INTRODUCTION TO THE SPECTROPHOTOMETER AND PIPETTING SKILLS

Section A: Intro to the spectrophotometer

A commonly used instrument in the analysis of cellular extracts is the Spectrophotometer. Today you will learn how to use the Spectronic 20 spectrophotometer, commonly referred to as the spec 20. Take the time to understand what you are doing, just like microscopy; spectroscopy is a skill you can master in this lab. <u>You will use these machines a lot this semester, and will write up lab reports based on such work.</u>

This instrument manipulates the fact that compounds absorb or scatter more light at specific wavelengths depending upon the chemical structure of the compound. For example, most proteins that contain the amino acids tyrosine and tryptophan have a relatively intense ultraviolet light absorption at 280nm. Nucleic acids, DNA and RNA absorb at 280nm as well, but peak at 260nm. Spectroscopy is used for a wide range of purposes, to identify and determine concentrations of cellular compounds, chemical products, biochemical reactions and cell growth.

Standard curves are used to determine concentration of the compound of interest. They are created by making **dilutions** of a known initial concentration. The absorption of each dilution is measured at a specific wavelength. (absorbance v. concentration) Which wavelength is used? This is determined plotting the absorbance of one known concentration at different wavelengths, known as the **absorption spectra**, (absorbance v. wavelength). The peak of this graph is the wavelength to use.

Today, you will

- learn how to use a spec 20.
- learn how to pipet correctly.
- review graphing skills: plot and use an absorption spectra and standard curve.
- review the concept of error, precision and accuracy in experimentation.
- review the concept of serial dilution.

More specifically:

1. You will first take absorption readings of the stock solution at various wavelengths and plot an **absorption spectra**, in order to determine the wavelength to use for the rest of the exercise.

2. You will make dilutions of the stock solution and plot a **standard curve** of absorbance v concentration at the peak wavelength that you determined from the absorbance spectra.

3. You will calculate the **grams / liter** of the stock solution and all of the dilutions plotted on the standard curve. (To know the grams needed for a particular absorbance reading, or the concentration in grams, from an absorbance reading.)

4. You will **determine the grams/ liter of an unknown concentration** of the stock solution using the spectrophotometer 20 and standard curve that you created.

5. You will make **serial dilutions** of the stock solution and plot a standard curve of absorbance v concentration at the peak wavelength that you determined from the absorbance spectra.

6. You will calculate the **grams/liter** of each serial dilution by multiplying the dilution factor by the grams/liter of the stock which you calculated already.

7. You will compare the results of the standard curve and the serial dilution standard curve and reflect on the differences between them.

Familiarize yourself with the various parts of the spec 20 and its internal mechanism.

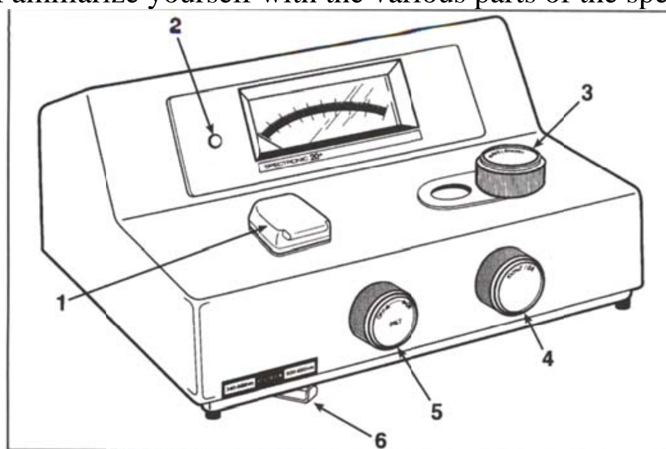


Figure 1-1 SPECTRONIC 20® spectrophotometer

KEY
1. Sample compartment
2. Pilot lamp
3. Wavelength control
4. Transmittance/Absorbance control (100%T/0A)
5. Power switch/Zero control
6. Filter lever

Figure.1 **The Spectronic 20.**

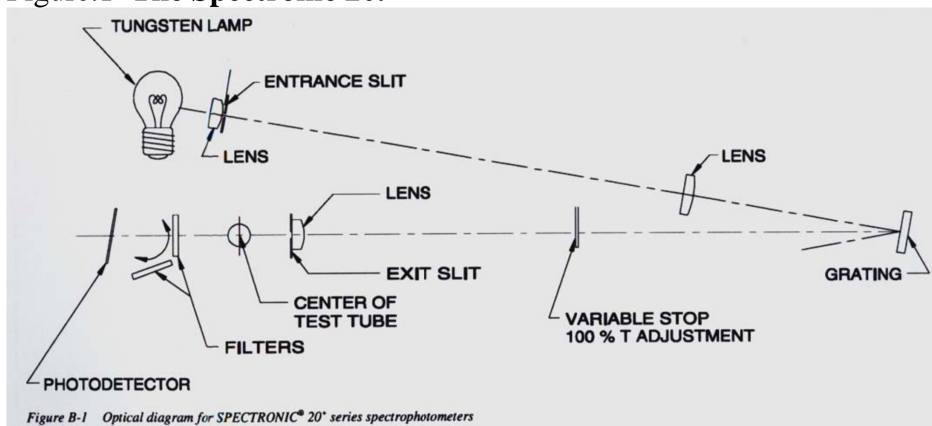


Figure B-1 Optical diagram for SPECTRONIC® 20® series spectrophotometers

Figure.2 **The internal mechanism of the Spectronic 20.**

The name of the dissolved compound: Methylene Blue

The Molecular Weight of the dissolved compound: 319.85 g/mol

How many grams are in one liter of a 1.0M solution? ______ (See *"Molarity "* in the appendix)

In a liter of a 0.1M solution?______________________________

In a liter of a 0.5M solution?______________________________

In a liter of a 0.02M solution?______________________________

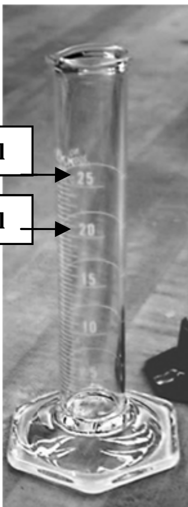
Part I. A. Turn on your spec 20 and let it warm up for at least 15minutes. During that time your instructor will review basic lab ware use, in particular how to use serological pipets.

Part I B. Solution Measurement, Containment and Transferring Review

1. Review the following types of lab ware: learn their specific names and functions. You should become familiar with the following if you are not already.

Beaker-often for work in progress (collection) where the wide mouth is useful, volume may be indicated but not the best for measurement.

Erlenmeyer Flask-good for pouring and preparing solutions. It is more controlled and limits evaporation, contamination or spillage and can hold a funnel well. Volume may be indicated but not the best for measurement. (*other types of flasks exist with modifications for particular purposes, i.e. vacuum flask, volumetric flask, …*)



Graduated Cylinder-the purpose is for measurement of volume. You should use one with the closest overall volume to that which you wish to measure to be most accurate. (*if you need 20ml it is best to use a 25ml cylinder not a 50ml*) If the cylinder in the picture on the left has a total measured volume of **25ml** and the next labeled line below it is 20ml, in between are 5 unlabeled lines, what is the volume between each line?

What then is the level of accuracy with this graduated cylinder?

Could you accurately measure 20.5 ml ?

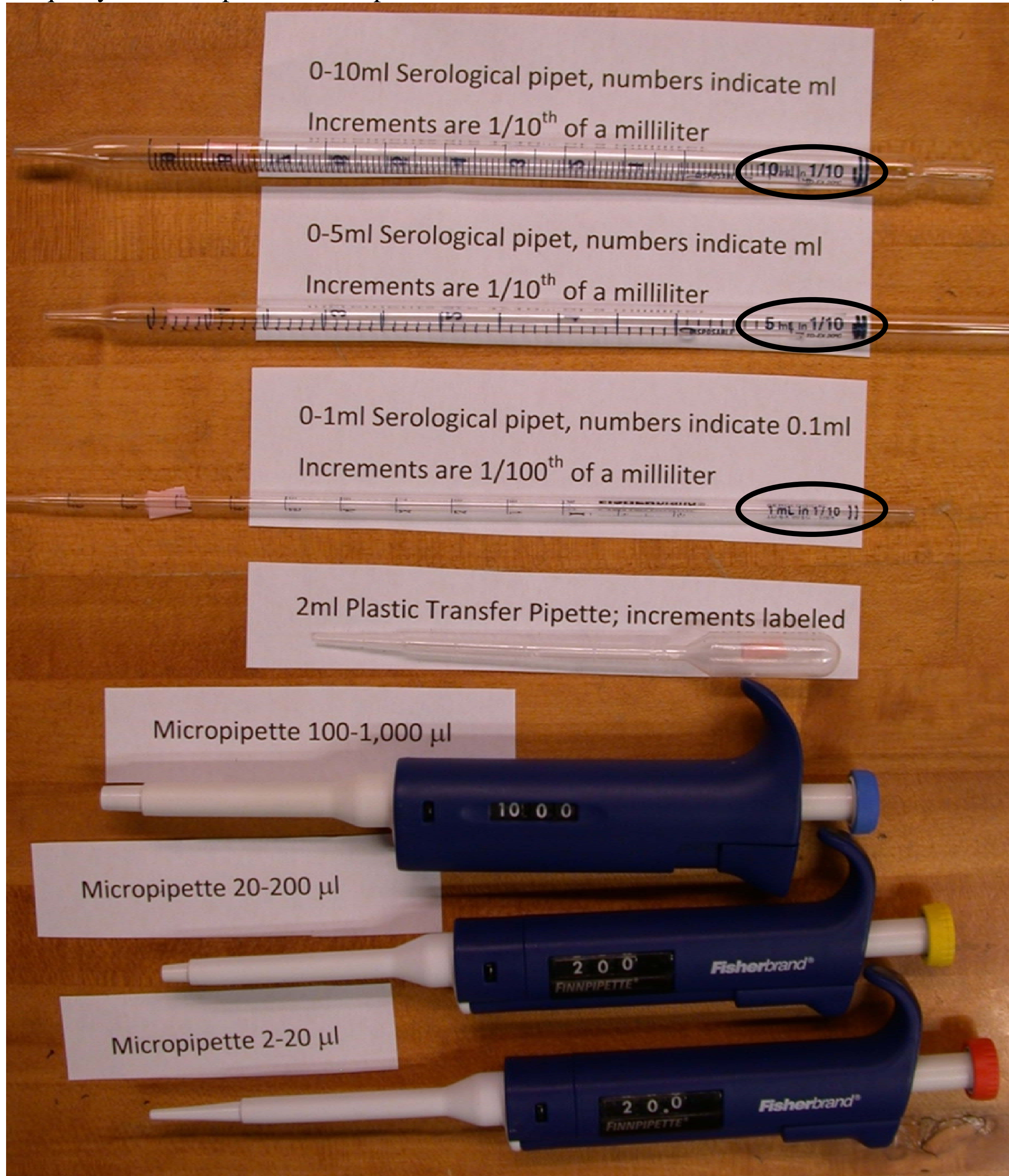
Pipettes are used to transfer smaller known amounts of liquid accurately. A variety of pipettes exist and they are simple to use once you know how, but you can incorrectly pipette so pay attention! Pipetting typically involves two things: a glass or plastic tube or tip and a device which creates vacuum used to draw up a known amount of liquid: a pump, bulb or micropipette.

In lab you will use and become familiar with: serological, disposable transfer pipettes and micropipettes. You should become confident in the use of each and able to choose the appropriate pipette to use when liquid transfer is needed.

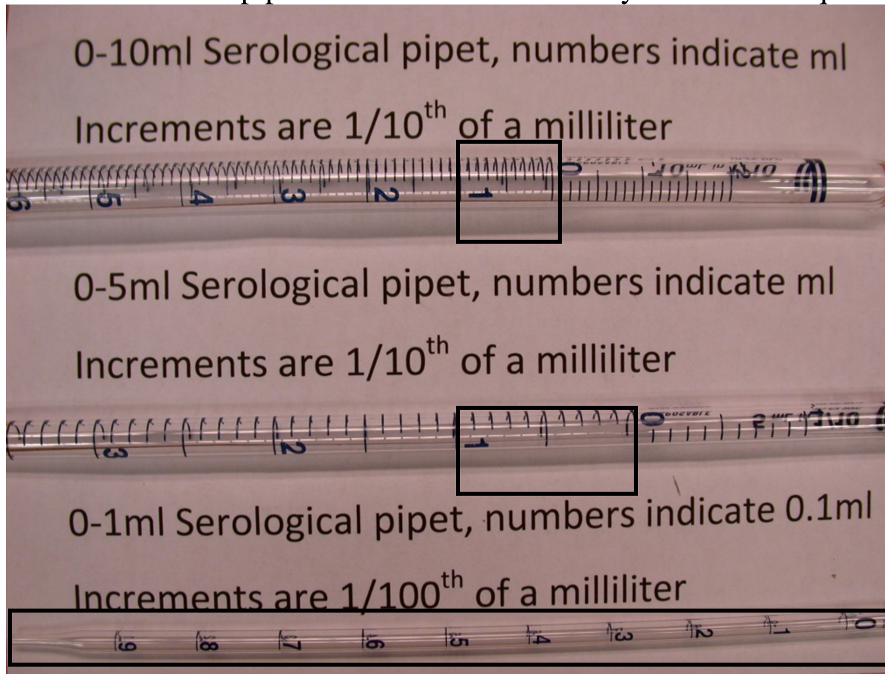
To pipette volumes less than one milliliter often involves micropipettes in which the operator sets the volume to transfer, attaches a tip, draws up and dispenses the liquid, then removes the tip.

Serological pipetting involves a larger glass pipette with gradations of measure and a pump or bulb which you attach. Like when using a micropipette, keep the apparatus vertical to keep the liquid in the pipette and out of the pump! Whichever type of pipette you use, keep it vertical as you draw up the liquid and the entire time until dispensed. Do not turn sideways or upside down. Otherwise you may not have the correct volume as you draw up and you will contaminate and possible break the pipetting device.

Serological glass pipettes come in different sizes. Below are three examples: a circle is drawn around that end of each pipette that indicates total volume and the finest incremental measure that is accurate. Like a ruler it has labeled gradations so that you can measure how much liquid you draw up into it, except it measures volume and the unit is milliliter (ml).



To become familiar with serological pipettes, look within each box drawn on the three pipettes below. The boxes start and stop at zero and one, you can see the volume is one milliliter. The larger the overall volume (10ml) the wider the pipet and the shorter the area that represents one milliliter. A narrower pipet allows for more accuracy with smaller quantities.



Part I C. Using a Serological pipet and pump (green goes with 10ml, blue with 1-2 ml)

1. Gently attach the appropriate pump to the appropriate pipette by grasping the pipet near the end that attaches; push and turn them into one another to create a seal. Be careful not to snap the pipet in half, in particular with 1ml pipettes. This is why it is important to hold it near where you place the pump as you create the seal.
2. Hold it vertically (always), place tip into stock liquid and with your thumb steadily roll the pump dial into action. You should see and feel that the liquid is being drawn up into the pipet. If your seal is ok it will hold the liquid in place when you stop and lift it out of the fluid. If it leaks, dispense and tighten the seal. Otherwise, double check your volume and dispense into the container you wish to transfer to by rotating the pump dial in the opposite direction. The dial gives you control over how much you draw up or dispense. Keep the pipette and pump vertical until no liquid is left inside of it, then remove the pipette and put it in the dirty pipette bin. If you are to reuse it with the same liquid, take care not to contaminate the tip that goes in and out of the liquid.
3. Take the time to look at the pipettes and understand how to read and use them.
4. **Label a spec 20 test tube "stock" and pipet 5ml of the stock solution into it.**
5. **Another person in your group should label a spec 20 tube "blank" and pipet 5ml of water into it, be sure to change pipets as you change solutions. The two tubes should have the same volume, compare them.**

6. If you have questions ask your instructor to check that you are pipetting correctly. To be sure the spec is warmed up continue to practice pipetting but now look at micropipettes and follow Part ID.

Part I. D Using a Micropipette –Always use with a tip!

Micropipette use is different in that it draws up all of a set volume in one motion and then dispenses it in a second motion. You must set the desired volume on the micropipette, attach a tip and manipulate the top of it in order to draw up and then dispense all of it plus a little air to ensure it all gets out.

This is why people incorrectly use the micropipette: as you press the top of the micropipette down you are pushing out the volume you will draw back up. If you go too far and that is possible every time, you will draw up the wrong amount. Be gentle since you must discern the first stop from the very end. The first resistance you feel is called the first stop and you must stop there or you will draw up an inaccurate amount of liquid.

1. Find the correct size range micropipette. Set the volume by turning the top of it, if it has a lock unlock it first! Do not go beyond the limits of the pipette or you will break it! If it is too hard to turn STOP turning!
2. Attach the correct size tip by pressing the micropipette down onto a tip to seat and seal it.
3. Hold the micropipette vertically; steadily push the top of the micropipette down until you feel the resistance of the first stop then place the tip into the liquid but only the end of the tip, do not submerse the entire tip in the liquid!
4. With slow controlled motion, gently release the top. When it is fully returned to its normal position wait for a second to be sure the liquid is fully draw up, then remove the tip from the stock liquid. Continue to hold the micropipette vertically.
5. Hold the micropipette so the tip is above or in the container you want to dispense into and once again steadily push the top of the micropipette down until you feel the resistance of the first stop but this time go beyond and push all of the sample out. Wait a second before you remove the tip, keeping your thumb pressed down, lift the tip out of the liquid if it was submersed.
6. Practice this a few times with different size micropipettes and use tubes that allow you to see the amount that you did transfer. Transfer the following into 5 different tubes: 1000µl, 300 µl, 100 µl, 50 µl, 15 µl. Note you need 3 different micropipettes to do this. Really look at how much is in the tube to develop a feel for the volumes.
7. Show your TA your tubes as well as demonstrate that you can do this by pipetting a 6th tube in front of your TA.
8. More information on micropipette use is found in the appendix. Refer to it.

Part I E. Absorbance Spectrum: readings of one concentration at various wavelengths.
Getting ready:

1. Your spec 20 should now be warm up: find your labeled "stock" spec 20 test tube that you pipetted 5ml of the stock solution into and the blank tube.

2. After you zero the Spec 20 light transmission, you will use this tube to blank the machine: telling it that the absorbance of the solvent that the compound is dissolved in, which in this case is water, equals zero.

3. Your initial wavelength will be 450, the end wavelength will be 850, and the increments in between are 25nm, as listed in the table below. (when you reach 600, remember to put the filter in place)

Collecting data:

*4. Set the wavelength and use the left knob to adjust the needle to 0% transmission/infinite optical density, which is the left most mark.

*5. Zero the spec 20: put the blank into the sample chamber, close the cover and adjust the needle to 100% transmission/0 Optical density, with the right-hand knob. Remove the blank.

6. Insert the tube labeled "Stock", close the sample chamber and record the absorbance reading into the chart below. Remove the "stock" tube, and close the sample chamber.

7. You now have a reading at one wavelength, to get the next reading, change the wavelength and repeat steps 4, 5 and 6 for as many wavelengths as your instructor tells you. (Zero the machine between every wavelength.)

Wavelength (nm)	(Absorbance) optical density(OD)
1 450	
2 475	
3 500	
4 525	
5 550	
6 575	
7 600 *filter	
8 625 *filter	
9 650 *filter	
10 675 *filter	
11 700 *filter	
12 725 *filter	
13 750 *filter	
14 775 *filter	
15 800 *filter	
16 825 *filter	
17 850 *filter	

1. Plot your results, absorbance (optical density) against wavelength.

2. Record the peak wavelength_________________________.

Part II. A Standard Curve: one wavelength and dilutions of the stock solution.

1. **Each student in your group must** label five test tubes and prepare each according to the chart below. Cap each tube and invert to mix the stock and water, wipe the tubes to keep them clean. Each student must use the spec 20 and will record their own data but the group will share the set of data.

Tube	Stock (ml)	Water (ml)	Molarity	grams/L	OD student 1	OD student 2	OD student 3
1	**5.0**	**0**	**3.5×10^{-5} M**				
2	**4.0**	**1.0**					
3	**3.0**	**2.0**					
4	**2.0**	**3.0**					
5	**1.0**	**4.0**					
10 * ____	Unknown sample **5.0**	**0**					

*Record the label of your unknown in the table above.

2. Set up the Spec 20 using the peak wave length from the absorbance spectrum and zero it with your water blank. You will not be changing the wavelength.

3. Record the absorbance of each standard tube and the unknown tube (#10) in one of the columns labeled “OD student #” of the chart above. Then do the calculations for the rest of the chart.

4. Calculate the molarity of each standard tube, convert it into grams/ liter. Review how to do this by reading the "Molarity" portion of the appendix and consult the instructor if you need help.

5. Using Excel, calculate the average absorbance reading and the standard deviation for each tube of the standard curve as well as the unknown sample. This shows you the error of technique within your group. What are the potential causes of error?

6. **Create the standard curve**:

   a. Plot the absorbance (OD reading) against the concentration of the stock solution (tubes 1-9) using the averages calculated.
   b. Insert a trendline and show the equation of the graph.
   c. Insert vertical error bars using the standard deviation (refer to the excel lab if you forgot how to do this). The error bars in this graph in step indicate the variation in your group’s pipetting technique.

7. Comparing Pipetting consistency:

a. Use a scatter plot to graph each group member's absorption readings v concentration separately on one graph. The consistency of pipetting by each person is seen by plotting your individual data, each as a series.
b. Insert a trendline for each series (each student's set of tubes). Show the equation and the R-value for each line. The closer the R-value is to 1.0 the more consistent or precise (reproducible) the pipetting.

8**. Can you be precise but still inaccurate? Specifically:**

a. What kind of experimental error would lead to alterations in the R-value?
b. What kinds of experimental error would affect the standard deviation?

9. **Determine the concentration of the unknown sample.** Using the standard curve, determine the concentration of your group's unknown sample.

a. Plot the average absorbance reading point on the best fit line of the standard curve.
b. Estimate the concentration by drawing a straight line to the axis.
c. Double-check this using the linear equation of the standard curve. Record the concentration in the chart above. Hand in all graphs, and have the instructor check your work before you leave.

Part II B: Serial Dilution Standard Curve

Serial dilution provides a method to reduce a concentration of a solution or cell culture while providing a mathematical framework to know the diluted concentration endpoint. When working with dilutions the concentration of original solution is kept track of by using a dilution factor. Simple dilution factors are calculated using such a formula:

$$D = \frac{V_1}{V_2}$$

V_1 is the volume of stock solution or cell culture being diluted and V_2 is the combined volume of the stock and diluent making up the dilution.

For example, if you added 1ml of stock solution to 9ml of water (diluent) the calculation of the dilution factor is:

$$D = \frac{V_1}{V_2} = \frac{\text{1ml stock}}{\text{1ml stock + 9ml water}} = \frac{\text{1ml}}{\text{10ml}} = \frac{1}{10} = 10^{-1}$$

Serial dilution typically involves multiple reductions in the proportion of the original stock solution or culture, compounding the dilution by that of the previous dilution factor.

$V_1D_1=V_2D_2$

V_1 is the intial volume you plan or need to use of the solution you wish to dilute, D_1 is the initial dilution of that starting solution you wish to dilute. V_2 is the combined volume of the stock and the diluent. D_2 is the end dilution factor. ($V_1C_1=V_2C_2$ is also used the C would be concentration instead of dilution)

For example, if you further diluted the previous example by using 1ml of the dilution created in 9ml of diluent the calculation of the dilution factor, $\mathbf{D_2}$ is:

$$D_2 = \frac{V_1 D_1}{V_2} \quad \text{or} \quad D_2 = \frac{V_1}{V_2} \times D_1 \qquad \text{So, } \mathbf{D_2} = \frac{1\text{ml}}{[9\text{ml}+1\text{ml}]} \times 10^{-1}$$

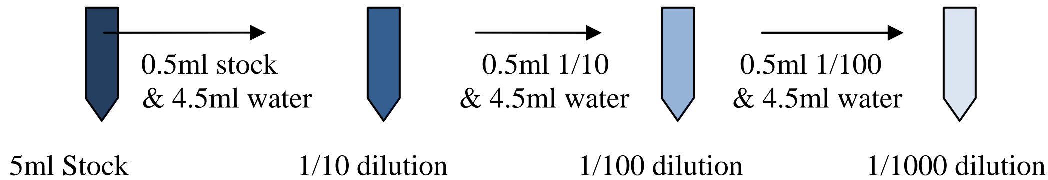
So, the example above is calculated

$$\mathbf{D_2} = \frac{1\text{ml}}{10\text{ml}} \times 10^{-1} = 10^{-1} \times 10^{-1} = 10^{-2}$$

If instead of a dilution factor you had a different unit of concentration, molarity for example, the relationship remains the same and you could solve for an unknown concentration.

Serial Dilution Standard Curve Procedure

1. Each student in your group must gather and label 5 test tubes and pipette following the table below. You will each make the following serial dilutions: 1/10, 1/100 and 1/1000. You use a portion of the previous dilution to make the next as depicted below:



2. Take absorbance readings using the spec 20 at the same OD that you used for your standard curve (peak wavelength of the spectrum from part I). Use serological pipettes for the 4.5ml and 5ml quantities but use a micropipette for the 500µl transfers. **See Part II. C** for how to use a micropipette and be sure to check with your TA that you can use it properly. Calculate the g/liter for each tube by multiplying that of the stock concentration by the dilution factor of each tube.

Sample	**Methyl blue**	**Water**	**g/liter of each dilution**	**OD student 1**	**OD student 2**	**OD student 3**
Blank	0 ml	5ml	0			
Full strength	5ml	0				
1/10	500µl stock	4.5ml				
1/100	500µl of 1/10	4.5ml				
1/1000	500µl of 1/100	4.5ml				

3. Using Excel, calculate the average absorbance reading and the standard deviation for each tube of the standard curve of your serial dilution. Determine the error of technique within your group. What are the potential causes of error? What are the differences between your serial dilution standard curve and the standard curve you first made?

4. **Create the serial dilution standard curve**:
    a. Plot the absorbance (OD reading) against the concentration of the stock solution using the averages calculated.
    b. Insert a trendline and show the equation of the graph. How does it compare to the standard curve you made in Part II A? Is the slope similar?
    c. Insert vertical error bars using the standard deviation (refer to the excel lab if you forgot how to do this). The error bars in this graph in step indicate the variation in your group's pipetting technique.

5. **Comparing Pipetting consistency of the serial dilution:**
    a. Use a scatter plot to graph each group member's absorption readings v concentration separately on one graph. The consistency of pipetting by each person is seen by plotting your individual data, each as a series.
    b. Insert a trendline for each series (each student's set of tubes). Show the equation and the R-value for each line. The closer the R-value is to 1.0 the more consistent or precise (reproducible) the pipetting.
    c. Does the pipetting consistency differ between the serial dilution standard and the standard curve from Part IIA?

6. **Determine the concentration of the unknown sample.** Using the standard curve, determine the concentration of your group's unknown sample.
    a. Plot the average absorbance reading point on the best fit line of the serial dilution standard curve, is it different from the other standard curve you made?
    b. Estimate the concentration by drawing a straight line to the axis.
    c. Double-check this using the linear equation of the standard curve. Record the concentration in the chart above. Hand in all graphs, and have the instructor check your work before you leave.

7. **Calculate the dilution factor for each tube from the standard curve in Part II A. Refer to the previous page for the formula.**

Tube	Stock (ml)	Water (ml)	Dilution factor
1	**5.0**	**0**	
2	**4.0**	**1.0**	
3	**3.0**	**2.0**	
4	**2.0**	**3.0**	
5	**1.0**	**4.0**	