Chromatography

- 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. [In chromatography, a cylinder, or column is densely filled with a "bed" of microscopic beads. These beads form a matrix through which proteins must pass before being collected. ]

- Molecules travel through the stationary phase at different rates because of their chemistry.
Common Types of Chromatography

• Gel filtration chromatography
  – size exclusion chromatography

• Affinity chromatography
  – a biomolecule (often an antibody) that will bind to the protein to be purified is attached to the beads.

• Ion exchange chromatography
  – the glass beads of the column have a charge on them (either + or -).
Principles of Size Exclusion Chromatography

- 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.

- Our 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.
• **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.
  – 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.

• **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.
  – 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 bloodstream, where it is eventually taken up by the liver.
Chromatography

- 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).
Experimental procedure

• 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”.

![Diagram of labeled collection tubes and column buffer tubes]
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. 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 µl 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. Begin to collect drops into tube 1.
6. Add another 250 µl 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.
Hydrophobic Interaction Chromatography (HIC)

• A chromatography column which is packed with hydrophobic beads is called a hydrophobic interaction matrix.

• When the sample is loaded onto the matrix in salt water, the hydrophobic proteins in the sample will stick to the beads in the column. The more hydrophobic they are the more tightly they will stick.

• When the salt is removed, the three dimensional structure of the protein changes again so that the hydrophobic regions of the protein now move to the interior of the protein and the hydrophilic ("water-loving") regions move to the exterior. The result is that the hydrophobic proteins no longer stick to the beads and drip out the bottom of the column, separated from the other proteins.
Purification of GFP

- Bacteria contain thousands of endogenous proteins from which GFP must be separated.
- The matrix has an "affinity" for the molecule of interest (GFP), but not for the other bacterial proteins in the mixture.
- GFP "sticks" to the column, allowing it to be separated from the bacterial contaminants.
- GFP has several stretches of hydrophobic amino acids, which results in the total protein being very hydrophobic.
Expression of the GFP gene is under the regulatory control of the arabinose promoter. Thus, when the bacteria were grown in LB containing arabinose (LB/amp/ara), GFP was expressed and the colonies appeared bright green.
Purification of GFP protein

• Collect cell
  – Centrifugation is a technique used to separate molecules on the basis of size by high speed spinning.
  
  – The heavier bacterial cells will be separated from the liquid growth media by a single centrifugation step.
  
  – Centrifugation results in a "pellet" of bacteria found at the bottom of the tube, and a liquid "supernatant" that resides above the pellet.
  
  – The collection and concentration of bacteria is a first step in the isolation of GFP from the bacteria that were grown in the liquid media.
• Lyse the cell
  – Lysozyme is an enzyme that functions to degrade (or lyse) the bacterial cell wall, by cleaving polysaccharide (sugar) residues in the cell walls.
  
  – The subsequent freeze-thaw step aids in the complete disruption of the wall and internal membrane.
  
  – Complete disruption or "lysis" releases soluble components, including GFP.
• This final centrifugation step serves to separate the large particles of lysed bacteria (such as the cell membrane and walls) from the much smaller proteins, including GFP.

• The larger bacterial debris are pelleted in the bottom of the microtube, while the smaller proteins remain in the supernatant. At this stage, the supernatant will fluoresce bright green upon exposure to UV light.
• **Equilibration buffer**
  A medium salt buffer (2 M (NH4)2SO4) which is used to "equilibrate" or "prime" the chromatography column for the binding of GFP.

• **Binding buffer**
  An equal volume of high salt Binding Buffer (4 M (NH4)2SO4) is added to the bacterial lysate. The end result is that the supernatant containing GFP has the same salt concentration as the equilibrated column. When in a high salt solution, the hydrophobic regions of proteins are more exposed and are able to interact with and bind the hydrophobic regions of the column.

• **Wash buffer**
  A medium salt Wash Buffer (1.3 M (NH4)2SO4) is used to wash weakly associated proteins from the column; proteins which are strongly hydrophobic (GFP) remain bound to the column.

• **Elution buffer**
  A low salt buffer (TE Solution; 10 mM Tris/EDTA) is used to wash GFP from the column. In low salt buffers (which have a higher concentration of water molecules), the conformation of GFP changes so that the hydrophilic residues of GFP are more exposed to the surface, causing the GFP to have a higher affinity for the buffer than for the column, thereby allowing the GFP to wash off the column.

The TE Solution will disrupt the hydrophobic interactions between the GFP and the column bed, causing GFP to let go and "elute" from the column. The GFP should pass down the column as a bright green fluorescent ring. This is easily observed using the UV light. If the column bed was disturbed in any of the preceding steps, the GFP will not elute as a distinct ring, but will elute with a more irregular, distorted shape. However, elution should still occur at this step.
• **Storage of tubes**
  - All of the collection tubes and their constituents can be parafilmed or covered and stored for approximately 1–2 weeks in the refrigerator.