Department of BSBE
Indian Institute Of Technology Guwahati

Techniques in Genetic Engineering

Gel electrophoresis- DNA, RNA, protein

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Gel electrophoresis- DNA

Electrophoresis is a process that enables the sorting of molecules based on size.

Using an **electric field**, molecules (such as DNA) can be made to move through a gel made of agarose or polyacrylamide.

The electric field consists of a negative charge at one end which pushes the molecules through the gel, and a positive charge at the other end that pulls the molecules through the gel.

The term "gel" in this instance refers to the matrix used to contain, then separate the target molecules. In most cases, the gel is a crosslinked polymer whose composition and porosity are chosen based on the specific weight and composition of the target to be analyzed.

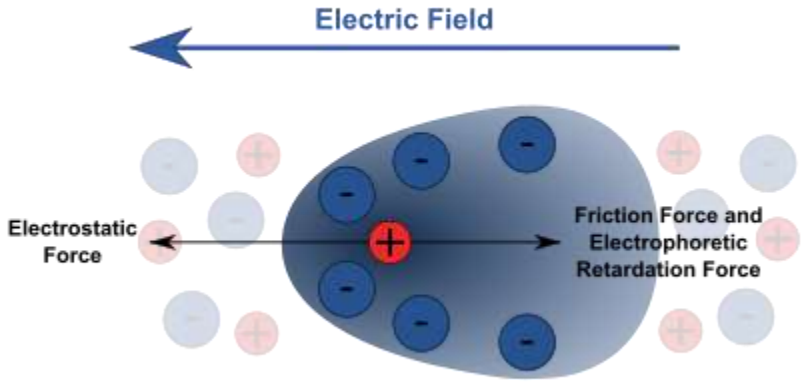
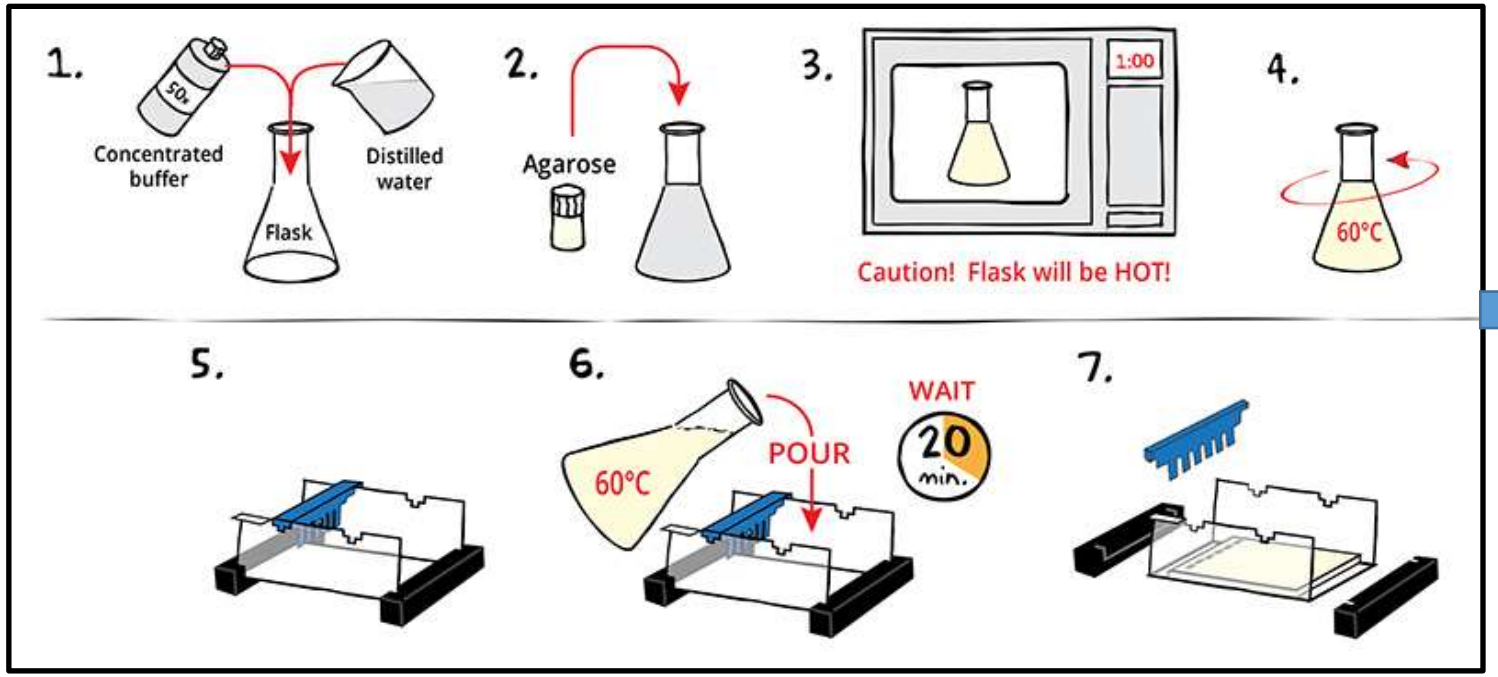
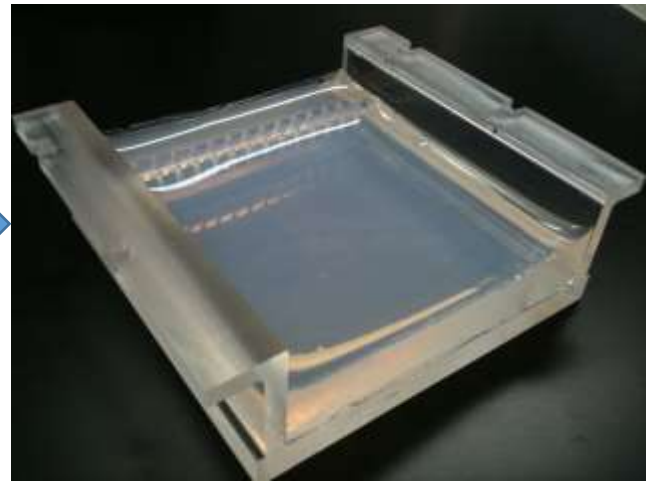


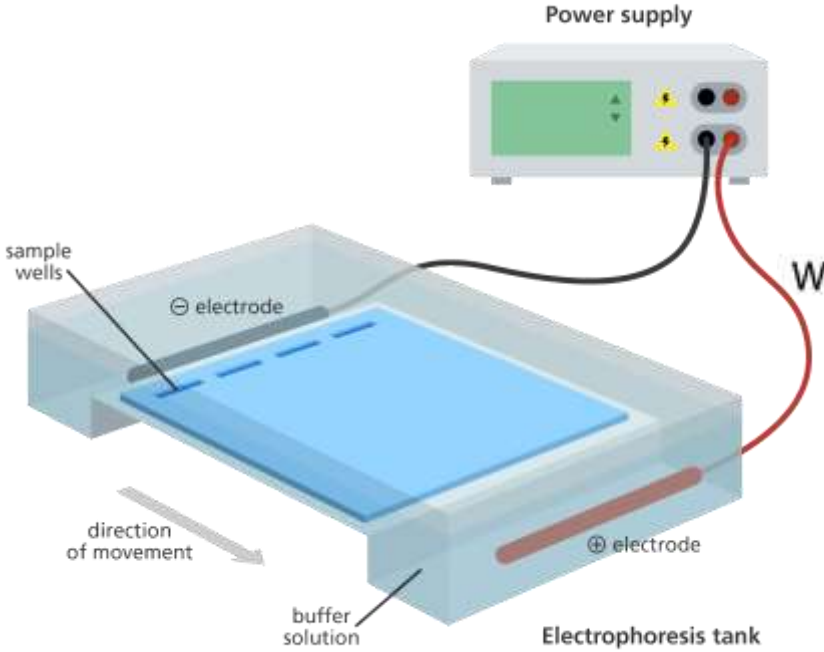
Illustration of electrophoresis

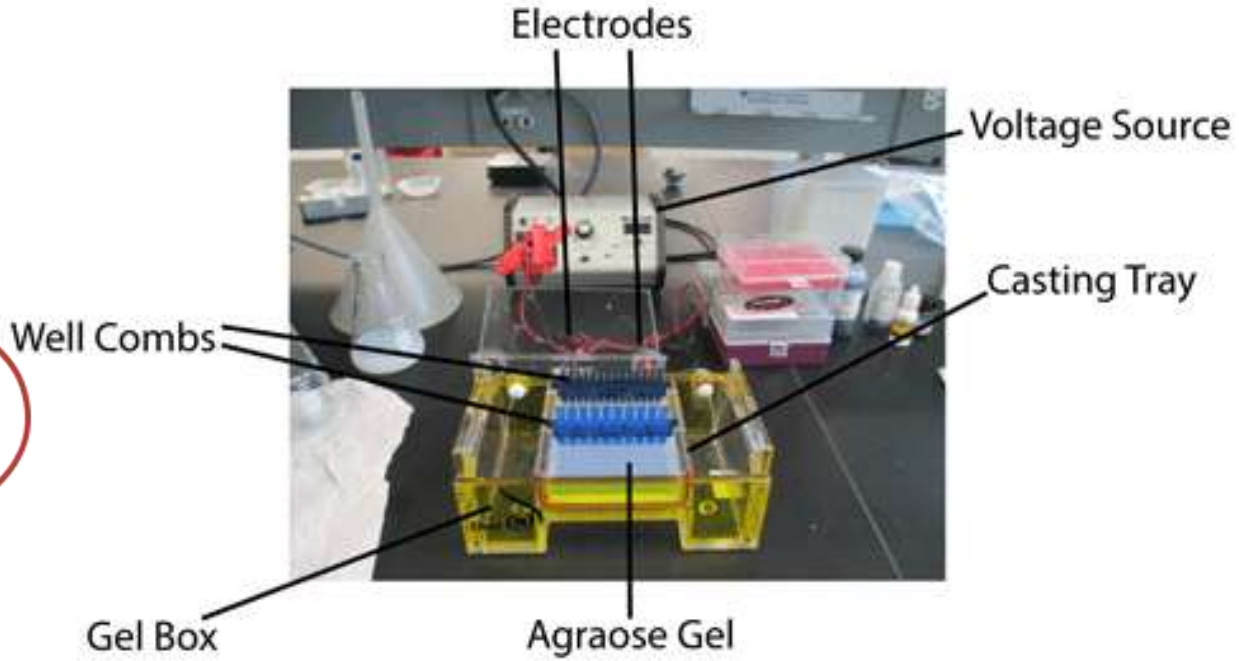
- Agarose gels are made from the natural polysaccharide polymers extracted from seaweed. Agarose gel is a three-dimensional matrix formed of helical agarose molecules in supercoiled bundles that are aggregated into three-dimensional structures with channels and pores through which biomolecules can pass. The 3-D structure is held together with hydrogen bonds and can therefore be disrupted by heating back to a liquid state.
- Agarose gels do not have a uniform pore size, but are optimal for electrophoresis. The distance between DNA bands of different lengths is influenced by the percent agarose in the gel, with higher percentages requiring longer run times, sometimes days.
- Instead high percentage agarose gels should be run with a pulsed field electrophoresis (PFE), or field inversion electrophoresis. "Most agarose gels are made with between 0.7% (good separation or resolution of large 5–10kb DNA fragments) and 2% (good resolution for small 0.2–1kb fragments) agarose dissolved in electrophoresis buffer.



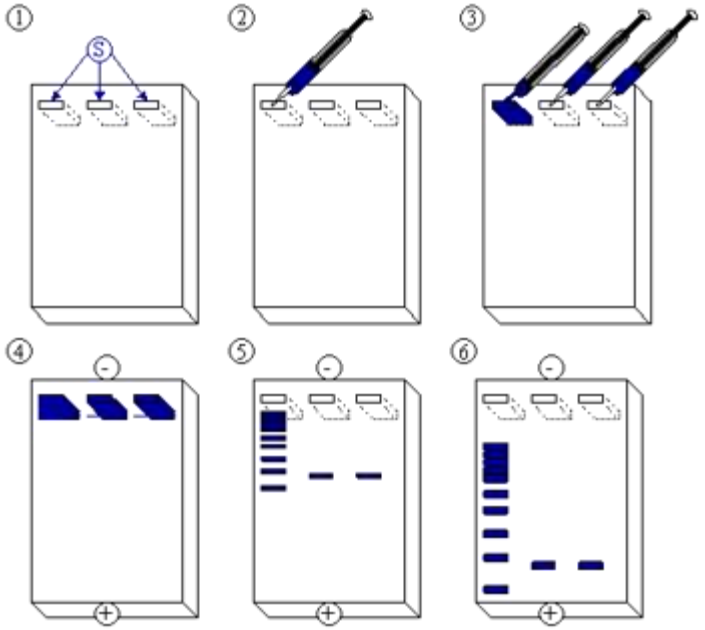


Setup of Agarose gel electrophoresis

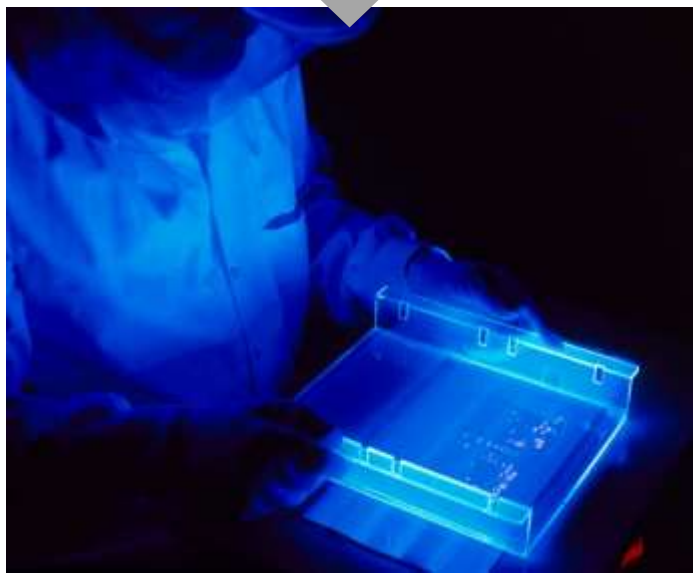




Loading of samples and Agarose gel electrophoresis

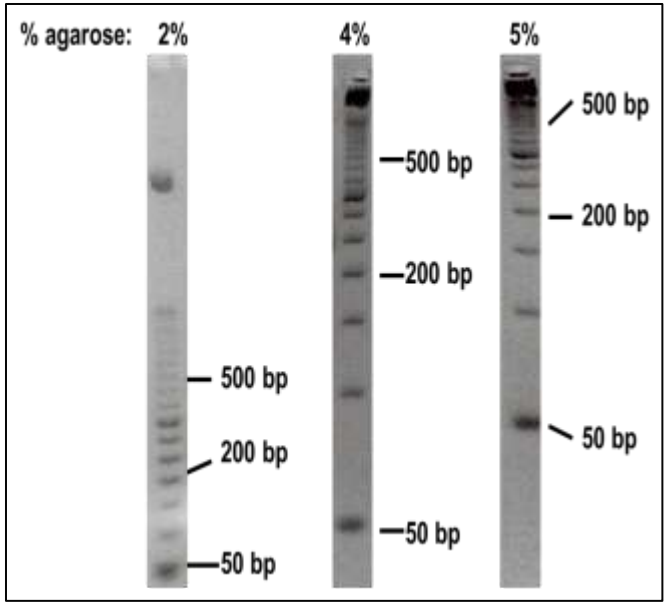


Visualization of Agarose gel in UV trans-illuminator



Resolution of linear DNA fragments in agarose gel

% Agarose (w/v)	Size Range (kb) for Optimal Separation
0.5	2 - 30
0.75	0.7 - 20
1.0	0.5 - 10
1.5	0.2 - 3
2.0	0.1 – 2



Buffer Systems

Weak acids and/or bases that do not dissociate completely.

Purposes of buffer:

- Maintains pH.
- Generate ions consistently to maintain current & keep resistance low.

1) TAE, pH 8.0, ~50 mM - Tris, Acetate, EDTA
2) TBE, pH 8.0, ~50 mM - Tris, Borate, EDTA

➢ TBE resolves low MW fragments better than TAE.

➢ TAE resolves high MW fragments better than TBE

Tris (T) is a weak base.

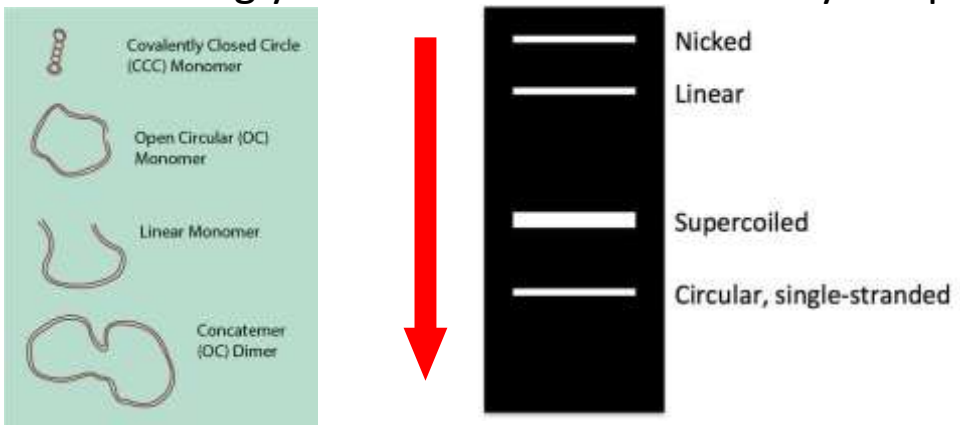
Acetic (A) acid & boric (B) acid are weak acids.

A number of factors can affect the migration of nucleic acids:

1. The dimension of the gel pores (gel concentration),
2. size of DNA being electrophoresed,
3. the voltage used,
4. the ionic strength of the buffer,
5. and the concentration of intercalating dye such as ethidium bromide if used during electrophoresis.

The movement of the DNA may be affected by the conformation of the DNA molecule

1. for example, supercoiled DNA usually moves faster than relaxed DNA because it is tightly coiled and hence more compact.
2. In a normal plasmid DNA preparation, multiple forms of DNA may be present.
3. Gel electrophoresis of the plasmids would normally show the negatively supercoiled form as the main band,
4. while nicked DNA (open circular form)
5. and the relaxed closed circular form appears as minor bands.
6. The rate at which the various forms move however can change using different electrophoresis conditions, and the mobility of larger circular DNA may be more strongly affected than linear DNA by the pore size of the gel.



Ethidium bromide(+vely charged) which intercalates into circular DNA can change the charge, length, as well as the superhelicity of the DNA molecule, therefore its presence in gel during electrophoresis can affect its movement.

The rate of migration of the DNA is proportional to the voltage applied, i.e. the higher the voltage, the faster the DNA moves.

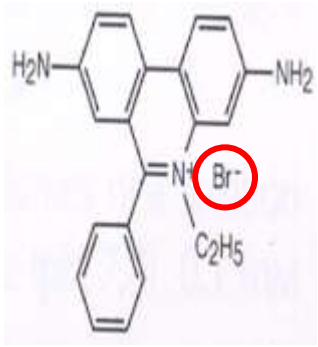
Visualization and DNA stains

- Monitoring the progress of the electrophoresis
- Tracking dyes are visible to naked eye during run
  - **Xylene cyanol** (migrates with ~5.0 kb fragments)
  - **Bromophenol blue** (migrates with 300 bp fragments)
  - **Orange G** (migrates with fragments of ~50 bp)
- But mobility of tracking dyes can vary substantially depending on agarose
  - Concentration
  - Type

DNA stain

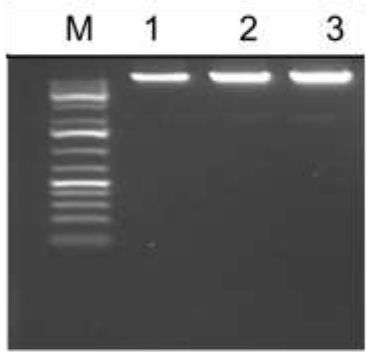
- Binds to ds DNA by intercalation between stacked bases.
- Used to visualize DNA with UV light.

E.g. Ethidium bromide, GelRed

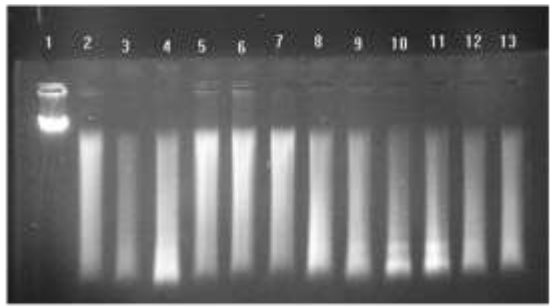


Variety of DNA can be run on Agarose Gel

Genomic DNA

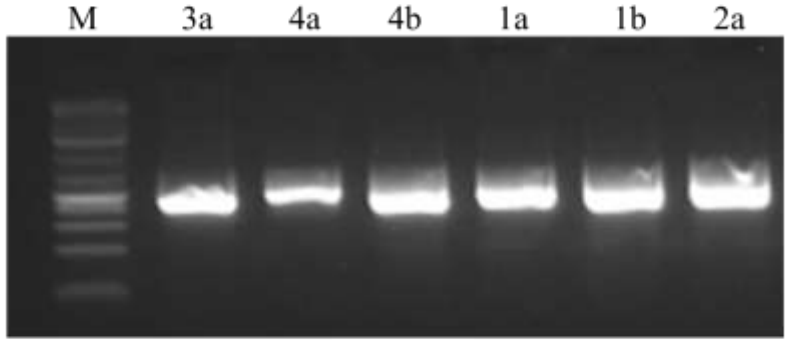


M = 1kb + DNA ruler
1 = Lambda DNA (control)
2 - 3 = gDNA



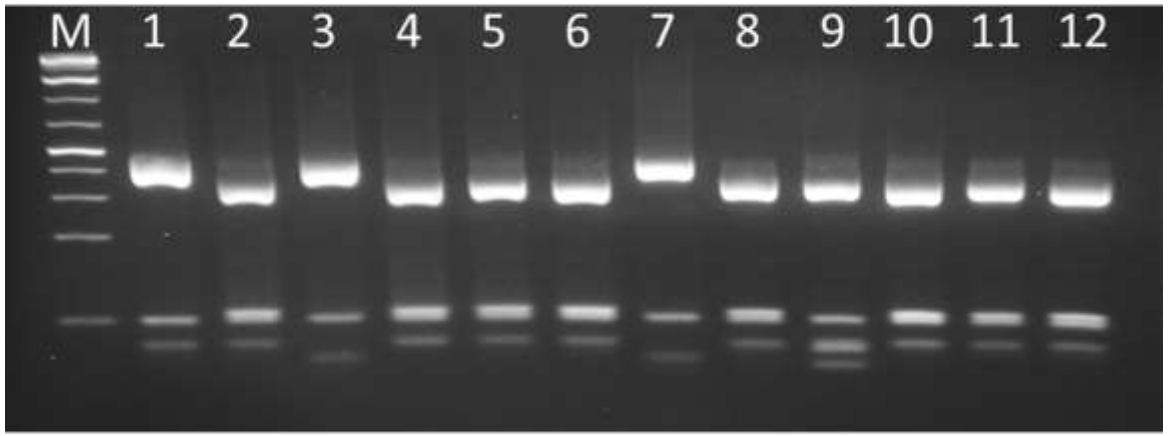
1 = Lambda DNA (control)
2 – 13 = gDNA

PCR products



M = 1kb + DNA ruler

Msel digestion of PCR products



M = 1kb + DNA ruler

About plasmid DNA and gel electrophoresis:

- Plasmid DNA can exist in three conformations:
- **supercoiled,**
- **open-circular (oc),**
- **linear** (supercoiled plasmid DNA is often referred to as covalently closed circular DNA, **ccc**).

- *In vivo*, plasmid DNA is a tightly supercoiled circle to enable it to fit inside the cell. In the laboratory, following a careful plasmid prep, most of the DNA will remain supercoiled, but a certain amount will sustain single-strand nicks.
- Given the presence of a break in only one of the strands, the DNA will remain circular, but the break will permit rotation around the phosphodiester backbone and the supercoils will be released

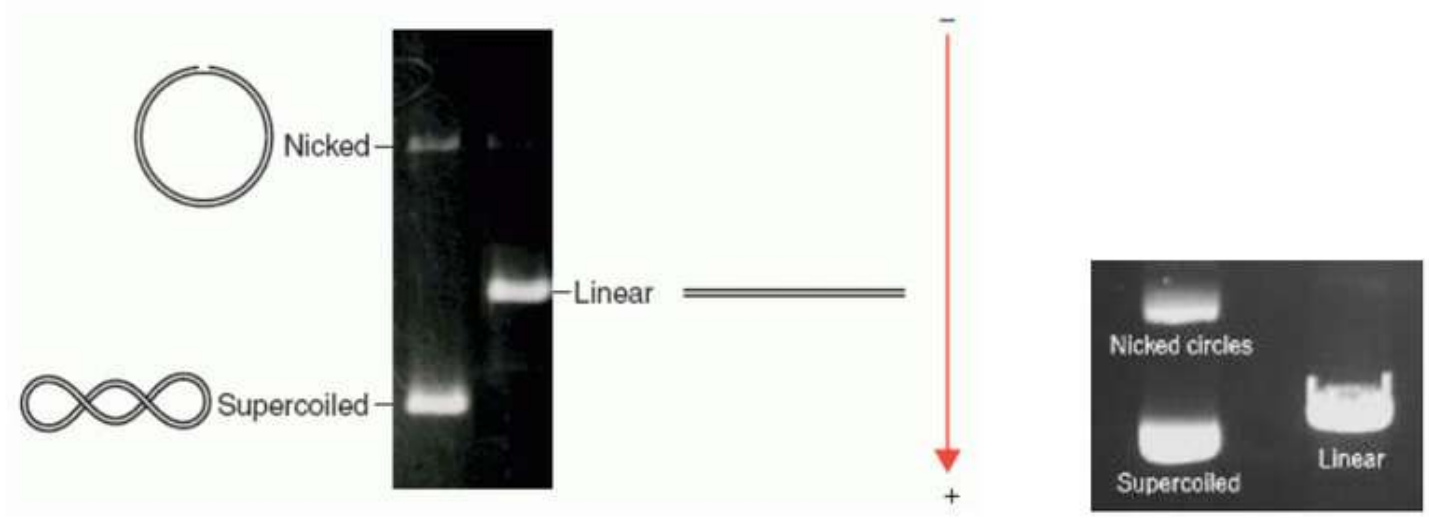
A small, compact supercoiled knot of ccc-DNA sustains less friction against the agarose matrix than does a large, open circle of oc-DNA.

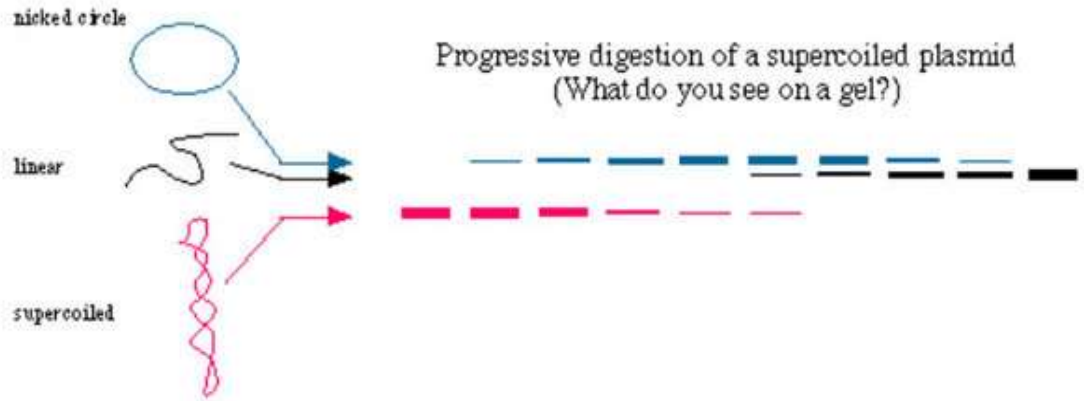
Therefore, for the same over-all size, supercoiled DNA runs faster than open-circular DNA.

Linear DNA runs through a gel end first and thus sustains less friction than open-circular DNA, but more than supercoiled.

Thus, an uncut plasmid produces two bands on a gel, representing the oc and ccc conformations. If the plasmid is cut once with a restriction enzyme, however, the supercoiled and open-circular conformations are all reduced to a linear conformation.

Following isolation, spontaneous nicks accumulate as a plasmid prep ages. This can clearly be seen on gels as the proportion of the two conformations change over time: plasmids preps that have been thawed and refrozen many times, show more oc DNA than fresh preps.





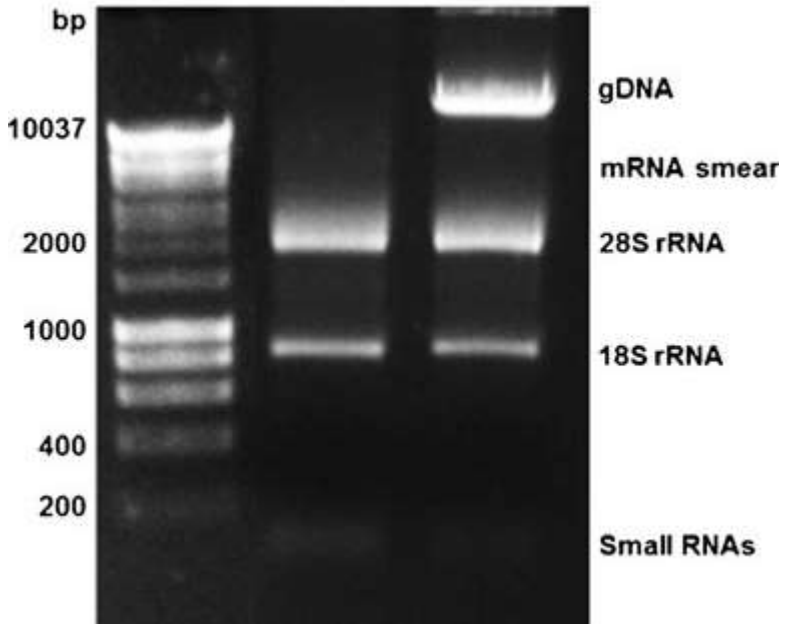
This order can change when increasing voltage and EtBr concentration.
Usually if you are working with a small plasmid that was purified by some kit, the bold line on your phoresis is cupercoiled DNA while thin line is open circular DNA.

- **size marker lane 1**.
- It is clear that in **lane 2** two fragments are present in non-equimolar amounts (the upper band must contain longer DNA molecules, but is less intense than the lower band..).
- In this particular case it's because they represent two circular forms of the same plasmid DNA (oc on top, and ccc below). The ratio of the amounts of DNA in both bands depends on the age and quality of the plasmid preparation.

  Note: the (*linear* DNA) bands in lane 1 cannot act as size markers for the *circular* DNAs in lane 2!!

  Lane 3 shows a comparable amount of that plasmid, digested with a restriction enzyme which linearised the circular DNA's

Gel electrophoresis- RNA



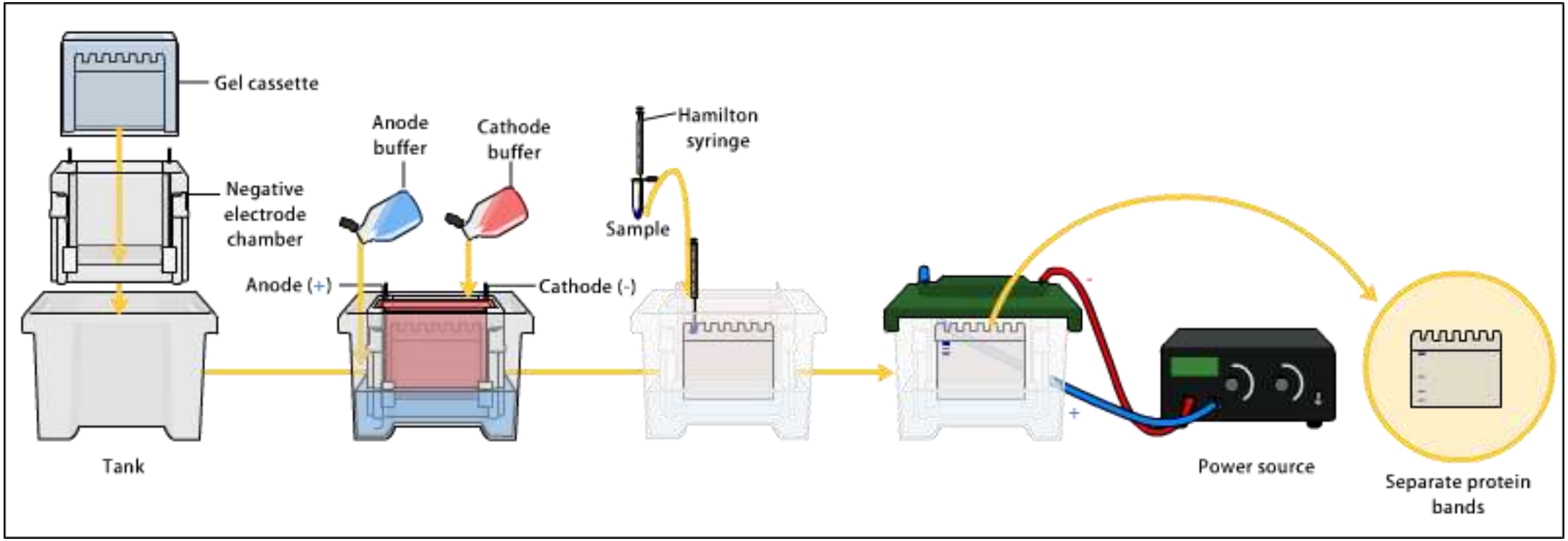
Example of agarose gel electrophoresis of total RNA isolated. Visualization of three intact RNA bands for 28 S RNA, 18 S RNA and 5 S RNA. Genomic DNA contamination can also be detected.

Applications of DNA RNA gel electrophoresis

1. Estimation of the size of DNA molecules following digestion with restriction enzymes, e.g., in restriction mapping of cloned DNA.
2. Estimation of the DNA concentration by comparing the intensity of the nucleic acid band with the corresponding band of the size marker.
3. Analysis of products of a polymerase chain reaction (PCR), e.g., in molecular genetic diagnosis or genetic fingerprinting
4. Separation of DNA fragments for extraction and purification.
5. Separation of restricted genomic DNA prior to Southern transfer, or of RNA prior to Northern transfer.
6. Separation of proteins, for example, screening of protein abnormalities in clinical chemistry.

Gel electrophoresis- protein

Polyacrylamide gel electrophoresis (PAGE) is a technique widely used to separate proteins or nucleic acids, according to their electrophoretic mobility.



Apparatus of SDS-PAGE

What is in the running buffer?

Tris, glycine, and SDS, pH 8.3. Tris is the buffer used for most SDS-PAGE. Its pKa of 8.1 makes it an excellent buffer in the 7-9 pH range.

Utilities of different components of SDS-PAGE

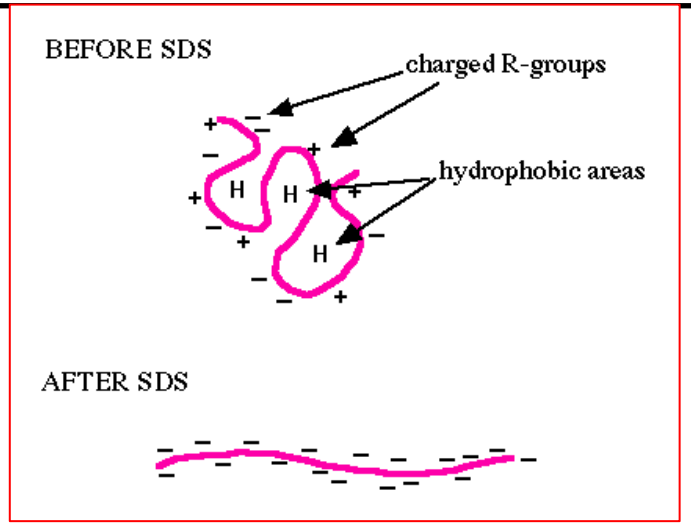
Acrylamide (C_3H_5NO; mW: 71.08) when dissolved in water, slow, spontaneous auto-polymerization of acrylamide takes place, joining molecules together by head on tail fashion to form long single-chain polymers. The presence of a free radical-generating system greatly accelerates polymerization. This kind of reaction is known as vinyl addition polymerisation.

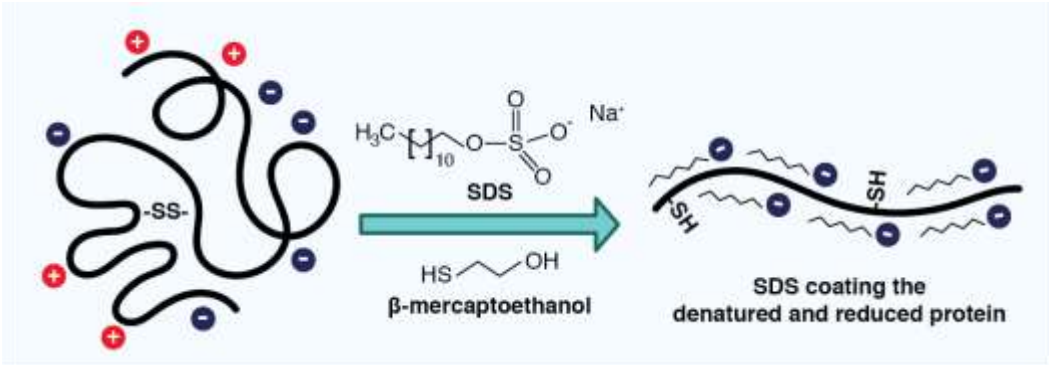
Bisacrylamide (*N*,*N*′-Methylenebisacrylamide) ($C_7H_{10}N_2O_2$; mW: 154.17) is the most frequently used cross linking agent for polyacrylamide gels.

Sodium dodecyl sulfate (SDS) ($C_{12}H_{25}NaO_4S$; MW: 288.38) (only used in denaturing protein gels) is a strong detergent agent used to denature native proteins to individual polypeptides. This denaturation, which is referred to as reconstructive denaturation, is not accomplished by the total linearization of the protein, but instead, through a conformational change to a combination of **random coil and α helix secondary structures**. When a protein mixture is heated to 100 °C in presence of SDS, the detergent wraps around the polypeptide backbone. It binds to polypeptides in a constant weight ratio of 1.4 g SDS/g of polypeptide. In this process, the intrinsic charges of polypeptides become negligible when compared to the negative charges contributed by SDS. Thus polypeptides after treatment become rod-like structures possessing a uniform charge density, that is same net negative charge per unit weight.

- SDS and β-mercaptoethanol denature the protein.
- SDS binds with denatured proteins and covers with negative charge
- β-mercaptoethanol is a strong reducing agent. It eliminates disulfide bonds in proteins by reducing them (adding hydrogen atoms) and breaks apart tertiary & quaternary structure further denaturing proteins

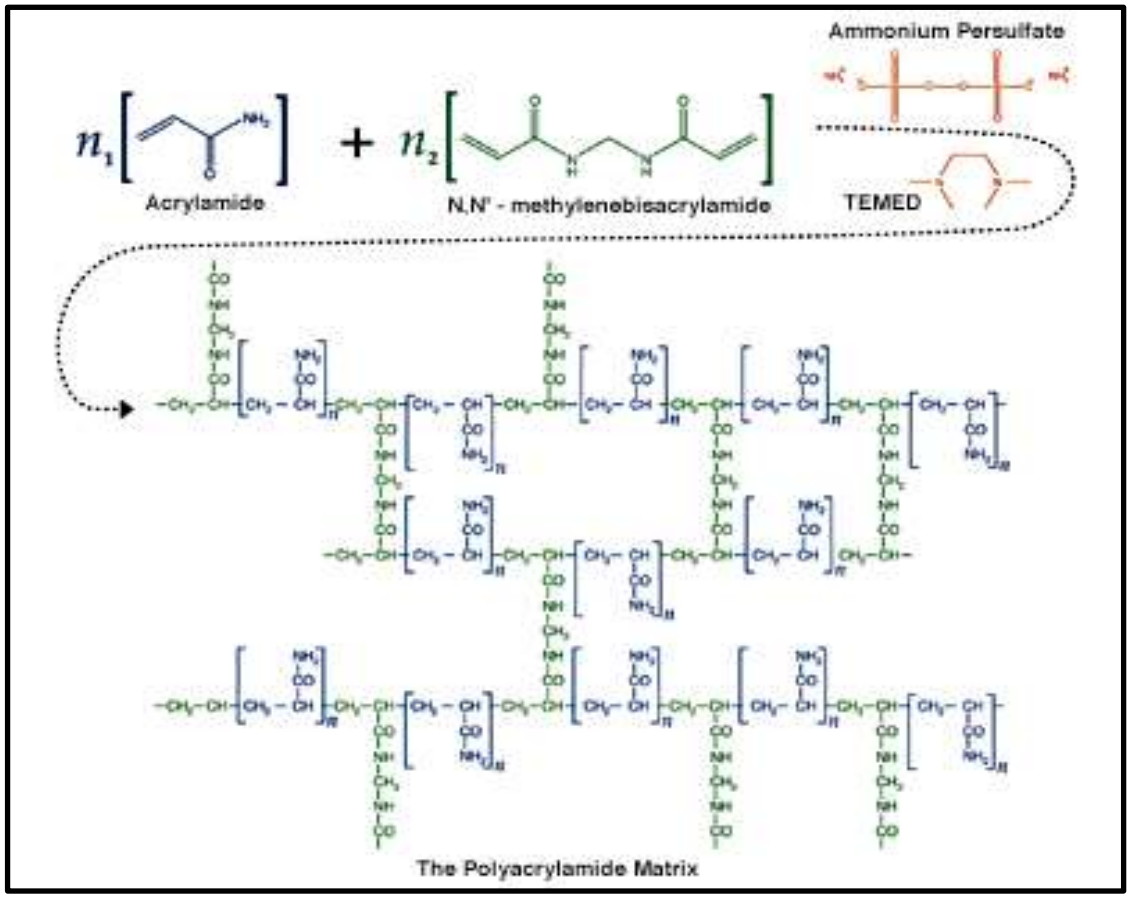
  Dithiothreitol (DTT) is an antioxidant that breaks disulphide bonds

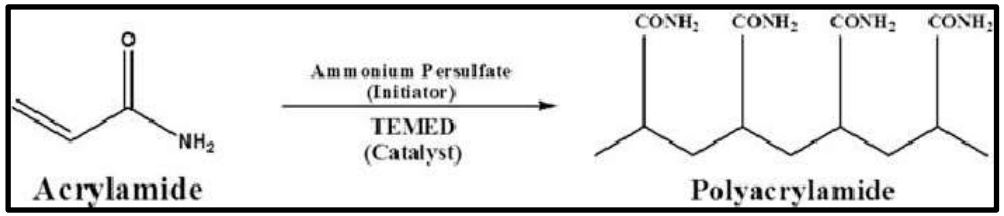




Ammonium persulfate (APS) ($N_2H_8S_2O_8$; mW: 228.2) is a source of free radicals and is often used as an initiator for gel formation. An alternative source of free radicals is riboflavin, which generated free radicals in a photochemical reaction.

TEMED (*N*, *N*, *N*′, *N*′-tetramethylethylenediamine) ($C_6H_{16}N_2$; mW: 116.21) stabilizes free radicals and improves polymerization. The rate of polymerisation and the properties of the resulting gel depend on the concentrations of free radicals. Increasing the amount of free radicals results in a decrease in the average polymer chain length, an increase in gel turbidity and a decrease in gel elasticity.



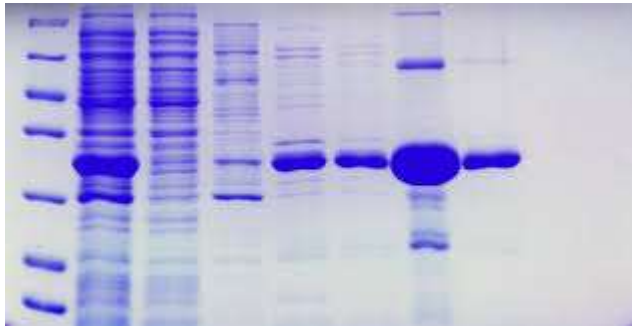


Chemicals for processing and visualization

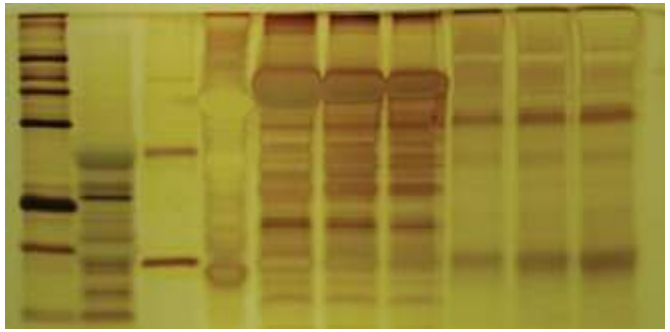
Tracking dye; as proteins and nucleic acids are mostly colorless, their progress through the gel during electrophoresis cannot be easily followed. Anionic dyes of a known electrophoretic mobility are therefore usually included in the PAGE sample buffer. A very common tracking dye is Bromophenol blue (BPB, 3',3",5',5" tetrabromophenolsulfonphthalein). This dye is coloured at alkali and neutral pH and is a small negatively charged molecule that moves towards the anode. Other common tracking dyes are xylene cyanol, which has lower mobility, and Orange G, which has a higher mobility.

Loading aids; most PAGE systems are loaded from the top into wells within the gel. To ensure that the sample sinks to the bottom of the gel, sample buffer is supplemented with additives that increase the density of the sample. These additives should be non-ionic and non-reactive towards proteins to avoid interfering with electrophoresis. Common additives are glycerol and sucrose.

Coomassie brilliant blue R-250 (CBB)($C_{45}H_{44}N_3NaO_7S_2$; mW: 825.97) is the most popular protein stain. It is an anionic dye, which non-specifically binds to proteins.



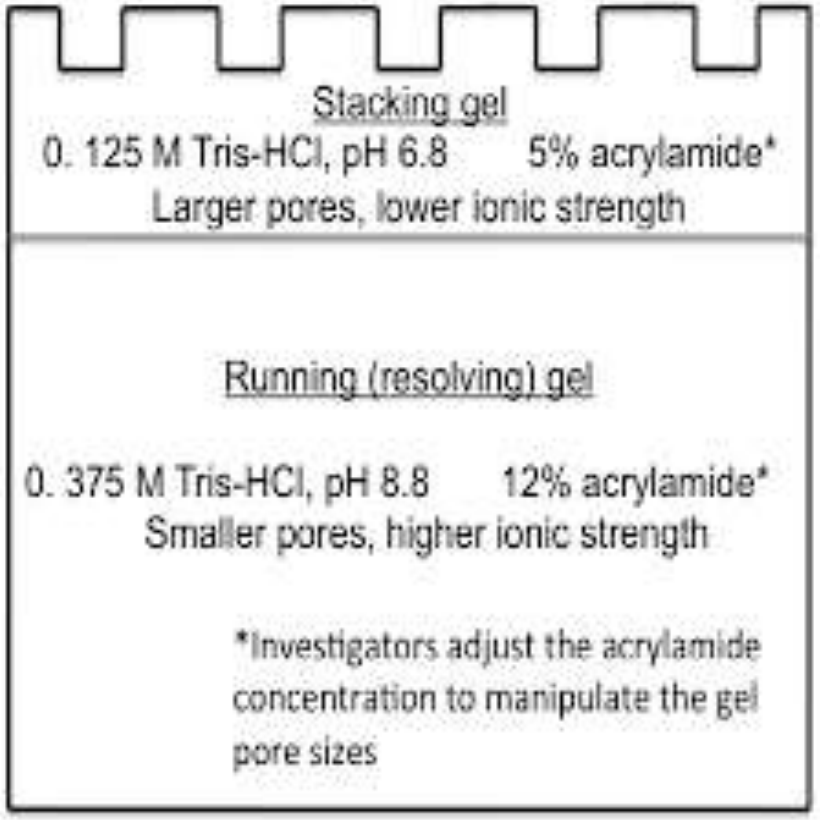
Silver staining is used when more sensitive method for detection is needed, as classical Coomassie Brilliant Blue staining can usually detect a 50 ng protein band, Silver staining increases the sensitivity typically 10-100 fold more.



Gel Layers: It Takes Two

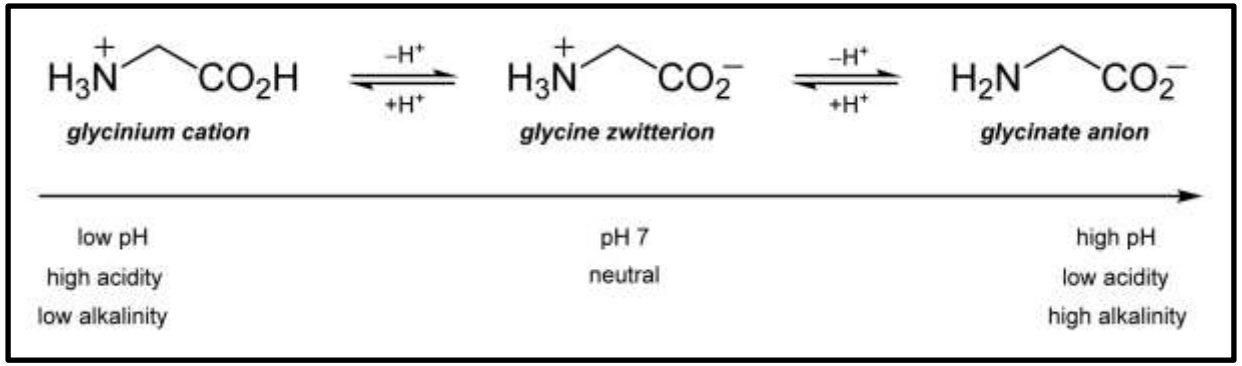
WHAT are there two layers in the gel?

The stacking layer and the resolving layer. The top (stacking) layer has a lower percentage of acrylamide and a lower pH (6.8) than the bottom (resolving) layer, which has more acrylamide and a higher pH (8.8). SDS PAGE is run in a discontinuous buffer system. There is discontinuity not only between the gels (different pH values and acrylamide amounts), but also between the running buffer and the gel buffers. The running buffer has different ions and a different pH than the gels.



What is the importance of glycine?

It is the ionic state of glycine that really allows the stacking buffer to allow the proteins to migrate. Glycine is an amino acid with the chemical formula NH2-CH2-COOH. The charge of its ion is dependent on the pH of the solution that it is in. In acidic environments, a greater percentage of glycine molecules become positively charged. At a neutral pH of around 7, the ion is uncharged (a zwitterion), having both a positive charge and a negative charge. At higher pHs, glycine becomes more negatively charged.



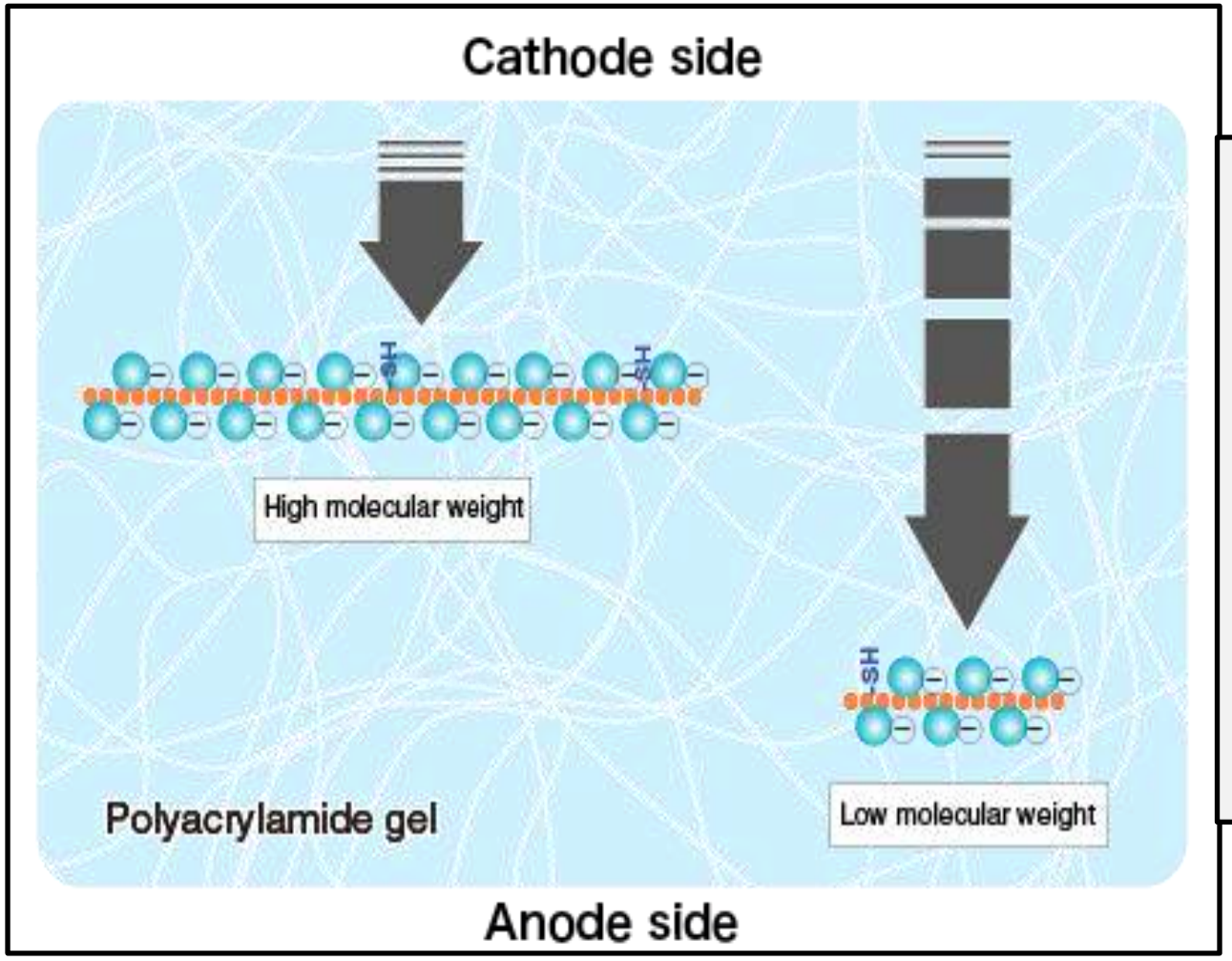
What is the importance of glycine in the stacking layer?

Glycine is in the running buffer, which is typically at a pH of 8.3. At this pH, glycine is predominately negatively charged, forming glycinate anions. When an electric field is applied, **glycinate anions** hit the pH 6.8 stacking buffer, and change to become mostly neutrally charged glycine zwitterions. That means they move slowly through the stacking layer toward the anode due to their lack of charge.

By contrast, the **Cl- ions** (from the Tris-HCl in the gel) move at a faster rate towards the anode. When the Cl- and glycine zwitterions hit the loading wells with your protein samples, they create a narrow but steep voltage gradient in between the highly mobile Cl- ion front (**leading ions**) and the slower moving, more neutral glycine zwitterion front (**trailing ions**). The electromobilities of the proteins in your sample are somewhere in between these two extremes, and so your proteins are concentrated into this zone and herded through the stacking gel between the Cl- and glycine zwitterion fronts.

What happens to glycine zwitterion in the resolving layer?

It gets real negative, real fast. When the Cl- and glycine zwitterion fronts hit the resolving layer at a pH of 8.8, the glycine ions gain a lot of negative charges. They are no longer predominately neutral and take off towards the positively charged anode as glycinate anions. Unaffected by polyacrylamide, they speed past the protein layer, depositing the proteins in a tight band at the top of the resolving layer.



What happens to the proteins in the resolving layer?

They slow way down and start to separate. Also, without the voltage gradient from the Cl- and glycine zwitterion fronts, they can separate.

The tight bands separated by molecular weight. The different sized proteins run at different speeds through the gel, the big ones taking longer as they try to navigate the polyacrylamide web. The molecular weight range of your protein of interest should be separated perfectly along the length of your gel!

Thank you