The authors readily acknowledge the many contributions by individuals who have participated in the teaching of these labs over the years. Specific recognition goes (in alphabetical order) to J. Bagarova, J. Chang, D. Cullinane, M. Gammons, J. Grimsby, T. Gulick, T. Karanja, S. Morey, A. Mozharova, S. Rassler, N. Shankar and D. Tsirelson.

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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Date</th>
<th>Description of Lab</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Introduction</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>How to Keep a Laboratory Notebook</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>How to Write a Laboratory Report</td>
<td>7</td>
</tr>
<tr>
<td>Sept 6&lt;sup&gt;th&lt;/sup&gt;, 7&lt;sup&gt;th&lt;/sup&gt;, 8&lt;sup&gt;th&lt;/sup&gt;</td>
<td>No labs</td>
<td></td>
</tr>
<tr>
<td>Sept 13&lt;sup&gt;th&lt;/sup&gt;, 14&lt;sup&gt;th&lt;/sup&gt;, 15&lt;sup&gt;th&lt;/sup&gt;</td>
<td>Lab 1: Mitosis and Meiosis: Modeling with beads</td>
<td>9</td>
</tr>
<tr>
<td>Sept 20&lt;sup&gt;th&lt;/sup&gt;, 21&lt;sup&gt;st&lt;/sup&gt;, 22&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>Lab 2: <em>Drosophila</em> as an Experimental Organism; Chi Square</td>
<td>19</td>
</tr>
<tr>
<td>Sept 27&lt;sup&gt;th&lt;/sup&gt;, 28&lt;sup&gt;th&lt;/sup&gt;, 29&lt;sup&gt;th&lt;/sup&gt;</td>
<td>Lab 3: Dihybrid Cross in <em>Drosophila</em>, Part 1: Cross F1s.</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Dihybrid Cross in Corn</td>
<td>39</td>
</tr>
<tr>
<td>Oct 4&lt;sup&gt;th&lt;/sup&gt;, 5&lt;sup&gt;th&lt;/sup&gt;, 6&lt;sup&gt;th&lt;/sup&gt;</td>
<td>Lab 8: Genome Wide Association (GWA) Study in Recombinant Inbred Lines (RILs) of lettuce; Mapping Functions</td>
<td>41</td>
</tr>
<tr>
<td>Oct 11&lt;sup&gt;th&lt;/sup&gt;, 12&lt;sup&gt;th&lt;/sup&gt;, 13&lt;sup&gt;th&lt;/sup&gt;</td>
<td>Lab 4: Plant Molecular Genetics, Part 1 – DNA Extraction</td>
<td>49</td>
</tr>
<tr>
<td>Oct 18&lt;sup&gt;th&lt;/sup&gt;, 19&lt;sup&gt;th&lt;/sup&gt;, 20&lt;sup&gt;th&lt;/sup&gt;</td>
<td>Lab 3: Dihybrid Cross in <em>Drosophila</em>, Part 2: Score F2s</td>
<td>35</td>
</tr>
<tr>
<td>Oct 25&lt;sup&gt;th&lt;/sup&gt;, 26&lt;sup&gt;th&lt;/sup&gt;, 27&lt;sup&gt;th&lt;/sup&gt;</td>
<td>Lab 4: Plant Molecular Genetics, Part 2 – PCR</td>
<td>57</td>
</tr>
<tr>
<td>Nov 1&lt;sup&gt;st&lt;/sup&gt;, 2&lt;sup&gt;nd&lt;/sup&gt;, 3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>Lab 4: Plant Molecular Genetics, Part 3 – Gel Electrophoresis</td>
<td>61</td>
</tr>
<tr>
<td>Nov 8&lt;sup&gt;th&lt;/sup&gt;, 9&lt;sup&gt;th&lt;/sup&gt;, 10&lt;sup&gt;th&lt;/sup&gt;</td>
<td>Lab 5: Bacterial Mutation, Part 1 –</td>
<td>63</td>
</tr>
<tr>
<td>UV Light Mutagenesis of <em>E. coli</em> Cells</td>
<td>Colony Counts and Killing Curves</td>
<td></td>
</tr>
<tr>
<td>Nov 15&lt;sup&gt;th&lt;/sup&gt;, 16&lt;sup&gt;th&lt;/sup&gt;, 17&lt;sup&gt;th&lt;/sup&gt;</td>
<td>The Use and Power of Semi-Log Graph Paper</td>
<td>73</td>
</tr>
<tr>
<td>Nov 22&lt;sup&gt;nd&lt;/sup&gt;, 23&lt;sup&gt;rd&lt;/sup&gt;, 24&lt;sup&gt;th&lt;/sup&gt;</td>
<td>No labs Thanksgiving Holiday</td>
<td></td>
</tr>
<tr>
<td>Nov 29&lt;sup&gt;th&lt;/sup&gt;, 30&lt;sup&gt;th&lt;/sup&gt;, Dec 1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>Lab 7: Gene Regulation: the <em>lac</em> operon</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Excel Tutorial – Inserting a Formula for Multiple Equations</td>
<td>88</td>
</tr>
<tr>
<td>Dec 6&lt;sup&gt;th&lt;/sup&gt;, 7&lt;sup&gt;th&lt;/sup&gt;, 8&lt;sup&gt;th&lt;/sup&gt;</td>
<td>Lab 6: Gene Structure: Map of the β-Globin Gene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(To be handed out)</td>
<td></td>
</tr>
</tbody>
</table>
LAB NOTEBOOKS DUE on last day of lab.

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Introduction

1. **Grading.** The laboratory grade constitutes 25% of the total course grade. It will be based on your laboratory notebook, laboratory write ups, worksheets, and the laboratory instructor’s evaluation of your performance. The latter includes attendance, participation in discussion, work habits, and evidence that you are seriously attempting to understand the experiments and problems. The instructor will give you more detailed information about what is expected from you.

2. **Cleanliness.** It is important to keep the laboratory clean. You will be maintaining long-term cultures of fruit flies, which are susceptible to mold infections. You will also be handling microorganisms under the usual aseptic conditions demanded of microbiology.

   Wash your hands before working with cultures. Leave cultures open to the room air as briefly as possible.

   _Leave laboratory tables and benches clear so that they can be wiped down after every session._

3. **Safety.** Food and beverages may not be consumed in the lab, and should never be placed on the lab benches. Wear shoes which cover your feet while in lab. The instructor will review safety procedures. On the few occasions when toxic chemicals are used, you will be instructed on how to handle them safely.

4. **Keeping track.** Label all cultures, plates, and tubes with enough information so that you know what’s in them, and can identify them again; include the date and your initials in the label. Write down this information in your notebook. Keep dated, neat, day-by-day records in a laboratory notebook. All laboratory scientists keep such notebooks. They function something like diaries so that one can go back and find out exactly what was done when. Remember that part of your lab grade will be based on how well you keep your notebook. (See next page for details).

5. **Working arrangements.** Students will work in groups of 2-3 that will be assigned randomly. It is much more efficient to work in pairs, so that manipulation of cultures and data collection can be divided up. Accuracy is also increased with this arrangement. Nevertheless, each individual must be responsible for the data presented in write-ups, and for the analysis of data. Each student must write his/her own lab write-ups; lab write-ups cannot be shared or written together with another student.

6. **Laboratory write-ups.** You will have several reports to hand in, each consisting of ONE graph or table and ONE page clearly written discussion of results and their meaning. The goal is to present your results and interpret them. Refer to “How to Write a Lab Report” in the appendix this manual. If you have questions, ask the instructor. Reports must be prepared using a computer and will be submitted through your Blackboard account. You can get instruction and access to the computers in the library, if needed.
How to Keep a Laboratory Notebook

Lab notes in all branches of science are a permanent record of what the scientist actually did and observed, written down as it happened. Your notebook must be done during lab and should not be put together later based on what you remember (or think you remember) about the experiment. The notebook should be well organized, with a neat table of contents at the front. PLEASE put your name and telephone number on the cover so that your work doesn’t get lost. All work conducted during the lab must be recorded during the lab. You should summarize what you accomplished during the lab at the end of the period.

What to include

Your notebook is the place to write questions and comments when reading the manual before a particular lab. Also use it for any notes you take on your instructor’s introduction to the lab. Especially, use it to record what you do while carrying out each experiment or exercise, step by step; your thoughts and calculations along the way; and all of your data, preferably organized in clear, well-organized tables. If you use specific protocols from the manual, you should state that clearly in your lab notebook and in any lab write-up. But you must record any changes that you make in the procedure and any problems that you encounter. You should also record all observations, unusual circumstances, and any doubts about the validity of your data. Always keep in mind that you will have to go back and use this information for writing up reports. You will find that task much easier if your data entries are neat and well annotated.

Every lab should be in the notebook. Some labs will need only a short entry; the lab on Drosophila as an Experimental Organism, in which we practice distinguishing male and female flies, is straightforward. For that lab, take a page to write down what you learned, and any problems you had. Other labs will require a lot of writing and analysis. For each of these labs, you should record in detail all relevant information such as what crosses you set up on what dates; numbers of flies of each sex and phenotype put into each vial; the dates you cleared and counted the flies, etc. Ideally, another experimenter should be able to use your notes to repeat the experiment exactly as you performed it. The questions at the end of specific labs are to help you understand the experiment and should be answered fully in your notebook. Similarly, the predictions you make for Drosophila gene mapping lab should be worked out in your notebook. There is no need to fit the whole semester’s work in the first 20 pages. Give each experiment the space it needs from the start, so you don’t have to cram data on 300 flies of 8 phenotypes into half a page, or record one experiment in four different places with other stuff sandwiched in between.

Lab notes should be kept in ink. The notebook must be neat enough for you (and your lab instructor) to read it. You’re not graded on handwriting, but please make an effort. The goal is a complete record, including mistakes. (Mistakes should be neatly crossed out, not obliterated, not erased. Sometimes knowing what mistakes you made is as important as reaching a conclusion.) Complete sentences are better than incomprehensible fragments.

Grading

Your notebook counts for 50 points of your lab grade. In terms of your overall course grade, that means it’s worth half of an hour exam. Lab notebooks will be collected and graded periodically.

One Page Laboratory Reports for Biology 252
This fall we are trying something new for the lab reports. We shall emphasize thinking, interpretation and conclusions as well as your ability to write coherently and precisely. A traditional lab report is modeled after a typical scientific paper that one finds in many journals. These traditional reports often contain an Introduction that describes the rational and background for the work; a Materials and Methods section that documents the precise protocols used; a Results section that reports the data collected and the analyses conducted; and finally a Discussion section in which the authors interpret the meaning of the results and fold it into the current knowledge in the field. Your lab notebook will still contain details about the Materials and Methods, and your collected data and analyses that make up a results section. This fall we will not have you rewrite these into your Lab Report. Instead, your lab report will consist of ONE TABLE, FIGURE or GRAPH which will present the essential results of your work and ONE PAGE of DISCUSSION in which you will explain and interpret your results that are presented in your table or figure. These must be your own, independent work!

The table or figure should show accurately what you observed, must be original and must be properly labeled. The tables and figures sometimes have multiple parts or insert figures, so that is possible but keep in simple and clear. The table or figure should have a legend that states what the table or figure is about and defines all abbreviations and analyses. You can examine a few figures or tables found in published scientific papers to get a sense of what these should look like or ask your instructor.

The one page of discussion must be original, clear and demonstrate that you understand the experiment. You can include whether the results fit with any hypotheses that you may have had and provide interpretations or explanations for why your results did or did not seem to fit your hypotheses; you can note any problems you had with the protocols that might explain your results if they did not match expectations. This discussion as those in any published scientific paper can have variable and flexible purposes. Depending on the results of the experiment, you could explain how it supports work of other studies or describe how the experiment may be improved to get more consistent results. Since this discussion is limited to a single page, you will need to think carefully about the critical ideas that you will want to include, and write concisely and precisely.

Within your discussion you should properly cite any articles, books or internet resources you used in the discussion, though these are not required. For example you may write “(Zimmer 2015)” at the end of a sentence if Zimmer’s paper contributed to your thinking in that sentence. You should then provide the full reference information at the end of your discussion; e.g. Zimmer, C. 2015. A weakness in bacteria's fortress. Scientific American. 312(1):40-45.

Notes on Plagiarism: Plagiarism refers to the copying of statements (whether exact or paraphrased words) or the ideas of others without quotations and acknowledging where you obtained them. It is a very serious crime in the world of academia. Original writing is not copying, pasting and then rearranging the ideas of others. You need to learn how to synthesize information that you read and properly acknowledge your sources of information. Often, you will simply acknowledge the source as we did above, showing where we found the details. If you use the exact words of the author you must make that clear by using quotes and cite the precise source and location of that quote immediately following the quote. A simple rule of thumb can help you develop your cognitive and writing skills (the point of these exercises) and often be used to avoid plagiarism. Read everything you want or need in preparation for your assignment, but when it comes time to write, close all books, papers, internet sources. For this particular assignment, the only thing you should have in front of you is the table or figure that you have created. Outline briefly what you think the key items you want to report and then begin to write.
The writing will of course utilize the information that you have been reading, but will be your interpretation and your words.

Plagiarism also refers to the copying of papers or lab reports from fellow students. You should not provide your report to others nor use the reports of others. Lab partners should never copy each other’s lab reports or even collaborate in the writing of the report. You may do an experiment together, and you can discuss the analysis of the data. However, each partner should be individually responsible for all data. Furthermore, the final analysis and creation of your table or figure and discussion should be done individually. Your report must clearly reflect your own attempt to make sense out of what you did in lab.

**Citing the lab manual:** To cite the lab manual in the reference section of your lab reports, use the following format and include the specific pages you are referencing:

LAB 1: DNA Structure, Replication, Mitosis and Meiosis

OBJECTIVE: To understand the nature of DNA which has the remarkable ability to carry an enormous amount of information AND be replicated and transmit that information accurately as cells divide through the processes of mitosis (cell division in somatic cells) and meiosis (cell division in sex cells to produce gametes). Students must thoroughly understand these processes and become familiar with and have working knowledge of the following terms: chromosomes, chromatids, centromeres, haploid (N), diploid (2N), allele, homozygous and heterozygous. To understand well these processes, one must also understand where the DNA is and what it is doing. One of the goals of the class this year will be “to build a gene” beginning with a fragment of DNA and follow the activities and processes that involve this fragment as cells divide and as genes are expressed.

BACKGROUND: In order to understand the genetics of eukaryotic organisms, it is essential to understand the essential features of the cell cycle with G1 (Gap 1), the S (Synthesis), G2 (Gap 2) phases when chromosomes with their coiled DNA molecules replicate and the division phases of mitosis and meiosis when chromosomes divide and separate. Now is the time to fix these fundamental activities of eukaryotic cells in your long-term memory. Before carrying out this lab exercise, you should have read the section on cell division in your genetics textbook. The lab offers 2 more ways to grapple with understanding the structure of DNA, replication and the patterns of cell division: 1) a model of the β Globin gene, 2) detailed charts that illustrate the successive stages of mitosis and meiosis, and 3) bead models that allow you to simulate mitosis and meiosis while monitoring the changes in the DNA. When you have questions, ask the instructor.

A. The β-Globin Gene Exercise (Adapted from 3-D Molecular Designs)

Introduction: Becoming familiar with the β-Globin gene map:

The rolls on your benches represent a sequence of double stranded DNA. This sequence happens to be the β-Globin gene, which is not critical for this lab but will be important for future exercises. Unroll your map of the β-Globin gene on your bench, propping at least the first several feet of the roll open with books. Please do NOT write on these rolls with anything except the dry erase marker provided.

The nucleotide sequences of both DNA strands are shown—the top sequence in red and the bottom sequence in blue, with opposite polarity (essentially orientated in the opposite direction); you do not need to pay attention in this lab to the rows of letters at the bottom of the roll representing encoded amino acids. The letters in the DNA sequence (A,G,T,C) represent the four different nitrogenous bases, Adenine and Guanine, are purines and Thymine, and Cytosine are pyrimidines respectively (see Figure 10-7 in your book to understand their general structure).

This sequence of DNA can be “read” in many ways. A sequence read is simply the order of bases on one strand starting at a particular point. In your DNA sequence, without additional information, you could start reading from any point on either the upper or lower strand and proceed in either direction (left or right). You will find that each strand is read in only one direction, but the starting point can and does vary.
1. Starting at any point, on either of the two strands, and reading in either direction, can you predict what the next base in the sequence will be?

2. How many reads are possible for a single strand sequence comprised of any 30 random bases? In other words, how many combinations of the bases are possible?

3. The length of the DNA in one of your haploid gametes (sperm or egg depending on your sex) is approximately 3 billion (3 x 10^9) base pairs long. How many different reads of 3 billion random bases of one strand are possible?

4. Starting at any point on either of the two strands can you predict the base on the complementary (opposite paired) strand?

5. For each single strand sequence of 30 random bases, how many different complementary strands are possible?

The first three questions above address the observation of Watson and Crick (1953) when they state “the sequence of bases on a single strand does not appear to be restricted in any way.” They are in essence stating that the information (number of possible reads in a sequence) is unlimited.

The last two questions address the observation of Watson and Crick when they state “However, if only specific pairs of bases can be formed, it follows that if the sequence of bases on one chain is given, then the sequence on the other chain is automatically determined.” Watson and Crick further state “It does not escape our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.”

You will now work with the beads in the second part of this lab that examines the structure of DNA, the organization of DNA into chromosomes and the replication of these chromosomes and subsequent division of these chromosomes that occurs as cells divide in mitosis and meiosis. Before you roll up your globin gene sequence, see if you can find and record in your lab note book, 10 base pairs (both strands) starting at base pair 62,201 and going to base pair 62,210. You will need this information and will be asked to locate the positions (approximately) of these bases in your bead constructions in the second part of this lab.

B. Chromosome Structure. The DNA of eukaryotic cells is organized as long, linear molecules (associated with proteins) called chromosomes. The typical chromosome consists of 1 chromatid (in G1 stage of the cell cycle) or 2 (in G2 stage) each with its own double stranded DNA molecule. At some point along the length of each chromosome is a specialized region called the centromere. In our chromosome models, plastic beads will represent the gene-bearing arms of the chromatids; the centromeres are represented by magnets.

1) Make a chromatid from the red beads. Make one arm of 12 beads, the other of 5 beads. Insert the ends of each arm into a centromere (Fig. 1). Make another identical chromatid from red beads, and join the two chromatids together at the magnetic centromeres. Now you have a chromosome consisting of 2 sister chromatids.

2) Using yellow beads make another chromosome that is similar to the first except for color. Now you have 2 homologous chromosomes. Such homologs are usually identical in length and position of the centromere, and each carries the same genes in the same order. However, the
specific DNA sequences of the corresponding genes may vary slightly. That is, each homolog may carry a different form of a particular gene. These different forms of a gene are called alleles. If a diploid individual carries two identical alleles at a given position (locus) in the two homologous chromosomes it is called homozygous. If it carries two different alleles on these homologous chromosomes it is called heterozygous.

3) Make 2 more homologous chromosomes as above. This time make one arm 10 beads long, and the other 3 beads long. Now you have 2 sets of homologous chromosomes. Let us call the long homologs chromosome number 1, and the short homologs, number 2. Chromosome 1 and 2 differ in length and position of the centromere, and also carry different sets of genes (Fig. 2).

4) Take small bits of tape to designate particular genes (A-D) and alleles (capital or lower case letters) on your chromosomes as illustrated in Fig. 2. These 4 genes are heterozygous in your model. Now go back to the 10 bp of the β-Globin gene sequence that you recorded from the previous section of this lab. Let’s assume that the β-Globin gene is located on your bead Chromosome 1 (in humans it is actually Chromosome 11), and that this chromosome is approximately 135 million base pairs (Mbp) long. Where would the strands with your globin gene sequence be located on your bead chromosome? Using strips of tape with the double stranded sequence of bases written on them, place the tape in all appropriate locations in your bead chromosome models. This gene is homozygous in your model as would be the case if the “A” gene had only capital “A” alleles; it is likely that most if not all students in this class are also homozygous for this region in their own genomes.

5) Now we are going to add some variation to your models. Recall homologous chromosomes are nearly identical but can carry different alleles of the gene. Your bead models are clearly heterozygous at the four loci (A-D) that you have marked, but homozygous at the β-Globin gene. As it turns out, one of the most well-known rare allele of the β-Globin gene is a change of base pair 62,206 which is in the middle of your recorded 10 bp sequence. When the A=T pair at position 62,206 is changed to a T=A pair the result is the allele for the human genetic disease sickle cell anemia! Make this change in your models so that your diploid individual is now heterozygous for this gene as well as the others and keep track of these different DNA sequences and alleles throughout the remainder of these exercises.

C. Ploidy and N. The nucleus of a germ cell (egg or sperm in animals) contains a fixed number of chromosomes that is typical of the particular species. This is called the haploid number (N). In Drosophila, for instance, N = 4; in humans N = 23. A haploid set of chromosomes contains one set of genes for constructing the organism. In animals, somatic cells (body cells) usually contain 2 sets of chromosomes, and therefore 2 sets of genes. They are diploid (2N). The complete 2N set of human chromosomes is shown in Fig. 3a, first as imaged directly from a mitotic cell, and then arranged as paired homologs. Nearly all eukaryotic organisms have alternating cycles of N and 2N generations. The situation differs a little among these organisms, some of which have very short haploid and dominant diploid generations (animals) and others with more equal or even dominant haploid generations (some plants and fungi), but the principles are the same.

Cluster the chromosomes you have made on the table in front of you. What is represented is the complement of chromosomes in the somatic cell of an organism for which 2N = 4. It has 2 pairs of homologous chromosomes, designated 1 and 2.
D. Mitosis. The function of mitosis is to replicate exactly the total complement of chromosomes in each cell division, so that all cells of the body will contain the same set of 2N chromosomes. Refer to the diagram of mitosis in your text, or on the wall chart, and simulate the process of mitosis. Line up the four chromosomes as in metaphase, in any order. Note the orientation of the chromosomes relative to the metaphase plate. Pull the chromatids apart at each centromere, separating them into two daughter cells. (What is this part of mitosis called?)

If you want, you can tie strings to the centromeres, as in Figs. 4 and 5, to represent the fibers of the mitotic spindle that provides the motive force for the separation of chromatids. Look closely at your daughter cells. (What phase did they just go through?) If each one contains a complete set of 4 chromosomes, then your mitosis went correctly. (What is the consequence of a missing or extra chromosome? How does this arise?) If a chromosome has been lost or added to a cell, then something went wrong, and the cell will probably die or malfunction.

Note that just before mitosis, each chromosome consists of 2 identical (sister) chromatids joined at the centromere. Just after mitosis, each chromosome consists of 1 chromatid. Before the next round of division, each chromatid will be copied again, in the process of DNA replication, during the S phase of the cell cycle (see text). If you wish, you can simulate DNA synthesis and go through another round of cell division, ending up with 4 identical sets of chromosomes in 4 cells.

D. Meiosis. Meiosis occurs only in the formation of germ cells. It consists of 2 sequential cell divisions. Meiosis results in 4 genetically unique cells in which the chromosome number is reduced to N. Egg and sperm cells must be haploid (N) because they unite in fertilization to produce a new diploid (2N) organism.

Start again with a complete set of 4 chromosomes, each with 2 sister chromatids. In the first meiotic division, homologous chromosomes pair, crossing-over occurs, and then they separate. Line up the homologs in pairs (Fig. 5). At this stage chromatids belonging to each homolog can exchange pieces, in a process called crossing-over. Do such a recombination of genetic material for each pair of chromosomes, as illustrated in Fig. 5. Now pull the homologous chromosomes apart into daughter cells. At the end of the first meiotic division (meiosis I), the chromosome number has been reduced from 2N to N. Each chromosome still consists of 2 chromatids, but in segments where crossing-over has occurred, the chromatids may be genetically different. The second meiotic division (meiotic division II) resembles mitotic division. Line the chromosomes up in each of the cells generated by division I. Now pull them apart at the centromere. You should now have 4 cells. Each cell should contain one copy of chromosomes 1 and 2. If any cell lacks one of the chromosomes, something has gone wrong. It will lack a set of essential genes and will probably die.

You should see that because of crossing-over, each of the 4 cells resulting from meiosis is genetically unique. As a result of fertilization (the union of an egg and a sperm cell), the new diploid organism receives one set of chromosomes from its mother and one from its father. Let us say that in the organism you have been simulating, the red chromosomes were maternal, the yellow chromosomes paternal. Note that crossing-over mixes up (recombines) the paternal and maternal genes. Thus the essential function of meiosis, crossing-over and fertilization, and the whole complicated business of sexual reproduction, is thought to be genetic recombination. The alleles within a population are reshuffled at each generation. Considering only the alleles A, a, B, and b, see if you can figure out how many genetically different germ cells can result from the meiosis you just simulated.

Clean up:
Please remove any tape from the beads and place them back on the tray in the configuration that they were in when you arrived. Push in your chair before you leave.

**Figure 3a.** Spectral karyotyping of human chromosomes. Chromosomes were hybridized with 24 different “painting” probe sets. The main image is view of the chromosomes in the nucleus. The inset image has matched the homologous chromosomes from the main image with each other.
Figure 3b. Human Chromosomes. Graphical representation of human karyotype staining with positions of several well-known genes.
Fig. 4. A way to simulate the mitotic apparatus with strings. Mitotic metaphase above; anaphase below.

Fig. 5. To the left, homologues of chromosome no. 1 (see Fig. 2) paired in meiotic prophase I. C indicates a chiasma (point of crossing over). To the right, the homologues as they pull apart after metaphase, showing the result of recombination.
To test your understanding of mitosis and meiosis, fill in the tables below. If you make a mistake, or find some aspect confusing, discuss the issue with other students or with the instructor.

The somatic cells of the mouse contain 40 chromosomes. With regard to the mouse, fill in the following blanks and tables with the appropriate numbers.

1) **Diploid** chromosome number: ____  **Haploid** chromosome number: ____

2) Write in the **number of chromatids per chromosome** at the end of each stage of cell division:

   **Mitosis**

<table>
<thead>
<tr>
<th>stage</th>
<th>prophase</th>
<th>metaphase</th>
<th>anaphase</th>
<th>telophase</th>
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<tr>
<td>chromatids per chromosome</td>
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   **Meiosis I**

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<th>metaphase</th>
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<tr>
<td>chromatids per chromosome</td>
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   **Meiosis II**

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<tr>
<td>chromatids per chromosome</td>
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</table>

3) Write in the **total number of chromosomes and chromatids per cell** at the end of each stage of the cell cycle or cell division:

   **Mitosis**

<table>
<thead>
<tr>
<th>stage</th>
<th>G1</th>
<th>end of S</th>
<th>G2</th>
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</tr>
<tr>
<td>chromatids</td>
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   **Meiosis I**

<table>
<thead>
<tr>
<th>stage</th>
<th>prophase</th>
<th>metaphase</th>
<th>anaphase</th>
<th>telophase</th>
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<tr>
<td>chromatids</td>
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</table>

   **Meiosis II**

<table>
<thead>
<tr>
<th>stage</th>
<th>prophase</th>
<th>metaphase</th>
<th>anaphase</th>
<th>telophase</th>
</tr>
</thead>
<tbody>
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<td>chromosomes</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chromatids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Lab 2: Drosophila as an Experimental Organism

OBJECTIVE: To become familiar with the life cycle of Drosophila; to become facile at manipulating experimental organisms; to learn to distinguish the sex of flies and identify wild-type and mutant traits.

BACKGROUND: Drosophila melanogaster, a common fruit fly, has been widely used in basic genetic research for almost a century. Drosophila are small and fecund, are easily reared in the laboratory, have short life cycles, and many other features you will learn about that make them ideal organisms for studying eukaryotic genetics.

Male and female flies can be distinguished by a number of sexually dimorphic features (Fig. 1). The female is larger than the male and has a more pointed abdomen. The male has a wide, dark stripe on the end of the abdomen on the ventral side. However, these characteristics may be hard to distinguish in recently emerged flies. There are two other sex differences that can always be used to tell the sex of a fly. The genital organs are located on the ventral posterior end of the abdomen. The ovipositor of the female is pointed, the claspers of the male are dark and surrounded by heavy, dark bristles that are not present in females. The male also has a distinctive spot of dark bristles, called the “sex comb,” on the first pair of forelegs. This characteristic is quite distinct even in recently emerged flies.

Figure 1. Male and female Drosophila, ventral view, 40x
The life cycle of *Drosophila* is characterized by 4 distinct stages: egg, larva, pupa, and adult (Fig. 2). A single adult female may lay as many as 500 eggs over a 10-day period. The larva hatches the day after the egg is laid. Like other insects, a fruit fly grows by periodically shedding its cuticular exoskeleton. Each successive molt is called an instar. There are 3 larval instars.

The cuticle of the 3rd larval instar hardens and darkens to become the pupal case. Within the immobile pupa, the maggot is remodeled to the adult fly. When metamorphosis is complete, the adult emerges (ecloses) from the puparium.

At first the fly is soft and light in color, the wings are folded, and the abdomen is enlarged. In a few hours, the fly pumps its appendages up with fluid from the abdomen, the wings expand, and its color darkens. A virgin female can be fertilized about 8 - 12 hours after eclosion. Female *Drosophila* can store and use sperm from a single insemination throughout their life. Therefore, when setting up crosses, females must be virgin to ensure that fertilization occurs from the sperm of the intended males. Two days after fertilization, she starts laying eggs. Adult *Drosophila* remain fertile as long as they live, which may be several weeks.

The rate of development from egg to sexually mature adult depends on temperature:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Larval Duration</th>
<th>Pupal Duration</th>
<th>Adults Appear At</th>
</tr>
</thead>
<tbody>
<tr>
<td>20°C (68°F)</td>
<td>8 days</td>
<td>6 days</td>
<td>15+ days</td>
</tr>
<tr>
<td>25°C (77°F)</td>
<td>5 days</td>
<td>4 days</td>
<td>10+ days</td>
</tr>
</tbody>
</table>

We incubate the Drosophila cultures at 21°C for a life cycle of about 14 days.
Figure 2. Life cycle of *Drosophila melanogaster*.
A. Mutants of Drosophila
The characteristics of Drosophila that are most commonly found in natural populations of flies are called “wild-type” features. A very large number of “mutant” flies with characteristics that differ from the wild-type have been discovered during the many years that Drosophila has been used as an experimental organism. These mutants are maintained as particular strains that can be purchased from suppliers or obtained from research laboratories. The white eye mutant is one of the earliest Drosophila mutants that was isolated in the laboratory of T.H. Morgan. Look at the flies under a dissecting microscope. The wild-type eye color of Drosophila is brick red; the white eye flies have pure white (really colorless) eyes.

Today you will look at 3 different pure-breeding strains of flies. They are in vials numbered 1, 2 and 3. Each group must determine the genotype of the flies in each vial by observing whether the flies have the mutant or the wild-type phenotype for each of 2 traits: eye color and wing size. The symbols for these traits are:

<table>
<thead>
<tr>
<th>TRAIT</th>
<th>WILD-TYPE SYMBOL</th>
<th>MUTATION SYMBOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye color</td>
<td>red</td>
<td>w+</td>
</tr>
<tr>
<td></td>
<td>white</td>
<td>w</td>
</tr>
<tr>
<td>Wing size</td>
<td>normal</td>
<td>m+</td>
</tr>
<tr>
<td></td>
<td>miniature</td>
<td>m</td>
</tr>
</tbody>
</table>

Use this notation in your notes. For example, the genotype of one strain may be “w m+” if it has white eyes and normal wing size. These strains are pure-breeding, that is, flies of a particular strain are homozygous and therefore genetically identical for the characteristic(s) in question.

All flies in the same numbered vials have the same genotype and all vials contain both male and female flies. Each student should identify the phenotypes of 3-4 flies from each vial and the group should decide together the genotypes of vials 1, 2 and 3. A vial of flies may have 0, 1, or 2 of the mutant traits.

B. Handling Drosophila:
1. Turn on your microscopes. Those that have two lights on their microscopes want only the top light on; the bottom light will not help you see the flies and will melt your ice. Put something on

Leica EZ 4

Oculars
Gross magnification knob
Fine magnification knob
On-off switch
Light selection touchpad

Leica Zoom 2000
the stage of the microscope to view and bring the magnification up high. The gross magnification knob is on the upper side or top of the microscope and the fine magnification knob is on the lower side of the microscope. Move these two knobs to become familiar with how to focus the microscope.

2. The flies are in vials on ice or at 8°C in the lab refrigerator, to immobilize them so you can work with them. They must be kept on ice. Chilled flies will quickly recover when returned to room temperature. Do not submerse the openings of the vials in ice or you will drown the flies.

3. Take your glass Petri dish and half fill the larger dish with crushed ice. Set the smaller dish onto the bottom one and push it gently to get a tight connection between the upper dish and the ice. Place a piece of filter paper in the top dish and place this whole setup on the microscope stage on top of a paper towel. As you look at your flies, the ice in your Petri dish will melt. When it does, take off the top dish, dump the water into the sink and refill with ice as needed.

4. Take one vial of flies, remove the stopper, and tap the vial gently to get 3-4 flies onto your filter paper. If you get too many flies, share them with other groups. Don’t put flies back into vials once they have been taken out because of the chance of mixing them up. Put the stopper on and put the vial back on ice so that the flies won’t wake up.

5. Use the paint brush to move the flies from one place to another, to manipulate the flies under the microscope and see the flies from different perspectives. Examine the flies for the 2 traits we are studying and draw them in your notebooks. Put the mutations next to the wild-type traits to compare the differences side by side. Distinguish between the males and females; separate them into two piles and have the TA check that you can tell the difference between the sexes.

6. When the flies start to wake up and walk around, it’s time for them to go. All flies that you look at today are going into the “morgue”. The morgue is the white plastic can containing soapy water that is on your tray. Pick up the fly with your paint brush and drop it into the morgue; replace the cover. Do not return any flies to vials. Try to avoid letting flies escape into the lab (and hall and offices).

7. When you are finished looking at the flies, wipe off the stage of the microscope with a paper towel, and be sure to turn off the lamp. Empty the water and ice from your Petri dish, rinse the dishes in water and place them on the tray next to the sink. Rinse your paint brush in water.

8. Set up your results in a chart like this; use the symbols discussed earlier for the traits. For example, regarding eye color, you will use “w” for white, and “w+” for wild type red eye color relative to the mutant white eye. For the miniature wing size mutant use “m” and “m+” for wild...
type wing which is longer and passes well beyond the end of the fly abdomen, relative to the mutant miniature wing.

<table>
<thead>
<tr>
<th>Vial number</th>
<th>Eye color</th>
<th>Wing Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

9. Pictures of the flies’ normal and mutant phenotypes will be shown at the end of the lab as well as pictures of male and female *Drosophila* to be sure all students can distinguish the differences.

Below you see examples of males flies, to the left is one with white eye and miniature wings (*w m*), to the right is one with wildtype eye and wing (*w+ m+*).

**Chi Square Analysis**

The chi square test \([x^2\text{, pronounced “kye (rhymes with sky) square”}]\) is a statistical test commonly used in genetics. It determines the “goodness of fit” of a data set which means it compares the *observed* frequencies of an experiment to the *expected* frequencies of an experiment. For example, the ratio of expected phenotypic frequencies of a monohybrid cross is 3:1; three out of four offspring will show the dominant phenotype and one out of four offspring will show the recessive phenotype. If we have an experiment of 1000 offspring and find 782 dominant phenotypes and 218 recessive phenotypes, can we statistically state that those offspring numbers are close enough to the expected numbers of 750 and 250 that they fit the 3:1 ratio? Are the differences in numbers due to chance? Or is another factor likely affecting the observed numbers? Chi square analysis will tell us.
The formula used in chi square analysis is:

\[ X^2 = \sum \frac{(o - e)^2}{e} \]

In this equation, \( o \) is the observed value, \( e \) is the expected value and \( \sum \) (sigma) is the “sum of”. Therefore, the chi square value will be the sum of the observed values minus the expected values squared, divided by the expected value for each category. In our example we have 2 categories, dominant phenotype and recessive phenotype. A chart is a good way to organize the calculations.

<table>
<thead>
<tr>
<th>Expected Ratio</th>
<th>Observed ((o))</th>
<th>Expected ((e))</th>
<th>Deviation ((o - e))</th>
<th>Deviation(^2) ((o - e)^2)</th>
<th>Deviation(^2)/ Expected ((o - e)^2/e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/4</td>
<td>782</td>
<td>(\frac{3}{4} \times 1000) = 750</td>
<td>782 – 750 = 32</td>
<td>32(^2) = 1024</td>
<td>1024 / 750 = 1.37</td>
</tr>
<tr>
<td>1/4</td>
<td>218</td>
<td>(\frac{1}{4} \times 1000) = 250</td>
<td>218 – 250 = -32</td>
<td>-32(^2) = 1024</td>
<td>1024 / 250 = 4.10</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
<td></td>
<td></td>
<td></td>
<td>(x^2 = 1.37 + 4.10 = 5.47)</td>
</tr>
</tbody>
</table>

When we begin a chi square analysis we state a null hypothesis. The null hypothesis asserts that the data set we are testing has no significant difference from the expected data. If the values of observed and expected data don’t match exactly, the difference can be accepted as chance variation. To determine if we can reject the null hypothesis or fail to reject it, we need to find the probability value \((p)\) associated with the chi square value. This is done using a chi square probability table.

Another factor needed to find the probability value is the degrees of freedom \((df)\). The degrees of freedom is equal to the number of categories we are using minus one \((n-1)\). In this instance, we are using two possible categories (dominant phenotype and recessive phenotype) so our \(df = 2 - 1 = 1\). Find the chi square value of 5.47 in the row for 1 \(df\), or find the two columns that the value lies between. Determine the probability by reading the value at the head of the column.
Chi Square Probability Table

<table>
<thead>
<tr>
<th>df</th>
<th>0.995</th>
<th>0.99</th>
<th>0.975</th>
<th>0.95</th>
<th>0.90</th>
<th>0.10</th>
<th>0.05</th>
<th>0.025</th>
<th>0.01</th>
<th>0.005</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>---</td>
<td>---</td>
<td>0.001</td>
<td>0.004</td>
<td>0.016</td>
<td>2.706</td>
<td>3.841</td>
<td>5.024</td>
<td>6.635</td>
<td>7.879</td>
</tr>
<tr>
<td>2</td>
<td>0.010</td>
<td>0.020</td>
<td>0.051</td>
<td>0.103</td>
<td>0.211</td>
<td>4.605</td>
<td>5.991</td>
<td>7.378</td>
<td>9.210</td>
<td>10.597</td>
</tr>
<tr>
<td>3</td>
<td>0.072</td>
<td>0.115</td>
<td>0.216</td>
<td>0.352</td>
<td>0.584</td>
<td>6.251</td>
<td>7.815</td>
<td>9.348</td>
<td>11.345</td>
<td>12.838</td>
</tr>
<tr>
<td>4</td>
<td>0.207</td>
<td>0.297</td>
<td>0.484</td>
<td>0.711</td>
<td>1.064</td>
<td>7.779</td>
<td>9.488</td>
<td>11.143</td>
<td>13.277</td>
<td>14.860</td>
</tr>
<tr>
<td>5</td>
<td>0.412</td>
<td>0.554</td>
<td>0.831</td>
<td>1.345</td>
<td>1.610</td>
<td>9.236</td>
<td>11.070</td>
<td>12.833</td>
<td>15.086</td>
<td>16.750</td>
</tr>
<tr>
<td>6</td>
<td>0.676</td>
<td>0.872</td>
<td>1.237</td>
<td>1.635</td>
<td>2.204</td>
<td>10.645</td>
<td>12.592</td>
<td>14.449</td>
<td>16.812</td>
<td>18.543</td>
</tr>
<tr>
<td>7</td>
<td>0.989</td>
<td>1.239</td>
<td>1.690</td>
<td>2.167</td>
<td>2.833</td>
<td>12.017</td>
<td>14.067</td>
<td>16.013</td>
<td>18.475</td>
<td>20.278</td>
</tr>
</tbody>
</table>

If the p-value (probability value) is 0.05 or less, we reject the null hypothesis. Our $x^2$ value lies between a p-value of 0.025 and 0.01 so it is less than 0.05. This means that there is less than a 5% chance that the difference in values is due to chance variation; we cannot say that 782 dominant phenotypes and 218 recessive phenotypes fit the 3:1 ratio. Something else is likely affecting those values.

The following chi square analysis of a dihybrid cross produces a p-value between 0.9 and 0.1 which is greater than 0.05 and therefore we fail to reject the null hypothesis. We can statistically state that there is no difference between the observed values and the expected values and that the values stated below fit the expected dihybrid ratio of 9:3:3:1. Any value differences can be attributed to chance.

<table>
<thead>
<tr>
<th>Expected Ratio</th>
<th>Observed</th>
<th>Expected</th>
<th>$e$</th>
<th>$(o-e)$</th>
<th>$(o-e)^2$</th>
<th>$(o-e)^2/e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9/16</td>
<td>552</td>
<td>567</td>
<td>-15</td>
<td>225</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>3/16</td>
<td>176</td>
<td>189</td>
<td>-13</td>
<td>169</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>3/16</td>
<td>210</td>
<td>189</td>
<td>21</td>
<td>441</td>
<td>2.33</td>
<td></td>
</tr>
<tr>
<td>1/16</td>
<td>70</td>
<td>63</td>
<td>7</td>
<td>49</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1088</td>
<td>1088</td>
<td></td>
<td></td>
<td></td>
<td>$x^2 = 4.40$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$df = 3$</td>
</tr>
</tbody>
</table>

For a more detailed explanation of chi square analysis, see your text.

Lab 3: Dihybrid Cross in *Drosophila*
Part I- Cross F1s
Part II- Score F2s

(Dihybrid Cross in *Drosophila*, Part 2- Scoring the F2 Population, will be 3 weeks from this lab)

PART I: Dihybrid Cross in *Drosophila*, Part I- Cross F1s
**OBJECTIVE:** To determine the recombinational map distance of two X-linked genes in *Drosophila* by generating an F2 population, and scoring their numbers and phenotypes.

**BACKGROUND:** The main emphasis of the first 50 years of genetics, and one that continues to be central today, was to determine the location of genes on chromosomes. The site of a gene is called its *locus* (plural, *loci*). Since the site of a gene is important in defining it, the term “locus” can be used as a synonym for “gene”. The process of determining the order of loci along a chromosome, and their distances from each other, is called “mapping”. That is what you will do in this exercise.

You will be working with 2 of the following sex-linked genes and their mutant phenotypes.

- **B:** *Bar*, narrow eye in male and homozygous female; kidney shaped eye in heterozygous female (partial dominant mutant allele)
- **cv:** *cross-veinless*, wings lack cross veins (recessive mutant allele)
- **f:** *forked bristles*, body bristles stubby with “split ends” (recessive mutant allele)
- **lz:** *lozenge*, eyes smaller, almond shaped, glossy (recessive mutant allele)
- **m:** *miniature wing* (recessive mutant allele)
- **sd:** *scalloped*, wing margin scalloped (recessive mutant allele)
- **v:** *vermillion* eyed; bright red (recessive mutant allele)
- **w:** *white* eyed (recessive mutant allele)
- **sn.** *singed bristles*; body bristles short and curled (recessive mutant allele)
- **y**

You will be making crosses between two strains that will segregate for the two genes. You need to become familiar with identifying mutants and annotating your notes properly. Your crosses involve white eye and miniature wing mutants, both are two of the genes listed above. The list of mutants above are all found on chromosome one. Determine if your strains have one or both mutants. If a strain has both mutants, it would be *w m* and the wildtype for both would be *w+ m+*. Or a strain may have only one mutant, *w’ m* or *w m’* (Note, since these are X linked genes we are showing the alleles on just one X chromosome that would be present in males along with the Y without these genes. Females with have 2 copies of each allele). As you can see, a wild-type locus is designated with this symbol + relative to the mutant.

Sex-linked traits can be determined by their differential outcome in reciprocal crosses. If you have a female with a mutant trait of interest crossed with a male that is wildtype for that trait, the reciprocal cross would be a male with the mutant trait crossed with a female wildtype. The crosses could be made so that P1 (parent 1) was female and P2 was male or the reverse. These are called reciprocal crosses.

In order to get this experiment going on time, you will be setting up the F1 crosses without fully understanding their rationale. You should think through this experiment during the next few
weeks in light of what you will be learning in lecture. The worksheet that follows will guide you in this process.

In three weeks, you will be continuing the experiment with the F2 generation that has emerged as the offspring of the F1 parents you set up today. In that lab, you will need to score a large number of F2 male flies to increase the chances that your genotype ratios will yield the expected results.

<table>
<thead>
<tr>
<th>Fly schedule</th>
<th>Task or Event</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>week 1</td>
<td>Set up F1 cross</td>
<td>0</td>
</tr>
<tr>
<td>week 2</td>
<td>Dump F1s</td>
<td>7</td>
</tr>
<tr>
<td>week 3</td>
<td>F2s start to emerge</td>
<td>14</td>
</tr>
<tr>
<td>week 4</td>
<td>Score all F2s</td>
<td>21</td>
</tr>
</tbody>
</table>

Background - Three weeks ago, virgin female flies of one strain were crossed with male flies of another strain and became the P1 generation (P stands for parental) for our cross. Reciprocal crosses were made so that in vials “A” labeled with red tape, the females were red-eyed and the males were white-eyed. In vials “B” labeled with white tape, the females were white-eyed and the males were red-eyed. These flies mated and the females laid eggs for seven days and then all adult flies were removed from the vials. The eggs in the vials hatched and developed into larva, then into pupa and most have emerged from their puparium as adult flies. These flies constitute the F1 generation (F stands for filial) and they are the flies in your vials today.

Your TA will tell you what the genotypes of the P1 generation were. Record this information and predict what the expected genotypes and phenotypes are of the F1 males and females in vials “A” and “B” that you are given today. In all vials the males and females will have different phenotypes. Why? You can use the chart at the end of this lab to predict what the F1 genotypes should be.

Procedure Week 1 – Setting up the F1 cross

1. Each student group will take 1 vial of “A” flies and one vial of “B” flies. Place those vials 2/3 in the ice to knock out the flies.
2. Label three fresh vials containing media with tape, “a”, your name, and today’s date.
3. Label three fresh vials containing media with tape, “b”, your name, and today’s date.
   Label the black box with your group name, lab day and time, and your TA’s name.
4. Using the dissection microscope, check the phenotypes of both males and females of “A” and “B” to see if they have the phenotypes that you predicted. If you find any fly that does not show the correct phenotype, you must see your TA for assistance. What are some possible reasons that the F1s have the wrong phenotype?
5. Place 3-4 females and 1-2 males into each of the 3 vials labeled “a”.
6. Repeat the process of checking the phenotypes of the flies in your “B” vial. Place 3-4 females and 1-2 males of vial “B” into each of the 3 vials labeled “b”.
7. Return box with the 6 lower-case lettered vials in it to the incubator, return microscope and wash out dishes and brush. Save any leftover F1 flies that you transferred into vials without media in your ice bucket; we will take them. Place any unused F1 vials or F1 vials with adult flies in them back into the boxes they were in. Don’t put any loose flies back into vials with media as a mistake could mix up the flies.
8. The F1 flies will mate and the females will lay eggs for 7 days. We will remove the F1s from the vials next week.

![Diagram of F1 generation setup](image)

Figure 1. The F1 generation setup: vials “a” and “b” contain F1 flies from reciprocal crosses.

Procedure – Week 2 – Dumping the F1 flies

1. Take your flies from the incubator. Have a morgue ready beside you to dump the flies into. Holding the fly vial in one hand, tap the vial on the table to knock any flies off the stopper. Quickly, pull off the stopper and gently shake the vial to dump out the flies. Tap the edge of the top of the vial against the edge of the top of the morgue for flies still in the vial. The TA or the lab tech will demonstrate this process for you. Cover the morgue. Replug the vial and check to see that all living flies came out. If there are still some in the vial, repeat the process. Single flies may be picked up with a paint brush and put into the morgue or pushed under the media.

Why is it necessary to remove the F1 flies from the vials before the F2s emerge?

There is a possibility that the media could fall out of the vial into the morgue and taking with it most of your F2s so don’t shake the vial too hard. If this does happen to you, save the vial with the media that’s left in it. There will be some eggs and larvae in the vial.

2. Return the vials to the incubator. Your F2 population will start to emerge next week.

Procedure – Week 3 – F2s Start to Emerge

Procedure – Week 4 – Score the F2s. See the procedure in the “Dihybrid Cross of Drosophila, Part 2: Score F2s in this lab manual.

Eye Color Comparison:

- Wildtype wing (m+); Wildtype red eye (w+)
- Wildtype wing (m+); Mutant white eye (w)
Wing Size Comparison:

Eye-color and wing mutation:
Notice how the miniature wing mutant wings extend just beyond the abdomen, and they are slightly narrower than wildtype wings.
Miniature wing (m), white eye mutant (w).

Wildtype wing length and eye color:
Notice how the wings extend well beyond the abdomen.
Wildtype wing (m+), wildtype red eye (w+).

Worksheet
The following is an example meant to guide you, with traits that differ from your flies.
Predicting possible outcomes based on a hypothesis is an essential feature of any well-planned experiment in biology. Experiments should test hypotheses. This is particularly true of genetics. What are the hypotheses you are testing in this experiment? The following exercise and questions will help to guide you through this mapping experiment, and make predictions about your results. This should be recorded in your lab notebook during class.

1) Previously you recorded genotypic information given to you about the parental (P1) lines of this cross. Diagram these reciprocal crosses and predict the resulting F1 genotypes and phenotypes AND now predict the expected genotypes and phenotypes in the F2 population of the next generation.

Here is an example, using a different set of traits, the cross between yellow body females (wildtype for all other traits including lozenge) and lozenge males (wildtype for all other traits including yellow body). You know that all the genes involved are sex-linked and that these inbred lines are homozygous.

<table>
<thead>
<tr>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>y lz+</td>
<td>y+ lz y lz+ Y</td>
</tr>
</tbody>
</table>

2) Predict what your F1 results should be. With the example above, the female parent would make one kind of X chromosome (y lz+) and the males being the heterogametic sex would make two types of gametes, (X chromosome with y+ lz and a Y). The F1 generation would therefore have females with an X chromosome from its mother and an X from its father while the males would have the X from its mother and the Y chromosome from its father. This “unequal” situation with males getting one copy of the gene and females getting two, is unique to genes on sex chromosomes and causes the unusual phenotypic patterns in F1 and F2 populations.

<table>
<thead>
<tr>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>y lz+</td>
<td>y+ lz y lz+ Y</td>
</tr>
</tbody>
</table>

3) A. Predict what the F2 genotypes and phenotypes would be if the genes were unlinked (you can do this with a branch diagram). For the above cross, females will segregate for yellow and wild-type body, but all will be normal eye (not lozenge) since the paternal X carries that wild-type allele. Males will receive any combination of alleles from the F1 mother and a functionally recessive Y chromosome from its father.

<table>
<thead>
<tr>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>y lz+</td>
<td>y+ lz Y</td>
</tr>
</tbody>
</table>

B. Predict what the F2 phenotypes and genotypes would be if crossing over did not occur in the F1 females. For the above case, the F2 females would be:

yellow and wild-type eye wild-type for all traits Males would be:

<table>
<thead>
<tr>
<th>y lz+</th>
<th>y+ lz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

yellow and wild-type eye lozenge eye and wild-type for other traits

Note, crossing over does not occur in Drosophila males and the Y chromosome does not carry any of these genes so it is functionally recessive.

4) With the two predictions above, (A. if the genes are unlinked and B. if there is no recombination in the female and the genes are completely linked) you now have the extreme
results that could occur with this cross. Your results will likely land somewhere between these two extremes.

5) You should also notice that the male F2 progeny and the female F2 progeny give you very different results (as is often the case with X-linked traits). Since the Y chromosome of the F1 father is functionally recessive, scoring the F2 sons, is basically like scoring a testcross (recall a test cross could be AaBbCc x aabbcc). In our example, the F1 mother is a double heterozygote and her gamete is being matched with a recessive gamete, the Y, from the father.

**How useful for mapping will the females of this cross be?** The female F2 will not segregate for any gene that was dominant in the F1 father (lozenge in this case) and thus the female F2 progeny are not that useful and you will only need to score the males.

6) You will find it most convenient if you score your data (F2 male phenotypes) in terms of the predicted genotypes from step 3A) arranged as reciprocal/complimentary classes (We are not showing the recessive Y chromosome). For example:

```
y   lz+    y+   lz
y+ lz    y   lz+
y+ lz+ y+ lz+ y lz y lz
```

The pair that should be most common would be the non-crossover category (NCO, the non-recombinant chromosomes of the F1 female, identified in 3B above). This is also called the parental class, because these are the original chromosomes contributed to the F1 female from the parental generation. In this example it would be which pair? The first pair, y lz+, y+ lz, is the NCO category. The remaining pair is the recombinant category.

7) You will find that arranging your data in this way will help you to organize your calculations of the distances between loci. Then you will be able to work out your map of the X chromosome.

The chart on the following page will help you keep track of the genotypes of the male and female flies of each generation of your experiment and the identifying letter of their vials. Fill in the genotypes and phenotypes of the flies as you set up each cross.
## Dihybrid Drosophila Cross Chart

<table>
<thead>
<tr>
<th>Vial ID</th>
<th>Red Tape (Red-eyed Female, white-eyed male) Cross 1</th>
<th>White Tape (White-eyed female, red-eyed male) Cross 2 (Reciprocal of Cross 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotype</td>
<td>A</td>
</tr>
<tr>
<td>P1</td>
<td>♀</td>
<td>--------------------------</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Y</td>
</tr>
</tbody>
</table>

Legend: Y = Yellow
<table>
<thead>
<tr>
<th>F1</th>
<th>♀</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Y</td>
<td>Y</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Y</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vial ID</th>
<th>A Genotype</th>
<th>B Genotype</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>F2</th>
<th>♀</th>
<th>A</th>
<th>B</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Y</td>
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<tr>
<td></td>
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<td>Y</td>
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</table>

|    |    | Y | Y |
|    |    | Y | Y |
|    |    | Y | Y |
Lab 3: Dihybrid Cross of *Drosophila*, Part II: Score F2 flies

**OBJECTIVE:** To determine the map distance of two X-linked genes in *Drosophila* by performing a dihybrid cross. In this lab, the phenotypes of the F2 flies will be scored in order to determine the frequency of recombination between the two genes and relate that to map distance.

**BACKGROUND:** In the previous part of this lab, you set up an F1 cross and its reciprocal cross. (The crosses were labeled with different colored tape.) One week later, you removed all F1 flies from the vials and kept the eggs and larvae of the F2 generation. The F2 flies have now emerged in your vials, and today you will score the phenotypes of a large sample of F2 flies.

In this particular cross, you will score the phenotypes of male flies only. Since the males have one “X” chromosome and one “Y” chromosome, and our genes of interest are located on the “X” chromosome, the phenotypes of male flies indicate their genotypes. A male fly with white eyes must have the “w” mutation for eye color because he has only one allele of this gene, and it must be expressed. A male fly with long (wildtype) wing size must have the “m+” allele because he shows that phenotype: a white-eyed, wildtype (long) wing size male would have the genotype “w m+”.

In some dihybrid crosses using X-linked genes, you can use phenotype data from both male and female F2 progeny to calculate the frequency of recombination. You should diagram a cross using X-linked genes in which this approach would be possible.

Fill in the expected F2 phenotypes in the table below to make data collection more efficient.

<table>
<thead>
<tr>
<th>Reciprocal Crosses</th>
<th>Genotypes</th>
<th>Phenotypes</th>
<th>Parentals or Recombinants</th>
<th>number of male flies with this genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cross 1</strong></td>
<td></td>
<td>fill in below</td>
<td></td>
<td></td>
</tr>
<tr>
<td>red tape</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cross 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>white tape</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: SCO = Single crossover.
PROCEDURE:

1. Place a vial of F2 flies horizontally on the ice to slow them down. If you put the vial in the ice vertically, the flies will fall to the bottom and get stuck in the medium.

2. Use the following steps to transfer flies from their original vial to a clean, empty vial:
   - Take a clean, empty vial and label it with the letter of the vial you want to score, “a” or “b”.
   - Place the new vial vertically in your ice bucket, leaving only 1 inch of the vial out of the ice; let it chill for 5 full minutes. Don’t get any ice in the vial.
   - Tap the original vial of flies against your hand a few times to knock the flies off the stopper. Take the stopper off quickly and invert the vial with the flies over the chilled vial on ice. Holding the two vials together, tap the vials into the ice knocking the live flies into the chilled vial. Replace the stopper on the live fly vial and stopper the chilled vial as well.

3. The flies will be immobilized in about 5 minutes, and then you can tap out 7-8 out at a time. Don’t take out more than this at one time; you don’t want your data flying away before you have time to score! Put females directly into the morgue. Score the phenotypes of male flies, and put the scored flies into the morgue.

4. Score as many male flies as you can during the lab. (Note: Females tend to emerge before males so don’t be alarmed if early in the week, the flies are mostly female.)

5. When you have finished, please return the microscope to the cabinet, dump out your ice, rinse the Petri dishes and brushes, and return your flies to the incubator.

6. Take some time at the end of the lab to combine your data within your group; also, take the data of the class as a whole.

Do you expect the reciprocal crosses to show any difference in their genotypic ratios? Test each set of data using Chi Square analysis to see if your group’s data or the class data give a result closer to the expected data.

You will write a detailed report on this lab. The instructor will discuss how it should be organized. The report should include a clear presentation of your data, a calculation of the map distance, and a comparison of your map with the well-established map of the Drosophila X-chromosome. To do this final step, you will determine the expected number of progeny in each of the 2 categories, parental or non-crossover (NCO) and single crossover (SCO), using the map distances from the book. You will also use the total number of male progeny scored by you and your partners.

For example, suppose you are looking at hypothetical genes “a” and “b”. You score 300 flies and know the map distance between two genes “a” and “b” is 20 map units: $a \ 20 \ b$

Since 20% of the progeny (or 0.20 x 300 = 60 flies) are expected to have recombination between $a$ and $b$, then the expected SCO category is 60. That is, (SCO)/Total = (60)/300 = 20%, the map distance between “a” and “b”.

The expected NCO category would be the remainder, 300 – (60) = 240.
Please note that determining the distance between genes from the reference map in your textbook you will need to convert from cM (centimorgans) to recombination frequency percentage in order to complete the calculations. A conversion table is provided on the next page.

**Converting between recombination and centimorgans (cM).**

The Drosophila X-chromosome map in your text depicts distance between genes in centimorgans (cM). You will need to refer to that map to determine the distance between the loci associated with the mutants used in this experiment and then find the associated recombination frequency Map Units in the table below and use that in your calculations in your report. A centimorgan (cM) is a measure of genetic linkage. It is not a standard physical distance except in that it represents the distance between the loci for which the expected average number of crossovers in one generation is 0.01.

Below is a mapping function conversion table. Use it to convert distance from centimorgans (cM) to recombination frequency map units for the Drosophila-X chromosome. Within each row a frequency is associated with a map distance in cM. The Haldane column (right column) is a list of distances in the unit of centimorgan (cM). The left column is the Recombination frequencies.

**Using the example on the previous page**, realize that the distance between gene a and b of that example happened to be 0.2554 cM on the map in the textbook.
From the table below one would read the recombination frequency between those two genes 0.2554 cM apart on the map is expected to be 0.20 in one generation. **It is the recombination frequency that is used in the calculations of the SCO and NCO.** (in that problem it was 0.2)
Lab 3: Dihybrid Cross in Corn (Zea mays)

OBJECTIVE:
You will characterize the segregation pattern, resulting from a simple cross in Zea mays or Brassica differing for two distinct traits and test the fit to expectations predicted by Mendel’s Laws.

BACKGROUND:
Maize or corn (Zea mays), like Drosophila, has been a very important organism for the study of genetics. Dr. Nina Fedoroff, a member of the National Academy of Science called corn “man’s first and perhaps his greatest feat of genetic engineering” (Federoff, Science 2003). Humans have drastically altered the appearance of this species over the last 10,000 years to produce a plant with huge cobs of densely packed and highly nutritious kernels and resulting in a major food staple for much of the world (Figure 1). This species also has several features that make it a good model system for genetics. Different lines with unusual traits are easy to cross and large progenies can be produced. The species also has mutants known to affect embryo, endosperm or seedling characteristics that can be viewed early in development and readily scored to assess heritable patterns. Traits expressed in adults such as flowering and fruit development require more space for growing larger plants and more time before scoring; note, Mendel had far greater sample sizes (> 7000) for the embryo traits that are expressed in early development and viewed in the kernels. We will discuss several of these traits and you will examine crosses segregating for a few. Research on Maize has not only aided our understanding of fundamental genetic processes, but has also contributed to the improvement of corn as an agricultural product. Plant and animal breeders are constantly developing new lines by making crosses with wild or divergent relatives and recombining traits into new and unique individuals. In this lab, you will be examining the results of a cross between two inbred lines of corn that differ for two major morphological traits. You will identify the new variants created by this cross and characterize the segregation patterns that result in light of your understanding of Mendelian “laws.”

SUMMARY: This experiment involves a simple dihybrid cross specifically designed to be examined in a class room situation. You should see that the offspring of the cross often have phenotype combinations that were not present in the parents. You should be able to predict the ratio of these various phenotypic combinations in the offspring and test your predictions.

Figure 1. Teosinte, the wild progenitor species of corn and a primitive variety of modern corn (from Federoff, Science November 2003).
### Table of Maize Genes and Morphological Traits Examined

<table>
<thead>
<tr>
<th>Locus</th>
<th>Location&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mutant Phenotype</th>
<th>Wildtype Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>d5</td>
<td>2S-56 dwarf</td>
<td>normal height</td>
<td>5L-128 white seedling green seedling</td>
</tr>
</tbody>
</table>

Information in table retrieved from http://www.maizegdb.org/

<sup>a</sup> Location refers to chromosome number (1-10), arm (Long or Short) and the current recombination distance from one end.

### PROCEDURE

**Cross 1, F2 in corn:**

A. You will be given germinated corn kernels to score seedling traits depicted in the table above. **Make a prediction:** what do you expect the phenotypic ratios to be in the following dihybrid cross, based on Mendel’s Laws and the location information provided? You should predict the expected ratios of each locus separately (e.g. white and green for the lw3/LW3 locus in the first cross) and then the two loci together (e.g. the white and dwarf phenotypes for the LW3/lw3 and D5/d5 loci together). **Also predict phenotypes and the genotypes of the inbred parents for each F1 of your crosses** (The inbred parents of this generation are often called P1 and P2).

For the corn seedling traits, the following cross was made.

1) F<sub>1</sub> shown below was intercrossed to produce F<sub>2</sub> $D5d5$ $LW3lw3$ $X$ $D5d5$ $LW3lw3$

B. **Predict the phenotypic ratios for each individual locus and for the combined loci resulting from the above cross and test your predictions by scoring seedlings distributed in the lab.** Be gentle with the seedlings as you score them because other labs will use these same seedlings. Return the boxes of seedlings to the appropriate area.

C. **Use the chi square test to evaluate your data.** What is your null hypothesis in each case?

D. **Points to follow up with in your notebook:**

1) Several loci located in different parts of the genome affect the same character. **Can you give some simple explanations for this occurrence?**

2) Some phenotypes which may appear similar (e.g. dwarf) are caused by defects in specific genes. **Can you imagine a mechanism by which it might occur? Without doing further experiments can you tell?**

3) The phenotypes that we are observing affect different tissues (e.g. whole plant, seedlings, leaf texture). **How might changes in the seedling color affect leaf color?**
E. Push in your chair before you leave.

**Genome Wide Association (GWA) Studies in Recombinant Inbred Lines (RILs)**

**OBJECTIVE:** The objective of this lab is to examine the role of segregation and recombination in the generation of variation in populations and to apply our understanding of these processes to the problem of identifying the genetic basis of phenotypic traits. In an earlier experiment this semester, you examined segregation patterns of two simple traits in maize. Here you will dissect the genetic basis of phenotypic differences in a cross involving wild and domesticated iceberg-like lettuce segregating for hundreds of traits. You will locate the position of major genes affecting a trait of your choice to the chromosomes of your experimental organism, lettuce.

**BACKGROUND:** Because genome sequencing technologies have advanced so much in the last 10 years, many species of microbes, plants and animals with their unique features are proving to be useful genetic model systems. One such species is lettuce. Lettuce, like maize, has been “genetically engineered” by humans who have often selected, directly or indirectly, for specific traits during the last several thousand years of agricultural practice. Wild lettuce and wild maize look nothing like their close domesticated relatives. Yet they can be crossed to the domesticated varieties in order to understand the domestication process and to identify the genetic basis of key economically important traits such as yield, disease resistance, kernel size (in maize) or heading (in lettuce), not to mention taste.

Plant and animal breeders are constantly developing new lines by making crosses with wild or divergent relatives. In this lab, you will examine a complex cross involving wild, weedy lettuce and common iceberg lettuce. While “Mendelian laws” generally hold, the phenotypic patterns become complicated because of the many interactions among genes and the role of the environment in the control of phenotypes. You will define a trait that distinguishes the wild from the domesticated lettuce (Fig 1), and then attempt to map the location of the gene(s) that control that trait. You will also be able to examine the influence of the environment on your traits of interest and begin to see how a phenotype depends on both on genetic and environmental factors.
Figure 1. Comparison of wild and domesticated lettuce. Photo by Dina Tsirelson and Rick Kesseli, University of Massachusetts, Boston

Wild *Lactuca serriola* (on left) and the crisphead cultivar of domesticated *Lactuca sativa* (on right).

**Lettuce Recombinant Inbred Lines**

Recombinant Inbred Lines (RILs) are segregating populations used in current studies by many eukaryotic geneticists. RILs have been produced for nearly all model systems from mouse and zebrafish to *Drosophila*, yeast and plants (not for humans of course). Here you will see many traits segregating in these RILs of lettuce. These RILs were created by crossing a wild, weedy lettuce and a domesticated iceberg lettuce (Figure 1).

The F1 hybrid was then self-pollinated (sib mating is used for mouse and other species with separate sexes) to produce the F2 population. Each member of the F2 was subsequently self-pollinated for many generations until each line was inbred and virtually homozygous at all loci (see Figure 2). Each member of a given RIL should therefore be genetically identical to other members of that specific line. Each RIL, however, differs from all other RILs. The different RILs will all have received approximately half the alleles (variant version of a gene) for its genes from each of the original parents. However, because of recombination during the breeding process, the alleles have been shuffled, and each RIL has a different combination of these alleles.

The weedy parent is not particularly edible; it has tough and odd shaped leaves, thorns, and a variety of other characteristics. You will be able to view some of these traits, like hypocotyl length (see Figure 3), in early seedling stages. Other traits, like spines on different parts of the plants or flowering time, can be observed only in later adult stages.

**Figure 2. How RILs are created.** For each pair of homologous chromosomes (and the figure shows just one pair), the F1 has one that it received from the domesticated parent, *L. sativa* (pink) and one received from its wild parent, *L. serriola* (green). Thus at every gene location, the F1 has one allele from the domesticated and one from the wild parent. The F1 would therefore be heterozygous at all loci that differed for these two parents. After the inbreeding process, in F6, the two homologous chromosomes are now identical (that is, homozygous at all loci). However, each chromosome has a mixture of genes that came from the original domesticated (pink) and wild (green) parents.
The hypocotyl is the part of the seedling stem between the attachment of the embryonic leaves (cotyledons) and the root. The hypocotyl elongates at germination to push the leaves to the surface of the soil. The hypocotyl length differs greatly between the wild species (left) and domesticated (right).

**Genome-Wide Association (GWA) Studies**

Your task is to identify different morphological traits in the RILs and to note whether the differences can be viewed in the parents or are novel and found only in some RILs. **What might be the explanation for finding new variants in the RILs that were undetected in the parents?** (Hint, is every trait that you see in yourself exactly like that in one or the other of your parents?) Once you have identified traits, you will scan the genome of lettuce to search for associations between regions of the genome and the observance of your trait. This procedure of scanning for genome-wide associations (GWA) is relatively new as it takes advantage of the powerful high-throughput next-generation sequencing technologies now available. GWA studies are used to identify the genetic basis of complex traits in many species (including humans).

Here, you will be given an Excel spreadsheet showing many genes that differ between the original wild and domesticated parents. These genes segregated in F2 and the later generations used to produce these RILs, and were subsequently mapped on to the 9 chromosomes of lettuce.
The alleles for these genes are DNA sequence differences that were identified by sequencing the genomes of these parents. These single base-pair differences are called single nucleotide polymorphisms (SNPs). These SNP alleles in the Excel file are designated (labeled and color coded) as coming from one or the other of these parents. These SNPs serve as signposts for us to locate the position of other genes segregating in the RILs that affect the important phenotypic differences distinguishing the wild and domesticated parents.

**PROCEDURE**

**RILs of Lettuce:**

There will be sets of RILs on each bench. Depending on life stage being examined this semester, the RILs may be seedlings growing in petri plates or juvenile or adult stages growing in pots. In total, there are approximately 80 different RILs as well as the parents in each set. There will also be replicate individuals of each of these RILs planted side by side in the petri plates or flats. You should identify the parents in each set labeled as SER for *Lactuca serriola* (wild) and SAT for *Lactuca sativa* (domesticated iceberg).

1. **Identify traits that differ between the two parents.** Describe these traits and record the information in your notebook.

2. **Now view the collection of RILs.** Recall that the individuals within a given RIL are genetically identical. **How variable are these individuals phenotypically? Do some RILs show variation while others do not?** Record observations in your notebook.

3. **What is the cause of this phenotypic variability?** If these plants were grown under different conditions (change the light, add more or less water, plant them outside…) how might these changes affect your ability to determine the genetic basis of your trait?

4. **Can you identify traits that appear in some RILs but not in either parent?**

5. **Choose two traits that your group plans to analyze.** These traits might be clearly defined with contrasting phenotypes such as tall vs short plants or having stems with spines vs no spines. Other traits might be unique and not have an obvious alternative state or not be found in either parent but present in a collection of the RILs such as an odd leaf shape or early flowering. In this situation you would have just one category.

6. **Carefully define each trait that you are scoring.** Explain how you are scoring that trait in either a quantitative or qualitative manner.

7. **Define the origin of the different contrasting states of the trait if you can.** That is, can you determine which parent contributed the trait to the subsequent generations? If you chose a unique trait, can you identify features of the trait that might have been derived from the different parents?

8. **Photograph your trait so that it can be readily identified by others who examine these RILs.** The photos should be well-lit and in focus. When composing each photo, include a ruler (provided) and a legible label in the shot. Be sure the ruler and the part of the plant you're focusing on are in the same plane relative to the camera. The label should include the genotype of the plant (e.g. *L. serriola*, *L. sativa*, RIL 309, etc), your team's name and the date.

9. **Record up to 6 different RILs that have each extreme or alternative versions of your trait** (longest vs shortest; thorns vs no thorns; serrated edges vs smooth…). If you chose a unique trait not found in the parents such as an odd leaf shape, try to identify as many RILs as possible (more than 6 if you can).
10. Carefully review Figure 4 and the Excel spreadsheet that you have been given. The figure shows how to read the Excel file. The parents followed by the different RILs are in the columns and numbered at the top. Each row defines a different single nucleotide polymorphism (SNP) marker. The SNPs with the “A” allele (pink) represent the DNA sequence that came from the domesticated parent and the “B” allele (green) represent the DNA sequence that came from the wild parent. Notice that the 3rd and 4th columns with the SAT and SER parent are all pink or all green respectively. The genes with these SNPs have been previously mapped on the lettuce genome. These SNP markers have been lined up with the markers at one end of Chromosome 1 (Chr1) found at the top of the file, followed by the SNPs in order to the bottom of Chr1. The recombination distances, in cM, from the top end of Chr1, are given in column 2. Each of the remaining chromosomes, Chr2 through Chr9, are lined up in a similar manner below Chr1. (Chr3 and 3A are different arms of the same chromosome but with enough recombination between them that they act unlinked). You can quickly see that each RIL is different from all others.

Figure 4. How to read the Excel file of RIL mapping data.
11. **Copy the entire worksheet into a second and third page.** Keep the original page as a back-up in case you accidently delete something; name and use your other two pages as worksheets for your two different scored traits you identified in step 5 above.

12. **In your first trait worksheets, insert a series of 10 to 12 blank columns at the front** (between the parents, which are shown in columns 3 & 4, and the RILs shown in columns 6 - ). The number of blank columns depends on the number of RILs that you identified in step 9 for your trait.

13. **Copy the column of each RIL that belongs to each extreme group together, side by side in these blank columns.** For example, if you were scoring height, you would copy and paste each of the 5 or so “Tall” RILs you scored into the first five blank columns and follow those by the five “Short” RILs.

14. **Now map the genomic region(s) responsible for your trait.** Starting at the top of the file (the top of Chr1), you can search down through the different regions of the different chromosome to **see if you can identify a region of the genome that is “associated” with your trait.** A candidate region would have a higher than expected distribution of alleles derived from one parent in one extreme category and the alleles from the other parent in the other category. Using the example of Tall and Short again, you might find that all your Tall RILs have the B (Green) alleles for a region (several rows of the mapped SNPs) on Chr2, and the short all have the A (Pink) alleles in that same region. You might find multiple regions, and you might find regions with alternative patterns (e.g. the Tall and Short RILs might have the pattern on Chr2 described above but also in another region have the Tall RILs with Pink and the short with Green alleles. Depending on the complexity of the trait and the size of your sample set, you might discover regions that are suggestive of being associated; (e.g. all the Talls have the Green, and all but one of the Shorts have the Pink).

15. **Discuss the genetic basis of any patterns you see. Be sure to discuss the probability of getting your patterns by chance alone.**

16. **Repeat this operation (Steps 12-15) for a second trait that you identify in your second work sheet.**

**Depositing your data:** Along with your photos, deposit your Excel file data. The RIL columns that you have identified as representative of the contrasting traits will be submitted to the Blackboard site. TAs will provide instructions.

**SUMMARY:** An earlier experiment with maize involved a simple cross specifically designed to be examined in a classroom situation. This experiment with lettuce involves mapping populations derived from distinct genotypes segregating for many complex traits. This experiment gives you the general feel for what a real study aimed at uncovering the genetic basis of phenotypic traits would look like. There are some short cuts in this experiment to keep it small and manageable. For example, to pin down the genetic basis of your traits we would likely need many more replicates of each RIL to limit environmental “noise.” We might also need to screen more RILs to increase the number in each of the defined phenotypic classes. As you answer the “thought questions” below, you might note how the experiment could be improved to obtain stronger and significant results that might be worthy of publication! Most of these traits have yet to be defined genetically or at the molecular level. Your trait could conceivably be something new, and your experiment and analysis could be considered a pilot experiment for future studies. You are participating in a long-term experiment that we hope will lead to the identification of many traits of interest in this important agricultural species. We will
be collecting data from many individuals across many environments and use these data to
dissect the environmental and genetic contributions to phenotypic differences.

**Thought Questions:**

1. Did you identify any candidate regions/SNPs for control of your traits? List the relevant
   SNPs/SNP ranges for each trait. What do these SNPs look like? For example, is there a
   perfect correlation with the trait or not? Which parent contributes each extreme? Does
   this fit the pattern you expect based on the parents’ phenotypes and genotypes?

2. Do you think it’s likely to get these results by chance? How could we reduce the
   likelihood of obtaining results that are caused solely by chance?

3. What do you think your results would look like if we had scored more RILs? Do you
   think we would have more or fewer candidate regions/SNPs?

4. Were all of your regions for your extremes of each trait equal and opposite? What might
   it mean if a region for one extreme of a trait does not have the opposite alleles for the
   other extreme?

5. If you didn’t find any regions that are perfectly correlated with your trait, propose an
   explanation. Give at least 2 reasons.

6. How effective do you find this method of analysis? How do you think we could increase
   the effectiveness of this kind of experiment?
Lab 4: Plant Molecular Genetics – Part I
DNA Extraction
Using Micropipettes and Microcentrifuges

**Silene latifolia**

**OBJECTIVE:** To determine the sex of dioecious plants through DNA extraction, PCR and gel electrophoresis analysis and to expose you to several important tools commonly used in molecular biology

**BACKGROUND:**
While most plants are hermaphroditic (possessing flowers with both male and female sex organs), about 5% of flowering plants are dioecious (individual plants are either male or female). Some well-known dioecious species are: asparagus, kiwi, *Cannabis sativa*, mulberries and ash trees. *Silene latifolia*, a common herbaceous weed, is also dioecious. The sex determination system in *S. latifolia* is similar to that found in mammals; males are XY and females are XX.

In this lab, each group will collect a small sample of leaf material from two plants of known sex (one male and one female) and also from two plants of unknown sex. You will extract DNA the first week. The second week you will set up a polymerase chain reaction (PCR) to amplify (make millions of copies) a specific small sequence (between 300 and 800 base pairs in size depending on which region is targeted) on the Y chromosome. You will visualize the amplified Y specific DNA as a single band of specific size by agarose gel electrophoresis in the third week. You cannot be certain that any of the steps work until the last week, but there are various controls in place so that you should be able to tell where the experiment failed (if that happens). For your unknown samples, you will see no band on the gel in the last week if any one of the following occurs: no or poor DNA extraction the first week, no or poor amplification by PCR the second week, the gel is made improperly on the third week or both unknowns are female and did not have the Y chromosome sequence to amplify. You should be able to distinguish between these alternatives by examining the results obtained by the various controls. Detailed protocols follow.

You must also first learn to use the equipment in the lab properly. One important item is the micropipette please read and carry out the following tutorial.
**Using Micropipettes & Microcentrifuges:** familiarization with the correct use of micropipettes. Update using Jocelyn’s method

**Introduction:** Frequently in genetics, molecular biology, or cell biology labs, we need to measure or transfer very small volumes of liquids. Micropipettes are precise measuring devices used for this purpose. There are many different brands of micropipettes, the most common being the Pipetman produced by the company Gilson. Although the details of various models differ, the basic components of micropipettes are similar; these are depicted in Figure 1. The tiny volumes we typically measure often range from less than 1 to 1,000 microliters (written as µL). Recall that 1 microliter (µL) is a millionth of a liter (L) and one thousandth of a milliliter (mL). Table 1 shows the three Pipetman models that we will be using and their recommended ranges of volumes.

![Figure 1 Parts of the Micropipette](image)

<table>
<thead>
<tr>
<th>Model of Micropipette</th>
<th>Recommended Range of Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>P20</td>
<td>2 to 20 µL</td>
</tr>
<tr>
<td>P200</td>
<td>20 to 200 µL</td>
</tr>
<tr>
<td>P1000</td>
<td>200 to 1000 µL</td>
</tr>
</tbody>
</table>

**Table 1. Pipette models used in this lab.** Many more models are available.

Prior to using a micropipette, it is critical to know what model you are using, how to set the volume, how to read the volume, and how to attach a tip.

Micropipettes usually contain a label at the top of the device indicating the model as seen below.

![Figure 2. Images of the tops of a P20, P200, and P1000.](image)
**Figure 3. Volume Indicator Dials:** The volume indicators are shown for a P20, P200, and P1000.

In **Figure 3** above, the **P20**, which has a range of 2 to 20 μL, is set to measure 6.86 μL. The dial contains 3 slots for numbers, with the bottom slot in red. The first slot, which is set to zero in the tens place. The second slot, which is set to 6 in the ones place. The red slot indicates the tenths (whole number) and hundredths place (notches). Each red notch measures 0.02 μL. **The P200**, which has a range of 20 to 200 μL, is set to measure 132.4 μL. The dial contains 3 slots for numbers. The first slot, which is set to 1, sets the hundreds place. The second slot, which is set to 3, sets the tens place. The third slot sets the ones (whole numbers) and tenths place (notches). Each notch measures 0.2 μL. **The P1000**, which has a range of 200 to 1000 μL, is set to measure 262 μL. The dial contains 3 slots for numbers, with the top slot in red. The red slot, which is set to zero, sets the thousands place. This slot should never be set to a number other than 0 or 1! The second slot, which is set to 2 in Figure 3, sets the hundreds place. The third slot sets the tens place (whole number) and the ones place (notches). Each notch measures 2 μL.

**HOW TO ATTACH A TIP PROPERLY:**

1. Find the correct tips. Different models of the micropipettes require different sizes and/or types of tips. If you use the wrong size tip you may not measure the volume accurately. The P20 and P200 use small yellow tips, and the P1000 uses large blue tips.
2. Leave clean tips in their tip boxes, keep them covered as much as possible.
3. Firmly attach the tip to the shaft, without touching either the shaft or the tip, as depicted in the image to the right.
4. To remove a tip, use the tip ejector button, as shown in Figure 4, below.
5. Always use fresh clean tips for each sample in order to prevent contamination.

**Figure 4. Attaching and removing tips:** To the left below see the proper method for attaching
When using a micropipette, there are a few guiding principles to keep in mind:

* Be consistent with speed and smoothness when using the plunger.
* Hold consistent pressure on the plunger at the first stop.
* Maintain vertical positioning of the micropipette.
* Avoid air bubbles.
* Change tips in order to prevent contamination.

Never drop a micropipette.
Never rotate the volume adjuster either below or above the range of the instrument. Never lay a filled micropipette on its side. (This will contaminate the shaft.) Never immerse the barrel of a micropipette in a liquid above the tip.
Never allow the plunger to snap up when liquid is being drawn into the tip.

Procedure

**Step 1.** Push down plunger to the **first stop**. Use your thumb!!

**Step 2.** Insert pipette tip into solution. (Make sure the tip is fully submerged in the solution.)

**Step 3.** Slowly release the plunger with your thumb. As you do this, you will see the solution rise up the pipette tip.

**Step 4.** Remove the pipette tip from the solution. (Make sure you do this before step 5!)

**Step 5.** Dispense the fluid inside the pipette tip by pushing down on the plunger all the way (to the **second stop**).

**Step 6.** Remove tip by pushing down on the tip ejector button with your thumb and ejecting tip into a used tip collection beaker.

**Figure 5. Using the Plunger:** Left, the plunger is not depressed. Middle, the plunger is at the first stop. Right, the plunger is at the second stop.
Figure 6. Using the tip ejector: pushing the ejector button will remove the used tip from the micropipette.

References: This handout is based on materials from the University of Michigan-Dearborn (http://umdearborn.edu/casl/naturalsciences/) and from STL Biochimie Genie Biologique (http://stlbgb.apinc.org/spip.php?article8).

PROBLEMS

1. Each image below represent a micropipette volume indicator, record the volume the device is set to transfer on the line below.

<table>
<thead>
<tr>
<th>p20</th>
<th>p200</th>
<th>p1000</th>
<th>p20</th>
<th>p200</th>
<th>p1000</th>
<th>p1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>

2. Finish drawing the diagram of each volume indicator below, include the notches, for each of the following volumes and micropipettes.

<table>
<thead>
<tr>
<th>p20</th>
<th>p200</th>
<th>p1000</th>
<th>p20</th>
<th>p200</th>
<th>p1000</th>
<th>p1000</th>
</tr>
</thead>
</table>

55
3. Each student must pipet the following volumes of liquid into separate tiny test tubes (microfuge tubes) provided prior to starting DNA extractions. Select the proper Pipetman, set the volume, and transfer the proper volume into a test tube. Use the smaller tubes, 0.7 mL for the first 5 volumes and the larger tube, 1.5 mL for the last volume.

<table>
<thead>
<tr>
<th>Volume</th>
<th>Volume</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5 μL</td>
<td>46.5 μL</td>
<td>522 μL</td>
</tr>
<tr>
<td>15.4 μL</td>
<td>153.8 μL</td>
<td>878 μL</td>
</tr>
</tbody>
</table>

**Proper Micro-centrifuge use:**

Centrifuges are commonly used in labs. They can be broken or your samples ruined if not used properly. Be sure to always keep them level, and when loading your tubes make sure the caps are snapped closed then place them so they are balanced and equally spaced as seen in figure 7 below.

1. Be sure the centrifuge is turned on and press door button to open.
2. Set the rpm and time to the appropriate settings.
3. Load tubes so they are balanced, they must be placed opposite to one another or equally spaced in the tube holders of the rotor.
4. Screw the cover onto the rotor. Close the door to the centrifuge.
5. Press start.
6. Most centrifuges automatically open the door when the run is over or indicate that you should press the door button to do so, when it is completely stopped. Unscrew the rotor cover and remove your tubes.
7. Leave the door open and the cover next to the centrifuge for the next person to use, or lightly screw it onto the rotor to store. If you spill anything inside tell your TA; it must be properly cleaned by staff.

**Figure 7. Centrifuge (top image) and loaded rotor (lower image).**

**DNA Extraction [Week 1]**

**Note:** In step “9” and in step “11” you will discard the supernatant. In step “14” you will save the supernatant and transfer it to a new tube. This solution contains your DNA. Read the procedure carefully so you only have to do the extraction once. Do not discard the pellet pestles!
1. **Each lab team will extract DNA from a total of 4 plants**: 1 known male, 1 known female and 2 unknowns (one from each of the unknown groups). Label microcentrifuge tubes for the 4 plants you are going to test including a group symbol.

2. **Sample the leaf tissue.** Take a labeled 1.5mL microcentrifuge tube to the corresponding plant and put the leaf between the tube and the cover of the tube. Push down on the tube cap to punch out a sample of leaf tissue and let it fall into the tube. Punch out another piece of tissue so you have 2 pieces in each tube. Repeat this procedure for your other 3 samples. Close the tubes and put them on ice.

3. **Clean the 4 blue pellet pestles** by spraying ethanol onto a Kim wipe and wiping each one down. Stand them upside down in a small beaker to dry for one minute before you use them.

4. **Add 500μL of Cell Lysis Solution (CLS) and 115μL of PPS (Protein Precipitation Solution) to each of your 4 sample tubes.** Use a new tip for each sample.

5. Push the plant tissue gently to the bottom of the tube and **Grind the plant tissue with the pellet pestle to break open the leaf cells.** Use a different pestle for each sample! The solution should be green. In this step you have broken open the cells. The cell lysis solution and the PPS contain buffers and inhibitors to prevent enzymes (including DNAses) from degrading components in the solution.

6. Check that your tubes are closed tightly and **centrifuge samples for 5 min. at 14,000 rpm to pellet plant debris.** Be sure that the centrifuge is properly balanced, which means the tubes are opposite one another, before you turn it on and review how to use it properly with your TA.

7. **For each sample: Transfer about 500μL of the supernatant to a new labeled 1.5 mL microcentrifuge tube and add 500μL of Binding Matrix (BM).** Shake the tube of Binding Matrix very well until it is totally dissolved **before** you use it and shake it between each use.

8. **Incubate the tubes at room temperature with gentle agitation for 5 minutes.** Slowly invert each tube back and forth by hand about every 30 seconds for 5 minutes. DO NOT
use a vortex at this step, it could shear the DNA. The five minutes of inverting should result in the DNA bound to the binding matrix.

9. **Centrifuge at 14,000 rpm for one minute to pellet Binding Matrix. Discard supernatant by pouring it off** into your waste solution flask.

10. **Add 500μL Ethanol Wash and gently resuspend the pellet using the force of the liquid from the pipet tip.** Continue drawing up the liquid and pipetting it over the pellet until the pellet is completely resuspended. This will take a few minutes.

11. **Centrifuge at 14,000 rpm for 1 minute and discard the supernatant by pouring it off.**

12. **Centrifuge again at 14,000 rpm for 1 minute and remove residual liquid with a small pipet tip.**

13. **Elute the DNA by gently resuspending the pellet in 100μL of DNAs-free water (H₂O).** Continue drawing up the liquid and pipetting it over the pellet until the pellet is completely resuspended; this will take a few minutes. When the binding matrix has been completely resuspended, incubate the tubes for 5 minutes at 55°C in a heat block.

14. **Centrifuge at 14,000 rpm for 1 minute and transfer ~ 100 μL eluted DNA (in the supernatant) using a P200 Pipetman to a clean labeled 0.6 mL microcentrifuge tube.** Avoid transferring particles of binding matrix with the eluted DNA. Bring your DNA to your TA to be stored until next week. Make sure your tubes are labeled with your group symbol and closed tightly.

**Clean up:**

Save the blue pellet pestles; they will be reused. Used tubes and Pipetman tips can be thrown in the “Used Tips” box. Waste solution must be collected; it cannot go down the drain. Pour it into the large jar labeled “Waste Solution with Ethanol” on the counter. Return all unused materials to your tray. Ice can be dumped in the sink. Push in your chair before you leave.

**Thought Question:**

1. If enzyme **inhibitors** were not added with the Cell Lysis Solution, what would be the effect?
2. DNA is a carbohydrate. There are many other carbohydrates in a cell (can you name some?). What is it about this procedure that allows us to extract the DNA and not other carbohydrates from the cell?

**Glossary:**

Centrifuge – v. - to rotate a solution at high speed to separate it by density using centrifugal force
Elute – v. - to extract one material from another, usually with a solvent
Heat block – n. – a block of metal with holes drilled out to bring contents of tubes to a specific temperature; a “dry” bath
Incubate – v. – to maintain an organism or a biochemical system at specific conditions for its growth or reaction
Pellet – n. – a small rounded or spherical mass of solid material precipitated by centrifugation

v. – to produce a small rounded or spherical mass of solid precipitated material by centrifugation
Supernatant – n. - the liquid overlying sediment or precipitate from centrifugation
Vortex – v. – to spin the contents of a tube at varying speeds
    n. – a machine that spins the contents of a tube at varying speeds

Figure 5. Centrifuge, left and middle, vortex, right

Figure 6. Male silene flower showing stamen on the left and female silene flower showing pistils and ovary on the right.

LAB 4: Plant Molecular Genetics - Part II
Polymerase Chain Reaction (PCR)
BACKGROUND: This week you will use PCR to increase the number of copies of a specific fragment of DNA that is known to reside on the Y chromosome. Your extractions from last week contain the nuclear, chloroplast and mitochondrial DNA from the leaf samples that you chose (about 6 billion base pairs in this diploid). There are not however, very many copies of any one gene or sequence. If you make millions of copies of one specific sequence and not amplify the remainder of the genome, you should be able to visualize (on a gel next week) that DNA as a distinct band that is the size of the sequence that you amplified. This is what PCR does. It is the exact process that is now used routinely in forensic studies where a small amount of DNA is available.

PCR uses a special enzyme, Taq polymerase. This is a DNA polymerase just like others that you have studied except that it comes from the bacteria Thermus aquaticus that grow in hot springs. The DNA polymerase of this bacterium is heat stable. In PCR, DNA is first denatured (becomes single stranded) by heating to near boiling (94 ºC) [recall that in vivo, DNA during replication becomes single stranded as Helicases swing into action]. The reaction is then rapidly cooled, allowing the primers (short sequences of DNA or RNA) to anneal to the complementary region of the DNA [recall again, that in vivo Primase makes RNA primers during replication, here we are supplying a specific set of pre-made primers that flank the target gene]. Lastly, the Taq DNA polymerase synthesizes a new single strand of DNA starting with the primer and using the original denatured single strands as template (you should know how this works). We have synthesized specific primers (remember that DNA polymerase needs a primer) that are complementary to sequences that flank a gene on the Y chromosome. The primers flank that sequence so that we are amplifying our target sequence on both denatured single strands of DNA. One primer will bind to one of the two strands of DNA on the “left” side of the target gene and allow synthesis 5’ to 3’ through the target gene and the other primer binds to the other strand on the opposite, “right” side of the target region and allows the synthesis (again 5’ to 3’) of the target. This sequence of events (heating to denature the DNA and cooling to replicate it) is repeated many times to amplify exponentially (copies are doubled with each cycle) our specific gene so that we can visualize it on a gel.

PROTOCOL:
1. **Note:** This step may be done by the staff prior to this lab; ask your TA. **Dilute the extracted DNA with sterile water according to your TA’s instructions into 4 clean labeled 0.5 mL tubes.** Put your original extraction tubes aside; you’ll be using your dilution tubes now. Too much DNA can make it more difficult for the primers to find and bind to the target DNA in the one minute of time they have, so we dilute the DNA.

2. **Label new 0.5 mL tubes with a marker, no tape.** You will need four plant DNA samples plus one negative control with water instead of DNA and a positive control containing diluted male Silene DNA (the negative “−” and positive “+” controls may be provided). **Keep all 6 tubes on ice.**

3. **Add 5μL of diluted DNA from your samples to its appropriate new labeled tube using a new tip for each sample.** Close tubes and leave on ice.

4. **Prepare a single cocktail of the components listed below in the tube labeled “C” which already contains both primers.** Make enough cocktail for 7 tubes (one more than the number of samples so that you do not run out of the mixture). Use the volumes listed in the “x 7” column. Each group will make one cocktail. Once made, keep your cocktail on ice until ready to use. The TA will add the TAQ to your cocktail last.
NOTE: The enzyme, Taq polymerase, is viscous and tends to sink to the bottom of the tube, so mix the cocktail well but gently by pipetting; don’t vortex.

Cocktail | 1 reaction | \( \times 7 \) 
--- | --- | --- 
sterile dH\(_2\)O | 6.8 \( \mu \text{L} \) | 47.6 \( \mu \text{L} \) 
5X PCR green flexi buffer | 5 \( \mu \text{L} \) | 35 \( \mu \text{L} \) 
dNTPs (2.5mM) | 2.5 \( \mu \text{L} \) | 17.5 \( \mu \text{L} \) 
MgCl\(_2\) (25mM) | 2.5 \( \mu \text{L} \) | 17.5 \( \mu \text{L} \) 
primer 1- forward (10 \( \mu \text{M} \)) | 1.5 \( \mu \text{L} \) | (10.5 \( \mu \text{L} \)) – already in tube “C” 
primer 2 - reverse (10 \( \mu \text{M} \)) | 1.5 \( \mu \text{L} \) | (10.5 \( \mu \text{L} \)) – already in tube “C” 
*Taq polymerase* (5 units / \( \mu \text{L} \)) | 0.2 \( \mu \text{L} \) | 1.4 \( \mu \text{L} \) - TA adds this to your tube 
| | | 20.0 \( \mu \text{L} \) | 140 \( \mu \text{L} \)

5. **Pipette** 20 \( \mu \text{L} \) of the cocktail into each of the 6 sample tubes containing 5\( \mu \text{L} \) diluted DNA and mix. You made extra so you should have a little left over. Centrifuge your tubes for 10 seconds to make sure that all liquid is in the bottom of the tubes.

6. Your instructor will place your samples into the thermocycler. Each cycle of steps 2, 3 and 4 will double the number of copies of your target DNA until you have enough that you can easily see it on a gel (next week). The program will take 3 hours so we will continue next week. See cycles below:

**PCR cycles:**
- Step 1: 94\(^\circ\)C - 4 minutes
- Step 2: 94\(^\circ\)C - 1 minute – denature the DNA
- Step 3: 60\(^\circ\)C - 1 minute – primers anneal to the DNA template
- Step 4: 72\(^\circ\)C - 1 minute – DNA polymerization
- Step 5: Return 35X to step 2
- Step 6: 72\(^\circ\)C - 5 minutes
- Step 7: Hold at 10\(^\circ\)C

**Clean up:**
Pipetman tips and tubes used for dilutions can be thrown in the “Used Tips” box. Ice can be emptied in the sink. Push in your chair before you leave.

**Thought Questions:**
1. What is your annealing temperature? ____ °C

2. What might happen in a PCR if the annealing temperature is too high or is too low?

3. Draw what happens to a single double stranded piece of DNA through two cycles of PCR.

4. If you assume that you start with a single piece of DNA for your target region (say you extracted the DNA from just one cell), how many copies of your target would you have after 30 cycles of PCR? If you were a forensic scientist (investigator of crime scenes) does this help explain why a single drop of blood at a crime scene provides enough material to look at an individual’s genes and “fingerprint” the individual?

Glossary:

Aliquot – v. – to divide a solution into equal volumes

Thermocycler - n. - a machine that can be programmed to cycle through various lengths of time at certain temperatures in order to allow polymerase chain reaction (PCR) to take place on DNA samples.

![Thermocycler](image-url)  
Figure 1. Thermocycler

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LAB 4: Plant Molecular Genetics Part - III

Agarose Gel Electrophoresis

BACKGROUND:
Recall that last week you amplified by PCR a specific sequence of DNA that is found on the Y chromosome. We now want to see the millions of copies of that sequence in those samples (males) that had a Y chromosome. Agarose gel electrophoresis is a method used to separate DNA molecules according to size and charge. The agarose forms a matrix or weave of strands with holes through which DNA migrates; long pieces of DNA travel slowly while short pieces travel fast. Refer to your text for a description of this process. DNA molecules are visualized by staining with GelRed, a proposed nonmutagenic nucleic acid stain, and excitation of the GelRed molecules with UV light. Full studies of GelRed are not complete; we will work with it as if it were a mutagen, using gloves and disposing of all gel waste through Environmental Health and Safety (EH&S).

Procedure
1. Each group makes a 1.5 % agarose mini gel.
   a. Have gloves on throughout this experiment. Add 0.6 g agarose to small flask with 40 mL 1X TBE (Tris-Boric Acid-EDTA) buffer
   b. Heat in microwave until boiling (about 1 min.; specific directions for each microwave will be given). Make sure the agarose is dissolved.
   c. Cool the flask for about 10 minutes until you are able to comfortably hold it in your hand. While it cools check that your comb is level and will be 2-3mm from the bottom of the gel not more or less or the wells will not be made properly.
   d. Your TA will add a small amount (4μl) of Gel Red stain into your flask containing the melted gel. Swirl the flask to distribute the Gel Red stain throughout the gel and pour the solution into a gel tray with an 8-10-well comb. Let the gel sit for ~ 20 minutes to solidify.
   e. When the gel is solidified and opaque, remove the black baffles on either side of the gel and gently remove the comb leaving wells in which your samples will be loaded.

2. The instructor will fill the electrophoresis chambers with 1X TBE buffer.

3. Load 20 μL of each of your samples to the wells of the gel using a new Pipetman tip for each sample. Put your pipette tip almost to the bottom of the tube and take only the sample. Draw up the solution very slowly; the green buffer is very viscous and air bubbles will occur if you try to draw it up too quickly. You should have a little of your sample left over. Load the samples in this order: 1 – known male, 2 – known female, 3 – unknown 1, 4 – unknown 2, 5 – positive control, 6 – negative control, 7 – ladder. If you have more than 7 wells, leave the outside ones empty; record the sample order in your notebook. Be careful not to puncture the bottom of the well with your pipette tip. Practice loading gels with the materials provided before you load your own samples.

4. To well # 7 (or the last well of your series), add 10 μL of 100 base pair ladder (it has blue dye in it). This is a mixture of DNA of standard sizes (100 bp, 200bp, 300bp etc) and
can be used to determine the size of your amplified DNA and to show that the gel has run properly.

5. Set the power source to 120V and run the gel for approximately 60 minutes. DNA is negatively charged so you want it to run to the positively charged electrode. **Your gel has finished running when the yellow dye reaches ¾ the length of the gel.** Unplug your gel from the power supply. Lift the cover off the gel by putting your thumbs on the white posts and pulling the cover up with your fingers. Don’t pull it off by the black and red electrical leads!

6. **View gel on the ultraviolet light transilluminator in the prep room. Wear gloves and safety glasses to protect your eyes. Ultraviolet light can burn your cornea.** Be sure to take a picture of your gel, to be included with full labels and description in your lab report.

**Clean up**

The 1X TBE buffer can be disposed of into a container. Wash the flask you used to make the gel; save any leftover gel that comes out of the flask and put it in the hazardous waste bag on the counter. Wash the gel rig with all its parts. Turn off and unplug power supply. Leave all used tubes on your tray. Throw away used pipet tips in the “Used Tips” box. Dump ice in sinks. Push in your chair before you leave.

**Thought Questions:**

1. What molecular structure of DNA makes it negatively charged?
2. If you do not see a band for one or both of your unknowns, what evidence do you have that this is because the samples were female and not because either the gel was made improperly, that the PCR failed or that your DNA extractions were poor? In other words, what supports the conclusion that one or both of the unknowns are female?
3. Suppose that you found a species *S. ambivala* that is closely related to *S. latifolia*, such that they share the same Y chromosome marker. However, *S. ambivala* is hermaphroditic. What would you expect to see on your gel, if you had taken twenty randomly chosen *S. ambivala* plants, and performed the same experiment?

**Glossary:**

Buffer solution – n. – a solution that is capable of neutralizing both acids and bases to keep the pH of a system constant.

UV transilluminator – n. – a light box in which DNA fragments in a gel, stained with ethidium bromide, will be visible.
Ultraviolet Light Mutagenesis of E. coli Cells

Mutagenizing Cells, Serial Dilutions and Plating of Cells [Week 1]

BACKGROUND:
Ultraviolet (UV) light can be used to induce genetic changes (mutations) in bacteria cells. To accomplish this, one must first determine the sensitivity of a bacterial strain to UV light. In this laboratory, this sensitivity will be established quantitatively by treating bacterial cells with UV light for different lengths of time and calculating the proportion of cells that have been killed by each treatment. This experiment will demonstrate the killing effects of UV light, the quantitative relationship between UV dose and response, and the proper dose of UV light that would be required in order to generate mutants. The experiment will also provide experience in handling bacteria, performing serial dilutions and viable cell counts, and graphing and interpreting data on a semi logarithmic scale.

Relevant sections from textbook (Klug and Cummings, Concepts of Genetics): Chapter on DNA: Mutation, Repair, and Transposable Elements

INTRODUCTION:
A gene mutation is a heritable change in the gene’s nucleotide sequence. Such genetic changes are a major source of genetic variability within populations. In addition, mutations are important tools for geneticists. By studying organisms bearing mutations, geneticists can identify the genes that control particular phenotypes, can study the transmission of these genes, and can analyze the cellular processes that produce these phenotypes.

Mutations can be either spontaneous or induced. Spontaneous mutations are those that arise “naturally,” without intervention of any artificial mutagenic agents. Induced mutations, on the other hand, result from either chemical or physical mutagenic treatments. Because spontaneous mutations occur at very low frequencies, geneticists commonly induce mutations in order to obtain an ample supply to use for experimental purposes. Mutations can be induced by a broad
range of chemical agents. Some of these chemical mutagens are described in the Mutation chapter of your textbook. In addition, mutations can be induced by physical factors such as ultraviolet light, gamma rays, and X-rays. All of these agents have characteristic mechanisms for inducing mutations and thus induce particular types of mutations.

In this laboratory, we will examine the mutagenic effects of UV light. This form of radiation is a common tool used by microbial geneticists for inducing mutations experimentally. While ultraviolet radiation has lower energy content than ionizing radiations such as gamma rays and X-rays, it can produce a mutagenic or lethal effect in cells exposed to wavelengths of 210 to 300 nanometers. Within the cell, UV light is absorbed primarily by the nucleic acids; in particular, UV light damages the pyrimidine bases within DNA. On exposure to UV light, adjacent pyrimidine (especially thymine) molecules can bond covalently to one another, forming pyrimidine dimers. This dimer formation distorts the DNA structure, interfering with proper DNA replication and transcription.

Besides exploring the mutagenic effects of UV light, this lab will give you experience working with the bacterium E. coli. E. coli cells offer many advantages for inducing and analyzing mutations. For example, they are small and easy to grow in very simple medium; in addition, they have short generation times (about 30 minutes) and thus can be grown in large numbers in short periods of time. (A typical “saturated” culture can have as many as 10^9 bacterial cells per milliliter.) Furthermore, individual cells give rise to visible colonies on solid medium, which allows many phenotypes to be visualized easily. Finally, a wealth of information has accumulated about the genetics and physiology of E. coli; as a result, highly sophisticated genetic techniques have been developed for studying this organism. Safety note: This strain of E.coli is not virulent; it is the common form that is found in most mammal intestinal tracts, including our own. Wash your hands when you are finished with the lab.

In this lab, we will treat cells with UV light in order to cause random mutations. Some of these random mutations will occur in genes that are essential for survival. Cells that undergo mutations in essential genes will be killed. In order to determine the sensitivity of our strain of E. coli to UV light, we will treat the cells with various doses of UV light. As the UV dose is increased, the mutation rate will rise, as will the death rate of the cells. We will then determine the percentage of cells that have survived and the percentage that have been killed at these different UV doses. When graphed, this information produces a killing curve, which shows the relationship between UV dose and its lethal effects. Biologists often use the information from a killing curve to establish optimal conditions for inducing mutations in subsequent experiments.

Materials Needed:
Glass beads 4 mm for spreading E. coli on plates
Test tubes (in racks) for dilutions (filled with 10 or 1 ml 0.1M MgSO4)
Vortex mixer
P200 Pipetman and tips
Petri plates (empty), 5 for each group

LB plates, 19 for each group
Black markers
Safety glasses
Disposable gloves
Timers sterile 5 ml
pipettes
pi-pumps for 5 ml pipettes
UV lamps

*E. coli* cells resuspended in 0.1 M MgSO4
*E. coli* waste jars
Jar for used beads with funnel
Empty trays to carry plates of bacteria to UV light in prep room

**Safety Considerations:** UV is a mutagen as well as an eye-damaging agent. Never look directly at the UV lamp! Always wear safety glasses when using UV.

**Although your skin is not highly sensitive to small doses of UV, you should also wear plastic gloves to minimize exposure to your hands.**

**Procedure:**

1. Prior to class, your instructor will grow *E. coli* cells in 500 mL of nutrient medium overnight at 37°C. This gives a concentration of approximately 10⁹ cells per milliliter. Just prior to class, the bacteria will be diluted 1/5 in a solution of 0.1 M MgSO4. You will receive a sample of these cells in a sterile test tube. All manipulations in this lab should be performed using sterile technique. Your instructor will demonstrate these methods and describe serial dilutions to you. You will label your test tubes with tape for these serial dilutions.

2. **Label 5 empty, sterile Petri dishes on the bottom:** B - 3 sec, C - 6 sec, D - 15 sec, E - 30 sec, and F - 50 sec. (A – 0 sec, are the control cells and will receive no treatment.) Vortex your sample of cells and use a 5 ml pipette and a green “pi-pump” to pipette 5 mL of the cell suspension into each of these dishes. These cells will be used for UV treatment for the times marked.

3. **Mutagenize your cells.** When your dishes of cells are ready, place them on a tray singly, not stacked, and take them into the prep room where UV light source is set up. When you are working in this room, remember to wear safety glasses and plastic gloves. Your instructor will demonstrate how the cells will be mutagenized. The important points are as follows: UV does not penetrate plastic, so the lid of the Petri dish must be removed during UV treatment. The uncovered plate will be slid into the UV apparatus and shaken gently on a tray to minimize UV exposure to your hands. One student should move the plates in and out, while another student in the group acts as timekeeper. After the cells have been mutagenized for the designated numbers of seconds, the plates should be covered. Dilutions and plating of the cells will then be carried out in the main lab.

4. In order to calculate the percentage of cells that have survived each treatment, it is necessary to count the number of viable cells in all the treated cultures, as well as in the untreated control (that is, the cells from the original test tube.) Such **viable cell counts** can be made by diluting cells for each condition, then spreading the cells on agar plates. During an incubation period, each cell on the plate will divide and eventually produce a **colony** of cells visible to the naked eye. During the next lab period, these colonies can then be counted. After taking into account the dilution factors that were used, the number of viable cells per milliliter in the various cultures can then be calculated.

By calculating:
# viable cells per ml in treated culture X 100,
# viable cells per ml in untreated control we can determine the percentage of cells that survived that particular treatment.

5. In order to count the number of separate bacterial colonies on an agar plate, it is necessary to dilute the cell suspensions so that a countable number of colonies (a few hundred at the most) grow on each plate. If we keep track of our dilution factors, we can calculate later the number of cells we started with. In order to dilute the cells to this extent, **serial dilutions must be carried out.** In this method, an initial dilution is made, then this dilution is diluted a second time, this second dilution diluted a third time, etc. What are the advantages of this serial method of dilution?

6. Since we do not know ahead of time which dilutions will yield the appropriate numbers of colonies, several dilutions will be made and spread on plates. You will be provided with test tubes filled with either 10 or 1 milliliter (mL) of 0.1 M MgSO4 for making dilutions. To dispense 0.1 mL (=100 μL), use the P200 Pipetman provided. **After each step of the serial dilution, be sure to vortex the solution to evenly distribute the cells in the tube!**

Your instructor will demonstrate the use of these items. See the following page for a guide to making the appropriate dilutions. Label your test tubes with treatment, for ex. “B” and dilution, for ex. “1/104” and line up the test tubes needed to make the dilutions for each treatment.

7. Once appropriate dilutions are made, **0.1 mL of these dilutions (vortex again) should be spread on an agar plate.** Label each agar plate on the bottom with the letter denoting the UV exposure, the dilution factor and your group name. To spread the *E. coli* cells, take a tube of sterilized glass beads and pour them into one agar plate, lifting the lid only high enough to add the beads. Add the beads slowly because they bounce and roll easily. Close the lid. Use a Pipetman to transfer 0.1 mL of the appropriate, diluted cells to the surface of the agar. Close the lid. Holding the lid, move the plate back and forth on the bench sending the beads crisscrossing the plate and spreading the cells on it. Turn the plate once in a while and shake back and forth again to make sure you have all of the surfaces covered evenly. Shake each plate for one full minute. The plates should be spread until all the liquid soaks in. When you are finished shaking your plate, hold both parts of the plate together as you turn it upside down. Hold onto the bottom plate containing the agar and pour the beads into the “bead waste” jar by briefly separating the top and bottom plates. Keep the bottom plate upside down so no contaminating elements fall into it. When you have removed the beads and liquid, put the top cover back onto the bottom plate. Place the plate with the top on it, on the table, right side up to let the plate dry, as you process the next. Your instructor will demonstrate this technique. Plate all of the treatments and dilutions asked for.

8. **The dried and spread plates should then be stacked upside down in two piles, taped together and placed in a 37°C incubator.** *Do not place plates close to the walls of the incubator, or on the floor of the incubator. After the colonies have grown, your instructor will refrigerate the plates until the next lab period.

**Serial Dilution Notes:**
*As seen in the picture to the right, you have two different sized tubes for dilution preparation. **Be sure to use the correct tubes for each dilution step, examples are depicted below:** The small tube with the white cap holds no more than 10mL, and has 1mL in it. The large tube with the red cap holds 15-20 mL and has 10 mL in it. The small tubes have 1mL and the larger have 10mL. *Be sure to label all tubes with tape before making dilutions. All volumes are in mL.
After adding the appropriate number of cells to the MgSO4 solution in each dilution tube, be sure to mix the solution well using a vortex mixer. After dilutions are made, spread 0.1 mL samples on labeled agar plates.

Clean up: Empty all liquid from tubes and original Petri dishes into the E.coli waste jars. Used tips, Petri plates, paper towels and gloves go into the labeled clear autoclave bags and NOT into the regular trash. Place all used empty test tubes into the large tub of water to soak; remove all tape on the tubes or caps first. Push in your chair before you leave.

A. Control Cells (No UV treatment):

1/102 = 0.1 mL control cells + 10 mL MgSO4 (Use New Tip)
1/104 = 0.1 mL of 1/102 + 10 mL MgSO4 (Use New Tip)
1/105 = 0.1 mL of 1/104 + 1 mL MgSO4 (Use New Tip)
1/106 = 0.1 mL of 1/105 + 1 mL MgSO4 (Use New Tip)
1/107 = 0.1 mL of 1/106 + 1 mL MgSO4 (Use New Tip)

Of these control dilutions, plate 1/104, 1/105, 1/106, 1/107.

B. 3 Second UV:

1/102 = 0.1 mL cells + 10 mL MgSO4 (Use New Tip)
1/104 = 0.1 mL of 1/102 + 10 mL MgSO4 (Use New Tip)
1/105 = 0.1 mL of 1/104 + 1 mL MgSO4 (Use New Tip)
1/106 = 0.1 mL of 1/105 + 1 mL MgSO4 (Use New Tip)
1/107 = 0.1 mL of 1/106 + 1 mL MgSO4 (Use New Tip)

Of these dilutions, plate 1/105, 1/106, 1/107

C. 6 Second UV:

1/102 = 0.1 mL cells + 10 mL MgSO4 (Use New Tip)
1/104 = 0.1 mL of 1/102 + 10 mL MgSO4 (Use New Tip)
1/105 = 0.1 mL of 1/104 + 1 mL MgSO4 (Use New Tip)
1/106 = 0.1 mL of 1/105 + 1 mL MgSO4 (Use New Tip)

Of these dilutions, plate 1/104, 1/105, 1/106

D. 15 Second UV:

1/102 = 0.1 mL cells + 10 mL MgSO4 (Use New Tip)
1/103 = 0.1 mL of 1/102 + 1 mL MgSO4 (Use New Tip)
1/104 = 0.1 mL of 1/103 + 1 mL MgSO4 (Use New Tip)
1/105 = 0.1 mL of 1/104 + 1 mL MgSO4 (Use New Tip)
Of these dilutions, plate 1/103, 1/104, 1/105

E. 30 Second UV:
1/1   (plate directly) (Use New Tip)
1/10  = 0.1 mL cells + 1 mL MgSO4 (Use New Tip)
1/102 = 0.1 mL of 1/10 + 1 mL MgSO4 (Use New Tip)
Plate all dilutions.

F. 50 Second UV:
1/1   (Plate directly) (Use New Tip)
1/10  = 0.1 mL cells + 1 mL MgSO4 (Use New Tip)
1/102 = 0.1 mL of 1/10 + 1 mL MgSO4 (Use New Tip)
Plate all dilutions.

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### Ultraviolet Mutagenesis of Bacterial Cells Worksheet

**Key:** TMTC = Too many to count; ND = No data; C = Contaminated plate

<table>
<thead>
<tr>
<th>UV Exposure (sec)</th>
<th>Dilution</th>
<th># Colonies from 0.1 ml</th>
<th>Overall dilution factor</th>
<th># Viable cells / ml after mutagenesis</th>
<th>% Survival</th>
<th>Mean % survival of each treatment</th>
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<td>0</td>
<td>1/10⁴</td>
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Lab 5: Bacterial Mutation Part - II
Ultraviolet Light Mutagenesis of E. coli Cells

Counting Colonies and Graphing on Semi-log Paper [Week 2]

During this second lab period, you will examine the plates that were spread with cells during the last lab. If certain plates have fewer than about 500 colonies, then you should count the colonies. Your instructor will demonstrate some tricks for counting bacterial colonies, such as marking off each colony as it is counted or dividing the plate into quadrants and counting only one quadrant. From these counts you should theoretically be able to calculate the number of viable cells per milliliter (mL) in the original cell suspensions.

Don’t worry if your plates show unexpected results or if the colonies did not grow. In case this is true, we are also providing you with sample data which were generated by your instructor. You can use these data to compute the numbers of viable cells per ml that were in each sample. As part of this exercise, you should think carefully about the dilutions that were made and about how that information must be used in your calculations. Also remember to figure in that you plated only 0.1 mL rather than 1.0 mL. You can use the worksheet provided to record the numbers of colonies and organize your calculations.

Even if you don’t use your own data, you should examine your own plates carefully. Are the colonies evenly spread? Are the numbers of colonies within a single dilution series consistent with each other? Think about possible sources of error in the procedures that were used.

Once the cell titers of the original untreated and UV-treated samples have been calculated, you can use this information to determine the percentage of cells that survived a particular UV treatment. Specifically, the percent survival is calculated as follows:

\[
\text{Percent Survival} = \left( \frac{\text{# viable cells per mL in treated culture}}{\text{# viable cells per mL in untreated control}} \right) \times 100
\]

We are interested in estimating what dosage of UV light results in 99% killing of the culture. To make this estimate, you should graph the mean % survival vs. time of UV treatment. This type of graph is called a “killing curve.” For this graph, it is necessary to use semi-log graph paper, which will be provided. You should examine this paper carefully, noting that the numbers along the Y-axis increase geometrically instead of arithmetically. You should plot the mean % survival on the Y-axis and seconds of UV treatment on the X-axis. What do you expect to be the relationship between these values? Your instructor will explain further how to graph your data on semi-log paper. Think about why this type of graph paper is useful for this purpose.

Clean up:
All plates, gloves, and paper towels should be put into the autoclave bags taped to the counter.
Push in your chair before you leave.
You should include your worksheet as well as answers to the following questions in your lab notebook:

1. What is the relationship between UV dose and cell killing?
2. What do you suppose is the relationship between killing rate and mutation rate?
3. Why is it useful to estimate the UV dose that will result in 1% survival?
4. What are the advantages of the serial dilution method for diluting cells?
5. What are the possible sources of error when generating viable cell counts and killing curves?
6. Suppose you are working in a genetics laboratory and want to select bacterial mutants of a certain type. What are the advantages and disadvantages of using UV light as your mutagenic agent? (You may want to consult your textbook.)
7. Many procedures suggest that when mutagenizing bacterial cells with UV light, the dish of cells should be shaken gently during the actual mutagenesis. Can you speculate on the reason for this?
8. When geneticists mutagenize cells with UV light in order to select mutants, they often keep the cells in the dark after the mutagenesis step. Can you speculate on the reason for this? You will need to consult your textbook.

![Figure 1. Plates of bacterial colonies in the UV Mutagenesis Lab.](image)

Plates in horizontal rows show the decreasing number of colonies in a series of serial dilutions from left to right. Treatment “A” plates are in the top row, treatment “B” plates are in the middle row and treatment “C” plates are in the bottom row.

Plates in vertical columns show the decreasing number of colonies with increased UV exposure to the culture at the same dilutions. The plates in the left-hand column have 1/10^4 dilution, the plates in the next column have 1/10^5 dilution and the plates in the third column have 1/10^6 dilution.
This kind of graph paper allows you to graph exponential data without having to translate your data into logarithms. The paper does it for you! At stationery and university bookstores, you can buy semi-log graph paper with anywhere from 1 to 5 or 7 cycles. "Cycles" will be explained in the next paragraph. For those of you who are likely to use many different kinds of graph paper, including semi-log and log-log graph papers, you might be interested in purchasing a book, from which you can photocopy the specific type you need at the moment.

Shown here is what is known as 3-cycle semi log graph paper. You will notice that the vertical axis is very peculiar as the numbers only go up from 1 to 9 and start all over with 1 again, over and over. This is because the distances indicate logarithmic distances. And you will remember that there is no log of zero! (If you want to print out a full-page copy of the graph, click on the graph to the right.)

As shown in the next figure, you MIGHT consider the line across the bottom as equal to one, and the next horizontal line labeled as 1 should be ten, and the next 1 is 100 and the top line is 1000. The lines' basic numeric value may not be changed. The only thing allowed is the placement of the decimal point - and they always differ by only one decimal place per cycle. Thus you might have 0.001, 0.01, 0.1 and 1.0, or you might have $10^5$, $10^6$, $10^7$ and $10^8$. So you can see that semi-log graph paper can plot both very small numbers as well as astronomical ones.

Let's see how this works and what the supposed power of the semi log graph is all about. In brief it is terrific for plotting anything that is exponential - such as compound interest.

Suppose that you make a series of numbers such that each one is double the one before it (black). (Or you might make each one 3 times the value of the previous one (red); or, for fun, 1.5-times the previous one (blue).) Starting with the series that is of the powers of two (the black line), we find $2^0 (=1)$ and mark it at the lower left corner; then move to $2^1 (=2)$, and plot that on the second major vertical line; then move to $2^2 (=4)$ and plot that on the next major vertical line, and so on with $2^3 (=8)$, $2^4 (=16)$, and so on. And - AMAZINGLY - we get a straight line. Scientists love
straight lines! If something gives a straight line on semi log graph paper, we call it an exponential function.

You will see that the $3^x$ and the $1.5^x$ also give straight lines. Wonderful! Any type of exponential function can be plotted to give a straight line!

For any values that increase exponentially with time, one can use these graphs to easily determine doubling times (the blue figures associated with the red line). All you have to do is take any point on the red line, and then go move upwards to a point that is double that value and see how much horizontal distance was gained. That is called the doubling time, or $t_{1/2}$. Of course, for convenience, you wouldn't choose a value like 22.86 and have to find twice that. Rather you would choose 1.00 and easily find 2.00, as was done in this diagram. Also shown are some successive doublings to 4 and then to 8. You will notice that the horizontal distances remain the same for each of the sections.

In reality, you are more likely to get data that plot like the green line. This is called a growth curve in microbiology. For a while, in the "log phase" the microbes are growing exponentially (see the straight portion of the line?), but then they begin to slow down because on any number of reasons or combination of reasons - buildup of waste products, exhaustion of food, etc. Soon growth ceases and the line runs horizontally in "stationary phase." Quiz: what is the doubling time for this microbe during exponential growth? (Answer: it doubles about once per hour.) Another name for doubling time is "generation time" or just plain "g".

**SURVIVAL and Radioactive DECAY CURVES**

The difference between growth curves as shown above and survival curves is from which corners the line or lines radiate. Above they have come from or near the bottom left and gone upwards. Survival curves come from the upper left and go downwards. That corner is defined as "1" or 100%. (And remember that the log of 1 = zero!) If you are using three cycle semi log paper, that means the upper cycle goes from 100% to 10%, the middle cycle is from 10% to 1%, and the lower cycle from 1% to 0.1%.
SURVIVAL CURVES and MINIMUM LETHAL HITS

This collection of "dose plots" or "survival curves" starts with it origin at the red "1". From it arise three plots, a, b, and c. Plot "a" is of the most sensitive critter, while that of "c" is of the least sensitive critter.

Game theorists have proven that if you extend asymptotes from the straight portions of the curves back to the vertical axis, where that line crosses indicates the minimum number of lethal hits.

Let's discuss the meaning of that for a moment. It has been shown in battle scenarios that the average soldier is usually shot several times and still doesn't die - although it is plausible that one bullet in just the right place - brain, heart, can kill. Thus the minimum number of hits to be lethal for a soldier is one. On the other hand, if we talk not about bullets but gamma rays, then the minimum number of lethal hits in a human must be much larger for immediate death. Perhaps there are a critical 1,000 cells in the body such that if ALL of them are knocked out - each by a single gamma ray, then the minimum number of lethal hits is 1,000. Thus if you were to irradiate a large population of people with gamma rays and make your dose plot, you would find the extrapolated asymptote crossing the vertical axis at 1,000. (Again, remember that most people will be hit with millions of gamma rays - but most of them would be in inconsequential places.)

Thus when you look at this figure, you see that both "a" and "b" are critters for which the minimum number of lethal hits is one. But for "c" the line extrapolates to "2", and so that critter requires at least two hits to kill it.

Another consideration that you can derive from these plots is the LD$_{50}$ ("lethal dose for 50%"; the dose that kills 50% of the critters). For "a" the LD$_{50}$ is about 5. You find this by moving down the red line to where it crosses the horizontal 0.5 line and at that point look down at the bottom to see how many hits that indicates.

For "b" LD$_{50}$ is about 10, but you run into a problem with "c" because its curve doesn't start declining immediately. So, for "c", you must go to a straight portion and then determine how many hits it takes to decrease the number to a half. So go down the blue line where it is straight to some convenient place and then measure off how many more hits it takes to drop in half. You should get something like 16 hits.
LAB 7: GENE REGULATION: the lac operon

OBJECTIVE: To understand the control of the lac operon by monitoring the response of E. coli upon induction with lactose in the presence and absence of glucose.

INTRODUCTION
Genes are transcribed into messenger RNA, which is then translated into proteins. While most E. coli genes exist in only one copy, the amounts of proteins produced from different genes are highly variable. These amounts range from a few molecules per cell to tens of thousands of molecules per cell. Furthermore, the rate of production of certain proteins is constant throughout the life cycle of E. coli, whereas the rate of production of other proteins varies enormously, depending on the environment and the cell’s needs. These variations in amounts of proteins produced from different genes result from differential gene expression. The specialized cell types in higher organisms show even more dramatic examples of differential gene expression. For example, all the various cell types in a given mammal contain the same set of genes, but different cell types select different subsets of these genes to synthesize different subsets of proteins. Thus, large amounts of digestive enzymes are synthesized in pancreatic acinar cells but not in muscle cells. Conversely, large amounts of contractile proteins are synthesized in muscle cells but not in pancreatic acinar cells.

The lactose operon of E. coli is one of the best understood bacterial systems in which the expression of genes is controlled by the chemical environment. The lactose operon is an inducible system in which three proteins are synthesized when lactose is present, but not when lactose is absent. Thus their synthesis is “induced” by lactose. Lactose is a disaccharide sugar which is used as a carbon source for energy and for synthesis of various cellular molecules. The first step in the metabolism of lactose is breakdown into two monosaccharides, galactose and glucose, by the enzyme beta-galactosidase (ß -galactosidase). The levels of ß -galactosidase are almost nondetectable in E. coli cells that are grown without lactose. However, when lactose is added to the growth medium, the levels of ß -galactosidase increase about a thousand-fold within a few minutes. The induction of ß -galactosidase is coordinated with two other proteins that function in lactose utilization: a permease which transports lactose through the cell membrane, and a transacetylase which probably inactivates toxic compounds similar to lactose. Also note that ß -galactosidase has a second minor enzymatic activity, which catalyzes the conversion of lactose to a closely related disaccharide, allolactose.

When lactose is not available in the environment of E. coli, then cellular production of proteins involved in utilizing lactose would waste cell resources. Thus it makes perfect biological sense that ß -galactosidase, permease, and transacetylase are synthesized only when lactose is present. Furthermore, the lac operon has a second level of control that also helps the cell conserve resources: if glucose is present, then ß -galactosidase, permease and transacetylase cannot be induced by lactose. This also makes biological sense since glucose is metabolized more easily than lactose; thus it is inefficient to utilize lactose when glucose is available as a carbon source. The suppression by glucose of the induction of ß -galactosidase is called catabolite repression. In theory, the enzymes of the lactose operon could be regulated at any of the steps that influence the activity of gene products: the transcription of the gene by RNA polymerase, the translation of mRNA, the degradation of mRNA, activation of the proteins after translation, or inactivation or decay of the proteins. However, in the lactose operon, induction of proteins is controlled exclusively by varying the rate at which RNA polymerase transcribes the
mRNA. The mechanisms by which lactose induces the coordinate synthesis of these three proteins were elucidated by Jacob and Monod in their famous operon model. Detailed discussion of the lactose operon can be found in your genetics textbook.

Briefly, the lactose operon includes 6 components, which are found in the following positions on the *E. coli* chromosome.

\[
i \quad p \quad o \quad z \quad y \quad a
\]

---

i = lactose repressor
p = promoter (binding site for RNA polymerase)
o = operator (binding site for the repressor)
z = ß-galactosidase
y = permease
a = transacetylase

I. The **structural genes** encoding ß-galactosidase, permease, and transacetylase are adjacent to each other on the *E. coli* chromosome. In this diagram, transcription occurs from left to right. The expression of all three genes is controlled by a single promoter (p), and a single **polycistronic mRNA** encoding all three proteins. The polycistronic mRNA ensures that levels of ß-galactosidase, permease, and transacetylase are regulated coordinately.

II. The transcription of the polycistronic mRNA is regulated by interactions of three components: the inducer (lactose), the lactose repressor, and the operator. The repressor is an **allosteric protein** with two binding sites: one site binds specifically to the operator DNA sequence; the other site binds allolactose, the derivative of lactose mentioned above. When lactose is absent from the medium, the lactose repressor binds to the operator. Note that the operator is located between the promoter (the site of binding of the RNA polymerase), and the transcription start site of the genes controlled by that promoter. Thus, the lactose repressor binds to the operator in a position which obstructs transcription of the polycistronic mRNA. When lactose is present, allolactose binds to the repressor, causing the repressor to change shape. The repressor then releases the operator, the RNA polymerase transcribes the polycistronic mRNA, the mRNA is translated, and the levels of ß-galactosidase, permease and transacetylase increase. The lactose repressor is considered to exert **negative control** over the lactose operon because it inactivates the operon when it binds to the operator DNA sequence.

III. As noted above, the induction of ß-galactosidase, permease, and transacetylase by lactose is suppressed if glucose is present in the *E. coli* environment. This suppression involves a second mechanism of regulation superimposed on the repressor-operator. Briefly, glucose must be absent for RNA polymerase to bind to the promoter. The mechanism of catabolite repression is described further in your genetics text.

In this lab, we will treat cells with lactose and measure induction of ß-galactosidase. In addition, we will test the effect of glucose on the induction of ß-galactosidase. To measure ß-galactosidase activity in cells, we add to cells a colorless compound, o-nitrophenylgalactoside (ONPG). When ß-galactosidase is present, this enzyme breaks down ONPG, yielding o-nitrophenol (ONP), which has a yellow color. The amount of yellow color (from ONP) can be measured by testing the absorbance (optical density, O.D.) of the solution at 420 nanometers.
(wavelength) in a spectrophotometer. The amount of yellow color is proportional to the level of the ONP product, which in turn is proportional to the level of β-galactosidase activity.

**Materials:** (for each group of students)

1. Approximately 50 mL of a log-phase culture of *E. coli* B, grown in a chemically defined medium with glycerol (M9-glycerol).
2. Approximately 50 mL of a log-phase culture of *E. coli* that has been grown in a chemically defined medium with glycerol (M9-glycerol), then 0.2% glucose for 30 minutes. This culture will be shared by the entire class. 3. 2.5 mL of 5% lactose
4. 4 mL of o-nitrophenylgalactoside (ONPG) at 4 mg/mL
5. 1 mL toluene (This is kept in the hood.)
6. 9 mL 1 M Na CO$_3$
7. 2 mL pipettes and pi-pumps
8. 10 test tubes for the spectrophotometer, one filled ¾ with deionized water
9. Visible light spectrophotometer
10. Shaking water bath set at 37°C with brackets to hold 125 mL Erlenmeyer flasks.
11. Markers for labeling tubes
12. Ice buckets
13. Water bath set at 37°C
14. Test tube racks to hold spectrophotometer tubes
15. Pipetman and tips, 1000 μL
16. Timer

**Safety Precautions:**

Note that toluene is volatile and toxic. Keep the stock bottles of toluene in the fume hood, and avoid breathing the fumes.

Sodium carbonate (Na$_2$CO$_3$) is corrosive and will burn your skin and eyes; wear gloves and safety glasses when using it.

This strain of *E.coli* is not virulent; it is the common form that is found in most mammal intestinal tracts, including our own. Wash your hands when you are finished with the lab.

**Experimental Procedures**

*(Note: Before starting, read the entire lab handout to be sure you understand the reasons for each experimental step.)*

In this experiment, you will measure the induction of β-galactosidase in cells after the cells are provided with lactose. The levels of β-galactosidase will be measured at exactly the point that lactose is added (time zero), and at 5 minute intervals up to 30 min. For this experiment, you will use cells grown with glycerol as a carbon source. In addition, you will use cells grown in glycerol plus glucose to test whether glucose inhibits the ability of lactose to induce β-galactosidase.

These experiments have two stages. First, lactose will be added to cells to induce β-galactosidase. Second, we will measure the levels of β-galactosidase in the cells.

You will use a spectrophotometer to make your measurements, see figure 1 and instructions for use below. Familiarize yourself with this instrument using the provided blank and sample of blue solution.
Starting up the instrument and learning to use it

When you turn on your GENESYS 20 spectrophotometer, it performs its power-on sequence, which is checking the software revision, initializing the filter wheel and the monochromator. This takes about 2 minutes to complete. Allow the instrument to warm up for 30 minutes before using it.

Note: Be sure that the cell holder is empty before turning on the instrument.

This takes about 2 minutes to complete. Allow the instrument to warm up for 30 minutes before using it.

How to use the spectrophotometer:

Next to your spectrophotometer are two sample tubes provided for you to learn how to properly use and test out the spec. One is a water blank and the other is Methyl Blue dissolved in water. Other groups will use the sample tubes so keep them intact after you use them.

Measure the OD of the Methyl Blue sample solution at 650nm. Set the wavelength, zero the spec with the blank and measure your methyl blue sample following the instructions below. When you have measured the sample dye tube correctly, the OD₆₅₀ should be close to 0.6. If you have issues, questions, or if your measurement has a very different value, ask your TA to check your operation of the spectrophotometer. You will use different wavelengths in lab today, change them when instructed to do so for the experiment.

To operate the spectrophotometer:

1. Press \textbf{nm ▲} or \textbf{nm ▼} to select the wavelength (in nanometers).
   
   \textbf{Note:} Holding either key will cause the wavelength to increase or decrease more quickly.

2. Insert your blank into the cell holder and close the sample door.

3. Press \textbf{0 ABS/100\%T} to set the blank to 0A or 100\%T.

4. Remove your blank and insert a sample into the cell holder. The sample measurement appears on the LCD display. Do not hesitate to ask for more instruction if you are not sure you are using the machine properly.
5. Fingerprints on the test tubes can alter the optical density readings so wipe down your tubes each time before you put them in the spectrophotometer with a Kim wipe.

**Part I. Induction of β-galactosidase:**

Summary: You will be given a 50 mL log-phase culture of *E. coli* that was grown with glycerol as the only carbon source. You will be adding lactose to induce β-galactosidase, then removing samples at regular intervals to check for enzyme activity. You will also use a culture grown with glycerol plus glucose, and will remove a sample 30 min after the addition of lactose.

1) For the cells grown in glycerol, label the top of the spectrophotometer tubes with a marker as follows: 0, 5, 10, 15, 20, 25 and 30. For the glucose experiment, label one tube, G-30. Add two drops of toluene to each of the 8 tubes, and place the tubes in an ice bucket. These tubes will receive samples of *E. coli* cells after different induction times. Be sure you understand the plan of the experiment. What is the purpose of the toluene?

2) When you are ready to start, choose which numbered flask you will use (1-9) and write your group name on the sign-up sheet for that flask. The 5% lactose has been measured exactly (2.5 ml) so you can dump the entire contents of that tube into your *E. coli* culture and mix well. Immediately start your timer (this is time zero), and immediately transfer 2 mL of the *E. coli* culture with a sterile pipette and a blue pi-pump to the chilled tube marked “0”. Mix gently and return the tube to the ice bucket. Put the remaining *E. coli* culture in the shaking water bath at 37°C so that induction can occur.

3) At 5, 10, 15, 20, 25, and 30 min after the addition of lactose to the glycerol culture, take your flask to your bench and transfer 2 mL of this culture to the appropriately labeled tube with a sterile pipette. Swirl gently to mix the cells with toluene, and place in the ice bucket. Swirl the culture in the flask immediately before you take each sample as the cells tend to settle to the bottom when the flask is not moving. Used pipettes can be stored in the *E. coli* waste jars until the end of the lab. Return your flask to the shaking water bath to culture at the proper temperature.

4) During the sampling time, the TA will add 5 mL of 5% lactose to the 50 ml culture of *E. coli* grown with glucose. This culture will be shared by the entire class to test whether a culture grown with glucose will induce β-galactosidase when lactose is added.

5) 30 min after the TA added the lactose to the glucose culture, transfer 2 mL of this culture to the tube labeled “G-30”. Again mix cells gently with the toluene, and put the tube on ice.

**Part II. Measurement of β-galactosidase:**

6) After all the bacterial samples have been mixed with toluene in the tubes, again swirl each tube gently. To prepare these tubes for enzyme measurement, set the tubes in a test tube rack, in a 37°C water bath for approximately 10 min to increase their temperature.

7) We are now ready to test the levels of β-galactosidase in these induced cell samples. Add 0.4 mL ONPG to each tube as precisely as possible using the 1000 μl Pipetman. Start your timer. The ONPG can be added to the tubes while they are in the water bath. Mix gently but thoroughly; then incubate at 37°C for 5-15 minutes. The point at which ONPG is added is time zero, since you are timing the reaction from that point. Periodically examine your tubes in the water bath. When any of the tubes show a slight yellow color, note the time and let the tubes incubate for 5 more minutes. After the 5 minutes, take the tubes out of the water bath and bring them to your bench. Add 1.0 mL of 1 M Na CO to all eight spectrophotometer tubes and mix gently. Stop your timer; this is the time of induction with ONPG that you will use in your calculations. The
first tube to show color will be the one with the highest level of β-galactosidase. Can you predict which tube this will be?

8) Blank the spectrophotometer for 420 nm using the tube of DI water (deionized water). Measure the absorbance of all of your experimental tubes at 420 nm. Next, blank the spectrophotometer for 550 nm with the tube of DI water and measure the absorbance of all of your experimental tubes at 550 nm. Wipe down each test tube with a Kim wipe before inserting it into the spectrophotometer to make sure it is clean. The procedure for using the spectrophotometer is described at the end of this lab.

There are two reasons for reading the optical density of each sample at both 420 and 550 nm. First, the optical density reading at 420 nm is actually due to a combination of absorbance by the ONP and optical density due to light scattering by E. coli cell debris. It is possible to correct for the light scattering of cellular debris at 420 nm by the following formula:

\[
\text{OD}_{420} = 1.75 \times \text{OD}_{550}
\]

Second, we wish to measure the amount of β-galactosidase / cell. Since the light scattering (turbidity) at 550 nm is proportional to cell density, it provides an estimate of the number of E. coli cells in each sample.

9) The amount of enzyme activity per cell in each sample can be calculated from the formula below, where \( t \) is the length of incubation with ONPG (in minutes).

\[
\text{Enzyme Units} = \frac{100 \times \text{OD}_{420} - (1.75 \times \text{OD}_{550})}{t \times \text{OD}_{550}}
\]

10) When you have finished analyzing your data, the kinetics of induction of β-galactosidase by lactose should be graphed, with the number of enzyme units per cell on the Y-axis vs. time of incubation with lactose on the X-axis. Plot the enzyme units per cell of the glucose sample (G30) as a single point and don’t connect it to the line of the rest of the data.

Clean up

Please pour your E. coli samples from your test tubes into the E. coli WITH toluene waste jar on the counter. This E. coli must be treated as hazardous waste. Empty your E. coli culture flask into the E. coli WITHOUT toluene waste jar on the counter. Place your empty test tubes and flask into the container to soak. The DI water test tube can be reused; leave it in your rack. Place all used pipettes, Pipetman tips, gloves and paper towels in the orange autoclave bag. Turn off the spectrophotometers, unplug and leave on the bench. Push in your chair before you leave.

Notes on Procedures

1. The bacteria are grown on chemically defined medium containing glycerol as the only carbon source. Obviously, if the growth medium contained glucose, it would suppress induction of the operon by lactose.

2. Either lactose or isopropylthiogalactoside (IPTG) can be used to induce the lactose operon. IPTG is a “gratuitous inducer” of the lactose operon. It can mimic allolactose and interact with the lac repressor to induce the lactose operon, but it cannot be metabolized or used as a carbon source by E. coli.

3. The o-nitrophenol solution is yellow. Therefore, it transmits yellow light and absorbs violet light. The spectrophotometer is used to measure the amount of light that is absorbed as it
passes through the solution of ONP. The absorption of light by a solution is described by the Beer-Lambert Law

\[
\text{Absorbance} = \text{O.D. (optical density)} = \log_{10} \left( \frac{I}{I_0} \right) = E l c o
\]

where \(I_0\) = the intensity of incident light, \(I\) = intensity of transmitted light, \(c\) = concentration of the absorbing substance, \(l\) = length of light path through the solution, \(E\) = extinction coefficient.

In these measurements, \(l\) is constant since we always use the same diameter tubes, and \(E\) is constant for any given substance. Thus, the absorbance or O.D. of the solution is directly proportional to the concentration of absorbing molecules.

4. In measuring \(\beta\)-galactosidase activity, a sample of \(E. coli\) is first mixed with a few drops of toluene. The toluene permeabilizes (makes holes in) the \(E. coli\) cell membrane. This kills the bacteria and prevents further changes in \(\beta\)-galactosidase levels. The holes in the cell membrane also enable the enzyme to react with the ONPG. If the cell membranes were not permeabilized, beta-galactosidase would remain inside the cells, ONPG would remain outside, and the enzyme could not break down the ONPG.

5. The formula above shows that the amount of ONP produced is proportional to the number of cells and the time of incubation. Thus, both time and cell number must be measured in each enzyme measurement. After allowing the reaction between ONPG and \(\beta\)-galactosidase to proceed, the reaction is terminated by the addition of \(\text{Na}_2\text{CO}_3\). The \(\text{Na}_2\text{CO}_3\) not only develops the color, but it also stops the reaction by changing the pH of the reaction mixture to \(pH = 11\) where the enzyme is not active. This allows the experimenter to precisely control the length of the enzyme reaction, and then measure the absorbance in the spectrophotometer at his/her convenience.

**Thought Questions:**

Record the answers to the following questions in your notebook.

1. Review Jacob and Monod’s operon model seen below. Then list all the metabolic changes which lead to synthesis of \(\beta\)-galactosidase when lactose is added to \(E. coli\) cells growing in M9-glycerol medium.

   
   ![Operon Model](image)

<table>
<thead>
<tr>
<th>i</th>
<th>p</th>
<th>o</th>
<th>z</th>
<th>y</th>
<th>a</th>
</tr>
</thead>
<tbody>
<tr>
<td>lactose repressor</td>
<td>promoter (binding site for RNA polymerase)</td>
<td>operator (binding site for the repressor)</td>
<td>(\beta)-galactosidase</td>
<td>permease</td>
<td>transacetylase</td>
</tr>
</tbody>
</table>

2. It is possible to estimate how long the cells took to synthesize \(\beta\)-galactosidase after adding the inducer by extrapolating the linear, increasing portion of the curve to the time-axis. This interval is determined by the rate of addition of bases by the RNA polymerase and the rate of polypeptide chain elongation. How well do your observations agree with calculations that RNA polymerase adds bases at
a rate of about 30 per sec, amino acids are added at a rate of about 6 per sec in *E. coli* cells at 37°C, and β-galactosidase is 1170 amino acids long?

**Excel Tutorial – Inserting a formula to solve multiple equations**

Above is the Excel template for the lac operon experiment. In column “A”, the times of incubation have already been entered. These are the approximate times you sampled your *E. coli* culture in minutes.

1. Enter the absorbance values of your samples that you determined using the spectrophotometer in columns “B” and “C”.

2. Enter the time that your samples were incubated with ONPG in the water bath in column “D”. This value will be the same for all samples. Your table should look like the one below.
3. You are now ready to calculate the number of enzyme units of beta-galactosidase per cell that were produced by the *E. coli* in each of your sample tubes. You could solve each equation using a calculator or you can have Excel solve them for you.

4. To enter a formula in a cell for Excel to solve, you first enter a “=”. This tells Excel that you are entering a formula. If you only had one equation to perform, you could enter your OD 420, OD 550 and “t” values yourself but since you have 8 equations to perform it is easier to tell Excel where the values are and have Excel plug them in for you.

5. In cell “E5” enter “=”, “100”, “*”, “(“. Instead of entering the value of the OD 420, you can now click on the cell which contains the value you want here, which is “B5”. You will see “B5” in your formula. You can continue entering the formula, “-”, “(“, “1.75”, “*”, “)”,”/”, “(“. For “t”, click in cell “D5”, “*” and in cell “C35” again for OD 550. Your entered formula should look like the one below.
6. When you hit enter, Excel will do the calculation and put the answer of 2.27 in the cell. To find the answers for the other samples, click in the “E5” box. Put your cursor on the bottom right corner of this box until you see the black cross. Click and drag the cursor down through all the boxes that you have data for, this will be through box “E12”. Excel will fill in the answers to all of your equations!
<table>
<thead>
<tr>
<th>time of incubation</th>
<th>OD 420</th>
<th>OD 550</th>
<th>time of incubation</th>
<th>enzyme units per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>with lactose (m)</td>
<td></td>
<td></td>
<td>with ONPG (m)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.160</td>
<td>0.080</td>
<td>100*(OD420-1.75<em>OD550))/(t</em>OD550)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.181</td>
<td>0.101</td>
<td>11</td>
<td>2.272727273</td>
</tr>
<tr>
<td>10</td>
<td>0.212</td>
<td>0.109</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.290</td>
<td>0.115</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.480</td>
<td>0.121</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0.462</td>
<td>0.135</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.598</td>
<td>0.156</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>glucose culture</td>
<td>0.163</td>
<td>0.171</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** The formula used to calculate enzyme units per cell is:

\[
100\times\frac{(\text{OD}_{420}-1.75\times\text{OD}_{550})}{(t\times\text{OD}_{550})}
\]
The number of enzyme units of beta-galactosidase that each of your *E. coli* samples produced can now be graphed. Use the time of incubation with lactose as your “x” value and enzyme units per cell as your “y” value.

**Graphing the data and making 2 different series**

Copy your “X” and “Y” values from Columns “A” and “E” in the screenshot including your values for the glucose culture and put them in 2 columns next to each other (when copying the enzyme units, paste as “paste values”). Highlight both columns and from the toolbar click on “Insert” and then on “scatter” and then on the “scatter with smooth lines and markers” choice. The graph will pop up looking like this:

![Graph example](image)

Under “chart tools”, “layout”, add “chart title” and “axis titles”.

To make 2 series out of the data, click on the chart to bring up the chart menu on the toolbar. On the “chart tools” section, click on “Design”, and then “Select Data”. Click on “series 1” on the table that pops up and “edit”. Here you can change the name of series 1 to “lactose”. Click the small box next to the text box for “Series X Values”. Take the cursor and click in the box
where your X-values are and highlight all of the values from 0-30, but not the glucose 30 minute value. Click the small box. Click the small box next to the text box for “Series Y Values” and highlight the “Y” values. Click the small box. Then click “ok” in the edit box.

In the “Select Data Source” box again, click “add”. Add your series name for the glucose data and click the small boxes again. Highlight the “30” minute box for glucose “X” value and your value for enzyme units for the glucose culture as your “Y” value. Click ‘ok’ and you should see 2 series of data on your graph, one is a line of multiple points and one is a single point, as you can see in the chart below and both series described in the legend: