Purification of Green Fluorescent Protein

Biomanufacturing Steps in Product Purification

- UPSTREAM PROCESSING

  Genetically engineered protein produced by an organism (ex. E.coli) are grown to yield the optimal amount of product

- DOWNSTREAM PROCESSING
  - Cells which do not export product need to be, isolated, homogenized, and crude fractions obtained by centrifugation
  - Chromatography of partially purified product is then carried out

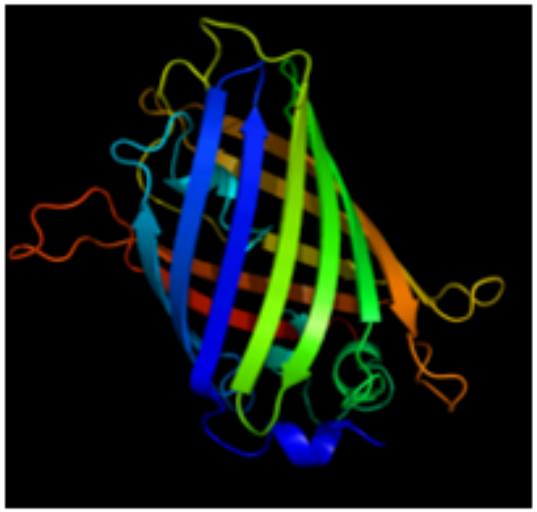
Green Fluorescent Protein

Why we use GFP production to exemplify Biomanufacturing Process

- Useful in following the purification process by using UV light detection of GFP

- In the presence of arabinose, GFP gene is transcribed into mRNA and translated into protein product

Green Fluorescent Protein Structure

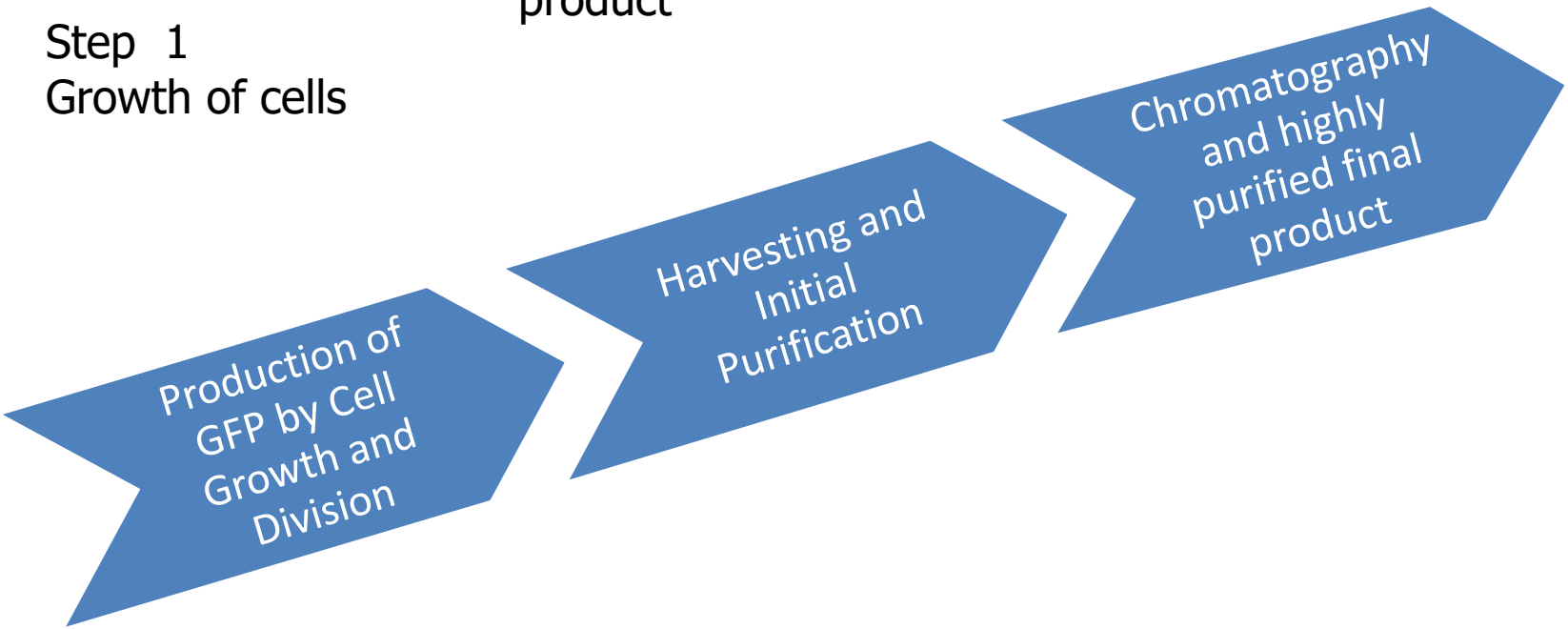


Flow Chart for GFP Product in Process

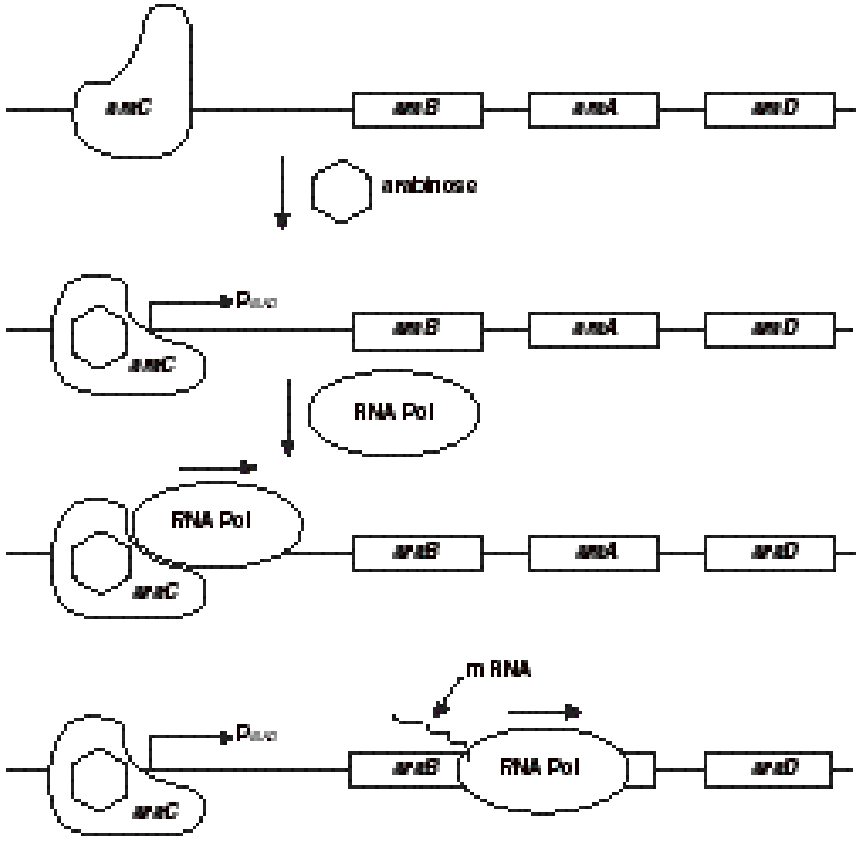
Step 1
Growth of cells

Step 2
Crude separation of product

3. Step
Purification by Chromatography



STEP 1 - Cell Growth of Transformed E.coli Results In GFP Product



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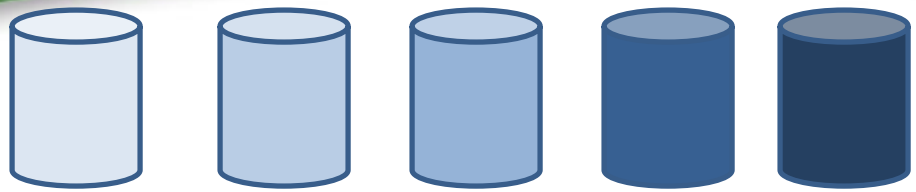
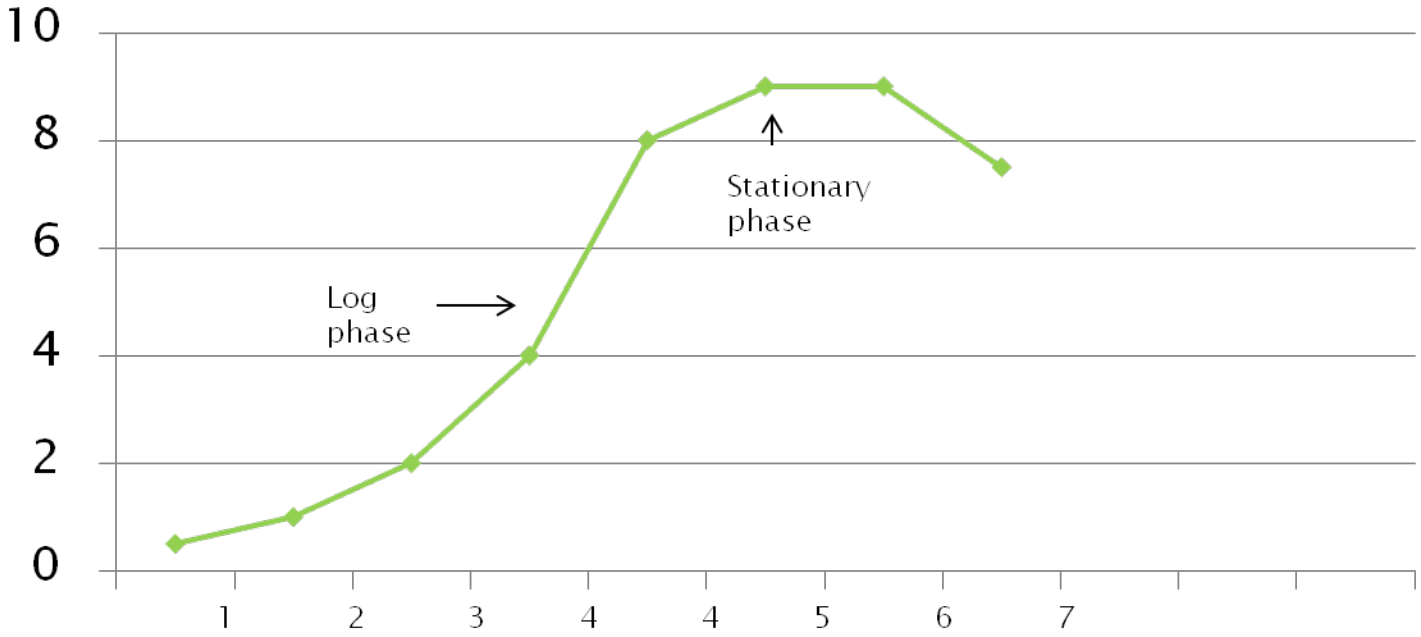
Growth of Transformed Cells in Culture



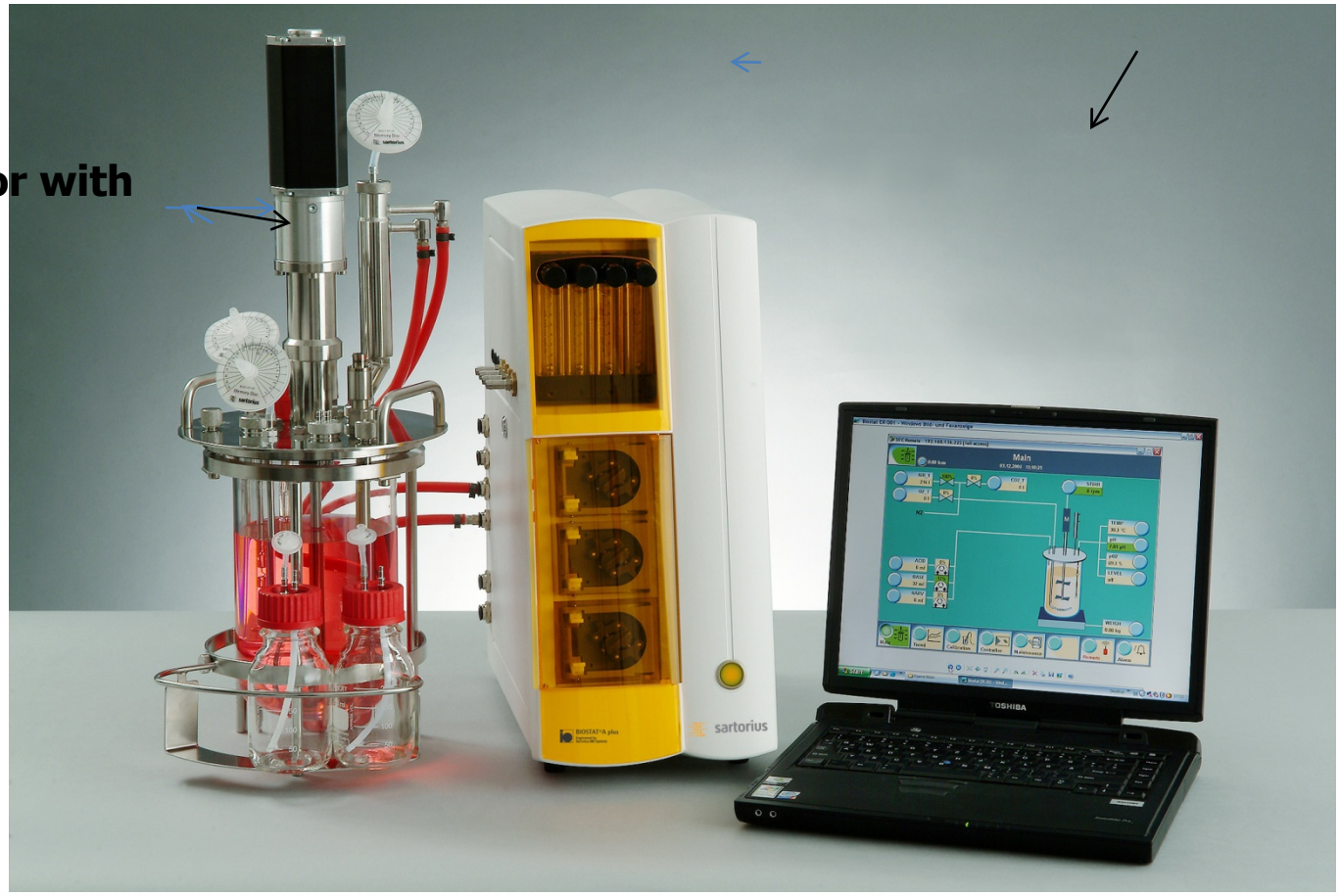


time of E. coli growth in culture (hours)

Controller supplying gas, acid or base etc

Computer controlling bioreactor

Bioreactor with cells



Step 2- : Harvesting and Initial Purification Steps

- **Location of Product**
- Is it inside the cell?
  - Can be separated from media by centrifugation and concentrated in the cell pellet E.coli product (GFP) is kept
  -
- Is it outside the cell?
  - Product is in the media and is diluted
  - It often needs to be concentrated

Mammalian cells transport product out of cell

Isolation of GFP from Transformed E. Coli

Step1 - If product is retained in cell:

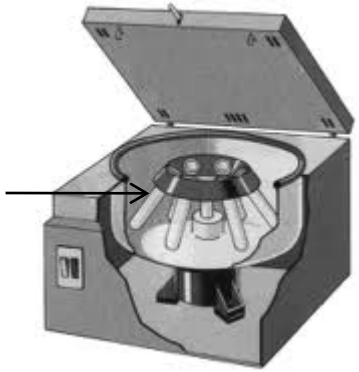
- Transfer all contents of media + cells into a centrifuge tube once cell growth is achieved
- Place this tube plus balancing tube into centrifuge and spin at selected speed
- Remove tube with cells and a pellet should present (since GFP glows, check tube with UV light to determine the location of GFP)

Centrifugation and Tangential Flow Filtration (TFF)

Separates by centripetal force acting on different sized particles

Centrifuge

rotor



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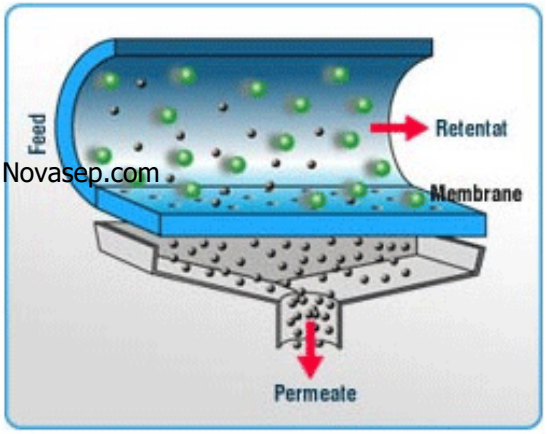
TFF process

Tangential Flow Filtration separates molecules by membrane pore size



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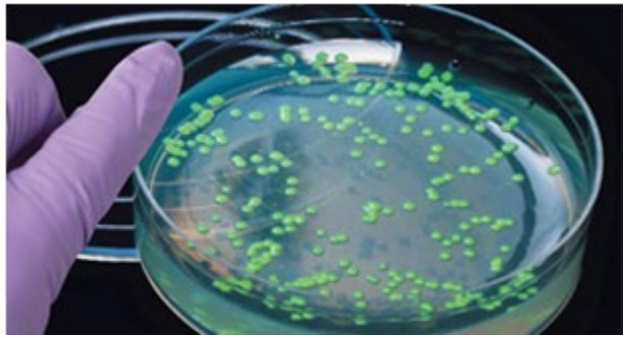
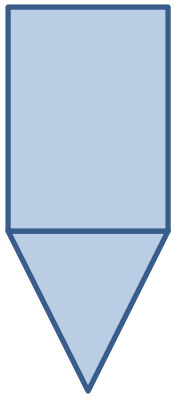
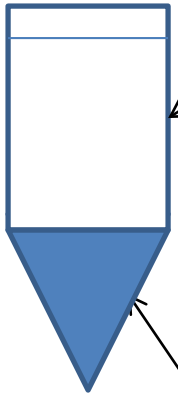
Pelleted Cells Will Glow under UV After Centrifugation



Colony is placed into media containing penicillin and arabinose and grown

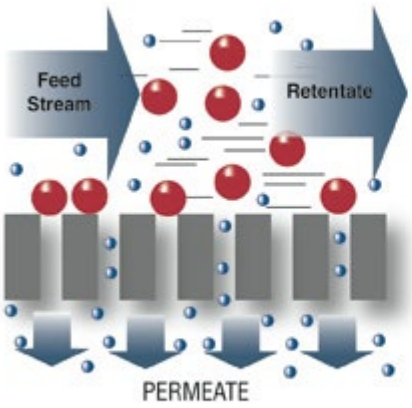


After spinning

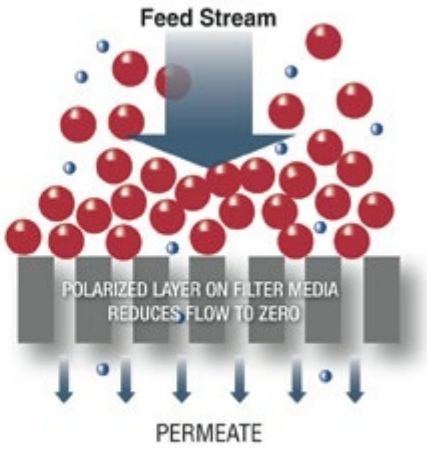


Tangential Flow Filtration Downstream Processing

This technique allows large volumes of media to be concentrated, removal or exchange of salts in solutions etc.



Tangential (Cross) Flow Filtration
(high permeate rate)



Dead-End Filtration
(low permeate rate)

Lysing Cells to Release Product

Cells must be broken open to release product from the intracellular space . This can be done by the following means:

1. **Freeze/thawing of cell pellet** will break open the cell wall. (requires freezing pellet). Addition of lysozyme facilitates process
2. **Homogenize pellet** by grinding with specially designed homogenizers (is frequently used and requires less time)
3. **Sonication** will break open membranes in an efficient way

Basics of Chromatography

- What is it?
  - Components are distributed between two phases
    - Stationary phase
    - Mobile phase which moves across the stationary phase
  - A Physical method that gently separate mixtures
- Different rates of moving over the stationary phase separates different molecules
  - It is a very gentle process: does not denature proteins
  - Large amounts of product can be reliably isolated
- Proteins that differ by one amino acid can be separated by this method

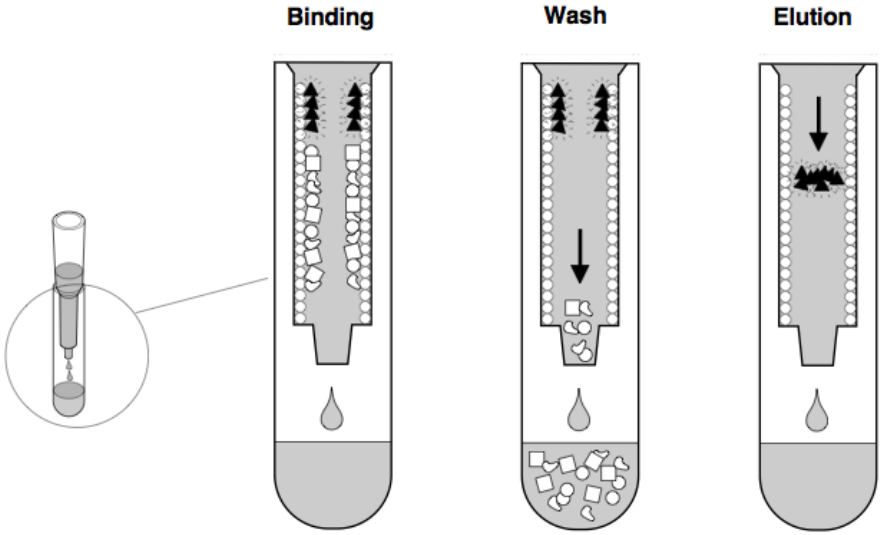
Types of Low Pressure Liquid Chromatography (LPC)

- Adsorption
- Ion Exchange
- Molecular Size Exclusion
- Affinity

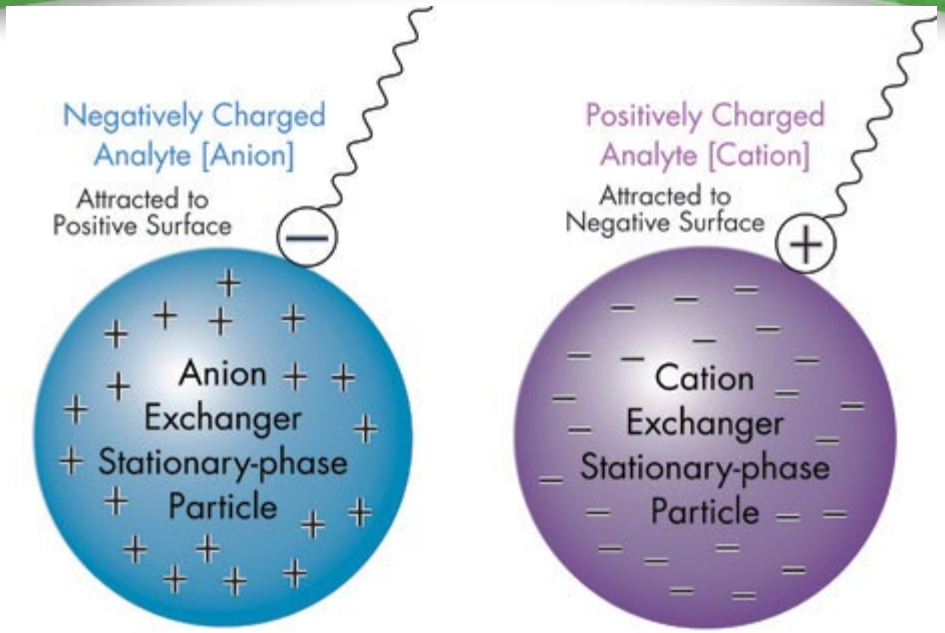
Adsorption Chromatography

Hydrophobic Interaction Chromatography

- uses a mobile liquid or gaseous phase that becomes adsorbed to the stationary phase
- differences in adsorption separate molecules

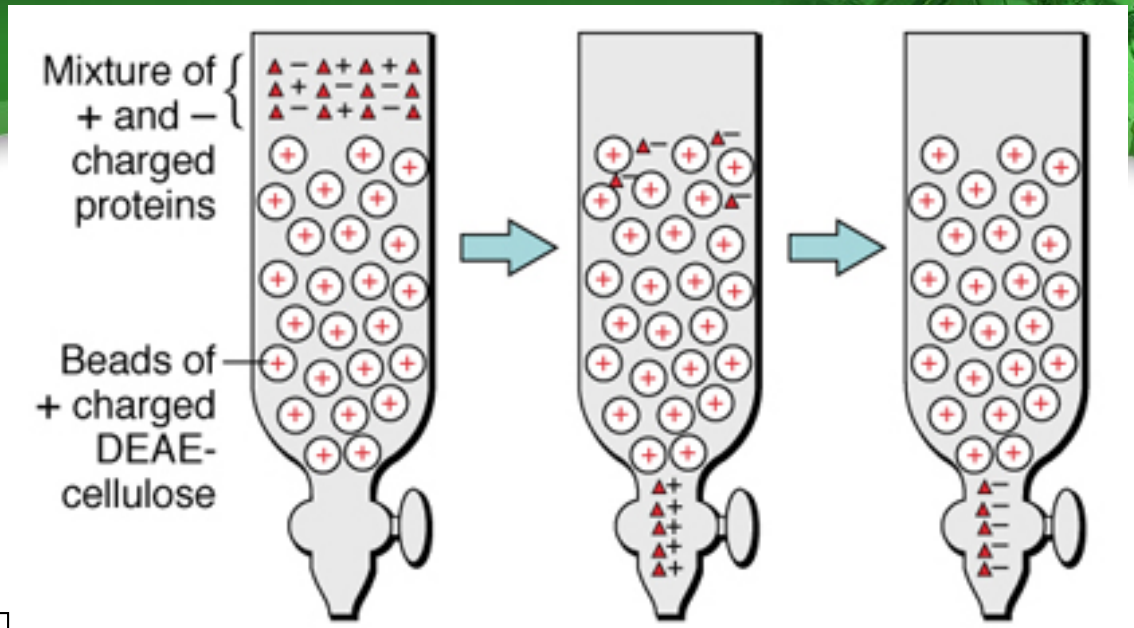


Ion Exchange Chromatography

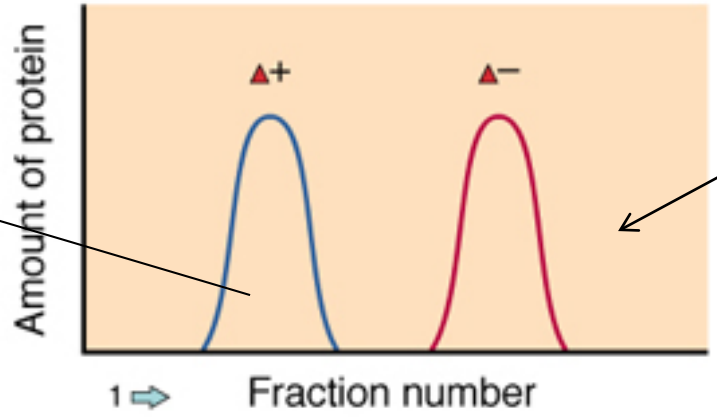


- Electrostatic forces attract oppositely charged ions between molecules in mobile and the stationary phase
- The strength of the attraction determines how long a molecule will stays on the resin resulting in separation

Anion Exchange Columns and How They Work cont'd

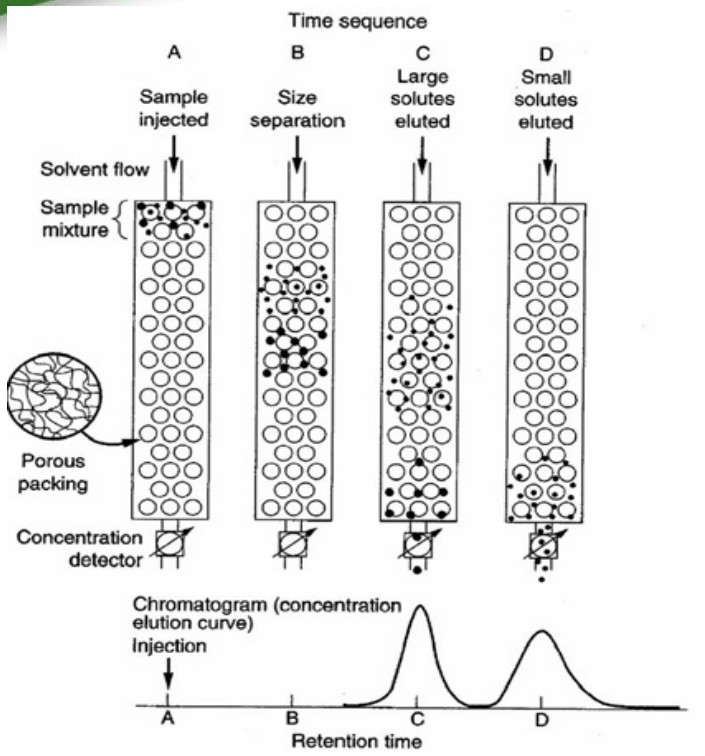


Positively charged Ions do not attach to resin & pass through



Negatively charged ions attach to the column

Molecular Size Exclusion Chromatography



Bead size allows only small molecules into the bead

Small molecules are last to emerge from the column

- Mixtures of differently sized molecules are added to the column
- Large molecules will be eluted first, smallest last
- Column resin must not be disturbed since all sized molecules will pass through the channels

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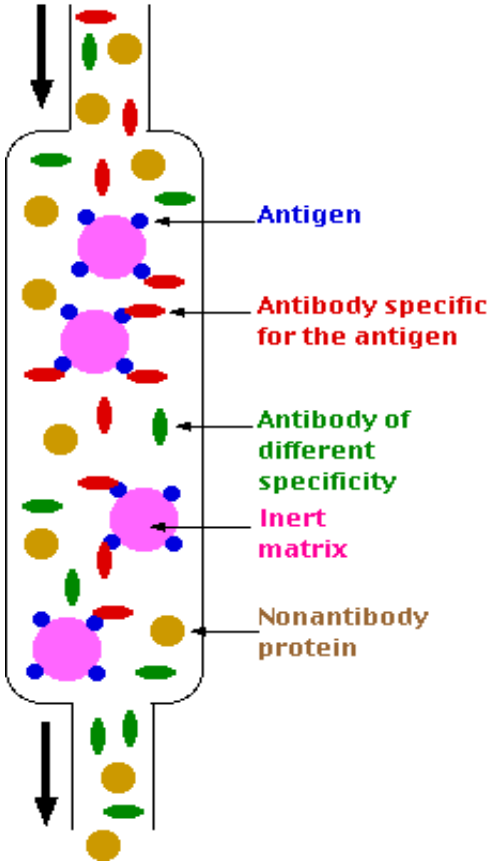
Affinity Chromatography

Affinity Column



How it is used

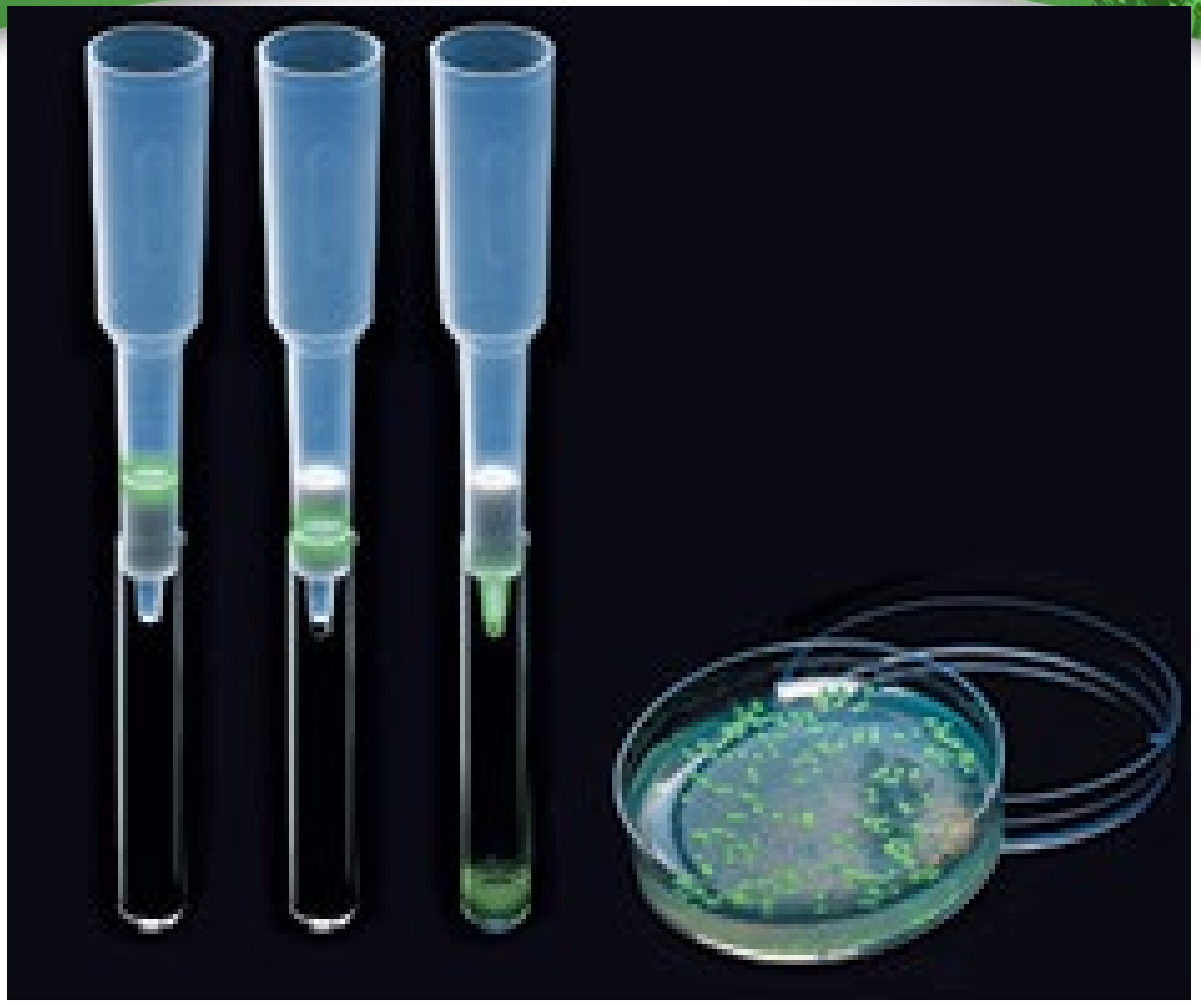
1. Stationary phase has an antigen covalently attached to the fixed resin

2. A mixture containing the antibody that can recognize the antigen will attach to it non-covalently

3. The substances in the mixture not wanted are washed out from column

4. The elution step releases the antibody from the antigen on the column by high salt or low pH resulting in a pure antibody being collected from column

Column Chromatography of GFP



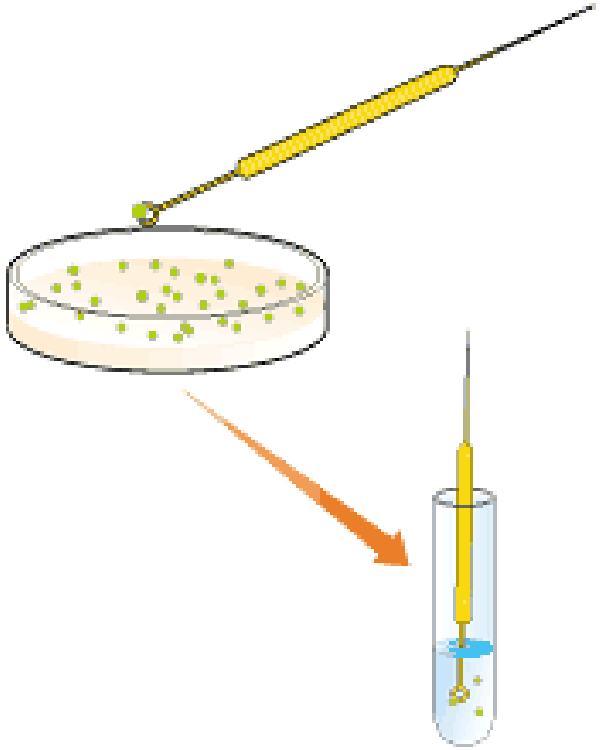
Standard Operating Procedures

- You will be purifying the original mixture from GFP transformed E.coli cells using column chromatography
- Harvest cells by centrifugation and add lysozyme. Place in freezer overnight
- Centrifuge the treated cells after defrosting them to collect the supernatant for columns
- SOP's for each step is found in your workshop book, starting on pages

Day 1-Growing Transformed Cells

Start with bacterial colonies transformed with pGLO plasmid DNA

Pick a single fluorescent green colony from the agar plate using a sterile inoculation loop

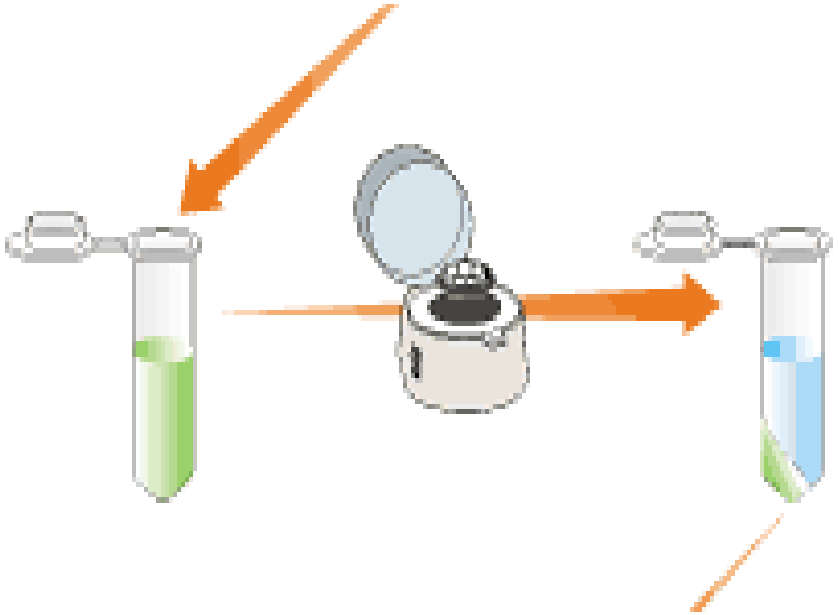


Inoculate in nutrient broth containing ampicillin and arabinose

Grow overnight at 32°C or 2 days at room temperature with shaking

Day 2

Transfer cell culture to microtube then centrifuge and pellet cells

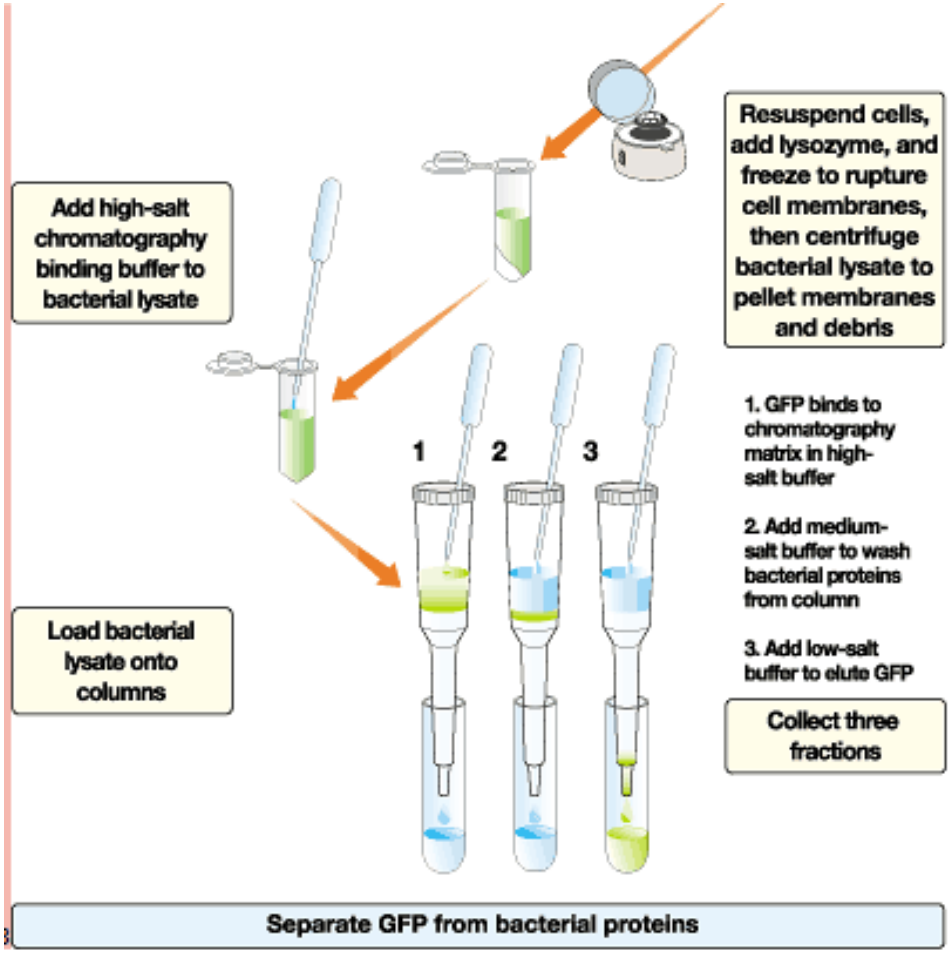


Cells must be lysed; freezing and thawing works well.

HIC Columns

- Some proteins are hydrophobic or water hating
- High salt concentration causes protein to fold exposes hydrophobic regions to outside and proteins stick to the column
- When low salt buffer is added, hydrophilic areas are exposed – protein is released

Day 3

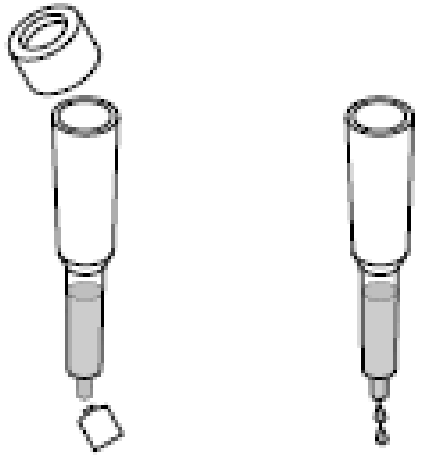

Resuspend cells, add lysozyme, and freeze to rupture cell membranes, then centrifuge bacterial lysate to pellet membranes and debris
Add high-salt chromatography binding buffer to bacterial lysate
1. GFP binds to chromatography matrix in high-salt buffer
2. Add medium-salt buffer to wash bacterial proteins from column
3. Add low-salt buffer to elute GFP
1
2
3
Load bacterial lysate onto columns
Collect three fractions
Separate GFP from bacterial proteins

Four Different Buffers for Use in Column

- Equilibration buffer- 2M ammonium sulfate
- Binding buffer- 4M ammonium sulfate
- Wash buffer- 1.3 M ammonium sulfate
- Elution buffer- low salt TE buffer

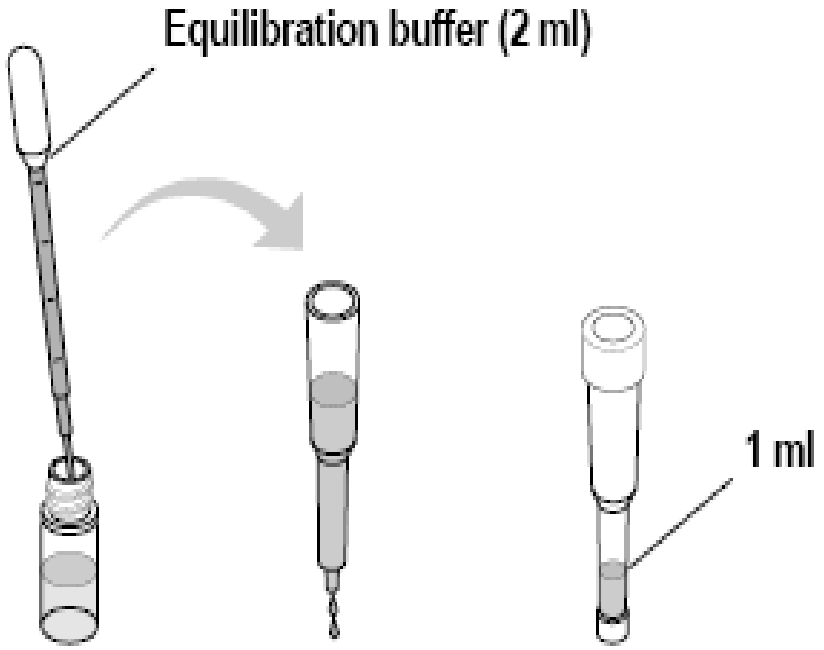
Preparation of HIC

While your tube is spinning, prepare the chromatography column. Remove the cap and snap off the bottom from the prefilled HIC column. Allow all of the liquid buffer to drain from the column (~3–5 minutes).



Equilibration Buffer

Prepare the column by adding 2 ml of Equilibration Buffer to the top of the column. This is done by adding two 1 ml aliquots with a rinsed pipette. Drain the buffer to the 1 ml mark on the column. Cap the top and bottom and store the column at room temperature until the next laboratory period.



Purification Step 1

- Label three collection tubes 1,2,3
- Place column into tube 1 and allow liquid to run through column
- Place 250 ul of supernatant onto the top of the column
- Let the liquid empty from column
- Move column to tube 2

Purification Step 2

- Add 250 ul of wash buffer to the column and allow all liquid to empty into tube 2
- This allows most of the cellular proteins to be rinsed through column
- GFP protein should still be attached to column

Purification Step 3

- Move column to test tube 3
- Finally, add 750 ul of TE buffer (low binding) to column and allow it to run completely through into test tube 3
- This buffer should elute the GFP protein

Examination of test tubes

- Examine all three test tubes by viewing under a UV light
- Which test tubes should contain the GFP?
- Compare your results with the expected ones on next slide
- Secure all your column fractions needed for SDS PAGE analysis