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A Geno Technology, Inc. (USA) brand name

Protein Fractionation

Teacher's Guidebook

(Cat. # BE-413)

MATERIALS INCLUDED

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

- 6 vials Tissue Extract & Pestle
- 1 vial Protein Buffer
- 1 vial Precipitant-Mes
- 1 bottle Salt Solution (Amm. Sulfate)
- 2 bottles Protein Assay: CB Reagent
- 1 bottle Protein Assay: Protein Standard (2mg/ml BSA)
- 200 Centrifuge Tubes (1.5ml)

SPECIAL HANDLING INSTRUCTIONS

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

ADDITIONAL EQUIPMENT REQUIRED

- Fresh Liver Tissue (optional)
- Razor Blades (optional)
- Forceps (optional)
- Microcentrifuge
- Spectrophotometer and cuvettes

TIME REQUIRED

- 2-3 hours

OBJECTIVES

- Learn strategies of protein purification.
- Learn principles of protein fractionation.
- Protein fractionation by changing pH.
- Protein fractionation by increasing salt concentration.

BACKGROUND

Protein fractionation utilizes the varied properties of proteins to separate a complex biological sample into more basic, enriched and concentrated samples. There are numerous properties of protein that can be utilized to fractionate proteins, including size, shape, sedimentation velocity, ability to bind to various ionic groups, affinity for substrates or pseudo-substrates, solubility, stability, and many more. Basically fractionation of a protein of interest can use any protein property that differs from unwanted proteins. For the fractionation of a protein of interest from a complex biological sample, numerous fractionation properties are routinely used.

The Protein Fractionation kit teaches protein fractionation using protein properties that are affected by changing the pH and ionic strength of the protein solutions. The use of acid and/or salt fractionations concentrates and enriches proteins into defined fractions, depending on their precipitation at differing pH and salt concentrations. Students understand the importance of enrichment of target proteins in the scheme of purification of protein molecules.

Adjusting the pH has been used as a simple and efficient way to precipitate proteins. Proteins have their lowest solubility at their isoelectric point. When the pH is gradually changed the pH of the solution passes through the isoelectric point of some proteins, causing some proteins to precipitate. Isoelectric precipitation is often used to precipitate unwanted proteins rather than to precipitate the protein of interest.

Precipitation of proteins by increasing the ionic strength of the solution is also knows as protein salting out. Salting out is dependent on the hydrophobicity on the surface of the protein. Hydrophobic regions are enriched in the hydrophobic amino acids, phenylalanine, tyrosine, tryptophan, leucine, isoleucine, methionine and valine. Proteins with more hydrophobic regions will aggregate and precipitate before those with smaller and fewer hydrophobic regions, thus resulting in fractionation. Salting out is usually performed at 4°C to decrease the risk of inactivation.

Salting out proteins from a crude extract using ammonium sulfate is a convenient purification step. Salts affect the electrostatic and non-polar properties of proteins in a reversible manner. At concentrations above 0.2M, salts not only neutralize the electrostatic forces on the protein surface but also affect the three dimensional structure of proteins, making them less soluble.

Salts, such as ammonium sulfate, have the tendency to disrupt the water structure, increase the water surface tension and increase the hydrophobic effect in the solution (i.e. decrease the solubility of non-polar molecules) and promote protein aggregation by association of hydrophobic surface. Ammonium sulfate is the most frequently used salt for salting-out experiments and can precipitate ~70% of proteins in a complex sample.

This lab activity is provided with a sample of tissue extract, an acidic buffer and a saturated solution of ammonium sulfate solution. Students in this lab activity will study the effects of pH and salt concentration on a complex tissue protein extract. Students will examine the extent to which fractionation and purification are achieved with these two methods and their value in purifying target proteins from complex samples. Students are challenged to consider how to use the fractionation techniques for purifying proteins from complex samples.

TEACHER'S PRE EXPERIMENT SET UP

1. For optimal results, and for more realistic experiment, we recommend supplying each group of students with approximately 50mg fresh chicken or bovine liver tissue. If fresh tissue is provided, empty the supplied Tissue Extract tube of the content and wash the tube with lab water. Supply each group with the pestle & tube for grinding fresh tissue.

2. If using fresh tissue, supply each group with a razor blade and a hard surface, such as a plate, to chop the tissue on.

3. 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 vial Tissue Extract & Pestle or 50mg fresh liver tissue & 1 grinding tube (tissue extract tube) & Pestle
- 3.5ml Protein Buffer
- 100µl Acid Precipitation Buffer
- 1ml Salt Solution (Ammonium Sulfate)
- 1 bottle CB Protein Assay Reagent (shared with whole class)
- 1 bottle Protein Standard (2mg/ml BSA) (shared with whole class)
- 1 Razor Blade (for fresh tissue)
- 32 1.5ml Tubes

PROCEDURE

Always wear gloves and protective clothing throughout the whole experiment.

I. Protein Extraction and Fractionation

1. If using the Tissue Extract go to step 4, if using fresh tissue start at step 2.

2. Chop the piece of tissue into small pieces with the razor blade. The smaller the pieces the better. Transfer the small pieces of tissue to the grinding tube, the one with the green pestle.

3. Add 200µl Protein Buffer to the fresh tissue and use the pestle to grind the tissue to form a uniform homogenate. Go to step 5.

4. Add 200µl Protein Buffer to the Tissue Extract vial, incubate at room temperature for 10 minutes. Use the pestle and grind the extract for 2 minutes. Go to step 5.

5. Centrifuge the homogenate at 5,000xg for 5 minutes.

6. Transfer 100µl supernatant containing the soluble proteins to a new tube without disturbing the pellet.

7. Add 100µl Acid Precipitation Buffer and mix the content by inverting the tube a few times or vortexing briefly.

8. Centrifuge the tube at 5,000xg for 5 minutes. The pellet is your Fraction 1: Acid precipitation.

9. Carefully transfer the supernatant to a new tube. Add 100µl saturated Ammonium Sulfate solution and mix the content by inverting the tube a few times or vortexing briefly.

10. Centrifuge the tube at 5,000xg for 5 minutes. The pellet is your Fraction 2 [Represents ~33% Ammonium Sulfate solution].

11. Carefully transfer the supernatant to a new tube. Add 200µl saturated Ammonium Sulfate solution and mix the content by inverting the tube a few times or vortexing briefly.

12. Centrifuge the tube at 5,000xg for 5 minutes. The pellet is your Fraction 3 [Represents ~60% Ammonium Sulfate solution].

13. Carefully transfer the supernatant to a new tube. Add 500µl saturated Ammonium Sulfate solution and mix the content by inverting the tube a few times or vortexing briefly.

14. Centrifuge the tube at 5,000xg for 5 minutes. The pellet is your Fraction 4 [Represents ~80% Ammonium Sulfate solution].

15. Carefully transfer all supernatant to a new tube. This is your Fraction 5.

Estimation of Fractionated Proteins

Determine protein content of each fraction using the following method of protein estimation, the CB Protein Assay, as follows.

II. CB Protein Assay

1. Resuspend the pellets from fractions 1 and 2 in 100µl Protein Buffer and from fractions 3 and 4 in 200µl Protein Buffer.

2. Add 0.7ml BSA protein standard (2mg/ml) to a tube and dilute with 0.7ml Protein Buffer to make 1mg/ml solution.

3. For a standard curve, prepare a range of protein standards with the diluted BSA (1mg/ml) from step 2, as indicated in Table 1.

Table 1

BSA Concentration (mg/ml)	0.0	0.1	0.3	0.5	0.8	1.0
Diluted BSA/ml	0.00	0.05	0.15	0.25	0.40	0.50
Protein Buffer	0.50	0.45	0.35	0.25	0.10	0.00

4. Label two sets of tubes with 0, 0.1, 0.3, 0.5, 0.8 and 1.0 and transfer 0.05ml of the appropriate standard to the tubes.

5. Label two tubes for each of Fractions 1-5.

6. Pipette Protein Buffer and fraction solutions into the appropriate tubes as indicated in Table 2. Each sample is done in duplicate

Table 2

Tube	**Protein Buffer (ml)**	**Fraction Vol. (ml)**
Fraction-1	0.03	0.02
Fraction-2	0.03	0.02
Fraction-3	0.04	0.01
Fraction-4	0.04	0.01
Fraction-5	--	0.05

7. Mix the CB Protein Assay Reagent by gently inverting the bottle several times.

To avoid foaming, DO NOT SHAKE THE BOTTLE.

8. Add 1ml CB Protein Assay Reagent to each tube and vortex briefly to mix the content. Incubate the tubes at room temperature for 5 minutes.

9. In the meantime, turn on the spectrophotometer and allow it to warm up. Adjust the wavelength to 595nm.

10. 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 595nm.*

III. Determination of the Protein Concentration

Determine the protein concentration with a traditional paper plot.

1. Draw the points with protein concentrations as *x* values and the average absorbance as *y* value on a grid or graph paper.

2. Draw a straight line through the points.

3. Lookup the Test Sample concentration from the plot using the absorbance value of the Test Sample.

4. Calculate the actual protein concentration:

$$\frac{\text{Protein concentration from the plot X 0.05ml (BSA standard volume)}}{\text{Test fraction volume (ml)}}$$

Determine the protein concentration using Microsoft Excel

1. Input the BSA standard concentration in a column and the absorbance value in a second column.
   Highlight the data in the two columns
   From the menu bar, choose **Insert** then **Chart.**
   A "Chart Wizard" Menu will appear

2. Under the "Standard Types" tab:
   For "Chart type" select: XY (Scatter)
   For "Chart sub-type" select the top chart
   (The one without any lines connecting the points) Click *Next>*

3. Under series in select columns
   Click *Next>*

4. Under the "Titles" tab: Type in appropriate titles (names) for the graph (chart) and the x and y-axes
   (e.g., Chart title: Protein Fractionation, x-axis: Absorbance, y-axis: Protein Concentration (mg/ml))
   Under the "Legend" tab: Click on the checkmark next to "Show legend"
   (The checkmark should disappear)
   Click *Next>*

5. Click on circle to left of "As object in" (a dot should appear in the circle)
   Click *Finish.*
   Your graph should appear on the sheet where your data is.

6. Highlight the chart by clicking inside the chart area.
   From the menu bar, choose **Chart** then **Add Trendline**
   The “Add Trendline” window will appear

7. Under the “Type” tab: For “Trend/Regression type” select: Linear

8. Under the “Options” tab:
   Click on box to left of “Display equation on chart” (a checkmark appears)
   Click on box to left of “Display R-squared on chart” (a checkmark appears)
   Click *ok.*

9. Move the equation and R-squared value to a suitable location on the graph. Save and print the data sheet.

10. Use the equation on the chart to calculate the concentration of your unknown sample.

RESULTS, ANALYSIS & ASSESSMENT

Tube #	Absorbance I (595nm)	Absorbance II (595nm)	Average	Protein Conc. (mg/ml)
0				0
0.1				0.1
0.3				0.3
0.5				0.5
0.8				0.8
1				1
Fraction 1				
Fraction 2				
Fraction 3				
Fraction 4				
Fraction 5				

1. Calculate the actual protein concentration:

$$\frac{\text{Protein concentration from the plot X 0.05ml (BSA standard volume)}}{\text{Test fraction volume (ml)}}$$

2. Determine the percentage (%) of total protein in each fraction.
   Take the mg/mL concentration for each fraction and then multiply by the volume of each fraction to get the amount of protein in mg/ fraction. Divide this by the total soluble proteins in the tissue extract times 100 to get the percentage protein in each fraction:

$$\frac{Mg\ protein\ in\ Fraction}{Total\ Soluble\ Protein\ in\ Tissue\ Extract} \times 100$$

   Total soluble proteins in tissue extract ----------
   % of total proteins in:
   Fraction 1_____ Fraction 4_____
   Fraction 2_____
   Fraction 3_____ Fraction 5_____

Q. Briefly describe the principles of protein fractionation used in this lab activity

A. Proteins have their lowest solubility at their isoelectric point. Lowering the pH of the crude extract with acid treatment reduces solubility of some proteins in the tissue extract, which in turn precipitates and removes some proteins from the tissue extract. The remaining soluble proteins are fractionated by incremental increases in the salt concentration. Salt, such as ammonium sulfate, has the tendency to disrupt the water structure and promote protein aggregation by association of hydrophobic surface.

Q. Describe the advantage of acid fractionation in the scheme of protein purification.

A. A simple way to remove unwanted protein from a complex protein sample.

Q. Describe the advantage of an ammonium sulfate fractionation*.*

A. Most widely used fractionation method that has the potential to precipitate up to 70% of the proteins in a complex sample. Ammonium sulfate fraction is generally used as an enrichment step for purification of proteins from complex tissue extracts.

Q. Describe the importance of fractionating complex tissue extracts.

A. Fractionation of tissue extracts allows removal of unwanted proteins and enrichment of target protein species from complex protein samples.

Q. List some possible applications of these fractionation techniques.

A. Enrichment of target proteins, one of the steps during purification of target proteins, and simplification of complex samples.

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Salting out proteins from a crude extract using ammonium sulfate is a convenient purification step. Salts affect the electrostatic and non-polar properties of proteins in a reversible manner. At concentrations above 0.2M, salts not only neutralize the electrostatic forces on the protein surface but also affect the three dimensional structure of proteins, making them less soluble.

Salts, such as ammonium sulfate, have the tendency to disrupt the water structure, increase the water surface tension and increase the hydrophobic effect in the solution (i.e. decrease the solubility of non-polar molecules) and promote protein aggregation by association of hydrophobic surface. Ammonium sulfate is the most frequently used salt for salting-out experiments and can precipitate ~70% of proteins in a complex sample.

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PROCEDURE

Always wear gloves and protective clothing throughout the whole experiment.

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4. Add 200µl Protein Buffer to the Tissue Extract vial, incubate at room temperature for 10 minutes. Use the pestle and grind the extract for 2 minutes. Go to step 5.

5. Centrifuge the homogenate at 5,000xg for 5 minutes.

6. Transfer 100µl supernatant containing the soluble proteins to a new tube without disturbing the pellet.

7. Add 100µl Acid Precipitation Buffer and mix the content by inverting the tube a few times or vortexing briefly.

8. Centrifuge the tube at 5,000xg for 5 minutes. The pellet is your Fraction 1: Acid precipitation.

9. Carefully transfer the supernatant to a new tube. Add 100µl saturated Ammonium Sulfate solution and mix the content by inverting the tube a few times or vortexing briefly.

10. Centrifuge the tube at 5,000xg for 5 minutes. The pellet is your Fraction 2 [Represents ~33% Ammonium Sulfate solution].

11. Carefully transfer the supernatant to a new tube. Add 200µl saturated Ammonium Sulfate solution and mix the content by inverting the tube a few times or vortexing briefly.

12. Centrifuge the tube at 5,000xg for 5 minutes. The pellet is your Fraction 3 [Represents ~60% Ammonium Sulfate solution].

13. Carefully transfer the supernatant to a new tube. Add 500µl saturated Ammonium Sulfate solution and mix the content by inverting the tube a few times or vortexing briefly.

14. Centrifuge the tube at 5,000xg for 5 minutes. The pellet is your Fraction 4 [Represents ~80% Ammonium Sulfate solution].

15. Carefully transfer all supernatant to a new tube. This is your Fraction 5.

Estimation of Fractionated Proteins

Determine protein content of each fraction using the following method of protein estimation, the CB Protein Assay, as follows.

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Protein Buffer	0.50	0.45	0.35	0.25	0.10	0.00

4. Label two sets of tubes with 0, 0.1, 0.3, 0.5, 0.8 and 1.0 and transfer 0.05ml of the appropriate standard to the tubes.

5. Label two tubes for each of Fractions 1-5.

6. Pipette Protein Buffer and fraction solutions into the appropriate tubes as indicated in Table 2. Each sample is done in duplicate

Table 2

Tube	Protein Buffer (ml)	Fraction Vol. (ml)
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Fraction-2	0.03	0.02
Fraction-3	0.04	0.01
Fraction-4	0.04	0.01
Fraction-5	--	0.05

7. Mix the CB Protein Assay Reagent by gently inverting the bottle several times.

To avoid foaming, DO NOT SHAKE THE BOTTLE.

8. Add 1ml CB Protein Assay Reagent to each tube and vortex briefly to mix the content. Incubate the tubes at room temperature for 5 minutes.

9. In the meantime, turn on the spectrophotometer and allow it to warm up. Adjust the wavelength to 595nm.

10. 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 595nm.*

III. Determination of the Protein Concentration

Determine the protein concentration with a traditional paper plot.

1. Draw the points with protein concentrations as *x* values and the average absorbance as *y* value on a grid or graph paper.

2. Draw a straight line through the points.

3. Lookup the Test Sample concentration from the plot using the absorbance value of the Test Sample.

4. Calculate the actual protein concentration:

$$\frac{\text{Protein concentration from the plot X 0.05ml (BSA standard volume)}}{\text{Test fraction volume (ml)}}$$

Determine the protein concentration using Microsoft Excel

1. Input the BSA standard concentration in a column and the absorbance value in a second column.
   Highlight the data in the two columns
   From the menu bar, choose **Insert** then **Chart.**
   A "Chart Wizard" Menu will appear

2. Under the "Standard Types" tab:
   For "Chart type" select: XY (Scatter)
   For "Chart sub-type" select the top chart
   (The one without any lines connecting the points) Click *Next>*

3. Under series in select columns
   Click *Next>*

4. Under the "Titles" tab: Type in appropriate titles (names) for the graph (chart) and the x and y-axes
   (e.g., Chart title: Protein Fractionation, x-axis: Absorbance, y-axis: Protein Concentration (mg/ml))
   Under the "Legend" tab: Click on the checkmark next to "Show legend"
   (The checkmark should disappear)
   Click *Next>*

5. Click on circle to left of "As object in" (a dot should appear in the circle)
   Click *Finish.*
   Your graph should appear on the sheet where your data is.

6. Highlight the chart by clicking inside the chart area.
   From the menu bar, choose **Chart** then **Add Trendline**
   The “Add Trendline” window will appear

7. Under the “Type” tab: For “Trend/Regression type” select: Linear

8. Under the “Options” tab:
   Click on box to left of “Display equation on chart” (a checkmark appears)
   Click on box to left of “Display R-squared on chart” (a checkmark appears)
   Click *ok.*

9. Move the equation and R-squared value to a suitable location on the graph. Save and print the data sheet.

10. Use the equation on the chart to calculate the concentration of your unknown sample.

RESULTS, ANALYSIS & ASSESSMENT

Tube #	Absorbance I (595nm)	Absorbance II (595nm)	Average	Protein Conc. (mg/ml)
0				0
0.1				0.1
0.3				0.3
0.5				0.5
0.8				0.8
1				1
Fraction 1				
Fraction 2				
Fraction 3				
Fraction 4				
Fraction 5				

1. Calculate the actual protein concentration:

$$\frac{\text{Protein concentration from the plot X 0.05ml (BSA standard volume)}}{\text{Test fraction volume (ml)}}$$

2. Determine the percentage (%) of total protein in each fraction.
   Take the mg/mL concentration for each fraction and then multiply by the volume of each fraction to get the amount of protein in mg/ fraction. Divide this by the total soluble proteins in the tissue extract times 100 to get the percentage protein in each fraction:

$$\frac{Mg\ protein\ in\ Fraction}{Total\ Soluble\ Protein\ in\ Tissue\ Extract} \times 100$$

   Total soluble proteins in tissue extract ----------
   % of total proteins in:
   Fraction 1_____ Fraction 4_____
   Fraction 2_____ Fraction 5_____
   Fraction 3_____

Q. Briefly describe the principles of protein fractionation used in this lab activity

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Q. Describe the advantage of acid fractionation in the scheme of protein purification.

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Q. Describe the advantage of an ammonium sulfate fractionation.

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Q. Describe the importance of fractionating complex tissue extracts.

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Q. List some possible applications of these fractionation techniques.

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