Lab 6+7: Bacterial Counting

In the study of bacteriology, there are numerous occasions when it is Necessary to either estimate or determine the number of bacterial cells in a broth culture or liquid medium. Determination of cell numbers can be accomplished by a number of direct or indirect methods. The methods include **standard plate** counts, **turbid metric** measurements, and visual comparison of turbidity with a known standard, **direct microscopic counts**, **cell mass determination**, and **measurement of cellular activity**. In this lab, you will compare three methods of bacterial enumeration: the **standard plate** count, **turbidimetric** measurement and **direct microscopic counts.**

1. Standard Plate Count (Viable Counts)

A **viable** cell is defined as a cell which is able to divide and form a Population (or colony). A viable cell count is usually done by diluting the Original sample, plating aliquots of the dilutions onto an appropriate culture medium, and then incubating the plates under proper conditions so that colonies are formed. After incubation, the colonies are counted and, from knowledge of the dilution used, the original number of viable cells can be calculated. For accurate determination of the total number of viable cells, it is critical that each colony comes from only one cell, so chains and clumps of cells must be broken apart. However, since one is never sure that all such groups have been broken apart, the total number of viable cells is usually reported as **colony-forming units** (CFUs) rather than cell numbers. This method of enumeration is relatively easy to perform and is much more sensitive than turbidimetric measurement. A major disadvantage, however, is the time necessary for dilutions, plating's and incubations, as well as the time needed for media preparation.

A. Spread Plate Technique

Material:

1. Seven 9-ml dilution tubes of sterile saline

2. Seven nutrient agar plates

3. 1.0 ml and 0.1 ml pipets

4. Glass spreader aka .hockey sticks.

5. 95% ethyl alcohol in glass beaker (**WARNING**: Keep alcohol away from flame!!)

6. Mixed overnight broth culture of *Staphylococcus aureus* and *Serratia Marcescens*

Procedure

1. Prepare serial dilutions of the broth culture as shown in the figure from a previous lab exercise (Isolation of Pure Cultures). Be sure to mix the nutrient broth tubes before each serial transfer. Transfer **0.1** ml of the final three dilutions (10-5, 10-6, 10-7) to duplicate nutrient agar plates, and label the plates.

2. Spread the 0.1 ml inoculum evenly over the entire surface of one of the
A nutrient agar plate until the medium no longer appears moist. Return the
Spreader to the alcohol.

3. Repeat the flaming and spreading for each of the remaining five plates.

4. Invert the six plates and incubate at room temperature until the next lab
Period (or ~ 48 hours, whichever is the shortest). At the end of incubation period, select the petri plate containing between 25-250 colonies plate with more than 250 colonies cannot be counted and designed **t**oo **n**umerous **t**o **c**ount (TNTC) . Plates with fewer than 25 colonies are designed **t**o **f**ew **t**o **c**ount (TFTC)

so, colonies fewer than 25 are not acceptable for statistical reasons and colonies more than 250 on plate are likely to produce colonies too close to each other to distinguishes as distinct Colony forming unit CFUs so, the assumption is that each viable bacterial cell is separate from all other and will develop in to a single discrete colony .

5. Calculate the number of bacteria CFU per millilitre of sample as follow

Suppose the plate of 10^{-6} diluent yield count of 130 colonies, then the number of bacteria in 1ml of original sample can be calculated as follow:

- **dividing the number of colonies by dilution factor, so**
- Bacteria \ ml= $130 \div 10^6 = 130,000,000$

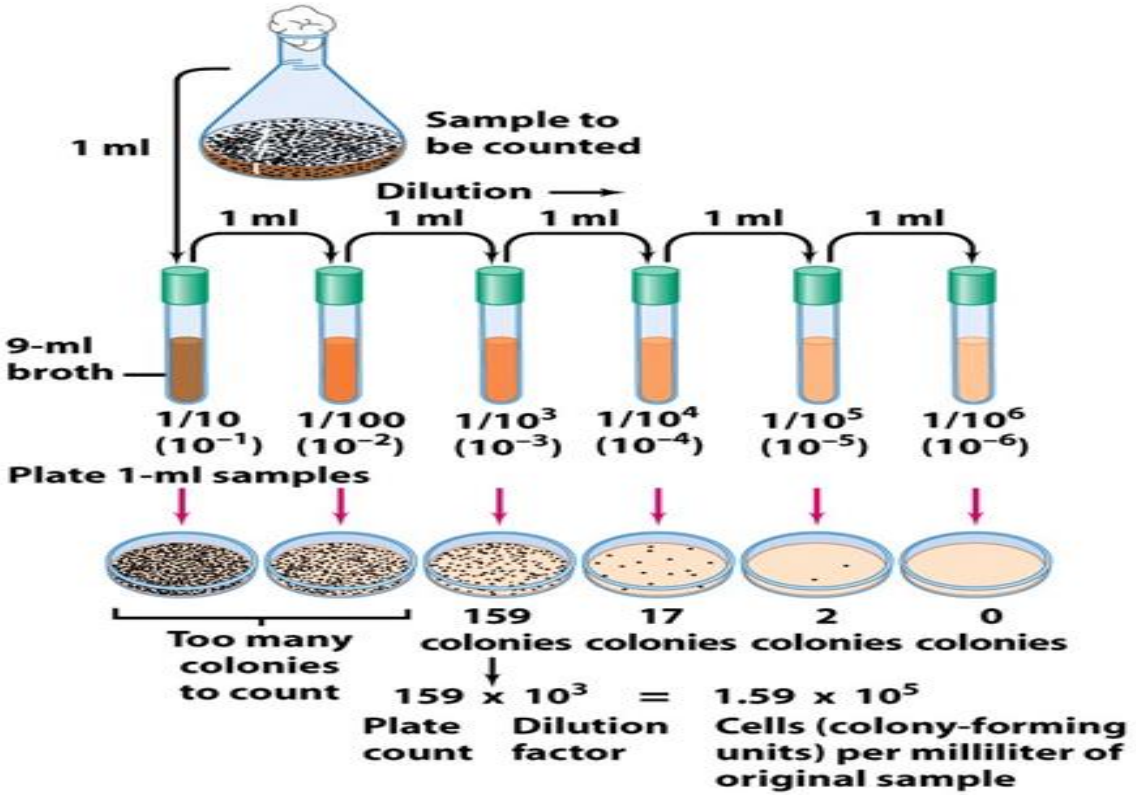

Figure 1: Spread Plate Technique

- This method used for counting the **number of bacterial (cell \ml) accurately**,
- **if** we want to count the **number of bacterial** (cell \ml) **in accurately ,** we used **Mac Far Lands tubes procedure:**

❖ Mac far lands far lands tubes procedure:

This procedure consist of using 10 tubes containing different amount of mixture of 1% $BaCL_2$ and 1% H_2SO_4

- The first tubes contain 9 ml of H_2SO_4 + 1ml of $BaCL_2$ that give high concentration

We take the sample bacterial suspension and compared with **Mac far lands** land tubes to detect the concentration of bacterial sample depended on opacity unties we reach to the same opacity in **Mac far lands** land tubes and bacterial sample, then we say that the concentration of bacteria like tubes for example number 7 so we detect the number of bacteria incorrectly.

B. Streak Plate Technique

1. Observe plates. Did you obtain isolated colonies on the agar plates which were streaked with *Serratia marcescens*? Which streaking technique do you prefer? If you did not obtain isolated colonies, what changes should you make in your technique to ensure isolated colonies?

C. Exposure Plates

1. Observe plates. Describe the morphology, size and colour of representative colonies.

1. Count the number of colonies on each plate and record.

DILUTION	Red Colonies	White Colonies	Total Number
10^6			
10^7			
10^8			

2. Turbid metric Measurement

A quick and efficient method of estimating the number of bacteria in a Liquid medium is to measure the turbidity or cloudiness of a culture and translate this measurement into cell numbers. This method of enumeration is fast and is usually preferred when a large number of cultures are to be counted. 34 Although measuring turbidity is much faster than the standard plate count, the measurements must be correlated initially with cell number. This is achieved by determining the turbidity of different concentrations of a

given species of microorganism in a particular medium and then utilizing the standard plate count to determine the number of viable organisms per millilitre of sample.

A standard curve can then be drawn (e.g., this lab protocol section), in which a specific turbidity or optical density reading is matched to a specific number of viable organisms. Subsequently, only turbidity needs to be measured. The number of viable organisms may be read directly from the standard curve, without necessitating time-consuming standard counts.

Turbidity can be measured by an instrument such as a colorimeter or Spectrophotometer. These instruments contain a light source and a light detector (photocell) separated by the sample compartment. Turbid solutions such as cell cultures interfere with light passage through the sample, so that less light hits the photocell than would if the cells were not there. Turbidimetric methods can be used as long as each individual cell blocks or intercepts light; as soon as the mass of cells becomes so large that some cells effectively shield other cells from the light, the measurement is no longer accurate. Before turbidimetric measurements can be made, the spectrophotometer must be adjusted to 100% transmittance (0% absorbance). This is done using a sample of uninoculated medium. Present transmittance of various dilutions of the

Bacterial culture is then measured and the values converted to optical density, based on the formula:

Absorbance (O.D.) = 2 - log % Transmittance.

A Wavelength of 420 nm is used when the solution is clear, 540 nm when the solution is light yellow and 600-625 nm is used for yellow to brown solutions.

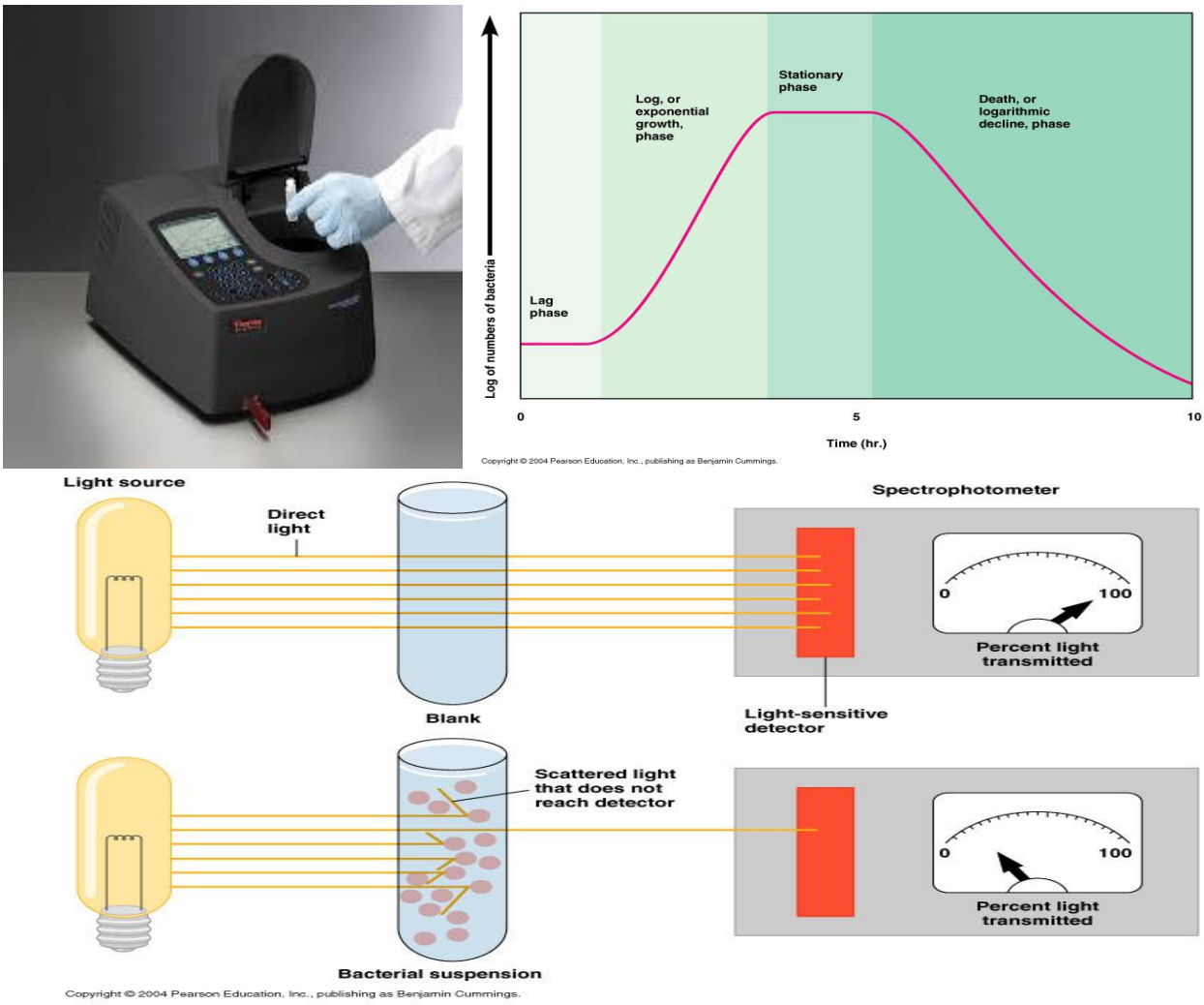

Figure 2: Turbid metric Measurement of bacterial cell

3. Direct Microscopic Count

Petroff-Hausser counting chambers can be used as a direct method to Determine the number of bacterial cells in a culture or liquid medium. In this procedure, the number of cells in a given volume of culture liquid is counted directly in 10-20 microscope fields. The average number of cells per field is calculated and the number of bacterial cells ml-1 of original sample can then be computed. A major advantage of direct counts is the speed at which results are obtained. However, since it is often not possible to distinguish living from dead 35 cells, the direct microscopic count method is not very useful for determining the number of viable cells in a culture.

Material:

1. Petroff-Hausser counting chamber

2. Cover slips

3. Sterile diluent (nutrient broth or sterile saline)

4. Pasteur pipets

Procedure: (work in pairs)

- ✓ **Note: Be extremely careful handling Petroff-Hausser counting chambers!**

1. Clean P-H counting chamber with 70% alcohol and let air dry.

2. Mix culture well and apply a single drop to counting chamber with Pasteur pipet. Examine the counting chamber using high power, oil immersion objective.

3. Make a preliminary estimation of the concentration of cells from the Overnight culture of *Serratia marcescens* using the following formula:

Total cells counted x 2.0x107 x dilution factor = cells/ml small squares counted

Therefore, if you counted an average of 15 cells per Small Square, then you would have a final concentration of 3.0 x 108 cells/ml.

4. You may have to adjust downward using one of your initial serial dilutions so that the counts per Small Square are in the 5 to 15 cell range.

5. Once this is done, make sure to allow time for cells to settle and move Focus through the suspension (i.e., up and down) so as to count all cells within the small square "box". Most cells will have attached to the bottom and/or top glass interface. You can also check the depth, which is 20 µm. The small square should also be 50 by 50 µm.

6. Count the number of bacterial cells in at least 10 small squares. Variability should be less than +/- 10%.

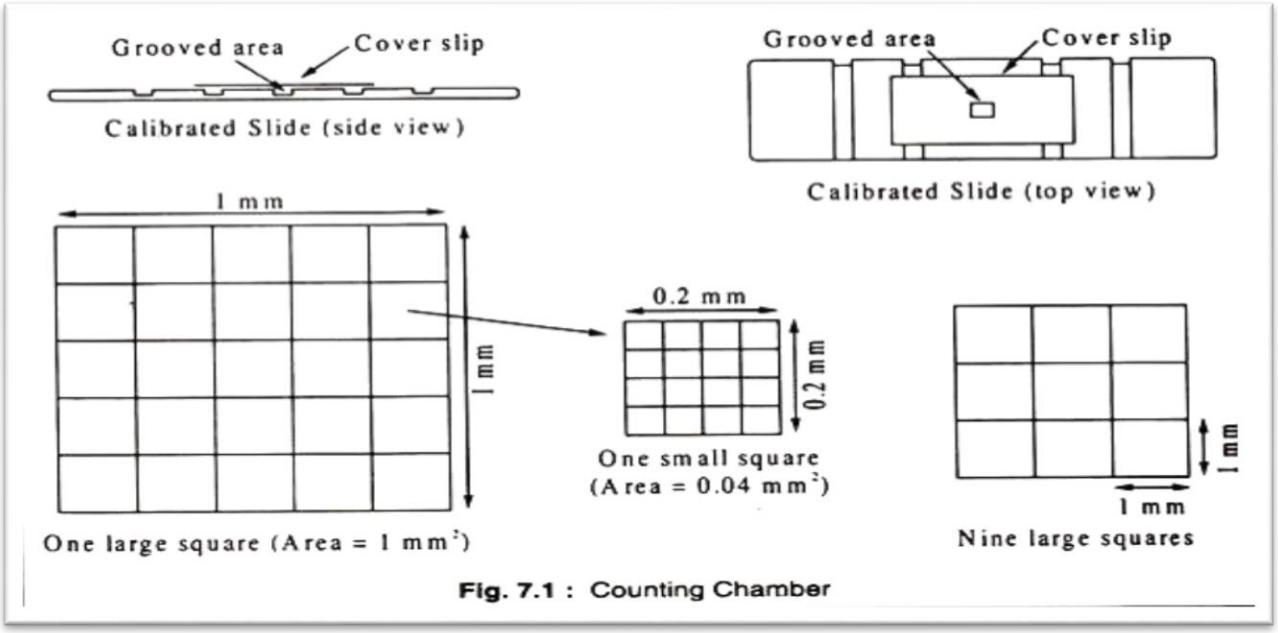

Fig. 7.1 : Counting Chamber

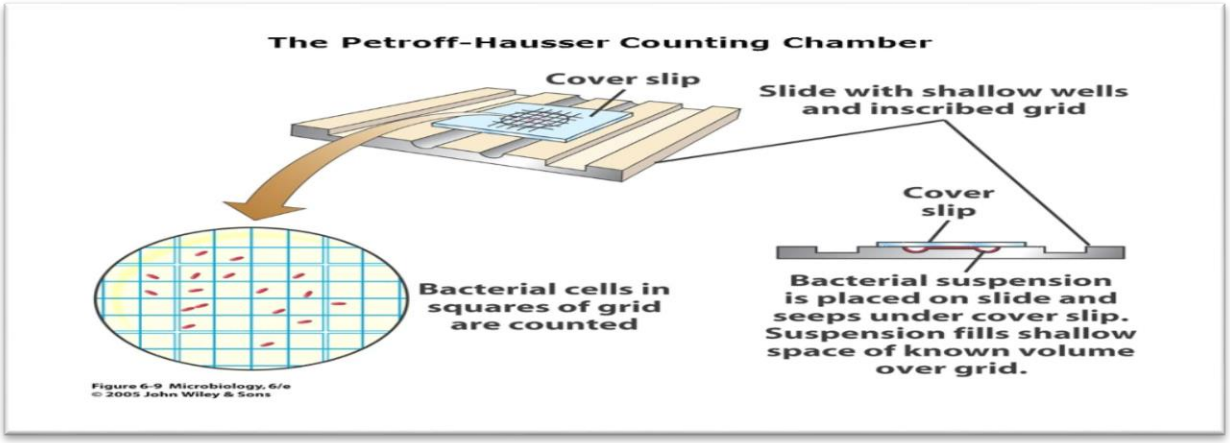

Figure 3: counting bacterial cell by Petroff-Hausser counting chambers

❖ Counting the number of bacterial colony by Colony counter devices

Procedure:

1. Remember to pull plates and refrigerate after 48 hours max. Either then or next lab period, count the number of colonies on each plate, calculate an average and record results.

2. Compare results from the standard plate counts with P-H direct microscopic counts.

3. Compare results from the standard plate counts and direct microscopic counts with that of optical density while considering the graph provided. Which data are the most robust and why? Which data yields the highest counts and why?

Results: Dilutions

Dilutions	10^6	10^7	10^8
plate 1			
Plate 2			
Average			

Figure 4: Counting bacterial cell by colony counter devices