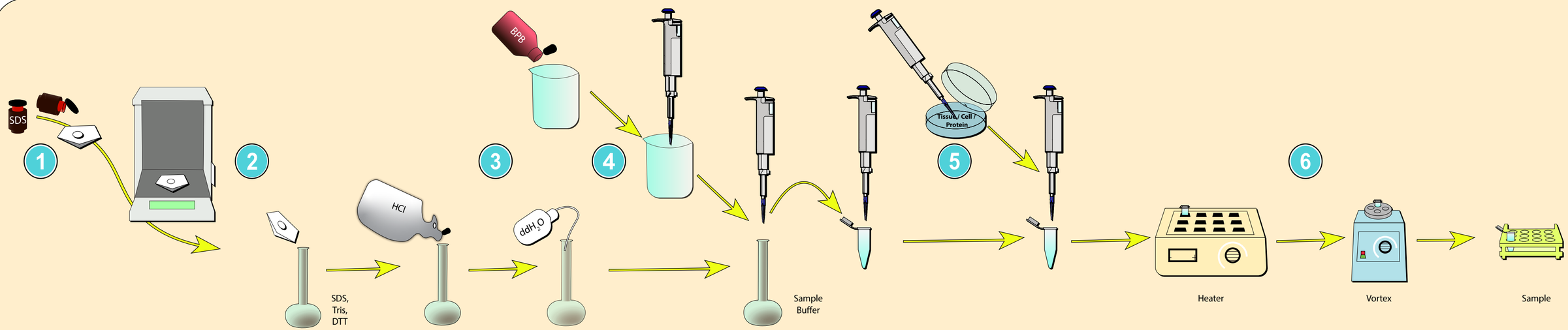
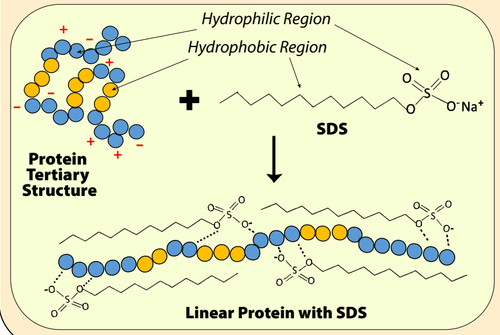


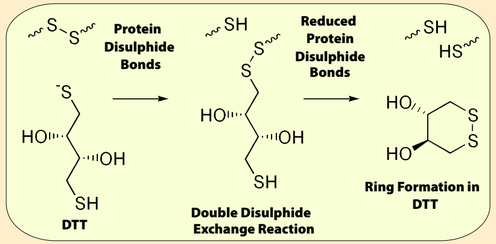
1. Sample buffer is mixed with a chemical denaturant like **sodium dodecyl sulphate (SDS)** for proteins or urea for nucleic acids. SDS denatures secondary and non-disulphide-linked tertiary structures rendering a constant **charge-to-mass ratio** to the protein (by adding about 1.4 g of the molecule to 1 g of the protein or 1 SDS molecule for about 2 amino acids). This renders the molecular weight (MW) of the protein as the logarithmic function to the relative migration distance (Rf).

$$\log MW = -A R_f + B$$

where A and B are numerical constants.



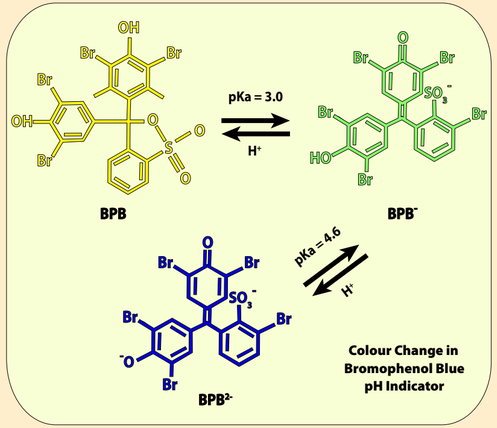
2. Sample buffer is usually **Tris buffer** with pH > 9 that inactivates proteases that can degrade proteins. Buffer is also mixed with a reducing agent like 5% (~ 100 mM) **2-mercaptoethanol (ME)** or 5-10 mM **dithiothreitol (DTT)** to reduce the disulphide linkages. The former is volatile and required in large quantity as it reacts slowly while the latter is less volatile and required in less quantity while also taking lesser time owing to an instantaneous ring structure formation with disulphide bonds.



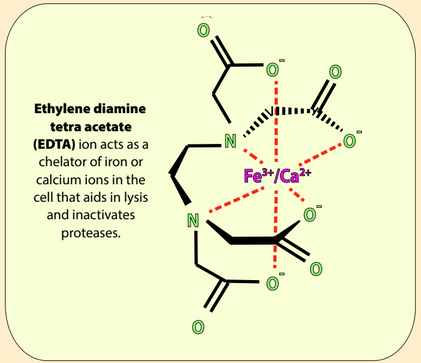
3. Adding **HCl** to the SDS buffer system provides the **leading chloride ions** that are essential for the stacking of proteins in the stacking gel. **Glycerol**, which is denser than water, is also added to the buffers to make the sample sink to the bottom of the well rather than flow out and mix with the buffer in the upper reservoir.

4. A tracking dye such as **bromophenol blue (BPB)** is added to the sample buffer to move in the same direction as the separating proteins and demarcate their leading edge. It is the distance to the dye front that is used in calculating the R_f of the protein band as it lies only slightly behind the non-detectable leading ions.

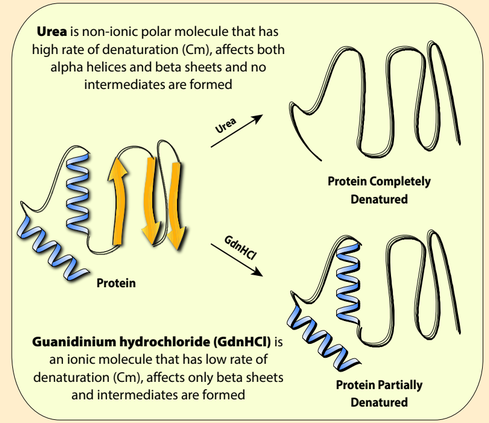
R_f = (distance to band) / (distance to dye front)

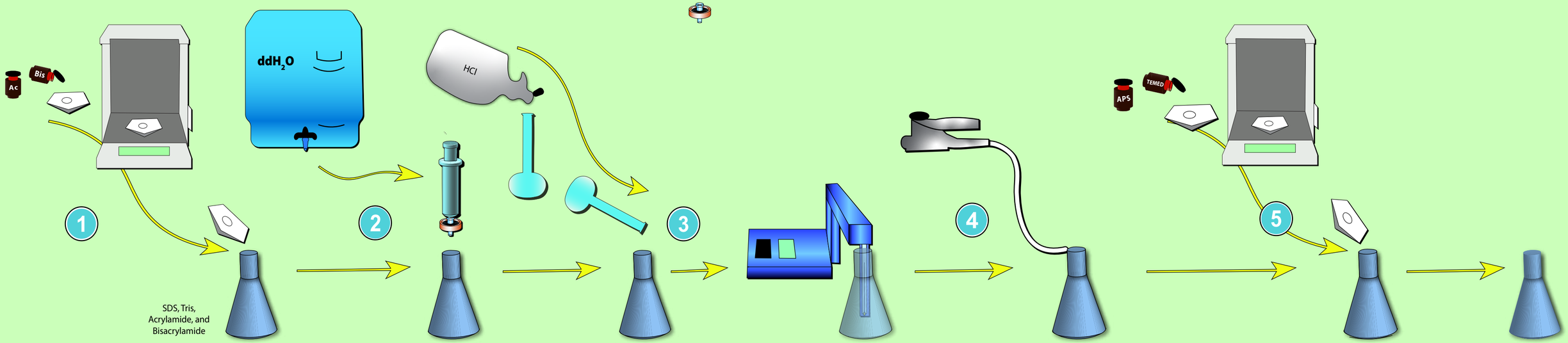


5. Samples are usually **cells, tissues, or purified proteins** that are broken down **mechanically** (by ad mill, french press, homogenizer, sonicator, or microfluidizer), **non-mechanically** (decompression, osmotic shock, freeze thaw, dessication or electroporation), **biochemically** (permeabilizers, antibiotics, detergents, chaotropes {guanidine chloride, urea}, chelators {EDTA}, peroxides, hypochlorides, oligonucleotides) or **biologically** (autolysis, lytic ezymes or phage lysis). These are added to the sample buffer to trigger straight chain formation of the proteins.



6. Sample is heated and vortexed for 3-5 minutes at 95 degrees to enable protein denaturation and inactivation of proteases. Sonication is not used at this point to prevent mechanical degradation.





1. Gels typically contains **acrylamide (Ac), bisacrylamide (Bis), SDS (or urea)** and a buffer with its pH adjusted. Ratio of Bis to Ac is usually 1 part in 35. Acrylamide concentration varies between 5% to 25%. Lower percentage gels are useful to resolve high MW proteins and vice versa. **Pore diameter** of the gel is a function of

(i) **% T** = Total concentration (w/v) of Ac and Bis

(ii) **% C** = Bis concentration (w/w) within T

Pore size is inversely proportional to % T and is parabolic with % C with a minima at 5%.

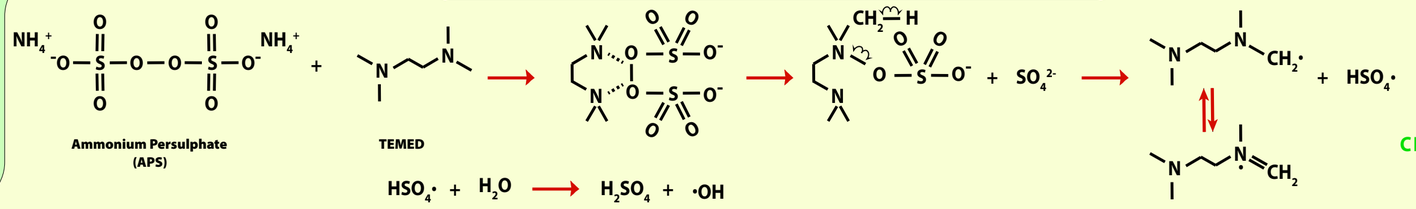
2. Diluting the buffer with **double distilled water (ddH_2O)** and filtering through **cellulose** or **cellulose acetate** membrane filters is necessary to remove fouling and insoluble impurities, and to allow SDS permeability.

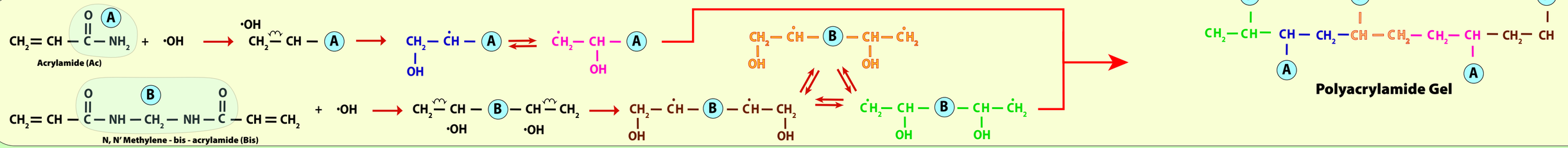
3. Adding **HCl** to the SDS buffer system helps in adjusting pH of the gel solutions. Typically, **stacking gel** has bigger pores with 4% T and pH 6.8, while **resolving gel** has smaller pores with 7.5% to 15% T and pH 8.8.

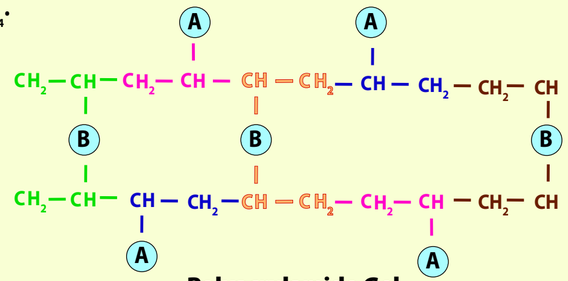
4. In order to de-gas acrylamide electrophoresis gels, 0.8 µm **Cellulose Nitrate (CN)** membrane can be used to rapidly filter and remove gas bubbles. Alternatively, the solution is degassed with reduced pressure in a vacuum.

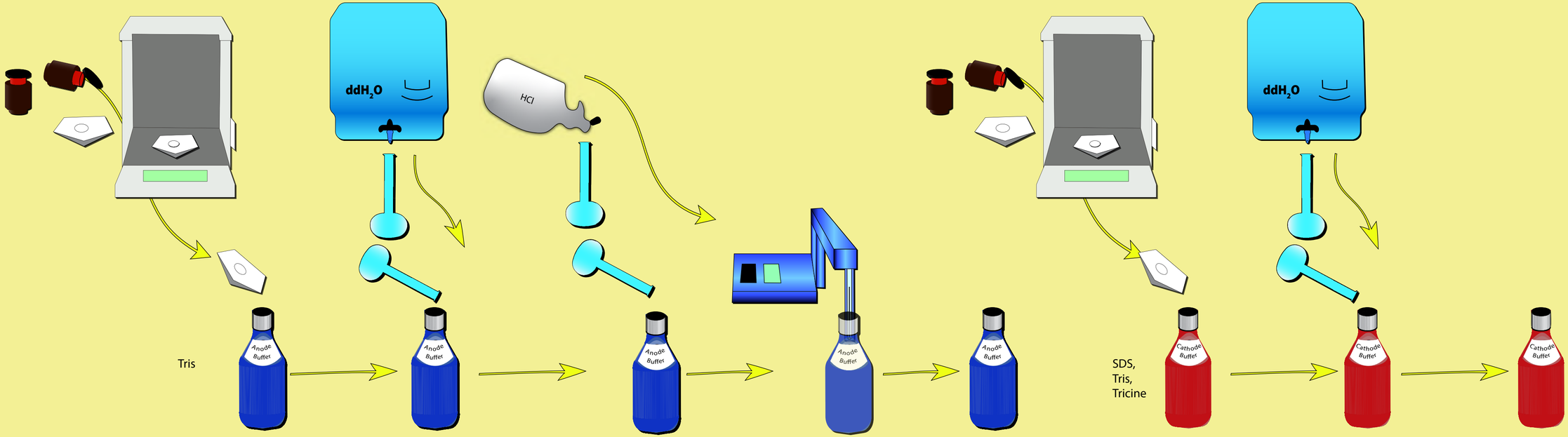
5. Mixture of Ac and Bis is then subjected to a **source of free radicals (ammonium persulphate or APS)** and a **stabilizer (tetramethylethylenediamine or TEMED)** that instantly initiates the polymerization reaction to form **polyacrylamide gel**. The gel is formed due to the cross-linking properties of Bis to the Ac monomer. It is thus essential to add APS and TEMED just before casting the gel and not anytime sooner.

MECHANISM OF POLYACRYLAMIDE GEL FORMATION









1. **Anode buffer,** or positive electrode buffer, is present in the **lower reservoir** of the gel electrophoresis unit.

2. It is usually **Tris buffer** whose pH is adjusted using HCl to about 8.9, which is slightly above the pH of the resolving gel (8.8). This favors the migration of negatively charged proteins towards the positively charged anode.

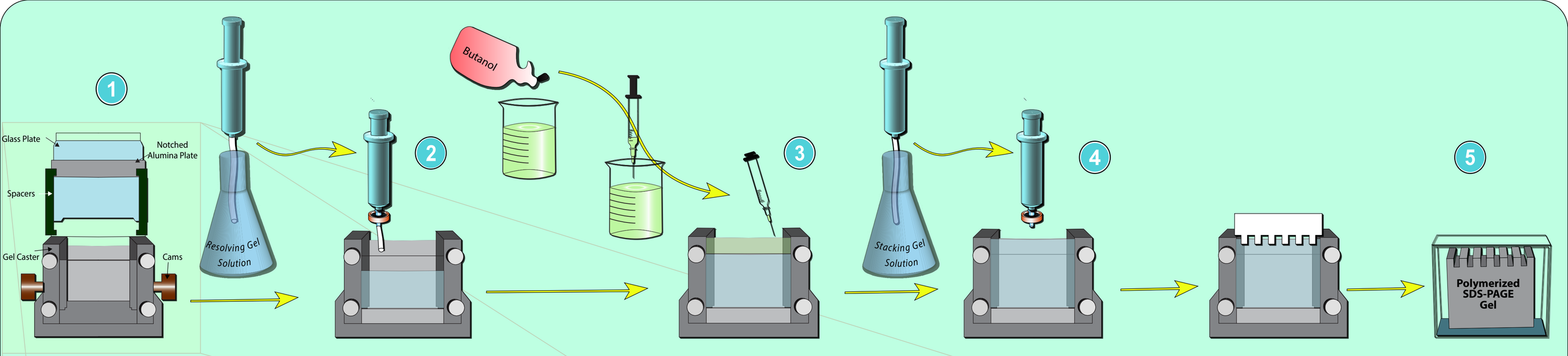
3. **Cathode buffer**, or negative electrode buffer is present in the **upper reservoir** adjacent to the gel. It is either **Tris/glycine/SDS** buffer or **Tris/tricine/SDS** buffer, with a pH adjusted to 8.25, which is slightly less than the pH of the resolving buffer. Excess SDS runs as a large front at the low molecular weight end of the separation. For smaller polypeptides, replacing glycine with Tricine impoves resolution of broad bands of SDS-polypeptide micelles.

4. Alternatives to a typical cathode buffer include **Bis-Tris** system that employs chloride as the leading ions, and 2-(N-morpholino)ethanesulfonic acid (**MES**) adjusted to pH 7.2 and used for smaller proteins or 3-(N-morpholino)propanesulfonic acid (**MOPS**) adjusted to pH 7.7 used for mid-sized proteins as the trailing ions.

5. Compared to Tris/glycine gel, neutral gel pH improves protein stability and result in sharper bands. It also reduces the risk of **protein deamination** or **alkylation**. It also lowers the risk of glycine ions and sulfhydryl groups reacting with free non-polymerized acrylamide.

Comparison of SDS-PAGE Buffer Systems

	Glycine (trailing ion) / Protein/SDS complex (stacked proteins) / Chloride (leading ion) / Common ion is Tris present in gel and running buffers	MES or MOPS (trailing ion) / Protein/SDS complex (stacked proteins) / Chloride (leading ion) / Common ion is Bis-Tris present in gel	Tricine (trailing ion) / Protein/SDS complex (stacked proteins) / Acetate (leading ion) / Common ion is Tris present in gel and running buffers
Gel buffer ion	$Tris^+$. Cl^- (pH 8.7)	Bis-$Tris^+$. Cl^- (pH 8.7)	$Tris^+$, $acetate^-$ (pH 8.7)
Running buffer ions	$Tris^+$, Gly^-, SDS (pH 8.3)	$Tris^+$, MES^-, $MOPS^-$, SDS (pH 8.3)	$Tris^+$, $tricine^-$, SDS (pH 8.3)
Gel operating pH	9.5	7.0	8.1



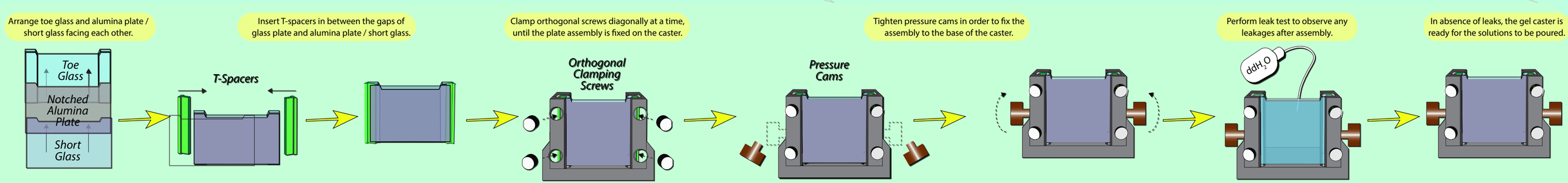
1. Ensure that all glass plates are clean and dry. Prepare the glass plates and assembled cassette for gel casting. Place the casting tray upright, with pressure cams in open position. Select the spacer plate of the desired gel thickness and place a short plate on the top. Slide the two glass plates in the casting frame, ensuring that both plates are flush on a level surface to avoid leakage. Screw tight the caster and engage the pressure cams to lock the glass plates in place. Perform a leak test with ddH_2O to ensure everything is tightly fit.

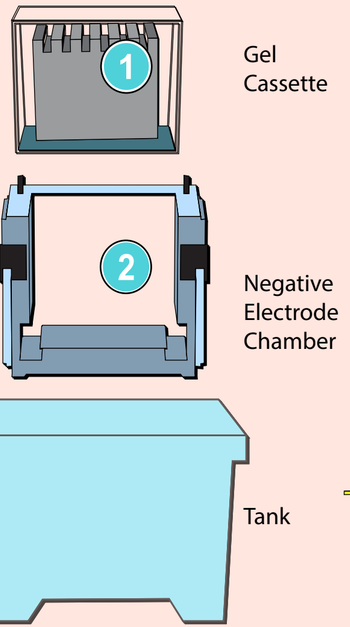
2. Add **resolving gel solution** till the mark designated for the stacking gel, and deep enough to apply a comb and have the samples stack up during electrophoresis. Wait for a sufficient time for the resolving gel to polymerize (usually 40 minutes or less).

3. Remove air bubbles while filling the resolving gel solution by adding **butanol** using a **Hamiltonian syringe**, since butanol is nearly immiscible with the hydrophilic gel solution and fills any air gaps in it by floating over. Drain the butanol once both layers settle, and wash the top surface of the gel with ddH_2O.

4. Once resolving gel solidifies, add the **stacking gel solution** over it. Insert an appropriate **gel comb** that fits the spacing in the glass plates and that is deep enough to add appropriate amount of the sample solution, while also considering the migration distance in the stacking layer.

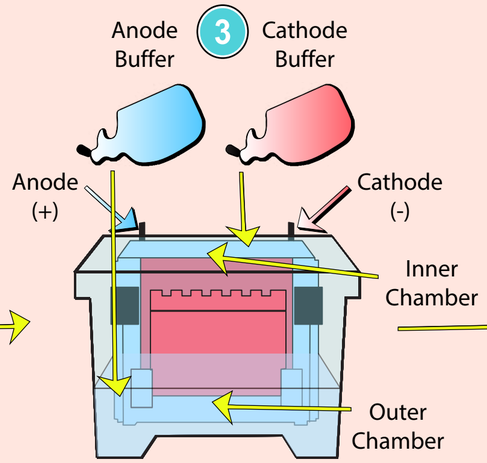
5. Once solidified, wash the gel with ddH_2O and keep it wet between the glass plates, until used for electrophoresis.



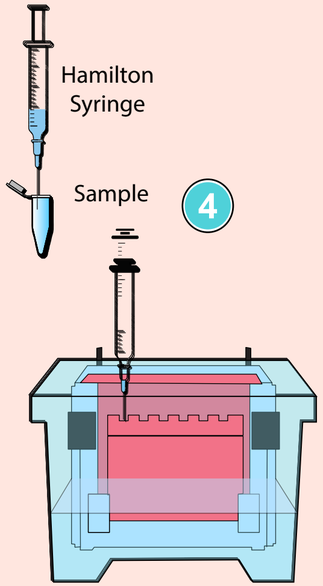


1. After gently removing the comb from the gel, Wrap the gel in paper towels and wet everything with ddH_2O. If the gel is required to be stored overnight, overlay it with **1X pH 8.8 Tris buffer** and store at **4˚C**. It is advisable to prepare resolving gel before the stacking gel if the gel is to be stored for longer periods, in order to improve its shelf life and prevent pH-based diffusion. Take ddH_2O and pour it into the lanes. Grasp the glass plate firmly and flick to remove the water from the lanes. Repeat the procedure multiple times.

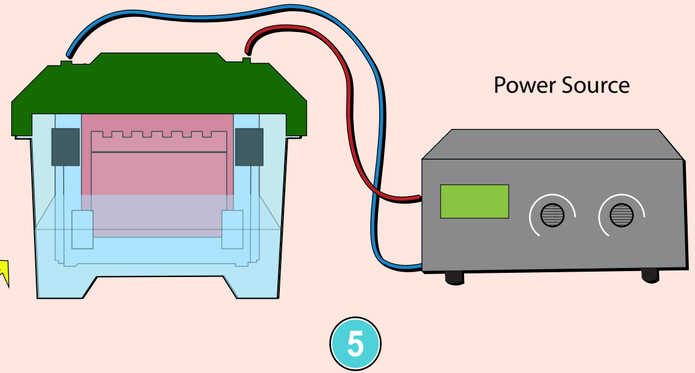
2. To assemble the inner chamber, seal the electrophoresis apparatus on the running buffer tray. **Short plate** of the gel is placed facing the interior of the apparatus. A **buffer dam** is placed alongside the **gasket** which is present on the opposite side of the apparatus. This creates the necessary spacing to pour the cathode buffer. Finally, fix both corners of the apparatus to create the upright inner chamber.



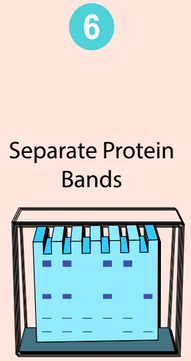
3. Insert the inner chamber into the outer chamber, while matching the colours of the electrodes to the colours of the junctions. Add the **running buffer (cathode buffer)** in the **inner chamber** followed by the e**lectrophoresis buffer (anode buffer)** in the **outer chamber**. The running buffer must be filled till it passes the wells and sinks the gel.



4. To facilitate loading samples, a **guide comb** can be used. In order to load the protein samples, take the pipette and angle it away from you. Slowly release the sample until all the sample sinks into the well.



5. Remove the guide comb and cover the box with the wiring apparatus. Connect the leads into the power supply and set it to at least **200 V**. Run the electrophoresis for about **30-45 minutes**. **Bubbles** are observed near the electrodes as power is supplied, Ensure that the liquid in the inner chamber submerges the short plate all the time, in order to prevent short-circuit which is confirmed when no bubbles are observed. Also, the current in the power supply unit drops down to 0.



6. Tracking dye runs through the gel and indicates its completion, while protein bands are observed only after staining. However, **pre-stained protein ladder** and standards are observable while the gel runs. For long term storage, dry stained gels in a 10% glycerol solution, place between cellophane sheets and store at **4˚C.**

THERMODYNAMICS OF GEL ELECTROPHORESIS

Regulated direct current (DC) power supplies control the three modes for electrophoresis -,

1. **Constant voltage (V) or electric field (E)**
2. **Constant current (I)**
3. **Constant power (P)**

$$I = V / R$$

$$P = VI = I^2R = V^2/R$$

$$E = V/d$$

where **R** is the **resistance** and **d** is the length of the electrophoresis system which is determined by ionic strength of the buffer, gel conductivity etc.

Most vertical electrophoresis chambers operate at a field strength of 10-20 V/cm for a 1 mm thick PAGE gel. This is limited by the heat generated in the system

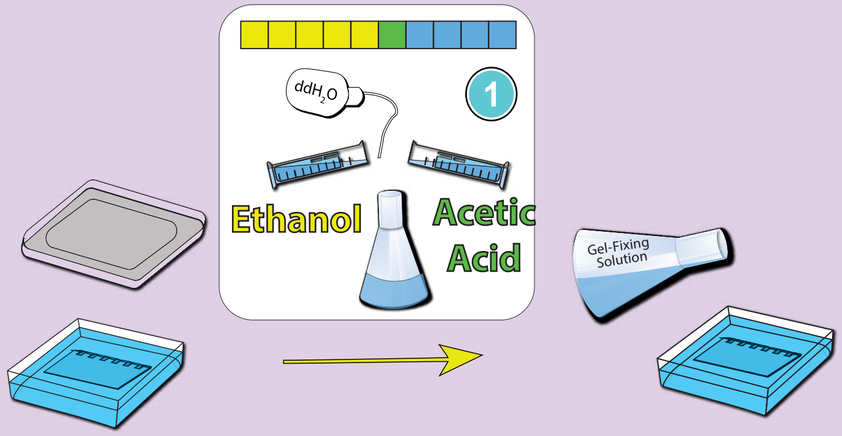
$$Heat = P / 4.18 \text{ cal/sec}$$

Excess Joule heating increases with increase in the number of gels, thickness of the gel, buffer volume and temperature. The voltage, however, remains constant over the gel thickness, while it increases over the gel length. In discontinuous systems, resistance increases as the run progresses, which can impact Joule heating.

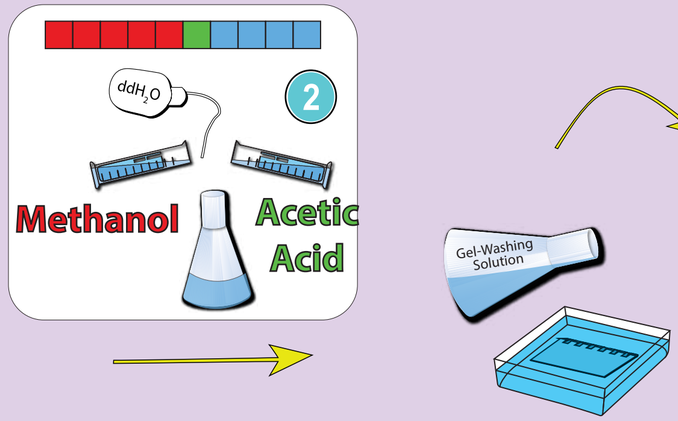
I and P decrease as R increases under **constant voltage**. This increases the run times and leads to lower diffusion rates. This leads to consistent runs even if multiple gels are run in a single unit.

Under **constant current** mode, P and V increase as R increases, leading to increased diffusion rates.This reduces the run time, but also impacts resolution and heats up the system quickly.

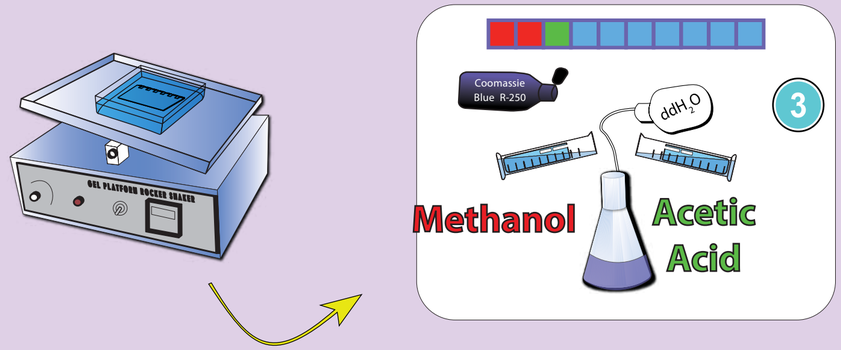
Constant power minimizes the risk of overheating, whereby both run times and resolution can be intermediate of constant voltage and constant current.



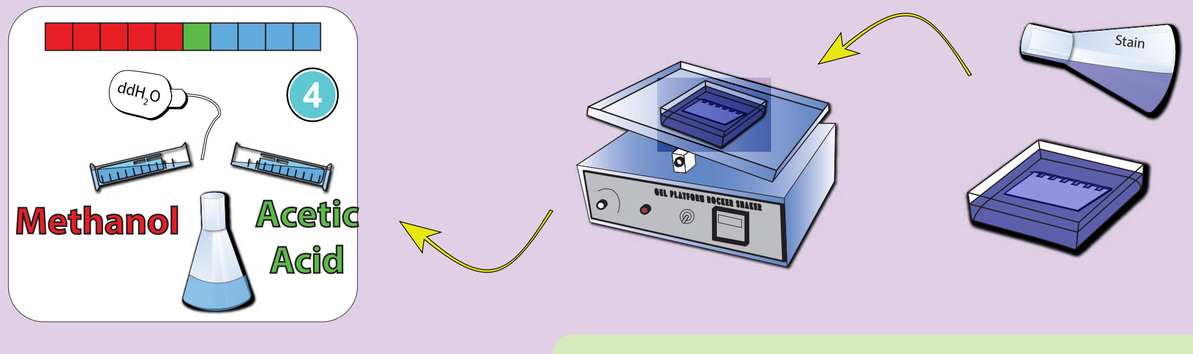
1. After electrophoresis, wash the gel off the glass plates with the **gel-fixing solution (50% v/v ethanol, 10% v/v acetic acid)** and soak in that solution for 1hr. The purpose of this step is to gently remove the gel from the plate and begin washing the SDS-containing gel buffers out of the gel. Aspirate the solution.



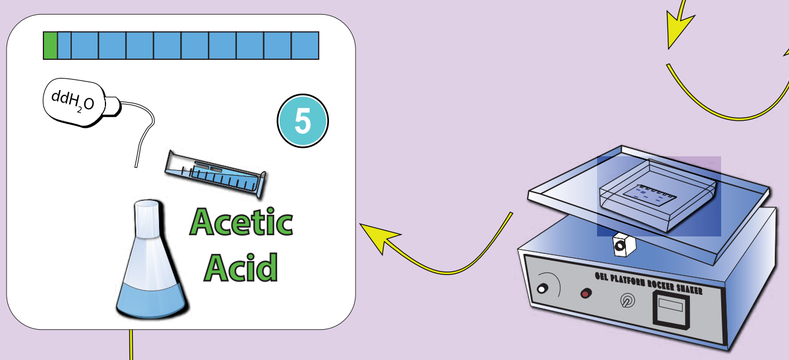
2. Cover the gel with **gel-washing solution (50% v/v methanol, 10% v/v acetic acid)**, and continue to fix the proteins in the gel by incubating overnight at room temperature with gentle agitation using a **gel rocker**. The gel should be covered during this process to avoid contamination and to prevent the evaporation of the solution. Aspirate the solution.



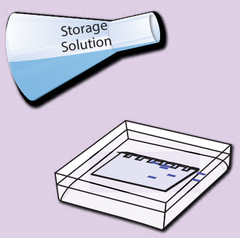
3. Cover the gel with **Coomassie stain (0.1% w/v Coomassie R350, 20% v/v methanol, 10% v/v acetic acid).** Stain the gel at room temperature for 3 to 4 hr with gentle agitation. Aspirate the stain.



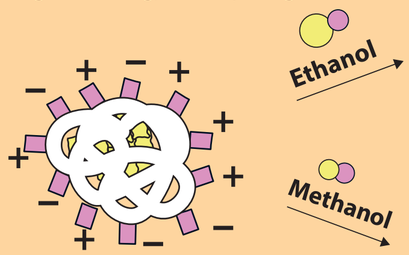
4. Cover the gel with the **destain solution (50% v/v methanol, 10% v/v acetic acid)** and allow the gel to destain with gentle agitation. The destain solution should be changed several times. Continue the destaining until the protein bands are seen without the background staining of the gel. Methanol tends to compress the gel at this point.



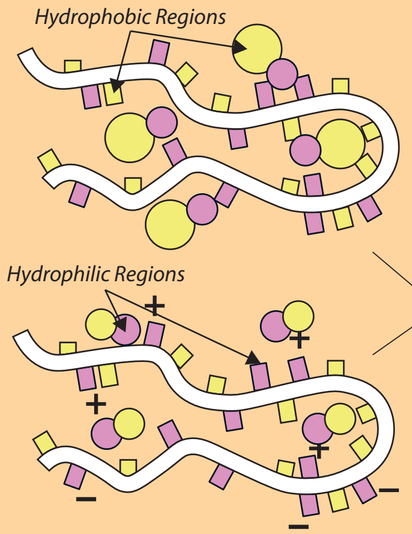
5. Equilibrate the gel in the **storage solution (5% v/v aceic acid)** for at least 1 hr. The gel returns to its original dimensions during this process. Store the gel in the storage solution as needed. It might be convenient to carefully transfer the gel to a heat-sealable bag for longer-term storage.



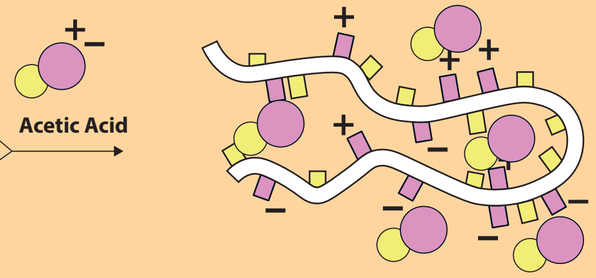
Ethanol and **methanol** both denature proteins. They replace water in the tissue environment disrupting hydrophobic interactions and hydrogen bonding, thus exposing the internal



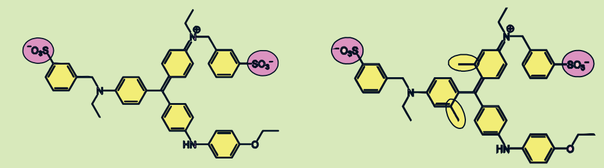
hydrophobic groups of proteins and altering their tertiary structure and solubility in water. Methanol is closer to the structure of water than ethanol, so ethanol interacts more strongly with hydrophobic areas than methanol.



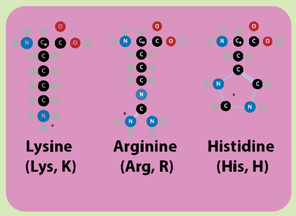
Acetic acid being a charged polar molecule, aids in providing the necessary interactions in hydrophobic regions as well as hydrophilic regions. It provides acidity in the environment,

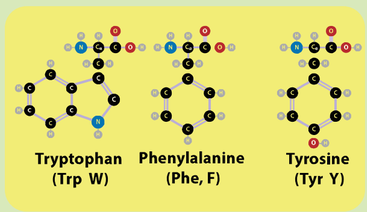


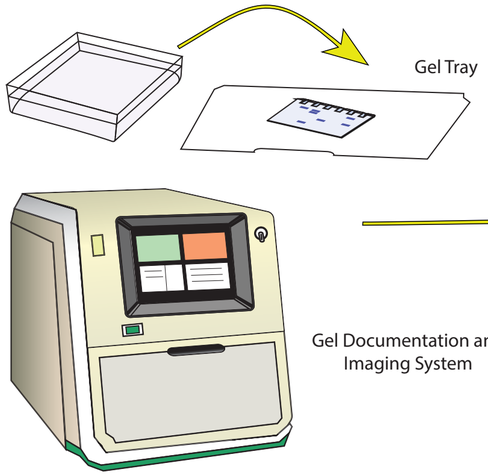
that exposes the charges on polar charged amino acid residues whose pKa is less than the pH of acetic acid. This helps the stain molecules to chemically interact with both hydrophobic and charged regions of the protein.

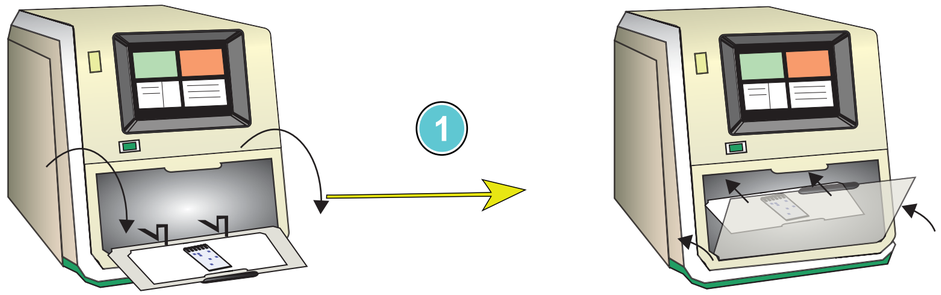


The staining molecule **Coomassie Brilliant Blue** has negatively-charged functional groups that bind to the positively-charged residues in the protein like **lysine, arginine and histidine** ,while the hydrophobic aromatic rings interact with the amino acids containing ringed structures or similar aromatic rings like **tryptophan, phenylalanine and tyrosine**. The dye's rings absorb visible light, thereby appearing blue. The more aromatic rings the protein has, the more profound the band will be. Owing to additional methyl groups in **G-250** monomer, absorptivity increases and proteins are resolved more effectively than **R-250** monomer of Coomassie Blue stain.

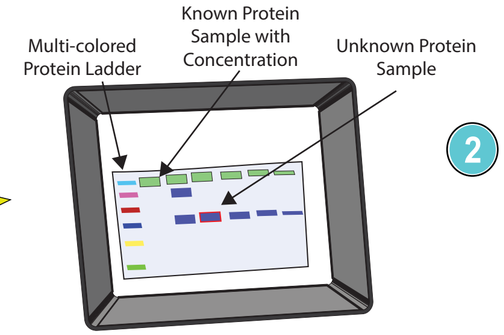




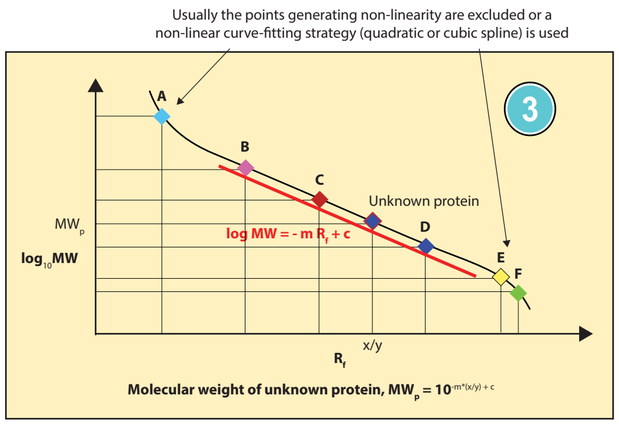




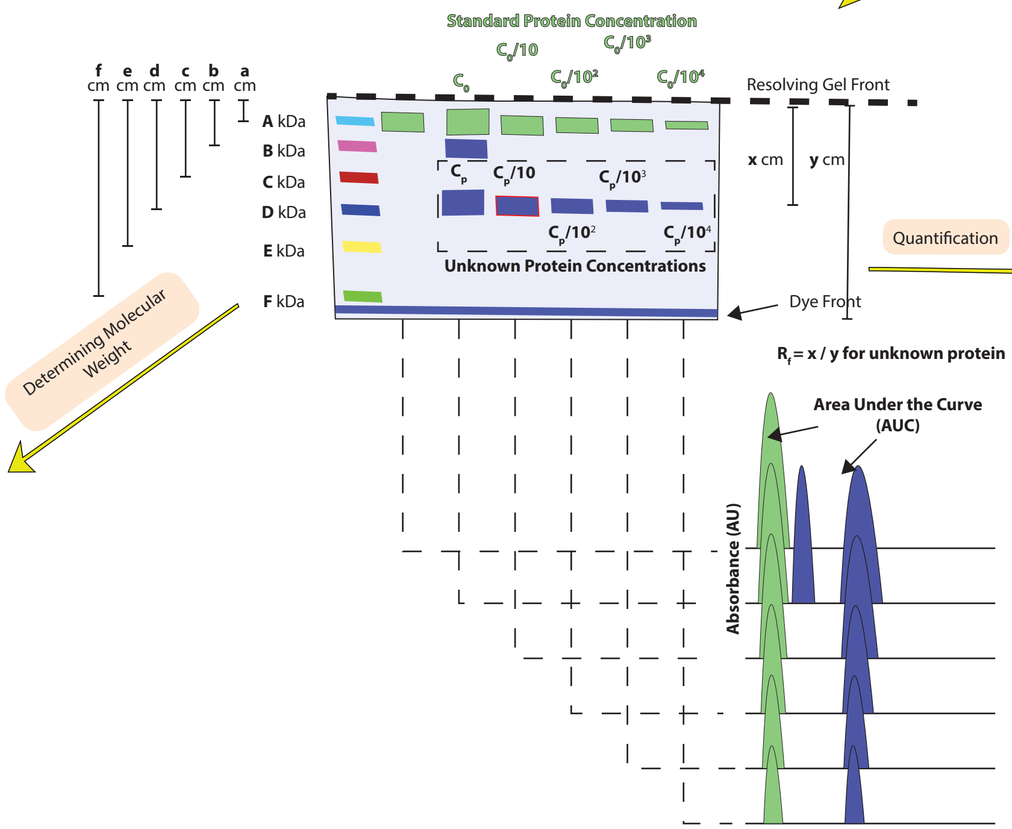
1. After the gel is stained, destained, washed and equilibrated, use the designated **gel documentation and imaging system** based on the **gel type, stain type, protein ladder type, and wavelength of UV absorption** appropriate for the gel. Usually the type of tray for imaging under UV light is pre-defined for standard and special gels.

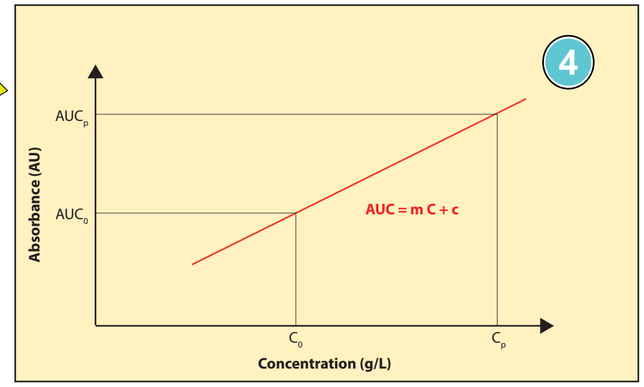


2. Usually, in addition to the unknown protein of interest, an optional **standard protein** of similar molecular weight and given concentration is used for generating linear curves of **$\log_{10}$MW versus R_f**. An unstained, pre-stained unicolour, or pre-stained multi-colour **protein ladder** containing known proteins of different molecular weights is used to determined the standard curve in order to find out the unknown protein's molecular weight.



3. Once the linear curve is generated using the protein ladder, R_f of the unknown protein can be calculated by physically measuring the distance of the protein band from the **starting end of the resolving gel** and dividing it with the distance of the **dye front**. Using the value in the linear equation, its molecular weight is determined within an accuracy of 5%.





4. Another application is the **quantification** of the unknown protein of interest. If the protein's standard solution iof known concentration is available, its standard curve is generated by diluting it usually with a factor of 10. A **linear curve** is generated between its **absorbance (AUC)** and **concentration (C)**, which is called the **Beer Lambart's Law**. This helps in determining the concentration of the unknown protein, and also in finding its **extinction coefficient (ε)** if the path length of the UV detector is known.

COLORS OF THE PROTEIN LADDER

A study by Compton et. al. provides an insight into the colors that we observe in the protein ladder.
A in the hypothetical gel above is the protein with a very high molecular mass, like **bovine serum albumin (BSA) (MW 66 kDa)** which is label using **Remazol Turquoise**.

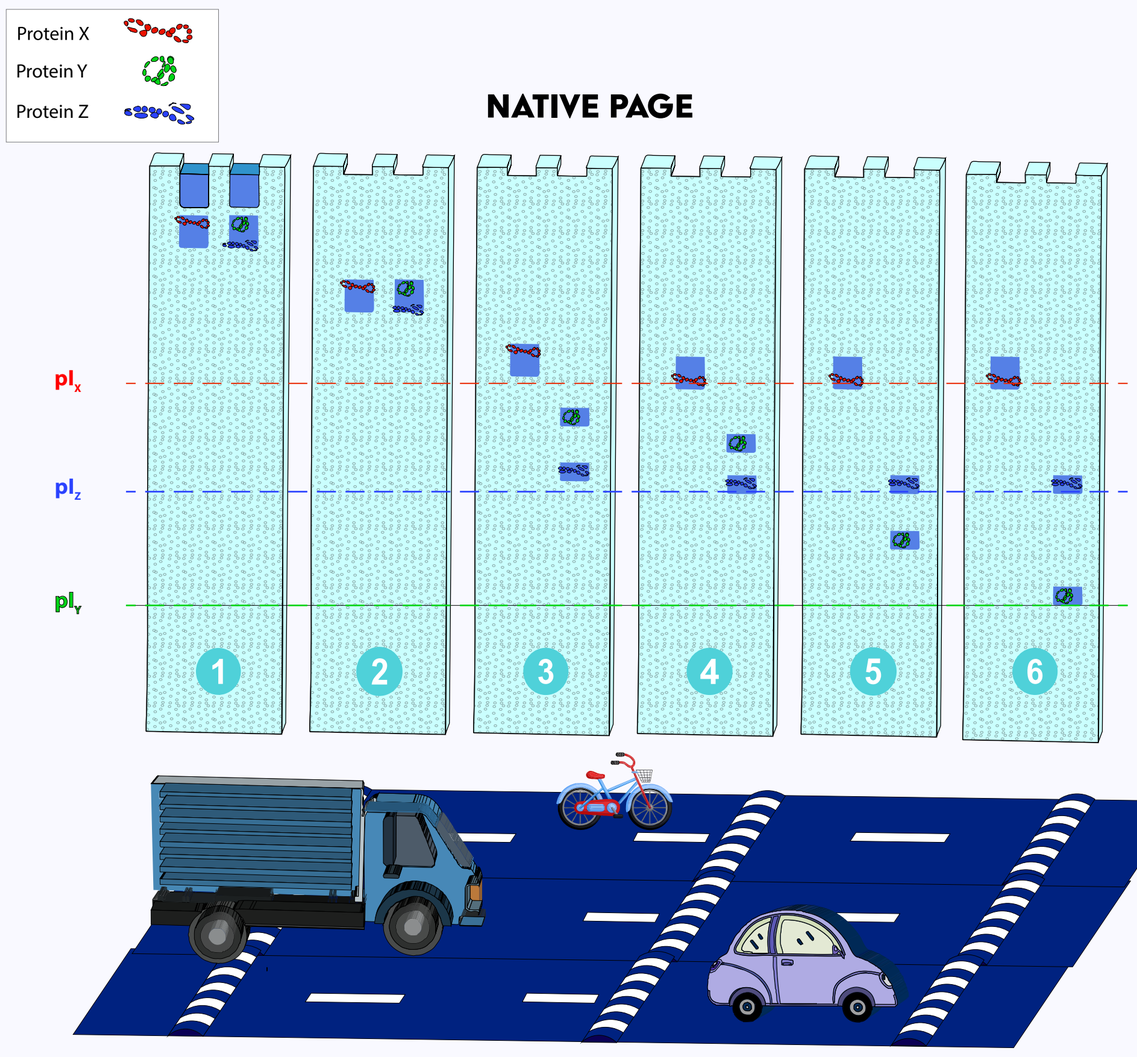
B may be a lighter yet heavier protein, like **egg albumin (MW 45 kDa)** which is labelled using **Remazol Brilliant Red F3B**.

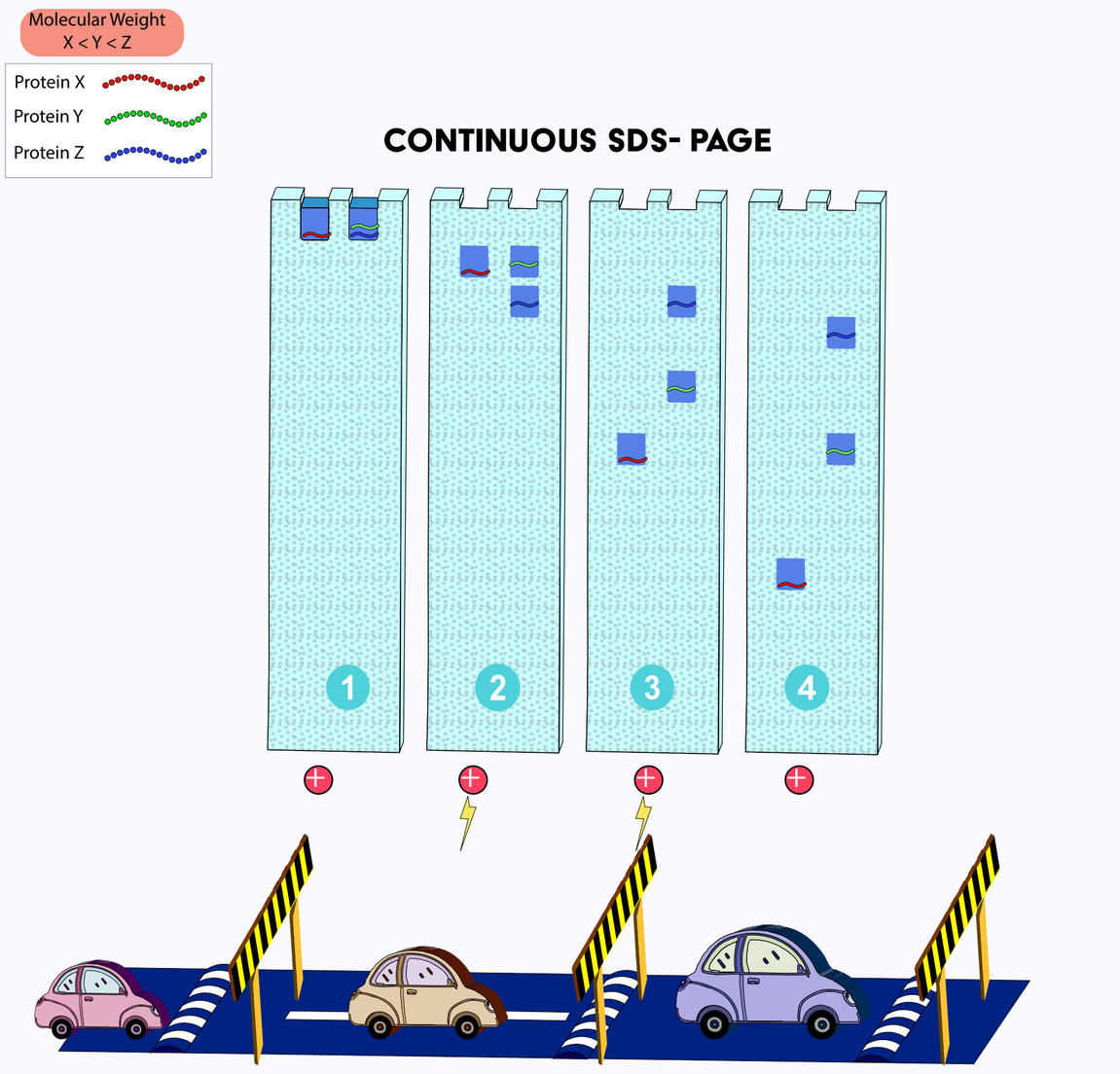
C may be a medium-weight protein, like **carbonic anhydrase (MW 29 kDa)** which is labelled using **Remazol Brilliant Orange 3R**.

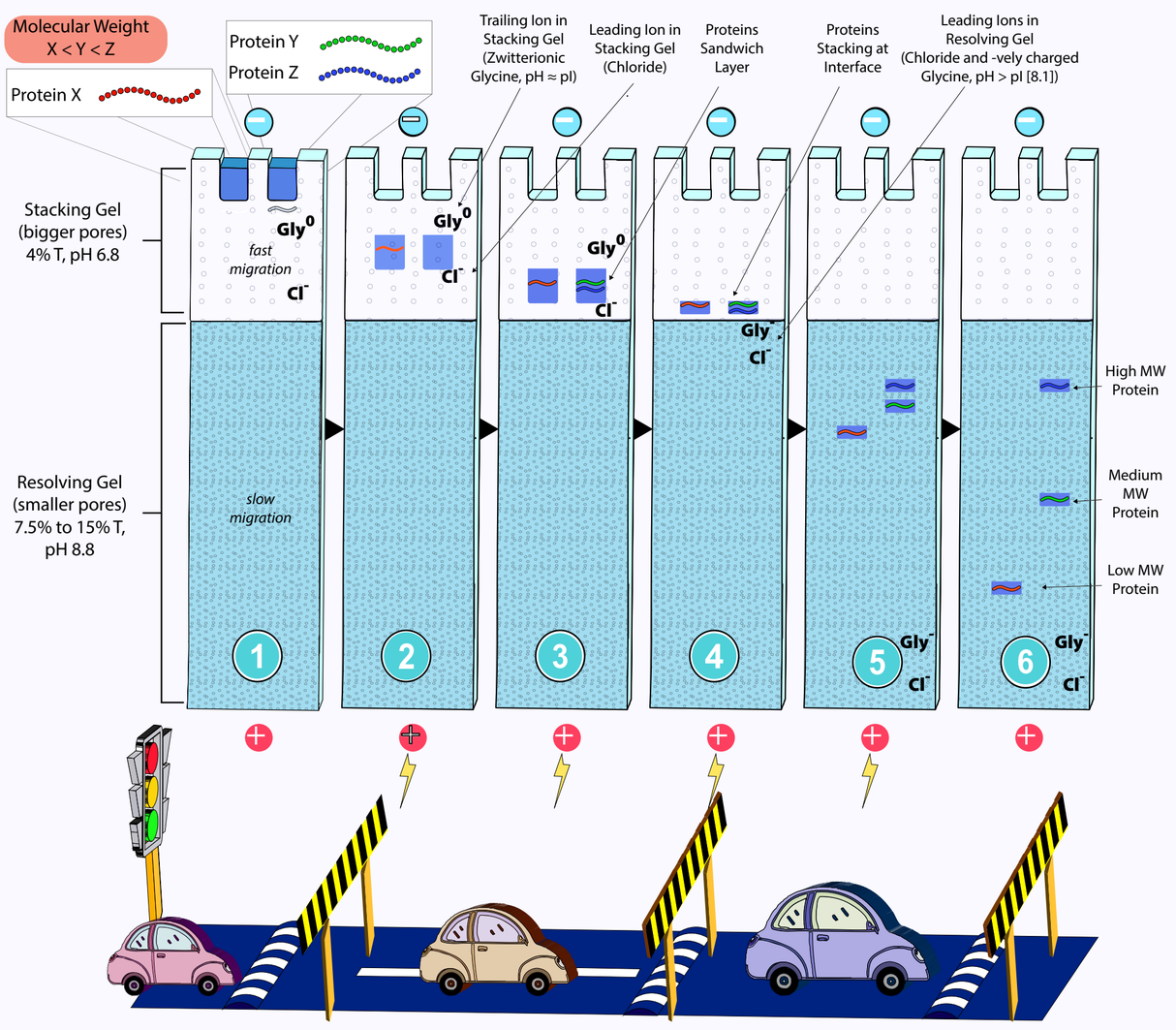
D may be yet another medium-weight protein, like **trypsin inhibitor (MW 20 kDa)** which is labelled using **Remazol Brilliant Blue R**.

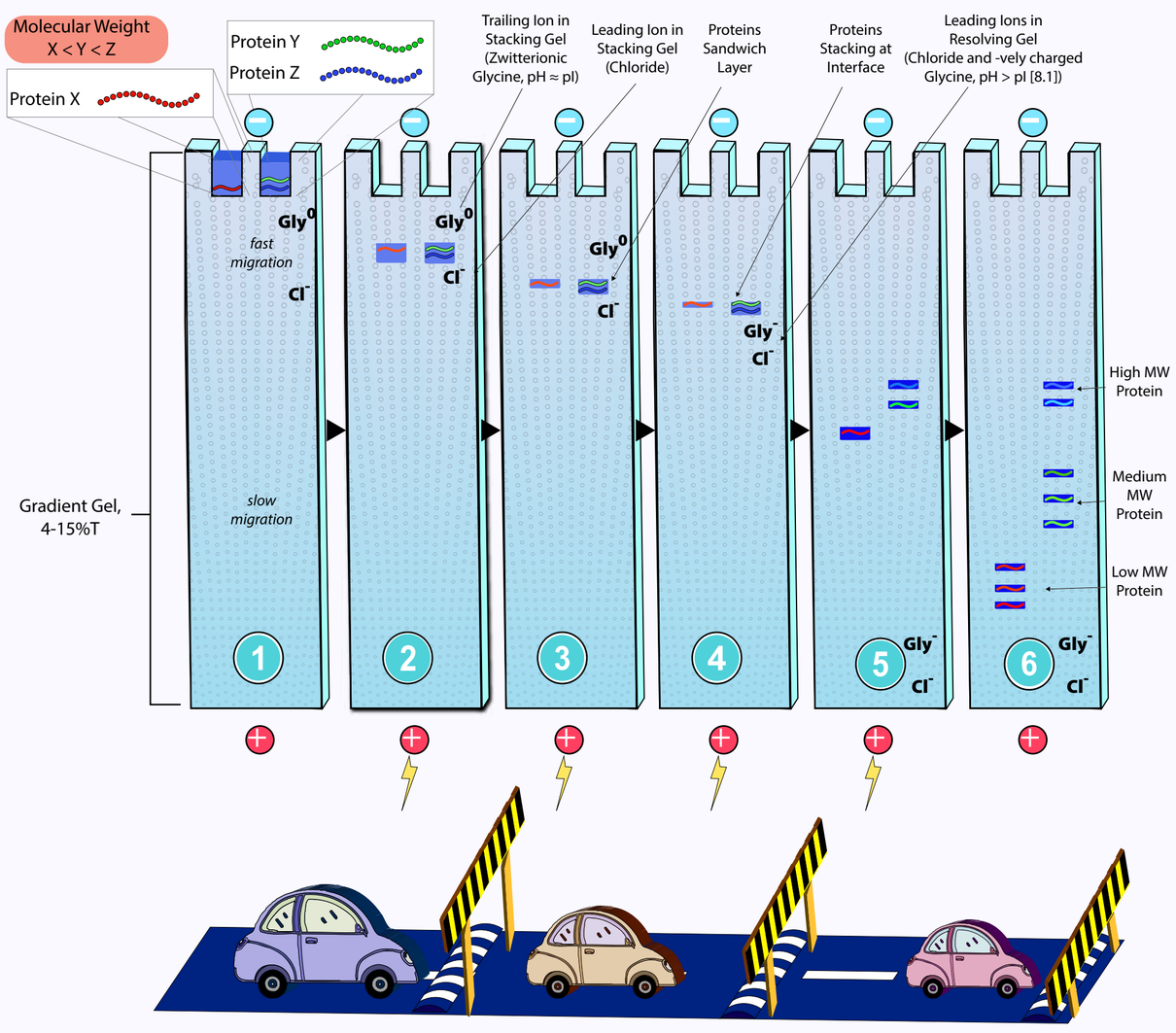
E may be a lightweight protein like **lactalbumin (MW 14.2 kDa)** which is labelled using a 4:1 ratio of **Remazol Golden Yellow RNL** and **Remazol Brilliant Orange 3R**.

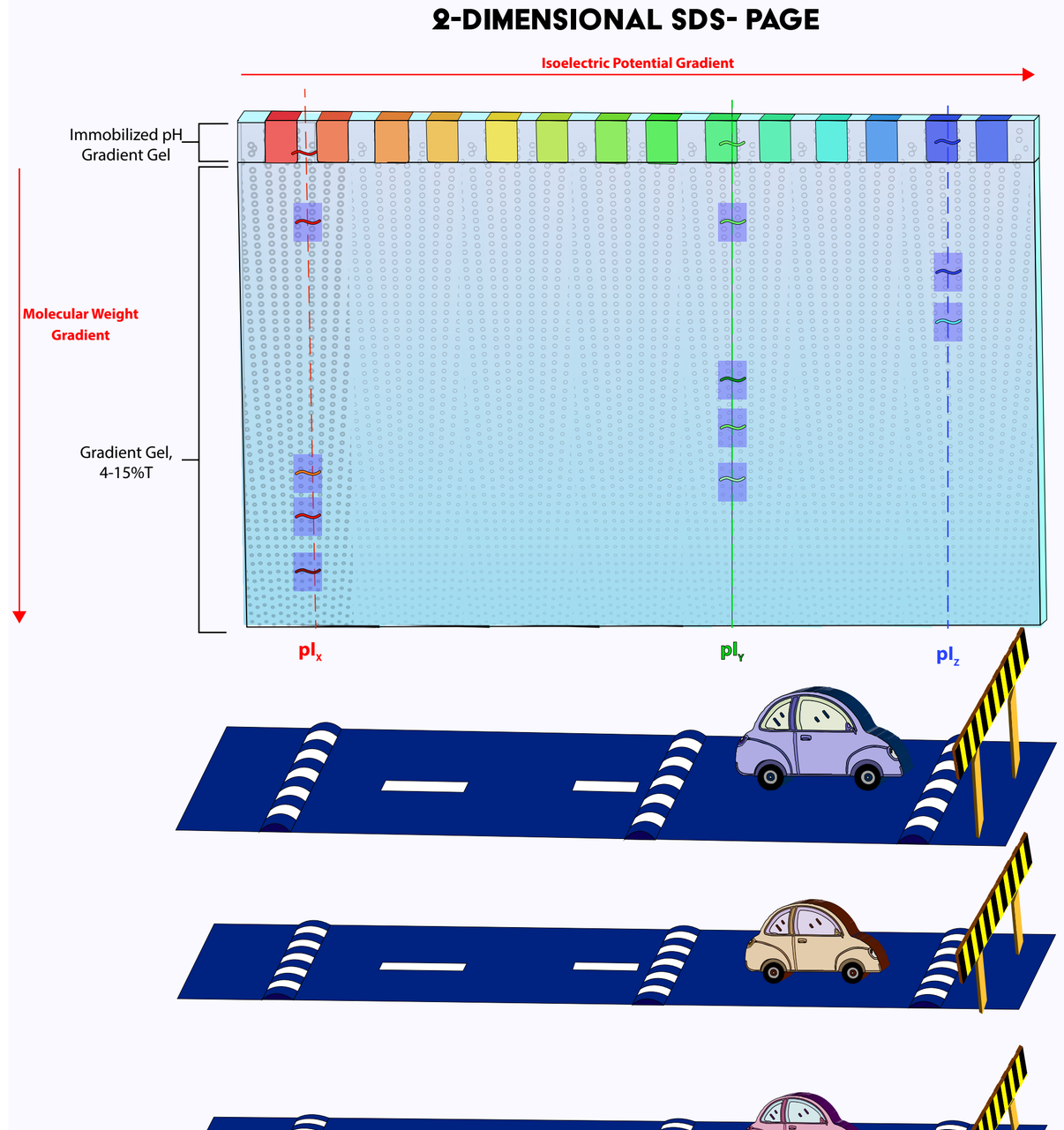
F may be the lightest protein in the pool like **aprotinin (MW 6 kDa)** which is labelled using 4:1 ratio of **Remazol Brilliant Blue R** and **Remazol Golden Yellow RNL**.











Different types of gel electrophoresis methods for protein purification can be considered like road traffic rules. Lets consider each gel phenomenon as a part of the road traffic :

Road bumper = polyacrylamide with bigger pores

Height barrier = polyacrylamide with variable pores

Car = linear protein

Bike, Truck = different proteins in native structure

Traffic lights = junction between stacking and resolving gels

1. **Native PAGE :** It is like a freeway for all vehicles, with only road bumpers. Proteins in their native state are separated based on their isolectric potential only. Charge-to-mass ratio varies, so it is not strictly based on molecular weight. Useful when protein in question is known and required in bulk for further analysis.

2. **Continuous SDS-PAGE :** It is like a car-only route with road bumpers and similar height barriers height barriers. Proteins are denatured into linear chains and their charge to mass ratio is rendered constant using SDS. This separation of proteins is purely a function of molecular weight. Owing to variable retention force on each protein, their rates of migration and diffusion vary and it is difficult to predict the protein based on only their location.

3. **Discontinuous SDS-PAGE :** It is like a car-only route with road bumpers and similar height barriers, but with traffic lights to stop the cars at the junction. All proteins stack together owing to a sandwich layer between a leading ion and a trailing ion which occurs at the junction of the stacking gel and the resolving gel. This ensures similar rates of migration and diffusion leading to better resolution in the resolving gel.

4. **Gradient SDS-PAGE :** It is like a car-only route with road bumpers and variable height barriers. The resolution of proteins, if done in a discontinuous SDS-PAGE, is increased, as the step change in retention force on each protein increases upon migration as the incoming pore size decreases constantly, which also affects diffusion. This leads to higher resolution than a single %T resolving gel.

5. **2-D SDS-PAGE :** It corresponds to unique lanes for cars of every size driven on a car-only road with road bumpers and variable height barriers. The proteins are first segregated on an immobilized pH gel (IPG) where proteins migrate based on their pI values. Later, a gradient SDS-PAGE is run while stacking the IPG over a gradient gel. This resolves the proteins as shown in gradient SDS-PAGE. Thus a two-factor(isoelectric potential and molecular weight) segregation of proteins leads to higher resolution.