

**Determining the number of cells per ml (colony forming units per ml) of the original undiluted sample using plate counts.**

The ability to quantitate the number of bacteria in a sample is an important procedure for many areas of microbiology, including food, dairy and beverage microbiology, water microbiology, cosmetics, and the pharmaceutical and biotechnology industries. Many studies require the quantitative determination of bacterial populations. Some of the most widely used methods for determining bacterial numbers are the standard, or viable, plate count method, spectrophotometric (turbidometric analysis), membrane filtration, and most probable number methods. Although the methods are somewhat similar in the results that they yield, there are distinct differences. For example, the standard plate count method is an indirect measure of cell density and reveals information related only to live bacteria. The spectrophotometric analysis is based on turbidity and indirectly measures all bacteria (cell biomass), dead or alive. In many of these procedures it is important to be able to make accurate dilutions of the original sample, to correctly use algebraic equations pertaining to dilutions, and to correctly use standard scientific notation.

In this exercise, we will use the standard plate count method. Other methods will be the subject of future exercises. The standard plate count method consists of diluting a sample with sterile diluent (often saline or phosphate buffer, but this varies) until the bacteria are dilute enough to count accurately. That is, the final plates in the series should have between 25 and 250 colonies per plate. Fewer than 25 colonies are not acceptable for statistical reasons, and more than 250 colonies on a plate are likely to produce colonies too close to each other to be distinguished as distinct **colony-forming units (CFU)**. The assumption is that each viable bacterial cell is separate from all others and will develop into a single discrete colony (CFU). Thus the number of colonies should give the number of live bacterial that can grow under the incubation conditions employed. A wide series of dilutions (e.g.  $10^{-4}$  to  $10^{-9}$  or  $10^{-10}$ ) is normally plated because the exact number of live bacteria in the sample is usually unknown. Greater precision is achieved by plating duplicates or triplicates of each dilution.

**Dilution Ratios:**

According to the *American Society for Microbiology Style Manual* dilution ratios may be reported with either colons (:) or division signs (/), but note that there is a difference between them. A division sign indicates the ratio of a part to a whole; for example  $\frac{1}{2}$  means 1 of 2 parts, with a total of 2 parts. Using 1:2 with a colon indicates the ration of 1 part to 2 parts, with a total of 3 parts. Thus  $\frac{1}{2}$  equals 1:1, but 1:2 equals  $\frac{1}{3}$ . Therefore, according to the American Society for Microbiology:

- Dilutions written as a fraction (e.g.  $1/10$  or  $1/100$ ) use the division sign [numerator is volume added, denominator is the total volume].
- Dilutions written as a ratio (e.g. 1 part to 9 parts) use a colon (1:9). [Note: the total volume is 10 parts]
- A 1:9 dilution and a  $1/10$  dilution are the same, but a 1:10 dilution written with a colon would be a  $1/11$  dilution, written as a fraction.
  - 1 : 1 “one to one” or “one part to one part” =  $1/2$  dilution
  - 1 : 2 “one to two” or “one part to two parts” =  $1/3$  dilution
  - 1 : 3 “one to three” or “one part to three parts” =  $1/4$  dilution
  - 1 : 9 “one to nine” =  $1/10$  (or  $10^{-1}$ )
  - 1 : 99 =  $1/100$  (or  $10^{-2}$ )

**Making a Dilution Series (serial dilution):**

Accuracy in making dilutions is of paramount importance in the determination of bacterial numbers. Make sure to pipette accurately. **Remember to mix the original undiluted sample before transferring and mix each tube or bottle well after making the addition and before transferring any volume.** See the figure showing the dilution scheme to be used for this exercise. It involves 99 ml dilution blanks and 9 ml dilution blanks. Other common dilution blank volumes used in microbiology are indicated at the end of the exercise.

Prior to making dilutions, it is a good idea to check that your pipette and pipetting device can deliver the desired volume without leaking. Make sure you understand the calibration markings of the pipette you will be using. The pipettes will be sterile. Also remember that each transfer must be made aseptically. You do not want to introduce contaminants during a plate count procedure. Therefore a pipette should be used close to the area of transfer, and not carried from one end of the bench to another (or across the room). Be careful not to inadvertently touch the pipette tip to your skin or other surfaces. (If this does happen, get a new pipette before making the transfer). Discard used pipettes in the proper disposal container containing Amphyl disinfectant.

A different pipette should be used for each transfer during serial dilutions. However, when plating it is acceptable to use one pipette (handled with good aseptic technique) to plate all dilutions as long as you start with the most dilute suspension first and progressively work your way up to the more concentrated suspensions. Be careful not to inadvertently touch the pipette tip to your skin or other surfaces. (If this does happen, get a new pipette before making the transfer). Discard used pipettes in the proper disposal container containing Amphyl disinfectant.

Prior to plating the appropriate dilutions, check with your instructor to determine which dilutions should be plated, and how many plates should be inoculated for each dilution.

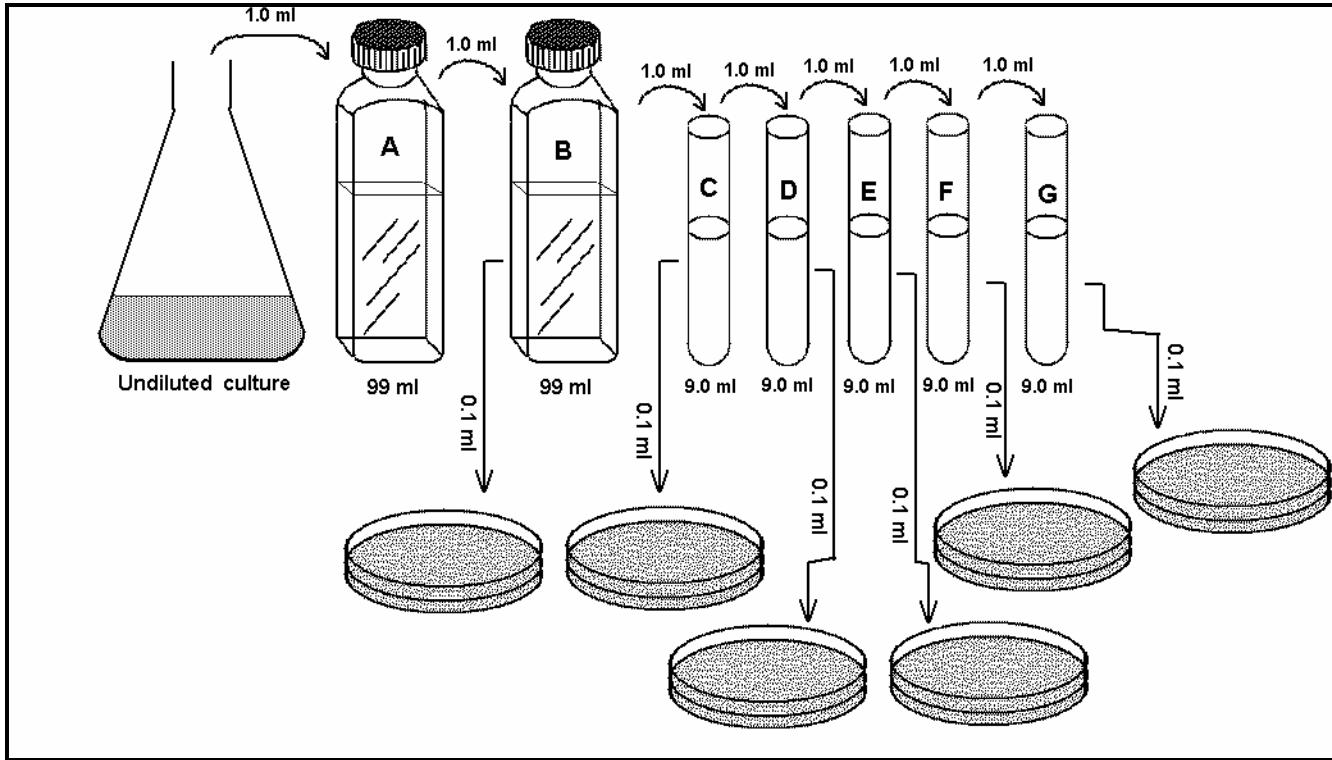
**Plating and determining the number of CFU per ml of the original culture suspension:**

1. When plating, note the dilution and the volume plated directly on the plate. Remember to label the bottom of the plate, not the lid.
2. With pour plates, volumes of 0.1 ml or 1.0 ml of the appropriate dilutions are acceptable. *With spread plates 0.1 ml is typically plated since a larger volume would take a long time to soak into the agar.* Allow pour plates to solidify or spread plates to soak in before inverting the plates for incubation. Normally 2-3 plates are inoculated per dilution. In some instances the protocol may specify that 0.2 ml or 50  $\mu$ l (0.050 ml) should be plated. See step #6 to correct for the volume plated, if something more or less than 1 ml was plated. **For this exercise you will use the spread plate technique (0.1 ml per plate).**
3. Incubate the solidified, “dried” and properly labeled plates as directed by your instructor.
4. After incubation, select those plates with a countable number of colonies: 25 minimum; 250 (depending on colony size) maximum. Fewer than 25 colonies is not statistically accurate and when there are more than 250 colonies, some of the colonies may overlap, reducing accuracy of the count.

- Lower than 25: plates are marked TFTC, “Too few to count”
  - More than 250: plates are marked TNTC, “Too numerous to count”
5. Count the colony-forming units (CFU) on the selected plates (those with 25 to 250 CFU).
  6. Adjust the count for the volume plated. For example if 235 CFU are present on a plate and 1 ml was plated, the # of CFU per ml of the dilution plated is 235. If 127 CFU are present on a plate and only 0.1 ml was plated, the # of CFU per ml of the dilution plated is 1,270. If you divide the CFU on the plate, by the volume plated (in mls), you will end up with the number of CFU per ml for that plate.
  7. Multiply the # of CFU per ml X the dilution factor (DF) of the total dilution used to inoculate that particular plate. For example if 1,270 CFU per ml was obtained with the total  $10^{-6}$  dilution ( $DF = 10^6$ ), the corresponding number of CFU per ml of the undiluted sample is  $1,270 \times 10^6$  CFU/ml
  8. Express the result in correct scientific notation (remember significant figures):  
 $1,270 \times 10^6$  CFU/ml =  $1.3 \times 10^9$  CFU/ml.
  9. Finally obtain the average count in CFU/ml if more than one plate yielded a countable number of colonies. (Before taking the average make sure all numbers are written in the same power of 10). [For example: when averaging  $5.4 \times 10^6$ ,  $9.2 \times 10^5$ , and  $7.7 \times 10^5$  you should write these as either  $54 \times 10^5$ ,  $9.2 \times 10^5$  and  $7.7 \times 10^5$  – or –  $5.4 \times 10^6$ ,  $0.92 \times 10^6$ , and  $0.77 \times 10^6$ .]

Dilution: volume of addition / total volume of bottle or tube after addition (often expressed as a fraction or in exponential form if a power of 10 is involved)

Dilution Factor (DF) = 1 / Dilution (usually expressed in exponential form if a power of 10 is involved)



Remember to mix each tube or bottle well after making the addition and before transferring any volume.

The following table refers to the dilution scheme shown on the previous page.

Bottle or Tube	A	B	C	D	E	F	G
Volume of Dilution Blank	99 ml	99 ml	9.0 ml	9.0 ml	9.0 ml	9.0 ml	9.0 ml
Volume of Addition	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Dilution of <u>this</u> step	1/100 or $10^{-2}$	1/100 or $10^{-2}$	1/10 or $10^{-1}$	1/10 or $10^{-1}$	1/10 or $10^{-1}$	1/10 or $10^{-1}$	1/10 or $10^{-1}$
Total Dilution	1/100 or $10^{-2}$	1/10,000 or $10^{-4}$	1/100,000 or $10^{-5}$	1/1,000,000 or $10^{-6}$	etc. $10^{-7}$	$10^{-8}$	$10^{-9}$
Dilution Factor	$10^2$	$10^4$	$10^5$	$10^6$	$10^7$	$10^8$	$10^9$

**Common dilution blanks and addition volumes used in microbiology:**

<b>Volume of Dilution Blank</b>	<b>Volume Added</b>	<b>Total Volume</b>	<b>Dilution</b>	<b>Dilution Factor</b>
999 ml	1 ml	1000 ml	$10^{-3}$	$10^3$
99 ml	1 ml	100 ml	$10^{-2}$	$10^2$
90 ml	10 ml	100 ml	$10^{-1}$	$10^1$
9.9 ml	0.1 ml	10 ml	$10^{-2}$	$10^2$
9 ml	1 ml	10 ml	$10^{-1}$	$10^1$
4.5 ml	0.5 ml	5 ml	$10^{-1}$	$10^1$
0.9 ml	0.1 ml (100 $\mu$ l)	1 ml	$10^{-1}$	$10^1$
0.99 ml	0.01 ml (10 $\mu$ l)	1 ml	$10^{-2}$	$10^2$