Scientific Practice-MCBG 2036
2018
Lecture 3-4

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Consultation Hours:
Thursday-Friday: 13H30- 14H00

Quick Quiz

Gel electrophoresis separates molecular products based on:

A. Shape
B. Size
C. Charge
D. Evolutionary similarity

Quick Quiz

When traveling through an agarose gel, larger molecular products will migrate _____ smaller molecular products.

A. Faster than
B. Slower than
C. At the same rate

Quick Quiz

As DNA has a net _____ charge, it migrates towards the gel box's _____.

A. Positive; cathode
B. Positive; anode
C. Negative; cathode
D. Negative; anode

Quick Quiz

The size of the molecular product is determined by:

A. The intensity of the band
B. The percentage of agarose gel
C. Comparison with a molecular weight “ladder”
D. Being familiar with your gel and “eyeballing” it

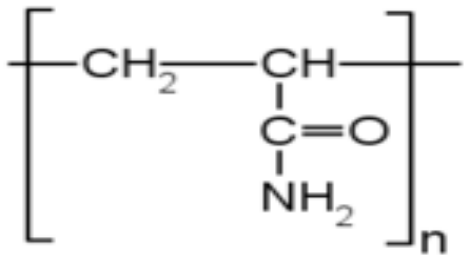
Quick Quiz

Buffer is used instead of water when making and running gels because:

A. Buffer enhances the transmission of electric currents in water
B. Buffer is more homogeneous than water
C. Buffer neutralizes the charge on molecular products prior to electrophoresis
D. Buffers can be customized based upon the electrophoresis protocol

Polyacrylamide Gels

- Acrylamide monomers polymerize into long chains that are covalently linked by a crosslinker
- Polyacrylamide is chemically complex, as is the production and use of the gel

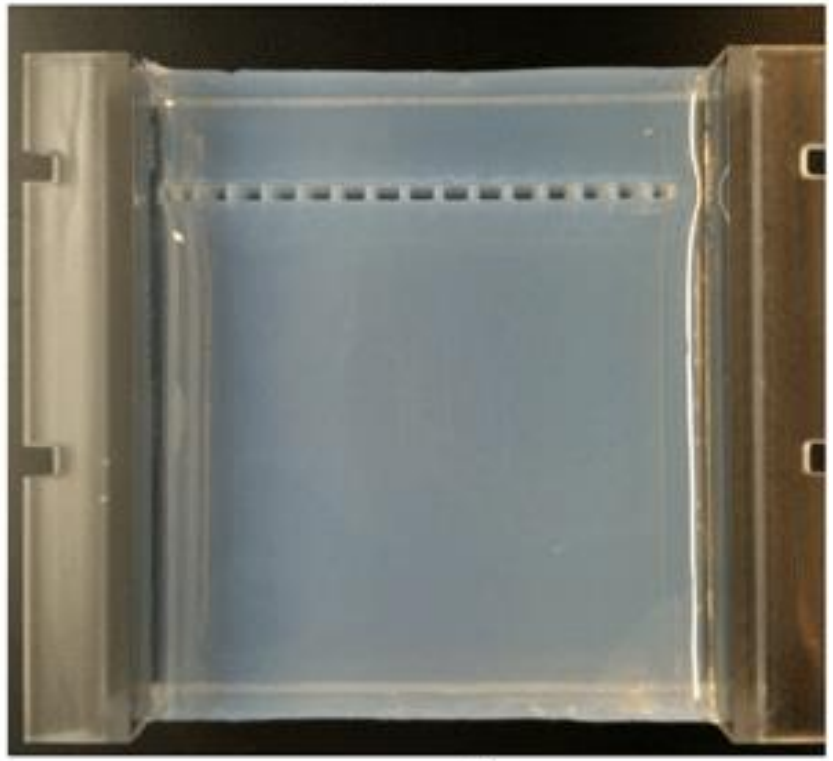


Other advantages over agarose gel:

- Chemically inert
- Stable over wide range of pH, Ionic strength and temperature
- Transparent

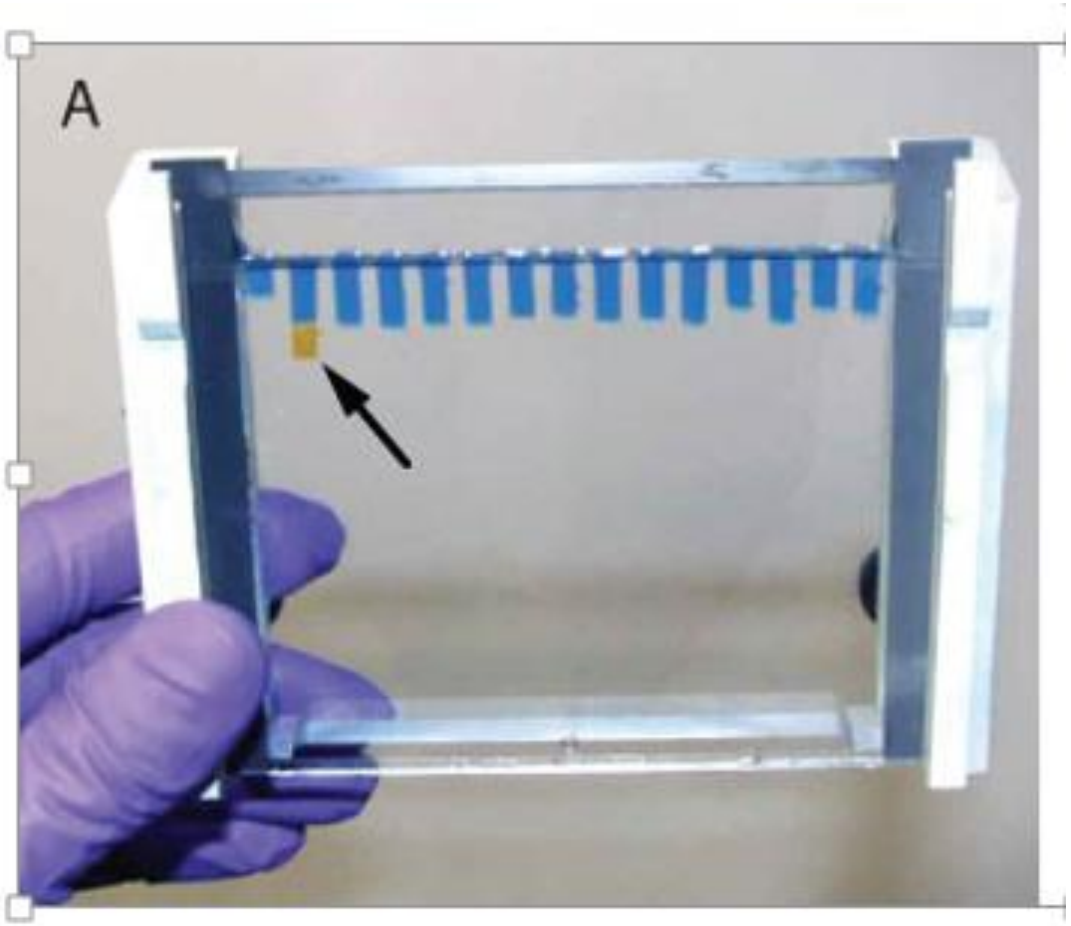
Charge: —

Wells



Charge: +

Agarose



Polyacrylamide

Polyacrylamide gels- advantages

- Have smaller pores than agarose, therefore high degree of resolving power.
- Can separate DNA fragments which range in size from 10-500 bp.
- DNA fragments which differ in size by one nucleotide can be separated from each other.
- Polyacrylamide gel electrophoresis is also

used to separate protein molecules.

ACRYLAMIDE (%)	RANGE OF SEPARATION (kDa)
8	25-200
10	15-100
12.5	10-70
15	6-60
20	4-40

Protein Electrophoresis

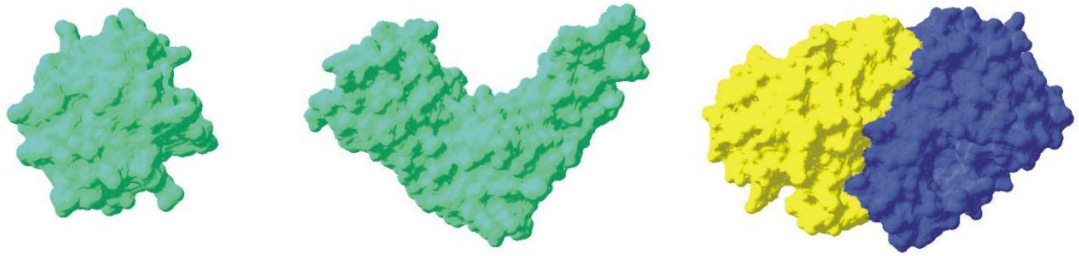
- **Separate proteins based on**
  - Size (Molecular Weight - MW)
- **Allows us to**
  - characterize
  - quantify
  - determine purity of sample
  - compare proteins from different sources
- And it is a step in Western blot

Protein Electrophoresis

- Proteins, unlike DNA, do not have a constant size to charge ratio
  - In an electric field, some will move to the positive and some to the negative pole, and some will not move because they are neutral
  - Native proteins may be put into gel systems and electrophoresed
  - An alternative to native protein gels forces all proteins to acquire the same size to charge ratio

Native PAGE

Separates folded proteins and protein-protein or protein-ligand complexes by charge, size, and shape



Useful for:

1. Examining protein-protein protein-ligand interactions
2. Detecting protein isoforms/conformers

Native proteins are **proteins** which are in their folded and/or assembled form, and are fully functional

While native (nondenaturing) PAGE does not provide direct measurement of molecular weight, the technique can provide useful information such as protein charge or subunit composition. Native PAGE also has the potential for separating proteins of identical molecular weight which cannot be resolved with SDS-PAGE.

https://www.nationaldiagnostics.com/electrophoresis/article/native-protein-electrophoresis

SDS-PAGE

- **SDS-PAGE** (sodium dodecylsulphate-polyacrylamide gel electrophoresis)

- The purpose of this method is to separate proteins according to their size, and no other physical feature

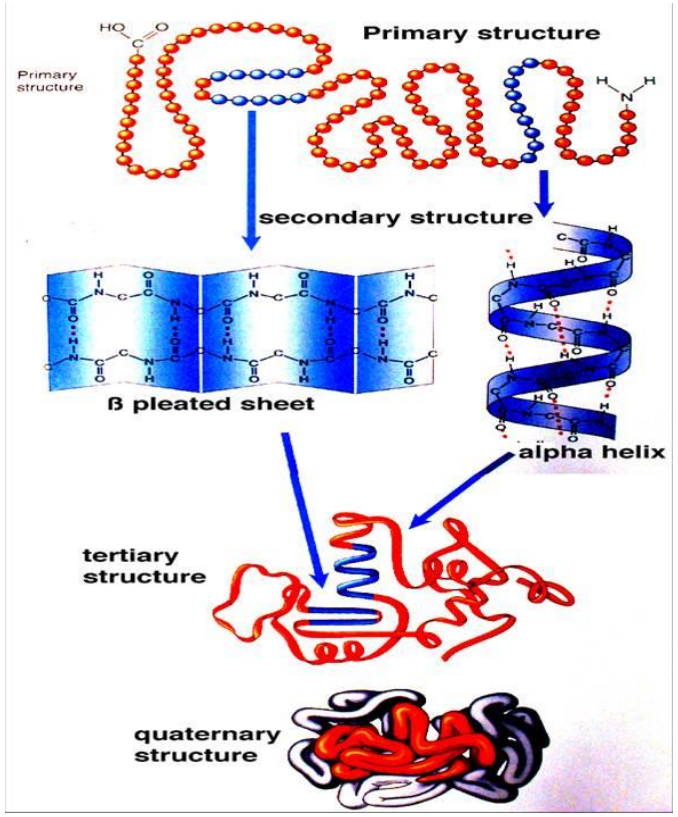
- In order to understand how this works, we have to understand the two halves of the name: **SDS** and **PAGE**

Goals

- To understand the principle of SDS-PAGE
- To become familiar with the SDS-PAGE setup
- To be able to analyze the results

SDS-Sodium Dodecylsulphate

- Since we are trying to separate many different protein molecules of a variety of shapes and sizes,
  - we first want to get them **to be linear**
  - no longer have any secondary, tertiary or quaternary structure (i.e. we want them to have the same linear shape).
- Not only the mass but also the shape of an object will determine how well it can move through and environment.
- So we need a way to convert all proteins to the same shape - we use SDS.



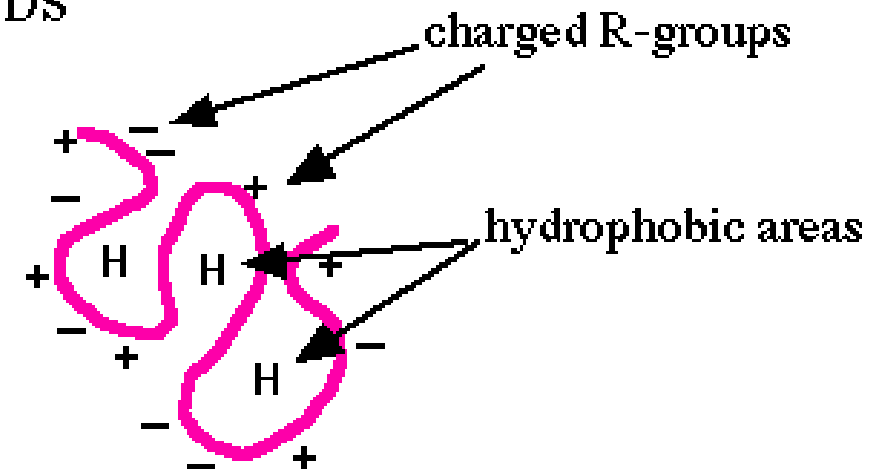
SDS- Sodium Dodecylsulphate

- SDS (sodium dodecyl sulfate) is a detergent that can dissolve hydrophobic molecules but also has a negative charge (sulfate) attached to it.

- If SDS is added to proteins, they will be solubilized by the detergent, plus all the proteins will be covered with many negative charges.

Sodium Dodecylsulphate

BEFORE SDS



AFTER SDS

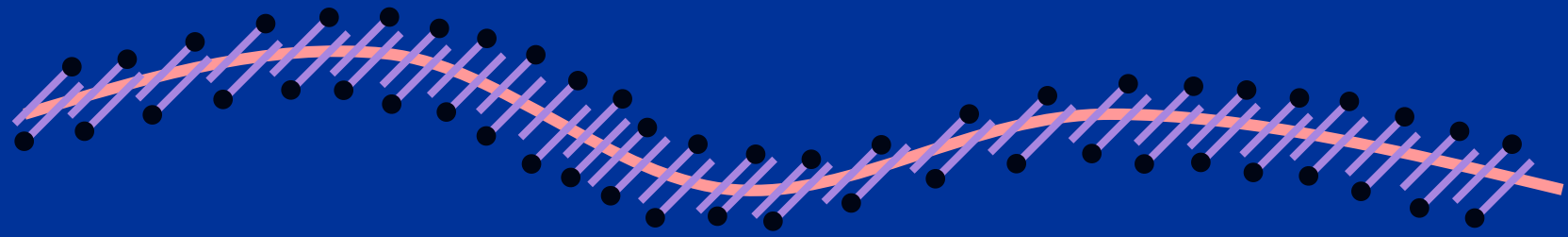
Sodium Dodecylsulphate

- A sample of protein, often freshly isolated and unpurified, is boiled in the detergent sodium dodecyl sulfate and beta-mercaptoethanol
  - The mercaptoethanol reduces disulfide bonds
  - The detergent disrupts secondary and tertiary structure
- The end result has two important features:
  1. all proteins contain only primary structure and
  2. all proteins have a large negative charge which means they will all migrate towards the positive pole when placed in an electric field.
- They migrate through a gel towards the positive pole at a rate proportional to their linear size
  - Molecular weights with respect to size markers may then be determined

SDS and Proteins

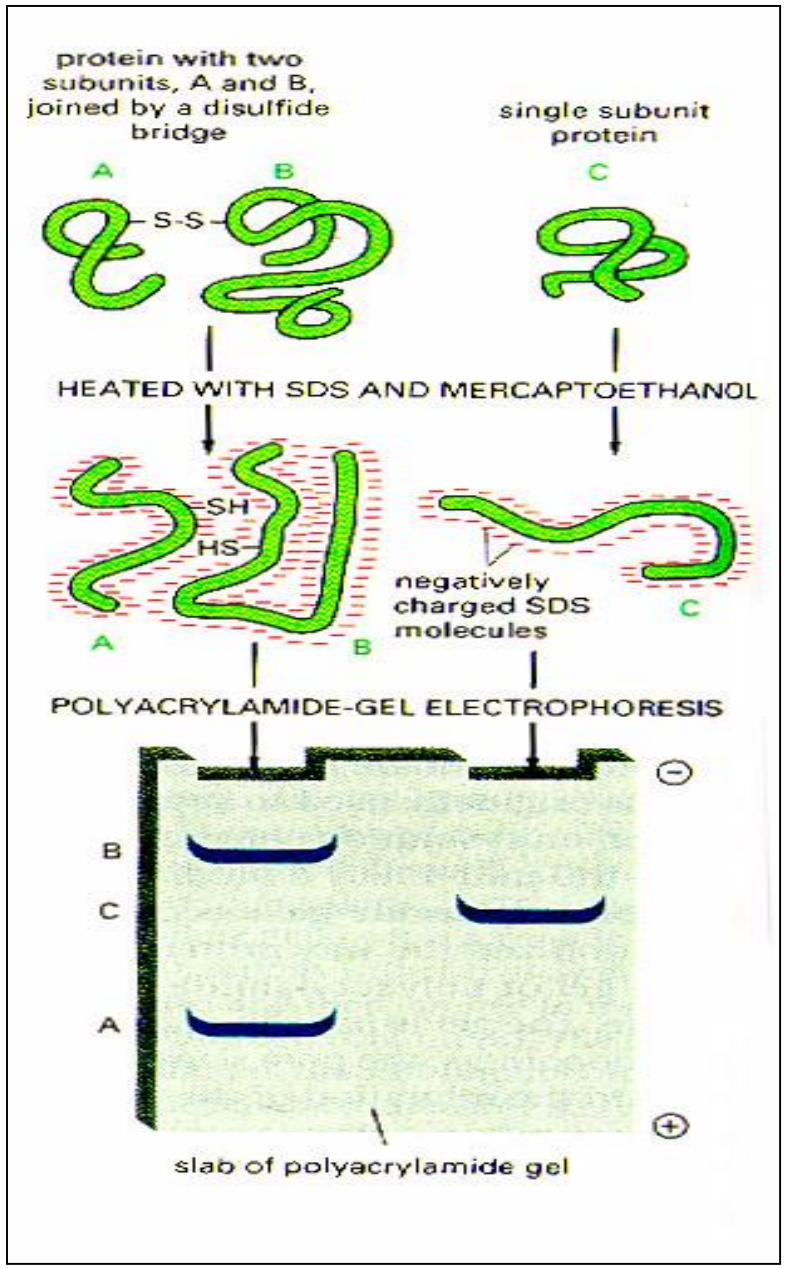
SDS and Proteins

- SDS nonpolar chains arrange themselves on proteins and destroy secondary tertiary and quarternary structrure



- So much SDS binds to proteins that the negative charge on the SDS drowns out any net charge on protein side chains
- In the **presence of SDS all proteins have uniform shape and charge per unit length**

SDS Gel Electrophoresis



Now we are ready to focus on the second half - PAGE.

Polyacrylamide Gel Electrophoresis (PAGE)

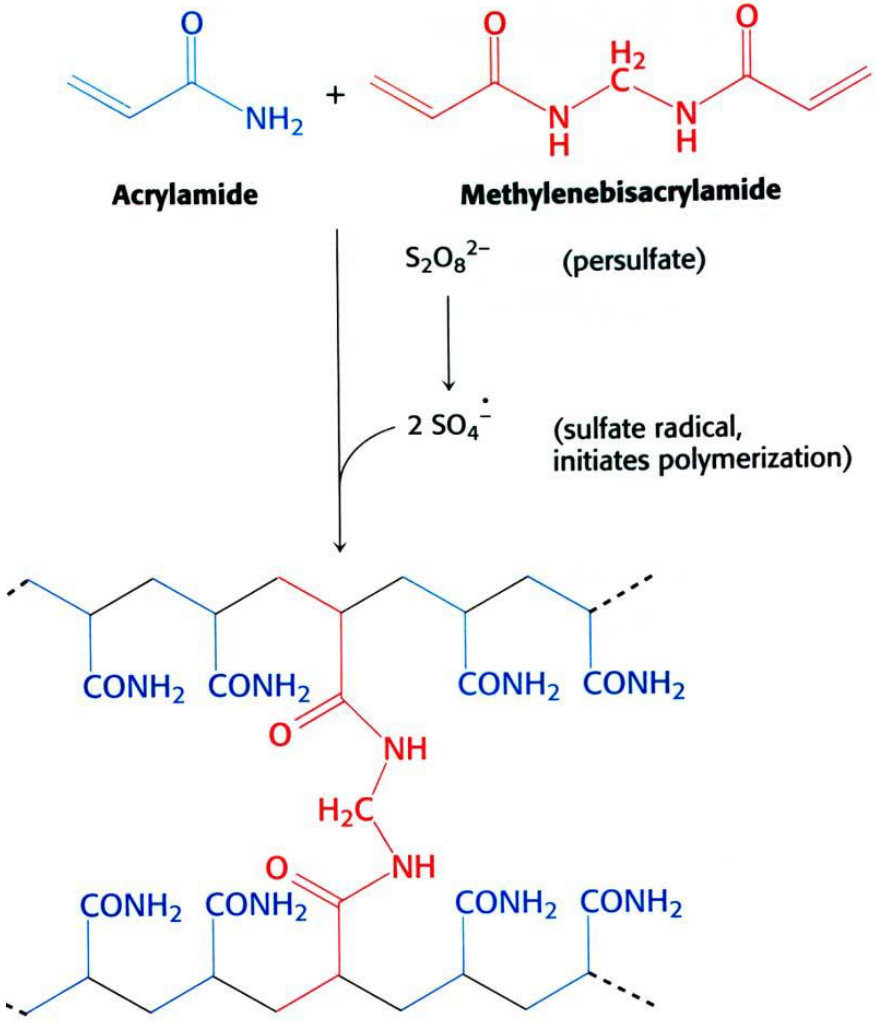
- PAGE is the preferred method for separation of proteins

- Gel prepared immediately before use by polymerization of acrylamide and N,N'-methylene bis-acrylamide.

Polymerization of acrylamide

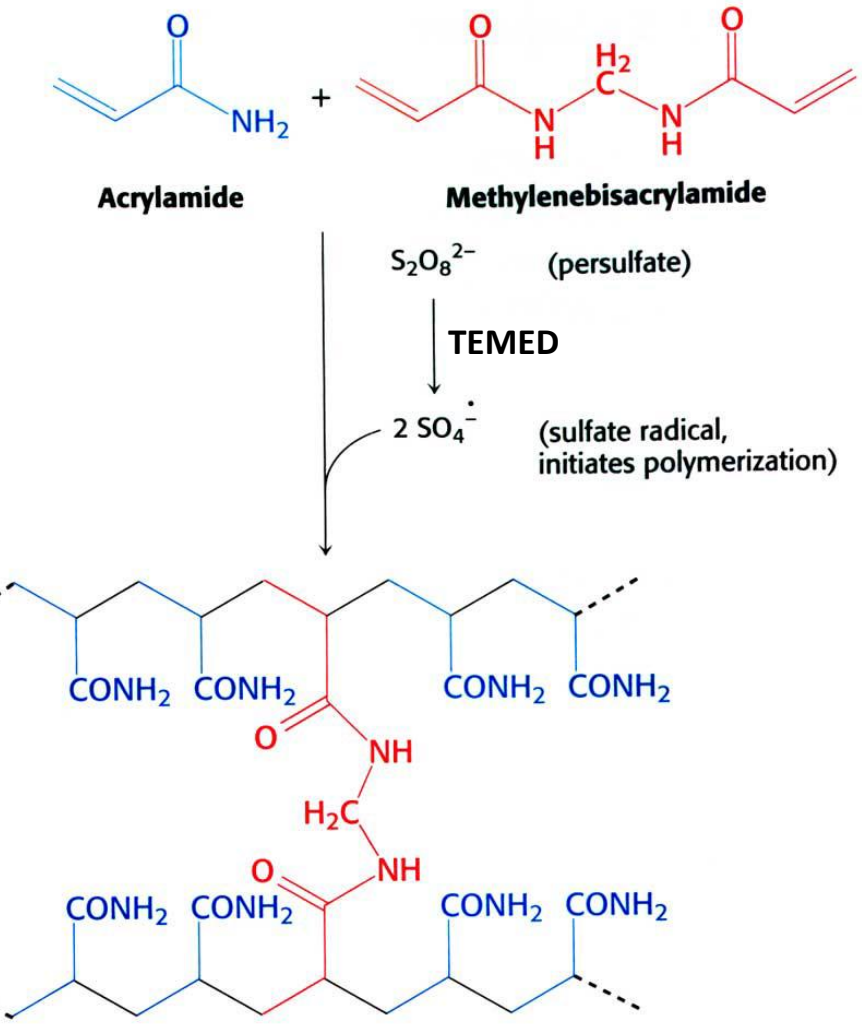
- Cross-linked polyacrylamide gels are formed from the polymerization of acrylamide monomer in the presence of smaller amounts of *N,N'*-methylenebisacrylamide (bis-acrylamide)

- Bisacrylamide is the most frequently used cross linking agent for polyacrylamide gels



Catalyst of polymerization

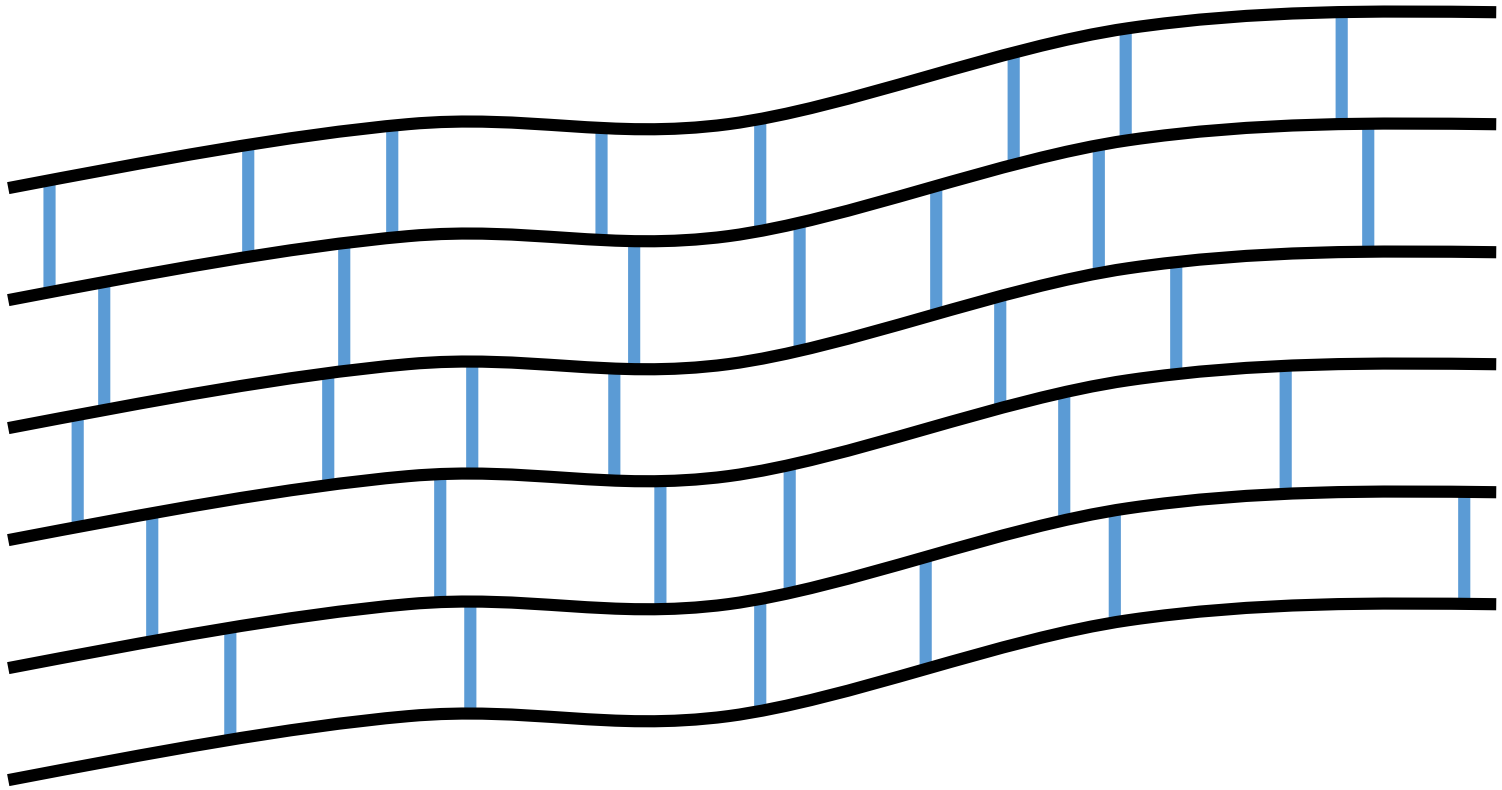
- Polymerization of acrylamide is initiated by the addition of ammonium persulphate and the base *N,N,N',N'*-tetrametyhlenediamine (TEMED)
- TEMED catalyzes the decomposition of the persulphate ion to give a free radical



$$S_2O_8^{2-} + e^- \rightarrow SO_4^{2-} + SO_4^{-\cdot}$$

Polyacrylamide Gels

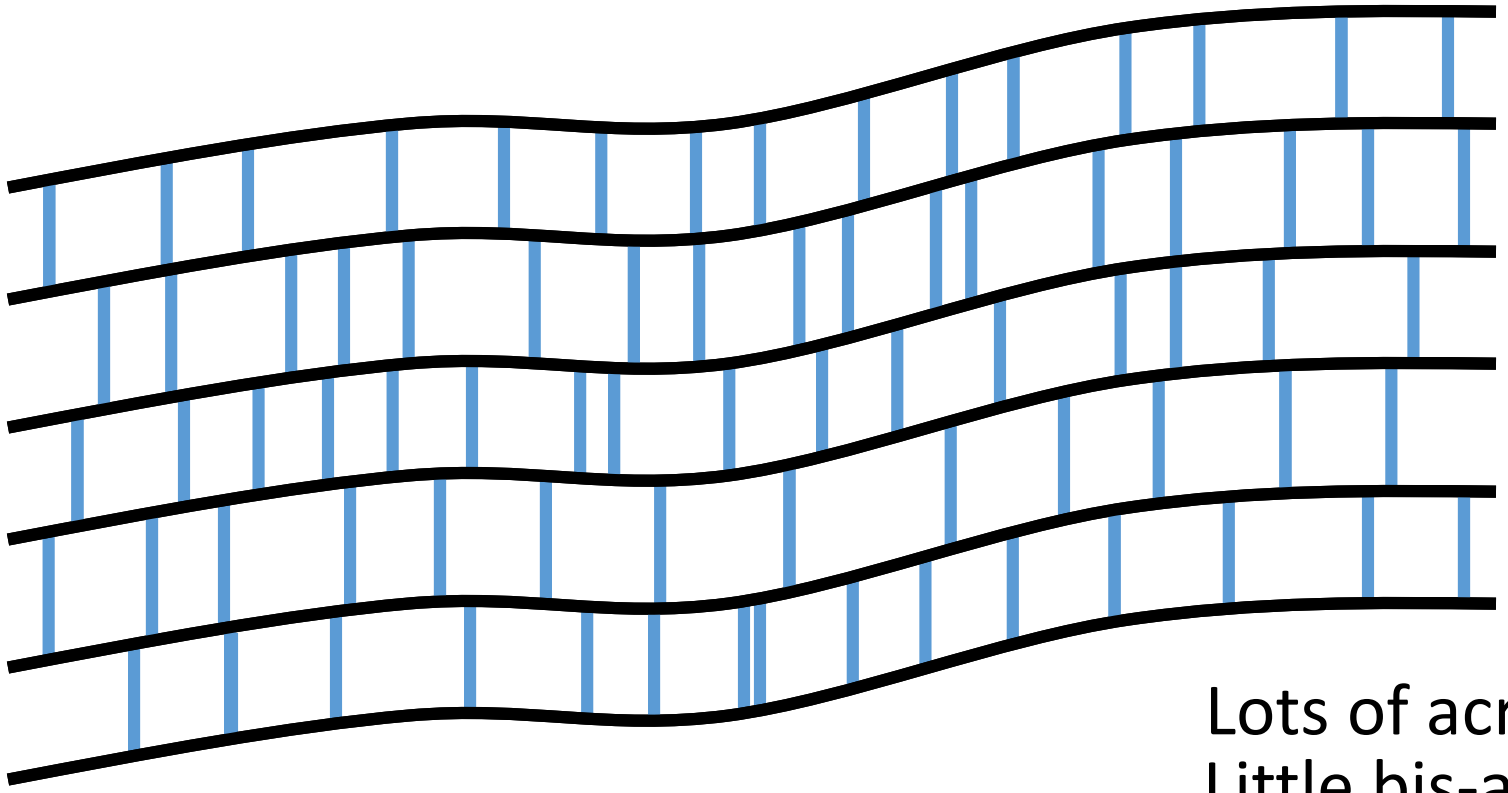
Bis-Acrylamide polymerizes along with acrylamide forming cross-links between acrylamide chains



Polyacrylamide Gels

Pore size in gels can be varied by varying the ratio of acrylamide to bis-acrylamide

Protein separations typically use a 29:1 or 37.5:1 acrylamide to bis ratio

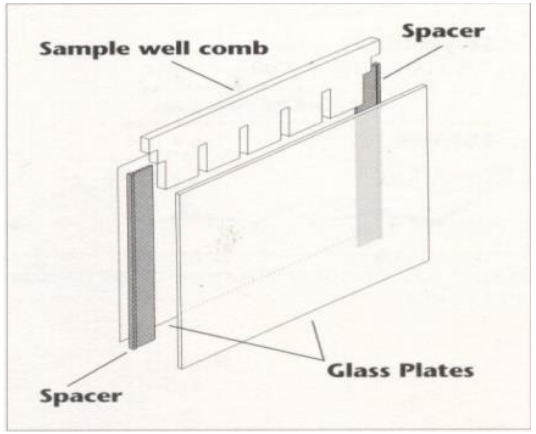


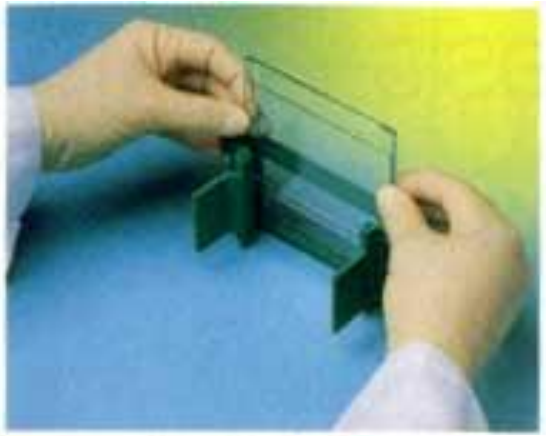
Lots of acrylamide
Little bis-acrylamide

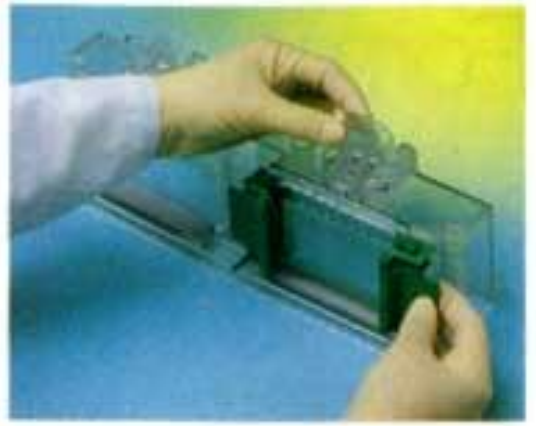
Components of the System

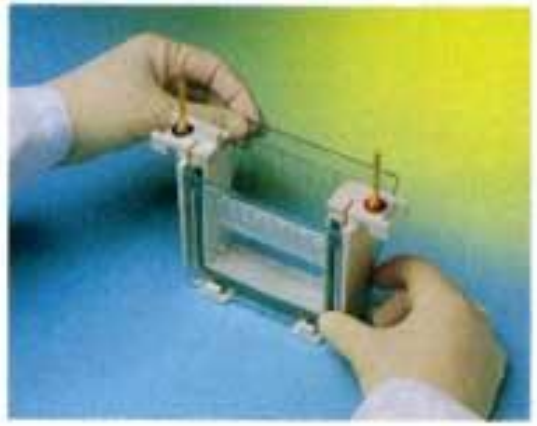
- DC Power Source, Reservoir/Tank, Glass Plates, Spacers, and Combs
- Support medium
  - Gel (Polyacrylamide)
- Buffer System
  - High Buffer Capacity
- Molecules to be separated
  - Proteins
  - Nucleic Acids

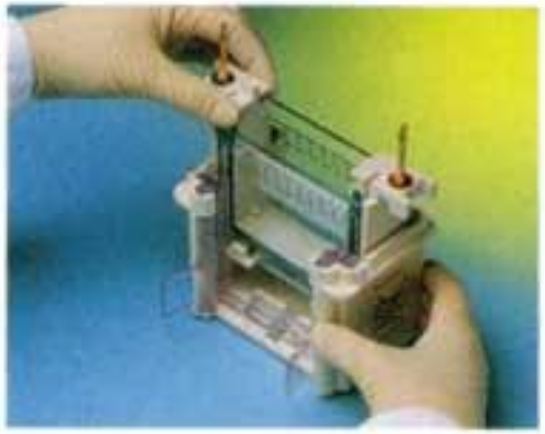
Step by Step Instructions on how to assemble the polyacrylamide gel apparatus











Procedure

- Prepare polyacrylamide gels
- Add diluted samples to the sample buffer (containing SDS and mercaptoethanol)
- Heat to 95°C for 4 minutes
- Load the samples onto polyacrylamide gel
- Run at 150 volts for 30-40 minutes
- Stain

Gel Preparation

Reagent	8% (Running Gel)	5% (Stacking Gel)
Acrylamide/ Bisacrylamide (40%) *	4.0 mls	2.5 mls
1 M Tris-HCl pH 8.8	7.5 mls	7.5 mls
water (distilled)	8.2 mls	9.7 mls
10% SDS	200 µl	200 µl
10% Ammonium Persulfate	100 µl	100 µl
TEMED (added last)	10 µl	10 µl
* = 19:1 w:w ratio of acrylamide to N,N'-methylene bis-acrylamide		

Gel Preparation

- Mix ingredients **GENTLY!** in the order shown in previous slide, ensuring no air bubbles form.
- Pour into glass plate assembly CAREFULLY without bubbles.
- **Overlay gel with isopropanol** to ensure a flat surface and to exclude air.
- Wash off isopropanol with water after gel has set (±15 min).

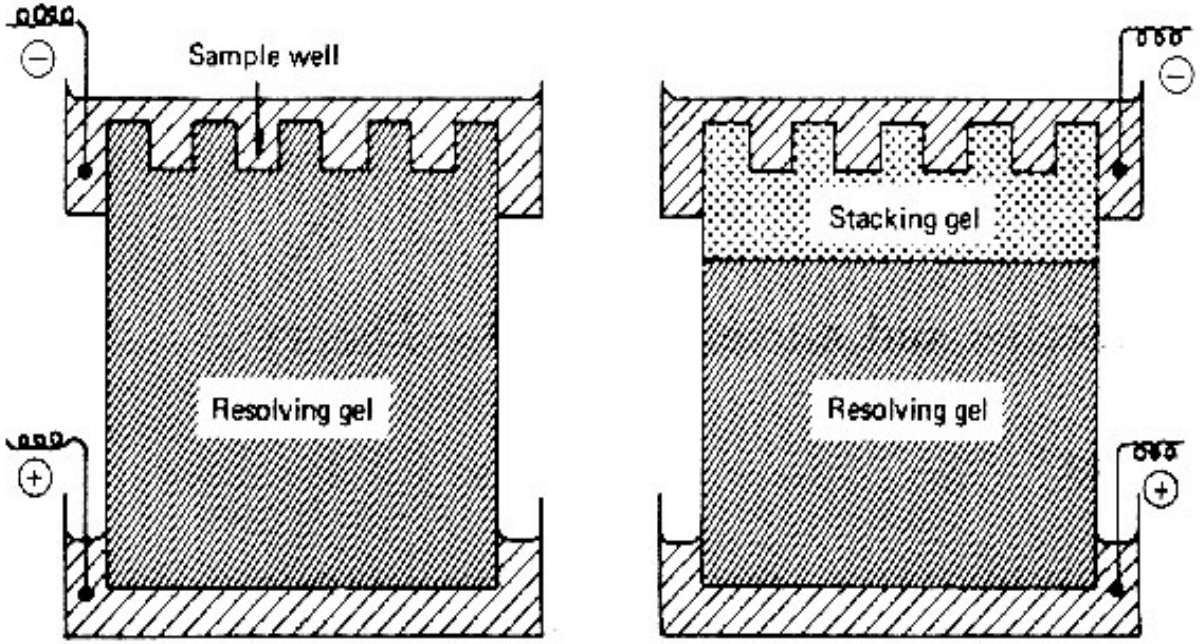
- Polymerization is quicker and more uniform if you degas the first three solutions.
- Molecular oxygen inhibits polymerisation by reacting with the free radical SO_4^- ions
- Oxygen can also lead to oxidation of protein products, which might be crucial if you then want to extract the products and use them for something else (e.g. Sun & Anderson, 2004).
- Finally, having bubbles in your gel can distort the results and make them less reproducible, as the bubbles will not form consistently with each repetition and they disrupt the physical medium of the polyacrylamide. So another purpose of degassing is to ensure repeatability.

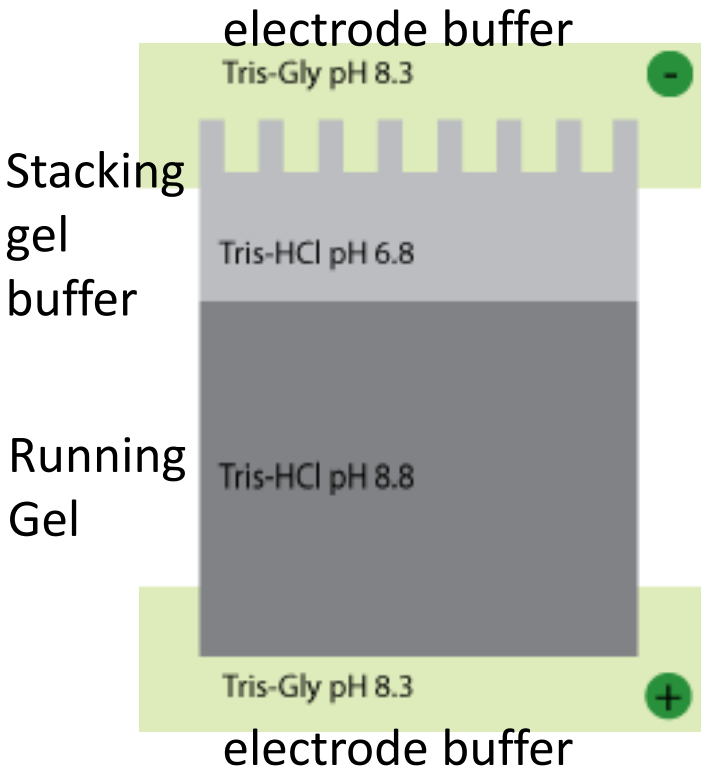
Continuous and Discontinuous Buffer Systems

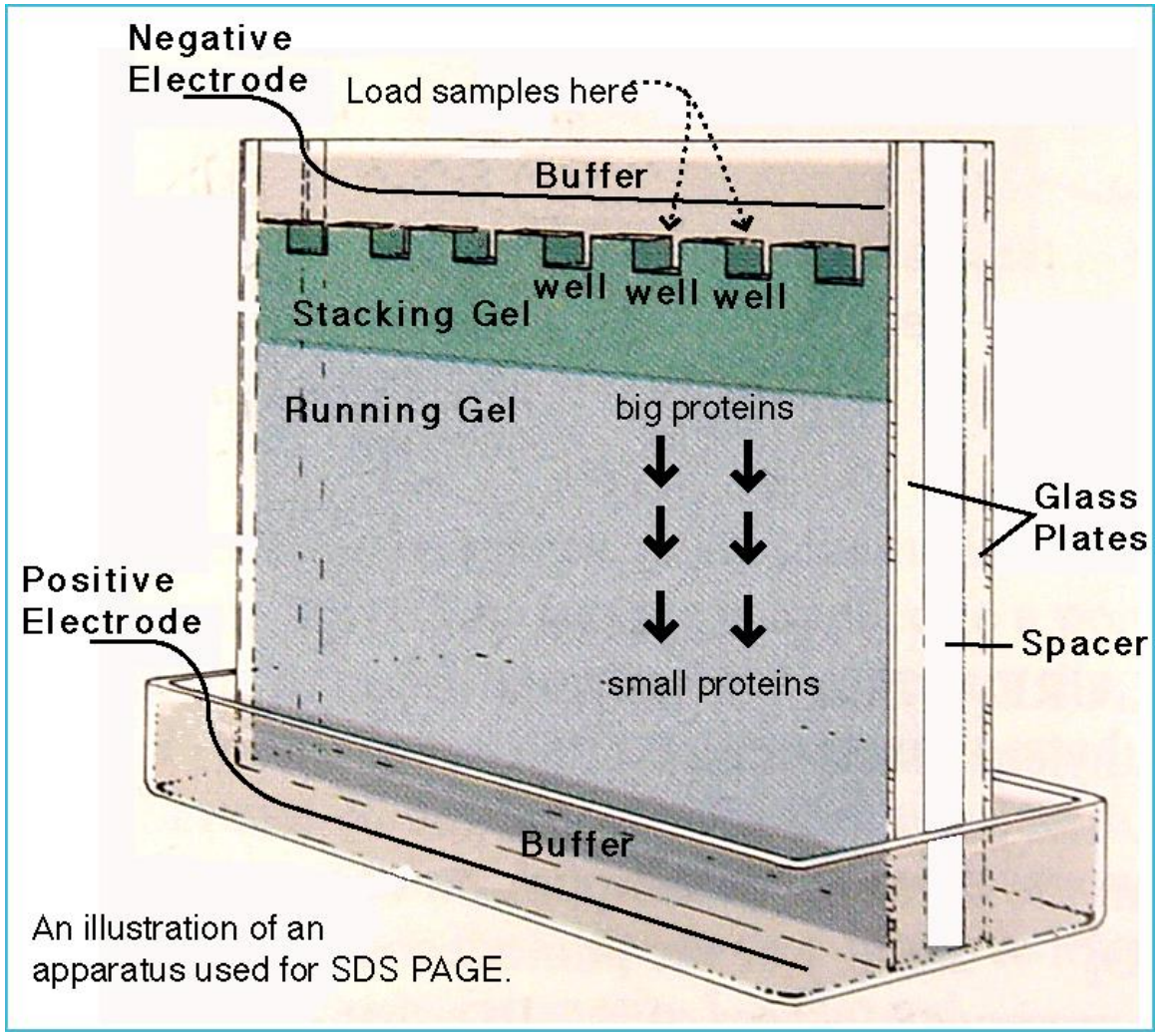
- A continuous system has only a single separating gel and uses the same buffer in the tanks and the gel

- In a discontinuous system a nonrestrictive large pore gel, called a stacking gel, is layered on top of a separating gel

- The resolution obtainable in a discontinuous system is much greater than that obtainable in a continuous one. However, the continuous system is a little easier to set up







Proteins are trapped in between glycine and Cl-ions. They are collected in a very narrow band at the interface of the stacking and running gels.

The negatively-charged glycine ions in the pH 8.3 electrode buffer are forced to enter the stacking gel, where the pH is 6.8. In this environment glycine switches predominantly to the zwitterionic (neutrally charged) state. This loss of charge causes them to move very slowly in the electric field.
The Cl- ions (from Tris-HCl) on the other hand, move much more quickly in the electric field and they form an ion front that migrates ahead of the glycine.

Sample Buffer

- **SDS** (sodium dodecyl sulphate) detergent to dissolve proteins and give them a negative charge
- **Mercaptoethanol** to break disulphide bonds
- **Tris** buffer to provide appropriate pH
- **Glycerol** to make samples sink into wells
- **Bromophenol Blue** dye to visualize samples

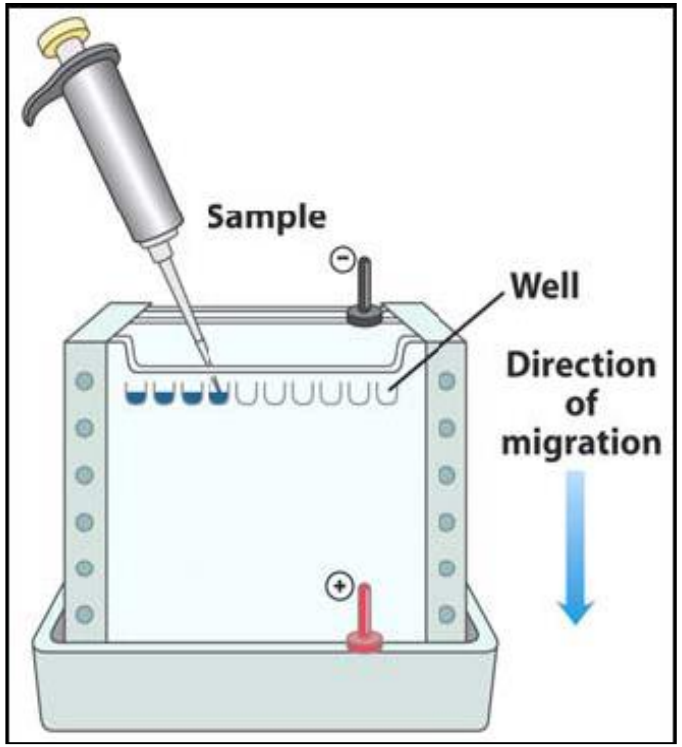
Loading Samples & Running the gel

- Run at 100-150 volts for 30-40 minutes

- **Running Buffer, pH 8.3**

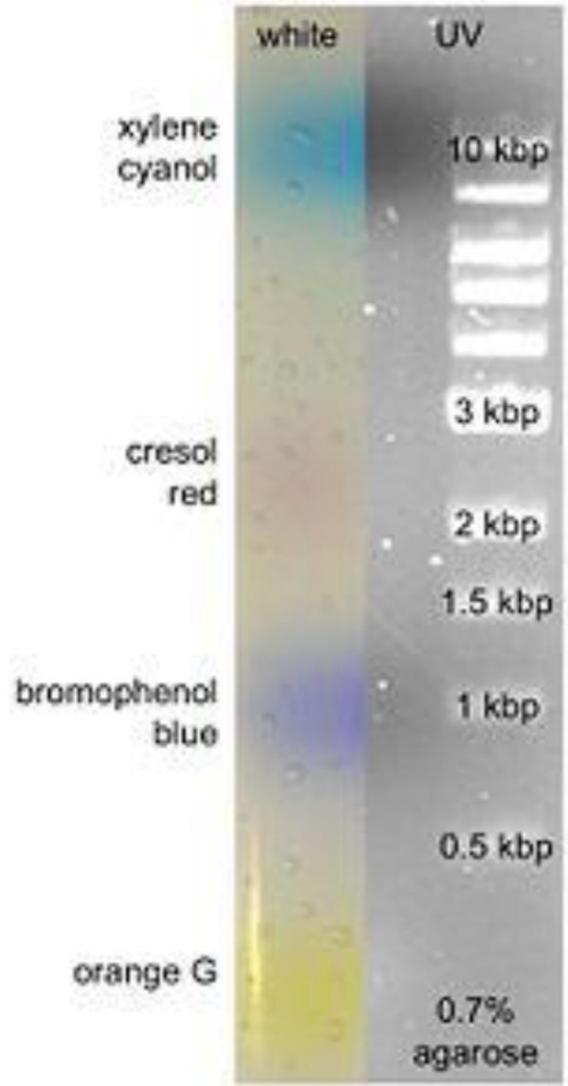
  Tris Base 12.0 g
  Glycine 57.6 g
  SDS 4.0 g

  distilled water to 4 liter



Different loading dyes

- **Ficoll & Orange G (6x)**
  1.5g Ficoll 400
  Orange G dye
  dH_2O to 10mL
- **Sucrose & xylene cyanol / bromophenol blue (6x)**
  4g sucrose
  25mg bromophenol blue or xylene cyanol (0.25%)
  dH_2O to 10mL
- **Glycerol & bromophenol blue (6x)**
  - 3ml glycerol (30%)
  - 25mg bromophenol blue (0.25%)
  - dH_2O to 10mL

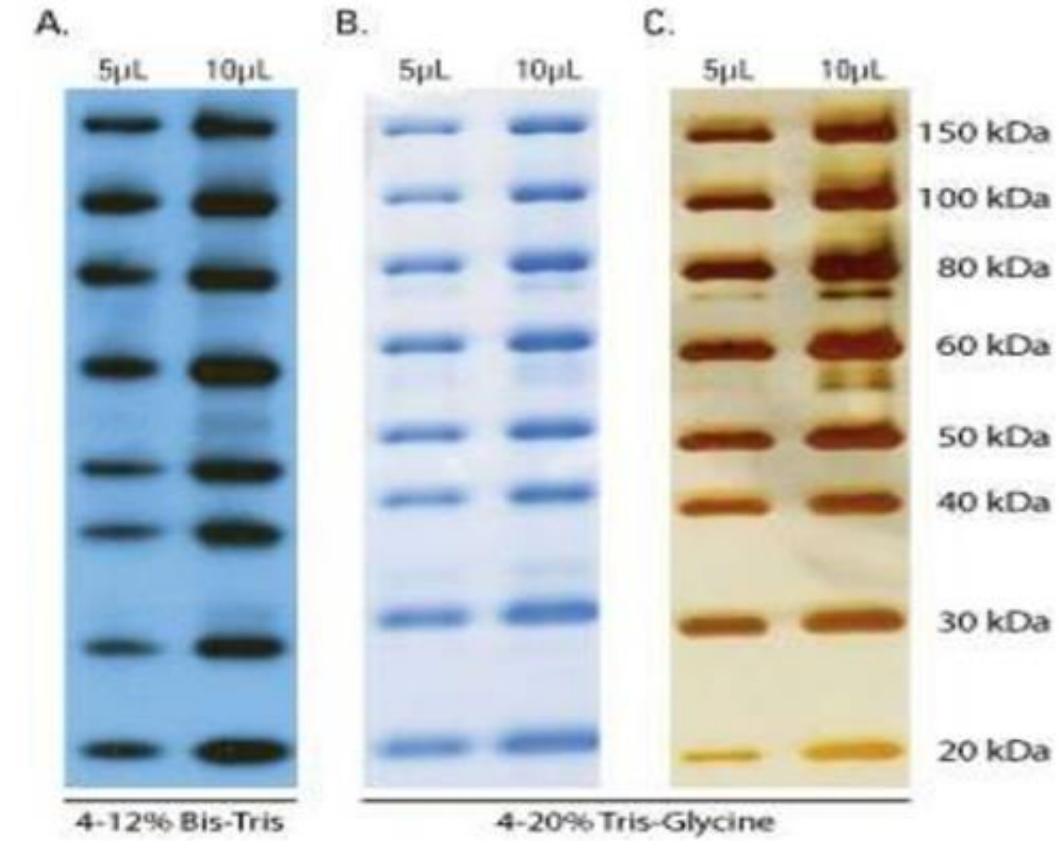


Staining Proteins in Gels

- Chemical stains detect proteins based on differential binding of the stain by the protein molecules and the gel matrix.
- They are nonspecific in action, detecting proteins without regard to their individual identities.
- The important characteristics for a useful stain are: low background, high sensitivity, large linear range and ease of use.

The Coomassie dye binds to proteins through ionic interactions between dye sulfonic acid groups and positive protein amine groups as well as through Van der Waals attractions.

Visualization of protein bands

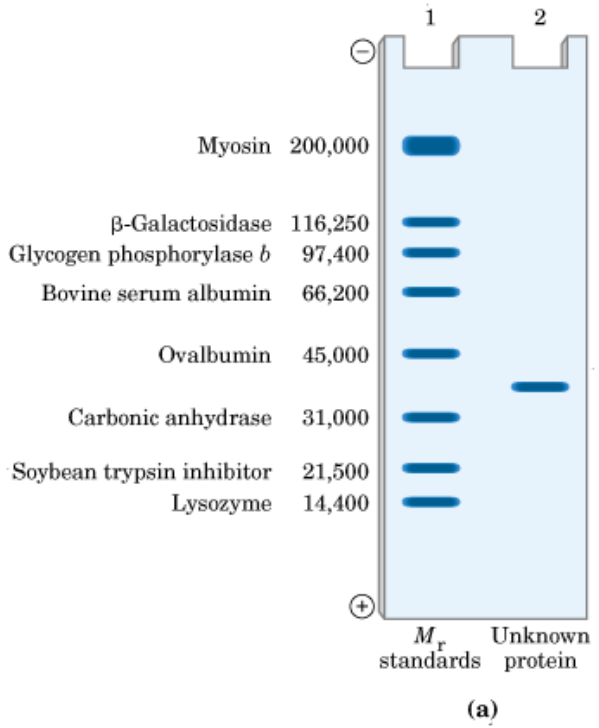


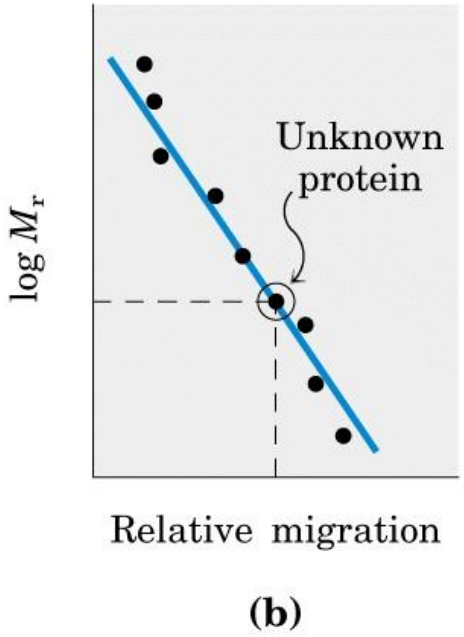
A. Staining band with Western blot; B. Coomassie blue stain; C. Silver stain

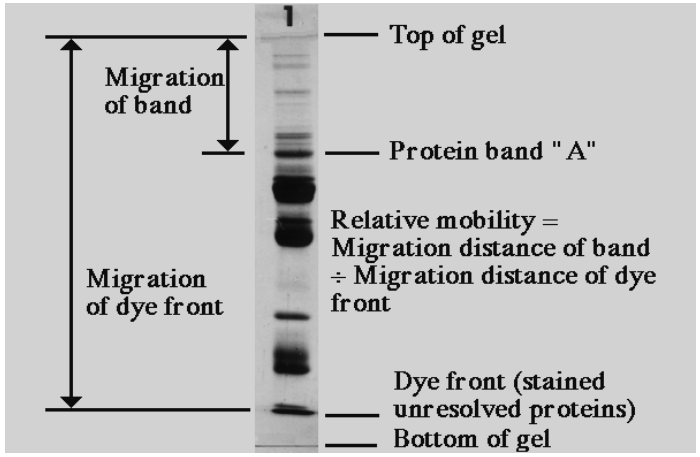
https://www.google.com/search?q=coomassie+blue+staining&source=lnms&tbm=isch&sa=X&ved=0ahUKEwjk_Y62s97KAhWGthoKHcguAQsQ_AUIBygB&biw=1184&bih=539#imgrc=DlUFYwTLZ1dpqM%3A

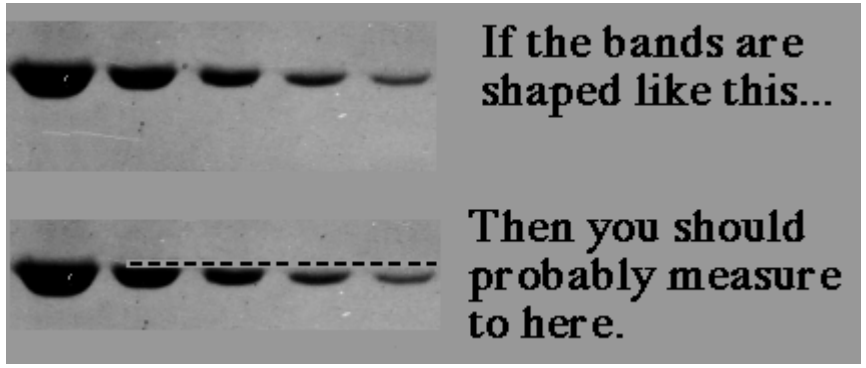
Determining Molecular Weights of Proteins by SDS-PAGE

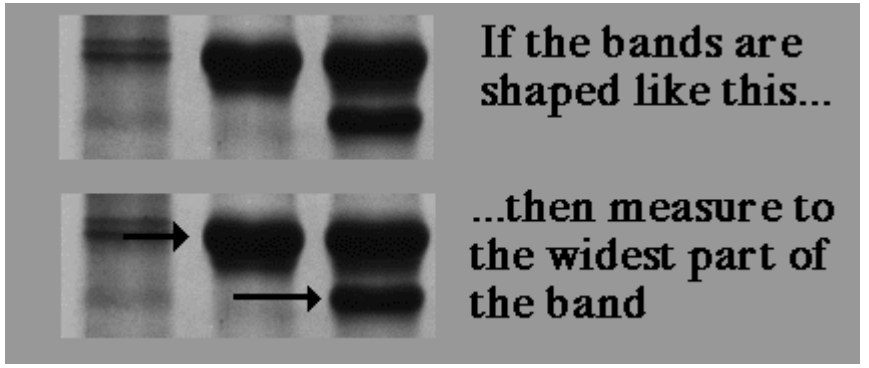
- Run a gel with standard proteins of known molecular weights along with the polypeptide to be characterized
- A linear relationship exists between the log10 of the molecular weight of a polypeptide and its Rf
- Rf = ratio of the distance migrated by the molecule to that migrated by a marker dye-front
- The Rf of the polypeptide to be characterized is determined in the same way, and the log10 of its molecular weight is read directly from the standard curve





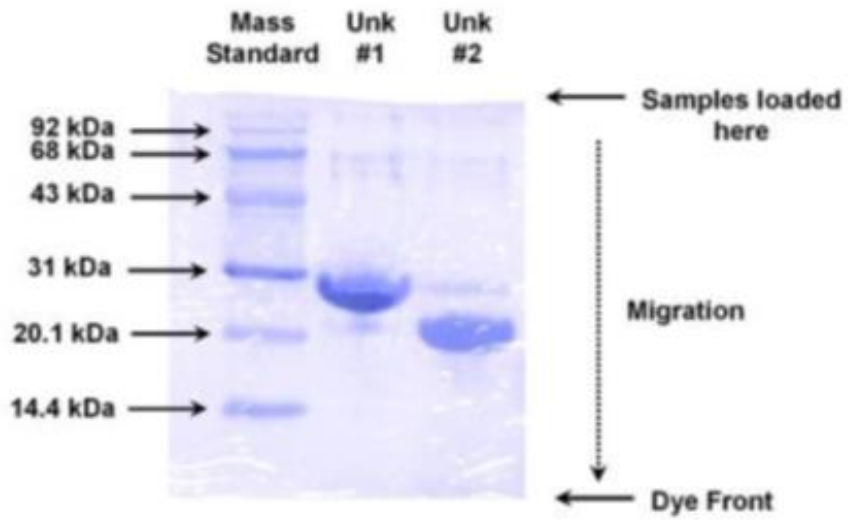


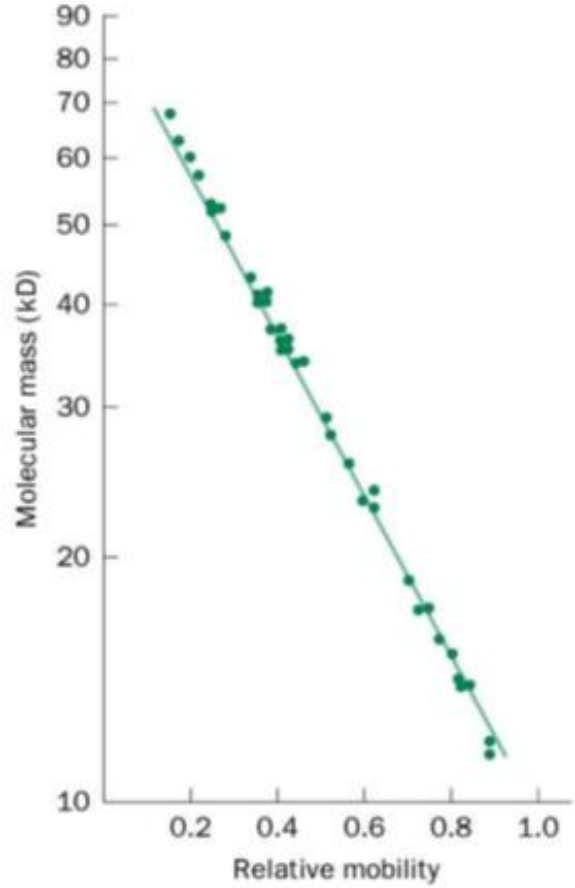




Determining molecular mass

- Plot relative mobility value (x-axis) vs log of MW (y-axis)
- This should normally give linear plot.





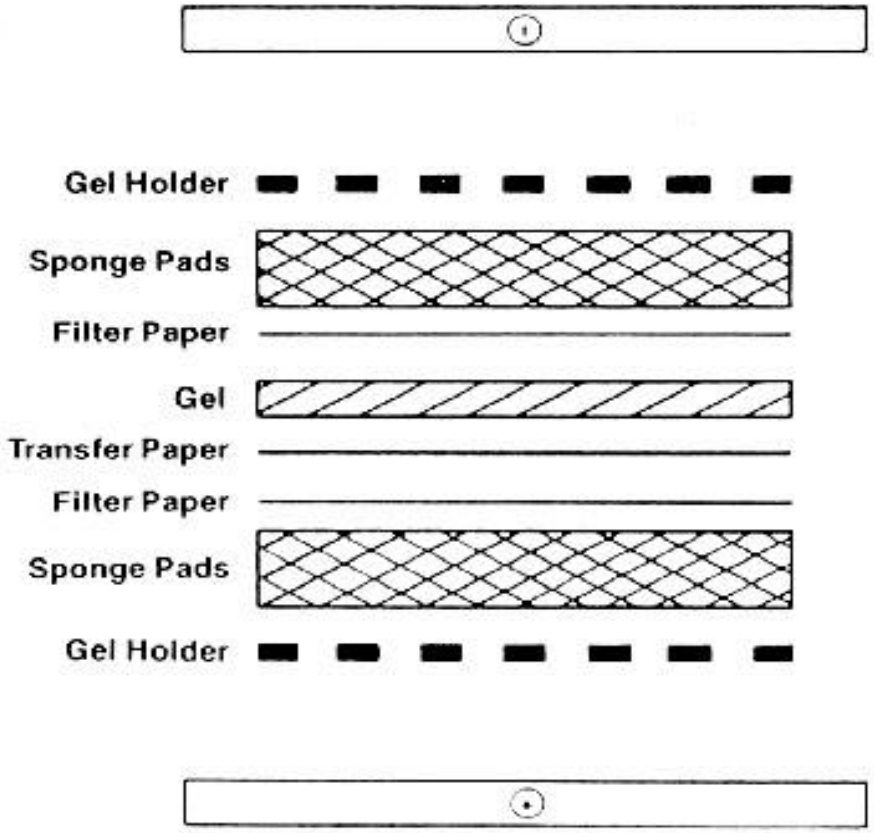
http://www.siumed.edu/~bbartholomew/images/chapter6/F06-25.jpg

Blotting

- Blotting is used to transfer proteins or nucleic acids from a slab gel to a membrane such as nitrocellulose, nylon, etc.

- The transfer of the sample can be done by capillary or Southern blotting for nucleic acids (Southern, 1975) or by electrophoresis for proteins or nucleic acids

Electrophoretic Blotting

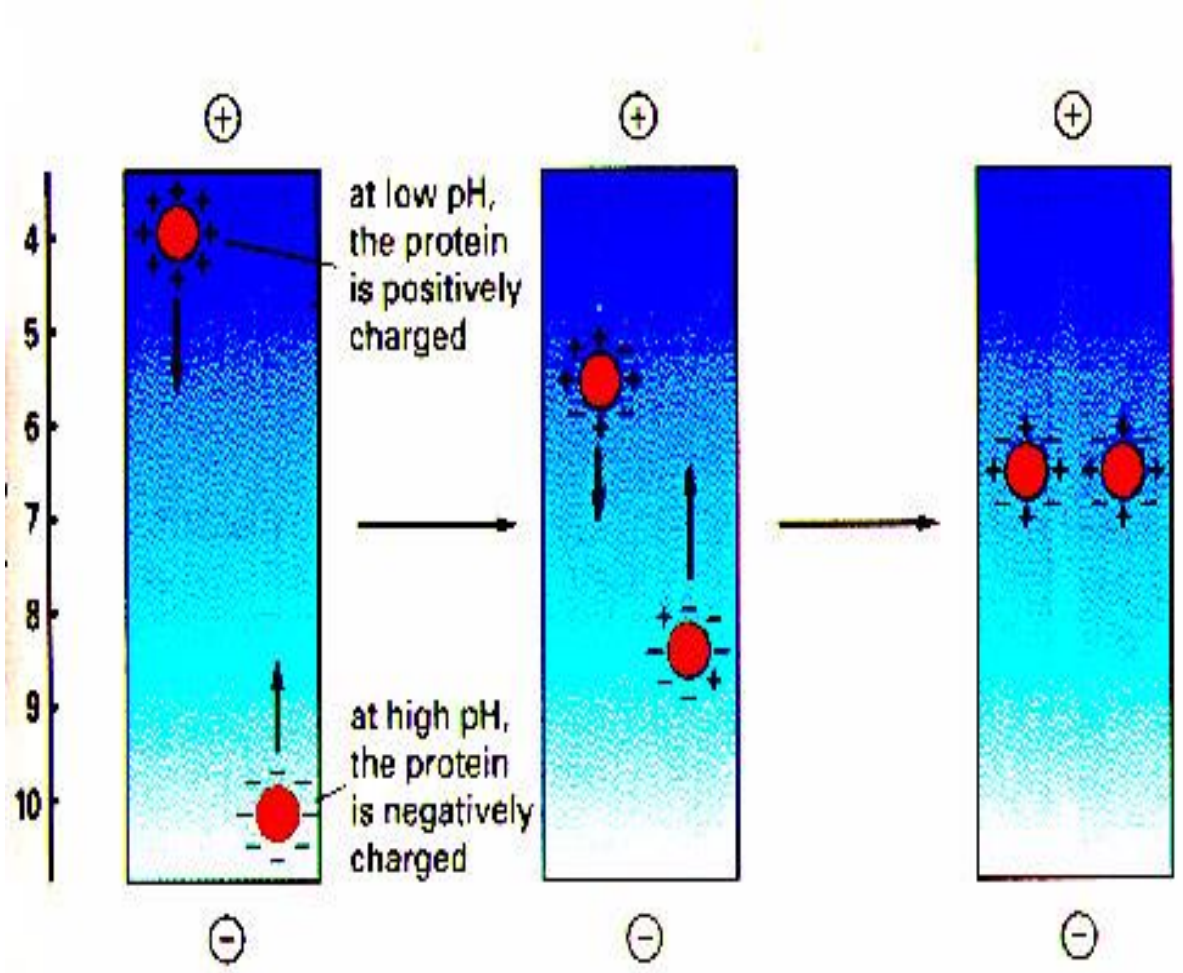


Transblot Turbo from BioRad

http://www.bio-rad.com/en-za/product/trans-blot-turbo-transfer-system?ID=LGOQBW15

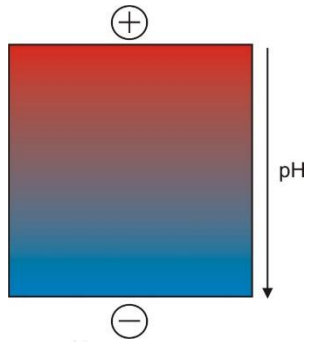
Isoelectric Point

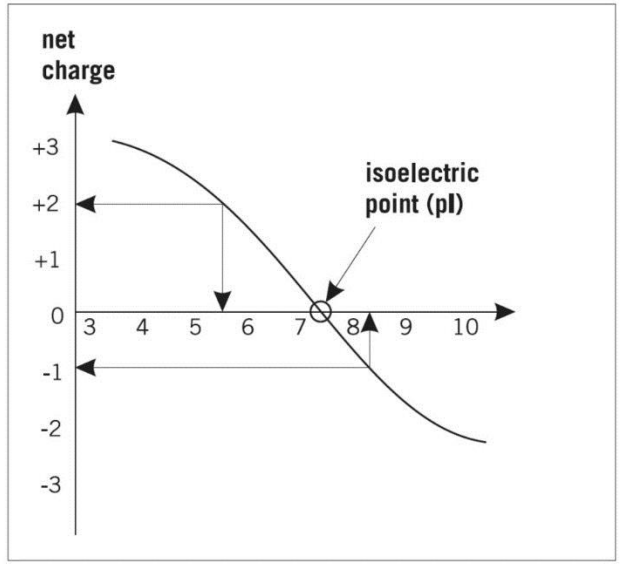
- There is a pH at which there is no net charge on a protein; this is the isoelectric point (pI).
- At pH above its isoelectric point, a protein has a net negative charge and migrates toward the anode in an electrical field.
- At pH below its isoelectric point, the protein is positive and migrates toward the cathode.

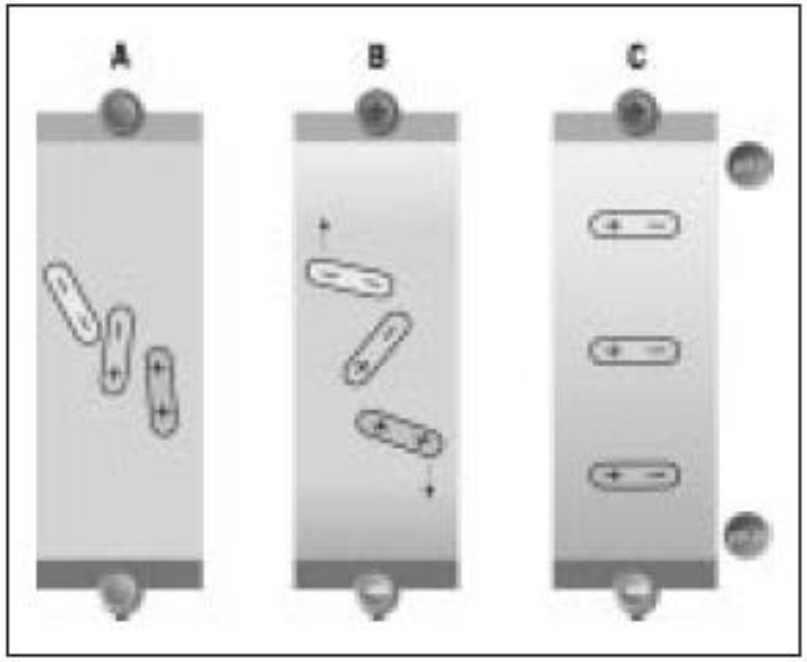


Isoelectric Focusing

- Isoelectric focusing is a method in which proteins are separated in a pH gradient according to their isoelectric points
- Focusing occurs in two stages; first, the pH gradient is formed
- In the second stage, the proteins begin their migration toward the anode if their net charge is negative, or toward the cathode if their net charge is positive
- When a protein reaches its isoelectric point (pI) in the pH gradient, it carries a net charge of zero and will stop migrating







Limitations of SDS-PAGE or IEF

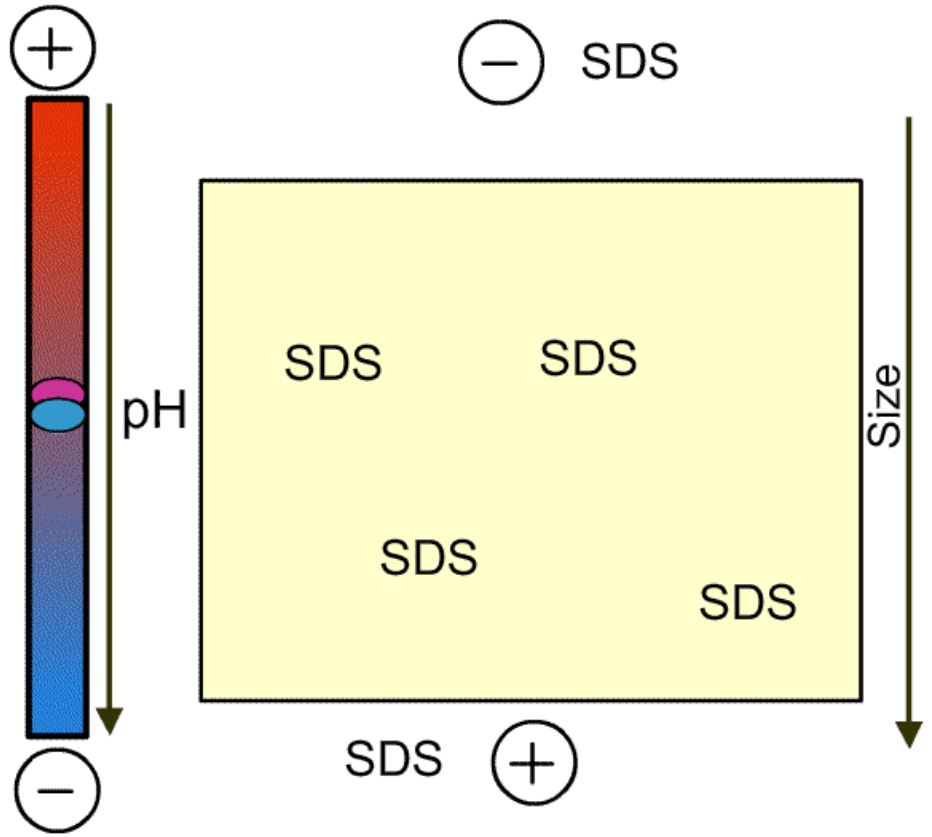
-A limited number (20~30) proteins may be separated by SDS-PAGE/IEF.
-Some proteins may have either same pI or same molecular weight.
-Cellular proteome consists of about 2-3 thousand proteins.

Solutions

- Combination of SDS-PAGE/IEF
- The technique is called 2-D electrophoresis

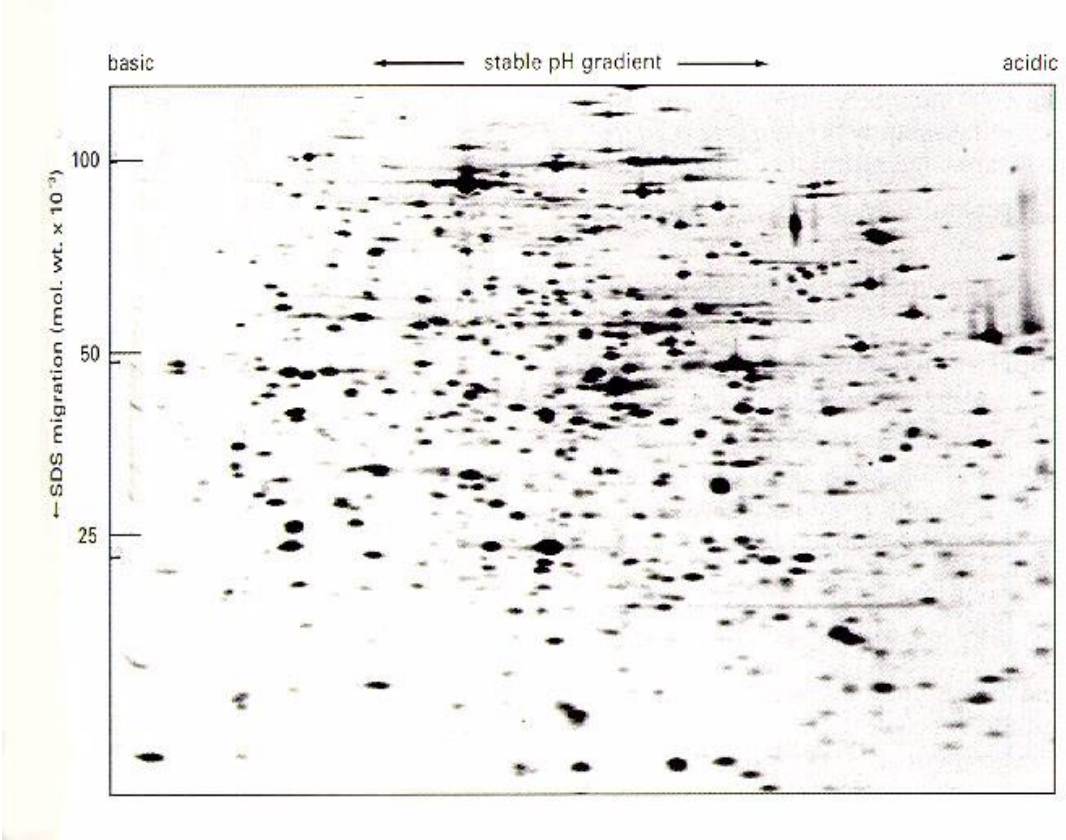
Two-Dimensional Gel Electrophoresis

- Two-dimensional gel electrophoresis is widely used to separate complex mixtures of proteins into many more components than is possible in conventional one-dimensional electrophoresis
- Each dimension separates proteins according to different properties



O'Farrell 2D Gel System

- The first dimension tube gel is electrofocused (IEF)- charge, pH
- The second dimension is an SDS slab gel- Size
- The analysis of 2-D gels is more complex than that of one-dimensional gels because the components that show up as spots rather than as bands must be assigned x, y coordinates

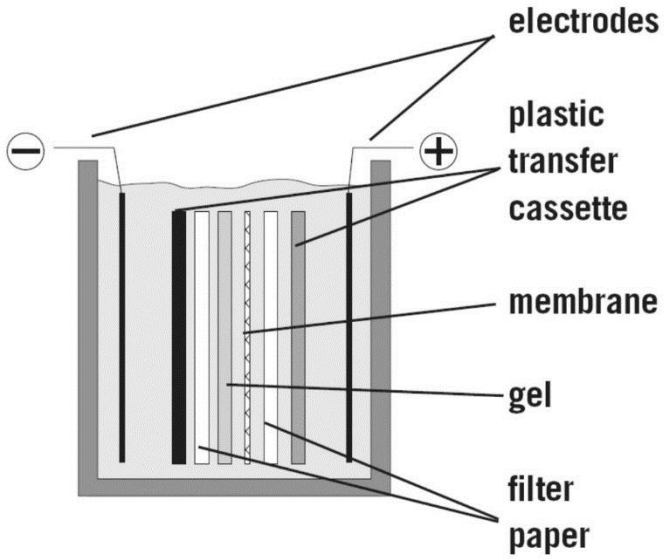


Western Blotting (WB)

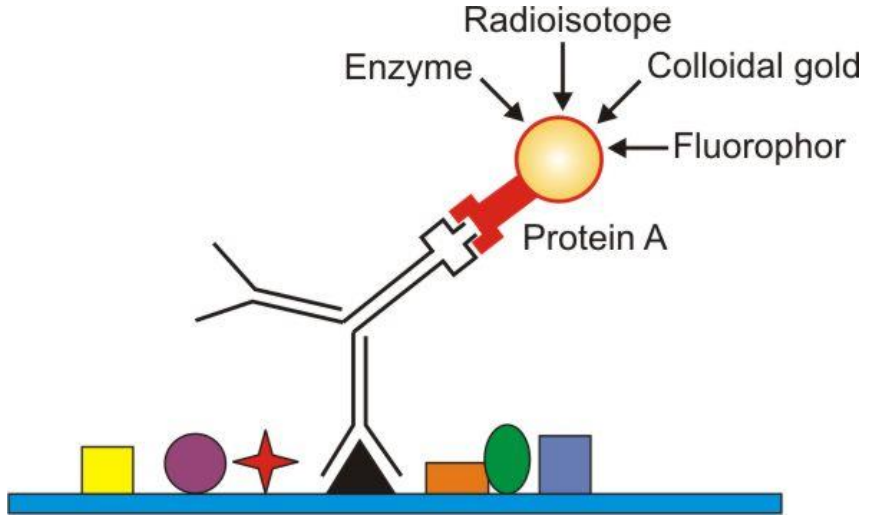
- WB is a protein detection technique that combines the separation power of SDS PAGE together with high recognition specificity of antibodies
- An antibody against the target protein could be purified from serum of animals (mice, rabbits, goats) immunized with this protein
- Alternatively, if protein contains a commonly used tag or epitope, an antibody against the tag/epitope could be purchased from a commercial source (e.g. anti-6 His antibody)

WB: 4 steps

1. Separation of proteins using SDS PAGE
2. Transfer of the proteins onto e.g. a nitrocellulose membrane (blotting)
3. Immune reactions
4. Visualization



Step 2: Blotting



Step 3 & 4: Detection

Considerations with PAGE

- Analysis of Gel
  - Staining or autoradiography followed by densitometry
  - Blotting to a membrane (by electrophoresis), for nucleic acid hybridization, autoradiography or immunodetection

Purpose of buffers and reagents in electrophoresis

- **1. N, N, N', N'-tetramethylethylenediamine (TEMED)-**it catalyzes the acrylamide polymerization.
- **2. Ammonium persulfate (APS)-**it is an initiator for the acrylamide polymerization.
- **3. Tris-HCl-** it is the component of running and gel casting buffer.
- **4. Glycine-** it is the component of running buffer. It serves as a buffering agent, maintaining pH and preventing sample damage during electrophoresis.
- **5. Bromophenol blue-** it is the tracking dye to monitor the progress of gel electrophoresis.
- **6. Coomassie brilliant blue -**it is used to stain the polyacrylamide gel.
- **7. Sodium dodecyl sulphate-**it is used to denature and provlde negative charge to the protein.
- **8. Acrylamide-** monomeric unit used to prepare the gel.
- **9. Bis-acrylamide-** cross linker for polymerization of acrylamide monomer to form gel.

Extra Resources/animation

- http://learn.genetics.utah.edu/content/labs/gel/
- https://www.youtube.com/watch?v=3CrzY7jb9fQ