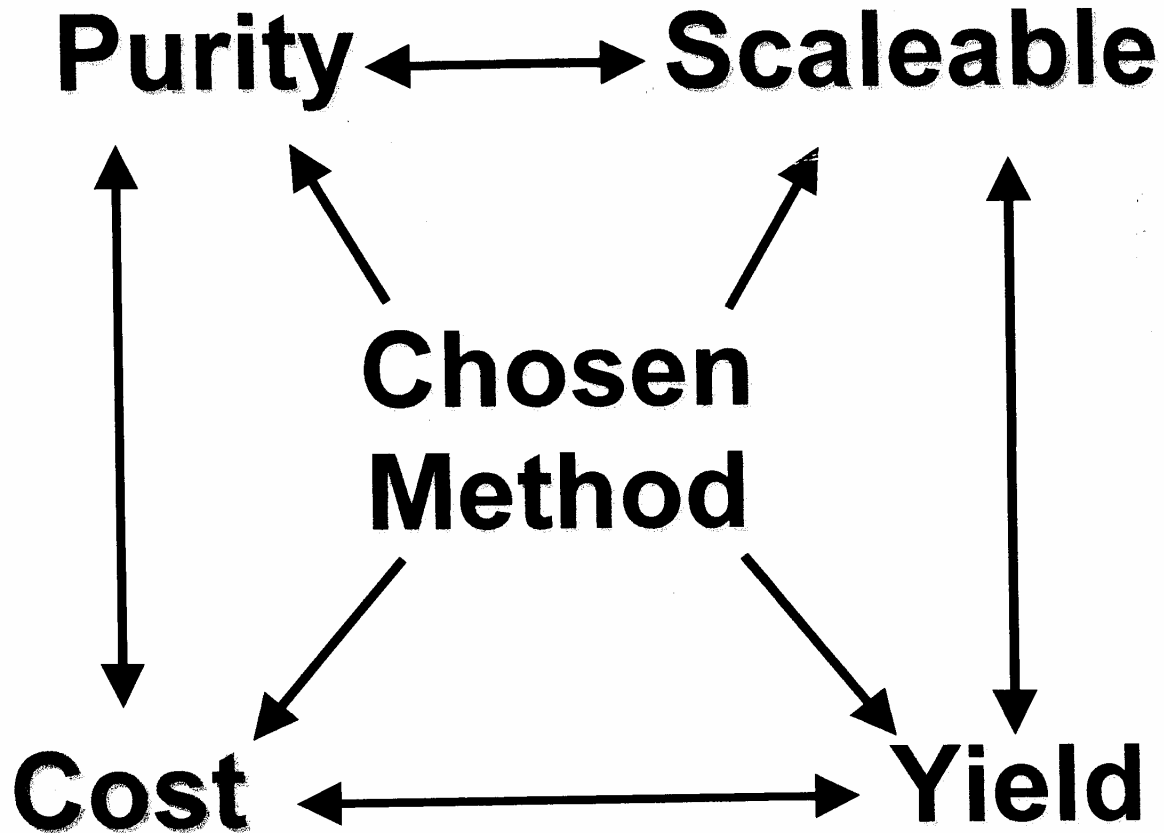
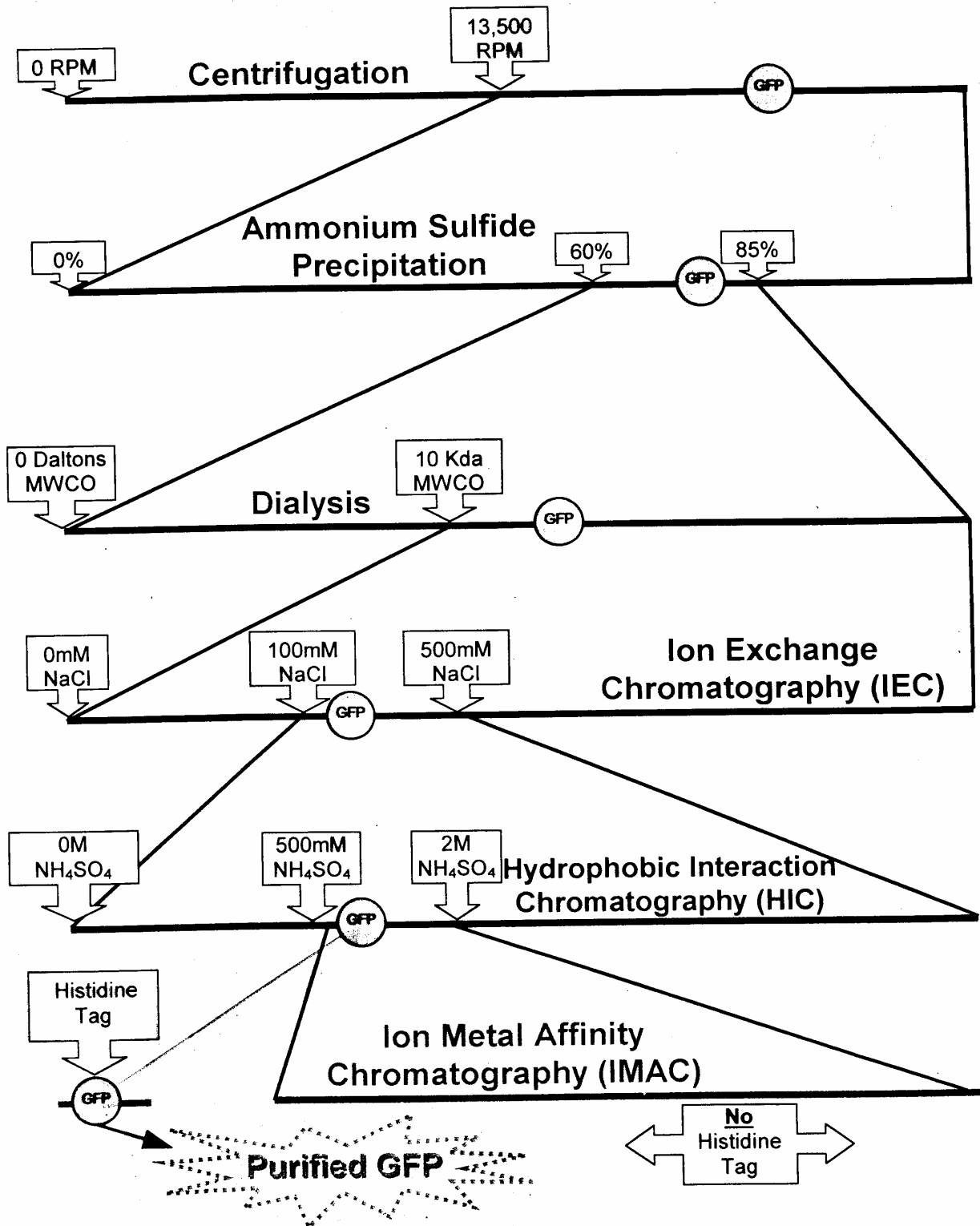


Selection of a Purification Technique

*Steve Harris – Biotechnology Instructor
SPASH*



GFP Purification Sequence



What is Biochromatography?

Steve Harris
Biotechnology Instructor
SPASH

Biochromatography Techniques

Biomolecules may be purified from naturally occurring sources such as animal or plant tissue, or, in the case of recombinant proteins, from microbial host cells. Some knowledge about the molecule of interest is helpful in choosing an effective purification scheme. In order to purify a molecule from other proteins present in a sample, scientists study how groups of molecules differ from one another and how these differences can be used to separate them. Data about the sequence or class of molecule can be used to estimate the size, charge, or hydrophobicity of the molecule to be purified and to determine which chromatography technique might be the most efficient choice.

Theoretically, all molecules have unique chemical characteristics that allow them to be separated from other molecules in a mixture using one or more chromatography techniques. Chromatography involves packing a cylindrical column with a resin or support medium consisting of microscopic adsorbent beads. A variety of bead types are available that have selective affinities for particular classes of molecules.

When a liquid sample is loaded at the top of a column and allowed to flow through, individual molecules in the sample may have an affinity for, or interact with, the support medium. Depending on the support medium, molecules may be adsorbed to or may enter the beads. Components in a mixture become separated as they migrate at different rates through a given medium. As they elute or exit the bottom of the column, the separated components can be collected in multiple liquid fractions. The fractions are then assayed to evaluate the efficiency of fractionation and to determine the location and purity of the target molecule of interest.

What is Biochromatography?

Steve Harris
Biotechnology Instructor
SPASH

Biochromatography Techniques

Biomolecules may be purified from naturally occurring sources such as animal or plant tissue, or, in the case of recombinant proteins, from microbial host cells. Some knowledge about the molecule of interest is helpful in choosing an effective purification scheme. In order to purify a molecule from other proteins present in a sample, scientists study how groups of molecules differ from one another and how these differences can be used to separate them. Data about the sequence or class of molecule can be used to estimate the size, charge, or hydrophobicity of the molecule to be purified and to determine which chromatography technique might be the most efficient choice.

Theoretically, all molecules have unique chemical characteristics that allow them to be separated from other molecules in a mixture using one or more chromatography techniques. Chromatography involves packing a cylindrical column with a resin or support medium consisting of microscopic adsorbent beads. A variety of bead types are available that have selective affinities for particular classes of molecules.

When a liquid sample is loaded at the top of a column and allowed to flow through, individual molecules in the sample may have an affinity for, or interact with, the support medium. Depending on the support medium, molecules may be adsorbed to or may enter the beads. Components in a mixture become separated as they migrate at different rates through a given medium. As they elute or exit the bottom of the column, the separated components can be collected in multiple liquid fractions. The fractions are then assayed to evaluate the efficiency of fractionation and to determine the location and purity of the target molecule of interest.

Four levels of Organization of Protein Structure

Steve Harris - Biotechnology Instructor

SPASH

1° Primary Structure refers to the linear sequence of amino acids that make up the polypeptide chain. This sequence is determined by the genetic code, the sequence of nucleotide bases in the DNA. The bond between two amino acids is a peptide bond. This bond is formed by the removal of a H₂O molecule from two different amino acids, forming a dipeptide.

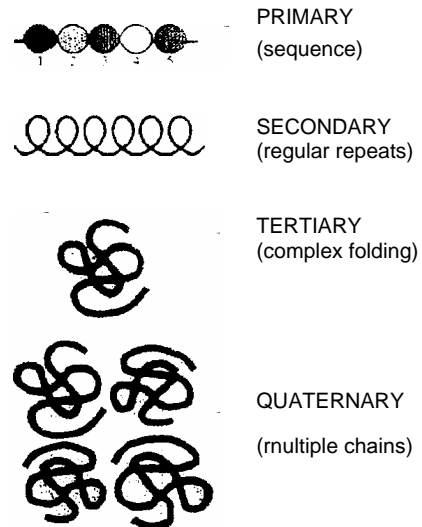
The sequence of amino acids determines the positioning of the different chemical groups of each building block which determines which groups will interact with each other and therefore determines the final structure and function of the molecule.

2° The secondary structure of protein molecules involves the coiling of the peptide chain into a helix or some other regular pattern of twists or kinks of the polypeptide chain. The regularity is due to hydrogen bonds forming between the atoms of the amino acid backbone of the polypeptide chain. The two most common types of secondary structure are called the alpha helix and pleated sheet.

3° Tertiary structure refers to the three dimensional globular structure formed by bending and twisting of the polypeptide chain. This process often means that the linear sequence of amino acids is folded into a compact globular structure. The folding of the polypeptide chain is stabilized by multiple weak, non-covalent interactions. These interactions include:

- Hydrogen bonds that form when a Hydrogen atom is shared by two other atoms.
- Electrostatic interactions that occur between charged amino acid side chains. *Electrostatic interactions are attractions between positive and negative sites on macromolecules.*
- Hydrophobic interactions: During folding of the polypeptide chain, amino acids with a polar (water soluble) side chain are often found on the surface of the molecule while amino acids with non polar (water insoluble) side chain are buried in the interior. This means that the folded protein is soluble in water or aqueous solutions.
- Disulfide Bonds: The polypeptide chains of some proteins are linked by disulfide bonds. Insulin, for example, is made from two polypeptide chains that are joined by two disulfide bonds.

4° Quaternary structure. Proteins that contain more than one polypeptide chain have an additional level of structural organization, quaternary structure, which refers to the association of the polypeptide chains. Each polypeptide chain in the protein is called a subunit. The subunits can be the same polypeptide chain or different ones. For example, the enzyme (β-galactosidase is a tetramer, meaning that it is composed of four subunits, and, in this case, the subunits are identical--each polypeptide chain has the same sequence of amino acids. Hemoglobin, the oxygen carrying protein in the blood, is also a tetramer but it is composed of two polypeptide chains of one type (141 amino acids) and two of a different type (146 amino acids). In chemical shorthand, this is referred to as α₂ β₂. For some proteins, quaternary structure is required for full activity (function) of the protein although that is not the case for β-galactosidase.



Protocol: Green Fluorescent Protein (GFP)

Extraction and Purification

Kelly Korbisch and Kelly Nomberg –2002
Steve Harris -Instructor

Green Fluorescent Protein (GFP), from the jellyfish Aequorea victoria, can be used as a fluorescent marker. GFP is an extraordinary protein molecule in many aspects. It is fluorescent, and its fluorophore is made up of modified amino acid residues. It has recently been discovered that GFP has a crystal structure motif called the beta-can. Moreover, it is the first known example of a Forster cycle within the core of a protein. It is an extremely stable protein made up of 238 amino acids.

Extraction of GFP from E. coli transformed with recombinant Plasmid DNA

1. Transfer 45mls of each overnight flask into a 50ml centrifuge tube.
2. Centrifuge at 6,000 revolutions per minute for 5 min.
3. Check cell supernatant and pellet under black light for fluorescence.
4. Decant liquid supernatant down drain.
5. Repeat steps 1-4 until flask(s) are empty.
6. Check cell pellet under black light for green fluorescence.
7. Add 5 ml of T8/PMSF buffer and resuspend cells by scraping it free with an inoculating loop and vortexing (setting 6-7).
8. Add 1 mg of crystallized lysozyme and gently vortex (setting 6).
9. Water bath incubate for 20 min. @ 37°C, 170 strokes per minute. (Use a rubber band to hold the tube horizontally in water bath attached to one of the flask clamps).
10. Refrigerate overnight or continue with lab.

(Optional)

- a. Sonicate the cell for 10 seconds at 70% power.
- b. Quick-freeze in liquid nitrogen for 1 minute or until frozen.
- c. Quick-thaw by shaking it (by hand) in a 37 degree water bath.
- d. Repeat steps 1-3 three times.

11. To a mortar and pestle add 8g of alumina and E.coli cells.
12. Add 1 ml of T8/PMSF buffer to 50ml tube as a rinse and then add it to mortar.
13. Grind for 15 minutes.
14. Pour ground E. coli cells into a washed 15ml centrifuge tube.
15. Add 1 ml of T8/PMSF buffer at a time to the mortar as a rinse and then add it to tube. Repeat, as needed using black light to check mortar, but do not exceed 13mls as your final volume.

(Optional)

- a. Add 10ul of RNase A.

16. Add 25ul of Dnase.
17. Mix gently and incubate for 20 minutes @ 37°C as previously described in step 9.
18. Centrifuge 5 minutes @ 6000 rpm.
19. Check both supernatant and pellet for fluorescence under black light. 20. In 1 ml lots transfer by pipette supernatant into 1.5ml tubes.
20. Centrifuge all tubes at 13,500 rpm for 25 minutes.
21. Check both supernatant and pellet for fluorescence under black light.
22. Combine by decanting all supernatants into a washed/clean 15ml tube.

Ammonium sulfate precipitation of GFP

*Kelly Nornberg & Kelly Korbisch - Independent Study
Steve Harris Biotechnology Instructor
SPASH*

Background: Proteins vary greatly in their solubility depending on their amino acid composition and the characteristics of the solvent such as ionic strength and temperature. One of the most common approaches for separating proteins is using increasing concentrations of a salt to "salt out" the proteins. Salting out is often used as an early step in a purification strategy. Ammonium sulfate is commonly chosen as the salt since high concentrations can be achieved and since it tends to be gentle. Even if a significant increase in purity is not achieved, an ammonium sulfate "cut" can be used to concentrate a large volume of extract for the subsequent purification steps.

"Salting out" is thought to work by "dehydrating" the environment around the protein. When ammonium sulfate is added to the protein solution, a large number of water molecules bind to the sulfate ion, which reduces the amount of water available to bind to the protein. If a protein is not hydrated by binding to water molecules, it will precipitate. Different proteins precipitate at different concentrations of ammonium sulfate. We can take advantage of this difference to remove some contaminating proteins from our crude extract.

Protocol:

1. Using the 100-1000 and the 20-200 pipette measure and record the exact volume of GFP.

Exact volume: _____ ml

1. Save 100ul of extract for spectral analysis. Label tube with group # and "spec. extract"
2. In a second tube save 100ul of extract for a PAGE gel. Label tube with group # and "gel extract"
3. Calculate and record the remaining exacted volume of GFP.

Exact volume: _____ ml

4. Chill extract on ice for 5 minutes.
5. Calculate the gram quantity needed of ammonium sulfate to bring the extract from 0% saturation to 60% at 0° C by adding 361 g/L. (*Note: You do not have a liter of extract.*)

SHOW CALCULATION:
$$\frac{361g}{1000mL} = \frac{Xg}{exactvolume}$$

6. Using the analytical scale, weigh out your calculated gram quantity of ammonium sulfate.
7. In a 50ml beaker on an ice pack add GFP extract, over a 10-minute period using a gentle swirling motion; slowly add ammonium sulfate (*be sure all of the salt dissolves*)
8. Pour GFP extract back into 15ml centrifuge tube and centrifuge at 6,000g for 10 minutes.
9. Check both supernatant and pellet for fluorescence.
10. Decant supernatant into a new 15ml centrifuge tube. (*If supernatant still glows*)
11. Measure and record the exact volume of GFP.

Exact volume: ___ ml

12. Save 100ul of 60% ammonium sulfide GFP for spectral analysis. Label the tube with group # and "spec 60%".
13. In a second tube save 100ul of 60% ammonium sulfide GFP for PAGE gel. Label tube with group # and "gel 60%"
14. Continue to chill the 60% ammonium sulfide GFP on ice.
15. Calculate the gram quantity of ammonium sulfate needed to bring the 60% ammonium sulfide to 85% saturation at 0° C by adding 201g/L. (*Note: You do not have a liter of extract.*)

SHOW CALCULATION:
$$\frac{201g}{1000mL} = \frac{Xg}{\text{exactvolume}}$$

16. Using the analytical scale, weigh out your calculated gram quantity of ammonium sulfate.
17. In a 50ml beaker on an ice pack add 60% ammonium sulfide GFP, over a 10-minute period using a gentle swirling motion, slowly add ammonium sulfate. (*Be sure all of the salt dissolves*)
18. Pipette GFP extract into 1.5ml microcentrifuge tubes and centrifuge at 13,500rpm for 10 minutes.
19. Check both supernatants and pellets for fluorescence.
20. Pipette supernatants into 15 ml tube and pour down drain. (*If it doesn't glow*).
21. Re-dissolve precipitates in 1 ml lots (total volume of 5-10ml) of T8/PMSF buffer by gentle agitation.
22. Measure and record the exact volume of 85% ammonium sulfate GFP.

Exact volume: ___ ml

23. Save 100ul of 85% ammonium sulfate GFP for spectral analysis. Label the tube with group # and "spec 85%".
24. In a second tube save 100ul of extract for PAGE gel. Label tube group # and "gel 85%"

Dialysis of re-dissolved "salted-out" GFP

1. Prep a "Slide-A-Lyzer" dialysis cassette (10,000-dalton MWCO membrane) with duct tape and label with waterproof marker.
2. Using syringe and needle, load GFP into a "Slide-A-Lyzer" dialysis cassette.
3. Check for leaks & fluorescence under black light.
4. Suspend unit into a beaker with 1 liters of T8 buffer (**not PMSF T8 buffer**) and add stir bar in bottom.
5. Place beaker on stir plate inside refrigerator overnight.
6. End of day replace T8 buffer with a fresh 1 liter of T8.
7. Incubate overnight again T8 buffer under cold conditions.

-----Day Two-----

8. Replace T8 buffer with fresh T8.
9. Incubate overnight again T8 buffer under cold conditions

-----Day Three-----

10. Using syringe and needle remove solution from unit and put in new tube
11. Check for fluorescence
12. Measure and record the exact volume of extract to nearest .1 ml.

Exact volume: _____ ml

13. Save 100ul of extract + 900µl nano-water for spectral analysis. Label tube group # and "Dialysis".
14. Save 100ul of extract for Polyacrylamide gel. Label tube group # and "Dialysis".
15. Store sample in a refrigerator overnight

Ion Exchange Chromatography

IEC

Anne Simonis-2002

Instructor: Steve Harris

Ion exchange chromatography separates molecules based on their net charges. Negatively or positively charged molecules are covalently bound to a solid support matrix, yielding either a cation or anion. When a charged molecule of opposite charge is applied, it is adsorbed, while neutral ions or ions of the same charge are eluted from the column. Binding of the charged molecules is reversible, and adsorbed molecules are commonly eluted with a salt or pH gradient. Ion exchange media are available in various particle sizes, ionic forms, and purity ranges.

*** Note: Before beginning IEC it is extremely important that the protein of interest (GFP) be completely dialyzed of all salts before beginning.**

1. Setup fraction collector and chromatography column as demonstrated by instructor. *(It is important that the chromatography column not be allowed to run dry during any phase of purification. This can lead to irreversible pitting of the matrix in the support material and decrease the exchange capacity of the column.)*
2. Using a 1 ml pipette, gently slide 4-8 mls of T8 buffer down the inside wall of the chromatography tube onto support material until final volume meets the top of the glass viewing area. *(Being careful not to disturb the support material.)*
3. Connect the column to the buffer reservoir and wash the support material with 30 mls of T8 buffer at a rate of 1 ml/min. into 5 ml glass tubes.
4. Allowing the column to continue to run, reduce the T8 volume to within 2 mm of the support material bed. *(Reminder. It is important not to allow the bed to go dry!)*
5. Carefully slide GFP onto the support material using the same method as step # 2 above.
6. Allowing the column to continue to run at a rate of 1 ml/min. Run the GFP into the support material. Collect the follow through and check for fluorescents. *(If the GFP is not completely dialyzed it will not bind to support material and pass through the column.)* Run the GFP until the volume of GFP is within 2 mm of the support material.
7. Carefully add 4-8 mls of T8 buffer in the same gentle method as described above in step # 2.
8. Wash bound GFP with 30 mls T8 buffer at a rate of 1 ml/min. Continue to run until the volume is reduced to 2 mm above the support material. *(Collect the follow through in 5 ml glass tubes while observing GFP with a black light.)*
9. As demonstrated by your instructor, set up a 100 mM/500 mM NaCl gradient, using 30 mls each. Add 4-8 mls of 100 mM NaCl to the chromatography column up to the top of the glass viewing area.
10. Allow the gradient to run and collect the follow through at a rate of 0.5 ml/min. into 1.5 ml microcentrifuge tubes. *(Note: The fraction collector's drop count will need to be changed at this point)*
11. After the gradient has completely run, combine the three or four brightest fractions. Measure and record the exact volume.

EXACT VOLUME: _____ ml

12. Save 100 μ l of IEC - GFP for spectral analysis, label tube: "Spec-IEC" along with group #.
13. Save 100 μ l of I EC - GFP for the PAGE gel, labeled tube: "gel-IEC" along with group #.

Hydrophobic Interaction Chromatography

HIC

Anne Simonis-2002

Instructor: Steve Harris

Hydrophobic interaction chromatography (HIC) separates molecules based on their hydrophobicity. Molecules that contain both hydrophobic and hydrophilic regions are applied to an HIC column in a high-salt buffer. The salt in the buffer reduces the solvation of the sample. As overall solvation decreases, hydrophobic regions that become exposed are adsorbed by the medium. The more hydrophobic the molecule, the less salt is needed to promote binding. Usually a decreasing salt gradient is used to elute samples from the column in order of increasing hydrophobicity.

1. Calculate the amount of ammonium sulfate (NH_4SO_4) required to make a 2M solution with GFP volume.
2. In a 50ml beaker on an ice pack add IEC-GFP. Over a 10-minute period using a gentle swirling motion slowly add ammonium sulfate (NH_4SO_4) to raise molarity to 2M. *(If the NH_4SO_4 is added quickly and not allowed to dissolve, the GFP will precipitate and you will have to start over!)*
3. Set up fraction collector and chromatography column as demonstrated by instructor. *(It is important that the chromatography column not be allowed to run dry during any phase of purification. This can lead to irreversible pitting of the matrix in the support material and decrease the hydrophobicity capacity of the column)*
4. Using a 1 ml pipette, gently slide 4-8 mls of 2M NH_4SO_4 down the inside wall of the chromatography tube onto support material until final volume meets the top of the glass viewing area. *(Being careful not to disturb the support material.)*
5. Connect the column to the NH_4SO_4 reservoir and wash the support material with 30 mls of 2M NH_4SO_4 at a rate of 1 ml/min. into 5 ml glass tubes.
6. Allowing the column to continue to run, reduce the NH_4SO_4 volume to within 2 mm of the support material bed. *(Reminder. It is important not to allow the bed to go dry!)*
7. Carefully slide GFP onto the support material using the same method as step # 4 above.
8. Allowing the column to continue to run at a rate of 1 ml/min. run the GFP into the support material. Collect the flow through and check for fluorescents. *(If the GFP does not have a high enough salt content, it will not bind to the support material and pass through the column.)* Run the GFP until the volume of GFP is within 2 mm of the support material.
9. Carefully add 4-8 mls of 2M NH_4SO_4 in the same gentle method as described in step # 4.
10. Wash bound GFP with 30 mls 2M NH_4SO_4 at a rate of 1 ml/min. Continue to run until the volume is reduced to 2 mm above the support material. *(Collect the flow through in 5 ml glass tubes while observing GFP with a black light.)*
11. As demonstrated by your instructor, set up a 2M/500mM NH_4SO_4 gradient, using 30 mls each. Add 4-8 mls of 2M NH_4SO_4 to the chromatography column.
12. Allow the gradient to run and collect the flow through at a rate of 0.5 ml/min. into 1.5 ml microcentrifuge tubes. *(Note: The fraction collector's drop count will need to be changed at this point)*
13. After the gradient has completely run, combine the three or four brightest fractions. Measure and record the exact volume.

EXACT VOLUME: _____ ml

14. Save 100ul of HIC-GFP for spectral analysis, label tube: "Spec-HIC" along with group #.
15. Save 100ul of HIC - GFP for the PAGE gel, labeled tube: "gel-HIC" along with group #
16. Continue to run the column with 30 mls of nano-water to completely clean the column.
17. Recharge the column for storage with 30 mls 2M NH_4SO_4 .

Polyacrylamide Gel Staining Procedure

The following preparations are adequate for staining two mini gels (8 x 10 cm), 0.75-1.0 mm thick. Refer to Table A for staining gels of other sizes.

1. Fixative Step - 20 min

Fixative Enhancer Solution Preparation (for 2 mini gels)

Reagent Grade Methanol	200 ml	50% v/v
Reagent Grade Acetic Acid	40 ml	10% v/v
Fixative Enhancer Concentrate	40 ml	10% v/v
Deionized Distilled Water	120 ml	30% v/v
Total	400 ml	100% v/v

After gel electrophoresis, place gels in the Fixative Enhancer Solution. With gentle agitation fix the gels for 20 minutes. Refer to Table A for fixing times and solution volumes for larger gels.

2. Rinse Step - 20 min

Decant the Fixative Enhancer Solution from the staining vessel. Rinse the gels in 400 ml deionized distilled water for 10 minutes with gentle agitation. After 10 minutes, decant water and replace with fresh rinse water. Rinse for an additional 10 minutes. Decant rinse water. For larger and thicker gels, rinse with 800 ml water for 40 minutes.

3. Staining and Developing Step - 20 min

Staining Solution Preparation and Procedure

(prepare within 5 minutes of use)

Place 35 ml deionized water into a large beaker or Erlenmeyer flask and stir with a Teflon coated stirring bar. Add the following to the beaker in this order

5.0 ml Silver Complex Solution
5.0 ml Reduction Moderator Solution
5.0 ml Image Development Reagent

Immediately before use quickly add 50 ml of the room temperature Development Accelerator Solution to the beaker. Swirl well. Add the contents of the beaker to the staining vessel. Stain with gentle agitation.

Stain both mini and large format gels for approximately 20 minutes or until desired staining intensity is reached. It may take at least 15 minutes before the bands first become visible. Note: Staining time is dependent on the sample and the quantity loaded. After the desired staining is reached place the gels in 5% acetic acid to stop the reaction.

4. Stop Step - 15 min

Prepare a 5% acetic acid solution to stop the staining reaction.

Place gels in stop solution for a minimum of 15 minutes. After stopping the reaction rinse the gels in high purity water for 5 minutes. The gels are then ready to be dried or photographed.

GREEN FLORESCENT PROTEIN (GFP) PURIFICATION

Stage	Total Volume	A ₃₉₅	A ₂₆₀	Total GFP yield (mg)	Total protein yield	% purity	Step % increase	Overall % yield
Initial Extract								
Ammonium Sulfide Precipitation 80% (ASP)								
Ammonium Sulfide Precipitation 85% (ASP)								
Dialysis								
Ion Exchange Chromatography (IEC)								
Hydrophobic Interaction Chromatography (HIC)								
Ion Metal Affinity Chromatography (IMAC)								
Enzyme-Linked Immunosorbent Assay (ELISA)								

Total GFP yield (mg) = (total volume) x (A₃₉₅) x (dilution factor)

Total Protein yield (mg) = (correction factor 1.23) x (total volume) x (A₂₆₀) x (dilution)

% purity = ((Total GFP in mg.) / (Total Protein yield in mg.)) x 100

Step % Increase = (Purity at previous step / Purity at this step) x 100

Overall % yield = (Yield of GFP at last purification step) / (yield of GFP at extract) x 100