10/03-04 Laboratory: Mutagenesis Part II

This week you will analyze the results from the Ames test that you conducted last week and share your findings with the class. You will also continue to develop as a microbiologist, learning techniques that are commonly used to examine bacteria quantitatively. Lastly, you will gain practice at developing good hands in the lab. We do not expect you to become a microbiologist overnight. However, we do expect that you will develop with every lab, so do not forget the skills that you acquired last week!

PART I – Analyzing your Ames Test results
Review your laboratory notes and then find your plate from last week. Record your qualitative observations first and then begin to count the individual colonies on each spot. Do this by circling the outer region of your spot with a sharpie, and then marking the colonies inside the circle as you count. If you have problems analyzing the results, then we will come around and give you some tips. Once you have counted all the colonies in each spot, post your results on the board. Be sure to list the number of colonies observed for the particular strain you used and test chemical (including the negative control). We will then discuss the results as a group. Write down the class results in your notebook.

PART II – Mutagenesis continued!
After discussing the results we will continue the analysis by conducting experiments as groups. The groups will be assigned by the instructors. The following experiments will be done by the number of groups designated:

Groups A, B, C, and D will test both *Salmonella* Ames strain and the 1996 strain for UV sensitivity. Groups A and B will do the same experiment, as will Groups C and D. However, all four groups must communicate prior to the experiment because each group will be given a limited amount of reagents. The basic premise is that we would like you to test these bacterial strains for UV sensitivity (UV as a mutagen). Two groups will conduct identical experiments for reproducibility. People who have been assigned to Groups A, B, C, and D will first meet as a large group to design their experiments. Use the pooled results in your planning! They will then work with their partners to conduct the experiment.

Groups E, F, G, and H will test the mutagenic range and toxicity of sodium azide (NaN3) on the *Salmonella* Ames strain. Groups E and F will do the same experiment, as will Groups G and H. However, all four groups must communicate prior to the experiment because each group will be given a limited amount of reagents. Two groups will conduct identical experiments for reproducibility. Groups E, F, G, and H will first meet as a large group to design their experiments. Use the pooled results in your planning! They will then work with their partners to conduct the experiment. Note: you still need to have a negative control!

Groups I, J, K, and L will test the toxicity of ethanol on the *Salmonella* Ames strain. This will also help us to determine the spontaneous rate of reversion of the strain we are using. Groups I and J will do the same experiment, as will Groups K and L. However, all

four groups must communicate prior to the experiment because each group will be given a limited amount of reagents. Two groups will conduct identical experiments for reproducibility. Groups I, J, K, and L will first meet as a large group to design their experiments. Use the pooled results in your planning! They will then work with their partners to conduct the experiment. Note: you will still need to have a positive control!

The protocols and reagents for these experiments are the same as last week. Please remember to use caution when working with *Salmonella*. Good sterile techniques and disinfecting practices should be used accordingly.

PART III – Revertants or Not ? – Quadrant Streaks

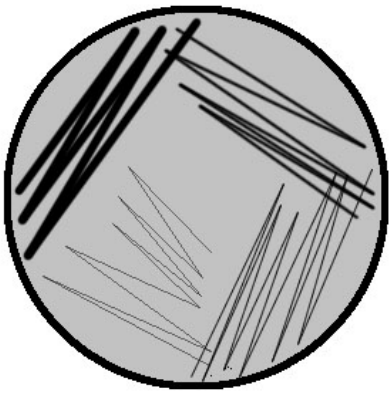
One way to determine if your colonies are real revertants is to streak them out onto a new plate! This exercise also teaches you how to do a **quadrant streak**. This technique allows sequential dilution of the original microbial material (broth culture or colonies on a plate) over the entire surface of a fresh plate. The original sample is diluted by streaking it over successive quadrants, decreasing the number of bacteria. Usually by the third or fourth quadrant only a few bacteria are transferred on the inoculating loop and these produce a few isolated colonies.

1. Obtain your plates that you poured last week. Examine them for contamination before proceeding. If they are contaminated, ask us for new ones!

2. Select a colony that you would like to test and streak it onto a plate using the quadrant streak method. If you do not have any colonies, then ask a neighbor for one. Record where you got it from and what caused the original reversion!

SAFETY LESSON! Loops are sterilized by holding them under a flame until they lightly glow, be careful not to burn yourself.

Quadrant Streaking



a) flame the inoculating loop until it is red hot and then allow it to cool. Avoid killing your bacteria by testing the loop on the inside wall of the agar plate – if it sizzles, then it is TOO hot.

b) remove a small colony with the sterile loop

c) immediately streak the loop very gently **over a quarter** of the plate using a back and forth motion

d) flame the loop again and allow it to cool. Go back over the edge of the **first** streaked area and extend the streaks into a new quadrant.

e) flame the loop again and allow it to cool. Go back over the edge of the **second** streaked area and extend the streaks into a new quadrant.

f) flame the loop again and allow it to cool. Go back over the edge of the **third** streaked area and extend the streaks into the last quadrant.

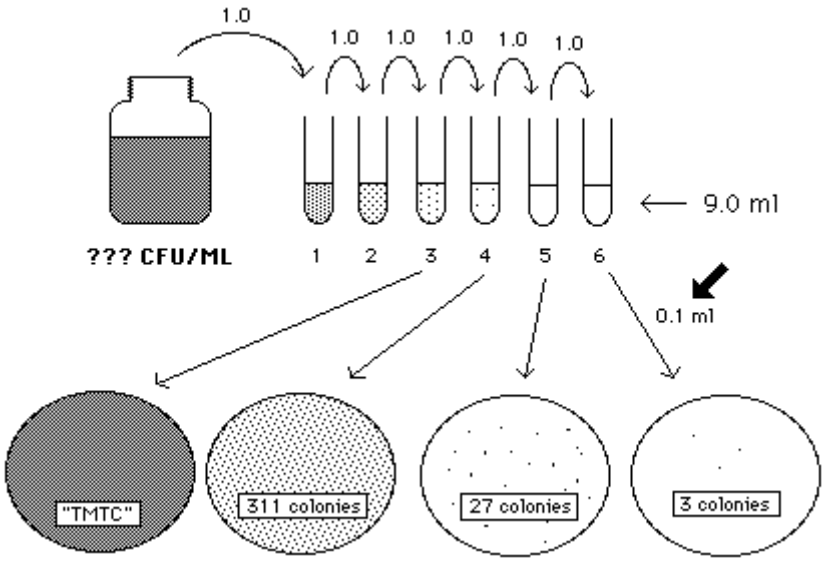
g) sterilize your loop by flaming it again, close the lid, and let the plate sit a few minutes. It should be incubated upside down to avoid condensation.

PART IV – Serial Dilutions

A *serial dilution* is simply a series of simple dilutions which amplifies the dilution factor quickly beginning with a small initial quantity of material (i.e., bacterial culture, a chemical, dye, etc.). The source of dilution material for each step comes from the diluted material of the previous. In a serial dilution the *total dilution factor* at any point is the *product* of the individual dilution factors in each step up to it.

Final dilution factor (DF) = DF1 * DF2 * DF3 (etc. . .)

Example: In the following, a bottle contains an unknown concentration of bacteria ("cfu/ml" means "colony forming units per milliliter", where each cfu equals one LIVE bacterium). One milliliter from the bottle is transferred into a tube containing 9 ml of broth, water, or saline (0.15% NaCl is called "normal saline" or "NS"). Thus the concentration of the bacteria in the tube is exactly 1/10 that in the bottle. If 1 ml of that is transferred into a second tube of 9 ml NS, another 10-fold dilution is accomplished. It is now 1/100 or 10^{-2} the concentration in the bottle. Hint: note that in this case it is $10^{-(\text{tube\#})}$.



So, if you know the dilution factor and want to determine the cfu/ml, then you work backwards by counting the number of colonies on any given plate from a particular dilution, and multiplying it by the dilution factor. The amount you plate each time must be taken into consideration when completing your calculations. The recommended amount is 0.1 ml. More than that would allow the bacteria to grow and move around plate before the liquid soaked into the agar. Less than that will not provide you with enough

liquid to spread around the plate and the colonies would grow into each other. The 0.1 ml recommended amount will both evaporate and soak into the agar within a minute or two to become a "dry" surface that will not allow most bacteria to move away from the growing colonies. Thus every colony that can be counted arose from a single bacterium. So the final calculation would take the plating factor into account:

Flask's **cfu/ml** = (colonies on the plate) x $10^{1+\text{Tube\# (for this example)}}$. Where did the "1" come from? It takes into account the need to multiply by an extra 10 because you added only 0.1 ml onto the plate from the tube! So really it is:

cfu/ml = colonies on the plate x final dilution factor

Thus, if you know the concentration of cells in a solution and want to dilute it to a particular concentration, then you just use serial dilutions.

$$\frac{\textbf{Original concentration of cells/ml}}{\textbf{Dilution Factor}} = \textbf{New concentration of cells/ml}$$

Practice Exercises
Try these calculations BEFORE you come to lab so that you have an idea of what we are doing in the serial dilution activity.

1. You are given a test tube containing 10 mL of a solution with 8.4×10^7 cells/mL. You are to produce a solution that contains less than 100 cells/mL. What dilutions must you perform in order to arrive at the desired result?

2. You have a tube containing 1 mL of a solution with 4.3×10^4 cells/mL and you are to produce a solution that contains 43 cells/mL. What dilutions must you perform?

3. You are given a container with 5 mL of a solution containing 5.1×10^3 cells/mL. You are to produce a solution that contains approximately 100 cells/mL.

4. You are given a container of yeast cells in a concentration of 2.6×10^6 cells/mL. You are to prepare a suspension which, when you spread 0.1 mL of the suspension on appropriate media, will result in about 100 cells. How will you do this?

Serial Dilution Protocols
You will first use food coloring to practice serial dilutions and then do dilutions with a *Salmonella* culture. In molecular biology it is very important to learn how to consistently transfer amounts of anything – cells, enzymes, dyes, etc. . ., so use this as an opportunity to assess your pipetting skills. Today we will use eppendorf tubes for our serial dilutions. Thus, you need to use the appropriate amount of liquid in each. I recommend using a <u>total</u> volume of 1000 µL in any given tube. It is easier to transfer 10 µL or 100 µL accurately than it is to transfer 1 µL or even 0.1 µL!

A. Food Coloring Practice
1. Prepare eppendorf tubes with **water** that will allow you to dilute the food coloring dye sample to a final dilution factor of 10^9.

2. Draw out a schematic of what you did in your laboratory notebook.

B. Plating Serial Dilutions of *Salmonella typhirium*
1. Prepare enough eppendorf tubes with **tryptic soy broth** (TBS) for diluting out a log phase culture of *Salmonella typhirium* so that when you plate three different dilutions, you will get between 10 and 100 cells on one plate, as well as 10 and 100 fold more on the other two plates. Do not forget the plating factor of 100 µL in your calculations. Show your work in your notebook.

2. Complete your dilutions and label your three plates.

3. In order to plate the bacteria you will need to have your pipetteman set to 100 µL and have a beaker of ethanol handy. You will also need a Bunsen burner flame and a glass wand.

SAFETY LESSON! Flaming a wand for plating bacteria is accomplished by dipping it in ethanol and exposing it to a Bunsen burner flame. You can avoid burning yourself and others by following a few rules:

- **DO NOT LET THE ETHANOL RUN DOWN THE WAND TOWARD YOUR HAND**
- **DO NOT PUT THE WAND BACK INTO THE BEAKER BEFORE ALL OF THE ETHANOL HAS BURNED OFF**
- **IF YOU START AN ETHANOL FIRE IN THE BEAKER, THEN COVER IT WITH THE LID PROVIDED**

4. Be sure to mix the eppendorf tubes by flicking them several times prior to adding the 100 µL sample to your plate. Flame the wand, let it cool, and then add the bacteria to the plate. Next, use the wand to spread the bacteria out evenly on the plate by turning the plate. Be careful that you do not gouge the plate!

5. Incubate the plates upside down at 37˚C overnight.

C. A New Plating Technique
Although the technique you just finished is commonly used in the lab, it requires several plates for most experiments that entail the counting bacterial cultures. So, one way to cut down on the numbers of Petri dishes used is to plate the dilutions on one Petri dish as indicated in the diagram on the next page.

Protocol

1. Use your previous serial dilutions to perform this plating technique. Plate the last four dilutions that you made by adding 10 µL drops to one side of the plate and then tilt it so that the drops run most of the way down. Avoid mixing the drops.
2. Incubate the plates upside down at 37˚C overnight.

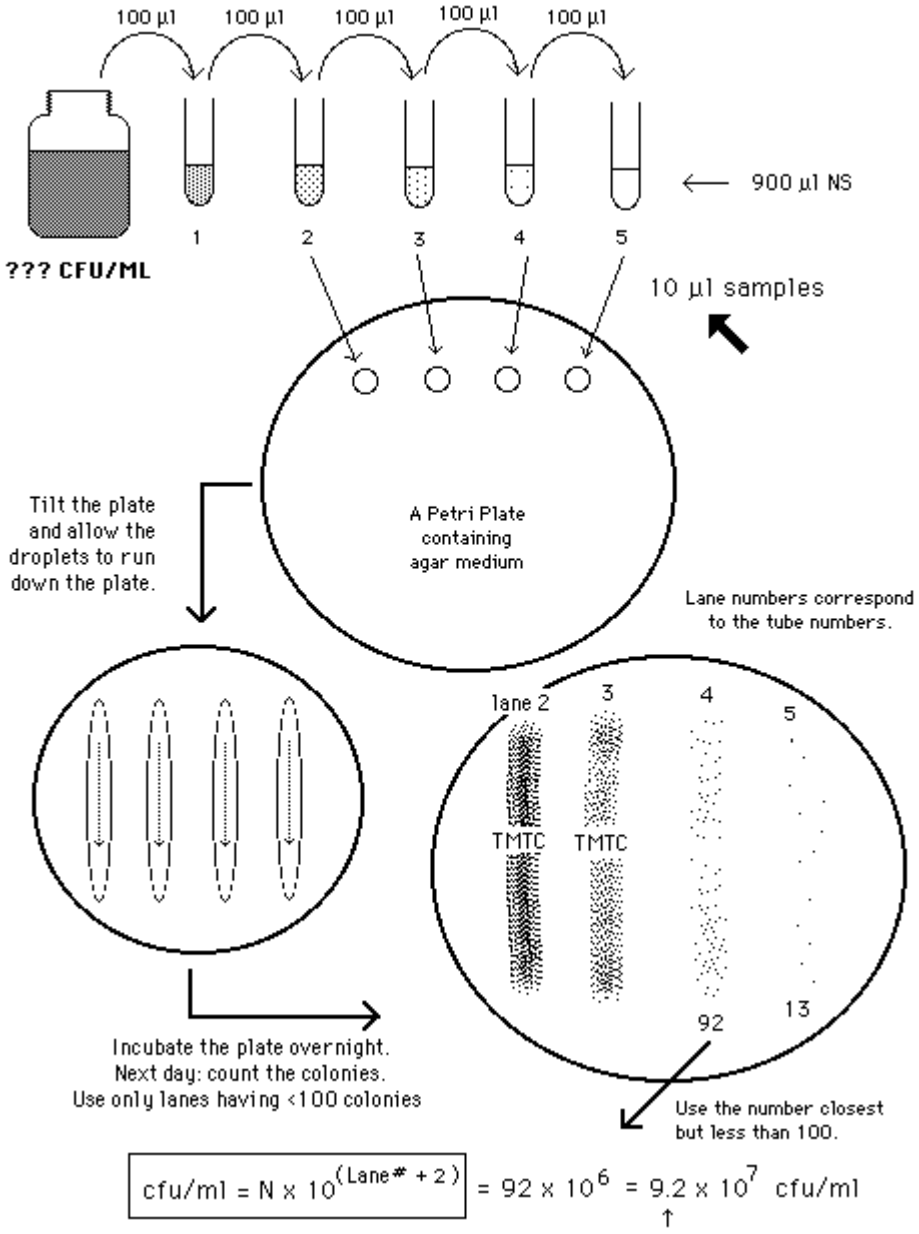


Figure borrowed from Science Projects Co.

NOTE: As you transfer the liquids down through the series of tubes, you MUST use a different pipet or pipet tip for each transfer. However, when transferring liquids from the tubes, you may use the same pipet or tip IF you start transferring from the most dilute tube and work your way upwards.