



# **Biotechnology Explorer™**

## **Size Exclusion Chromatography**

### **Instruction Manual**

**Catalog Number  
166-0008-EDU**

**[www.bio-rad.com](http://www.bio-rad.com)**

***BIO-RAD***

## **To the Teacher**

One of the biggest challenges for those studying biotechnology for the first time is that many of the events and processes they are studying are invisible. Bio-Rad's Explorer products offer a unique solution. All of our educational kits use colored or fluorescent molecules so that the biological processes that are being studied can be clearly and easily visualized.

This Size Exclusion Chromatography (SEC) kit is designed to teach basic gel filtration chromatography techniques. This kit utilizes the colored molecules hemoglobin and vitamin B12 to illustrate the principles of SEC. Students can easily visualize the separation of these molecules as they pass through the chromatography column.

### **A Complete Teaching Guide**

Developed over five years, Biotechnology Explorer kits and curricula have been written for teachers, by teachers, and have been extensively field-tested in a broad range of classroom settings from high school through the undergraduate level. Easy-to-use Biotechnology Explorer kits are the perfect way to bring the excitement of biotechnology into the classroom. Each kit contains an innovative step-by-step protocol, which makes the kits the perfect choice for both experts and beginning teachers.

The curriculum contained within the manual for each kit makes our products unique. Each kit contains its own unique curriculum package which is divided into a Teachers Guide and Student Manual. The Teachers Guide is divided into three sections. One section contains background information, lecture topics, and suggested references which will enable each teacher, both the experienced and the newcomer to biotechnology, to prepare and design lectures and lessons which can precede the actual labs. This advance preparation will virtually insure that the labs run smoothly and that the students will understand the concepts behind each lab. There is a detailed section on the laboratory set up, complete with simple procedures which contain graphic diagrams detailing the advance preparation for the labs. This makes the set up for each lab simple and straightforward. In addition, this section contains time tables which will help you plan your schedule. Each lab can be performed in a 50 minute class period, which should fit into most schedules.

Finally, we provide a detailed Teachers Answer Guide which contain answers to all of the questions posed in the Student Manual. The teacher can use these answers as a guide when reviewing or grading the questions presented in the student section of the manual.

The Student Manual is designed to maximize student involvement in both the labs and the thought questions embedded in each lesson. Student involvement in this process will result in an increased understanding of the scientific process and the value of proceeding into a task in an organized and logical fashion. Students who engage in the science curriculum found in the Bio-Rad explorer kits develop a positive sense of their ability to understand the scientific method.

We strive to continually improve our curriculum and products. Your input is extremely important to us. Incorporation of your ideas, comments, critiques, and suggestions will enable the Explorer products to evolve into even better teaching aids.

You can find the catalog and curriculum on the internet. Look up our home page at [www.bio-rad.com](http://www.bio-rad.com) or call us at 1-800-424-6723.

Ron Mardigian  
Director, Biotechnology Explorer Program  
[ron\\_mardigian@bio-rad.com](mailto:ron_mardigian@bio-rad.com)

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## Student Objectives

- Compare and contrast the use of different types of column chromatography in the purification of proteins.
- Explain how naturally occurring or recombinant proteins are separated and purified using column chromatography.
- Discuss how the structure and biochemical properties of proteins relate to purification using column chromatography.
- Apply the scientific method to solve a problem\*
  - \* **Problem:** Can Hemoglobin (molecular weight of 65,000 daltons) be separated from vitamin B12 (molecular weight of 1,350 dalton) by gel filtration chromatography?

## Pre-Lab Activities

The following activities are recommended before chromatography is conducted:

1. Cover Biology text on protein structure.
2. Review DNA structure and function and protein synthesis.
3. Conduct library and online research studying the functions of some common proteins.

## Background Lectures Ideas

Our bodies contain thousands of different proteins which perform many different jobs. Digestive enzymes are proteins; some of the hormone signals that run through our bodies and the antibodies protecting us from disease are proteins. The information for assembling a protein is carried (in code) in our DNA. The section of DNA which contains the code for making a protein is called a gene. There are thousands of genes on each chromosome. Each gene codes for a unique protein. The gene which makes a digestive enzyme in your mouth is different from one which makes an antibody.

Proteins are often products sought to be used for medical purposes. Some of these proteins are purified in large quantities from a naturally-occurring source. Recently, many proteins for medical purposes have been made through genetic engineering and recombinant DNA technology. No matter what the source, a protein of interest is found in a mixture of a cell's other proteins. Some cells, such as bacteria, produce large quantities of up to two thousand different kinds of proteins.

Since 75% of the dry matter in living things is protein, biologists must often purify a protein of interest from other proteins in a cell. Determining the procedures for the purification of a particular protein is a challenging task for the biotechnology industry. To separate any of the macromolecules, scientists utilize their knowledge of the chemistry of these molecules, including: the molecular weight of the protein (size), its charge, and its shape.

## Instructors Guide

### Kit Inventory Check (✓) List

This section lists the components provided in the Size Exclusion Chromatography kit. It also lists required accessories. Each kit contains sufficient materials to outfit eight student workstations. Use this as a checklist to inventory your supplies before beginning the experiments.

| <b>Kit Components</b>     | <b>Number/Kit</b> | <b>(✓)</b>               |
|---------------------------|-------------------|--------------------------|
| Protein Mix               | 1 vial            | <input type="checkbox"/> |
| Hemoglobin                |                   |                          |
| Vitamin B12               |                   |                          |
| Poly-Prep® sizing columns | 8                 | <input type="checkbox"/> |
| Column end-caps           | 25**              | <input type="checkbox"/> |
| Column buffer             | 50 ml             | <input type="checkbox"/> |
| Pipettes (1 ml)           | 10                | <input type="checkbox"/> |
| Collection tubes          | 100               | <input type="checkbox"/> |
| Manual and Quick Guide    | 1                 | <input type="checkbox"/> |

\*\* Several extra are supplied with the kit.

| <b>Required Accessories</b>         |   |                          |
|-------------------------------------|---|--------------------------|
| Test tube rack for holding 12 tubes | 8 | <input type="checkbox"/> |
| Black marking pen                   | 8 | <input type="checkbox"/> |

## Implementation Timeline

The active lab session is designed to be carried out in a single 50 minute period. The detailed laboratory protocol can be found in the Student Manual.

**Lesson 1 Lesson on Chromatography**

Lesson on hemoglobin, RBCs, vitamin B12, protein biochemistry.

Analysis and thought questions.

**Lesson 2 Run the Laboratory**

**Lesson 3 Analysis of results**

Analysis questions

## Lesson 1

# Introduction to Chromatography

This investigation is intended to teach basic techniques of size exclusion chromatography. This laboratory activity integrates well into both basic and advanced biology curricula. The two molecules used in this activity, hemoglobin and vitamin B12, are both compounds essential to functions in the human body; thus this laboratory activity can be linked to basic lessons in biology, human physiology, and biochemistry.

This section describes the experimental and conceptual points which may prove challenging to students. These points are extremely important to the overall outcome of the activity. Instructors should direct their students attention to these points, and when possible, demonstrate the technique before the students attempt the procedure.

Chromatography is commonly used in biotechnology for purifying biological molecules, like proteins, for medicine or other uses. Chromatography separates individual components from complex mixtures. Chromatography consists of a mobile phase (solvent and the molecules to be separated) and a stationary phase either, in paper (in paper chromatography) or glass beads, called resin, (in column chromatography), through which the mobile phase travels. Molecules travel through the stationary phase at different rates because of their chemistry.

### Some Common Types of Chromatography

In **gel filtration chromatography**, commonly referred to as **size exclusion chromatography (SEC)**, microscopic beads which contain tiny holes are packed into a column. When a mixture of molecules is dissolved in a liquid and then applied to a chromatography column that contains porous beads, large molecules pass quickly around the beads, whereas smaller molecules enter the tiny holes in the beads and pass through the column more slowly. Depending on the molecules, proteins may be separated, based on their size alone, and fractions containing the isolated proteins can be collected.

In **affinity chromatography**, a biomolecule (often an antibody) that will bind to the protein to be purified is attached to the beads. A mixture of proteins is added to the column and everything passes through except the protein of interest, which binds to the antibody and is retained on the solid support. To get the protein to elute from the column, another buffer is used to disrupt the bond between the protein of interest and the antibody. Often this elution buffer contains high concentrations of salt or acid.

In **ion exchange chromatography**, the glass beads of the column have a charge on them (either + or -). A mixture of protein is added to the column and everything passes through except the protein of interest. This is because the charge of the beads is picked to have the opposite charge of the protein of interest. If the charge of the beads is positive, it will bind negatively charged molecules. This technique is called **anion exchange**. If the beads are negatively charged, they bind positively charged molecules (**cation exchange**). Thus, a scientist picks the resin to be used based on the properties of the protein of interest. During the chromatography, the protein binds to the oppositely charged beads. After the contaminant is separated from the protein of interest, a high salt buffer is used to get the desired protein to elute from the column.

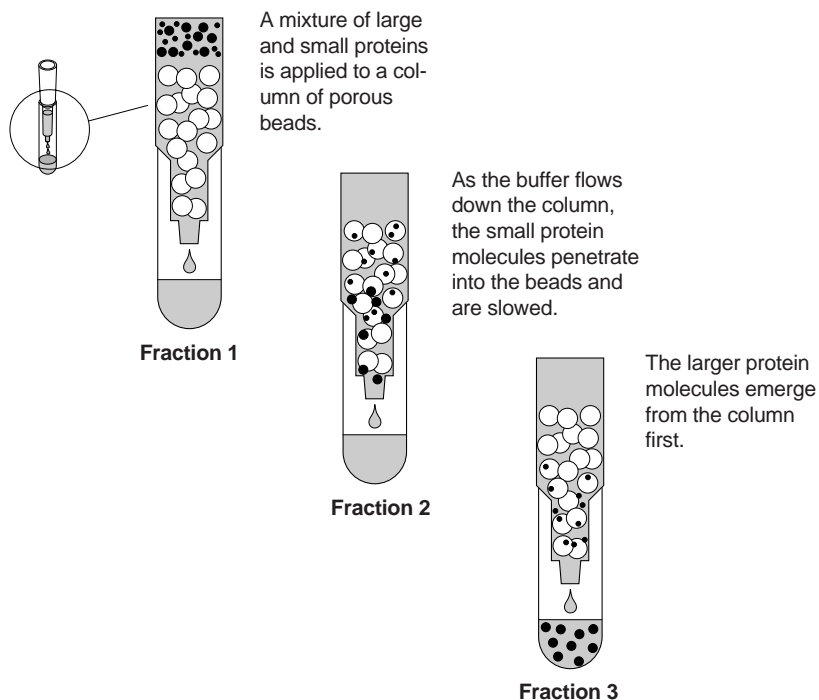
This kit is designed to teach basic principles of size exclusion chromatography (SEC), a technique which allows the separation of molecules on the basis of size. The kit uses the colored molecules hemoglobin and vitamin B12 to illustrate the principles of SEC. Hemoglobin (reddish-brown) is much larger than vitamin B12 (pink), and thus passes through the column more quickly than vitamin B12. The students can easily visualize the separation of these molecules as they pass through the column and into collection tubes.

## Principles of Size Exclusion Chromatography (SEC)

The mass of beads within the column is often referred to as the **column bed**. The beads act as “**traps**” or “**sieves**” and function to filter small molecules which become temporarily trapped within the pores. Larger molecules pass around, or are “**excluded**” from, the beads. This kit contains eight columns which are prefilled with beads that effectively separate or “**fractionate**” molecules that are below 60,000 daltons. As the liquid flows through the column, molecules below 60,000 daltons enter the beads and pass through the column more slowly. The smaller the molecules, the slower they move through the column. Molecules greater than 60,000 pass around the beads and are excluded from the column—also referred to as the **exclusion limit** of a column.

The liquid used to dissolve the biomolecules to make the mobile phase is usually called a **buffer**. The mixture of biomolecules dissolved in the buffer is called the **sample**. The sample is placed on the column bed and the biomolecules within the buffer enter the top of the column bed, filter through and around the porous beads, and ultimately pass through a small opening at the bottom of the column. For this process to be completed additional buffer is placed on the column bed after the sample has entered the bed. The mobile phase liquid is collected, as drops, into collection tubes which are sequentially ordered. A set number of drops is usually collected into each tube. The larger molecules which pass quickly through the column will end up in the early tubes or “**fractions**”. The smaller molecules which penetrate the pores of the stationary phase end up in the later fractions.

Hemoglobin and vitamin B12 are the two molecules which are being separated in this lab activity. Hemoglobin, which is brown, has a molecular weight of 65,000 daltons and is thus **excluded** from the column. Hemoglobin will pass more quickly through the column and appear in the early collection tubes, or **fractions**. Vitamin B12, which is pink, has a molecular weight of 1,350 daltons and is thus **fractionated** by the column. The vitamin B12 molecules penetrate the pores of the beads, becoming temporarily trapped. As a result, they pass much more slowly through the column and appear in the later **fractions**. The schematic below illustrates the differential fractionation of large and small molecules on a size exclusion column.



## The Sample—Hemoglobin and Vitamin B12

### Hemoglobin

Hemoglobin, a protein found in red blood cells, functions to transport oxygen to the tissues of the body. The hemoglobin used in this experiment is bovine hemoglobin. The use of bovine hemoglobin (rather than the human counterpart) avoids the potential health hazard presented when using human blood products. Hemoglobin is made up of four polypeptides (small proteins) which associate to form a large, globular protein. Hemoglobin gets its name from the heme group, the iron-containing component of hemoglobin which physically binds oxygen. The iron-containing heme group is responsible for the red-brown color of hemoglobin. The closely related protein, **myoglobin**, is found in muscle and is responsible for delivering oxygen to muscle tissue. Muscles which are very active and require a lot of oxygen are dark in color because of a high myoglobin content. An example would be the red-brown color of the dark meat of chicken.

Hemoglobin is the main component of red blood cells (RBCs), the oxygen carrying cells of the body. Again, it is the heme group of hemoglobin which gives RBCs their distinctive red color. Different forms of hemoglobin are produced during different stages of development. Fetuses produce a form of hemoglobin which has a higher **affinity** (tighter binding) for oxygen than does adult hemoglobin. Because fetuses depend upon their mothers for their oxygen supply, it is important that maternal hemoglobin can easily give up its oxygen to the fetal hemoglobin. For this reason, obstetricians advise their patients to avoid vigorous exercise during pregnancy. Vigorous exercise depletes the tissues of oxygen, which sets up a competition between the transfer of oxygen to maternal tissues or to fetal hemoglobin.

In addition to oxygen, hemoglobin can also bind carbon monoxide. Hemoglobin actually has a higher affinity for carbon monoxide than for oxygen. Suffocation from carbon monoxide occurs when oxygen bound to hemoglobin is displaced by carbon monoxide, which in turn deprives body tissues of oxygen.

The body can adapt to environmental changes which require increased amounts of oxygen delivery to tissues. At high altitudes, where the amount of oxygen in air is decreased, the body responds by increasing the number of red blood cells produced. This effectively increases the number of molecules of hemoglobin in the blood supply, which has the effect of increasing the oxygen supply to the tissues. For this reason, athletes will train at high elevation to increase the amount of RBC, and thus increase their oxygen capacity, which is needed for rigorous exercise.

Sickle cell anemia is a molecular disease of hemoglobin. A single change or mutation in the gene which encodes hemoglobin results in a mutation in the amino acid sequence. This mutation changes the three dimensional structure of the polypeptides of hemoglobin, causing them to “stick” together as rod-like structures. The abnormal rod-like hemoglobin molecules distort the structure of red blood cells, causing them to have a **sickle** shape. Unlike their round counterparts, the sickle-shaped RBC can not freely pass through capillary beds, and thus the capillary beds become blocked. The blocked capillary beds of organs and tissues make delivery of oxygen difficult, resulting in extreme fatigue and even death. Because sickle-cell anemia is a genetic disorder which results from a mutated genetic sequence, at this time there is no cure. However, the side effects of sickle cell anemia can be alleviated by frequent blood transfusions from people who have normal hemoglobin and red blood cells. Sickle cell anemia is a genetic disease in which the individual has inherited a defective mutant hemoglobin gene from both parents. Individuals with the **sickle cell trait** have received an abnormal gene from only one parent, and the single defect actually confers an evolutionary advantage. In Africa, expression of the sickle cell gene positively correlates with malaria infections. Malaria is a deadly disease caused by a mosquito-borne parasite. The parasite infects and ultimately

kills RBCs. The parasite can infect normal RBCs, but can not infect sickle cell RBCs. Thus, the sickle cell trait helps confer resistance to malaria and results in a positive evolutionary adaptation. Unfortunately, expression of two copies of the gene is deleterious.

### **Vitamin B12**

Vitamin B12 is a vitamin that is essential to humans and other vertebrates. Vitamin B12 is an essential cofactor of several biochemical reactions which occur in the human body. One function of vitamin B12 is the breakdown of fats. Sources rich in vitamin B12 include eggs, dairy products, and meats. Vitamin B12 is not found in plants and vegetable foods. Thus people who have strict vegetarian diets are often deficient in vitamin B12, unless they take some supplementary vitamin.

Pure molecules of vitamin B12 can not be absorbed by the intestines. Vitamin B12 must bind to a carrier protein in the intestinal tract. When vitamin B12 binds to this **carrier protein**, the complex is able to pass through the intestine and into the blood stream, where it is eventually taken up by the liver.

Because vitamin B12 is only required in minute quantities (humans require  $\sim 3 \mu\text{g/day}$ ), vitamin B12 deficiencies are extremely rare. However, some individuals have a genetic disorder in which the gene that codes for the carrier protein is mutated. Individuals with this mutation do not synthesize the carrier protein necessary for absorption into the blood stream. Thus, even though these people have adequate intakes of vitamin B12, they still show signs of deficiency because they lack the required carrier protein.

## Laboratory Workstation (✓) Checklist

**Student Workstations.** Materials and supplies that should be present at each student workstation prior to beginning each lab experiment are listed below. The components provided in this kit are sufficient for 8 student workstations.

**Instructors (Common) Workstation.** A list of materials, supplies, and equipment that should be present at a common location that can be accessed by all student groups is also listed below. It is up to the discretion of the teacher as to whether students should access common solutions, or whether the teacher should aliquot solutions.

| <b>Student workstation items</b>      | <b>Number required</b> | <b>(✓)</b>               |
|---------------------------------------|------------------------|--------------------------|
| Collection tubes                      | 12                     | <input type="checkbox"/> |
| Size exclusion chromatography columns | 1                      | <input type="checkbox"/> |
| Column end-caps                       | 1                      | <input type="checkbox"/> |
| Pipette                               | 1                      | <input type="checkbox"/> |
| Lab marker                            | 1                      | <input type="checkbox"/> |
| Test tube rack                        | 1                      | <input type="checkbox"/> |

| <b>Instructor workstation items</b> | <b>Number required</b> |                          |
|-------------------------------------|------------------------|--------------------------|
| Protein mixture                     | 1 vial                 | <input type="checkbox"/> |
| Column buffer                       | 1 bottle               | <input type="checkbox"/> |

## Advance Laboratory Preparation

This section describes the preparation that needs to be performed by the instructor before the laboratory. An estimation of preparation time is included in each section.

### **Advance Preparation**

**Objectives**      Rehydrate protein mixture  
Set up student and instructor workstations  
Photocopy Quick Guides for students

**Time required**    Twenty minutes to 1 hour

**Procedure**        Approximately 15 minutes before the start of the laboratory, use one of the pipettes in the kit and add 0.5 ml of distilled water to the vial of protein mix. Mix gently several times over the course of 15 minutes. Keep on ice or in the refrigerator until the start of the experiment.

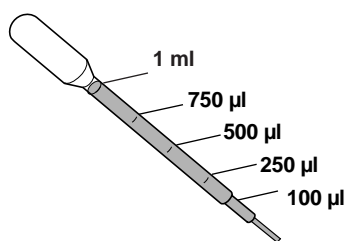
## Lesson 2 Laboratory Instructors Lab Manual

This version of the lab protocol contains detailed notes and helpful hints for setting up and running the lab.

### Techniques to Highlight

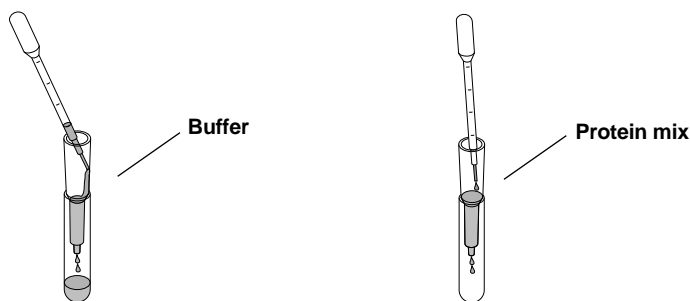
#### Pipetting

Before beginning the experiment, point out to the students the graduation marks on the pipet. The 250  $\mu\text{l}$  and 1 ml marks will be used for measurements in this exercise. Have the students practice with volumes of water to acquaint themselves to precision pipetting.



#### Chromatography

Also stress that it is important not to disturb the column bed. When loading sample or buffer onto the column bed, the pipette should be inserted close to the bed against the wall of the column. Liquid should be gently expelled from the pipette down the wall of the column (for the buffer) or onto the top of the bed (for the protein mix).



#### Important hints for successful chromatography

1. Snap, do not twist, the bottom tab from the prefilled column.
2. Place the column gently into the collection tubes. Jamming the column tightly into the collection tubes will create an air tight seal and the sample will not flow through. You can create a "paper crutch" by folding a small piece of paper, about the size of a match stick, and wedging it between the column and the collection tube. This crutch makes it impossible for an air tight seal to form, and insures that the column will flow.
3. The columns are designed to drip slowly. The entire chromatography procedure should take 20 to 30 minutes. It is important not to remove the column more than needed from collection to collection tube, as motion can cause major disturbance to the column bed.

## Setting Up and Running the Lab

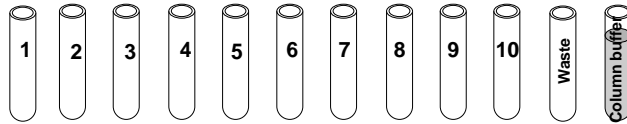
1. Each student team will require 12 **collection tubes**. Have each team label 10 collection tubes sequentially from 1 to 10. The last two tubes are labeled “waste” and “column buffer”. Place the tubes in the rack. Label either the tubes or the rack with your name and laboratory period.
2. Pipet 4 ml of **Column Buffer** into the tube labeled column buffer. There is only one stock bottle of column buffer provided in this kit. The teacher may aliquot the 4 ml into each of the labeled collection tubes, or one student from each group may aliquot their own 4 ml of column buffer.
3. Have the students remove the top cap and snap off the end of their Poly-Prep sizing column. Drain the buffer into the “waste” collection tube. Then recap the bottom of the column with the **column end cap**.
4. Place the column onto tube 1. The students are now ready to load (or the teacher may choose to load) the protein sample onto the column. There is one vial of protein mix in the kit—it may be most convenient to approach individual student groups with the vial and load a drop onto the column.
5. Have students remove the end cap from the column. Observe the top of the column bed; all of the buffer should have drained from the column. This is best observed by looking directly over the column—the “grainy” appearance of the column beads should be visible. If any residual buffer remains on top of the column, the protein sample will be diluted when a drop is applied, which will result in poor separation. Carefully load **one drop** of protein mix onto the top of the column bed. The pipette should be inserted into the column and the drop should be loaded just above the top of the column bed so that application of the protein sample minimally disturbs the column bed.
6. Allow the protein mix to enter the column bed. This is best observed by looking directly over the column. Then, carefully add 250  $\mu$ l of column buffer to the top of the column. This is best done by inserting the pipet tip into the column so that it rests just above the column bed. Carefully let the buffer run down the side of the tube and onto the top of the bed. Begin to collect drops into tube #1.
7. When all of the liquid has drained from the column, add another 250  $\mu$ l of column buffer to the top of the column. Add the buffer as before, by placing the pipette just above the top of the column and letting the buffer run down the side of the tube. Continue to collect drops into tube 1. The number of drops that are collected into tube 1 do not need to be counted.
8. When all of the liquid has drained from the column, add 3 ml of column buffer to the top of the column. This can be done by adding 1 ml from the pipette three times. At this time the protein mix has entered the column far enough so that slight disturbances to the column bed will not affect the separation. Transfer the column to tube 2 and begin to count the drops that enter into each tube. Collect 5 drops of buffer into tube 2. Collect 5 drops into each tube, with the exception of tube 10, into which 10 drops will be collected. The teacher can point out that as one student loads the column, another student can count the drops as they drop into the collection tubes.
9. When 5 drops have been collected into tube 2, transfer the column onto tube 3. Collect 5 drops of buffer into each collection tube. When 5 drops have been collected into a tube, lift it off and transfer it to the next tube.
10. Continue collecting 5 drops into each tube. When you reach tube 10, collect a total of 10 drops. After the last 10 drops have been collected, cap the column.

11. The collection tubes containing the column fractions can be parafilmmed or covered and stored in the refrigerator. If tightly sealed, the fractions can be stored for ~ 1 week for future observations/discussions. The column can also be capped with the top and end caps and stored in the refrigerator for ~ 1 week.
12. It may be interesting for the students to compare the starting mix with their individual fractions. You can take the remaining column buffer and add ~ 5 drops of protein mix to the bottle. You can then aliquot 5 drops of this “starting mix” into each of the students “waste” tube. The student groups can then compare the starting mixture with the size-fractionated samples.

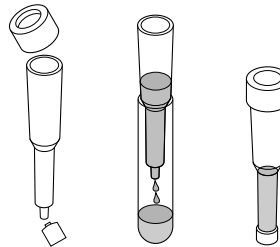
# Laboratory Quick Guide

## Size Exclusion Chromatography Kit

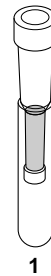
1. Obtain 12 collection tubes and label ten sequentially from 1 to 10. Label the tubes with your name and laboratory period. Label the final two tubes “Waste” and “Column Buffer”. Using a clean pipette, transfer 4 ml of column buffer into the tube labeled “Column Buffer”.



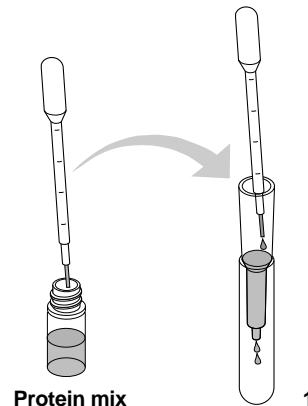
2. Remove the cap and snap off the end of the sizing column. Allow all of the buffer to drain into the waste tube. Observe the upper surface of the matrix and insure that all of the buffer has entered the column. Looking directly over and into the column, you should see the “grainy” appearance of the column matrix. Cap the bottom of the column.



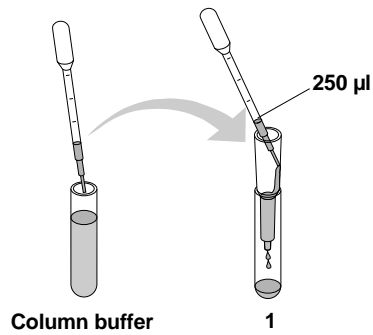
3. Carefully place the column onto tube 1. You are now ready to load (or the teacher may load) the protein sample onto the column.



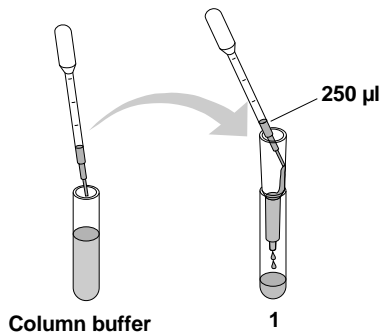
4. When you are ready to load the protein mix, uncap the column. It is important to uncap the column only when you are ready to load your protein—you do not want your column to run dry. Using a pipette, add one drop of protein mix onto the top of the column bed (your teacher may do the loading for you). The pipette should be inserted into the column and the drop should be loaded just above the top of the column so that it minimally disturbs the column bed.



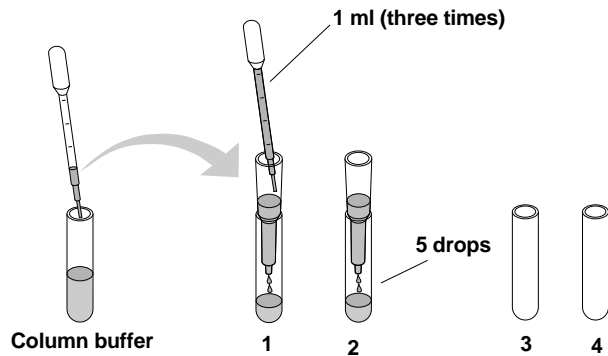
5. As soon as the drop of protein mix enters the column bed, carefully add 250  $\mu$ l of column buffer to the top of the column. This is best done by inserting the pipette tip into the column so that it rests just above surface of the column matrix. Carefully let the buffer run down the side of the tube and onto the top of the bed. (Note: The size separation will work best when the column bed is left undisturbed). Begin to collect drops into tube 1.



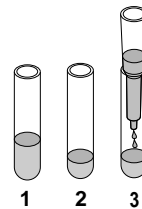
6. Add another 250  $\mu$ l of column buffer to the top of the column. Add the buffer as before, by placing the pipette just above the top of the column and letting the buffer run down the side of the tube. Continue to collect drops into tube 1.



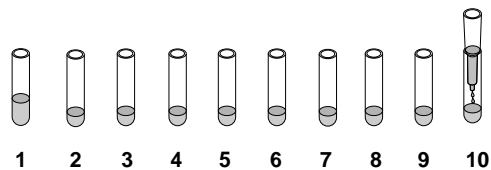
7. Add 3 ml of column buffer to the top of the column matrix. This can be done by adding 1 ml three times from the pipette. At this time the protein mix has entered the column far enough so that slight disturbances to the column bed will not affect the separation. Transfer the column to tube 2 and begin to count the drops that enter into each tube. Collect 5 drops of buffer into tube 2.



8. When 5 drops have been collected into tube 2, transfer the column onto tube 3. Collect 5 drops of buffer into each collection tube. When 5 drops have been collected into a tube, lift it off and transfer it to the next tube.



9. Continue collecting 5 drops into each tube. When you reach tube 10, collect a total of 10 drops. Cap the column and if your teacher instructs you to do so, parafilm or cover your fractions until the next laboratory period. Store the fractions in the refrigerator. Sketch your results.



# Student Manual

## Size Exclusion Chromatography

### Lesson 1A Introduction to Chromatography

Chromatography is commonly used in biotechnology for purifying biological molecules, like proteins, for medicine or other uses. Chromatography allows the separation of individual components from complex mixtures. Chromatography consists of a mobile phase (solvent and the molecules to be separated) and a stationary phase either paper (in paper chromatography), or glass beads, called resin (in column chromatography), through which the mobile phase travels. Molecules travel through the stationary phase at different rates because of their chemistry.

#### Some Common Types of Chromatography

In **gel filtration chromatography**, commonly referred to as **size exclusion chromatography (SEC)**, microscopic beads which contain tiny holes are packed into a column. When a mixture of molecules is dissolved in a liquid and then applied to a chromatography column that contains porous beads, large molecules pass quickly around the beads, whereas smaller molecules enter the tiny holes in the beads and pass through the column more slowly. Depending on the molecules, proteins may be separated, based on their size alone, and fractions containing the isolated proteins can be collected.

In **affinity chromatography**, a biomolecule (often an antibody) that will bind to the protein to be purified is attached to the beads. A mixture of proteins is added to the column and everything passes through except the protein of interest, which binds to the antibody and is retained on the solid support. To get the protein to elute from the column, another buffer is used to disrupt the bond between the protein of interest and the antibody. Often this elution buffer contains high concentrations of salt or acid.

In **ion exchange chromatography**, the glass beads of the column have a charge on them (either + or -). A mixture of protein is added to the column and everything passes through except the protein of interest. This is because the beads are picked to have the opposite charge of the protein of interest. If the charge of the beads is positive, it will bind negatively charged molecules. This technique is called **anion exchange**. If the beads are negatively charged, they bind positively charged molecules (**cation exchange**). Thus, a scientist picks the resin to be used based on the properties of the protein of interest. During the chromatography, the protein binds to the oppositely charged beads. When the contaminant is separated from the protein of interest, a high salt buffer is used to get the desired protein to elute from the column.

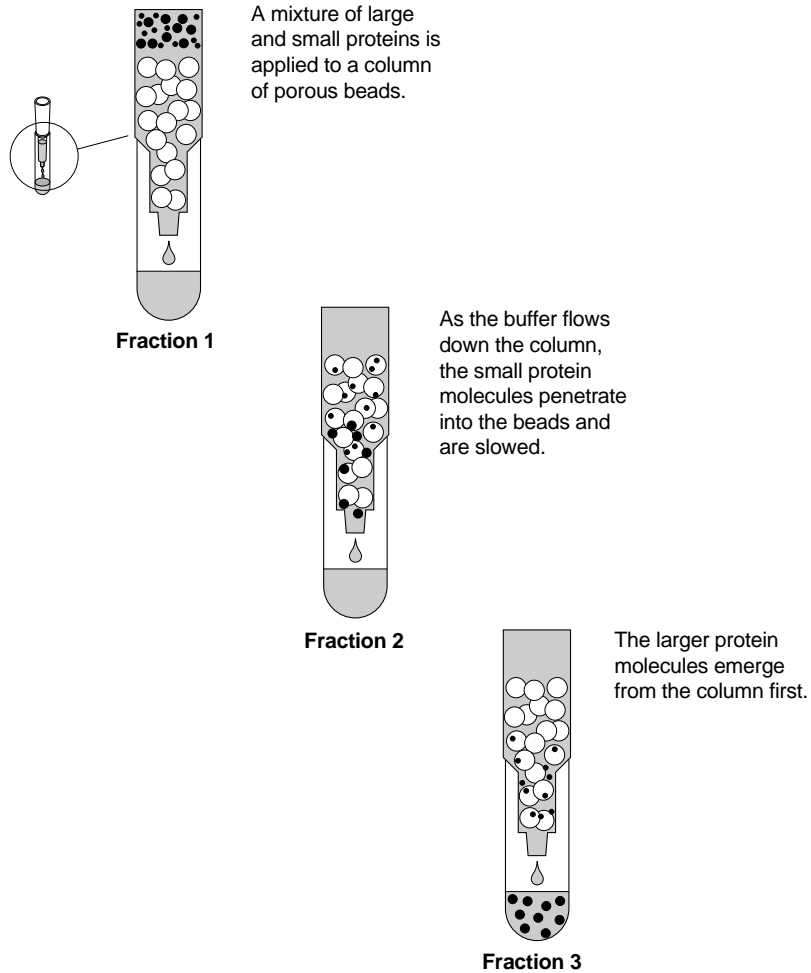
#### Principles of Size Exclusion Chromatography (SEC)

In this laboratory you will investigate the principles of size exclusion chromatography. Size exclusion chromatography is a very powerful technique for the physical separation of molecules on the basis of size. In this procedure, a mixture of molecules dissolved in liquid (the mobile phase) is applied to a chromatography column which contains a solid support in the form of microscopic porous spheres, or “**beads**” (the stationary phase). The mass of beads within the column is often referred to as the **column bed**. The beads act as “**traps**” or “**sieves**” and function to filter small molecules which become temporarily trapped within the pores. Larger molecules pass around or are “**excluded**” from the beads. This kit contains eight columns which are prefilled with beads that effectively separate or “**fractionate**” molecules that are below 60,000 daltons. As the liquid flows through the column, molecules below 60,000 daltons enter the beads and pass through the column more slowly. The smaller the molecule, the slower they move through the column. Molecules greater than 60,000 pass

around the beads and are excluded from the column—also referred to as the **exclusion limit** of a column.

The liquid used to dissolve the biomolecules to make the mobile phase is called a **buffer**. The mixture of biomolecules dissolved in the buffer is called the **sample**. The sample is placed on the column bed and the biomolecules within the buffer enter the top of the column bed, filter through and around the porous beads, and ultimately pass through a small opening at the bottom of the column. For this process to be completed, additional buffer is placed on the column bed after the sample has entered the bed. The mobile phase (liquid) is collected as drops into a series of collection tubes. A set number of drops is collected into each tube. The larger molecules which pass quickly through the column will end up in the early tubes or “**fractions**”. The smaller molecules which penetrate the pores of the stationary phase end up in the later fractions.

Hemoglobin and vitamin B12 are the two biomolecules in your sample. Hemoglobin, which is brown, has a molecular weight of 65,000 daltons. Vitamin B12 is pink and has a molecular weight of 1,350 daltons. The schematic below illustrates the differential fractionation of large and small molecules on a size exclusion column.





## Lesson 1B The Sample

### Hemoglobin

Hemoglobin, a protein found in red blood cells, functions to transport oxygen to the tissues of the body. The hemoglobin used in this experiment is bovine hemoglobin. The use of bovine hemoglobin (rather than the human counterpart) avoids the potential health hazard presented when using human blood products. Hemoglobin is made up of four polypeptides (small proteins) which associate to form a large, globular protein. Hemoglobin gets its name from the heme group, the iron-containing component of hemoglobin which physically binds oxygen. The iron-containing heme group is responsible for the red-brown color of hemoglobin. The closely related protein, **myoglobin**, is found in muscle and is responsible for delivering oxygen to muscle tissue. Muscles which are very active and require a lot of oxygen are dark in color because of a high myoglobin content. An example would be the red-brown color of the dark meat of chicken.

Hemoglobin is the main component of red blood cells (RBCs), the oxygen carrying cells of the body. Again, it is the heme group of hemoglobin which gives RBCs their distinctive red color. Different forms of hemoglobin are produced during different stages of development. Fetuses produce a form of hemoglobin which has a higher **affinity** (tighter binding) for oxygen than does adult hemoglobin. Because fetuses depend upon their mothers for their oxygen supply, it is important that maternal hemoglobin can easily give up its oxygen to the fetal hemoglobin. For this reason, obstetricians advise their patients to avoid vigorous exercise during pregnancy. Vigorous exercise depletes the tissues of oxygen, which sets up a competition between the transfer of oxygen to maternal tissues or to fetal hemoglobin.

In addition to oxygen, hemoglobin can also bind carbon monoxide. Hemoglobin actually has a higher affinity for carbon monoxide than for oxygen. Suffocation from carbon monoxide occurs when oxygen bound to hemoglobin is displaced by carbon monoxide, which in turn deprives body tissues of oxygen.

The body can adapt to environmental changes which require increased amounts of oxygen delivery to tissues. At high altitudes, where the amount of oxygen in air is decreased, the body responds by increasing the number of red blood cells produced. This effectively increases the number of molecules of hemoglobin in the blood supply, which has the effect of increasing the oxygen supply to the tissues. For this reason, athletes will train at high elevation to increase the amount of RBC's, and thus increase their oxygen capacity, which is needed for rigorous exercise.

Sickle cell anemia is a molecular disease of hemoglobin. A single change or mutation in the gene which encodes hemoglobin results in a mutation in the amino acid sequence. This mutation changes the three dimensional structure of the polypeptides of hemoglobin, causing them to "stick" together as rod-like structures. The abnormal rod-like hemoglobin molecules distort the structure of red blood cells, causing them to have a **sickle** shape. Unlike their round counterparts, the sickle-shaped RBC can not freely pass through capillary beds, and thus the capillary beds become blocked. The blocked capillary beds of organs and tissues make delivery of oxygen difficult, resulting in extreme fatigue and even death. Because sickle-cell anemia is a genetic disorder which results from a mutated genetic sequence, at this time there is no cure. However, the side effects of sickle cell anemia can be alleviated by frequent blood transfusions from people who have normal hemoglobin and red blood cells. Sickle cell anemia is a genetic disease in which the individual has inherited a defective mutant hemoglobin gene from both parents. Individuals with the **sickle cell trait** have received an abnormal gene from only one parent, and the single defect actually confers an evolutionary advantage. In Africa, expression of the sickle cell gene positively correlates with malaria infections. Malaria is a deadly disease caused by a mosquito-borne parasite. The parasite infects and ultimately

kills RBCs. The parasite can infect normal RBCs, but can not infect sickle cell RBCs. Thus, the sickle cell trait helps confer resistance to malaria and results in a positive evolutionary adaptation. Unfortunately, expression of two copies of the gene is deleterious.

### **Vitamin B12**

Vitamin B12 is a vitamin that is essential to humans and other vertebrates. Vitamin B12 is an essential cofactor of several biochemical reactions which occur in the human body. One function of vitamin B12 is the breakdown of fats. Sources rich in vitamin B12 include eggs, dairy products, and meats. Vitamin B12 is not found in plants and vegetable foods. Thus people who have strict vegetarian diets are often deficient in vitamin B12, unless they take some supplementary vitamins.

Pure molecules of vitamin B12 can not be absorbed by the intestines. Vitamin B12 must bind to a **carrier protein** in the intestinal tract. When vitamin B12 binds to this carrier protein, the complex is able to pass through the intestine and into the blood stream, where it is eventually taken up by the liver.

Because vitamin B12 is only required in minute quantities (humans require  $\sim 3 \mu\text{g/day}$ ), vitamin B12 deficiencies are extremely rare. However, some individuals have a genetic disorder in which the gene that codes for the carrier protein is mutated. Individuals with this mutation do not synthesize the carrier protein necessary for absorption into the blood stream. Thus, even though these people have adequate intakes of vitamin B12, they still show signs of deficiency because they lack the required carrier protein.



## Lesson 2 Chromatography Lab

### Workstation Check (✓) List

**Your workstation.** Materials and supplies that should be present at your workstation prior to beginning this lab are listed below.

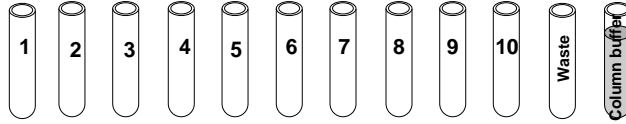
| <b>Student workstation items</b> | <b>Number required</b> | <b>(✓)</b>               |
|----------------------------------|------------------------|--------------------------|
| Collection tubes                 | 12                     | <input type="checkbox"/> |
| Sizing chromatography columns    | 1                      | <input type="checkbox"/> |
| Column end-caps                  | 1                      | <input type="checkbox"/> |
| Pipette                          | 1                      | <input type="checkbox"/> |
| Lab marker                       | 1                      | <input type="checkbox"/> |
| Test tube rack                   | 1                      | <input type="checkbox"/> |

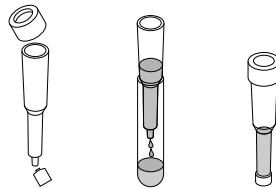
| <b>Instructor workstation items</b> | <b>Number required</b> | <b>(✓)</b>               |
|-------------------------------------|------------------------|--------------------------|
| Protein mixture                     | 1 vial                 | <input type="checkbox"/> |
| Column buffer                       | 1 bottle               | <input type="checkbox"/> |

## Laboratory Protocol

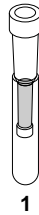
1. Place the 12 collection tubes in your test tube rack. Label 10 collection tubes sequentially from 1 to 10. Label the last two tubes “waste” and “column buffer”. Label either the tubes or the rack with your name and laboratory period.
2. Pipet 4 ml of Column Buffer into the tube labeled column buffer.



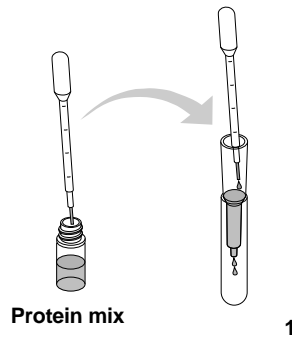
3. Remove the cap and snap off the end of the Poly-Prep sizing column. Drain all of the buffer into the “waste” collection tube. Cap the bottom of the column with the column end cap.



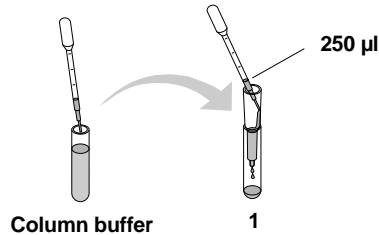
4. Gently place the column onto collection tube 1 (Do not jam the column tightly into the collection tubes—the column will not flow). You are now ready to load the protein sample onto the column.



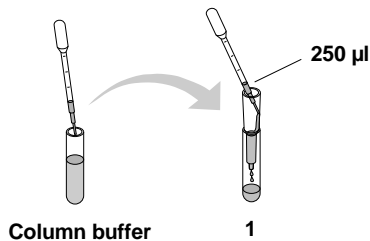
5. Remove the end cap from the column. Observe the top of the column bed; all of the buffer should have drained from the column. This is best observed by looking directly over the column—the “grainy” appearance of the column beads should be visible. Carefully load one drop of protein mix onto the top of the column bed. The pipette should be inserted into the column and the drop should be loaded just above the top of the column so that it minimally disturbs the column bed.



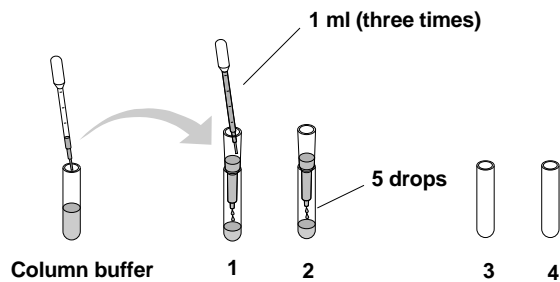
6. Allow the protein mix to enter the column bed. This is best observed by looking directly over the column. Carefully add 250  $\mu$ l of column buffer to the top of the column. This is best done by inserting the pipette tip into the column so that it rests just above the column bed. Carefully let the buffer run down the side of the tube and onto the top of the bed. Begin to collect drops into tube 1.



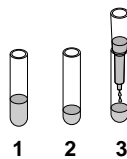
7. When all of the liquid has drained from the column, add another 250  $\mu$ l of column buffer to the top of the column. Add the buffer as before, by placing the pipette just above the top of the column and letting the buffer run down the side of the tube. Continue to collect drops into tube 1.



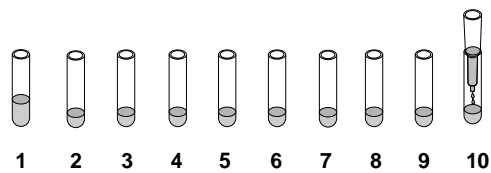
8. When all of the liquid has drained from the column, add 3 ml of column buffer to the top of the column. This can be done by adding 1 ml from the pipette three times. At this time the protein mix has entered the column far enough so that slight disturbances to the column bed will not affect the separation. Transfer the column to tube 2 and begin to count the drops that enter into each tube. Collect 5 drops of buffer into tube 2.



9. When 5 drops have been collected into tube 2, transfer the column onto tube 3. Collect 5 drops of buffer into each collection tube. When 5 drops have been collected into a tube, lift it off and transfer it to the next tube.



10. Continue collecting 5 drops into each tube. When you reach tube 10, collect a final 10 drops. Cap the column when finished collecting drops. Store your samples and column according to your teachers instructions.



### **Lesson 3 Analysis of Laboratory Results**

#### **Review Questions**

1. Think about the color of the protein mix that was applied to the column—why was the mixture a reddish-brown color?
2. Why do you think the column needed to be “dry” (the absence of buffer on the top of the column bed) when the protein mix was loaded?
3. Why did you need to add more buffer after the protein mixture was loaded onto the column (at step 7 and 8 of the protocol)?
4. Examine the 10 fractions that you collected.  
Which tube contains the “peak” fraction for hemoglobin and vitamin B12? The peak fractions contain the highest concentration of protein/vitamin and will be the most intense in color.
5. Which molecule, hemoglobin or vitamin B12, exited the column first? Would this molecule be the larger or smaller of the two?

## Appendix A Teachers Answer Guide

### Lesson 1

#### Review Questions Chromatography

- 1a. Name two different types of column chromatography techniques.

**Affinity chromatography, ion-exchange chromatography, and size exclusion chromatography are common types of chromatographic techniques.**

- 1b. Using one of the chromatographic techniques that you mentioned above, can you think of an example in which two molecules could be purified using liquid phase chromatography?

**Examples of affinity chromatography would include the purification of an antibody over a solid support containing the antigen (antibody-antigen interactions would be a good background lecture topic). Also, the purification of an enzyme over a solid support containing the substrate would be another example of affinity chromatography (enzyme-substrate interactions would be another lecture topic).**

**Examples of ion-exchange chromatography would include the separation of two proteins with very different isoelectric points—for example at pH 7.2, lysozyme is (+) charged, whereas albumin is (-) charged (background lecture topics would include isoelectric points, amino acids contributing to a proteins' charge, pH, etc.).**

- 2a. What type of chromatography is being used in this lab activity?

**Size exclusion chromatography is being used in this lab activity.**

- 2b. Briefly explain how the chromatography in this activity functions.

**Molecules can be separated on the basis of size by passing a mobile-phase of the mixture over a column of porous beads (solid phase) which act to filter a mixture. This filtering causes smaller molecules to pass through the column more slowly than large molecules (which are excluded from the beads). Thus, large molecules exit the column first and are present in “early” fractions, whereas smaller molecules exit the column later and are present in “later” fractions.**

3. If the following mix of molecules was 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 containing: hemoglobin: 65,000 daltons, myoglobin: 17,000 daltons, myosin: 180,000 daltons.

First molecule to appear: **myosin (180,000—it is the largest)**

Second molecule to appear: **hemoglobin (65,000—it is intermediate)**

Third molecule to appear: **myoglobin (17,000—it is the smallest)**

- 4a. If a size exclusion chromatography column is said to have an exclusion limit of 40,000 daltons, would hemoglobin (60,000 daltons) be fractionated or excluded from the column?

**Hemoglobin would be excluded from the column. It is larger than the pores of the beads; thus it is excluded from the pores and would pass through the column quickly.**

- 4b. Would vitamin B12 (1,350 daltons) be fractionated or excluded from the column?

**Vitamin B12 would be fractionated by the column—its molecular weight is much lower than the exclusion limit, causing it to be filtered through the pores.**

## Lesson 1 Review Questions

1. What gives hemoglobin and myoglobin their distinctive reddish-brown colors?

**It is the heme or iron-containing group of both molecules that give them their distinctive red color. Iron is naturally colored red—think of iron pills or red, iron-containing soil.**

2. If athletes wanted to increase their endurance by increasing their oxygen-carrying capacity, how might they accomplish this increase?

**RBCs, which contain hemoglobin, are responsible for delivering oxygen to tissues. An athlete could increase oxygen capacity by increasing RBC number, thereby increasing the overall number of hemoglobin molecules in the body. Athletes accomplish this by training at high altitudes, where the body responds to the decrease of oxygen in the air by producing more RBCs. Additionally, some athletes resort to “blood-doping”, which involves infusing RBCs directly into the blood stream. This raises the RBC concentration, raising the oxygen capacity. However, blood-doping can be extremely dangerous, since too high of a concentration of RBCs can thicken the blood, resulting in strokes or heart attacks.**

- 3a. Would you expect a flightless bird, such as an ostrich or turkey, to have a preponderance of dark meat (dark meat being rich in myoglobin) in its wings or legs?

**The preponderance of dark meat would be in the legs. Active muscles require high myoglobin contents to supply oxygen to the working muscles.**

- 3b. What about a bird of flight, such as a duck or hawk?

**The preponderance of dark meat would be in the wings. In this case, the wings are the more active muscles and would require higher myoglobin contents.**

- 4a. How could gene therapy, where a normal gene is substituted for a defective copy of a gene, help individuals afflicted with sickle-cell anemia?

**Gene therapy involves the placement of a normal gene into the body of an individual afflicted with a genetic disorder. Because patients with sickle-cell anemia have a defective copy of the gene for hemoglobin, the substitution of the defective gene with a normal gene could, in theory, “cure” the sickle-cell individual. It should be mentioned that the normal gene is contained in the individual as a supplemental gene, usually carried by a virus, and does not physically replace the defective gene.**

- 4b. Similarly, how could gene therapy be used for those individuals who have vitamin B12 carrier protein disease?

**Again, gene therapy could be used to place a normal copy of the gene for the carrier protein into individuals with defective carrier protein genes.**

## Lesson 2 Review Questions

1. Think about the color of the protein mix that was applied to the column—why was the mixture reddish-brown?

**The iron-containing heme group of hemoglobin contributes the majority of red-brown color to the protein mix. Vitamin B12 is dark pink. The mixture of both produces a dark reddish-brown solution.**

2. Why do you think the column needed to be “dry” (the absence of buffer on the top of the column bed) when the protein mix was loaded?

**Slight diffusion naturally occurs as the molecules pass through the SEC column. For this reason, it is desirable to apply highly concentrated solutions of biomolecules to the column. Having the top of the column bed “dry” when the protein mix is loaded decreases the overall amount of diffusion that occurs during the purification process.**

3. Why did you need to add more buffer after the protein mixture was loaded onto the column (at step 7 and 8 of the protocol)?

**In order to keep the mixture of hemoglobin and vitamin B12 in the fluid phase, additional buffer needs to be added to the column after the protein mixture was applied. The buffer insures that the fluid phase will move through the column, resulting in completion of the chromatographic separation procedure.**

4. Examine the 10 fractions that you collected.

- a. Which tube contains the “peak” fraction for hemoglobin and vitamin B12? The peak fractions contain the highest concentration of protein/vitamin and will be the most intense in color.

**The peak fractions will vary slightly between the groups of students—the peak can be affected by diffusion, disruption of the column bed when loading of the sample, etc. In general, the peak hemoglobin fraction will be found in tube 3, 4, or 5. The peak vitamin B12 fraction should be found in tube 7, 8, or 9.**

5. Which molecule, hemoglobin or vitamin B12, exited the column first? Would this molecule be the larger or smaller of the two?

**Hemoglobin exited the column first, followed by vitamin B12. Hemoglobin (65,000) is much larger than vitamin B12 (1,350), and so it would be expected to elute first from the size exclusion column.**

## **Appendix B Glossary of Terms**

### **Biotechnology**

Applying biology in the real world by the specific manipulation of living organisms, especially at the genetic level, to produce potentially beneficial products.

### **Buffer**

The liquid which is used to dissolve the biomolecules which will be applied to the chromatography column.

### **Chromatography**

A process for separating complex liquid mixtures of proteins or other molecules by passing a liquid mixture over a column containing a solid matrix. The properties of the matrix can be tailored to allow the selective separation of one kind of molecule from another. Properties include solubility, molecular size, and charge.

### **Column**

A round, plastic cylinder which is densely filled with small, porous spheres or beads.

### **Column bed**

The mass of beads packed within the chromatography column.

### **Exclusion limit**

The upper size limit for molecules that can not penetrate the pores of the porous beads.

### **Fraction**

A tube which contains material that has flowed through the chromatography column. Multiple tubes or fractions are collected during each chromatography run. Because large molecules pass quickly through the column, they are found in the early fractions. The small molecules which penetrated the beads are found in the later fractions.

### **Fractionate**

The ability to separate molecules of different sizes. Small molecules are able to penetrate the porous beads which make up a size exclusion chromatography column; large molecules can not penetrate the pores and pass around the beads. Thus, molecules of different sizes can be fractionated or separated from a complex mixture.

### **Sample**

A mixture of biomolecules that is dissolved in a buffer and which is applied to a chromatography column.



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**Website** [www.bio-rad.com](http://www.bio-rad.com) **Bio-Rad Laboratories Main Office** 2000 Alfred Nobel Drive, Hercules, CA 94547, Ph. (510) 741-1000, Fx. (510)741-5800  
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