Serial Dilutions

All three bacterial plate count methods described in lab require you to serially dilute your samples until you have 30-300 colony forming units (CFU) on the plate. Plates with more than 300 CFU are very difficult to count. Plates with less than 30 CFU are not statistically reliable. If you can see turbidity in a broth culture you have millions more bacteria than you need. If you plated straight from this turbid broth, all the CFU would grow together into a confluent mass. You would not be able to distinguish one colony from another.

Traditionally serial dilutions were done with 1mL pipettes and 99mL sterile dilution bottles because they were the most accurate measuring devices available. With the advent of accurate and reliable micropipettors, smaller volumes can be used (0.1mL in 9.9mL or less of diluent). The important part of both methods is to ensure there is adequate mixing of your dilutions. In this lab we will be using water as the diluent. It is cheap and the organisms we use survive fine in it. Alternatively, phosphate buffered saline may be used. You will do either the large or small volume dilution procedure. Please ask your instructor which dilution procedure you will use.

Procedure:

The tricky part of doing serial dilutions is determining the correct dilution to get 30-300 CFU's per plate. If you start out with a broth culture and do a 10^{-6} dilution, you should be in the right ballpark.

Large Volume Serial Dilutions:

Materials:

- 3 - 99mL sterile water bottles
- 100µL micropipettor with sterile tips
- 3 – 1mL sterile pipettes with blue pipetting aid
- 4 TSA plates (or other plate appropriate for your organism)
- 1 broth culture of organism

Diluting:

1. Label your three bottles 1:100, 1:10,000 and 1:1,000,000.
   - In scientific notation this would be 10^{-2}, 10^{-4}, and 10^{-6}.
2. Using a sterile 1mL pipette, transfer 1mL of your broth culture into the 10^{-2} bottle.
   - Each bottle contains 99mL of sterile water.
3. Tightly cap the bottle, grab it in your hand, rest your elbow on the table and rapidly move your arm with the bottle in an arc, up and down, 25 times.
   - This should adequately disperse the bacteria evenly throughout the bottle and break up bacterial clumps.
   - This first bottle now has a 1:100 dilution of your original broth culture. There are still way too many bacteria in here to count if you were to plate them, so further dilution is necessary.
4. Using a new sterile 1mL pipette, transfer 1mL out of the first bottle (1:100 or 10^{-2}) and add this to your bottle labeled 1:10,000 (10^{-4}). Repeat the bottle shaking procedure.
   - There are probably still too many bacteria in this dilution for you to successfully count, so the dilution process needs to be repeated once more.
5. Using a new sterile 1mL pipette, tranfer 1mL from the 1:10,000 (10^{-4}) bottle and add it to the 1:1,000,000 (10^{-6}) bottle. Repeat the shaking procedure.

Discard all used pipettes and used micropipettor tips in the Vesphene/Cavicide containers

Plating:

Since you do not know which of your dilutions will yield countable results, you will plate from two of the three bottles.

1. Label two plates "0.1mL of 10^{-4}" and "1mL of 10^{-4}".
2. Label the other two plates "0.1mL of 10^{-6}" and "1mL of 10^{-6}".
3. Quickly shake the dilution bottle and aliquot the indicated amount from the appropriate tube onto the center of the plate.
   - You can use either a 1mL pipette or your 100µL micropipetter, depending on the volume you are plating. Disperse it in 2-3 drops around the center of the plate.
4. Working quickly, use a sterile blue L-shaped spreader (looks like a little hockey stick) to spread the inoculum evenly around the plate. This is easier to do with the smaller inoculum volume.
   - Do **NOT** invert the plates until all the liquid has absorbed into the surface of the agar.
5. Incubate the plates for the appropriate time and temperature.
   - This is usually 24 hours at 35-37º Celsius.

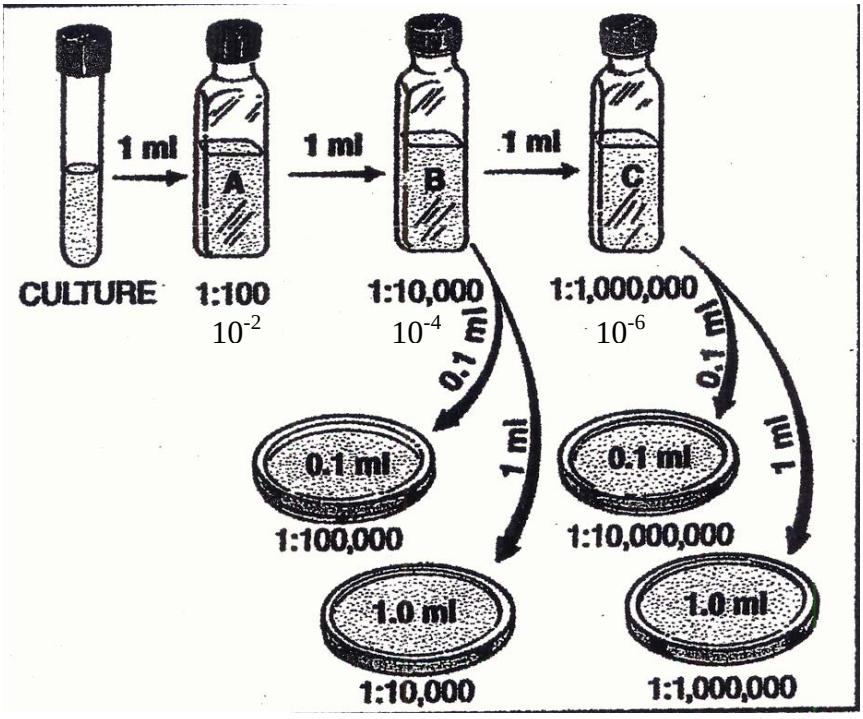

Figure 1: Large Volume Serial Dilution Scheme[i]

<u>Small Volume Serial Dilutions:</u>

Materials:

- 1 - 99mL sterile water bottle
- 3 sterile test tubes
- 100µL micropipettor with sterile tips
- 10mL sterile pipettes with green pipetting aid
- 1 broth culture of organism
- 4 TSA plates or other media that will support the organism

Diluting:

1. Label your three screw-capped tubes 1:100, 1:10,000 and 1: 1,000,000.
   - In scientific notation this would be 10^{-2}, 10^{-4}, and 10^{-6}.
2. Using a sterile 10mL pipette, aliquot 9.9mL of sterile water into each tube.
3. Using a 100µL micropipettor and sterile tip, transfer 0.1mL of your broth culture into the 10^{-2} tube.
4. Cap the tube.
5. Either mix it for a few seconds on a vortex mixer or vigorously flick the tube to adequately disperse the bacteria evenly throughout the tube and break up bacterial clumps. Do NOT shake the test tube!
   - This first tube now has a 1:100 (10^{-2}) dilution of your original broth culture. There are still way too many bacteria in here to count if you were to plate them, so further dilution is necessary.
6. Using a new sterile tip, transfer 0.1mL from the first tube (1:100 or 10^{-2}) and add it to your tube labeled 1:10,000 or 10^{-4}.
7. Repeat the tube mixing procedure.
   - This second tube now has a 1:10,000 (10^{-4}) dilution of your original broth culture. There are probably still too many bacteria in this dilution for you to successfully count, so the dilution process needs to be repeated once more.
8. Using a new sterile tip, transfer 0.1mL from the second tube (1:10,000 or 10^{-4}) and add it to your tube labeled 1:1,000,000 or 10^{-6}.
9. Repeat the tube mixing procedure.

Discard all used pipettes and used micropipettor tips in the Vesphene/Cavicide containers

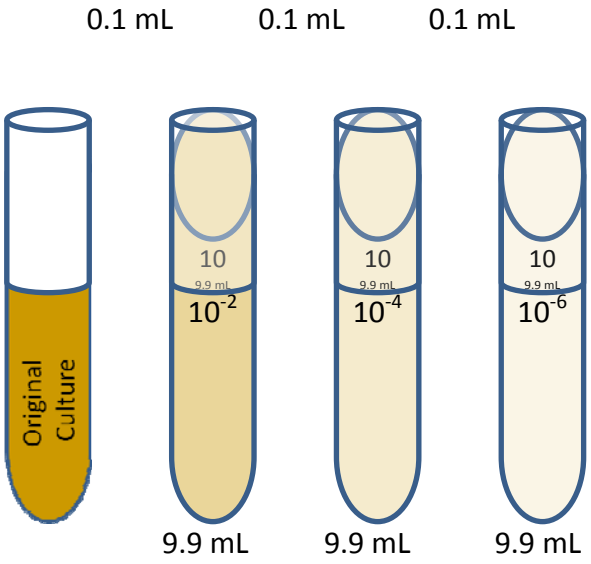

Figure 2: Small Volume Serial Dilution Scheme

Plating:
Since you do not know which of your dilutions will yield countable results, you will plate from two of the three tubes.

1. Label two plates "0.1mL of 10^{-4}" and "1mL of 10^{-4}".
2. Label the other two plates "0.1mL of 10^{-6}" and "1mL of 10^{-6}".
3. Quickly mix or flick the dilution tube and aliquot the indicated amount from the appropriate tube onto the center of the plate.
   - You can use either a 1mL pipette or your 100µL micropipetter, depending on the volume you are plating. Disperse it in 2-3 drops around the center of the plate.
4. Working quickly, use a sterile blue L-shaped spreader (looks like a little hockey stick) to spread the inoculum evenly around the plate. This is easier to do with the smaller inoculum volume.
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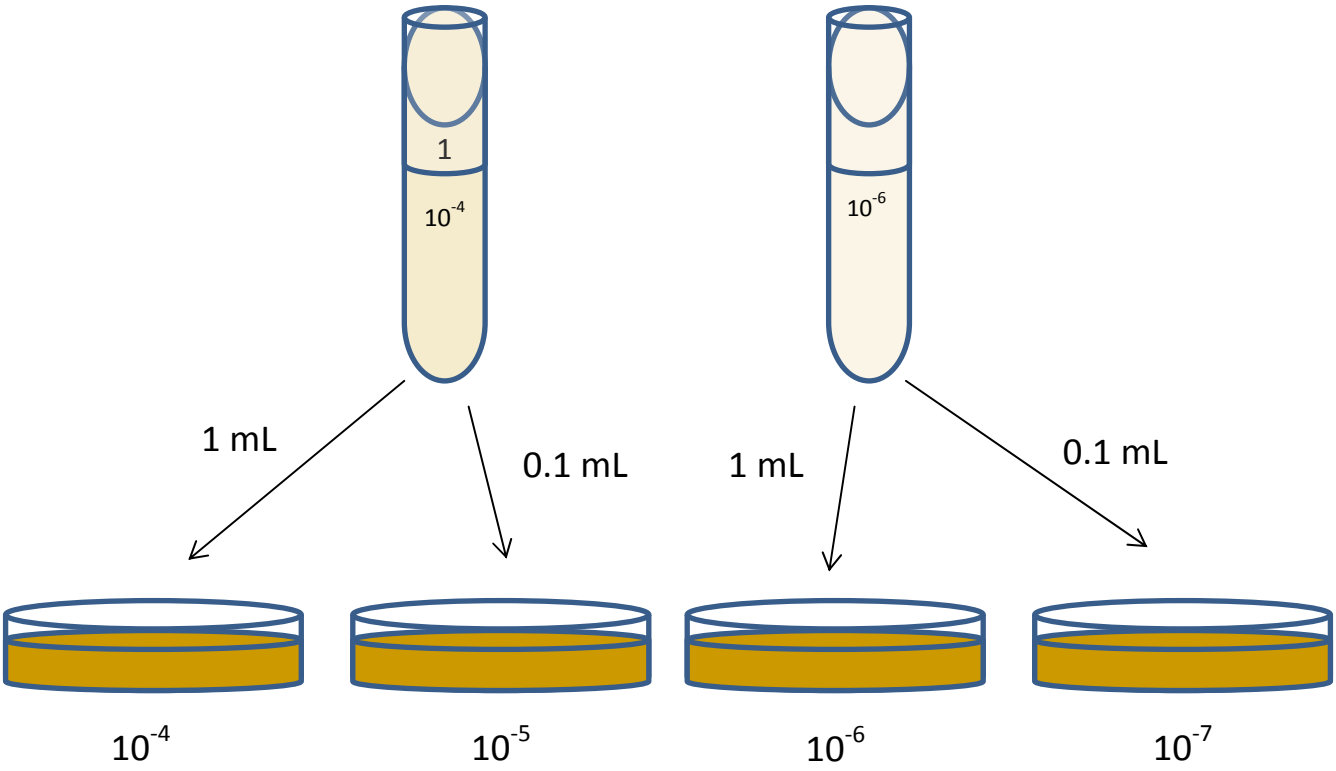

Celsius.

Figure 3: Small Volume Serial Dilution Scheme

Counting:
Pick the plate which has between 30-300 CFU. Count every colony on the plate. Clinical and research laboratories have expensive automated counters to accomplish this. We do it the low tech way! It is easy to loose track of which colonies you have counted. There are two ways to keep track of this.

- Flip your plate over, bottom side up, and put a felt tip pen dot on the back of each colony. This keeps you from counting the same colony more the once, or recounting the same colonies after an interruption.
- Alternately you can place your plate on the gridded and lighted surface of a Quebec colony counter. You can use the grid to keep track of which areas you have counted. There are hand held tally counters available.

Calculations:
You can now calculate the original number of CFU per mL of solution.

$$\frac{\#\ \text{CFU}}{\text{Amount Plated}} \quad \text{X} \quad \text{Dilution factor} \quad = \quad \text{CFU/ml}$$

Your answer should be expressed in scientific notation with 2 significant figures.

For example: say you counted 150 CFU on the 1:10,000 plate with an inoculum of 0.1mL.

$$\frac{150\ \text{CFU}}{0.1\text{mL}} \quad \text{X} \quad 10{,}000 \quad = \quad 15{,}000{,}000 \text{ or } 1.5 \times 10^{7}$$

Compare the values you obtained with the rest of the class.

How would your calculations change if you were using the 4.9mL diluent volume instead if the 9.9mL volume?

[i] Benson, H. J. *Microbiological Applications* 8th Edition. New York: McGraw Hill, 2002. Fig. 21-1. Pg. 87. *Bacterial Population Counts*. ICBN# 0-07-231889-9.