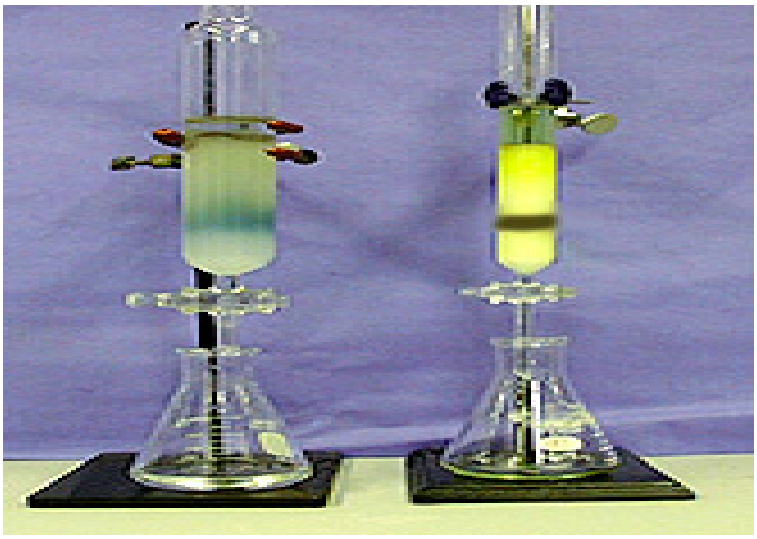
College of the Canyons: "Introduction to Biotechnology" Custom Lab Exercises



Gel Filtration: AKA Column Chromatography

Version 05-07-12

- **Chromatography is used by scientists to separate one substance from another. All chromatography rely on the "differential attraction" to one of two phases. Think about HPLC. The stationary phase was non polar, the liquid phase was a series of alcohols variable polarities. The dyes in Grape Kool Aid were eventually pulled off the non-polar column once a non-polar enough solvent was used.**
- **There are dozens of chromatographic techniques that rely on this "differential affinity".**
- **In this experiment you will use gel filtration to separate a mixture of protein and salt.**
- **A plastic column is filled with tiny porous beads. Small molecules running through the column temporarily get trapped in the pores in the beads. Big molecules do not fit in the pores and flow between the beads and therefore exit the column before the small molecules. The process is largely physical, in that the beads act like a type of filter, hence the name "gel filtration". It has been called chromatography due to its similarity to separation seen in chromatographic techniques.**
- **The experiment will be *repeated* to help clarify the role of various factors on data accuracy as well as expand on the technique of gel filtration.**
- **Purification of molecules based on size is used in the manufacture of foods and medicines as well as in scientific research and medical labs.**

For more information on College of the Canyons' Introduction Biotechnology course, contact Jim Wolf, Professor of Biology/Biotechnology at (661) 362-3092 or email: jim.wolf@canyons.edu. Online versions available @ www.canyons.edu/faculty/wolfj

I. OBJECTIVES:

1. To separate a protein from salt using a Sephadex column.
2. To understand the principles of gel-filtration.
3. To examine factors influencing data accuracy in colorimetric and other quantitative analysis.
4. To refine gel filtration techniques by repeating essential elements of the experiment as directed.

II. BACKGROUND

In the first experiment, you separated the colored dyes in Grape Kool-Aid using a C_{18} HPLC column (AKA Sep-Pak). You learned that dye molecules could be separated because one dye was more polar than the other. Another method of separating mixtures is using gel filtration, which separates molecules according to their size. The chromatography column in this case is filled with microscopic beads with pores running through them. As a liquid solution containing a mixture of different sized molecules flows through the column, molecules small enough to enter the pores in the beads move slowly within beads and from bead to bead, while molecules too large to enter the pores travel more quickly between and around the beads. Since larger molecules are excluded from the interior of the beads, the separation technique is sometimes called **size-exclusion chromatography**. As mentioned on the cover sheet, this activity is more correctly called a filtration technique. Filtration is the sieving of particles under pressure. The pressure is gravity and the small spheres act as "reverse" filters" holding the smaller particles back while the larger particles move past (opposite of how a coffee filter works). It has acquired the name chromatography as it is similar in many respects to many chromatographic techniques in results and set-up. On a molecular level, the idea of filtration (which is a physical process) becomes blurred with chromatography (which is a chemical process). As a result, the process has acquired a number of names and in-the-end, it is an example of scientists falling victim to giving "common names" to a process that is not entirely correct (using common names to name organisms is the best example, with many animals having dozens of common names, but only one scientific name).

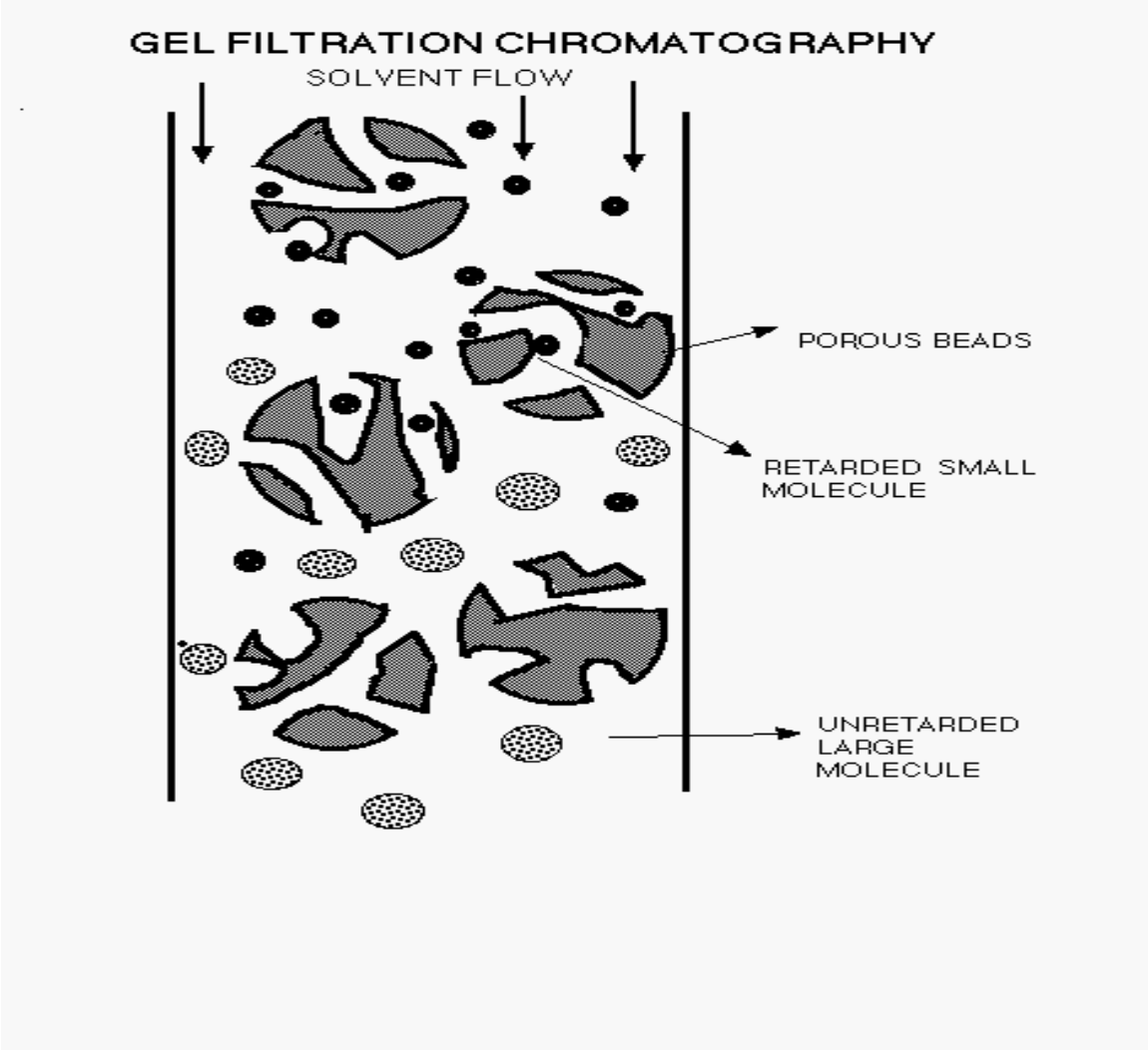
Sephadex (the commercial name for the beads used in this lab) and similar products are available with many different sized pores, so that a scientist can select a pore size just right for a particular job. To confound issues further, sometime these beads are coated with functional groups that can attract or repel other functional groups seen on the surface of misc. molecules (i.e. proteins, sugars, etc.). So this type of bead would have both filtration like elements (with its pores) and a chromatographic elements (with the functional groups and the subsequent attraction/ repulsion aspect). Gel filtration is important because it can separate molecules gently so that they are undamaged for further study. Gel filtration is used in research, industrial testing, and medical laboratories. It plays a key role in the purification of enzymes and other proteins, polysaccharides, nucleic acids and other molecules.

In today's experiment, you will be using a pre-packed Sephadex column to separate a protein from the salt solution in which it is dissolved. The protein is albumin from hen's egg, dissolved in a solution of NaCl. Separating a protein from a salt is called "desalting," a common procedure. For example, a scientist may wish to purify certain proteins from blood. Blood contains salts similar to those in sea water, so one of the first tasks in purifying blood proteins is

to free them from the salts. The albumin molecules are thousands of times larger than the sodium and chloride ions present in solution. Both the salt and the protein are colorless, so detection will involve the use of a conductivity meter and colorimetric assay. Since NaCl ions conduct an electrical current, you will detect the salt quantity by measuring the degree of electrical conductivity in each sample. In the Bradfords's assay for protein, a small amount of a red indicator solution is added to each sample. If no protein is present, the solution turns brownish or a light blue. If protein is present, the solution turns an intense blue because the indicator color is changed as it binds to a protein.

Colorimetric analysis is a very common method to quantify proteins. Visual assessment of the resulting colors will be used in the first phase of this experiment. A color chart will be provided as a way of quantifying the albumin. Based on preliminary data, the separation of the salt and protein will be graphed. This graphical approach will be used to further investigate how a gel filtration column is used to separate proteins of different sizes. The data should then be discussed with the instructor and the second phase of the experiment undertaken. The following protocol has BOTH experiments outlined. ***To encourage a sense of discovery, it is very important you complete the first phase of the experiment entirely. After the first phase is completed, proceed with the protocol as directed to in SOP: Phase Two.***

Diagram of a Gel Filtration Column in Process of Separating Salt from Albumin (not to scale).



III. SOP/Lab Activities: AKA PHASE ONE:

Important: Always note any addenda that are posted by the instructor.

1. Check-List of Materials for a Lab Station

A. _____ 12, 2.0 ml microfuge tubes (round bottom)
B. _____ marking pen
C. _____ test tube rack (long, linear to allow for easy fraction collection).
D. _____ Sephadex G-25 column mounted on support/ring stand.
E. _____ 2 plastic transfer pipets
F. _____ albumin/NaCl solution: 1.8 ml in a plastic tube
G. _____ Bradfords reagent in a plastic tube
H. _____ conductivity/salt meter
I. _____ cup to clean electrodes, for waste from the column
J. _____ squeeze bottle of deionized water
K. _____ Bradford color chart

2. PROCEDURE - PRELIMINARY STEPS:

A. Fill the squeeze bottle with deionized water.

B. Remove top and bottom caps from the column, and let the deionized water left in the column during storage drain out of the column into a waste cup. Place the caps where they won't be lost. **STOP the column flow when the liquid is just below the bead bed. This step is VERY important, so ask if not sure. The liquid should slowly drain and once is has disappeared below the top level of the column contents (AKA bead bed). Once this occurs, place the bottom cap on and move on to the later steps.**

Regarding adding samples to bead bed. This idea is an extension of "Bernoulli equations". These are observations of a physicist (Bernoulli) who did work on water flow. By adding slowly, you avoid turbulence and thus can attain laminar flow (movement of particles in a side by side manner, as opposed to chaotic, where particle move in a erratic direction). This "laminar flow" then allows for the samples of liquid to move through the bead bed in a very slow, controlled and more predictable manner. As the liquid moves through the bead bed, the first fractions in will be the first fractions out and the only thing holding back or altering the movement of the "parcels" through the bead bed is their interactions with the beads. Chaotic flow or other issues can alter the flow and thus, cause less reliable separation.

NOTE: If you column is dripping EXTREMELY slowly, you may have a clogged column. All columns drip slowly (as a fast drip would not allow for adequate separation). This said, really slow columns may suggest the column is indeed clogged. This happen because columns get some protein and salt left behind, and this is an ideal surface area for bacteria. Trouble is, you can not predict exactly when this will happen. The best you can do is to keep your eyes peeled and discard columns once they get clogged. So….keep your eyes peeled for clogged columns and ask if not sure…

C. Label the 10 test tubes 1 to 10, and line them up in the test tube rack.

3. THE SEPARATION

A. When the column has drained (again, just below the bead bed), carefully position the test tube rack with its marked test tubes directly under the column. Test tube #1 should be ready to collect the first drops from the column.

B. Using your transfer pipette **gently transfer** one ml of the albumin/NaCl mixture to the top of the column. SLOWLY dribble the mixture down the interior side of the column. Try to minimize disturbing the column bead bed. This step is also **VERY** crucial to overall success. If added quickly or turbulently, the bead bed may be disturbed. This can **REALLY ADVERSLY** affect results. See post labs questions for more insights into this idea).

C. Remove the cap and start to collect 19 drops (0.9 ml) in tube #l and then move on to tube #2.

D. Continue to collect 19 drop samples in the correct sequence in the test tubes. When the column stops dripping see step E.

E. By the second or third tube, the entire original sample will have entered the beads, and the column will stop dripping. At this stage, carefully add deionized water from the squeeze bottle onto the column to a height of about three centimeters. Keep counting 19 total drops per tube. When you have collected more drops and the column has again stopped dripping, carefully add more deionized water onto the column to fill it. Once you have collected all ten 0.9 ml (19 drops) samples, replace the caps on the column, trapping the remaining water in the column for storage. Be sure there is at least one centimeter of deionized water stored in the column.

4. TEST FOR ELECTRICAL CONDUCTIVITY TO INDICATE THE PRESENCE OF NaCl.

Note: The salt meters are old and cranky. Trouble is, new ones are VERY hard to find and few probe models are small enough to fit into the narrow opening of a 2.0 ml microfuge tube. So, be patient and treat your salt meter with tender, loving care. You may need to swap out meters and trade with someone who has a working unit that gives reliable values. Ask the instructor for clarification if needed.

A. Close tube # 1, invert it 2 or 3 times to mix, then immerse the conductivity meter electrode into the liquid. Read and record the salt concentration (mg/oz) on your data table. If the meter jumps between two values, record the average of the two.

B. Rinse the electrodes off by squirting deionized water from the squeeze bottle over them. Let the drops fall into a waste cup. Gently shake off clinging water drops.

C. Measure and record the salt concentration for each of the other tubes by repeating above procedure. Remember to squirt the electrodes with deionized water between samples.

5. TEST FOR PROTEIN: THE BRADFORD TEST

A. With a transfer pipet add exactly 10 drops of Bradfords reagent to each sample. Be careful not to contaminate the pipet by allowing it to touch the samples.

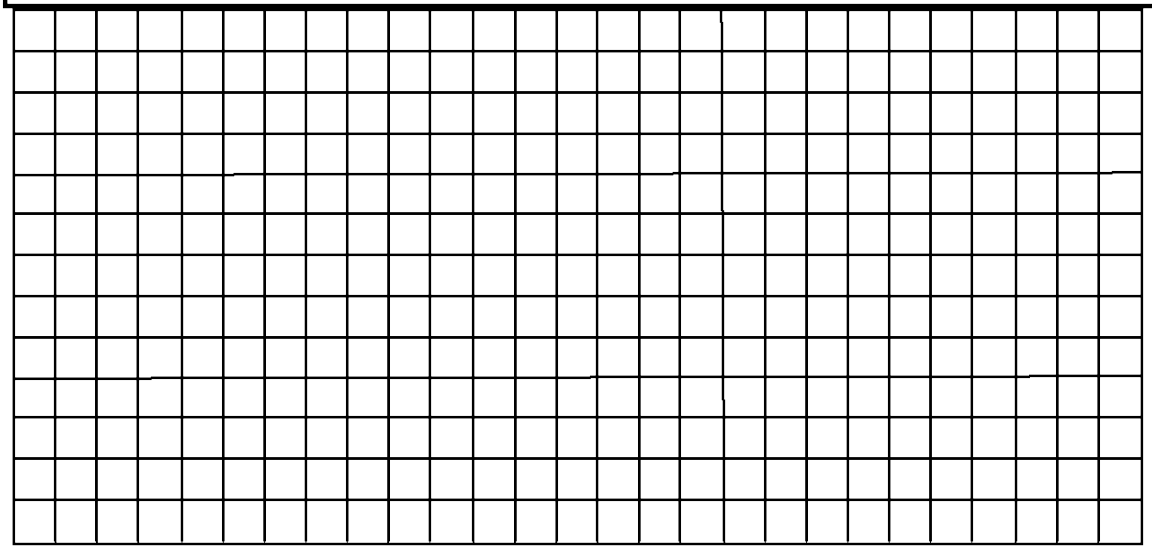
B. Close each tube securely. Invert each sample several times to thoroughly mix the indicator with the sample. On your data table record your impression of the color, and record the approximate protein concentration for each tube, using the color chart. Use color descriptions such as dull blue, bright blue, blue gray, gray brown, and so on. If your sample is between two colors on the chart, estimate the protein concentration. Make sure to save all of your samples for later analysis when you redo the experiment in the second phase of this lab.

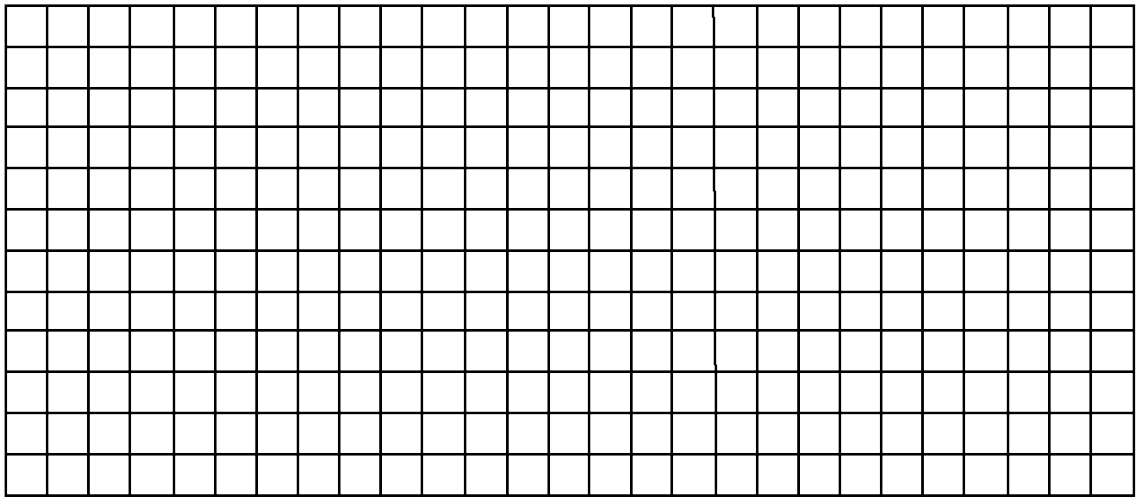
6. INTERPRETING YOUR DATA

Make two graphs of your data with the tube number along the X axis. Put salt concentration (mg/oz) along the Y axis of one graph and protein concentration (μg/ml) along the Y axis of the other graph. These graphs or the table of data should be in your lab notebook. It is not necessary to reproduce both the graph and table in your lab notebook.

COMPLETE ALL OF THIS FIRST PHASE BEFORE READING AHEAD AND COMPLETING THE SECOND PHASE. THIS IDEA HAS BEEN REVIEWED IN THE LAB LECTURE, IN THE HANDOUT, ETC. SO…BE PATIENT AND FOLLOW THE PROTOCOL IN THE PRECISE ORDER.

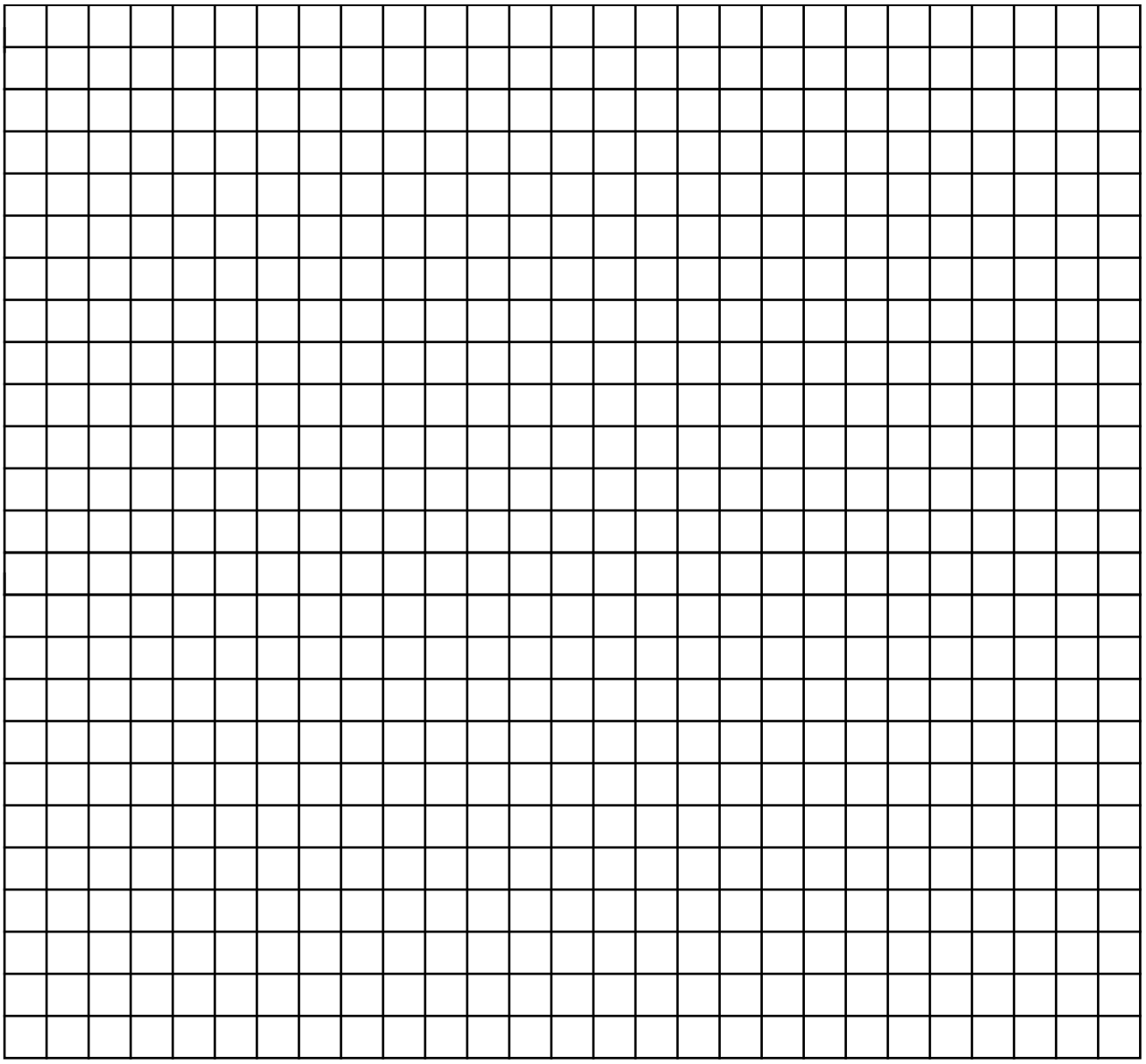
Tube #	Salt Concentration	Bradford Color	Protein Concentration
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
Controls			





Exercise: A scientist used a Sephadex G-100 column to determine the molecular weight of a newly discovered protein. The G-100 Sephadex beads have a larger pore size than the G-25 beads that you have just used. In the G-100 beads the pore size is large enough so that some of the proteins can enter the beads. The scientists ran three standard proteins (A, B, and C) with known molecular weights through the column and measured the elution volume. The elution volume is the total volume of water that drips from the column after adding the sample until the most concentrated protein sample is collected. Using the data below plot the data for the standard proteins. Note: the following example is a practice exercise, and should not appear in your lab notebook. It is still fair game for quizzes, exams, etc…

	Molecular Weight	**Elution Volume**
Protein A	95,000 A.M.U.	10.0 ml
Protein B	70,000 A.M.U.	25.0 ml
Protein C	13,000 A.M.U.	70.0 ml



SOP: Phase Two:

COMPLETE ALL OF THE FIRST PHASE BEFORE READING AHEAD, COMPLETING THE SECOND PHASE, ETC.

Well, did your experiment work? Hopefully the answer is yes. Some of your samples will have salt, but no protein and others have protein, but no salt. This said, how precise is your data?

A. Look at your data (especially the table). Assume that the BSA/salt mixture has a concentration of 200 ug/ml for the BSA and the salt conc. is 50 mg/oz. How does this affect your data? The total of all of the fractions *cannot exceed* the amount added. Total your samples and see if this is the case.

B. Take your BSA salt mixture and create a positive control and a negative control. For the negative salt control, simply immerse the device into fresh water. For a positive control, take a sample from the original BSA/salt mixture and immerse a salt probe in it. For BSA positive control, collect 19 drops of the BSA/salt and add 10 drops Bradford. Label the tube "positive control". For a negative control, place 19 drops of DI water into a microfuge tube and add 10 drops of Bradfords. Label and store accordingly.

C. Repeat the experiment (as outlined in steps 2-5 in the first phase). Use your new insights into the range of possible answers, positive and negative control. Yes…you need to do the whole thing again….but reflect on your technique as well as this related theory that accompanies the range of your actions.

This second experimental treatment should give you different numbers from the firs experiment for a few simple reasons. You know what to expect, and you completed a few controls. As you revisit the experiment for the second time. Notice how this makes you look at your data differently. In the previous experiment, key ideas were intentionally withheld to help demonstrate the need for key information. Scientists know what to expect and an experiment without a control is potentially VERY problematic. While not always conducted, positive and negative controls and additional key steps are essential to helping someone interpret the data.

Enter your data on the next page. Yes, you need to enter all of your new data and graph again… Relax…graphing is good for you! As you set up your experiment for a second time, try to think of ways to improve your results. The idea of solution concentrations amounts, positive and negative controls are a start. Try to name 5 more things that may help increase the accuracy of your data and write them below:

1.

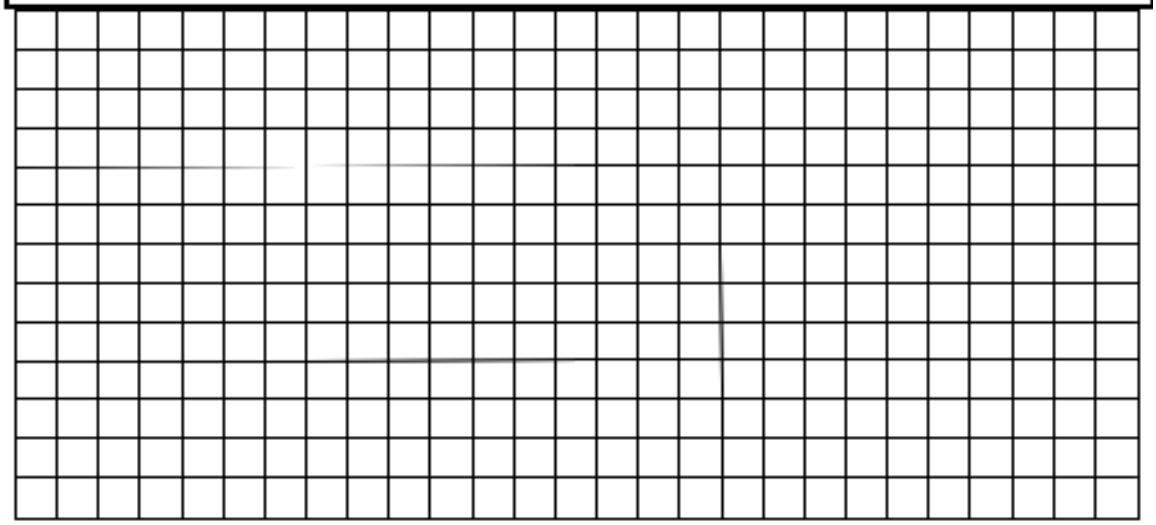
2.

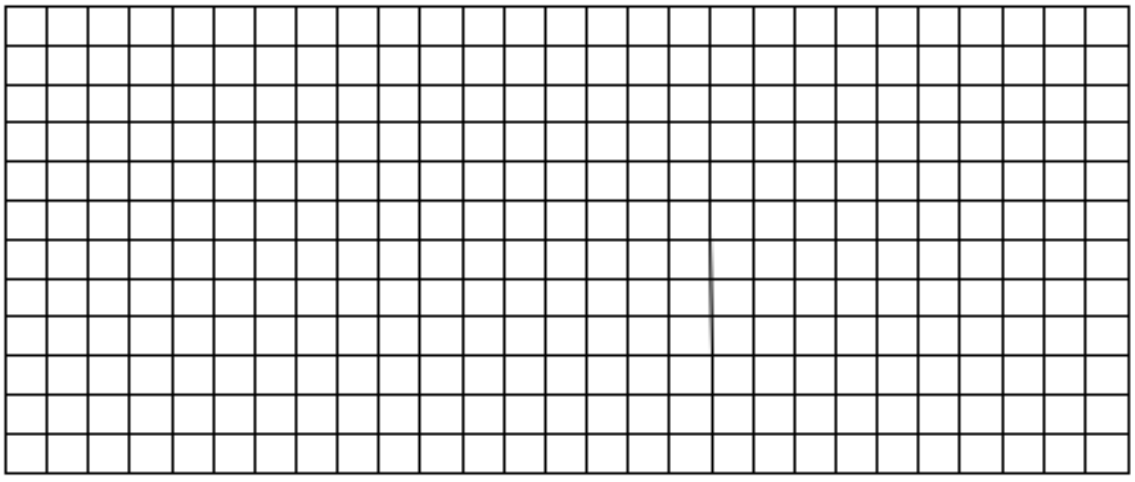
3.

4.

5.

Tube Number	Salt Concentration	Bradford Color	Protein Concentration
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
Controls			





Now that you have done TWO gel filtrations, you should be ready to publish your manuscript! Seriously though, this idea is a HALLMARK of biotechnology. WE REPEAT THINGS IN SCIENCE AND BIOTECHNOLOGY IS ALL ABOUT REPEATING, REFINING and UNDERSTANDING LABS AND ASSOCIATED TECHNIQUES.

So try one more filtration experiment. Try to separate the pigments in Grape Kool-Aid. Sound familiar? Check out their structures (on the old HPLC lab) and predict which sample should emerge first. Use only water to elute (separate the fractions) and only add a few drops (3 drops of Kool Aid maximum) to the column. Remember to make sure the bead bed level is just even with the water, carefully add the Grape Kool Aid and try to avoid dribbling too much down the side (so load the sample right above the bead bed, using the tip of the pipet to deliver the sample right above the bead bed). Collect 10 drop fractions in order as they come out. See if your predictions and insights from the last lab have any bearing on the results of this experiment. Be sure to add some DI water to the column to a height of about 1 cm above the bead bed and recap (both caps) before storing.

IV. Post-Lab Questions/Activities: The following post lab questions are for your benefit. The questions will help you to address a range of topics relating to the lab activity. Along with the post lab handouts, these questions will help to ensure that you have both correct information regarding the lab data and crucial lab processes. Complete the post lab questions at the end of the lab and post lab handouts (keys for both of these are available from your instructor) **before making any lab-notebook entries.**

1. Were you able to separate the protein from salt? Explain how you came to this conclusion.

2. Which of your tubes contains the most concentrated protein that is salt free? Explain how you concluded this.

3. Which of your tubes contains the most concentrated salt solution with the least amount of protein? Explain how you concluded this.

4. Is there one of your samples that you might choose to run through the column a second time? Which one and why?

5. Which tube (only one tube) proves that the original solution contained a high concentration of protein and a high concentration of salt?

6. Why did the salt and protein tube total concentrations (1-10) not equal the concentration of the original amount added to the column? FYI, the concentrations added were 50 mg/oz for salt and 200 µg/ml. Discuss the discrepancies in the salt and protein concentration data separately.

V. Notebook Entries:

Data from the lab should be the focus of this section and if there are any incorrect results, you should discuss this as well as expected results. Section V will contain both your results and discussion. Your data should drive the discussion. An informed discussion is dependent on understanding the post lab questions/activities.

Your intro should:

- Define gel filtration and basis of size and pressure source.
- Albumin and salt solution: that each substance was quantified.

Results should be:

- Table of data or graph, (table preferred), NOT BOTH.

Discussion should consider the following:

- Effectiveness of the process.
- Evidence that separation did occur.
- Did control vials equal total sum of fractions (both salt and albumin)?
- Ideas as to how to improve protocol.
- Do not mention supplemental Kool Aid experiment.

References:

California Lutheran University Enriched Science Program: www.clunet.edu