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A Geno Technology, Inc. (USA) brand name

Ion Exchange Chromatography

Teacher's Guidebook

(Cat. # BE-415)

MATERIALS INCLUDED WITH THE KIT

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

CHECKLIST

- 6 Column: Cationic Chromatography Columns
- 6 vials Protein: Protein Mix
- 2 bottles Equilibration Buffer
- 1 bottle Elution Buffer
- 1 bottle Protein Assay: RED660 Reagent
- 120 Centrifuge Tubes (2ml)

SPECIAL HANDLING INSTRUCTIONS

- Store Cationic Chromatography Columns and Protein Mix 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 Material Safety Data Sheets (MSDS).

ADDITIONAL EQUIPMENT REQUIRED

- Low speed centrifuge for 1.5-2ml tubes
- Spectrophotometer and Cuvettes or Microplate Reader and Microplate
- Stands and clamps

TIME REQUIRED

- 1-2 hours

OBJECTIVES

- Learn the principle of ion exchange chromatography.
- Understand the immobilization of protein on ionic charged columns.
- Learn factors influencing binding and elution of proteins during ion exchange chromatography.
- A hands-on ion-exchange chromatography lab activity.
- Separate Cytochrome-C from hemoglobin.

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.

Ion exchange chromatography is used to separate charged molecules, including proteins, from complex biological samples. Charged substances are separated by column chromatography with resins that carry charged ionic groups. Biomolecules, such as proteins, with an opposite charge will bind to the resins. The ionic groups of the columns are covalently bound to a gel matrix and are protected by small concentrations of counter ions that are present in the buffer. When a sample is added to the column, an exchange with the weakly bound counter ions takes place and the charged molecules bind to the solid support.

Proteins contain regions of charged groups on their surface that are formed by the side groups of charged amino acids, the α-amino and α-carboxyl termini of the polypeptide chains, and other interacting groups. These charged groups are available for interaction and exchange with ionic groups of the ion exchange columns.

Proteins are multivalent anions or cations and the protonation (addition of a proton) of protein molecules changes with changes in pH. Under strongly acidic pH conditions, all proteins are present as cations as a result of the suppression of the dissociation of the carboxy groups and protonation of the amino groups. At pH values above 12, proteins are present as anions due to the amino group being a free base and the carboxy group is dissociated. Depending on the total (net) charge of a protein and the wide range of ion

exchange columns available it is simple to bind proteins of interest to a corresponding charged stationary phase for purification.

During the practical application of ion exchange chromatography it is important to use pH values that ensure the ionic exchange resins are in an ionized state and the proteins contain an excess of positive or negative charges, i.e. they are not near their pI (isoelectric point) value, the net charge is zero.

Increasing the salt concentration results in the shielding of the charges on the protein's surface and effective binding to an exchanger is inhibited. Also changing the pH of the binding buffer changes the ionization of the protein charged groups and results in the breaking of the interaction with the ion exchange columns. As a result, proteins immobilized on an ion exchange column can be eluted either by increasing the salt concentration or by altering the pH of the binding buffer, or a combination of the two.

This lab activity involves using a protein mix, consisting of hemoglobin and Cytochrome C, and running ion-exchange chromatography to separate the proteins.

TEACHER'S PRE EXPERIMENT SET UP

1. Air bubbles may be introduced into the resin of columns during shipping and these may result in poor or inhibited buffer flow. To remove bubbles from the resin bed, vortex vigorously to resuspend resin. Check that bubbles are no longer present and allow resin to settle.

2. 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 Cationic Chromatography Column
- 1 vial Protein Mix
- 16ml Equilibration Buffer
- 1.5ml Elution Buffer
- 1 bottle RED660 Reagent (shared with whole class)
- 20 Centrifuge Tubes (2ml)

PROCEDURE

Always wear gloves and protective clothing throughout the whole experiment.

I. Ion Exchange Chromatography

1. Add 0.2ml Equilibration Buffer to the Protein Extract vial. Periodically vortex the tube until the protein completely dissolves.

2. Centrifuge the tube for 2 minutes at maximum speed in a microcentrifuge to remove the froth.

3. Clamp the Cationic Chromatography Column in an upright position to the stand.

4. Open the top cap first and then the bottom cap of the column to prevent air entering the resin. Allow the buffer to drain out of the column, under gravity, to a waste container. Make sure that the column resin evenly settles down in the column.

5. ***Equilibrate the column*****:** Apply 2 column volumes (1ml each) of Equilibration Buffer. Add 1ml Equilibration Buffer, allow the buffer to drain out and then apply the second volume. Let the buffer drain out into a waste container.

    *Add the Equilibration buffer slowly to avoid disturbing the resin in the column.*

6. Carefully load all the Protein Extract prepared in step 2 to the column.

7. Once the protein sample has entered the column, wash the column 4 times (2ml each) with Equilibration Buffer to remove unbound protein from the column: Collect 2ml wash fractions as the buffer freely drains into labeled 2ml collection tubes. Change to a fresh tube before applying the next wash volume.

8. ***Elute the sample using a salt gradient*:** Prepare a salt gradient by mixing Equilibration Buffer and Elution Buffer, a high salt buffer, as detailed in the table below.

Fraction #	Salt Concentration (mM)	Equilibration Buffer (µl)	Elution Buffer (µl)
1	100	950	50
2	200	900	100
3	300	850	150
4	400	800	200
5	500	750	250
6	600	700	300

9. Apply 1ml of each prepared gradient elution buffer to the column, starting with the lowest salt concentration (Fraction #1) first.

10. Collect 1ml fractions as the buffer freely drains into labeled 2ml collection tubes. Change to a fresh tube before applying the next elution buffer. Collect all 6 elutions in 6 separate 2ml tubes.

11. Record the color changes of the fractions.

12. Determine the protein concentration of the fractions by following the protocol in Section II.

II. RED660 Protein Assay

1. Label 10 tubes and transfer 50µl elute from each fraction (four washes and six elutions).

2. Mix the RED660 Reagent gently by inverting the bottle several times.

    *To avoid foaming, DO NOT SHAKE THE BOTTLE.*

3. Add 1ml RED660 Reagent to each tube and vortex briefly to mix the content. Incubate the tubes at room temperature for 5 minutes.

    *If using a spectrophotometer, proceed to step 5. If not, examine the tubes closely, the stronger the color the higher the concentration of protein.*

4. In the meantime, turn on the spectrophotometer to allow it to warm up. Adjust the wavelength to 660nm.

5. Add 1ml distilled water or RED660 Reagent to a cuvette to zero the absorbance of the spectrophotometer. Measure the absorbance of each tube and record the values.

    *The absorbance can be measured with a microplate reader instead of using a spectrophotometer. Transfer 250µl from each assay tube to a microtiter plate well. Add 250µl distilled water to a well as reference blank. Read the absorbance at 660nm.*

RESULTS, ANALYSIS & ASSESSMENT

Prepare a graph showing protein elution profile of ion-exchange chromatography. If using a spectrophotometer, plot the absorbance against the fraction number. If a spectrophotometer was not used then plot color intensity against fraction number

Q. Observe the RED660 protein assay tubes. Which fraction contains the most proteins?

A. *Make this observation without the use of any instrument and write down the tube where you observe maximum color. If using a spectrophotometer, plot the curve of absorbance against fraction number to determine the fraction with the highest protein concentration.*

Q. Describe briefly how proteins are immobilized on an ion-exchange column.

A. *When a protein sample is added to the column, an exchange with the weakly bound counter ions within the exchange column takes place. The charged protein molecules bind to a solid support carrying an opposite charge to the protein molecule.*

Q. Briefly describe the process of elution of immobilized proteins in ion-exchange chromatography.

A. *Changing of the pH of binding buffer changes ionization of the protein charged groups, which results in breaking of the interaction with the ion exchange columns and elution of proteins from the ion exchange columns. Or the salt concentration of the binding buffer is increased and the salt ions compete for the ions on the exchanger as well as shielding of the charges on the protein surface resulting in elution of the proteins.*

Q. What will be the elution profile of the chromatography if you alter the pH of the Elution Buffer?

A. *Changing the pH of Elution Buffer will alter the elution profile of the chromatography and consequently the position of elution of the target protein will change.*

Q. How do you select the operating pH for ion-exchange chromatography?

A. *Chromatography should be operated at pH values where the exchangers are mostly ionized and the proteins contain an excess of positive or negative charges to go through ion-exchange process during chromatography.*

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