Dilutions in Biomanufacturing: In Two Parts
SCENARIO 1: DILUTIONS IN THE MEDIA PREPARATION DIVISION

PROBLEM STATEMENT

There are many situations in biotechnology/biomanufacturing where some substance must be diluted with water or an aqueous buffer. 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.

SCENARIO DESCRIPTION AND SPECIFIC EXAMPLE

Many companies have a media production division whose role is to prepare and provide buffers, culture media, and other reagents to the rest of the company. Frequently these aqueous solutions are initially prepared in a concentrated form (called the "stock solution") and then must be diluted, either by the media production staff or at the point of use in the company. Technicians must be able to accurately determine how much diluting substance (usually water or buffer) and how much of the concentrated stock solution must be combined to obtain the correct final concentration and volume.

As an example, observe that Box 1 shows a short portion of a standard operating procedure (SOP) that is followed by technicians as they manufacture an antibody pharmaceutical product. (For example, the monoclonal antibodies that are sometimes used to treat COVID-19 are an antibody pharmaceutical product.) (Don't worry if you do not fully understand the SOP; the details are not important right now.) Note that to perform this portion of the SOP, the technician will need two chemicals and water. Figure 1 shows how $NaHCO_3$ and PBS were supplied by the media prep department to the production staff who are following this SOP. Questions:

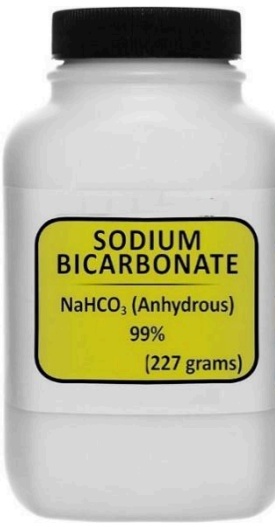
Question 1. Do any of the supplied reagents require dilution by production staff?

Question 2. If so, how should this be accomplished?

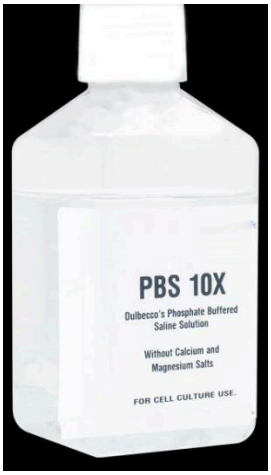
BOX 1
Portion of SOP Used When Manufacturing an Antibody Product

6.5.1. Prepare a bottle with 150 mL of 1M $NaHCO_3$ in ultrapurified water as follows:
- 6.5.1.1 Weigh out 12.6 ± 0.1g of $NaHCO_3$ and transfer to a 250 mL beaker.
- 6.5.1.2. Measure 145 mL ultrapurified water and add to the 12.6 ± 0.1g of $NaHCO_3$ in the beaker. Add a magnetic stir bar and stir on a magnetic stirrer to dissolve.
- 6.5.1 3. Transfer the dissolved $NaHCO_3$ solution to a 250 mL graduated cylinder and bring to 150 mL volume with ultrapurified water.
- 6.5.1.4. Label the bottle as 1M $NaHCO_3$ with date, preparer initials, storage: room, temp, disposal: drain.

6.5.2. Add 100 mL of 1X PBS to the bioreactor vessel where cells will be cultured.



a.



b.

Figure 1. a. Sodium Bicarbonate Reagent Bottle. If you could look inside this bottle, you would see that this chemical has been supplied as a powder. **b. 10X PBS (Phosphate Buffered Saline) Reagent Bottle.** If you could look inside the bottle, you would see that it is a clear liquid.

ISSUES TO BE ADDRESSED AND MATHEMATICS

The first question asked in this scenario is whether, given the SOP, preparing either the sodium bicarbonate ($NaHCO_3$) or the PBS requires a dilution step. Beginners sometimes have difficulty identifying situations where something is diluted. It is important to realize that dilutions (in our context) begin with a concentrated aqueous solution, not with a chemical in powdered form. Thus, making the sodium bicarbonate does not require dilution. In contrast, the PBS has been supplied as a concentrated stock solution. The label on the PBS container says that it is "10X," which means that the reagent has been supplied in a form that is ten times more concentrated than is required for normal use. Thus, the answer to the first question is that production staff preparing the sodium bicarbonate do not perform a dilution while they do so when preparing the PBS.

Answering the second question in this scenario requires determining how much of the PBS stock solution is required and how much diluent must be added to it to obtain 100 mL of 1X PBS. There is a very convenient formula, Equation 1, to do this calculation. It is:

Equation 1
THE $C_1V_1 = C_2V_2$ EQUATION
HOW TO MAKE A LESS CONCENTRATED SOLUTION FROM A MORE CONCENTRATED SOLUTION

$(Concentration_{stock})\ (Volume_{stock}) = (Concentration_{final})\ (Volume_{final})$
This equation can be abbreviated:
$C_1V_1 = C_2V_2$

In our scenario, the stock solution of PBS is labeled "10X." This SOP requires 100 mL of the PBS at a concentration of "1X."

Applying Equation 1:

C_1 is 10X C_2 is 1X V_1 is the unknown V_2 is 100 mL

Thus:

(10X) (?) = (1X) (100 mL)

? = **10 mL**

To make the PBS, take **10 mL** of the concentrated stock and **bring to a final volume of 100 mL.** This is what will be added to the bioreactor.

The mathematics required to solve this equation is simple algebra – solving an equation with one unknown. The major difficulty for beginners is recognizing situations where this equation applies. Also, as will be discussed below, technicians must have a clear understanding of the meaning of "concentration" and the conventional ways of expressing the concentration of a solution. They also must understand the terminology used in the equation, particularly the use of "10X" to show that a reagent is supplied at 10 times the normal concentration.

SUPPORTING MATERIALS

Why do beginners find dilution challenging?

First, beginners sometimes confuse "concentrations" with "amounts." Concentrations are ratios; amounts are not. Before solving calculation problems, such as shown above, it is essential that students can distinguish between an amount (such as a gram, liter, or mole) and a concentration (such as grams/liter or moles/liter). Observe that in biological/chemical terminology, the symbol "M" for molarity, indicates a concentration (moles/liter), although it does not appear to be a ratio at first glance.

A second issue that confuses beginners in biology (and chemistry), is there are multiple ways to express the concentration of molecular solutes in a solution. Concentration can be expressed as molarity, percent, molality, or as a simple ratio. The calculations involved with the different expressions of concentration are somewhat different and therefore confusing.

Terminology can be a source of confusion. In this scenario, the convention of using an "X" is shown. 10X means that a solution is ten times more concentrated than it is commonly used. For example, if a solution is routinely used at a concentration of 0.1 molar, then 10X means the solution is 1 molar. Technicians need to be familiar with this convention and understand its meaning.

Another unfortunate source of confusion relating to terminology is that the terminology of dilutions 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). Another example is that people sometimes use the term "dilution factor." If someone says the dilution factor = 2, do they mean 1 part analyte + 1 part solvent or 1 part analyte + 2 parts solvent? There is inconsistency that can cause problems, particularly for unsuspecting beginners, and can lead to calculation errors.

At a basic math level, technicians need to have a clear understanding of ratios. They should see that 10 mg of substance in 100 mL of total solution is the same *concentration* as 1 mg of substance in 10 mL of total solution.

Also note that, in the workplace, calculations often are required as technicians move through a procedure. In this scenario we show an SOP that requires knowledge to perform, even though SOPs are generally explicit. In this example, the PBS was not supplied at the required concentration and the technician might not discover this until getting to that step in the procedure (though advance preparation is always a good idea). Hence, the necessity to perform a calculation in the workplace often occurs when the technician is preoccupied with other task(s).

Another issue that might be confusing is that it is important to understand what each variable is in the $\mathbf{C_1V_1 = C_2V_2}$ equation. Observe that V_2 is the *final total* volume. Biologists will typically bring a solution to the final volume by adding diluent to the amount calculated as V_1 using a graduated cylinder or volumetric flask. In the carbon fiber example below, the volume of diluent required is calculated by subtraction, a different strategy. In yet other situations, the volume of stock required, V_1, is so small that the diluent is measured out and the stock added to it directly. The technician needs to know what strategy is appropriate in each situation.

For a technician, the $\mathbf{C_1V_1 = C_2V_2}$ is a handy calculation tool that applies in a great many situations. However, this also means that sometimes beginners try to apply the equation where it is not relevant. For example, in the scenario above, it is not relevant when asked to make 150 mL of 1M $NaHCO_3$. This means that the technician needs to understand what a dilution is. Also, as will be described in the second part of this two-part scenario, there are other ways to think about dilutions that are useful in other situations.

There is more than one way to think about these types of problems. Here is an example from Maddie, a practicing technician. Observe Maddie's use of the term "dilution factor," which is a term she understands in a particular way, and her intuitive understanding that C_1/C_2 = the dilution factor = 250.

"When I prepare selection media for my HCT116 cells, I calculate the volume of antibiotic to add based on the dilution factor listed on the antibiotic bottle. I am trying to make 15 mL of DMEM [the selection medium] with an unknown amount of the antibiotic. Hygromycin [is the antibiotic] which we use to kill non-resistant cells and only keep the hygromycin-resistant cells […the genetically modified cells that carry a gene of interest]. The bottle of hygromycin says that a dilution factor (DF) of 250× is desirable for selection. I can use $C_1V1=C_2V_2$ to calculate the volume of hygromycin I need:

Formulas needed: DF = (C_1/C_2) and $C_1V_1=C_2V_2$

We know that DF = 250 and V_2 = 15 mL, so we can set up $C_1V_1=C_2V_2$ in a way that lets us add the known information:

Solve for V_1:

$C_1V_1=C_2V_2$

$V_1=(C_2V_2)/C_1$

Rewrite the equation to include DF:

$V_1=(1/DF)(V_2)$

Input known values and solve:

$V_1=(1/250)$ (15 mL)

V_1=0.06 mL

Convert 0.06 mL to microliters:

0.06 mL (1000) = 60 µL of hygromycin"

More Examples

Here is another example from biomanufacturing, this time from beer brewing, provided by Tyler, a quality control technician in a craft beer manufacturing facility. Yeast is essential in the manufacture of beer. Yeast uses sugars to create alcohol, to carbonate the beer, and to create compounds that give a beer its particular flavor and aroma. It is necessary to add the correct amount of yeast cells to the fermenter where the beer is to be brewed. Tyler writes: "Say I have a fermenter that has 700 hL of beer in it and I want that beer to be pitched at a rate of 12 million

cells of yeast per mL. If I have a tank that has a yeast population of 120 million cells per mL, how much yeast do I need to pitch to reach my target of 12 million cells per mL?" Observe again that contextual knowledge is required. Hectoliter, abbreviated hL, is a metric unit of volume equal to100 liters. This unit of volume is widely used in the brewing industry but is seldom used elsewhere. Also, Tyler uses the term "pitch," which means to add yeast to the fermentation tank. He applies the $C_1V_1=C_2V_2$ equation as follows:

$C_1 = 120 \times 10^6$ cells/mL $V_1 = ?$ $C_2 = 12 \times 10^6$ cells/mL $V_2 = 700$ hL

Substituting into the equation:

$(120 \times 10^6$ cells/mL) (?) = $(12 \times 10^6$ cells/mL) (700 hL)

? = 70 hL

Tyler concludes: "So in the above example we would need to pitch 70hL of yeast to the 700hL of beer in order to achieve our target pitch rate."

The concept of "dilution" has a great many applications beyond biomanufacturing and in many situations the $C_1 V_1=C_2V_2$ equation is a valuable tool to handle these calculations. For example, carbon fiber products are used in the manufacture of many items, such as aircraft, bicycle frames, and sporting goods. "Sizings" is a term used for coatings that are applied to the surface of carbon fibers during the manufacturing process. A manufacturer of carbon fiber products provided the following example of a scenario that is conceptually much like the biomanufacturing scenario, but might appear different to a beginner:

> "The technician is asked to prepare a 1.5% concentration sizing bath (sizing is the chemical treatment applied to the surface of the carbon fiber product) by diluting a 20% resin/80% water mixture."

In this case, the desired volume of a 1.5% sizing bath is not specified and the technician would need to know what size is required for the application. The absence of this necessary piece of information means that contextual knowledge is required to perform the task. The term

"20% resin/80% water mixture" is used by the supervisor in this manufacturing setting. In a biotechnology setting, the terminology would usually simply be "20%" and water would be assumed as the solvent. Here, slightly different terminology might obscure the fact that the underlying concept is the same in the biomanufacturing and carbon fiber manufacturing situations.

The supervisor provided calculations for his carbon fiber manufacturing scenario based on a 50-gallon sizing bath:

> (20%) (?) = (1.5%) (50 gallons)
> ? = **3.75** gallons (amount of the original resin required)
> 50 gallons – 3.75 gallons = **46.25** gallons (amount of water required to get final 50-gallon volume)

Thus, the calculation tool used in the biotechnology and carbon fiber manufacturing settings is the same but the context, terminology, and assumed knowledge is different.