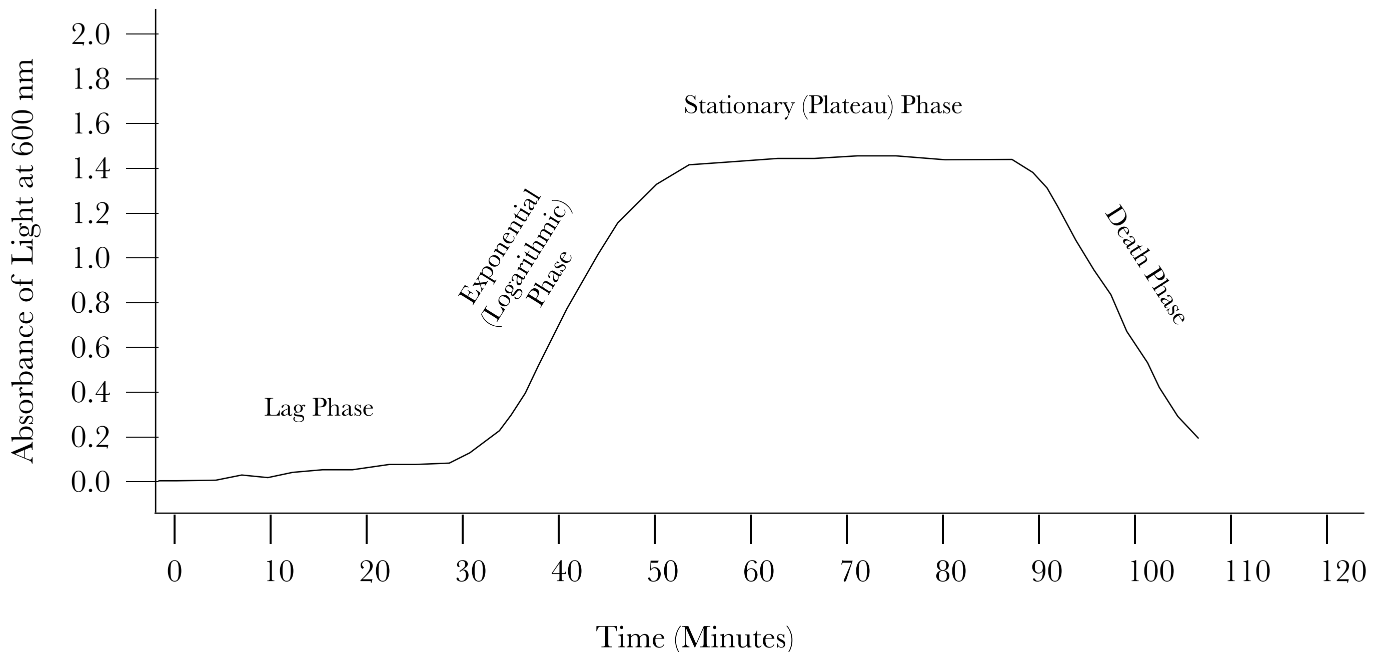
Part 1: Bacterial Growth

**Theory**:

**Growth** of microorganisms can be measured a number of ways. The asexual process of growth that will be measured in this lab is **binary fission**, where the daughter cells will predictably increase in mass till they each reach maturity and undergo binary fission as well. At the cellular level, bacterial growth is an increase in the number and size of the organelles, the cytoplasm volume, and the amount of cell wall and cytoplasmic membranes. **Direct physical measurement** of the volume of cells (by dry weight, or wet weight) can be measured. **Direct chemical measurement** of the contents of the cells (total protein, total DNA content) can also be measured and reflect the amount of growth. **Indirect measurement of chemical activity** of cells (such as the rate a gas is produced or consumed) will also reflect growth but the size of the cell (new daughter cell versus mature cell) can affect the results. **Indirect Turbidity Measurements** use a instrument to measure the amount of light that is scattered as it passes through a tube of nutrient broth and cells, called a **spectrophotometer**. Any particulate in the liquid media will block the light, and this **turbidity** or **optical density** is directly related to to the cell mass or cell number. This method is simple and does not destroy the growing cells, but after about 10 million cells per mL have grown, the sensitivity of this method is not as accurate.

Many bacterial will double their population at regular time intervals (i.e., every 60 minutes) as long as the growth conditions are optimal. This rapid type of growth is not additive (i.e., linear) but is **exponential growth**—a rate of growth really only seen with bacteria and not capable with other microorganisms. This rate of growth can easily be graphed as seen below.



The **Lag Phase** begins immediately after the sterile nutrient broth is inoculated with bacterial cells. However, it make take some time till cell division begins. The transfer to the sterile nutrient broth can physically "shock" the microbes, and new enzymes many need to be synthesized before cell division can begin. This is why the growth curve initially appears flat.

The **Exponential** (**Logarithmic**) **Phase** is a "regular pattern" of binary fission and cell growth such that the population of bacteria is doubled at a constant rate. This is the **generation time**, and is also known as the **doubling time**. During this time the growth conditions are optimal. To estimate the generation time, take two absorbency measurements on the Y-axis (where one is double the other) and where both fall in the exponential phase. Follow them over to the "data line" then down to the time on the X-axis. Subtract the smaller time from the larger time to get the generation time.

The **Stationary** (**Plateau**) **Phase** occurs at there are limitations on the amount of time growth conditions are optimal. Nutrients get used up, metabolic byproducts build up, and space to grow decreases. At this point the number of cells growing and dividing equals the number of cells dying. It is during this phase that secondary metabolites (such as antibiotics) are produced, or spores begin to form.

The **Death Phase** is where the number of viable cells decreases exponentially. Because a "dead" cell and a spore can still block light like the "live" cell, turbidity measurements cannot detect this phase.

**Plate Counts** can also be done (along with the **serial dilution**) technique to estimate the number of bacteria per mL. This simple method involves spreading a small amount of bacteria solution (0.1 mL) onto a Petri dish evenly then observing for the number of **Colony Forming Units** on the plate. Serial dilutions are done so a manageable, yet statistically valuable number can be arrived at on the plate—the ideal number of CFUs is in the range of **30 to 300**.

**Materials**:

| **Amount per group (table of 3 to 6 students)** | **Materials** |
|---|---|
| 1 | Large Test Tube: 5 mL of Nutrient Broth Culture with ***Escherichia coli*** |
| 4 | Large Test Tubes: 2.9 mL Sterile Nutrient Broth in each. |
| 1 | Small Test Tube (empty): *will be used to repeatedly for each spectrophotometer test* |
| 1 | Small Test Tube: 3 mL Sterile Nutrient Broth covered by Parafilm (this is your "**BLANK**") |
| 1 | Flask: 50 mL of Sterile Nutrient Broth (with added "dash" of casamino acids, and 2 mL of 1M glucose solution.) |
| 1 | green Pipette aid |
| 1 | blue Pipette aid |
| 6 | Large Pipettes (10 mL) for Turbidimetric Measurements (1st experiment) |
| 5 | Small Pipettes (1 mL) for Serial Dilution (2nd experiment) |
| 1 | beaker of alcohol with glass hockey stick |
| 4 | Nutrient Agar Plates |
| 1 | Spectrophotometer |
| 1 | test tube rack |

**Procedures**:

*Experiment 1 — Turbidimetric Growth Measurement*:

1. Spectrophotometer should be on (left front knob) for at least 15 minutes to warm up light bulb.
2. Set the wavelength dial (right top knob) to 600 nm.
3. With the **sample compartment empty** and the lid closed, turn the **left front knob** till the arrow is at **0% Transmittance** (look strait at the needle to avoid parallax errors).
4. Wipe the outside of the "**Blank**" test tube of sterile nutrient broth free of any oily fingerprints and place it in the compartment such that the marks on the tube and compartment are lined up. Close the lid. Adjust the **right front dial** till it reads **0 Absorbance** (look strait at the needle to avoid parallax errors). *Note that the absorbance scale on the spectrophotometer is logarithmic while the percent transmittance is linear.*
5. *REPEAT steps 3 and 4 BEFORE EVERY Absorbance measurement with the spectrophotometer.*
6. Obtain an absorbance measurement by adding **3 mL of *E. coli* culture** to the empty small test tube using the **large 10 mL pipette**. Don't worry about adding a test tube top, and insert it into the compartment, close the lid, and observe the number for the absorbance. Record your value. *Don't be too surprised if your first 2 or 3 measurements are very, very similar—this is after all the Lag Phase.*
7. Dump the culture into the sink, rinse the small test tube with tap water, shake it dry. You will use it again for every absorbance measurement.
8. Between samples, you will return your flask to the shaker incubator which helps to aerate the bacteria so that the *E. coli* is well oxygenated for optimal growth. Having only 50 mL of broth to start will keeps the volume low so the surface area when shaken will be higher. Also, the nutrient broth will have a "dash" of **casamino acids** and 2 mL of 1 molar **glucose** solution to optimize the growth rate.
9. Wait 15 minutes between each measurement for a total of 6 or 7 measurements. While we wait for the bacteria to grow, a discussion of math surrounding serial dilutions will be done on the white board.

Absorbance at time 0 minutes:
Absorbance at time 15 minutes:
Absorbance at time 30 minutes:
Absorbance at time 45 minutes:
Absorbance at time 60 minutes:
Absorbance at time 75 minutes:
Absorbance at time 90 minutes:

*Experiment 2 — Serial Dilution*:

| Amount per Student | Material |
|---|---|
| 1 tube | bacterial sample |
| 4 | Nutrient Agar Plates |
| 4 | Sterile Test Tubes with 2.9 mL of Nutrient Broth |
| 4 | 1 mL Pipettes |
| 1 | Pipette Bulb |
| 1 | Beaker of 95% ethanol with glass spreader rod. |

1. As soon as you get your last absorbance measurement, you can begin the serial dilution experiment.
2. Label the 4 large test tubes pre-filled with 2.9 mL of sterile nutrient broth #1, #2, #3, and #4. You don't need to put your group's name on these tubes as they will be discarded at the end of lab.
3. Label the bottom of the 4 nutrient agar Petri dishes with your group's name, the date, and label them #1, #2, #3, and #4.

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4. *Use the smaller 1 mL pipette and pipette aid for the remaining steps. Each step should use aseptic technique.*
5. With a new pipette, transfer 0.1 mL of *E. coli* from the flask to test tube #1. Gently mix.
6. With a new pipette, transfer 0.1 mL of test tube #1 solution to test tube #2. Gently mix.
7. With a new pipette, transfer 0.1 mL of test tube #2 solution to test tube #3. Gently mix.
8. With a new pipette, transfer 0.1 mL of test tube #3 solution to test tube #4. Gently mix.

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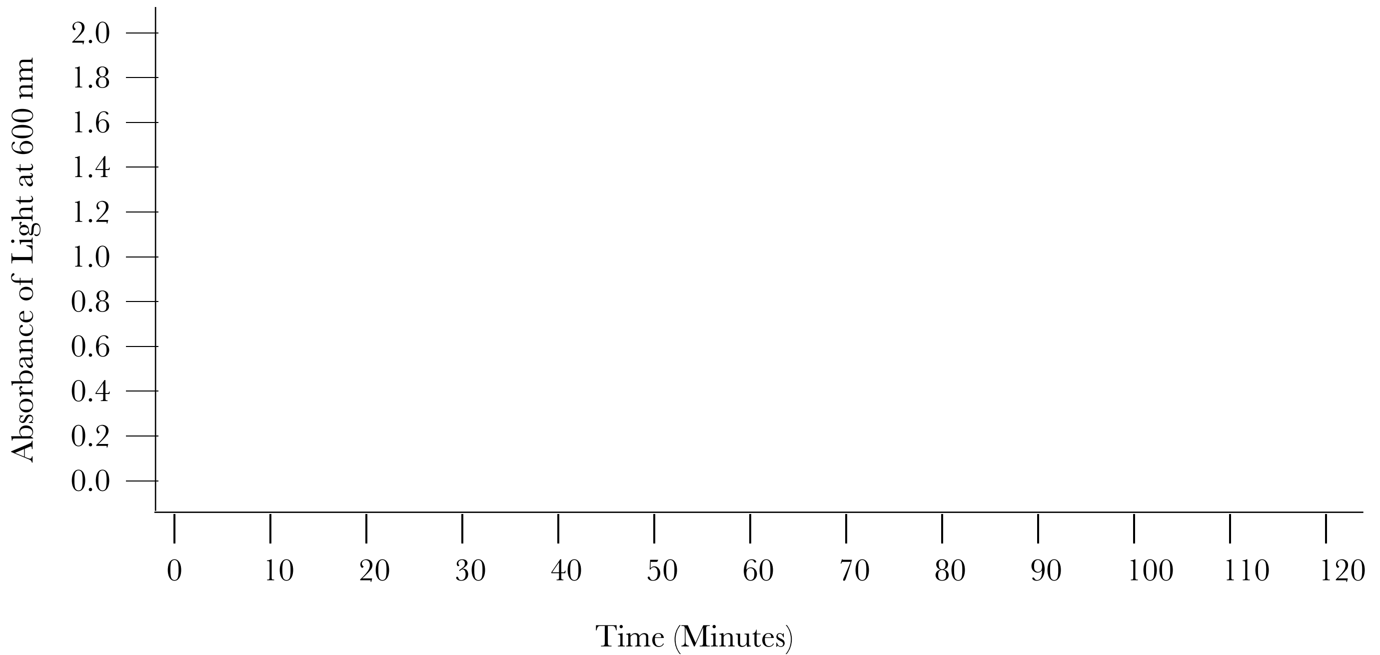
9. With a new pipette (*your last new pipette by the way*), transfer 0.1 mL of test tube #4 solution to Petri dish #4, then with a flame sterilized glass hockey stick, evenly spread the sample over the nutrient agar's surface.
10. With the **same pipette**, transfer 0.1 mL of test tube #3 to Petri dish #3, then with a flame sterilized glass hockey stick, evenly spread the sample over the nutrient agar's surface.
11. With the **same pipette**, transfer 0.1 mL of test tube #2 to Petri dish #2, then with a flame sterilized glass hockey stick, evenly spread the sample over the nutrient agar's surface.
12. With the **same pipette**, transfer 0.1 mL of test tube #1 to Petri dish #1, then with a flame sterilized glass hockey stick, evenly spread the sample over the nutrient agar's surface.
13. Incubate the plates at 37 ºC for 24 to 48 hours. I will move them to the fridge afterwords.
14. At the next lab, count the number of CFUs on each plate, then calculate the cell concentration per mL in the flask you started with at the beginning of experiment #2.
15. Put all the used test tubes and flasks in the discard area.

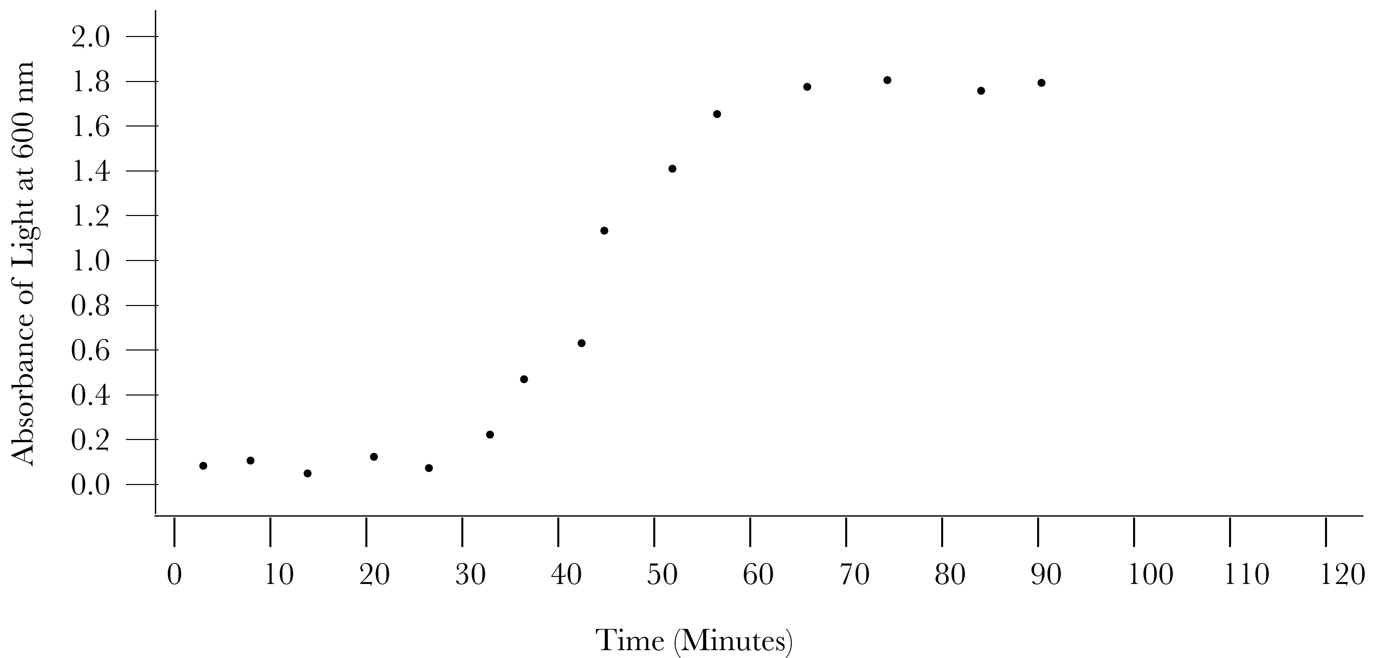
**Lab 8 Questions (Due at the end of lab)**

**Name: ______________________________ Grade: _______ of 10 points**

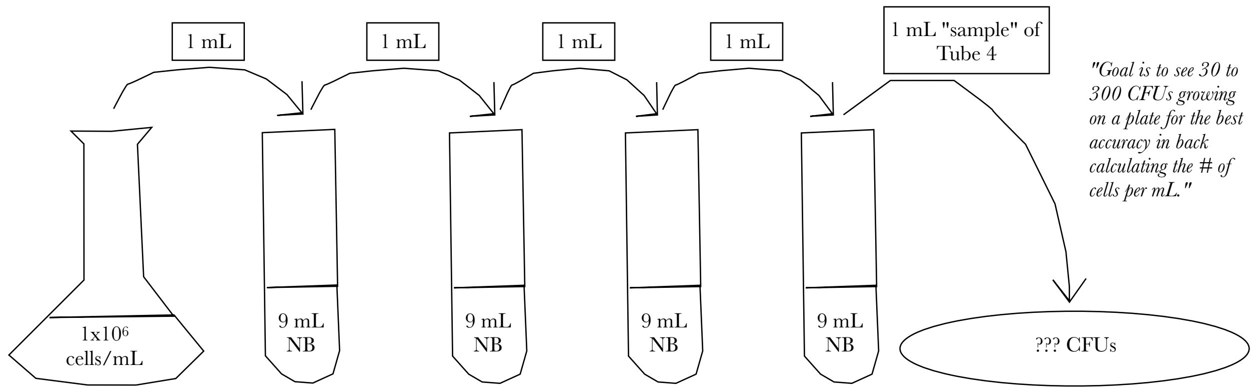
1. (5 points) Transfer your absorbance data to the chart below (use a ruler to improve accuracy). Since this is data that will vary from experiment to experiment you should NOT connect the dots, but rather "*draw a line free-hand through the points*". Calculate your generation time in minutes for *E. coli*. Be sure to show your work (i.e., draw lines to show where you got the times, show your math, include your units, etc…) to get full credit.




2. (5 points) Below is another problem like the one you just did with data points provided. Calculate the generation time for this microbe. Be sure to draw a "free-hand" line and do the same work as you did for problem #1.

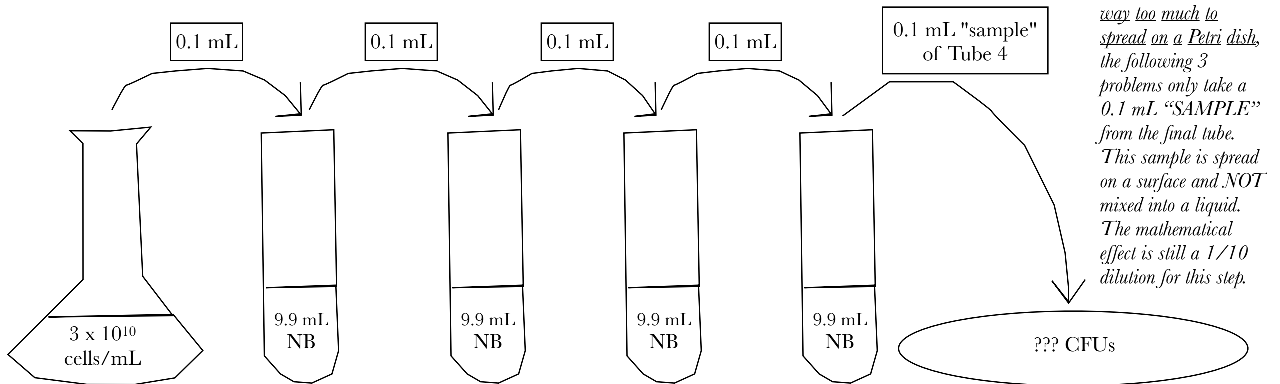


# Serial Dilution Practice Problems:



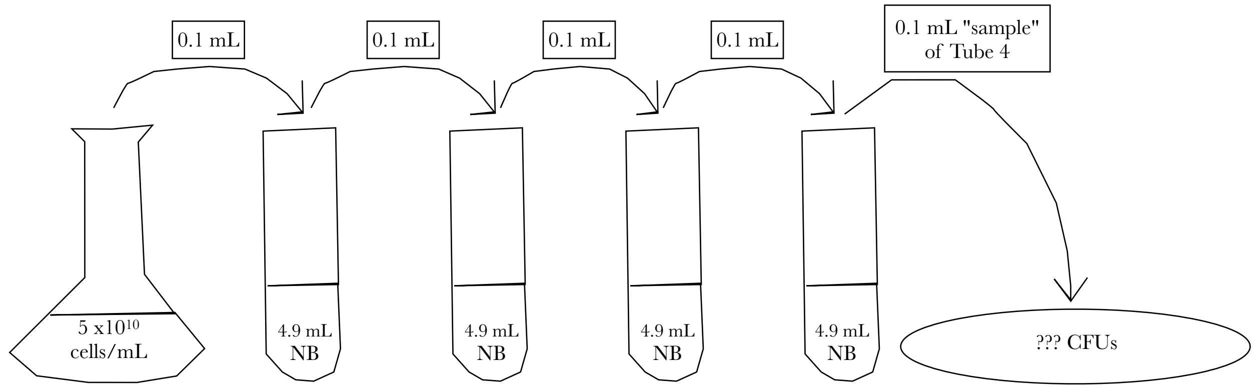

*"Goal is to see 30 to 300 CFUs growing on a plate for the best accuracy in back calculating the # of cells per mL."*

| Stock Concentration in Flask | Flask | Tube 1 | Tube 2 | Tube 3 | Tube 4 | Number of Colony Forming Units (CFUs) grown from Tube 4 |
|---|---|---|---|---|---|---|
| Dilution "per Transfer" | | 1/10 | 1/10 | 1/10 | 1/10 | 1 mL to 1 plate = 1/1 = 1* **note 1 mL has no effect here. 1mL is also TOO much to spread on a plate!!!* |
| Total Dilution | | 1/10 | 1/100 | 1/1,000 | 1/10,000 | 1/10,000 |
| Dilution Factor | | 10 or 1 x$10^1$ | 100 or 1 x$10^2$ | 1,000 or 1 x$10^3$ | 10,000 or 1 x$10^4$ | 10,000 or 1 x$10^4$ |
| # of Cells per mL | 1 x $10^6$ cells / mL or 1,000,000 cells / mL | 1 x $10^5$ cells / mL or 100,000 cells / mL | 1 x $10^4$ cells / mL or 10,000 cells / mL | 1 x $10^3$ cells / mL or 1,000 cells / mL | 1 x $10^2$ cells / mL or 100 cells / mL | 1 x $10^2$ CFUs inoculated on plate (same as 100 CFUs) *GOOD! 100 is between 30 and 300 CFUs!* |

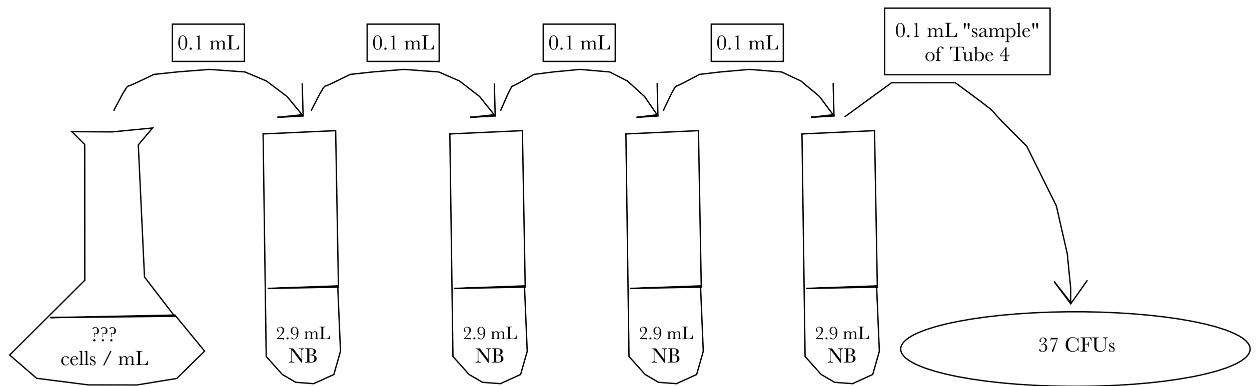



*"Because 1 mL is way too much to spread on a Petri dish, the following 3 problems only take a 0.1 mL "SAMPLE" from the final tube. This sample is spread on a surface and NOT mixed into a liquid. The mathematical effect is still a 1/10 dilution for this step.*

| Stock Concentration in Flask | Flask | Tube 1 | Tube 2 | Tube 3 | Tube 4 | Number of Colony Forming Units (CFUs) grown from Tube 4 |
|---|---|---|---|---|---|---|
| Dilution "per Transfer" | | | | | | |
| Total Dilution | | | | | | |
| Dilution Factor | | | | | | |
| # of Cells per mL | | | | | | |




| Stock Concentration in Flask | Flask | Tube 1 | Tube 2 | Tube 3 | Tube 4 | Number of Colony Forming Units (CFUs) grown from Tube 4 |
|---|---|---|---|---|---|---|
| Dilution "per Transfer" | | | | | | |
| Total Dilution | | | | | | |
| Dilution Factor | | | | | | |
| # of Cells per mL | | | | | | |




| Stock Concentration in Flask | Flask | Tube 1 | Tube 2 | Tube 3 | Tube 4 | Number of Colony Forming Units (CFUs) grown from Tube 4 |
|---|---|---|---|---|---|---|
| Dilution "per Transfer" | | | | | | |
| Total Dilution | | | | | | |
| Dilution Factor | | | | | | |
| # of Cells per mL | | | | | | $3.7 \times 10^1$ cells inoculated on plate = 37 CFUs growing on plate |