# ADVANCE MICROBIOLOGY
## Module 23

## Enumeration of bacterial numbers by serial dilution & plating

## Academic Script

Microorganisms rarely occur in nature as a single species. Rather, they are mixed with other species, in a so-called mixed culture most often as a **biofilm.** Therefore, to study a microbial species, microbiologists and laboratory technologists must use a **pure culture** — that is, a population consisting of only one species. This is particularly important when trying to identify a pathogen. The most important thing is, the bacterial culture has to be diluted to decrease the bacterial cell load, which is done by the process of serial dilution of the sample. 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, with the help of calculation of dilution factor of the dilution used, the original number of viable cells can be calculated.

It is very essential to count or enumerate the number of bacteria in a sample, especially in food stuff and water, to assess it fit for human consumption. The sanitary control of food quality is primarily concerned with testing food products for the presence of specific microorganisms. Food products are the primary vehicle responsible for the transmission of microbial diseases of the gastrointestinal system. For this reason, food products are routinely examined for the presence of bacteria. The **heterotrophic plate count** can be used to determine the number of viable bacteria in a food sample. The larger the count, the greater the likelihood that specific pathogens capable of causing disease will be present and also that the food will spoil. Normally, raw hamburger should not contain over 106 bacteria per

gram. One of the limitations of the heterotrophic plate count is that only bacteria capable of growing in the culture medium under the environmental conditions provided will be counted. As a result, a medium that supports the growth of most heterotrophic (requiring organic carbon) bacteria is commonly used.

A **serial dilution** is the stepwise dilution of a substance in solution which is done before plating method. Usually the dilution factor at each step is constant, resulting in a geometric progression of the concentration in a logarithmic fashion. Serial dilutions are used to accurately create highly diluted solutions as well as solutions for experiments resulting in concentration curves with a logarithmic scale. Serial dilutions are widely used in experimental sciences, including biochemistry, pharmacology, microbiology, and physics. In microbiology, serial dilution may also be used to reduce the concentration of microscopic organisms or cells in a sample. As, for instance the number and size of bacterial colonies that grow on an agar plate in a given time is concentration-dependent, and since many other

diagnostic techniques involve physically counting the number of micro-organisms or cells on specials printed with grids (for comparing concentrations of two organisms or cell types in the sample) or wells of a given volume (for absolute concentrations), dilution can be useful for getting more manageable results.

**Steps involved in Serial dilution**:

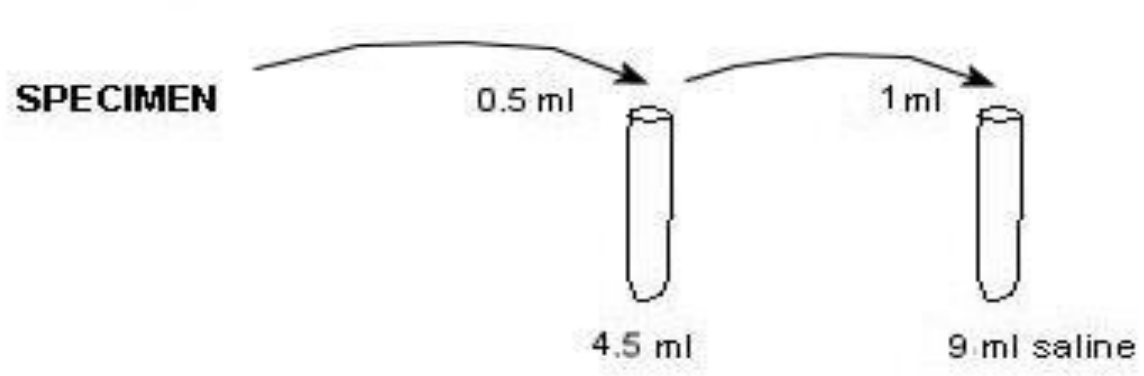
1. With a wax pencil, label the bottom of six Petri plates with the following dilutions: $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, $10^{-8}$, and $10^{-9}$. Label four bottles of saline or phosphate buffer $10^{-2}$, $10^{-4}$, $10^{-6}$, and $10^{-8}$.
2. Using aseptic technique, the initial dilution is made by transferring 1.0 ml of liquid sample or 1 g of solid material to a 99-ml sterile saline blank. This is a 1/100 or $10^{-2}$ dilution. Cap the bottle.
3. The $10^{-2}$ blank is then shaken vigorously 25 times by placing one's elbow on the bench and moving the forearm rapidly in an arc from the bench surface and back. This serves to distribute the bacteria and break up any clumps of bacteria that may be present.

4. After the $10^{-2}$ blank has been shaken, let it settle down for sometime, uncap it and aseptically transfer 1.0 ml to a second 9-ml saline blank. Since this is a $10^{-2}$ dilution, this second blank represents a $10^{-3}$ dilution of the original sample. Cap the bottle.
5. Shake the $10^{-3}$ blank vigorously 25 times and transfer 1.0 ml to the third 9-ml blank. This third blank represents a $10^{-4}$ dilution of the original sample. Cap the bottle. Repeat the process to produce till $10^{-8}$ dilution.

In this way the sample is diluted so that it could be enumerated on the surface of the solid agar media.

**Calculation of dilution factor:**

**i. Dilution factor** = amount of specimen transferred divided by the total volume after transfer [amount of specimen transferred + amount of diluent already in tube].




*Pic 1: Determination of the dilution factor of each tube in the set.*

**ii. Dilution factor for a tube** = amount of sample volume of specimen transferred + volume of diluent in tube.

But after the first tube, each tube is a dilution of the previous dilution tube.

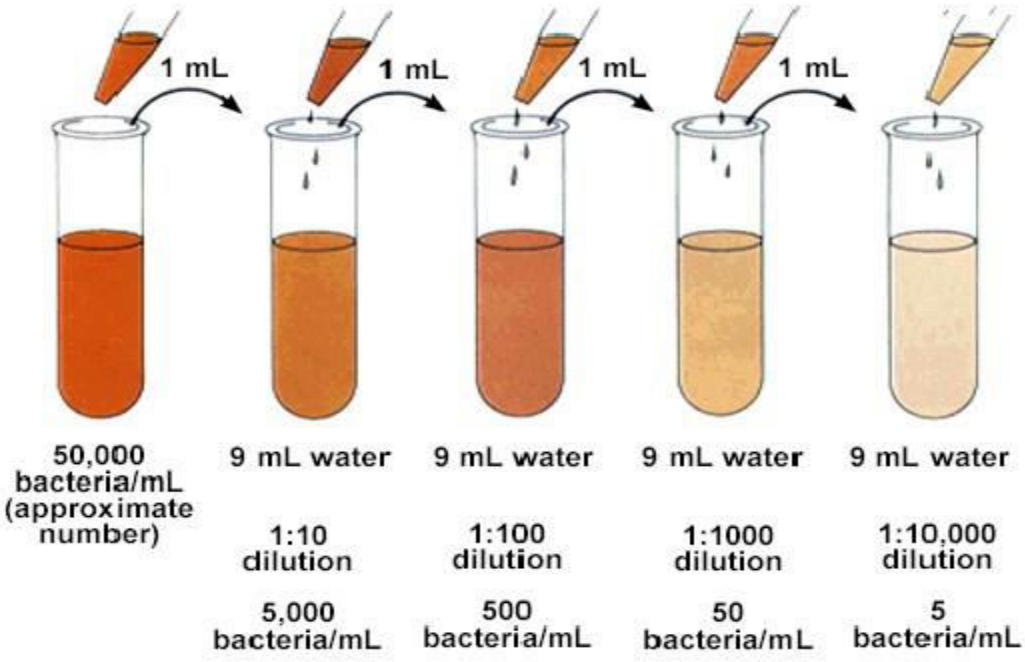
SO,

**Total dilution factor** = (previous dilution factor of tube) **X** (dilution of next tube)

**FOR THE ABOVE DILUTION SERIES:**

0.5 ml added to 4.5ml = 0.5/5.0 = 5/50 = 1/10 for 1st tube

1ml added to 9ml = 1/10 (2nd tube) **X** previous dilution of 1/10 (1st tube) = total dilution of 1/100 for 2nd tube.




*Pic 2: Serial Dilution of the sample*

## Standard Plate Count or Pour Plate method

After serial dilution of the sample we perform the standard plate count method which is a reliable method for enumerating bacteria and fungi. Pour Plate Method is also known as Total Viable Count (TVC) or The Standard

Plate Count Method (SPC). This method is extensively used for obtaining pure cultures with prokaryotes and fungi, a pour plate also can yield isolated colonies.

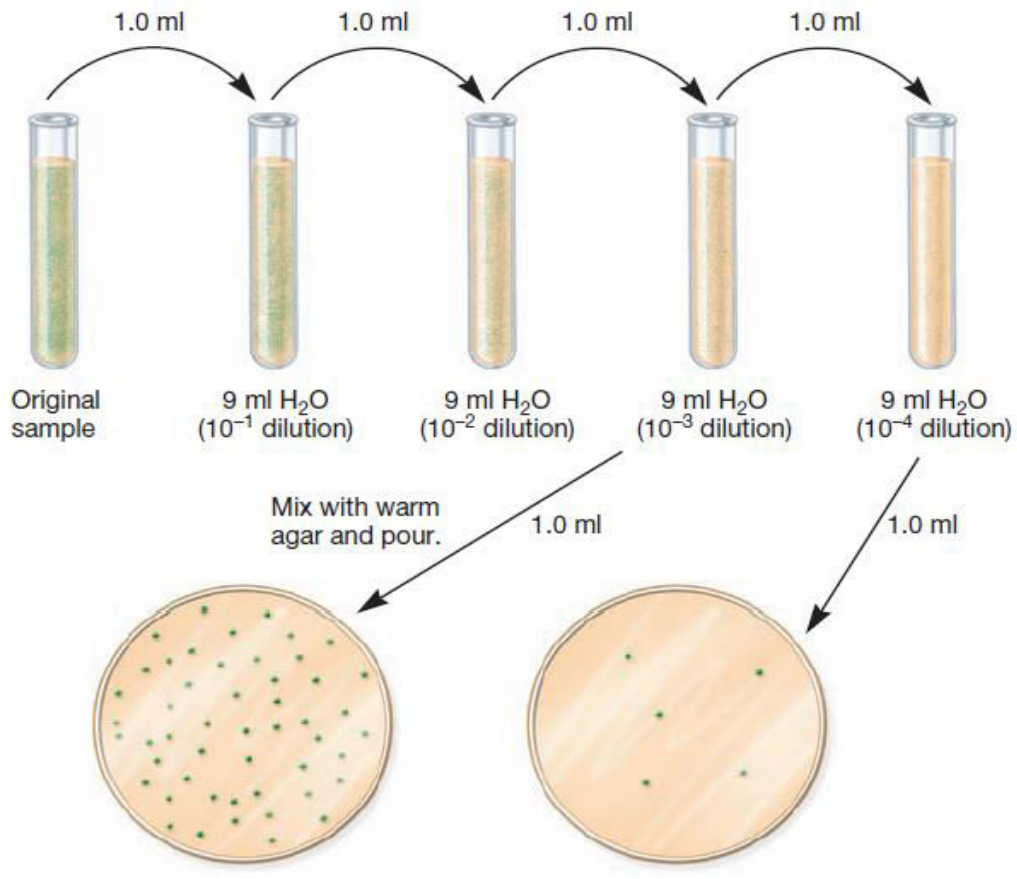
The procedure starts with the serial dilution of the sample followed by Pour Plating.

**Steps involved in pour plate method:**

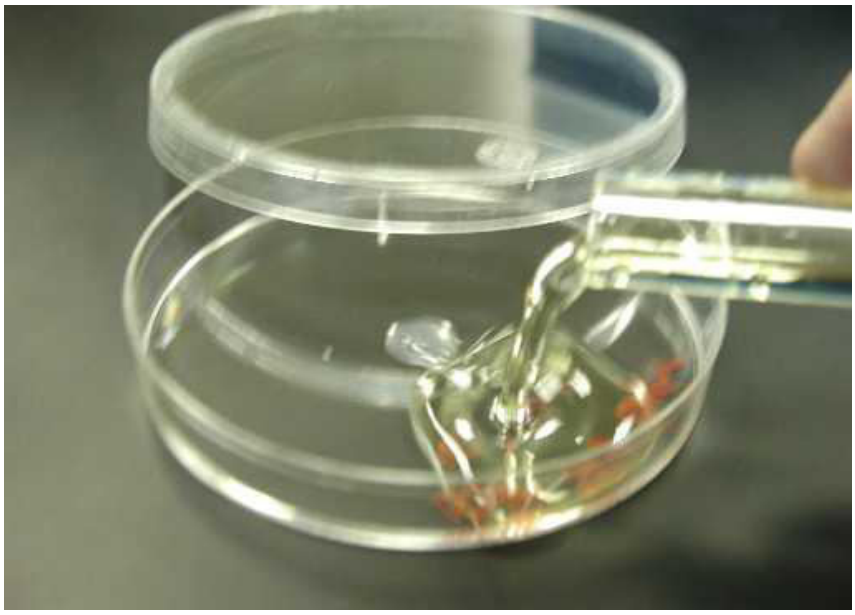
1. The original sample is diluted several times by serial dilution to reduce the microbial population sufficiently to obtain separate colonies when plating.
2. Then small volumes of several diluted samples as inoculum are mixed with liquid or molten agar that has been cooled to about 45°C in the test tubes, before it gets solidified.
3. It is also mixed thoroughly by shaking the contents of the liquid cooled agar medium to allow uniform distribution of microorganisms. The mixtures are then poured immediately into sterile Petri dishes.
4. Most bacteria and fungi are not killed by a brief exposure to the warm agar.
5. After the agar has hardened in the Petri plate, each cell is fixed in place and forms an individual colony.

6. Calculate the number of bacteria (CFU) per milliliter or gram of sample by dividing the number of colonies by the dilution factor.

7. The number of colonies per ml reported should reflect the precision of the method and should not include more than two significant figures. For example, suppose the plate of the $10^{-6}$ dilution yielded a count of 130 colonies. Then, the number of bacteria in 1 ml of the original sample can be calculated as follows: Bacteria/ml = (130) ÷ ($10^{-6}$) = $1.3 \times 10^{8}$ Or 130,000,000.




*Pic 3: Serial dilution and Pour Plating*



*Pic 4: Pouring of the molten medium along with the bacterial inoculum*

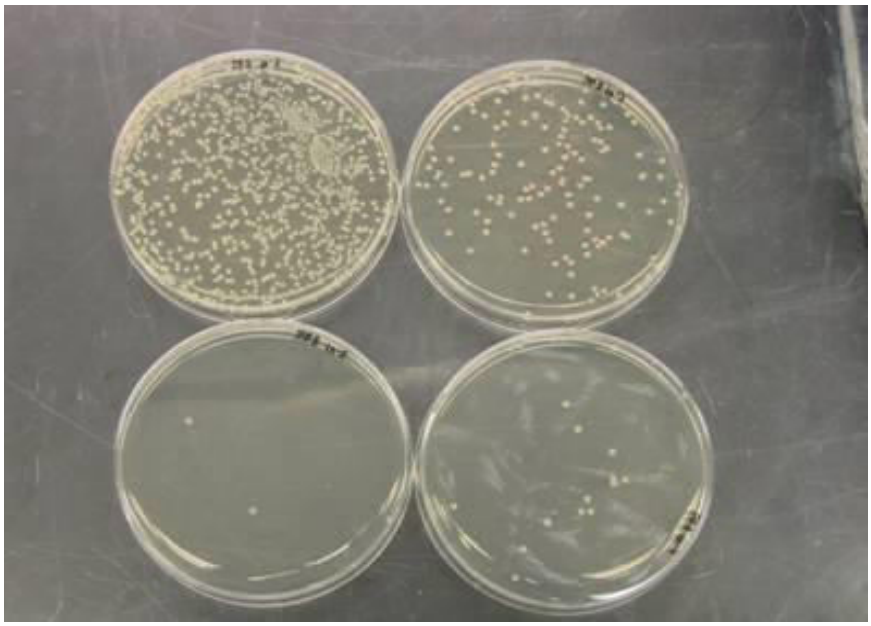
**Result of serial dilution and pour plate**

Upon incubation, the colonies that are developed on the plates are of three different types-

(1) Surface Colonies,

(2) Sub-surface Colonies, and

(3) Bottom Colonies



*Pic 5: Bacterial colonoies Pour plated after serial dilution*

**However, Pour Plate** Method has several important features to be noted:

(1) This method takes the advantage of the liquefying (at 92°-95°C temperature) and solidifying (at 45°-48°C

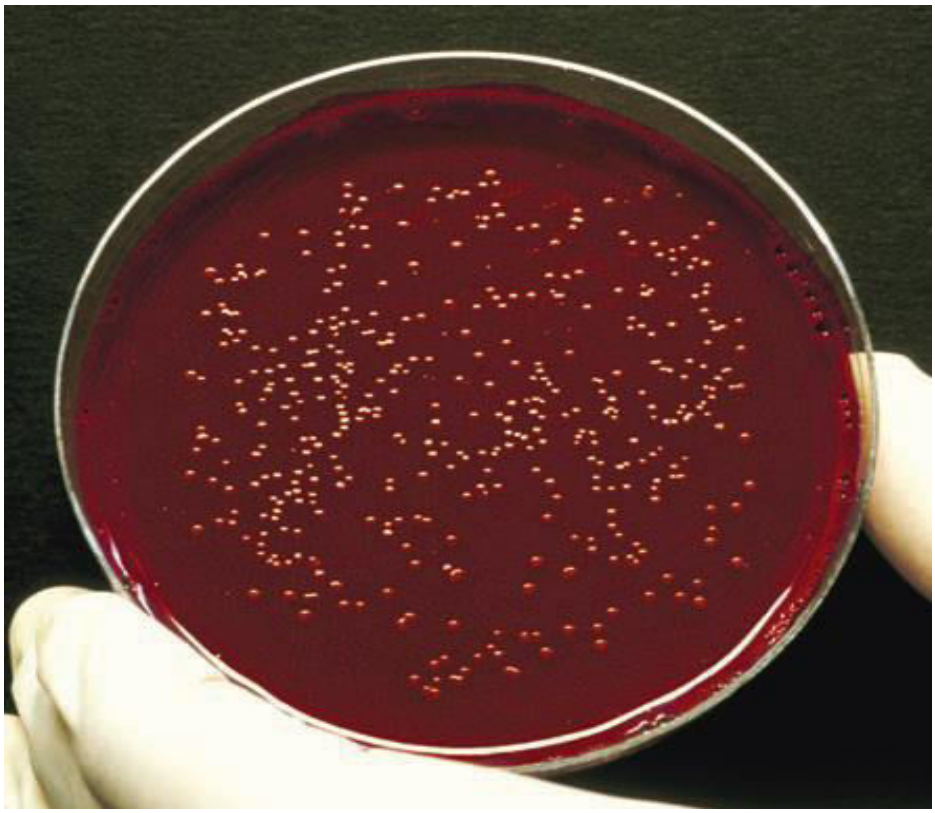
temperature) characteristics of Agar Agar Powder present in the nutrient agar medium.

(2) Psychrophilic organisms escape detection in this method, as they are easily killed even at the gelling temperature of agar.

(3) Numbers of colonies does not represent the numbers of microorganisms, instead they are referred to as the CFUs (Colony Forming Units), as a colony may be a result of a single cell, a pair of cells, a chain of cells, a cluster of cells or even a bunch of cells which are adjacently place on the medium.

(4) In case, if you are performing SPC for water, milk, food, soil or any other samples, it needs to count the exact number of colonies after the incubation of 24 hrs at 37 C. The colony count between 30-300 is used for further calculation purpose. If the number of colonies are 30, it is referred to as TLTC (Too Less To Count). And if the count of colonies exceeds 300, it is referred to as

TNTC (Too Numerous To Count). And these types of results are simply omitted from the further calculations.



*Pic 6: Individual bacterial colonies have grown. Each colony represents a 'Colony Forming Unit' (CFU) since it developed from single bacterial cell.*

The Pour Plate Method is one of the most suitable methods for the quantification or enumeration of total number of microorganisms in the given sample. The pour plating after serial dilution can be used to determine the number of cells in a population. Plates containing between 30 and 300 colonies are counted. The total

number of colonies equals the number of viable microorganisms in the sample that are capable of growing in the medium used. Sterile technique should be used in the transfer of microorganisms from tube to tube, as well as in the production of the pour plates. Colonies growing on the surface also can be used to inoculate fresh medium and prepare pure cultures.

**Spread Plate Technique**

Another technique which can be performed after serial dilution of the sample is spreading with the use of sterile spreader over the solid agar plates.

Following are the steps involved in the spread plate technique:

**1.** Prepare serial dilutions of the broth culture or any solid sample with the help of sterile water.

**2.** Transfer **0.1** ml of the final three dilutions ($10^{-5}$, $10^{-6}$, $10^{-7}$) to duplicate nutrient agar plates, and label the plates.

**3.** Spread the 0.1 ml inoculum evenly over the entire surface of one of the nutrient agar plates until the

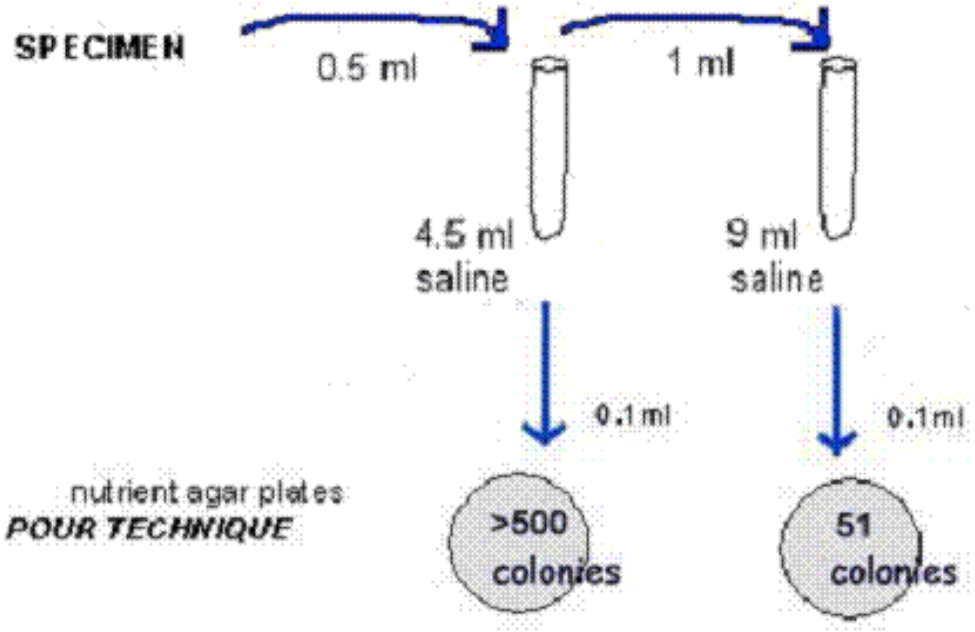
medium no longer appears moist. Return the spreader to the alcohol.

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

**5.** Invert the plates and incubate at 37°C for overnight.

The isolated colonies which appear on the agar plate are counted as CFU and number of bacteria in the original sample is calculated as per the dilution factor.

## Calculation of CFU:



1. The countable plate is the one with **51** colonies.
2. The total dilution of the 2nd tube from which that pour plate was made = $1/10^2$
3. The amount used to make that pour plate = **0.1ml** (convert to 1/10 - it is easier to multiply fractions and decimals together).

**51 colonies** = 51 X $10^3$ = **5.1 X $10^4$** (scientific notation) OR **51,000 CFUs/ml** **$1/10^2$ X 1/10**

**45 colonies** = 45 X $10^4$ = **4.5 X $10^5$** (scientific notation) OR **450,000/ml**
**$1/10^3$ X 1/10**