

Laboratory Seven: Enumeration of Bacteria

Bacterial enumeration is the measurement of the number of bacterial cells per milliliter, gram, or cubic meter of a sample (the units depends on the nature of the sample). There are a number of methods that can be used to determine the number of cells/unit and these methods can be divided up into different categories.

Viable counts involve counting cells that can be cultured and/or are metabolically active. **Total counts** involve counting all cells including dead or inactive cells. **Direct methods** of enumeration involve counting actual cells or colonies and **indirect methods** involve estimating the number of cells based on cell mass, scattering of light through a culture (spectroscopy), or a statistical method called the MPN (most probable number) technique.

The table below gives examples of different methods.

Table 1. Examples of Different Bacterial Enumeration Methods

Category	Method of Enumeration	Description
Indirect and viable	MPN	Make statistical estimates numbers of cells by their patterns of growth in liquid culture media.
Direct and viable	Standard plate count	Dilute a sample in saline, spread on solid media, and count colonies. Calculate the number of cells in original sample from counts and dilutions.
Indirect and total	Spectroscopy	Measure the amount of light that passes through a liquid culture using a spectrophotometer and estimate the number of cells/ml based on amount of light that passes through culture.
Direct and total	Fuorescent staining and microscopy	Stain the cells with fluorescent dyes, which make them visible in raw samples (i.e. soil). Count the number of cells using a fluorescent microscope.

In this lab we will be using two methods to enumerate the number of cells per ml of a liquid culture, the spectroscopy and the standard plate count methods.

Standard Plate Count Method: A Viable and Direct Count

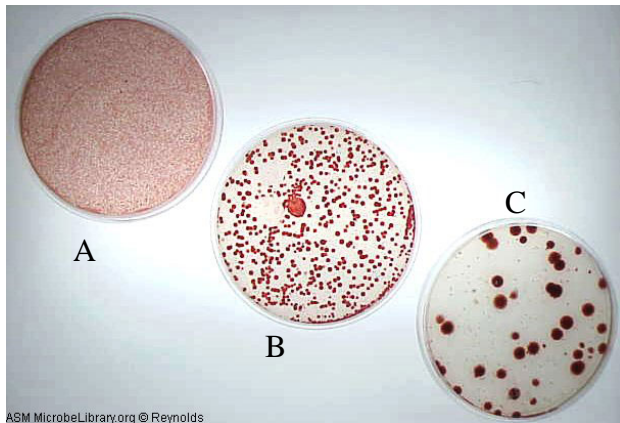
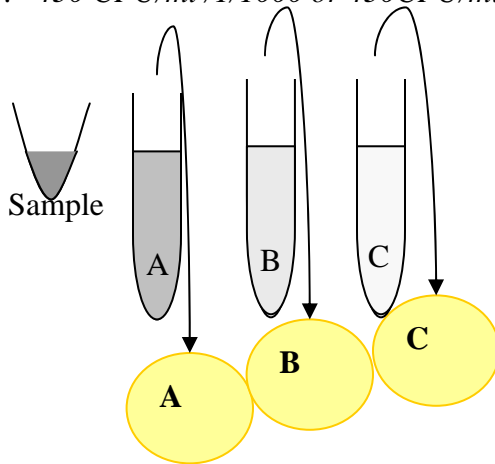
A standard plate count method is used to determine the number of viable bacterial cells per unit volume of a sample using agar plate media. For example, if a biologist was interested in determining the number of viable bacterial cells per milliliter of pond water he or she would transfer a fixed volume of pond water to a plate, spread the solution across the plate and count the colonies that form after incubation. The colonies are referred to as colony forming units (CFU). Once he/she determines the number of CFUs on the plate he/she can divide by the volume plated to determine the concentration of cells in the sample. If a sample contains over one thousand cells per unit volume then it will produce too many CFUs to count accurately on the plate. These samples should first be diluted in sterile media before transferring to plate media so that a countable number of colonies appear. Since the actual concentration of the sample is unknown it is common practice to dilute the sample serially (for example 1/10, 1/100, 1/1000, etc.) then spread-plate the multiple serial dilutions. The highest dilutions will produce the lowest number of CFUs and the lowest dilutions will produce the highest number of CFUs. The plate with the countable number of colonies should be selected to count. When using standard size Petri dishes, a countable plate would be one with between 30 and 300 CFUs. Dilutions with fewer than 30 colonies are easy to count, but often produce inaccurate results since one or two contaminating colonies can cause a significant overestimate of the cell count. After the colonies are counted the concentration of cells in the plated dilution can be determined by dividing by the amount plated. Once the concentration of cells at

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the specific dilution is determined the concentration in the original sample can be calculated by dividing by the total dilution. See the example provided below.

Observe the figure below. A sample was serially diluted as follows:

1. One milliliter from the sample was transferred to 9 ml of sterile media in tube A and mixed. This was a 1/10 serial dilution.
2. One milliliter from tube A was transferred to 9 ml of sterile media in tube B and mixed. This was another 1/10 serial dilution. The total dilution up to this point is 1/100.
3. Then 1 ml from tube B was transferred to 9 ml of sterile media in tube C and mixed. This is a 1/10 serial dilution. The total dilution up to this point is 1/1000.
4. 0.1 ml from each tube was plated. Plates A and B contained too many colonies to count. Plate C is countable. If plate C contained 43 colonies we can determine the concentration in the sample.
 - a. $43 \text{ CFU}/0.1 \text{ ml} = 430 \text{ CFU/ml}$
 - b. $430 \text{ CFU/ml} / 1/1000$ or $430 \text{ CFU/ml} / 1 \times 10^{-3} = 4.30 \times 10^5 \text{ CFU/ml}$



Spectroscopy Enumeration Method: Optical Density

In indirect method of enumerating the cell concentration in a bacterial culture involves using a spectrophotometer. Bacterial cells absorb light well at the wavelength of 686 nm when grown in standard media. A spectrophotometer can be used to measure the amount of light at a wavelength of 686 nm that is transmitted through a bacterial culture. The more bacteria in the culture, the more light absorbed by the culture and the less light transmitted through the culture. The spectrophotometer will measure the percentage of light transmitted through the culture and this number can be converted to optical density (OD). OD is a quantitative way of describing the turbidity of a culture. OD is inversely proportional to the percent transmittance ($OD = 2 - \log \%T$). As the turbidity of a culture increases the cell concentration increases and the OD increases (the percent transmittance decreases). In order to relate OD to an actual cell concentration a standard curve has to be

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set up. A culture is serially diluted and the OD is calculated for each dilution using a spectrophotometer. Next a viable count is made for the original culture that was diluted. Calculations are made to determine the viable count of cells for each serial dilution. A graph is used to plot the OD of each dilution on the X axis and the log viable cell concentration on the Y axis.

In this laboratory exercise you are going to do a standard plate count to determine the viable cell concentration of a culture. The you will dilute that same culture and determine the OD of each serial dilution. Finally you will create a standard curve for that culture.

Learning Objectives

After completing this laboratory activity you will be able to

- Enumerate the viable cell concentration of a sample using the standard plate count method.
- Estimate the total cell concentration of a sample using a spectrophotometer.
- Create a standard curve to estimate cell concentrations at various optical densities.

Procedure:

Part A (dilutions)

1. Obtain 4 tubes of sterile media (each contains 9ml), 1 bottle of sterile media, 1 tube of culture, 1 p-1000 pipettor, and blue box pipette tips. See figure 1 for assistance.
2. Label the tubes with the following: B, C, D, and E
3. Label the culture SC.
4. Label the sterile media bottle A.
5. Aseptically transfer 1.0 ml of your stock culture (sc) into the 9.0 ml of media in Bottle A.
6. Pump the pipette up and down to rinse out the inside of the pipette into your new media. Cap the bottle and shake.
7. Use a new pipette to aseptically transfer 1.0 ml from bottle A into the 9.0 ml of media in tube B. Pump the pipette up and down before flaming and recapping.
8. Vortex tube B and use a new pipette to aseptically transfer 1.0 ml from tube B into the 9.0 ml of media in tube C. Pump, flame and cap.
9. Vortex tube C and use a new pipette to aseptically transfer 1.0 ml from bottle C into the 9.0 ml of media in tube D. Pump, flame and cap.
10. Vortex tube D and use a new pipette to aseptically transfer 1.0 ml from tube D into the 9.0 ml of media in tube E. Pump, flame and cap.

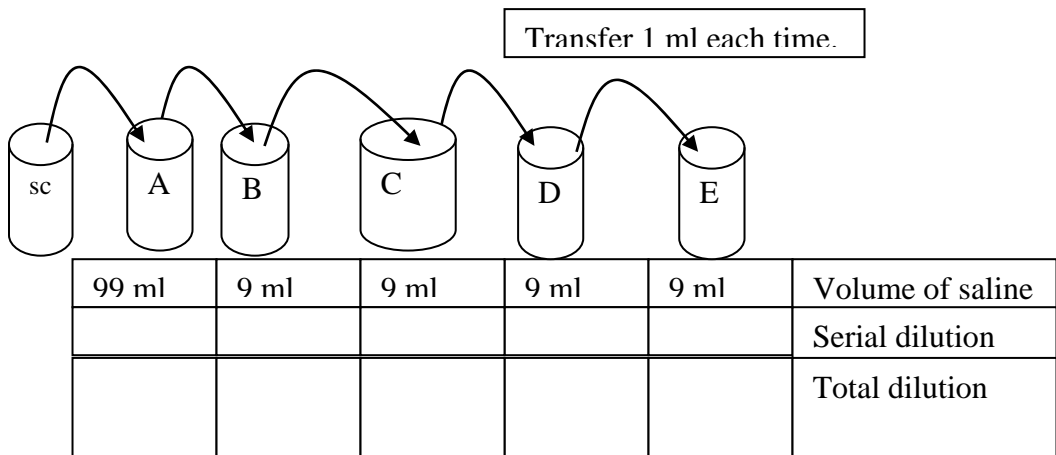


Figure 1. Dilution Series

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Part B (Spread plate)

1. Obtain 12 plates of media, 1 p-100 pipettor, 1 yellow box tips, 1 glass spreader, some ethanol, and 1 glass Petri dish. See figure 2 for assistance.
2. Transfer some ethanol to the glass Petri dish.
3. Label 3 plates B, 3 plates C, 3 plates D and 3 plates E. Also label with your group name, date and any other pertinent information.
4. Aseptically pipette 0.1 ml of culture from tube B to a properly labeled TSA plate.
5. Dip a glass spreader into a glass petri dish of ethanol then pass the spreader briefly through the burner flame. Allow the flame to burn out.
6. Use the flamed spreader to uniformly spread the transferred liquid over the entire plate. Turn the plate as you spread to help ensure uniformity. Return the spreader to the beaker.
7. Repeat steps 1-3 two more times so that there are 3 replicates for tube B.
8. Repeat steps 1-3 in triplicate for tubes/bottles C, D and E.
9. Make sure your plates are labeled properly (Group #, dilution factor or tube letter, and date). Incubate at 37 degrees C for 24 to 48 hours.

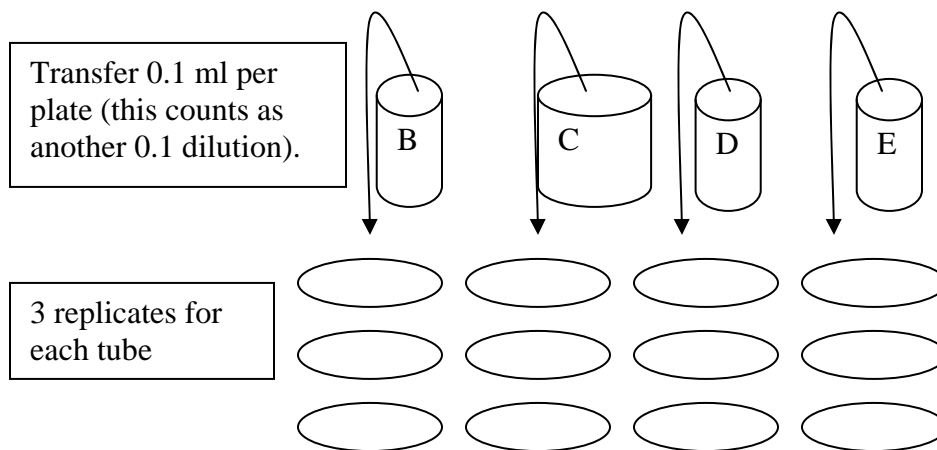


Figure 2. Spread plating

Part C (Spectroscopy measurements)

1. Turn on the Spec 20.
2. Obtain 6 cuvettes and label them **BL**, **SC**, **0.50**, **0.25**, **0.13**, **0.06**.
3. Obtain 2 2ml pipettes and a blue pipettor.
4. Use a fresh 2 ml pipette to transfer 4 ml of sterile media (from media stock bottle labeled "part C") into **BL cuvette**. This will be your blank.
5. Transfer 8 ml of stock culture (the same stock culture you used in Part A) to **SC cuvette**.
6. Put 4 ml each of sterile media in the other 4 cuvettes.
7. Now transfer 4 ml from the **SC** to cuvette **0.50** and mix gently by pipetting the liquid up and down into the tube. Be careful not to stick the entire pipette into the tube or it will overflow and get everywhere. This is a $\frac{1}{2}$ dilution (0.50).
8. Next transfer 4 ml from **0.5** to **0.25** and mix gently.
9. Next transfer 4 ml from **0.25** to **0.13** and mix gently.
10. Finally, transfer 4 ml from **0.13** to **0.06** and mix gently.
11. Go to the Spec 20. Make sure it has warmed up for 15 minutes. Use the wavelength knob to adjust the wavelength to 686 nm.

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12. Now use the zero knob to set the transmittance to zero (called “zeroing the spec”).
13. Wipe cuvette **BL** with a kimwipe. Insert your **BL** tube and turn the blanking knob to 100% transmittance . This tube has no bacteria in it so theoretically it should read 100% T.
14. Wipe cuvette **SC** with a kimwipe. Measure the % transmittance. Record data in Table 3.
15. Next measure the percent transmittance for the 4 dilutions and record. You don’t need to blank again if the spec 20 continues to be zeroed out as you measure. If you see that it is not then you need to zero again.
16. Record your data in table 3.
17. Now calculate the OD for each sample using the following formula:
$$OD = 2 - \text{Log} (\%T)$$
18. You previously calculated the viable cell count for the SC so record this in table 3 in the appropriate space. Next multiply this number by the various dilutions you performed here (i.e. 0.5, 0.25, etc) to determine the viable cell count for the other tubes you measured. Record your data in table 3.
19. Finally convert the viable cell counts to log number of cells and plot the values on the graph provided. Put the OD on the X axis and the log cell number on the Y axis. This is a standard curve that can be used to estimate the cell concentration of a FRESH culture of the same species, growing in the same media using optical density.

Part D (viable Count calculations)

1. Count the number of colonies on each plate. Plates that have over 300 colonies are “too numerous to count” (TNTC). Don’t bother counting them; record those at TNTC. Record your colony counts for each plate in Table 1. Each colony is a “colony forming unit” or CFU and represents what was once a single cell inoculated on the plate.
2. Pick a set of plates to do your calculations (set B, C, D or E). You want to choose the set where each plate in the set has more than 30 CFUs and less than 300. If you do your calculations with a set that has fewer than 30 colonies or greater than 300, your counts will be statistically invalid or inaccurate.
3. Calculate the average number of colonies per plate for the set that you chose and put this in table 2. Also fill in the serial and total dilutions for each tube/bottle in Table 2.
4. Now calculate the number of cells/ml that occurred in the tube that you plated. Divide the average CFUs counted by the number of milliliters you plated (0.1ml). For instance if you counted plate C and the average CFUs were 61 then tube C would have 617 cells/ml (CFUs become equivalent to cells in the tube).
$$61 \text{ cells} / 0.1 \text{ ml} = 617 \text{ cells/ml}$$
5. Now calculate the number of cells/ml that occurred in the sc. Divide the concentration of cells you found in the tube you plated (above) by the dilution factor for that tube. Using the example above for tube C:

$$617 \text{ cells/ml} / [1 \times 10^{-5}] = 6.17 \times 10^7 \text{ cells/ml in SC}$$

6. Once you found this for the SC you can estimate the number of viable cells/ml for each dilution level by simply multiplying the SC concentration by the total dilution for each tube. Record these viable counts for all tubes in table 2.

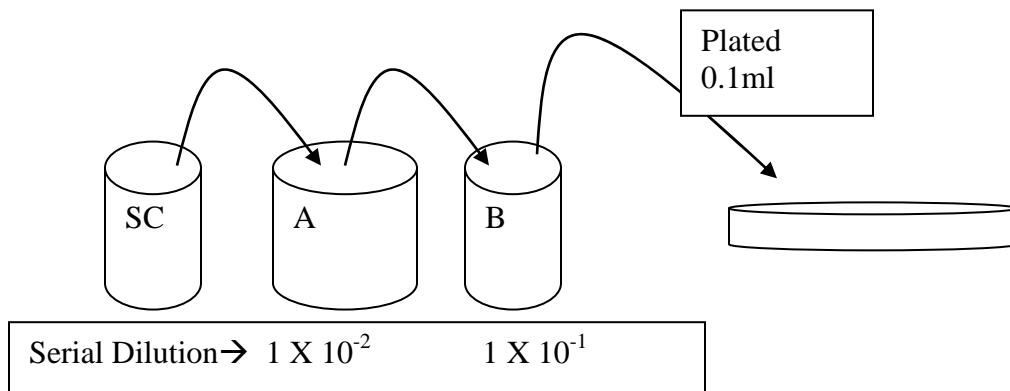
Questions to ponder

1. Why can a standard curve be used only to estimate cell concentration of a fresh culture of the same species grown in the same media? For instance why can’t it be used for other species, or same species grown in other media? Why can’t it be used on older cultures?
2. How does the concentration for sc using the viable direct count compare to the concentration for sc using the total indirect count method? Why do these numbers differ?

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3. If the stock culture you were given was old, would you expect the heterotrophic plate count to be higher or lower? Why?
4. Why bother doing 3 replicates?
5. Why would it be inappropriate to use a plate that had only 10 colonies on it to calculate the number of cells in the sc?
6. Why do you need to blank the spectrophotometer with culture media and not water?
7. Which method would you use (heterotrophic plate count or spectrophotometer) if you had enumerate 200 different samples? Why?
8. Which method would you use (streak plate or spread plate method) if you needed isolated colonies from a soil sample? Why?

Use these sample questions below to make sure you can do the dilution calculations for the practical.



1. What is the dilution factor for tube B?
2. What is the dilution factor for the plate?
3. If you counted 250 cells on the plate, how many cells per ml would be in tubes B, A, and SC?