

EFFECTS OF UV IRRADIATION ON MICROBIAL NUMBERS AND POPULATIONS

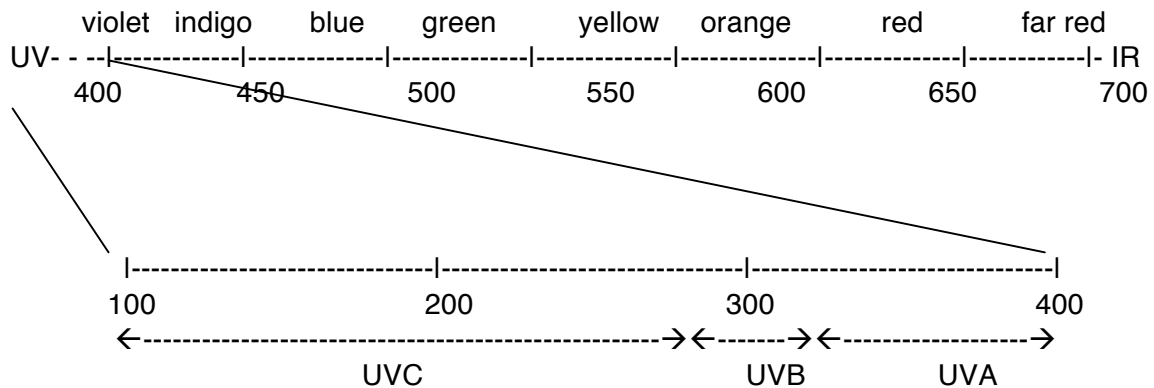
Objectives

- To learn the effects of UV irradiation on different bacterial species.
- To learn if there is a correlation between the length of exposure to UV irradiation and the number of bacteria killed.
- To learn the different techniques used to count the number of microorganisms in a sample.
- To have more practice at dilution series and calculations.

Introduction

Effects of UV Irradiation

Light is a form of energy that travels in rhythmic waves. The distance between the crest of two waves is called the wavelength and is expressed in metric units. The light spectrum covers a wide range of rays with different wavelengths. At one extreme are the gamma rays with wavelengths of less than a nanometer ($1 \text{ nm} = 10^{-9}$ meter) while at the other extreme are the radio waves with wavelengths of more than a kilometer. The most important part of the light spectrum is a narrow band called the visible light (400-700 nm). These wavelengths, taken together, are seen as homogeneous white light, but if passed through a prism, component wavelengths (in nm) are perceived as the colors of the visible spectrum.



Light wavelengths between 100 and 400 nm are considered ultraviolet (UV). Wavelengths below 100 nm are extremely hazardous (i.e., ionizing radiations such as gamma rays and X-rays). Beyond 800 nm, the radiation is in the form of infrared (IR or heat rays), microwaves (10^6 - 10^9 nm) and radio waves (10^9 - 10^{12} nm).

The spectrum of UV (100-400 nm) is further subdivided into three categories:
 1- UVA (also called long wave UV or black light) has a wavelength of 320-400 nm. These waves have comparatively low energy levels and low penetrability and do not cause sunburn, although they are able to produce a tan on the skin. It is estimated that about 99% of UV from the sun that reaches the earth surface is in the UVA form. The other forms of UV get absorbed by the

ozone layer. [en.wikipedia.org/wiki/Ultraviolet].

The skin is made up of an upper layer (epidermis) and a lower layer (dermis). Dermis is a fibrous, tough and durable layer. Epidermis contains several layers of cells. The top layer is called "stratum corneum" and is covered with several layers of dead cells containing a waxy, water-repelling substance called keratin. This layer sloughs off constantly and gets replaced from below. The next layer is composed of epithelial cells, interspersed with melanocytes. Melanocytes, when exposed to UV light, excrete a substance known as melanin (responsible for the tan color). So a tan is usually the skin's response to UV damage.
[http://pubs.acs.org/cen/whatstuff/stuff/8025_sunscreens.htm]

Dermis and epidermis sit on top of a final layer called the basal layer, which is responsible for constantly producing epithelial cells. The skin is a dynamic tissue in that cells are continuously moving from the basal layer to the stratum corneum and getting shed off.

Sunning salons and solariums claim to use only UVA and many people visit them to get a tan. However, strict quality control measures should be practiced in such establishments as the lamps used could be producing other harmful wavelengths. Thus their prolonged use is questionable as far as skin aging and skin cancer are concerned.
[http://www.nevdgp.org.au/info/std_misc/SOLARIUMS.htm]

2- UVB are wavelengths of 280-320 nm and can damage epithelial cells of the skin. When there is such damage, the body tries to increase the blood flow to the area and thus swelling, heat and redness ensue which are translated into sunburn. Of course, UVB can also cause the release of more melanin pigments in the skin to protect the skin from further effects of UV and as a result, the skin gets a more pronounced tan.

3-UVC has wavelengths of 100-280 nm and is the most penetrating and dangerous among the three categories. Fortunately no UVC from the sun reaches the earth surface, although UVC can be created artificially by special lamps.

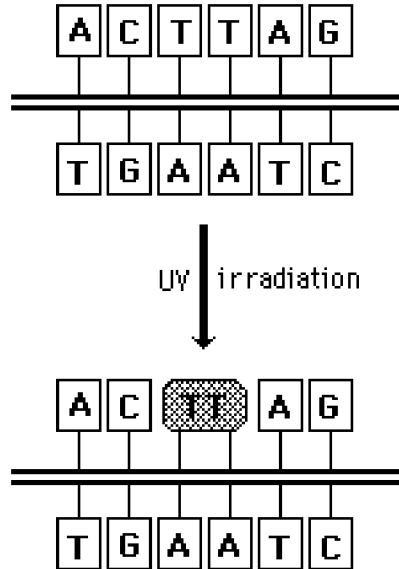
Now that we have discussed the categories of UV rays, we will give the rest of our discussion to generalities about UV. One thing that should be mentioned here is that not all effects of UV are bad. UV actually has an important benefit in that it induces body cells to produce vitamin D. Lack of vitamin D is known to cause various cancers, bone pain and bone fracture, to name just a few.

One other benefit of UV is its use as a sterilizing agent for materials that would otherwise be damaged by high heat (e.g., autoclaving) or materials that are hard to sterilize by other methods.

Although its rays have a very low power of penetration and are not as harmful as the rays of lower wavelengths, they are still quite powerful mutagens. Their main effect on biological systems stems from the fact that they are absorbed by the DNA molecules in the cells and cause alterations that are harmful to cell survival.

To understand the mutagenic effects of UV irradiation on a molecular level, it is a well-known fact that short wavelength rays (such as UV) interact with water molecules in the cell to produce free radicals (-OH). Such free radicals lack one or more electrons and attack other molecules such as cell proteins or DNA to rob them of electrons. Attack from a large number of free radicals on the same macromolecule could render it nonfunctional.

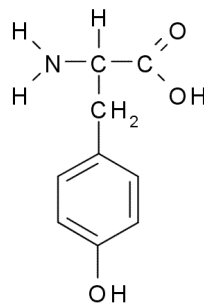
Except the production of free radicals, UV can directly affect DNA. The most common effect is at locations on the DNA molecule where two thymine (T) bases occur adjacent to each other. UV irradiation causes the two T bases to covalently fuse together. Such structures are called thymine dimers and cause a distortion in the shape of DNA.



Thus, when it is next time for DNA replication, a wrong base may be incorporated at the thymine dimer position on the strand being synthesized. This would constitute a site of mutation and if it involves a protein that plays a role in cell survival, it may be lethal.

In humans, skin cancer is the most common effect of UV-induced mutations. The interaction of the UV with skin cell DNA causes unrestricted growth of some skin cells that ultimately produce a tumor. Although skin cancer is the most common type of cancer, its prevention is rather easy. Protecting the skin from sunrays that contain UV irradiation by wearing proper clothing or sunscreen cream or lotions will definitely reduce skin cancer risks.

Since melanin absorbs UV and reduces its harmful effects, a chemical that resembles melanin may be ideal for use as a sunscreen. Melanin is synthesized from the amino acid tyrosine which has a benzene ring as seen below [<http://commons.wikimedia.org/wiki/Image:Tyrosine.png>]:



Tyrosine combines with DOPA (dihydroxyphenylalanine) to produce dopaquinone which goes through further steps to finally change to melanin. [<http://omlc.orgi.edu/spectra/melanin>]. It is thought that when UV waves hit a benzene hexagon, the vibration of this ring dissipates the energy of the wave to render it harmless. [Evaluating the effectiveness of sunscreens. 2005. T&S Educational, Inc.]. Of course there are other chemicals that can also absorb the UV wave such as zinc oxide, titanium dioxide, octyl methoxycinnamate (OMC), 4-methylbenzylidene camphor (4-MBC), avobenzene, oxybenzone, and homosalate. [<http://pubs.acs.org/cen/whatstuff/stuff/8025sunscreens.html>].

Sunscreens are rated by their degree of effectiveness called SPF (sun protection factor); the higher the SPF value, the more protection they offer. SPF is actually the ratio of the time of exposure to UV with and without the sunscreen to produce a similar degree of effect on skin. For example, if it takes 20 minutes to produce skin redness without UV protection but 200 minutes to get the same degree of redness with sunscreen application, then the SPF of that sunscreen is $200/20=10$. Usually sunscreen chemicals are dissolved in an oily or creamy base to spread better on the skin. The higher the concentration of the chemical in such a base, the higher would be its SPF value.

In the lab experiment today, we want to find the effects of UV irradiation on two species of bacteria (*Serratia marcescens*, a Gram negative rod and *Staphylococcus aureus*, a Gram positive coccus) with and without different SPF sunscreens. *S. marcescens* is a member of the bacterial *Enterobacteriaceae* family that inhabits our intestines and produces round pink colonies, if grown at 30°C or lower temperatures on agar plates. However, the colony color will be tan when grown at higher temperatures than 30°C. *Staph. aureus* is the major cause of nosocomial (hospital environment) diseases and is resistant to many antibiotics. [Incidentally, the strain that is also resistant to methicillin, called MRSA (pronounced Mersa) has recently been in the news. We are not using that strain in our labs!!]. Since the two bacteria mentioned above could become pathogenic, especially in immunosuppressed individuals, it is important to use sterile techniques as demonstrated by your TA and take utmost care not to infect yourself, your lab partners and/or your lab environment.

We will use only 15 seconds of irradiation from a UV lamp to see the percentage kill as well as noting any observable genetic changes (such as colony color change, colony smoothness, colony shininess, etc). Then we will determine if different sunscreens protect bacteria from the deleterious UV effects, as determined from the number of bacteria surviving after irradiation. This takes us to the next topic of the lab today, namely "Enumeration of Bacteria" that will be described below.

Enumeration of bacteria

Determining the number of microorganisms in a given sample is sometimes very important. For example, the safety of many foods and beverages depends on knowing the levels of microorganisms present in them. A variety of methods have been developed for the enumeration of microbes. These methods either measure cell number directly or measure cell mass, or cell constituents that are proportional to cell number. The four general approaches used for estimating the size of microbial populations are:

1-Direct count of cells using a counting chamber or tagging the cells with fluorescent material and counting them under a fluorescent microscope.

2-Indirect cell count which involve serial dilutions of the sample and the spread of a sample from each dilution on agar surface or inoculation of indicator tubes. Viable plate count and most probable number (MPN) are two common methods in this category.

3-Direct measurement of microbial mass (i.e. weight) and correlating it with cell number by reference to a standard curve.

4-Indirect measurement biochemical components of microbial cells or cell products, (such as protein, ATP, peptidoglycan, lipopolysaccharides, etc.) and correlating it with cell number by reference to a standard curve.

Since we are going to use the viable plate method of counting cell numbers in this experiment, we will limit our discussion of enumeration to this method only.

Viable Plate Count Method of Enumerating Microorganisms

The most common procedure for enumeration of microorganisms such as bacteria is the viable plate count. In this method, serial dilutions of a sample containing viable microorganisms are plated onto a suitable growth medium. The suspension is either spread onto the surface of agar plates (spread plate method), or is mixed with molten agar, poured into plates, and allowed to solidify (pour plate method). The plates are then incubated under conditions that permit microbial reproduction so that colonies develop that can be seen without the aid of a microscope. If we assume that each bacterial colony arises from an individual cell that has undergone cell division, then by counting the number of colonies and accounting for the dilution factor, the number of bacteria in the original sample can be determined.

The viable count is an estimate of the number of cells. Because some organisms exist as pairs or groups and because mixing and shaking of the sample do not always separate all the cells, we actually get a count of the colony forming units. One cell or group of cells will produce one colony, therefore when we record results for a viable count, it is customary to record the results as colony forming units per ml (cfu/ml) or per gram (cfu/g) of test material.

Because we generally have no idea of the number of bacteria in a sample, it is necessary to prepare a dilution series to ensure that we will obtain a dilution containing a reasonable number of bacteria to count. Dilutions in the range 10^{-1} (1/10) to 10^{-8} (1/100,000,000) are generally used, although with particular types of samples the range of dilutions can be decreased; for example, for water that is not turbid, the maximal dilution needed is 10^{-6} because we know that if there were 10^7 or more bacteria per milliliter, the water would be visually turbid.

The major disadvantage of the viable count is that it is selective and therefore biased. The nature of the growth conditions, including the composition of the medium used and the conditions such as temperature and pH, determine which bacteria in a mixed population can grow. Since there is no universal set of conditions that permits the growth of all microorganisms, it is impossible to enumerate all microorganisms by viable plating. This same disadvantage, however, becomes advantageous when one is interested in only a specific microbial population.

Laboratory Supplies

<i>Serratia marcescens</i> grown O/N	1 ml/table
<i>Staphylococcus aureus</i> grown O/N	1 ml/table
UV lamp	1/lab
Goggles	4/lab
BHI plates	40/section
Saline tubes (0.85% NaCl), tubes of 10 ml	1/table
Pipets, 1 ml	8/group
Vortexer	1/table

Pipetmans: P20, P200 & P1000	1 set/tableside
Eppendorf tubes	1 beaker/table
Saran wrap	1 roll/lab
Tape	1 roll/table
Sunscreen (Clear Spray, No Rub; SPF4, SPF15 & SPF30)	1 set/lab

Procedures

For enumeration, students at the two right tables of the lab will work on one species and the students at the two left tables will work on the other. For UV experiment, the students at the right side of each table will work with one species and the other side of the table with the other species.

Session 1

Enumeration of bacteria

1. For each species, obtain 3 Eppendorf tubes and label them 10^{-2} , 10^{-4} and 10^{-6} . Using a P-1000 Pipetman, turn the knob to read 099 to add 990 μl of saline to each tube. These are the dilutions of the overnight (O/N) culture of bacteria that we want to enumerate. For the 10^{-2} dilution, take 10 μl of the undiluted O/N culture and add to the 990 μl saline in the tube labeled 10^{-2} . Close the lid and vortex vigorously to mix well. Now take 10 μl out of this tube and add to the saline in the 10^{-4} tube. Again mix well by vortexing. For the 10^{-6} tube, take 10 μl out of the 10^{-4} tube, add to saline in the 10^{-6} tube and repeat the mixing.
2. Next obtain 2 fresh Eppendorfs for each species and label them 10^{-3} and 10^{-5} and add 900 μl saline to each. Take 100 μl of the 10^{-2} dilution tube and add to the 10^{-3} tube. Similarly, take 100 μl out of the 10^{-4} dilution tube and add to the 10^{-5} tube. Mix contents of these tubes by vortexing.
3. Obtain 4 BHI plates and label them -4, -5, -6 and -7. Add your table number to the label. Also add SM if you are working with *Serratia marcescens* or SA if your species is *Staphylococcus aureus*.
4. Using a P-200 Pipetman, transfer 100 μl of the 10^{-3} dilution tube to the plate labeled 10^{-4} . Quickly spread the inoculum on the surface of the agar using a sterile spreader. After the spreading is finished, re-sterilize the spreader.

Note 1: **Important.** To sterilize the spreader, dip it in the 95% ethanol jar, touch the spreader to the inside of the jar above the alcohol level to get rid of the extra alcohol and then quickly take the spreader through a flame. Do not hold the spreader in the flame for more than a second. (Our purpose is to kill any bacterium present via the alcohol and not via the heat.) Be extra careful as flaming alcohol drops may fall on objects and cause a fire hazard. Also while the alcohol is burning off, keep the spreader head down so the dripping alcohol will not spread to your fingers and hand.

Note 2: Since we are using one tenth of 1 ml, we are in effect diluting our sample 10 times in this step. That means that, as long as 0.1 ml is spread, the dilution number of the plate is 10 times less than that of the tube, If we had spread 1 ml of the solution of the tube on the plate, then the dilution factor of the plate would be 1/1 and not 1/10.

5. Repeat the above procedure to spread 100 μ l from the 10^{-4} , 10^{-5} and 10^{-6} dilution tubes onto the 10^{-5} to 10^{-7} plates, respectively. Let the plates sit at room temperature without being moved for 15 minutes for the liquid to be absorbed by the agar.
6. Make sure the agar surface is dry, invert the plates and incubate them at 30°C.
7. Your TA will remove the plates from the incubator after 48 hours and transfer them to a refrigerator for storage till the next session.

Effects of UV Irradiation

1. Obtain 6 BHI plates and label SM if you are working with *Serratia marcescens* or SA if your species is *Staphylococcus aureus*. Also add your Table Number and the following designations to the label depending on the use of the plate:
 - Plate C: plastic lid left on the plate during UV radiation (C=Covered)
 - Plate NC: plastic lid taken off during UV radiation (NC=Not Covered)
 - Plate SW: plastic lid replaced with Saran wrap (SW) during UV radiation
 - Plate SPF4: plastic lid replaced with SW sprayed with SPF4 sunscreen
 - Plate SPF15: plastic lid replaced with SW sprayed with SPF15 sunscreen
 - Plate SPF30: plastic lid replaced with SW sprayed with SPF30 sunscreen
2. Using a P-200 Pipetman, transfer 100 μ l of the 10^{-5} dilution tube to each of these plates. Quickly spread the inoculum on the surface of the agar using a sterile, alcohol-flamed spreader. After the spreading is finished, re-sterilize the spreader.
3. Very carefully take the lid off of SPF designated plates and cover the plates individually with Saran wrap. Make sure no wrinkles are present on the agar side. You may use pieces of tape on the bottom of the plate to hold the Saran wrap in place (although we have found this not to be necessary). Put the lids back on over the Saran wrap.

Note: Do the above step one plate at a time and try to decrease the possibilities of contamination to a minimum.

4. For the plates that get a specific SPF treatment, remove the lid. Then, hold the spray can that has the same SPF as the plate about 6 inches away from the plate and spray the Saran wrap as uniformly as possible for about 1-2 seconds. Uniformity of application is very important. Excess sunscreen should be avoided. It takes about 15 minutes for the sunscreen to dry on the surface of the Saran wrap. During this time and until the end of irradiation treatment, do not touch the Saran wrap.
5. When all plates are ready, take your plates to the center table.

Note: WEAR PROTECTIVE GLOVES AND GOGGLES. BE AWARE THAT UV RAYS ARE HARMFUL and may cause serious skin burns and blindness. Do not look at the lamp directly.

6. Place plate C under the UV lamp. Do not take off the lid. Turn the lamp on and let it irradiate the plate for exactly 15 seconds with the lid on. Turn the lamp off. Take out plate C and set it aside.

7. Now, take the lid off of plate NC and place it under the UV lamp. Turn the lamp on and let it irradiate the plate for exactly 15 seconds. Turn the lamp off. Take out plate NC, replace its lid and set it aside.
8. Take the lid off of plate SW that is now covered with Saran wrap and place it under the UV lamp. Turn the lamp on and let it irradiate the plate for exactly 15 seconds. Turn the lamp off. Take out plate SW, remove and discard the Saran wrap, replace the lid and set it aside.
9. Repeat the above step for plates SPF4, SPF15 and SPF30. Make sure UV lamp is off.

Incubate the inverted plates at 30°C for 72 hours. Your TA will transfer the plates to the refrigerator to be studied in the next lab session.

Note: Our experience shows that if the plates are incubated one or three days (and not longer), mutant colonies would show their characteristics in a more pronounced way and it would be easier to study them.

Session 2

Enumeration of Bacteria

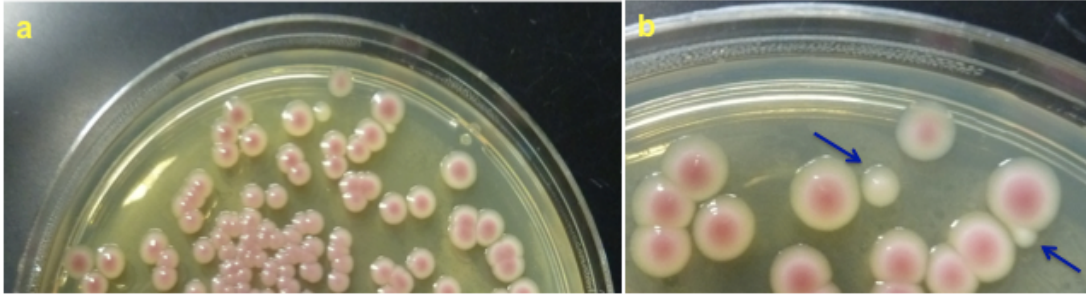
1. Count the colonies on each plate. This process is facilitated by holding the plate towards a light source and marking the position of each colony on the back of the petri plates with a marking pen to keep track and avoid recounts. If a plate has more than 300 colonies, record it as TNTC (too numerous to count).
2. From the plate count data, calculate the concentration of bacteria in the original sample. For statistical reasons, use data only from that have between 30 and 300 colonies. Assume that each colony forming unit (cfu) represents the progeny of a single cell. Therefore, the number of bacterial cells in the original sample is determined by multiplying the number of colonies on a dilution plate by the corresponding dilution factor. For example, if there were 200 colonies on the 10^{-6} plate, then there were $200 \times 1,000,000 = 200,000,000$ colonies or 2×10^8 cfu/ml in the original sample. Generally, replicates of each dilution are plated, and the mean count recorded. We will use the whole class data as replicates to fill the table in question 1 of the Results sheets.

Note: Choosing numbers between 30 and 300 is arbitrary but useful limits. If there are very few colonies on the plate (i.e. below 30), then a small error would be highly aggrandized. If there are a large number of colonies on the plate (i.e. over 300), then chances of colony overlap and counting errors increase.

Effects of UV Irradiation

1. Count the colonies on each plate and record the results in the table in question 4 of the Results sheets. Note any special changes in the characteristics of colonies.

Figures below show (a) a plate of *Serratia marcescens* after UV irradiation and part of the same plate enlarged (b). Arrows show two possible mutants.



Use of any section of this Lab Manual without the written consent of Dr. Eby Bassiri, Dept. of Biology, University of Pennsylvania is strictly prohibited.

Results of the Effects of UV Irradiation on Microbial Numbers and Populations Exercise

Name _____ Date _____ Section _____

Partner(s) _____

Which species did your group use? _____

1. Fill this table with enumeration data obtained from all groups:

Serratia marcescens

Plate/Dilution	Lab Table				Average	Standard error
	1	2	3	4		
10^{-4}						
10^{-5}						
10^{-6}						
10^{-7}						

Based on above results, the best estimate of cell number per ml of the original tube is:

Staphylococcus aureus

Plate/Dilution	Lab Table				Average	Standard error
	1	2	3	4		
10^{-4}						
10^{-5}						
10^{-6}						
10^{-7}						

Based on above results, the best estimate of cell number per ml of the original tube is:

2. Write a main advantage and a main disadvantage of the viable plate enumeration method.

3. From the work you did in this exercise, is there evidence that Saran wrap is not able to stop UV rays? Explain.

4. Fill this table with UV irradiation data obtained from all groups:

Serratia marcescens

Plate #	Lab Table				Average	Standard error
	1	2	3	4		
C						
NC						
SW						
SPF4						
SPF15						
SPF30						

Staphylococcus aureus

Plate #	Lab Table				Average	Standard error
	1	2	3	4		
C						
NC						
SW						
SPF4						
SPF15						
SPF30						

Based on above results, answer the following questions:

-Does the plastic lid stop UV rays?

-Does Saran wrap stop UV rays?

-Compare the different sunscreens. Which is the most effective?

-Which is the least effective?

5. Do the sunscreens behave similarly on the two species? Explain.

6. Did you observe any white colonies in UV-treated *Serratia* plates? Assuming you find a few such colonies, can you suggest a way they originated?

7. Compare the effect of UV radiation on the two species you used (Plates NC). Which one is more sensitive? Calculate the percentage of cells surviving after 15 seconds of UV irradiation. Could you give a biological reason for the differences you observe between the two species?

8. We saw in the lab that a short duration of UV radiation could kill a large number of the cells. We also know that a very large number of bacteria exist in the soil. Based on these two pieces of information, explain whether bacteria could survive under sunshine? Give a logical reason.

Use of any section of this Lab Manual without the written consent of Dr. Eby Bassiri, Dept. of Biology, University of Pennsylvania is strictly prohibited.