



PR104-02

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A Geno Technology, Inc. (USA) brand name

Recombinant Protein Purification

Teacher's Guidebook

(Cat. # BE-419)



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MATERIALS INCLUDED

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

- 1 vial GB Lysate
- 6 Hydrophobic Columns
- 1 bottle 2X HP Loading Buffer
- 1 bottle HP Elution Buffer
- 1 bottle RED660 Protein Assay Reagent
- 130 Centrifuge Tubes

SPECIAL HANDLING INSTRUCTIONS

- Store GB Lysate and Hydrophobic Columns at 4°C.
- All other reagents can be stored at room temperature.
- Briefly centrifuge all small vials before opening to prevent waste of reagents.

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).

The following items need to be used with particular caution.

Part #	Name	Hazard
C111	Column: Hydrophobic Column	Flammable

ADDITIONAL EQUIPMENT REQUIRED

- Stand and clamps
- UV light box

TIME REQUIRED

- 2-3 hours

AIMS

- Learn step by step approach to protein purification.
- Learn protein purification strategies.
- Understand the benefits and importance of affinity 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". The "Protein Purification" kits aim to cover many of the common techniques used in protein purification.

One of the important aspects of biotechnology is the use of bacteria as living factories to produce genetically engineered proteins, known as recombinant proteins. The advantages of producing recombinant proteins, as opposed to purifying specific proteins from biological samples, is that vast quantities can be produced and scientists can attach *affinity tags* that allow for the rapid and specific purification of the recombinant proteins.

The science behind the purification of recombinant proteins with a specialized affinity tag is the use of affinity chromatography. Affinity chromatography involves the attachment of a specific ligand to a solid support or resin. The ligand will bind the specific tag and all the untagged, non specific proteins are washed from the solid support. The solid support is then treated with an elution buffer that breaks the interaction between the ligand and the tagged recombinant protein. Below are a few examples of the more common affinity tags used in science today, with information on the tag, the ligand and the elution buffer:

Glutathione S-Transferase (GST)

The use of GST as a tag is one of the more common, and oldest, purification strategies for recombinant protein purification. The open reading frame (ORF) for the GST enzyme is placed in frame with the ORF of the protein of interest and the subsequent recombinant protein is purified on a glutathione resin. The recombinant protein is eluted by competitive binding with excess glutathione. Several disadvantages to this method are that the GST enzyme must fold correctly in order to be an effective tag. The folding of GST can be severely inhibited by the protein it is attached to. The large size of the GST tag (~220 amino acids) often affects the solubility of the recombinant protein,

which makes it difficult to purify and also distorts the protein's natural confirmation making it inappropriate for structural studies. One solution is to use a specific protease to cleave the GST moiety from the recombinant protein after purification.

Poly Histidine Tag

The poly histidine tag has become a preferred replacement for the GST tag as it overcomes many of the disadvantages of the GST tag. The poly histidine tag is usually a short sequence of histidine amino acids, normally 6, in frame with the recombinant protein. The recombinant His protein is purified with the IMAC (immobilized metal ion affinity chromatography) technique. This system exploits the property of the histidine side chains that have high affinity for metal ions, such as nickel, zinc and cobalt. The recombinant protein can be eluted with low pH or by competitive adsorption with imidazole. Scientists favor the histidine tag over GST because of its small size, six amino acids as opposed to 220 amino acids.

GST and the poly histidine tags are the more common tags used in the laboratory today, however other options exist and new and improved tags are consistently being developed.

Calmodulin Binding Protein (CBP)

The calmodulin binding tag is a small (4kDa) tag that allows researchers the option of purifying delicate proteins under mild conditions. The CBP tag binds to a calmodulin resin and the proteins can be eluted with a neutral buffer containing low concentrations of the calcium chelator, EGTA.

Green Fluorescent Protein (GFP)

The green fluorescent protein (GFP) is more routinely used as a marker for proteins in living systems, but can be used to purify proteins on a hydrophobic column, due to the protein properties of the tag. The green fluorescent protein is extremely hydrophobic compared to host bacterial proteins, which are relatively hydrophilic. This allows the use of hydrophobic chromatography for the purification of recombinant proteins containing green fluorescent protein as a tag. This tag will be used to introduce student to affinity purification as fluorescent nature of the protein allows for the constant monitoring of the purification process and the visualization of the protein throughout the experiment.

Many other tags are available on the market and the selection is dependent on the protein to be purified and personnel preference. A new technique, known as the TAP (Tandem Affinity Purification), has recently been developed and employed with great success. The basic principle is to use two affinity tags followed by a sequential purification of the protein. This technique greatly reduces contamination of the purified proteins by proteins that bind non-specifically to affinity columns.

This lab activity is provided with reagents and supplies to purify a recombinant protein in a single hydrophobic chromatography step.

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. Add 300 μ l 1X HP Loading Buffer to the GB Lysate vial. Soak the lysate for 5-10 minutes with periodic vortexing. Centrifuge the vial at 16,000xg for 5 minutes and check under UV light for fluorescence in the supernatant. Aliquot 50 μ l of the green supernatant to each student group.
3. Aliquot reagents for each student group according to the next section.

MATERIALS FOR EACH GROUP

Each group is supplied with the following components.

- 50 μ l GB Lysate
- 1 Hydrophobic Column
- 8ml 1X HP Loading Buffer
- 5ml HP Elution Buffer
- 10ml RED660 Protein Assay reagent
- 20 Centrifuge Tubes

PROCEDURE



Always wear gloves and protective clothing throughout the whole experiment.

I. Hydrophobic Chromatography

1. Clamp the Hydrophobic Column in an upright position on to a stand.
2. 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.
3. *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 in to a waste container. Repeat until all 4ml has been applied.
4. Carefully load 50 μ l GB Lysate to the column.
5. 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.

6. 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.
7. Observe the fractions under UV light to discover the fraction containing the highest concentration of fluorescent protein.

II. RED660 Protein Assay

1. Label 10 tubes and add 100µl from each elute fraction to the tubes.
2. Mix the RED660 Protein Assay Reagent gently by inverting the bottle several times.



To avoid foaming, DO NOT SHAKE THE BOTTLE.

3. Add 1ml RED660 Protein Assay Reagent to each tube and mix by inverting 5-6 times.
4. Observe the tubes. The tube with the greatest concentration of color contains the most protein.

NOTE: *The absorbance of each assay can be measured with spectrophotometer or plate reader at 660nm.*

RESULTS, ANALYSIS & ASSESSMENT

1. Observe the fraction tubes under UV light box. Which fraction contains the most fluorescence?
2. Describe the benefits of using a tagged recombinant protein.

A tagged recombinant protein is specifically designed so that the protein can be rapidly and simply purified with an established purification technique.

3. Describe some affinity tags and their respective ligands and elution conditions

GST (glutathione S-transferase). Ligand: Glutathione. Elution: Free glutathione.

His tag. Ligand: Nickel, Cobalt or Zinc ions. Elution: Low pH or Imidazole.

CBP (Calmodulin Binding protein). Ligand: Calmodulin. Elution: Calcium chelator.

GFP (green fluorescent protein). Ligand: Hydrophobic hydrocarbons. Elution: Ionic buffer.



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