Edvo-Kit # 277

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Affinity Chromatography of Glucose Binding Protein

Experiment Objective:

Students will learn the principle of affinity chromatography by isolating a carbohydrate-binding lectin protein from an extract of jack bean meal.

See page 3 for storage instructions.

Version 277.221205

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Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Experiment Components

Components		Storage	Check (✓)
A	Affinity Gel	Refrigerator	❑
B	Jack Bean Meal	Room temperature	❑
C	NaCl	Room temperature	❑
D	Dextrose	Room temperature	❑
E	Con A Control	Refrigerator	❑
F	Horseradish Peroxidase	Refrigerator	❑
G	ABTS (lyophilized)	Refrigerator	❑
H	Standard Dilution Buffer	Refrigerator	❑
I	ABTS Reaction Buffer	Refrigerator	❑

The following components can be stored at room temperature.

- Membrane ❑
- Columns ❑
- 50 mL Conical tubes ❑
- Transfer pipets ❑
- Petri dishes ❑
- Microcentrifuge tubes ❑

Experiment #277 contains material for up to 10 lab groups.

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

Requirements *(NOT included in this experiment)*

- Vortex (recommended)
- Clinical centrifuge
- Ring stands with clamps for columns
- Microtest tubes or small glass test tubes for collecting fractions
- Test tubes (15 mL) to collect elutant
- 50 mL, 100 mL beakers or flasks
- Graduated cylinders: 10 mL, 100 mL, and 250 mL
- 10 mL pipets
- Micropipettes (1-10 µL or 1-100 µL)
- Forceps or tweezers
- Distilled water
- Filter paper
- Shaking, rotating, or rocking platform (recommended)
- Incubator (recommended)
- Paper towels

Background Information

Chromatography is a collection of laboratory techniques that separate a mixture into its components. This is accomplished by passing the mixture through a medium in which the components move at different rates. The method was initially used for the separation of plant pigments and dyes, hence its name which is Greek for "to write in color" (Figure 1). Fast forward to today and chromatography is an essential tool in biology, chemistry, and industry. There are many kinds of chromatography. All involve dissolving a sample into a mobile phase that then passes through a secondary substrate called the stationary phase. Separation occurs because different biomolecules in the sample mixture have different properties such as size, charge, hydrophobicity, and biorecognition.

Affinity chromatography is one of the most powerful and effective chromatography methods. This method takes advantage of highly specific binding interactions between certain molecules (Table 1). Affinity chromatography requires careful design and an understanding of the interactions between all molecules involved but the result is a selective and high-yield purification. A protein's biorecognition is determined by its binding sites which are influenced by its primary, secondary, tertiary, and quaternary structures.

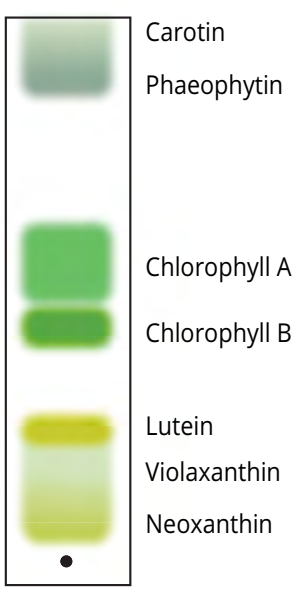


Figure 1: Chromatography of Plants

Drawing based on image by Florian Siebeck Chromatography_of_chlorophyll_results.jpg

Table 1: Examples of biological interactions used in affinity chromatography

Ligand	Target Molecule
Antibody	Antigen
Enzyme	Substrate
Nucleic Acid	Complementary base sequence
Hormone, Vitamin	Receptor, Carrier Protein
Lectin	Polysaccharide, Glycoprotein

PROTEINS

Proteins are large biological compounds responsible for many functions within a cell. They consist of specific sequences of amino acid residues linked to each other by peptide bonds (Figure 2). The sequence of residues in a polypeptide chain is called the primary structure. The sequence and distribution of amino acids have profound effects on the solubility, the three-dimensional shape (conformation), and the biological activity of a protein.

Chemical varieties of amino acid side chain functional groups - such as hydroxyl, carboxylic acid, amino, guanidino, phenolic, and sulfhydryl - are largely responsible for the chemical activity, binding specificities, and electrical properties of a protein. For example, the non-polar hydrocarbon groups of amino acids such as valine and alanine are important in maintaining the overall structure of a protein and creating the appropriate chemical environments within that are not in contact with the aqueous environment.

While the backbone of the polypeptide chain consists of peptide bonds, the folding path of the backbone through space is called the secondary structure of a protein. The folding patterns are complex, having bends, twists, and spirals. Secondary structures are mainly determined by hydrogen bonds between backbone oxygens, nitrogens, and hydrogens. Well-known examples of secondary structure include alpha-helices and beta-pleated sheets.

Protein secondary structure is influenced by the type of amino acids present in that part of the polypeptide chains. The complete three-dimensional folding pattern of a polypeptide chain, including the positioning of the amino acid functional groups relative to each other, is called the tertiary structure. Examples of bonds that stabilize the tertiary structure of proteins are ionic bonds, hydrogen bonds, disulfide linkages, Van der Waals interactions, and hydrophobic interactions. The tertiary structure creates three-dimensional crevices and pockets unique to its conformation and affects solubility. Most importantly, the precise tertiary structure is necessary for the biological activity of proteins.

Some proteins consist of several polypeptide chains that are specifically associated with each other by non-covalent and covalent bonds. The three-dimensional arrangement of polypeptide chains to each other in a protein is called the quaternary structure. The individual polypeptide chains that make up the protein are often called subunits. The subunits of a protein can be identical, similar, or completely different from one another. Different subunits can be responsible for different functions within a protein. Taken together, the primary, secondary, tertiary, and quaternary structure of a protein is what specifies its binding properties. These features are utilized in affinity chromatography for protein purification.

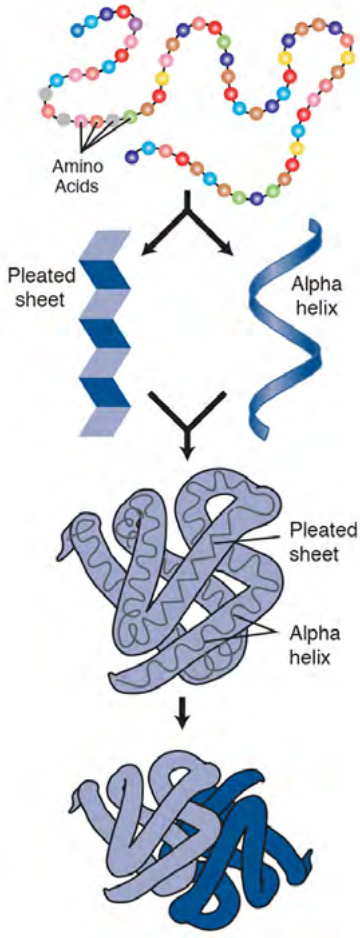


Figure 2

Image Courtesy: National Human Genome Research Institute. www.genome.gov

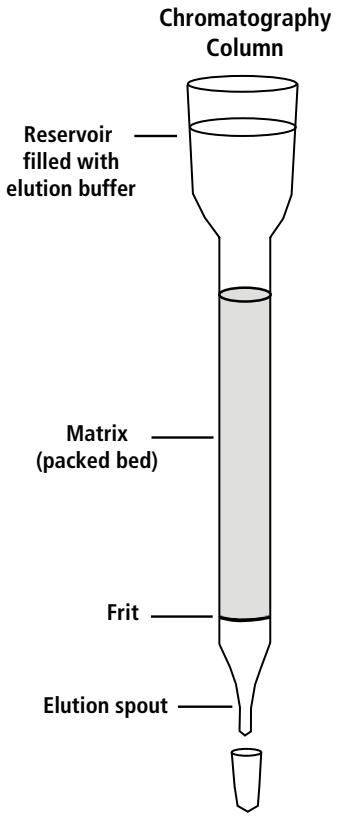


Figure 3

AFFINITY CHROMATOGRAPHY

Affinity chromatography uses the reversible interaction between a specific ligand and a target protein to purify the latter. Often this reaction is described as a lock and key interaction. Within the mobile phase is the "lock", the target protein with an affinity for the "key" protein. This "key" protein, also known as the ligand, is immobilized on a solid support to create the stationary phase. When the mobile phase passes through the stationary phase the "key" ligand catches the "lock" protein and holds it. Washing follows where other components of the mobile phase are removed. Finally, the affinity between the lock and key is reduced which allows the target protein to pass through the stationary phase and be collected.

The basic components of an affinity chromatography experiment are the chromatography column, matrix, and elution buffer (Figure 3). The column is a tube with a frit and elution spout fitted at the bottom. The frit is a membrane or porous disk that supports and retains the matrix in the column but allows water and dissolved solutes to pass.

The matrix is the separation material that sits in the column; the sample must pass through the matrix before eluting out the bottom of the column. Filling the chromatography column with matrix is referred to as packing. The packed matrix is called the bed and the volume it occupies is referred to as the bed volume. It is very important not to allow the bed to run dry. Otherwise, cracks and fissures develop that reduce the purification power of the column.

When a sample is added to a column the column is said to be charged. After a sample is charged on the affinity column it is washed with a suitable buffer to remove proteins from the extract that do not bind to the column.

The bound protein is eluted from an affinity column using the elution buffer. The elution buffer can work in two ways. In most cases, a solution of purified ligand is used to elute the bound protein. The concentration of the ligand in the eluting buffer is high enough so that the binding equilibrium for the ligand-protein pair shifts from the column-bound ligand to the ligand in the eluting solution. This results in the elution of the affinity column-bound protein. In cases where it is prohibitive to use high concentrations of purified ligand to elute the affinity column, the affinity-bound protein can be eluted by disrupting the ligand-protein interaction with an acidic pH buffer. This non-specifically disrupts the protein-ligand interaction, and the protein elutes from the column. Rapid neutralization of fractions that contain the eluted protein will help maintain the biological activity of the bound protein when using this low pH method.

PURIFICATION OF CON A

Concanavalin A (Con A) is a carbohydrate-binding protein widely used in biology to characterize cell surfaces and in biochemistry to purify glycosylated macromolecules. Scientists are also investigating its abilities both to activate lycophytes and inhibit certain tumor growths. Con A has a quaternary structure of four identical subunits (Figure 4). It binds to several sugars including mannose (strong bond), mannose-containing oligosaccharides (strong bond), and glucose (weak bond). The protein is primarily harvested from *Canavalia ensifrmis* (jack bean), a drought-resistant legume native to south and central America.

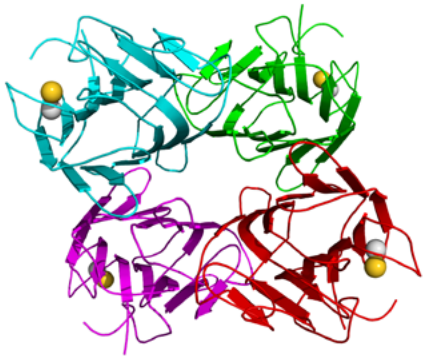


Figure 4: Crystallographic structure of a tetramer of jack bean concanavalin A

Image File: Boghog2, Public domain, via Wikimedia Commons

In this experiment, Con A will be purified from jack bean meal. The extraction begins through simple rehydration and vortexing of powdered jack bean meal followed by affinity chromatography through a Sephadex matrix (Figure 5). The rehydrated jack bean meal is added to the column and Con A proteins bind to the dextran of Sephadex. Next, the column is washed with a salt buffer to remove proteins from the extract that did not bind. Bound proteins are then eluted from the column by adding a one molar (1M) solution of dextrose. The concentration of the dextrose in this eluting buffer is high enough so that the binding equilibrium for the ligand-protein pair (in this case dextrose and Con A) shifts from the column-bound ligand to the ligand in the eluting solution. This results in the elution of the affinity column-bound Con A.

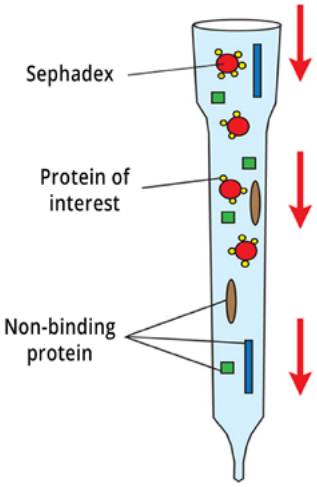


Figure 5

Next, an assay will be used to determine which fraction(s) contain the highest concentration of Con A. Fractions collected from several points in the experiment along with a control are applied to a nylon membrane. The binding activity of Con A is maintained even when bound to a nylon membrane. This means that Con A on the membrane will also bind to horseradish peroxidase, a mannose-containing glycoprotein with enzymatic activity. Because Con A binds to the core of horseradish peroxidase, this binding does not affect the enzymatic activity of the horseradish peroxidase. Consequently, the horseradish peroxidase can simultaneously be bound to Con A and catalyze a reaction. In this experiment, this reaction involves ABTS, a substrate for peroxidase which changes color when it reacts with the peroxidase enzyme. When ABTS is added to the Con A-horseradish peroxidase complex, it allows for the visualization of Con A in each elution fraction.

Experiment Overview

EXPERIMENT OBJECTIVE

In this experiment, students will learn the principle of affinity chromatography by isolating a carbohydrate-binding lectin protein from an extract of jack bean meal.

LABORATORY SAFETY

Wear gloves and safety goggles

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
4. Exercise caution when using any electrical equipment in the laboratory.
5. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.

LABORATORY NOTEBOOKS

Address and record the following in your laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Write a hypothesis that reflects the experiment.
- Predict experimental outcomes.

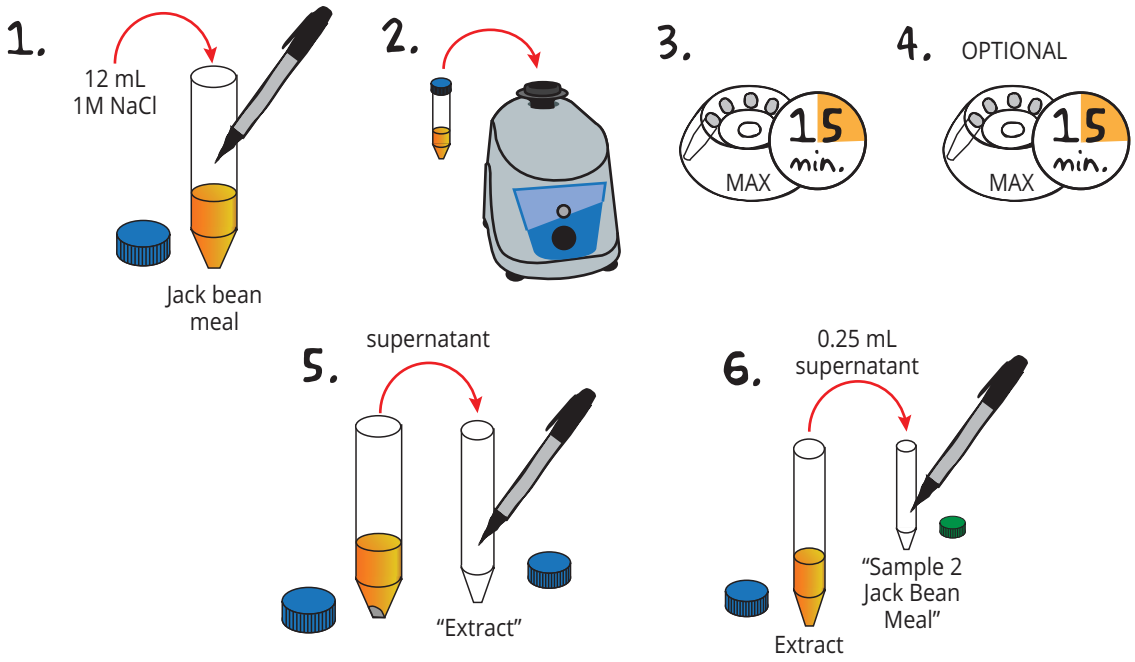
During the Experiment:

- Record (draw) your observations, or photograph the results.

After the Experiment:

- Formulate an explanation from the results.
- Determine what could be changed in the experiment if the experiment were repeated.
- Write a hypothesis that would reflect this change.

Module I: Column Chromatography

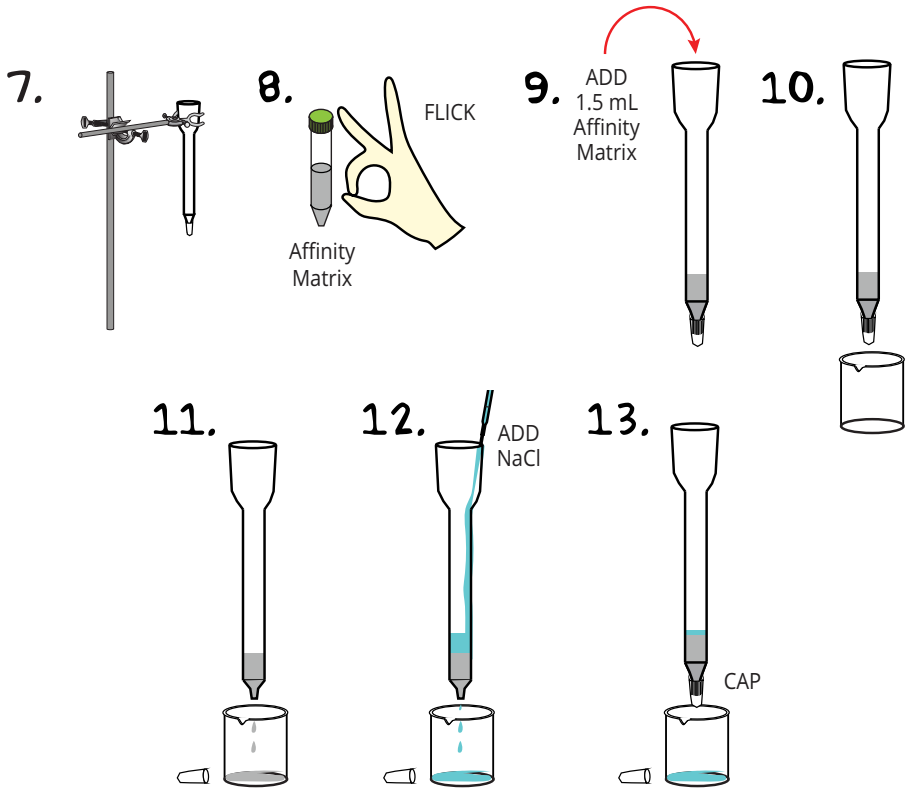


PREPARE MOBILE PHASE

1. **ADD** 12 mL of 1M NaCl to the 50 mL tube containing 0.5 g of jack bean meal. **LABEL** with your group ID.
2. **VORTEX** for 30 seconds or until the Jack bean meal is fully suspended and partially dissolved. If a vortex is unavailable **SHAKE** vigorously for at least 60 seconds.*
3. **CENTRIFUGE** your sample at 2000 rpm or at max speed for 15 minutes. Remember to counterbalance with another group's sample(s) and to always load samples symmetrically.*
4. (OPTIONAL) If there are pieces of undissolved jack bean meal that are not part of the pellet, **TRANSFER** the supernatant to a clean conical centrifuge tube and repeat step 3.
5. **TRANSFER** the supernatant to a clean conical tube. **LABEL** with your group ID and "Extract". **DISCARD** the used centrifuge tube and pellet.
6. **LABEL** a microtest tube "Sample 2: Jack bean meal extract". **TRANSFER** 0.25 mL of the supernatant from step 5 to the tube.

**During these steps you or your group members can begin to prepare the column (steps 7 – 13 on page 9).*

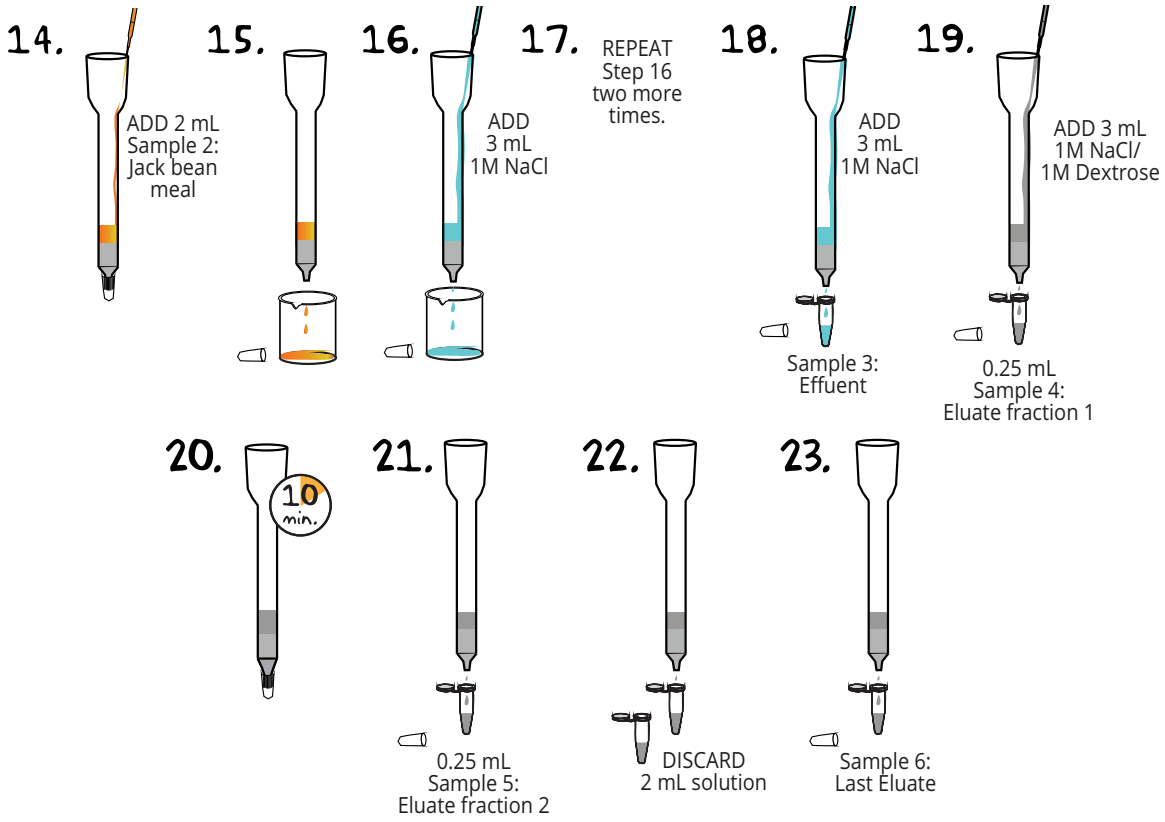
Module I: Column Chromatography, continued



PREPARE THE COLUMN

7. Vertically **MOUNT** the column on a ring stand. Make sure it is straight and that the white cap is firmly attached to the bottom of the column.
8. **COLLECT** the affinity matrix from your teacher and **RESUSPEND** it by flicking the tube several times.
9. **ADD** 1.5 mL of the affinity matrix into the column by letting it stream down the inside wall of the column.
   NOTE: If the flow is stopped by an air pocket, stop adding the matrix and firmly tap the column until the air is removed and the matrix continues to flow down the side of the column.
10. **PLACE** an empty beaker under the column.
11. **REMOVE** the cap from the bottom of the column and allow the matrix to pack into the column. When the surface of the column is moist and no liquid is visible ***IMMEDIATELY PROCEED*** to step 13.
12. Gently **ADD** 3 mL of 1 M NaCl (wash solution) to the column by letting it stream down the inside wall of the column.
13. **ALLOW** the wash to flow through. When the last of the wash enters the column, **STOP** the flow by reattaching the cap. The surface of the gel should be moist, not dry, and a very small amount of NaCl solution should remain.

Module I: Column Chromatography, continued

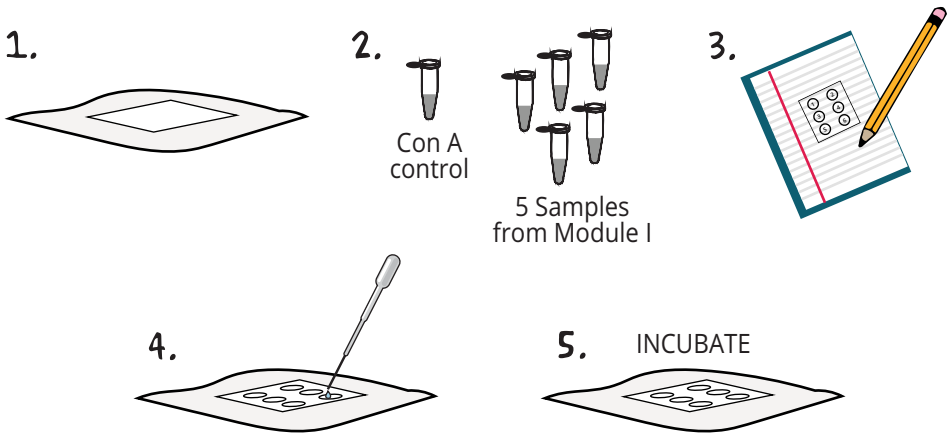


RUNNING THE COLUMN

14. Charge the column by gently **ADDING** 2 mL of extract.
15. **REMOVE** the cap from the bottom of the column and **ALLOW** the effluent to flow through and into a beaker. As the last of the extract enters the column, **STOP** the flow by reattaching the cap. The surface of the gel should be moist, not dry and no extract should be present on the surface of the gel.
16. Gently **ADD** 3 mL of 1 M NaCl, **REMOVE** cap, and allow solution to flow through and into beaker. **REPLACE** cap.
17. **REPEAT** step 16 twice more for a total of 3 washes.
18. **LABEL** a microtest tube "Sample 3: Effluent". **ADD** an addition 3 mL of 1 M NaCL to the column, **ALLOW** to flow through, and **COLLECT** at least 0.25 mL in the labeled tube (any remaining wash can flow into the beaker). **REPLACE** cap.
19. **LABEL** a microtest tube "Sample 4: Eluate fraction 1". Gently **ADD** 3 mL of 1 M NaCl / 1 M Dextrose (elutant) to the column, **ALLOW** to partially flow through, **COLLECT** the first 0.25 mL fraction in labeled tube. ***IMMEDIATELY REPLACE*** cap.
20. Let the column **SET** for 10 minutes.
21. **LABEL** a microtest tube "Sample 5: Eluate fraction 2". **UNCAP** column, **ALLOW** solution to partially flow through, and **COLLECT** a 0.25 mL fraction in labeled tube. ***IMMEDIATELY REPLACE*** cap.
22. **UNCAP** column, **ALLOW** solution to partially flow through, and **COLLECT** a 1 mL fraction in an unlabeled tube. **DISCARD** solution. **REPEAT** for a total of 2 mL discarded solution.
23. **LABEL** a microtest tube "Sample 6: Last elute". **UNCAP** column, **ALLOW** remaining solution to flow through, and **COLLECT** in labeled tube. **REPLACE** cap.

OPTIONAL STOPPING POINT:
Samples can be stored in the refrigerator until the next lab period.

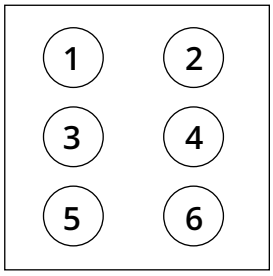
Module II: Sample Assay



PREPARATION OF THE MEMBRANE

1. **PLACE** a piece of membrane on a paper towel.
2. **COLLECT** a sample of Con A Control (1 mg/mL) from your teacher and the five samples previously prepared.
3. **RECORD** in your lab book the application location of each sample. For example:

   Sample Numbers:
   #1 Con A Control, 1 mg/ml
   #2 Jack bean meal extract
   #3 Effluent
   #4 Eluate fraction 1
   #5 Eluate fraction 2
   #6 Last eluate fraction collected



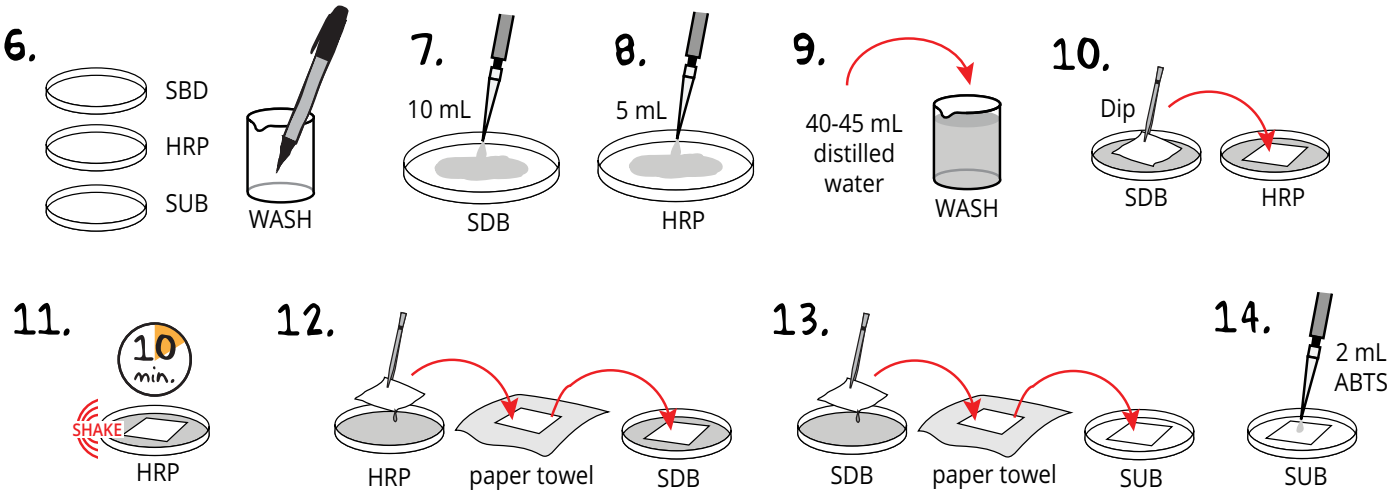
All samples are applied as shown in the diagram above.

4. Using a micropipet, slowly **APPLY** 10 µL of each sample to the membrane. Allow sufficient time for the sample to be absorbed by the membrane. The size of each 10 µL spot should be between 10-11 mm in diameter.
5. **INCUBATE** the membrane at 37°C for 10 minutes or at room temperature for 15 minutes.

STOP

OPTIONAL STOPPING POINT:
The membrane can be stored at room temperature until the next lab period.

Module II: Sample Assay, continued



DETECTION OF CON A PROTEIN

6. **OBTAIN** three 60 mm petri dishes. **LABEL** them "SDB" (Standard dilution buffer), "HRP" (Horseradish peroxidase), and "SUB" (Substrate). **OBTAIN** a 50 mL beaker and **LABEL** as "Wash".

7. **ADD** 10 mL of Standard Dilution Buffer to the SDB dish.

8. **ADD** 5 mL of Diluted Horseradish Peroxidase to the HRP dish.

9. **ADD** 40-45 mL of distilled water to the Wash beaker.

10. Using tweezers, **DIP** the dry membrane into the SDB dish and then **TRANSFER** it to the HRP dish.

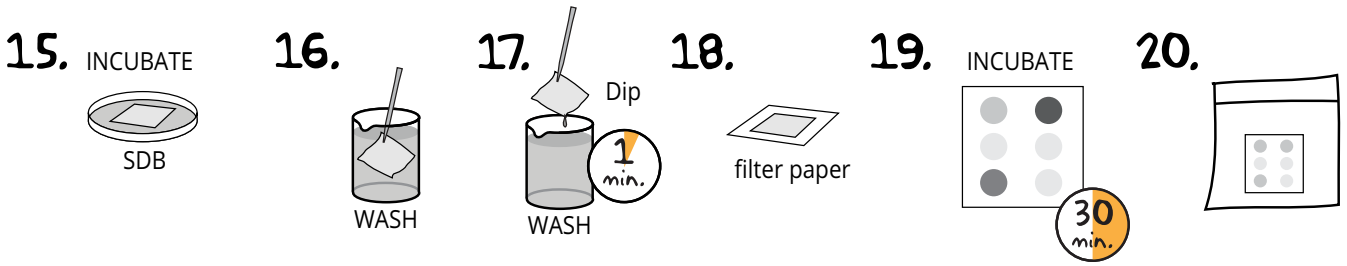
11. **INCUBATE** the membrane in the HRP dish for 10 minutes at room temperature on a shaking or rotating platform. ***NOTE: If a shaking platform is unavailable manually mix by swirl or rotated the petri dish. Manual mixing must be done frequently and carefully throughout the incubation period.***

12. Using tweezer, **LIFT** the membrane from the HRP dish. **DRAIN** excess liquid from the membrane by placing membrane on a paper towel (sample side up). **PLACE** the membrane into the SDB dish.

13. Using tweezer, **LIFT** the membrane from the SDB dish. **DRAIN** excess liquid from the membrane by placing membrane on a paper towel (sample side up). **DIP** the membrane into the empty SUB dish.

14. Carefully **PIPET** 2 mL of the ABTS substrate overtop of the center of the membrane (the substrate should spread evenly over the entire membrane). **AVOID** disturbing the dish and causing the substrate to spill off the membrane.

Module II: Sample Assay, continued



15. **INCUBATE** the membrane undisturbed for 30-90 seconds or until the color is sufficiently developed. ***Do not allow the membrane to become too dark.***

16. Using tweezer, **LIFT** the membrane from the SUB dish and ***IMMEDIATELY DIP*** the membrane into the wash beaker.

17. **WASH** the membrane by successive dipping for one minute.

18. Using tweezer, **LIFT** the membrane from the water and DRAIN excess liquid from the membrane. **PLACE** membrane on a piece of filter paper.

19. **INCUBATE** the membrane at 37°C for 10 minutes or at room temperature for 30 minutes.
NOTE: Initially, precipitation dots will appear very dark but will become lighter after the membrane dries.

20. **OBSERVE** and **RECORD** the color intensity of the precipitated substrate in samples 1 - 6. Membranes can be photographed and/or stored in a sealable plastic bag.

Study Questions

1. What pattern of enzyme (horseradish peroxidase) binding activity would you expect if you assayed the 0.5 mL effluent fractions?

2. Dextrose is used to elute the bound Con A from the affinity gel column, yet the bound dextrose was not removed from the Con A-containing eluate fractions before adsorbing the fractions to the membrane. Why does Con A bind to horseradish peroxidase if the dextrose is still present in the Con A binding site?

3. Con A adsorbs strongly to the membrane, yet the horseradish peroxidase protein binds only slightly. If horseradish peroxidase bound as strongly to the membrane as Con A, then the assay may not be possible unless an intermediate step was done. What would that intermediate step be?

Instructor's Guide

ORGANIZING AND IMPLEMENTING THE EXPERIMENT

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in the planning and the implementation of this experiment with your students. These guidelines can be adapted to fit your specific set of circumstances.

If you do not find the answers to your questions in this section, a variety of resources are continuously being added to the EDVOTEK web site: **www.edvotek.com**

In addition, Technical Service is available from 8:00 am to 5:30 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800-EDVOTEK (1-800-338-6835).

Safety Data Sheets can be found on our web site: **www.edvotek.com/safety-data-sheets**

Pre-Lab Preparations

MODULE I: COLUMN CHROMATOGRAPHY

1. For each group, **WEIGH** out 0.5 g of jack bean meal (Component B) and **PLACE** in 50 mL conical tubes.
2. **DISSOLVE** the NaCl powder (Component C) in 550 mL distilled or deionized water. **DISPENSE** 45 mL for each group. **SAVE** 60 mL for preparation of 1 M NaCl / 1 M Dextrose.
3. **ADD** all of Dextrose (Component D) to 60 mL 1 M NaCl (saved from previous step). **STIR** to dissolve and **DISPENSE** 5 mL of 1 M NaCl / 1 M Dextrose for each group.
4. **MIX** the Affinity Gel (Component A) to obtain an even suspension and **DISPENSE** 1.75 mL into tubes or beakers for each group.

EACH GROUP WILL RECEIVE:

Components	Amount
• Jack bean meal	0.5 grams
• 1 M NaCl	45 mL
• 1 M NaCl/1 M Dextrose	5 mL
• Affinity Gel	1.75 mL
• Membrane strip (3.5 x 3.5 cm)	1 piece
• Filter Paper (4 x 4 cm)	1 piece
• Con A Control	15 µL
• SDB	10 mL
• Horseradish Peroxidase	5 mL
• ABTS Substrate	2 mL
• Petri plates	3
• Column/ring stand	1
• Small beakers	2

MODULE II: SAMPLE ASSAY

5. **CUT** membrane strips into 3.5 x 3.5 cm pieces to fit into 60 mm Petri dishes. **DISTRIBUTE** one piece of membrane per group. Wear gloves when handling the membranes.
6. **CUT** filter paper into 4 x 4 cm pieces. **DISTRIBUTE** one piece of filter paper per group.
7. **DISPENSE** 15 µL of Con A Control (Component E) for each group.
8. In a beaker or flask, **DILUTE** 10x Standard Dilution Buffer (Component H) by mixing 20 mL of 10x SDB with 180 mL of distilled or deionized water. This is 1x SDB. **DISPENSE** 10 mL 1x SDB to each group. **RESERVE** 50 mL 1x SDB for preparation of Horseradish peroxidase (below).
9. **DILUTE** Horseradish peroxidase stock (Component F) by mixing entire contents with 50 mL of 1x SDB (saved from previous step). **DISPENSE** 5 mL of diluted enzyme to each group.
10. Prepare ABTS.
    a. **TRANSFER** 22 mL ABTS Reaction Buffer (Component I) into a 50 mL conical tube. **LABEL** "ABTS".
    b. Carefully **REMOVE** the stopper from the vial of lyophilized ABTS (Component G) and **TRANSFER** approximately 0.5 mL of the ABTS Reaction Buffer from step 10a to the vial.
    c. **CLOSE** the stopper and gently shake the vial to **MIX**.
    d. **TRANSFER** the entire contents of re-hydrated ABTS to the 50 mL conical ABTS tube.
    e. **CLOSE** the lid and shake the tube to **MIX** well.
    f. **ALIQUOT** 2 mL of ABTS to each group.

NOTE:
For best results, perform steps 9 and 10 on the day of the assay.

Experiment Results and Analysis

Dots of precipitated product will appear immediately upon immersing the membrane in the peroxidase substrate solution. Their intensity on the membrane is optimal after 30-90 seconds.

1. The Con A control at 1 mg/ml will show precipitate. This is a control to indicate that the Con A - HRP assay is working.

2. The jack bean meal extract will give a spot of variable intensity, from no precipitate to very light precipitate. This is dependent upon the concentration of the Con A in the extract plus the concentration of other non-specific jack bean meal proteins that bind to the membrane.

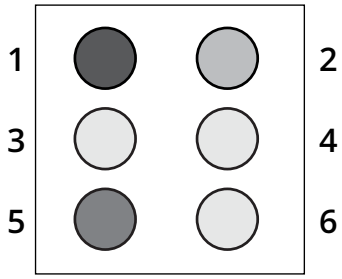
3. There should be no precipitate in the effluent if the column is thoroughly washed. There may be a very light spot of precipitate due to a slight amount of endogenous peroxidase activity in jack bean meal.

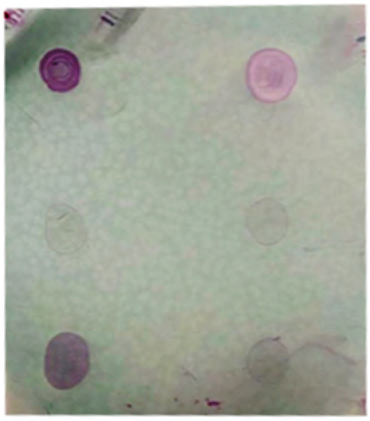
4. There will be no precipitate for eluate fraction 1 since the elutant has not passed through the column.

5. The eluate fraction 2 will have an intense amount of precipitate. Eluate fraction 2 contains the greatest amount of pure Con A.

6. The last fraction will vary in intensity depending on how much Con A is in this last eluate fraction. In general, the last elute fraction will have a less intense spot of precipitate the elute fraction 2.

1: Con A Control
2: Jack bean meal extract
3: Effluent
4: Eluate fraction 1
5: Eluate fraction 2
6: Last eluate fraction collected





Please refer to the kit insert for the Answers to Study Questions