Laboratory Manual
Davidson College
Biology Department

Principles of Biology I (Bio 111)

Cell and Molecular Biology

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Table Of Contents

Hints for Your Oral Presentations

Oral presentations are an important means of communicating scientific information. Oral presentations often are used to present experimental findings at colleges and universities (where they also are known as "seminars"), and at scientific meetings. Therefore, it is important that you gain experience with this presentation format.

Everyone realizes speaking in front of a group can be uncomfortable, and it is especially hard the first time. You will make some mistakes - that's part of the learning process. Please realize that any questions that you are asked by your classmates or instructor are not meant to be taken personally. So, don't be afraid of questions - they are intended to further our understanding of your scientific investigation. The best preparation for presentations is to understand what you did, especially why you set the experiment up the way you did in order to answer a specific scientific question.

Each group will give an oral presentation about their experiment. The presentation should be organized in a manner similar to your scientific reports, with general categories such as: Introduction, Material and Methods, Results, and Discussion/Conclusion. For groups of four, each person should present one of the following sections.

1) The Introduction can include things such as background information, the reasons for doing the experiment, and your hypothesis.

2) The Materials and Methods should include your experimental design, where you describe the samples you are testing and the controls you have incorporated into the experiment. In addition, you can do a very brief overview of the major procedures you performed. Remember to consider your audience: all the groups did a basic enzyme laboratory, so there is no need to repeat "standard" protocols. Include procedures that are different from the standard protocol, and be sure to present enough of your protocol so that everyone is clear as to exactly what you did.

3) The Results should be a clear and concise display and explanation of your data. Your data should be distilled down to the important facts, and not necessarily every piece of data you collected. However, don't make the mistake of showing a figure and saying, "This is what we got." and then sitting saying nothing else. Walk us through the figure. Point out important parts of each figure.

4) Finally, the Discussion will be your interpretation of your results. What do your data mean? Discuss whether your data support your hypotheses. Do you have reason to believe your data were inaccurate? What would you do next time to investigate the problem further?

Your group's presentation should last no more than 15 minutes, because there must be time for questions and discussion with the rest of the class afterward. Each person in your group must speak during the presentation. The use of visual aids is very important; we suggest you **print very small figures** and then use the digital projection system for the class to see.

In preparing your presentation, you may find it helpful to keep the following questions in mind:

1. Do you clearly state the question(s) you are trying to answer?

2. Is it clear what you did to try and answer your question?

3. Do you explain your results, especially inconsistent or unexpected results?

4. Do you convey why you did the different conditions in your experiment?

5. Did you explain what your data mean? Can you answer the question from number 1 above?

Your group will be critiqued in two ways. First, your classmates will review your presentation. You will not be graded by your classmates - these comments are to help you. Each person will review every group by responding to the following two questions:

1) What were the strengths of this group?

2) What improvements could be made by this group?

When making comments about the presentation of others, keep in mind the four questions listed above, as well as other things such as whether the group was organized, if everyone participated, if their conclusions were valid, etc. These comments are meant to be helpful suggestions and not a slap in the face.

I will be interested in similar categories, especially how clearly you present your material, whether you display understanding of what you did and why you did it, and if the data support your conclusions. You will receive a group grade, but the most important aspect of this exercise is to become comfortable talking in front of a group and to have fun with your presentation.

Writing an Abstract

An abstract is a single paragraph summary of your experiment. Like a paper (or lab report), an abstract should contain an introduction, methods, results, and conclusion. Every scientific paper has an abstract at the beginning to let the reader know what the paper is about and to make an informed decision whether the entire paper is worth reading. Abstracts also are printed in reference books where the whole article does not appear, and are used to decide which articles you need to obtain. A third use of abstracts is to summarize the work you will be presenting at a meeting, so people will know if they should come to see your complete presentation.

Guidelines for writing good abstracts

Revise, revise, revise. The Abstract should be clearly and concisely written. Try to address each of the questions below (under ABSTRACT). Use plain English whenever you can, active voice when you can, and use simple sentences. It is not necessary to refer to any literature (if you do, list the references below the abstract). State only your most important conclusion(s). Remember, the Abstract probably will be the only portion of your report that most people read. Make sure it is well written.

ABSTRACT

1. Title: The title should indicate the question you investigated, or the method, if that is important. Example: Effect of Owner Education Level on Number of Cats per Household.

2. Author(s) and address(es). Example: Mary Darwin and John D. Helix, 1994, Introductory Biology, Davidson College.

3. What is the general topic you were investigating and why is it important? One to two sentences. Example: Education level may affect choices people make about their personal lives and habits.

4. What are the specific questions you are addressing with this project? The Abstract should not include your complete methods. Provide a one or two sentence overview. Example: We investigated the relationship between education level and the number of cats per household for residents of a small town.

5. How did you do this experiment? For a single paragraph abstract, one or two sentences are needed. You are not trying to be complete, just give a general idea of how you did it. Example: The residents of a small town in North Carolina were polled as to the number of years of education for adults in households and the number of cats associated with the household.

6. What did you observe? One sentence should be enough: state only your main point(s). Example: Adults with either low education levels (0-10 years of school) and those with high education levels (more than 16 years of school) had significantly more cats per household than those with intermediate education levels (11-16 years of school). Include your most important data (mean values, standard deviations, number of samples you studied, etc.) which influenced your conclusion.

7. What did you find out about the general topic or question (see #3 above)? One sentence, 2-3 sentences for a longer abstract. Example: We concluded that education level can affect choices not directly associated with academic pursuits.

Here is the final abstract from the example above:

Effect of Owner Education Level on Number of Cats per Household. Anna Author and Aaron Associate, Biology Department, Davidson College, Davidson, NC 28036.

Education level may affect choices people make about their personal lives and habits. We investigated the relationship between education level and the number of cats per household for residents of a small town. The residents of a small town in North Carolina were polled as to the number of years of education for adults in households and the number of cats associated with the household. Adults with either low education levels (0-10 years of school) and those with high education levels (more than 16 years of school) had significantly more cats per household than those with intermediate education levels (11-16 years of school) when analyzed by the statistical test ANOVA, ($p<.005$). This finding is highlighted by noting that those people with high or low education levels were more likely to have four or more cats (23%) than those people with intermediate education (4%). We concluded that education level affects whether a household will have pet cats.

With the method outlined above, you should be able to produce a good abstract in less than an hour. If you haven't clearly and carefully thought through what you did in the experiment, writing the abstract should help you do so. It is shorter than a lab report, but includes most important points. (For your information, the study and abstract above was invented for this lab and does not reflect an authentic study.) Other sample abstracts will be available in the lab for your viewing pleasure. Also, consult the posters on display in Watson and Dana.

NEWS ITEM: If you are under the mistaken impression that the research you do is unimportant, then take a lesson from Emily Rosa. Emily published her research results in *JAMA - the Journal of the American Medical Association*. She conducted her research while in the fourth grade! She was curious whether there was any validity to a new form of alternative medical therapy called “touch therapy”. She and her mom, a nurse, conducted an experiment which Emily designed. The end result demonstrated that touch therapy was not able to discern as much information as the practitioners claim. You can read her article in the April 1, 1998 issue of *JAMA* in our library (Rosa L, Rosa E, Sarner L, Barrett S. A close look at therapeutic touch. JAMA. 1998 Apr 1;279(13):1005-10).

Guidelines for Scientific Papers

I. Scientific Laboratory Report

Writing a laboratory report is like writing an original research paper. The most common format for a scientific research paper is:

Abstract
Introduction
Materials and Methods
Results
Discussion
References

Introduction

The "Introduction" of the report should explain why the work was done. What were the objectives of the research? How does the research help to fill a hole in our knowledge? The Introduction should include a clear statement of the problem or question to be addressed in the experiment. It is always helpful to put this question into some context by stating why this question needs to be answered or why you found this question to be particularly interesting. Any background material that is particularly relevant to the question should be included in this section.

Materials and Methods

The "Materials and Methods" section tells how the work was done. It should NOT be a simple list of the materials used. What procedures were followed? What research materials were used: the organism, special chemicals, instruments? In some of the experiments you will be doing, many of the procedures are given in great detail in the handouts. It is not necessary to retype these verbatim, but rather summarize them and cite the laboratory manual in your references. Provide details only about changes from the handout and about your individual project. The most important feature of this section should be to include enough detail in your description of how your experiment was set up and run so that anyone reading the Materials and Methods could repeat your experiment.

Results

The "Results" section explains in words what you found, the data that you generated, explained succinctly in the body of the report and presented in detail as tables or graphs. The results section should be written so that any college student could read the text to learn what you have done. For example, you might use a paragraph to explain what is seen on a particular graph; “When the enzyme was soaked in sulfuric acid, no change in absorbance was observed (Table 1)” Do not make the common mistake of writing, “We performed the experiment, see figures 1-4.” That is too brief and does not convey to a novice what you have done. When stating your results in the body of the text, refer to your graphs and tables. Do not attempt to discuss the interpretation of your data - explanations should be included in the “Discussion” section. Each table and figure should be numbered sequentially for easy reference in the text, and all figures must have a brief description called a legend, which provides the reader enough information to know what you did to produce the data.

Discussion

The "Discussion" section typically includes your appraisal of what your research means, including its success in meeting the objectives stated in the introduction, and its significance in advancing your knowledge of the subject. This section also is the place to explain discrepancies or difficulties with

experiments, as well as suggestions for future work. For example, if you had known initially what you know now, how might you have changed your experiments? Most importantly, the Discussion provides an opportunity to compare your results with those of others. What previous information exists that is relevant to your research? Do your results support or supplement that information? Once again, when providing your interpretation of the data, direct the reader to specific tables and graphs to prove your point.

References

Finally, it is important to place your work in perspective with the published work of other scientists. We will not have much opportunity to use references in Introductory Biology, but references are an important component of any report. Scientific journals usually require specific reference formats. We will discuss the preferred format for your reports.

II. Presentation of Results in Scientific Reports: Figures and Tables

Data that have been collected need to be presented clearly and succinctly. As a result, two forms of presentation are most commonly used in scientific papers: figures and tables. Which method to use depends somewhat on the data, but in general anything that can be displayed pictorially (e.g. a graph or diagram) is more desirable, because the reader can immediately see the trends in the data. In the paper itself, graphs are referred to as "Figures", and are numbered sequentially in the order of presentation (Figure 1, Figure 2, etc). Tables also are numbered sequentially. Although figures and tables often are placed directly into the middle of scientific papers. For simplicity, you may include them at the end of your report, with one figure or table per page.

Graphs

Graphs can be made using a graphing program such as Excel. Remember to label each axis, including units of measurement, and clearly identify the data you are displaying (e.g. label each line in a graph). In addition, every graph must have a short description below it to tell the reader some basic information about that data and the way it was obtained. This description is known as a legend. The legend starts with the figure number, followed by a one sentence title. The text of the legend should be no more than one short paragraph and should be as brief as possible. Following is an example of a graph with legend:

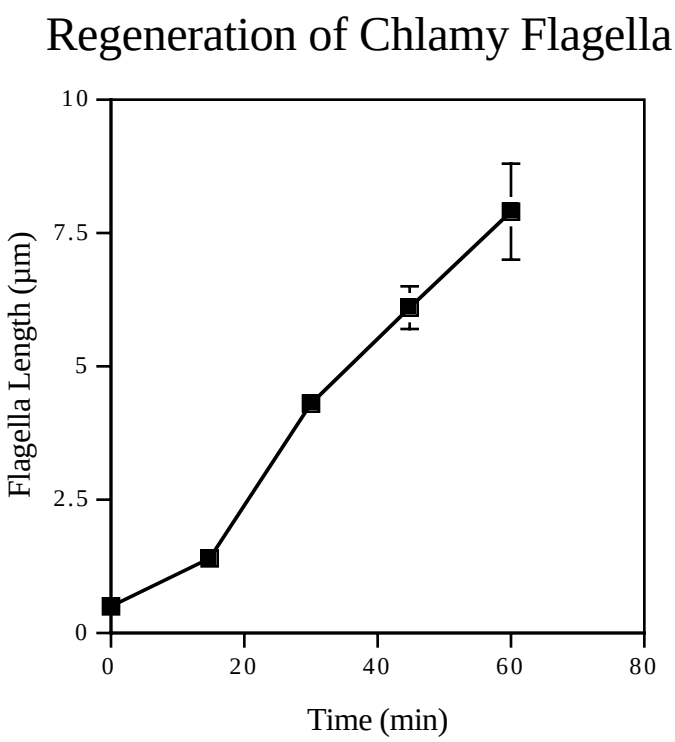


Figure 1. Regeneration of *Chlamydomonas* flagella as a function of time. Flagella were removed from cells using the pH shock method and allowed to regrow under constant light. Each data point represents the averaged result of 20 individual cells and the error bars represent the 95% confidence interval.

This graph was made using the statistical program Excel. Notice how the axes are labeled, and the figure is numbered and titled (bold type). The legend explains how the data were obtained. Please also look at the sample scientific papers posted in the laboratory as other examples of legends.

Tables

Tables should be made using the same principles outlined for graphs, though the format is different. Tables can be created with Word, using tabs to create the different columns. Tables are numbered, but this number usually appears at the top of the table. The title usually follows the table number:

Table 2. **List of students who loved Intro Biology (1837 - present)**

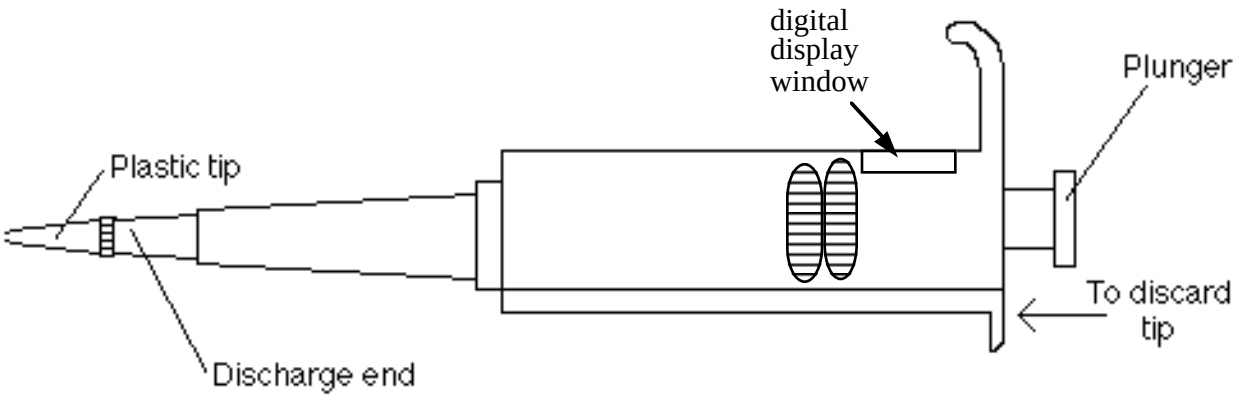
Name	Date Class was Taken	Final Grade (%)
Alfred Nobel	Fall, 1923	94
Charles Darwin	Spring, 1897	81

Tables generally do not contain legends. Often, though, footnotes are included under a table to provide explanatory information. Of course, all column headings should be clearly labeled to describe the data listed below them.

When preparing your data for a presentation, think about the most effective way of showing your data to the audience. Some information can be conveyed most effectively in a table. Other information can be conveyed most effectively in a figure. If you do decide to use a figure, then consider what type of figure will be most effective.

How to Use a Micropipettor

The micropipettor is used to transfer small amounts (< 1 ml) of liquids. The scales on micropipettors are in microliters (1000 µl = 1 ml). The micropipettors we will be use are made by Rainin and called "Pipetmen". They come in three sizes which are capable of pipetting three ranges of volumes: **P20** = 0.5-20 µl, **P200** = 20-200 µl, and **P1000** = 200-1000 µl. They are used in conjunction with disposable (often sterile) plastic tips; the smaller two micropipettors (P20 and P200) require the yellow tips and the P1000 pipettor uses the larger blue tips. The following is an illustration of a micropipettor:

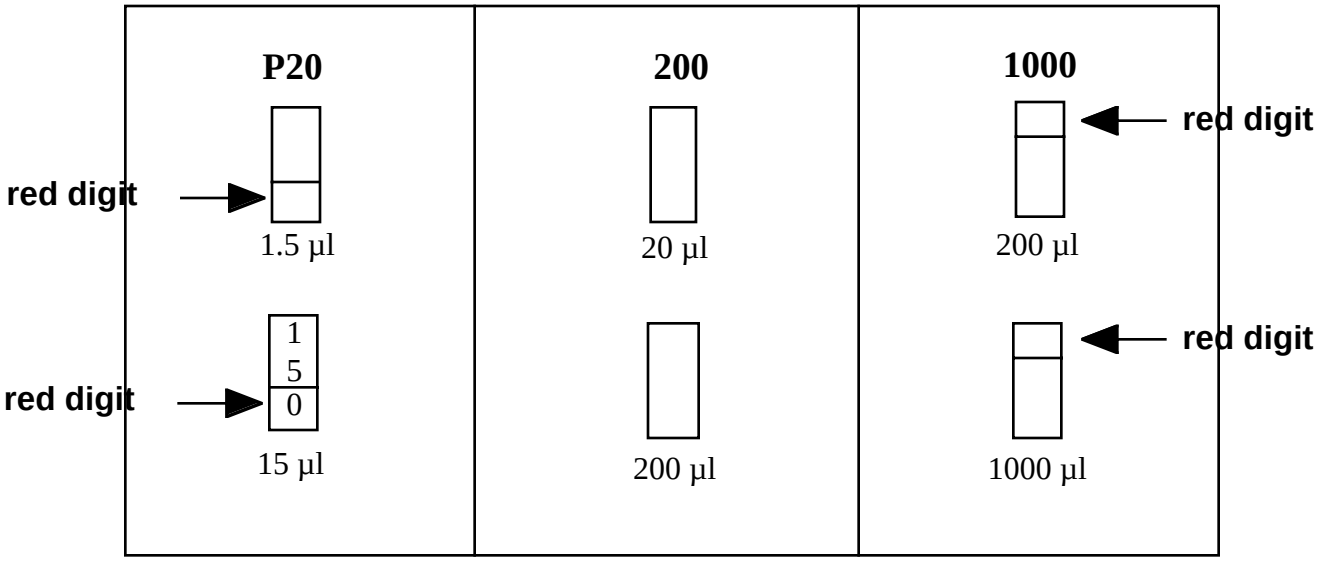


Directions for use of the micropipettor:

1. **Never exceed the upper or lower limits** of these pipettors. They are very expensive and delicate instruments which we cannot afford to damage. The limits are:

P20:	0.5 to 20.0 µl
P200:	20 to 200 µl
P1000:	200 to 1000 µl

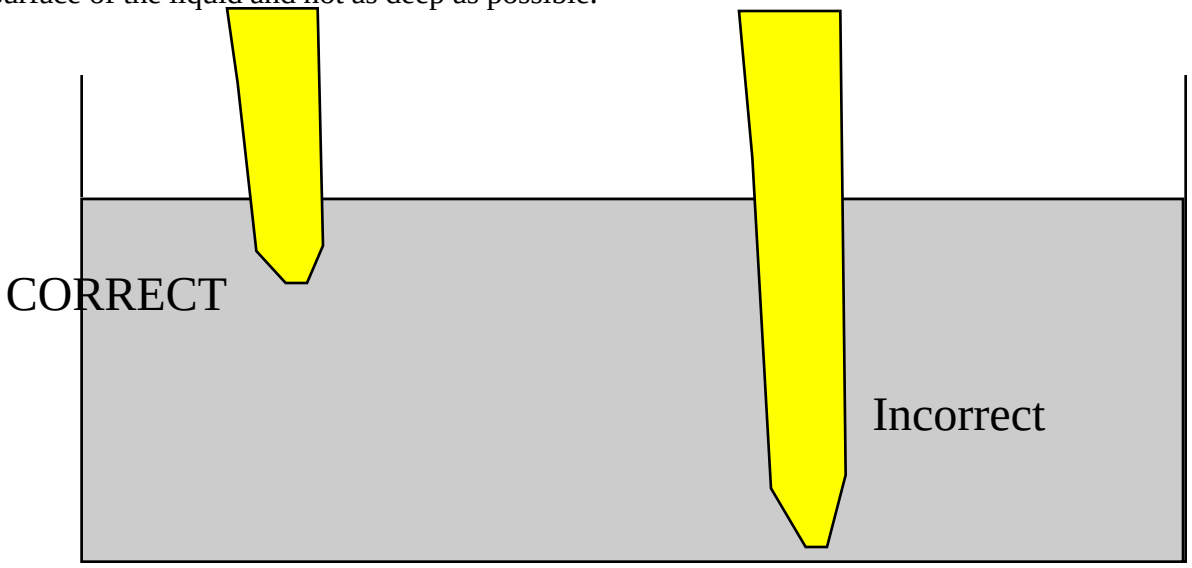
2. Set the desired volume by turning the centrally located rings clockwise to increase volume or counterclockwise to decrease volume. Some examples are provided below:



3. Place a tip on the discharge end of the pipettor. NOTE: If sterile conditions are necessary, do not allow the pipet tip to touch any object (including your hands).

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 and insert tip into the solution, just barely below the surface of the liquid and not as deep as possible.



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. Remove tip by pressing down on the tip discarder.

REMEMBER TO CHANGE TIPS BETWEEN SOLUTIONS TO AVOID MIXING OR CONTAMINATING THE SOLUTIONS USED!

Introduction to Spectrophotometry

Focused Reading: "Properties of Light"… pg. 138 – 140. Stop @ "Light absorption and biological…"
Bring a calculator to lab

Introduction
The purposes of this laboratory are to introduce you to:

1. Conventions used in making solutions: molarity, and per cent.
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: Remember to record your data, answers to questions and problems, and notes on the blank pages of this lab manual. Keep it beside you and write in it as you work.

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 back of your textbook (front inside cover of Purves text).

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:

- 1.0 M solution of NaCl has 58.54 g NaCl dissolved in dH_20 with a final volume of 1000 ml (or 5.85 g in 100ml).
- 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.
- 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. Note the differences in results as you think through the following mental exercise.

Experiment 1:

Here is a demonstration experiment for you to observe. The directions in Step 1 were followed exactly. Examine the graduated cylinder on the front bench and record the results in your lab manual.

Step number one has been done for you:
1. 5 grams (g) of sodium chloride (NaCl) was obtained and added to exactly 50 ml of distilled water (dH_20) in a graduated cylinder. The salt was stirred vigorously to dissolve it.
2. Determine the excess volume by reading the volume of the graduated cylinder. This procedure was a failed attempt to make a 10% w/v solution. The number of ml in excess of 50 represents the volume displaced by the 5 g of dissolved NaCl.
3. What is the actual percentage of NaCl (w/v)?

Concentrations in Percent (Volume/Volume = v/v)
Aqueous solutes may be specified as percent solutions volume/volume (v/v). 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 dH_20 (95 ml) to make a total of 100 ml (or by diluting 5 liters 100% ethanol with 95 liters dH_20).

Experiment 2:

On the front bench is a stock solution (1.0 M) of a dye, neutral red. Make 2 ml of a 4% v/v solution from this neutral red stock using dH_20. Label your tube and save this solution; you will use it later today.

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 passes (the incident light, I_0), a sample solution holder, a photosensitive tube which 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.

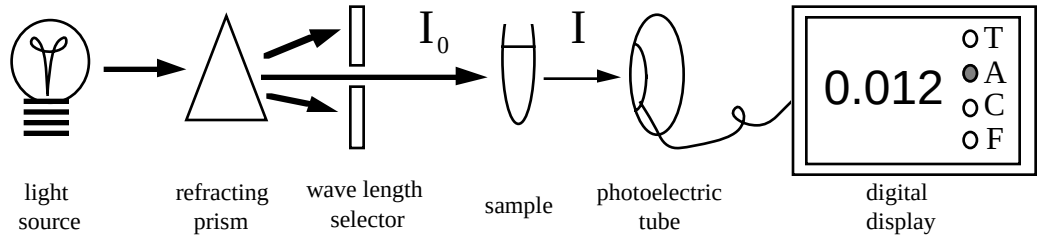


Figure 1. *A schematic diagram of the components of a spectrophotometer 20.* The arrows indicate the pathway of light.

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

$$\%T = (I \div I_o) \cdot 100 \qquad Abs. = \log_{10} (100/\%T)$$

Microplate Reader

Above 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 about 8 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 8 fiber optics light sources and 8 phototubes. Each row of 8 is scanned and then the plate advances by one row and the process continues until all 12 rows are scanned. The absorbance data then are displayed on a screen in an 8 x 12 array. These data can be saved in the memory to be printed later. This technology is based on the same principles as older spectrophotometers, but now we can measure more samples in less time. 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.

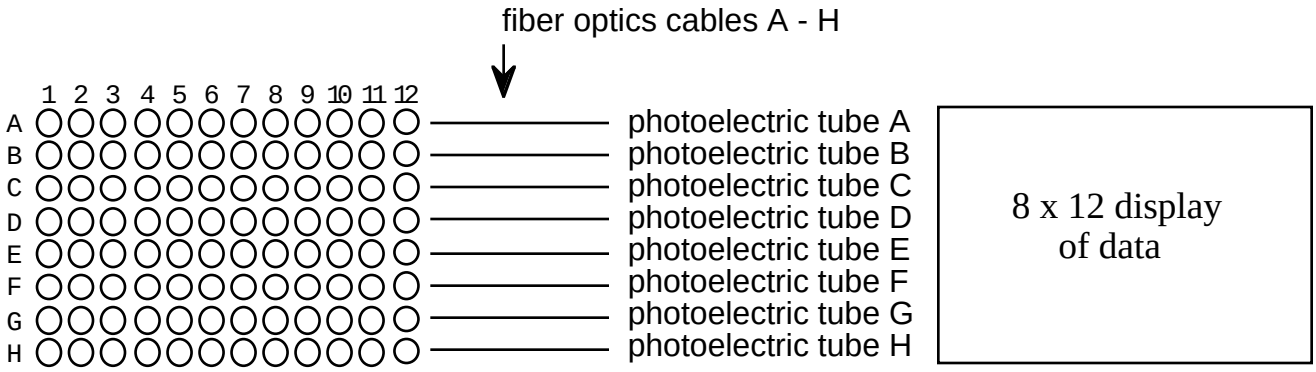


Figure 2. *Schematic diagram of a microplate reader.* Samples are placed in the 96 microwells, analyzed by the 8 channel spectrophotometer, and the absorbance data are displayed in the large LED window.

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.

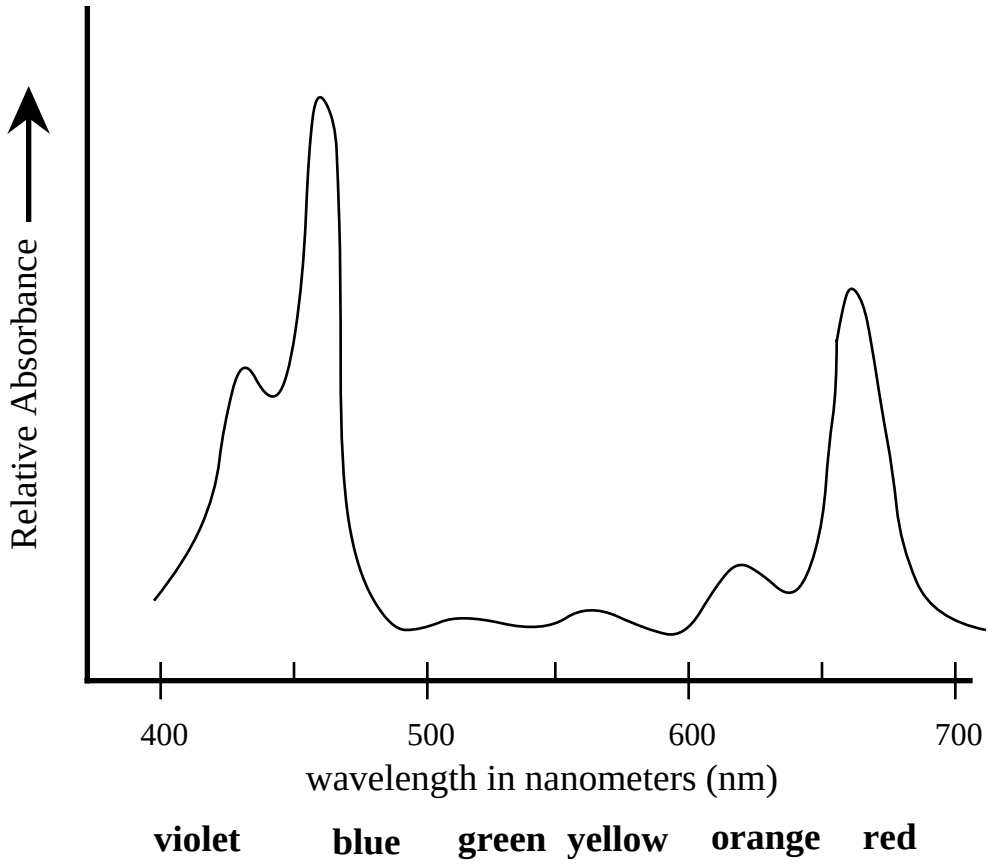


Figure 3. *The absorption spectrum of chlorophyll a.* The absorbance of visible light by chlorophyll a is measured spectrophotometrically as a function of wave length. The absorption maximum is about 460 nm. Compare with figure 8.7 on page 140 of Purves.

How to use a spectrophotometer to Answer Biological Questions

Standard Concentration Curve and Determining the Concentration of a Characterized 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 which 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.

Two 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.

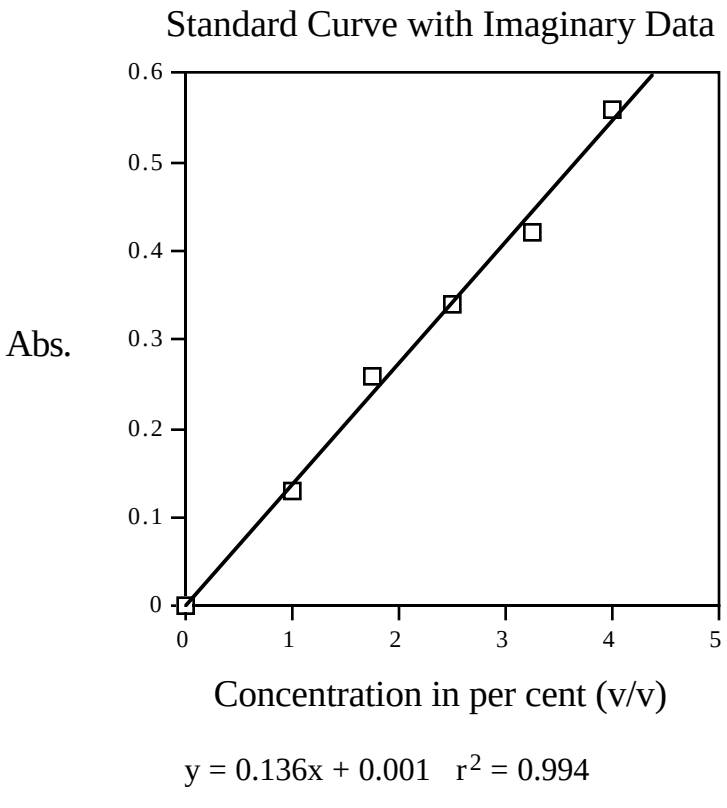


Figure 4. *This is an example of a standard curve.* A best fit line has been generated and the resulting equation and r^2 value are shown below the X-axis label.

Experiment 3:

Using the 4% (v/v) neutral red solution you prepared in experiment #2, set up the following solutions in microfuge tubes using distilled water available on the lab benches.

TABLE 1 Volumes of neutral red and distilled water used to prepare solutions for tubes 1 - 6. You must calculate the volumes and fill in the table for the first two columns before you begin the experiment.

Tube Number	Distilled Water (µl)	Neutral Red Solution (4%) (µl)	% Neutral Red Solution (v/v)	Concentration of Neutral Red (µg/µl) **	Concentration of Neutral Red (M) **
1	0.0	400	4.0		
2			3.25		
3			2.5		
4			1.75		
5			1.0		
6	400	0.0	0.0		

** These parts of the table should be completed when answering question #6 at the end of the lab.

Experiment 4:

Determination of absorption maximum.
Use tubes 1 - 6.
Put 200 µl of the six different dilutions of neutral red into six different wells (wells H7 - H12) of your 96-well plate.
You will collect the data for experiments 4 and 5 at the same time (see below).

Experiment 5:

Determination of a standard concentration curve for neutral red.
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.

Operation of the Microplate Reader

1. Turn on the spectrophotometer and let it warm up for 15 minutes.

There are two main variables which you must pay attention to while you use the plate reader: the **ANALYSIS NUMBER** and the **FORMAT NUMBER**. **Format** is easy because we will always use format #1, which simply means that all of the wells are to be analyzed for absorbance. **Analysis** is the list of options you want to use when performing your spectrophometric analysis of your samples. Specifically, you tell the machine which wavelength of light to use, to calculate the absorbance, to use a single wavelength of light, and to make only one reading for each time you press the START key. Today, we will use ANALYSIS NUMBERS 1- 6.

2. Before you collect any data, clear the memory of the plate reader. To do this:
press FUNCTION, CLEAR ALL; when it asks you if this is OK?,
press FUNCTION, CLEAR.

3. Start the reading of your samples by using all six wavelengths of light that the plate reader can use. At the end of each reading, you will get a printout of your absorbance data. When you have finished all six, tear off your paper, clean up any mess you created and remove your 96-well plate. To execute the analysis with all 6 wavelengths (1 = 340; 2 = 405; 3 = 450; 4 = 490; 5 = 595; and 6 = 655 nm):

a. press ANALYSIS, 1, ENTER, START. When the reading is complete,
b. press ANALYSIS, 2, ENTER, START. When the reading is complete,
c. press ANALYSIS, 3, ENTER, START. When the reading is complete,
d. press ANALYSIS, 4, ENTER, START. When the reading is complete,
e. press ANALYSIS, 5, ENTER, START. When the reading is complete,
f. press ANALYSIS, 6, ENTER, START. When the reading is complete,
g. remove your paper printouts, remove your plate and clean up any mess.

Analysis of the Data for Experiments 4 and 5

Experiment 4:
Now that you have several pages of data, what do you do next? Follow the order of the experiments, beginning with number 4; determine the absorption maximum for neutral red. Find the wavelength that produced the highest absorbance values. This wavelength is the absorption maximum for neutral red and all subsequent experiments should use only the data generated with this wavelength of light. To generate an abbreviated absorption spectrum, plot the absorbance for tube number 3 in a graph with the Y-axis as the absorbance value and the X-axis as the wavelength of light. This graph will be graph number 1.

Experiment 5:
Using the absorbance data that were generated with the absorption maximum, subtract the absorbance of the blank from the values for the samples. This subtraction corrects for the amount of light absorbed by the plastic and the water. Now, construct a graph with the X-axis as per cent neutral red (increasing from left to

right), and the Y-axis as absorbance at your selected absorbance maximum (e.g. Abs $_{666nm}$) using your adjusted absorbance values. Plot the results from tubes 1 through 6 and draw a straight line (best fit) to generate the standard concentration curve. This graph will be graph number 2.

After completing these analyses and generating two graphs, continue with experiment number 6 below.

Experiment 6:

Determination of the concentration of the unknown neutral red solutions.

Obtain four neutral red solutions of unknown concentration from your instructor and record the identifying letter in your lab manual. Determine the concentration of your unknowns by putting 200 µl of it in a well, and record the well's identifying letter and number (e.g. H3 - H6). Use the plate reader at the wavelength of light that is absorbed the best 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.

Analysis of the Data for Experiment 6

Using the standard curve (graph number 2), 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 absorbance (after subtraction) 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.

Experiment 7:

Determine the absorption maximum for NADPH and $NADP^+$.

Over the next two weeks, we will use NADPH and $NADP^+$, and we need to know which wavelength of light to use. You will want to perform a series of experiments similar to experiments 4 and 5. Record which wavelength is absorbed the best and verify your results with the instructor.

Experiment 8:

Clean up. Leave your workstation as you found it. Although this step may seem like a stupid thing to list in a protocol, 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).

Before you leave lab:

Make sure you have recorded all data and observations and plotted both of your graphs for this week:
1) Maximum absorption wave length of neutral red,
2) Standard curve, and determination of concentration of unknown solutions.
3) Determine the absorption maximum for NADPH.

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. Does it matter whether the proper volume of solute is added first or second (relative to the water) to the graduated cylinder?
6. The aqueous stock solution of neutral red is 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 µg/µl are contained in your 4% (v/v) solution? Calculate the concentration of neutral red in each tube (µg/µl) 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_2C0_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 #2
Isocitrate Dehydrogenase: Measurement of IDH Activity

Assigned Reading: "Enzymes…" pp. 102-106 Stop @ "Molecular Structure. . ."
Pg. 116, Fig 7.3 [reduction of NAD^+]

Bring a calculator to lab

Goals of this exercise:
With this session, we begin a three week study and discussion 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 the amount of substrate in the assay mixture affects rates of activity of an enzyme.
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 Mg^{2+} or Mn^{2+} 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 CO_2 from the six-carbon substrate to generate a five-carbon product, α-ketoglutarate .

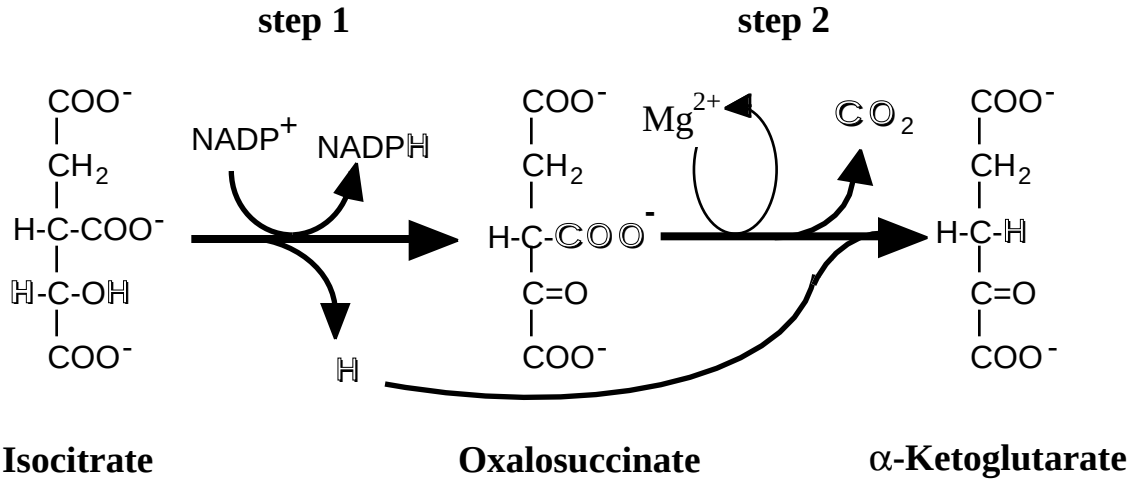


Figure 1. IDH catalyzes the sequential dehydrogenation and decarboxylation of isocitrate to α–ketoglutarate.

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 $NADP^+$ as its coenzyme (Figure 2). This $NADP^+$-dependent form of IDH is considered to be the only IDH in bacteria and is the most prevalent form of IDH in most plants and animals. In higher organisms, this form of IDH appears to be found in all organs and tissues. This form of IDH is used in lipid synthesis. The NAD^+-dependent form of IDH is limited to eukaryotic organisms and is localized in mitochondria. You may be familiar with this form of IDH from previous study of the Krebs cycle. Both forms of IDH require a divalent metal ion.

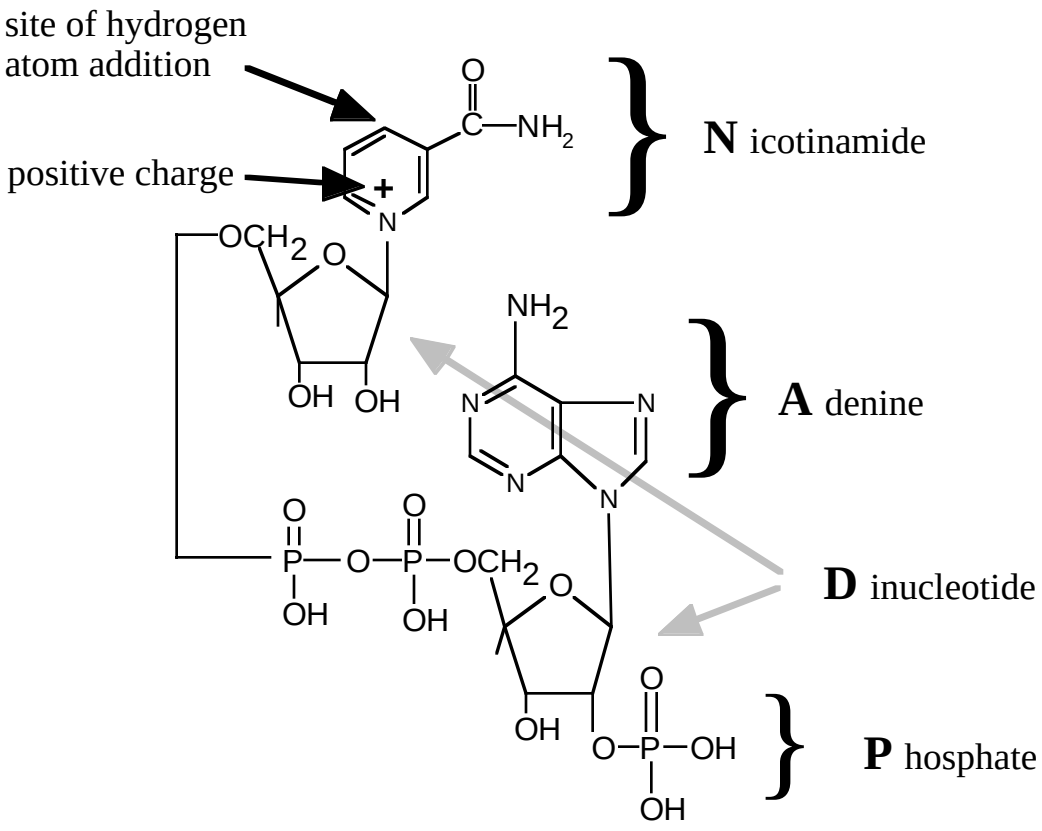


Figure 2. The molecular structure of $NADP^+$. The active site is where the hydrogen atom will be added to convert $NADP^+$ to NADPH. In NAD^+, the phosphate group is replaced with an H^+. This diagram illustrates what the letters N-A-D-P represent.

NEWS ITEMS: In June, 1996, 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. (See summary in *Science*. Vol 273. 19 July, 1996)

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.

Protocols

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.

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. How to Perform IDH Assays

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

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 **Table 1**. You should ask yourself "What is the purpose of each assay?" You also should ask why Assays 5 - 7 are identical.

Table 1: How to perform IDH assays.

Wells	Buffer	$NADP^+$	IDH	Isocitrate
A 1	200	**0**	**0**	**0**
A 2	180	10	10	**0**
A 3	180	10	**0**	10
A 4	180	**0**	10	10
A 5	170	10	10	10
A 6	170	10	10	10
A 7	170	10	10	10

All volumes are in µl. In this experiment, you will 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.

Step-by-Step Procedure

1. Use the P-200 micropipet to add Assay Buffer to the indicated wells.
2. Use the P-20 micropipet to add 10 µl of $NADP^+$ to all wells, except A1 and A4.
3. Use the P-20 micropipet to add 10 µl of IDH to all wells, except A1 and A3.
4. Place the microplate in chamber of the plate reader.
5. Use the Multi-8 micropipet to add 10 µl of isocitrate to all wells, except A1 and A2.
6. Activate the plate reader and use Analysis 7.
7. After printing, remove your plate from the plate reader.
8. Retrieve your data from the printer.

9. Return to your station and organize your data in the **Table 1a** (below).
10. Prepare a graph of your data.

Table 1a: Data from triplicate IDH assays.

Time, min	Well A1	Well A2	Well A3	Well A4	Well A5	Well A6	Well A7
0							
0.5							
1							
1.5							
2							
2.5							
3							

Considerations - Experiment 1

Compare your data from wells A1 through A7. Was there activity in wells A1 - A4? Was there activity in wells A5 - A7? Was activity the same in wells A5 - A7? Was activity linear for three minutes? If not, explain your observations. Do your data support your hypotheses? If not, how will you change the protocol? Determine the "corrected" reading for each assay by subtracting the reading of the "control", well A1 from the other readings. (Would well A2, A3 or A4 provide better "control" data?)

Construct a graph that visually portrays your data from wells A2 - A7 by plotting absorbance as a function of time (in minutes). The initial rate of a reaction may be determined from the slope of the line joining each successive point. This graph will be graph 1.

Use the directions for Excel in Appendix A in the back of the lab manual.

Experiment 2. Effects Of Varying Enzyme Concentration

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 B 1, B 2 and B 3 in Table 2 are triplicate assays containing 5 µl of IDH. Set up reactions as shown in **Table 2**.

Table 2: The effects of varying enzyme amounts.

Wells	Buffer	$NADP^+$	IDH	Isocitrate
B 1-3	175	10	5	10
B 4-6	170	10	10	10
C 1-3	160	10	20	10
C 4-6	150	10	30	10
C 7-9	180	10	0	10

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the indicated wells.
2. Use the Multi-8 micropipet to add 10 µl of $NADP^+$ to all wells.
3. Use the correct micropipettor to add appropriate volume of IDH to each well.
4. Place the microplate in chamber of the plate reader.
5. Use the Multi-8 micropipet to 10 µl of isocitrate to the wells.
6. Activate the plate reader.
7. After printing, remove your plate from the plate reader.

8. Retrieve your data from the printer.
9. Return to your station and organize your data in **Table 2a** (below).
10. Prepare a graph of your data.

Table 2a: Data from varied enzyme amounts.

Time, min	5 µl of IDH	10 µl of IDH	20 µl of IDH	30 µl of IDH	0 µl of IDH
0					
0.5					
1					
1.5					
2					
2.5					
3					

Considerations - Experiment 2

Compare the data from wells B1 through C3. Was there activity in all wells? Did activity vary with the amount of enzyme in each assay? Was activity the same 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 your observations. Do your data support your hypothesis?

Determine the mean activity for each set of triplicate assays. Construct a graph to portray your data. Compare activity with the volume of enzyme in the assay solution. [Hint - take advantage of Excel's ability to generate a formula for the best fit line: **y = mx + b**; **b** is the Y intercept and **m** is the slope or change in absorbance over time which is the definition of activity.] This graph will be graph 2A.

Construct another graph that compares volume of enzyme the slope of the three lines (slope equals enzyme activity) from your previous graph. You may use the table below to collect and organize the data. This new graph will be graph 2B. What conclusions can you reach from your results?

Volume of IDH (µl)	Slope	r squared value
0		
5		
10		
20		
30		

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.

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. **Table 3** 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.

Table 3: The effects of varying isocitrate concentration.

				Isocitrate	
Wells	Buffer	IDH	$NADP^+$	Sol*	Vol
E 1-3	170	10	10	1	10
E 4-6	170	10	10	2	10
F 1-3	170	10	10	3	10
F 4-6	170	10	10	4	10
G 1-3	180	10	10	-	0
G 4-6	190	0	10	-	0

*****The concentration of these isocitrate solutions will be provided by the instructor. The second number refers to the volume (µl) to be used.

Procedure

1. Use the P-200 micropipet to add Assay Buffer to the indicated wells.
2. Use the P-20 micropipet to add 10 µl of the different concentrations of isocitrate to the wells, as indicated.
3. Use the Multi-8 micropipet to add 10 µl of $NADP^+$ to all wells.
4. Place the microplate in chamber of the plate reader.
5. Use the Multi-8 micropipet to add 10 µl of IDH to all wells.
6. Activate the plate reader.
7. After printing, remove your plate from the plate reader.
8. Retrieve your data from the printer.
9. Return to your station and organize your data in **Table 3a** (below).
10. Prepare a graph of your data.

Table 3a: Data from varied isocitrate concentrations.

Time, min	____mM	____mM	____mM	____mM	____mM
0					
0.5					
1					
1.5					
2					
2.5					
3					

Considerations - Experiment 3

Compare the data from your experiment. Determine the activity for each concentration of isocitrate by constructing a graph and generating the best-fit lines and equations – this graph will be graph 3A. Next, construct a graph that compares activity as a function of isocitrate concentration. This graph will be graph 3B. Do your data support your hypothesis? Is the relationship between activity and concentration of substrate linear? Explain this relationship, referring to graphs 3A and 3B.

Preparation for Next Week's Lab:

In 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:

1. What are the effects of temperature on the stability of IDH?
2. What are the effects of pH of the assay solution?
3. What are the effects of $NADP^+$ concentration?
4. What are the effects of different divalent metal ions?
5. What are the effects of varying salt concentrations?
6. Which species or tissues have the most activity?

Before leaving lab today, each group will complete the following:

1. Develop a clear, concise and simple hypothesis about the effects of one of the above environmental conditions upon enzyme activity.
2. Design an experiment to test that hypothesis.
3. Prepare a protocol to carry out that experiment.

Lab #3
Parameters that Affect IDH Activity

Focused Reading: "Molecular Structure…" pp 106-108. Stop @ "Metabolism and. . ."
"Enzyme activity…" pp 108-109. Stop @ "Allosteric enzymes…"
"Enzymes and their…" pp 111-112. Stop @ "Summary…"

Bring a calculator to lab.

Goals for this Lab:

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

I. Introduction

Last week we:

1. Learned how to perform isocitrate dehydrogenase (IDH) assays.
2. Examined the relationship between activity and amount of enzyme in an assay.
3. Examined the relationship between activity and substrate concentration.
4. Learned how to present experimental data in graphic form.
5. Chose one of the following experiments (Options A –F) to complete.
6. Designed an experimental protocol for that experiment.

II. Methods and Materials

We will use the same general methods that we used in Lab #2. All equipment, solutions and supplies required to carry out the experiments have been prepared and are ready for use. You may wish to review your protocol again and assign specific tasks before you start your experiments.

Option A: The Effects of pH on IDH Activity

Hypothesis: pH of the assay buffer will have no effect on IDH activity.

To test this hypothesis, we will need to follow a protocol that holds all conditions constant except the pH of the assay buffer.

Table 4: The effects of varying pH of the assay buffer.

Wells	pH	Buffer	$NADP^+$	IDH	Isocitrate
A 1-3					
A 4-6					
B 1-3					
B 4-6					
C 1-3					
C 4	Blank				

Table: Data from varying pH of the assay buffer.

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

Considerations - Experiment on pH

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?

Hypothesis: IDH activity does not require a divalent metal in the assay solution.

To test this hypothesis, we will need to follow a protocol that holds all conditions constant except for the presence or absence of divalent metal ions in the assay solution.

Table 5: Does IDH Have a Metal Ion Requirement?

Wells	**Buffer ****	**Metal, µl**	$NADP^+$	IDH	Isocitrate
D 1-3		None			
D 4-6		EDTA 10			
E 1-3		Mg^{2+} 10			
E 4-6		Mn^{2+} 10			
F 1-3		Ca^{2+} 10			
F 4-6		Zn^{2+} 10			
G 1		Blank			

****You will want to use a buffer that does not contain any added Mg^{2+}**

Table 5a: Data from cofactor experiment.

Time	None	EDTA	Mg^{2+}	Mn^{2+}	Ca^{2+}	Zn^{2+}
0						
0.5						
1						
1.5						
2						
2.5						
3						

Considerations - Experiment on metal ions

Does IDH require a divalent metal ion for activity? Does additional Mg^{2+} added to the standard assay buffer increase activity? What does this observation mean? Does the addition of Mn^{2+} added to the standard assay buffer increase activity? What does this observation mean? How can the effects of Ca^{2+} and Zn^{2+} on IDH activity be explained?

Option C: The Effects of Temperature on IDH Stability

Hypothesis: Exposure to 37° C will have no effect on IDH stability.

To test this hypothesis, we will need to follow a protocol that holds all conditions constant except temperature. We can incubate samples of IDH at 37°C for 0, 2, 4, 6, 8, 10 min prior to assaying activity. Keep all samples on ice until incubations are complete and assay at the same time.

Table 6: Does exposure to 37°C affect IDH stability?

Wells	**Min, 37°**	Buffer	$NADP^+$	IDH	Isocitrate
D 1-3					
D 4-6					
E 1-3					
E 3-4					
F 1-3					
F 4-6					
G 1	Blank				

Table 6a: Data from temperature experiment.

Time, min	**___Min**	**___Min**	**___Min**	**___Min**	**___Min**	**___Min**
0						
0.5						
1						
1.5						
2						
2.5						
3						

Considerations - Experiment on temperature pre-treatment

Is IDH stable at 37°C? How can these results be explained? How could this experiment be altered to test the hypothesis further?

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

Problem: 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 an hypothesis and sketch a graph predicting the relationship of activity *vs.* coenzyme concentration.

Table 7: The effects of varying the concentration of $NADP^+$.

Wells	Buffer	**$NADP^+$**	IDH	Isocitrate
D 1-3				
D 4-6				
E 1-3				
E 4-6				
F 1-3				
F 4		0		

*The concentration of these $NADP^+$ solutions will be provided by the Instructor. The second number refers to the volume [µl] to be used. NOTE: In these assays, we will initiate reactions with isocitrate. Do not add isocitrate until your plate is in the plate reader.

Table 7a: Data from varying the concentration of $NADP^+$.

Time, min	**_____mM**	**_____mM**	**_____mM**	**_____mM**	**_____mM**
0					
0.5					
1					
1.5					
2					
2.5					
3					

Considerations - Experiment varying $NADP^+$ concentration

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: The Effects of NaCl on IDH Activity

Hypothesis: Sodium chloride will have no effect on IDH activity.

To test this hypothesis, we will need to follow a protocol that holds all conditions constant except concentration of NaCl in the assay solution.

Table 8: The effects of NaCl (0 – 1M) on IDH activity.

Wells	Buffer	5M NaCl	[NaCl] M	$NADP^+$	IDH	Isocitrate
D 1-3						
D 4-6						
E 1-3						
E 4-6						
F 1-3						
F 4-6						
G 1			Blank			

Table 8a: Data from the effects of NaCl on IDH activity.

Time (min)	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5	Conc. 6
0						
0.5						
1						
1.5						
2						
2.5						
3						

Considerations - Experiment on salt concentrations

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: Does IDH activity vary among different organisms or tissues?

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. We 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 Assay Buffer, using a kitchen blender.
3. Filter the homogenate through two layers of cheesecloth into a small beaker that is on ice.
4. Transfer 1 ml samples to 1.5 ml microfuge tubes, spin for 5 minutes.
5. Transfer the supernatant to clean 1.5 ml microfuge tubes, on ice.
6. Use standard conditions to assay IDH activity.

Before You Leave Lab

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.
3. Schedule a meeting of your group to analyze your results and prepare slides for your group's oral presentation.

LAB #4

What is your genotype? A PCR Answer

Focused Reading: "The polymerase…" pp 214-217
"DNA fingerprinting…" 328-329
"Gel electrophoresis…" pp312-313

WWW Reading: http://www.bio.davidson.edu/courses/bio111/Bio111.html
View the two PCR movies.
The first PCR movie requires that your computer have QT 3.0
The second PCR movie requires the plugin for Shockwave.

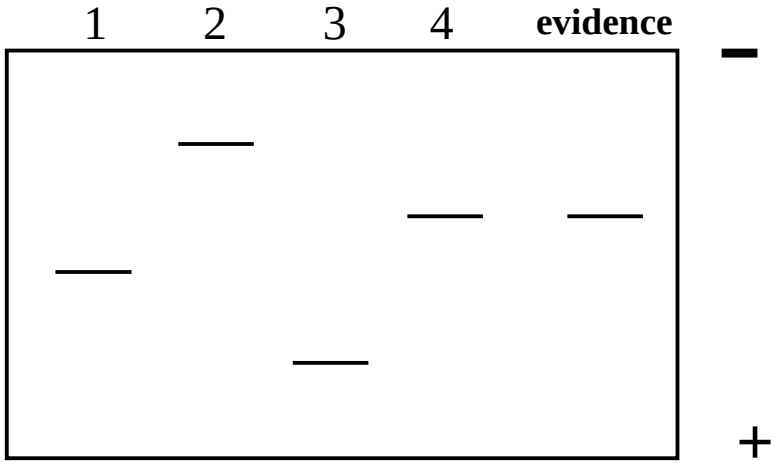
I. Introduction

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 have discussed Southern blots repeatedly in class but have not covered PCR in much detail. 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 D1S80. D1S80 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 D1S80 locus from 4 different DNA sources, we might see the following:

1) ATGCCGTATTACG**CG**CG**CG**CG**CG**CGCCTATTAGGTATTAG
2) ATGCCGTATTACG**CG**CG**CG**CG**CG**CG**CG**CG**CG**CCTATTAGGTATTAG
3) ATGCCGTATTACG**CG**CG**CG**CCTATTAGGTATTAG
4) ATGCCGTATTACG**CG**CG**CG**CG**CG**CG**CG**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?

II. Protocol

Week 1

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:

1) Follow the protocol as carefully as possible.
2) Do not contaminate your hair or DNA with that of others (remember one cell contains enough to be amplified).
3) 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 should see a more dense of the solution at the bottom of the tube as it mixes with the less dense water.

4) The most common mistakes are pipetting errors. Be sure to check that you are transferring about the right volumes and always use clean tips; when in doubt a new one.
5) Be very careful loading the gel. We will have time to practice this week so that when you are loading your real sample next week, you will be a pro.

DNA extraction[1]

1) Pluck a hair so that a large portion of root is removed from your head (yikes!) For most of you, the root will be white/translucent in appearance. People of African heritage will have roots that are dark. Regardless of the color, it 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 (~5mm). 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:

Reagent	Volume	Final concentration
Extracted DNA	15.0 µl	~ 100 ng of DNA
Reaction mixture	10.0 µl	see below **

**the reaction mixture contains the following cocktail:

Reagent	Volume	Final concentration
H_2O	4.00 µl	
10X PCR buffer (without Mg)	2.50 µl	1.5 mM $MgCl_2$
DMSO	1.25 µl	5% v/v
20X dNTPs (dATP, dTTP, dCTP, dGTP	1.25 µl	200 µM each
#1 primer	0.50 µl	100 ng primer
#2 primer	0.50 µl	100 ng primer
Total Volume	**10.00 µl**	

PCR

The D1S80 locus requires hot start PCR. This term means that the Taq DNA polymerase is not added to the PCR mixture until the mixture has been heated to 95˚C. This hot start is necessary because the D1S80 primers have a tendency to anneal to each other rather than to

the template while the mixture is heating up for the first time. This tendency allows the DNA polymerase to generate "primer dimers". If addition of the DNA polymerase is delayed, then inappropriately annealing primers are denatured as the kinetic energy increases, so no replication occurs until the temperature is lowered later in the procedure, allowing the primers to anneal to the proper portion of the template DNA. DMSO has been included in the reaction mixture to enhance the specificity of the primers.

The PCR temperature conditions are as follows:

Step 1: 5 minutes at 95˚ C (pause during this step for hot start – see below)
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

5) To initiate hot start PCR, denature the DNA by incubating the tubes for 5 minutes at 95˚ C (Step 1), maintain the tubes at 95˚ C while you add 0.4 µl Taq DNA polymerase to each tube. Do not allow the tubes to cool and do not take time to mix the reaction mixture after adding the Taq polymerase.
6) Resume the same PCR program with the heated lid enabled.
7) When the PCR is completed, the tubes are removed and stored at 4˚ C until next lab meeting.

D1S80 factoids

- >80% of all populations 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 sequences[2]:

#1 5' GAAACTGGCCTCCAAACACTGCCCGCCG 3'
#2 5' GTCTTGTTGGAGATGCACGTGCCCCTTGC 3'

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 D1S80 by the PCR followed by high-resolution PAGE. *American Journal of Human Genetics* 48: 137 – 144.

LAB #5

The PCR Results

Add 2.5 µl of the 10X loading dye to each PCR reaction tube and 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 and appropriate conditions can be refined accordingly.

0.5X TBE
45 mM Tris-borate
1 mM EDTA use 0.5 M stock that is pH 8.0

While the gel is running, we will learn how to calculate the molecular weights of bands on a gel. Turn to the next page to begin this portion of the experiment.

How to calculate the MW of a molecule that has been separated in a gel.

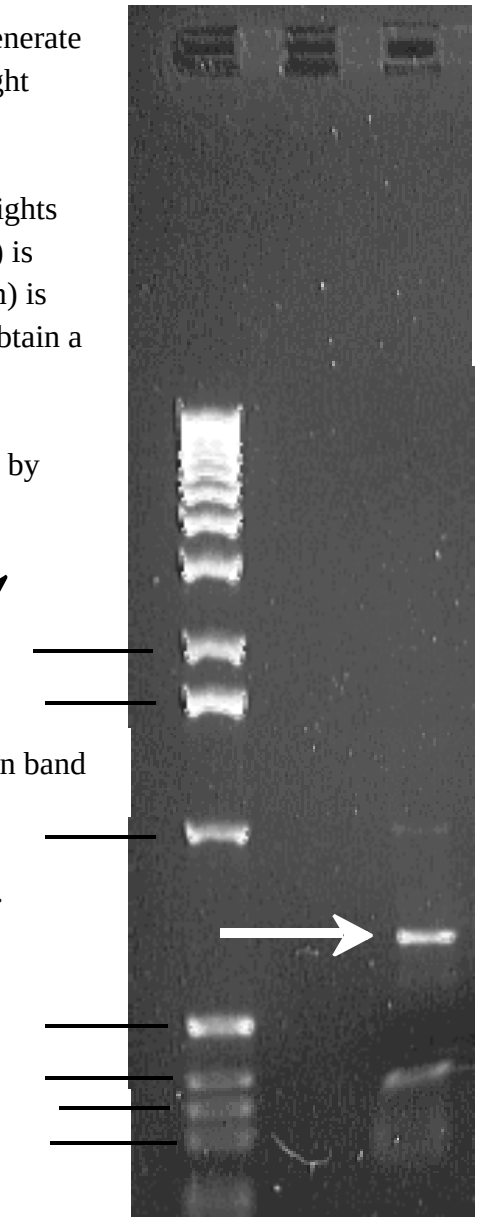
The $\log_{10}$ of a molecule's molecular weight is proportional to the distance that molecule has migrated. Therefore, the first step is to generate a standard curve using molecules of known size (the molecular weight markers).

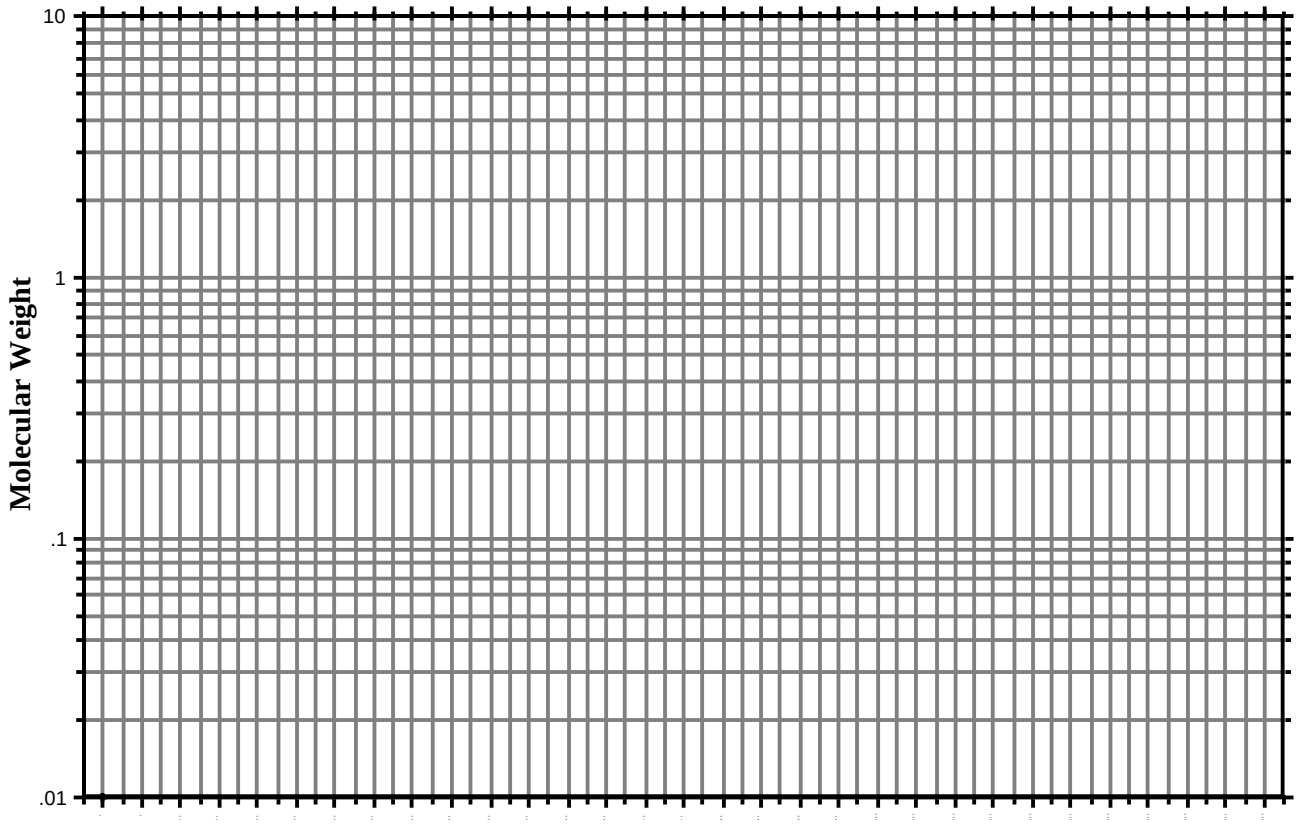
When using semilog paper (see the next page), the molecular weights (in units of base pairs (bp) for DNA; kiloDaltons (kDa) for proteins) is plotted on the Y-axis and the distance the molecule migrated (in mm) is plotted on the X-axis. When generating a standard curve, you will obtain a straight line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

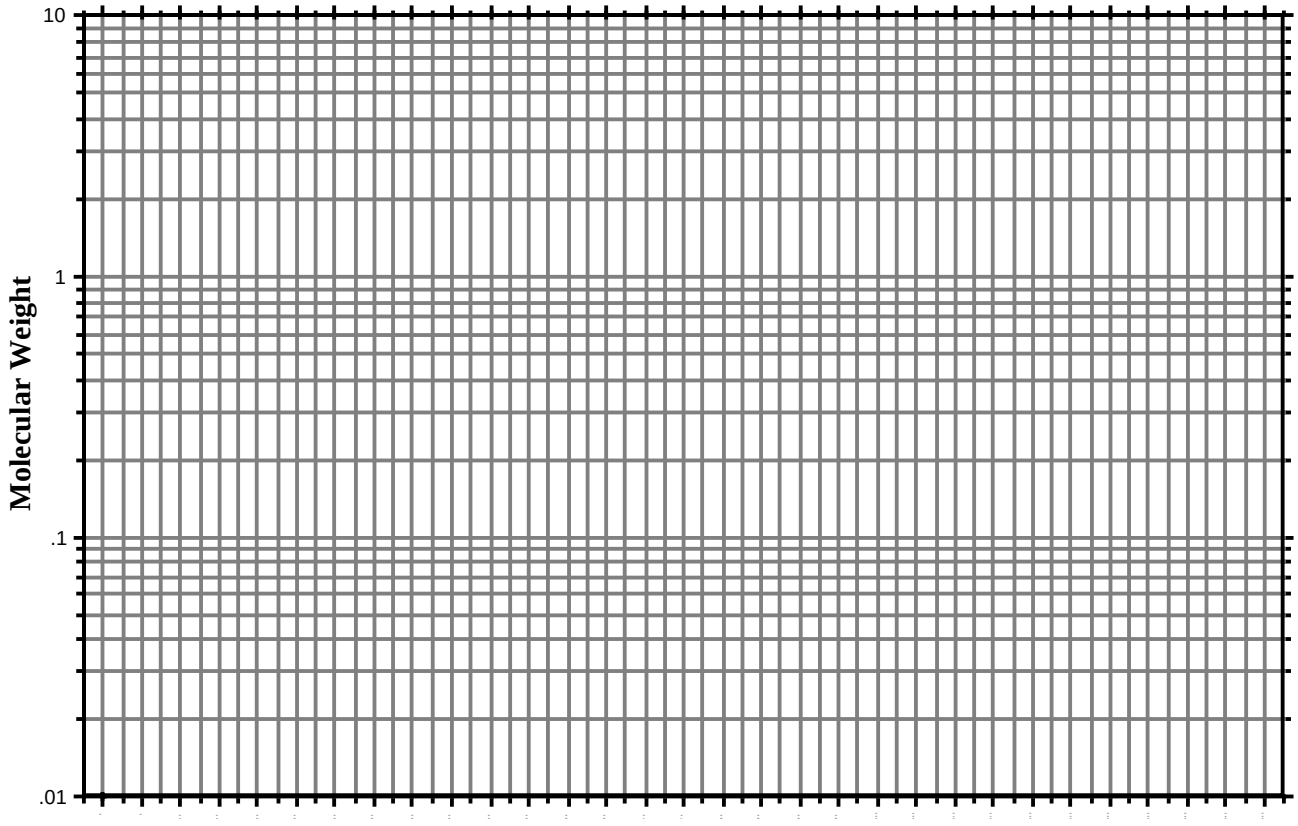
Once your standard curve is ready, measure the distance traveled by your molecule of interest. Find that distance on the X-axis, and go up until you intersect with your standard curve. Move over to the Y-axis and that will indicate the molecular weight of the molecule you are studying.

Use the first graph paper 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 second graph is for you to use on your PCR DNA "fingerprint".




10
1
.1
.01
Molecular Weight


10
1
.1
.01
Molecular Weight

LAB #6
Using Microscopes

Focused Reading: **"**Microscopes:…" pp 56-57.
Figures 4.1 (pg. 56) and 4.3 (pg. 57).

Goals for This Exercise

During this session, we will learn how to use a compound microscope that has the ability to view specimens in bright field, dark field, and phase-contrast illumination. We 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.

I. 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 his or her microscope and to gain proficiency in the use of the instrument.

Dos and Don'ts

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.

II. 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 scopes 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.

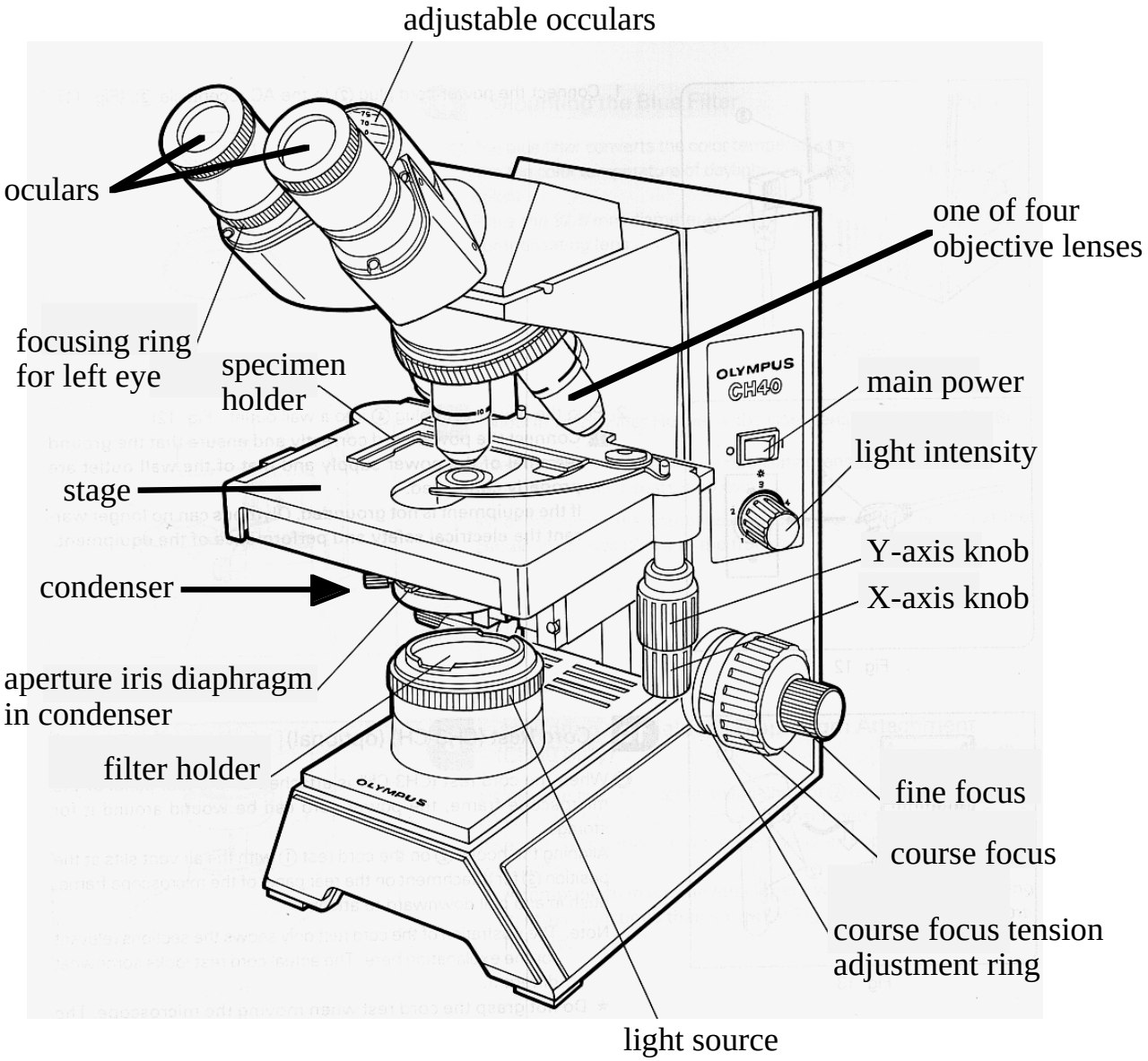


Figure 1. Diagram of a compound phase-contrast microscope, with labeled parts. Although microscopes are built to be rather sturdy, they should be handled carefully. The microscopes that we will be using are new and of high quality. They were purchased with grant money from the National Science Foundation.

Different Types of Microscopy: Bright Field, Dark Field, and Phase-Contrast

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.

III. 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.

<u>Bright Field</u>

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

11. 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 4 objective lenses.

What structures can you see now that you could not see in bright field?
What is difficult to see in dark field that was easy to see in bright field?

Phase-Contrast

12. 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.

13. Select the appropriate objective lens and phase ring pair. You might need to increase the amount of light since images do not appear as bright in phase. Once you have done this, you should adjust the

condenser vertically with the knob on the left side of the condenser. Once these adjustments are made, using a phase-contrast microscope is similar to using a bright field scope.

What structures can you see now that you could not see in bright field? Dark field?
What is difficult to see in phase-contrast that was easier to see in bright field?
Do you see the same colors in phase that you saw in bright field? Dark field?

A Series of Experiments on *Chlamydomonas* Mating

Focused Reading: Pp. 492-494 "*Chlorophyta.*"
"Cell Adhesion" pp. 82-84. Stop @ "Specialized Cell Junctions."
Figures 27.26, 27.27 (pg. 493) and 5.5 (pg. 83).

Overview

Over the next three weeks we will become comfortable with a fundamental tool in biology - the compound microscope. We will conduct a series of experiments on a unicellular green alga, *Chlamydomonas reinhardtii*, or Chlamy for short. Chlamy is a biflagellated green plant that reproduces asexually (by mitosis) and sexually (via meiosis, mating, and zygote formation).

A Word About Cooperative Learning

The laboratory is a place where scientists (that includes you) come together to work as teams and talk about methods, results, and conclusions. It also is a place to assert yourself, take responsibility for your own education, and trust your common sense. For example, we will view cells that are immobilized which requires us to kill the cells by chemically **cross-linking** the all proteins, euphemistically called **fixing** the cells. The other three people in your group may suggest that you pour the Lugol's fixative solution into your one and only supply of cells even though you also are supposed to mate LIVING cells later in the lab. Unfortunately, you have been cursed with short-sighted lab mates. To you, it seems obvious that you cannot kill all the cells in step 3 if you need live ones for step 7. So, you assert yourself and persuade your lab mates that they have made a miscalculation (this technique works better than calling them idiots, even if they are). This situation is not confrontation but cooperative learning. Each of you can be both student and teacher if you **think** while you are in the laboratory; don't just hurry through in order to finish. What you learn from each other in the lab is just as important as what you learn in the class. That is why laboratory material is tested in the "lecture" reviews.

Background Information on Chlamy

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 2 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 since 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.

Why would anyone want to know how efficient Chlamy sex is? If you are trying to study the process of gametogenesis at the molecular level, you would need to be able to compare wild-type mating to abnormal, or mutant, mating. To compare these two, you might use mating efficiency as an indicator of the ability of a gamete to mate. Because human subjects are reluctant to submit to experiments such as these, especially experiments on mating efficiency, you would be forced to find an alternative organism to study and one that has a short generation time. For example, imagine you are trying to learn how gametes fuse and you decide to generate a mutant strain of Chlamy that cannot fuse. (There are several strains like this and some Davidson students are conducting their honors research on them.) Once the mutant is generated, you can try to clone the gene that has been altered, which would allow you to identify the gene that encodes the "fusing gene". Maybe a new contraceptive could result from this research.

Chlamy cells come in 2 sexes called **mating-type plus** (**mt^+**) and **mating-type minus** (**mt^-**). When a mitotically dividing cell is deprived of nitrogen, it differentiates into gametes; mt^- cells differentiate into *minus* gametes and mt^+ cells differentiate into *plus* gametes. These two gametes of opposite sexes will fuse to form a diploid zygote that becomes a metabolically inactive spore. When conditions are favorable for mitotic growth (i.e. there is enough nitrogen), the zygote spore undergoes meiosis and germination to produce a tetrad of four haploid progeny: two mt^+ cells and two mt^- cells. We will be working with *plus* and *minus* gametes in today's exercise.

Protocol

A reminder

Objective Lens	Phase Ring
10X, 20X (red lines)	10
20X (no red lines)	cannot be used with phase
40X (red line)	40

Each person should:

A) Place 25 µl of *minus* gametes on a clean (use a Kimwipe) glass microscope slide and cover with a coverslip. Do not press down on the coverslip or else 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?
4. Do you see any other colors besides green? If so, where and what organelle could this structure be? (Hint: "The better to see you with, my dear.") Try all 3 forms of illumination.

C) On the same slide but separate from the previous sample, place 12.5 µl of mt⁻ cells into 12.5 µl 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]. Examine this preparation of stained cells under the microscope. View the cells at all three magnifications with each form of illumination.

1. What structure(s) can you see better with fixed cells than with live, unstained cells? Give 2 possible reasons why.

As a group:

D) In a microfuge tube, mix 150 µl of *plus* and 150 µl *minus* gametes and record the time.

Each person should:

1. Take out a 25 µl subset, or **aliquot**, of the mating cells shortly after you have mixed them together and observe these mating cells (still alive) under the microscope using the 10X and 40X objectives. Choose the form of illumination that will allow you to see the flagella the best.
2. What is going on? Describe how Chlamy cells mate. Pay special attention to the tips of the flagella.
3. Using phase-contrast and the 40X objective, look carefully for some round gray objects floating around. What are these?
4. After the cells have mated for at least 15 minutes in the original tube, take an aliquot of mating cells and fix them in Lugol's stain. Record the amount of time the cells have been mating. Observe these stained cells using the 40X objective lens and phase-contrast.
5. Do you see any cells that look like diploid **zygotes** instead of haploid gametes?
6. What 2 or 3 features are noticeably different in zygotes? (Look carefully for a cell that is different from the haploids you have looked at until now.)
7. Each person should count the first 25 cells with flagella that you see and "score" them as either gametes or zygotes. Record the number of each type of cell. Try to be random in your selection of cells to count; do not hunt for one kind of cell over the other.
8. Compare your data with the data from the other 3 people in your group. Did you all get the same numbers?
9. Determine the % mating efficiency using the formula below. Write your results on the board.

% mating efficiency = 2(no. of zygotes) ÷ [2(no. of zygotes) + (no. of gametes)]

or written in words:

% mating efficiency = no. gametes fused ÷ [total of all gametes in original mixture]

10. Do your numbers agree well with your colleagues in other groups?

11. Can you imagine any reason why your results would vary significantly from another person's?
12. What modification to your technique would you make to avoid this problem next time (like next week!)?

Turning off the scope

1) Turn the light down to zero.
2) Turn off the power.
3) Rotate the 10X objective in place and turn the condenser to bright-field.

Before you leave the lab, you should know the following:

- How can you see flagella better without staining them?
- What colors do you see in a Chlamy cell and what structures are responsible for the colors?
- How do you calculate the total magnification you are using on a microscope?
- With what appendage(s) do Chlamy cells mate? How efficient was this mating process?
- How can you standardize your methodology so that 2 people can get similar results when counting cells?

Before next week, your lab group should meet so that you can be prepared for next week's lab. Timing will be very important. You can meet at the end of session today or later.

Lugol's Fixative (protect from light and made fresh each semester)
1 g iodine
2 g K^+ Iodide
12 ml H_2O
dissolve the KI first, then add the iodine
filter undissolved crystals and store in a dark container

LAB #7

How long does it take for *Chlamydomonas* to regenerate its flagella?

Adapted from Vanderwalle and Heyes, *Journal of Biological Ed.*, (1993) 27(2): 125-129.

Focused Reading: "Microtubules…" pp 74-75. Stop @ "Centrioles. . ."
Figures 4.21 (pg. 72) and 4.24 (pg. 74).

Goals for this Session:

During this session, you will collect data on the regeneration of flagella on Chlamy. These cells will have been deflagellated before you come to lab and you will measure the length of the flagella over a one hour time period. You will learn how to use a version of NIH Image (called Scion Image) and the computer to capture images of the cells and measure their flagella on the computer monitor.

This set of experiments requires a lot of teamwork. You should have decided who will do which job(s) in order to make the necessary observations, and record all the information. [However, in the interest of your own edification and getting more bang for the buck, each person should sneak time to make observations for yourself because there will be test questions which are based on your laboratory work.] Do not waste time at the beginning of lab. The cells have just lost their flagella which means they cannot follow the best light in order to eat, or escape predators. They will start regenerating their flagella ASAP. On the other hand, do not begin the experiments below until you are ready - you may have to repeat the entire process if you begin before organizing yourselves because, "I thought you were keeping the time!"

You will want to measure the length of the flagella as a function of time. At each time point, two people should each measure the length of 20 flagella (total of 20 flagella on 20 cells). Once the cells have been fixed with Lugol's solution, the data are safe and you do not need to rush. With this point in mind, you might want to rotate the job of measuring flagella. You should organize yourselves so that aliquots are fixed every 15 minutes. You will use the same ingredients, **reagents**, as last week so stain them with Lugol's to fix the cells and flagella and to enhance visualization.

Detailed Protocol:

A) I have laboriously removed the flagella from about 5.0 X 10^{12} cells

How many flagella did I pile up? (For those with time on their hands, how close to the moon would this stack go if they were placed end to end?)

B) Because there are 4 people in a group, each person should have a job:

#1 - One person should record all the data and make sure that no time points are missed.

#2 - One person should be the time keeper and fix all the aliquots at the right time.

#1 and #2 should prepare all the slides for #3 and #4. Do not make the slides until they are ready for them. If you leave Chlamy cells under a cover slip too long, they will pop off their flagella again.

#3 and #4 - Two people should measure the length of the fixed flagella.

Turn to Appendix B to read how to use NIH Image to measure flagella length.

C) Person #1: set up 5 microfuge tubes and label them 0 - 60 at intervals of 15. In each tube there should be 50 µl of Lugol's fixative. Have a table ready that has spaces for all the data he or she will need to record.
Person #2: As soon as your group is ready, add 50 µl of deflagellated cells to the appropriate microfuge tube and move the flask back to the light shelf. Call this time point time zero. This measurement is the starting length of the flagella.
People #3 and #4: measure the flagella lengths from the fixed cells; 20 flagella from 20 cells.

D) This cycle of events should happen every 15 minutes, so there will be a total of 5 time points and data for each. **DO NOT** record averaged data in your lab book. Enter the raw data; there will be time to average the results later and a good scientist keeps all data, not just the averaged results. If you were doing AIDS research and you recorded only averaged results, you might find yourself in jail.

E) After one hour has passed, you should have collected all the data. The last step in any experiment is to clean up. You should clean your area, turn off the microscopes, throw away any trash, and return any equipment to where you found it. This step is important because so many people use this equipment and room. If you get an opportunity to work in a research lab, you had better clean up and return things properly or you may find yourself unemployed.

time	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																				

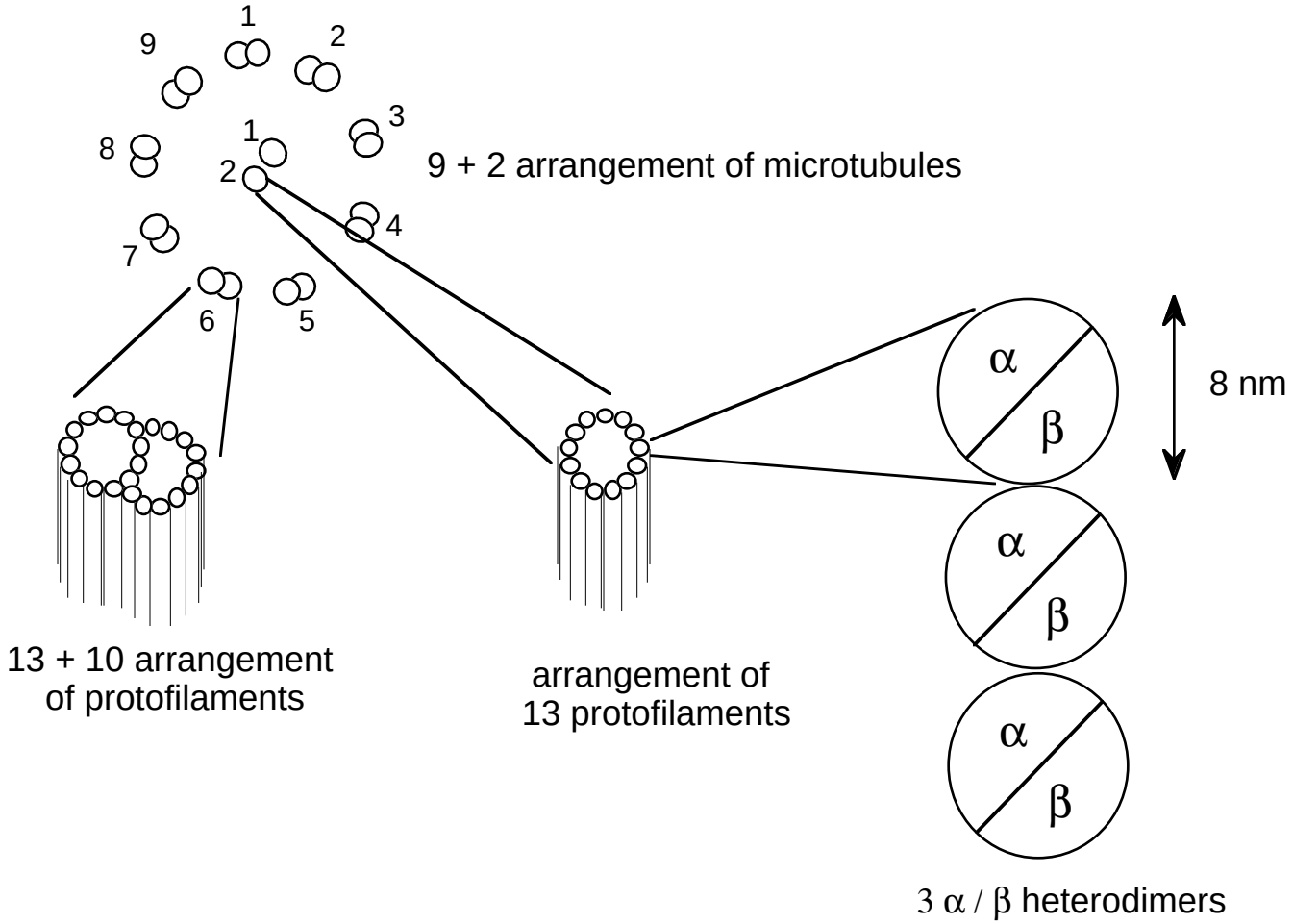
General Information about *Chlamydomonas* Flagella

To remove the flagella of Chlamy, you can either have great eye-hand coordination, or use the pH shock method. As it so happens, Chlamy 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 Chlamy 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 Chlamy cells, they shed their flagella. Hypothesize what is going on when Chlamy 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 how long it takes pond scum to regrow its flagella. Chlamy 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 Chlamy flagella.

There is an interesting story of colossal proportions that you must figure out. As you read in Purves, the flagella are comprised of many different proteins (about 200 different proteins are required to make a normal flagellum) but the predominant protein is **tubulin**. Each Chlamy flagellum is built upon the 9+2 structure of microtubules (see the figure below). The outer 9 microtubels 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 (9x(13+10)) + (2x13) 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 Chlamy is haploid so one allele = one gene = one locus.

Diagrams of Flagellar Structure at Increasing Magnifications



Compare with Figures 4.21 and 4.24 in the Purves text.

Here are some questions for you to answer:

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.

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 they grow at 0.17 μm per minute. Once you have determined the rate in your experiment, you can try the calculations again.

Before the end of lab

At this time, each laboratory group has to 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? Would gametes (in G_0) regenerate flagella faster or slower than vegetative (mitotically active) cells? What would happen in the presence of added ATP? caffeine? glucose? amino acids? EGTA? Once you have found a question that interests you, you then should use your knowledge of molecular and cellular biology to formulate an answer to your question. For example, if we block translation with cycloheximide, then you might hypothesize that flagella will not grow at all. This hypothesis is a good one; a good hypothesis can be tested. A bad hypothesis might be, "Chlamy cells do not like to have their flagella removed and are happier when the flagella are regenerated." How could this hypothesis be tested? Formulate your hypothesis so that you can design an experiment to test it.

Here are some reagents you might want to use next week:

NAME	**FUNCTION**	**STOCK**	**FINAL**
Cycloheximide	translation inhibitor	2 mg/ml	10 μg/ml
Actinomycin D	transcription inhibitor	5 mg/ml	50 μg/ml
Caffeine	cyclic nucleotide (cAMP) phosphodiesterase inhibitor	66 mM	6.6 mM
Arginine	essential amino acid	10 mg/ml	100 μg/ml
Calcium	signal transduction/ second messenger	100 mM	1 mM
Lithium chloride	disrupts production of IP_3 **(NOTE: lithium is a teratogen)**	1M	20 mM

Remember to include good controls in your 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 2 experimental conditions:
1) Cells regenerating their flagella in the presence of disco music
2) Control cells regenerating their flagella in the presence of pleasant, non-disco music
(Notice the difference between the control and experimental is only one variable - the presence or absence of disco muisic.)

Your hypothesis probably would 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 (plus and minus disco). Let's look at a hypothetical set of results. When the cells are subjected to disco, the flagella did not grow. When the cells were grown in the presence of normal music, they 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 answer next week, formulate an hypothesis, design the experiment, and discuss the protocol with me. This meeting will give us a chance to answer any major questions you might have and order the reagents you will need.

Your written protocols must be turned in today and they should be specific enough so that next week, you can come into the lab and begin immediately by following your own directions. We will look over your protocols and give them back to you at the next lab meeting.

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.

LAB #8

Testing the effects of "environmental" changes on the regeneration of *Chlamydomonas* flagella

Focused Reading: Review pp 108-112
Review pp 270-273, pp. 276-277.

You will perform the experiments you have designed in your protocols. If any modifications were made to your written protocols, you should talk to me before you begin the experiment.

Before the end of today's session, you should:

1) Obtain all the data you need to draw a conclusion. We will spend the next period working with Excel to analyze your data from today.

Control Data

time	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																				

Experimental Data A

time	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																				

Experimental Data B

time	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																				

READ THIS!

Before you leave lab today:

Start thinking about the next set of experiments we will be conducting. 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, peanut butter - **be creative**! We will prepare these materials for you so you can test their mutagenicity.

When your 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. Do 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.

Lab #9
A Beginner's Guide to Descriptive Statistics

Your instructor will give you directions for making YFPM (your favorite potential mutagen).

Once you have collected a large set of data, 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 are:

1) The central tendency

2) The spread of your data

Mean

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

Mean = sum of all the data ÷ sample size (often called **n**)

For example, with the data set (1,1,1, 5), n = 4; the mean is 8 ÷ 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:

Range = value of maximum data point minus value of minimum data point

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

Variance

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:

Variance = the sum of (each data point minus the mean)^2 ÷ sample size

For example, with the data set (1,1,1, 5): $(1-2)^2 + (1-2)^2 + (1-2)^2 + (5-2)^2 = 12$

The variance is 12 ÷ 4 = 3.

Standard Deviation

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:

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

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:

$$\text{Standard error} = \sqrt{\text{variance} \div n} \quad (n = \text{sample size})$$

For example, with the data set (1,1,1, 5), the standard error is 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.

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

The same variance of 3 gave different standard errors (if **n = 4: 0.866** versus if **n = 30: 0.316**) because of the difference in sample size. However, if you look closely at standard error and standard deviation, 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. Many scientists use standard error to make their data look better than it really is. What we would like to be able to say is we are ≥ 95% sure that if we were to repeat a particular experiment another time, the mean value would fall within a certain range. Excel can generate a 95% confidence interval as well. To calculate the 95% confidence interval, the following formula is used:

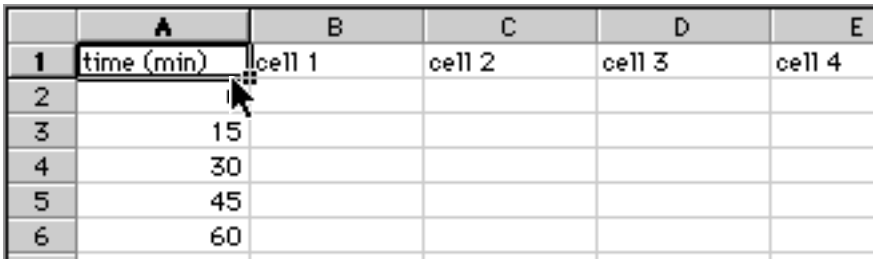
$$\chi \pm 1.96(\sigma / \sqrt{n})$$

where χ is the average, σ is the standard deviation, and n is the sample size.

Excel for Office ‘98

Rather than calculating averages, standard deviations, etc. manually, we will enter our data into a Excel, a spreadsheet software program, and have this program calculate these values for us. Once you have entered data in Excel, you also can use this program to graph the results.

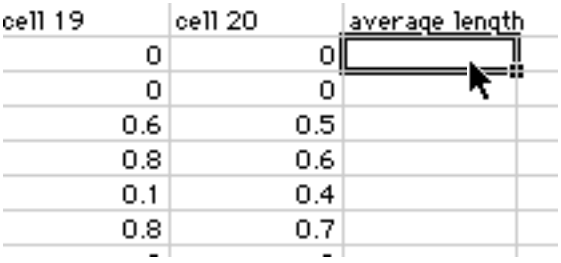
1) Enter your data with the times listed down the first column and with your flagella measurements going across in rows.



	A	B	C	D	E
1	time (min)	cell 1	cell 2	cell 3	cell 4
2					
3	15				
4	30				
5	45				
6	60				

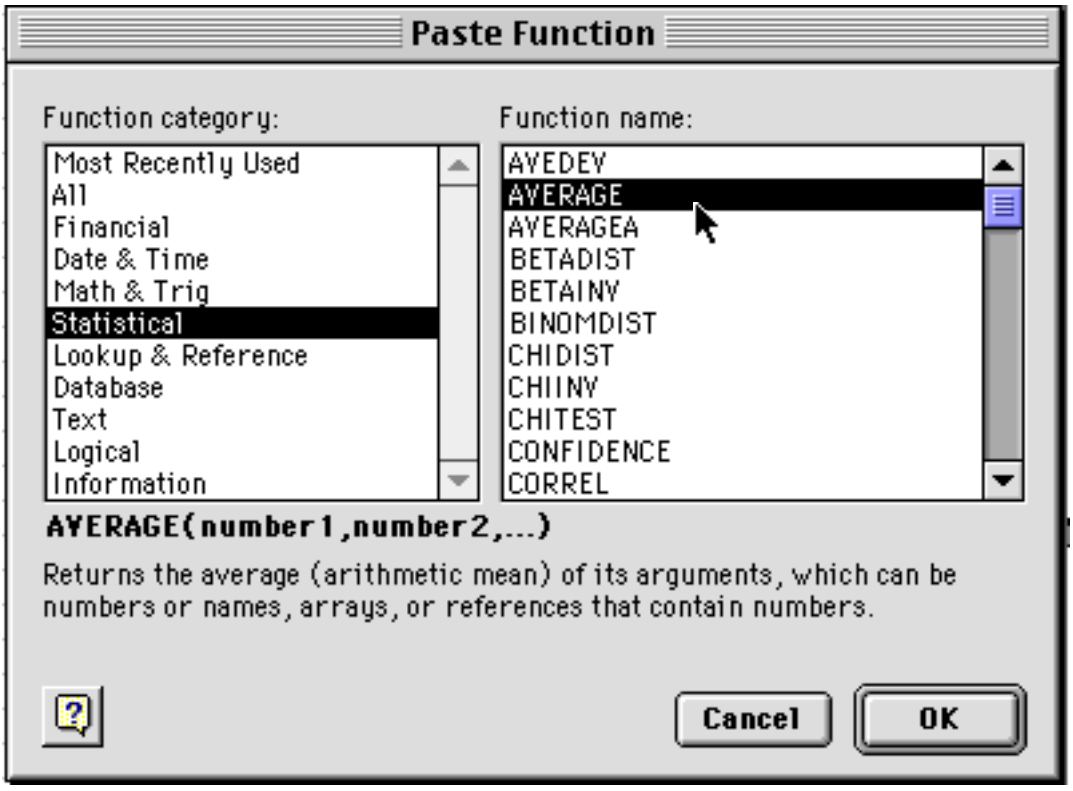
Save these data: give the file a good name and have the file saved to the Bio111 folder and your section’s lab folder. When entering the same number many times, you can save yourself some time with a short cut. Enter the number once, highlight that number, then click and drag the small box in the lower right hand corner to fill as many boxes as you need with the same number.

2) Once all the data are entered, save the file again.
Start a new column called average length and click on the first box:

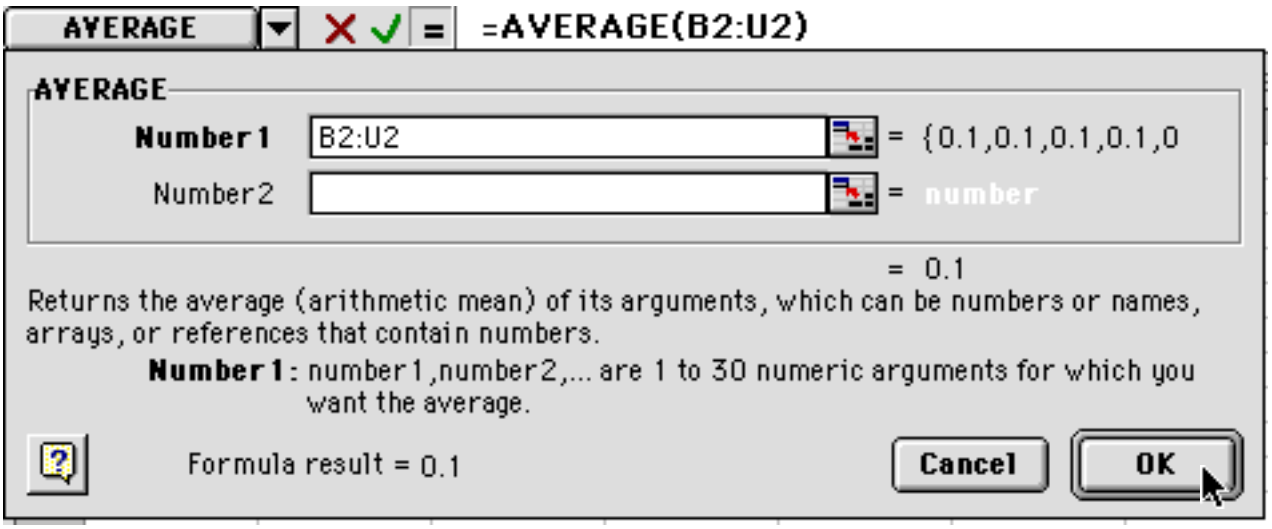


cell 19	cell 20	average length
0	0	
0	0	
0.6	0.5	
0.8	0.6	
0.1	0.4	
0.8	0.7	

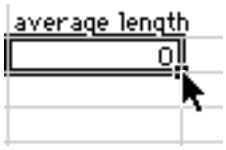
Click on the Insert menu and select “Function…”



3) You will see a window like the one shown above. Select Statistical and then Average and click on OK. You will get a dialog box like the one below. At the top is the formula for averaging the data entered in boxes B2 through U2. You want to make sure all the boxes that you want to have averaged are within the range you have selected. Do not select A2 because that has your time values in it. Then click OK.

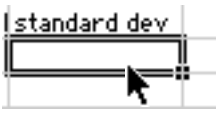


4) When you hit OK, you will see this:

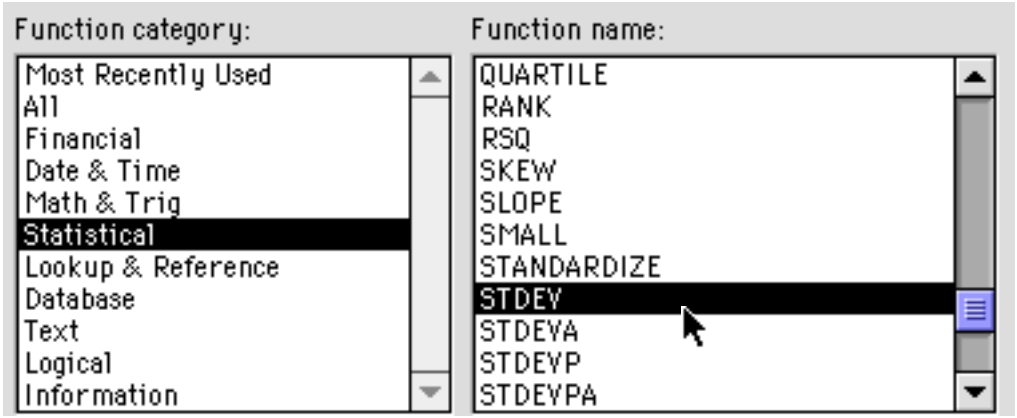


Put your cursor on the box as shown and click and drag down until you reach the 60 minute row. When you let go, all the averages will be calculated.

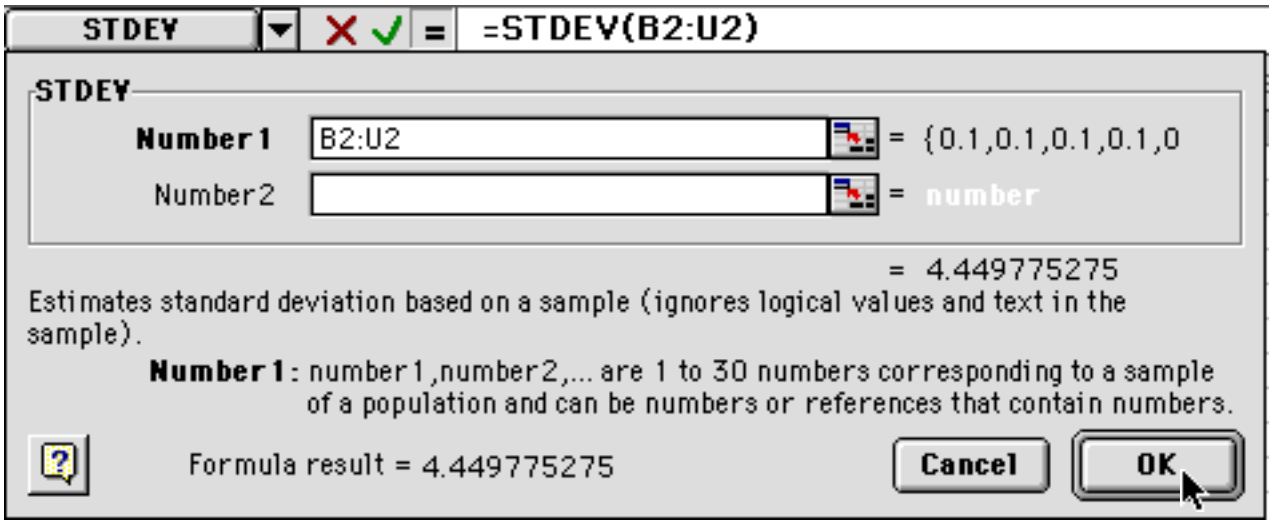
5) Create a new heading called Standard Deviation:



Click on the box below. Go to the insert menu and select "Function…" Select **Statistical** and **STDEV** as shown:

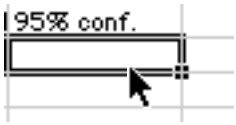


6) You will get another dialog box. Enter the same range as before. In our example, we are using B2- U2 so we would type "B2:U2"

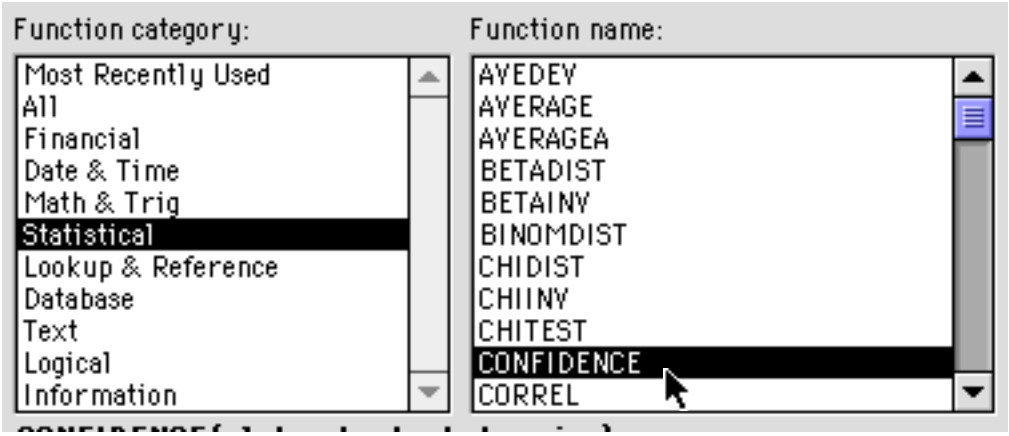


When you click on OK, the value will be entered (probably close to 0). Then click on the lower right hand corner and drag it down to the 60 minute level. The program will calculate the standard deviation for all of your averages.

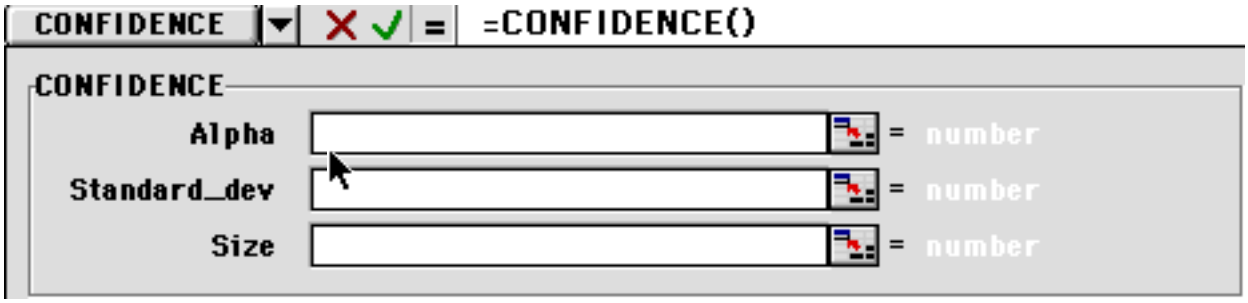
7) Enter a new heading called 95% confidence. Under this heading, click on the next box.



Go to the Insert menu and select "Function…" as you did earlier. You will see this dialog window. Select statistical and confidence as shown.



7) Now you will get a new dialog box:

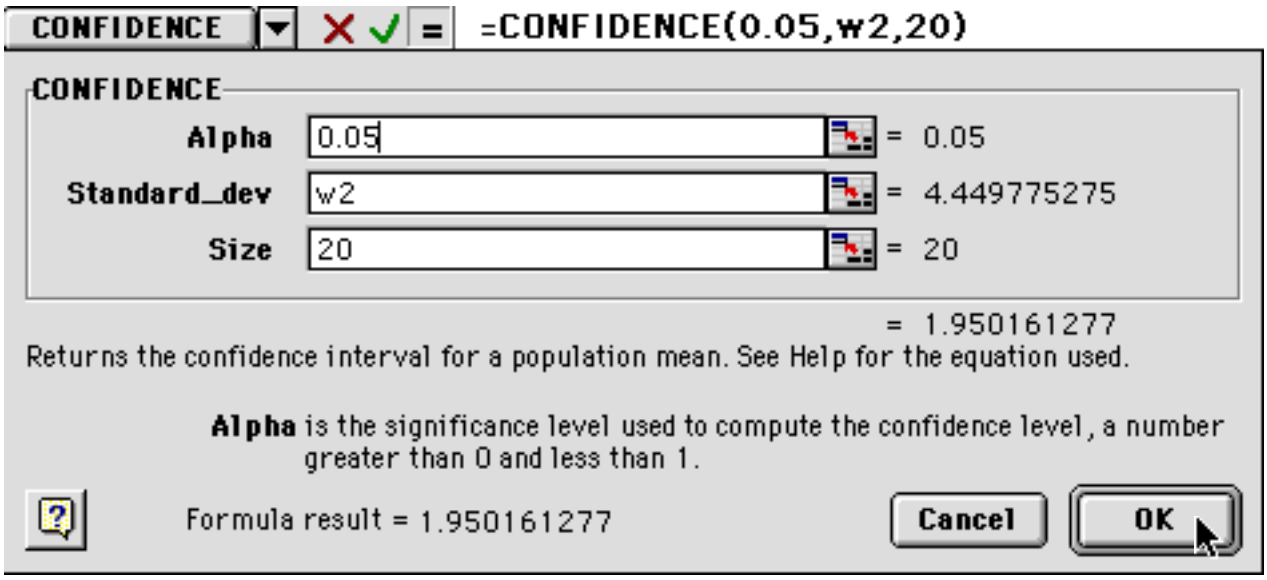


You will need to enter some information:

1) The alpha number is the percentage of confidence you want. Enter **0.05**.
2) The Standard_dev is can be obtained by typing in the box location where the first standard deviation was pasted **W2**.
3) The size is your sample size, or n value. For us, it will be **20**.

8) When you have done this step, you will see something like the dialog box below. Notice that the values are entered in the white boxes, but the numbers are shown to the right, near the cursor in the figure below. The

standard deviation has to be greater than zero to calculate the confidence value, but don't worry about this for now.



9) When you click OK, you will get either a number or a "#NUM!" if a standard deviation was zero. This prompt is Excel's way of telling you that zero is an unacceptable value for standard deviation. Ignore that and drag the box down to calculate all the confidence values through 60 minutes. After you have obtained all the values, change your "#NUM!" values to zero.

10) You now are done analyzing your data. Obviously, though, the data are not easy to present in this format. Rather, it is convenient to present such data as a graph. If you are note familiar with the graphing capabilities of Excel, follow the directions found in Appendix A in the back of this manual.

Lab # 10 – Part I

Pouring Agar Plates for Next Week's Lab

Focused Reading: See Figure 13.6 on page 245.

NOTE: You do not need to wear gloves while pouring the agar plates, because no mutagenic chemicals or bacteria are involved in this procedure.

1. Use the work area with a 2.5-3 foot long piece of absorbent lab counter paper. Label the petri dishes you will use with your initials and your lab time (e.g. Tues. am). I recommend you put your initials on the bottom, along the perimeter.

2. Look in the water bath on your table for a flask labeled DMA. This flask contains Davis Minimal Agar that has been autoclaved to make it sterile, and is being kept at 47° C to keep it liquefied.

3. Think about these important points in pouring a petri plate before doing it:
a) You must work quickly, because once the container of minimal agar is removed from the bath, it will start to harden within 2-3 minutes.
b) When pouring agar into the petri dish, pour just enough to fill the dish about half way.
c) Although you must work fairly quickly, pour the agar gently to minimize the number of bubbles (bubbles look amazingly similar to colonies when the agar hardens).

4. 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.

5. Let the plates harden 15 minutes before moving them. These plates will be stored "upside down" until next week's lab.

Lab #10 – Part II
The Spot-Overlay Ames Test of YFPM
(Your Favorite Potential Mutagen)

Developed from: Dorothy M. Maron and Bruce N. Ames. Revised methods for the *Salmonella* mutagenicity test. *Mutation Research*. Vol. 113: 173-215, 1980.

Focused Reading: "Point Mutations…" pp 234-237. Stop @ "Chapter Summary"

Web Reading: http://ntp-server.niehs.nih.gov/htdocs/LT-studies/tr389.html

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 College and allows us to screen more compounds quickly and cheaply. Next week, we will use the traditional method to quantify the degree of mutagenicity for selected compounds.

NEWS ITEM: April 25, 1997. Dr. Bruce Ames was awarded the Japan Prize ($210,000 cash) for his lifelong work with carcinogens. "When people ask me if I'm the 'Ames' of the Ames test, I say: 'That was so long ago, that was my father.'" Dr. Ames is professor of biochemistry and molecular biology at UC-Berkeley http://mendel.berkeley.edu/Center.homepage.html and his research is focusing on the relationship of aging, nutrition, and cancer. You can find 4 papers by his group in the April 1, 1997 issue of *Proceedings of the National Academy of Sciences* in the library.

I. 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 they 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 shows the

pathway for histidine synthesis, which begins with catabolic intermediate **C** and uses nine enzymes (numbered 1-9 in figure 1) to convert **C** into histidine.

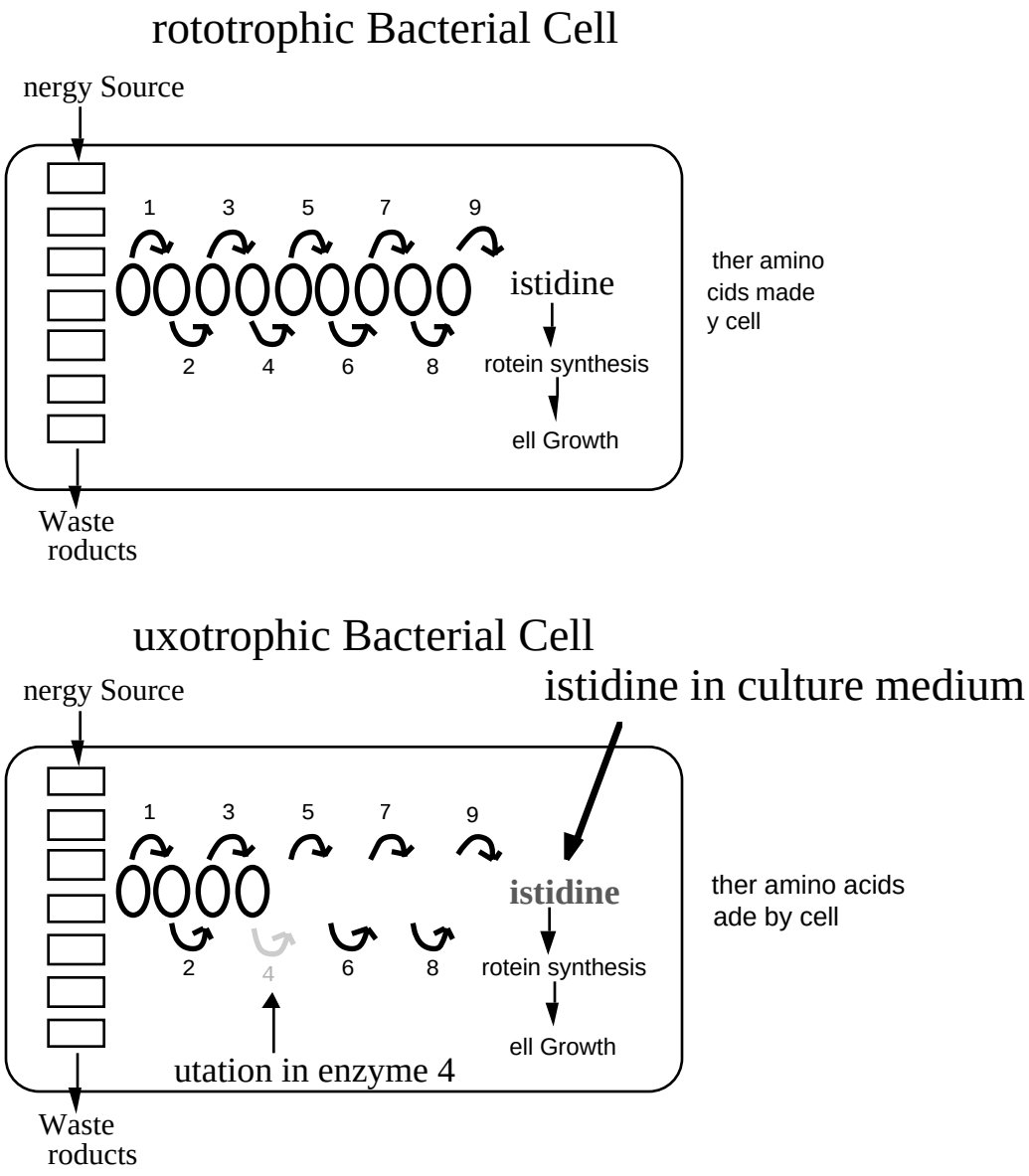


Figure 1. Schematic overview of *Salmonella typhimurium* metabolism and the effect of a nonrepaired point mutation in the synthesis of histidine.

TOP) Metabolism of a normal (prototrophic) *S. typhimurium* which can make its own histidine. The catabolism (breakdown) of the food source produces precursor C which is needed for histidine synthesis. The formation of each intermediate (a-i in ovals) in the histidine synthesis pathway is catalyzed by a different enzyme (arrows 1-9). Each enzyme is a protein whose synthesis is encoded by a separate gene. The absence of any one of the nine enzymes would prevent the synthesis of histidine.

BOTTOM) Metabolism of an auxotrophic (His minus) mutant that cannot make its own histidine. Here the catabolism of food still produces the precursor C but enzyme number 4 is not made due to a point mutation in the encoding DNA. This results in an interruption of histidine synthesis because intermediate (e) cannot be made which prevents the rest of the enzymes from being able to bind to their substrates and synthesizing the appropriate products. In order for this auxotroph to grow, histidine must be supplied in the medium.

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* which 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. Here is a list of the available strains that we can use and what 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 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 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 1538 but is supposed to detect more mutagens than 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 traits shared by each of these strains. 1) These mutant strains lack a DNA excision-repair 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 they are consumed by an animal. One job of the liver is to detoxify harmful chemicals, but in the process some chemicals are converted into very potent

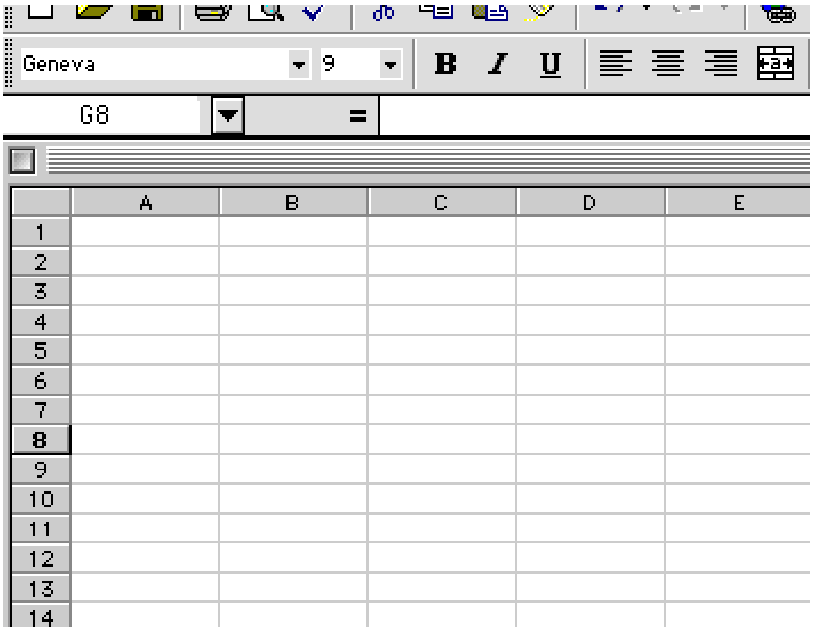
mutagens. For this reason, all test chemicals used in the Ames test by the FDA are routinely incubated with rat liver extract in an oxygenated environment so that liver enzymes, such as oxygenases, will "activate" the chemical being screened; the "activated" chemical then is added to the bacteria. Due 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 (page 12 of 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.….

Appendix A
Beginners Guide to Graphing with Excel

Double click the Excel icon in the "Bio111 Folder".

When Excel opens, you will see a spreadsheet like this one:

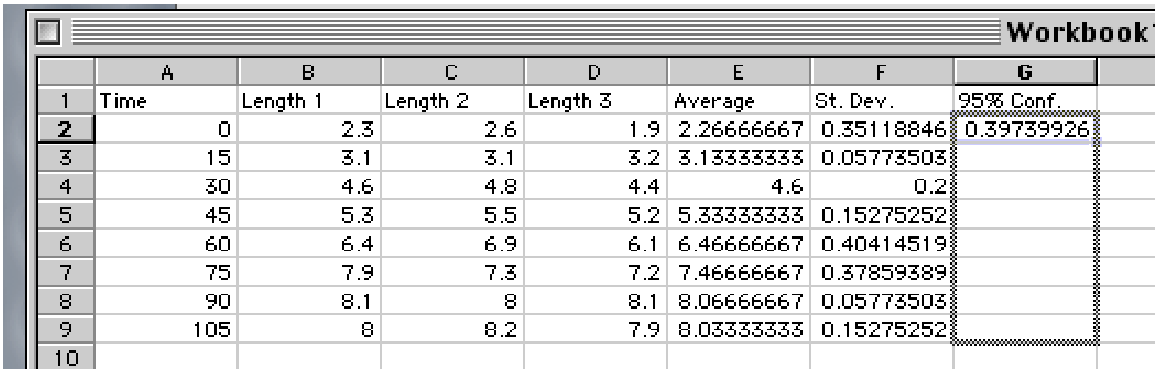


Click on cell A1 and enter a name for your X axis values (for instance, type Time). Enter the appropriate values in separate cells of column A. Click on cell B1 and enter a name for your dependent variables (for instance, type Length). Enter the appropriate values in separate cells of column B. If you have multiple replicates of each experimental condition (e.g. lengths of 3 flagella measured at each time point), enter these data in columns C, D, E, etc. At this point, your spreadsheet may look like this one:

	A	B	C	D
1	Time	Length 1	Length 2	Length 3
2	0	2.3	2.6	1.9
3	15	3.1	3.1	3.2
4	30	4.6	4.8	4.4
5	45	5.3	5.5	5.2
6	60	6.4	6.9	6.1
7	75	7.9	7.3	7.2
8	90	8.1	8	8.1
9	105	8	8.2	7.9
10				

At this point, you can use Excel to analyze your data. As discussed previously, you can calculate the average, standard deviation, and 95% confidence values by using the Insert menu and the Function key.

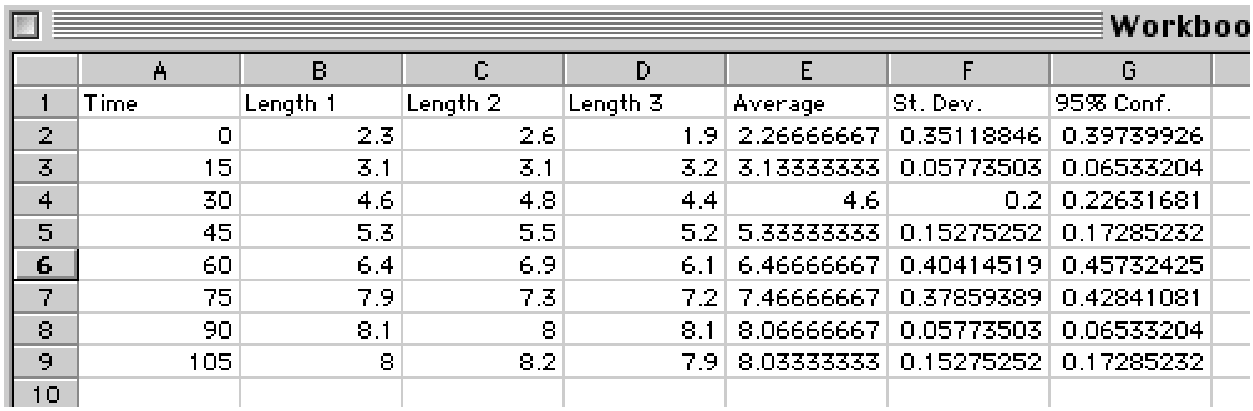
Remember: if you insert a function into one cell, you can transfer that function to adjoining cells. Place the cursor on the lower right-hand corner of the cell (the cursor should become a filled-in plus sign), click and hold the mouse, and drag the cursor to cover the cells you want to contain the formula.



Workbook

	A	B	C	D	E	F	G
1	Time	Length 1	Length 2	Length 3	Average	St. Dev.	95% Conf.
2	0	2.3	2.6	1.9	2.26666667	0.35118846	0.39739926
3	15	3.1	3.1	3.2	3.13333333	0.05773503	
4	30	4.6	4.8	4.4	4.6	0.2	
5	45	5.3	5.5	5.2	5.33333333	0.15275252	
6	60	6.4	6.9	6.1	6.46666667	0.40414519	
7	75	7.9	7.3	7.2	7.46666667	0.37859389	
8	90	8.1	8	8.1	8.06666667	0.05773503	
9	105	8	8.2	7.9	8.03333333	0.15275252	
10							

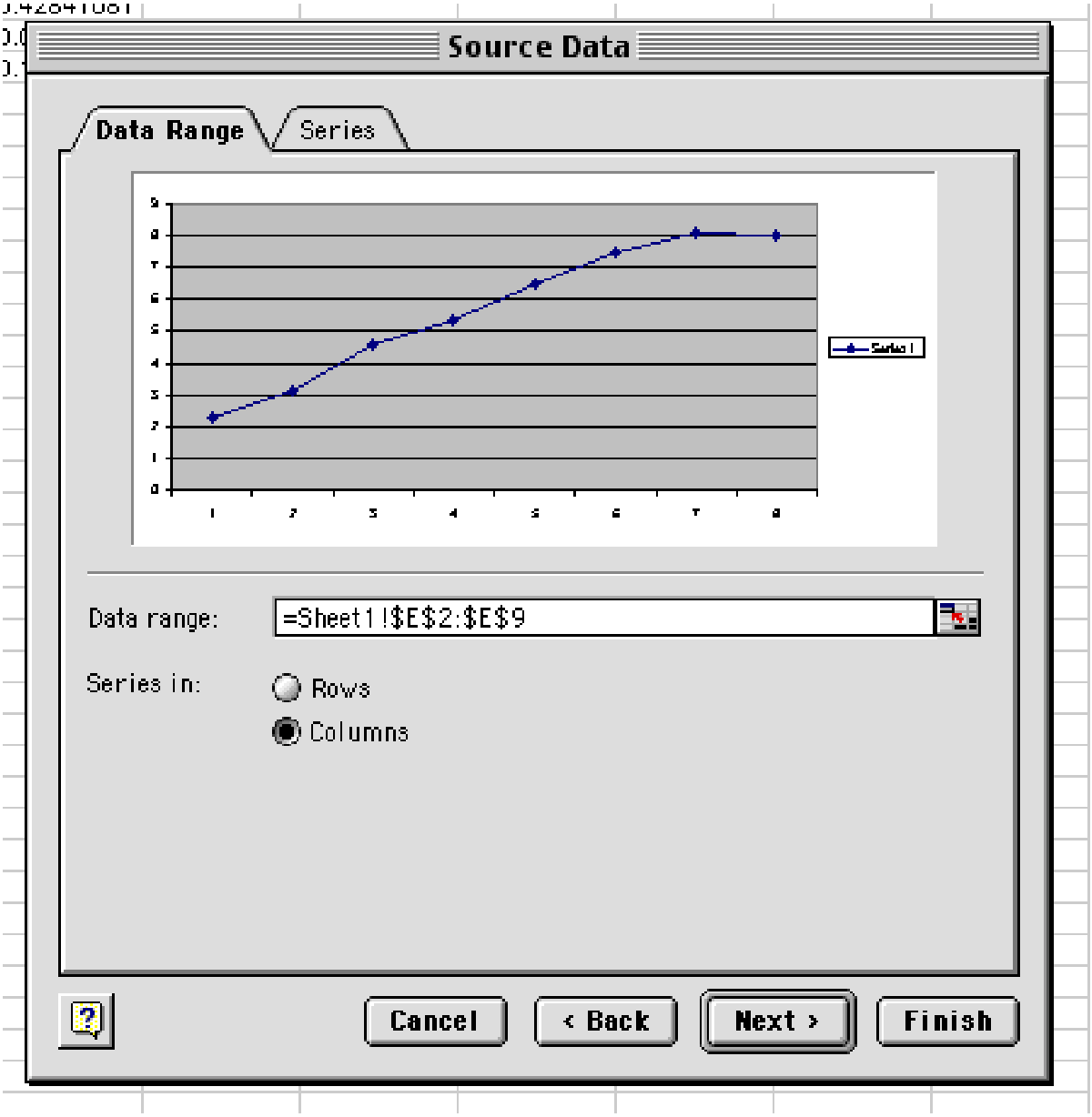
At this point, your spreadsheet may look like this one:



Workboo

	A	B	C	D	E	F	G
1	Time	Length 1	Length 2	Length 3	Average	St. Dev.	95% Conf.
2	0	2.3	2.6	1.9	2.26666667	0.35118846	0.39739926
3	15	3.1	3.1	3.2	3.13333333	0.05773503	0.06533204
4	30	4.6	4.8	4.4	4.6	0.2	0.22631681
5	45	5.3	5.5	5.2	5.33333333	0.15275252	0.17285232
6	60	6.4	6.9	6.1	6.46666667	0.40414519	0.45732425
7	75	7.9	7.3	7.2	7.46666667	0.37859389	0.42841081
8	90	8.1	8	8.1	8.06666667	0.05773503	0.06533204
9	105	8	8.2	7.9	8.03333333	0.15275252	0.17285232
10							

To graph your data, click on the Chart Wizard icon on the top menu bar. Choose the type of graph you want and then click the Next button. The next window should ask for a data range. The data range is the experimental values you wish to graph. In this example, we are interested in graphing the length averages. Click on the data range box and then use the cursor to cover the cells containing your experimental values. When you release the mouse clicker, the desired cell locations should appear in the data range box. For our example, the data range box should contain the following information: =SHEET1!$E$2:$E$9

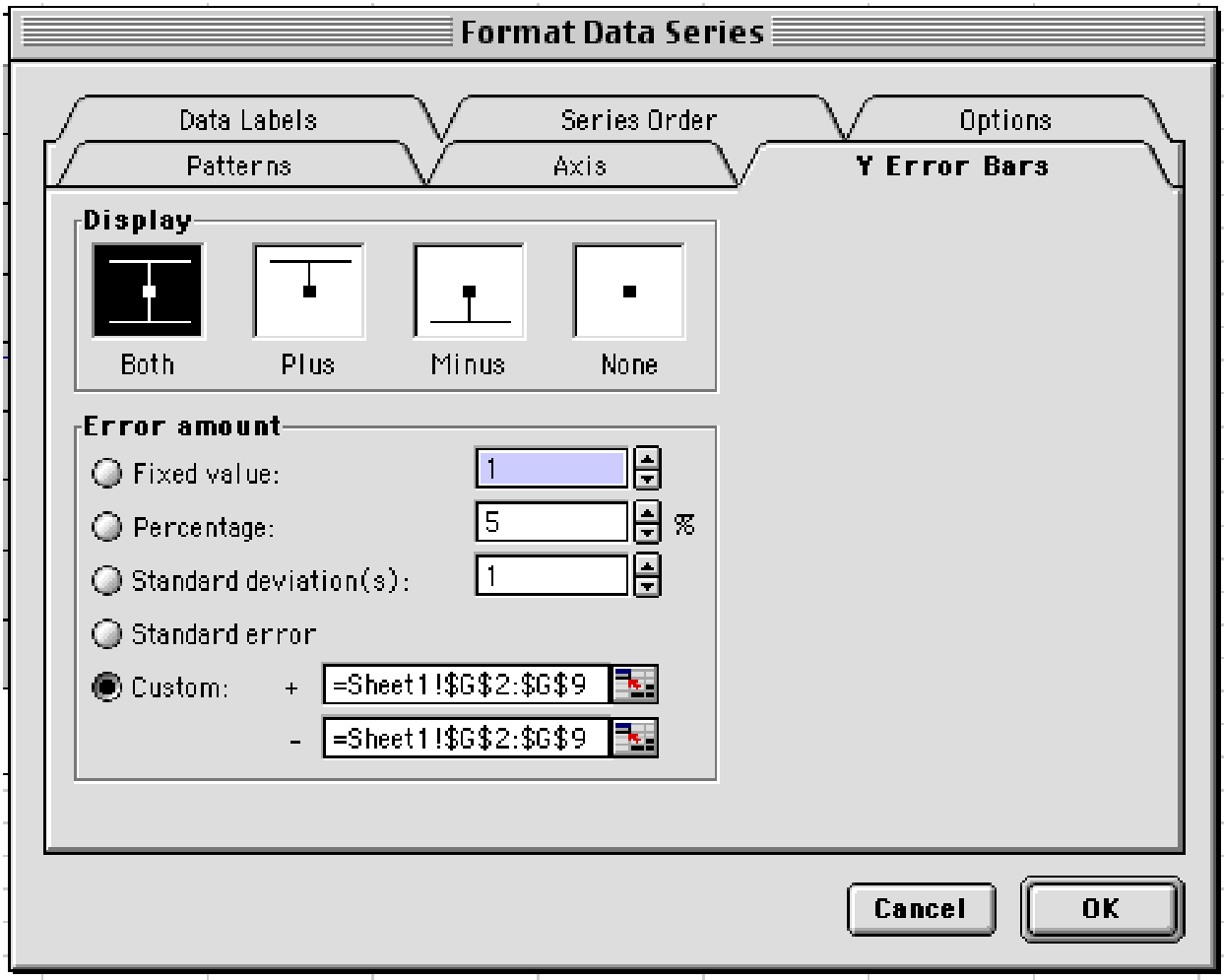


Now select the Series tab. Click on the Category (X) Axis Labels box. This box allows you to specify the values for the X axis. In this example, we wish to graph the average length versus time. As a result, column A contains the X axis values. Insert the locations of these cells into this box following the same procedure used to specify the Y axis values. In the example provided, the box should contain the following information: =SHEET1$A$2:$A$9

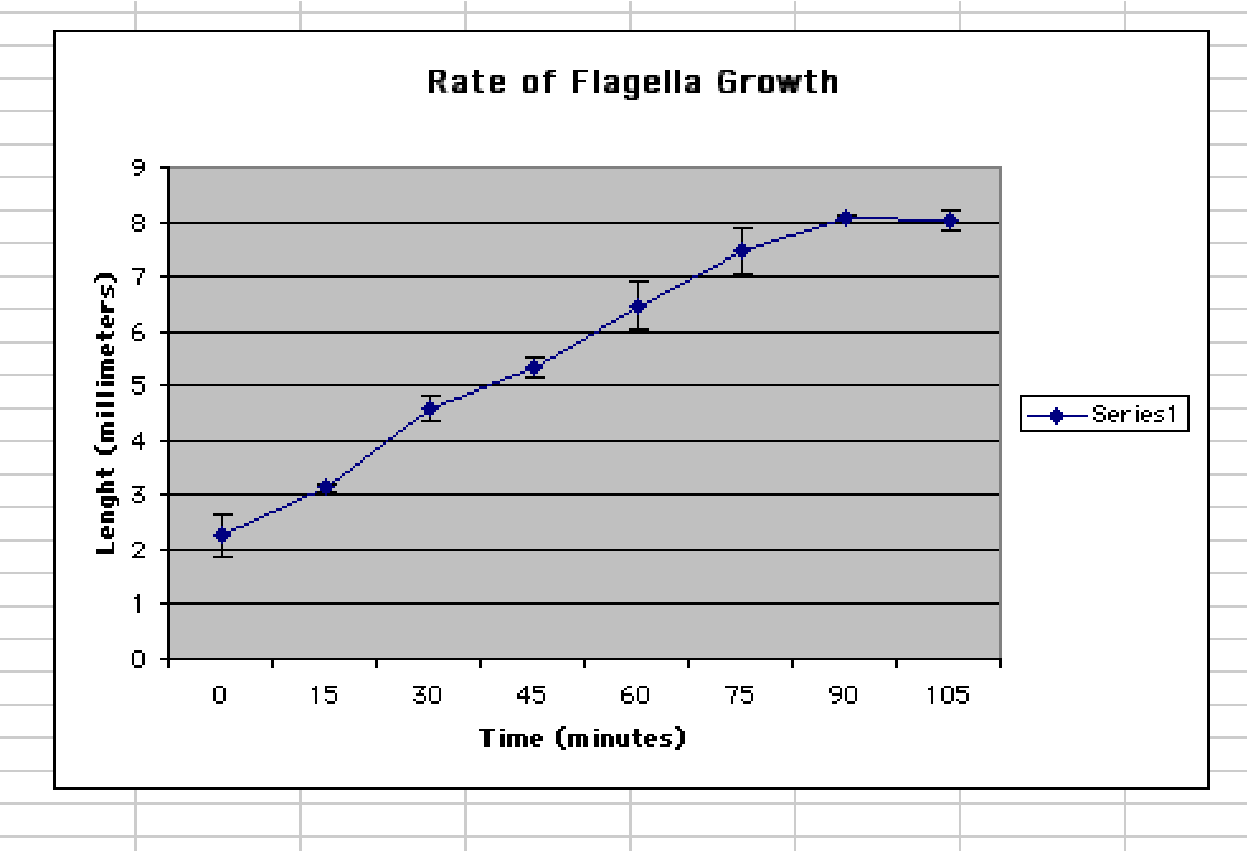
After specifying the X and Y values in this manner, click the Next button. The following window allows you to develop a title, legend, axes names, etc. for the graph.

When you have finished inserting these items, click the Next button, you will be asked to save the finished chart as either an independent chart, or as part of the worksheet. Remember to save your work in your group's folder.

As a final step, you may want to include error bars on your graph. To accomplish this task, use the Format menu and choose Selected Data Series. Select the Y Error Bars tab. If you want to use, for instance, 95% confidence values for your error bars, click on Custom: + box. Use the cursor to select the cells on your worksheet containing this information. When you release the clicker, this information will be transferred to the Custom: + box. Repeat this process for the Custom: - box if you want the error bars to extend in both directions.



Save your modified graph.


Rate of Flagella Growth
Lenght (millimeters)
0
1
2
3
4
5
6
7
8
9
0
15
30
45
60
75
90
105
Time (minutes)
Series1

Appendix B

Use NIH Image, a computer, and a CCD camera to measure the length of flagella

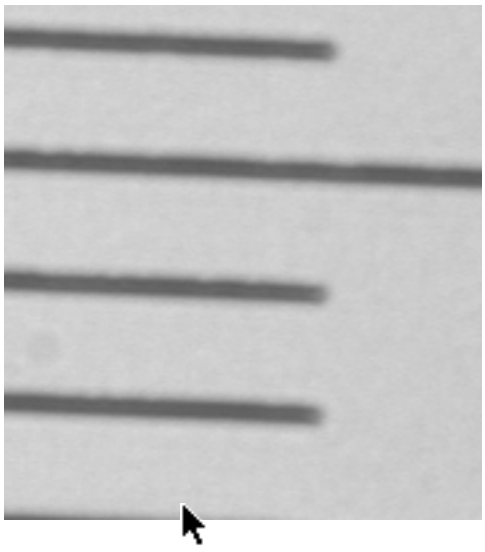
1) Turn on the computer. Find the icon of a microscope in the 111 folder. Double click on this icon. It will launch the program and turn the screen into funny shades of gray.

2) Put the stage micrometer on the stage and turn on the microscope. Use bright field optics to view the micrometer with the 10X objective. Switch up to the 40X objective, still in bright field.

3) The camera should be plugged in, and connected to the computer. All you have to do is pull out the black knob that is on the right side of the adjustable oculars. This knob allows light to travel to the cameras. Notice at the top of the screen, it will say Camera (Live).

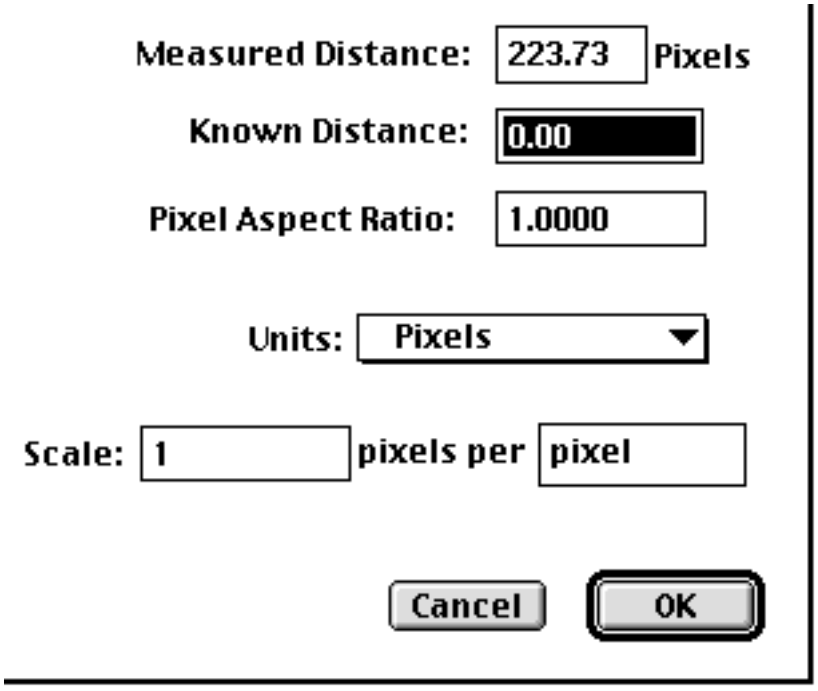
4) While in NIH Image, go to the special menu and select "Start Capturing". The "¸ G" symbol tells you that if you hold down the Apple key (⌘) and G at the same time, you will accomplish the same task. Notice at the top of the screen it will say Camera (1:1).

5) Now the image from the scope is being shown on your screen. If you do not see it, then adjust the light. If the screen looks dark, turn up the light. More likely, it looks white, so turn down the light. You should see something like this :



6) To the left of the image is a list of icons, some of which we will use. Notice that the cursor is pointing to the "line tool" Click once on this. This will let you draw a line on the image of the stage micrometer and tell the how long this line is. Since the distance between each dash on the micrometer is 10 microns, you should draw a line that is 50 microns long. After you have drawn the line, double click on the "line tool" icon. You will see a new dialog box that will let you define the distance you just drew:

Change the units to micrometers and enter the "Known Distance" as 50. Now you have calibrated the computer. You are ready to measure the lengths of the flagella now. Save the camera settings and call it "Calibrated at 40X". This step will permit you to reopen this file and retain the calibration every time you need to measure something using the 40X objective (e.g. next week).

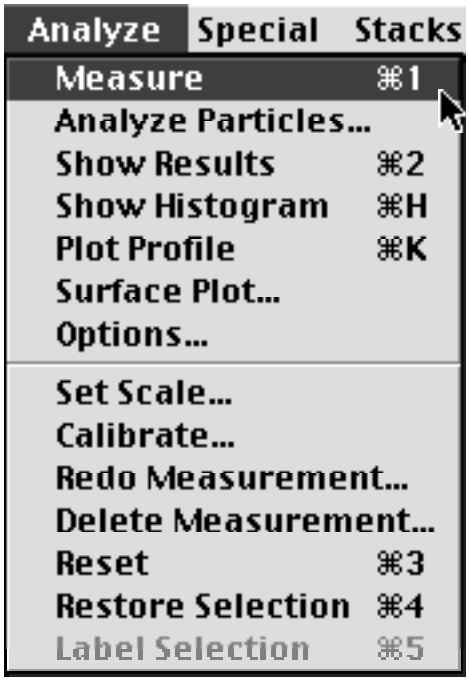


7) Remove the stage mircometer and store it in the box it came in. Prepare a wet mount of the cells you want to measure, and start capturing new images. When you see a cell that you want to measure, stop capturing (hit G). This step should give you a "frozen" image of the cells.

8) If you need to enhance the image, you can click and drag the diagonal line that is in the "Map" portion at the bottom of the list of icons. This step will be demonstrated in class.

9) Click on the magnifying glass and then click on the cell you want to measure. You should zoom in once or twice to magnify the cell. Now click and hold on the "line tool". You will get three options. Select the "Freehand Line" option.

This option will let you trace the length of the flagellum. When you think you have traced it pretty well, go to the Analyze menu and select "Measure", or type "Apple 1". This command will instruct the computer to measure the line you just drew. Go to the Analyze menu (see)
and select the "Show Results" option or type "Apple 2". Notice that if you make a mistake, you can "Redo Measurement..." or "Delete Measurement...".



10) When you are finished measuring this cell, go to the Edit menu and select “Unzoom “or type “Apple U”. Now you are ready to find another cell to measure. If you do not see one on your screen, then type “Apple G” to start capturing new images. Look at the top to make sure it says “Camera (Live)”. If not, then hit “Apple G” again.

11) Continue this process until you have measure 20 flagella for each time point. Enter the measurements in your lab notebook in the tables provided for you. You will analyze these data using Excel.

12) When you have recorded 20 measurements, you may hit 3 to delete all measurements and start over.