FRED HUTCHINSON CANCER RESEARCH CENTER
SCIENCE EDUCATION PARTNERSHIP

MOLECULAR MIXTURES

Using Size Exclusion Column Chromatography
For Molecular Separation

This is a new protocol and your input is requested. PLEASE LET YOUR TEACHER KNOW THE POSITIVE AND NEGATIVE EXPERIENCES YOU HAVE WHILE USING THIS LAB GUIDE AND NEW REAGENTS. THANK YOU.

PURPOSE

- To gain experience with chromatography as a biomedical research tool used to separate molecular mixtures.
- To learn and understand the basics of how this method works by running a sample mixture containing two molecules through a size exclusion column.
- To learn and experience a method for detecting protein molecules.

FIRST, THE BIG PICTURE OR WHY SEPARATE MOLECULES?

William Bateson, the "father of genetics" who translated and revived Mendel's works, advised, "***treasure your exceptions***."

Molecular biologists, geneticists, and biochemists are always trying to figure out how living things work. Doctors compare the diseased with the healthy. Several basic strategies are used in these quests:

1) narrowing the system studied down to its basic components, and
2) comparing two or more variants or forms to understand how they differ (hence, "treasure your exceptions").
3) Often some combination of both approaches is used.

There are the research questions (variations of "how does this work?") and then there are the tools or techniques that can be used to work toward answering the questions. Chromatography (translated means roughly "writing with color") is a research tool. It has been around for a long time and has many, many variations.

What are some examples of how chromatography is used?

Example 1. A classic example is separation of the pigments in plant leaves. The leaves are ground up and the components are separated, most commonly by paper chromatography, and then characterized according to how they behave in the system. Often some molecules of known characteristics (for example, known sizes) are included to use as a gauge for estimating the sizes of the unknown molecules. The pioneer scientists first studying something, such as the pigments in plant leaves, get to name all the molecules they identify.

Example 2. In biomedical research, we often want to "tag" or label molecules for use in experiments. These tags are usually small molecules (the idea is that small tags interfere less with the normal functions of the molecule being studied). We might label big DNA molecules with fluorescent tags for use in determining the DNA sequence. The first step is the labeling—binding the tags to the DNA, and the second step involves removing any tags that didn't bind to DNA. This cleanup step is important because in the experiment we follow the tag and infer

that it is attached to the DNA. A size exclusion column can separate the DNA (big) from the small, unbound tags.

Example 3. Many new medicines produced by biotechnology are made in cellular systems and then purified. At first this is done on a small scale for research and development. After a new medicine proves effective, the challenge becomes purifying it in a manufacturing scale process using big vats of growing cells. In many cased this purification process involves a series of chromatography steps, often using column chromatography with separations based on size on one column and on affinity in a second column step. Insulin production is currently done this way.

What are some common forms of chromatography?

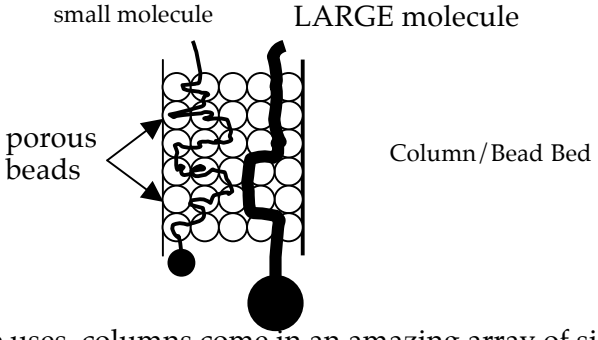
You may already be familiar with paper chromatography; two close relatives are affinity chromatography and size exclusion chromatography.

Paper chromatography depends heavily on the solubility of the molecules and their absorbance to the paper.

Affinity chromatography—as its name implies—depends on the affinity or binding of molecules to the "matrix." Depending on the purpose, the matrix may be the paper in paper chromatography or may be some form of beads or resin held in a column. In this type of system the molecule of interest is retained in the matrix and all other molecules are eluted into a waste container. Another step using a different buffer releases the retained molecule from the matrix.

This lab is an exploration of size exclusion column chromatography. It's called column chromatography because it is done in a column (rather than on paper or on a thin layer matrix on a glass plate). The name **size exclusion** describes the mechanism for the separation: molecules larger than a designated size are excluded from the solid column material—in this case tiny porous beads (also called the column bed, resin, or matrix).

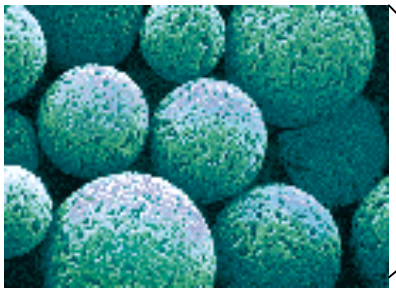
Molecular mixtures separate on a size exclusion column because smaller molecules spend more time (travel a longer path) going through the column. You might think of the column beads as somewhat like whiffle balls. The molecules that are small enough to enter the holes of the whiffle balls bounce around inside the balls and take a while to escape. Molecules bigger than the holes just flow around the whiffle balls and thus travel a shorter, faster path in going through the column.



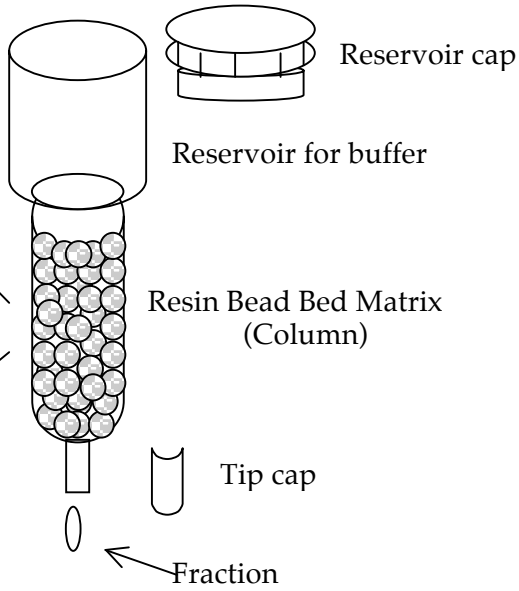
With all these possible uses, columns come in an amazing array of sizes and materials (just like cars, screwdrivers, and pens……). One size does not fit all. The resin used is based on type and size of the molecule(s) to be separated and the degree of resolution required. The resolution depends on particle size, pore size, flow rate, column length, and sample volume.

Below is a labeled diagram of the columns provided in this kit and an electron microscope image of the size exclusion chromatography beads that make up the matrix of these columns.

Chromatography Column



Electron micrograph of resin beads.



COLLECTING FRACTIONS

If we collect all of the liquid coming out of the column in a beaker, all the molecules get mixed up again. So we "collect fractions" as the liquid comes out of the column. Generally we use a rack set up with numbered tubes and collect a set number of drops or a set sample size in each tube (for example, 10 drops per tube or 0.5 ml per tube).

ASSAY FRACTIONS (detecting colorless proteins)

The job of the column is separation. Sometimes you may be lucky enough to study molecules that are naturally colored, like the protein hemoglobin or the vitamin B_{12}. But most molecules are colorless, so following separation is the challenge of detecting the molecules of interest. One way to detect proteins is based on a color change when the dye Coomassie Blue, in the Bio-Rad protein detection reagent, binds to proteins. The dye binds to primarily basic (especially arginine) and aromatic amino acid residues.

The Bio-Rad protein detection reagent is a murky brown/blue color. When the dye in it binds to proteins, it turns a stronger, brighter blue color. This color change can be compromised if a molecule already has color such as the vitamin B_{12} (orange) we are using.

DESCRIPTION OF REAGENTS:

Ovalbumin
Ovalbumin is a glycoprotein and the major protein of egg white. It has a molecular weight of 43,000 daltons. It is used as a source of amino acids for the developing bird enbryo. Ovalbumin is used in conjugo-immuno-determinations and drug and pharmaceutical processing.

Vitamin B_{12}
Vitamin B_{12}, so known as cyanocobalamin, is a non-protein molecule with a molecular weight of 1,355 daltons. It is found in all animal foods – meat, meat products, milk, dairy foods, fish, eggs – yeast extract and certain algae, such as seaweed. This complex structured compound with its cobalt content forms part of the B group vitamins, and the body needs very small amounts. It is required in the manufacturing and maintenance of red blood cells and it stimulates appetite, promotes growth and release of energy.

BSA - Bovine Serum Albumin
BSA is a large molecule with a molecular weight of 67,000 daltons. It is a major protein found in bovine blood serum which functions as a carrier molecule for the transport of certain small MW compounds in the blood. Some of the molecules that bind to serum albumin include bilirubin, fatty acids, hormones and some synthetic dyes.

Bio-Gel P-60 beads
Bio Gel is the registered trademark name of the resin that is packed into the columns. The beads are made of polacrylamide. Bio Gel beads can be ordered in different sizes-that is, different size beads have different pore sizes, making them better able to sort different sized molecules. The SEP columns use Bio Gel P-60 beads, which best separates molecules of molecular weight 3,000-60,000 daltons.

Phosphate Buffered Saline (1X PBS)
Phosphate Buffered Saline is the column buffer being used. It is a salt solution that helps to maintain the pH of the system and preserve the molecules natural shape and function.

Protein Detection Reagent (PDR)
PDR is a reagent that can detect the presence of protein in a solution. The reagent contains Coomassie brilliant blue, a dye that binds to proteins. We purchase the reagent from Bio-Rad (catalog # 500-0006). The dye works by binding to primarily basic and aromatic amino acids, causing a color change (to bright blue).

You might have students look up further information about the other two molecules that were chosen for Mixture B, including their functional roles in nature.

STUDENT LAB:

YOUR EXPERIMENTAL SAMPLE

Sample Mixture A that you will use contains vitamin B_{12}. and the colorless protein ovalbumin. The size exclusion column separates molecules based on the molecular weights (mass) of the molecules in the mixture. Since ovalbumin is colorless you will not be able to observe it until using the Protein Detection Reagent (PDR) in the analysis step.

The ovalbumin and vitamin B_{12} mixture is a good starting place because the molecular sizes of the two molecules have a good size differential appropriate for the beads or matrix type used in our columns; these work for the range of ~3000 to 60,000 daltons.

TO GET PROFESSIONAL REPRODUCIBLE RESULTS:

1) review the mechanism for size exclusion column chromatography (how it works)
2) review and flow chart the lab protocol
3) predict your results for Mixture A (ovalbumin + vitamin B_{12}). Think about your experimental set up. If all molecules will flow (elute) out by test tube numbers 10-12, which numbered tubes would you predict contain each of the different molecule fractions?
4) using the Molecule Characteristics Chart as a team or class choose two molecules to create a Mixture B and predict the expected results
5) review essential tips for getting good results with this technique
6) prepare and run the molecular mixtures on a column, collecting fractions
7) analyze the fractions; record the data
8) add the protein detection reagent, analyze the contents of the fractions and record the data
9) summarize your results and what you learned
10) ask the next question – what experiment would you want to do next?

Materials:

For each team of students:
16 tubes
tube rack
ring stand and clamp
chromatography column
beaker for buffer
3 transfer pipets
    1 for loading sample
    1 for buffer solution
    1 for PDR
1 Data Recording Sheet
colored pencils (1 set for two teams)
permanent marker

For the class:
sample mixture A
sample mixture B
Protein Detection Reagent (PDR)
column buffer (1X PBS)
positive control reagent (BSA)

PROCEDURE

1. Record on your data sheet which mixture you and your partner are separating. Use the internet and or available classroom resources to find the characteristics of each of the molecules in your mixture.

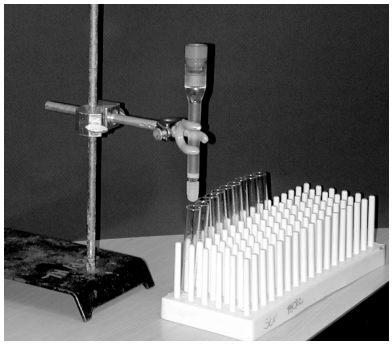
2. Obtain all needed equipment, reagents and your test sample. Set up your equipment like the one at the Teacher's Station.

3. Label your collection tubes and transfer pipets with the permanent marker.
   - Label 12 of the tubes from 1-12, label two more tubes "waste" and "PDR"
   - Label your pipets "buffer", "sample", and "PDR"

   It is important not to mix your pipets.

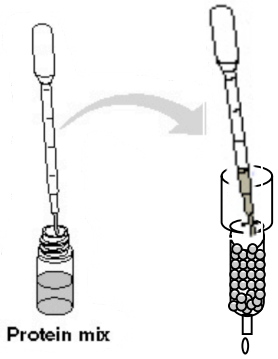
4. Adjust the chromatography column on the ring stand at your station so that it sits just above the collection tubes in your rack. Be sure the column sits high enough for the tubes to easily move underneath, but not so high that the drops *splash.* For the next step place the column over the "waste" tube.

5. Carefully remove the top cap from the column reservoir first, then the bottom cap from the tip. Allow the column buffer (the clear liquid above the gel bed) to elute (flow) out into a waste tube. (This helps to equilibrate the column bed.) It elutes (drips) slowly…….so be patient and watchful. You do not want the bead bed to get dry.



   - How do you tell if the bead bed is getting dry? When the buffer gets close to the bead bed begin looking into the column from the top. While there is buffer on the top of the gel it will appear shiny. When the last of the buffer in the reservoir begins to be absorbed (percolate) into the bead bed, it will begin to look dull and grainy from the top.

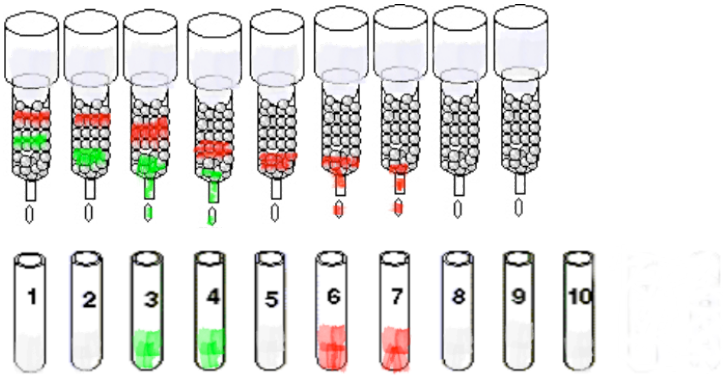
6. When the column buffer elutes to just the point you can see the top of the gel bed get grainy or dull looking, quickly and gently add one drop of your ***sample*** onto the center of the bead bed. The pipet should be inserted close to the bed, and the liquid drop should be gently expelled so as not to let the pipet physically touch or disturb the beads.



7. When the sample has completely entered the bead bed, again with the pipet close to the bead bed, add one drop of column buffer ***gently*** down the sides of the reservoir. Count to 5 slowly under your breath and then add more buffer **gently** down the side of the reservoir to fill the reservoir full without disturbing the bead bed .

8. Allow two more drops of the buffer to elute into the waste tube and then move the column over your tube labeled #1.

9. Collect 10 drops into tube 1. Now, move the rack so that the next 10 drops flow (elute) into tube 2. Continue this process until you have collected 10 drops into each of the 12 labeled tubes.



10. Keep consistently adding buffer to the column reservoir as you collect all of the column fractions. You want to maintain constant pressure and steady flow of the buffer eluting through the column.

11. When you finish collecting 10 drops in tube 12, move the column back over the top of the waste tube. (Make sure you have emptied the tube first.) Allow 10 more drops to elute. Now, cap the bottom first, then top of your column, (be sure to do it in this order), making sure that the column is still full with buffer.

12. ***Immediately record your initial results using color pencils***. Use the middle row of tubes on the Data Sheet provided. If any of your tubes has a colorless fraction, just draw a line to show the volume in the tube with your regular pencil and do not shade or color in.

13. Note how many colored fractions you have, the tube number(s) they are in, the color plus its intensity.

14. Fill the Protein Detection Reagent tube.

15. Using two clean tubes make these samples:

    - Negative Control: 10 drops of PBS buffer + 2 drops protein detection reagent
      Positive Control: 1 drops of BSA (known protein) + 9 drops PBS buffer + 2 drops of protein detection reagent

16. Now, add 2 drops of the PDR to each fraction in tubes 1-12. Compare each tube to the control tubes.

17. Record the results using color pencils in the third row of tubes on your data sheet. Note how many colored fractions you have, the tube number(s) they are in, the color plus its intensity.

ANALYSIS :

Use what you have learned about chromatography and protein detection to analyze your results.

1. What molecules did the class/team choose for Mixture B? What was the reasoning for use of these particular molecules?
2. What molecules did your lab group separate? Were the molecules all proteins? What evidence do you have?
3. Which fraction tubes had a colored molecule present? (Be sure to also describe the color before and after adding PDR.)
4. Which fraction tube(s) had colorless proteins that were detected with the Protein Detection Reagent? Which tube had the greatest concentration of protein present? Look for the tube number that contains the "peak" blue fraction to tell this or not. The peak fraction contains the highest concentration of protein, and will be the most intense in color (deepest blue).
    - Remember, blue is the color change that will occur when the PDR reacts with a protein.

5. Of the molecules that exited the column which is the largest? Smallest? Which eluted from the column first?
6. How does your data analysis compare/match up to your prediction?
7. Summarize your understanding of how column chromatography can be used as a tool to separate a molecular mixture.
8. What would you predict the outcome of this same experiment to be if
    a. the size of the holes in the beads were larger?
    b. the size of the holes in the beads were smaller?

9. If the following mix of molecules were purified using size exclusion chromatography, what would be the order in which the molecules pass through the opening in the bottom of the column? Mixture contains: hemoglobin: 65,000 daltons, myoglobin: 17,000 daltons, myosin: 180,000 daltons. Explain your answer.
10. Do you think that the shape of the molecule has an affect on the molecules movement through the column? Explain.

Extension

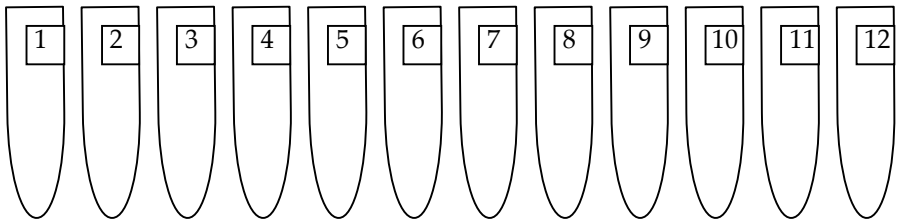
Draw a graph to represent the colors, with tube number on the x-axis and color intensity on the y-axis. You can use colored pencils to shade under the graph to represent the colors observed.

Chromatography Lab Data (Student Mixture)

I. Prediction

Observations/Comments:

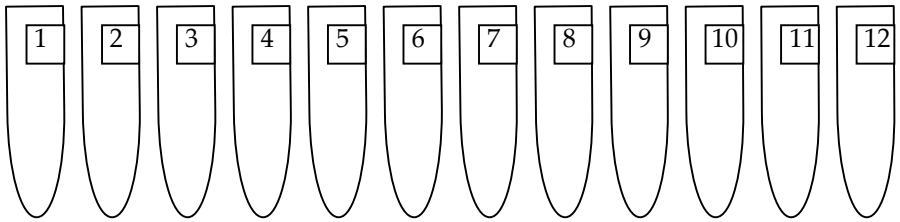
Using your color pencils, color in where you predict the _____ and _____will fractionate into the test tubes.



II. Fractionate Results

Observations/Comments:

Using your color pencils, color in the initial experimental results. If there is no color use your regular pencil to draw a line to represent volume only.



III. Fractionate + Protein Detection Reagent

Observations/Comments:

After adding the protein detection reagent to each of the fractionates, use your color pencils to show the results.