

## Bacterial Enumeration

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.

Category	Method of Enumeration	Description
<b>Indirect and viable</b>	MPN	Make statistical estimates numbers of cells by their patterns of growth in liquid culture media.
<b>Direct and viable</b>	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.
<b>Indirect and total</b>	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.
<b>Direct and total</b>	DAPI or AODC 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 heterotrophic plate count methods.

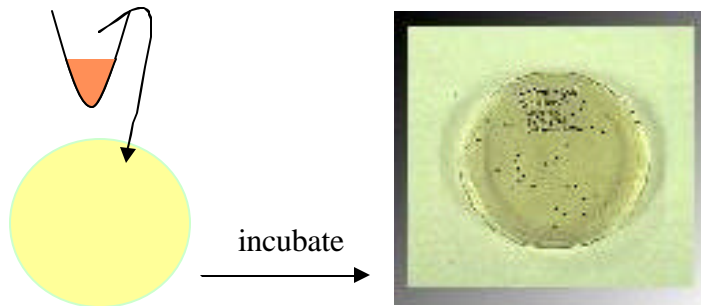
### Standard Plate Count Method: A Viable and Direct Count

When healthcare providers (HP) treat infectious disease sometimes it is important for them to know the number of infectious organisms in the tissue or bodily fluid before they prescribe a treatment regime. Since the HP is only interested in determining the number of live pathogens he/she would most likely choose a **viable** method of enumeration.

A popular method used by HPs to enumerate cells is a called the standard plate count (SPC) method. The SPC method is a *direct count* method used to determine the number of viable, heterotrophic cells per volume (or per milliliter) of fluid. It involves diluting down a sample (if the sample contains > 3,000 cells/ml) and evenly spreading a small amount across an agar plate. Each cell that ends up on the plate will form a colony overnight. The colonies can then be easily counted and a few calculations can help the HP determine the number of cells/ml of sample.

### How to do the Calculations

The calculations are quite simple. For example, let's say the HP took a blood sample from a septicemic patient (a patient who is infected with a microorganism that is actively growing in his/her blood). The HP transferred 0.1 ml of blood to an agar plate, spread the blood evenly across the plate and let the plate incubate for 24 hours. The next day the HP counted 35 colonies on the plate. How many infectious cells exist per ml of blood?

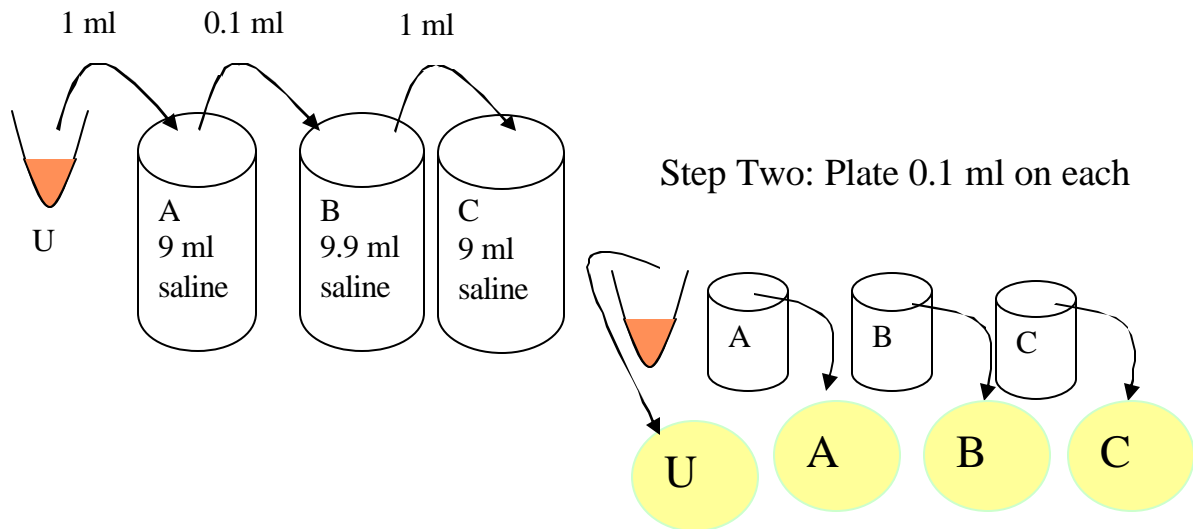


$35 \text{ colonies} \div 0.1 \text{ ml of blood} = 350 \text{ cells/ml of blood.}$

*Note: Keep in mind that each colony represents a single cell that existed in the sample and grew into a colony overnight so we can use colony and cell interchangeably in this example.*

Here is another example that is a little more complicated. A patient came in with a urinary tract infection. The HP had no idea how many cells were in the urine, but she guessed that there were too many to plate the urine directly. She figured she would have to perform a dilution series and plate diluted samples to get countable number on the agar plate. The HP transferred 1ml of urine (tube U below) to 9ml of sterile saline and mixed the solution (tube A below). Next, the HP transferred 0.1 ml of from tube A to 9.9 ml of sterile saline in tube B mixed the contents. She then transferred 1 ml from B to 9 ml of saline in C. Next, she transferred 0.1ml from the urine tube to an agar plate and spread the fluid evenly across the plate. She did the same from tubes A, B and C. She let the plates incubate for 24 hours. The next day the HP counted the plates and saw that she couldn't count the number of colonies on plates U and A because their were so many on the plate they were piled on top of one another. She recorded these as TNTC. She counted 50 colonies on plate B and 3 colonies on plate C.

## Step One: Dilute



### Working through the Calculations

- The first step in this process is to choose which plate to count.
  - U** and **A** cannot be counted because the HP would have to make inaccurate estimations since the colonies are growing on top of one another. We score these plates as too numerous to count (TNTC).
  - C** is not counted because it has less than 30 colonies, which would give inaccurate results.
  - B** can be easily counted and there are enough colonies to give statistically accurate results (i.e. the count is between 30 and 300 colonies).
    - So we choose B
- The second step is to determine the concentration of cells in the tube from which we prepared the plate.
  - Plate B has 50 colonies so how many cells/ml is there in tube B (the tube we plated from)?
    - $50 \div 0.1 \text{ ml} = 500 \text{ cells/ml}$  is the **concentration of cells** in Tube B
- The next step is to determine the **serial dilutions** (SD) in each tube.
  - $SD = AT \div TV$ ; where AT is the amount transferred and TV is the total volume in that tube after transfer.
    - Lets do tube A first
      - TV for tube A is  $1 \text{ ml} + 9 \text{ ml} = 10 \text{ ml}$
      - AT is 1ml
      - So  $SD = 1 \text{ ml} \div 10 \text{ ml} = 0.1$
    - Now B
      - $TV = 0.1 \text{ ml} + 9.9 \text{ ml} = 10 \text{ ml}$
      - AT = 0.1 ml
      - So  $SD = 0.1 \text{ ml} \div 10 \text{ ml} = 0.01$
    - SD for C
      - $TV = 1 \text{ ml} + 9 \text{ ml} = 10 \text{ ml}$

2.  $AT = 1 \text{ ml}$
3.  $SD = 1 \text{ ml} \div 10 \text{ ml} = 0.1$
4. Now we must determine the **total dilution** (TD) at the tube from which we transferred to the plate that we counted. We counted B so we want to know the TD at tube B.
  - a. To get the TD at B all we need to do is multiply all the SDs that have occurred up to that point. In our case, we had 2 SDs before we plated from tube B; the SD at A and the SD at B.
    1.  $TD = SD_A \times SD_B = 0.1 \times 0.01 = 0.001$
5. The final step is to divide the concentration of cells in the tube we plated (Tube B) by the total dilution at that tube and this will tell us the concentration of cells in the original sample or the number of cells/ml of urine.
  - a.  $500 \text{ cells/ml} \div 0.001 = 500,000 \text{ cells/ml urine.}$

## Spectroscopy Method

The Spectroscopy method of enumeration is useful to get a quick and dirty **total** count of the number of cells in a culture. It is less accurate than the plate count method because it is an indirect method of counting cells, but it is useful to get a fast count of cells in a liquid culture.

The more cells in a culture the more turbid it becomes, correct? That is precisely correct and that is how the spectroscopy method works. As the **turbidity** (or cloudiness) of a culture increases the number of cells per milliliter increases in the culture. The **optical density (OD)** is a mathematical term used to describe the turbidity of a culture. As the OD of a culture increases it gets more turbid. The Spec 20 is a device that can quantify the OD of a culture. It does this indirectly, however. The Spec 20 measures the amount of light that can pass through a culture; this is called the **percent transmittance (%T)**. The more cells in the culture the less light can pass through. This is because the cells will cause the light to scatter. Now think about it, the greater the optical density (or turbidity) the less light can pass through the culture; therefore, the lower the %T.

The higher the OD the lower the %T (indirectly proportional)

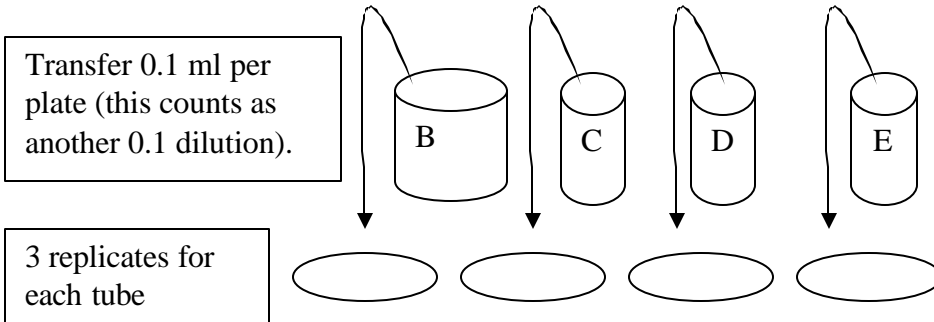
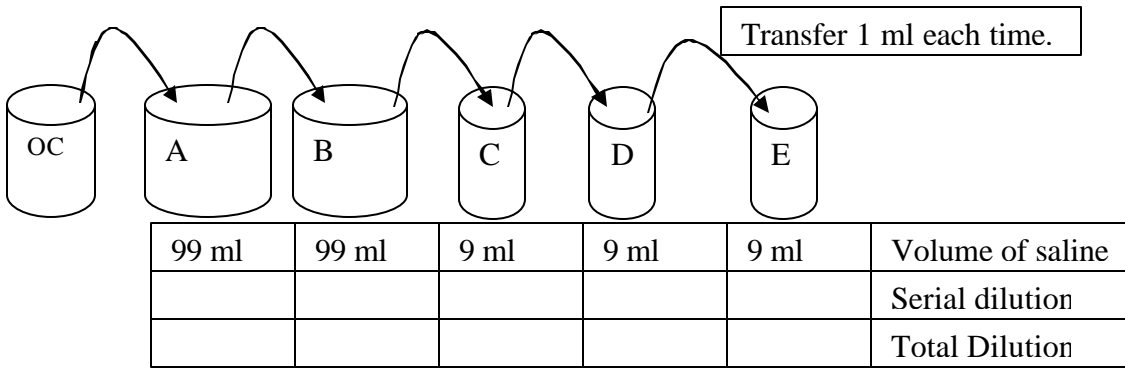
The higher the OD the higher the turbidity (directly proportional)

Remember this equation so you can convert OD to %T:  $OD = 2 - \log \%T$   
OD can be converted to cells per ml by a simple conversion.

## Procedure

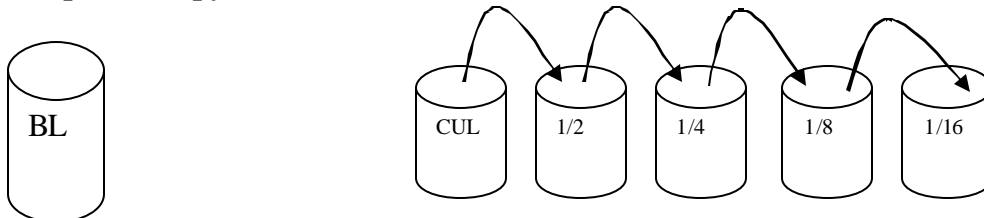
### Part A (Standard plate count method)

1. Observe Dr. Furlong do the dilutions of a culture on day 1.
2. She will dilute the culture and plate them using the following scheme shown on the next page. Determine the serial dilutions and Total dilutions at each tube.



- Next she will spread plate 0.1 ml of each tube (B, C, D and E) 3X and incubate the plates overnight.
- Why did she do it 3 X? \_\_\_\_\_
- The next day you come to lab you will count the plate that have between 30 and 300 colonies and determine the number of cells/ml in the OC (Original Culture).
- Record your results here \_\_\_\_\_.

**Part B (Spectroscopy measurements)**



- Obtain 6 cuvettes and label them BL, CUL,  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$ ,  $\frac{1}{16}$ .
- Obtain 2 2ml pipettes and a blue pipettor.
- Get a fresh 2 ml pipette. Put 4 ml of sterile media (from media stock bottle) into BL. This will be your blank.
- Put 2ml each of sterile media in  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$ , and  $\frac{1}{16}$  cuvettes.
- Transfer 4 ml of culture to CUL.
- Now transfer 2 ml from CUL to  $\frac{1}{2}$  and mix gently by pipetting the liquid up and down into the tube.
- Next transfer 2 ml from  $\frac{1}{2}$  to  $\frac{1}{4}$  and mix gently.
- Next transfer 2 ml from  $\frac{1}{4}$  to  $\frac{1}{8}$  and mix gently.
- Finally, transfer 2 ml from  $\frac{1}{8}$  to  $\frac{1}{16}$  and mix gently.
- Take your cuvettes to the Spec 20.
- Adjust transmittance to 0.

12. Use a Kimwipe to wipe the dust and fingerprints from the tube. Add BL to spec reader and close lid. Adjust transmittance to 100.
13. Remove BL.
14. Wipe each tube (CUL, 1/2, 1/4, 1/8, and 1/16) and put into spec reader and record percent transmittance in Table 1.
15. Calculate the O.D.  
O.D. =  $2 - \log$  of percent transmittance
16. Calculate the number of cells/ml in your culture  
Culture concentration = O.D.  $\times 1.25 \times 10^6$  cells/ml

Table 1. Comparison of Viable to Total counts

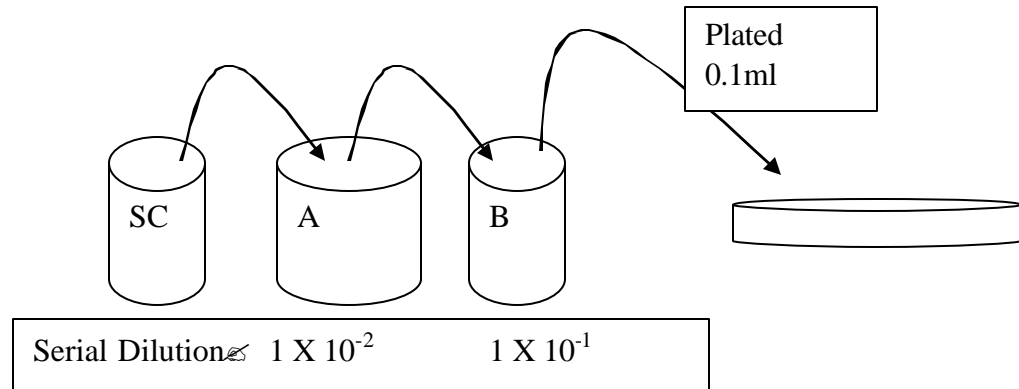
Cuvette	Percent transmittance	OD	Culture concentration
CUL			
1/2			
1/4			
1/8			
1/16			

1. What happened to the percent transmittance as the culture became more dilute? Did it increase or decrease?

## Questions to ponder

1. 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?
2. If the stock culture you were given was old, would you expect the heterotrophic plate count to be higher or lower? Why?
3. Why bother doing 3 replicates?
4. 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?
5. Which method would you use (heterotrophic plate count or spectrophotometer) if you had enumerate 200 different samples? Why?
6. 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.

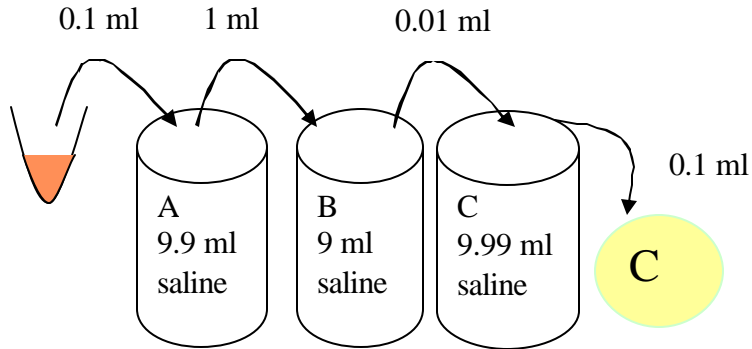


1. What is the dilution factor for tube B?
2. What is the dilution factor for the plate?
3. If you counted 250 cells on the plate, how many cells per ml would be in tubes B, A, and SC?

**Now Try this one!**

A lab technician took a fecal sample and did the following dilution.

1. Transferred 0.1 ml from fecal sample to 9.9 ml saline in A
2. Transferred 1 ml from A to 9 ml saline in B
3. Transferred 0.01 ml from B to 9.99 ml saline in C.
4. Transferred 0.1ml from C to an agar plate
5. Counted 65 colonies on plate C



Questions:

1. What is the serial dilution at A?
2. What is the serial dilution at B?
3. What is the serial dilution at C?
4. What is the total dilution at C?
5. What is the concentration of cells in tube C?
6. What is the concentration of cells in the fecal sample?