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

A Geno Technology, Inc. (USA) brand name

Affinity Chromatography

Teacher's Guidebook

(Cat. # BE-417)

MATERIALS INCLUDED WITH THE KIT

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

- 6 Column: Albumin Affinity Columns
- 1 vial Protein: ASA (Animal Serum Albumin)
- 1 bottle Affinity Binding Buffer
- 1 bottle Affinity Elution Buffer
- 1 bottle Elution Buffer
- 1 bottle Protein Assay: RED660 Reagent
- 140 Centrifuge Tubes (2ml)

SPECIAL HANDLING INSTRUCTIONS

- Store Affinity Columns and Protein: ASA (Animal Serum Albumin) at 4°C.
- All other reagents can be stored at room temperature.
- Briefly centrifuge all small vials before opening to prevent waste of reagents.

ADDITIONAL EQUIPMENT REQUIRED

- Low Speed Centrifuge for 1.5-2ml tubes
- Spectrophotometer and cuvettes or Microplate reader and microplate (Optional)

TIME REQUIRED

- 1-2 hours

OBJECTIVES

- Learn the principles of affinity chromatography.
- Understand immobilization of proteins on affinity columns.
- Discover the factors influencing binding and elution of proteins on affinity columns.
- A hands-on affinity chromatography lab activity.
- Learn to perform spin column chromatography

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 scientists 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.

Affinity chromatography is a powerful tool for the purification of specific biomolecules, including proteins. The basic principle is that a biospecific ligand is immobilized to a solid support or resin of a column matrix, such as cellulose, agarose or polyacrylamide. A solution containing the protein of interest is passed over the column and the proteins interact with the ligand, whilst other proteins are washed away... Ligands are often based on biological functional pairs, such as enzymes and substrate, antigens and antibodies, receptor and hormone, or lectins to sugars chains on proteins.

The specific ligand binds the protein of interest and all non-specific molecules are washed away. Recovery of the bound protein can be achieved by changing experimental conditions to favor desorption. The protein is eluted in a specific buffer, either by pH and/or ionic strength shift or by competitive displacement; a solution is passed through the column that has a high concentration of free ligand. This is a very efficient purification method since it relies on the biological specificity of your target protein, such as the affinity of an enzyme for a substrate.

Affinity Chromatography is commonly used for applications such as purification of fusion proteins, antibodies and glycoproteins.

The Affinity Chromatography kit teaches the basic principles of affinity chromatography utilizing a highly specific affinity column designed for purification of albumin from complex protein samples such as serum or biological extracts. This lab activity involves

preparation and running affinity chromatography for the isolation of fluorescent dye labeled albumin from a complex tissue extract.

In addition, the kit also teaches students how to use spin method for chromatography: a widely popular biotechnique. In spin methods, the flow of chromatography liquid media (buffers) is forced through the column by centrifuging the column.

TEACHER'S PRE EXPERIMENT SET UP

1. Add 600µl Affinity Binding Buffer to the ASA (Animal Serum Albumin) vial. Allow to soak 5-10 minutes at room temperature and periodically vortex the tube. Centrifuge the tube for 5 minutes at maximum speed in a microcentrifuge (~5000xg). Do not disturb the pellet. Aliquot 100µl supernatant to each student group.

2. Aliquot reagents for each student group according to 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 Albumin Affinity Column
- 1 vial 100µl ASA (Animal Serum Albumin)
- 2ml Affinity Binding Buffer
- 1ml Affinity Elution Buffer
- 1ml Elution Buffer
- 1 bottle RED660 Reagent (Shared with whole class)
- 22 Centrifuge Tubes (2ml)

PROCEDURE

Always wear gloves and protective clothing throughout the whole experiment.

I. Affinity Chromatography

1. Centrifuge the Affinity Column briefly (~500xg) for 5 seconds to bring the resin to the bottom of the column.

2. Open the cap and break off the bottom plug of the column. Place the column in a 2ml tube and centrifuge for 5 seconds to let the buffer drain out.

3. *Equilibrate the column*: Place the column in a 2ml collection centrifuge tube. Add 0.2ml Affinity Binding Buffer to the column and centrifuge for 5 seconds to let the buffer drain into the 2ml tube, discard the buffer collected in the collection tube (the flow through). Repeat this step twice to thoroughly equilibrate the column.

   OPTIONAL*: These chromatography steps (equilibration, binding, and elution) may also be performed by simply allowing the buffers to slowly drip into the collection tubes. However, the drip method will be considerably slower.*

4. Place the column in a clean 2ml tube. Carefully load 100µl ASA (Animal Serum Albumin) to the column. Incubate for 5minutes and then centrifuge for 5 seconds and collect the flow-through in the collection tube, label this tube Fraction 1.

5. *Wash the column 3 times with Affinity Binding Buffer*: Place the column in a new tube. Apply 0.2ml Affinity Binding Buffer to the column. Centrifuge for 5 seconds and label the flow through Fraction 2. Repeat this step twice in two separate tubes and label the flowthroughs Fractions 3 and Fraction 4, respectively.

6. *Elute the sample using a salt gradient*: A salt gradient elution buffer is prepared by mixing the Affinity Elution Buffer and the Elution Buffer, which has a high concentration of salt, as in the table below:

Fraction#	Salt Concentration (M)	Affinity Elution Buffer (ml)	Elution Buffer (ml)
5	0	0.20	0.0
6	0.2	0.18	0.02
7	0.4	0.16	0.04
8	0.6	0.14	0.06
9	0.8	0.12	0.08
10	1.0	0.10	0.10

7. For gradient elution, place the column into a clean tube. Apply 0.2ml of the lowest salt concentration buffer (Fraction 5). Centrifuge for 5 seconds and collect the fraction in a 2ml collection tube.

8. Place the column in a fresh, clean tube and apply the next elution buffer starting with fraction 6 through to 10 and repeat step 7, until all the elution buffers have been added. Collect all 6 fractions in 6 separate 2ml tubes.

II. RED660 Protein Assay

1. A protein assay can be undertaken to determine the distribution of the proteins.

2. Label 10 tubes and transfer 20µl sample from each fraction to the labeled tubes.

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

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

4. 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.*

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

6. Add 1ml distilled water to a cuvette to zero the absorbance of the spectrophotometer. Measure the absorbance of each tube and record the values in the results section.

 *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.*

OPTIONAL: *The purified protein can also be identified by polyacrylamide gel electrophoresis. Perform SDS-electrophoresis with crude protein extract and the purified fractions and examine the distribution of the protein bands.*

RESULTS, ANALYSIS & ASSESSMENT

Prepare a graph showing protein elution profile of affinity 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. *Fraction 6 or 7.*

Q. Describe briefly how proteins are immobilized within an affinity column.

A. *Protein is immobilized due to specific interaction between immobilized ligand on the affinity column and the target proteins.*

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

A. *Elution of molecules of interest can be achieved by changing experimental conditions to favor desorption. The protein is eluted in a specific buffer, either by pH and/or ionic strength shift or by competitively displacement elution, such as high concentration of free ligand.*

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