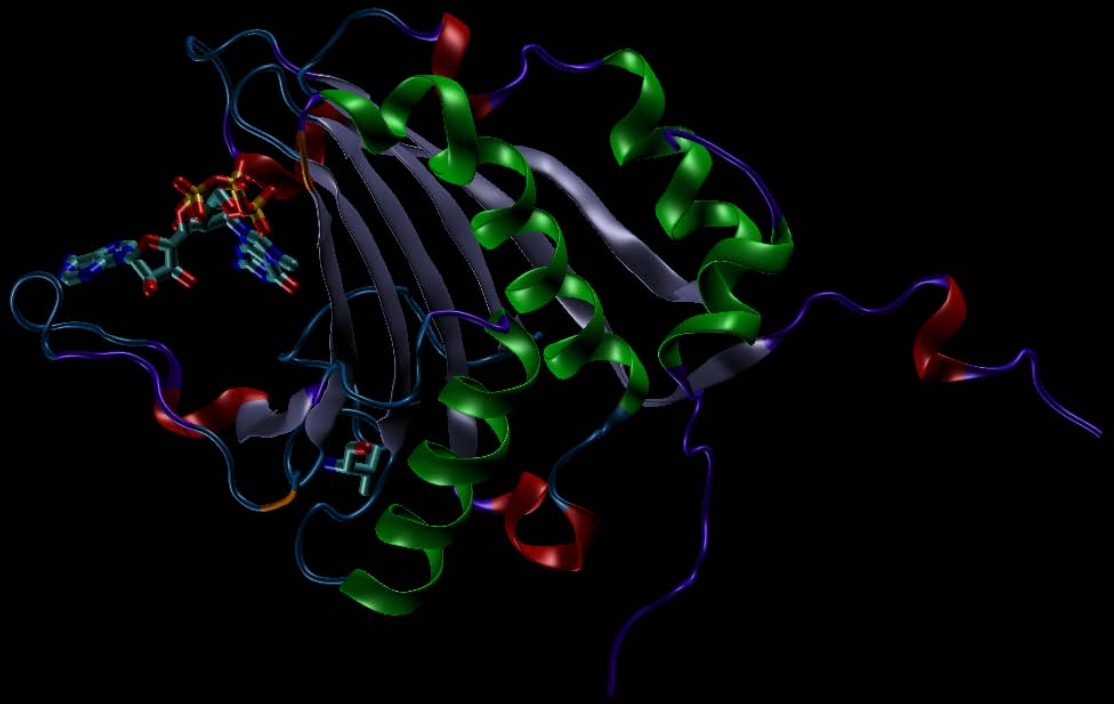
Experiment 3 :
Protein Purification & Translation



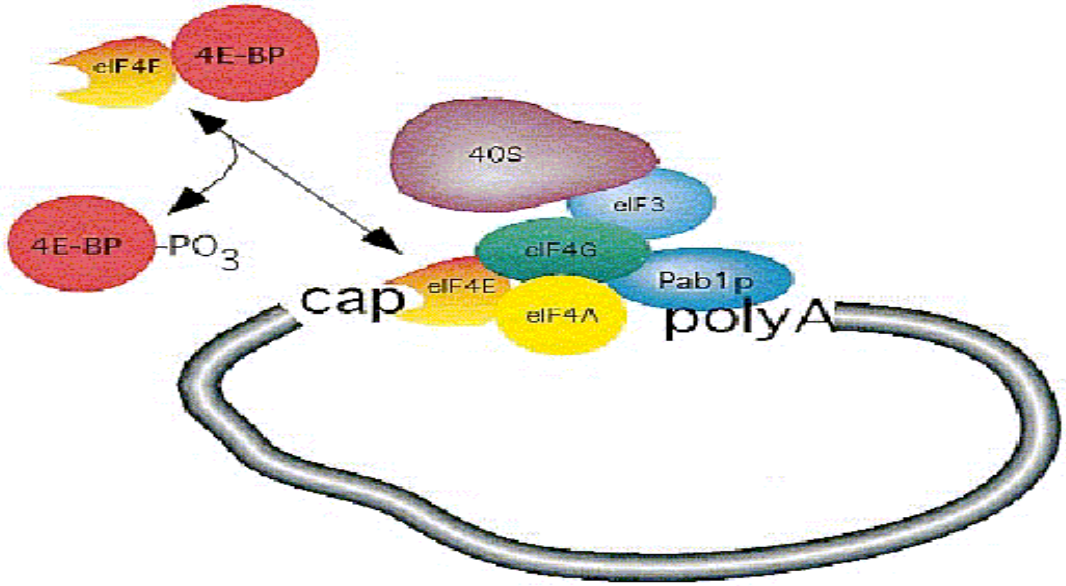
Jad Belle

BUGS

Translation

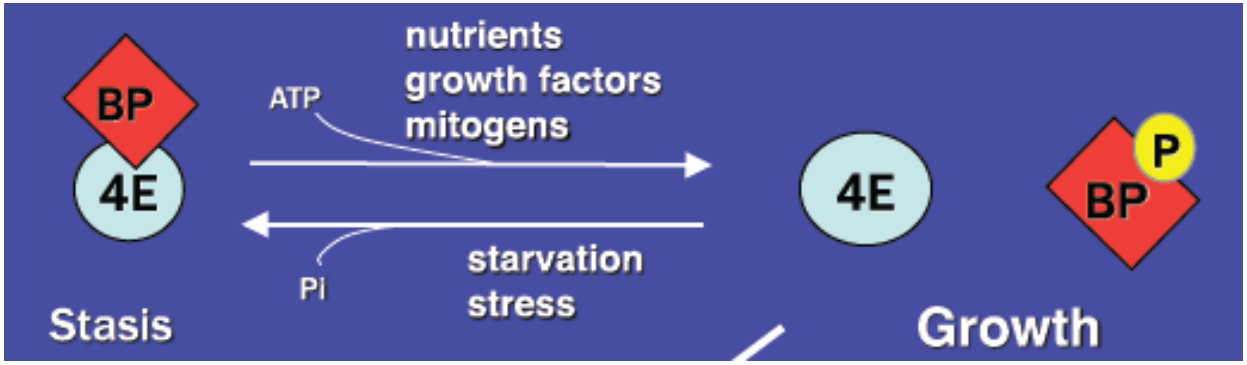
- mRNA → Proteins
- Process mediated by ribosome and other factors
- In most cases, methionine is the first amino acid incorporated in the nascent polypeptide
- Met encoded by AUG in eukaryotes and prokaryotes
  - Although GUG and AUU encode valine and isoleucine respectively, they can also encode methionine if preceded by the Shine-Dalgarno sequence in prokaryotes only.

Translation Initiation



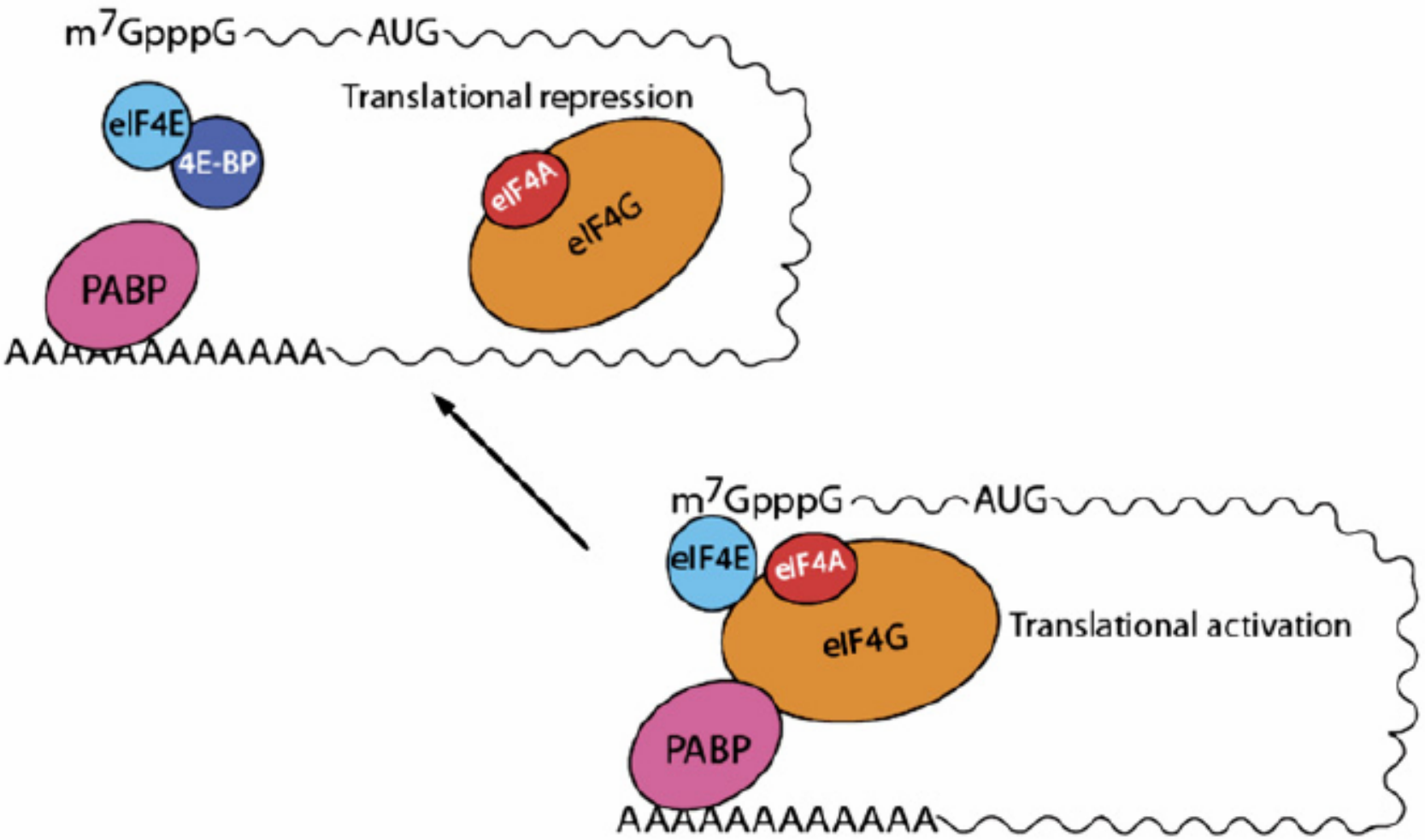
- Eukaryotic Translation Initiation Factors (eIFs) are proteins that bind the mRNA and participate in translation
- eIF4G is a scaffold – It connects the mRNA and associated proteins to the ribosome via eIF3 while also connecting with the polyA binding protein Pab1p to circularize the mRNA transcript.
  - Circularization facilitates multiple rounds of translation for the ribosome
- eIF4E, the cap binding protein, is also associated with eIF4G and is essential to translation initiation
- eIF4A is a helicase which allows the ribosome to resolve RNA secondary structures

Translation Initiation



- eIF4E-BP1 is a natural inhibitor of eIF4E that is upregulated when the cell lacks suitable conditions for growth.
- The inhibitor competes with eIF4G for eIF4E binding and thus prevents translation initiation by disrupting the complex.

- eIF4E is essential for cap-dependent translation. It directs the binding of the ribosome at the 5’ end so it can start scanning for a translation initiation site.


m7GpppG
AUG
Translational repression
eIF4E
4E-BP
eIF4A
eIF4G
PABP
AAAAAAAAAAA
m7GpppG
AUG
eIF4E
eIF4A
eIF4G
Translational activation
PABP
AAAAAAAAAAA

Protein Purification

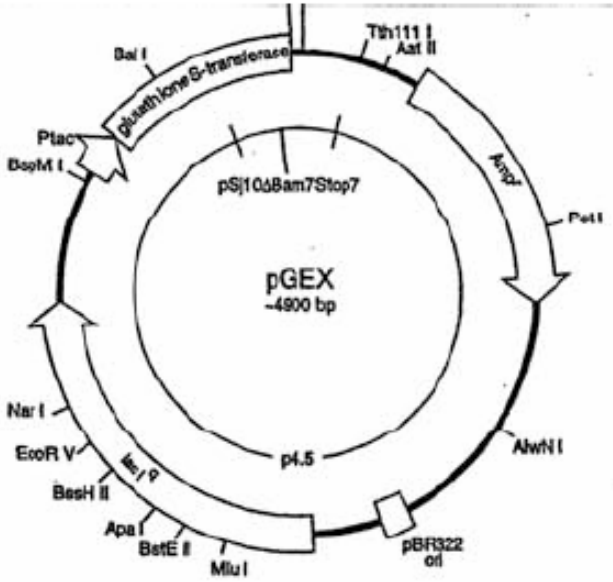
Stepwise procedure:

1. Transform BL21 with plasmid
2. Induce production of protein of interest with IPTG
3. GST purification
4. Measure protein concentration (Bradford)
5. SDS PAGE to evaluate purification and protein molecular weight

Protein Purification: Bacterial Expression

- BL21: Strain of E. Coli commonly used lacking proteases, serves as host for protein production.

- pGEX vector: Commonly used to express protein to be purified



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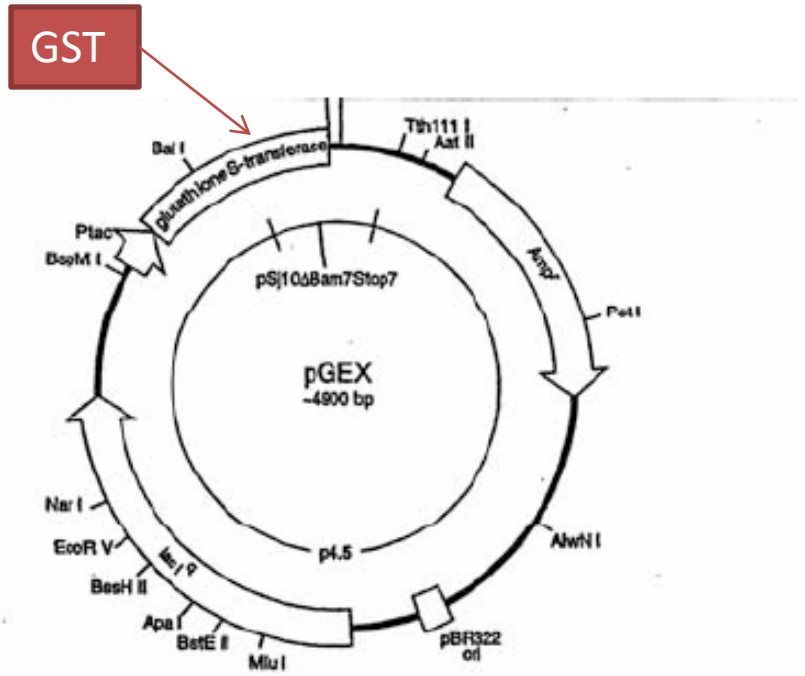

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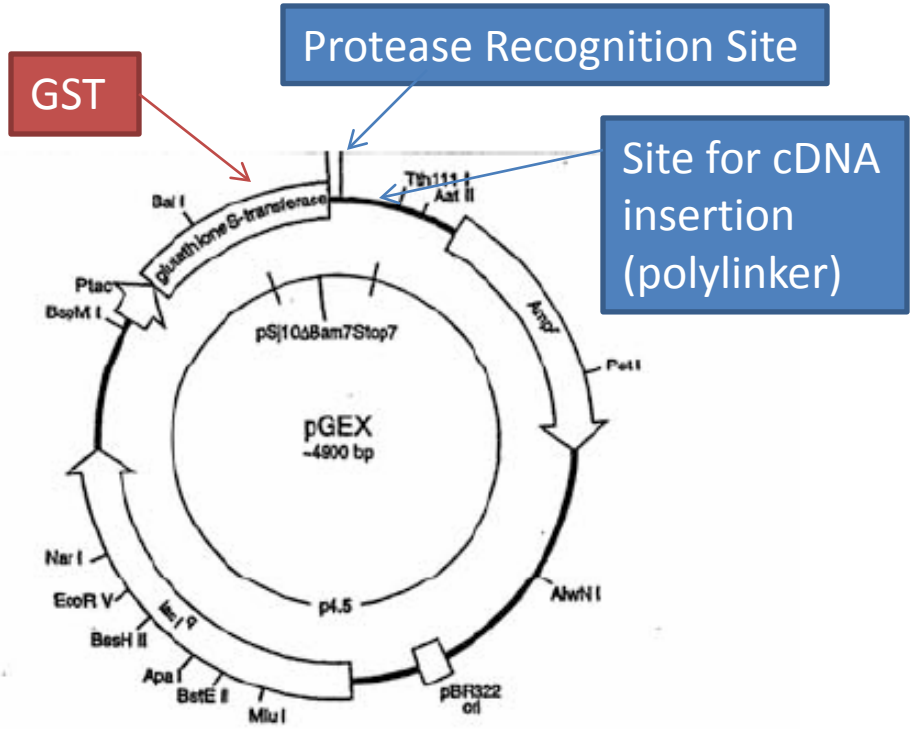

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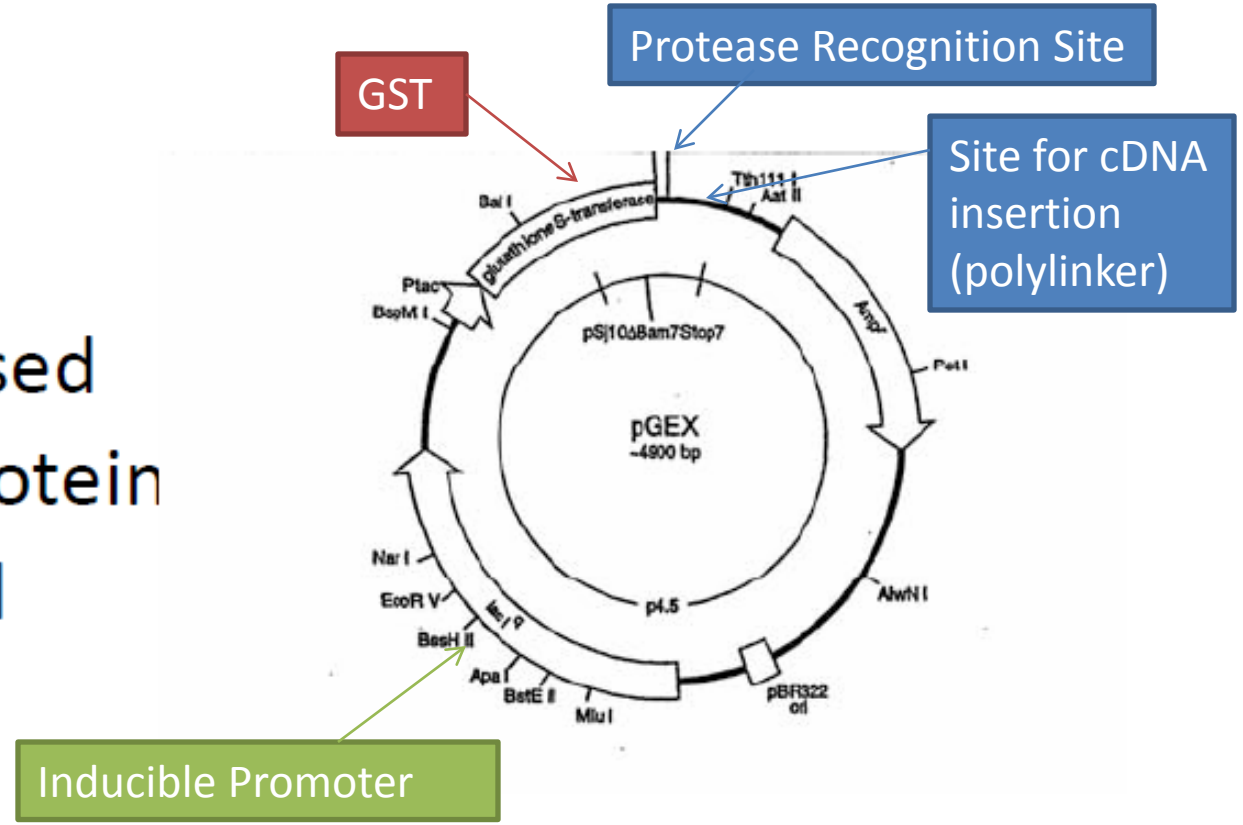

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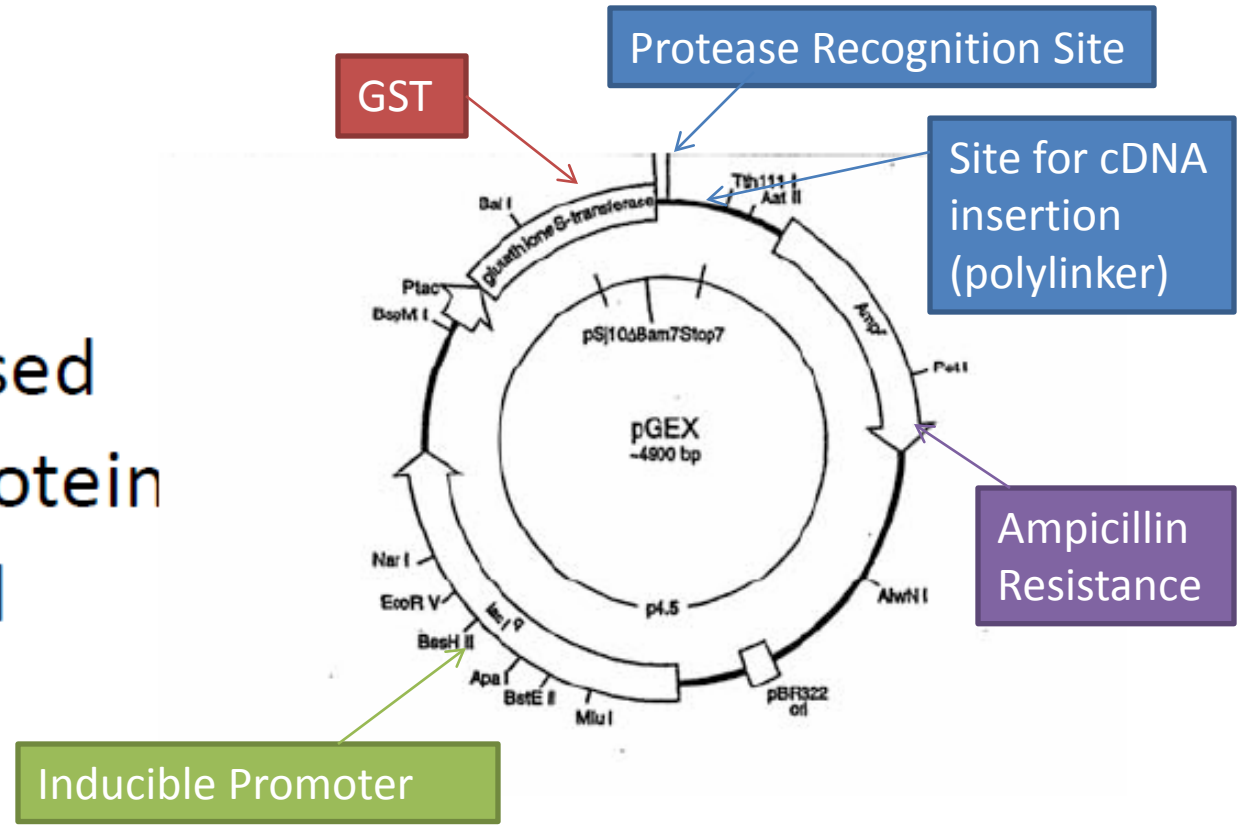

Protein Purification: Bacterial Expression

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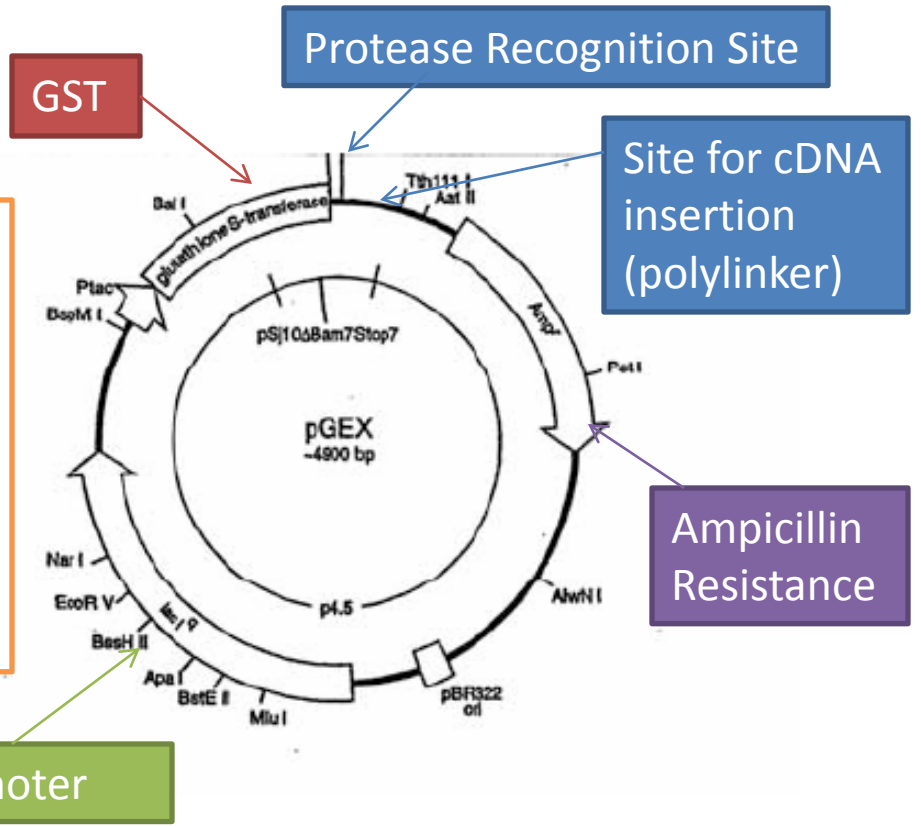
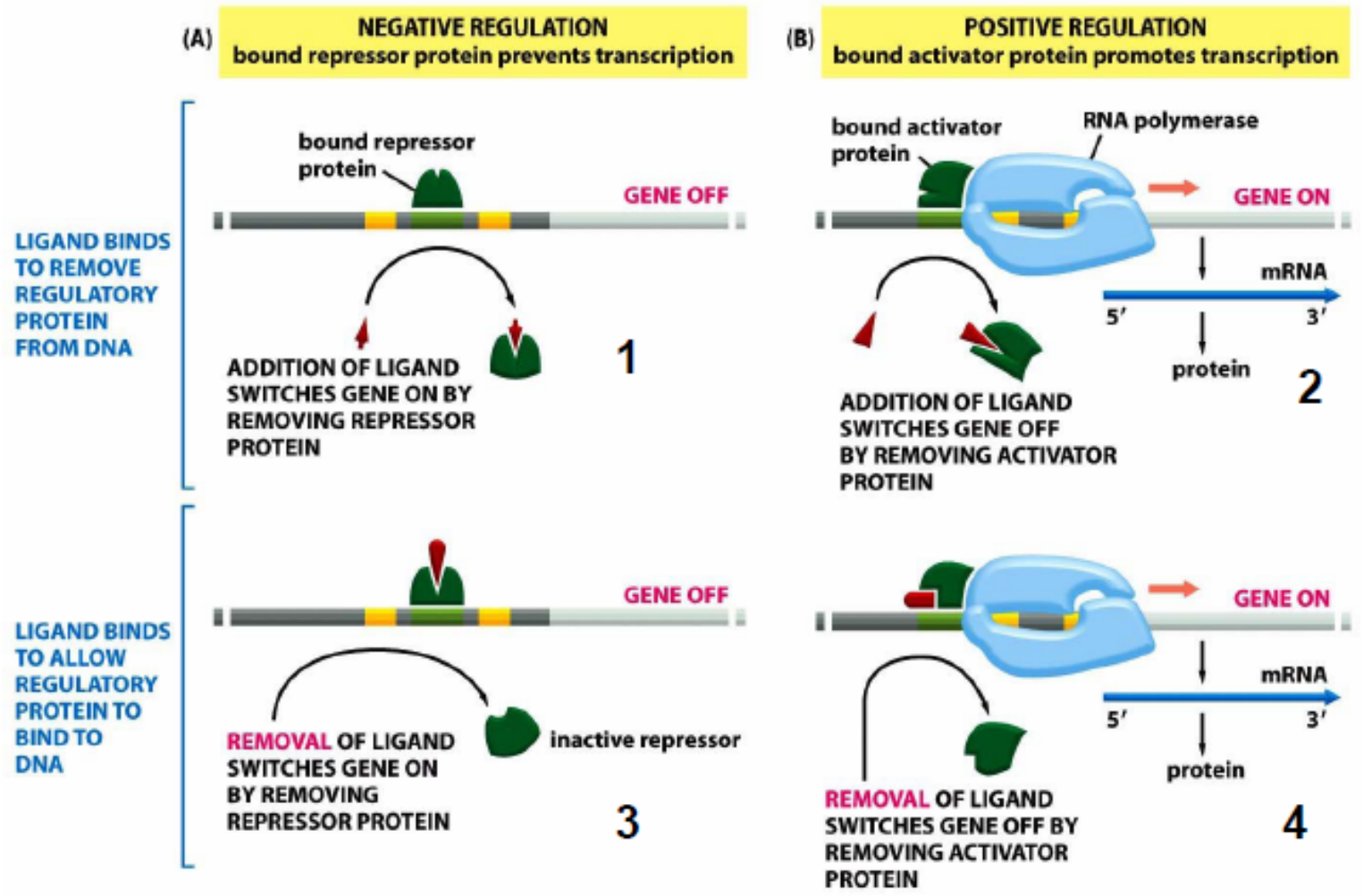
•2 Proteins are expressed in pGEX for this experiment:

- •GST
- •GST-4EBP fusion protein



Inducible Gene Promoters



Extraction from Cells

- After expression, need to extract total protein from E.coli cells.
- Methods:
  - Osmotic Shock
  - Ultrasonic Vibration (Sonication)
  - Force through a small orifice
  - Grinding in a blender

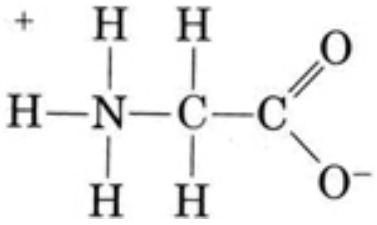
- Reagents
  - Lysozyme: Disrupts polysaccharide linkages in bacterial cell wall peptidoglycan (found in tears and saliva)
  - Sarkosyl: Detergent that disrupts cellular membranes
  - DTT: Disrupts disulfide linkages, prevents protein folding

Extraction from Cells

- Done by your TAs
  - Resuspended the cells in PBS (physiological buffer)
  - Added DTT to reduce non-native disulfide linkages
  - Added Protease inhibitors
  - Sonicated to disrupt bacterial cell wall
  - Triton detergent added to solubilize proteins
  - Centrifuged to bring down pellet of cellular debris
  - Supernatant containing protein given to students
    - GST sample (control)
    - GST-4EBP1 sample

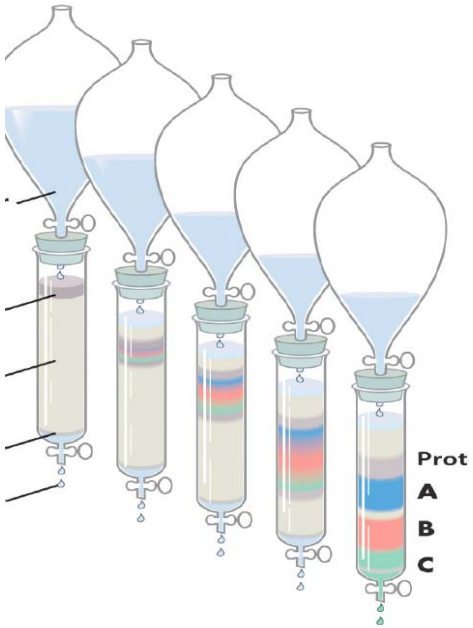
Protein Purification Techniques

- There are different ways to separate proteins in the lab by taking advantage of their physical and chemical properties governed by their amino acid sequence and arrangement.
  - Size
  - Shape
  - Isoelectric Point: The pH at which a protein has net neutral charge in which its ionizable group charges cancel out
  - Charge
  - Hydrophobicity
  - Density
  - Intermolecular interactions

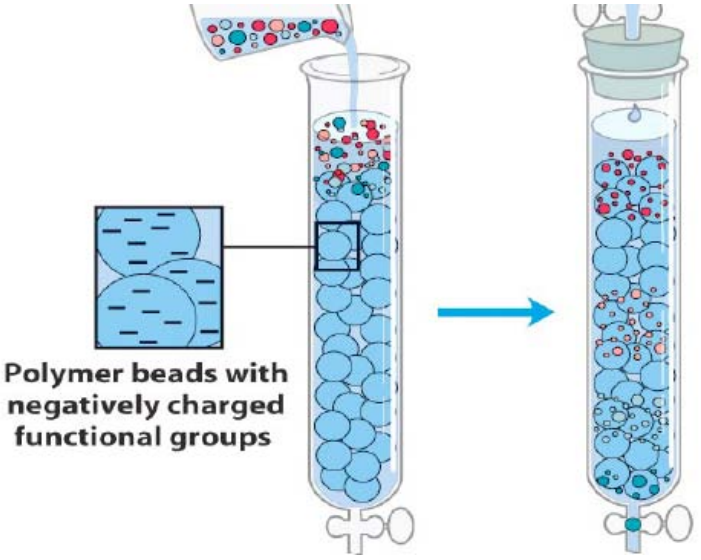


Chromatography

- Simple Column
  - Pour protein sample onto solid porous matrix, those proteins which interact most with the matrix are eluted last.

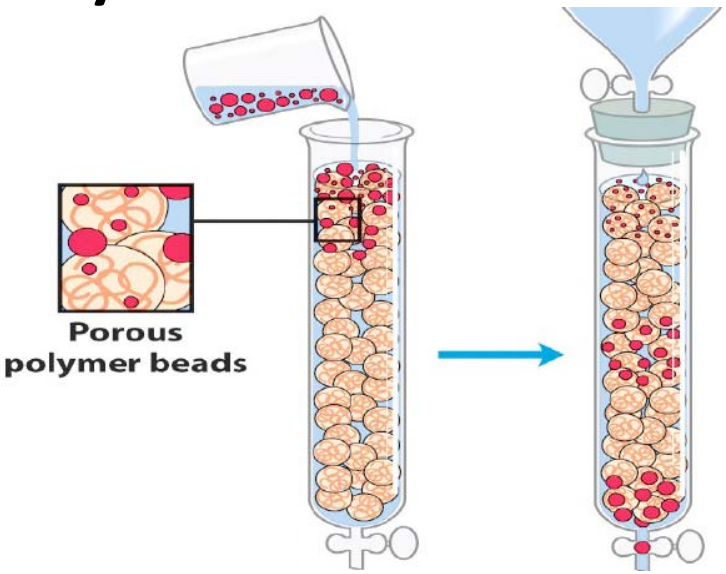


- Ion Exchange
  - Column filled with charged polymer beads, proteins movement through the column determined by their net charge at the pH used. For example, if column is negative, negative proteins will elute quickly but positive proteins will be hindered.

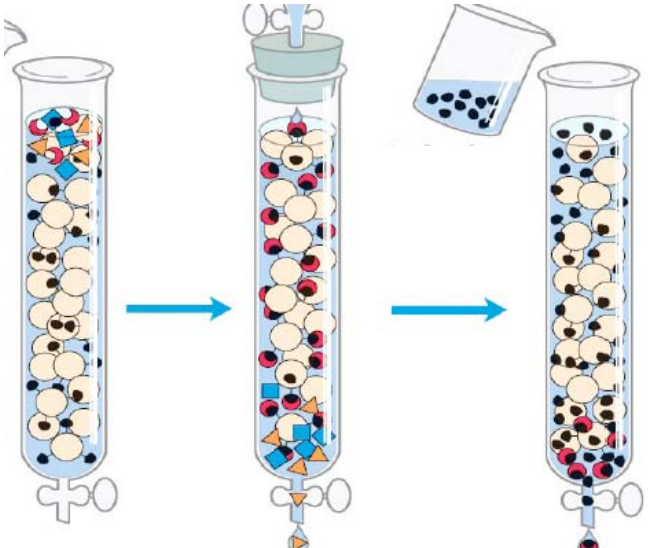


Chromatography

- Size Exclusion
  - Column filled with porous polymer beads, protein movement depends on size; larger molecules pass quickly while smaller proteins move through the beads and take longer to pass through.



- Affinity
  - Column is filled with beads attached to ligand or protein that interacts with your protein of interest. Protein will be trapped in the column while contaminants elute. To extract protein of interest wash the column with excess ligand to displace from the beads.

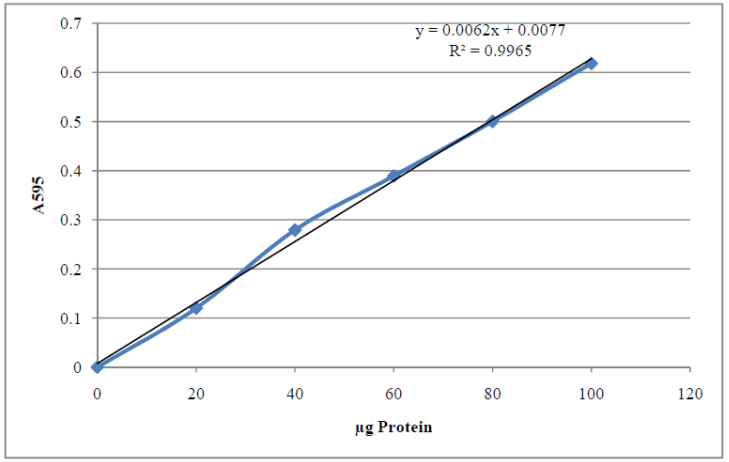


GST Affinity Purification

1. Wash GST-sepharose beads to remove their storage solution
2. Add bacterial protein extract to beads (total protein), incubate to allow binding. Beads will bind GST.
3. Discard supernatant containing bacterial proteins and nucleic acids
4. Add PBS to wash away impurities
5. Add excess reduced glutathione to compete with glutathione-sepharose and elute protein from beads.
6. Dialysis to exchange the buffer. Protein is too large to pass through tubing so only buffer is exchanged by free diffusion until equilibrium of concentrations.
7. Flash Freeze for storage

Assessing Protein Concentraion: Bradofrd Assay

1. Prepare standards, add Bradford reagent, incubate 5minutes, read A595 using the spectrophotometer
2. Prepare sample dilutions to attain absorbance values within the range of the standards
3. Prepare a standard curve and use it to calculate sample protein concentrations as a function of their A595 values



Assessing Protein Concentraion: Bradofrd Assay

- Bradford reagent complexes with protein shifting the maximal absorbance of the dye from 495 to 595. Therefore, more protein results in a higher absorbance value at 595nm.
- Ideally, the sample dilutions would place their absorbance values between those of the highest and lowest standards to reduce error
- Remember dilution factors

Assessing Protein Purity: SDS PAGE

- Sodium Dodecyl Sulfate (SDS) is a detergent that linerazies proteins and gives them a strong negative charge
- Experiment used Laemelli loading buffer which contains β-Mercaptoethanol to reduce disulfide bonds and prevent non-native protein folding
- The uniformly charged proteins migrate down the polyacrylamide gel driven by an electric potential, moving towards the positive terminal
- Separation will be based on protein molecular weight: smaller, lighter ones will migrate down faster.

Assessing Protein Purity: SDS PAGE

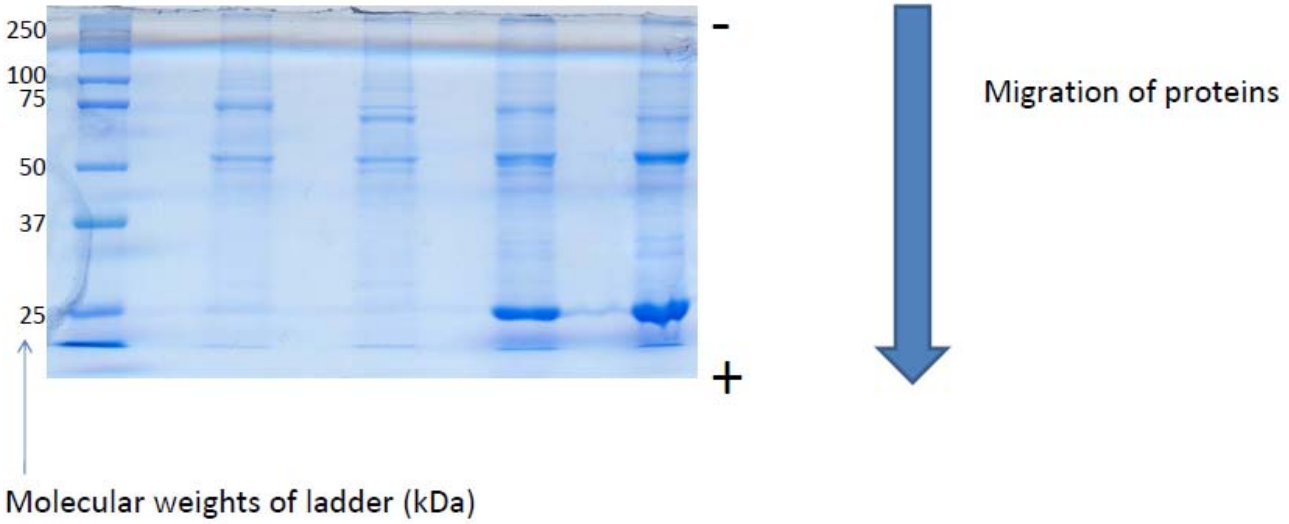
- Acrylamide polymerizes to form the matrix, a porous meshwork that hinders protein movement
- Make a resolving gel through which the proteins are separated and a stacking gel with lower percentage for loading wells.
- Ammonium persulfate (APS) triggers polymerization
- TEMED catalyzes the reaction

- The % of a gel is the % of acrylamide content. Higher percentage means a tighter meshwork and slower migration, used for better resolution of smaller proteins.

- Coomasie blue added to samples stains proteins and then non-bound dye is removed by de-staining with a solution of methanol and acetic acid.

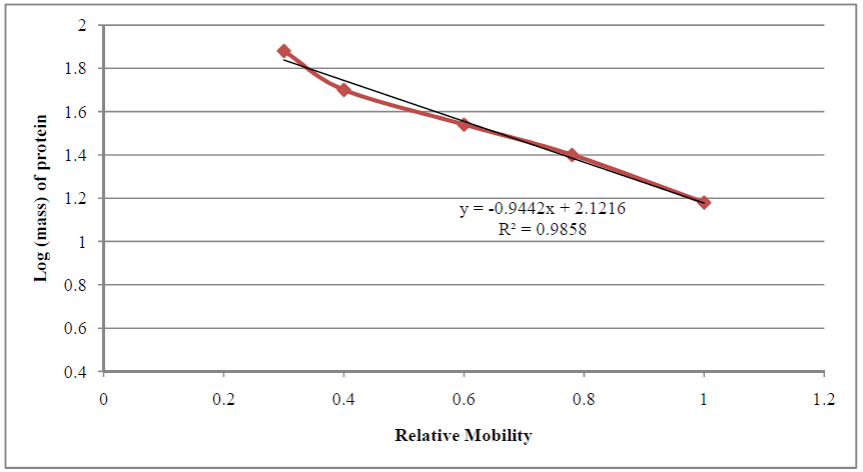
Assessing Protein Purity: SDS PAGE

- Compare the migration of your protein sample bands with those of the marker ladder to find their molecular weight.
- Should see many bands in the lysate lane due to contaminants
- In your GST and GST-4EBP lanes you will only see those proteins if purification was successful
- GST = 26kDa
- 4EBP1 = 40kDa



Assessing Protein Purity: SDS PAGE

- Calculate the relative mobility of all standards as a fraction of their migration over the migraiton of the furthest standard. Plot log(molecular weight) vs. relative mobility to get a standard curve
- From the standard curve you can find the molecular weight of unkown samples based on their relative mobility



Protein Identification : Alternative Methods

- Western blotting
  - Resolve protein samples by SDS PAGE, transfer to nitrocellulose membrane and then use antibodies to visualize protein of interest
- Isoelectric focusing
  - Resolve proteins in a pH gradient made of ampholytes. Proteins will migrate until they reach the region at which the pH = pI (isoelectric point)
- 2D Gel electrophoresis
  - Separation by isoelectric focusing followed by size separation on another axis

In Vitro Translation

- Now that the eIF-4E protein is ready, need an assay to test the effect of inhibitors on cap-dependent translation
- Rabbit reticulocyte lysate system is used. It consists of immature erythrocytes (RBCs).
  - The system has no nucleus so it cannot produce RNA
  - It is also highly translationally competent, the machinery is engaged in hemoglobin formation at this early developmental stage.
  - The reticulocytes contain all components needed for translation

Rabbit Reticulocyte Lysate Important Points

- Preparation
  - Micrococcal Nuclease
    - Degrades any pre-existent RNA
  - EGTA
    - Chelates Ca2+ ions to inactivate the nucleases (cofactors)
- Additives for Optimization
  - Creatine Phosphate + Creatine Phosphokinase
    - Energy generating system
  - Calf Liver tRNA
    - To expand the range of mRNA that can be translated
  - Hemin
    - To prevent inhibition of initiation

In Vitro Translation

- How can we quantify translation?
- Using the luciferase enzyme which oxidizes its substrate luciferin using ATP in a light emitting reaction.
  - Luciferase acts as a monomer. This means that its activity in oxidizing luciferin will directly correlate with translation levels.
- So you incubate the RRL with luciferase mRNA, luciferin, and whatever you want to test it with
  - GST
  - GST-4EBP1
  - Cycloheximide
    - Translation Inhibitor (Positive Control)
  - Buffer A
    - Negative Control
- The amount of luciferase expressed will correlate with luminosity measured in a luminometer and this will in turn allow the study of translation efficiency or block.

Things to keep in mind

- Know the function of reagents
- Be aware of alternative experimental methods
- Know general experimental procedure and the conditions used, don't focus on details like incubation times and amounts of reagents used.
- Know how to calculate unknowns from standard curves and how to determine protein masses form SDS PAGE
- Understand positive and negative controls in each experiment and why they're used

- Note: kept samples on ice throughout experiment to prevent protein denaturation and degradation (need functional 4EBP)