



BIO-RAD

Professional Development

Protein Electrophoresis

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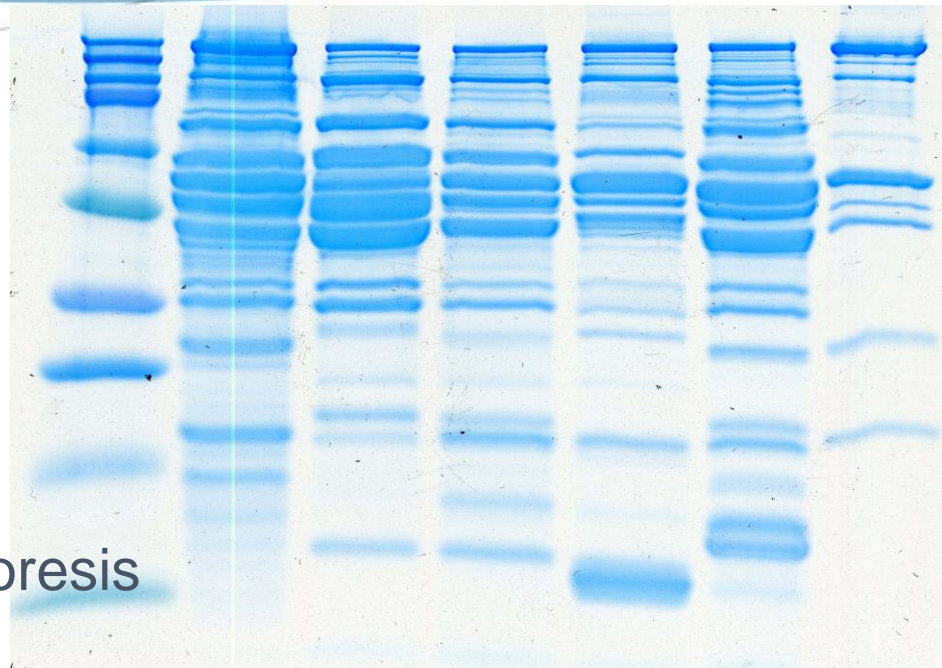
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Curriculum and Training Specialist
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Protein Electrophoresis

Workshop Outline

- 💧 Protein Introduction
- 💧 Protein Structure
- 💧 Set Up Electrophoresis
- 💧 Considerations for Electrophoresis
- 💧 Considerations for Classroom Teaching



What's the point? Proteins!



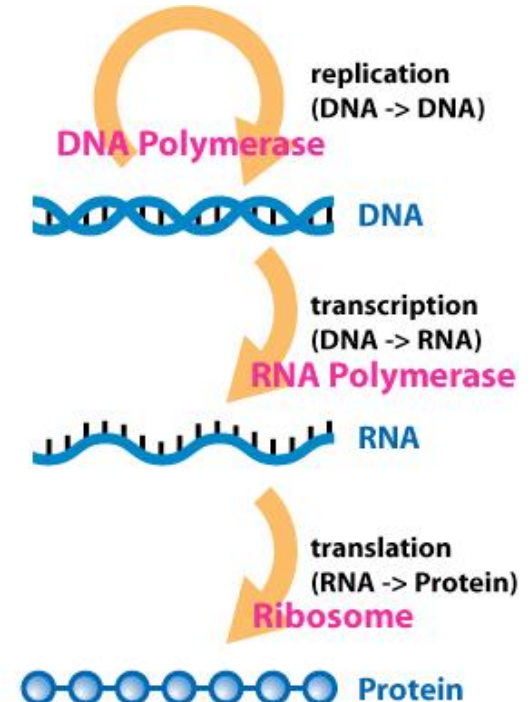
- Proteins are the work horses of the cell
- Proteins give us the structures that we see all around us



Where Do Proteins Come From?

DNA \Rightarrow RNA \Rightarrow PROTEIN \Rightarrow TRAIT

- ◆ Central dogma of molecular biology
- ◆ DNA codes for proteins
- ◆ Amino acid sequence determines protein structure which determines function



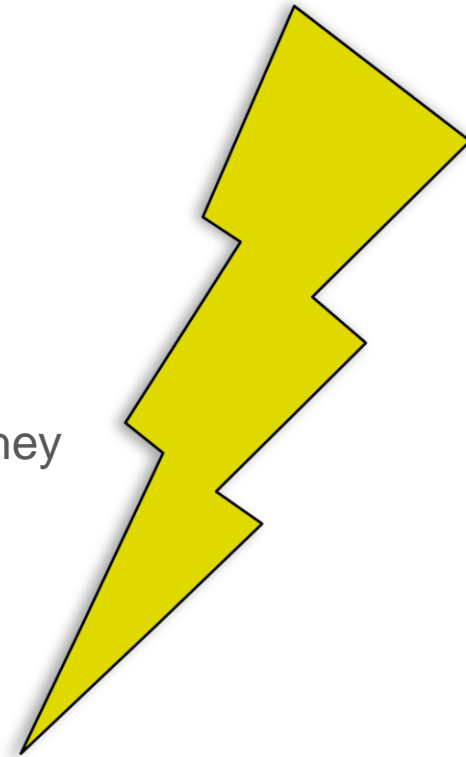
How Can We Study Proteins?

- 💧 Chromatography
- 💧 Electrophoresis

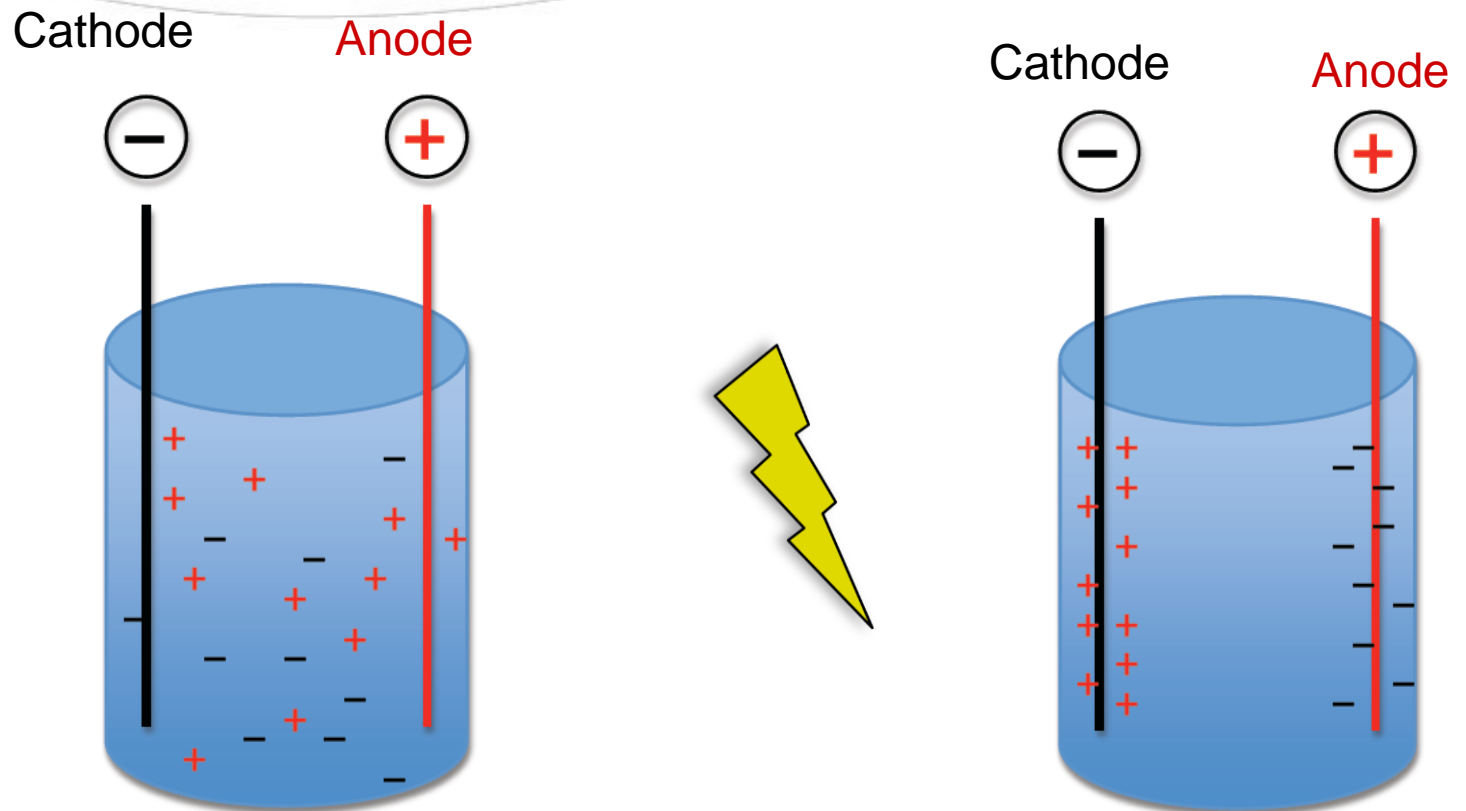
Electrophoresis

- Word origin:

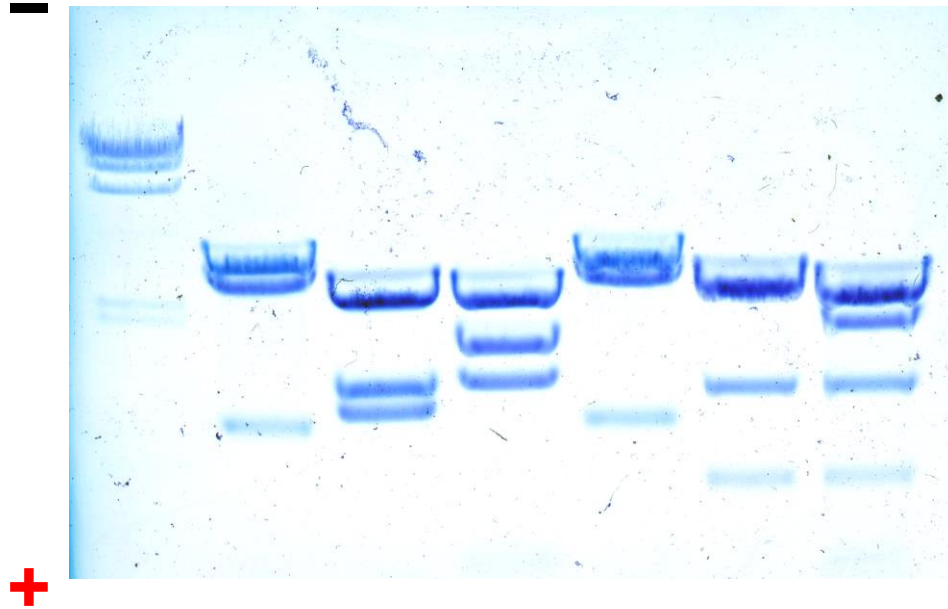
- Electro- electricity
- phoresis- carried by
- So, literally, ***carried by electricity***
- Charged molecules will migrate in an electric field and they migrate toward the electrode of opposite charge



Electrophoresis



Electrophoresis of DNA



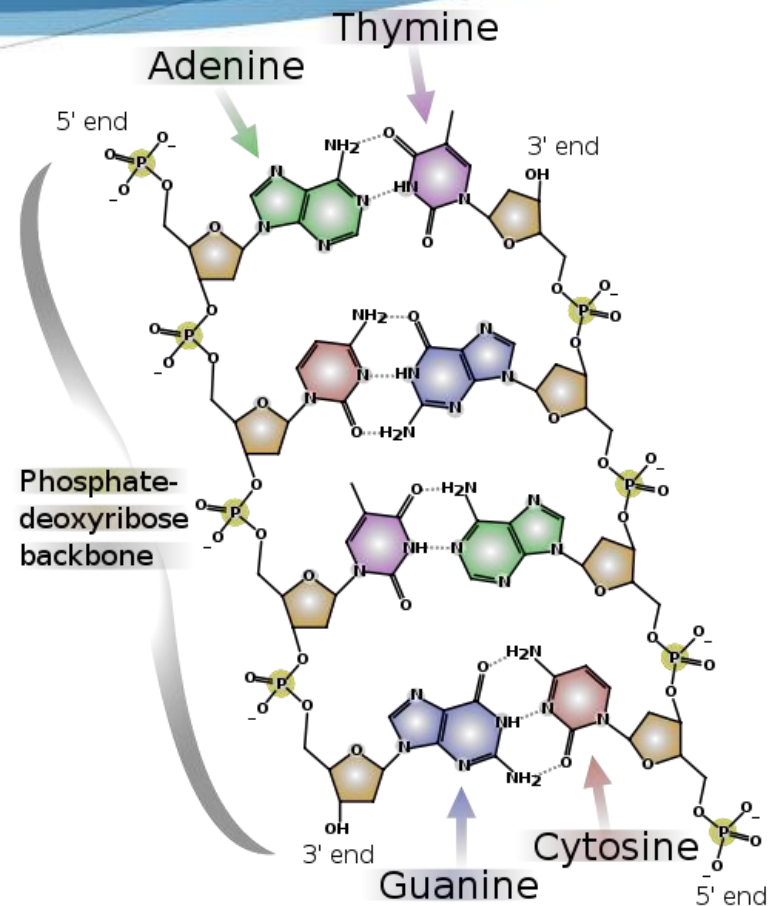
larger DNA molecules



smaller DNA molecules

DNA Molecules

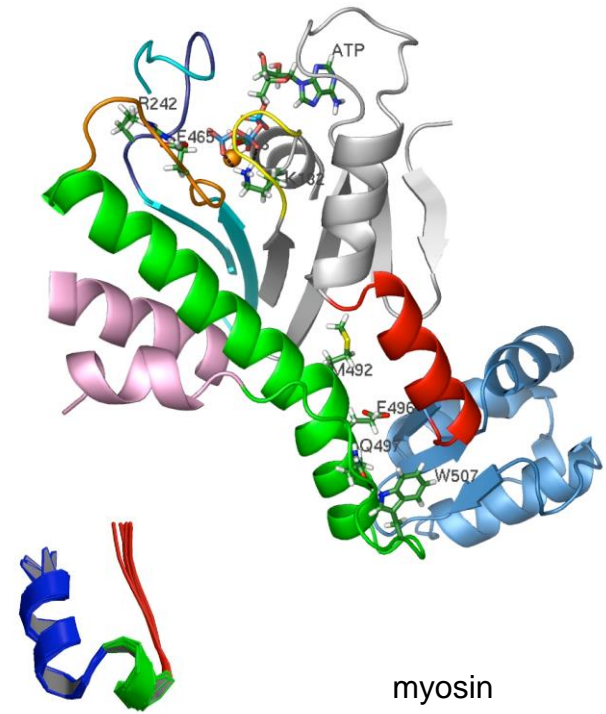
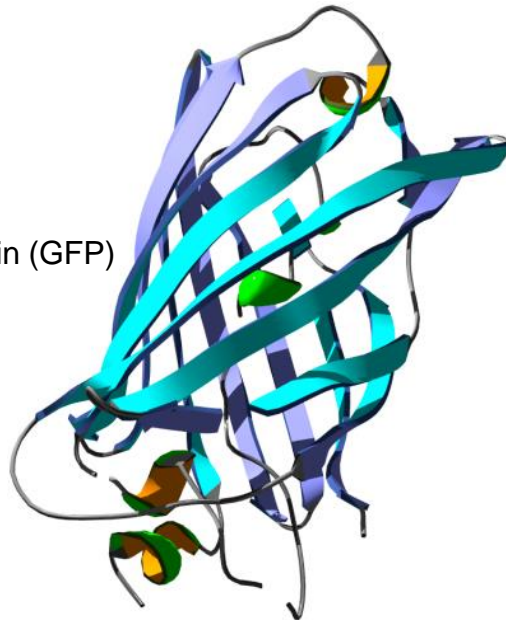
- ◆ Negative charge
- ◆ 4 bases forming a polymer
- ◆ Base pairs linked by hydrogen bonds
- ◆ Average base pair = 660 daltons (larger than proteins)
- ◆ Linear



Protein Molecules

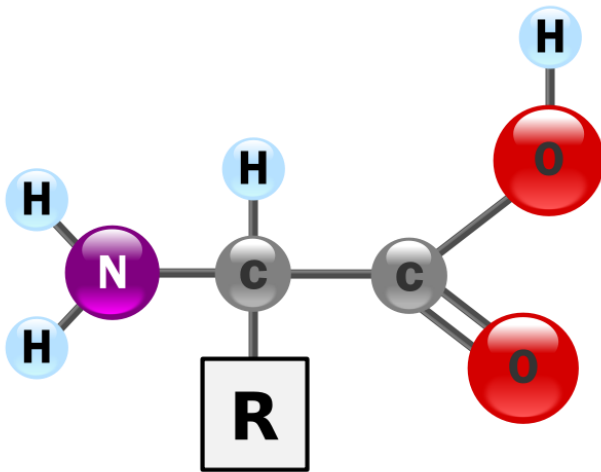
- Charge varies
- 20 amino acids form polypeptide polymers
- 3-4D structure

green fluorescent protein (GFP)



TRP-cage

Amino Acid Composition



Nonpolar, hydrophobic

alanine
isoleucine
leucine
methionine
phenylalanine
proline
tryptophan
valine

contain aromatic rings

contain sulfur

Polar, hydrophilic

asparagine
cysteine
glutamine
glycine
serine
threonine
tyrosine

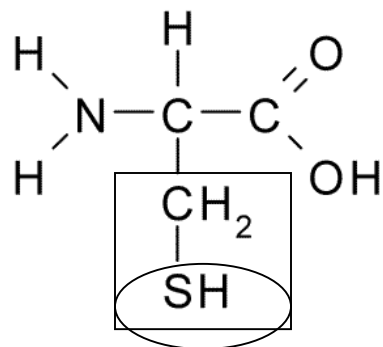
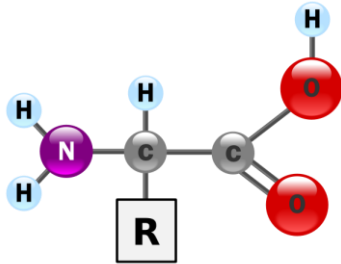
Acidic

aspartic acid
glutamic acid

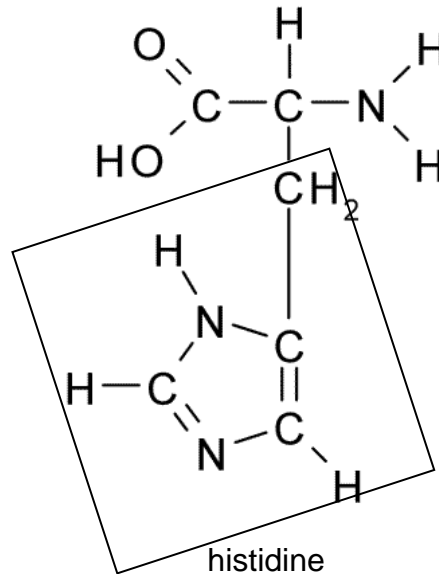
Basic

arginine
histidine
lysine

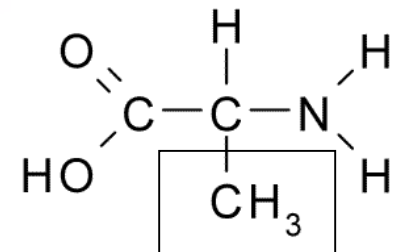
Amino Acids



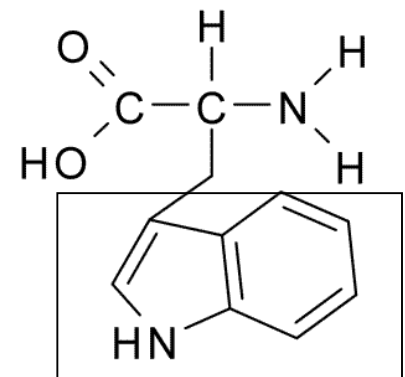
cysteine



histidine

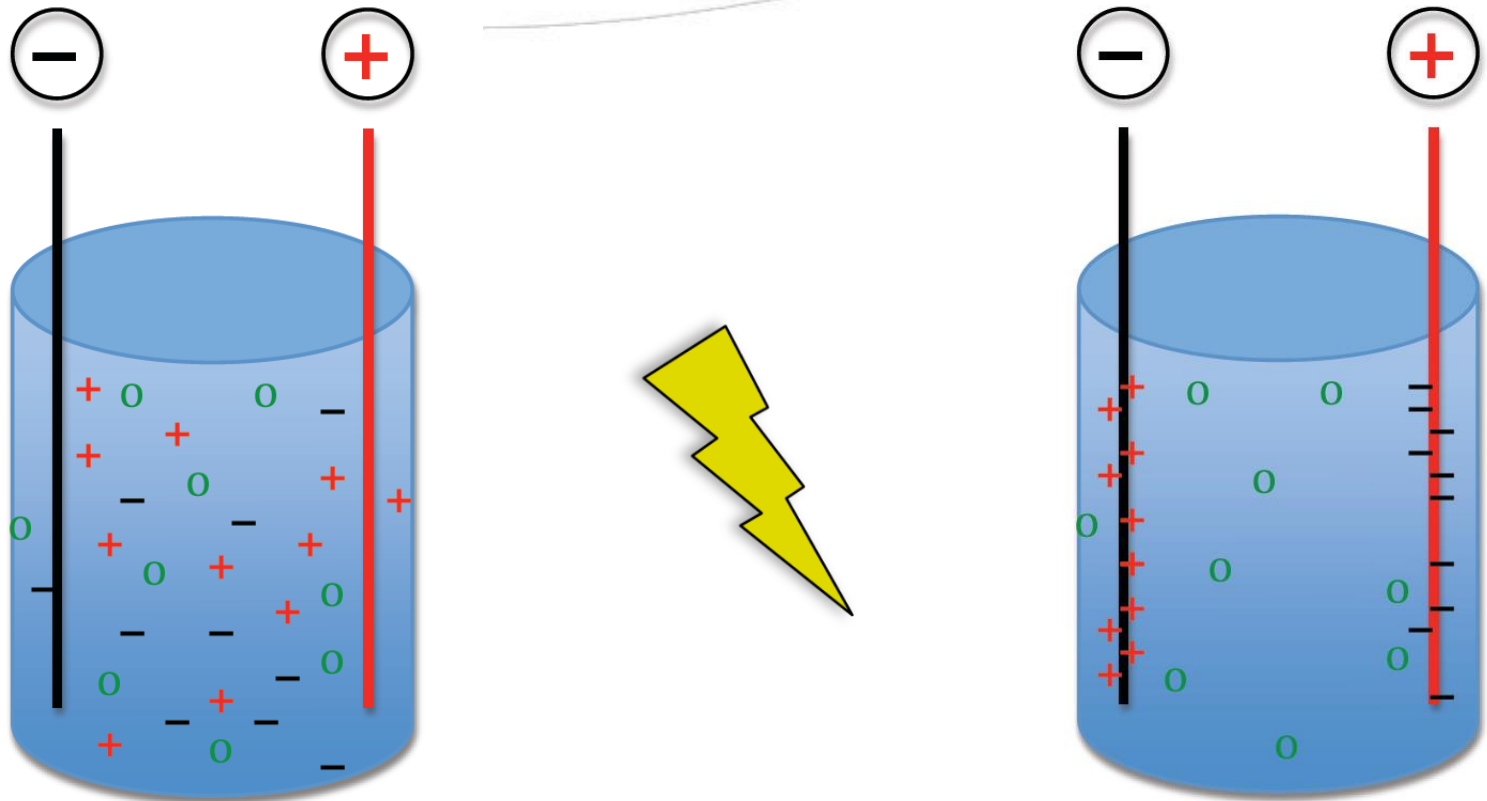


alanine



phenylalanine

Proteins in an Electric Field?

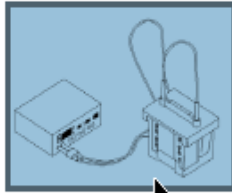


Loading Gels for Electrophoresis

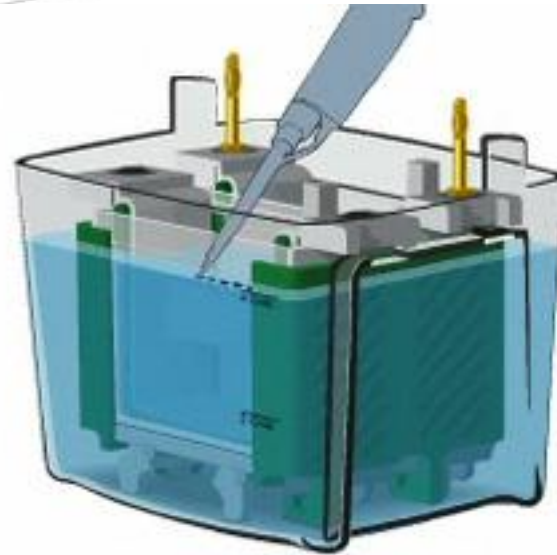
12. Load your gel.

Lane	Volume	Sample
1 & 2	empty	empty
3	10 μ l	Precision Plus Protein Kaleidoscope prestained standards (Sds)
4	10 μ l	fish sample 1
5	10 μ l	fish sample 2
6	10 μ l	fish sample 3
7	10 μ l	fish sample 4
8	10 μ l	fish sample 5
9	10 μ l	actin and myosin standard (AM)
10	empty	empty

13. Electrophorese for 30 minutes at 200 V in 1x TGS electrophoresis buffer.

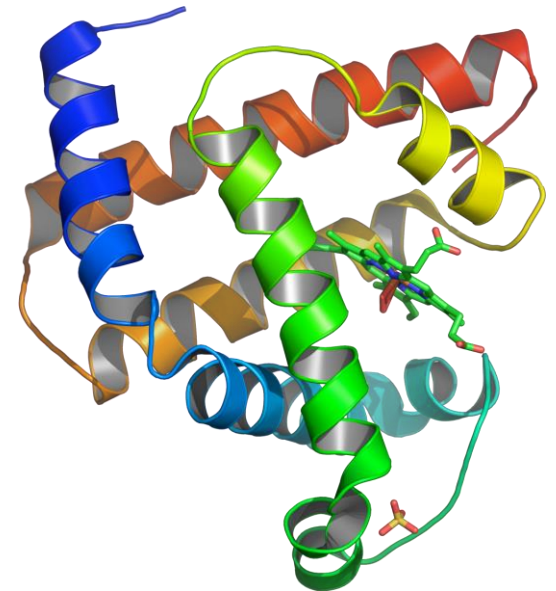


14. After electrophoresis, remove gel from cassette and transfer gel to a container with 40 ml Bio-Safe Coomassie blue stain and stain gel for 1 hour, with gentle shaking for best results.



Considerations for Separating Proteins by Electrophoresis

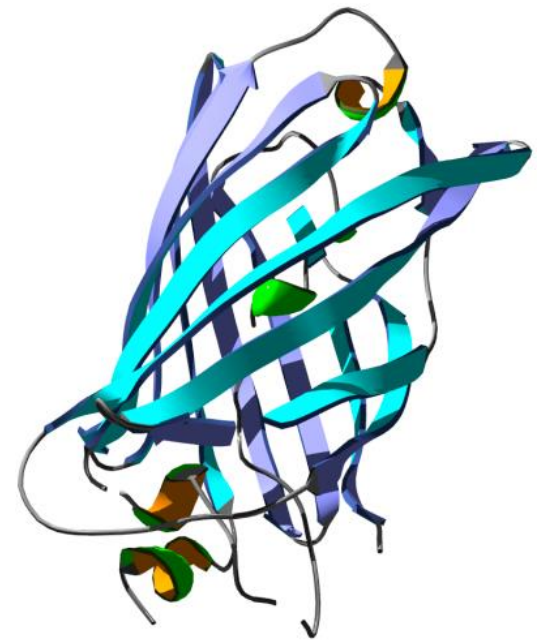
- Size
- Structure /conformation
- Charge



myoglobin

First: Size of Proteins

- ◆ Compare the mass of proteins and DNA molecules
 - ◆ Base pair 660 daltons
 - ◆ Amino acid 110 daltons
- ◆ So, for a protein of average size (300 amino acids): 33,000 daltons (33 kDa)



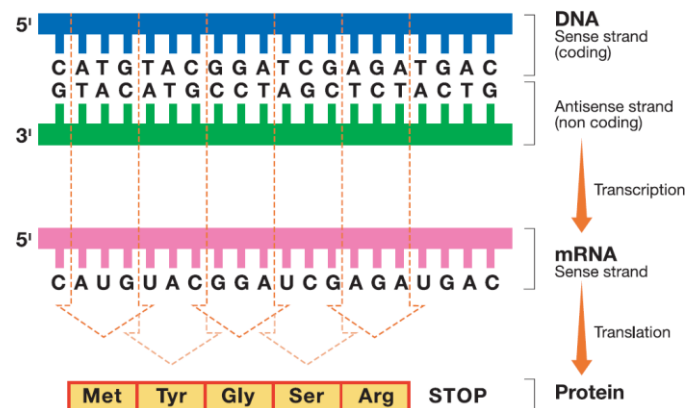
GFP

Size of Proteins

- How many base pairs (minimally) are needed to code for 300-amino acid protein?

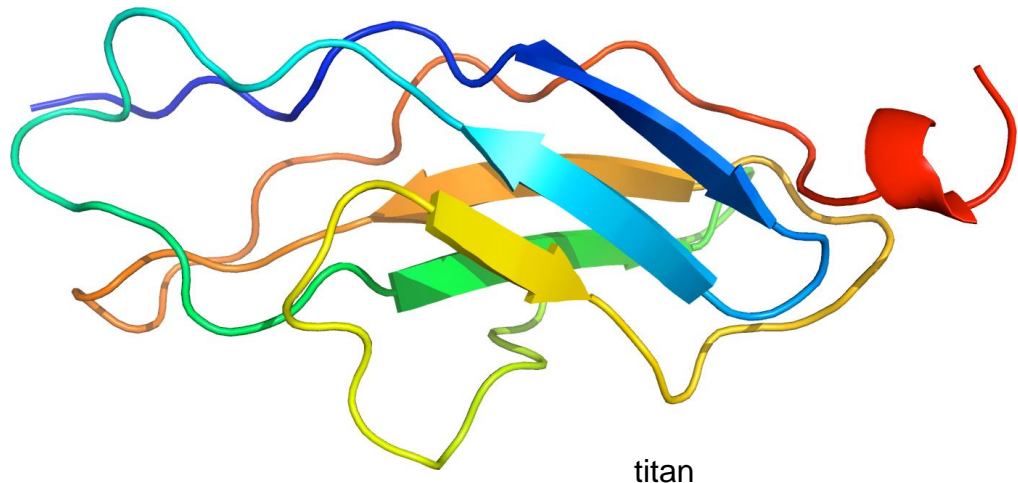
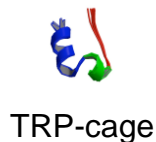
$$300 \text{ aa} \times 3 \text{ bp/aa} = 900 \text{ bp}$$

$$900 \text{ bp} \times 660 \text{ daltons/bp} = \sim 600,000 \text{ da (600 kDa)}$$



Protein Size

- Proteins range in size from ~20 amino acids (2.1 kDa) to 27,000 amino acids (~3,000 kDa)
- Average size of proteins is 300 amino acids (30-45 kDa)
 - Although, as a rule, eukaryotic > prokaryotic > archaeal proteins

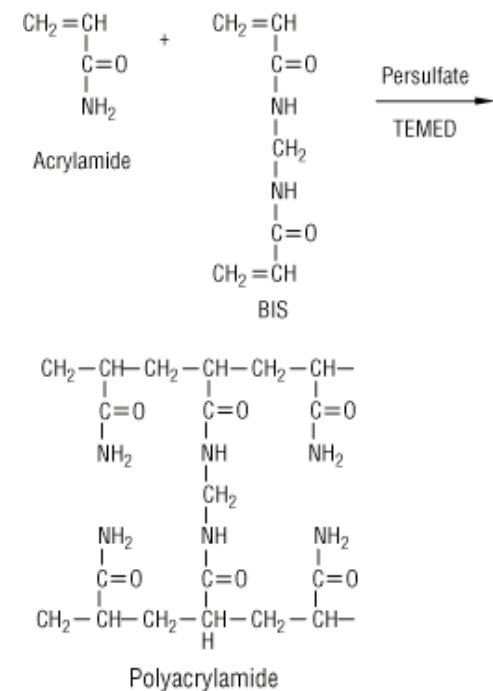


Solving for Protein Size Matrix for Electrophoresis

- ◆ Agarose is used as a matrix for separating DNA molecules by size.
- ◆ For proteins, polyacrylamide is used as the matrix.

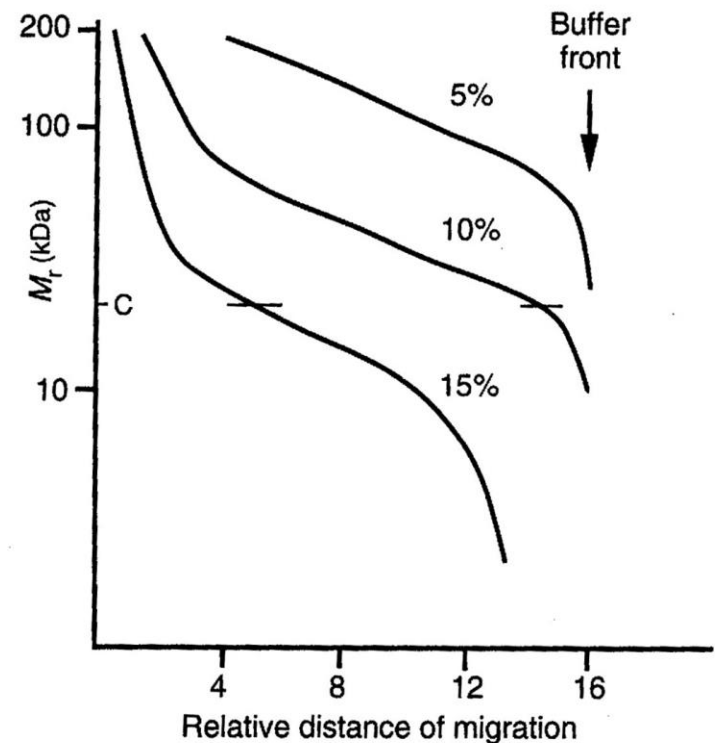
Polyacrylamide

- ◆ To prepare, acrylamide monomers are mixed with bis-acrylamide (a crosslinker) and a catalyst for polymerization.
- ◆ Acrylamide forms long polymers, and bis-acrylamide crosslinks those polymers, forming a matrix.

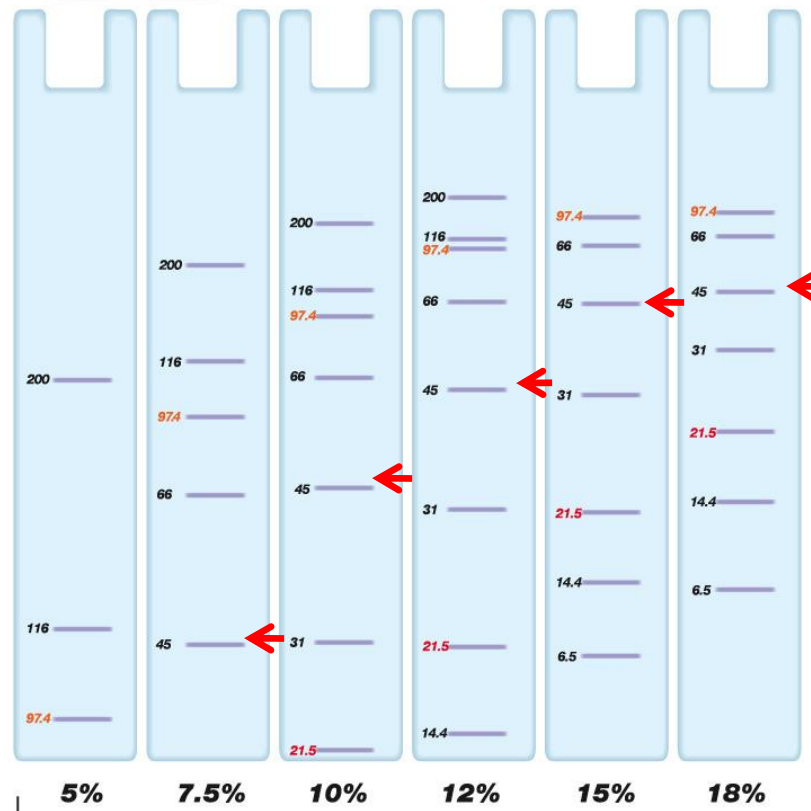


Acrylamide Concentration

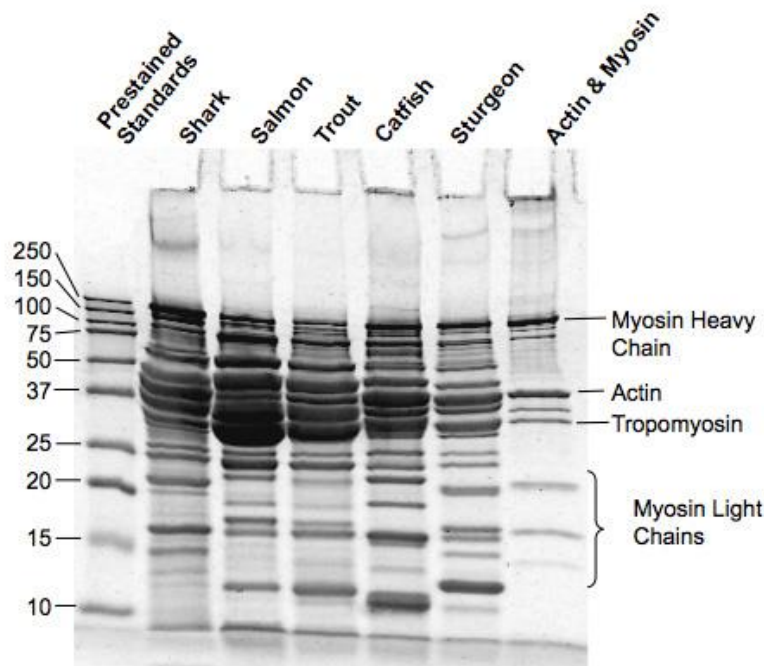
- Electrophoresis of DNA: agarose concentration is varied depending on size of DNA molecules of interest
- Higher concentrations → denser matrix for separating smaller molecules.
- Similarly, acrylamide concentrations are varied for different sizes of proteins



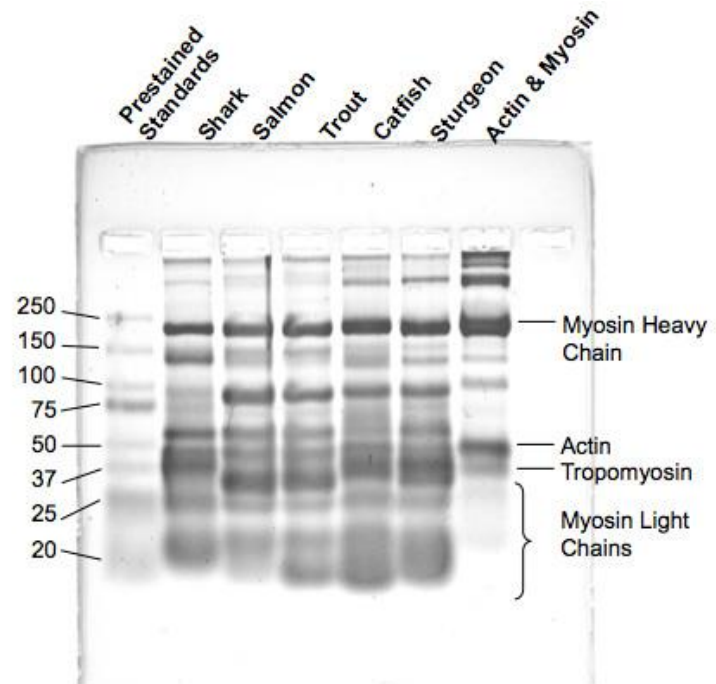
Changing Acrylamide Concentration



Agarose *Can* Be Used to Separate Proteins

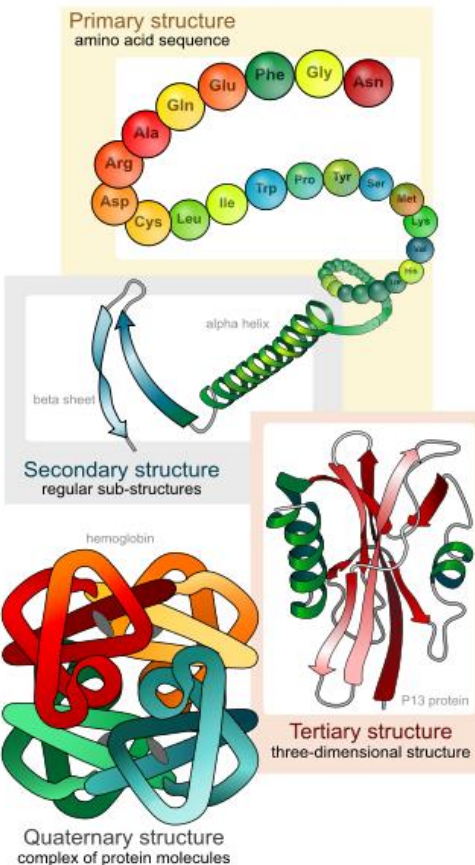


Polyacrylamide



Agarose

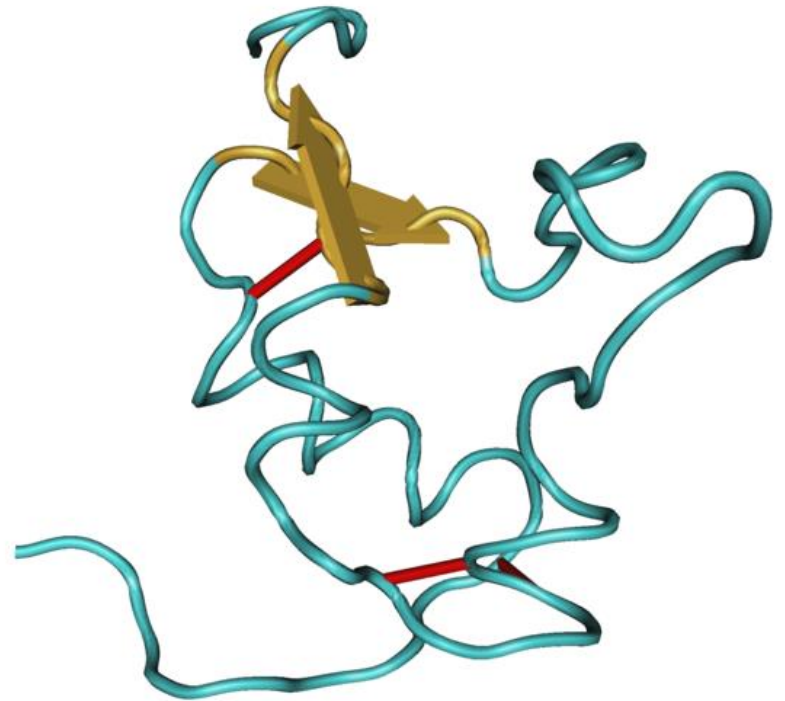
Next Consideration Protein Structure



- 1° structure: amino acid sequence
- 2° structure: regular substructures (β -pleated sheet, α -helix)
- 3° structure: folding of secondary structures into 3-dimensional structure
- 4° structure: folding together of subunits (individual polypeptide chains)

Protein Structure: Disulfide Bonding

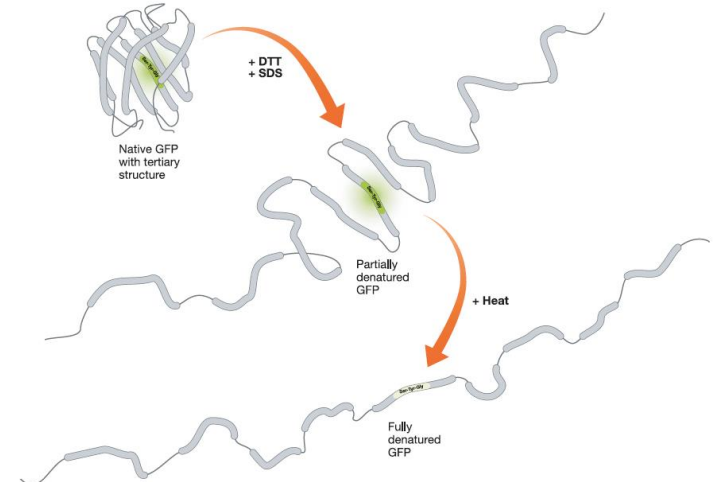
- ◆ In addition to the complex folding of one or more polypeptides, there may be disulfide (covalent) bonds:
 - ◆ Between amino acids within a polypeptide
 - ◆ Between amino acid in different polypeptides
 - ◆ Or both



Insulin-like growth factor

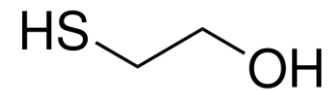
Solving Conformation

- Proteins have 2°, 3°, and 4° structure that would confound separation by size.
- Samples are placed in a sample buffer containing:
 - Detergent
 - Reducing agent
- Samples are heated
- Proteins are reduced and denatured

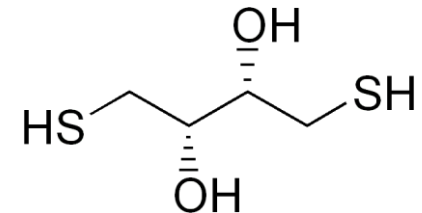


Reduction of Disulfide Bonds

- Reducing agents- molecules that disrupt disulfide bonds
 - β-mercaptoethanol (BME)
 - Dithiothreitol (DTT)
- DTT or BME reduce $-\text{C}-\text{S}-\text{S}-\text{C}-$ to $-\text{C}-\text{S}-\text{H}$ and $\text{H}-\text{S}-\text{C}-$



β-mercaptoethanol



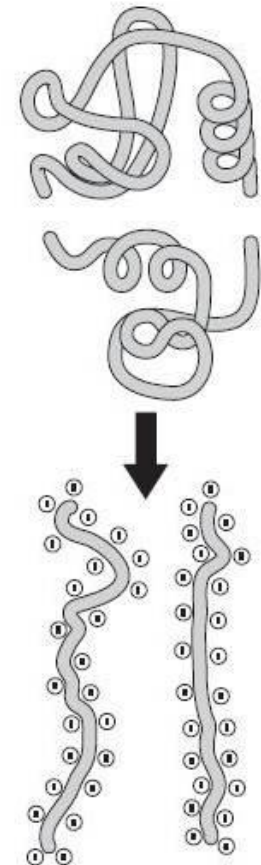
dithiothreitol

Next Problem: Charge

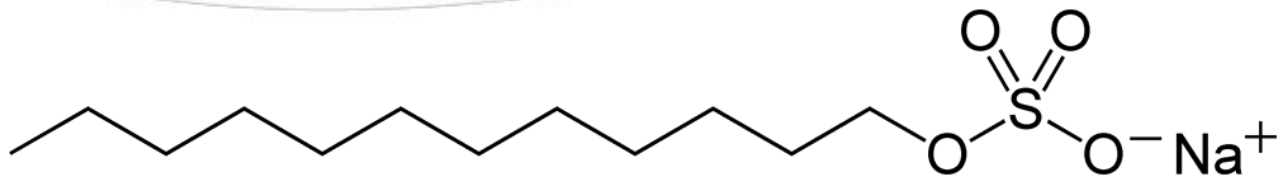
- 💧 Proteins can be overall negatively charged
- 💧 Proteins can be overall positively charged
- 💧 Proteins can be neutral
- 💧 We loaded at the top of the gel or at the cathode – how could we do that and separate our proteins?

Solving for Charge Variation

- SDS has 2nd role: when samples are heated, it binds to the denatured proteins
- SDS binds at fixed ratio: 1.4 g SDS : 1 g polypeptide
- Strong negative charge of the SDS outweighs any intrinsic charge of the proteins
- All proteins have same charge density (charge:mass), so they migrate according to mass in SDS-PAGE



Detergent: SDS



- ◆ Detergent normally used is SDS
 - ◆ Sodium dodecyl sulfate
 - ◆ Ionic detergent
- ◆ Disrupts non-covalent bonds
 - ◆ Protein loses 2° and 3° structure (denaturation)

DETERGENTS

💧 Are amphiphiles, containing a lipophilic portion and a hydrophilic portion.

💧 Lower the interfacial energy between unlike phases.

💧 Emulsify or solubilize aggregated particles.



I like fat!



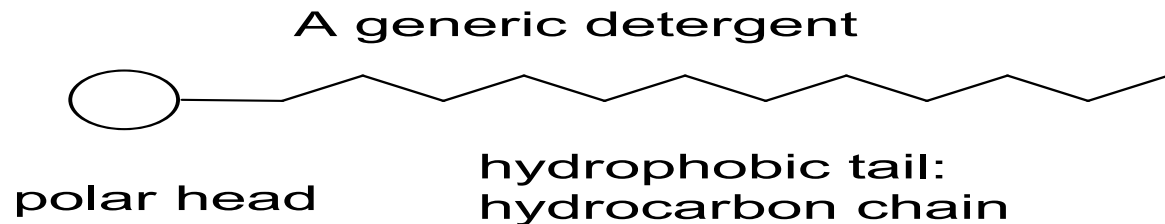
I like water!

More about detergent terms

💧 Lipophilic portion is also referred to as “hydrophobic” tail

💧 Hydrophilic portion is also referred to as “polar” head

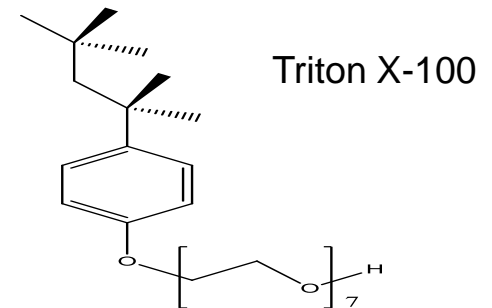
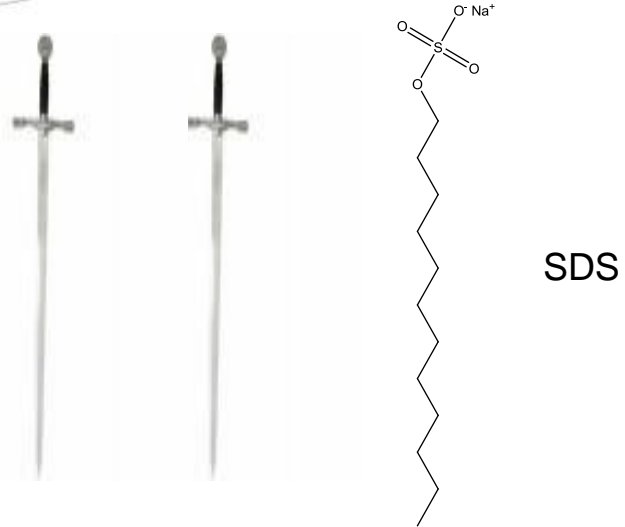
💧 Types: nonionic, anionic, cationic and zwitterionic



Detergents: Ionic vs non-ionic Denaturing vs non-denaturing

🔪 Swords (denaturing):
“pointy” hydrophobic
ends, ionic polar ends

🟢Gloves (non-denaturing): bulky, non-penetrating hydrophobic ends, non-ionic or zwitterionic polar ends.

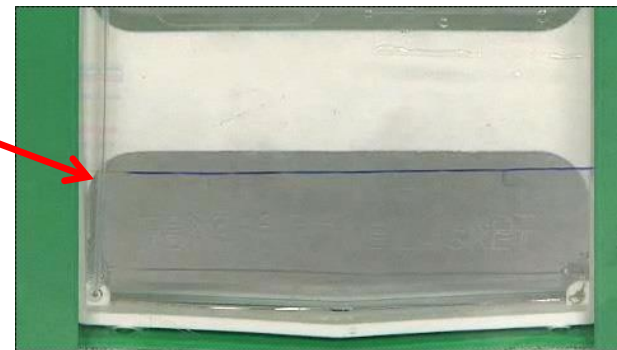


Typical Sample Buffer Components

- ◆ Tris buffer
- ◆ SDS
- ◆ β -mercaptoethanol or dithiothreitol
- ◆ Glycerol
- ◆ Tracking dye (bromophenol blue)

Typical Sample Buffer Components

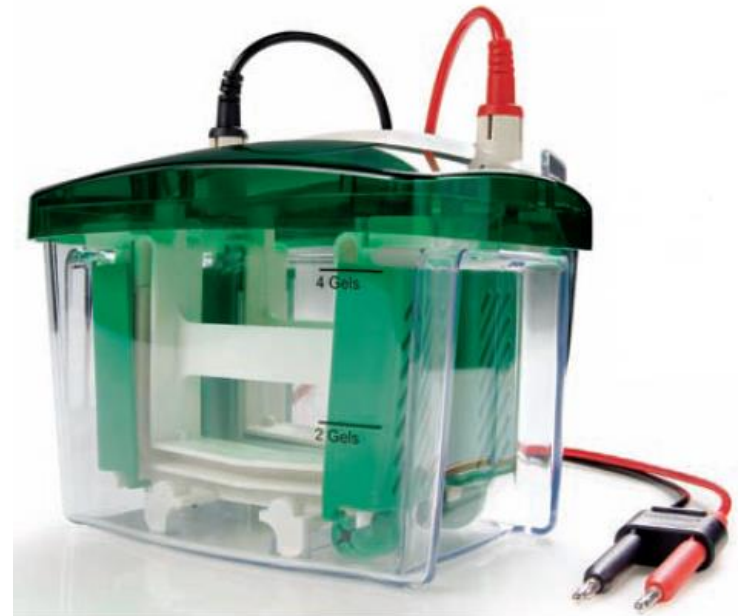
- ◆ Tris buffer
- ◆ SDS
- ◆ β -mercaptoethanol or dithiothreitol
- ◆ Glycerol
- ◆ Tracking dye (bromophenol blue)



670
daltons

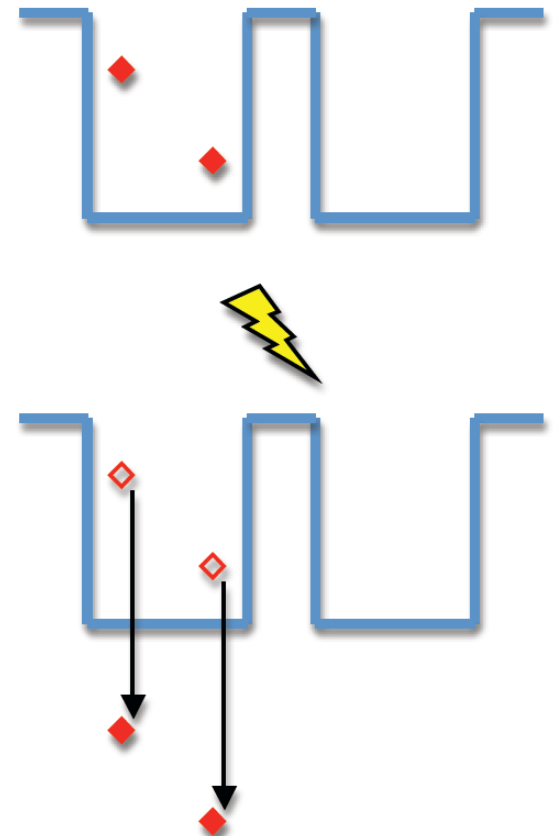
Vertical Gels

- ❖ Unlike agarose gel electrophoresis, SDS-PAGE gels are run vertically
- ❖ New problem: how to get all of the proteins to enter the gel at the same time



Loading Samples

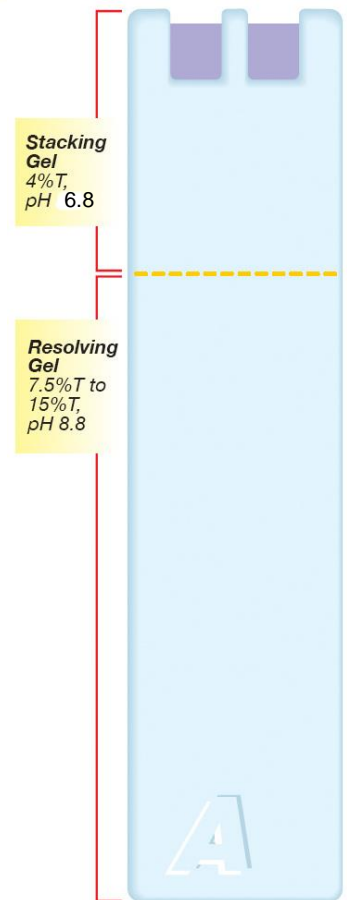
- ◆ We loaded in the wells at the top of the gel.
- ◆ When current was applied, all proteins begin to migrate toward the positive electrode
- ◆ Proteins at the top of the well entered the gel after proteins at the bottom of the well



How to make proteins in a sample migrate together

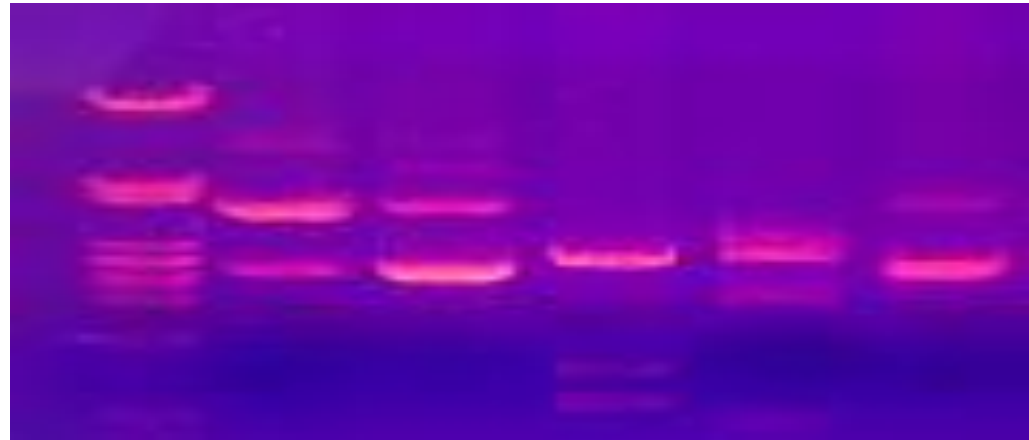
Discontinuous Buffer System

- Has both stacking and separating gels
- Stacking gel has lower acrylamide concentration than separating gel
- Stacking gel has lower pH than separating gel
- Running buffer yet a different pH, and contains glycine



Chemistry of electrophoresis and electrolysis

Electric fields
and electric currents



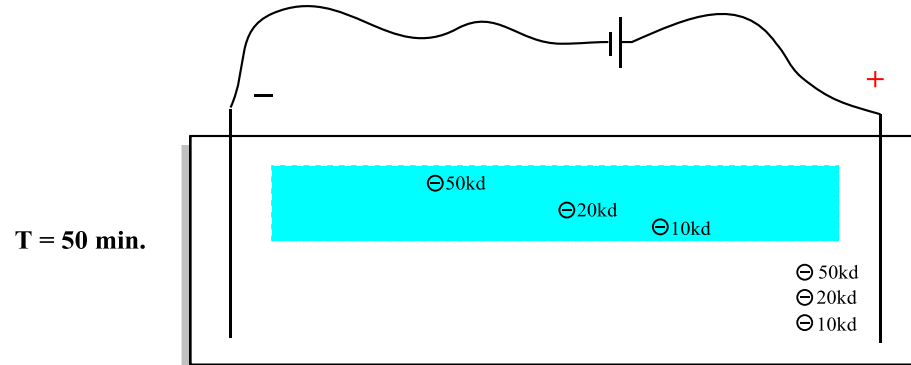
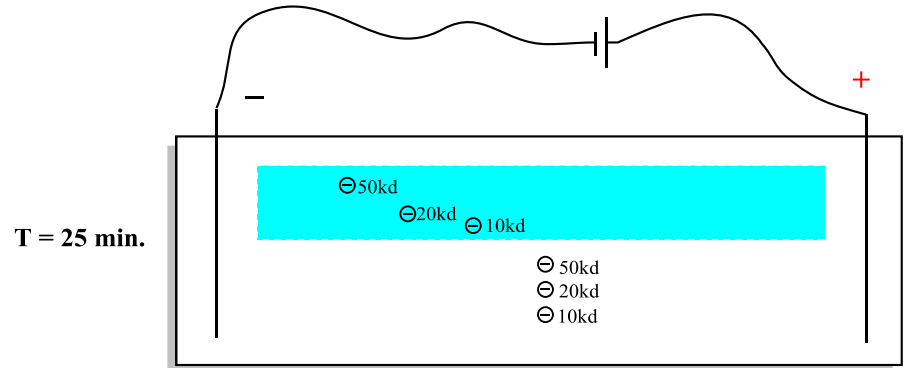
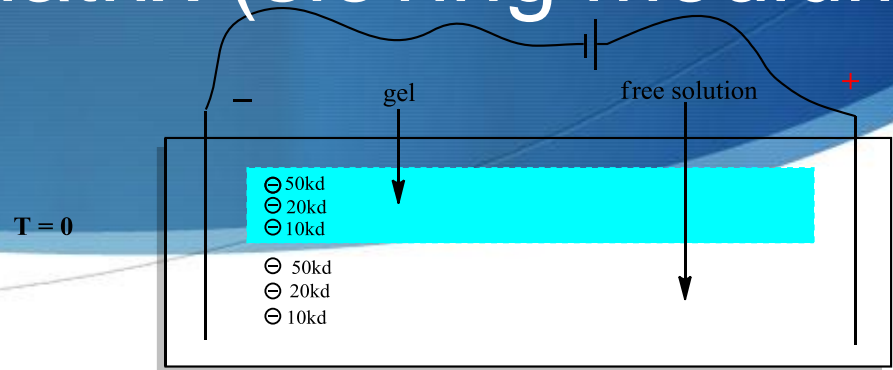
SDS-PAGE, DNA electrophoresis and the need for a gel matrix (sieving medium)

Without a viscous medium, all molecules move at ~ the same rate in electric field

Gel structure retards larger solute particles

DNA molecules, SDS-proteins have ~ equal charge/mass

Electrophoresis occurs between the electrodes (field-driven).



Electrolysis always occurs during electrophoresis.

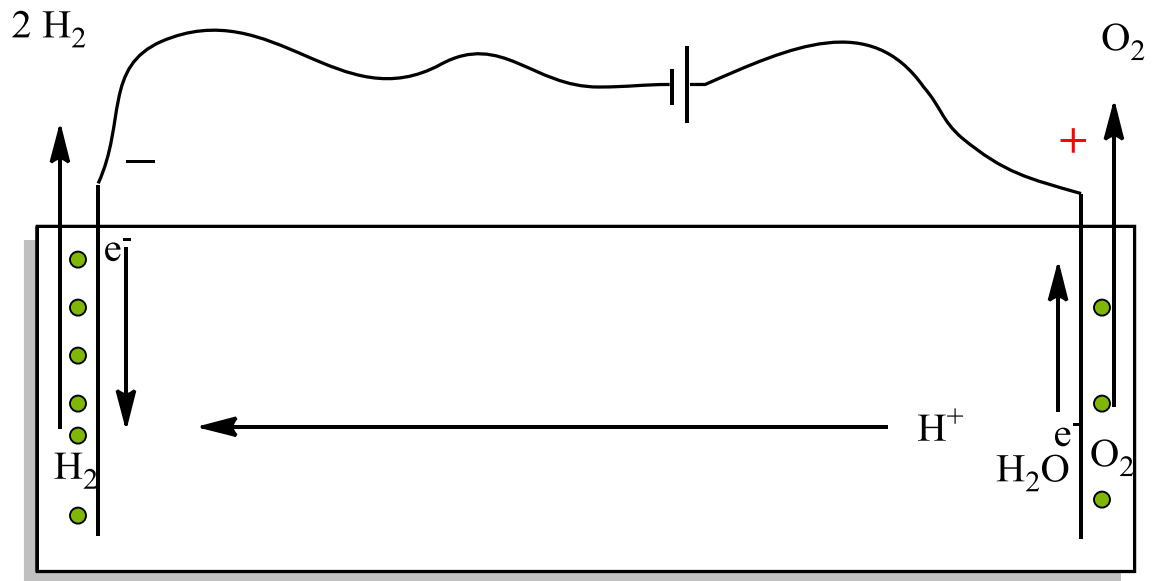
🟢 Cathode produces H_2 at twice the rate that anode produces O_2

🟢 Current is carried by solute ions. Electrons aren't soluble in H_2O .

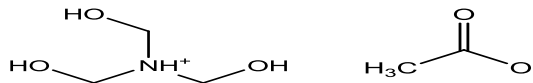
🟢 Example: TAE buffer; tris supplies cations (+), acetate supplies anions (-).

🟢 Electrolysis occurs at the electrodes.

Electrolysis of water (overall equation): $2 \text{H}_2\text{O} + \text{energy} \longrightarrow 2\text{H}_2 + \text{O}_2$



Cathode (reduction):



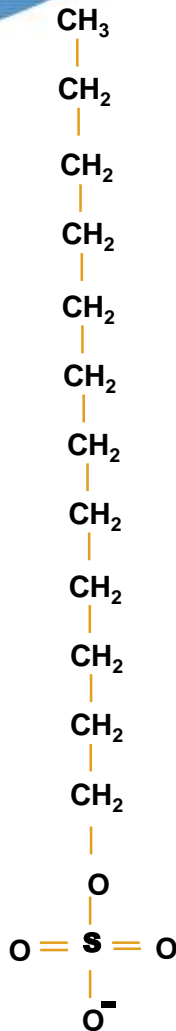
Anode (oxidation):



SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

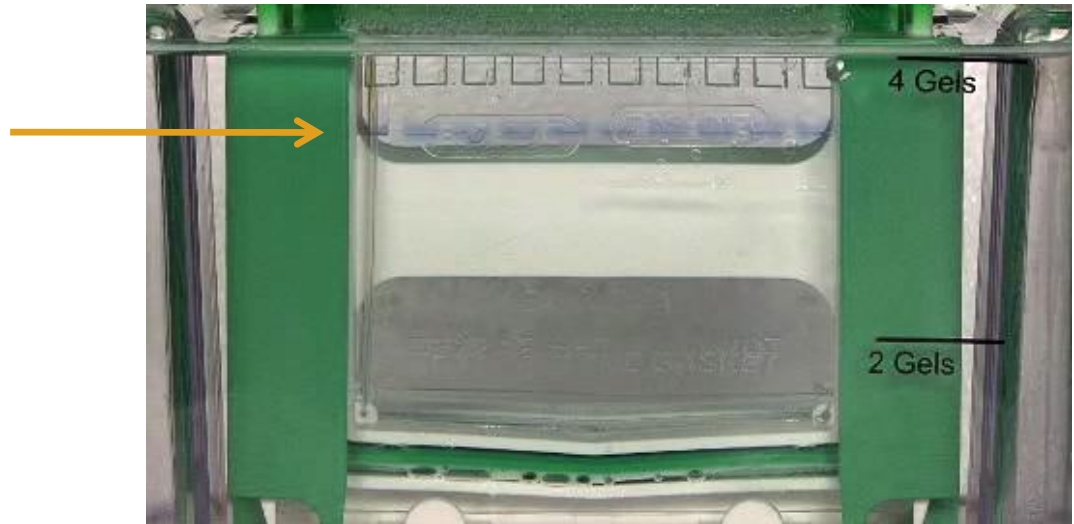
- SDS detergent (sodium dodecyl sulfate)
 - Solubilizes and denatures proteins
 - Adds negative charge to proteins
- Heat denatures proteins

SDS



Stacking in Action!

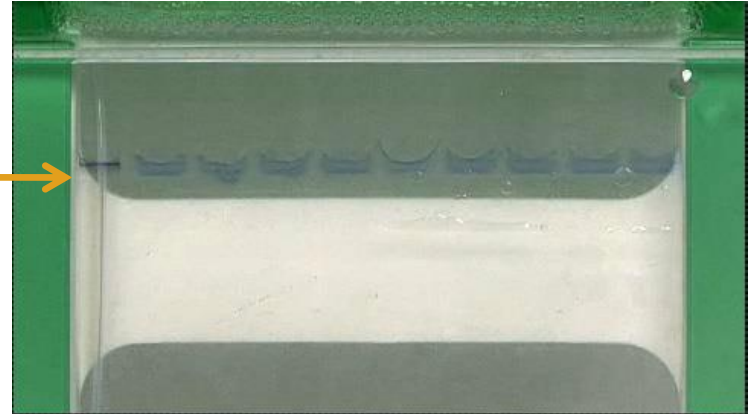
- Samples are loaded in the wells and current is applied to the gel



Stacking in Action!

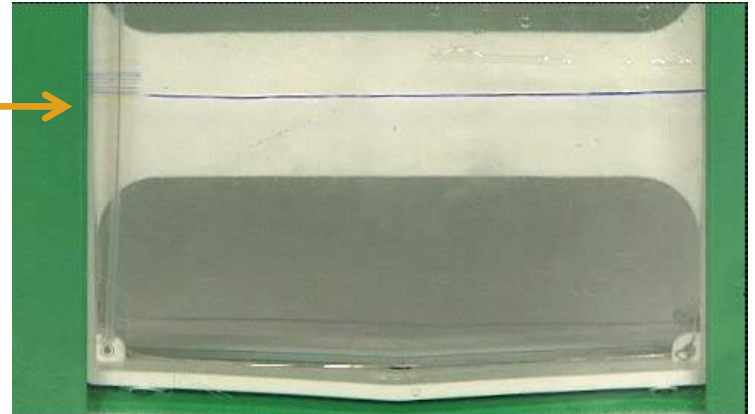
- As samples migrate into the gel, they can be visualized by the loading dye

①



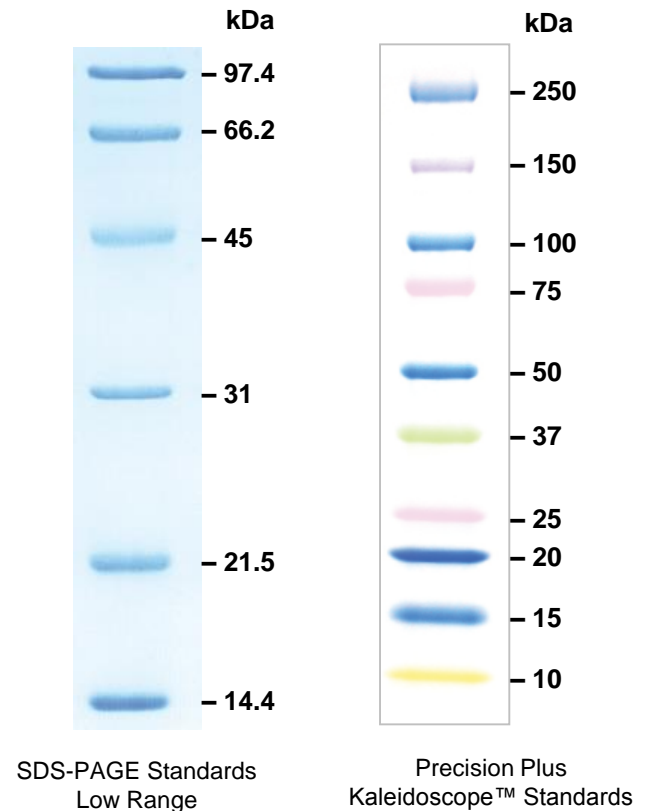
- As the samples move into the gel, they “stack” before they enter the separating gel

②



Ready to Go!

- So, the samples are ready and the gel apparatus is assembled- what's next?
- Just as with electrophoresis of DNA, a size standard is run along with the samples. In this case, it is a mixture of proteins of known molecular weight.
- Choice of molecular weight standard depends on the range of molecular weights of the proteins of interest
- Standards are available pre-stained, unstained, or with specialized tags



Running the Gel

- Most gel boxes will run from 1-4 gels concurrently
- Gels (and buffers) can be purchased or prepared in the lab
- Average running time for a 10 cm gel is 30-45 minutes

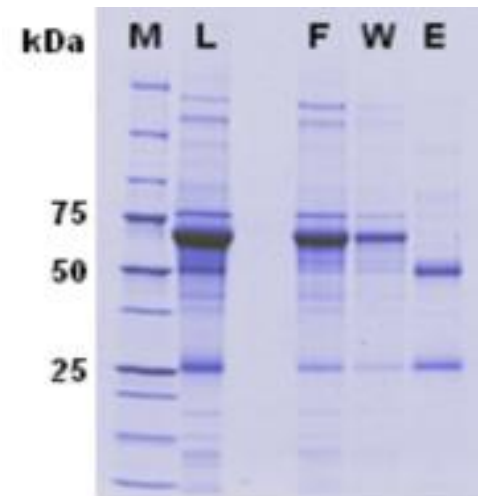


Visualizing the Samples

- Most proteins are not colored, so, how do we see them in the gel?

Visualizing the Samples

- Generally, a total protein stain is used. Gel is stained, then “destained” to remove non-specific stain.
- With Coomassie-based stains, blue-stained proteins appear blue on a clear background
- Staining solution also fixes proteins in gel

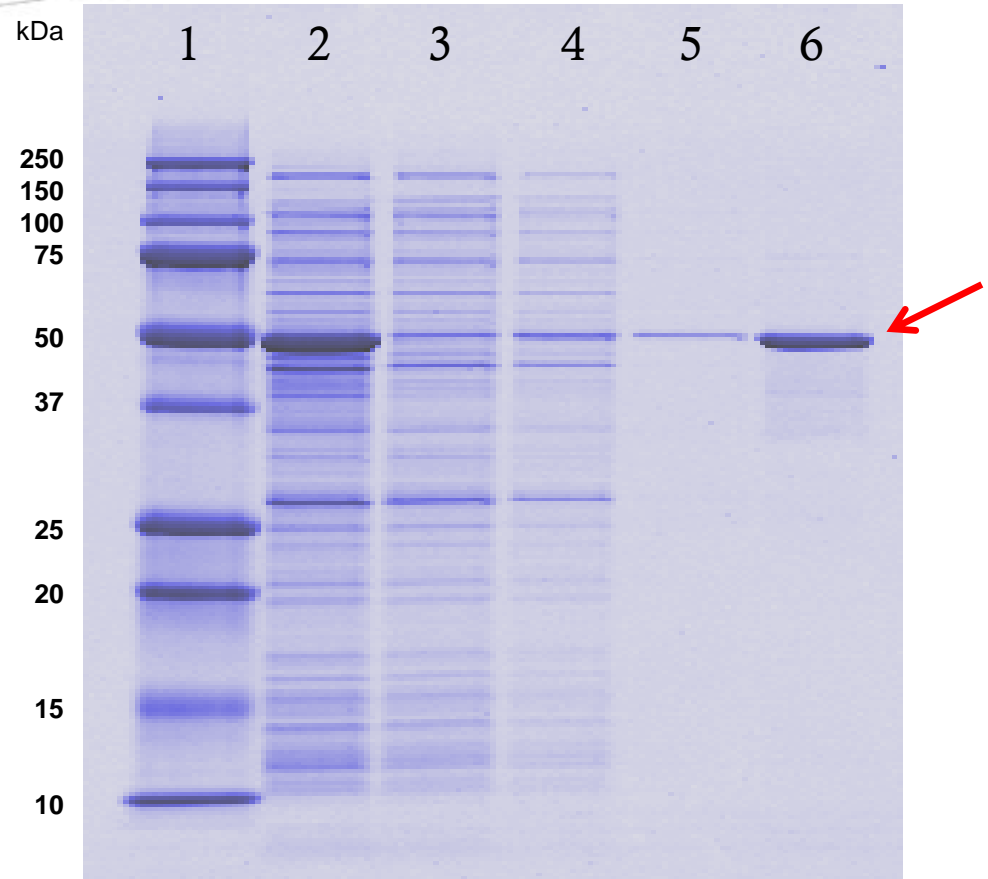


What can an SDS-PAGE gel tell you?

- Size of protein
- Number of proteins
- Quantity of protein
- Purity of proteins
- Changes in protein profile
- NOT protein identity

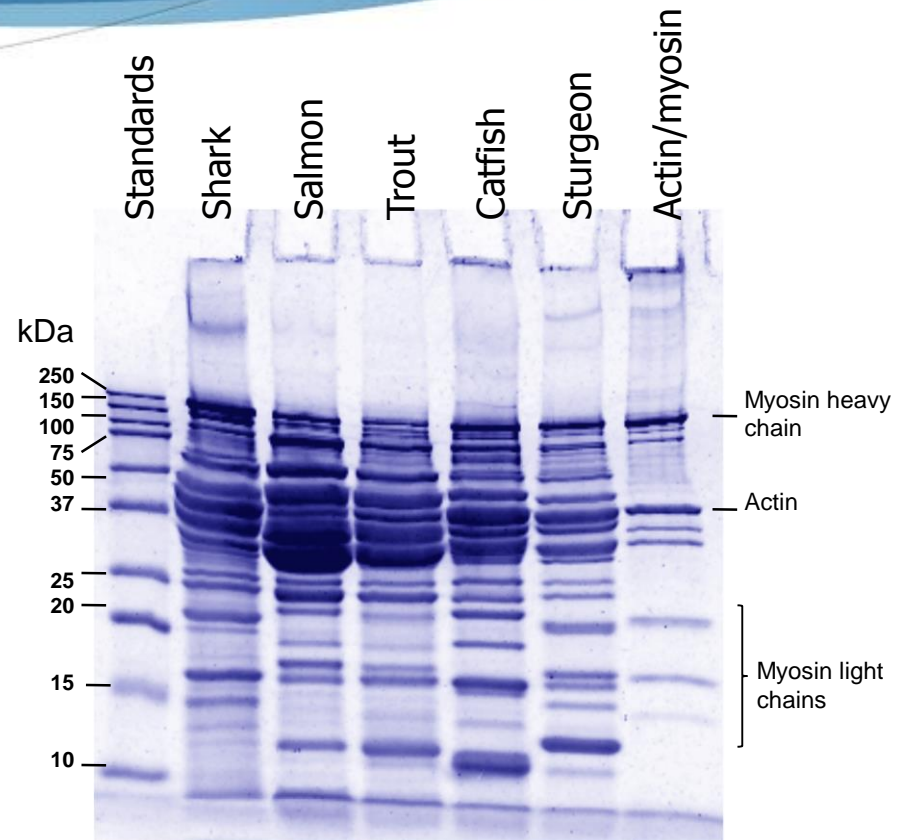
Lane 1: standards
Lane 2: column load
Lane 3: column flow-through

Lane 4: column wash
Lane 5: column wash 2
Lane 6: elution

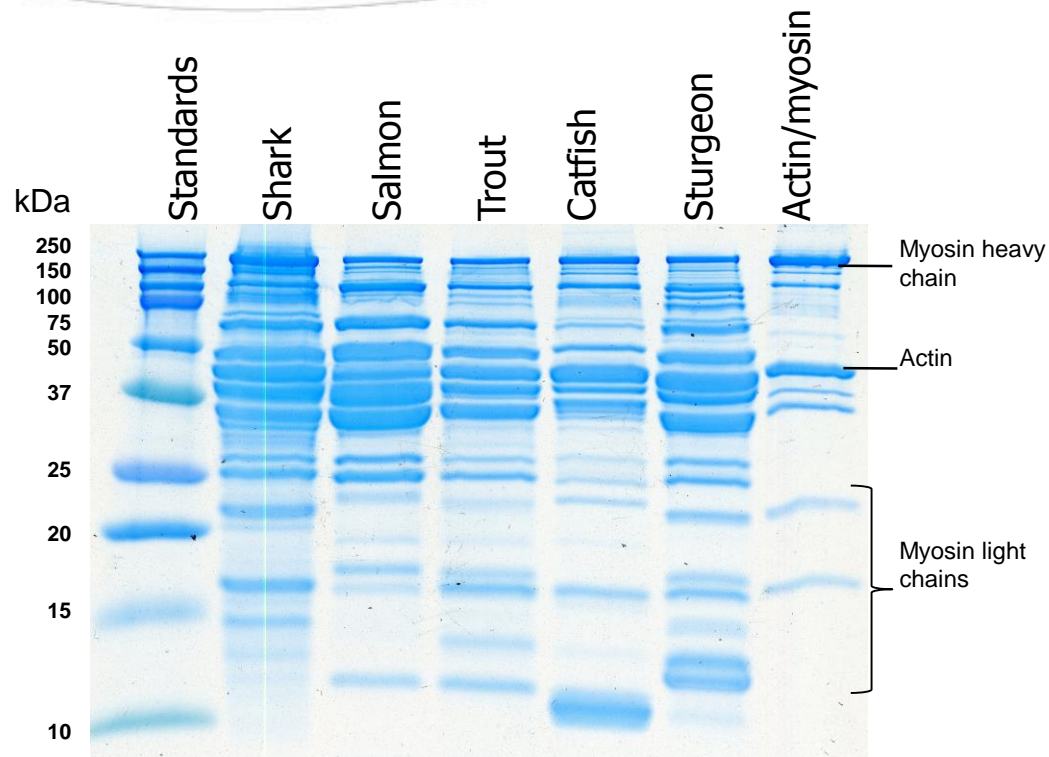


Protein Identity

- Results can be suggestive, for example, there is a protein of the expected size visible on the gel
- But identity must be confirmed by further experiments

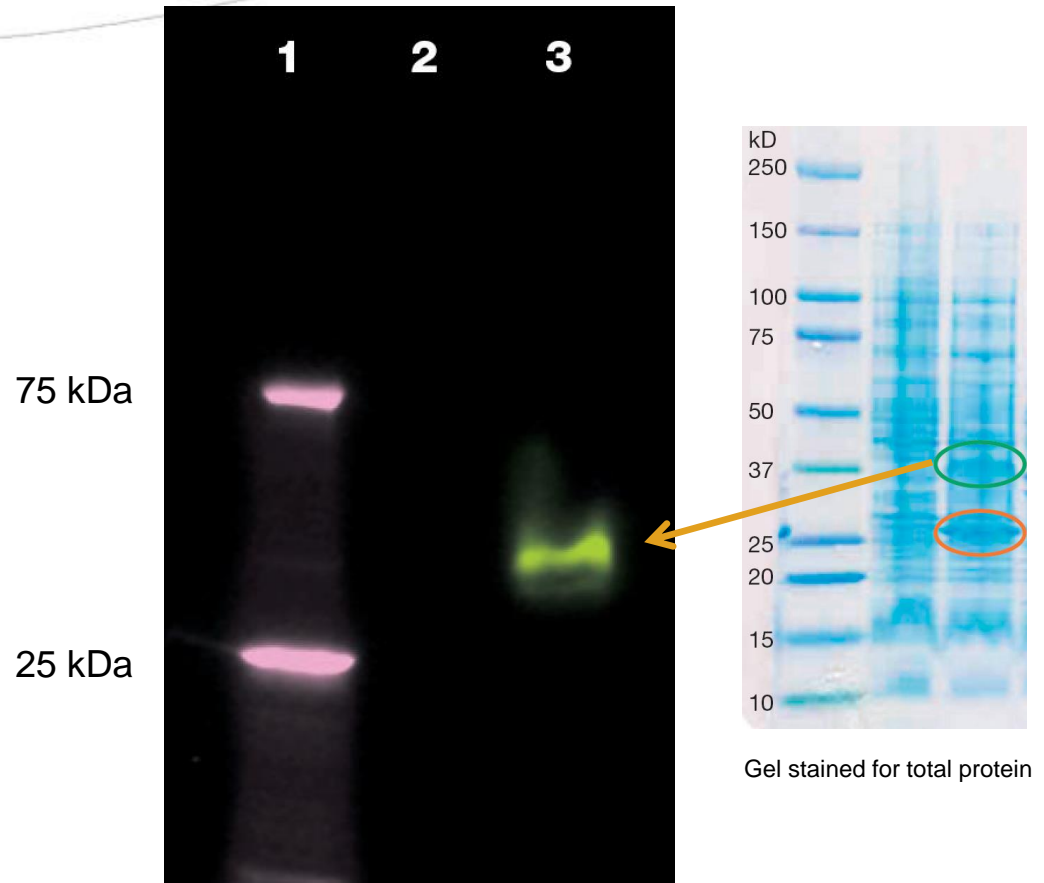


Protein Profiler Module



pGlo SDS-PAGE Extension

- There are some special cases in which samples can be visualized without staining the gel.
- GFP maintains its structure even in a gel and can be visualized using UV excitation.
- Some size standards contain proteins that fluoresce under UV light.



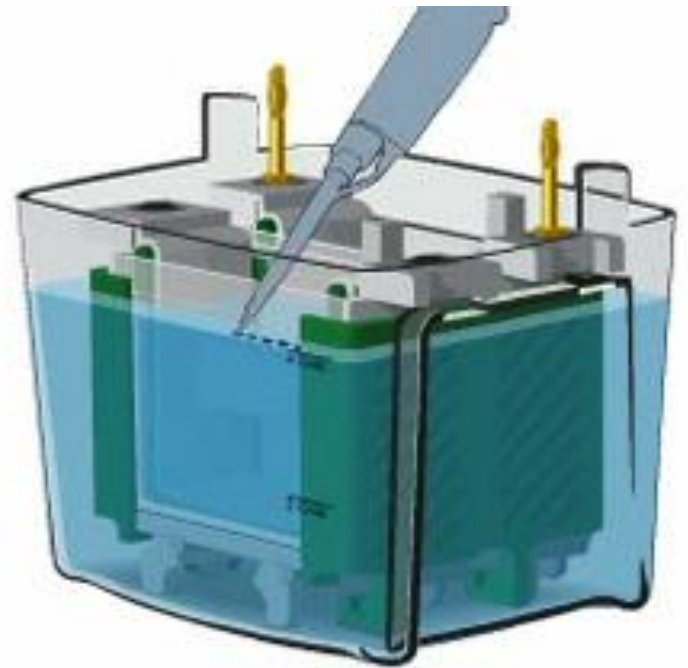
SDS-PAGE in the Classroom

- What do you need to get started?
 - Gel box
 - Power supply
 - Pipettors
 - Gels & buffers



SDS-PAGE in the Classroom

- Time management
 - Sample preparation
 - Loading and running the gel
 - Staining the gel
 - Discussion of results
- Sample choice



SDS-PAGE in the Classroom

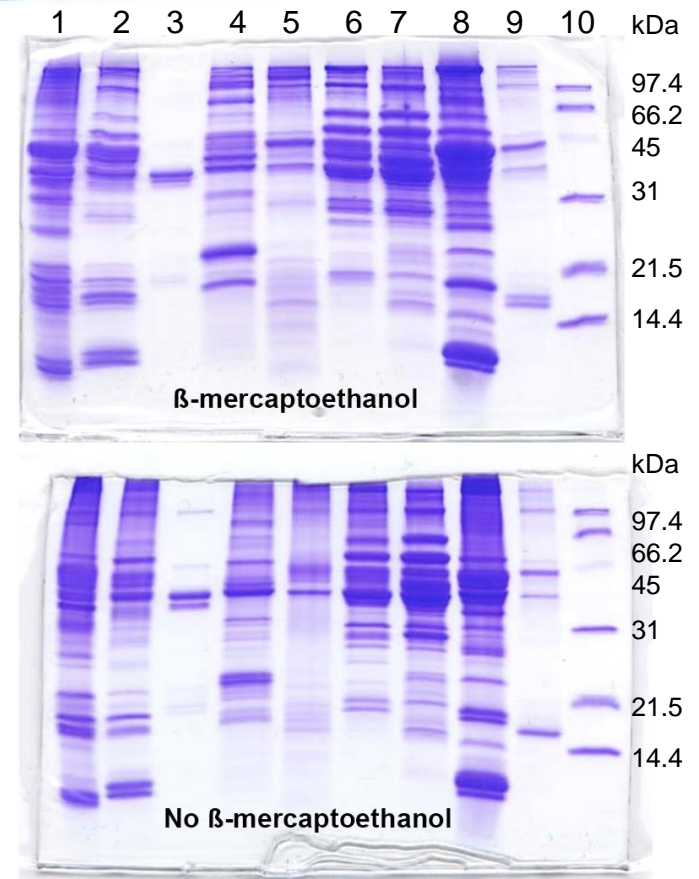
- 💧 Eliminating safety hazards
 - 💧 Use a non-hazardous stain like Bio-Safe
 - 💧 Use precast polyacrylamide gels
 - 💧 Use current research quality equipment to prevent shock hazards

Omitting a Reducing Agent

- Top gel: samples with β -me
- Bottom gel: the same samples without β -me

Lane 1: scallop
Lane 2: catfish
Lane 3: tuna
Lane 4: swordfish
Lane 5: shark

Lane 6: shrimp
Lane 7: lobster delite
Lane 8: orange roughy
Lane 9: flounder
Lane 10: standards



SDS-PAGE in the Classroom

- 💧 Problem areas
 - 💧 Pouring gels (if not purchased pre-poured)
 - 💧 Loading the gels (gel loading guide helps)
 - 💧 Leaking gel boxes (research grade equipment reduces or eliminates problem)



SDS-PAGE in the Classroom

- Problem areas
 - Filling the center buffer chamber
 - Running samples far enough into the gel
 - Blue fingers (wear gloves to avoid looking like a Smurf when staining for total proteins)



<http://www.smurf.com/>

QUESTIONS?

