G-Biosciences ♦ 1-800-628-7730 ♦ 1-314-991-6034 ♦ technical@GBiosciences.com

A Geno Technology, Inc. (USA) brand name

Hydrophobic Chromatography

Teacher's Guidebook

(Cat. # BE-416)

MATERIALS INCLUDED WITH THE KIT

This kit has enough materials and reagents for 24 students (six groups of four students).

CHECKLIST

- 6 Column: Hydrophobic Columns
- 6 vials Protein: Tissue Extract & Pestle
- 1 bottle HP Loading Buffer (2X)
- 1 bottle HP Elution Buffer
- 1 bottle Enzyme Substrate (starch)
- 1 bottle Enzyme Color Dye-I
- 1 vial Enzyme Color Dye-II
- 150 Centrifuge Tubes (1.5ml)

SPECIAL HANDLING INSTRUCTIONS

- Store Hydrophobic Columns and Tissue Extract at 4°C.
- All other reagents can be stored at room temperature.

The majority of reagents and components supplied in the *BioScience Excellence*™ kits are non toxic and are safe to handle, however good laboratory procedures should be used at all times. This includes wearing lab coats, gloves and safety goggles.
For further details on reagents please review the Safety Data Sheets (SDS).
The following items need to be used with particular caution.

Part #	Name	Hazard
C111	Column: Hydrophobic Column	Flammable
E141	Enzyme Color Dye-I	Corrosive

ADDITIONAL EQUIPMENT REQUIRED

- Low Speed Centrifuge for 1.5-2ml tubes
- Water bath or Beaker and thermometer
- Stands and clamps

TIME REQUIRED

- 2-3 hours

OBJECTIVES

- Learn the principle of hydrophobic chromatography.
- Understand the immobilization of protein on hydrophobic columns.
- Discover the factors influencing binding and elution of proteins on hydrophobic columns.
- A hands-on hydrophobic chromatography lab activity

BACKGROUND

A key step in proteomics, the study of proteins function and structure, is the purification of proteins. The ability to isolate and purify specific proteins is an essential feature of modern biochemistry as it allows scientist to study proteins in isolation from other proteins, which greatly aids the understanding of a particular protein's function.

Unfortunately there is no single ideal protein purification procedure and often the purification of a protein involves several techniques. The main idea behind protein purification is to select the best techniques to isolate a protein of interest, based on differences in its physical properties from other "unwanted proteins. One general separating technique, with many different approaches, is chromatography. The "Protein Chromatography" kits will aim to cover some of the chromatography techniques routinely used in protein purification.

The amino acid residues that make up proteins have many varied properties depending on their side chains, including different charged groups and hydrophobic properties. The hydrophobic regions of a protein are enriched in hydrophobic residues that include phenylalanine, tyrosine, tryptophan, leucine, isoleucine, methionine and valine. Hydrophobic chromatography is based on the binding of these hydrophobic regions to immobilized hydrophobic groups.

Hydrophobic chromatography (HIC) is designed on the hydrophobic attraction between the stationary phase (column) and the hydrophobic regions of the protein molecules. The more hydrophobic a protein is the stronger it will bind to the column. The stationary phase consists of small non-polar groups (butyl, octyl or phenyl) attached to a hydrophilic polymer backbone (cross-linked dextran or agarose, for example). These hydrophobic regions, in media favoring hydrophobic interactions, such as an aqueous solution with high salt concentration, can bind to the hydrophilic ligands coupled to an uncharged column matrix.

The sample is loaded on to the column in a buffer containing a high concentration of a non-denaturing salt (frequently ammonium sulfate). The proteins are then eluted as the concentration of the salt in the buffer is decreased. Elution is brought about by decreasing the salt concentration and in some cases decreasing the solvent polarity with PEG, non-ionic detergents, denaturants, urea or chaotropic ions. Elution of different

column-bound proteins can be controlled by reducing the salt concentration or by adding solvents.

The Hydrophobic Chromatography kit is designed to teach students the basic principle of hydrophobic chromatography. This lab activity involves running hydrophobic chromatography to isolate a starch hydrolyzing enzyme from a complex tissue extract, which can be assayed for with the supplied assay.

The Hydrophobic Column provided in this kit contains highly cross-linked agarose beads containing “phenyl” as non-polar hydrophobic group for interaction and purification of proteins. The sample loading buffer contains ammonium sulfate. After washing out unbound proteins, the bound hydrophobic target protein/enzyme is eluted. An elution buffer is provided for eluting the target enzyme by a gradient of decreasing ammonium sulfate concentration.

TEACHER'S PRE EXPERIMENT SET UP

1. To make 1X HP Loading Buffer, add 25ml HP Elution Buffer to the bottle containing 2X HP Loading Buffer. Mix the content by inverting 5-6 times.

2. Prepare the Enzyme Detection Dye: Take 1ml Enzyme Color Dye-I solution and add to the vial of Enzyme Color Dye-II. Mix the contents by inverting the tube until the tube contents have dissolved. This may take up to 10 minutes to completely dissolve. Transfer the dissolved Enzyme Color Dye-II into the remaining Enzyme Color Dye-I solution and briefly mix the solution. This is the Enzyme Detection Dye.

3. Aliquot reagents to each student group according to the amount needed in the next section.

MATERIALS FOR EACH GROUP

Supply each group with the following components. Several components will be shared by the whole class and should be kept on a communal table.

- 1 Hydrophobic Column
- 1 vial Tissue Extract & Pestle
- 8ml 1X HP Loading Buffer
- 5ml HP Elution Buffer
- 2.5ml Enzyme Substrate
- 2.5ml Enzyme Detection Dye
- 24 Centrifuge Tubes (1.5ml)

PROCEDURE

Always wear gloves and protective clothing throughout the whole experiment.

I. Hydrophobic Chromatography

1. Add 150µl 1X HP Loading Buffer to the Tissue Extract vial. Incubate at room temperature for 10 minutes and then use the pestle to grind the tissue extract for 2 minutes.

2. Centrifuge the tube for 5 minutes at maximum speed in a microcentrifuge. Do not disturb the pellet. You will use the supernatant for the chromatography.

3. Clamp the Hydrophobic Column in an upright position on to a stand.

4. Open the top cap first and then the bottom cap of the column to prevent air bubbles entering the column. Let the buffer drain out of the column under gravity to a waste container. Ensure the column resin evenly settles to the bottom of the column.

5. *Equilibrate the column*: Apply 2 bed volumes (4ml) of 1X HP Loading Buffer. Add 0.5ml 1X HP Loading Buffer and let the buffer drain out freely into a waste container. Repeat until all 4ml have been applied.

6. Carefully load 100µl supernatant from Step 2 to the column.

7. Wash the column 3 times with 0.5ml 1X HP Loading Buffer: Apply 0.5ml 1X HP Loading Buffer to the column and let the buffer drain into a waste container.

8. Elute the proteins with 10x0.5ml elution steps: Apply 0.5ml HP Elution Buffer to the column and let the buffer drain into a 1.5ml tube. Collect all 10 fractions separately.

9. Proceed to the next section to determine the enzymatic activity of each fraction.

II. Enzyme Detection Assay

1. Label 10 tubes fractions 1-10 and label one tube for the crude tissue extract as the positive control.

2. Add 200µl Enzyme Substrate (starch) to each tube.

3. Add 50µl of each fraction to the corresponding tube and add 5µl tissue extract supernatant to the control tube.

4. Close the tubes and invert 5-6 times to mix the contents.

5. Incubate the tubes for 20 minutes at room temperature.

6. Add 200µl Enzyme Detection Dye to each tube and mix the contents by inverting the tubes 5-6 times.

7. Place the tubes in warm water bath or beaker and thermometer (50-65°C) for up to 30 minutes to develop the assay color. Check every 5 minutes for color change.

8. Observe the tubes. The fraction with the most intense orange/ brown color contains the enriched starch hydrolyzing enzyme.

RESULTS, ANALYSIS & ASSESSMENT

Q. Observe the enzyme protein assay tubes. Which fraction contains the most enzymes?

A. Make this observation without the use of any instrument and write down the number of the tube with the most intense orange/ brown color.

Q. Describe briefly how proteins are immobilized within the hydrophobic column.

A. Protein is immobilized due to hydrophobic interactions between the phenol groups (a hydrophobic group) and the hydrophobic regions on the surface of the proteins in a buffer favoring hydrophobic interactions, which is ammonium sulfate (high salt concentration).

Q. Briefly describe the process of elution of immobilized proteins in hydrophobic chromatography.

A. Lowering the concentration of salt (ammonium sulfate), which favors hydrophobic interactions, decreases interaction of hydrophobic groups with the column resulting is elution of the proteins.

Q. What other methods of elution of immobilized proteins from hydrophobic columns?

A. *Elution may be achieved by decreasing the elution buffer polarity with PEG, non-ionic detergents, denaturants, urea or chaotropic ions.*

Q. Describe the types of protein that can be successful purified using hydrophobic chromatography.

A. Protein having one or more hydrophobic regions on their surface.

Q. Briefly describe hydrophobic regions of protein molecules.

A. Protein hydrophobic patches consist of the side chains of phenylalanine, tyrosine, tryptophan, leucine, isoleucine, methionine and valine.

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