FRED HUTCHINSON CANCER RESEARCH CENTER
SCIENCE EDUCATION PARTNERSHIP

TEACHER GUIDE
MOLECULAR MIXTURES
Molecular separation using size exclusion column chromatography

The kit looks the same, but appearances can be deceiving
New Protocol, New Column Matrix, New Background Information

- *Background information provides the big picture of "Why separate molecules?"*
- *Columns contain a different bead matrix; Bio-Gel P-60*
- *PBS (phosphate buffered saline) is used to run the columns instead of water*
- *Columns run slower but get better separation*

As soon as you get the kit back to school or home

The reagent box should be stored in the refrigerator.

FORWARD

This lab has been changed to improve the student experience. The focus is on molecules, (their characteristics and how they differ) and a tool (chromatography) that can be used to separate one molecule from a mixture of molecules for further study.

The chromatography columns now contain Bio-Gel P-60 beads that are made of polyacrylamide. The original Sephadex G-75 beads were made of agarose. The Bio-Gel beads give a more concentrated flow than the Sephadex beads. This provides a more defined separation of the molecules available in this kit, which visually assists the students.

The column buffer has been changed to 1X phosphate buffered saline (PBS), which is commonly used in cell cultures. PBS helps maintain the pH of the system and the molecules natural shape and function better than the water used in the previous protocol.

We have eliminated the use of BSA (bovine serum albumin) in a molecular mixture. BSA can bind to smaller molecules, thereby altering their normal flow rate through the column. We now use BSA only as the positive control reagent for protein detection.

Please note that this lab is still under revision. Some things to look forward to will be recommended extensions, assessments and a annotated bibliography of references and more information and focus on molecules. *In the mean time your continued input is requested. Please, upon returning the kit be very specific about what worked well and where problems, if any, occurred. It would be very helpful to know the quality of separation your students obtain and if you do further extensions what those were.*

Thank you.

SUMMARY AND PURPOSE

This lab is designed to give student researchers experience with chromatography as a biomedical research tool used to separate molecular mixtures. To learn the basics of how this method works, students run a sample mixture of two molecules through a size exclusion column. The size exclusion column separates molecules based on the molecular weights (mass) of the molecules in the mixture. The column matrix provided in the kit columns is useful for molecular separations within the molecular weight range of ~3000 to 60,000 daltons.

The recommended sample "Mixture A" contains the colorless protein ovalbumin (MW: 43,000 daltons) and vitamin B_{12}, a smaller, reddish/orange molecule which is not a protein (MW: 1,355 daltons). This is a good starting place because the molecular sizes of the two molecules have a size differential appropriate for the beads or matrix type used in our columns. Also, since ovalbumin is colorless, students will not observe this protein until using the protein detection reagent in the analysis step of the protocol.

After students gain a basic understanding through discussion of how the column separation works, the class will view the chart of available molecules in the kit and select a combination of two molecules for Mixture B. Several molecular options (proteins, dyes, polysaccharides) for this mixture are included in the SEP kit.

This lab is designed for each lab group (generally composed of 4 students) to be divided into 2 teams. One team will run Mixture A while the other will run Mixture B.

DISCUSSION SUGGESTIONS

Pre lab

- review molecular structures (shapes, sizes)
- molecules important in the cell and body relating their functions
- known techniques for the separation of molecules (gel electrophoresis, chromatography: paper, size exclusion, affinity, etc.)
- relative sizes and shapes of biomolecules and other molecules
- roles of proteins and other molecules being used
- reading of Dr. Hla Shain's article "Why purify proteins?"
- chromatography model included in kit (whiffle ball model)

Post lab

- compare predictions to results
- review which molecules elute off of the column first/last and why
- possible sources of error
- other forms of chromatography (see reference information section of notebook)
- possible applications (medical uses, etc.)

Some reference materials have been provided in the reference section of the notebook to assist you with the above recommendations.

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 cases 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?

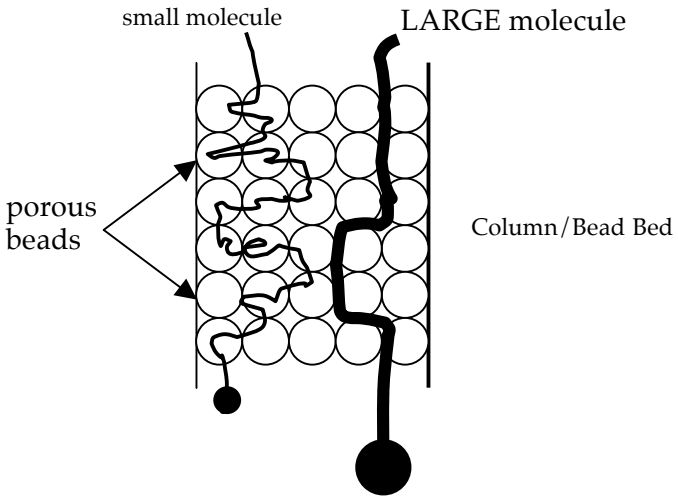
You may already be familiar with paper chromatography; two close relatives are affinity chromatography and size exclusion chromatography.

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.

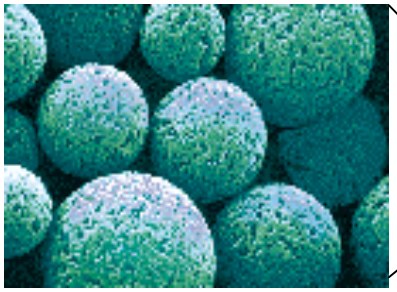
This lab is an exploration of size exclusion column chromatography. It's called column chromatography because it is done in a column (rather than on paper or on a thin layer matrix on a glass plate). The name **size exclusion** describes the mechanism for the separation: molecules larger than a designated 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.



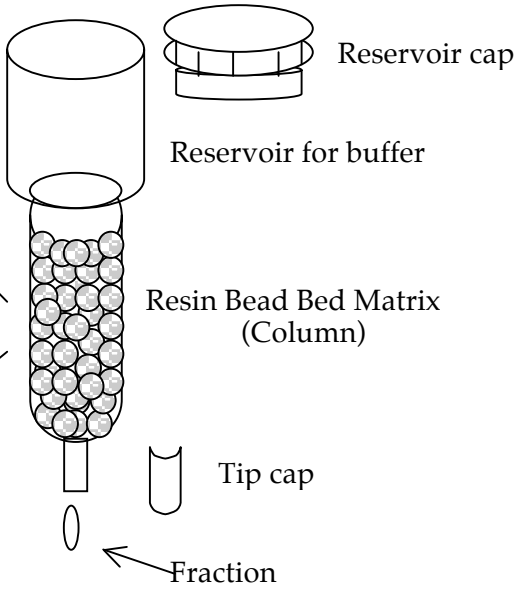
With all these possible uses, columns come in an amazing array of sizes and materials (just like cars, screwdrivers, and pens……). One size does not fit all. The resin used is based on type and size of the molecule(s) to be separated and the degree of resolution required. The resolution depends on particle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

On the next page, you will see 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.



THE PROCESS

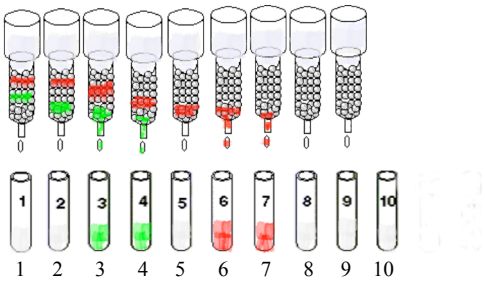
1. Collecting fractions

If we collect 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. A rack is set up with numbered tubes and a set number of drops or a set sample size is collected in each tube (for example, 10 drops per tube or 0.5 ml per tube).

Example

Bio Rad assay: Bio Rad's column separating hemoglobin and cytochrome C.
In looking at the Bio Rad example below, one can see the column chromatography results of separating hemoglobin (MW of 65,000 daltons) and cytochrome C (MW of 12,300 daltons). The hemoglobin was excluded from the column beads and the cytochrome C was not. Hemoglobin eluted into tubes 2 and 3 and cytochrome C eluted into tubes 5 and 6. Notice that both molecules are naturally colored.

When this same lab was run using the student protocol in the SEP kit, the hemoglobin fractions were present in tubes 3, 4 and 5 with a peak fraction in tube 4; the cytochrome C fractions were present in tubes 8-11 with the peak fractions being in tubes 9 & 10. Only tube 6 was completely clear. This is important to remember when students select molecules.



THE PROCESS ***continued***

2. Assay fractions (Detecting colorless molecules)

The job of the column is separation. Sometimes you may be lucky, as in the Bio Rad example, to study molecules that are naturally colored like the protein hemoglobin or the vitamin B12. Most molecules however are colorless. So, following separation comes the challenge of detecting the molecules of interest. One fairly good way to detect colorless proteins is based on a color change when the dye Coomassie Blue binds to proteins. [Bio-Rad's protein assay is based on the color change of Coomassie Brilliant Blue G-250 dye in response to various concentrations of protein. The dye binds to primarily basic (especially arginine) and aromatic amino acid residues.]

The Bio-Rad protein assay reagent is a murky brown/blue color. When the dye in it binds to proteins, it turns a stronger, brighter blue color.

Important points to keep in mind when teaching and carrying out this lab:

All molecules in this kit will elute out of the column by the 10-12th test tube when collecting 10 drops per tube. It is important therefore to try and separate molecules with large MW differences. If not, the molecules fractionate so close together that it becomes too difficult for students just learning about chromatography to analyze. Remember that all molecules around the MW of 60,000 or greater will come off the column together in ~ the 2nd or 3rd tube depending on how many drops eluted before you began collection in tube #1. Any molecules close to 60,000 MW……say in the 40,000's and greater will come off in the next tube or so.

One way this method can be used is to exclude your molecule of choice. A bead and pore size would be chosen so that the molecule you want to separate out is excluded (ie. size exclusion chromatography) and will be the ***first*** to come off of the column. To exclude the molecule of choice with the bead matrix used in this lab, the MW would need to be around 60,000 or greater. So, if you had a molecule with a molecular weight of 67,000 and one with a MW of 200,000 they would both be excluded and elute together through this column with no separation.

The method we are using is fractionation. Both of the molecules in Mixture A the (ovalbumin and the vitamin B_{12}) get caught up in the pores and therefore slowly percolate through the beads. The larger molecules get caught up less than the smaller ones. This enables the larger molecules to elute off of the column first. When the molecules MW is close to the bottom of the bead range (in this case 3,000), any molecules with a MW close to that or smaller will all elute together.

Once the fractions have been collected in the tubes, the scientist will look for "peak" fractions. These are the ones with the greatest color intensity (before adding the protein detection reagent if a colored non-protein and after adding the PDR if a protein). This "peak" fraction has the greatest concentration of the molecule of choice and will be the one used for further study or testing.

In industry the columns vary in length, bead pore size and matrix composition. Sometimes more than one separation process and type is necessary if the scientist is trying to purify the sample. Remember that there are many different chromatography protocols. We are only learning about size exclusion and the basis of how it works and practicing simple applications of the process.

TYPICAL STEPS IN THE SIZE EXCLUSION CHROMATOGRAPHY PROCEDURE AND WHAT STUDENTS WILL CARRY OUT

1) Review background and mechanism for size exclusion column chromatography
2) View and discuss Bio Rad example assay
3) Review SEP Column Chromatography protocol (flow chart)
   - predict the results for the Mixture A consisting of two molecules ovalbumin (colorless, MW 43,000) and vitamin B_{12} (reddish, MW 1,355)
   - using the Molecule Characteristics Chart have the class (or teams) choose two molecules to create a Mixture B. and predict the expected results
4) Review essential tips for getting good results
5) Prepare and run the molecular mixture on the column, collecting fractions
6) Analyze the contents of the fractions and record the data
7) Summarize your results and what was learned
8) Ask the next question

TIMELINE (Designed for 50 minute periods)

Day 1: Review with students
- molecular structures (shapes, sizes)
- molecules important in the cell and body relating their functions

Teach
- laboratory techniques and principles used to separate different molecules from each other with focus on size exclusion column chromatography
- demonstrate models in kit
- demo lab set up and flow chart procedure for lab
- discuss with students Bio-Rad example
- class determines molecules for Mixture B

Day 2: Run column chromatography lab
Each team runs one mixture

Day 3: Analysis and discussion of each teams results

Student Preparation for Lab:

1. The day before the review of molecular structures and methods used to separate and purify molecules, have the students read the background information and procedure to the column chromatography lab for homework.
2. The night after discussing molecules, their structures, "The Big Picture", and the lab procedure have the student's flow chart the procedure for homework.

Challenging Students to Think About the Process

After discussing the protocol and flow chart with students pose the question:
What happens if you are trying to separate two proteins of very similar size using this system?

Now, using the overhead of the molecules available in the kit and some of their characteristic properties have the class choose a second mixture of two molecules giving supportive reasoning for their choices.

PRE-LAB SET UP: TEACHER PREPARATION

THE DAY BEFORE THE LAB

1. Dilute Protein Detection Reagent (PDR).
   The PDR comes as a concentrated solution and needs to be diluted 1 part PDR to 4 parts deionized H_20. For 50 ml of diluted PDR (enough for 1 class) mix 10 ml concentrated PDR and 40 ml distilled H_20.
2. Prepare 1X PBS
   The PBS stocked with the kit is 10X and will need to be diluted to 1X. Determine the total amount of 1X needed and dilute accordingly (you will need approximately 1200 ml/class). For 1 liter of 1X PBS mix 100 ml of 10X PBS and 900 ml of distilled H_20.
3. Prepare the columns (1 column/2 students plus 2 – 4 columns as back-ups)
   Wash each column to remove the 20% alcohol storage solution. For all 20 columns, this takes about 1.5 hours. The flow rate is about 5-6 drops/minute with these columns and you cannot wash them all at the same time. ***Caution: For the columns to run properly the beads must remain wet. At most, wash 8 columns at a time.***
   1. Resuspend the beads by inverting the column until all of the beads have fallen to the reservoir. Once all the beads are resuspended turn the column upright.
   2. Wash each column twice with about 7.5ml of column buffer (1X PBS).
      - Set each of the columns on a ring stand. Arrange these side by side.
      - Place a beaker under each column to catch the waste storage solution.
      - Remove the top cap and then the tip cap from each column. This avoids creating a vacuum in the column.
      - Let the storage solution elute (drain) into the waste beaker until it just enters the bead bed. Watch from the top of the column as the solution gets close to the bead bed. It will appear to go from shiny to a dull/grainy appearance as the last of the storage solutions just enters the bed. To avoid drying the beads proceed immediately to the next step.
      - Fill the reservoir with column buffer using a clean transfer pipet. Run the buffer gently down the side of the reservoir so as not to disturb the bead bed. Let this elute into the waste container as you did the storage solution. When it is getting close to the gel bed refill the reservoir again with column buffer for the second wash.
      - When you have washed 2 reservoirs of buffer through the column (~15mL or so) fill the reservoir 1/2 full again with buffer, recap the column and leave it until the next morning.
4. Set up lab stations (With the exception of the color pencils, there is enough equipment and materials for 16 teams of 2.)

   <u>Student Stations</u>
   1 ring stand w/clamp and chromatography column (*not in kit)*
   1 permanent marker
   1 pack of color pencils (*2 teams will need to share 1 pack*)
   1 beaker for buffer
   1 beaker for waste

   <u>2 Reagent Stations</u> **Set out just before lab on ice.
   Racks with collection tubes (16 per student team)
   **diluted 1:4 Protein Detection Reagent (3 ml per team)
   **microtubes with Sample Mixtures (A and B)
   transfer pipets (3 per team)
   column buffer (1X PBS)
   **BSA for making control solution

DAY OF LAB

1. Resuspend the columns. This can be done up to one hour prior to class. If air bubbles form in the bead bed, resuspend again.
2. Make up sample mixtures, mix really well and aliquot to microtubes. Keep refrigerated or on ice.
   - Plan 2 drops (from a transfer pipet, 1 drop is ~30 μl) per group. This allows for error. Each pair of students will only need to use one drop for the lab.
   - Use equal number of drops for each molecule. For example:
     10 drops ovalbumin + 10 drops vitamin B_{12} = 20 drops (enough for 10 pairs of students).

     Mixture A: ovalbumin + vitamin B_{12}

     Mixture B: _____ + _____

     Have the class determine the contents of Mixture B after the discussion of method and available molecules. One half of the class can do Mixture A and the other half mixture B.
3. Put out all reagents and aliquots of molecule mixtures
4. Place safety reminder about Protein Detection Reagent being a strong acid on board.
5. Place a reminder on board of which student pairs are doing Mixture A and which are doing Mixture B.

*After students have run and recapped the columns, they are now ready for the next class. Columns **do not** have to be resuspended between classes. However, depending on the molecules used in Mixture B some columns may require additional washing.*

SEE STUDENT GUIDE FOR LAB PROCEDURE

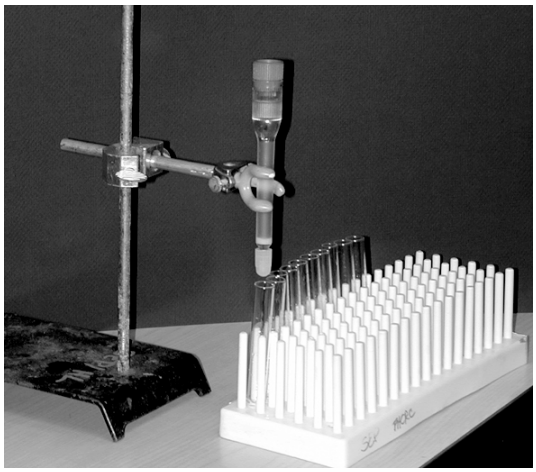
POST KIT-USE CLEANUP (TEACHER)

SEP will reuse the columns but not the beads. Therefore, before returning the kit please:

1. Empty and rinse each column well. Please do final rinse with distilled water.
2. Cap both ends.
3. Clean all tubes and pipettes. The protein detection reagent commonly stains the test tubes, please rinse well with 70% or 90% ethanol or isopropyl alcohol (if available), then once with water before returning them in the kit.

ESSENTIAL TIPS
Getting professional/reproducible results

1. Set up equipment at the Instructor's Station for demo and student reference.
2. Be sure the chromatography column is high enough on the ring stand for the rack of tubes to easily move underneath (see picture on right).



3. Always remove the cap from the reservoir first; then remove the cap from the tip. This prevents a vacuum from forming which would disturb the consistency of the bead bed.
4. It is important to always maintain this consistency/uniformity for even flow of molecules through the column. To do this
   a) Always apply the sample mixture as close to the bed as possible without touching it. See Flow Chart
   b) Add column buffer ***slowly*** placing the tip of the transfer pipette as close as possible to the column bed, or already present buffer, letting the buffer run down the side of the reservoir so as not to create a current. See Figure 2 below.
   c) Keep the column buffer at the same level for consistent constant pressure flow. Fig.3

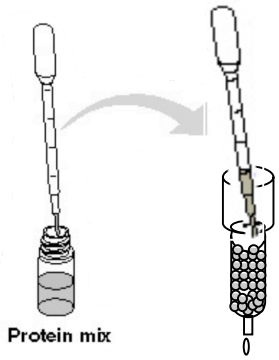


Figure 1

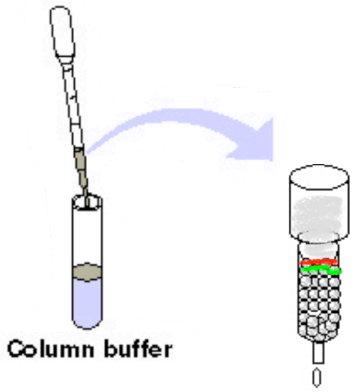


Figure 2

Figure 3

(Columns by C. McIntyre SEP, Other Copyright of BioRad Laboratoies)

5. Keep the gel bed wet at all times. If it dries, it cracks like dried mud and *all* of the molecules can move quickly through at the same time. (This defeats the purpose.)
6. Patience is important. Fractions collect 5-6 drops per minute, so….very slowly. It is important to be sure to always collect the same number of drops in each tube.

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.

Molecules included in the SEP kit:

In Refrigerator Box of Samples		
Quantity	**Item Description**	**Special Notes**
900 µl	Riboflavin (yellow)	MW 376.4 (vitamin)
900 µl	Cytochrome C (reddish)	MW 12,327 (protein)
900 µl	Blue Dextran (blue)	MW 2,000,000 (polysaccharide)
900 µl	Horseradish Peroxidase (tan)	MW 44,000 (protein)
900 µl	Myoglobin (brown)	MW 18,800 (protein)
900 µl	Vitamin B12 (orange)	MW 1,355.4 (vitamin)
900 µl	Ovalbumin (colorless)	MW 43,000 (protein)
In green crate		
1	Food Coloring Set	Small molecular weight dyes (MWs under 1000)
1	Dyes/Indicators	MWs under 1000

BIO-RAD CHROMATOGRAPHY KITS

Bio-Rad Laboratories sells two types of chromatography kits that work very well in the classroom. These kits are described in detail in Bio-Rad's Biotechnology Explorer Catalog as well as on their website (http://www.bio-rad.com Search for "Biotechnology Explorer"). You can also contact Bio-Rad at 1-800-4BIORAD.

Bio-Rad Kit #7 (How Can You Size up the Situation?) is a size separation chromatography kit similar to the SEP lab. Students separate hemoglobin and vitamin B_{12} by size exclusion chromatography. A kit that includes 8 workstations lists for $39.50. Be sure to inquire about the educational discount.

Bio-Rad Kit #2 (Protein purification) allows students to purify green fluorescent protein (GFP) from E. coli that has been transformed with a plasmid that carries the GFP gene. This experiment is designed to follow Kit #1 (Bacterial Transformation with pGlo). The type of chromatography used to separate GFP works by using a column that has an affinity for hydrophobic proteins. GFP is a hydrophobic protein and sticks to the column, while non-hydrophobic proteins in the bacterial cell pass through the column. GFP is released from the column by passing a buffer through the column that disrupts the interaction of the protein from the column, allowing the protein to be collected in tubes. This kit, which includes 8 workstations, is listed at $59.50. Be sure to inquire about the educational discount.

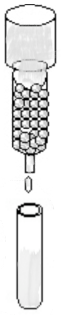
ACKNOWLEDGEMENTS

SEP gratefully acknowledges permission from Bio-Rad laboratories to reprint figures from the Biotechnology Explorer website.

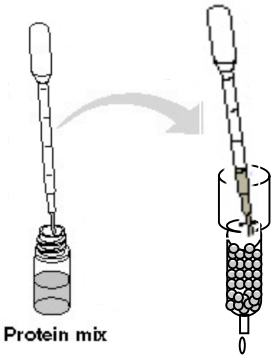
The 1999 SEP lead teachers provided substantial input for reworking the laboratory protocol.

Finally, SEP would especially like to thank Cindy McIntyre for the many long hours she dedicated to this project.

Chromatography Lab Protocol Overview

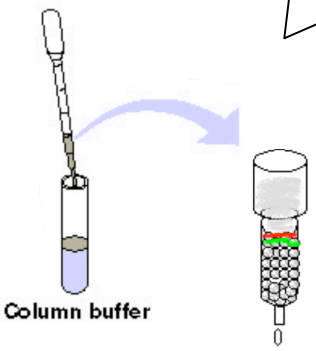
1. 

Equilibrate column by eluting the column buffer into the waste tube until the bead bed (peering into column from above) just appears to look dull/grainy.

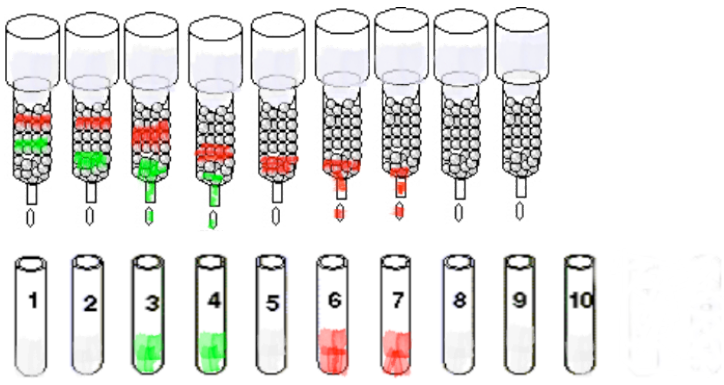
2. 

Load sample mixture, 1 drop, onto column

3. **Slowly add**



column buffer until reservoir is 1/2 full. Maintain this level for constant pressure and flow throughout the lab.



4. **Collect 10 drops of fractionate per test tube.**