

Dilution Theory and Problems

Microorganisms are often counted in the laboratory using such methods as the viable plate count where a dilution of a sample is plated onto (or into) an agar medium. After incubation, plates with 30-300 colonies per standard-sized plate are counted. This number of colonies (30-300) was chosen because the number counted is high enough to have statistical accuracy, yet low enough to avoid nutrient competition among the developing colonies. Each of the colonies is presumed to have arisen from only one cell, although this may not be true if pairs, chains, or groups of cells are not completely broken apart before plating. It is possible, but unlikely, for an original (undiluted) sample of microorganisms which is to be counted to have 30-300 cells/ml so that a pour plate using a 1 ml volume from the sample will give good results. More likely, a sample will have greater numbers of cells/ml; sometimes, as in the case of unpolluted water samples, the sample will have less. In either case, the sample must be manipulated so that it contains a number of cells in the correct range for plating. If the cell number is high, the sample is diluted; if too low, the sample is concentrated. Dilutions are performed by careful, aseptic pipetting of a known volume of sample into a known volume of a *sterile* buffer, water, or saline. This is mixed well and can be used for plating and/or further dilutions. If the number of cells/ml is unknown, then a range of dilutions are usually prepared and plated.

Concentration of samples is usually performed by filtration through a filter with pores small enough to retain the microorganisms. This way, the volume of the original sample can be reduced, so that the number of cells/ml increases to the 'countable' range. Since this procedure is rarely used in our classes, this exercise will focus on dilution theory.

In order to make the calculation of the number of cells/ml in the original sample easier, dilutions are designed to be easy to handle mathematically. The most common dilutions are 1/10, 1/100, and 1/1000. Looking first at the 1/10 (or 10^{-1}) dilution, it can be made by mixing 1 ml of sample with 9 ml of sterile dilution buffer. This gives the fraction:

$$\frac{1 \text{ ml of sample}}{1 \text{ ml of sample} + 9 \text{ ml of buffer}} = \frac{1 \text{ ml}}{1 \text{ ml} + 9 \text{ ml}} = \frac{1 \text{ ml}}{10 \text{ ml}} = \frac{1}{10} = 0.1 = 10^{-1}$$

Some alternative methods of obtaining a 1/10 dilution are:

$$\frac{10 \text{ ml of sample}}{10 \text{ ml of sample} + 90 \text{ ml of buffer}} = \frac{10 \text{ ml}}{10 \text{ ml} + 90 \text{ ml}} = \frac{10 \text{ ml}}{100 \text{ ml}} = \frac{1}{10} = 0.1 = 10^{-1}$$

-or-

$$\frac{0.1 \text{ ml of sample}}{0.1 \text{ ml of sample} + 0.9 \text{ ml of buffer}} = \frac{0.1}{0.1 + 0.9} = \frac{0.1}{1.0} = \frac{1}{10} = 0.1 = 10^{-1}$$

-or-

$$\frac{0.5 \text{ ml of sample}}{0.5 \text{ ml of sample} + 4.5 \text{ ml of buffer}} = \frac{0.5}{0.5 + 4.5} = \frac{0.5}{5.0} = \frac{1}{10} = 0.1 = 10^{-1}$$

What if the sample is not liquid, for example, food samples? Since 1 ml of water weighs 1 gram (under standard conditions), you can consider that 1 gram of any sample is equal to 1 ml. Therefore, 1 gram of pepper + 9 ml of dilution buffer = a 1/10 dilution of the pepper. It is recognized that 1 gram of pepper might not actually have a volume of exactly 1 ml but if the arbitrary assignment is recognized as standard, it can be used conveniently and reproducibly.

How is a 1/100 dilution obtained? Usually, 1 ml of sample + 99 ml buffer or 0.1 ml of sample + 9.9 ml of buffer are used to give this dilution. A 1/1000 dilution can be obtained by adding 0.1 ml of sample to 99.9 ml of buffer.

Once the dilution is made, an aliquot can be plated on an agar plate using the spread plate technique or in an agar medium using the pour plate technique. After incubation, the colonies are counted. How do the colonies on the plate relate to the number of cells (colony-forming units or CFU) in the original sample? Try a problem:

- One ml of a sample was mixed with 99 ml of buffer. One ml of this was plated (using the pour plate method) in nutrient agar. After incubation, 241 colonies were present on the plate. How many colony-forming units were present per ml of the *original sample*? State your answer in CFU/ml.

The dilution used was $1/(1+99) = 1/100 = 0.01 = 10^{-2}$. One ml of the dilution contained 241 colony-forming units. How much did one ml of the original sample contain? Obviously *more than* 241 colony-forming units! To arrive at the correct number, *either* divide the colony-forming units/ml of the dilution by the dilution (241 colony-forming units/ml divided by $10^{-2} = 241 \times 10^2 = 2.41 \times 10^4$ CFU/ml of original sample) *or* multiply by the *dilution factor*. The dilution factor is defined as the *inverse of the dilution*, therefore in this example the dilution factor would be $1/10^{-2} = 10^2$. Multiplying by the dilution factor: 241 colony-forming units/ml $\times 10^2 = 241 \times 10^2 = 2.41 \times 10^4$ CFU/ml of original sample. Either way, the answer you get is the same. Try another problem:

- One ml of a sample was mixed with 99 ml buffer. One-tenth of a ml of this was plated on nutrient agar. After incubation, 142 colonies were present on the plate. How many colony-forming units were present per ml of the original sample?

In this case, the mathematics of the dilutions is the same, but the number of colonies counted on the plate the represent number of colony-forming units in *only 0.1 ml* of the dilution.

Remember, you should always report your answers as CFU/ml. Therefore, the number of colony-forming units per ml of dilution is 142 colonies divided by 0.1 ml plated, or $142 \times 10 = 1420$ colony-forming units/ml of dilution. Dividing this number by the 10^{-2} dilution results in a final answer of 1420×10^2 or 1.42×10^5 colony-forming units/ml of original sample. Try another:

- One ml of a sample was mixed with 99 ml of buffer. One ml of this mixture was added to a sterile petri plate and mixed with 25 ml of molted agar (cooled to 45 degrees). The mixture was allowed to solidify undisturbed. After incubation, 241 colonies were present on the plate. How many colony-forming units were present per ml of the original sample:

The answer to this problem is *identical* to the answer to the first problem! The amount of agar used is not relevant because the number of organisms present depends only on the number of ml of the dilution which were added to the agar. Whether 25 ml or 35 ml of agar were used, the number of organisms added to the agar remains constant, so the colony count will be identical.

What if the sample requires more dilution? For example, what if the sample to be counted is a culture of *Escherichia coli* grown overnight in a rich medium? The number of cells/ml will be around 10^9 (1,000,000,000). Obviously, a single dilution would not be enough, so *successive dilutions* must be performed. An easy example of a successive dilution is making a 1/100 dilution using only two tubes of 9.0ml buffer. You would add 1 ml of sample to the first 9ml tube and mix well. This is a 1/10 dilution. Next, you would remove 1 ml from this dilution and add it to the second 9ml tube of buffer. This is also a 1/10 dilution, *but not of the original sample*.

Instead, this is a 1/10 dilution of a 1/10 dilution. To arrive at the final dilution, multiply the second dilution by the first dilution: $1/10 \times 1/10 = 1/100$. If you made another 1/10 dilution from the second tube, you would have a 1/1000 final dilution. Try a problem:

- An overnight culture of *Escherichia coli* is used as a sample. One ml of this culture is added to a bottle containing 99ml of buffer. This dilution is mixed well (as all dilutions are!), and one ml of this is mixed in 9 ml of buffer. This second dilution is diluted by three successive 1/10 dilutions. The last (fifth) dilution is plated, i.e. 0.1 ml is plated on nutrient agar. After incubating the plate, 56 colonies are counted. How many colony-forming units were present per ml of *E. coli* culture?

To solve this problem, try writing out the procedure. Then multiply the successive dilutions together (here is where scientific notation comes in handy, since it is easy to add the exponents without 'losing a zero').

$$\frac{1}{1+99} \times \frac{1}{1+9} \times \frac{1}{10} \times \frac{1}{10} \times \frac{1}{10} = \frac{1}{1,000,000}$$

OR

$$10^{-2} \times 10^{-1} \times 10^{-1} \times 10^{-1} \times 10^{-1} = 10^{-6}$$

The number of colony-forming units per ml of the dilution can then be divided by the final dilution:

$$\frac{56 \text{ colonies}}{0.1 \text{ ml plated}} \text{ divided by } 10^{-6} = 560 \text{ colony-forming units/ml} \times 10^6$$

$$= 5.6 \times 10^8 \text{ colony-forming units/ml}$$

Note that the 0.1 ml that was plated is treated as another 1/10 dilution. Some microbiologists treat it as such in their computations rather than dividing the colony count by the number of milliliters plated, as done here.

Whichever way it is done, the answer will be the same.

The only way to understand dilution theory well is to practice it, so you should work the problems given in this supplement. The answers to the problems will be given to you by your lab TA in the next lab period. Before you start, review the basic rules of dilution theory:

- Since dilutions result in *physically* lowering the number of cells per ml in a solution, the calculations must *mathematically* raise the number of cells per ml. Therefore, remember that the final answer should be *larger* than the original colony count, not smaller. It is not possible to have negative exponents in the final number of colony-forming units/ml (a negative exponent implies that there is less than one colony-forming unit/ml).
- The number of ml of agar into which a sample is added is irrelevant, unless of course, it is further diluted.
- The number of ml plated is relevant.
- The dilution is determined by dividing the number of ml of sample added to the dilution buffer by the *total* of the ml of sample plus the ml of buffer.
- Successive dilutions are multiplied to find the total dilution.
- Plates with 30-300 colonies on them should be used for the greatest accuracy in counting.

- The number of colony-forming units/ml of original culture is calculated by either dividing the number of colony-forming units/ml of the dilution which gave 30-300 colonies per plate by that dilution *or* by multiplying the number of colony-forming units/ml or the dilution which gave 30-300 colonies per plate by the inverse of that dilution (i.e. the dilution factor).
- If more than one plate of a dilution has been prepared and is counted, the colony counts (of that dilution only) should be averaged.

QUESTIONS

1. One ml of a water sample is added to 9 ml of sterile water. This is mixed well and further diluted by 4 successive 1/10 dilutions. One-tenth of a ml of each dilution is spread on a plate of nutrient agar. After incubation, the following data were obtained:

Dilution used for plating	Amount plated	Colony counts after incubation
first 1/10	0.1 ml	too many to count
second 1/10	0.1 ml	730
third 1/10	0.1 ml	67
fourth 1/10	0.1 ml	5
fifth 1/10	0.1 ml	0

What was the number of colony-forming units/ml of the original water sample that were capable of growing on nutrient agar?

2. Three grams of soil were added to 27 ml of sterile water and shaken vigorously. After the soil particles settled, 0.1 ml of this was added to 9.9 ml of sterile water. This was further diluted by 4 successive 1/10 dilutions. One ml from the last dilution was used to prepare a pour plate. After incubation, 289 colonies were present on this plate. What was the number of colony-forming units/gram of the soil?

3. A bacterial culture was diluted and results from duplicate plates were obtained as indicated below. What was the number of colony-forming units/ml of the original culture?

Dilution used for plating	Amount plated	Colony counts after incubation (results from duplicate plates)
10 ⁻²	0.1 ml	too many to count
10 ⁻³	0.1 ml	too many to count
10 ⁻⁴	0.1 ml	321 ; 403
10 ⁻⁵	0.1 ml	34 ; 42
10 ⁻⁶	0.1 ml	6 ; 1
10 ⁻⁷	0.1 ml	0 ; 0
10 ⁻⁸	0.1 ml	0 ; 0

4. Ten grams of hamburger were added to 90 ml of sterile buffer. This was mixed well in a blender. One-tenth of a ml of this slurry was added to 9.9 ml of sterile buffer. After thorough mixing, this suspension was further diluted by successive 1/100 and 1/10 dilutions. One-tenth of a ml of this final dilution was plated onto Plate Count Agar. After incubation, 52 colonies were present. How many colony-forming units were present in the *total* 10 gram sample of hamburger?

5. Devise a scheme to prepare a 10^{-6} dilution on a plate *using the least number of sterile water dilution tubes*.
6. Devise a scheme to prepare 1/20, 1/40, and 1/80 dilutions of a disinfectant.

Some convenient conversions to remember:

$$10^0 = 1 \quad 10^1 = 10 \quad 10^2 = 100 \quad 10^3 = 1000 \quad 10^4 = 10,000 \quad 10^5 = 100,000$$

$$10^6 = 1,000,000 \quad 10^7 = 10,000,000 \quad 10^8 = 100,000,000 \quad 10^9 = 1,000,000,000$$

$$10^{10} = 10,000,000,000$$

$$10^{-1} = 0.1 = 1/10 \quad 10^{-2} = 0.01 = 1/100 \quad 10^{-3} = 0.001 = 1/1000$$

$$10^{-4} = 0.0001 = 1/10000 \quad 10^{-5} = 0.00001 = 1/100000$$

$$10^{-6} = 0.000001 = 1/1000000 \quad 10^{-7} = 0.0000001 = 1/10000000$$

$$10^{-8} = 0.00000001 = 1/100000000 \quad 10^{-9} = 0.000000001 = 1/1000000000$$

Remember exponents are **additive** and fractions are **multiplicative**

$$10^{-1} \times 10^{-1} \times 10^{-1} \times 10^{-1} = 10^{-4}$$

which is the same as $1/10 \times 1/10 \times 1/10 \times 1/10 = 1/10,000$

and

$$10^{-1} \times 10^{-2} \times 10^{-3} \times 10^{-4} = 10^{-10}$$

which is the same as $1/10 \times 1/100 \times 1/1000 \times 1/10000 = 1/1000000000$