \text{NADH} \rightarrow 2 \text{NAD}^+
\]

\[
\text{glucose} \rightarrow \rightarrow \rightarrow \text{2 pyruvate} \rightarrow \text{2 fermentation products}
\]

\[
2 \text{ADP} + 2 \text{Pi} \rightarrow 2 \text{ATP}
\]

Glycolysis \hspace{1cm} Fermentation

As shown above, NAD⁺, an empty electron carrier, is converted to NADH, a full electron carrier (the electrons being “carried” are associated with the hydrogen atom) during glycolysis. Fermentation is simply one or more biochemical steps that transfer the H in NADH and an extra electron to a molecule of pyruvate. As a result, NADH is restored to NAD⁺, which is needed for glycolysis, and pyruvate is converted to a “fermentation product” which can be a variety of things depending on the organism.

Animals, including human beings, produce lactic acid when their cells ferment. In organisms from other kingdoms the fermentation products can be quite different. Some bacterial species produce acetic acid (vinegar) when they ferment, whereas others produce acetone (the main ingredient in nail polish) or other organic molecules. In the Kingdom Fungi, single-celled yeasts when
fermenting will produce CO$_2$ and ethanol instead. This process, known as *alcohol fermentation*, is the basis for beer and wine production. Regardless of the fermentation products, the purpose of fermentation is always the same – to regenerate NAD$^+$ so that glycolysis can continue to produce 2 ATP per glucose without interruption.

In the following exercise you will investigate alcohol fermentation in yeast under different conditions and measure the production of one fermentation product – CO$_2$.

**Exercise 1 – Observing and Measuring Alcohol Fermentation in Yeast**

1. On your worksheet, write a hypothesis addressing the effect of increasing concentrations of yeast on CO$_2$ production.

2. To test your hypothesis, plan an experiment combining various amounts of water, yeast solution and corn syrup (a source of sugar). You will plan 3 reactions that each total 12 ml in which the independent variable is the concentration of yeast. You should also have a control reaction. No reaction should contain more than 4 ml of yeast solution or corn syrup.

3. Assemble each reaction in small beakers, and once all 3 reactions are complete, transfer each mixture to a labeled saccharometer (as shown on the right). Simultaneously tilt all 3 saccharometers (gently) until no air is trapped inside the column. At this point you will begin the timing of your experiment (record start time on your worksheet).

4. At 5 minute intervals, use the graduations on the column to estimate the volume of gas trapped in the column in ml for a total of 30 minutes.

5. Record the data on your worksheet, graph the data, and answer the associated questions.

**Part 2: CELLULAR RESPIRATION**

While 2 ATP per glucose molecule is clearly better than nothing, it is not nearly enough to meet the energy needs of complex multicellular organisms such as plants and animals. To get the maximum ATP yield from molecules of glucose requires cellular respiration, which and produce up to 36 ATP per glucose molecule. In *aerobic* organisms, cellular respiration requires O$_2$ (which is why we breathe!), hence the term *aerobic respiration*. 
The overall process of cellular respiration can be summarized in the following equation:

\[
\text{glucose} \quad \text{oxygen} \quad \text{carbon dioxide} \quad \text{water} \\
C_6H_{12}O_6 + 6 O_2 \rightarrow 6 CO_2 + 6 H_2O \\
36 \text{ADP} + 36 P_i \rightarrow 36 \text{ATP}
\]

In eukaryotic cells, cellular respiration begins with glycolysis in the cytoplasm and continues in the mitochondria as outlined below:

**The Citric Acid Cycle** – This is a biochemical pathway involved in breaking pyruvate down to CO₂. In the process, energy rich electrons in hydrogen atoms are transferred to NAD⁺ and FAD producing NADH and FADH₂. In addition, 2 ATP per original glucose are also produced.

**Oxidative Phosphorylation** – This is the process by which the remaining 32 ATP molecules are produced involving two distinct stages:

- **Electron Transport** - electrons gathered by NADH and FADH₂ during glycolysis and the citric acid cycle are used to produce an H⁺ gradient within mitochondria in a process that requires O₂

- **Chemiosmosis** – the H⁺ gradient produced by electron transport provides energy for ATP synthase to make 32 ATP per original glucose

The importance of O₂ for cellular respiration cannot be overemphasized. O₂ is the final electron acceptor in the electron transport chain. Without O₂ electron transport does not occur, bringing cellular respiration to a halt, and the only option for ATP production is fermentation. This means 2 ATP per glucose instead of 36. The cell diagram below summarizes fermentation and cellular respiration in relation to O₂ and where each process occurs in eukaryotic cells, and the number of ATP molecules produced.
In the next exercise you will detect the oxidation of succinate, a metabolic intermediate in the Citric Acid Cycle, as evidence of cellular respiration. **Succinate dehydrogenase (SDH)** is an enzyme in the Citric Acid Cycle which catalyzes the removal of 2 hydrogens from succinate (i.e., the oxidation of succinate) which are transferred to the electron carrier FAD. This yields the products fumarate and FADH$_2$ as shown below:

\[
\text{FAD} + \text{succinate} \xrightarrow{\text{succinate dehydrogenase}} \text{FADH}_2 + \text{fumarate}
\]

FADH$_2$ in turn will donate the electrons from these 2 hydrogens to coenzyme Q in the electron transport chain. The compound DCPIP (di-chlorophenol-indophenol) is not normally found in cells, however when added to mitochondria it will substitute for coenzyme Q and receive electrons from FADH$_2$. Before receiving the electrons (in its oxidized state) DCPIP is a blue color, however after receiving the electrons (being reduced by FADH$_2$) DCPIP is colorless. Because of this color change, DCPIP is a good indicator of respiration as illustrated below.

In the next exercise you will add DCPIP to a mitochondrial suspension made from lima beans (yes, plants carry out cellular respiration too!) and detect the citric acid cycle step illustrated above by the loss of blue color in DCPIP.
**Exercise 2 – Detecting cellular respiration in a mitochondrial suspension**

1. Review the experiment below, write your hypothesis on your worksheet and identify the independent and dependent variables as well as the control.

2. Label 3 test tubes and add the components indicated in the chart below, in order:

<table>
<thead>
<tr>
<th></th>
<th>Tube #1</th>
<th>Tube #2</th>
<th>Tube #3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate Buffer Solution</td>
<td>4.0 ml</td>
<td>3.7 ml</td>
<td>3.4 ml</td>
</tr>
<tr>
<td>DCPIP</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Succinate Solution</td>
<td>0 ml</td>
<td>0.3 ml</td>
<td>0.6 ml</td>
</tr>
<tr>
<td>Lima Bean Extract</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>5.0 ml</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

3. Make sure each tube is mixed and score the color of each tube every 5 minutes for a total of 30 minutes using the scale shown below:

   ![Color Scale]

   0 1 2 3 4 5

4. Graph color score vs time for each tube and answer the associated questions.

---

**Part 3: DESIGNING AN EXPERIMENT**

Having investigated alcohol fermentation in yeast and cellular respiration in a mitochondrial suspension, you and your group will design and carry out a new experiment to expand on what you have already learned.

**Exercise 3 – Design an experiment**

1. Decide as a group to further investigate yeast fermentation or cellular respiration in lima bean mitochondrial suspension.

2. Identify an independent variable you have not already investigated (e.g., amount of corn syrup or mitochondrial suspension) and come up with a hypothesis with regard to this variable. Write the hypothesis on your worksheet.

3. Design an experiment to test this hypothesis. On your worksheet, briefly describe your experimental plan, and identify the independent variable, dependent variable and control.

4. Carry out your experiment, record and graph the results on your worksheet, and write your conclusion.
Fermentation & Cellular Respiration Lab Worksheet

Name: __________________________ Group: _______ Date: ______________

Exercise 1 – Yeast fermentation

State your hypothesis below and identify the indicated components of this experiment:

- Hypothesis:

- Independent variable:

- Dependent variable:

- Control:

Results:

<table>
<thead>
<tr>
<th></th>
<th>0 min</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>20 min</th>
<th>25 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tube 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tube 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

On the grid below, graph the results for each tube by plotting the amount of gas produced vs time.

- Did these results support your hypothesis? Explain.
Exercise 2 – Cellular respiration

Indicate the roles of each of the following components in your experiment:

- Lima bean extract:
- Succinate:
- DCPIP:
- Buffer:

State your hypothesis below and identify the indicated components of this experiment:

- **Hypothesis:**

- Independent variable:
- Dependent variable:
- Control:

Results:

<table>
<thead>
<tr>
<th></th>
<th>0 min</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>20 min</th>
<th>25 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tube 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tube 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

On the grid below, graph the results for each tube by plotting the color score vs time.
Did these results support your hypothesis? Explain.

Why was it important for this and the previous experiment to keep the total volume of each tube constant?

Exercise 3 – Design an experiment

State your hypothesis:

Provide the details of your experiment below:

Identify the indicated components of your experiment:

Independent variable:

Dependent variable:

Control:

Draw a chart or table and record the results of your experiment below:
Graph your results on the grid below:

- Did these results support your hypothesis? Explain.
Bio 6 – Photosynthesis Lab

Introduction

In order to survive, organisms require a source of energy and molecular building blocks to construct all of their biological molecules. The ultimate source of energy for almost all of life on Earth is the light that comes from the sun (see the box on the next page for an example of organisms that do not depend on light as the ultimate source of energy).

Photosynthesis and cellular respiration are two of the most important biochemical processes of life on Earth. Both are a series of reactions that are catalyzed by unique enzymes at each step. Although it is somewhat of an oversimplification to describe them as “opposite” sets of reactions, for introductory purposes we can think of them as such.

Photosynthetic (“light” “forming”) organisms are those that can take simple molecules from the environment such as carbon dioxide (CO₂) and water (H₂O), and using the energy of the sun, create their own biological macromolecules such as carbohydrates, proteins, lipids and nucleic acids. You will note that the reactions of photosynthesis are both endothermic and anabolic, in that they require energy and use small molecules to make larger ones. These reactions take place in the chloroplasts of plant cells.

We can summarize the series of reactions in photosynthesis in terms of the initial reactants and the final products - leaving out details of all the reactions in between. In introductory biology, we simplify what is happening by showing only the monosaccharide glucose as the ultimate organic molecule that is produced.

\[
\text{sunlight} \\
6 \text{ CO}_2 + 6 \text{ H}_2\text{O} \rightarrow \rightarrow \rightarrow C_6\text{H}_{12}\text{O}_6 + 6 \text{ O}_2
\]

carbon dioxide water glucose oxygen

In reality, the products of photosynthesis include the formation of all of the biological macromolecules the organism requires. For example, photosynthetic organisms must have a source of nitrogen (e.g. fertilizer) to use photosynthetic products to make proteins and nucleic acids. You should also note that one of the products of photosynthesis is oxygen. Essentially all of the oxygen in our atmosphere comes from the process of photosynthesis.

The chemical reactions of photosynthesis actually occur in two distinct stages – the Light Reactions and the Calvin Cycle:

1) The Light Reactions convert light energy to energy contained in ATP and NADPH while producing O₂ as a byproduct.
2) The **Calvin Cycle** (also known as the **Light-independent or Dark Reactions**) which uses energy from ATP, hydrogen from NADPH, and carbon from CO2 to produce energy-rich glucose and other organic molecules.

Both of these processes occur in chloroplasts and are summarized in the illustration below.

The exercises you will carry out today involve detecting evidence of O2 production and CO2 consumption as indicators of the Light Reactions and the Calvin Cycle, respectively. Be sure to refer to the illustration above as needed to ensure that you relate each exercise to the corresponding stage of photosynthesis.
Part 1: Photosynthesis & CO₂ Consumption

You have learned that photosynthesis involves the conversion of carbon dioxide and water into organic molecules such as glucose during the Calvin Cycle. In doing so, oxygen gas is produced during the Light Reactions while carbon dioxide is consumed during the Calvin Cycle.

In the first experiment, we will be using a plant called Java Moss. The experimental design involves observing changes in the concentration of dissolved CO₂ revealed by changes in pH. The variables to be examined in relation to carbon dioxide are the amount of light exposure and the presence of plant material.

When CO₂ concentrations increase in aqueous solution, it causes an increase in the concentration of H⁺ ions, thus decreasing the pH value. This occurs through the formation of an intermediary compound called carbonic acid, which forms by the combination of CO₂ and H₂O as shown here:

\[
\text{CO}_2 + \text{H}_2\text{O} \quad < \quad \text{H}_2\text{CO}_3 \quad < \quad \text{H}^+ + \text{HCO}_3^- \\
\text{carbonic acid} \quad < \quad \text{bicarbonate}
\]

Thus the pH indicator phenol red will be used to reflect the amount of CO₂ present in the vials. Phenol red is orange when slightly acidic and yellow when more strongly acidic. This occurs when the concentration of CO₂, and thus the concentration of carbonic acid, is high.

<table>
<thead>
<tr>
<th>Below pH 7 (acidic)</th>
<th>Neutral pH</th>
<th>Above pH 7 (basic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higher CO₂ Level</td>
<td>-----------</td>
<td>Lower CO₂ Level</td>
</tr>
<tr>
<td>YELLOW or ORANGE</td>
<td>RED</td>
<td>PINK</td>
</tr>
</tbody>
</table>

The relationship between dissolved CO₂ and pH can be summarized as “higher CO₂ concentrations result in higher H⁺ concentrations and thus lower pH values”. Conversely, “lower CO₂ concentrations result in lower H⁺ concentrations and thus higher pH values”.

As you carry out the following experiment, keep in mind that plants, like animals, have mitochondria and carry out cellular respiration which produces CO₂ as a byproduct. So a plant that is not actively photosynthetic (i.e., in the dark) will still carry out cellular respiration and thus may cause a net increase in dissolved CO₂ causing a drop in pH and a color change toward yellow. A plant that is actively photosynthetic, however, will consume more CO₂ in the Calvin cycle than is produced by cellular respiration. So if photosynthesis is occurring there should be a net decrease in dissolved CO₂ and a corresponding increase in pH as shown by a color change toward red.
Exercise 1 – Observing Photosynthesis via CO₂ Consumption

1. Label three screw cap tubes 1, 2 and 3 with a marker and line them up in order in a test tube rack.

2. Use a graduated cylinder to add 100 ml of tap water to a 250 ml Erlenmeyer flask, then add 5 ml of phenol red solution to the flask. Swirl to mix.

3. Take a clean straw and blow bubbles into the solution and stop once it turns orange (not yellow). *Note: orange will allow you to detect pH changes in either direction (i.e., more acidic or basic).*

4. Add CO₂ enriched phenol red solution from the previous step to each tube until almost full.

5. Place equal & generous amounts of *Java moss* in tubes 1 & 2. Tube #3 will not have any plant material.

6. Tightly screw the caps on each tube and record the color of the solution in each tube on your worksheet.

7. Wrap a piece of aluminum foil around tube #2, being sure to cover the bottom so that no light enters this tube.

The chart below summarizes the contents of each tube:

<table>
<thead>
<tr>
<th></th>
<th>Tube 1</th>
<th>Tube 2</th>
<th>Tube 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂ enriched phenol red solution</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Java Moss</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Aluminum foil (to block light)</td>
<td></td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

8. Place each tube side by side in an outside row of a rack and put a 1 liter beaker filled with tap water in front of the tubes. Position your lamp so that the light shines through the beaker of water before reaching the tubes (this will avoid overheating the samples). Turn on the lamp and make sure the light path is not blocked by labels on the beaker or any parts of the rack.

   *NOTE: make sure nothing is blocking the light path to the tubes (e.g., label on beaker or panel on rack)*

9. Record the starting time on your worksheet.

10. Write your hypothesis on your worksheet regarding which tube should show evidence of the most Calvin cycle activity, and identify the independent variable, dependent variable and control.

11. After at least 1 hour has passed, record the colors of each tube on your worksheet, analyze your data and answer the corresponding questions.

   *NOTE: As you examine your tubes, remember that photosynthesis, specifically the Calvin cycle, will decrease the concentration of dissolved CO₂, and cellular respiration will increase the concentration of dissolved CO₂.*

   *Move on to the next experiment while this experiment continues...*
Part 2: Photosynthesis & \( O_2 \) Production

Refer to the overall reactions of photosynthesis in the introduction and you will see that the only gaseous product of photosynthesis is oxygen gas or \( O_2 \). This is the result of splitting water during the Light Reactions of photosynthesis. Thus if you can detect gas production in plant material you can be confident that the gas is \( O_2 \), in particular if the gas production is dependent on light. The next exercise involves a very simple but clever method to detect gas production in plant material, specifically small pieces of spinach, as they are exposed to a light source.

To perform this experiment you will produce numerous small circular spinach discs, remove any residual gas from the spinach discs, expose them to various levels of light, and determine the proportion of discs that begin to float due to oxygen gas production.

Exercise 2A – Detecting \( O_2 \) Production during the Light Reactions of Photosynthesis

Preparing the spinach discs:

1. Obtain several spinach leaves from the front of the lab.

2. Place the spinach leaves on the cutting board at your lab bench, and using the metal corer at your lab bench punch at least 75 or so discs from the leaves (enough for Exercises 2A & 2B). This will go faster if you punch through multiple layers of leaves.

3. Transfer all of the spinach discs to the 250 ml filter flask (with spout on the neck) at your bench.

4. Add 0.2 % sodium bicarbonate (\( \text{NaHCO}_3 \)) to the flask up to the 100 ml line, and swirl to make sure all the spinach discs are in the liquid.

5. Place the rubber stopper on top of the flask and make sure the hole is sealed with a piece of masking tape.

6. Connect the flask to the vacuum spout with the rubber hose provided and turn on the vacuum.

7. Wait until the liquid begins to bubble vigorously and then stops. This may take several minutes, and when complete your spinach discs will be “degassed”.

   NOTE: Sometimes the vacuum pressure is quite weak. If are having trouble getting your flask to bubble, consult your instructor.

8. Turn off the vacuum and peel back the tape on the rubber stopper to let air into the flasks. Most of the spinach disks should then sink to the bottom.

9. Give the flask a swirl and then immediately pour the bicarbonate solution with the spinach discs into the glass bowl at your bench. If you pour after swirling the discs should not stick to the side.

10. Proceed to setting up your experiment, and store any leftover spinach discs in a dark place such as a drawer.
Setting up the experiment:

1. Label the three glass petri dishes (the base, not the lid!) A, B and C.

2. Fill the base of each petri dish ~2/3 full with 0.2% NaHCO₃.

3. Use tweezers to transfer 10 completely submerged (i.e., on the bottom of the bowl) spinach discs to each petri dish. Each disc should be completely flat on the bottom of the petri dish before beginning the experiment.

4. Place a lid on each petri dish and put them in the following locations:
   - A – in a closed drawer at your work bench (this is your “no light” control)
   - B – leave on your bench top far from the lamp
   - C – position directly under the lamp, cover with a one liter beaker filled with water*, and turn the light on

   *The beaker of water is essentially a heat filter preventing the lamp from increasing the temperature of your sample. Without this temperature would be a 2nd independent variable.

5. Leave each dish of spinach discs in their respective locations for 20 minutes.

6. Count the number of discs that are floating or on edge (i.e., more buoyant due to O₂ production).

7. Record the results on your worksheet, graph the data (% floating vs source of light), and answer any associated questions on the worksheet.

Exercise 2B – Design an experiment

In this exercise you and your group will design a new experiment based on the previous one. In this experiment you will test the effect of different colors (i.e., different wavelengths of visible light) on photosynthetic activity as assessed in the previous experiment.

1. As a group, come up with a hypothesis regarding the effect of the different color filters at your lab bench (red, green and blue) on photosynthetic activity. Write the hypothesis on your worksheet.

2. Design an experiment to test this hypothesis. On your worksheet, briefly describe your experimental plan, and identify the independent variable, dependent variable and control.

3. Carry out your experiment, record and graph the results on your worksheet, and write your conclusion.

   NOTE: Due to limited supplies you can use the results for dishes A and C from Exercise 2A as negative and positive controls, respectively.
Exercise 1 – Photosynthesis & CO₂ consumption

State your hypothesis below and identify the indicated components of this experiment:

- Hypothesis:
- Independent variable:
- Dependent variable:
- Control:

Results:

<table>
<thead>
<tr>
<th></th>
<th>Tube 1</th>
<th>Tube 2</th>
<th>Tube 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color at Start of Experiment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Color at the End of the Experiment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change in pH (more acidic, more basic, no change)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change in [CO₂] (increase, decrease, no change)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Explain any color change in each tube with regard to photosynthesis (or cellular respiration):

TUBE 1:

TUBE 2:

TUBE 3:

Did these results support your hypothesis? Explain.
Exercise 2A – Photosynthesis & O₂ production

State your hypothesis below and identify the indicated components of this experiment:

➢ Hypothesis:

➢ Independent variable:

➢ Dependent variable:

➢ Control:

Results:

<table>
<thead>
<tr>
<th>light source</th>
<th>total # of discs</th>
<th># of floating discs</th>
<th>% floating discs</th>
</tr>
</thead>
<tbody>
<tr>
<td>no light (dark)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>room light</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lamp</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Graph your results on the grid below:

➢ Did these results support your hypothesis? Explain.
Exercise 2B – Design an experiment

- Briefly describe or outline the design of your experiment below:

- State your hypothesis:

*Identify the indicated components of your experiment:*

- Independent variable:
- Dependent variable:
- Control:

*Draw a chart or table and record the results of your experiment below:*

*Graph your results on the grid below:*

- Did these results support your hypothesis? Explain.
Lab 19 - Assessing the Efficacy of Antiretroviral Drug Combinations in HIV Patients using ELISA

You are part of a team of clinicians and researchers running a clinical trial to assess the efficacy of two different antiretroviral drug “cocktails” in treating HIV patients. You will evaluate how well two patients, Patient “A” and Patient “B”, are doing after several months of treatment, each with a different antiretroviral cocktail. The levels of HIV virus in the blood (aka the viral load) is a key metric of how effective a drug cocktail is at preventing the virus from replicating and spreading. You make a qualitative measurement of the viral load in Patient A and Patient B using an enzyme-linked immunoabsorbant assay (ELISA). This assay uses an antibody that binds to the HIV capsid protein p24.

The yellow tubes are marked A for serum taken from Patient A or B for serum taken from Patient B (note – these are simulated, not real, samples). These are the samples you will test in the procedure beginning on page 55 of the BioRad lab manual (below).

Make a prediction about what your results will look like if one cocktail is more effective than the other:

Conclusion:
Based on your actual data, which patient was treated with a more effective antiretroviral cocktail?
Biology 6 - Assessing the Efficacy of Antiretroviral Drug Combinations in HIV Patients using ELISA

Introduction

Immunology is the study of the immune system and how the body protects itself against disease. Over 100 years ago, biologists found that animals’ internal immune systems respond to invasion by “foreign entities” or antigens. When an invader enters the body, it provokes an immune response that begins with the production of proteins called antibodies. Like magic bullets, antibodies seek out and attach themselves to invading entities (antigens), flagging the invaders for destruction by other cells of the immune system. The antigenic invaders may be any molecules foreign to the body, including components of infectious agents like bacteria, viruses, and fungi. Today, antibodies have become vital scientific tools, used in biotechnology research and to diagnose and treat disease. The number of different antibodies circulating in the blood has been estimated to be between $10^6$ and $10^{11}$, so there is usually an antibody ready to deal with any antigen. In fact, antibodies make up to 15% of your total blood serum protein. Antibodies are very specific; each antibody recognizes only a single antigen.

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

Where Is ELISA Used in the Real World?

With its rapid test results, the ELISA has had a major impact on many aspects of medicine and agriculture. ELISA is used for such diverse purposes as home pregnancy tests, disease detection in people, animals, and plants, detecting illegal drug use, testing indoor air quality, and determining if food is labeled accurately. For new and emerging diseases like severe acute respiratory syndrome (SARS), one of the highest priorities of the US Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) has been to develop an ELISA that can quickly and easily verify whether patients have been exposed to the virus.

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

**How Are Antibodies Made?**

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

![Antigen Detection ELISA](image)

**A)** Structure of IgG bound to the HIV capsid protein p24 as determined by X-ray crystallography (Harris et al. 1998, Momany et al. 1996). These structures can be downloaded from the Protein Data Bank (www.pdb.ufmg.br, Berman et al. 2000) using the PDB identification codes 1IGY and 1AFV and manipulated using free online software such as Rasmol and Protein Explorer. **B)** A commonly used representation of an antibody bound to an antigen.
Secondary antibodies recognize and bind to primary antibodies in an immunoassay. They are prepared by injecting antibodies produced by one species of animal into another species. This works because the antibodies produced by different species are different enough from each other that they will provoke an immune response. For example, if you want a secondary antibody that will recognize a human primary antibody, inject human antibodies into an animal like a rabbit. After the rabbit immune response, the rabbit serum will contain antibodies that recognize and bind to human antibodies. Secondary antibodies are frequently labeled to make them visible.

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

**Controls in Immunoassays**

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

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

Now let’s put this all together.

**The main steps in this antigen detection ELISA are:**

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

2. Add primary antibody to the wells and incubate. The antibodies will seek out the antigen from the many proteins bound to the well. If your sample contains the antigen, the antibodies will bind it tightly and remain in the well.
3. Detect the bound antibodies with HRP-labeled secondary antibody. If the primary antibodies have bound to the antigen, the secondary antibodies will bind tightly to the primary antibodies.

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

Exercise 1 - Assessing the Efficacy of Antiretroviral Drug Combinations in HIV Patients using ELISA

You are part of a team of clinicians and researchers running a clinical trial to assess the efficacy of two different antiretroviral drug “cocktails” in treating HIV patients. You will evaluate how well two patients, Patient “A” and Patient “B”, are doing after several months of treatment, each with a different antiretroviral cocktail. The levels of HIV virus in the blood (aka the viral load) is a key metric of how effective a drug cocktail is at preventing the virus from replicating and spreading. You will make a qualitative measurement of the viral load in Patient A and Patient B using an enzyme-linked immunoabsorbant assay (ELISA). This assay uses an antibody that binds to the HIV capsid protein p24.

Carry out the ELISA protocol on the following pages to determine how effective each antiretroviral cocktail has been in these patients. Before beginning, however, predict what the results should look like if 1) an antiretroviral cocktail was ineffective 2) an antiretroviral cocktail was effective. Write your predictions in your lab notebook.
Laboratory Procedure

1. Obtain the tubes marked A (for serum taken from Patient A), and those marked B (for serum taken from Patient B). Note – these are simulated, not real, samples.

2. Label the outside wall of each well of your 12-well strip. Two students may share a strip of 12 wells. On each strip label the first three wells with a “+” for the positive controls and the next three wells with a “−” for the negative controls. Label the remaining wells for patient A and patient B. When you are done your 12-well strip should look like this:

   ![Diagram of a 12-well strip labeled with positive and negative controls and initials for patient A and B]

3. Bind the antigen to the wells:
   a. Use a pipet to transfer 50 µl of the positive control (+) from the violet tube into the three “+” wells.
   b. Use a fresh pipet tip to transfer 50 µl of the negative control (−) from the blue tube into the three “−” wells.
   c. Use a fresh pipet tip for each sample and transfer 50 µl of each of your team’s samples into the appropriately initialed three wells.
4. Wait 5 minutes while all the proteins in the samples bind to the plastic wells.

5. Wash the unbound sample out of the wells:
   a. Tip the microplate strip upside down onto the paper towels so that the samples drain out, then gently tap the strip a few times upside down on the paper towels. Make sure to avoid splashing sample back into wells.
   b. Discard the top paper towel.
   c. Use a transfer pipet filled with wash buffer from the beaker to fill each well with wash buffer taking care not to spill over into neighboring wells. The same transfer pipet will be used for all washing steps.
   d. Tip the microplate strip upside down onto the paper towels so that the wash buffer drains out, then gently tap the strip a few times upside down on the paper towels.
   e. Discard the top 2–3 paper towels.

6. Repeat wash step 5.

7. Use a fresh pipet tip to transfer 50 µl of primary antibody (PA) from the green tube into all 12 wells of the microplate strip.

8. Wait 5 minutes for the primary antibody to bind.

9. Wash the unbound primary antibody out of the wells by repeating wash step 5 two times.
10. Use a fresh pipet tip to transfer 50 µl of secondary antibody (SA) from the orange tube into all 12 wells of the microplate strip.

11. Wait 5 minutes for the secondary antibody to bind.

12. Wash the unbound secondary antibody out of the wells by repeating wash step 4 three times.

   The secondary antibody is attached to an enzyme (HRP) that chemically changes TMB (the enzyme substrate), turning it from a colorless solution to a blue solution.

13. Use a fresh pipet tip to transfer 50 µl of enzyme substrate (SUB) from the brown tube into all 12 wells of the microplate strip.

14. Wait 5 minutes. Observe and record your results.

**Results Section**

Label the figure below with the same labels you wrote on the wells in step 1. In each of the wells, put a “+” if the well turned blue and a “−” if there is no color change.

Is your sample positive? Explain your answer.
ELISA – Study Questions

1. What does the acronym ELISA mean?

2. Of the different types of biological macromolecules, which ones most commonly serve as antigens to which antibodies will bind?

3. Describe the roles of the 1\textsuperscript{o} Antibody and the 2\textsuperscript{o} Antibody during an ELISA test.

4. Diagram a well containing all components necessary for a positive ELISA result.

5. After the addition of the 2\textsuperscript{o} Antibody, how did you determine which wells contain the Antigen bound to 1\textsuperscript{o} Antibody bound to 2\textsuperscript{o} Antibody?

6. Describe how you could use ELISA to determine if the human and cow versions of a specific protein (e.g. the blood protein albumin) have a similar epitope.
Bio 6 – Principles of Genetic Inheritance Lab

Overview

In this laboratory you will learn about the basic principles of genetic inheritance, or what is commonly referred to as “genetics”. A true appreciation of the nature of genetic inheritance will require solving of a variety of genetics problems, and to do so you will need to understand several related concepts, some which should be familiar and others which may be new to you. Thus you will begin this lab by examining the concepts of **genes**, **gamete** production by **meiosis**, and **probability**. You will then use these concepts to work through a series of genetics problems addressing various aspects of genetic inheritance in plants and animals.

Part 1: KEY GENETIC CONCEPTS

We all know that when living organisms reproduce, their offspring are much like their parents. Chickens don’t give birth to lizards and apple trees don’t give rise to pine trees. So what is the biological basis for this obvious reality? You probably already know this has to do with genes, genes one inherits from one’s parents. However the process of passing on genes from one generation to the next is more complex than it may appear.

The simplest form of genetic inheritance involves **asexual** reproduction. This is the case when a single parent organism passes its genes to offspring which are basically clones of the parent (i.e., genetically, and for the most part, physically identical). Although this mode of reproduction is quite convenient (imagine if you could simply have children identical to yourself, no partner necessary), it has one extremely significant shortcoming: **NO genetic diversity**. For some species asexual reproduction works quite well, however for most plants and animals (including humans) this just won’t cut it, genetic diversity is too important for the long term survival of species.

So how is genetic diversity produced? The answer is **sexual reproduction**: the production of **gametes** (sperm and eggs) by **meiosis** followed by the fusion of sperm and egg (fertilization) to form a new, genetically unique individual. Sexual reproduction essentially “shuffles” the genes of each parent producing a unique combination of parental genes in each and every offspring. This is the sort of genetic inheritance we will focus on, genetic inheritance based on sexual reproduction.

Through sexual reproduction, each offspring inherits a **complete** set of genes from each parent, however the study of genetic inheritance is generally limited to one or two genes at a time. Thus when you begin to work with genetics problems you will focus initially on a single gene at time, and then learn how to follow the inheritance of more than one gene. To focus on large numbers of genes would be rather complicated and is not necessary for our purposes.

Before you begin to examine genetic inheritance via genetics problems, you will need to understand some important concepts that are central to the process: the nature of **chromosomes**, **genes** and genetic **alleles**; the process of **gamete production** by **meiosis**; and the concept of **probability**. Once these concepts and their associated terminology are clear, you will then be ready to immerse yourself into the world of genetic inheritance.
Chromosomes, Genes and Alleles

As you learned in the previous lab, chromosomes are extremely long pieces of DNA in the nuclei of cells that contain up to a thousand or more genes each. Each species of organism has a characteristic number of chromosome types: distinct chromosomes each having a unique set of genes and a unique length. For example, the fruit fly Drosophila, an organism used in many genetic studies, has only 4 types of chromosomes – 3 autosomes (non-sex chromosomes) and the X and Y sex chromosomes. Human beings (Homo sapiens) on the other hand have 23 types of chromosomes – 22 autosomes and the sex chromosomes (X and Y) as illustrated in the human male karyotype shown below (notice the X and Y sex chromosomes):

Notice one more thing about this human karyotype: there are two of each autosome as well as two sex chromosomes. This is because human beings are diploid, which means having two of each chromosome type. Most plants and animals are in fact diploid, and as we investigate the process of genetic inheritance we will only concern ourselves with diploid species. However you should be aware that not all organisms are diploid. Some are normally haploid (one of each chromosome) such as the fungi, and some may have more than two of each chromosome (e.g., four of each = tetraploid, eight of each = octoploid) as seen in a fair number of plant species as well as a few animal species.

As shown in the diagram to the left, genes are discrete sections of chromosomal DNA responsible for producing a specific protein or RNA molecule. The process of gene expression, the production of protein or RNA from a gene, will be addressed in a future lab.

The functional protein or RNA molecule produced from a particular gene is its gene product. It is important to realize that the DNA sequence of a gene, and hence its gene product, can vary within a species. In other words, a particular gene in a species such as Homo sapiens can have different versions, what are referred to in genetics as alleles. The gene products produced from an organism’s genetic alleles account for its physical and behavioral characteristics, what we collectively call an organism’s traits. The specific traits an individual exhibits, whether physical or behavioral, are referred to as the individual’s phenotype. The specific genetic alleles an individual has for a particular gene is the individual’s genotype. As you shall soon see, an individual’s phenotype is largely determined by its genotype.
Since diploid organisms have two alleles for each gene, an individual can have two copies of the same allele for a gene or two different alleles. If the alleles are the same, the individual is said to be homozygous for that gene. If the alleles are different, the individual is said to be heterozygous for that gene. When an individual is heterozygous for a gene, one allele may override or “mask” the other allele by determining the phenotype regardless of the other allele. In this situation, the allele that determines the phenotype is said to be dominant while the other allele is said to be recessive. The terms dominant and recessive are relative terms just like the terms big and small. Something is only big or small in relation to something else, and in the same way an allele for a gene is only dominant or recessive in relation to another allele for the same gene.

Meiosis and the Production of Gametes

Sexual reproduction in diploid plant and animal species requires the production of haploid gametes by the process of meiosis which is illustrated below.

Since meiosis was covered in the previous lab, we won’t review the process in much detail other than to remind you of several key points that pertain to genetic inheritance:

1) Diploid organisms have two of each chromosome type, one haploid set of chromosomes inherited from the mother (maternal chromosomes) and another haploid set inherited from the father (paternal chromosomes), and thus two alleles for each gene, one maternal allele and one paternal allele.

2) The haploid gametes produced by meiosis (egg or sperm) contain one of each chromosome type (e.g., one of each autosome and one sex chromosome), and thus contain only one allele for each gene.

3) It is completely random which chromosome of a given type, maternal or paternal, ends up in a gamete produced by meiosis. Thus it is completely random which allele for a particular gene (maternal or paternal) ends up in a gamete.

4) Since an individual has only two alleles for each gene, there is a 50% chance that either allele (maternal or paternal) will end up in a given gamete.
These points are central to the study of genetics as it pertains to sexual reproduction. Diploid parent organisms produce large numbers of haploid gametes, any of which may fuse at the moment of fertilization to produce a diploid zygote, a genetically unique new individual. It is the combination of alleles inherited from each parent that determine the genotype and phenotype of each new offspring.

Given the variety of possible genotypes and phenotypes for the offspring of any two parents, genetics must also address the probability of each possibility. So before you begin working with genetic crosses it is important that you understand basic concepts of probability.

**Probability**

To understand genetic inheritance, you need to have a basic understanding of probability. Probability refers to the likelihood that something will happen as opposed to what actually happens. For example, we all know that a single coin flip has a 50% chance of being “heads” or “tails”, thus the probability of heads is 50% or 0.5 or ½ as is the probability of tails. However we also know that we cannot know what the outcome of a single coin flip will be. All we can do is predict the likelihood or probability of each possible outcome, in this case heads or tails.

When analyzing the inheritance of genes you will also be dealing with probabilities. When two organisms mate and produce offspring we cannot know what genetic alleles will be inherited by a given individual (genotype) or its physical characteristics (phenotype). We can only predict the likelihood of various characteristics based on the probability of inheriting particular genetic alleles from each parent. The next two exercises will help illustrate the nature of probability and help prepare you to solve genetics problems.

**Exercise 1A – Probability and sample size**

*In this exercise you will perform several sets of coin flips. This will be done to compare the predicted outcome based on probability to the actual outcome, and how this relates to sample size (i.e., the number of repetitions):*

1. On your worksheet, determine the expected numbers of heads and tails for sample sizes of 10 and 100 coin flips (the probability of heads is 50% or 0.50, as is the probability of tails).
2. Perform 10 sets of 10 coin flips, recording the results on your worksheet.
3. Combine the results for all 10 sets of coin flips on your worksheet to get a total number of heads and tails out of 100 coin flips.
4. On your worksheet, compare the actual results to expected results for each set of 10 coin flips as well as the combined set of 100 coin flips and answer the corresponding questions.
When flipping a coin there are only two possible outcomes, each with the same probability. Sometimes, however, you must deal with more than two possible outcomes, each with a different probability. This is the case, for example, when you roll a pair of dice, for which there are eleven possible outcomes in terms of the total sum: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12.

The probabilities of each possible sum of the dice are not the same. If you have ever played a board game or craps you know for example that a roll totaling 7 is much more likely than a roll totaling 2. So why are the probabilities different? It has to do with how many different combinations add up to a given sum. For example, to roll a pair of dice and get a sum of 2, both dice must show a “1”. There is no other way to roll a total of 2 with a pair of dice. There are six different ways, however, to roll a 7:

<table>
<thead>
<tr>
<th>Red die</th>
<th>Black die</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

So how do you determine the probabilities of each possible sum of the dice? As shown in the matrix below, there are $6 \times 6 = 36$ different combinations yielding 11 possible sums:

<table>
<thead>
<tr>
<th>red die</th>
<th>black 1</th>
<th>black 2</th>
<th>black 3</th>
<th>black 4</th>
<th>black 5</th>
<th>black 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>red 1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>red 2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>red 3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>red 4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>red 5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>red 6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>12</td>
</tr>
</tbody>
</table>

Each die has six possible outcomes (1 through 6), each of which are equally likely and each of which can be paired with any roll on the other die. Looking carefully at the matrix, you can see there is one combination out of 36 that yields a sum of 2 (probability = $1/36$ or 0.028 or 2.8%), two combinations that yield a sum of 3 (2/36 or 0.056 or 5.6%), three combinations that yield a sum of 4 (3/36 or 0.083 or 8.3%), and so on.

If you think about it, the rolling of a pair of dice is actually two independent events in combination. Whether you roll the red die or black first, or both at the same time, is irrelevant. The probability of rolling a sum of 2 (“snake eyes”) for example is still $1/36$, regardless of the timing. This leads to an important rule regarding probability commonly referred to as the “product rule”: 

263
The probability of a specific combination of two or more outcomes is the **product** of the probabilities of each individual outcome.

In our “snake eyes” example, the probability of rolling 1 on the red die is 1/6 and the probability of rolling 1 on the black die is 1/6, thus the probability of rolling 1 on both the red die **AND** the black die is 1/6 x 1/6 which equals 1/36. Another example would be the probability of flipping two coins and having both turn up “heads”, or in other words the probability of coin 1 **AND** coin 2 both yielding “heads”. Using the rule above the probability would be 1/2 x 1/2 which is 1/4.

Another important rule commonly called the “sum rule” or “addition rule” addresses probability when there are **multiple** ways to arrive at a particular outcome:

The probability of an outcome that can occur in multiple ways is the **sum** of the probabilities of each individual outcome.

For example, we have already determined that the probability of rolling a sum of 3 with a pair of dice is 2/36 since there are 2 different ways to obtain a sum of 3: red 1 plus black 2 (1/36) **OR** red 2 plus black 1 (1/36). Each outcome yields a roll of 3 and by adding both probabilities you arrive at an overall probability of 2/36.

Another example would be the flipping of a coin. The probability of flipping heads **OR** tails on a given toss is clearly 100%: 50% heads + 50% tails = 100%. What about the probability of flipping two coins and getting heads for one and tails for the other? There are actually two ways this can occur:

- heads from coin 1 and tails from coin 2 (50% x 50% = 25%)
- tails from coin 1 and heads from coin 2 (50% x 50% = 25%)

In this case the overall probability of getting one heads and one tails from two coin flips is 25% + 25% which equals 50%.

You are now ready for the next set of exercises in probability, and keep in mind that the probability of **X AND Y** occurring means you **multiply**, and the probability of **X OR Y** occurring means you **add**.

**Exercise 1B – Dice Baseball**

In this exercise you will play a game of baseball using a pair of dice. This will require that you consider the probabilities of various outcomes for an “at bat” and match these with probabilities in rolling a pair of dice:

1. On your worksheet, determine the probability of each possible sum for a pair of dice.

2. Consider the probabilities of each sum and use your worksheet to design your own dice baseball game in which a given sum of the dice equals a particular outcome of an “at bat” (e.g., 7 = out, 12 = home run). Do your best to be realistic – your instructor will explain this further.

3. Arrange teams of 1 to 2 people and play a nine inning game keeping score on your worksheet (you can go to extra innings if necessary).
Part 2: SOLVING GENETICS PROBLEMS

Important Tools for Solving Genetics Problems

Before you begin to solve genetics problems it is important that you are familiar with the types of symbols used to represent genetic alleles as well as two important tools you will use to solve genetic problems. Let’s first address the symbols for genetic alleles:

Symbolizing Alleles

Genetic alleles are commonly represented by a single letter, with the dominant allele being upper case and the recessive allele being lower case:

- dominant allele – A
- recessive allele – a

When working with more than one gene in a genetic problem, the alleles of each gene will be represented using a different letter:

<table>
<thead>
<tr>
<th>Gene 1</th>
<th>Gene 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>dominant allele – A</td>
<td>dominant allele – B</td>
</tr>
<tr>
<td>recessive allele – a</td>
<td>recessive allele – b</td>
</tr>
</tbody>
</table>

When writing the genotype for an individual it is best to keep the alleles for a particular gene next to each other and to list a dominant allele before a recessive allele:

Aa Bb

If an allele is unknown, indicate this with an underscore:

A_ bb

When there are more than two alleles for a gene, different superscripts on a single letter are commonly used to distinguish alleles:

- alleles – C\(^\alpha\), C\(^\gamma\), C\(^\iota\)
- genotype – C\(^\alpha\)C\(^\gamma\)

When a gene is located on the X chromosome (“X-linked gene”), alleles are represented by superscripts on a capital X. In males a “Y” without a superscript is used to symbolize the Y chromosome which would not have an allele for the X-linked gene:

- female genotype for an X-linked gene – X\(^\alpha\)X\(^\alpha\)
- male genotype for an X-linked gene – X\(^\alpha\)Y
Pedigrees

For some problems it will be helpful to diagram a pedigree of all family members relevant to the problem as shown here for three generations of a hypothetical family.

As you can see, males are represented by squares and females by circles. Individuals exhibiting the phenotype of concern have a filled-in symbol. Pedigrees are useful in that they allow you to represent all familial relationships visually so that you can fill in and deduce as many genotypes as possible. In addition, they also allow you to deduce the mode of inheritance of the condition in question (i.e., dominant vs recessive condition, sex-linked vs autosomal gene).

On many occasions you may find it simpler to diagram your pedigree without shapes and colors, and simply represent individuals in the pedigree by their genotypes (or as much of the genotype as you know) as shown to the right.

Punnett squares

Another extremely useful tool is the Punnett square, which is basically a grid or matrix showing all possible combinations of gametes from each parent to produce offspring. A Punnett square is useful if you know the genotypes of the parents being crossed. Once you determine all possible haploid gametes for each parent you simply fill out the Punnett square as shown below:

**cross:** Aa (female) x Aa (male)

**gametes:** A & a (female); A & a (male)

\[
\begin{array}{c|c|c}
\text{A} & \text{a} \\
\hline
\text{AA} & \text{Aa} \\
\text{Aa} & \text{aa} \\
\end{array}
\]

By placing the different gametes for each parent along either side of a Punnett square, it becomes easy to fill in all possible combinations of egg and sperm. All that’s left is to determine the probabilities of each genotype and/or phenotype. This gets a bit more complicated when dealing with two genes, however the principle is the same as shown in the next example:
cross: Aa Bb x Aa bb

gametes: AB, Ab, aB & ab; Ab & ab

<table>
<thead>
<tr>
<th>AB</th>
<th>Ab</th>
<th>aB</th>
<th>ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>AA Bb</td>
<td>AA bb</td>
<td>Aa Bb</td>
</tr>
<tr>
<td>ab</td>
<td>Aa Bb</td>
<td>Aa bb</td>
<td>aa Bb</td>
</tr>
</tbody>
</table>

Notice that the Punnett square doesn’t have to be a square at all, it simply needs to accommodate all parental gametes and their possible combinations, nothing more.

**Types of Genetics Problems**

Solving genetics problems involves using the known characteristics of some individuals to deduce the probability of unknown characteristics of other individuals. The characteristics given will relate to phenotype (physical description), genotype (genetic alleles), or both. In general, the genetics problems you will be asked to solve are of two basic types:

1) Characteristics of the parents are given and you must determine the probabilities of each possible type of offspring.

2) Characteristics of the offspring are given and you must deduce characteristics of the parents.

Regardless of which type of problem you are solving, you will need to use the principles we have addressed in Part 1 (genes, alleles, meiosis, gametes, probability), the tools reviewed in the previous section, and a bit of common sense. The approach to solving each type of problem, however, is slightly different.

**Solving Type 1 problems**

In Type 1 problems, you will be given characteristics of the parents being crossed and asked to figure out the probabilities of all possible genotypes and/or phenotypes in the offspring. Solving such problems will require 4 basic steps:

1) **Determine the genotypes of both parents and write out the cross.**
   - genotypes may be given in the problem or you may have to deduce them

2) **Determine the genotypes of all possible gametes produced by each parent, and write them on adjacent sides of a Punnett square.**
   - gametes are *haploid* and thus have one allele for each gene of interest

3) **Fill in all possible offspring genotypes in the Punnett square.**
   - all possible combinations of genetically different eggs and sperm must be accounted for

4) **Determine the probabilities of each possible genotype/phenotype in the offspring.**
To illustrate these steps let’s solve the sample problem below:

In pea plants, purple flower color is determined by a dominant allele \( P \) and white flower color is determined by a recessive allele \( p \). If two heterozygous pea plants with purple flowers are crossed, what are the probabilities of each possible genotype and phenotype in the offspring?

**Step 1: Write out the genotypes of each parent in the cross.**

Due to the phrase “two heterozygous pea plants with purple flowers are crossed” we know that the genotypes of the parents being crossed must be:

\[ Pp \times Pp \]

**Step 2: Determine the genotypes of all different gametes produced by each parent, and arrange them on adjacent sides of a Punnett square.**

Due to meiosis, each parent will produce haploid gametes containing either the \( P \) allele or the \( p \) allele, each in equal proportions (50% of each):

- genotypes of male gametes – \( P, p \)
- genotypes of female gametes – \( P, p \)

![Punnett square](image)

**Step 3: Determine all possible genotypes in the offspring.**

Simply fill in the Punnett square with the genotypes of all possible unions of gametes:

- \( P \times P \) = \( PP \) (1/4)
- \( P \times p \) = \( Pp \) (1/4)
- \( p \times P \) = \( Pp \) (1/4)
- \( p \times p \) = \( pp \) (1/4)

NOTE: The probabilities of each gamete and each resulting offspring genotype are shown in parentheses, though this normally would not be necessary.

**Step 4: Summarize the probabilities of each possible genotype/phenotype in the offspring.**

The Punnett square above reveals that there are only 4 different combinations of sperm and egg yielding 3 possible genotypes and 2 possible phenotypes. Probabilities are determined by following the **product rule** (e.g., \( P \) and \( P \) in a gamete = \( \frac{1}{2} \times \frac{1}{2} = \frac{1}{4} \)) and the **sum rule** (e.g., \( Pp \) or \( Pp \) = \( \frac{1}{4} + \frac{1}{4} = \frac{1}{2} \)):

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \frac{1}{4} PP )</td>
<td>( \frac{1}{4} ) purple flowers (( \frac{1}{4} + \frac{1}{4} + \frac{1}{4} ))</td>
</tr>
<tr>
<td>( \frac{1}{2} Pp (\frac{1}{4} + \frac{1}{4}) )</td>
<td>( \frac{1}{4} ) white flowers</td>
</tr>
<tr>
<td>( \frac{1}{4} pp )</td>
<td></td>
</tr>
</tbody>
</table>

268
Once you complete step 4 you will have your answer. Whether you represent the probabilities as fractions, decimals or percentages is up to you. All that matters is that the value for each probability, regardless of how it is presented, is correct. Now you are ready for the next exercise in which you will solve similar problems on your own...

**Exercise 2A – Type 1 genetics problems**

For the following two problems, tall plant height is determined by a dominant allele (T) and short plant height is determined by a recessive allele (t). Determine the probabilities for all possible *genotypes* and *phenotypes* of the offspring resulting from each cross.

1. homozygous dominant (TT) x heterozygous (Tt)
2. heterozygous (Tt) x homozygous recessive (tt)

**Solving Type 2 problems**

In Type 2 problems you will be given the characteristics of offspring and must figure out the characteristics of one or both parents. To solve such problems simply follow these two steps:

1) Diagram a pedigree containing all individuals in the problem, and indicate the genotypes of as many individuals as possible using the information provided.

2) Use the pedigree to deduce the information the problem asks for.

Let’s do a sample problem of this type:

In cats, black fur color is determined by a dominant allele B and white fur color is determined by a recessive allele b. A white cat gives birth to 3 white kittens and 2 black kittens. What is the genotype and phenotype of the father?

**Step 1: Diagram a pedigree showing the genotypes.**

```
bb x __
  
3 bb, 2 B_
```

The phenotypes given for the mother cat and her kittens indicate the genotypes above (for this problem a full pedigree is not necessary, a simple diagram such as this will suffice).

**Step 2: Deduce missing genotypes in pedigree to arrive at the answer.**

Since some of the kittens are white (bb), both parents must carry the white allele (b). Since some of the kittens are black (B_), at least one parent must have a black allele (B) which in this case can only be the father. Thus the father cat must be black with the genotype Bb, which is the answer to the problem. Though not asked in the problem, you can also deduce that the black kittens all have the genotype Bb since
all kittens receive a white allele (b) from the mother. Keep in mind, it is always a good idea to confirm your answer with a Punnett square based on the parental genotypes you have deduced.

\[ bb \times Bb \]

\[ 3 \text{ bb}, 2 \text{ Bb} \]

**Exercise 2B – Type 2 genetic problems**

*Albinism in human beings (lack of skin pigment) is due to a recessive allele. Use A to represent the dominant “normal” allele and a to represent the recessive albinism allele in solving the two problems below:*

1. An albino child is born to two parents with normal skin pigmentation. What are the genotypes of the parents?

2. An albino woman has 8 children with the same man, 3 with normal pigmentation and 5 that are albino. What can you conclude about the father?

### Incomplete Dominance, Codominance, and Multi-Allelic Inheritance

The problems you have solved so far are relatively straightforward as far as genetics problems go. In each case the problem involved only one gene, two alleles, and the alleles were either dominant or recessive. The next few problems you will solve involve some new concepts that are frequently encountered in genetics.

The first issue is **incomplete dominance**, which occurs when a heterozygous genotype results in a phenotype that is intermediate between the two homozygous phenotypes. For example, two different alleles for a single gene result in three different colors of carnation flowers. The \( R \) allele results in red pigment production (red) and the \( r \) allele results in a lack of pigment production (white). The three possible genotypes and phenotypes are shown below:

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR</td>
<td>red carnations</td>
</tr>
<tr>
<td>Rr</td>
<td>pink carnations</td>
</tr>
<tr>
<td>rr</td>
<td>white carnations</td>
</tr>
</tbody>
</table>

This clearly is not complete dominance, otherwise the heterozygotes (Rr) would have red flowers. The white allele is still considered recessive since it results in the lack of pigment. However there is less red pigment with one red allele (Rr) than with two red alleles (RR). Thus in this case we would say that the red allele is *incompletely* dominant over the white allele.

The nice thing about incomplete dominance is that the phenotype reveals the genotype, something you will appreciate when you solve problems such as those in the next exercise.
Exercise 2C – Incomplete dominance

In snapdragons, red flowers are produced by homozygous RR plants and white flowers are produced by homozygous rr plants. Heterozygous plants (Rr) produce pink flowers. Solve the following problems on your worksheet, being sure to show all your work:

1. Indicate the probabilities of each genotype and phenotype resulting from the a cross between two pink snapdragons.

2. A cross between two snapdragons produced 15 offspring with red flowers and 17 offspring with pink flowers. What are the genotypes and phenotypes of the parents?

Some traits involve a gene that has more than two alleles. One such example is human ABO blood type which is determined by a single gene with three different alleles. The “A” and “B” alleles each result in a different glycoprotein on the surface of red blood cells. When present in the same individual, both alleles are expressed equally resulting in the AB blood type. This is an example of codominance. The “O” allele produces no glycoprotein and thus is recessive to both the “A” and “B” alleles. Based on these relationships the three alleles are symbolized IA, IB, and i, with the various ABO genotypes and phenotypes summarized below:

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Blood Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAIA</td>
<td>A</td>
</tr>
<tr>
<td>IAi</td>
<td>A</td>
</tr>
<tr>
<td>IBIB</td>
<td>B</td>
</tr>
<tr>
<td>IBi</td>
<td>B</td>
</tr>
<tr>
<td>IAIB</td>
<td>AB</td>
</tr>
<tr>
<td>ii</td>
<td>O</td>
</tr>
</tbody>
</table>

Refer to the information above as you solve genetic problems involving human ABO blood type in the next exercise...

Exercise 2D – Multi-allelic inheritance and codominance

Solve the following problems on your worksheet, being sure to show all your work:

1. Indicate the probabilities of each genotype and phenotype in the children of a woman with blood type O and a man with blood type AB.

2. A woman with blood type A and a man with blood type B have 3 children, one each with blood types A, B and O. What are the genotypes of the parents?
Sex Linkage

All of the genetic problems you have solved up to this point involve genes on an autosome, i.e., any chromosome that is not a sex chromosome. For genes on autosomes, inheritance patterns are generally no different for males and females. This is not the case, however, for genes on the X and Y sex chromosomes. Females have two X chromosomes and thus two alleles for each gene on the X chromosome. Males have only one X chromosome along with a single Y chromosome. With very few exceptions, genes on the X chromosome are not found on the Y chromosome and vice versa. Thus males will have only one allele for each gene on the X chromosome.

As a result of this disparity, the inheritance patterns for genes on the sex chromosomes are typically different for males versus females. This phenomenon is called sex linkage, which refers to genetic inheritance that differs depending on the sex of the individual. Genes on the X chromosome, referred to as X-linked, and genes on the Y chromosome, referred to as Y-linked, exhibit sex linkage or sex-linked inheritance. Because the Y chromosome has so few genes, we will limit our focus to X-linked genes.

The approach to solving genetic problems involving X-linked genes is basically the same as for autosomal genes. The alleles for X-linked genes are symbolized using an “X” with a superscript representing the allele (e.g., X^b). In place of a second allele in males the symbol “Y” is used, with no superscript, to represent the Y chromosome. Let’s now look at a sample problem:

In humans, a genetic allele responsible for color-blindness is recessive and X-linked. If a color-blind woman and a man with normal color vision plan to have children, what sort of color vision would you predict in their children?

Step 1: Write out the genotypes of each parent in the cross.

Using X^b and X^a to represent the dominant normal and recessive color-blindness alleles, respectively, the only possible genotypes for “a color-blind woman and a man with normal color vision” are:

X^bX^b  x  X^aY

Step 2: Determine the genotypes of all different gametes produced by each parent.

By meiosis, the woman produces eggs all with the genotype X^b. The man produces sperm containing his X chromosome (X^a) or his Y chromosome:

genotype of female gametes – X^b

.genotypes of male gametes – X^a or Y

Step 3: Determine all possible genotypes in the offspring.

Simply draw a Punnett square accounting for all parental gametes and fill in all possible genotypes for the children:

<table>
<thead>
<tr>
<th></th>
<th>X^b</th>
<th>X^aX^b</th>
<th>X^bY</th>
</tr>
</thead>
<tbody>
<tr>
<td>X^b</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Step 4: Summarize the probabilities of each possible genotype/phenotype in the offspring.

Whenever the results of a cross differ for males and females, you should indicate the probabilities for male and female genotypes/phenotypes separately. The answer to this problem therefore is best expressed as follows:

100% of their girls will have normal color vision (though all will be carriers)
100% of their boys will be color-blind

Hopefully it is clear that the method of solving an X-linked genetic problem is really no different than solving any other problem. Some general features of X-linked genes you may have realized by solving this problem and should keep in mind are:

- sons always receive their X chromosome from their mother (dad contributes Y)
- daughters always receive their father’s X chromosome
- females are diploid for X-linked genes and can be carriers of recessive alleles
- males are hemizygous for X-linked genes (have only one allele) and thus show the phenotype of the single allele inherited from the mother

Exercise 2E – X-linked inheritance

Hemophilia, like color-blindness, is an X-linked recessive condition. Solve the following problems on your worksheet, being sure to show all your work:

1. A normal woman whose father is a hemophiliac marries a normal man. What are the odds that hemophilia will afflict any given male child? any given female child?
2. A couple has three children, a normal boy and a boy and girl each with hemophilia. What can you say about the parents?

The Inheritance of Multiple Genes

When following the inheritance of more than one gene, the approach is similar to single gene inheritance. The trick is to correctly determine all different parental gametes and keep track of the alleles for each gene. To illustrate how this is done, let’s do a sample problem:

In pea plants, flower color is determined by a dominant purple allele \( P \) and a recessive white allele \( p \), and plant height is determined by a dominant tall allele \( T \) and a recessive short allele \( t \). A tall pea plant with purple flowers that is heterozygous for both genes is crossed with a short plant with purple flowers that is heterozygous for flower color. What are the probabilities of each possible phenotype in the offspring?
Step 1: Write out the genotypes of each parent in the cross.

Tt Pp (tall purple plant) x tt Pp (short purple plant)

These are the only genotypes consistent with the information given in the problem.

Step 2: Determine the genotypes of all different gametes produced by each parent.

By meiosis, each parent will produce haploid gametes containing one allele for each gene. The different gametes produced by each parent are:

TP, Tp, tP, tp (tall purple plant)

tP, tp (short purple plant)

Step 3: Determine all possible genotypes in the offspring.

Draw a Punnett square showing the gametes for each parent and fill in the genotypes of all possible offspring:

<table>
<thead>
<tr>
<th></th>
<th>TP</th>
<th>Tp</th>
<th>tP</th>
<th>tp</th>
</tr>
</thead>
<tbody>
<tr>
<td>tP</td>
<td>Tt PP</td>
<td>Tt Pp</td>
<td>tt PP</td>
<td>tt Pp</td>
</tr>
<tr>
<td>tp</td>
<td>Tt Pp</td>
<td>Tt pp</td>
<td>tt Pp</td>
<td>tt pp</td>
</tr>
</tbody>
</table>

Step 4: Summarize the probabilities of each possible phenotype in the offspring.

Based on the Punnett square, 3 out of 8 genotypes give a “tall purple” phenotype, 3 of 8 “short purple”, 1 of 8 “tall white”, and 1 of 8 “short white”. Thus the probabilities of all possible phenotypes in the offspring are:

3/8 – tall with purple flowers
3/8 – short with purple flowers
1/8 – tall with white flowers
1/8 – short white flowers

Problems such as this can get rather complicated, however if you keep track of all alleles properly at each step, you can easily solve any such problem.

Exercise 2F – Genetics problems involving two genes

Solve the following problems on your worksheet, being sure to show all your work:

1. Indicate the probabilities of all phenotypes in the offspring of two tall pea plants with purple flowers, both of which are heterozygous for both genes.

2. A normal woman with an albino father and no family history of hemophilia plans to have children with a hemophiliac man with normal skin pigmentation whose mother is albino. Determine the probabilities of all possible phenotypes in their children.
Interpreting Pedigrees

As mentioned earlier, pedigrees can be a very useful tool to visualize phenotypes in a family lineage (see page 8 of this lab). Once a pedigree has been produced one can deduce the likely mode of inheritance of the genetic condition (i.e., dominant vs recessive, autosomal vs X-linked) and as a result determine the genotypes of various individuals in the pedigree. To illustrate this let’s consider some examples:

This pedigree reveals an **autosomal recessive** mode of inheritance, i.e., the gene responsible for the genetic condition is recessive and located on an autosome (non-sex chromosome). The condition is clearly recessive since unaffected couples have affected children. This would not be possible with a dominant allele. You will also notice that the condition “skips a generation” which also is only possible with a recessive allele. It also appears to be autosomal since males and females are affected equally (recall that males are far more likely to be affected by an X-linked recessive condition). You should also examine affected females such as the one at the top of the pedigree. If a condition is actually X-linked then all sons of affected females would have the condition. If this is not the case, as in this pedigree, the gene must be on an autosome.

Once the mode of inheritance has been determined, genotypes can then be deduced for everyone in the pedigree. For example, all affected individuals above must be homozygous recessive (aa) and if two unaffected parents have affected children, they both must be heterozygous (Aa). For some unaffected individuals the second allele cannot be deduced based on the pedigree and thus is unknown (A?).

Here is an example of a pedigree revealing a genetic condition to be **X-linked recessive**. This condition is recessive for the same reasons as the previous pedigree. It is most likely X-linked since only males are affected. However this cannot be said with 100% certainty since there is a slight chance the gene responsible for this condition is autosomal and by random chance only males are affected in this family.

The next example shows an **autosomal dominant** condition. Notice that, consistent with a condition caused by a dominant allele, there are affected individuals who show up every generation. Also, there are no unaffected couples having affected children as you would see with a recessive condition. This condition cannot be X-linked since, if it was, all fathers would pass the condition to their daughters since all daughters receive their fathers X chromosome. If you look carefully you will see one affected father having an unaffected daughter making it certain that the locus of this mutant allele is an autosome.
This last example below shows an **X-linked dominant** condition. It has all the characteristics of a dominant condition as indicated for the previous pedigree. You can tell if the condition is X-linked by looking at the offspring of an affected man and an unaffected woman (there are 3 such couples in this pedigree). Remember that fathers give their X chromosome to all daughters and their Y chromosome to all sons. Thus if the condition is X-linked and dominant, all daughters of these men should be affected and all of their sons should be unaffected. All 7 daughters of these men have the condition, whereas none of their 5 sons are affected. The odds of this occurring for an autosomal dominant condition would be $2^{-12}$ or 1 in 4096 since there would be a 50% chance for each daughter to inherit the condition and a 50% chance for each son to be unaffected. Therefore it is almost certain that this genetic condition is dominant and X-linked.

In the final exercise for this lab you will practice diagramming and interpreting pedigrees.

**Exercise 2G – Interpreting pedigrees**

*Solve the following problems on your worksheet, being sure to show all your work:

1. Examine the pedigree on your worksheet and determine the mode of inheritance (dominant or recessive, autosomal or X-linked). Based on the mode of inheritance, determine the genotypes of each individual in the pedigree representing any unknown alleles with a question mark (?).

2. A hypothetical mutation in a single gene results in a “zombie” phenotype. A normal man and woman have 3 children – a zombie girl, a normal girl and a normal boy. The man has a normal sister and mother but his father is a zombie. The woman has a normal brother and both her parents are normal. Diagram a pedigree of this family and determine the mode of inheritance for the zombie condition. Once you have done so, determine the genotypes of all family members and indicate any unknown alleles with a question mark (?).
Genetic Inheritance Lab Worksheet

Name ________________________

Exercise 1A – Probability and sample size

Probability of heads = ______   Probability of tails = ______

Fill in the table to the right with the expected numbers of heads and tails based on the probabilities above:

<table>
<thead>
<tr>
<th></th>
<th>10 flips</th>
<th>50 flips</th>
<th>whole class</th>
</tr>
</thead>
<tbody>
<tr>
<td>heads</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tails</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Record the numbers of heads & tails and the percentages for your actual coin flips:

<table>
<thead>
<tr>
<th>set &gt;</th>
<th>1</th>
<th>%</th>
<th>2</th>
<th>%</th>
<th>3</th>
<th>%</th>
<th>4</th>
<th>%</th>
<th>5</th>
<th>%</th>
<th>all</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>heads</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tails</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>10</td>
<td>100%</td>
<td>10</td>
<td>100%</td>
<td>10</td>
<td>100%</td>
<td>10</td>
<td>100%</td>
<td>10</td>
<td>100%</td>
<td>50</td>
<td>100%</td>
</tr>
</tbody>
</table>

Use the table below to record the results for the entire class (which should be on the whiteboard):

<table>
<thead>
<tr>
<th>class</th>
<th>percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>heads</td>
<td>%</td>
</tr>
<tr>
<td>tails</td>
<td>%</td>
</tr>
<tr>
<td>TOTAL</td>
<td>100%</td>
</tr>
</tbody>
</table>

Exercise 1B – Dice Baseball

Refer to the matrix on page 5 of the lab to fill in the probabilities for each sum of the dice:

<table>
<thead>
<tr>
<th>sum&gt;</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>prob (x/36)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>36/36</td>
<td></td>
</tr>
</tbody>
</table>

Determine the following “at bat” outcomes for each sum of the dice – single, double, triple, home run, out, walk, double play (if runner on first) – doing your best to be consistent with real baseball:

<table>
<thead>
<tr>
<th>sum&gt;</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>outcome for “at bat”</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Keep score for your baseball game in the table below. As you play, keep track of your baserunners using the props provided in your lab kit:

<table>
<thead>
<tr>
<th>team</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Exercise 2A – Type 1 genetics problems**

Solve the problems below in the space provided. Be sure to indicate the probabilities of each possible phenotype and genotype and to show your work:

1. homozygous dominant (TT)  x  heterozygous (Tt)

2. heterozygous (Tt)  x  homozygous recessive (tt)

**Exercise 2B – Type 2 genetics problems**

Solve the problems below in the space provided, and be sure to show your work:

1. An albino child is born to two parents with normal skin pigmentation. What are the genotypes of the parents?

2. An albino woman has 8 children with the same man, 5 of which are albino. What can you conclude about the father?
**Exercise 2C – Incomplete dominance**

*Solve the problems below in the space provided, and be sure to show your work:*

1. Indicate the probabilities of each genotype and phenotype resulting from the cross between two pink snapdragons.

2. A cross between two snapdragons produced 15 offspring with red flowers and 17 offspring with pink flowers. What are the genotypes and phenotypes of the parents?

**Exercise 2D – Multi-allelic inheritance and codominance**

*Solve the problems below in the space provided, and be sure to show your work:*

1. Indicate the probabilities of each genotype and phenotype in the children of a woman with blood type O and a man with blood type AB.

2. A woman with blood type A and a man with blood type B have 3 children, one each with blood types A, B and O. What are the genotypes of the parents?
Exercise 2E – Multi-allelic inheritance and codominance

*Solve the problems below in the space provided, and be sure to show your work:*

1. A normal woman whose father is a hemophiliac marries a normal man. What are the odds that hemophilia will afflict any given male child? any given female child?

2. A couple has three children, a normal boy and a boy and girl each with hemophilia. What can you say about the parents?

Exercise 2F – Genetics problems involving two genes

*Solve the problems below in the space provided, and be sure to show your work:*

1. Indicate the probabilities of all phenotypes in the offspring of two tall pea plants with purple flowers, both of which are heterozygous for both genes.

2. A normal woman with an albino father and no family history of hemophilia plans to have children with a hemophiliac man with normal skin pigmentation whose mother is albino. Determine the probabilities of all possible phenotypes in their children.
Exercise 2G – Interpreting pedigrees

1. Examine the pedigree shown and determine the mode of inheritance (dominant or recessive, autosomal or X-linked).

Based on the mode of inheritance, determine the genotypes of each individual in the pedigree representing any unknown alleles with a question mark (?)

2. A hypothetical mutation in a single gene results in a “zombie” phenotype. A normal man and woman have 3 children – a zombie girl, a normal girl and a normal boy. The man has a normal sister and mother but his father is a zombie. The woman has a normal brother and both her parents are normal. Diagram a pedigree of this family and determine the mode of inheritance for the zombie condition. Once you have done so, determine the genotypes of all family members and indicate any unknown alleles with a question mark (?)

---

281
Supplemental Problems

To ensure you understand the key aspects of genetic inheritance you will need to practice solving genetic problems much like the ones you’ve solved in this laboratory. What follows are sets of genetic problems grouped by category. Solve these problems on your own in order to complete this laboratory.

SINGLE GENE PROBLEMS

In Drosophila, a type of fruit fly, the long-wing allele (L) is dominant over the vestigial-wing allele (l):

1. If a heterozygous long-winged fly is mated with a homozygous long-winged fly, what percentage of the offspring would be expected to be homozygous long-winged? Would there be any vestigial-winged flies?

2. A long-winged fly mated with a fly having vestigial wings produces 35 long-winged and 33 vestigial-winged offspring. What are the genotypes of the parents and the offspring?

PROBLEMS INVOLVING TWO GENES

3. Two rough, black guinea pigs produce two offspring, one rough white and the other smooth black. If these same parents were to have additional offspring, what proportion of phenotypes would you expect?

4. In Drosophila, gray body color is dominant over ebony, and straight wings are dominant over curved. A gray-bodied female with curved wings is mated with a gray-bodied male with straight wings, yielding some ebony, curved-wing offspring. What other types of offspring could be produced and in what proportions?

For the next problem, assume that brown eyes (B) are dominant over blue eyes (b), and right-handedness (R) is dominant over left-handedness (r).

5. A right-handed, blue-eyed man marries a right-handed, brown-eyed woman. They have two children, one left-handed and brown-eyed and the other right-handed and blue-eyed. By a later marriage with another woman who is also right-handed and brown-eyed, the man has nine children, all of whom are right-handed and brown-eyed. What are the genotypes of this man and his two wives?

X-LINKED PROBLEMS

6. Yellow body color in Drosophila is an X-linked characteristic that is recessive to gray body color (Drosophila females are XX and males XY as in humans). A gray female mated with an unknown male and produced some yellow and some gray offspring of both sexes. What is the genotype of the original female? What is the phenotype of the male with which she mated?

For these problems keep in mind that colorblindness and hemophilia are both X-linked recessive conditions.

7. In humans, migraine is due to an autosomal dominant allele. A normal-visioned woman who has never suffered from migraine headaches takes her daughter to a doctor for an examination. In the examination the doctor discovers that the girl is color-blind and suffers from migraine headaches. What can the doctor conclude about the girl’s father?
8. In humans, aniridia (a type of blindness) is due to an autosomal dominant allele, and optic atrophy (another type of blindness) is due to a recessive X-linked allele. A man blind from optic atrophy marries a woman blind from aniridia. Assuming that the woman is homozygous for both genes, would any of their children be expected to be blind? If so, what type of blindness would they have?

9. A non-hemophiliac man who is blind from aniridia (see previous problem) and whose mother is not blind marries a non-hemophiliac woman who is not blind and whose father has hemophilia. What kinds of children might they have and in what proportions?

10. A normal-visioned man marries a normal-visioned woman whose father is color-blind. They have two daughters who grow up and marry. The first daughter has five sons, all normal-visioned. The second daughter has two normal-visioned daughters and a color-blind son. Diagram the family history in a pedigree and indicate the genotypes of all family members.

MULTIPLE ALLELE PROBLEMS

In rabbits, four alleles for a single gene affecting coat color have the following relationships:

\[ C > c^{ch} > c^{h} > c \]

Thus there are four possible phenotypes which are associated with the following genotypes:

- full color – \( C_\_ \) (\( C \) with any other allele)
- chinchilla – \( c^{c}c^{ch} \) or \( c^{ch}c^{h} \) or \( c^{ch}c \)
- Himalayan – \( c^{h}c^{h} \) or \( c^{h}c \)
- albino – \( cc \)

11. Would it be possible for a cross between two chinchilla rabbits to result in both Himalayan and albino offspring? (be sure to qualify your answer)

In mice, three alleles for a single gene affecting coat color have the following relationships:

\[ A^Y > A > a \]

There are four possible phenotypes which are associated with the following genotypes:

- yellow coat – \( A^YA \) or \( A^a \)
- agouti (gray) – \( AA \) or \( Aa \)
- lethal (mice die in utero) – \( A^YA^Y \)
- black – \( aa \)

12. For the following crosses indicate the phenotypes of the parents and the expected proportions of all possible offspring:
   a) \( A^ya \times Aa \)
   b) \( A^ya \times A^a \)
   c) \( AA \times Aa \)
   d) \( A^YA \times A^a \)
   e) \( A^YA \times A^A \)
**Bio 6 – Drosophila Genetics**

**Objectives**

Upon completion of this laboratory you will understand how to: 1) Use a biochemical assay to evaluate a biochemical phenotype, 2) Determine the pattern of inheritance for a gene using experimental data, 3) determine the parental genotypes by investigating the offspring, 3) Use the chi-squared test to test a hypothesis

**Introduction**

Our understanding of the principles of genetic inheritance have come from, in no small part, studies carried out using *Drosophila melanogaster*, commonly known as the fruit fly. Thomas Hunt Morgan chose *D. melanogaster* for genetic experimentation in 1907 and his work provided the first convincing experimental evidence that genes are located on chromosomes.

Since then it has become a model organism and “work-horse” for genetic research. Several characteristics of *Drosophila* make it an ideal organism for study. The flies are easy to culture, requiring little space and resources. Large numbers of *Drosophila* can be cultured readily, and they have a short generation time. A female can lay hundreds of eggs and a new generation can be produced every two weeks. In addition, the fruit fly has only four chromosomes (n=4, 2n=8). The male fruit fly has an X and a Y chromosome (XY) and the female fruit fly has two X chromosomes (XX).

**Sexing Flies**

Male and female flies can be identified by differences in their physical characteristics. Females are generally larger than males. The posterior end of the male abdomen is usually darker and rounder than that of females. Males can also be distinguished by the presence of a thick set of bristles on each foreleg called a sex comb. On the ventral (under) side of the male abdomen, the darkly pigmented genital arch is also clearly visible.
**Nomenclature**

Morgan’s lab developed a widely-adopted nomenclature to express the alleles in *Drosophila*. A **wild type** allele is the one most commonly found in nature. Genes are named for the first mutant (non-wildtype) phenotype associated with the gene. For example, in *Drosophila*, red eyes is wild type. The first fly mutant discovered by Morgan’s lab was a white-eyed fly. The allele for white eyes is given the symbol *w*. A wild-type allele is indicated by a superscript +. Thus, the wild-type allele for eye color in *Drosophila* is *w*⁺ whereas the mutant allele is indicated as *w*. Furthermore, if the mutation is dominant the first letter of the name or symbol is capitalized. If the mutation is recessive the first letter is given in lowercase. For example, one mutant gene results in red-brown eyes that darken to sepia and then black. This gene has been named *sepia* and given the symbol *se*. This is an autosomal recessive trait so the genotype of a sepia-eyed fly is *sepia sepia* (expressed using just the gene symbols as *se se*) whereas a red-eyed fly is *sepia*⁺*sepi*⁺ (se⁺ se⁺) or *sepia*⁺*sepi*⁺ (se⁺ se⁺).

**Aldehyde oxidase (AO)**

In this lab, the trait you will study is **aldehyde oxidase (AO)** activity. This enzyme is encoded by the *aldox* gene. Wild-type *Drosophila* have AO activity, however there are mutant *Drosophila* flies that lack AO activity. You will use a **spot assay** to assess flies for the presence of the enzyme. In the presence of AO, the assay mixture, benzaldehyde, nitroblue tetrazolium (NBT) and phenazine methosulfate (PMS), will turn **blue**. **Note that this assay mixture contains carcinogens and should be handled using gloves. If the mixture makes contact with your skin at any time, thoroughly wash the exposed skin with soap and water.**

**Part I – Sexing *Drosophila Melanogaster***

Identifying the sex of a fly is a prerequisite to breeding flies for genetic studies, interpreting genetic data, and maintaining fly cultures. You will need to familiarize yourself with the anatomy of the male and female fruit fly and practice sexing.

**Exercise 1 – Sexing Flies**

*You instructor will demonstrate how to anesthetize flies using FlyNap. Exposure to FlyNap for 2-4 minutes is sufficient to anesthetize the flies for 50 minutes or more.*

1. Anesthetize the flies in vials 1a and 1b.
2. Label and index card “Vial 1a" and draw a line to divide it down the center. Mark the left side of the card “♂” and the right side of the card “♀”.
3. Begin with the flies from vial 1a. Pour them out onto the index card. Using the dissecting microscope, sort the males and female flies to the appropriate side of the card.
4. Once you have sorted out three males and three females ask your instructor to check your identifications. When given the go-ahead, return the remaining un-sorted flies to the vial.
5. Prepare another index card for vial 1b. Sort out three males and three females.
Part II – The Spot Assay

You will familiarize yourself with the spot assay using the flies from Exercise 1 to setup the control reactions. Vial 1a contains fly known to have aldehyde oxidase (AO) whereas vial 1b flies do not. Before beginning the assay, on the worksheet, write a hypothesis regarding AO activity and prediction for the experiment.

Exercise 2 – The Spot Assay

1. Place one fly per well from vial 1a in one row of the 96-well plate. Make a note of the sex of the fly in each well.
2. Repeat this procedure with flies from vial 1b, place them in a second row.
3. Add one drop of assay mixture per well.
4. Using the end of the glass stir rod, homogenize the flies in each well. Be sure to clean the rod between each well.
5. Place the plate in the dark (e.g. the desk drawer) for five minutes.
6. Remove the plate and score each well for AO activity. Remember that AO will catalyze a reaction that results in a blue product.
7. Record your results in the table of the worksheet and answer the associated questions.

Part III – Determining the Pattern of Inheritance of the Aldox Gene

You will now determine whether the wild-type (AO present) or mutant (AO absent) allele is dominant and whether the gene is autosomal or sex-linked. The flies in vial 1a are true-breeding, wild-type flies. This means that when these flies breed each subsequent generation always has the AO enzyme. The flies in vial 1b are true-breeding for the mutant allele meaning that these flies always produce offspring lacking AO. The flies in vial 2 are the F1 progeny from a cross between a male from vial 1a and female from vial 1b.

Exercise 3 – The Pattern of Inheritance of Aldox

1. Complete questions 8-12 of the worksheet. Use this information to generate a hypothesis about the inheritance of this gene and predict the outcome of the cross.
2. Anesthetize the flies in vial 2.
3. Sex the flies as you did previously until you have ten males and ten females.
4. Perform the spot assay as you did in Exercise 2.* Include a fly from vial 1a as a positive control.
   When the positive control sample turns blue assess the results for all samples, recording your results in the worksheet table.
   * Note that the assay mixture is light sensitive. Cover the plate with an index card to protect it from light between steps.
5. Analyze your data and answer the remaining questions for Exercise 3.
Part IV – Determining Parental Genotypes Using Progeny

The flies in vial 3 are the F1 progeny of a cross between parents of unknown AO status. Given that you have determined the pattern of inheritance for the *aldox* gene you are now able to predict the possible outcomes of any particular cross. You will first assay the F1 offspring for AO activity. Then, using this data you will hypothesize the genotype of the parents and give an appropriate prediction. You will then use the chi-squared test to determine if the data support or falsify your hypothesis.

Exercise 4 – Determining Parental Genotypes

1. Anesthetize the flies in vial 3.
2. Perform the spot assay as given in Exercise 2 using 24 flies in total. Include a positive control from vial 1a.
3. Record the results in the table of the worksheet.
4. Collect the total class data and record it in the table for class data in the worksheet.
5. Based on the class data, generate a hypothesis regarding the genotypes of the parents. Record this on your worksheet. You may find it useful to draw Punnett squares for the possible crosses to help you come up with an appropriate hypothesis. Explain your rationale and hypothesis with your instructor before continuing.
6. Perform the chi-squared test to evaluate your hypothesis. Use the table in the worksheet to calculate the chi-squared value.
7. Evaluate your hypothesis and answer the remaining questions to draw a final conclusion.
Ex. 2: THE SPOT ASSAY

1. Propose a hypothesis about AO activity in flies from vial 1a and flies from vial 1b.

2. Predict the results of the experiment (test) based on your hypothesis (if/then).

3. Why is it necessary to homogenize the flies?

Record your results in the table below:

<table>
<thead>
<tr>
<th>Vial 1a (AO present)</th>
<th>Vial 1b (AO absent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fly #</td>
<td>Sex (F or M)</td>
</tr>
<tr>
<td>----------</td>
<td>--------------</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

4. Do your results match your predictions?

5. Does the sex of the fly appear to have an impact on the results of the assay test?

6. Which two characteristics are most useful to your group in determining the sex of the flies?

7. Considering that the mutant AO allele does not produce a functional enzyme, do you think this allele is likely to be dominant or recessive (be sure to qualify your answer)?
Ex. 3: DETERMINING THE PATTERN OF INHERITANCE OF THE ALDOX GENE

8. What is the genotype of the female parent (lacking the enzyme) in our cross?

9. What is the genotype of the male parent (producing the enzyme) if the gene is not sex-linked?

10. What is the genotype of the male if the gene is sex-linked?

11. What would be the genotypes of the F₁ progeny if the gene is not sex-linked (do a Punnett square)?

12. What would be the genotypes of the F₁ progeny if the gene is sex-linked (do a Punnett square)?

13. Propose a hypothesis about the inheritance of this gene (i.e., Is it sex-linked?).

14. According to your hypothesis, what are the genotypes of the parents?

15. Based on your hypothesis, predict the outcome of this cross.
Record your results in the table below:

<table>
<thead>
<tr>
<th>Fly #</th>
<th>Sex (F or M)</th>
<th>AO Activity (+/-)</th>
<th>Fly #</th>
<th>Sex (F or M)</th>
<th>AO Activity (+/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td>20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

16. What allele appears to be dominant?

17. What evidence of your results above supports your answer?

18. Based on your results, should the aldox gene be written beginning with a capital or lowercase letter (remember this is based on the mutant allele – lower case if recessive and upper case if dominant)?

19. Correctly write the names of the wild-type and mutant alleles using Drosophila nomenclature (wild-type alleles are written just like the mutant alleles with the addition of a “+” superscript).
Ex. 4: DETERMINING PARENTAL GENOTYPES USING EVIDENCE FROM PROGENY

Record your results in the table below:

<table>
<thead>
<tr>
<th>Fly #</th>
<th>AO Activity (+/-)</th>
<th>M or F</th>
<th>Fly #</th>
<th>AO Activity (+/-)</th>
<th>M or F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td>24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total the number of offspring in each phenotype category below:

<table>
<thead>
<tr>
<th></th>
<th>Your Totals</th>
<th>Class Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO-present</td>
<td>F - M -</td>
<td>F - M -</td>
</tr>
<tr>
<td>AO-absent</td>
<td>F - M -</td>
<td>F - M -</td>
</tr>
</tbody>
</table>

20. Is the trait sex-linked?

21. Which allele is dominant?

22. Using all observations, determine the genotypes of the parent flies, making sure to name the alleles correctly.
Complete the chi-square calculations in the table below:

<table>
<thead>
<tr>
<th></th>
<th>AO Activity (+)</th>
<th>AO Activity (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed Value (o)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expected Value (e)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deviation (o - e) or d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deviation$^2$ (d$^2$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$d^2/e$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chi-square $\chi^2 - \sum d^2/e$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degrees of freedom (df)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probability $(p)^*$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*see a $\chi^2$ table

23. What does the probability $(p)$ value you identified actually mean with regard to your conclusion about the genotypes of the parent flies?

24. Does the experiment support or contradict your conclusions concerning the pattern of inheritance derived from Exercise 3?

**REVIEWING YOUR KNOWLEDGE**

25. List the most obvious characteristics used to determine the sex of a fruit fly.

26. A fruit fly geneticist discovered a genetic mutation that resulted in pupae and young flies with dark pigment granules in the nuclei and cytoplasm of their fat cells. After studying the inheritance of the mutation, the geneticist named the gene Frd (for Freckled). What does this name tell you about the inheritance of this gene?
27. In another strain of fruit flies, geneticists discovered a mutation producing flies with no gut muscles. They named the gene controlling this phenotype jeb (for jelly belly). What does this tell you about the inheritance of this gene?