

Laboratory Safety

General Safety Rules

- Work in a laboratory only during regular, assigned period when an instructor is present, unless specific authorization has been given by the instructor to work in the laboratory at other times.
- Read carefully and observe fully all laboratory instructions. In case there is any doubt about any procedure, check with your instructor.
- Learn the location and proper use of emergency showers, fire extinguishers, and eye wash stations.
- Avoid inhaling chemical vapors or gases. Use fume hoods for hazardous materials.
- Immediately wash off and chemicals spilled on the skin with lots of water. In case of a serious spill, remove contaminated clothing immediately and flush affected area with lots of water.
- Do not eat, smoke, or drink in the laboratory. Do not bring any food items into the laboratory.
- Do not leave experiments in progress unattended without authorization.
- Keep working areas neat and clean at all times.
- Report all accidents to the instructor immediately.

Personal Protective Equipment

- All persons working with hazardous chemicals should wear gloves.
- All persons working with chemicals that could be splashed in the eyes are required to wear safety goggles or glasses.
- Contact lenses should not be worn in lab when hazardous chemicals or vapors are being used.
- Because of the danger of broken glassware or spilled chemicals, covered shoes should be worn in the laboratory. (No types of open toe shoes are permitted in labs.)

Chemical Safety

- Almost every chemical, whether solid, liquid or gaseous, is poisonous to the human body to some degree. Always use proper caution when handling chemicals.
- Consult a physician if you are pregnant or have any other medical condition that might render you susceptible to exposure to the chemicals used in this laboratory.
- When handling chemicals, keep your hands away from your face, eyes and body until your hands have been washed thoroughly.
- Do not taste any chemical. Label every container so items can be identified.
- When diluting acids, ALWAYS POUR ACID INTO WATER SLOWLY.
- Do not pipet anything into the mouth.

Waste Disposal

- Always treat laboratory glassware as if it were fragile. If glassware breaks, do not pick broken glass up with your hands. Use a broom and dustpan, then place pieces in the cardboard box labeled "Glass Disposal Box."
- Do not pour any chemicals down the drain. The instructor will advise you how to use the proper waste containers.
- Discarded animal parts must be placed in a red cardboard "Biohazardous Waste" box.
- Discarded sharp items including: scalpels, dissecting pins, probes, and needles must be placed in a red, plastic "Sharps Box."

Safety Agreement

Biology III

I have read the Lab Safety Rules and procedures for the prevention of injuries in the laboratory, and I will observe them in my lab work.

INSTRUCTOR'S NAME:

STUDENT'S NAME (PRINT)

STUDENT'S SIGNATURE

DATE

NOTE - There is a COPY of this safety agreement form in the last section of the appendix for this *Study Guide/Lab Manual* for you to tear out and sign. Bring your signed copy to the first lab session. (Keep this copy here in your lab manual for reference.)

How to Use a Micropipettor



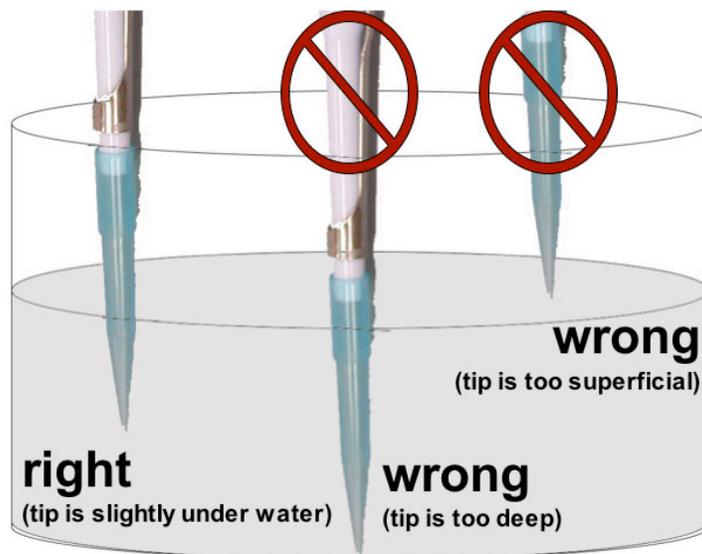
Micropipettors (a.k.a. pipette-men) are used to measure and transfer small amounts of liquids (≤ 1 mL). You will find them in almost every biology laboratory in the world. They are expensive instruments (\sim \$250/each) that must be shared by many scientists. Thus it is imperative that you treat our micropipettors as delicate and calibrated instruments. The scales on micropipettors are in microliters ($1000 \mu\text{L} = 1 \text{ mL}$). In this course you will use four different types of micropipettors. Their properties are summarized in the table below.

				
Name	P20	P200	P1000	Multichannel
Tip color	yellow	yellow	blue	yellow
Minimum Volume	1 μL	20 μL	200 μL	5 μL
Maximum Volume	20 μL	200 μL	1000 μL	50 μL
				note: red on top
	note: red on bottom			
	Setting shown = 10 μl	Setting shown = 100 μl	Setting shown = 1000 μl	

A few important directions for the operation of any micropipettor:

1. Know the limits of your micropipettor (and don't exceed those limits) of these pipettors. If you go above or below the minimum or maximum volume for a given pipettor, you will jeopardize the instrument's calibration. (Note: just because you can dial 210 on a P200, doesn't mean that you should!)
2. Set the desired volume by turning the centrally located rings clockwise to increase volume or counterclockwise to decrease volume.
3. Place a disposable plastic tip on the discharge end of the pipettor. NOTE: If sterile conditions are necessary, do not allow the yellow or blue plastic pipet tip to touch any object (including your hands, the bench, the side of a test tube, etc.).

4. The plunger will stop at two different positions when it is depressed. The first of these stopping points is the point of initial resistance and is the level of depression that will result in the desired volume of solution being transferred. Because this first stopping point is dependent on the volume that is being transferred, the distance you have to push the plunger to reach the point of initial resistance will change depending on the volume being pipetted. The second stopping point can be found when the plunger is depressed beyond the initial resistance until it is in contact with the body of the pipettor. At this point, the plunger cannot be depressed further. This second stopping point is used for the complete discharging of solutions from the plastic tip. You should not reach this second stop when drawing liquid into the pipettor, only when expelling the last drop. Before continuing, practice depressing the plunger to each of these stopping points until you can easily distinguish between these points.
5. Depress the plunger until you feel the initial resistance (first stop) and insert tip into your solution, just barely below the surface of the liquid and not as deep as possible. The wide (top) portion of the disposable pipette tip should never be underwater. Only the disposable pipet tip should touch the liquid; the pipettor should never touch any of the liquids. You should never rest the pipette tip on the bottom of the container (even if you have a shallow volume of liquid) because this could lead to inaccurate measurements.
6. Carefully and slowly release plunger. If you release the plunger too quickly, it will suck liquid up into the pipettor and damage it. NOTE: If the solution you are pipetting is viscous, allow the pipet tip to fill to final volume before removing it from solution to avoid the presence of bubbles in the plastic tip, which will result in an inaccurate volume.
7. Discharge the solution into the appropriate container by depressing plunger. This time, depress the plunger to the point of initial resistance, wait one second, and then continue pressing the plunger as far as it will go in order to discharge the entire volume of solution.
8. Discard the tip by pressing down on the tip discarder over a waste container.
9. Always change tips between solutions. You do not want to contaminate or mix your solutions with a dirty pipet tip.



Introduction to Spectrophotometry

Focused Reading

- p 163-64 “How does photosynthesis...” to “Photosynthesis uses...”

✓ Special Note

- Bring a calculator to lab today
-

Introduction

The purposes of this laboratory are to introduce you to:

1. Conventions used in making solutions: molarity, and percent.
2. Spectrophotometry and the use of the microplate reader.
3. Procedures for obtaining, recording, and analyzing data.
4. Conventions used in presenting data in graphs.
5. Procedures for planning and working through a series of related experiments.

Note: Record all your data, answers to questions and problems, and notes directly in this lab manual. Keep this lab manual beside you and write in it as you work. Good note taking skills are absolutely critical to successful scientific experiments.

Concentrations of solutions

It is important that you understand the units of the metric system (i.e., milli and micro). If you do not, please review these units of measurement as well as the Celsius (centigrade) temperature scale.

In the instructions below, the solute is the substance dissolved, the solvent is the liquid in which the solute is dissolved, and the resulting mixture is the solution.

Molar Concentrations

In technical terms, a mole of a compound is 6.02×10^{23} molecules of that compound. Practically speaking, a mole is the compound's molecular weight in grams. A one molar (1.0 M) solution has one mole of a compound (the solute) dissolved in solvent so that the final volume is 1000 mL (one liter). The molecular weight of NaCl is 58.54. Therefore:

- A 1.0 M solution of NaCl has 58.54 g NaCl dissolved in dH₂O with a final volume of 1000 mL (or 5.85 g in 100 mL).
- A 0.1 M solution of NaCl has 5.85 g in 1 liter - or 0.585 g in 100 mL - or 0.058 g in 10 mL.
- A 0.2 M solution of NaCl has 11.71 g in 1 liter - or 1.17 g in 100 mL - or 0.117 g in 10 mL.

Concentration in Percent (Weight/Volume = w/v)

By definition, percent means "in a hundred" and by convention, a 10% w/v solution contains 10 grams of a solute in a total volume of 100 mL of the solution. A w/v solution is not made by adding 10 grams of a solute to 100 mL of solvent, but instead by dissolving 10 grams solute in enough solvent to dissolve the solid and then more solvent is added to reach a total volume of 100 mL of the solution.

Concentrations in Percent (Volume/Volume = v/v)

Aqueous solutes may be specified as percent solutions volume/volume (v/v). Volume/volume solutions differ from weight/volume solutions – and it is important to understand the difference. One hundred mL of a 5% v/v solution will have 5 mL solute diluted to 100 mL with solvent. Thus, a 5% v/v aqueous solution of ethanol is made by diluting 5 mL 100% ethanol with enough dH₂O (95 mL) to make a total of 100 mL (or by diluting 5 liters 100% ethanol with 95 liters dH₂O).

A handy formula for calculating v/v solutions:

$$C_s V_s = C_w V_w$$

C_s = concentration of the stock (original) solution

V_s = volume of the stock solution (usually what you will solve for)

C_w = concentration of the working solution desired (more dilute than the stock)

V_w = total volume of the working solution desired (the solution you are making)

$V_w - V_s$ = volume of the solvent (often water) to be mixed with V_s

Suppose you have a stock bottle of 70% ethanol and you need to make 1 L of 50% ethanol.

$$C_s V_s = C_w V_w \quad 70\% \cdot V_s = 50\% \cdot 1 \text{ L}$$

$$V_s = (50\% \cdot 1 \text{ L}) / 70\%$$

$$V_s = 0.714 \text{ L}$$

$$V_w - V_s \quad 1 \text{ L} - 0.714 \text{ L} = 0.286 \text{ L}$$

Thus you would measure out 714 mL of 70% ethanol and mix it with 286 mL of water to get your 1 L of 50% ethanol.

This formula is simply determining proportions, so it can also be used to calculate how to dilute any solution. To make V_w units (mL, L, etc.) of a solution at C_w (M, mM, g/L, % etc.) from water and a stock solution of C_s you need to be certain you are using equivalent measures of volume for both V_s and V_w (i.e., make sure both are measured in mL or both are measure in L, etc.) and you need to be certain that you are using equivalent measures of concentration (i.e., make sure both C_s and C_w are both in mg/L or both in mM, etc.). Then you simply solve for V_s to determine how much stock solution you will need. Then you simply subtract V_s from V_w to determine how much solvent should be added. Here's an example. Suppose you have a 9.5 mM solution of KCl and want to make 927 mL of 4 mM KCl. You need to know how many mL of your 9.5 mM KCl solution you will mix with how many mL of H_2O in order to make 927 mL of 4 mM KCl. Using the $C_s V_s = C_w V_w$ formula:

$$C_s V_s = C_w V_w \quad 9.5 \text{ mM} \cdot V_s = 4 \text{ mM} \cdot 927 \text{ mL}$$

$$V_s = (4 \text{ mM} \cdot 927 \text{ mL}) / 9.5 \text{ mM}$$

$$V_s = 390.3 \text{ mL}$$

$$V_w - V_s \quad 927 \text{ mL} - 390.3 \text{ mL} = 536.7 \text{ mL}$$

Thus you would measure out 390.3 mL of 9.5 mM KCl and mix it with 536.7 mL of water to get your 927 mL of 4 mM KCl.

Experiment I: Evaluating a w/v solution

Experiment I is a demonstration experiment for you to observe and think about.

1. Locate the graduated cylinder with a NaCl solution in it. Before lab your instructor mixed 5 grams (g) of sodium chloride (NaCl) with exactly 50 mL of distilled water (dH_2O) and the solution was stirred vigorously to dissolve the salt.
2. Determine the excess volume by reading the volume of the graduated cylinder. This solution represents a failed attempt to make a 10% w/v solution. The number of mL in excess of 50 represents the volume of liquid displaced by the 5 g of dissolved NaCl.
3. What is the actual percentage of NaCl (w/v) in this solution?

Experiment 2: Making a v/v solution of neutral red

On the side or front bench is a stock solution (100%) of a dye, neutral red. Use this stock solution and water to make 6 mL of a 4% v/v solution. Label your tube and save this solution; you will use it later today. Make sure to mix the solution thoroughly and use parafilm to cover the top of the test tube during mixing.

C_s	V_s	C_w	V_w	$V_w - V_s$

_____ mL 100% neutral red + _____ mL H₂O = 6 mL 4% neutral red

Experiment 3: Diluting solutions

Using the 4% (v/v) neutral red solution you prepared in experiment 2, set up the following solutions in eight plastic microfuge tubes using distilled water and neutral red. Label the tubes with the concentrations in the left column of the table below. (Note: you can use a Sharpie marker to write directly on the tubes.)

You must calculate the volumes and fill in the table for the two unshaded columns before you make your solutions. Each tube should contain a final volume of 1000 μ L.

neutral red concentration	4% neutral red solution to add	distilled water to add	Complete these two columns later (when answering study question #6)	
			concentration of neutral red solution (mg/mL)	concentration of neutral red solution (M)
0 %	0 μ l	1000 μ l		
0.36 %				
0.84 %				
1.56 %				
1.96 %				
2.52 %				
3.4 %				
4 %	1000 μ l	0 μ l		

Spectrophotometry

A solution, such as neutral red, appears colored because it absorbs certain wavelengths of light in the visible spectrum and transmits or reflects others. Each solution with a different solute has its own characteristic absorption properties or "spectrum." A spectrophotometer is an optical machine that measures and lets you see (sense) how much light energy is transmitted by a substance in solution at different wavelengths of radiant energy. Biologists use the spectrophotometer for two different purposes (we will do both in the laboratory today):

1. to determine the absorption spectrum of a pure substance in solution
2. to determine the concentration of a solution.

A spectrophotometer consists of a white light source (light of all visible wavelengths), a prism or diffraction grating that separates the light into different wavelengths, a slit through which a narrow beam of the desired wavelength (I) passes (the incident light, I_0), a sample solution holder, a photosensitive detector that measures the energy of light transmitted through the solution (I), and a recording device that displays the amount of transmitted light energy digitally or on a dial. See Fig. 1 below.

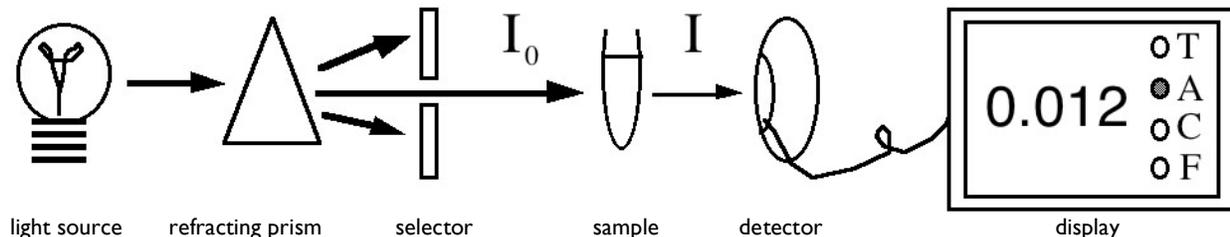


Figure 1. A schematic diagram of the components of a spectrophotometer. The arrows indicate the pathway of light.

Transmittance is the ratio of the transmitted light energy (I) to the incident light energy (I_0); percent transmittance is 100X that ratio. Transmittance, however, is not proportional to solute concentration, so it is usually converted into absorbance that is proportional to solute concentration. Digital spectrophotometers have readouts for both percent transmittance and absorbance, but we will always measure the absorbance.

$$\%T = (I \div I_0) \times 100$$

$$\text{Abs.} = \log_{10} (100/\%T)$$

Microplate Reader

Figure 1 is a simplified diagram of a spectrophotometer that can measure one sample at a time. In our experiments, we will be using a microplate reader that is capable of measuring the absorbance of 96 samples in just a few seconds. The basic design is exactly the same; a selected wavelength of light passes through the samples and a phototube measures the amount of light transmitted through the sample, which the plate reader converts to absorbance. However, the samples are located in microwells that are arranged in an 8 x 12 matrix in one plastic plate (see figure 2). You can put your samples in any or all of the microwells. The plate is moved over an array of eight fiber optic light sources and eight phototubes. Each row of eight is scanned and then the plate advances by one row and the process continues until all 12 rows are scanned. The absorbance data are then displayed on a screen in an 8 x 12 array. You also can program the plate reader to measure the absorbance of all 96 samples at time intervals of your choice (e.g., every 30 seconds). You should take advantage of these capabilities when you design your experiments for next week.

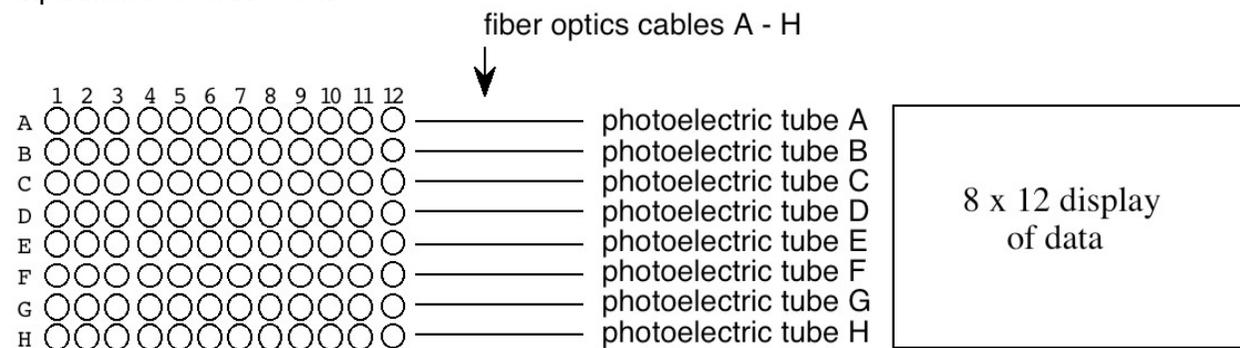


Figure 2. Schematic diagram of a microplate reader. Samples are placed in the 96 microwells, analyzed by the eight channel spectrophotometer.

The Absorption Spectrum

Because solutions of pure substances do not absorb the energy of all wavelengths of light equally, a substance may be identified by the unique pattern of wavelengths absorbed. The chlorophylls in plants absorb strongly in the blue wavelengths (about 450 nm) and red wavelengths (about 650 nm), but reflect the green wavelengths (about 525 nm). A plot of absorbance versus visible wave lengths (400 to 700 nm) for a solution of chlorophyll a shows two major peaks, one at 450 and one at 650 nm, and a valley from 500 to 625 nm (See Figure 3). This spectrum is characteristic for chlorophyll a and may be used as an aid in its identification.

By measuring the absorbance of an uncharacterized solution over a range of wavelengths and plotting the absorbance value on the Y-axis and the wavelength on the X-axis, one can determine the absorption spectrum of a sample. The absorption maximum of any pure substance in solution is the wavelength where absorption is the greatest.

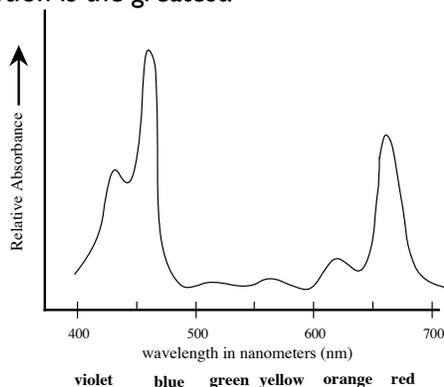


Figure 3. The absorption spectrum of chlorophyll a. The absorbance of visible light by chlorophyll a is measured spectrophotometrically as a function of wave length. The absorption maximum is about 460 nm. Compare with figure 8.6a on page 165 of your textbook.

Standard Concentration Curve & Determining the Concentration of a Substance

One can construct a standard concentration curve of a solution by measuring the absorbance of several different known concentrations of the solution and graphing the results by plotting absorbance on the Y-axis and concentration on the X-axis. Spectrophotometry can be used to measure the absolute or relative concentration of a characterized substance in solution. To determine the absolute concentration of a pure substance, one first constructs a standard concentration curve from known concentrations and then takes the absorbance reading of the unknown concentration. The unknown concentration can be determined from the standard curve by drawing a horizontal line on the graph parallel to the X-axis and through the point on the Y-axis that corresponds to the absorbance. This line will intersect the standard curve; at this intersection, a vertical line is dropped to the X-axis and the concentration read from the X-axis.

Three factors are important in determining unknown or relative concentrations. The absorption maximum should be used, and absorbance rather than percent transmittance should be plotted because absorbance is directly proportional to concentration and transmittance is not. You cannot measure outside the standard curve.

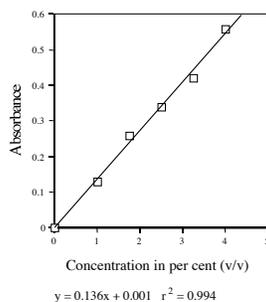


Figure 4. An example of a standard curve. A best fit line has been generated and the resulting equation and r^2 value are shown below the X-axis label.

Experiment 4: Determination neutral red's absorption maximum

For each of your eight different dilutions of neutral red (made in experiment 3) pipette 200 ul into three triplicate wells of your 96-well plate. Keep track of which concentration corresponds with each particular well. You will collect the data for experiments 4 and 5 at the same time (see below).

Experiment 5: Generating a neutral red standard curve

You will generate these data at the same time as those from Experiment 4. Use only the data from the wavelength that is the absorption maximum for neutral red. With the appropriate data, you will generate a curve to measure the unknown concentration of a solution. Follow the directions below to collect the data.

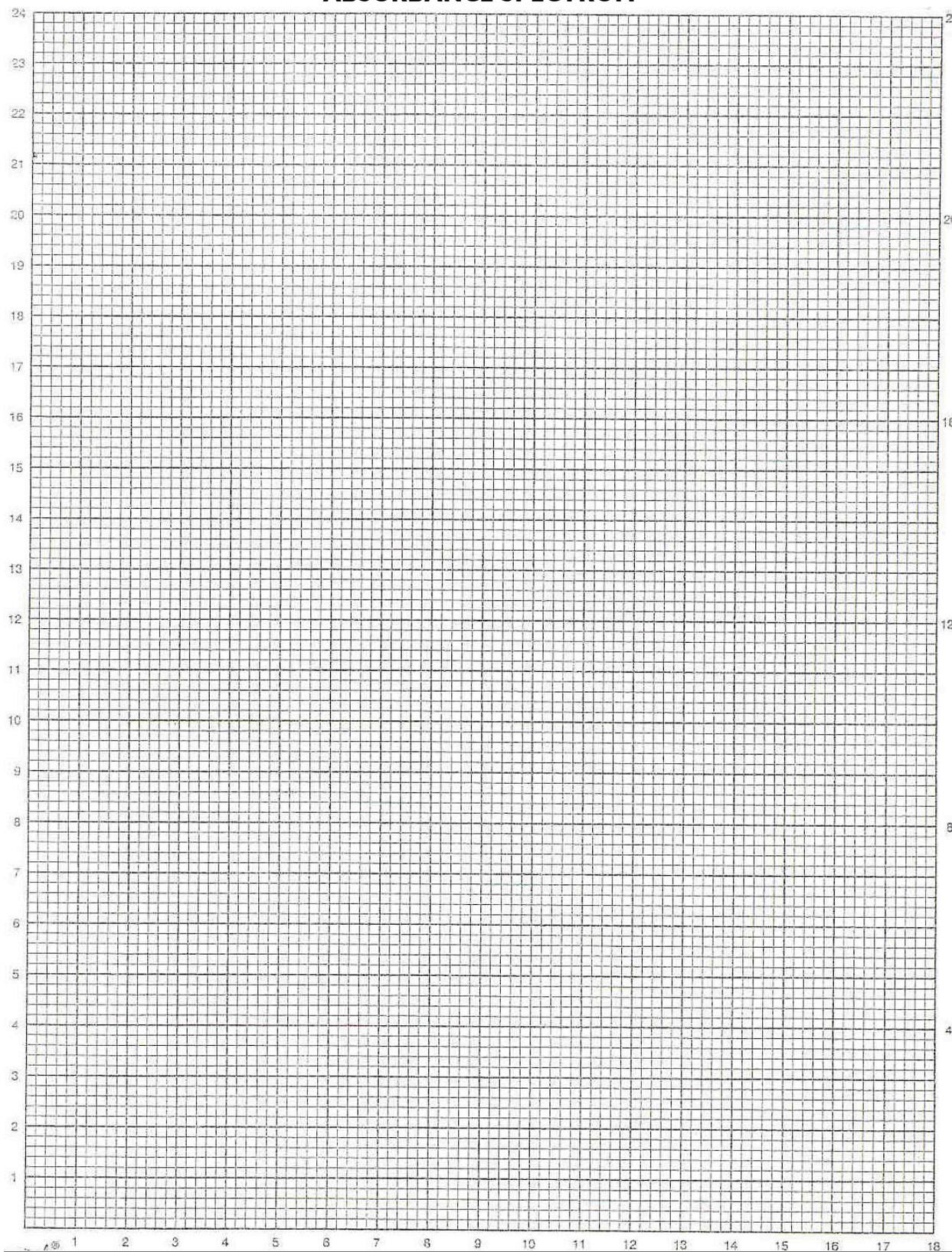
Using the Plate Reader (a type of spectrophotometer)

1. Your instructor will have turned on the plate reader and let it warm up. Open the KCjunior program on the PC and click OK – the software will start up.
2. Click on the “Open Protocol” button and select the “340 nm endpoint” option then click on the “open” button, then on the “read plate” button.
3. You will see a read plate dialog box. Click on the “read plate” button. (There’s no need to fill in the results ID or plate description boxes).
4. You will get another read plate dialog box. Place your 96-well plate in the carrier and make sure the lid is shut, then press the OK button.
5. You should hear the machine moving and in a few seconds you should see an 8x12 report of the absorbance values in all 96 wells.
6. Go up to “Results” in the upper left menu portion of the monitor and select the “print results” option from the drop-down menu. (Make sure “brother HL-514 series” is selected in the printer options). Click the “OK” box to print your data.
7. Close your results using the “close results” command under the “Results” menu in the upper left. (There is no need to save your results on the computer after you have your printout).
8. Now you will repeat this procedure for five more wavelengths of light, using protocols named “405 endpoint”, “450 endpoint”, “490 endpoint”, “595 endpoint”, and “650 endpoint”.
9. When you have collected data for all six wavelengths, remove your plate from the plate reader and take your six printouts back to your bench to analyze your data.

Wavelength (nm)	Absorbance values for 3.4% neutral red			
	Value 1	Value 2	Value 3	average
340				
405				
450				
490				
595				
650				

To generate an absorption spectrum that will reveal the absorption maximum, plot the average absorbance of the 3.4% neutral red solution on the graph paper on the next page. Use absorbance (which is a ratio and therefore has no units) on the y-axis and the wavelength of light (in nm) on the x-axis.

ABSORBANCE SPECTRUM



Data Analysis

Experiment 4:

Use your graph on the previous page to identify the wavelength that produced the highest absorbance values. This wavelength is the absorption maximum for neutral red.

Neutral red absorption maximum:	_____ nm
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All subsequent experiments should use only the data collected at the absorbance maximum wavelength.

What is the advantage of using this absorbance max for subsequent experiments with neutral red?

What can you predict about the absorbance maximum wavelength for a blue dye?

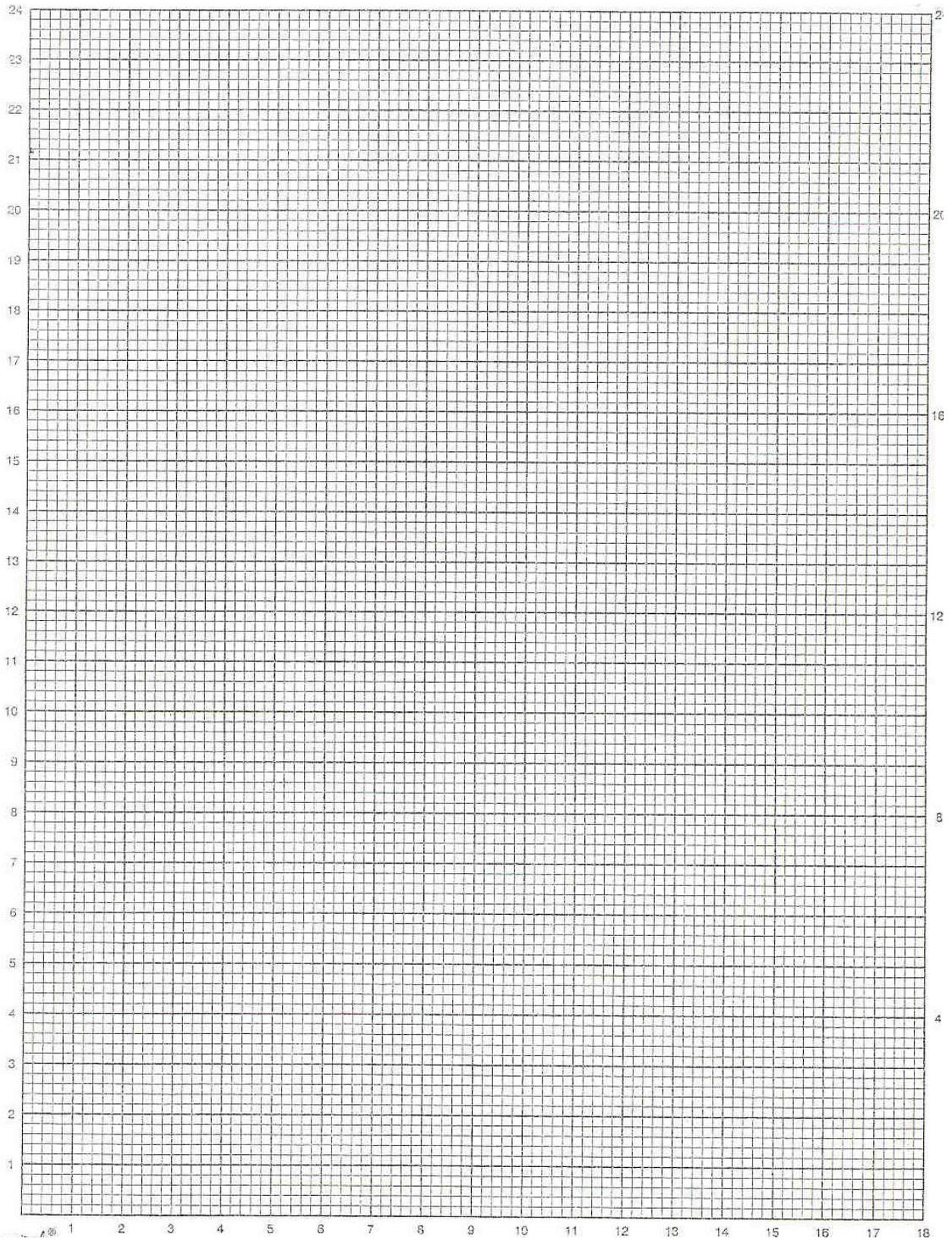
Experiment 5:

Using the absorbance data that were generated with the absorption maximum wavelength (determined in experiment 4), subtract the average absorbance of the blank (0% neutral red) from the values for the samples. Subtracting the blank corrects for the amount of light absorbed by the plastic and the water.

Well Names	Neutral Red Concentration	Average Absorbance at _____ nm (absorbance maximum)	Corrected absorbance value (0% blank subtracted)
	0 %		
	0.3 %		
	0.84 %		
	1.56 %		
	1.96 %		
	2.52 %		
	3.4 %		
	4 %		

Now, construct a graph (on the graph paper below) with the x-axis as percent neutral red (increasing from left to right), and the y-axis as absorbance at your selected absorbance maximum (e.g., Abs_{666nm}) using your corrected absorbance values. Draw a straight line (best fit) to generate the standard concentration curve.

STANDARD CURVE



Experiment 6:

Using your standard curve to determine concentrations

Obtain four neutral red solutions of unknown concentration from your instructor. Determine the concentration of your four unknowns by putting each unknown (A-D) into three individual wells (200 $\mu\text{L}/\text{well}$). Record well names (e.g., H3 - H6) corresponding with each unknown on the chart below. Use the plate reader at the wavelength of light that is absorbed maximally by neutral red. Subtract the absorbance of water (the blank) from these values and use these corrected values to determine the concentrations of the four unknowns. Using the standard curve, determine the concentration of your unknown dilutions of neutral red. For each unknown, the unknown concentration can be determined from the standard curve by drawing a horizontal line on the graph parallel to the X-axis and through the point on the Y-axis corresponding to the corrected absorbance for the unknown. This line will intersect the standard curve; at this intersection, a vertical line is dropped to the X-axis and the concentration read from the X-axis. When you have determined the estimated concentration ask your instructor to reveal the actual concentrations of the four unknowns. How well did your standard curve predict the concentration on the four unknown solutions?

Well Name	Neutral Red Unknown	Absorbance at _____ nm	Corrected Absorbance (subtract av. blank)	Estimated concentration (from your std. curve)	Actual concentration (provided by instructor)
	A				
	A				
	A				
	Average A				
	B				
	B				
	B				
	Average B				
	C				
	C				
	C				
	Average C				
	D				
	D				
	D				
	Average D				

Experiment 7:

Determining the absorption maximum for NADPH and NADP⁺

Over the subsequent two weeks, we will use NADPH and NADP⁺ as indicators of enzyme activity. Consequently, we will need to know which wavelengths of light will give the best information on NADPH concentration. You will want to perform a series of experiments similar to experiment four. Put 200 μL NADPH into each of three empty wells and 200 μL of NADP⁺ into another three empty wells. Record your averaged results in the table below.

	340 nm	405 nm	450 nm	490 nm	595 nm	650 nm
NADPH						
NADP⁺						

Look at your data above. Which wavelength is absorbed the best and the amount of light absorbed by each chemical. Record your responses in the table below. Verify your results with the instructor before you leave today.

	Absorbance maximum (nm)	Amount of absorbance at maximum
NADPH		
NADP⁺		

Cleaning Up

Leave your workstation cleaner than you found it. Although this step may seem obvious, it is quite important. It is basic laboratory courtesy to leave the workspace as clean as you found it with equipment back in its proper place(s).

❖ Study Questions (to answer on your own time):

1. How do you account for the excess volume you observed in Experiment 1?
2. The solution made in experiment 1 does not constitute a 10% w/v solution of sodium chloride but something less than that. Calculate the actual percentage from your data.
3. Why do you have to dissolve the solute in a volume of solvent less than the final volume you eventually want? Does it matter if the salt is added first or second to the graduated cylinder?
4. Describe precisely how you made up the 4% v/v solution of neutral red.
5. In Experiment 1, does it matter whether the solute is added to the graduated cylinder before or after the water?
6. The aqueous stock solution of neutral red was actually a 1.0 M solution. Determine the molar concentration of the six solutions you made. If neutral red has a molecular weight of 87, how

many $\mu\text{g}/\mu\text{L}$ are contained in your 4% (v/v) solution? Calculate the concentration of neutral red in each tube ($\mu\text{g}/\mu\text{L}$) and add these data to the table from experiment 3.

7. Describe how to prepare 50 mL of 70% ethanol when your only source is a stock container of 95% ethanol.
 8. What is the molarity of your 10% w/v NaCl solution?
 9. What is the percent concentration of a 2 M NaCl solution?
 10. The molecular weight of Na_2CO_3 is 106. Describe how you would make up 100 mL of a 0.15 M solution.
 11. What is the absorption maximum for NADPH?
-

Lab Notes

Measuring Enzyme Activity with Spectrophotometry

📖 Focused Reading

- p 125-129 start at “What are enzymes?” TO “Molecular Structure...”
- p 142 Fig. 7.4 (NAD is an Energy...)

✓ Special Notes

- Bring a calculator to this lab
- This is a particularly busy day in lab – preparation, organization, and efficiency are all essential

Goals of this exercise:

With this session, we begin to study and discuss of some of the properties of NADP⁺-dependent IDH. The goals of these laboratory sessions are:

1. Learn spectrophotometric analyses of enzyme activity
2. Determine how the amount of enzyme in the assay affects the rate of activity.
3. Determine how substrate amount in the assay mixture affects enzyme activity rates
4. Determine the effects of environmental conditions on enzyme activity
5. Learn how to organize our data into tabular and graphic form

I. Introduction

Enzymes are biological catalysts with remarkable power, increasing reaction rates by at least a million-fold. They increase reaction rates by lowering activation energies, allowing chemical reactions to proceed under physiological conditions. Enzymes are highly specific as to substrates and reactions catalyzed. They are usually proteins, although some enzymes are other types of biological molecules. Enzymes function best in dilute aqueous solutions under limited conditions of temperature, pH, and salt concentration. Some enzymes require one or more non-protein components called “coenzymes” and “cofactors”; a coenzyme is an organic molecule, while a cofactor may be a metal ion. Some enzymes simultaneously require both a cofactor and a coenzyme. Isocitrate dehydrogenase [IDH] is one of these enzymes, requiring both NADP⁺ as a coenzyme and Mg²⁺ or Mn²⁺ as a divalent metal cofactor,

IDH is a ubiquitous enzyme found in all living organisms and has two catalytic activities (Figure 1). As its name implies, IDH removes hydrogens from its substrate, isocitrate. In addition, it is a decarboxylase, removing a CO₂ from the six-carbon substrate to generate a five-carbon product, α-ketoglutarate.

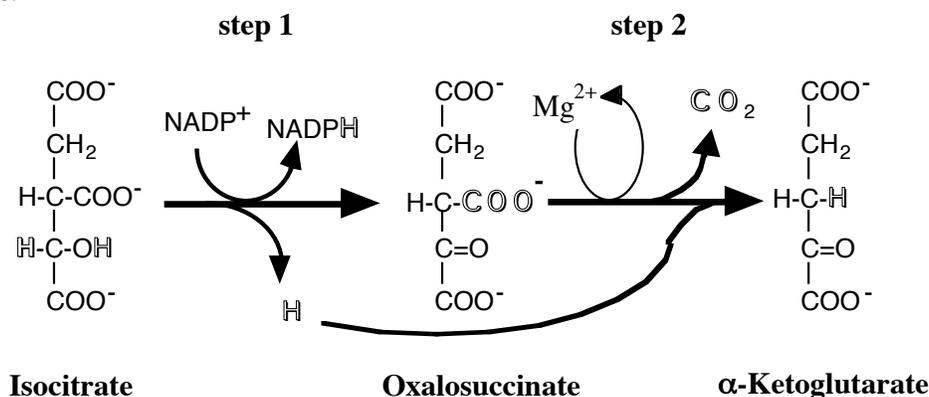


Figure 1. IDH catalyzes the sequential dehydrogenation and decarboxylation of isocitrate to α-ketoglutarate. Note that IDH catalyzes two sequential reactions to convert isocitrate into α-ketoglutarate.

Two distinct forms of IDH are found in higher organisms. They differ in their distribution within the cell and in their coenzyme requirements. The soluble form of IDH requires NADP⁺ (Figure 2) as its coenzyme. This NADP⁺-dependent form of IDH is thought to be the only IDH in most bacteria. In higher organisms, this form of IDH is found in the cytoplasm of cells within all organs and tissues, and it is used in lipid synthesis. The other form of IDH is NAD⁺-dependent; it is localized in the mitochondria of eukaryotic organisms. You may be familiar with this second form of IDH from previous study of the Krebs cycle (and if you're not, you will meet it again in the bioenergetics section of this course). Both forms of IDH require a divalent metal ion.

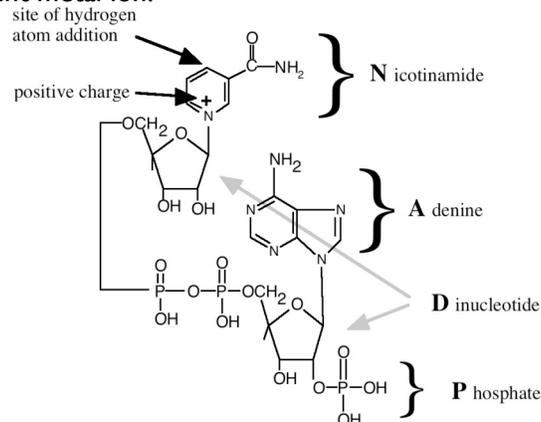


Figure 2. The molecular structure of NADP⁺. The active site is where the hydrogen atom will be added to convert NADP⁺ to NADPH. This diagram illustrates what the letters N-A-D-P represent.

📰 NEWS ITEMS: A team of researchers found a species of voles that was resistant to mutations caused by radiation. When they analyzed their cells, they found that the voles had elevated levels of IDH, which they believe is protecting them from radiation-induced mutations. (*Science* 273)

NADP⁺-dependent IDH activity is especially high in cardiac tissue and is often monitored in the blood of heart attack patients. Detectable IDH activity in the arterial blood suggests severe tissue damage with leakage of the soluble (cytosolic) IDH into the blood system.

How IDH activity is measured

IDH activity routinely is measured using a spectrophotometer to monitor the reduction of NADP⁺ to NADPH. While performing assays, the spectrophotometer is set at 340 nm, the absorption maximum of NADPH (and results from last week's lab). Assays are performed at a standard temperature, usually 25°C to 30°C.

Designing a protocol

Before a scientist begins an experiment, he or she must first define a problem and suggest possible explanations based upon previous knowledge or observations. In other words, develop an hypothesis, which might be considered an "educated guess" or a tentative explanation as to the cause and effects relating to that problem. A good hypothesis is one that is testable and fosters predictions that consider one variable at a time. The hypothesis may turn out to be incorrect, but it is a good hypothesis if it can be tested. In fact, an hypothesis that cannot be tested is useless to science - it may be good philosophy, but not good science. Hypotheses can not be proven to be correct - they may be tested extensively and rigorously and they may be proven to be incorrect, but an hypothesis can never be proven to be true.

A scientist must first define a problem and then develop an hypothesis. Next, one must devise predictions that will hold, or will not hold, if the hypothesis were true. These predictions lead to experiments. Many experiments may be possible, and all may be tried eventually; however, it is important to perform one discrete experiment at a time. After designing an experiment, our scientist must outline a series of logical procedures to be completed in the laboratory or in the field. This written sequence of steps is called a **protocol**. A well-planned protocol will include the following elements:

1. An outline of the sequence of detailed procedures.
2. Calculations of volumes, concentrations, etc., of all reagents to be used.
3. Tables constructed for recording data.
4. Procedures for testing and organizing data for presentation

Experiment 1: Performing an IDH assay

Hypothesis 1: A successful assay for IDH activity simultaneously requires the enzyme (IDH), the substrate (isocitrate), and the coenzyme (NADP⁺).

Hypothesis 2: Under ideal conditions, IDH activity will be linear for at least three minutes.

To test your hypotheses, you will need to set up assays as in the table below. You should ask yourself, “What is the purpose of each assay? And why are assays done in triplicate?”

Important notes for all experiments:

1) Bubbles and/or drops of liquid clinging to the sides of your wells (rather than mixed in the bottom) will yield unusual absorbance readings. Get in the good habit of ejecting your liquids into the bottom of the wells and then checking your plate for bubbles and drops on the sides before you place it in the spectrophotometer.

2) You will usually initiate the reactions by adding 10 µL of isocitrate solution as the last step. (You will use a multi-tip pipet, at the plate reader, to add isocitrate to all wells that get isocitrate.) **HOWEVER**, in later experiments, isocitrate will not always be the last reagent added. Look closely at the instructions!

		Coenzyme	Enzyme	Substrate
Well	Buffer	NADP⁺	IDH	Isocitrate*
A 1	200 µL	0 µL	0 µL	0 µL
A 2	200 µL	0 µL	0 µL	0 µL
A 3	200 µL	0 µL	0 µL	0 µL
B 1	170 µL	10 µL	20 µL	0 µL
B 2	170 µL	10 µL	20 µL	0 µL
B 3	170 µL	10 µL	20 µL	0 µL
C 1	180 µL	10 µL	0 µL	10 µL
C 2	180 µL	10 µL	0 µL	10 µL
C 3	180 µL	10 µL	0 µL	10 µL
D 1	170 µL	0 µL	20 µL	10 µL
D 2	170 µL	0 µL	20 µL	10 µL
D 3	170 µL	0 µL	20 µL	10 µL
E 1	160 µL	10 µL	20 µL	10 µL
E 2	160 µL	10 µL	20 µL	10 µL
E 3	160 µL	10 µL	20 µL	10 µL

FYI: Standard assay conditions in these experiments include 0.2 M Tris buffer at pH of 8.5 with 1 mM MgCl₂, 0.144 mM NADP⁺, and 0.23 mM isocitrate

Procedure

1. Use the appropriate pipettors to add the appropriate amounts of buffer, NADP+, and IDH to the appropriate wells. (You will add the isocitrate at the last second before you start the plate reader.)
2. Use KCjunior software to select the “IDH (340 nm every 30 sec)” option and click on the “open” button, then on the “read plate” button.
3. Click on the “read plate” button. (there’s no need to fill in the results ID or plate description boxes).
4. Place your 96-well plate in the carrier (with well A1 at the top left position). Add the last ingredient (isocitrate) to the appropriate wells as rapidly and simultaneously as you can. Then quickly shut the lid and press the OK button to start recording the absorbance.
5. You should hear the machine shaking the plate (to mix the solutions) and moving the plate. The plate reader will wait 30 seconds and repeat the procedure six more times for a total of three minutes (and seven total reads).
6. When the plate reader has finished all seven readings, go up to “Results” in the upper left menu portion of the monitor and select the “print results” option from the drop-down menu. (Make sure “Brother HL-514 series” is selected in the printer options). Click the “OK” box to print your data. You should get a two-page printout with seven sets of data.
7. Set up an Excel file as below (use Excel 2008 if multiple versions are present), and type your raw triplicate absorbance data into the appropriate cells. See the subsequent pages of the lab manual for how to use Excel to calculate the averages and corrected averages. The “corrected” reading for each assay is found by subtracting the average reading of a “control” experiment, in this case wells A1-3. (Would wells B1-3, C1-3, or D1-3 provide better “control” data?)

Time	A1 abs	A2 abs	A3 abs	A avg abs	Corrected A average absorbance	B1 abs	B2 abs	B3 abs	B avg abs	Corrected B average absorbance	C1 abs	C2 abs	etc...
0													
0.5													
1													
1.5													
2													
2.5													
3													

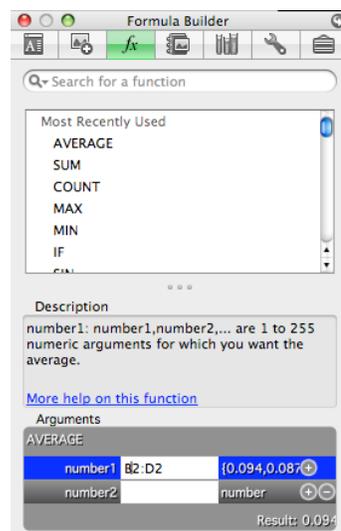
8. In Excel, prepare a graph of your data with time (independent variable) on the x-axis and average absorbance (dependent variable) on the y axis. Include data for all experimental conditions on one graph, so that the graph will have a separate line representing each experimental condition. See the subsequent pages for instructions.

A Beginner's Guide to Graphing with Excel

Click on the Excel 2008 icon in the tool bar. When Excel opens, you will see a spreadsheet. In the first cell, enter a name for your X axis values (for instance, "Time"). Enter the appropriate time values in the first column. Enter headings for dependent variables in subsequent columns. For example, at the top of the second column, enter "A1 abs." representing the absorbance values for well A1 of the 96 well plate. Enter the raw absorbance data for well A1 at each time point. Similarly enter raw data for all other wells. Leave two blank columns to the right of each set of triplicate data, and type headings for these columns as in the image below. At this point, you should have a spreadsheet whose first portion looks similar to the following image:

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Time	A1 abs	A2 abs	A3 abs	A avg abs	Corrected A average absorbance	B1 abs	B2 abs	B3 abs	B avg abs	Corrected B average absorbance	C1 abs	C2 abs	etc...
2	0	0.071	0.099	0.055			0.02	0.072	0.06			0.08	0.13	
3	0.5	0.075	0.088	0.059			0.114	0.111	0.92			0.174	0.17	
4	1	0.089	0.094	0.071			0.23	0.221	0.21			0.29	0.28	
5	1.5	0.064	0.09	0.069			0.341	0.337	0.345			0.401	0.4	
6	2	0.081	0.082	0.061			0.46	0.452	0.461			0.52	0.51	
7	2.5	0.076	0.099	0.053			0.6	0.611	0.597			0.66	0.67	
8	3	0.079	0.085	0.072			0.725	0.715	0.714			0.785	0.78	

Using Excel, we can determine the average values for our experimental replicates. Click on the empty cell for the average values of well A at time 0 (in Excel this is cell E2; don't confuse the Excel cell names with the well numbers from the 96 well plate). Next, click on the Insert menu at the top. Select the Function option and double click Average in the Formula Builder box that pops up. You might notice that Excel "wants" to calculate the average of the values in cells A2 to D2. Our experimental values, though, are only in cells B2 to D2. Using the mouse, make any necessary changes to these cell designations by highlighting the cells that you want to average (in this case B2, C2, & D2). Alternatively, type B2:D2 into the box at the bottom of the Formula Builder window. Hit the return key and the average value of your triplicates will be inserted into cell E2. To determine the averages for the other triplicate readings, click in cell E2 and then grab the small square in the lower right corner of the cell, dragging it down to the bottom of cell E8. When you let go you should have averages in every cell. Repeat this process for the raw data in the B wells, the C wells, etc. until you have computed the averages for all your data.



The main window should now look something like this:

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Time	A1 abs	A2 abs	A3 abs	A avg abs	Corrected A average absorbance	B1 abs	B2 abs	B3 abs	B avg abs	Corrected B average absorbance	C1 abs	C2 abs	etc...
2	0	0.071	0.099	0.055	0.075		0.02	0.072	0.06	0.051		0.08	0.13	
3	0.5	0.075	0.088	0.059	0.074		0.114	0.111	0.92	0.382		0.174	0.17	
4	1	0.089	0.094	0.071	0.085		0.23	0.221	0.21	0.22		0.29	0.28	
5	1.5	0.064	0.09	0.069	0.074		0.341	0.337	0.345	0.341		0.401	0.4	
6	2	0.081	0.082	0.061	0.075		0.46	0.452	0.461	0.458		0.52	0.51	
7	2.5	0.076	0.099	0.053	0.076		0.6	0.611	0.597	0.603		0.66	0.67	
8	3	0.079	0.085	0.072	0.079		0.725	0.715	0.714	0.718		0.785	0.78	

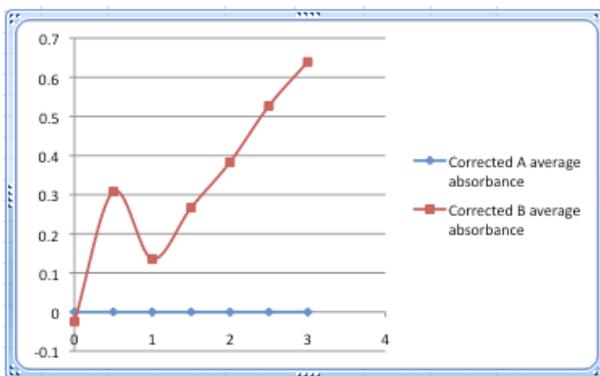
Now we will “correct” the data by subtracting the absorbance that is due to the buffer and the plastic. The remaining values will represent the true absorbances of just the substance we are interested in. Wells A1-3 contained just buffer. We will subtract the A averages from all averages to achieve the corrected values. Note that this will result in values of 0 for our corrected A averages. Click in the first open cell of the “corrected A average” column (cell F2). Type “=E2-\$E2” and hit enter. This tells Excel to subtract the value in cell E2 from itself and put the result in this cell. The dollar sign is important for later. Now click in cell F2 and grab the small square in the lower right corner, dragging it down to the bottom of cell F8. Notice that the formula is copied in a relative way—each time it refers to the cell just to the left.

Simply copy and paste cells F2:F8 into cells K2:K8 and into every other column where corrected values are required. The formula, not the absolute values, is transferred. The dollar sign tells the pasted formula always to subtract column E values from what is just to the left. You should now have corrected values in every column. Spot check the values by eye to make sure things look right. If not, go back and correct your formulas.

To graph the data, highlight column A (time values) as well as all the “corrected” columns. It’s OK to include the cells that contain the headings, as Excel usually recognizes those as labels. (Use the apple/command key to highlight multiple separate regions of the spreadsheet simultaneously.) Go to the Insert menu and select Chart. Select the Chart option (not Sheets or SmartArt Graphics) and then click the XY (Scatter) option at the far right. Choose one of these five graph types (which do you think is most appropriate?). At any time later you can switch to a different one of these five types.



You will then see a graph of your data as below (though with more than two data series). Note that Excel automatically used the first highlighted column as the X axis values, with the remaining columns as Y axis values in separate lines on the graph. If you ever want to change which data are on which axis, just right-click on the white area in the graph above the key and pick Select Data. Position the cursor in the “X values” or “Y values” box, and then highlight the cells whose data are meant for that axis.

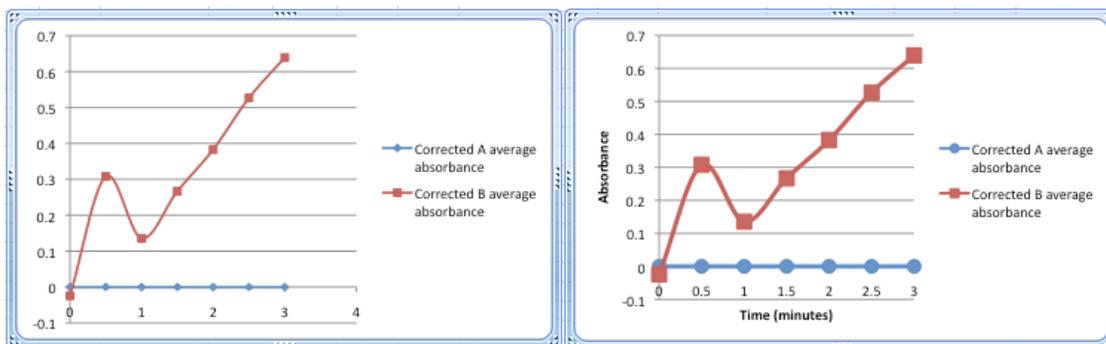


You can change the appearance of your graph by double-clicking and/or right-clicking various features on the graph and selecting the formatting options, or by using the Formatting Palette.

- *Remove gridlines:* Right-click the lines themselves and select Delete.
- *Remove or modify the key* (confusingly called a legend by Excel): If you have only one data series represented as a line on the graph, just highlight the "series 1" key and hit delete. However, if you

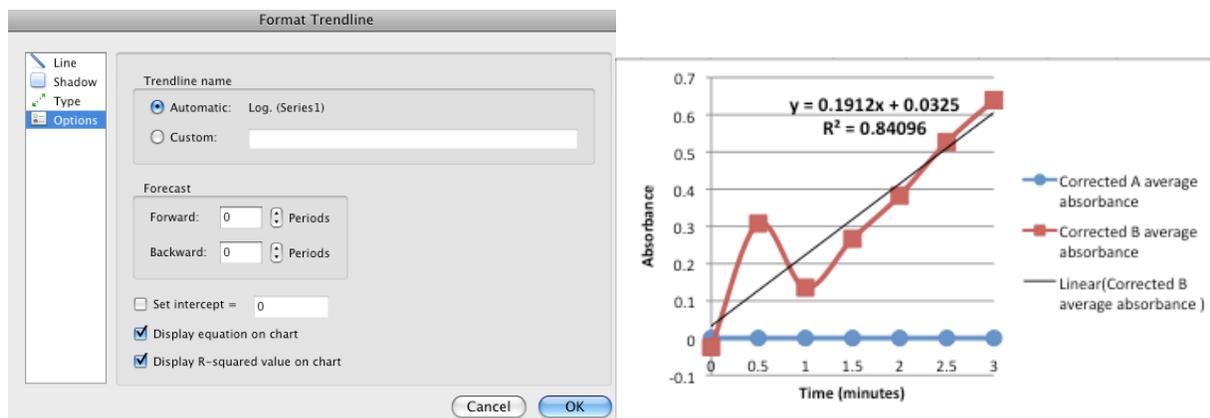
have a graph of several lines (as you will need to eventually), you should keep and properly label the keys to indicate what each line represents, if Excel hasn't done this automatically. Go back to the Select Data option as above, and type a new title in the "name" box for each data series that you can highlight at the left. (Note that you can also add data lines to the graph with the Add button; in the box that comes up, just highlight the proper data cells for the X and Y axis).

- *Label the axes and title the graph:* Click on the graph so that it has blue circles at the corners and blue squares along the sides, and make sure the Formatting Palette is visible (if not, find it in the View menu). Under Chart Options, the Titles section lets you easily add the appropriate information.
- *Modify colors, fonts, and sizes of graph features:* You can adjust many parameters simply by double-clicking on specific parts of the graph. For example, to change the color, size, or shape of the data points, double click on the data series within the graph and you will get a window that lets you change those parameters. To change the scale of either axis, double click on the line of the x- or y-axis and you can change the minimum, maximum, major/minor units, etc. To change the font, size, or orientation of the labels double click on the text itself.



Notice that the graph above at right includes the same data as before, but some of the cosmetic details have been changed to make the data more prominent and the graph easier to view.

Finally, you can use Excel to determine a best-fit line of your data and the line's slope. After you have completed your graph, right-click on any data point, and select "Add Trendline." To format the trendline, either double click it, or highlight it and go to the "Format" menu. Under "Type," "Linear" is the default, and that is what we will use. Under "Options," click the boxes for "Display equation on chart", and "Display R-squared value on chart". Click the "OK" button. The equation for your trendline (in $y = mx + b$ format where $m =$ slope and $b =$ the value of the y-intercept) and an R^2 values will then appear on your graph. Briefly, the closer your R^2 value is to 1.0, the better your experimental data fit the trendline, your best fit line.



Experiment I • Considerations

Using Microsoft Excel as according to the instructions in the previous pages, enter data from all the wells into a spreadsheet, and have Excel calculate averages and corrected averages. Construct a graph that visually portrays your data by plotting corrected average absorbance (y-axis) as a function of time (x-axis). Your graph should include five lines, one for the averaged data from wells A1-3 on the 96 well plate, one with averaged data from B1-3, etc. Was there activity in wells A1 - D3? Was there enzyme activity in wells E1 - E3? Was activity the same in wells E1, E2, and E3? Was activity linear for three minutes? If so, the slope of the best fit line tells you the rate of reaction. If not, explain your observations. The initial rate of a reaction may be determined from the slope of the line joining each successive point.

Do your data support your hypotheses? If not, how would you change the protocol?

Save your completed graph and data worksheet in an appropriate folder (such as Tuesday AM lab) with a descriptive name. Email the file to yourself, or place it in a shared folder on the public server (see instructions page LM 65) so that the raw data are preserved. Include your notes below on this page, and attach a copy of your graph (**Graph I**) to the next page. Make sure all aspects of the graph are properly labeled within Excel, and make sure to include an appropriately descriptive title.

Attach Graph I to this page:

Experiment 2: Effects of varying enzyme concentration on IDH activity

Problem: What is the relationship between the rate of a reaction and the amount of enzyme in the assay solution when substrate and coenzyme are abundant (non-limiting)? This question might become “In subsequent experiments, how much enzyme solution should I use in each assay?”

Hypothesis: IDH activity will vary directly with the amount of enzyme in each assay.

To test this hypothesis, you will need to follow a protocol that holds all conditions constant except the amount of enzyme added to each assay. All tests should be run more than once; routinely, enzyme assays are run “in triplicate.” For example, wells B4, B5, and B6 in the table below are triplicate assays containing 5 μL of IDH. Set up reactions as shown the table below.

Your instructor will tell you (or write on the board) that today’s IDH stock concentration today is: _____ . You will need to know this stock concentration for later calculations.

Wells	Buffer	NADP⁺	IDH	Isocitrate
A 4-6	180 μL	10 μL	0 μL	10 μL
B 4-6	175 μL	10 μL	5 μL	10 μL
C 4-6	170 μL	10 μL	10 μL	10 μL
D 4-6	160 μL	10 μL	20 μL	10 μL
E 4-6	130 μL	10 μL	50 μL	10 μL
F 4-6	100 μL	10 μL	80 μL	10 μL
G 4-6	200 μL	--	--	--

Procedure:

1. Use the appropriate pipettors to add appropriate amount of buffer, NADP⁺, and IDH to the appropriate wells.
2. Again use the “IDH (340 nm every 30 sec)” protocol on KCjunior to collect your data. Make sure to add the isocitrate at the plate reader, just before you start collecting your data.
3. When the plate reader has finished all seven readings, print your results.
4. Return to your station and organize your data in Excel in a similar manner as you did in Experiment I. Type in the raw triplicate data. Use Excel to calculate the averages absorbance values and corrected average absorbance values (with blanks subtracted). Create a single graph in Excel that includes a separate line for each experimental condition. Make sure all aspects of the graph are properly labeled within Excel, and make sure to include an appropriately descriptive title. Attach your graph (**Graph 2A**) to the next page of your lab notebook. Save the raw data file in your email or public shared folder.

Attach Graph 2A to this page:

Experiment 2 • Considerations

Look at the graph you just created to answer the following questions:

Was there activity in all wells?

Did activity vary with the amount of enzyme in each assay?

How consistent was the activity in the three wells with the same amount of enzyme?

Was activity linear for the first three minutes for each volume of enzyme? If not, explain.

Do your data support your hypothesis?

Return to Excel and use its ability to generate a formula for the best fit line for each of the five lines on the graph you just made. For each best fit line you will get an equation in the $y = mx + b$ format where **b** is the y intercept value and **m** is the slope or change in absorbance over time which is the definition of activity. Put the slopes in the table below.

IDH Volume/Well	IDH Concentration*	Slope (Abs/min) (a.k.a. activity)
0 μL		
5 μL		
10 μL		
20 μL		
50 μL		
80 μL		

*You will need to calculate the IDH concentration by multiplying the stock concentration by the proportion of IDH in the wells. (Hint: $C_s V_s = C_w V_w$)

Use the table above to construct another graph (**Graph 2B**) in Excel that compares the concentration of enzyme on the x-axis to the slope of the appropriate lines (slope indicates enzyme activity) from your previous graph. What conclusions can you reach from your results? Attach Graph 2B to the next page. Make sure all aspects of the graph are properly labeled within Excel, and make sure to include an appropriately descriptive title.

Why is it inappropriate to graph enzyme activity versus IDH volume/well for these data? Why is it far more appropriate to graph enzyme activity versus IDH *concentration*?

Attach a copy of Graph 2B to this page:

Experiment 3. Effects of varying isocitrate concentration

Problem: What is the relationship between the rate of a reaction and the amount of isocitrate in the assay solution when the amounts of IDH and NADP⁺ in the assay are held constant? Before you start this experiment, develop an hypothesis and sketch a graph predicting the relationship of activity vs. isocitrate concentration.

Write your hypothesis here:

Sketch a simple graph predicting the relationship between activity (y-axis) and isocitrate concentration (x-axis) here:

Procedure: To test your hypothesis, you will need to follow a protocol that holds all conditions constant except the amount of isocitrate added to each assay. The table below outlines such a protocol using five concentrations of isocitrate. Each concentration is tested in triplicate. Add reagents to your wells as listed from left to right.

***Important Note:** in this experiment you will add the enzyme (IDH) last.

Wells	Buffer	NADP⁺	Isocitrate	*IDH
A 7-9	170 μ L	10 μ L	0	20 μ L
B 7-9	167.5 μ L	10 μ L	2.5 μ L	20 μ L
C 7-9	165 μ L	10 μ L	5 μ L	20 μ L
D 7-9	160 μ L	10 μ L	10 μ L	20 μ L
E 7-9	150 μ L	10 μ L	20 μ L	20 μ L
F 7-9	130 μ L	10 μ L	40 μ L	20 μ L
G 7-9	90 μ L	10 μ L	80 μ L	20 μ L
H 7-9	200 μ L	--	--	--

1. Use the appropriate pipettors to add appropriate amount of buffer, NADP⁺, and isocitrate to the appropriate wells (NOTE – this experiment adds IDH at the plate reader instead of isocitrate.)
2. Again use the “IDH (340 nm every 30 sec)” program to collect your data. Make sure to add the IDH at the plate reader as simultaneously as possible and just before you collect your data.
3. When the plate reader has finished all seven readings, print your results.

- Return to your station and organize your data in Excel in a similar manner as you did in Experiments 1 and 2. Type in the raw triplicate data. Use Excel to calculate the average absorbance values and corrected average absorbance values (with blanks subtracted). Create a single graph in Excel that includes a separate line for each experimental condition. Make sure all aspects of the graph are properly labeled within Excel. Attach a copy of your graph (**Graph 3A**) to the next page of your lab notebook. Save the raw data file in your email or public shared folder.
- Determine the activity for each concentration of isocitrate by determining the slopes of the lines in the graph you just made above. Then, use the table below to construct another graph that compares activity (y-axis) as a function of isocitrate concentration (x-axis). Attach that graph (**Graph 3B**) to the next available page of your lab notebook.

Isocitrate Used	Isocitrate Concentration*	Slope (Abs/min) (a.k.a. activity)
none	0 mM	
2.5 μ L		
5 μ L		
10 μ L		
20 μ L		
40 μ L		
80 μ L		

*You will need to calculate the isocitrate concentration by multiplying the stock concentration by the proportion of isocitrate in the wells. (Hints: The isocitrate stock concentration is 4.6 mM; and $C_s V_s = C_w V_w$)

Do your data support your hypothesis?

Is the relationship between activity and concentration of substrate linear?

Attach a copy of Graph 3A to this page:

Attach a copy of Graph 3B to this page:

Preparation for Next Week's Lab:

During next week's lab, we will study the effects of environmental conditions on enzyme activity. Each group of students will design an experimental protocol to address one of the following questions. Hint: look at the following few pages of the lab manual to get a preview of these experiments. Your team will need to sign up for one of the following options by the end of lab today.

- Option A: Does pH influence IDH activity?
- Option B: Does IDH have a metal ion cofactor requirement?
- Option C: Does temperature influence IDH activity?
- Option D: Does NADP+ concentration influence IDH activity?
- Option E: Does NaCl concentration influence IDH activity?
- Option F: Does IDH activity vary among different organisms and/or tissues?

Now that you have selected a variable to test next week, you will need to think about the following things. (Reading forward to next week's lab notes will be helpful.)

1. Develop a hypothesis about the effects of the environmental condition you chose to vary upon enzyme activity.
2. Design an experiment to test that hypothesis.
3. Prepare a protocol to carry out that experiment. Write your protocol in the space below.

IDH Physical Models

✓ **Special Note**

When handling the IDH models please be very careful – they are fragile, one-of-a-kind objects that cannot be easily (or inexpensively) replaced.

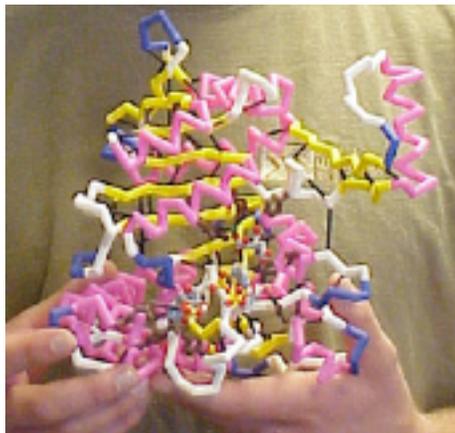
In lab today your group must:

A. Find on the space-filling and the wire models of IDH:

- the active site
- isocitrate
- Mg²⁺
- NADP⁺ and/or NAD⁺
- an alpha helix
- a beta sheet

B. Have one person randomly and gently tumble the two models and have another person move both models so they are sitting in the same orientation.

C. Answer the question: Why does IDH prefer NADP⁺ over NAD⁺ given they have the same structure on the reactive end of the molecule?



wire frame model of IDH



space-filling model of IDH

Parameters that Affect Enzyme Activity

Focused Reading

- p 129-131 “Molecular Structure...” to “How are Enzyme. . .”
- p 131-132 “Enzymes can be...” to “Allosteric enzymes...”
- p 133-135 “Enzymes are affected...” to “Chapter summary...”

✓ Special Note

- Bring a calculator to lab
-

Goals for This Lab:

This week, we will determine the effects of environmental perturbations of our standard IDH assay conditions. We will use the techniques and information we learned last week and apply that to this week's experiments.

Methods and Materials

You will use the same general methods that we used in the previous lab. All equipment, solutions and supplies required to carry out the experiments have been prepared for you. You should review your protocol and assign specific tasks to specific team members before you start your experiments. You will find helpful tables and suggestions for the different experimental options on the following pages. Find the specific page that relates to the experiment that your group chose to perform today.

The stock solutions provided for all groups are:

2.88 mM NADP⁺

4.6 mM isocitrate

_____ IDH (look on the board for the IDH concentration)

If you are using other reagent(s) for your experiment the concentration(s) will be provided on the reagent itself and/or in the notes.

Before You Leave Lab (All Groups):

1. Be certain that you have collected all of the data you need to make your experiment complete.
2. Be certain that each member of the group fully understands what was done and has a copy of all of your data recorded in her/his lab manual.
3. Be certain that you have analyzed your results and determine if your results support your hypothesis.

Option A: Does pH influence IDH activity?

Write out your hypothesis in this space:

To determine if pH influences IDH activity, we will need to follow a protocol that holds all conditions constant except the pH of the assay buffer. Remember to include blank(s).

Wells	pH	Buffer	NADP ⁺	IDH	Isocitrate

Time (min.)	pH ___					
0						
0.5						
1						
1.5						
2						
2.5						
3						

Considerations:

Does IDH activity vary when the pH of the assay mixture varies, or do levels of activity remain constant regardless of pH? Explain how the pH of the assay mixture might affect activity of an enzyme.

Option B: Does IDH Have a Metal Ion Requirement?

Write out your hypothesis in this space:

You will need to follow a protocol that holds all conditions constant except for the presence or absence of specific divalent metal ions. Your stock ion solutions were all prepared at 20 mM and your EDTA solution is 0.1 M. (EDTA is a chelator that removes ions from solutions.)

Wells	Buffer **	Metal	NADP ⁺	IDH	Isocitrate
		None			
		10 μL EDTA			
		10 μL Mg ²⁺			
		10 μL Mn ²⁺			
		10 μL Ca ²⁺			
		10 μL Zn ²⁺			
		Blank			

**You will use a special buffer that does not contain Mg²⁺ Do not share solutions with other lab groups.

Time (min.)	None	EDTA	Mg ²⁺	Mn ²⁺	Ca ²⁺	Zn ²⁺
0						
0.5						
1						
1.5						
2						
2.5						
3						

Considerations:

Does IDH require a divalent metal ion for activity? Does additional Mg²⁺ added to the standard assay buffer increase activity? What does this observation mean? Does the addition of Mn²⁺ added to the standard assay buffer increase activity? What does this observation mean? How can the effects of Ca²⁺ and Zn²⁺ on IDH activity be explained?

Option C: How does temperature influence IDH?

Write out your hypothesis in this space:

You will need to follow a protocol that holds all conditions constant except temperature. The temperature on the plate reader can be set for a variety of temperatures above ambient (room) temperature. Remember to make a blank.

Wells	Temperature	Buffer	NADP ⁺	IDH	Isocitrate

Time (min.)	____ ° C						
0							
0.5							
1							
1.5							
2							
2.5							
3							

Option D: Does enzyme activity vary with NADP⁺ concentration?

Write out your hypothesis in this space:

What is the relationship between the rate of a reaction and the amount of coenzyme in the assay solution when the amount of enzyme is held constant? Before starting this experiment, develop a hypothesis and sketch a graph predicting the relationship of activity vs. coenzyme concentration.

Wells	Buffer	NADP ⁺	IDH	Isocitrate	*NADP ⁺ concentration
		0			

*Note: 2.88 mM NADP⁺ stock provided.

Time (min)	0 mM NADP ⁺	___ mM NADP ⁺					
0							
0.5							
1							
1.5							
2							
2.5							
3							

Considerations:

Organize the data from the experiment. Determine the mean activity for each concentration of NADP⁺. Construct a graph that compares activity as a function of NADP⁺ concentration. Do the data support the hypothesis? Is the relationship between activity and concentration of NADP⁺ linear? Explain this relationship.

Option E: Does NaCl concentration influence IDH activity?

Write out your hypothesis in this space:

To test this hypothesis, you will need to follow a protocol that holds all conditions constant except concentration of NaCl in the assay solution. You will be given a 5 M NaCl solution to dilute to a wide variety of different concentrations. FYI – blood is ~0.9% salt and the FW of NaCl is 58.44. Remember to set up appropriate blanks.

Wells	Buffer	5M NaCl	NaCl Concentration	NADP+	IDH	Isocitrate
		0	0			

Time (min)	0 M NaCl	___ M NaCl						
0								
0.5								
1								
1.5								
2								
2.5								
3								

Considerations:

Were differences in activity between the treatments observed? What was the relationship between the concentration of NaCl and activity? Explain how salt might affect enzyme activity.

Option F: Is IDH present in specific organisms and/or tissues?

Write out your hypothesis in this space:

This experiment is “open ended”; we may design a single, additional experiment, comparable to the ones listed above, or expand these topics into a research project of wider magnitude.

1. You may chose to survey IDH activity:
 - i. in a wide variety of related species.
 - ii. in different tissues of a single species.
2. Homogenize the samples in cold TEM Buffer, using a kitchen blender.
3. Filter the homogenate through two layers of cheesecloth into a small beaker on ice.
4. Transfer 1 mL samples to 1.5 mL microfuge tubes, spin for five minutes.
5. Transfer the supernatant to clean 1.5 mL microfuge tubes, on ice.
6. Use standard conditions to assay IDH activity.

Wells	Buffer	Tissue	NADP ⁺	IDH	Isocitrate

Time (min)						
0						
0.5						
1						
1.5						
2						
2.5						
3						

Attach a copy of your graph on this page:

Lab Notes

Graphing Exercise: Introduction to Data Presentation

The enzyme wildcatase catalyzes the conversion of wildcatin to catin. Previously, researchers determined that catin absorbs maximally at 605 nm, while wildcatin has a minimal absorbance at this wavelength. To characterize the properties of wildcatase, you have conducted a series of experiments. The scientific community is extremely excited to hear about your results. In fact, the organizers of the 16th Annual International Wildcatase Convention ask you to present your findings at their meeting in Cancun. Normally, you would decline this invitation, preferring to stay in Davidson. Because your friend is one of the meeting organizers, though, you agree to attend and present your findings. Of course, now you must organize your data and make graphs and/or tables of your results for your presentation.

Brief descriptions of these experiments and the resulting data are provided on subsequent pages. For each experiment, determine the most effective graphical means of presenting your data and generate a graph in Excel that you can include in your lab notebook on the following pages.

Experiment I: After a series of experiments, you have concluded that two different enzymes can catalyze the conversion of wildcatin to catin. You have named these enzymes wildcatase A and wildcatase B. A study of 100 mammal species has revealed that 50 of these species contain wildcatase A and wildcatase B, 40 contain only wildcatase A, and the remaining 10 contain only wildcatase B.

Attach a copy of your graph on this page:

Experiment 2: Your studies indicate that both deer mice and field mice contain only wildcatase A. To investigate the location of wildcatase A within these organisms, you looked for the presence of this enzyme in various tissues. You discovered that, in the deer mouse, wildcatase A is present in lung, brain, spleen, and liver. It is not found in muscle, kidney, or heart tissues. In the field mouse, wildcatase is present in lung, brain, and spleen tissues. It is not found in liver, muscle, kidney, or heart tissues.

Attach a copy of your graph on this page:

Experiment 3: In examining the tissues of the deer mouse, you discovered that the different tissues containing wildcatase A contain different amounts of wildcatase A. Lung contains 350 units of wildcatase A per gram of tissue. Spleen contains 250 units of wildcatase per gram of tissue. Liver contains 100 units of wildcatase A per gram of tissue. Brain contains 500 units of wildcatase A per gram of tissue.

Attach a copy of your graph on this page:

Experiment 4: To investigate the kinetics of wildcatase A and wildcatase B activity, you combined 100 mM wildcatase A and 100 mM wildcatin. You then measured the absorbance at 605 nm at 30 second intervals for two minutes, resulting in the following data: 0 min: 0.010; 0.5 min: 0.100; 1.0 min: 0.20; 1.5 min: 0.300; 2.0 min: 0.400. You then did a similar experiment, but used wildcatase B instead, resulting in the following data: 0 min: 0.010; 0.5 min: 0.150; 1.0 min: 0.300; 1.5 min: 0.450; 2.0 min: 0.600.

Attach a copy of your graph on this page:

What is your genotype? A PCR Approach

Focused Reading

- p 245-247 “DNA polymerase...” to “Telomeres...”
- p 354-357 “Gel electrophoresis separates...” to “16.1 Recap”

Web Reading

- Movie of PCR method: <http://www.bio.davidson.edu/misc/movies/pcr2.mov>
 - Polymerase Chain Reaction: <http://www.dnalc.org/resources/animations/pcr.html>
 - Gel electrophoresis: <http://learn.genetics.utah.edu/content/labs/gel/>
-

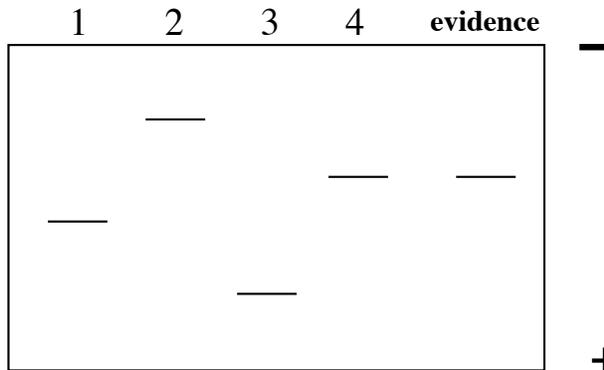
Introduction

Unless you spent the last few years in a cave, you have heard about the increasing use of “DNA fingerprinting” in court cases. The technology available is so sensitive that unbelievable sources of DNA have been used to convict criminals. In Minnesota, for example, DNA was extracted from the back of a postage stamp because some epidermal cells from a person’s tongue had been deposited on the glue when the stamp was licked. Most likely, a pivotal point of contention in future court cases will be the collection and handling of the evidence. For example, what if some DNA from the crime scene is proposed to have come from the accused? The defense attorney could suggest that the police collected some epidermal cells from the sidewalk (from a visit the day before) at the same time as the blood drops. What do you think of this possibility as a defense? If you want to read more about this area, the library has several books in the area of call numbers 614.1.

There are two standard methods for “DNA fingerprinting”: 1) Southern blots and, 2) PCR. We will discuss both of these techniques in class. Dr. Kary Mullis, the inventor of PCR, was awarded a Nobel Prize in 1993 for his revolutionary innovation. As you know, PCR allows you to amplify a single copy of DNA into millions of copies, provided the ends of the DNA of interest have been sequenced because you have to supply DNA polymerase with primers that will specifically hybridize to the target gene and no other DNA. Over the next two weeks, we will use PCR to determine the genotype of every student in class. We are using a hair root as our source of genomic DNA and are looking at a locus called DIS80. DIS80 contains a Variable Number of Tandem Repeat sequence (VNTR). As the term implies, there is a section of DNA that is repeated to varying degrees in each person. As a simplified example, let’s say the repeat unit is the two nucleotides sequence CG. If we were to sequence this portion of the DIS80 locus from four different DNA sources, we might see the following:

- 1) ATGCCGTATTACG**CGCGCGCGCGCG**CGCCTATTAGGTATTAG
- 2) ATGCCGTATTACG**CGCGCGCGCGCGCGCGCGCGCG**CGCCTATTAGGTATTAG
- 3) ATGCCGTATTACG**CGCGCG**CGCCTATTAGGTATTAG
- 4) ATGCCGTATTACG**CGCGCGCGCGCGCGCGCGCG**CGCCTATTAGGTATTAG

In this example, there are four alleles of this VNTR with four different lengths. If we electrophoresed these four segments of DNA on a gel, we would observe bands of four different sizes (2>4>1>3). In a criminal case, we might have four suspects and one DNA sample from the crime scene. The resulting gel might look like this:



Questions:

- 1) So, “who done it?”
- 2) What is wrong with the above gel? Why is this example too easy?

Now it is time for us to determine our genotypes. You should be forewarned – this technique is a delicate procedure that does not always work for everyone. In order to process this kind of evidence for a criminal case, a technician usually has a master’s degree in Forensic Science, and a few years of on-the-job training. Nevertheless, even these experts sometimes make mistakes. So, do not be discouraged if your sample does not work, but try to avoid this situation by observing these guide lines:

- Follow the protocol as carefully as possible.
- Do not contaminate your hair or DNA with that of others (remember one cell contains enough DNA to be amplified).
- Immediately after the DNA extraction is finished, visually check to verify that you have extracted DNA by gently removing the tube from the thermocycler and flicking the tube holding it up to a light and looking very carefully. You might be able to see a denser region of the solution at the bottom of the tube as it mixes with the less dense water.
- The most common mistakes are pipetting errors. Be sure to check that you are transferring the correct volumes and always use clean pipette tips; when in doubt use a new tip.
- Be very careful loading the gel. We will have time to practice loading gels this week so that when you are loading your real sample next week, you will be a pro.

DNA extraction

- 1) Pluck a hair so that a large portion of root is removed from your head (yikes!) For people of Caucasian heritage, the root will be white/translucent in appearance. People of African heritage will have roots that are dark. Regardless of the color, the root will be sticky so you can test it by touching it to the bench top to see if it adheres. Check to make sure you got some root and not all shaft.
- 2) Put the hair into a small microfuge tube with the root at the bottom of the tube. Cut off most of the hair but keep the root (~5 mm). Be careful, sometimes the root will jump away when you cut the hair.

- 3) Incubate the root in 100 μL digestion buffer (which contains 6 μg of proteinase K) for 1 hour at 55°C, then 10 minutes at 95°C (what is the purpose of this step?). Use thermocycler program HAIR 1 - lid disabled.

During this waiting period, we will practice loading gels so you will be ready for next week.

PCR Reaction Mixtures

4) When the DNA extraction cools, **vortex the tubes for 30 seconds** and then set up a new 500 μL microfuge tube by adding the following: 15.0 μL of your DNA and 10 μL of reaction mixture. The reaction mixture contains H₂O, 10X PCR buffer, dimethylsulfoxide (DMSO; a solvent), dNTPs (dATP, dTTP, dCTP, & dGTP), the two primers, and Taq DNA polymerase. (The Taq is added immediately before distribution by your instructor, 0.4 μL Taq per 10 μL mixture.)

PCR

The PCR temperature conditions are as follows:

- Step 1: 5 minutes at 95° C
- Step 2: 1 minute at 95° C
- Step 3: 1 minute at 65° C
- Step 4: 1 minute at 72° C
- Step 5: repeat steps 2 - 4 twenty-nine more times
- Step 6: hold at 4° C

When the PCR is completed, the tubes are removed and stored at 4° C.

DIS80 factoids

- >80% of all people tested are heterozygous
- 28 alleles have been published
- Repeat unit is 16 nucleotides long
- If there were zero repeat units, the PCR product would be 142 bp long
- PCR products range from 430 to 814 base pairs long
- 41 repeated units have been observed in the largest allele
- Primer sequences²:
 - #1 5' GAAACTGGCCTCCAAACACTGCCCGCCG 3'
 - #2 5' GTCTTGTTGGAGATGCACGTGCCCTTGC 3'

The PCR Results

Add 2.5 μL of the 10X loading dye to your PCR reaction tube. Load 15 μL of your DNA/dye mixture into a lane on the gel. It is important to note which lane contains your DNA. We will also add DNA of known sizes to several lanes on the gel. This DNA often called a “ladder” will provide a ruler of sorts for us to determine the size of your DNA. Electrophorese the DNA on a 1.5% agarose gel using 0.5X TBE and 200 ng/mL ethidium bromide. We usually run these gels at 90-100 volts for 1 - 1.5 hours. The exact time and voltage will depend on the gel box configuration. Appropriate conditions can be refined accordingly.

While the gel is running, we will learn how to calculate the molecular weights of bands.

How to calculate the molecular weight (MW) of a molecule that has been separated in a gel

The \log_{10} of a molecule's molecular weight is proportional to the distance that molecule has migrated. Therefore, the first step is to generate a standard curve using molecules of known size (the molecular weight markers).

When using semilog paper (see the next page), the molecular weights (in units of kilobases (kb) for DNA) is plotted on the Y-axis and the distance the molecule migrated (in mm) is plotted on the X-axis. When generating a standard curve, you will obtain a straight line (use a best-fit line).

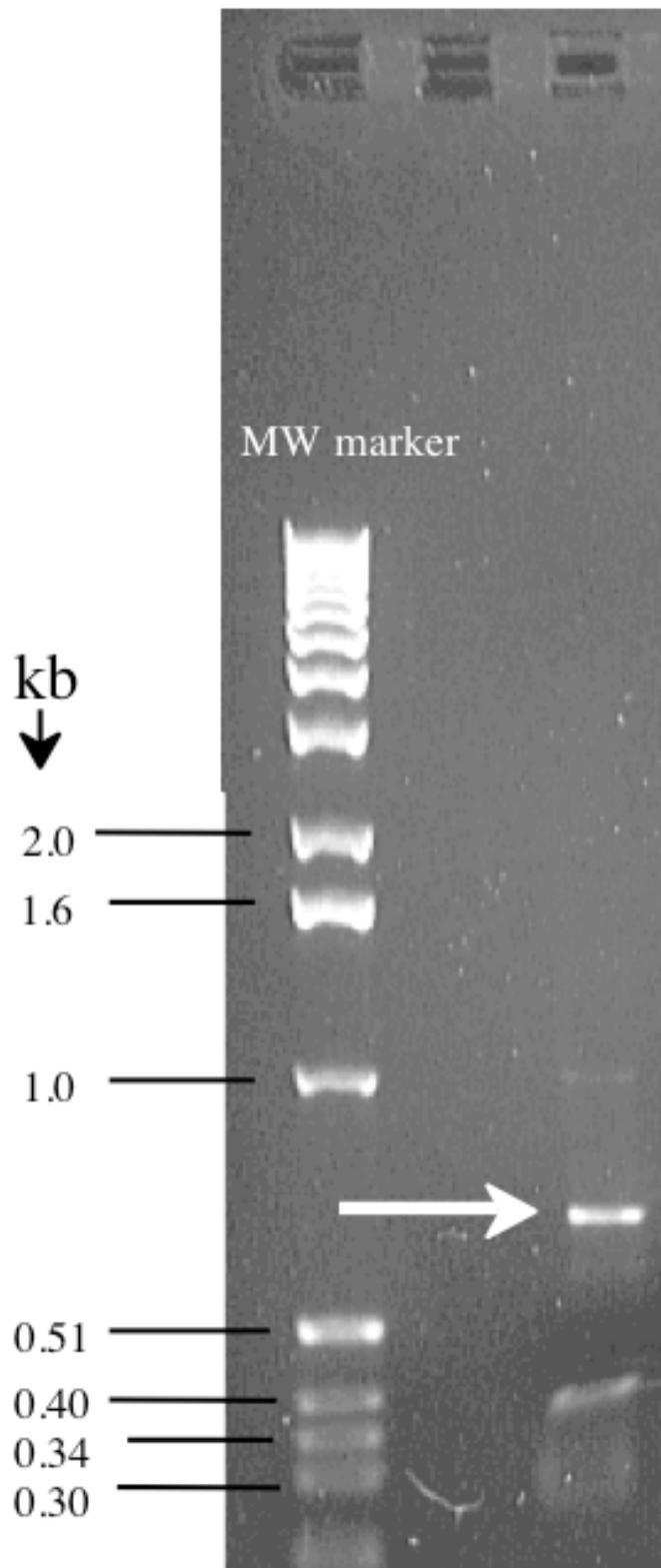
When your standard curve is ready, measure the distance traveled by your molecule of interest. Find that distance on the X-axis, and go up until you intersect with your standard curve. Move over to the Y-axis and that will indicate the molecular weight of the molecule you are studying.

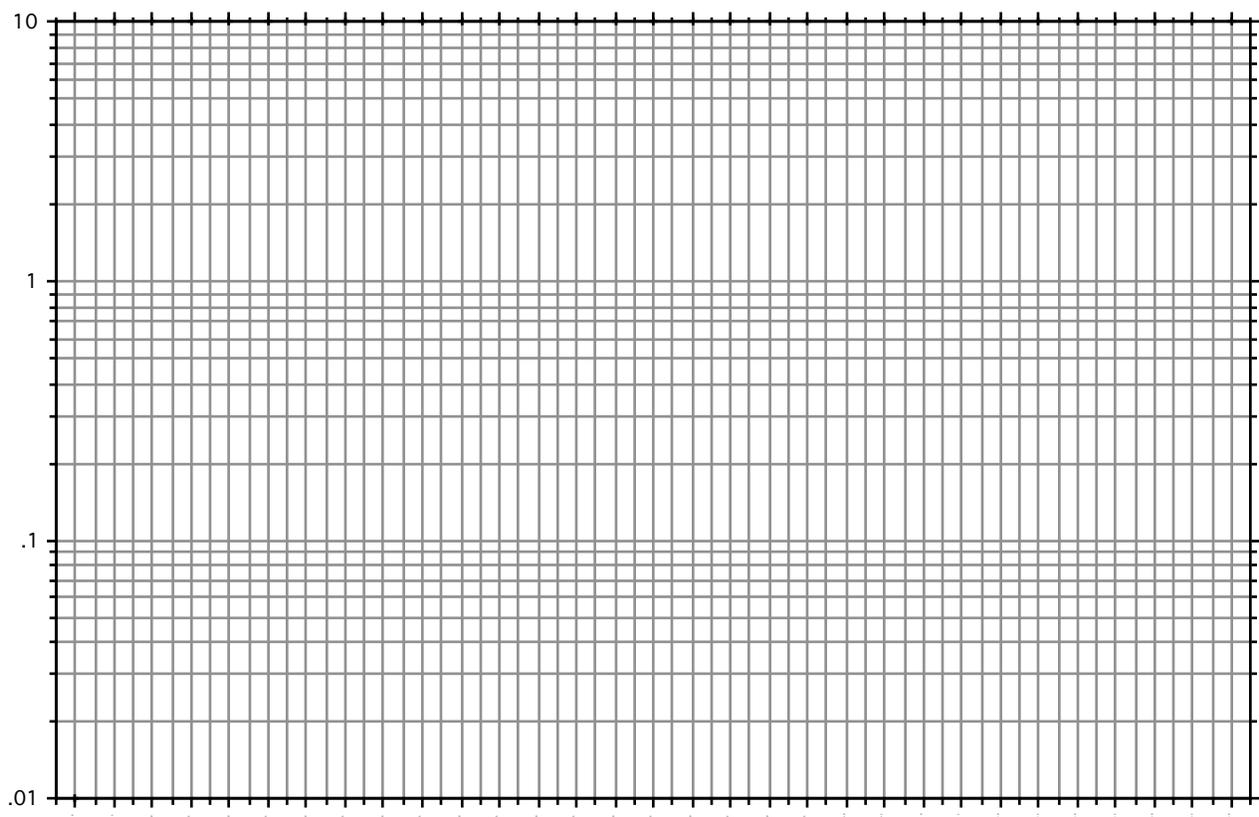
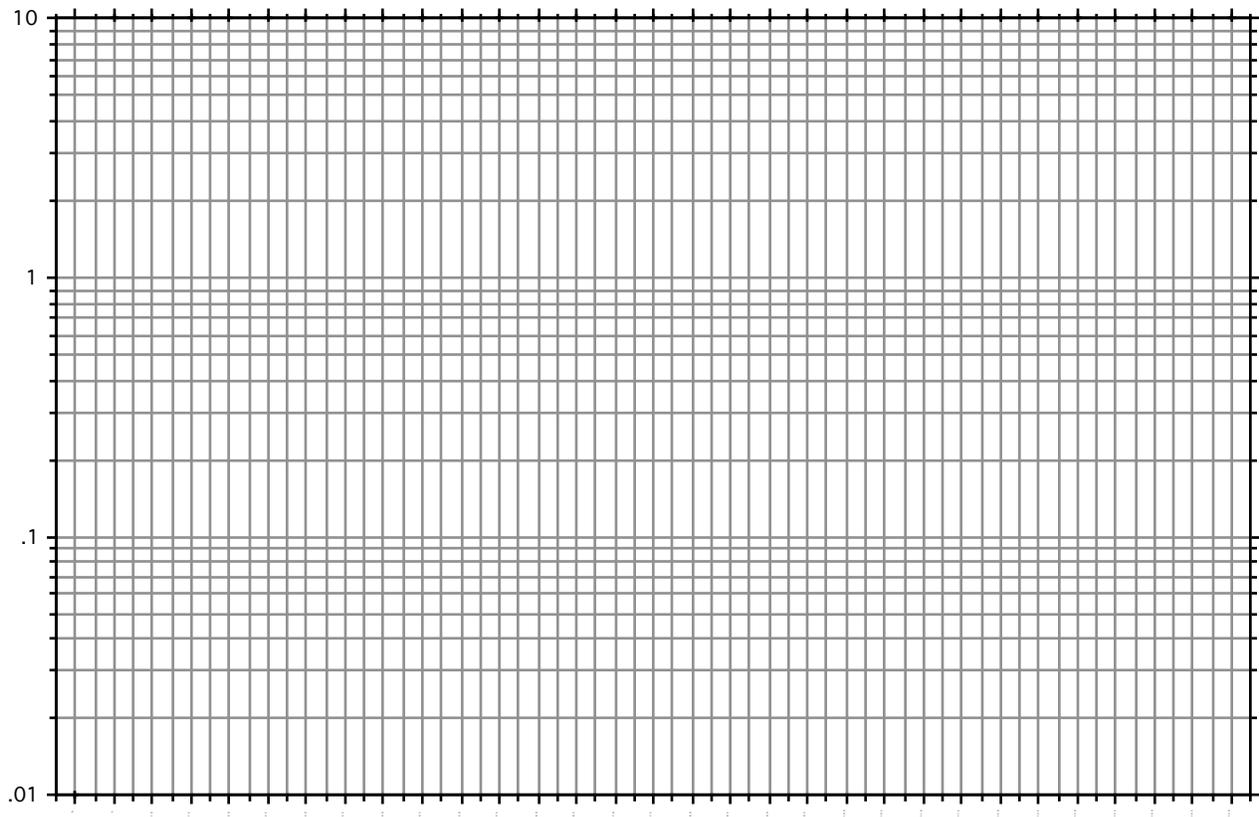
Use the upper graph on the next page and the DNA gel shown to the right to determine the molecular weight of the unknown band indicated with an arrow.

The lower graph is for you to use on your PCR DNA "fingerprint."

Footnotes:

1. (Adapted from: *PCR Technology* by Henry A. Erlich, W. H. Freeman and Co., NY, 1992, pp. 35-37.)
2. Budowle, B., Chakraborty, R., Giusti, A. M., Eisenberg, A. J., and Allen, R. C. (1991) Analysis of the VNTR locus DIS80 by the PCR followed by high-resolution PAGE. *American Journal of Human Genetics* 48: 137 – 144.





Lab Notes (Including Picture of *YOUR* DNA)

VNTR Online Exploration

1. Go to: www.ncbi.nlm.nih.gov
2. Make sure "All Databases" is selected in the pull-down menu at the top.
3. Enter DIS80 in the search box and click Search. (Note: enter D"one"S80)
4. List the databases that have matches to DIS80. (In other words, which databases show a number instead of "none" to the left of the name?) What kind of information do you expect to find in each (e.g., nucleotide sequences, protein sequences, journal articles, etc.)?
5. Click on the number in the box next to the Nucleotide sequence database (42 as of January 2010).
6. Look at the description of each entry. (Make sure to view all 42 entries.) How many species are described to have this VNTR? How many alleles are there in the database? Give the accession number of the largest (highest number of repeat units) and smallest VNTR alleles.
7. Click on any of the entries that specify the number of repeat units; scroll to the bottom of the page and copy the DNA sequence into a Word document.
8. Can you identify the repeat unit? (Hint: look at the FEATURES part of the database entry you opened; it identifies the repeat region.) How many base pairs (how big) is the repeat unit? Bold the last repeat unit (the 3'-most one) in your Word document. Are all repeat units identical? If they are not, will that impair your ability to estimate the number of repeat units you have?
9. On the sequence in your Word document, underline the sequences that represent the primers you are using for the PCR reaction. What size fragment do you expect to see on the gel, if this is the sequence of one of your two alleles?
10. Put square brackets around the nucleotides that are not primers and are not considered a part of the repeats (note, they may be at more than one place in the sequence). How many of them are there? Your text says that there are 142 extra base pairs (bp) that you need to subtract from the total size of the PCR product to get the size of the repeat region. What accounts for the other 57 bp?

Using Microscopes

Focused Reading

- p 70-72 “Microscopes are needed...” to “Cells are surrounded...”
- p 70 fig. 4.1 (The Scale of Life)
- p 71 fig. 4.3 (Looking at Cells)

Web Reading

- How to Use Your Compound Microscope Properly
www.bio.davidson.edu/Courses/Bio111/Bio111LabMan/LL/scopes.html
- Molecular Expressions Optical Microscopy Primer
(optional – a great place to learn about microscopes, try interactive animations, and see cool images)
<http://microscopy.fsu.edu/primer/index.html>

✓ Special Note

- You will be working with iodine in this lab and there is a risk of staining your clothing, so you may want to wear old clothes.
-

Goals for This Exercise

During this session, you will learn how to use a compound microscope that has the ability to view specimens in bright field, dark field, and phase-contrast illumination. You also will learn about a model research organism, *Chlamydomonas*. *Chlamydomonas* is a unicellular green alga that has two flagella and can reproduce asexually by mitosis, or sexually after undergoing gametogenesis.

Care and Use of the Compound Microscopes

A compound microscope is illustrated in Figure 1 and can magnify from 40 to 2000 times (40 – 2000X). Microscope quality, however, depends on resolving power in addition to magnification. Resolving power is the ability to distinguish between two points in the field of view. Thus, if you can magnify 1000-fold yet cannot resolve detail, then your microscope would be of little value. Even more important may be the abilities of the microscopist to learn the capabilities of her/his microscope and to gain proficiency in the use of the instrument.

Important things to know when using a microscope:

1. Always carry a microscope with both hands, one grasping the handhold in the back and one grasping the bottom.
2. Do not swing the microscope and do not bang it onto the bench top.
3. Never place the microscope near the edge of the bench and keep electrical cords out of the way.
4. All of our compound microscopes are parfocal, which means that the objects remain in focus as you change from one objective lens to another. Examine your material first using the lower power objective (i.e., 10X); then use a higher power objective (i.e., 20X or 40X). Because the objectives are parfocal, you need to use only the fine focus knob to fine-tune your image. Never use the coarse adjustment to focus downward. Replace and remove a slide only after the lowest power objective has been rotated into viewing position.
5. Never attempt to repair a microscope or force an adjustment knob. You may severely damage the instrument.

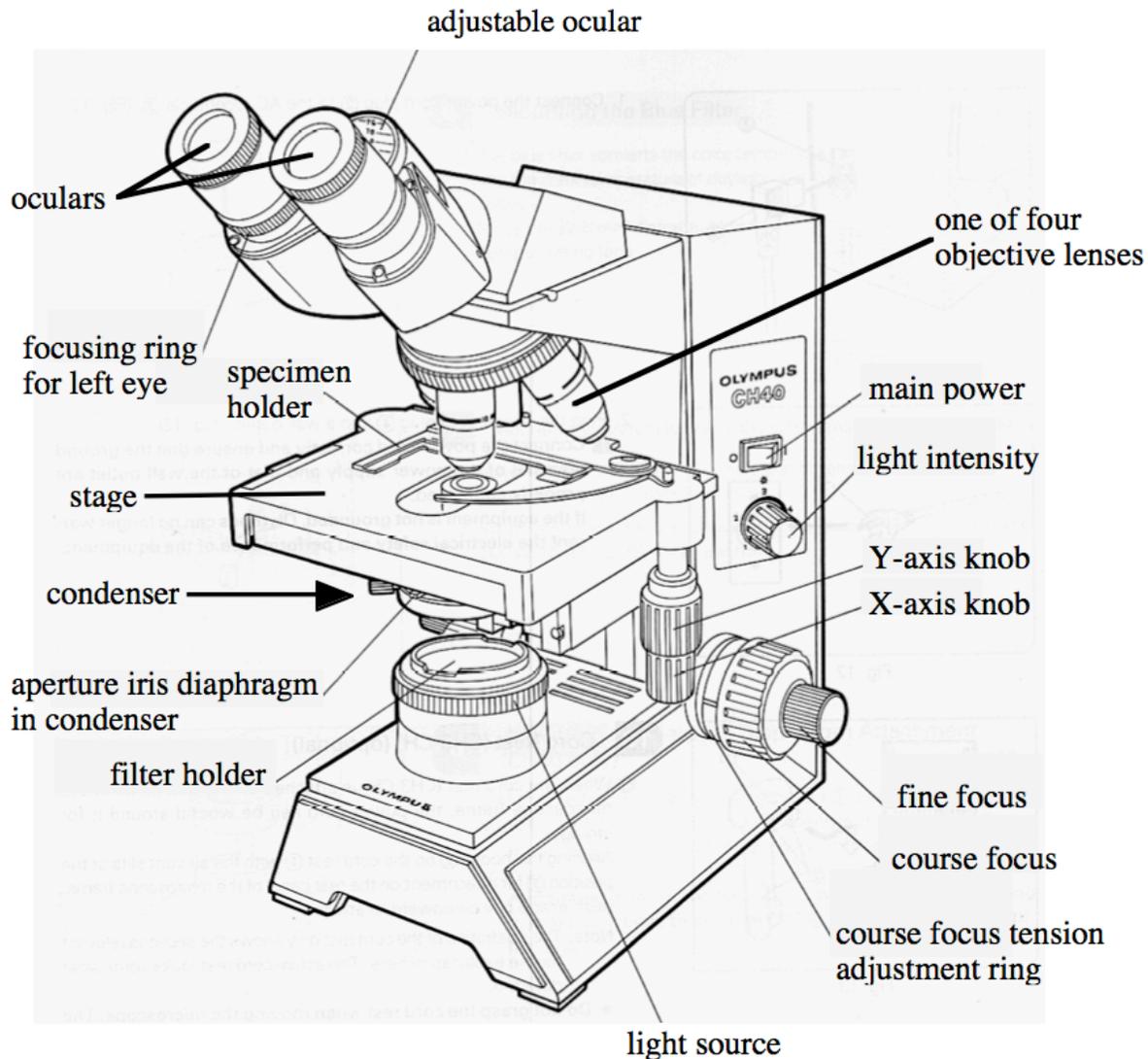
Parts of a Microscope:

Ocular: The piece you look through. Sometimes called an ocular lens or eyepiece, this unit is really a series of lenses. Our microscopes are binocular, having two oculars. Learn to use both eyes; focus your

eyes as if you were looking at an object about five to ten meters in front of you. You should adjust the width of the oculars to match the width of your eyes.

Objective lens: Sometimes called the objective; a set of self-contained lenses. The objective gathers light and directs it through the tube to the oculars. These microscopes have three phase contrast objectives (10X, 20X, and 40X with red lines on them) and one bright field objective (20X with no red line).

Nosepiece: The rotating turret to which objectives are mounted. There are preset positions for each objective, detected by slight pressure changes while turning the nosepiece and usually a clicking noise. You should not grab the objectives to turn the nosepiece – use the black ring instead.



Stage: The flat surface upon which slides are placed. On your microscopes, the stage moves up and down and the slide is manipulated by a geared device. A moveable stage is sometimes called a mechanical stage. The slide is moved left/right and front/back by two knobs projecting downward from the stage.

Condenser: A lens system under the stage that gathers light from the light source and focuses it on the specimen. There is a diaphragm in one part of the condenser that can be adjusted to allow the viewer

to see different parts of the cell when using bright field illumination. You should experiment with this control. These condensers also have phase rings but you should not have to make any adjustments to them.

Condenser Adjustment Control: Under the stage on the left side is a small knob that is used to adjust the height of the condenser. Usually, the condenser always will be all the way up.

Light Switch Control: The light switch and intensity controls are on the right side of the microscope base, about half way up the side. There is an on/off switch as well as a brightness control. Use only as much light as necessary to illuminate the specimen.

Light Source: On our microscope the light source is built into the base and is directly under the condenser.

Adjustment (Focus) Knobs: Both coarse (large) and fine (small, inner) adjustment knobs are found on both sides of our microscopes. Remember that the coarse adjustment is used only with the low-power objective. These knobs control a gear mechanism that raises and lowers the stage.

Types of Microscopy

There are three different ways that we can view specimens with these microscopes. The type of illumination with which people are most familiar is called Bright Field. Think of the light source as producing a solid tube of light that travels up to and through the condenser. When you view specimens with all of this light, you are using bright field illumination.

Dark Field: Dark field illumination seems like an oxymoron, but in this case it describes an unusual way of viewing specimens in some compound microscopes. The light that passes directly through the condenser does not enter the objective lens. Only light that has been scattered or reflected by the specimen enters the objective. As a result, you wind up seeing bright objects on a dark background.

Phase-Contrast: Phase-contrast microscopy allows us to see otherwise transparent organelles and structures. We will make extensive use of this for viewing flagella. In a phase-contrast scope, the light hits the specimen and some of the light continues in a direct path. Other portions of the light pass through membranes that redirect the light. This redirected light is slowed down by $1/4$ a wavelength (a phase shift of $1/4$) by passing through a special filter. This special filter is shaped like a doughnut and is called a phase ring. The redirected and out of phase light eventually reaches your eyes but not at the same time as the unaltered light that passed straight through. The end result is that you can see transparent structures because they altered the pathway of light as it went through the structures. This phase shift allows us to view subcellular structures within living cells.

Viewing A Specimen:

Everyone will follow the procedure for viewing a specimen as a group. Your instructor will demonstrate how to make a wet mount (see below) and show you the differences between bright-field, dark-field, and phase-contrast microscopy using a microscope that is equipped with a camera and projector. In this session, we will be looking primarily at wet mounts. A “**wet mount**” is a specimen mounted in an aqueous solution but you do not expect to keep the slide for very long. If your preparation begins to dry out while you are working with it, make a new one.

Every time you work with a microscope:

- 1) Position the scope so it is directly in front of you and your chair is adjusted so that you do not have to strain to view a specimen.
- 2) Make sure the light intensity control is turned all the way off before turning on the microscope.

- 3) Make sure the 10X objective is in place over the specimen.
- 4) If you are making a wet mount, clean the microscope slide by fogging it with your breath and then wiping it with a Kimwipe.

Bright Field

1. Switch on the light source and then dial the adjustment knob to about 4.0. Start with the oculars set so they are at equal heights.
2. Turn the condenser so that the “ O” is facing you. This position is the bright field slot on the condenser.
3. Position the low-power (10X) objective over the specimen and, looking from the side, raise the stage as high as possible. Notice how close to the objective the stage is.
4. Use the coarse adjustment to lower the stage away from the glass slide while looking through the oculars until the specimen comes into focus. Adjust the focus to its sharpest with the fine adjustment knob.
5. Now it is time to make sure both oculars are focused. Use the fine focus while looking through the right ocular and close your left eye. Pick one object to focus on. Then close your right eye and focus the left ocular by turning it up and down with the focusing ring for the left eye but do **not** touch the fine focus control during this time.
6. Readjust the light intensity to reduce glare and center the specimen in the field of view by moving the stage.
7. Use the knob on the left side of the condenser to move the condenser up as high as possible. You may also want to adjust the condenser’s diaphragm to maximize the resolution but minimize the “graininess” of the image.
8. Place the 20X objective (no red line) over the specimen and sharpen the focus with the fine adjustment knob (only!) as necessary. Readjust light.
9. Adjust the condenser’s diaphragm to maximize the resolution of the structure you are trying to see. The actual setting will depend on what you are trying to see. Small translucent objects will be seen more easily with the diaphragm closed substantially while large pigmented structures are easier to see with the diaphragm wide open.
10. Repeat steps 8 and 9 but use the 40X objective instead of the 20X.

Dark Field

1. Turn the condenser ring clockwise so that the “D” is facing you. This position will permit you to see objects in dark field illumination. You also must adjust the condenser so that it is as high as it can go - use the knob on the left side of the scope. You can use dark field illumination with any of the four objective lenses.
2. What structures can you see now that you could not see in bright field?
3. What is difficult to see in dark field that was easy to see in bright field?

Phase-Contrast

1. When you use phase-contrast, you must match the objective lens with the phase ring in the condenser. Therefore, you must follow this table:

Objective Lens	Phase Ring
10X, 20X (red lines)	10
40X (red line)	40

A Reminder: only the objectives with red lines can be used for phase. The 20X objective that does not have a red line on it is not equipped with phase rings.

2. Select the appropriate objective lens and phase ring pair. You might need to increase the amount of light because images do not appear as bright in phase. When you have done this, you should adjust the condenser vertically with the knob on the left side of the condenser. When these adjustments are made, using a phase-contrast microscope is similar to using a bright field scope.
3. What structures can you see now that you could not see in bright field? Dark field?
4. What is difficult to see in phase-contrast that was easier to see in bright field?
5. Do you see the same colors in phase that you saw in bright field? Dark field?

Lab Notes

The Dynamics of *Chlamydomonas* Flagellar Regeneration

Adapted from Vanderwalle & Heyes, *J Bio. Ed.*, (1993) 27: 125-29.

Focused Reading

- p 602 “Chlorophytes, charophytes, and...” to “Excavates”
 - p 88-89 “Microtubules...” to “Centrioles. . .”
 - p 87 fig. 4.20 (The Cytoskeleton)
-

Overview:

Over the next several weeks we will become comfortable with a fundamental tool in biology - the compound microscope and the use of digital cameras and digital imaging software to measure very small structures. We will conduct a series of experiments on a unicellular green alga, *Chlamydomonas reinhardtii*. *Chlamydomonas* is a biflagellated green plant that reproduces asexually (by mitosis) and sexually (via meiosis, mating, and zygote formation).

Background Information on *Chlamydomonas*

There are several reasons why *Chlamydomonas* is such a useful model organism. It is a haploid organism, which means there is only one copy of each chromosome. Therefore, the genotype is always expressed in the phenotype (unlike diploids that may have a recessive mutation that is not revealed in the phenotype). It has a generation time of two weeks (from mating of one generation to when the next generation can mate). Finally, there are hundreds of mutant strains (stored at Duke University) that have been generated over the years and can be used for research. For example: *ac-17* cannot fix carbon during photosynthesis, *arg-7* requires the amino acid arginine to be added to the medium because it cannot synthesize its own; *act-1* is resistant to the translational inhibiting drug cycloheximide; and *pf14* has straight and paralyzed flagella so it cannot swim.

Each person should:

A) Place 25 μL of *Chlamydomonas* on a clean (use a Kimwipe) glass microscope slide and gently cover with a cover slip. (If you press down on the cover slip you will crush the cells.) Place the slide on the **stage** of the microscope and use the 10X **objective lens** to observe the cells swimming around. Start with bright field, then try dark field and phase-contrast.

1. Can you see the flagella?
2. Which form of illumination allows you to see them the best?
3. What is the total magnification you are using with a 10X objective lens and the 10X oculars?

B) Increase the magnification by using the 20X objective lens. Again, view the cells in bright field, dark field, and phase-contrast. Remember to use lenses with the red ring for phase and the 20X without the red ring for bright-field.

1. What is your total magnification now?
2. Can you see the flagella? Which form of microscopy is the best for seeing flagella?
3. Can you see any other organelles in these cells? (Try all three forms of illumination.)
4. How can you see flagella better without staining them?
5. What colors do you see in *Chlamydomonas* cells?
6. What structures are responsible for the colors?
7. How do you calculate the total magnification you are using on a microscope?

C) On a new slide (or on your original slide if there's space), place 12.5 μL of cells into 12.5 μL of Lugol's fixative. Lugol's fixative is a dye that stains the sugars that are covalently bound to the proteins (sugar-coated proteins are called glycoproteins) on the surface of the flagella. Cover with a cover slip. Examine this preparation of stained cells under the microscope. View the cells at all three magnifications

with each form of illumination.

What structure(s) can you see better with fixed cells than with live, unstained cells? Give two possible reasons here:

Goals for this Session:

During this session, you will collect data on the regeneration of flagella on *Chlamydomonas*. These cells will be deflagellated, and you will immediately measure regrowing flagella over a 90 minute time period. You will learn how to use software called Visicapture (to capture images of the cells) and ImageJ (to measure flagella within your images).

This set of experiments requires teamwork and efficiency. Assign tasks ahead of time (see next page) but make observations for yourself because some test questions may cover lab work. Be organized and ready to start immediately when the cells are delivered to you, because cells will already be starting to regenerate their flagella.

You will measure the length of the flagella as a function of time. At each time point, cells will be "fixed" with Lugol's fixative, an iodine-based substance that kills, stains, and immobilizes cells. You will capture images of fixed cells and then measure the flagella on the image files. For each time point, you should measure the length of 20 flagella (total of 20 flagella on 20 cells).

Chlamydomonas untreated with acid (retaining their original flagella, to allow you to determine normal flagellar length. You must fix cells with Lugol's to immobilize and allow for visualization.)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	

Deflagellated Chlamydomonas (cells must stay alive until the specified time point, at which you treat with Lugol's to immobilize and allow for visualization.)

time (min.)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
0																					
15																					
30																					
45																					
60																					
75																					
90																					

Detailed Protocol:

- A) About 5.0×10^{12} *Chlamydomonas* cells will be deflagellated and delivered to you.
- B) In a four person group:
- **Person #1** should fill microfuge tubes with 50 μ L Lugol's fixative, labeling with time points 0, 15, 30, etc.. Then, for most of the time (with help from person #2) measure flagellar lengths on ImageJ, once images are received from persons #3 and #4. Record all the data and make sure that no time points are missed.
 - **Person #2** should, at time 0 and every 15 minutes thereafter, add 50 μ L of deflagellated *Chlamydomonas* to the appropriate microfuge tubes already containing Lugol's fixative. Move the container of live cells back to the light shelf each time. Make a slide for each fixed sample; to avoid dried out slides, do not make the slides until persons #3 and #4 are ready to measure them. In your extra time, help person #1 measure flagellar lengths and record data.
 - **Persons #3 & 4** should capture images of fixed cells on the computer using VisiCapture. For each slide, capture an image for each of the first 20 cells that you see whose full periphery is visible (i.e. not clumped). If flagella are visible, make sure that the longest flagellum for a given cell is in focus. Transmit the image files via flash drive or Bio111 shared folder (see instructions on the next page) to persons #1 and #2 so that they can make measurements.
- C) In a three person group:
- **Person #1** should fill microfuge tubes with 50 μ L Lugol's fixative, labeling with time points 0, 15, 30, etc.. Then, for most of the time, measure flagellar lengths on ImageJ once images are received from person #3. Record all the data and make sure that no time points are missed.
 - **Person #2** should, at time 0 and every 15 minutes thereafter, add 50 μ L of deflagellated *Chlamydomonas* to the appropriate microfuge tubes already containing Lugol's fixative. Move the container of live cells back to the light shelf each time. Make a slide for each fixed sample; to avoid dried out slides, do not make the slides until person #3 is ready to measure them. In your extra time, either help person #1 with measurements or help person #3 with image capturing, depending on who is running behind.
 - **Person #3** should capture images of fixed cells on the computer using VisiCapture. For each slide, capture an image for each of the first 20 cells that you see whose full periphery is visible (i.e. not clumped). If flagella are visible, make sure that the longest flagellum for a given cell is in focus. Transmit the image files via flash drive or shared folder (see instructions on the next page) to person #1 so that he/she can make measurements.
- D) Enter **RAW DATA** (not averages) in your lab book and/or Excel file initially. A good scientist keeps all data in her/his notebooks, not just the averaged results. It's always better to take too many lab notes than too few. In the real world, scientists are required to keep on hand (for future scrutiny) all raw data that underlie any averages published. Otherwise, the scientists could be accused of fraud.
- E) When you are done, clean your area, turn off the microscopes, throw away any trash, and return equipment to where you found it. It is good lab etiquette to clean up and return things properly when many people share equipment and space.

Instructions for adding a BIO111 shared folder alias to your desktop

Connect to the Server

1. Click on the desktop or on the **Finder** icon on the left end of the dock (the series of icons depicting computer applications)
2. On the Finder menu, select the **Go > Connect to Server..** commands
3. In the Server Address field, type `smb://louise/public/biology/111 Shared Files` and then click Connect. If prompted, enter Davidson username and password.
4. When the 111 Shared Files folder is opened, click on the appropriate lab for your group. For example, click **Monday PM** if you are in Monday afternoon lab.

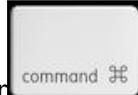
Create the alias

5. When the appropriate folder is open, right click on the appropriate lab for your group. For example if your group is nearest the main door to the lab, click **Near Door**. On the resulting pop up menu, left click on the Make Alias command.
6. A new alias will appear in the server window.

Move the alias to the desktop.

7. Point to the new alias and click-and-hold with the left mouse button.
8. Drag the mouse pointer to your desktop but DON'T release the mouse button yet. You will notice a green + sign.



9. With your other hand, hold down the command key (looks like  or ) and the green + will disappear.
10. Release the left mouse button, and the alias will be moved to your desktop. (Holding down the command key changes the drag-and-drop from a *copy* to a *move*.)

General Information about *Chlamydomonas* Flagella

To remove *Chlamydomonas* flagella we use the pH shock method. As it so happens, *Chlamydomonas* is very sensitive to changes in its environment. If we manipulate the pH of the growth medium by adding acetic acid until the pH decreases from about 7.2 to 4.5, the cells shed their flagella. Scientists have investigated why this event happens. We know that cells will not shed their flagella if there is no calcium in the growth medium. (Calcium can be removed from any solution by adding in a compound commonly referred to as EGTA. EGTA has a very high affinity for calcium and acts as a chelator, like a molecular sponge, to absorb ionically all the calcium, which means *Chlamydomonas* cells can not use or sense any calcium ions if EGTA is present.) Other researchers have shown that if one can experimentally elevate the level of calcium in the cytoplasm of *Chlamydomonas* cells, they shed their flagella. Hypothesize what is going on when *Chlamydomonas* cells shed their flagella when pH shocked in the presence of calcium. Can you devise an experiment to test your hypothesis?

By now you may be wondering why anyone would care about the dynamics of pond scum flagellar regeneration. *Chlamydomonas* is a model organism for studying flagella and much of our understanding of cilia and flagella is due in large part to our understanding of *Chlamydomonas* flagella. As you read in your textbook, the flagella are comprised of many (~200) different proteins, but the predominant protein is **tubulin**. Each *Chlamydomonas* flagellum is built upon the 9+2 structure of microtubules (see the figure below). The outer nine microtubules are “doublets”, consisting of a complete circle of 13 protofilaments fused with a partial circle of 10 protofilaments. The two centrally located microtubules consist of a “singlet” of 13 protofilaments. Therefore, each flagellum contains $(9 \times (13 + 10)) + (2 \times 13)$ protofilaments. Each protofilament is composed of dimers of α **tubulin** and β **tubulin**. Each monomer of a globular tubulin molecule has a 4 nm ($4 \cdot 10^{-9}$ meters) diameter and is comprised of 450

amino acids. Therefore, the α/β dimer has a diameter of 8 nm and is made of 900 amino acids. Although there are multiple genes for tubulin, for the sake of simplicity let's assume a single gene for each form of tubulin (one for α and one for β). These prototypical genes are about 1800 bases long. Remember that *Chlamydomonas* is haploid so one allele = one gene = one locus.

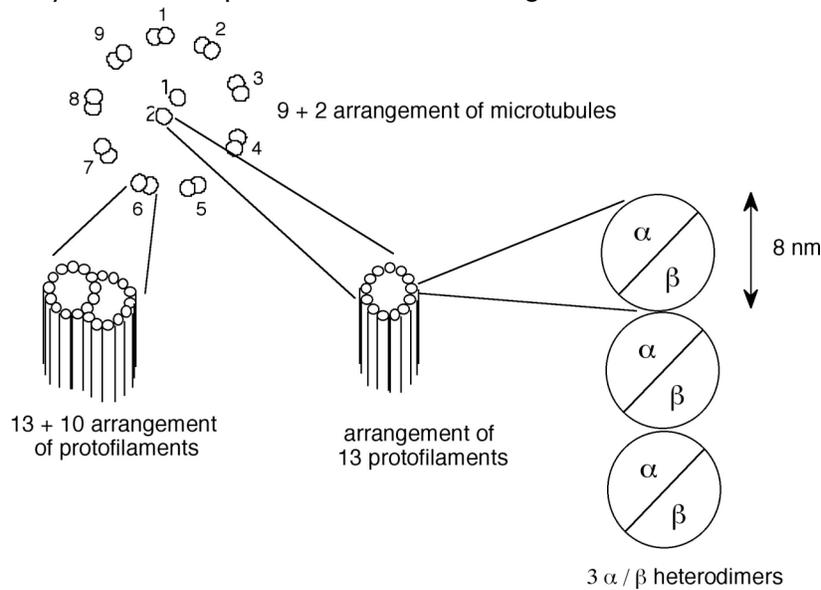


Diagram of tubulin structure in flagella. Compare with Figs 4.20 (page 87) & 4.22 (page 89) in your text.

Study questions:

- 1) Based on your results for flagella regeneration, calculate how many amino acids are being polymerized per minute into the tubulin component of the regenerating flagella.
- 2) Assume that all of the mRNA needed for this process is being synthesized **de novo**, from scratch. How many mRNA bases (assume no introns) must be transcribed per minute if every mRNA is translated only once? What if each mRNA is translated 100 times?
- 3) If RNA polymerase can travel no faster than 2500 bases per minute, is it possible for all of the RNA to be transcribed *de novo*? Explain your answer.

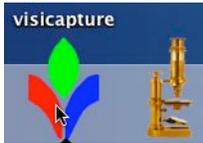
Note: You will not be able to answer these questions with your experimentally determined rate because that rate will not be determined for another two weeks. However, make the assumption that flagella regenerate at 0.17 μm per minute. When you have determined the rate in your experiment, you should try the calculations again.

NEWS ITEM: How can studying the flagella of pond scum be useful for understanding human health? A team of researchers found that mutant *Chlamydomonas* lacking the *IFT88* gene appear normal except for the striking absence of flagella. *IFT88*, also called *polaris*, encodes for a protein involved in intraflagellar transport, the process of moving cargo up and down the microtubule tracks within flagella and cilia. Humans and mice have a gene called *Tg737* that is orthologous to the *Chlamydomonas IFT88* gene. Knockout mice, engineered to have defects in both copies of *Tg737* die shortly after birth from polycystic kidney disease (PKD). Humans also die from PKD where kidneys enlarge due to overproliferation of kidney epithelial cells. Scientists now think that malfunctioning cilia on the side of the epithelial cells urine flows past bend when urine flows and this bending sends a signal to the epithelial cells cell that inhibits cyst formation. In patients with PCK, their kidney epithelial cells have abnormal cilia and their kidneys are filled with cysts that ultimately compromise the kidney's ability to function. [*HHMI Bulletin* 18(2): 33]

Measuring *Chlamydomonas* flagellar lengths digitally

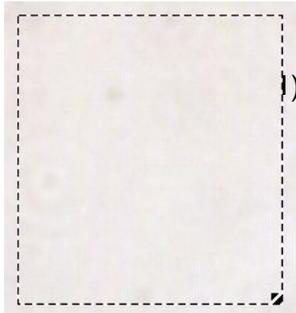
Wake the computer from sleep mode by tapping the space bar a couple times. If the computer is off, turn on the computer by pressing the power button.

Visicapture Instructions

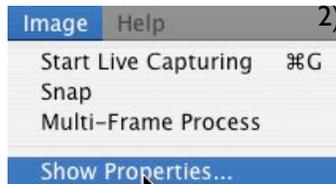


Find the visicapture icon in the tool bar at the bottom of the viewing area. Click once on this icon to launch the program. Visicapture is the tool that allows you to capture images from the microscope.

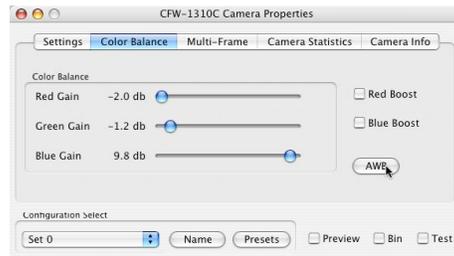
Put the stage micrometer on the stage and turn on the microscope. Use bright field optics to view the micrometer with the 10X objective. When the microscope is on and you have a slide in place with the 10X objective, pull out the lever (sometimes called a stop) that sends light up to the digital camera. Find the object and focus the microscope. Now hold down the Apple (⌘) key and type a “G” (abbreviated as “⌘G”). When you simultaneously hit these two keys you activate the camera if it is off or take a picture when the camera is on. You can use ⌘G as a toggle switch between live and captured image modes.



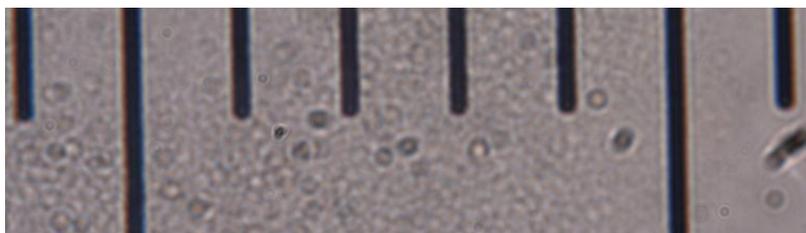
Because these cameras can capture color images, you want to set the white balance properly. To set the white balance, move the microscope stage until you reach a clear part of the slide (with no *Chlamydomonas* or dirt) have a white area showing. Turn up the light to the brightness you want. Now click and drag to form a box in the white area, as shown at left.



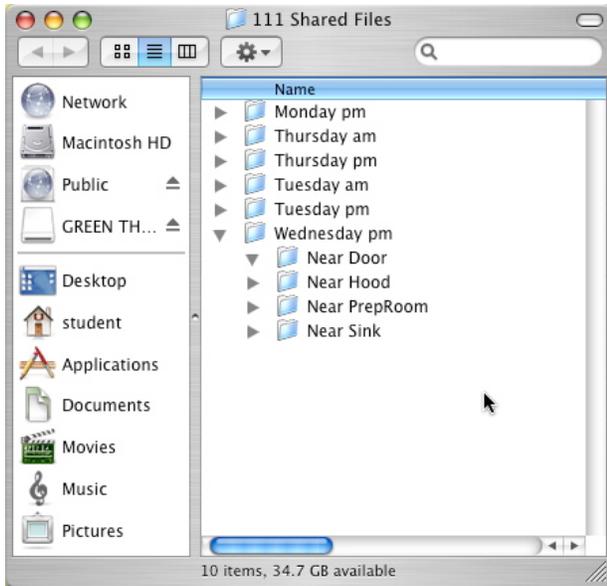
Next, select “Show Properties...” from the Image AWB button if you have already selected an area to “white.” This action will take a few seconds in order camera. When white balancing is complete, you adjust the white balance any more. (If colors look can reset the AWB as needed.)



Now change the objective lens to the 40X or 60X power one your microscope is equipped with) and fine focus knob. When the stage micrometer is in place, you will see something similar to the image below. Notice that you can distinguish the long and short lines in this image. Make sure you can see the difference in the long and short lines in your image. Move the stage if necessary.

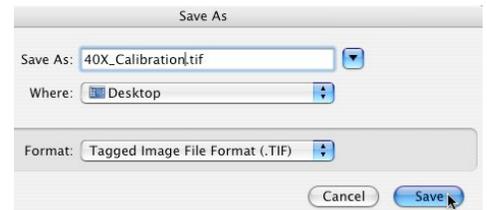


When you have captured an image you want to use, you need to save it in a way that your lab mates can access the file easily. We have created a community of folders that each student in Bio111 can access. Go to the desktop and click on the icon called “111 Shared Files alias.” This action will bring up a login window. Use your normal username and password to access the community of folders. You will see each lab section has its own folder, organized by day of the week and time of day (AM or PM). Inside each lab time are four folders. The folders are named according to where you are sitting. You should only work with files inside your group’s folders. When you save the captured images, you want to save them directly to your lab’s community folder.



Use your normal username and password to access the community of folders. You will see each lab section has its own folder, organized by day of the week and time of day (AM or PM). Inside each lab time are four folders. The folders are named according to where you are sitting. You should only work with files inside your group’s folders. When you save the captured images, you want to save them directly to your lab’s community folder.

In the File menu, choose “Save As.” You will see a dialog box similar to the one shown here. Save the image as a tiff file, which is the most complete form of the image. Do not use punctuation marks, underscores, or spaces in your file names. If you wanted to post the image on a web site or use in a PowerPoint presentation, save as a jpeg file (.jpg). Save the image to your lab’s community folder directly so your lab mates who will measure the flagella can access the files. You can move it another folder later, if you want.



One note about the community folders. The folders may not refresh instantly, so if your lab mates have added a new image to your folder but you cannot see the new image listed on your computer, just right click on the folder or resize the window just a bit. This adjustment will cause the folder to refresh and show all files inside the folder.

Image J Instructions

It is time to calibrate the computer so it can apply real, physical dimensions to the number of pixels on the computer monitor. To measure images, you will launch ImageJ. You can either click on the icon in the dock one time or drag the tiff file you just created onto the icon. If ImageJ is already launched, then you can still “drag and drop” the file onto the ImageJ icon in the dock.



Now it is time to draw a line on your calibration image. To do this, select the straight line tool from the ImageJ menu window:

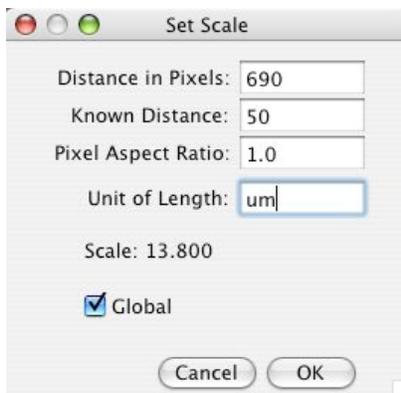


If you cannot find this menu, just hit the return key and it will reappear.

Draw a straight line that is perpendicular to the micrometer markings from the middle of one long bar to the middle of the next long bar:



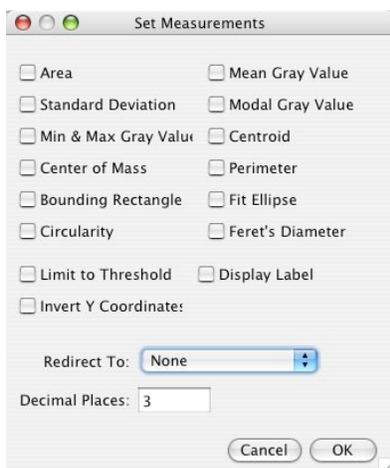
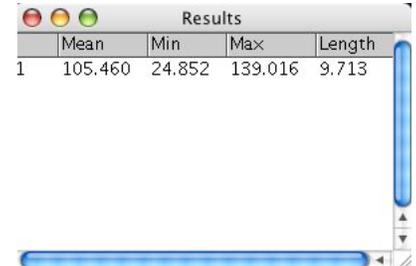
Notice that the yellow calibration line barely touches the tops of the four short bars. This placement helps ensure that the calibration line is fairly straight, even though the image is at an angle. Also notice that the yellow calibration line goes from the *middle* of the long bar on the left to the *middle* of the long bar on the right. (Think about why it would not be accurate to draw a calibration line from the middle of one bar to the left or right side of the other bar.)



Tell ImageJ that the line you have just drawn is exactly 50 μm (micrometers or microns; often abbreviated as microns) long. To do this, select “Set Scale...” from the Analyze menu. You will see a dialog box similar to the one at left.

This action converts pixels on the monitor to microns. When you have calibrated ImageJ for this session at a given magnification, you do not need to recalibrate. (Make sure to check the “global” box so that your calibration will apply to all subsequent images!)

Test your calibration by drawing a line from the middle of one short bar to the middle of the next short bar. Then type “⌘M” or select “Measure” from the Analyze menu. A new window should appear that shows you the length of the line you just drew.



However, notice there are three columns that are irrelevant (Mean, Min, & Max). To get rid of these three columns, while the Results are still displayed, choose “Set Measurements...” from the Edit menu. Deselect all the options as shown:

Close the Results window by clicking on the red dot in the top left corner. Then type “⌘M” again. If you do not see the Results window, choose “Results” under the Window menu. Your data should just appear as length in microns, as you have calibrated ImageJ.



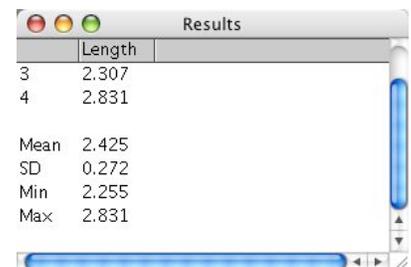
Now you are ready to measure flagella. Rotate the objective to 10X, remove the stage micrometer slide and store it safely. Put a wet mount of *Chlamydomonas* cells on your stage and find the cells in 10X before increasing magnification to 40X or 60X. When you see a flagellum you want to measure, type “⌘G” to capture the image and then ImageJ to measure the flagellum. However, because flagella are not straight, **you will need to select the Free Hand tool** from the ImageJ options:



Trace the flagellum from one end to the other, then type “⌘M”. **CAUTION:** Sometimes Java programs are unstable and may crash (true on all platforms). Therefore, we recommend that someone record the lengths in a notebook (or Excel file that you save frequently) as they are measured so you won’t have to repeat any measurements if ImageJ crashes.

You will need to capture at least 20 images of flagella (only count one flagellum per cell and measure only the longer of the two). Each time you capture the image in Visicapture, you will need to measure it in ImageJ. Repeat this process until all your measurements are collected. Luckily, ImageJ can perform some calculations for you. When you have all your measurements collected, choose “Summarize” from the Edit menu:

You will find the mean, standard deviation as well as the maximum and minimum lengths you measured.



When you are ready to measure another round of flagella, you can choose “Clear Results” from the Edit menu to clean out the results table.

Preparing for Next Week's Experiment:

At this time, each laboratory group will formulate an hypothesis and design an experiment to test that hypothesis. To formulate an hypothesis, you might just wonder aloud, "What if we....?" For instance, what if we prevent the cells from transcribing any new RNA? What if we prevent the cells from translating any new proteins? What if these plant cells are put in the dark? What would happen in the presence of added ATP? caffeine? glucose? amino acids? EGTA? When your lab team has agreed upon a question that interests you, you then should use your knowledge of molecular and cellular biology to formulate a probable answer to your question. For example, if we block translation with the drug cycloheximide, then you might hypothesize that flagella will not regenerate at all. This hypothesis is a good one because it can be tested. A bad hypothesis might be, "*Chlamydomonas* cells do not like to have their flagella removed and are happier when the flagella are regenerate." This hypothesis is practically impossible to test. Formulate your hypothesis so that you can design an experiment to test it. To help you formulate a testable hypothesis consider the reagents that we can make available to you in lab next week in the table below. Your team must sign up for an option before leaving lab today.

<u>FUNCTION</u>	<u>DRUG/ REAGENT(S)</u>	<u>STOCK Concentration</u>	<u>FINAL/WORKING Concentration</u>
translation inhibitor	Cycloheximide	2 mg/mL in ethanol	10 µg/mL
transcription inhibitor	Actinomycin D	5 mg/mL in ethanol	50 µg/mL
phosphodiesterase (PDE) inhibitor	Caffeine	66 mM in water	6.6 mM
elevated and/or reduced Ca ⁺⁺	Calcium EGTA (ion chelator)	100 mM in water 100 mM in water	1 mM 1 mM
disrupts IP ₃ production	lithium chloride	1 M in water	20 mM
prevent photosynthesis	darkness (use foil)	--	--
alter metabolism	temperature variation (requires different settings on the spectrophotometer)	--	--

Remember to include good controls in your experimental design. A good control is an experimental condition that will give you a standard or predictable result against which you can compare the results of the condition you are actually interested in studying. For example, if you wanted to see the effects of disco music on the regeneration of flagella, you would design an experiment that had two experimental conditions:

- 1) Cells regenerating their flagella in the presence of disco music
- 2) Control cells regenerating their flagella in the presence of non-disco music at the same volume, beat, etc. (*Notice the difference between the control and experimental is only one variable - the presence or absence of disco music.*)

Your hypothesis probably might be that disco will prevent flagella from growing. This hypothesis is a testable hypothesis because you can measure the length of the flagella in the two situations (with and without disco). Let's look at a hypothetical set of results. When the cells are subjected to disco, the flagella did not regenerate. Flagella in the presence of normal music did not grow either. How should these results be interpreted? Did disco prevent the regeneration? What do the results of your control condition tell you? Why must every experiment have good controls?

Each group should decide upon a question to ask next week, formulate an hypothesis, design the experiment, and discuss the protocol with your instructor. This meeting will give us a chance to answer any major questions you might have and order the reagents you will need. Next week's lab will be very busy – so careful preparation this week is particularly critical.

Your protocol must be written today and it should be specific enough so that next week, you can come into the lab and begin immediately by following your own directions. Your instructor will give you feedback on your protocol. Check to see how your instructor will review your protocol – you may need to submit a written protocol.

You also should have the following in your lab notebook:

- 1) The data from today's experiment.
- 2) Answer all the questions asked of you in the protocol above (with the exception of the optional question regarding the moon).
- 3) You should note any observations you think note worthy - use your best judgment.

📖 **NEWS ITEM:** In lab we study the flagella of the volvocine green algae *Chlamydomonas reinhardtii*. The multicellular volvocine *Volvox* is like 1,000- 5,000 *Chlamydomonas* all hooked up in a sphere with their flagella sticking on the outside. This arrangement is rudimentary multicellularity and introduces new hurdles including the fact that the organism (without a circulatory system) needs to figure out how to get nutrients to all of those cells. Diffusion isn't fast enough. A study published in 2006 showed that *Volvox* have evolved a mechanism that coordinates flagella beating. In this manner not only can the organism move within its environment but it can also move its environment (*i.e.*, food) across more of its surface area and facilitate feeding. [PNAS 103:8315]

Lab Notes

Parameters That Affect *Chlamydomonas* Flagellar Regeneration: Gene Expression and Protein Localization

📖 Review Reading

- p 131-135 “How are enzymes.....” to end of chapter
- p 318-320 “How is Eukaryotic...” to “Regulators, Enhancers, and Silencers”
- p 326 “How is Gene Expression...” to end of p 326

✓ Special Note

- You will be working with iodine so you may want to wear old clothes.
- This is a particularly busy day in lab and requires exceptionally organized and efficient teamwork.
- You must have your solutions mixed and be ready to go shortly after the start the lab period.

You will perform the experiments you have designed and record your results in the tables in the following page. If any modifications were made to your written protocols, you should talk to your instructor before you begin your experiment. Today will be a busy session of performing your experiment and collecting data. Do not worry about analyzing your data today. You will spend the next lab session analyzing data.

Your protocol:

Note – You may not need to use all the cells in these data tables – check with your instructor to determine how many flagella you should measure and/or for how long you should conduct your experiment.

Untreated *Chlamydomonas* (a.k.a. *Chlamydomonas* that were never deflagellated - so you can determine if regeneration was complete)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Control Data

time (min.)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
0																				
15																				
30																				
45																				
60																				
75																				
90																				

Experimental Data

time (min.)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
0																				
15																				
30																				
45																				
60																				
75																				
90																				

Experimental Data

time (min.)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
0																				
15																				
30																				
45																				
60																				
75																				
90																				

Before you leave lab today:

Start thinking about the next set of experiments we will be conducting. Read over the description of the Ames test on subsequent pages of this lab manual. Using a modified version of the Ames test, we will be testing the mutagenicity of various compounds. By next week's laboratory session, bring something (Your Favorite Potential Mutagen - YFPM) you have been curious about or have heard "might cause cancer": tobacco, hair dyes, smoked meats, fried bologna, charcoal, pesticides, insecticides, rat poison, UV light, caffeine, ethidium bromide, mustard, green M&Ms, tailpipe residue, etc. - be creative! (Remember most foods have been tested for safety.) We will prepare these materials for you so you can test their mutagenicity.

Web Reading

- Cancer Biology Videos: Gene Mutation
http://www.cancerquest.org/videoseries1_english.html

When you read about the Ames test, be sure to think about controls (positive and negative) and how you can determine the dose response of your agent. Formulate your protocol as before: state a hypothesis, determine how many conditions you are going to test (and thus how many Petri plates you will use), and be detailed enough in your outline of procedures so that you know what you are doing.

📖 **NEWS ITEM:** Since 1990, cancer death rates in the US have gone down for males in every category of cancer. The same is true for females (including breast cancer), with the exception of lung/bronchus cancer. Death from lung/bronchus cancer in women has multiplied by eight since 1960. The American Cancer Society estimates that in 2006, 26% of all cancer deaths in women will be caused by lung/bronchus cancer while breast cancer will result in only 15% of cancer deaths in women. Smoking accounts for 30% of all cancer deaths, and 87% of all lung/bronchus cancer deaths. Smoking is associated 15 different kinds of cancer. Contrary to popular misconception, "light" or low tar" cigarettes offer no protection against cancer. Can you think of a behavioral change in US culture that may explain why lung/bronchus cancer in women is the only type of cancer to not decrease in frequency? [American Cancer Society Cancer Facts, 2006]

Lab Notes

A Beginner's Guide to Descriptive Statistics

Statistics

When you have collected a large set of data as you did last week for flagellar regeneration, you need to use some descriptive statistics to convey the important aspects of the distribution of your data. Two features of the distribution that you should describe include:

- 1) The central tendency
- 2) The spread of your data

Mean (a.k.a. average)

A simple measure of the central tendency of the data is the mean (or average):

$$\text{Mean} = \text{sum of all the data values} \div \text{sample size}$$

For example, with the data set (1, 1, 1, 5), $n = 4$; the mean is $8 \div 4 = 2$

Range

The simplest measure of the spread of your data is the range, which tells you the distance between your most extreme data values, but does not address the issue of how frequent these extreme values are. The formula for calculating the range is:

$$\text{Range} = \text{value of maximum data point} - \text{value of minimum data point}$$

For example, with the data set (1, 1, 1, 5):
the range is $5 - 1 = 4$.

Variance (Var)

The variance of your data is a measure of spread that will take into account both the deviations of your data (away from the mean) and how frequently these deviations occur. The formula for calculating variance is:

$$\text{Variance} = \text{the sum of (each data point - the mean)}^2 \div \text{sample size}$$

For example, with the data set (1, 1, 1, 5):
the mean = 2
the variance = $((1-2)^2 + (1-2)^2 + (1-2)^2 + (5-2)^2) / 4 = 3$

Standard Deviation (SD)

The standard deviation of your data is the square root of the variance, and therefore it reflects both the deviation from the mean and the frequency of this deviation. Standard deviation often is used instead of the variance because the scale of the variance tends to be larger than the scale of the raw data, while the standard deviation is on the same scale as most of the data. The formula for standard deviation is:

$$\text{Standard deviation} = \sqrt{\text{variance}}$$

For example, with the data set (1, 1, 1, 5):
the standard deviation is the square root of 3, which is 1.73.

Standard Error of the Mean (SEM)

The standard error of the mean is another common way to describe the deviation from the mean and the frequency of this deviation, but it also takes into account the size of your data set. The formula for standard error is:

$$\text{Standard error} = \sqrt{\text{variance}/n}$$

For example, with the data set (1,1,1, 5):

the standard error = the square root of $(3 \div 4) = 0.866$.

To see why standard error is a useful statistical description, let's consider another data set where the variance equals 3 but $n = 30$.

$$\text{Standard Error} = \text{square root of } 3 \div 30 = 0.316.$$

The same variance of 3 in the two examples above gave different standard errors (if $n = 4$: variance = 0.866 versus if $n = 30$: 0.316) because of the difference in sample size.

If you look closely at standard error and standard deviation formulas, you will notice that standard error has taken the sample size into account twice. To some extent, this calculation is statistics at its worst. Standard error is a statistical analysis of one set of data treated as if you had actually repeated the same experiment many times and gotten a range of means. In other words, standard error is a statistical approach that attempts to look at the variance of this imaginary range of means and determine the variance of these means. Some scientists use standard error to make their data look better than they really are.

\bar{x}	indicates mean
n	indicates sample size
SD	indicates standard deviation
SEM	indicates standard error of the mean (sometimes abbreviated as SE for standard error)

Using Microsoft Excel for Statistical Analysis

Rather than calculating averages, standard deviations, etc. manually, we will enter our data into Excel and have Excel calculate these values for us. As you already know, you also can use this program to graph the results.

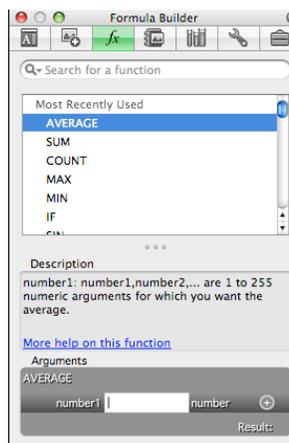
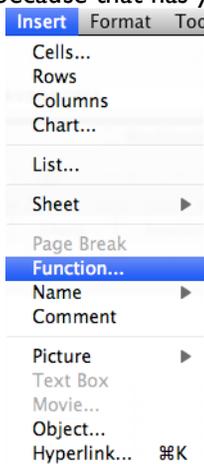
Enter your data and save the file.

time (min)	1	2	3	4	5	6	7
0	0	0	0.1	0	0.2	0	0.1
15	0.7	0.7	0.8	0.7	0.9	0.7	0.8
30	2.2	2.4	0	2.5	2.3	2.5	2.2
45	3.8	3.3	5.1	5.6	3.5	4.1	4.2
60	5	4.1	4.2	3.7	5.5	6	3.8
75	6.3	0	5.3	4.6	6.9	7.5	4.5
90	6.2	6	5	5.8	6.7	6	7

Start a new column called average length and click on the first open cell:

	15	16	17	18	19	20	average
1	0	0	0	0	0	0	
	1.7	0.7	1.1	0.7	0.7	0.5	
	2.2	2.2	2.2	2.2	2.2	2.2	
	2.9	4.5	5.4	4.7	3.9	2.9	
	5.5	4.5	4.4	4.9	5.8	5.1	
	6.9	5.6	5.5	6.1	7.3	6.4	
	6.1	6.7	6	6.5	6.1	6	

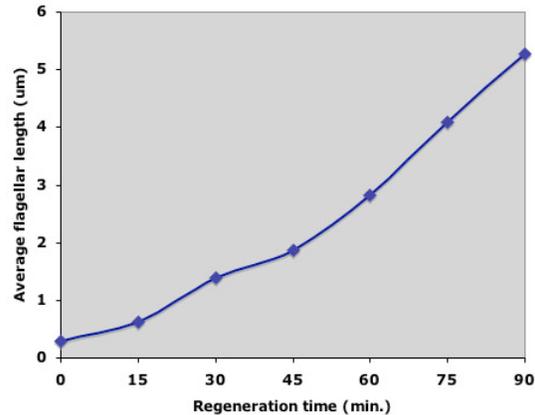
Click on the Insert menu and select “Function...” as shown in the image below left. You will then get a Formula Builder window like the one shown at right. Double click “Average,” and then position the cursor in the white box at the bottom. Either type into the box the range of cells containing the data you want (for example B2:U2), OR select the cells on the spreadsheet. Hit the return key. (Do not select A2 because that has your time values in it.)



Put your cursor on the box in the lower right corner of the box that just calculated your average length at time zero. If you drag the cursor down to your bottom row and then let go, you will have calculated the averages for your remaining rows.

length 18	length 19	length 20	Average	Std Dev
0.25	0.5	0.7	0.24671202	
1	0.95	0		
1.9	0.9	1.6		
2.25	2.1	1.75		
3.1	2.75	3.5		
4.2	4.4	5		
4.9	5.5	5.25		

These data are not so easy to present in this tabular format. It is much more clear to present such data as a graph. To graph your data, go to the Insert menu, pick Chart, and make subsequent selections as you've done before. Be sure to define the X axis values properly. After some additional formatting, your graph might look something like this image.

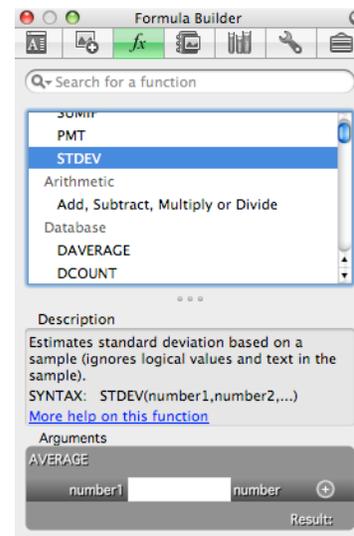


Currently, your averaged data points do not include any indication of the variation in your data. By adding “y error bars” to the averaged data points you can indicate how “tight” or “broad” your averaged values were. Very small/short error bars indicate that the averaged values were very similar, while large/tall error bars indicate that values averaged were quite different.

Most often in published scientific papers you will see that the error bars indicate the standard error of the mean (SEM), which is related to standard deviation (SD). To calculate the SEM you just need to divide the SD by the square root of your sample size (n). You can set up two new columns in Excel to do these calculation on all your data. (FYI the square root of 20 is 4.472).

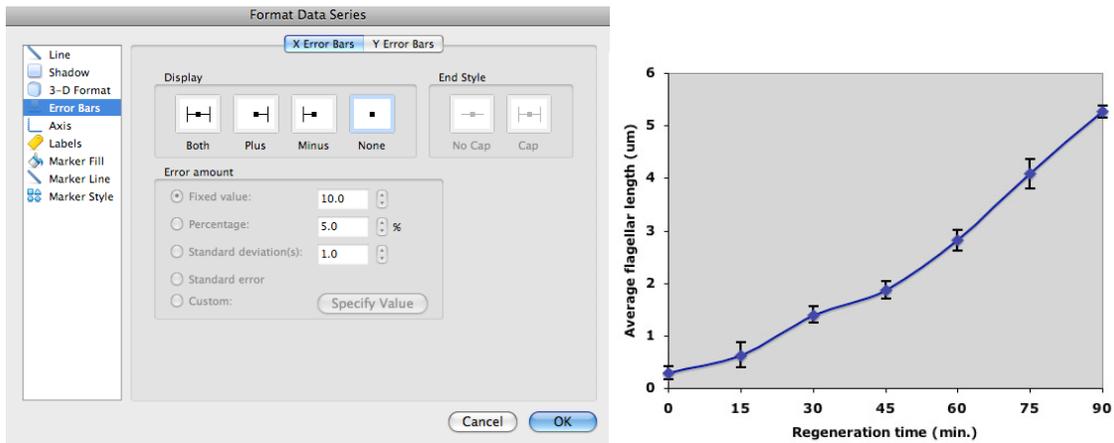
First, create a new heading called Standard Deviation in the column next to your averages. Click on the first empty box in this column and insert a function called STDEV. Enter the same range as before, B2:U2.

When you hit return, the standard deviation for your data will appear in that cell. Again, you can apply the formula to all the rows simply by highlighting the first cell and dragging down to the bottom row.



Now add a new column called SEM. In the first cell, type $=W2/4.472$. This will divide the SD (from cell W2) by the square root of the sample size. Copy the SEM formula down to all the rows.

Finally, to add error bars, double click on any one of the data points within the graph. You will get a window similar to the one shown below left. Select the Y Error Bars tab and pick “both” under the display options. Select the Custom option and click Specify Value. For the Positive Error Value, use the cursor to select all the cells on your worksheet containing the SEM values. Repeat this process for the Negative Error Value, making certain that you selected the very same cells. You will see that error bars have been added to the data points on your graph as illustrated in the graph below right. Remember that error bars in various situations can potentially represent SD, SEM, or even other values (that we haven’t discussed) so your figure legend should include a short sentence that indicates what exactly your error bars represent.



How do you interpret error bars? When SEM error bars from two data sets overlap, the population averages are statistically not different from each other. It's a little more tricky when SEM bars do NOT overlap. To make it simple, we'll say here that non-overlapping SEM error bars mean that the two population averages are indeed statistically significantly different. In reality, to meet the commonly accepted threshold for statistical significance ($p < 0.05$), SEM error bars would have to not overlap AND be separated a bit further, by about 50% the width of the bars.

Insert your flagellar regeneration graph (with error bars) here:

Ames Test Potential Mutagen Preparations

✓ Special Reminder

- You must bring your potential mutagen to lab today
-

Ames Test Preparations

At the start of lab your instructor will give you directions for preparing YFPM (your favorite potential mutagen) for the Ames test that you will perform during the next lab session. It is essential to record the concentration of YFPM as you prepare it.

Flask Letter	Potential Mutagen	Concentration in Flask

Lab Notes

Pouring Agar Plates (preparation for next week's lab)

Focused Reading

- p 291 fig. 13.6 (Growing Bacteria in the Laboratory)
-

1. Wash your hands with soap and water to reduce the possibility of getting stray bacteria from your hands onto your plates.
2. Use the work area with absorbent bench paper. Label your Petri dishes with a small identifying mark on the edges of the dishes.
3. Look in the water bath for a flask of Davis minimal agar (DMA). It has been autoclaved to make it sterile and is being kept at 47° C to keep it liquefied.
4. Think about these important points before pouring your Petri plates:
 - a) You must work quickly, because when the container of minimal agar is removed from the warm water bath, it will start to harden within 2-3 minutes.
 - b) When pouring agar into the Petri dish, pour just enough agar to cover the surface.
 - c) Pour the agar gently to minimize the number of bubbles (bubbles look confusingly similar to colonies when the agar hardens). Do not shake the agar
5. When you are ready to pour:
 - a) Pull out the container of DMA and remove the cap.
 - b) Open the cover of the Petri dish halfway and pour in the agar to just cover the bottom of the dish. Try to minimize the introduction of bubbles.
 - c) Repeat for all the dishes.
 - d) Immediately rinse the flask with warm water to facilitate washing the flask.
6. Let the plates harden ~10 minutes before moving them. These plates will be stored “upside down” until next week’s lab.

The Spot-Overlay Ames Test

Developed from: Maron & Ames (1980) Revised methods for the *Salmonella* mutagenicity test. *Mutation Research*. 113: 173-215.

Focused Reading

- p 275-278 “Point Mutations...” to end of p 278

Web Reading

- Sodium Azide Information: <http://ntp.niehs.nih.gov/index.cfm?objectid=E87F08EB-BDB5-82F8-F368EC83462317FB> and <http://www.bt.cdc.gov/agent/sodiumazide/basics/facts.asp>
-

Goals for This Session:

During this session, you will pour agar plates to be used in next week’s experiments. You will become familiar with the Ames test, which is a worldwide standard for testing new compounds to determine if they are mutagenic. The method you will use this week was developed here at Davidson and allows us to screen many compounds quickly and inexpensively. Next week, we will use the traditional method to quantify the degree of mutagenicity for selected compounds.

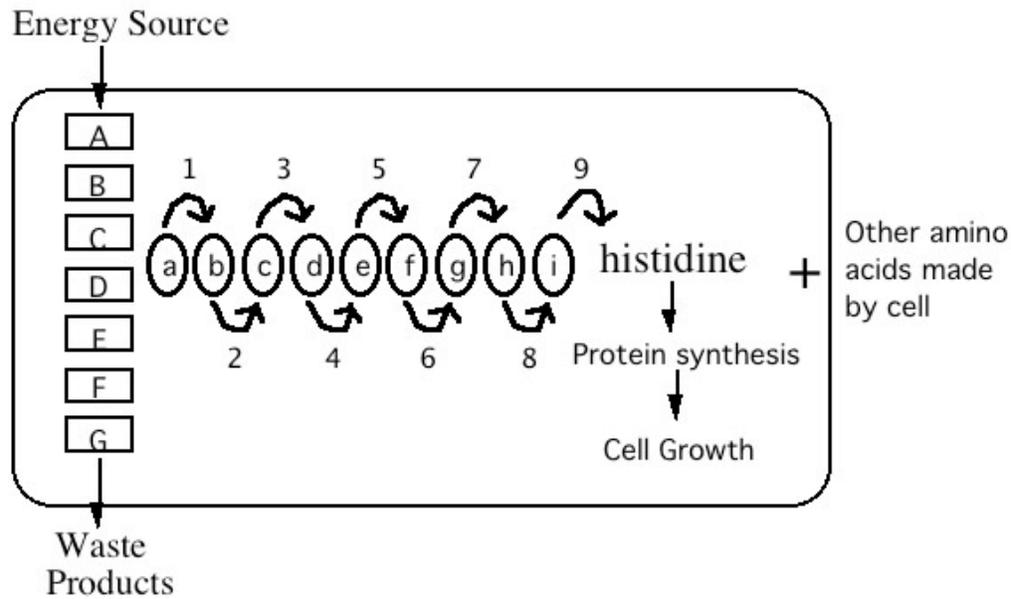
Background

Our environment is full of potential carcinogens (cancer-causing agents) such as UV light, industrial pollutants, pesticides, food additives, and natural products such as tobacco. These carcinogens can induce cancers because they are mutagens (chemicals that cause mutations), which change the nucleic acid sequence of DNA. It is important to have a rapid and inexpensive assay for testing chemicals we suspect are carcinogenic, including the large number of new synthetic chemicals being produced each year.

It is estimated that 90% of all carcinogens are also mutagens, and with this thought in mind, Bruce Ames and his colleagues developed a test in the 1970s that uses special bacteria that are very sensitive to mutagenic agents. The Food and Drug Administration (FDA) now uses the Ames test to screen many chemicals rapidly and inexpensively. Those few chemicals that appear to be mutagenic by the Ames test are tested further in animals to assess their ability to cause cancer.

Wild-type cultures of the bacterium *Salmonella typhimurium* grow in media without the addition of any amino acids. This growth is possible because the *Salmonella* bacteria have metabolic pathways for making all of their own amino acids. Each amino acid has a separate pathway for its synthesis. For example, Figure 1 diagrams histidine synthesis, which begins with catabolic intermediate C and uses nine enzymes (numbered 1-9 in figure 1) to convert C into histidine.

Prototrophic Bacterial Cell



Auxotrophic Bacterial Cell

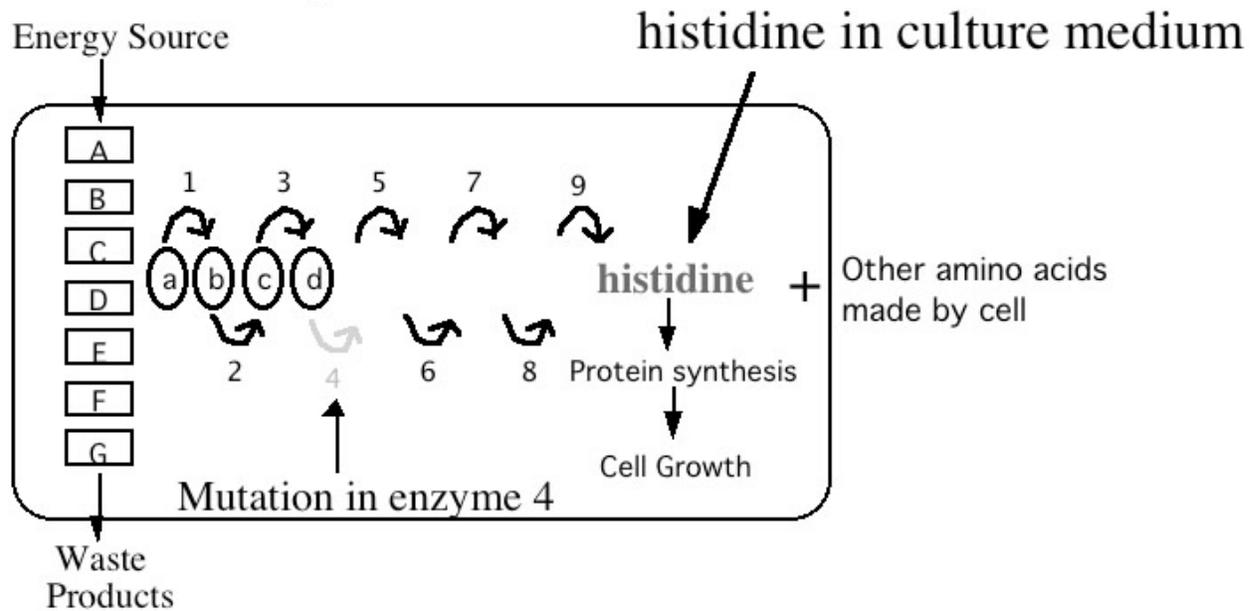


Figure 1. Schematic overview of *Salmonella typhimurium* metabolism and the effect of a non-repaired point mutation in the histidine synthesis pathway. (Top) Metabolism of a normal (prototrophic) *S. typhimurium* that can make its own histidine. The catabolism (breakdown) of the food source produces a precursor (C) that is needed for histidine synthesis. The formation of each intermediate (a-i) in the histidine synthesis pathway is catalyzed by a different enzyme (arrows 1-9). Each enzyme is a protein encoded by a separate gene. The absence of any one of the nine enzymes would prevent histidine synthesis. (Bottom) Metabolism of an auxotrophic (*His*⁻) mutant that cannot make its own histidine. Here the catabolism of food still produces the precursor (C), but enzyme 4 is not made due to a point mutation in the encoding DNA. Consequently histidine synthesis is interrupted because intermediate (c) cannot be made, preventing the rest of the enzymes from binding to their substrates and synthesizing the appropriate products. In order for this auxotroph to grow histidine must be supplied in the medium it is growing in.

The Ames test uses a mutant strain of *Salmonella typhimurium* that cannot grow in the absence of the amino acid histidine because a mutation has occurred in a gene that encodes one of the nine enzymes necessary for histidine biosynthesis (see Figure 1, bottom). The mutation prevents translation of a functional enzyme #4, and thus the cell cannot complete the conversion of the catabolic intermediate **C** to histidine. Therefore, the Ames mutants only can grow if histidine is supplied in the growth medium. These **auxotrophic** mutants are called *histidine-dependent* or his⁻ (pronounced hiss-minus) mutants because they depend on an external source of histidine to grow. Auxotrophs are mutant individuals that cannot make all the metabolic products that wild-type (**prototrophic**) individuals of the same species can make.

There are several different mutant strains of *S. typhimurium* that have different mutations in their DNA. We will use a variety of different mutant strains that have important distinctions that make them suitable for detection of different types of mutagens. Below is a list of the available strains and the type of mutation each strain carries:

- **TA 1535** has a base substitution that produces a **missense mutation** in the gene coding for the first enzyme of histidine synthesis. The mutant enzyme has a proline where a leucine is in the wild-type enzyme.
- **TA 100** is very similar to TA 1535, but is supposed to detect a different range of mutagens.
- **TA 1537** has a frameshift mutation (**deletion of one nucleotide**) in a different gene than is mutated in TA 1535.
- **TA 1538** has a different frameshift mutation (**insertion of one nucleotide**) in the same gene that is mutated in TA 1537.
- **TA 98** is similar to TA 1538 but is supposed to detect more mutagens than TA 1538 does.
- **TA 102** is significantly different from the others. It has an *ochre* mutation, which means that it has a **nonsense mutation**. This mutation occurs in the same gene that is mutated in the strain TA 1535.

In addition to the mutations listed above, there are two other important traits shared by each of these strains. 1) These mutant strains lack a DNA excision-repair (proof-reading) mechanism that exists in wild-type bacteria and would normally repair any new mutations in the DNA that are caused by exposure to mutagens during our experiments. The result of this defect is that DNA errors are not corrected, thus enhancing the strain's sensitivity to mutagens. 2) These strains have a defective lipopolysaccharide layer that allows chemicals to penetrate more easily into the cell than is true with wild-type bacteria.

In summary, we have mutant strains of *Salmonella typhimurium* that cannot synthesize histidine, are very susceptible to additional mutations because they lack the normal repair mechanisms found in bacteria, and are more permeable than wild-type bacteria to external chemicals, including potential mutagens. In order for these cells to survive on a plate that lacks histidine, they must “learn” how to synthesize histidine by undergoing another mutation that corrects the original mutation that prevented the production of the missing enzyme. This type of mutation is known as a back mutation, or **reversion**, because this second mutation returns the mutant to the wild-type genotype. This reversion can happen spontaneously or as the result of a mutagen. To be considered a mutagen, a compound must result in a mutation rate more than

double the spontaneous mutation rate. Note that many mutations, in many different genes, may be occurring in the bacteria. We, however, can detect only the mutations that result in a phenotypic reversion.

A brief note about mutations: a mutation is any change in a DNA sequence from the original sequence of nucleic acids, and mutations happen all the time in your cells. Sometimes it is because a mutagen comes from the outside of the cell and in some manner creates changes in the DNA. Often the mutations are just errors that occur during DNA replication when cells divide. In fact, there is an average of nearly one mutation (error) in your DNA every time one cell divides. Your cells have ways to repair the mutated DNA, and they usually do, but if the mistake is overlooked, the change in the DNA is maintained in future replications in the cell. This scenario represents one way that a “spontaneous” mutation can occur; there was no obvious cause on which to blame the mutation.

To determine the number of **revertants** following exposure to a mutagen, we must have a way to differentiate the mutant strain we started with (*his⁻* auxotrophs) and the new mutants we may generate (*his⁺* revertants). The Ames test uses a chemically defined medium for this purpose, meaning the amounts of each ingredient are known, and the medium is lacking one nutrient necessary for bacterial growth. If a *his⁻* culture is placed on a chemically defined minimal agar lacking histidine, only those cells that have mutated to *his⁺* (revertants), will grow and form colonies. In theory, the number of colonies that revert and grow is proportional to the mutagenicity of the test chemical.

The chemically defined medium used for the Ames test actually has just a trace (growth limiting) amount of histidine added only to the soft agar overlay. Trace amounts of histidine in the medium are necessary because some mutagenic agents act preferentially on actively replicating DNA. When Ames mutants are plated on this medium, they grow until they run out of histidine (only 2-3 cell divisions lasting about one hour), and the result is a faint, nearly invisible lawn of growth within the overlay. Conversely, revertant bacteria should form large colonies because their growth is not limited because they can produce their own histidine. Each large colony represents one revertant bacterium and its offspring.

By definition in the Ames test, a mutagen is any chemical agent that results in twice the number of mutants as occurred spontaneously, and thus is potentially carcinogenic for humans.

What you have read is an overview of the theory behind the Ames test that we will use. Many chemicals in nature, however, are not carcinogenic/mutagenic until after an animal consumes them. One job of the liver is to detoxify harmful chemicals, but in the process some chemicals are converted into very potent mutagens. For this reason, all test chemicals used in the Ames test by the FDA are routinely incubated with rat liver extract in an oxygenated environment so that liver enzymes, such as oxygenases, will “activate” the chemical being screened; the “activated” chemical then is added to the bacteria. Due to the potential hazards associated with this step, we will not pre-treat our potential mutagens with liver extracts. Instead, we determine if various agents are mutagenic in their “unactivated” states.

📖 NEWS ITEM: Remember the vole found near Chernobyl that was resistant to mutations and also had high levels of IDH (mentioned earlier in the lab manual)? It is speculated that the particular allele carried by these mutation-resistant voles is more effective at inactivating oxygen radicals formed by the radiation. Oxygen radicals can “activate” chemicals and cause them to become mutagenic. Hmmmmm.....

Overview of the Experimental Series with the Ames Test

The ultimate goal of the laboratory series on the Ames test is to have you design experiments to test an unknown potential mutagen. For the first week, we will use a new variation of the Ames test that was developed at Davidson College. This variation is called the **Spot-Overlay Assay** and it is designed to allow us to screen a number of different chemicals quickly and inexpensively. Every lab group will use sodium azide as one of its potential mutagens because we know that this chemical is a powerful mutagen

(positive control). The second week, we will use the standard overlay assay developed by Dr. Ames to quantitate any mutagens detected by the spot-overlay assay. To perform the Ames test successfully, you will have to be careful to maintain sterile conditions, because you want only *Salmonella typhimurium* in your Petri dish and not other contaminating strains from the air, your fingers, lips ... you get the picture. Furthermore, you must be very careful in this laboratory series:

THIS LAB HAS POTENTIAL HAZARDS!

- 1) Does “*Salmonella*” sound familiar to you? It is the bacteria that turns your stomach inside out after eating bad potato salad.
- 2) You will be handling potential or known carcinogenic/mutagenic materials.

YOU MUST WEAR GLOVES AT ALL TIMES WHEN HANDLING THE CHEMICALS AND BACTERIA USED IN THESE EXPERIMENTS.

We have faith in your abilities to use your common sense and be careful in what you do with test tubes, pipet tips, etc. that have come in contact with the bacteria and chemicals. Enough warnings, now on to the good stuff.

In your experimental designs for both weeks, the use of controls will be important, just as it is in every experiment. A **positive control** is a condition that will test positive in your assay; in this case, that means adding a chemical (a known mutagen) that should allow the cells to grow and reproduce, thus bacterial colonies will grow in a minimal media lacking histidine. **Negative controls** are conditions that should not cause anything to happen. For our experiments, this control would be a condition where no mutations should occur above the rate of spontaneous reversions (such as water). It is very possible that more than one positive and negative control might be needed for each chemical and bacterial strain tested.

Perhaps the best way to think about what controls are needed is to look at the possible results for your “test condition” (i.e., what happens when you add your favorite potential mutagen?) and make sure that you could explain the results. For example, what would happen if you saw no colonies in any of your spot-overlays? What if there were bazillions of cells in every spot-overlay? Your control condition spot-overlays should be designed so that you can interpret these results.

How will you determine the “spontaneous” mutation rate? When you develop an answer to this question, you can look at the possible outcomes of this experiment. There are only two: either you will see more colonies with a potential mutagen present, or you won't. Take the first possible outcome: if you see more colonies in your spot-overlay with a potential mutagen (due to new mutations) than you see in the spot-overlay with no added chemicals, can you be sure that it was due to the potential mutagen and nothing else? What control(s) do you have to perform to make sure this interpretation is true?

Now the second scenario: let's say you get few, if any, colonies with your potential mutagen present. Can you be sure that this chemical is not a mutagen? What other variables could be different, or go wrong, to give the result of no colonies? If you were testing bleach and no colonies grew, what could you conclude?

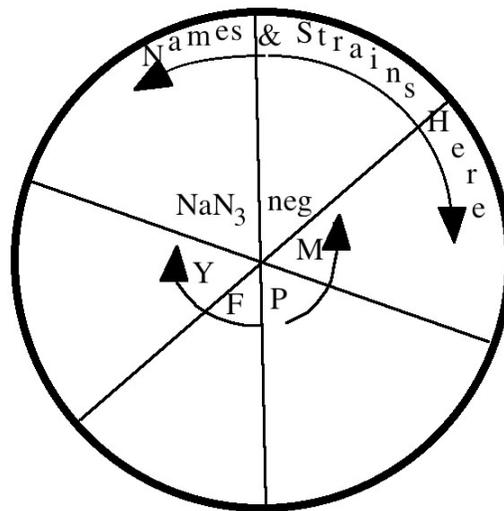
After thinking through the possibilities and discussing them in class, we will have several controls, both positive and negative, to include in our experimental design for this first week.

CAUTION! (Do we have your attention?)

Pay attention to what you are handling. If it is a container containing bacteria or chemicals, be sure and wear gloves. PLEASE have only those people handling these things wear gloves, to conserve their use. You can consume only one pair for the entire lab period if you think.

1) Be aware of where you should throw away used gloves, pipet tips, etc. that have come in contact with bacteria and chemicals **BEFORE** you use them. All trash, including tubes with bacteria and pipet tips go in the orange biohazard bags, which will be autoclaved. The metal caps are cleaned and reused.

2) When handling anything that is supposed to be sterile, such as the pipet tips, Petri plates, tubes of distilled water, bacteria, agar, etc., be sure to uncover or uncap items for as brief a time as possible. Also, be sure and keep the caps of tubes and lids of Petri plate facing down towards the floor when you are holding them to reduce the possibility of contamination.



Spot-Overlay Protocol

1. Obtain six pre-poured Petri plates containing minimal agar (this medium contains no histidine at all). Label on the bottom of the plate your group name, the date, and what strain of cells will be tested. Divide each plate into six equal parts. Label each pie-shaped section with the chemical to be tested in that area. Labeling Petri dishes is tricky: we recommend writing small, and around the perimeter.

2. Locate the tubes that contain the soft-agar overlay in the water bath on your table - each group will have **36** (six plates with six spots on each) of these. These tubes have been autoclaved to sterilize them and put at 47°C to keep them liquefied.

3. Locate the tubes containing the different strains of *Salmonella* that will be kept at room temperature on the lab bench.

4. If you will be touching tubes, pipets, or plates that contain bacteria, you must **PUT ON GLOVES**.

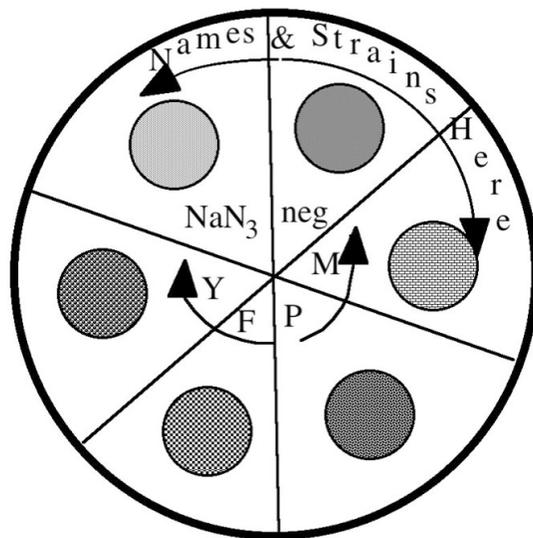
5. Locate your chemicals to be assayed and any solvents you may be adding to the overlay soft agar tubes.

	TA 98	TA 100	TA 102	TA 1535	TA 1537	TA 1538
Negative Control	___ μl dH ₂ O 60 μl bacteria					
Positive Control	___ μl dH ₂ O ___ μl NaN ₃ 60 μl bacteria	___ μl dH ₂ O ___ μl NaN ₃ 60 μl bacteria	___ μl dH ₂ O ___ μl NaN ₃ 60 μl bacteria	___ μl dH ₂ O ___ μl NaN ₃ 60 μl bacteria	___ μl dH ₂ O ___ μl NaN ₃ 60 μl bacteria	___ μl dH ₂ O ___ μl NaN ₃ 60 μl bacteria
YFPM #1	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria
YFPM #2	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria
YFPM #3	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria
YFPM #4	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria	___ μl dH ₂ O ___ μl mutagen 60 μl bacteria

NOTE: Now you must work quickly to make sure the soft agar overlay does not harden in the tube. Make sure you understand all aspects of step 6 *before* proceeding.

6. Sterilely add your test chemical and bacteria to the overlay tube and spot this mixture onto the minimal agar.

- a) Set your pipettor to the appropriate volume of test chemical (usually ~ 60 μl , but check with your instructor). Open the box of sterile pipet tips, aseptically put one on, and take an aliquot of your test chemical (or solvent used as a control) into the micropipettor.
- b) Remove the cap from the overlay tube, and discharge your aliquot of the test chemical into the overlay tube.
- c) Using one strain at a time, swirl the tube containing the bacteria, remove **60 μL of *S. typhimurium* strain** and aseptically add it to the overlay tube from step b above.
- d) After swirling the tube to mix the bacteria and test chemical, withdraw **150 μL of the mixture**.
- e) Lift the top of the Petri plate (open end facing down) and quickly but gently discharge the bacteria/chemical/agar onto the minimal agar - you should create a small puddle.
- f) Repeat this step until each strain has been tested with each potential mutagen.
- g) Invert the plates (*i.e.*, store with the lid on the bottom) and place in a 37°C incubator (not the frig) for 48-72 hours.



Analysis of Results (see the start of next week's lab)

References

Ames B, McCann J, Yamasaki E (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. *Mutation Research* 31: 347-364.

Ames B, Yanofsky C (1971) The Detection of Chemical Mutagens with Enteric Bacteria, pp. 267-282. In: *Chemical Mutagens-Principles and Methods for their Detection*, Vol. 1, A. Hollander (Ed.), Plenum Press, N.Y.

Lab Notes

Assessing Potential Mutagens

Focused Reading

- p 387-390 “Most cancers...” to end of p 390
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Goals for This Session:

This week, you will count the colonies on each spot on each plate from last week. Each group will post its results on the board and all results will be discussed as a group. We will determine which strains and YFPM suggest that a compound is mutagenic. Then, each group will design a new experiment to quantify the potential mutagen.

Introduction

We will start this week by looking at each group’s data from last week’s spot overlay experiments. Each group should list their results on the front whiteboard. List the number of colonies observed for each strain and test chemical on the board. We will discuss these results together, then each lab group will determine which substance(s) will be quantitatively analyzed with further experimentation today.

Analyzing Your Results

After an appropriate incubation period, you removed your plates from the warm incubator and stored them at 4° C. Today you will count the number of visible colonies in each spot-overlay and put those numbers in the table below. Even though the size of the colonies may vary greatly, each colony still arose from only one bacterium that has reverted, thus “all colonies are created equal.” A colony consists of a distinct white spot that you clearly can distinguish from a bubble or other similar looking phenomena. Your definition of a colony (anything that shows up versus only really big ones, etc.) is less important than being *consistent* in counting colonies from one plate to another. When you have finished counting, record your results in the table provided. Each plate should be counted by at least two members of your team to improve accuracy. Discard your plates in the orange biohazard bags provided in the lab.

	Negative Control Water	Positive Control NaN₃	Potential Mutagen #1	Potential Mutagen #2	Potential Mutagen #3	Potential Mutagen #4
TA 98						
TA 100						
TA 102						
TA 1535						
TA 1537						
TA 1538						

NOTE: Be prepared to list the number of colonies in your test conditions and controls for the class to analyze today.

Design a Follow-Up Experiment

Your team should follow up on the most interesting results with another experiment. Develop an experimental protocol and check with your instructor before you proceed to the next steps. Use the space below to write out and/or diagram your experiment before you conduct it. Be sure to include positive and negative controls.

Tube #	Overlay Agar	Mutagen	dH₂O	<i>Salmonella</i>	Total Volume*	Final mutagen concentration
1	5 mL	_____ : __ μL	__ μL	TA_____ : 60 μL		
2	5 mL	_____ : __ μL	__ μL	TA_____ : 60 μL		
3	5 mL	_____ : __ μL	__ μL	TA_____ : 60 μL		
4	5 mL	_____ : __ μL	__ μL	TA_____ : 60 μL		
5	5 mL	_____ : __ μL	__ μL	TA_____ : 60 μL		
6	5 mL	_____ : __ μL	__ μL	TA_____ : 60 μL		
7	5 mL	_____ : __ μL	__ μL	TA_____ : 60 μL		
8	5 mL	_____ : __ μL	__ μL	TA_____ : 60 μL		
9	5 mL	_____ : __ μL	__ μL	TA_____ : 60 μL		
10	5 mL	_____ : __ μL	__ μL	TA_____ : 60 μL		
11	5 mL	_____ : __ μL	__ μL	TA_____ : 60 μL		
12	5 mL	_____ : __ μL	__ μL	TA_____ : 60 μL		

*The total volume in each tube should be the same value.

Plate Protocol

This method is somewhat different from last week's, though the principle is the same.

1. Each group will need at least 12 plates that you poured last week. Label them with your group name, and what condition will be tested on it. We recommend writing small, and around the perimeter of the bottom. Experimentals and controls will be on different plates.
2. Locate the tubes of the soft-agar overlay tubes (the volume will be 5 mL instead of last week's volume of 1 mL) in the water bath on your table - each group will have exactly 12 of these. These tubes have been autoclaved to sterilize them and put at 47°C to keep them liquefied.
3. Locate the tubes containing the appropriate strains of *Salmonella* that will be kept at room temperature on the lab bench. You will probably test of strains strain that were the most sensitive based on your results from last week.
4. If you will be touching tubes, pipets, or plates that contain bacteria, you must WEAR GLOVES.
5. Locate your chemical to be assayed and any solvents you may be adding to the overlay soft agar tubes.

NOTE: Now you must work quickly to make sure the soft agar overlay does not harden in the tube. Make sure you understand all aspects of step 6 before proceeding.

Step 6 is not the same as it was last week.

6. Sterilely add your test chemical to the overlay tube.
 - a) Set your pipet to the appropriate volume of test chemical. Open the box of sterile pipet tips, aseptically put one on, and take an aliquot of your test chemical (or solution being used as a control) up into the pipet.
 - b) Remove the cap from the overlay tube, and discharge your aliquot of the test chemical into the overlay tube.
 - c) Sterilely add **60 µL of the proper strain of *Salmonella typhimurium*** to the appropriate overlay tube. Mix the bacteria, chemical and agar by thumping the tube for a few seconds or so.
 - d) Lift the top of the Petri plate and quickly but gently pour the entire contents of the overlay onto the agar surface (try to do it gently and prevent the introduction of bubbles).
 - e) Gently swirl the plate with the soft agar overlay until it completely covers the surface of the agar base layer.
 - f) Put the plate down on a flat surface and allow the agar to harden at least five minutes before moving the plate again.
 - g) Repeat steps a-f.
 - h) Invert the plates (*i.e.*, store with the lid on the bottom) and place in a 37°C incubator for 48-72 hours.

- i) Make plans to return to the lab to count colonies in the next few days – your instructor may have a sign-up sheet for this purpose.

Analyzing Your Results

After the appropriate incubation period, make plans with your instructor to count the number of visible colonies on each plate. An exact size definition of a colony is less important than each group being consistent in counting colonies from one plate to another.

Plate	# of Colonies

Make plans with your teammates to prepare your oral presentation and/or lab report.

❖ Study Questions:

1. Suppose that you tested different concentrations of sodium azide. Would you expect the mutagenicity of sodium azide proportional to the amount present? Explain your answer.
2. In your experiment testing the mutagenicity of your unknown substance(s), explain the ingredients and the purpose of the positive and negative controls.
3. If you added a known mutagen such as sodium azide to the rich medium that contained histidine, what would you expect to see? Explain.
4. If you knew that 5 μM of sodium azide was highly mutagenic, what would you expect to see if you added 50 mM sodium azide? (The exact amount is not the critical issue here. The main point is that you added a whole lot more.)

5. Davis Minimal Agar (DMA) has none of the 20 amino acids in it. Sodium azide causes base substitution mutations at locations all over the bacterial DNA, not only at the single nucleotide that is wrong in the his-gene of this mutant bacteria. What if sodium azide caused a base substitution mutation in a gene coding for an enzyme needed to make a different amino acid, such as leucine, instead of the base substitution for the wrong nucleotide in the gene for the enzyme needed to make histidine? What would you see on your Petri dish of mutant bacteria on DMA and why.

Lab Notes