BCHEM 365
Lecture 5
September 18, 2018

Other support media for gel electrophoresis

- Polyacrylamide, starch
- Polyacrylamide gels: most commonly used gel for the following reasons:
- i) They are very stable
- ii) They can be prepared in a wide range of concentrations to give a variety of pore sizes
- iii) They can be made to have a gradient of concentrations in one gel slab
- iv) Polyacrylamide gels are good for separating proteins of size 5 to 2,000 kDa because unlike agarose it gives uniform pore size even at high concentration
- Polyacrylamide gel was widely used in the Maxam-Gilbert or Sanger DNA sequencing for separation of small fragments up to 1 bp difference
- Electrophoresis in acrylamide gels is often referred to as PAGE (**p**oly**a**crylamide **g**el **e**lectrophoresis)

Polyacrylamide gels

Preparation of polyacrylamide gel

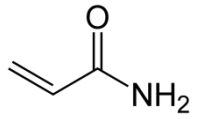
- Two methods are used to polymerize acrylamide: chemical polymerization and photopolymerization

Chemical polymerization

You need the following reagents:

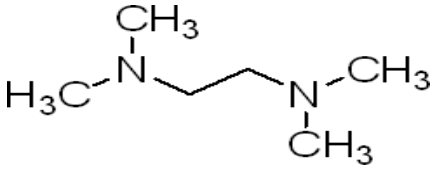
- Acrylamide as the monomer
- A cross-linking agent: N,N’-methylene bis-acrylamide (normally referred to as bis-acrylamide)
- An initiator of the polymerization process: ammonium persulfate
- A catalyst: N,N,N’,N’-tetramethylenediamine (TEMED)

Reagents for chemical polymerization of acrylamide



Acrylamide: molecular formula C_3H_5NO

Molar mass: 71.08 g/mol. A white odorless crystalline solid. A known neurotoxin and suspected carcinogen

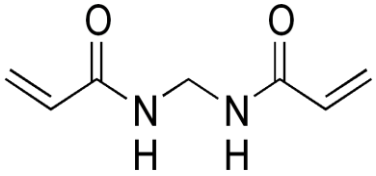


N,N,N' N'-tetramethylenediamine (TEMED)- the catalyst

$(NH_4)_2S_2O_8$

Ammonium persulfate: molar mass 228.2 g/mol. Free radical is produced in this way:

$S_2O_8^{2-}$ + initiator catalyst → $SO_4^{\bullet -}$ + $SO_4^{\bullet -}$



N,N'-methylene-bis-acrylamide: molecular formula $C_7H_{10}N_2O_2$

Molar mass 154.17 g/mol

A crosslinking reagent

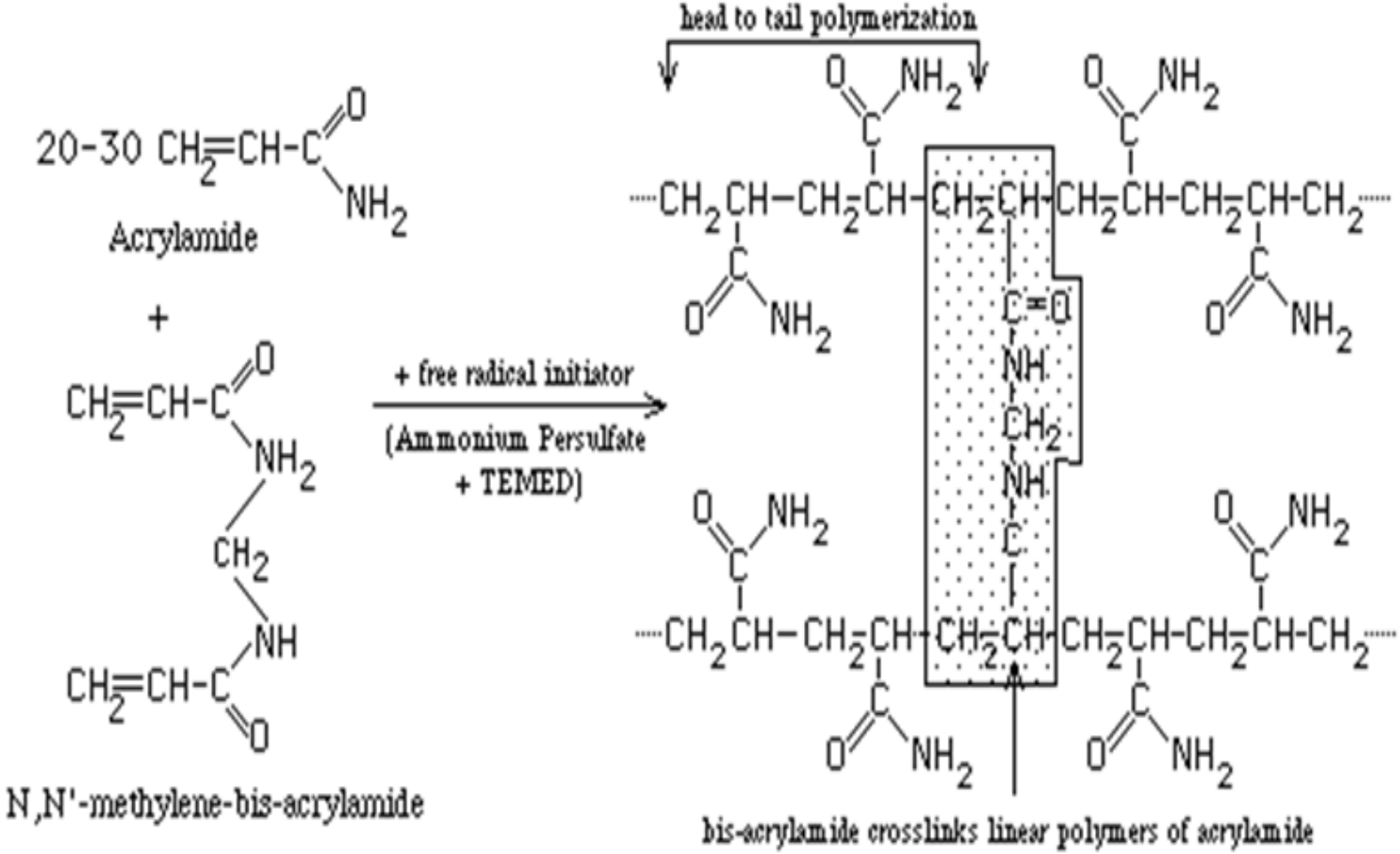
Chemical polymerization

- Proceeds by a free radical polymerization process. Upon addition of ammonium persulfate to acrylamide, the ammonium persulfate dissolves in the water used to preprae the acrylamide reagent to form free radicals (free radical is a molecule with an unpaired electron represented by a dot). This process is short-lived, slow, hence needs a catalyst
- An additional catalyst/initiator TEMED is added to enhance the free radical production from persulfate and continue the polymerization process
- TEMED catalyzes the decomposition of persulfate ion to give a free radical
- $S_2O_8^{2-}$ + initiator => $SO_4^{\bullet -}$ + $SO_4^{\bullet -}$
- The free radical initiates polymerization process through **vinyl addition** of the monomers

Chemical polymerization

- Vinyl addition of acrylamide monomers occurs in a **head-to-tail fashion** to build long and linear chains of polyacrylamide
- Addition of bis-acrylamide leads to **copolymerization** of the straight polymer chains to produce a cross-linked polyacrylamide
- Bis-acrylamide is essentially two acrylamide molecules linked by a methylene group and therefore is able to set up a copolymerization reaction in which long and linear polyacrylamide chains are occasionally interspersed with bis-acrylamide molecule, thereby introducing a second site for chain extension

Head-to-tail and copolymerization of acrylamide to form a polyacrylamide network (gel) held together by bis-acrylamide crosslinks



Formation of polyacrylamide gel from acrylamide and bis-acrylamide

- The result is a fairly well-defined three dimensional network of crosslinked polyacrylamide having a range of **pore sizes** and **rigidity**
- The pore size and rigidity can be controlled by the experimenter to enable separation of a wide range of sample sizes.
- Pore size is controlled by changing the **concentration** and **ratio** of acrylamide monomer and bis-acrylamide

Rate of polymerization of acrylamide gels

- The rate of polymerization is dependent on

(1) The net concentration of monomers and initiators

(2) Temperature

(3) Purity of the reagents

All three should be controlled for reproducibility. Reagents should be electrophoresis grade and deionized water should be used

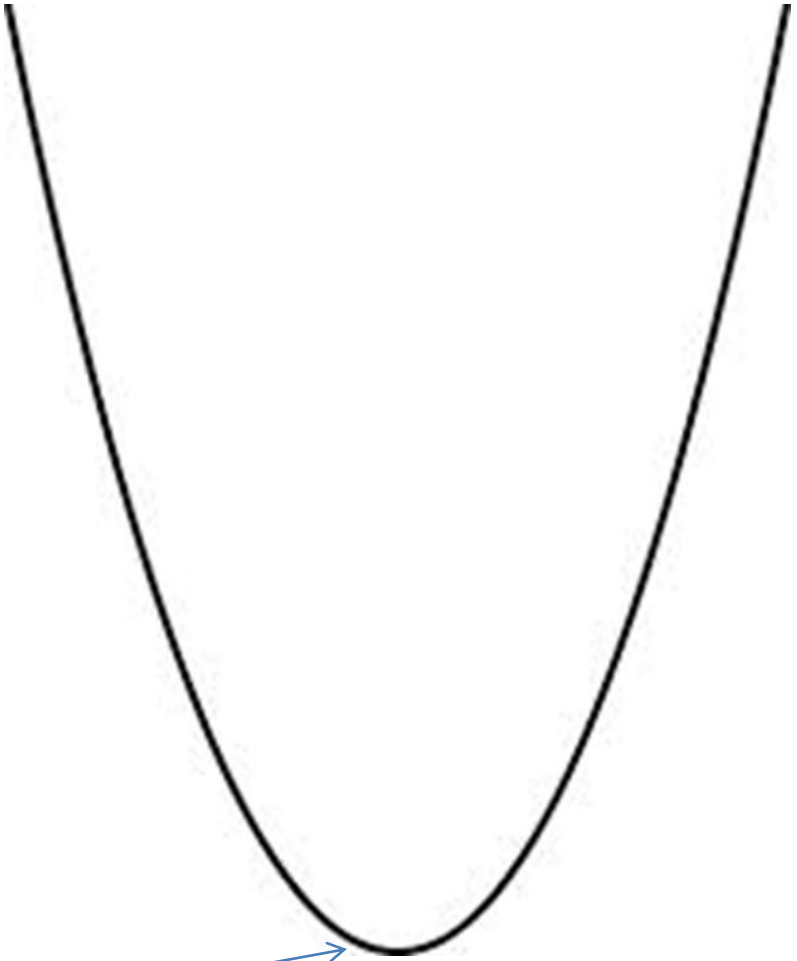
- For highest quality results, dissolved oxygen should be removed from the monomer mixtures by degassing them, since oxygen decreases the rate of polymerization. This is done by briefly placing it under vacuum to remove dissolved air prior to use . Degassing of the gel also serves to remove heat that is liberated during polymerization.
- Polymerization of acrylamide is an exothermic reaction. Warming up of the gel solution as it sets can liberate air bubbles that become trapped in the polymerized gel.
- Air bubbles in the gel affect resolution of the sample

Polyacrylamide gels: pore size and rigidity

- The ratio of bisacrylamide to acrylamide, as well as the total concentration of both components affect the pore size and rigidity of the final gel matrix. These, in turn, affect the range of protein sizes (molecular weights) that can be resolved

- By convention, polyacrylamide gels are characterized by a pair of values, %T and %C, where %T is the concentration of total monomer (acrylamide + bis) in g/100 ml and %C is the proportion of **bis** alone as a percentage of total monomer.

- The effective pore size of a polyacrylamide gel is an **inverse function** of the total monomer concentration (%T) and a **biphasic function** of %C. When %T is increased at a fixed %C, the number of chains increases and the pore size decreases continuously. On the other hand, when %T is held constant and %C is increased from low values, pore size decreases to a minimum at about 5%C.



Pore size

At 5% Bis-concentration, pore size is minimum

Bis concentration

Polyacrylamide gels: pore size and rigidity

- With further increases in %C from the minimum of 5%, pore size increases, probably due to the formation of shorter, thicker bundles of polymer chains.
- A plot of pore size against %C should give a parabola
- Typically 5-20% by weight (5%, 7.5%, 10%, 12.5%, 15%, 20% are commonly used values). To vary pore size, fix concentration of BIS at 5% and vary monomer concentration. Implies gel is mostly water.
- Gels with low %T (e.g., 7.5%T) are used to separate large proteins, while gels with high %T (e.g., 15%T) are used with small proteins.

Properties of polyacrylamide gels

- Polyacrylamide gels are hydrophilic and electrically neutral at the time they are cast. They are transparent to light at wavelengths above 250 nm and do not bind to protein stains.
- The gel has both solid (rigid fibers) and entrapped liquid components which create a three-dimensional shape for the gel. Without the liquid, the gel will dry to a thin film. At the same time, the polymer fibers hold the liquid firmly and prevent it from escape.
- Polyacrylamide gels are well suited for protein electrophoresis

Polyacrylamide gels

- TEMED is a base, hence polymerization is most efficient at alkaline pH. Polymerization efficiency falls rapidly at pH values below 6
- Photopolymerization with riboflavin is used for low-pH gels

Polyacrylamide gels

Photopolymerization

- In this method, riboflavin replaces ammonium persulfate and TEMED. When the gel is poured, it is placed in front of bright light for 2-3 hours
- Riboflavin is decomposed by light to generate free radicals that initiate polymerization of acrylamide

RE-cap on pore size of acrylamide gels

- Polyacrylamide gels can be made to have varying pore sizes
- The size of the pores within the gel can be varied/controlled by changing the concentrations of both the acrylamide and bis-acrylamide

Gel electrophoresis of proteins

- Gel electrophoresis is a broad subject encompassing many different techniques
- There are many variations of gel electrophoresis of proteins
- Gel electrophoresis reveals information about molecular weight, subunit structure, purity, and charges on proteins
- The most common use of gel electrophoresis is preparative and qualitative analysis of complex mixtures of proteins
- When combined with microanalytical methods and sensitive, linear image analysis systems, gel electrophoresis can be used for quantitative studies of proteins
- The technique provides the highest resolution of all methods available for separating proteins. Polypeptides differing in molecular weight by as little as a few hundreds of Daltons and proteins differing by less than 0.1 pH unit in their isoelectric points are routinely resolved on gels.

Websites for electrophoresis equipment and knowhow

- www.cleaverscientific.com
- www.biorad.com
- www.cbdscientific.com

Variations in gel electrophoresis of proteins

- Tube vs. slab gel electrophoresis
- One dimensional (1D) vs. two dimensional (2D) gel electrophoresis
- Native vs. denaturing electrophoresis
- Continuous vs. discontinuous gel systems

Tube vs. slab gel electrophoresis

- Acrylamide can be polymerized into any desired shape
- Two shapes used for electrophoresis
  - **Tube Gels** : polymerize in glass tubing to give cylindrical shaped gel
  - **Slab Gels:** polymerize between glass plates

Tube gel electrophoresis

- Polymerization is carried out in glass tubes of dimensions 7 mm × 10 cm in length
- For isoelectric focusing (I.E.F.) gels and 1st dimension of 2-D electrophoresis
- **Advantages of tube gels:**
- Tube gels are easy to load
- Technique requires minimum use of apparatus
- **Disadvantages:**
- Only one sample can be run per tube, however, there are many places for 12, 24, etc. tubes per vacutainer
- Inaccurate comparison among different samples as different tubes would have different conditions

Apparatus includes solid vacutainer stoppers for variable size tubes. Features cooling jacket for temperature control, leveling base and interlocking safety cover

Slab gel electrophoresis

- Vertical gel slab sizes range from small (2 cm × 3 cm) to large (15 cm × 18 cm)
- **Advantages** of **s**mall vertical gels
  - Require less time and reagents than large gels
  - They are good for rapid screening of samples
- **Advantages** of large vertical gels
  - Provide better resolution
  - Good for separating proteins of similar sizes , as well as a large number of proteins
- Precast slab gels are commercially available for techniques such as SDS-PAGE, native gels, and iso-electric focusing

Slab gel electrophoresis

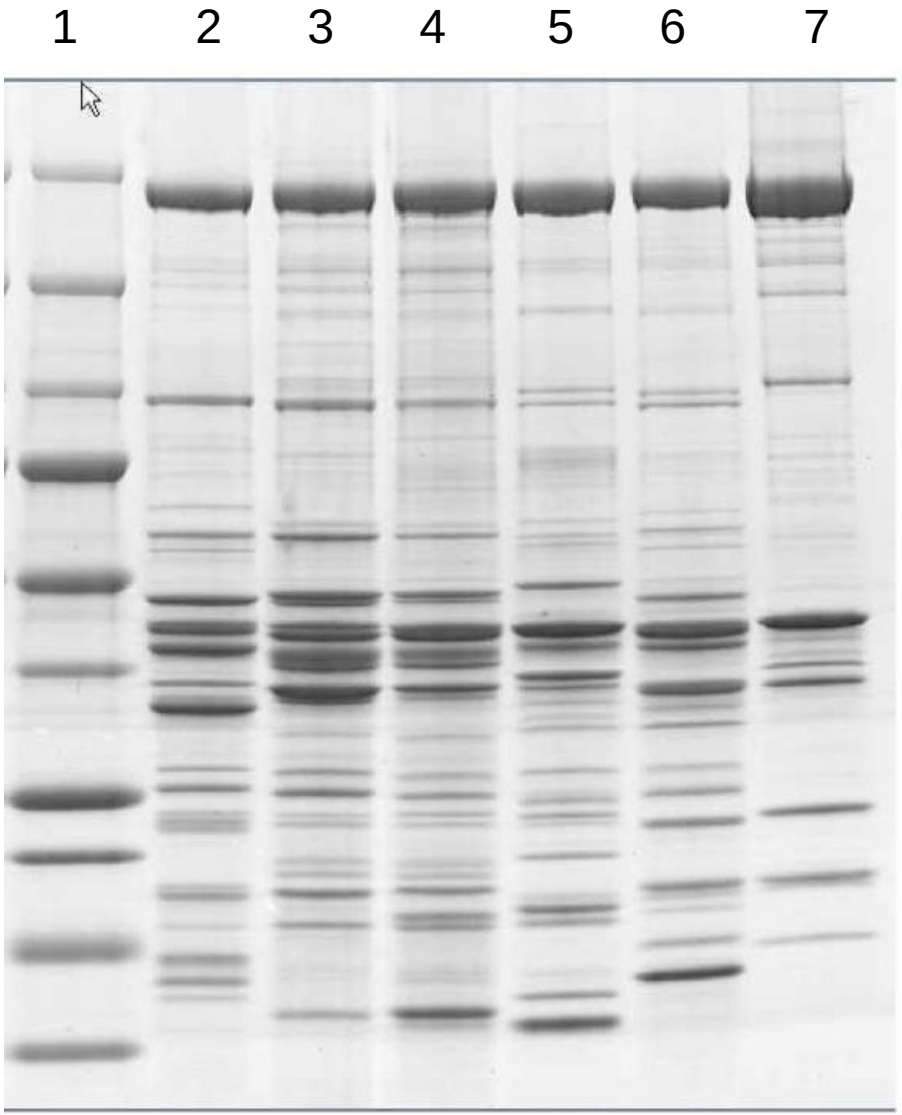
- **Slab gels offer the following advantages**
  - Permit analysis of up to 20 samples on a single gel slab having identical gel conditions
- Applications of vertical gel slabs
  - For routine analysis of proteins
  - For separation of DNA fragments during DNA sequence analysis

One dimensional vs. two dimensional electrophoresis

- Electrophoresis can be one dimensional (1D) (i.e. one plane of separation) or two dimensional (2D)
- One dimensional electrophoresis is used for most routine protein and nucleic acid separations
- It is also used for comparative analysis of multiple samples
- Gel sizes range from 2 cm × 3 cm (tiny) to 15 cm × 18 cm (large format). The most popular size (8 cm × 10 cm) is usually referred to as a "Mini-gel“
- An example of a one-dimensional separation of proteins is shown below. In this configuration, the protein pattern is one of multiple bands with each band containing one protein or a limited number of proteins with similar molecular weights

A typical analytical SDS-PAGE gel showing muscle proteins from five fish varieties and a control from rabbit muscle extract. Samples were separated by SDS-PAGE in a precast mid-size-gel and stained with colloidal CBB G-250.

Lanes are from left to right: marker proteins, shark, salmon, trout, catfish, sturgeon, and rabbit (actin and myosin). The salmon and trout patterns (lanes 4 and 5) are very similar, as expected given the close evolutionary relationship between the two species. All of the fish samples appear to contain muscle proteins similar to those of rabbit.



One dimensional vs. two dimensional electrophoresis

- Samples that are loaded in adjacent wells and electrophoresed together are easily compared to each other after staining or some other detection step.
- The intensity of staining and "thickness" of protein bands are indicative of their **relative abundance**.
- The position (height) of bands measured from the start point within their respective lanes indicates their **relative sizes** (and/or other factors affecting their rate of migration through the gel, such as shape).
- Below, we find an electrophoregram of **protein lanes and bands in 1D SDS-PAGE.** Photograph of a mini-gel after removal from the cassette and staining with Coomassie dye (Thermo Scientific GelCode Blue Stain Reagent). The mini-gel has ten lanes, each containing many protein bands of varying abundance.

Precast protein gels for SDS-PAGE

A Thermo Scientific Pierce Precise Protein Gel Cassette. The plastic cassette contains a mini-gel that is 1 mm thick. Dividers along the top provide 10 wells for loading protein samples and molecular weight markers (ladder). Ordinarily, protein bands would not be visible until after electrophoresis, disassembly of the cassette and staining of the gel.

One dimensional vs. two dimensional electrophoresis

- Two dimensional separation of proteins is used for fingerprinting, and when properly constructed can be extremely accurate in resolving all of the proteins present within a cell (greater than 1,500).
- Two-dimensional separation of nucleic acids is used to resolve over 3,000 transcripts in gene expression studies

Native vs. denaturing gel electrophoresis

- Native: electrophoresis is carried out in the absence of denaturing agents such as heat, reducing agents. Also called **nondenaturing** gel electrophoresis
- The buffer system used is nonreducing and nondenaturing which maintains the protein's secondary structure and native charge density
- In native –PAGE most proteins have a slightly positive charge and migrate toward the negative pole, the cathode
- Some proteins may have pI greater than 8 or 9 and will migrate toward anode. In this case, it may be necessary to reverse the anode
- In nondenaturing gels, proteins are not denatured and are therefore separated based on their charge-to-mass ratio

Native Gel Electrophoresis

- There are two methods:

(1) Nondenaturing polyacrylamide gel electrophoresis of proteins

(2) Nondenaturing polyacrylamide gel electrophoresis of nucleic acids

- For proteins, the nondenaturing gel retains the activity of native proteins, because the folded structure, charge, shape and size are preserved allowing the structure of the protein to drive the mobility in the gel. For DNA, the small pore sizes of the polyacrylamide gel is used for small amplified DNA sequences in fingerprinting (10 bp to 3,000 bp)
- Native PAGE does not provide direct measurement of molecular weight, but can be used to estimate the charge on a protein or subunit composition

Native Gel Electrophoresis

- Native PAGE is good for separating proteins of identical molecular weight which cannot be resolved with SDS-PAGE.
- Because proteins on native PAGE usually retain their activity, the technique can be used to detect enzymes in their biologically active form
- Conversely, electrophoresis of proteins in the presence of a reducing agent, or a detergent, such as sodium dodecyl sulfate (SDS) unfolds the protein.
- Electrophoresis of DNA in the presence of urea and formamide denatures DNA
- Urea and formamide disrupt the hydrogen bonds between the two strands so that migration of DNA on a gel does not depend on its shape

Native Gel Electrophoresis

- Separation of proteins on native gel depends on the mobility of the protein
- Mobility on native gels is a function of charge, mass, and isoelectric point of the protein and is governed by the equation

$$\log R_m = \log Y_0 - K_R T \ldots\ldots (1)$$

where R_m = relative mobility, normalized to the tracking dye front or some other standard

Y_0 = relative mobility of the protein in free solution, i.e., in the absence of any sieving matrix or a gel. Y_o is related to the charge on a protein

K_R = retardation coefficient or mechanical resistance. It measures the extent to which a gel matrix impedes mobility. It is elated to MW

T = % monomer (acrylamide)of the gel matrix

Native Gel Electrophoresis

- In the presence of SDS, all proteins have the same charge-to-mass ratio and identical relative mobilities, Y_0
- In such a case, a simple relationship exists between R_m and K_R when %T is known. In a free solution therefore, SDS-treated proteins will migrate at the same speed. Knowing K_R means that one can calculate mobility
- K_R is directly related to molecular weight and so molecular weight can be calculated from K_R.

Native Gel Electrophoresis

- In native gels, the situation is more complicated. Both Y_0 and K_R can vary between proteins. Y_0 is related to the charge, while K_R varies with molecular mass or size of the protein.
- Y_0 and K_R are obtained from graphs known as **Ferguson Plots**
- To plot such graphs, protein mixtures and standards are separated on native polyacrylamide gels of varying percentages of acrylamide (%T)

Native Gel Electrophoresis

- The distance travelled by a protein, log R_m , is plotted against %T.

$$\log R_m = \log Y_0 - K_R T \ldots\ldots (1)$$

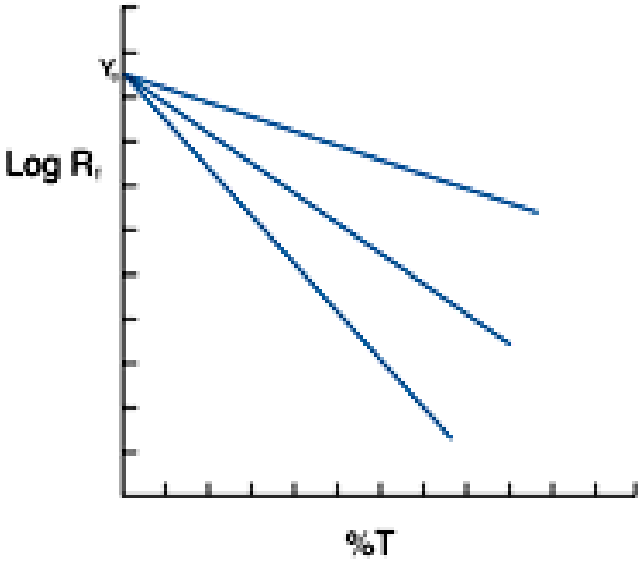
- From equation (1), the graph would have a slope of

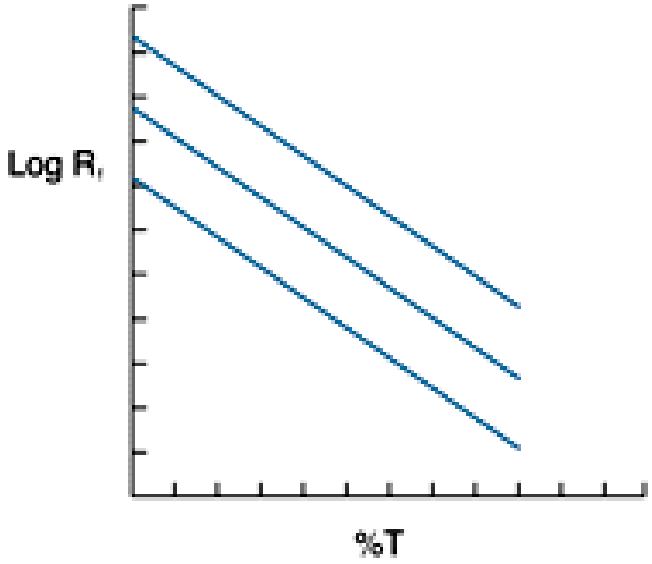
$-K_R$ and a Y intercept (when %T = 0) of Y_o

- Comparison with standards of known charge and size allows determination of the charge and molecular weight of the unknown samples.

Native Gel Electrophoresis

Ferguson Plots





Three proteins of the same charge (Y_0) but different mass

Three proteins of different charges but same mass, i.e., slope (K_R)

Native Gradient PAGE

- Native gradient gel electrophoresis enhances the process by using the pore size of the gel to estimate the molecular weight of a sample
- A gradient gel is made with increasing acrylamide concentration along the length of the gel, hence pore size gradually decreases
- As proteins migrate they enter smaller pore size range and their mobility decreases
- Eventually, each protein reaches its "pore-limit", at which point they stop migrating

Native Gradient PAGE

- At the pore limit, there is no movement and the relative positions are a direct reflection of their molecular weight.
- A plot of log MW against the distance moved by each protein (R_m) gives a sigmoid curve. Within the linear gradient range of this curve, where the log MW is proportional to log R_m

Native Gradient PAGE

- Proteins separated by native gradient gels may be visualized by the general protein staining reagents as well as **activity stains** where the protein is likely to be an enzyme. These are stains which enable visualization of enzyme activity.
- Activity staining involves the use of **chromogenic** or **chemiluminescent** compound which reacts with the enzyme (the protein separated) to deposit a colored, insoluble compound in the gel, or a luminous product, respectively.
- If the reaction is a direct involvement with the enzyme to produce color, we have a positive stain. If the colored compound is formed in a reaction inhibited by the enzyme, then we have a negative stain

Native gel electrophoresis for DNA

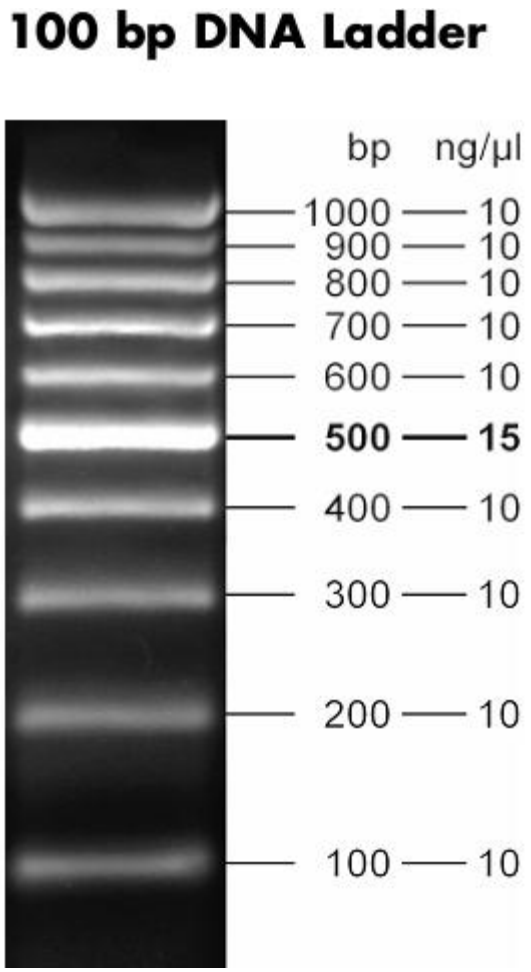
Nondenaturing polyacrylamide gel electrophoresis is used for DNA fingerprinting in marker systems such as RAPDs and SSRs to examine small fragment sizes (10-300 bp) amplified by the primers

RAPDs = Random Amplified Polymorphic DNA sequences

SSRs = Simple Sequence Repeats

It is recommended that the researcher begins with 12% of 29:1 acrylamide/bis-acrylamide as the starting point

Concentration may be reduced (e.g., to 8%) or increased (e.g., to 12%) for larger or smaller fragments, respectively. For nondenaturing gels, Tris-glycine buffer (25 mM Trizma-base, 192 mM glycine) may be used.

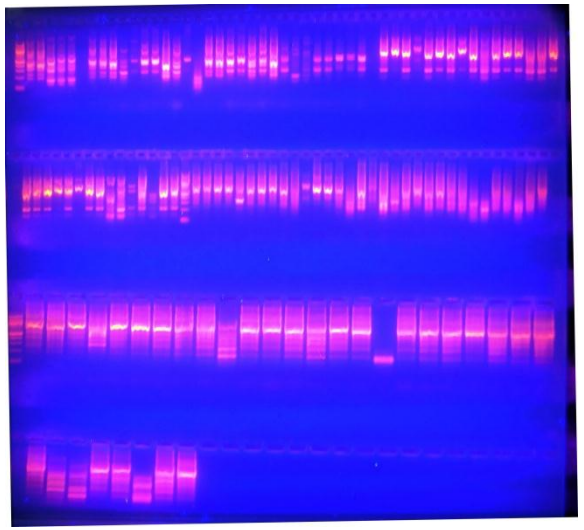


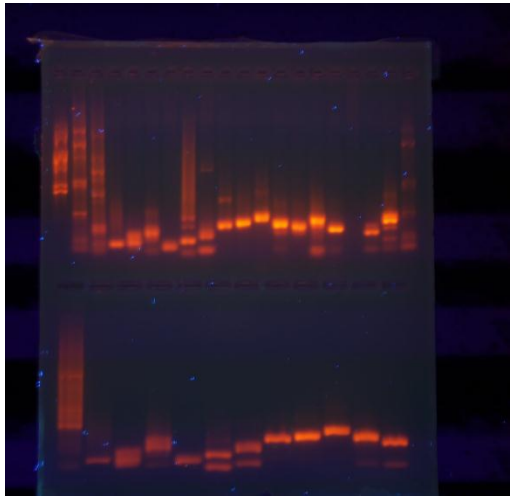
Nondenaturing gels for DNA

Run the gels at constant volatge of 250 V for 2-5 h, depending on the acrylamide concentration.

Generally it takes 2 h for 8%, and 3 h for 12%, 5 h for 16% gels when the tracking dyes, bromophenol blue and xylene cyanol reach the bottom of the gel

RAPDs on agarose gel





SSRs on PAGE

Buffers for electrophoresis

- The electrical current in an electrophoresis cell is carried largely by ions
- The current-carrying ions are supplied by the compounds in the buffer (Note that proteins constitute only a small portion of this current)
- Other roles of the buffer systems include:

  -maintenance of desired pH

  -provide a medium for heat dissipation

  -in native systems, buffers maintain the pH environment needed for protein activity

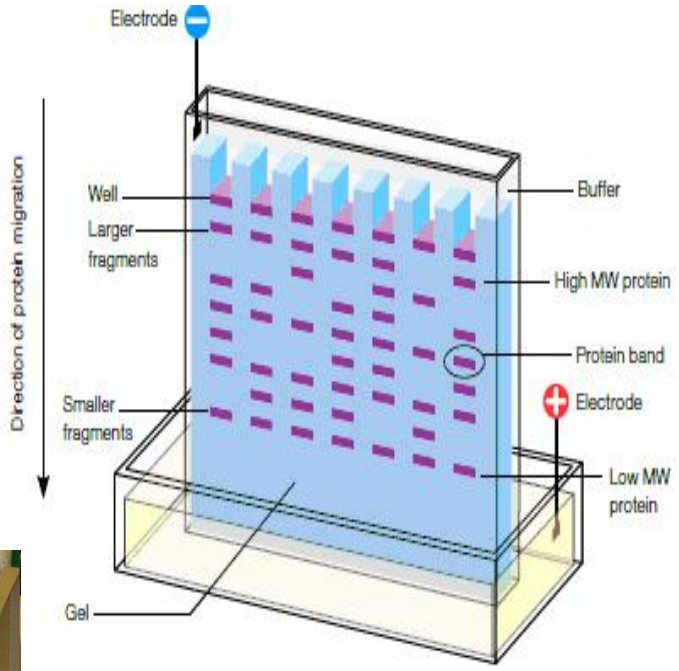
Buffers for electrophoresis

Two classes of buffer systems are commonly used in electrophoresis: continuous or discontinuous

Continuous buffer systems

Continuous systems use one and the same buffer ions at constant pH in the gel, sample, and electrode reservoirs/tank. A **single** separating/resolving gel is used

- Schematic for continuous gel electrophoresis



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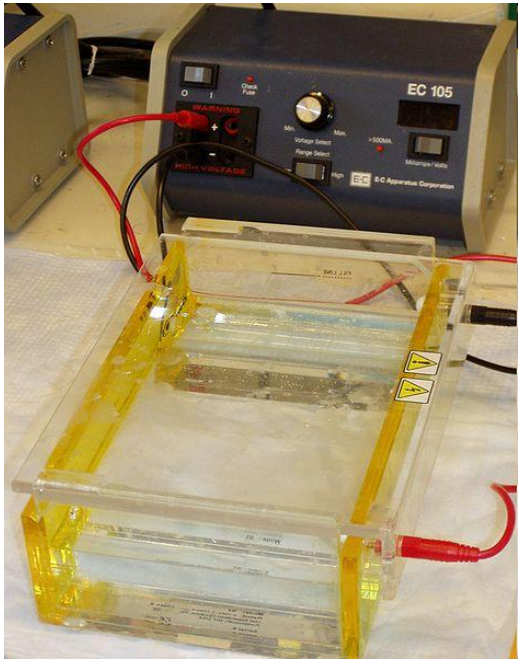


Agarose gel

Continuous gel buffers

- In continuous systems, the sample is loaded directly on the gel in which separation will take place, like a resolving gel.
- Continuous gels are easy to prepare and give adequate resolution for some applications, however, bands tend to be broader and resolution consequently poorer
- To improve on resolution, the strength of the sample buffer is diluted. Dilution leads to decreased buffer ions, a decrease in conductivity and hence localized voltage drop across the sample
- Voltage drop in the sample helps drive proteins into the gel

Continuous gel buffers

- As proteins migrate through the pores of the gel they are separated on the basis of electrophoretic mobility differences
- The width of bands formed are highly dependent on the height of the applied sample volume
- To prevent spreading of bands, small sample volumes (5-15 microliter) must be applied. This can be achieved by using highly concentrated samples
- In continuous systems, molecular charge density and gel pore size are the only factors that control mobility and resolution
- Larger molecules are better resolved on continuous buffer systems than small molecules because of a small difference between their free solution mobility and their mobility in a gel. Continuous buffer systems work best with highly concentrated samples

Buffers for electrophoresis

- Almost any buffer can be used for continuous gel electrophoresis, but for best results, solutions of relatively low ionic strength are used
- Buffers of low ionic strength keep heat generation at a minimum. On the other hand, protein aggregation may occur if the ionic strength is too low.
- Buffers for DNA/RNA include TAE or TBE. TAE offers a lower buffering capacity, hence conducts lower current and requires more time, but offers better separation.
- Different buffers exist for different proteins : TRIS-glycine, TRIS-tricine, etc.
- Concentrations of electrophoresis buffers are in the range of from 0.01 to 0.1 M

Buffers for electrophoresis

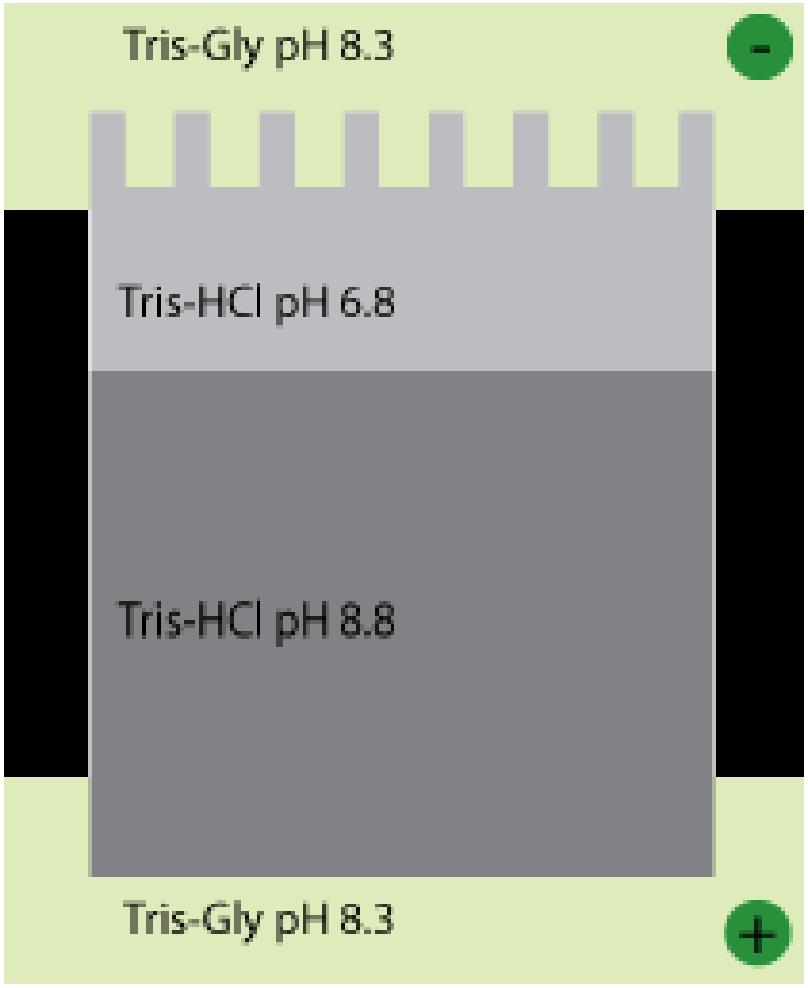
Discontinuous buffer systems

- Discontinuous buffer systems employ different buffers and pH for tank and gel, and often two different buffers within the gel
- Discontinuous systems are designed to enhance the sharpness of the bands by sharpening the starting zones. This is achieved in a process called **"stacking"**. It involves concentrating protein samples into a very narrow and tight zone prior to separation. It results in improved band sharpness and resolution.
- Stacking is an electrochemical phenomenon that establishes an ion gradient in the early stage of electrophoresis on the basis of mobility differences between (1) proteins, (2) leading buffer ions and (3)trailing buffer ions is formed that cause all the proteins to focus into a single sharp band

Discontinuous buffer systems

- Samples are diluted in gel buffer and sandwiched between the gel and the electrode buffer.
- When the electric field is applied, leading ions from the gel move ahead of the sample proteins while trailing ions from the electrode buffer migrate behind the proteins
- Proteins in the sample become aligned between the leading and trailing ion fronts in the order of decreasing mobility.
- In this way, proteins are said to be stacked between the two buffer ion fronts.

A discontinuous gel system



Discontinuous buffer systems

- The width of the stack therefore becomes very thin and compact, having about 100 µm thickness and with protein concentrations of about 100 mg/ml
- Electrophoretic stacking concentrates proteins into regions narrower than can be achieved by mechanical means. This has the effect of minimizing spreading of overall band widths and increasing resolution.
- In order to allow the stack to develop, gels used with discontinuous systems are usually divided into two distinct segments.

Discontinuous buffer systems

- The smaller, upper portion is called the stacking gel. It is cast with appreciably larger pores than the lower resolving gel (or separating gel) and serves mainly as an anticonvective medium during the stacking process. In the stacking gel, no sieving effect occurs.
- Separation takes place in the resolving gel, which has pores of roughly the same size as the proteins of interest. Once proteins enter the resolving gel their migration rates are slowed by the sieving effect of the small pores

Discontinuous buffer systems

- In the resolving gel, the trailing ions pass the proteins and electrophoresis continues in the environment supplied by the electrode buffer. The proteins are said to become “unstacked” in the resolving gel. They separate there on the basis of size and charge.
- Runs are monitored and timed by means of the buffer front. Migration of the buffer front as it moves through the gel can be followed by the change in refractive index between the regions containing the leading and trailing ions. Addition of a tracking dye that moves with the buffer front aids in visualization of the protein front.
- The major advantage of a discontinuous buffer system is increased resolution and sharpness of the sample band.

Gel electrophoresis of proteins

Many techniques are available

- **S**odium-**d**odecyl **s**ulfate-**p**olyacrylamide **g**el **e**lectrophoresis (SDS-PAGE)

✓ Native (buffer) gels

✓ Gradient gels

- Iso-electric focusing
- Two-dimensional polyacrylamide gel electrophoresis
- Cellulose acetate electrophoresis
- Western blotting

SDS-PAGE

- SDS – PAGE stands for **S**odium **D**odecyl **S**ulfate **P**olyacrylamide **G**el **E**lectrophoresis
- SDS-PAGE is the most widely used method for analyzing **protein** mixtures qualitatively
- It is an example of **denaturing** gel electrophoresis
- For DNA, denaturing gels with urea and formamide are used.
- A demerit of native PAGE is that the native structure of a protein affects mobility in a gel matrix such that the rate of migration is not a true estimate of the charge-mass ratio nor an accurate reflection of the molecular weight.
- In their native structure, proteins fold into a variety of shapes to produce compact or elongated forms. Nature of folding depends on the amino acid sequence of the protein
- To overcome structural effects on mobility, denaturation of the protein is carried out

SDS-PAGE

- Denaturation allows separation of proteins on a true charge/mass ratio basis. It also separates proteins into their individual subunits and permits analysis of large, complex protein aggregates.
- Sodium dodecyl sulfate (also called sodium lauryl sulfate) is the most common denaturant used. Other denaturants are Triton X-100 and β-octylglucoside. Both are nonionic.
- The molecular formula of SDS is given below:

SDS-PAGE

- MW: 288.38

$$(CH_3-(CH_2)_{10}-CH_2OSO_3^-Na^+)$$

- It denatures proteins by binding to the protein chain with its hydrocarbon tail, exposing normally buried regions and coating the protein chain with surfactant molecules.
- Proteins bind the SDS detergent uniformly along their length to a level of 1.4 g SDS/g protein. This creates a charge/mass ratio which is uniform/consistent between proteins.
- For this reason, separation on a polyacrylamide gel in the presence of SDS occurs by **mass alone**.

- SDS PAGE offers a rapid and relatively accurate way to determine protein molecular weights. Molecular mass determined by SDS-PAGE is usually accurate within 5 - 10% error rate

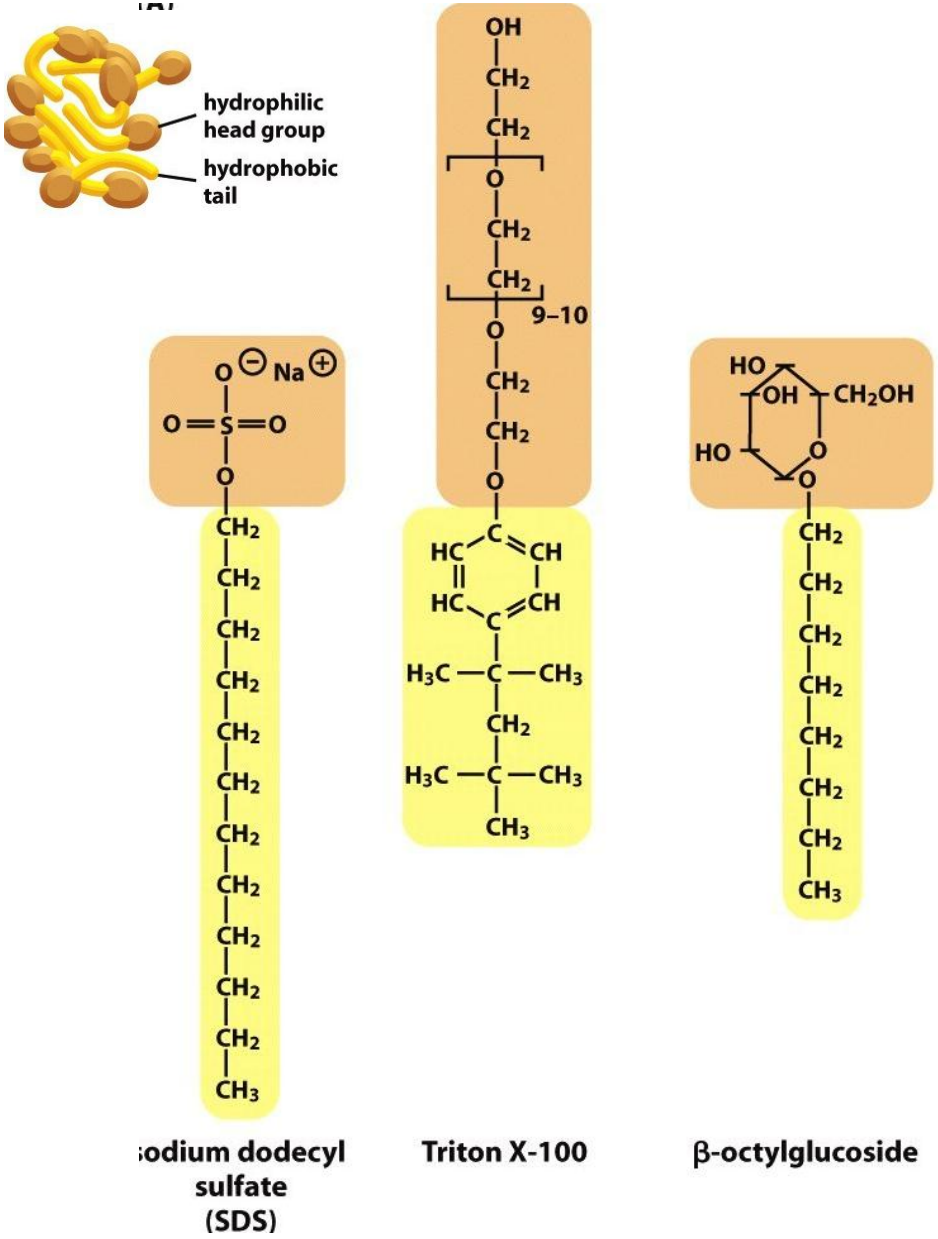
SDS-PAGE

- An exception: some proteins, such as, histones may not be fully denatured and may retain enough secondary structure or contain sufficient charged groups to migrate anomalously so that molecular weight will not be a direct reflection of their mobility. In such a case, Triton X-100 combined with urea and acetic acid are used to enhance denaturation

Denaturation of proteins for SDS-PAGE

- Complete dissolution of sample is required. Undissolved particles present in the sample will lead to clogging of the gel and streaking of the protein band from the well to the end of the gel.
- Complete denaturation of protein sample is required. Incomplete denaturation will not fully saturate the proteins with SDS and will lead to blurred bands or altered mobilities

Structure and function of detergent micelles. The three commonly used detergents are sodium dodecyl sulfate (SDS), an anionic detergent; Triton X-100 and β- octylglucoside are two nonionic detergents. Triton X-100 is a mixture of compounds in which the region in brackets is repeated 9 or 10 times. The hydrophobic portion of each detergent is shown in yellow, and the hydrophilic portion is shown in orange.



SDS-PAGE

- SDS is a strong detergent which completely dissolves many cells and tissues by heating to 95°C in loading buffer
- To solubilize more difficult samples such as plant tissue, a stronger loading buffer, containing more SDS and dithiothreitol (DTT) at high pH is used
- **Reagents: sample buffer for tissue culture cells and soft tissue**

  0.5M Tris-HCl, pH 6.8
  4.4% SDS
  300 mM Mercaptoethanol
  10mg/ml Bromophenol blue
- Mix sample with an equal volume of 2× sample buffer and heat to 95° C for 10 min, cool to room temperature and centrifuge at 14,000 rpm for 5 min in a microcentrifuge to remove all particles. Load the gel

SDS-PAGE

- **Reagents: sample buffer for plant and hard tissue – CHES buffer**

  1% CHES (N-cyclohexyl-2-aminoethanesulfonic acid), adjust pH to 9.5 with NaOH
  2% SDS
  1% DTT
  10% glycerol
  Can be stored at -20° C up to 6 months

Homogenize sample in 5-15 volumes of CHES buffer in a homogenizer and heat to 95° C for 10 minutes, cool to room temperature and centrifuge at 14,000 rpm for 15 minutes. Load the gel

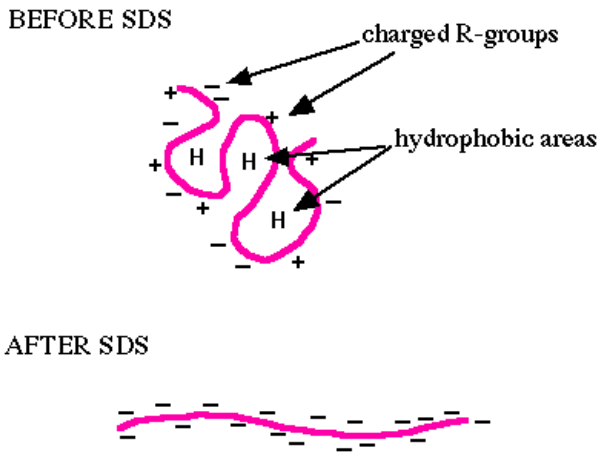
The lipopolysaccharide (LPS) layer of the capsule of yeasts, fungal and Gram negative bacteria cell walls requires enzymatic digestion with lysozyme or zymolyase prior to homogenization.

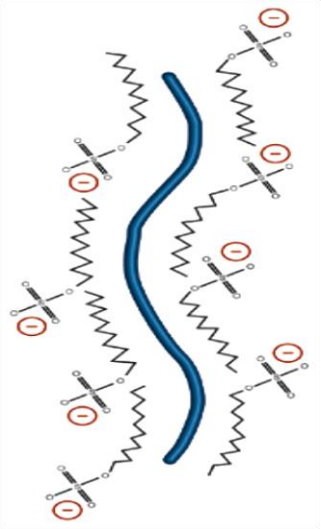
SDS-PAGE

- Mercaptoethanol reduces any disulfide bridges present that are holding together the protein tertiary and quaternary structure
- SDS binds strongly to protein and disrupts most noncovalent bonds, thereby decreasing protein folding. By so doing, SDS denatures the protein and confers a net negative charge in proportion to polypeptide length.
- Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod-shaped structure with a series of negatively charged SDS molecules along the polypeptide chain.

SDS-PAGE

- On average, one SDS molecule binds for every two amino acid residues. The original native charge on the molecule is therefore completely buried by the negatively charged SDS molecules. All proteins treated this way therefore act as negatively charged linear molecules and can be electrophoretically separated by size alone
- In SDS gel electrophoresis of samples SDS imparts identical charge:mass ratio to all proteins
- The rod-like structure remains stable as any attempt that tends to fold up the protein chain would result in repulsion between negative charges on different parts of the protein chain, returning the conformation back to the rod shape





SDS attaches to protein by its hydrophobic chains. Negatively charged SO_4^{2-} groups are exposed to the medium

SDS-PAGE

- The sample buffer also contains the following:
- An ionizable tracking dye, usually bromophenol blue These allow the electrophoretic run to be monitored so samples do not run beyond the plate
- Sucrose or glycerol, which gives the sample solution some density, allows the sample to settle easily through the electrophoresis buffer to the bottom when injected into the well

SDS-PAGE

History of development of SDS-PAGE

- The SDS-PAGE electrophoresis is the most widely used method for elucidating protein subunit, molecular weight, purity, and when combined with Western blot is very useful in protein identification in gene expression studies and in some diseased conditions.
- Although SDS-PAGE electrophoresis was already in use, Laemmli (1970) developed protocols that improved the resolution and protein identification of the SDS-PAGE method.

History of SDS-PAGE

- Starch gels that were first used in protein separation were replaced with a convenient and versatile polyacrylamide in 1960s (Ornstein, 1964; Davis, 1964). Furthermore, Shapiro et al.(1967) developed SDS-PAGE by including sodium dodecyl sulfate (SDS) to the gel and the sample.
- In 1970,Laemmli added an improvement to the SDS-PAGE method to achieve a tremendous improvement in resolution and power of protein identification. He did so in two ways:
- (a) By introducing discontinuous electrophoresis system comprising
- (i) Stacking gel
- (ii) Resolving (separating) gel
- having different pore sizes.
- (b) By adding glycine and TRIS to improve the ionic strengths and pH of the buffer.

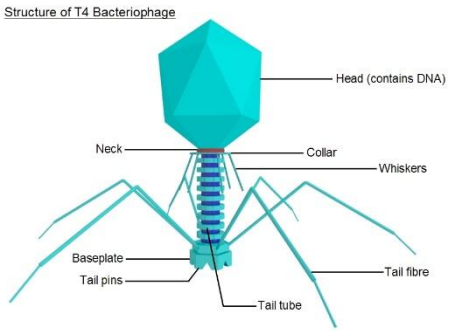
Denaturing gels

- The upper stacking gel is prepared with low acrylamide percentage (i.e. large pore size to allow proteins to move freely and concentrate into a tight band) and low pH of 6.8 [low %T, low pH]
- The lower resolving gel is prepared with high acrylamide percentage (means smaller pores) with a pH of 8.8 [high %T, high pH]
- Both gels contain only Cl^- as the mobile anion. The tank buffer has glycine as its anion, at a pH of 8.8.

Preparation of Denaturing Protein Gels

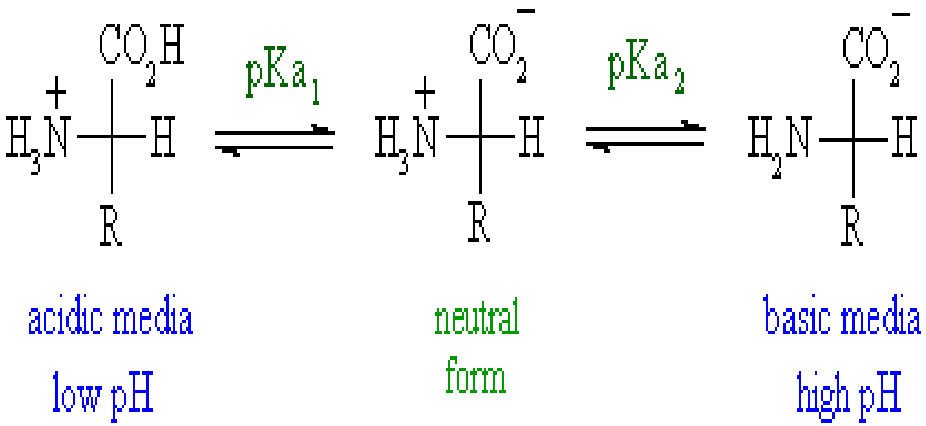
U. K. Laemmli

Professeur Emeritus
University of Geneva, Switzerland.
Department of Molecular Biology



- SDS-PAGE system was developed by Laemmli (article in Nature, 1970).
- The classic Laemmli SDS-PAGE method uses a discontinuous gel system
- Discontinuous buffer systems use different buffers for tank and gel, and often two different buffers within the gel, with a third buffer in the tank.
- Because discontinuous systems concentrate, or stack the protein samples into a very narrow zone prior to separation, two gels are prepared: stacking gel and resolving gel

Laemmli SDS-PAGE



Glycine can exist in three different charge states, namely, positive, neutral or negative, depending on pH

- Three buffer systems: (i) stacking gel, pH 6.8 TRIS-HCl; (ii) resolving gel pH 8.8 TRIS-HCl; (iii) tank or electrode buffer pH 8.3 TRIS-glycine
- The stacking gel has a low concentration of acrylamide and the running gel a higher concentration capable of retarding the movement of proteins.
- Control of the charge state of glycine by the different buffers drives the stacking process

Laemmli SDS-PAGE

- The isoelectric point is the average of the two pKas, *i.e.* **pI = 1/2 (pKa_1 + pKa_2)**= ½(2.34 + 9.6), pI = 5.97. At very acidic pH (below pKa_1) the amino acid will have an overall +ve charge and at very basic pH (above pKa_2) the amino acid will have an overall -ve charge.
- Laemmli method promotes stacking and prevents smearing of bands which occurs in poorly resolved gels. Smearing reduces resolution and detection of proteins.

Laemmli SDS-PAGE

- The stacking gel works like this: When the power is turned on, 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 of relatively lower pH close to the pI, glycine switches predominantly to the zwitterion state causing them to move very slowly in the electric field.
- The Cl^- ions (from Tris-HCl) on the other hand, with their small size and negative charge move much more quickly in the electric field and form an ion front (leading ion) that migrates ahead of the almost neutral to positive glycine (trailing ion).

Laemmli SDS-PAGE

- The separation of Cl^- from the TRIS counter-ion (which is now moving toward the anode) creates a highly mobile Cl^- front and a slowly migrating glycine front with a narrow zone of steep voltage gradient which also pulls the glycine along but behind it.
- The abrupt voltage drop causes the proteins in the gel sample having an electrophoretic mobility that is intermediate between the extreme of the mobility of the glycine and Cl^-, to move rapidly (sweeping) to fill the gap and prevent a break in the circuit.
- By this, proteins are concentrated into a tight band between the Cl^- and glycine fronts. Stacking significantly improves resolution

SDS-PAGE

Photograph showing samples being loaded into the wells of an SDS-PAGE minigel. Six wells that have been loaded can be identified by the blue dye (bromophenol blue) added to the loading buffer

Gel electrophoresis apparatus and reagents

- Many variations in apparatus available from different manufacturers: Bio-Rad PROTEAN, Mini Plate

Apparatus

1) A set of 2 glass plates: a long plate (rectangular), a short plate (notched)
2) Spacer (1mm thick): 2 are needed, one for each opposite side. You can use a flexible tape for this
3) Gel casting system (casting frame to sandwich glass plate; casting stand for glass plates, or casting frame)
4) Clamping frame and electrode assembly for precast gel
5) Comb (1 mm thick) for glass plates; precast gels already have comb attached (gel cassette)
6) Electrophoresis tank or Gel box with lid; lid brings the power from the power supply
7) Micropipettes and slender tips

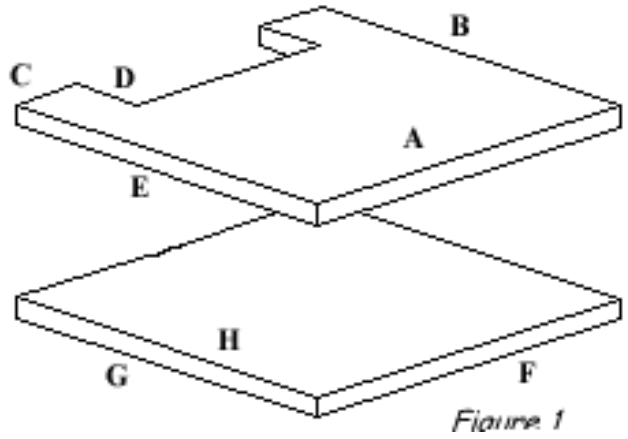
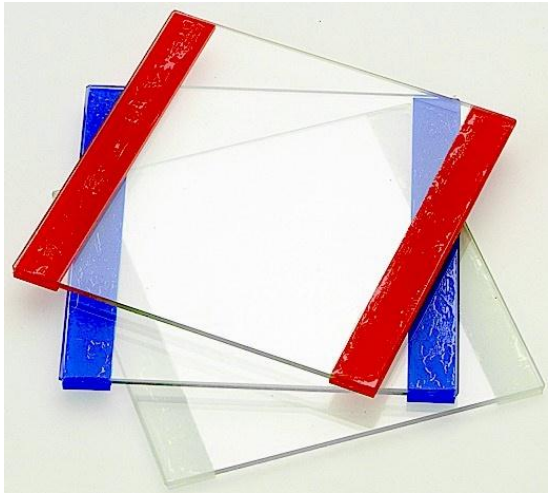


Figure 1

Rectangular and notched plate

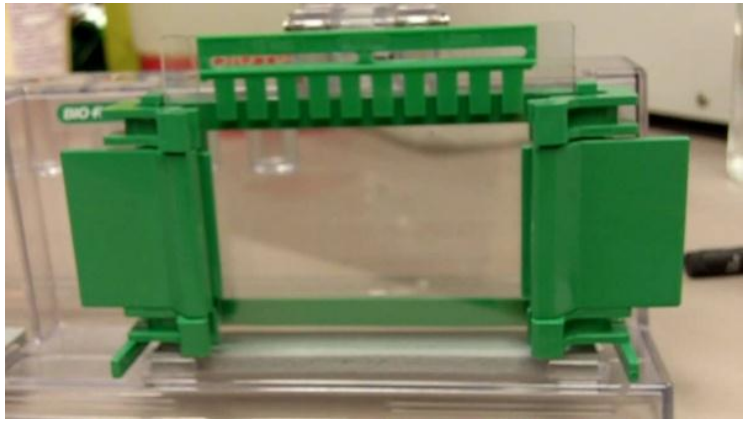
Two spacers



Glass plates with two spacers attached

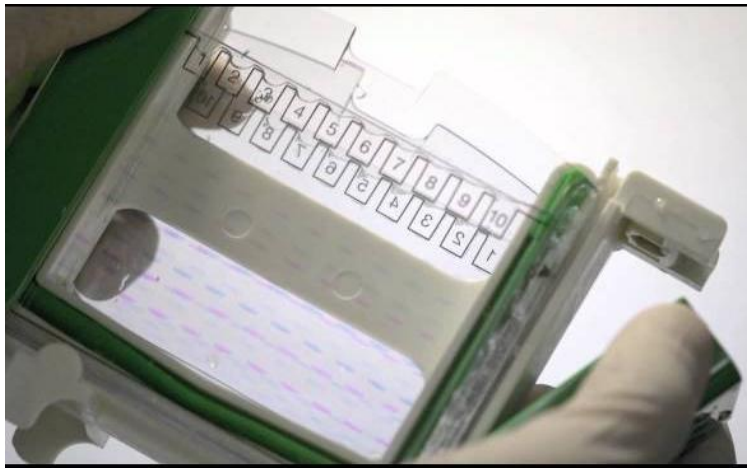
Casting frame (bottom) and casting stand (up)

Combs



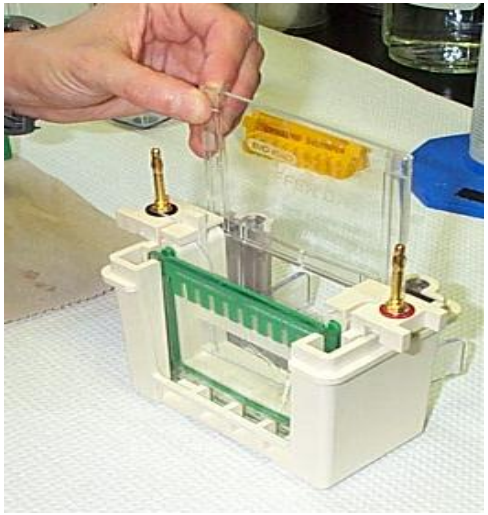
Casting frame with comb fitted to a casting stand

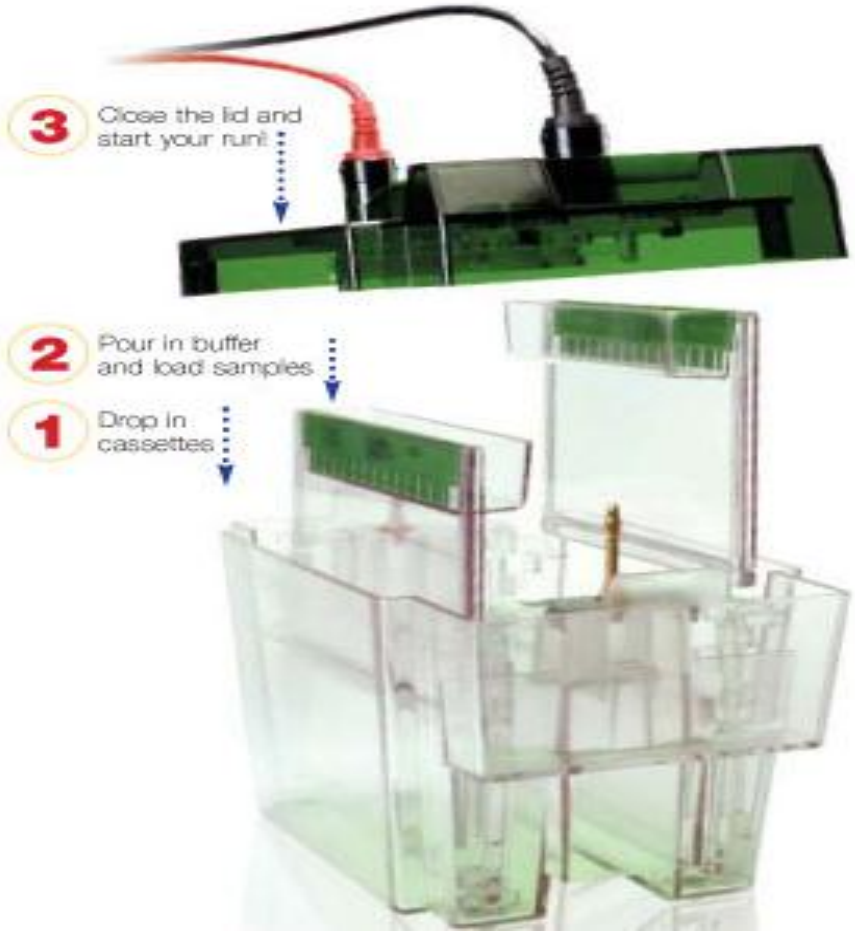
Gel cassette



Clamping frame and electrode assembly with gel cassette in place (comb removed to show numbered wells

Buffer dam

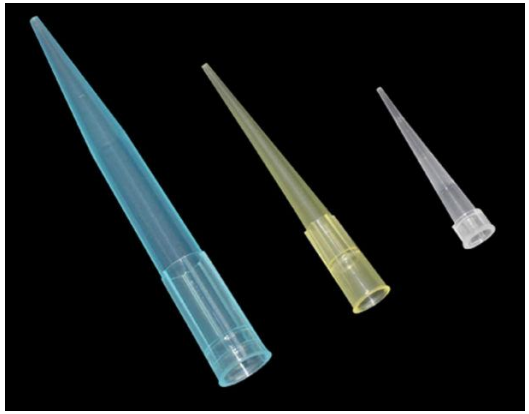




Clamping frame and electrode assembly with casting gel or gel cassette in front and buffer Dam behind; has two ears with red and black coding

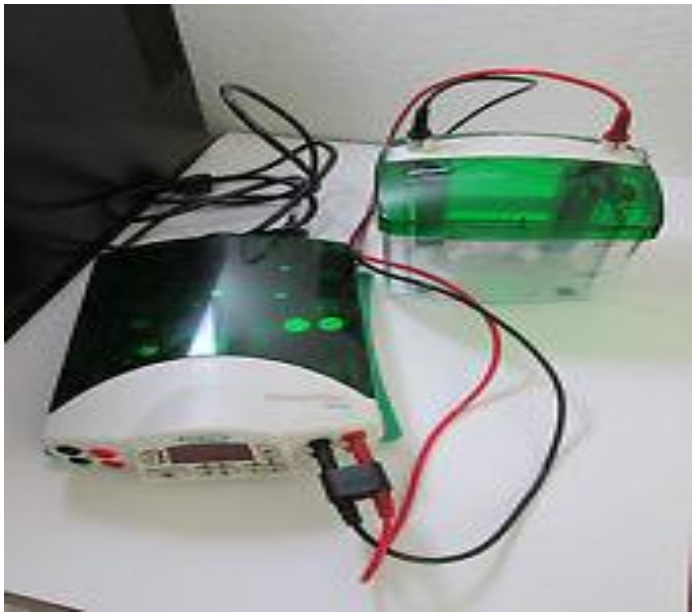
Gel box or electrophoresis tank with lid

Pipette tips with slender ends for loading a gel



Normal pipette tips

Power pack



Gel box connected to power pack

Reagents for vertical gel electrophoresis

1) Acrylamide
2) Laemmli sample buffer – to prepare protein samples for SDS-PAGE
3) Running buffer

   Acts as an electrolyte for running the gel (counteracts changes in pH arising from electrolysis of water and has enough electrolytes to conduct electricity to run gel in a short time. It is sold as a 10x concentrate.

4) Stacking gel
5) Resolving gel
6) Ethanol
7) SDS (10%)
8) Staining solution
9) Destaining solution and reservoir

Wear gloves at all times during reagent preparation and casting gel. Acrylamide is a neurotoxin; precast gel in gel cassette is stored in sodium azide, a bacterial inhibitor and toxic to humans

Reagents for vertical gel electrophoresis

Stock solutions

Solution A: Acrylamide

- 30 g acrylamide/0.8 g Bis to 100 ml with Super Q water, sterilize by filtration through 0.2 µm membrane filter

Solution B:

1.5 M Tris, pH 8.8 = 36.3 g Tris in 100 ml water. Adjust pH to 8.8 and adjust volume to 200ml.

Solution C:

- 0.5 M Tris, pH 6.8 = 6 g Tris in 40 ml water. Adjust pH to 6.8 and adjust volume to 100 ml.

Solution D: ammonium persulfate (APS)

- 10% APS = 0.1 g in 1 ml water

Solution E: 10% SDS

3% Stacking gel

6.3 ml water/2.5 ml soln C/0.1 ml 10% SDS/1.2 ml soln A/10 µl TEMED/100 µl APS, (lower pH of 6.8)

5X Tray or Running Buffer/liter (Tris-Glycine-SDS)

- 15 g Tris/72 g glycine/5 g SDS. Dilute 1:5 for upper tray and 1:10 for lower tray. This is equivalent to 25 mM Tris /192 mM Glycine/0.1 % SDS (Higher pH of 8.8)

Reagents for vertical gel electrophoresis

- **2x Laemmli Bio-Rad sample buffer** – to prepare protein samples for SDS-PAGE. Based on Tris-glycine-SDS running buffer. Ensures optimal band resolution

<u>Contains:</u>

- 2.5 mM Tris-HCl, pH 6.8
- 25 % glycerol to make sample denser so it can sink to bottom of well
- 2 % SDS
- 0.01 % Bromophenol Blue

<u>To use: (50:50 with sample)</u>

- Dilute 1 part sample with 1 part Laemmli sample buffer.
- Add reducing agent to Laemmli : ß -mercaptoethanol (β-ME) or dithiothreitol (DTT)

The reducing agent breaks disulfide linkages in proteins

Add 50 µl of β-ME per 950 µl of sample

buffer to a final concentration of 5 % ß –ME equivalent to 710 mM. Alternatively, use DTT at a final concentration of 350 mM (54 mg/ml).

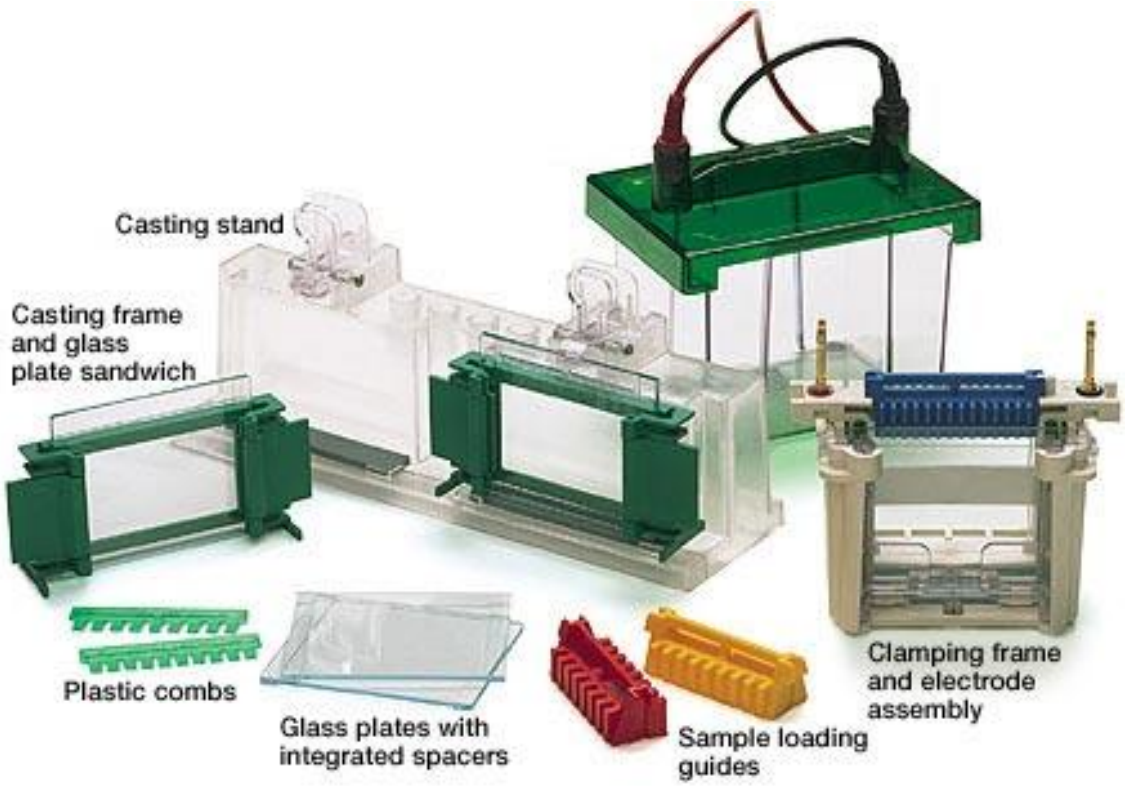
Add 1 mM EDTA to disperse cells

Resolving gel composition

29 ml				45 ml		
Component	5%	7.5%	10%	5%	7.5%	10%
Soln. A (ml)	5	7.5	10	7.5	11.25	15
Soln. B (ml)	7.5	7.5	7.5	7.5	7.5	7.5
SDS (ml)	0.3	0.3	0.3	0.45	0.45	0.45
TEMED (µl)	15	15	15	20	20	20
APS (µl)	150	150	150	200	200	200
Water	16.1	13.6	11.7	24.15	20.4	17.55

Preparation of PAGE gels

- To obtain optimal resolution of proteins, two gel systems are used: a "**stacking**" gel which is cast over the top of the "**resolving**" gel
- Stacking gel: has lower concentration of acrylamide (e.g., 7% for larger pore size), lower pH of 6.8 and different ionic content
- These properties of the stacking gel allow proteins in a loaded sample to be concentrated into a tight band during the first few minutes of electrophoresis before entering the resolving portion of a gel
- If using gradient gel, the use of a stacking gel is not necessary as the gradient, with its continually decreasing pore size performs this function
- To make stacking gel: Mix all solutions, add TEMED last
- To make resolving gel: Mix all solutions, add TEMED last



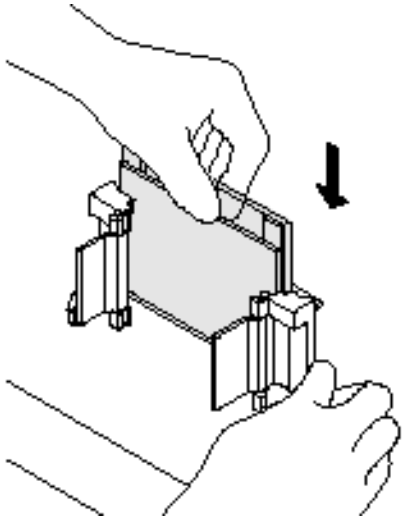
Vertical gel electrophoresis

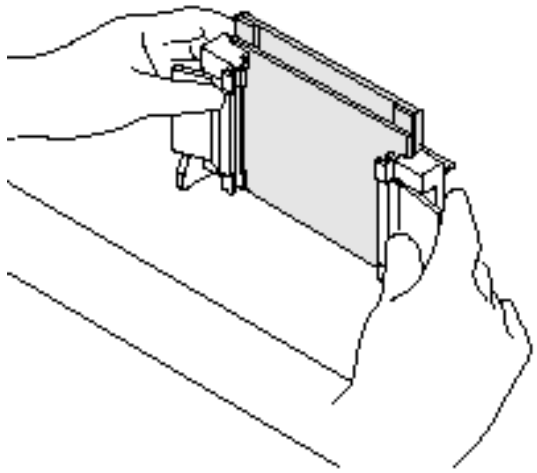
Assembly of apparatus and casting a gel

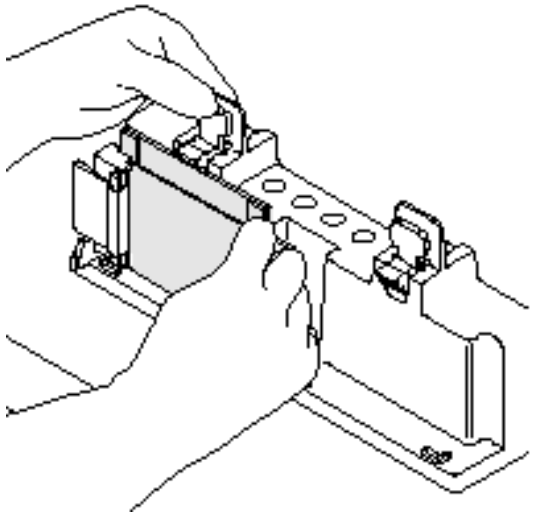
- The gel is cast between two glass plates, separated by spacers, typically less than 2mm thick

<u>To assemble</u>

- Place the spacers on the long plate (spacer plate). Place short notched plate on top of spacer plate so spacer is between the two glass plates. Keep the short plate in front. Top side of spacer plate is marked by manufacturer
- Slide both plates into the casting frame and secure it by locking the pressure clamps. To tighten and lock, move clamps through 90^{o}
- Insure that both plates reach the bottom and align them perfectly. A gasket in the casting frame makes a seal. Check that the bottom is flat, i.e., glass plates do not protrude at the base of casting frame. Place casting frame on the casting stand
-

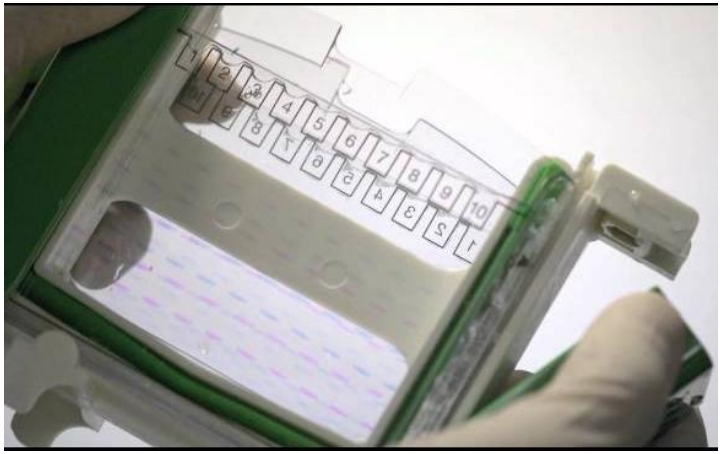






Assembly of apparatus and casting a gel

- Place comb in between glass plates. Make a mark on the right of glass plate, about 1 cm down the comb. Check for leakages by spraying water into the space between the glass plate. If no leakages, blot the water with filter paper. Remove the comb and cast the resolving gel



Precast gel in clamping frame and electrode assembly

Assembly of apparatus and casting a gel

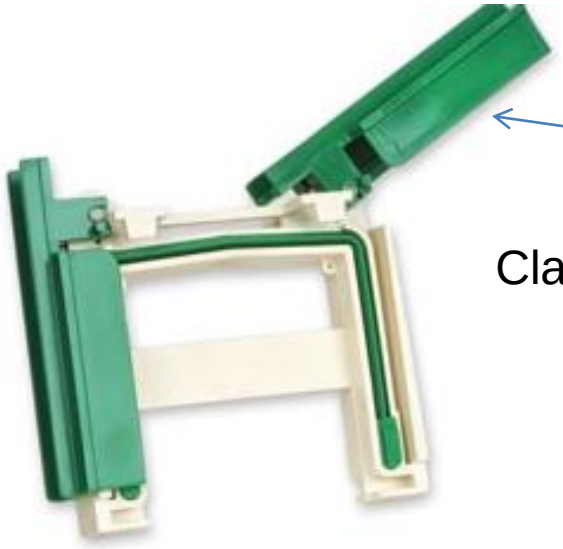
- To cast the resolving gel, pour gel mix between the two glass plates to the black mark you made outside the glass, avoiding bubbles. Gel must be poured right away after adding TEMED
- Remove any bubbles by adding a layer of isopropanol on top of the gel way up to the top.
- Wait for gel to polymerize. Blot out the isopropanol using a strip of filter paper or pour it off. After drying the isopropanol, cast the stacking gel immediately after adding TEMED
- Pour stacking gel on top of resolving gel to fill up the plates. Be careful not to introduce any air bubble. Place comb in the stacking gel which will create wells for samples. Wait for gel to polymerize. Remove the comb and place unit in a gel box.

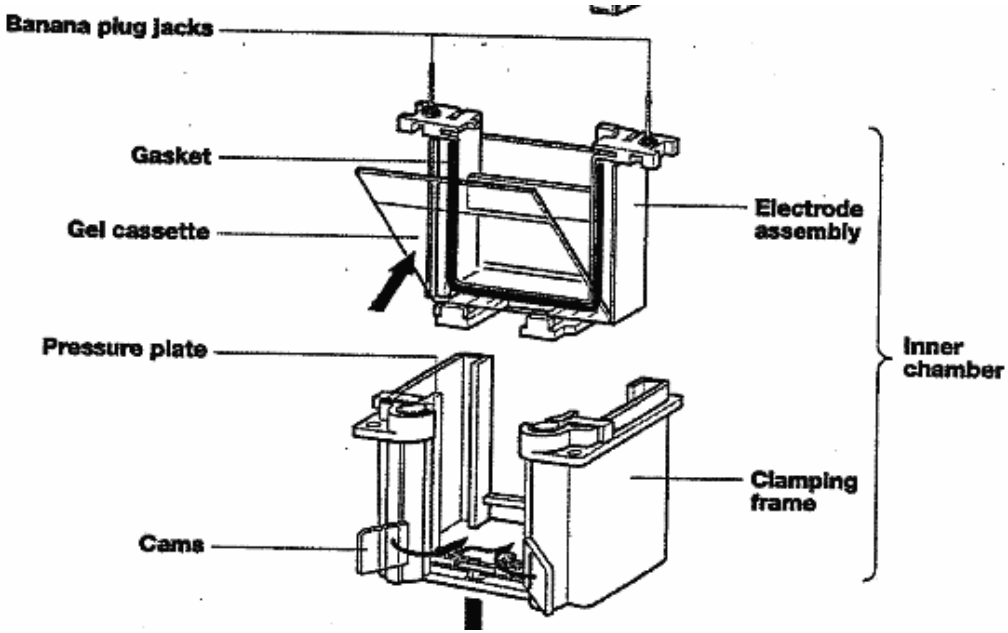
Using a precast gel

Use a gel casting clamping frame. Place buffer dam in the gel casting frame having two sides and gaskets to hold plates in place. This frame can run 2 gels, one on each side, or run one gel with the other side occupied by the buffer dam.

Place buffer dam in frame and clamp side arms to secure the plate. Remove gel cassette from storage pouch and remove the protective tape from the bottom to prevent insulation of the gel cassette.

This cassette has a comb already in place, and when placed in the frame must face the inside of the gel compartment.





Electrophoresis

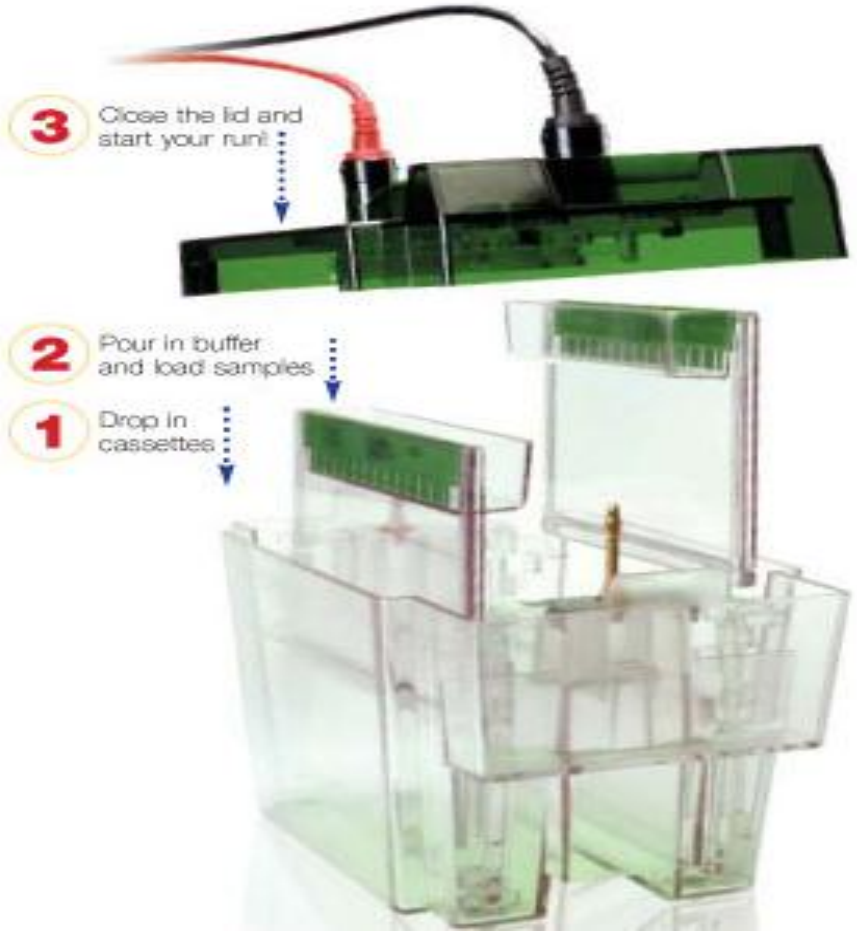
- Remove the glass plates from the stand and clamp and place in gel box/electrophoresis tank
- Add buffer to outer and inner reservoir. Fill the outer compartment with buffer to the mark indicated on gel box.
- Fill the inner compartment with buffer to the mark on gel box. The level of buffer on the inner compartment will be higher than that of the outer. This is used to ensure that there is no leakage, since as if there is, the level of buffer in the inner will drop down. Remove the comb gently and straight up
- Add more buffer to cover the inner glass plate to flood the wells with the buffer
- Load gel: prepare samples with the loading buffer (Laemmli loading buffer) and, if required, denature by heating a sample and adding denaturing agents.

Electrophoresis

- Use micropipette tips with slender ends to load sample wells. Load 1st and 2nd wells with molecular marker and positive control. Load sample wells
- After loading attach lid to gel box, red to red and black to black. Connect to power pack, red to red and black to black.
- Turn on power supply and choose either constant current or constant voltage. Press the run icon on power supply and observe the development of a line of bubbles in the tank- a sign that electrophoresis has began.
- The bubbles come from electrolysis of water and so the bubbles are hydrogen gas.

Electrophoresis

About 20 min into running of the gel, you want to see sample compressed into a narrow line caused by the sample moving through the fairly low percentage of the stacking gel into the high percentage and stiffer resolving gel causing the sample to compress into a tight line.



Electrophoresis

- Run electrophoresis till the dye front reaches appreciable length down the gel, before bromophenol blue reaches end
- When the electric field is turned off, the proteins stop moving. The gel matrix holds the proteins at their final positions long enough for them to be stained to make them visible.
- The gel material can also withstand high voltage gradients, feasible for various staining and destaining procedures, and can be cut to extract separated fractions or dried for autoradiography and permanent recording

Polyacrylamide gels

- Separation is achieved by (1) net charge carried by the protein and (2) sieving effect of the gel matrix.
- Proteins segregate into discrete zones, corresponding to their individual gel-mediated mobilities
- Lanes of protein bands are created

Polyacrylamide gels

- Separation by charge: in native PAGE, migration occurs because most proteins carry a net negative charge at slightly basic pH. The higher the negative charge, the faster the protein will migrate
- Separation by sieving effect of the gel: the frictional force created by the pore sizes of the gel matrix creates a sieving effect, retarding the movement of proteins according to their size.
- Small proteins encounter small frictional force while large proteins face larger frictional force and are retarded the most

Gradient gels

- Gradient gels can be applied to protein and nucleic acid separation
- Main features of a gradient gel
- Low percentage of acrylamide at the top (beginning of sample path) and high percent-acrylamide at the bottom (end) unlike homogeneous gels
- Pore size increases linearly as proteins move down the gel, hence migration rates decreases down the gel
- Are required for separation of both large and small molecular weight proteins on a single gel slab
- Used for a quick estimate of the range of protein sizes in a mixture permitting proper selection of a single concentration gel
- Resolution is much better in homogeneous gel

Gradient gels

- Another form of gradient gel

- Increasing concentration of denaturing reagent (e.g SDS for protein; urea and formamide for DNA).
- Denaturing gradient gel electrophoresis is used to resolve PCR amplicons up to a single base changes, and separate out DNA on the basis of variations in GC content.
- Find applications in elucidating molecular diversity among microbial organism (Microbial ecology) (in later lectures)

Properties of proteins

- Since proteins are charged molecules, they migrate under the influence of electric fields. In electrophoresis, the two most important physical properties of proteins are their

  (1) Electrophoretic mobilities : this depends on the charge, size, and shape of the protein

  (2) Isoelectric points: depends only on the net overall charge of the protein

- Various electrophoresis systems have been developed to exploit differences between proteins in these two fundamental properties.
- Electrophoretic mobility, μ, is the rate of migration of a protein per unit of field strength , $\mu = v/E$

- Units of electrophoretic mobility are those of velocity (cm/sec) divided by the units of electric field (V/cm), which gives $cm^2/V\text{-}sec$.

Properties of proteins

Electrophoretic mobility of a protein is dependent on

- Its charge
- Frictional coefficient (shape) $f=(6\pi r\eta)$ where r=radius of the particle, η= viscosity of the medium
- The ratio of charge: mobility
- Both charge and frictional coefficients are established by the composition of the protein and by the makeup of the surrounding medium.
- Electrophoretic mobilities are influenced by factors such as pH and the amounts and types of counter ions and denaturants that are present in the medium.
- Proteins are amphoteric molecules, that is, they can carry positive, negative, or zero net charge depending on the pH of their environment.
- For every protein there is a specific pH at which its net charge is zero. This pH is called the "isoelectric point", or pI, of the protein. A protein is positively charged in solution at pH values below its pI and negatively charged when the pH is above its pI.

Properties of proteins

- This pH dependence on charge affects the mobilities of proteins in terms of both magnitude and the direction of migration and is exploited in gel electrophoresis, especially in the technique of isoelectric focusing
- The electrophoretic mobilities of proteins are very different in gels than in free solution.
- Gels can act as molecular sieves for molecules such as proteins. They consist of three-dimensional networks of solid material and pores. During electrophoresis in gels the polymeric material acts as a barrier to the motion of proteins forcing them to move between the buffer-filled pores of the gels.