ISOLATION OF PURE CULTURES

A **pure culture** theoretically contains a single bacterial species. There are a number of procedures available for the isolation of pure cultures from mixed populations. A pure culture may be isolated by the use of special media with specific chemical or physical agents that allow the enrichment or selection of one organism over another. The *differential* and *selective* procedures will be utilized later in this course. Simpler methods for isolation of a pure culture include: (i) spread plating on solid agar medium with a glass spreader and (ii) streak plating with a loop. The purpose of spread plating and streak plating is to **isolate** individual bacterial cells (colony-forming units) on a nutrient medium.

Both procedures (spread plating and streak plating) require understanding of the aseptic technique. **Asepsis** can be defined as the absence of infectious microorganisms. However, the term is usually applied to any technique designed to keep unwanted microorganisms from contaminating sterile materials.

FIRST PERIOD

Material:

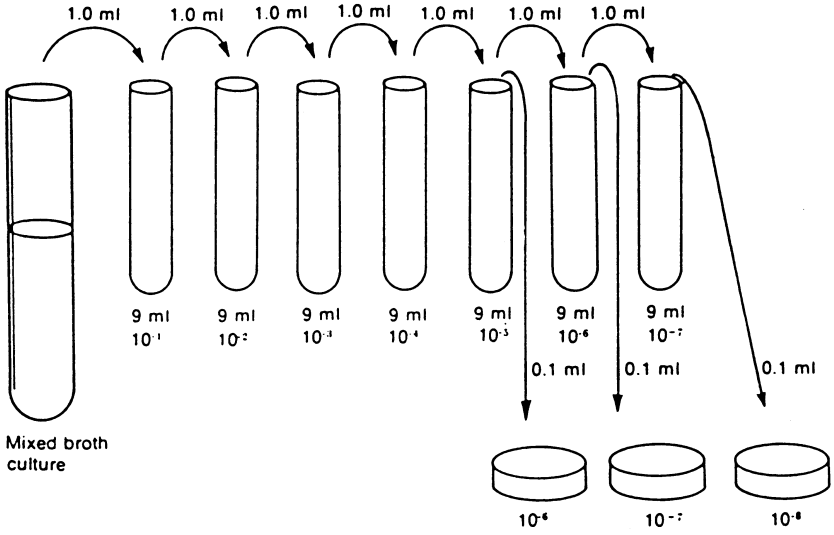
1. Seven 9-ml dilution tubes of sterile saline
2. Seven nutrient agar plates
3. 1.0 ml and 0.1 ml pipets
4. Glass spreader aka “hockey stick”
5. 95% ethyl alcohol in glass beaker (**WARNING**: Keep alcohol away from flame!!)
6. Mixed overnight broth culture of *Staphylococcus aureus* and *Serratia marcescens*

Procedure: (work in pairs)

A. Spread Plate Technique

In this technique, the number of bacteria per unit volume of sample is reduced by serial dilution *before* the sample is spread on the surface of an agar plate.

1. Prepare serial dilutions of the broth culture as shown below. 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 each of three nutrient agar plates, and label the plates.



Preparation of 10^{-6} to 10^{-8} dilutions utilizing seven 9 ml sterile nutrient broth blanks.

2. Position the beaker of alcohol containing the glass spreader **away from the flame**. Remove the spreader and very carefully pass it over the flame just once (lab instructor will demonstrate). This will ignite the excess alcohol on the spreader and effectively sterilize it.

3. 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.

4. Repeat the flaming and spreading for each of the remaining two plates.

5. Invert the three plates and incubate at room temperature until the next lab period.

B. Streak Plate Technique

The streak plating technique isolates individual bacterial cells (colony-forming units) on the surface of an agar plate using a wire loop. The streaking patterns shown in the figure below result in continuous dilution of the inoculum to give well separated surface colonies. Once again, the idea is to obtain isolated colonies after incubation of the plate.

1. Label two nutrient agar plates No. 1 and No. 2.

2. Prepare two streak plates by following two of the 3 streaking patterns shown in the figure below. Use the $\mathbf{10^{-1}}$ dilution as inoculum.

3. Invert the plates and incubate at room temperature until the next lab period.

Various methods of preparing streak plates for the individual organisms.

C. Exposure Plates

Exposure of sterile media to the environment will demonstrate the importance of aseptic technique.

1. Label two nutrient agar plates as "Exposure I" and "Exposure II."

2. Uncover the plate marked "Exposure I" and allow it to remain exposed in the lab for about 5 minutes.

3. Expose the plate marked "Exposure II" to a source of possible contaminants. Use your imagination: cough or sneeze, place your fingers on the surface of the agar, etc.

4. Invert the plates and incubate at room temperature until the next lab period.

SECOND PERIOD

Material:

1. Colony counter

Procedure:

A. Spread Plate Technique

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

DILUTION	Red Colonies	White Colonies	Total Number
10^{-6}			
10^{-7}			
10^{-8}			

B. Streak Plate Technique

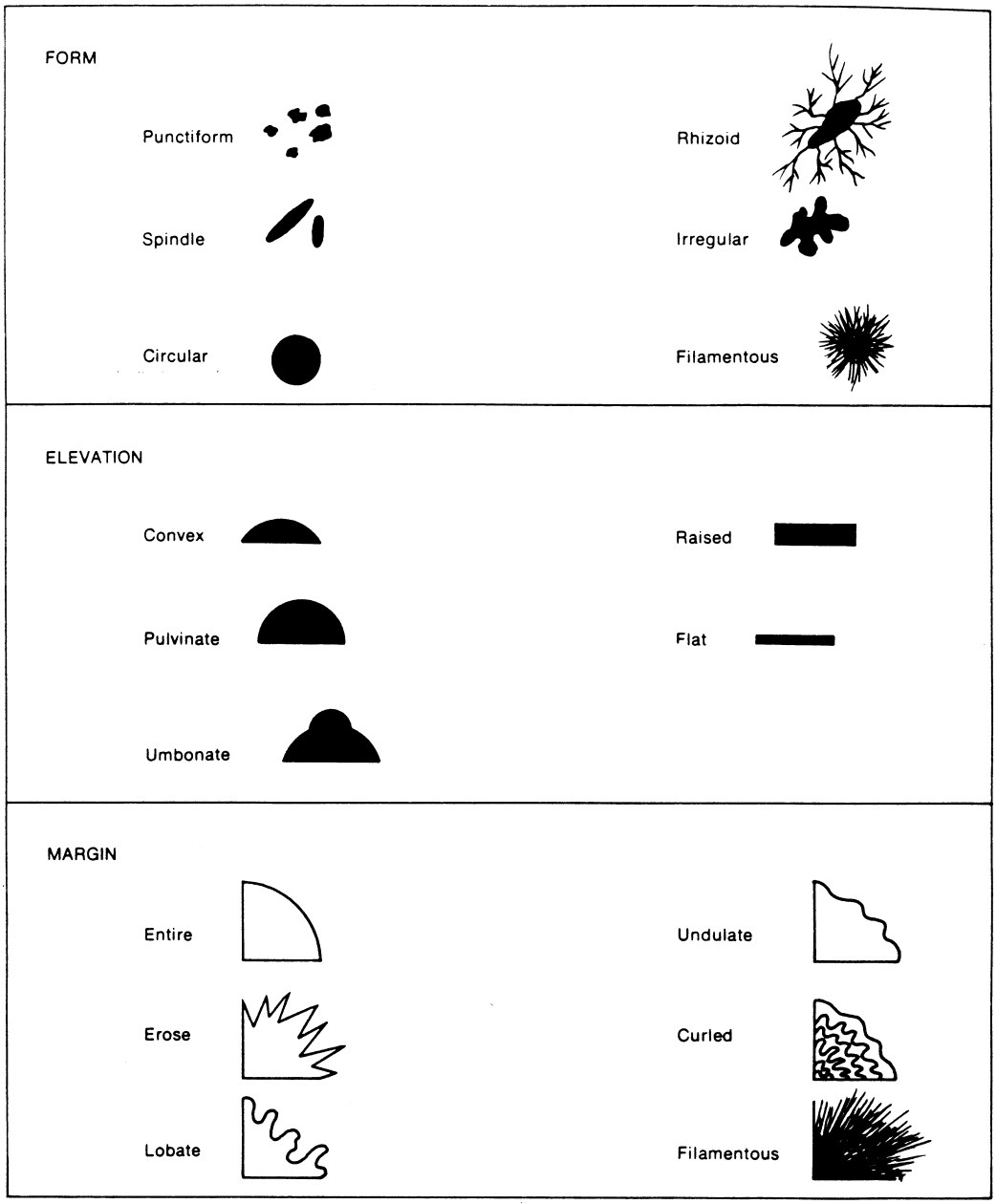
1. Observe plates. Did you obtain isolated colonies on the agar plates which were streaked with *Serratia marcescens*? Which streaking technique do you prefer? If you did not obtain isolated colonies, what changes should you make in your technique to ensure isolated colonies?

C. Exposure Plates

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

COLONIAL MORPHOLOGY

Both colonial and cellular morphology are characteristic of each species of bacteria and are sometimes useful in the identification of an unknown microorganism. When a bacterium grows on a solid agar surface, the number of cells increases until a visible mass of cells, called a colony, appears. It is usually inferred that each colony arises from the division of a single cell. The most useful culture characteristics are morphology, size and pigmentation of the colony. The figures presented below illustrate some of the morphological characteristics of bacterial colonies and provide helpful terminology for the description of colony morphology.



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 (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. 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.) = 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.

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

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 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 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 the 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 broth culture at 600 nm. Note, you may have to use one of your serial dilutions of the broth culture to get a good reading.

2. Record results.

C. Direct Microscopic Counts

Material:

1. Petroff-Hausser counting chamber
2. Cover slips
3. Sterile diluent (nutrient broth or sterile saline)
4. Pasteur pipets

Procedure: (work in pairs)

Be extremely careful handling Petroff-Hausser counting chambers!

1. Clean P-H counting chamber with 70% alcohol an 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:

   $$\frac{\text{Total cells counted} \times 2.0\times10^{7} \times \text{dilution factor}}{\text{\# small squares counted}} = \text{cells/ml}$$

   Therefore, if you counted an average of 15 cells per small square, then you would have a final concentration of 3.0×10^{8} 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%.

SECOND PERIOD

Material:

1. Colony counter

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

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

Number of colony-forming units per ml _____

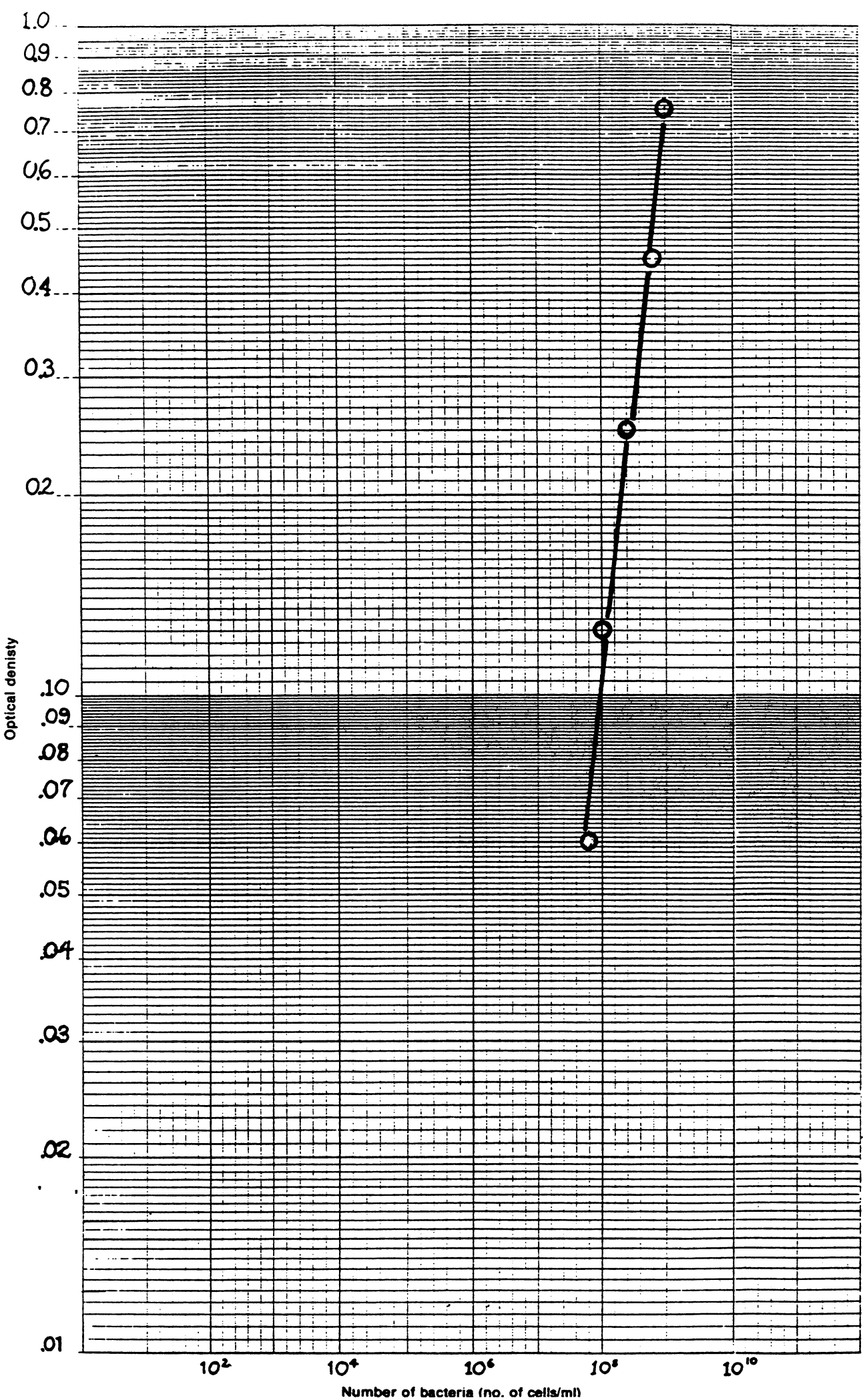


Figure 13-1. Standard curve comparing the O.D. (turbidimetric) of a broth culture with the number of viable cells/ml (standard plate count).