Needed Math Project
Award # 2100062

Dilutions in Biomanufacturing: in Two Parts
SCENARIO 2: IN THE ANALYTICAL LABORATORY
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1. Problem Statement

There are many situations in biomanufacturing where some substance must be diluted. It is sometimes confusing to technicians to determine how much diluting substance to add to the substance to obtain a final solution with a particular volume and concentration.

In the previous scenario we introduced the $C_1V_1 = C_2V_2$ equation and showed how it is a convenient tool that is often used when preparing buffers and other solutions. The $C_1V_1 = C_2V_2$ equation is a valuable calculation tool, but there are times when the $C_1V_1 = C_2V_2$ equation provides an answer that is mathematically correct but is not practical. For example: suppose there is a suspension of cells at a density[1] of 10^6 cells/mL and the analyst wants to dilute them with cell culture medium to obtain 5 mL of cells with a concentration of 10^1 cells/mL.

Substituting into the $C_1V_1 = C_2V_2$ equation:

$$C_1 \quad V_1 \quad = \quad C_2 \quad V_2$$

$$(10^6 \text{ cells/mL})\ (?) = (10^1 \text{ cells/mL})\ (5 \text{ mL})$$

$$? = 0.00005 \text{ mL} = \mathbf{0.05\ \mu L}$$

[1] In these scenarios we use the terms “concentration” and “density” interchangeably in reference to items such as bacterial or mammalian cells that are suspended in a liquid and are not actually dissolved. This terminology is acceptable in a biological setting. A chemist might avoid the term “concentration” to refer to suspended cells.

This volume, 0.05 μL, is too low to accurately measure with normal measuring devices. It is therefore necessary to dilute this cell suspension using more than one step, that is, with a series of dilutions. In this situation, it is useful to have strategies in addition to the $C_1V_1 = C_2V_2$ equation to handle dilution calculations.

2. Scenario Description and Specific Example

For a specific example, consider an analyst in a testing laboratory who is performing a viable cell count of bacteria. (Viable cell counting will be explained later.) As shown in Figure 1, there is an original broth containing bacteria suspended in a nutritional substance (broth). The broth has an unknown density of bacteria, and the analyst needs to determine that density. The analyst dilutes the culture as shown in the diagram and plates 0.1 mL of the last three dilutions onto three Petri dishes with nutrient agar. The following day the analyst counts the number of colonies on the plates with the results shown in the illustration. What was the approximate concentration of bacteria in the original broth?

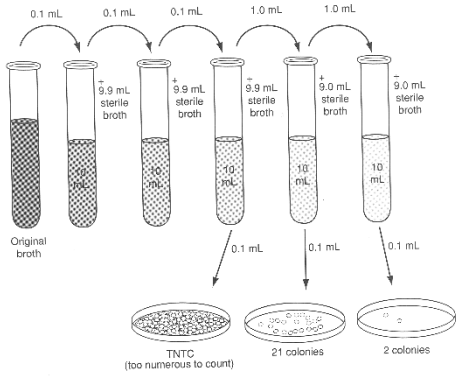

Figure 1. Viable Cell Count Scenario.

3. Issues to be Addressed

Counting seems like a simple mathematical process. However, counting microorganisms is not so simple. For one thing, microorganisms, such as bacteria, are not visible to the eye. Another problem is that bacteria may be present in extremely large numbers in a sample. For example, in nutrient broth, there might be 1×10^9 bacterial cells per mL. Therefore, microbiologists have

devised various methods to count bacterial cells. One such method is called viable cell counting. To perform a viable cell count, a sample of bacterial cells is first diluted through a series of dilutions. (A series of dilutions is required because the original concentration of cells is typically quite high.) Then, 0.1 mL of diluted cells are spread on a petri dish that contains nutrient agar. It is assumed that every living cell in the 0.1 mL placed on the agar divides to form a colony of bacterial cells. A colony contains so many individual cells that it is visible to the eye. It is also assumed that each colony originates from a single cell. It is possible to count the colonies and therefore to estimate the number of bacteria in the original broth.

4. Mathematics (Calculations)

The original bacterial broth was diluted using a series of dilution steps. Figure 2 shows how each tube was progressively diluted. The first agar plate (the one plated with the least diluted suspension of bacteria) contained too many colonies to count. The third agar plate was plated with a suspension that was too dilute. The middle plate had 21 colonies, so it is the one the analyst used for calculations. The concentration of cells added to this plate was about 21 cells/0.1 mL = 210 cells/mL. The dilution tube from which these cells were drawn had been diluted 1×10^7. This means that there were originally about

$210 \times 10^7 = 2.10 \times 10^9$ bacterial cells/mL in the original broth.

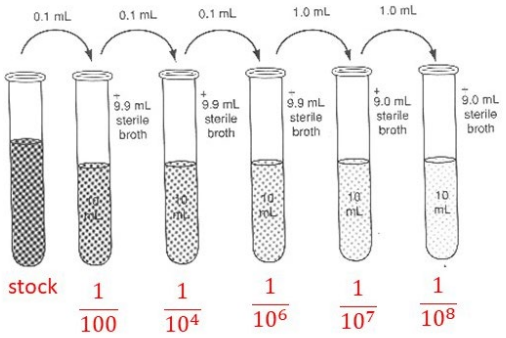

Figure 2. The numbers in red are the dilutions in each tube. For example, observe that the first dilution tube was made by taking 0.1 mL of the original bacterial culture and adding 9.9 mL of sterile broth. This is a 1/100 dilution. The second dilution was similarly made by taking 0.1 mL of the original bacterial culture and adding 9.9 mL of sterile broth. In this second

dilution tube the original bacterial culture has been diluted $\frac{1}{10^2} \times \frac{1}{10^2} = \frac{1}{10^4}$ ***. In general, to calculate the final dilution in a dilution series:***

Dilution in final tube = (Dilution in first tube) (Dilution in second tube)….(Dilution in last tube)

5. Extensions Beyond This Example

Counting bacteria is just one situation where a series of dilutions is used in a biological or biomanufacturing setting. Here is another scenario that looks somewhat different to a beginner but is mathematically related.

Antibodies are often used in assays to locate and tag biological molecules. For example, as explained by a biomanufacturing expert, assays involving antibodies are used routinely to determine 1) the production level of a therapeutic protein during the manufacturing process and 2) the level of proteins in patient samples.

Antibodies are purchased as concentrated stock solutions that are diluted right before use. The optimal dilutions of antibodies are best determined by experimentation. For example, an antibody manufacturer might suggest a 1/1000 dilution as a starting point for a particular application. But the analyst needs to experiment to see if this is, indeed, the best dilution. Suppose an analyst decides to try dilutions of 1/500, 1/1000, 1/2000, and 1/4000 to see which dilution is best in a particular setting. Suggest a strategy to prepare these dilutions.

As always, contextual knowledge is required to strategize because the volumes required are not specified. Let's assume that the analyst needs at least **2 mL** of each of these dilutions as an example. Antibodies are expensive, so the analyst will want a strategy that minimizes, or close to minimizes, the use of antibody stock. There is more than one way to prepare these dilutions; one possibility is as follows, Figure 3.

Dilution Tube A. **8 µL original stock + 4 mL diluent.** (Technically 3992 µL of diluent is needed, but that is close enough to 4 mL that most people would not measure 3992 µL.) This tube has a 1/500 dilution of antibody, and it uses relatively little of the valuable antibody stock.

Dilution Tube B. **Remove 2000 µL from Tube A and add 2000 µL of diluent.** This is a ½ dilution of Tube A.

Dilutions Tube C. **Remove 2000 µL from Tube B and add 2000 µL of diluent.** This is a ½ dilution of Tube B.

Dilution Tube D. **Remove 1500 µL from Tube C and add 1500 µL of diluent.** This is another ½ dilution.

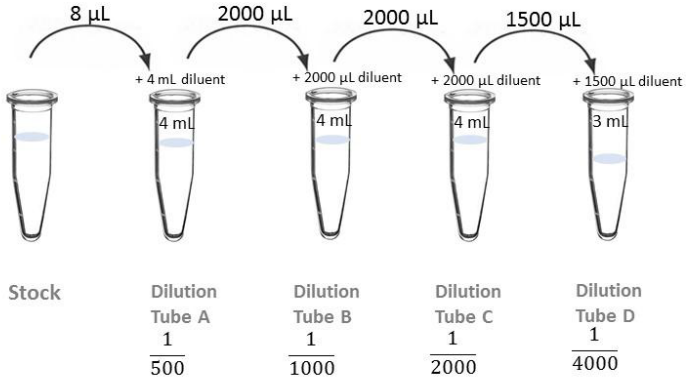

Figure 3. Antibody Example Showing How the Desired Dilutions are Achieved.

Yet, another example of a situation where dilutions are frequently used is when standards are prepared from a concentrated stock. For example, for an assay, an analyst might need standards that are 2 ppm, 4 ppm, 10 ppm, 50 ppm, and 100 ppm. These five standards might be prepared by diluting a 500 ppm standard. This type of activity is not limited to biological settings; standards are prepared in quality control laboratories across many sectors.

Further Notes

Why do beginners find these kinds of dilution situations difficult? As noted in Part I of this two-part scenario, the concept of concentration must be understood. When discussing chemical reagents (as we were in Part I), we are concerned with the concentration of molecules in a liquid, but the same idea applies to cells in suspension.

When biologists are working with situations that require a series of dilutions, the $\mathbf{C_1V_1 = C_2V_2}$ equation is seldom mentioned. This makes a dilution series feel like different than the dilutions that occur when making buffers and other solutions.

An unfortunate source of confusion relating to dilutions is that the terminology in the biological literature is inconsistent. For example, if 1 mL of stock is combined with 9 mL of diluent, people might call this a 1 to 10 dilution, or a 1 to 9 dilution, or a 1 in 10 dilution, or a 1:10 dilution, or a 1:9 dilution or a 1/10 dilution (as we do in these scenarios). This inconsistency can cause problems, particularly for unsuspecting beginners, and can lead to calculation errors.

It is seldom apparent to beginning students how to think about and prepare a specific dilution with a specific volume. For example, beginners struggle to find a strategy to prepare 50 mL of a 1/5 dilution of a stock. Since this is not intuitive to most students, it is important to take time to discuss how this is calculated. Similarly, students do not intuitively see that the final dilution, after a series of dilutions is the product, not the sum, of all the dilutions.

The viable cell count scenario requires reasoning “backwards” to find the density of cells in the original broth. Again, this reasoning is seldom obvious to students and requires time and discussion.

The bottom line for dilution is that it takes time to explore with beginners the strategies and complexities involved. It is necessary for instructors and supervisors to allow time for beginners to develop the insights necessary in these varied situations.