

## CHEM 461 – Lab #1

### Essential Laboratory Skills: Solutions, Dilutions and Beer's Law

Students will work individually on this experiment.

#### Introduction

During a typical working day, biochemists (and many other scientists) will prepare and use a number of solutions. Many days they will also perform one or more colorimetric assays which rely on Beer's Law. All of these topics are covered in "freshman" chemistry. However, one of the oddities of our educational system is that these essential laboratory skills are only touched upon in the lecture. This laboratory exercise is designed to jog your memory about the topics and to begin the process of moving "theoretical" knowledge from your brain cells to your muscle cells where they must reside in order to be a successful bench scientist in biochemistry and related fields.

In preparation for this lab, please take the time to refresh your brain cells by picking up a general chemistry book and reviewing the sections on the concentration of solutions (especially molarity), dilutions and Beer's Law.

Another key laboratory skill is the habit of planning ahead. Successful scientists spend time at the desk planning the details of an experiment before heading to the lab. Calculations of quantities to be measured (mg of this, mL of that) and protocols laying out each step of the experiment thought about and written down in the lab notebook ahead of going to the lab drastically cut down on errors and make time in the laboratory more productive.

#### Part A – Solutions and Dilutions

Most working scientists prepare stock solutions of various compounds and then mix and dilute these stock solutions to prepare working solutions that are actually used in an experiment.

For example, if one needed a solution for an experiment which contains 100 mM NaCl and 5 mM MgCl<sub>2</sub>, an efficient scientist would probably prepare stock solutions of 1 M NaCl and 100 mM MgCl<sub>2</sub> (experience tells one that these compounds are both stable and soluble at these concentrations) by weighing out an appropriate amount of the solid and dissolving it in the correct quantity of water. (Obviously, one would need to decide what volume of the stock would be appropriate based on the needs of the experiment and lab in general.) Once these stocks were prepared one would make the desired volume of working solution by diluting the appropriate volumes of each stock with water. Thus for the example above (i.e. 100 mL of the 100 mM NaCl, 5 mM MgCl<sub>2</sub> solution) one would mix 10 mL of the 1 M NaCl stock, 5 mL of the 100 mM MgCl<sub>2</sub> stock and 85 mL of water.

A big advantage of working from stock solutions is flexibility. For instance, if one needed a set of four solutions all of which contain 100 mM NaCl and either 0, 1, 2.5 or 5 mM MgCl<sub>2</sub>, one would need to weigh out appropriate amounts of NaCl four times and MgCl<sub>2</sub> four times for a total of eight different trips to the balance. Using the stock solution approach as outlined above one would need to weigh out each compound only once (to make two stock solutions). These stocks would then be used to make the four working solutions. The latter approach is much more efficient.

Another advantage of stock solutions is that you can prepare small volumes of solutions, where it is physically impossible to weigh out the required amount of solute. The best balances commonly available are only accurate to 0.1 mg and it is generally best to never weigh less than 1 mg of a solid.

Thus, if one needed 200  $\mu\text{L}$  of 100  $\mu\text{M}$  cAMP you would need to weigh out 0.00742 mg (the MW for the disodium salt is 371) of the solid which could not be done. To make the desired solution, one could make a 2.0 mM stock solution (1 mg /1.35 mL) and then dilute this out appropriately (10  $\mu\text{L}$  stock + 190  $\mu\text{L}$  water) to get what one needed. Experience tells us that this stock solution is stable for a month or so if one stores it at  $-20\text{ }^\circ\text{C}$  (i.e. in a regular freezer). Thus, one might store it away to save effort (and money, as cAMP is not cheap) for the next time one needed a similar solution.

Your goal for Part A is to prepare four “working solutions” containing  $\text{CuSO}_4$  and sodium p-nitrophenolate. You will be provided with a stock solution of sodium p-nitrophenolate but you will need to prepare your own stock solution of  $\text{CuSO}_4$ .

You will turn the working solutions into your instructor in labeled and covered tubes for grading. Accuracy will count!

You must arrive for lab with all of the quantities you will need pre-calculated so that you can get to work straight away. (Turn in a copy of your work sheet showing all of the calculations at the beginning of the lab.)

#### I) Stock Solutions

A) Buffer (20 mM borate, pH 9.0) – made for you

B) 0.5 mM Sodium p-nitrophenolate (Na p-NP) in water – made for you

C) 0.1 M  $\text{CuSO}_4$  in water – each lab group will need to make 10 mL of this solution; solid  $\text{Cu}_2\text{SO}_4 \cdot 5 \text{H}_2\text{O}$  will be available.

Show all the calculations needed to prepare this solution in your lab notebook. Turn in a copy of these calculations at the beginning of the lab period.

#### II) Working Solutions

Prepare each of the following solutions by dilution of the stock solutions given above:

Solution A: X mL of Y mM  $\text{Cu}_2\text{SO}_4$ , Z  $\mu\text{M}$  Na p-NP in buffer

Solution B: X mL of Y mM  $\text{Cu}_2\text{SO}_4$ , Z  $\mu\text{M}$  Na p-NP in buffer

Solution C: X  $\mu\text{L}$  of Y mM  $\text{Cu}_2\text{SO}_4$ , Z  $\mu\text{M}$  Na p-NP in buffer

Solution D: X  $\mu\text{L}$  of Y mM  $\text{Cu}_2\text{SO}_4$ , Z  $\mu\text{M}$  Na p-NP in buffer

Please note: each student will be assigned different values of “X”, “Y” and “Z” for each solution.

Make sure to label each tube with the name of the solution (i.e. "A", "B", etc.), your name and the date.

Turn in all of the solutions you prepare at the end of the lab period.

All calculations need to prepare these solutions must be shown in your lab notebook and you must turn in a copy of these lab notebook pages at the beginning of the lab period.

### **Part B – Colorimetric Assays (The Bradford Assay for Total Protein)**

Quantitative analysis (the determination of the amount of something) is very common in the biochemistry lab. There are a large number of methods for doing this type of analysis. One of the most common methods is the colorimetric assay where one uses a spectrophotometer to determine the absorption of light by a colored analyte (or analyte derivate, for analytes that are not intrinsically colored). Colorimetric assays are generally quick and inexpensive and are generally at least moderately sensitive.

Colorimetric assays rely on the fact that the higher the concentration of a colored compound in solution is the deeper (or more intense) the color is. Think Kool-Aid... the more powder you put in the glass of water the deeper the color gets. Quantitatively, this relationship is described by Beer's Law which basically says that (over at least a limited concentration range) the absorbance of a solution of a colored compound is directly proportional to the concentration of the compound.

Performing a colorimetric assay involves two parts: the construction of a "standard curve" (also called a "calibration curve") and the analysis of "unknowns" (i.e. the samples whose concentration you want to know about). The standard curve is then used to infer the concentration of analyte in the unknowns.

One "problem" with colorimetric assays is that they require "colored" (i.e. absorbing in the visible or UV regions of the spectrum) analytes and many "interesting" analytes are not colored. Additionally, in a complex mixture (say a biological or environmental) sample there might be more than one absorbing compound and thus specifically analyzing a single component becomes a problem. Thus colorimetric assays often involve a derivitization step where the sample (both the standards and the unknown) are incubated with a reagent that reacts with the analyte to make a colored product.

In the case of the Bradford's assay for total protein, the sample is incubated with a dye, Coomassie Blue G-250 in an acidic solution. The absorbance maximum of the dye bound to protein is different that the dye free in solution. Thus, the absorbance at 595 nm in this system follows Beer's Law.

Your goal in Part B is to determine the concentration of protein in each of two unknown samples. As in part A, you will need to prepare ahead of time and come to lab with all of the quantities needed for the standard curve figured out ahead of time.

One question that will arise as you prepare, is "How much should I dilute out the unknowns?" This is important because in order for the experiment to be valid, the concentration of protein (and therefore the absorbance) of the unknowns must be in the same range as the standards. The answer here (as it often is in "real life") is "I don't know." So how do you proceed? Simple, just do something and see what happens! In this case, dilute out

the sample (use a nice and round ratio such as 1:10 or 1:5 or 1:2, etc.), run the assay, see what the result is and react accordingly.

There are three possible results: 1) the absorbance of the unknown could be less than your lowest standard, 2) the absorbance could be between your lowest and highest standards or 3) the absorbance could be greater than your highest standard. If you have obtained the second result you might want to play the lottery that day because you got lucky. You guessed right and are done! If you got either of the other two results, adjust your dilution based on the result you obtained and rerun that sample. If you still do not obtain the second result, make further adjustments to the dilution and rerun the unknown again. In this case, you might also want to stay away from the lottery!!!

(Note: make sure that you keep the incubation time roughly constant. That is, if you have read the absorbance of your standards 10 minutes after adding the reagent, don't wait 30 minutes before reading the unknowns, read them at about 10 minutes also.)

Use the data sheet shown below (reproduce it in your lab notebook) to help organize your thoughts.

Figure out all of the volumes needed for the standard curve before coming to lab and turn in a copy of your calculations at the beginning of the lab period.

You will need to write a short lab report that includes the standard curve for the Bradford and the concentration of protein in each unknown sample.

*Solutions Provided:*

1 mg / mL bovine serum albumin (BSA) – standard

Dye Reagent Concentrate (as it comes from the manufacturer)

Unknown solutions (two per group; make sure to record their identity)

*Microassay Procedure* (slightly modified from BioRad's literature)

1. Prepare five dilutions of a protein standard which is representative of the protein solution to be tested. The linear range of the assay for BSA is 1.2 to 10.0  $\mu\text{g/ml}$
2. Pipet 800  $\mu\text{l}$  of each standard and sample solution into a clean, dry test tube. Protein solutions are normally assayed in duplicate or triplicate.
3. Add 200  $\mu\text{l}$  of dye reagent concentrate to each tube and vortex.
4. Incubate at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour.
5. Measure absorbance at 595 nm ( $A_{595}$ ).

Notes:

The concentration (and thus the absorbance) of your unknowns must be in the same range as your standards. You will need to dilute the unknown solutions before assaying them. Therefore, you will also need to take the dilution into account when you report the final results.

Prepare and assay each sample (standards and unknowns) in triplicate.

Use a spreadsheet to do all of the calculations. Print out a copy of this spreadsheet and tape or staple it into your lab notebook. Also make sure to note the file name in your notebook.

Prepare a standard curve by plotting  $A_{595}$  vs. the [BSA] and fitting this data to a straight line using linear regression.

Use the slope and intercept of the standard curve to calculate the concentration of protein in each unknown sample. Remember to take into account the dilution you made before reporting the final result.

Significant figures are important!

Data Tables for Bradford Assay (an example)

Standards:

Tube	[BSA]*	$\mu\text{L BSA}^\dagger$	$\mu\text{l H}_2\text{O}$	Total Volume ( $\mu\text{l}$ )	
A	0	0	800	800	
B				800	
C				800	
D				800	
E				800	
F				800	

\* mg/mL      † 1 mg / mL stock

Unknowns:

Tube	Sample ID	$\mu\text{L sample}$	$\mu\text{l H}_2\text{O}$	Total Volume ( $\mu\text{l}$ )	Dilution Factor*
G				800	
H				800	
I				800	
J				800	
K				800	
L				800	

\*  $\mu\text{L sample} / \text{total volume } (\mu\text{L})$