BACTERIAL ENUMERATION

In the study of microbiology, 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, turbidimetric measurements, visual comparison of turbidity with a known standard, direct microscopic counts, cell mass determination, and measurement of cellular activity. In this exercise, you will compare three methods of bacterial enumeration: the standard plate count, turbidimetric measurement and direct microscopic counts.

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, then incubating the plates under proper conditions so that colonies are formed. After incubation, the colonies are counted and, from a 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, platings and incubations, as well as the time needed for media preparation.

Turbidimetric 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.

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 milliliter of sample. A standard curve can then be drawn (see example in this handout), 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. Percent 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.) = - 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.

Direct Microscopic Count

Epifluorescence microscopy 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 cells, the direct

microscopic count method is not very useful for determining the number of viable cells in a culture.

FIRST PERIOD

Material:

1. Seven 9-ml dilution tubes of nutrient broth
2. Six nutrient agar plates
3. 1.0 and 10 ml pipets
4. Glass spreader
5. 95% ethyl alcohol in glass beaker (**WARNING:** Keep alcohol away from flame!!)
6. Overnight broth culture of *Serratia marcescens*

Procedure: (work in pairs)

A. Spread Plate Technique

1. Prepare serial dilutions of the diluted (1/2; 1/4; 1/8; or 1/16) 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. Save all dilutions for next protocol.

2. Spread the 0.1 ml inoculum evenly over the entire surface of one of the nutrient agar plates 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 shortest). Remember that only plates with 30 to 300 colonies are statistically valid!

B. Turbidimetric Method

1. Using the spectrophotometer, determine the optical density (O.D.) of the assigned dilution (1/2; 1/4, 1/8; or 1/16) at **600 nm**. Note: you will want to use your serial dilutions of the broth culture to get as many readings as you can from between 0.5 and 1.0 O.D. units (n = 4 or 5 measurements).

2. Record results. See sample graph and plot your data accordingly.

C. Direct Microscopic Counts

Material:

1. Diluted *Serratia marcescens* culture (preserved with 1% glutaraldehyde)
2. Irgalan black in 2% acetic acid
3. Particle-free acridine orange (AO) solution
4. Polycarbonate filters (0.2 µm pore-size)
5. Sterile water
6. Sterile diluent (nutrient broth or sterile saline)
7. Filtration apparatus and vacuum pump

Procedure: (work in pairs)

Dye Filters and Prepare "Blanks"

1. Place polycarbonate filters in the irgalan black solution for about 5 minutes. Rinse the dyed filters 2x in sterile water to remove excess dye. The sterile water will be in plastic Petri dishes.

2. Filter acridine orange daily with a 0.2 µm pore-size Acrodisc filter assembly. Prepare *blanks* as you would for a sample (see detailed procedure below). The number of cells per microscope field for a blank or background sample should be zero; however, < 2 cells per field is acceptable. If there are more than 2 cells or fluorescent "particles" per microscope field, the acridine orange should be filtered again.

Staining

1. Rinse the 15-ml, 25-mm glass filter assembly with sterile water. Cover the ground glass base with sterile water and place a predyed *black* polycarbonate filter on the bubble of water. Turn on vacuum to remove excess water. The filter should lay flat on the base of the filtration assembly. Secure the filtration tower.

2. Mix the preserved sample, and pipet a known volume of the sample into the filter unit. Sample volume should be adjusted for dilution of the original *Serratia* culture to give the appropriate number of cells per microscope field.

   Note: The final volume of liquid to be filtered must be equal to or greater than 2 ml. You may need to add sterile diluent so that the volume is at least 2 ml. That volume will ensure more random distribution of cells on the filter.

3. Add 200 µl of acridine orange to the sample in the filtration tower. Mix and allow to stain for 3 - 5 minutes.

4. Filter the stained sample under vacuum pressure < 250 mm Hg. Allow the filter to dry, otherwise it will cause blurring when immersion oil is added.

5. Make a "sandwich" as follows:

   (i) glass slide;
   (ii) drop of low-fluorescence immersion oil;
   (iii) dry filter;
   (iv) drop of low-fluorescence immersion oil on the center of the filter;
   (v) glass cover slip;
   (vi) another drop of low-fluorescence immersion oil.

6. Examine the slide using an epifluorescent microscope. Count the number of bacterial cells in at least 10 microscope fields per filter.

SECOND PERIOD

Material:

1. Colony counter
2. Semi-logarithmic, 2-cycle graph paper (see website)

Procedure:

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

2. Obtain results from the other groups and record their results.

3. Plot the O.D. vs the standard plate count.

4. Plot the O.D. vs the direct microscopic count (same graph).

5. Compare results from the standard plate count with direct microscopic counts while considering the graph provided. Which data are the most robust and why? Which data yields the highest counts and why?

Results:

ORIGINAL DILUTION FACTOR: _____

Dilutions

	10^{-6}	10^{-7}	10^{-8}
Plate #1			
Plate #2			
Average			

Number of colony-forming units per ml _____

Culture	O.D. at 600 nm	Direct Count	Plate Count
1/2 dilution			
1/4 dilution			
1/8 dilution			
1/16 dilution			

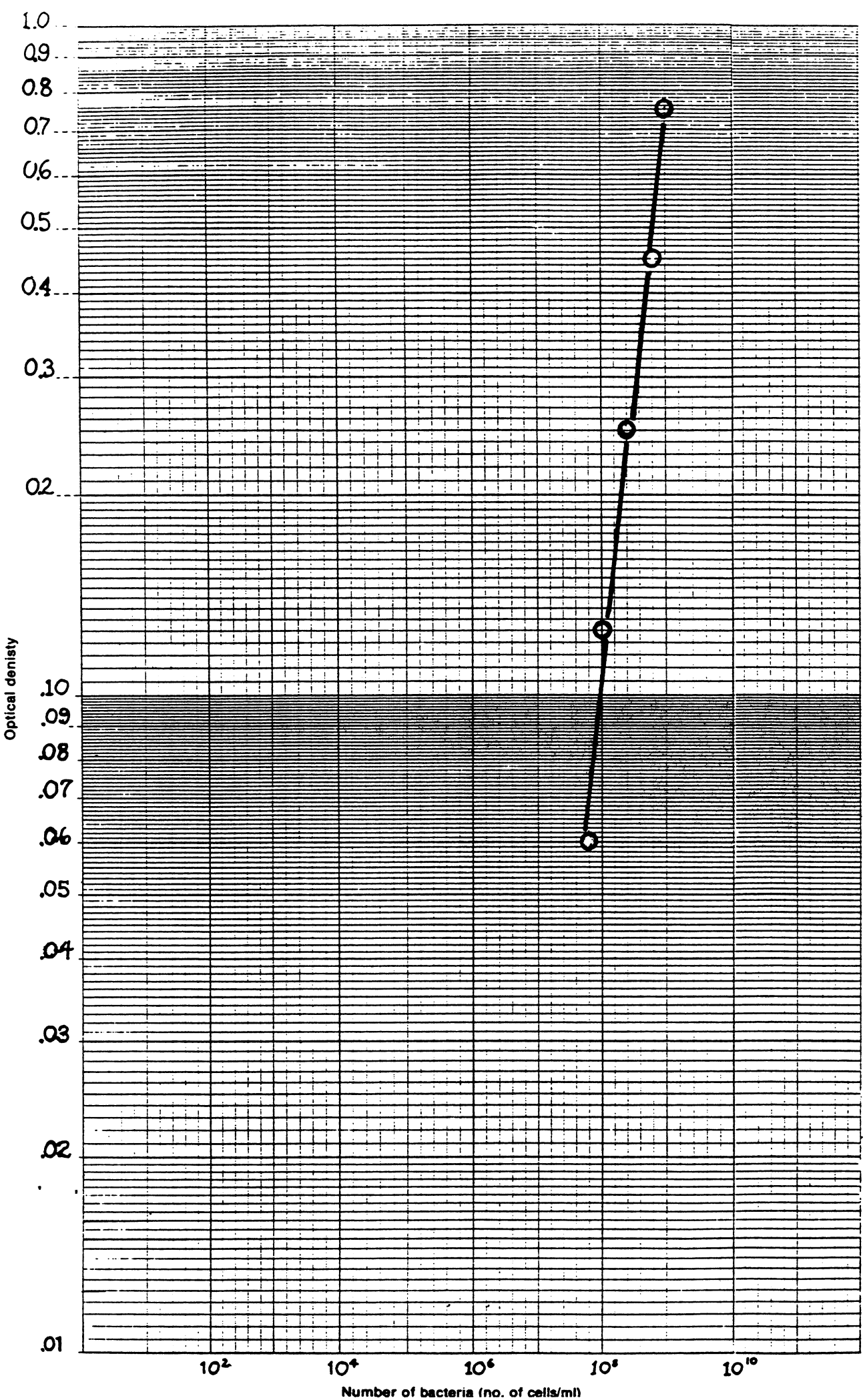

Figure 13-1. Standard curve comparing the O.D. (turbidimetric) of a broth culture with the number of viable cells/ml (standard plate count).