Biology 196 Laboratory
Protein Chromatography

Laboratory Objectives

After completing this lab you should be able to:

- Define chromatography and explain how it works
- Perform size-exclusion chromatography and predict the outcome.
- Identify which amino acids are hydrophobic in nature
- Perform hydrophobic interaction chromatography on a cellular mixture.
- Utilize a centrifuge

Experiment #1: Size Exclusion Chromatography:

Chromatography is a useful method to separate molecules such as sugars, amino acids, proteins, etc. This separation can be based on a number of physical properties of the molecules involved including size, charge (positive or negative; **hydrophobic** or **hydrophilic**), and even the three dimensional conformation of the molecule itself. Today you are going to separate a mixture of proteins on the basis of their size through a process known as size exclusion chromatography (aka gel filtration chromatography).

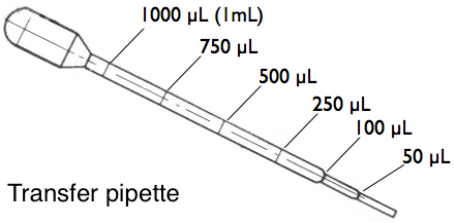
In size-exclusion chromatography a mixture of proteins is added to a column containing microscopic porous beads. Think of these beads as very, very small wiffle balls. Any molecule that is smaller than the holes in the beads (or wiffle balls) will be able to enter and exit the beads at will and will take a long time to travel from the top to the bottom of the column in their extended zig-zag journey.

Molecules that are larger than the holes in the beads will travel down the column in a direct journey very quickly since they are too large to enter into the holes. Since small molecules travel slowly and large molecules travel quickly through the column material, a method of separation is established based on size.

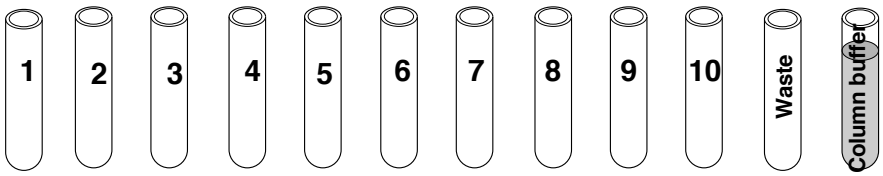
Procedure:

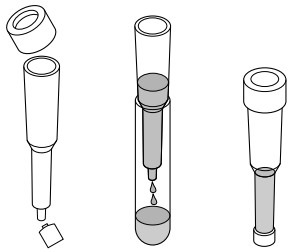
Students should work in groups of two for this experiment.

1. Obtain 12 collection tubes, label the first 10 as 1-10, and the remaining two as waste and buffer. Add 4 ml of column buffer to the tube marked buffer using transfer pipettes.

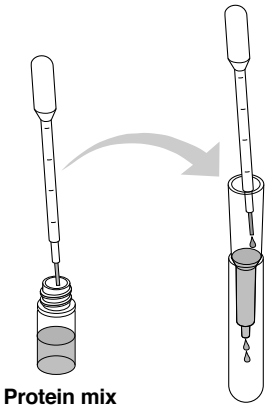


Transfer pipette

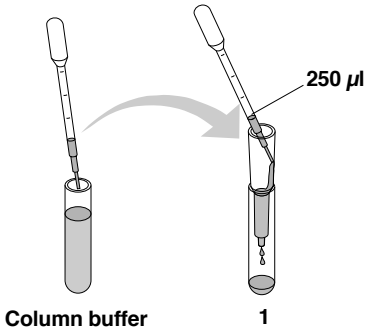




2. Obtain one prepared column, remove the top cap and snap off the bottom end. Allow the liquid buffer to drain from the column into the tube marked waste. *Tip: Do not let the column make an airtight seal with the tube or your column will stop dripping.*



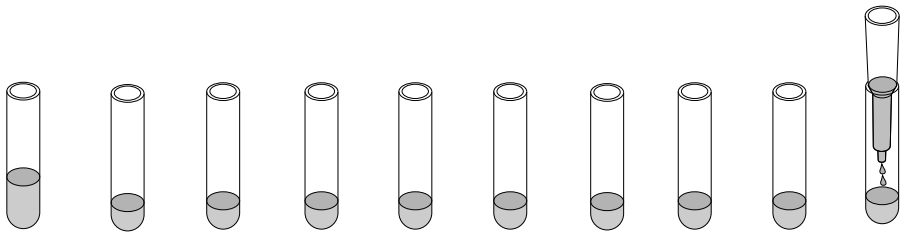
3. Place the column into tube 1 and carefully add 3 drops of the protein mix to the top of the column matrix with the supplied pipettes. Do this very slowly so as not to disrupt the column matrix!



4. Once the protein mix has entered the column matrix, carefully add 250 ul (*µl = "microliter"*) of column buffer to the top of the column matrix. Begin to collect drops in tube 1.

   Once the 250 ul of column buffer from step 4 has entered into the column matrix, carefully add 3 ml of column buffer to the top of the column matrix. Transfer the entire column to tube 2 and collect 5 drops.

5. After collecting 5 drops in tube 2, transfer the entire column to tube 3 and collect 5 drops again. Keep repeating this until you have 5 drops in all of your collection tubes.



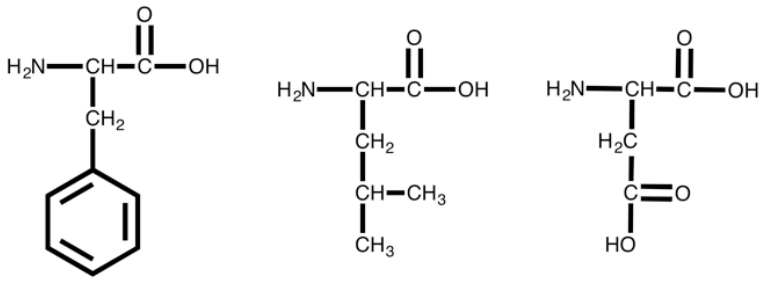
Examine the results. The protein mixture utilized in this experiment contained red-brown hemoglobin (MW 65,000) and dark pink Vitamin B12 (MW 1,350).

Which molecule exited the column first? _____

Which molecule is smaller? _____

Experiment #2: Hydrophobic Interaction Chromatography (HIC):

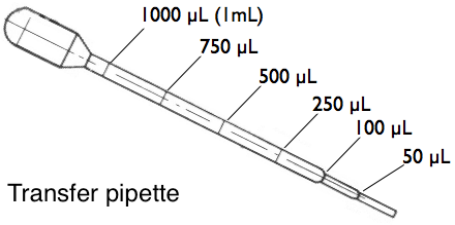
To complete this experiment you need to have a background in amino acids and protein folding. Use chapter five of your textbook to review the structure of amino acids. Some of the amino acids that make up proteins are very *hydrophilic* ("water loving"), while others are *hydrophobic* ("water hating"). Hydrophobic amino acids tend to reside near the interior of a protein when folded. Look at the diagrams of amino acids below and label them as being hydrophobic or hydrophilic amino acids:



_____ _____ _____

In this experiment we will isolate our protein via *affinity* to a hydrophobic matrix. Conditions that affect protein folding can break the bonds holding the hydrophobic amino acids in the interior of the protein. An extreme unfolding event would be **denaturation**. For example, by increasing the **tonicity** of the solution using high salt we can cause the protein to unfold, exposing the hydrophobic amino acids. This will cause the protein to "stick" to the column matrix. To remove (**elute**) the protein we will add a low salt buffer that will cause the protein to refold, hiding the hydrophobic amino acids in the interior of the protein once again.

You will be given the task of purifying a protein from bacteria. This strain of bacteria has been modified by the addition of a gene from the jellyfish, *Aequorea victoria*. If this gene is transcribed and translated, a hydrophobic fluorescent protein will be produced that glows under UV light.

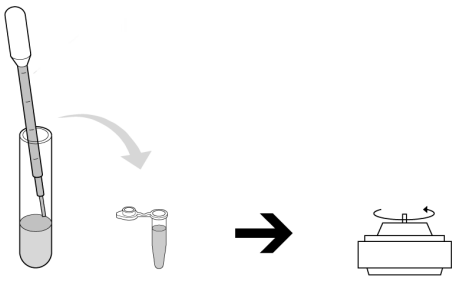


Transfer pipette

Procedure:

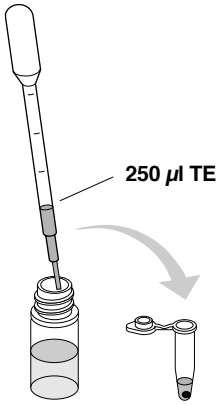
Part A: Sample preparation. Students should work in groups of two for this experiment.

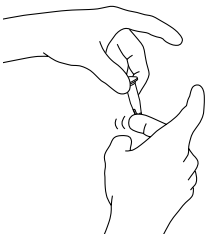
1. Transfer 1.5 ml of bacterial overnight growth to an eppendorf tube, labeled on top with your initials. Spin in the microcentrifuge for 3 min. (**Important:** *See the appendix at the end of this documenton the proper balancing of the centrifgue.*)



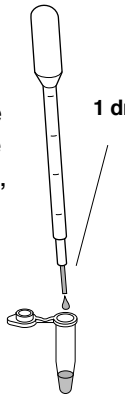
2. Carefully remove all of the **supernatant**. Because it will still contain some bacteria, be sure to dispose of it in the biohazardous waste container.

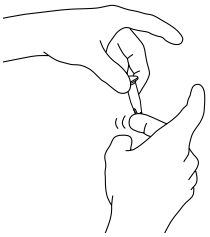
3. Add 250 ul **TE buffer** to the pellet and vortex until all of the pellet is dissolved.





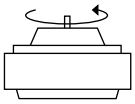
4. Add 2 drops of **lysozyme**, mix, place at 32ºC for 20 min (*in the water bath designated by your instructor*). The lysozyme will burst open the E. coli so the protein can be removed. This is a very important step in the procedure. If the cells do not burst open, the protein cannot be isolated! If the lysozyme works, you should notice that the solution has become more viscous.





5. Spin in the microcentrifuge for 10 min. Add 250 µl of binding buffer to a test tube to prepare for step 6.

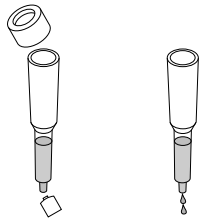
(*While the samples are still in the centrifuge you can also start step one of part B below*)



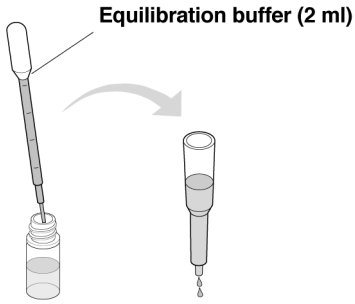
6. This time save the **supernatant** and transfer it to a tube which already has 250 ul of **binding buffer** present. The binding buffer is a high salt buffer which causes the proteins to unfold exposing their hydrophobic residues.

Part B: Purification

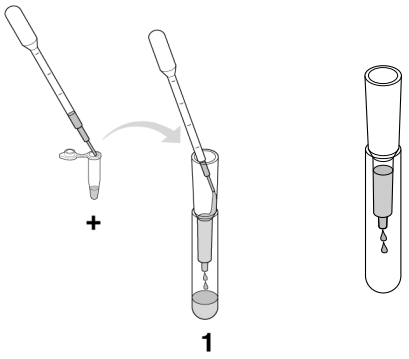
1. Remove the bottom and top cover from the column. Set the column in a collection tube (labeled 1) and place in the test tube rack. Let all the liquid buffer drain from the column (~3-5 minutes)



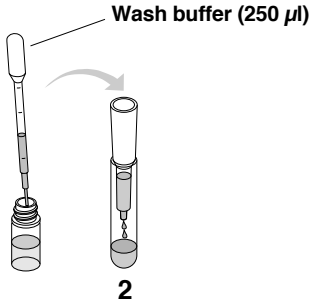
2. Add 2 ml equilibration buffer to the column and let the solution pass through the column slowly drop by drop. Depending on how the column is packed this could occur fast or slow. If you are still waiting for Part A when this finishes, add more buffer. Never let your column dry out.

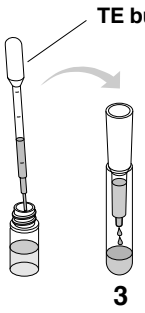


3. Add all of the solution from PART A to the column. Carefully and gently add this solution to the top of the column matrix (the white material in the column). Let the solution pass through slowly drop by drop. Collect all of the flow through in the collection tube labeled 1. Hydrophobic proteins should now be bound to the column while hydrophilic proteins have passed through.

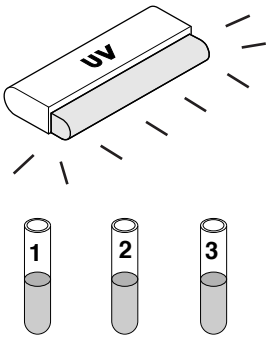


4. Transfer the column to a new collection tube labeled 2 and add 250 ul of **wash buffer** and let this solution pass through drop by drop. Again, add this solution directly to the top of the column matrix gently. Wash buffer is a medium salt buffer which should allow some moderately hydrophobic proteins to begin to re-fold into their normal shape. As they refold, their hydrophobic regions will again be buried and thus cannot bind to the column. During this stage, proteins that are only moderately hydrophobic will pass through.





5. Add 750 ul TE gently to the top of the column matrix and collect the flow through in a collection tube labeled 3. TE is a very low salt buffer, in which all proteins will refold burying their hydrophobic residues, so all proteins should be removed from the column. This flow through should contain only the most hydrophobic proteins that were present in the bacteria.



6. Place tubes 1, 2, and 3 side by side and examine with a hand-held UV light source. If you have performed the experiment correctly, only tube 3 should show any fluorescent activity. You may want to hold the UV lamp up to the column during step 5. If performed correctly you can watch see the fluorescent protein going down the column, forming a drop at the tip of the column, and dripping into the solution in collection tube 3..

Study Checklist

To perform well on the next quiz, you need to have a thorough knowledge of the following:

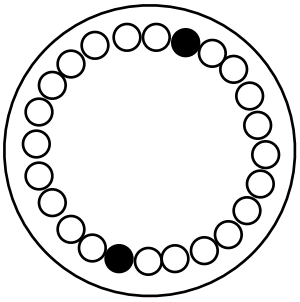
- You should know and understand all of the terms which appear in **boldface** type.
- What chromatography is and its basic principles
- The mechanism of *size exclusion* chromatography.
- Which proteins were separated by size exclusion chromatography and the relative size of the two proteins.
- Identify hydrophobic vs. hydrophilic amino acids and the principles of protein folding.
- The mechanism of *hydrophobic interaction* chromatography.
- The purpose of each of the reagents used to prepare the green fluorescent protein (GFP) from the bacteria.
- What is happening to the GFP at each step of the column chromatography section.
- The proper way to balance a centrifuge

Appendix: Centrifugation

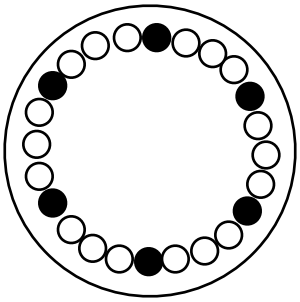
A **centrifuge** is a device used to separate materials based on their relative densities. It uses a rotor that spins very fast - sometimes tens of thousands of times per minute. Due to the extreme speed, the centrifuge can present a safety concern when unbalanced. You cannot put one tube into a centrifuge. The rotor must have symmetrical density. This is often achieved by adding tubes of equal weight on opposite sides of the rotor.

Failing to properly balance a centrifuge will result in poorer performance, extreme noise, possible damage to the machine and/or the samples. In large, fast centrifuges improper balance can cause the rotor to break and could cause human safety issues.

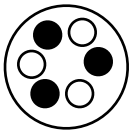
Samples of **balanced** rotor configurations:



2 tubes in a 24-place rotor



6 tubes in a 24-place rotor



3 tubes in a 6-place rotor