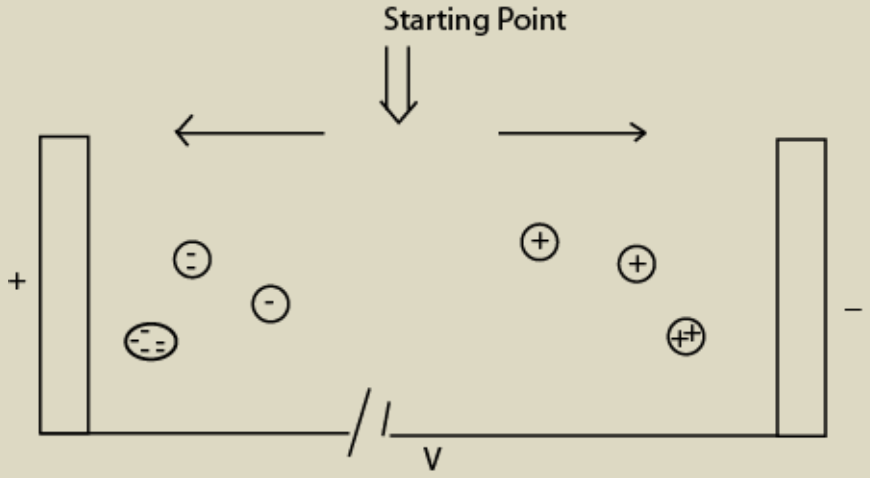
Lecture 9: Polyacrylamide gel electrophoresis;

Some basic concept: If a mixture of electrical charges molecules are kept in electric filed for field strength E, they shall move toward opposite electrode. This process is called electrophoresis.



Velocity of movement (v) is defined by following equation:

$$v = \frac{Eq}{f}$$

E → Field Strength

q → net charge on the molecule

f → Frictional coefficient

$f = 6\pi \eta \Upsilon$

Υ → radious of the molcule **(Size)**

η → coefficient of viscocity

f - depends on buffer viscocity.

If electrophoresis is being taken place in matrix, this depends on porOsity of the matrix

-Also depends on size of molecule

As clear from the equation, mobility depends on various factors like net charge (q) and frictional coefficient (f). Size of biological molecule is proportional (not strictly as we see during next lecture) to the mass. Molecule with equal charge /mass ratio have identical electrophoretic mobility in a given medium.

Now let us assume the electrophoresis is being taken place in liquid medium. What happens when voltage is removed? All molecules are diffused and mixed up again and electrophorectic mobility is not detectable soon after voltage was removed. If we want to see the position of molecule after electrophoresis, we need to minimize the diffusion. For this purpose, solid matrix is required in the process of electrophoresis. Polyacrylamide is commonly used matrix is protein electrophoresis while polymerized agarose is used for DNA electrophoresis.

Electrophoresis at constant current or constant voltage?

Ohm's law relates V to current, I by electrical resistance, R as follow

$$I = \frac{V}{R}$$

We might think that increasing V would result in increase in current which in turn give faster electrophoretic mobility and doing experiment at fixed high voltage is better (??). However, large current results in sufficient power generation (watts law). The power generated during electrophoresis is given by

$$W=I^2 R$$

Heat generation is not desirable in electrophoresis as this result in bad resolution of separation due to convection of buffer causing mixing of separated molecule. Moreover, temperature (heat) decreases viscosity of buffer. Decrease in viscosity results in easier movement of ions resulting in decrease in resistance. According to Ohm's law decrease in resistance at fixed voltage result in further increase in current and head generation. Additionally, at high temperature thermolabile molecule like protein may breakdown during electrophoresis and may not be separated in native form. Thus, except specify applications, constant current may be a better option.

Experimental set-up for poly acrylamide gel electrophoresis

As explained above a solid support during electrophoresis helps in separation of molecules in process of electrophoresis. A very common method for separating proteins by electrophoresis uses a polyacrylamide gel as a support medium. The process is called polyacrylamide gel electrophoresis. Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide and N,N'-methylene- bis-acrylamide. N,N'-methylene- bis-acrylamide is a cross-linking agent for the gels. The polymerization is initiated by the addition of freshly prepared ammonium persulfate along with N,N,N^1,N^1,- tetramethylethylenediamine (TEMED). The TEMED has ability to exist as a free radical and acts as an additional catalyst for the polymerization (Fig. 1). The polymerization is done in gel polymerized between two plates of glass. Details experimental set-up is shown in series of figures (Fig.2).

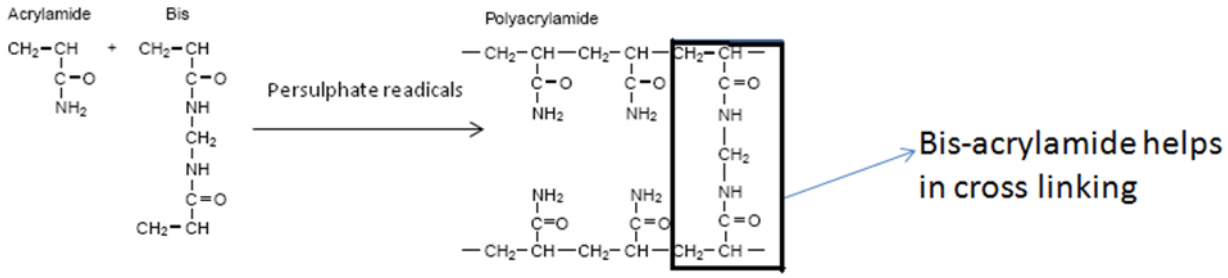


Figure 1: Polymerization reaction of acrylamide and bis-acrylamide

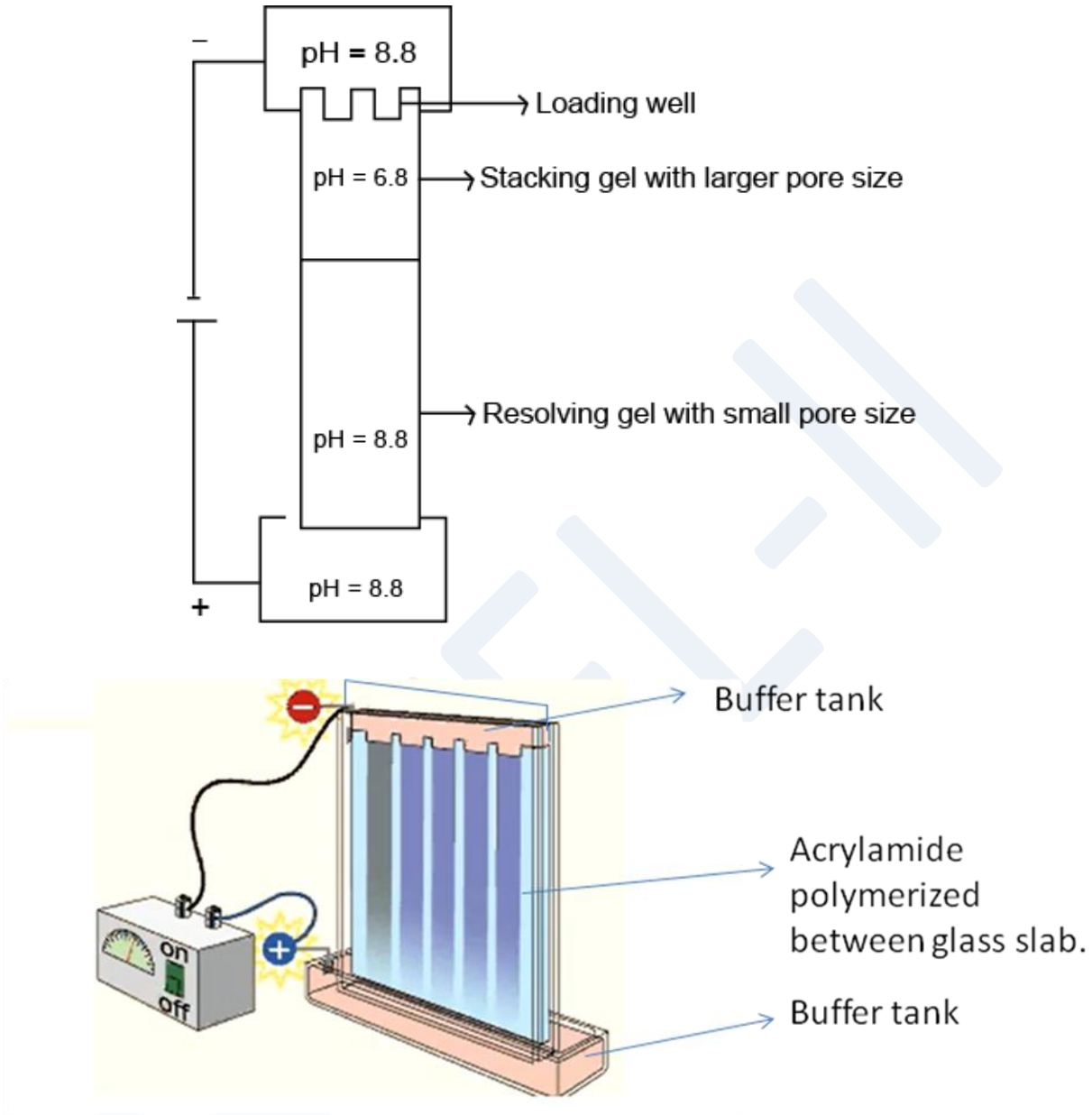


Figure 2 A: Experimental set-up for a polyacrylamide gel electrophoresis

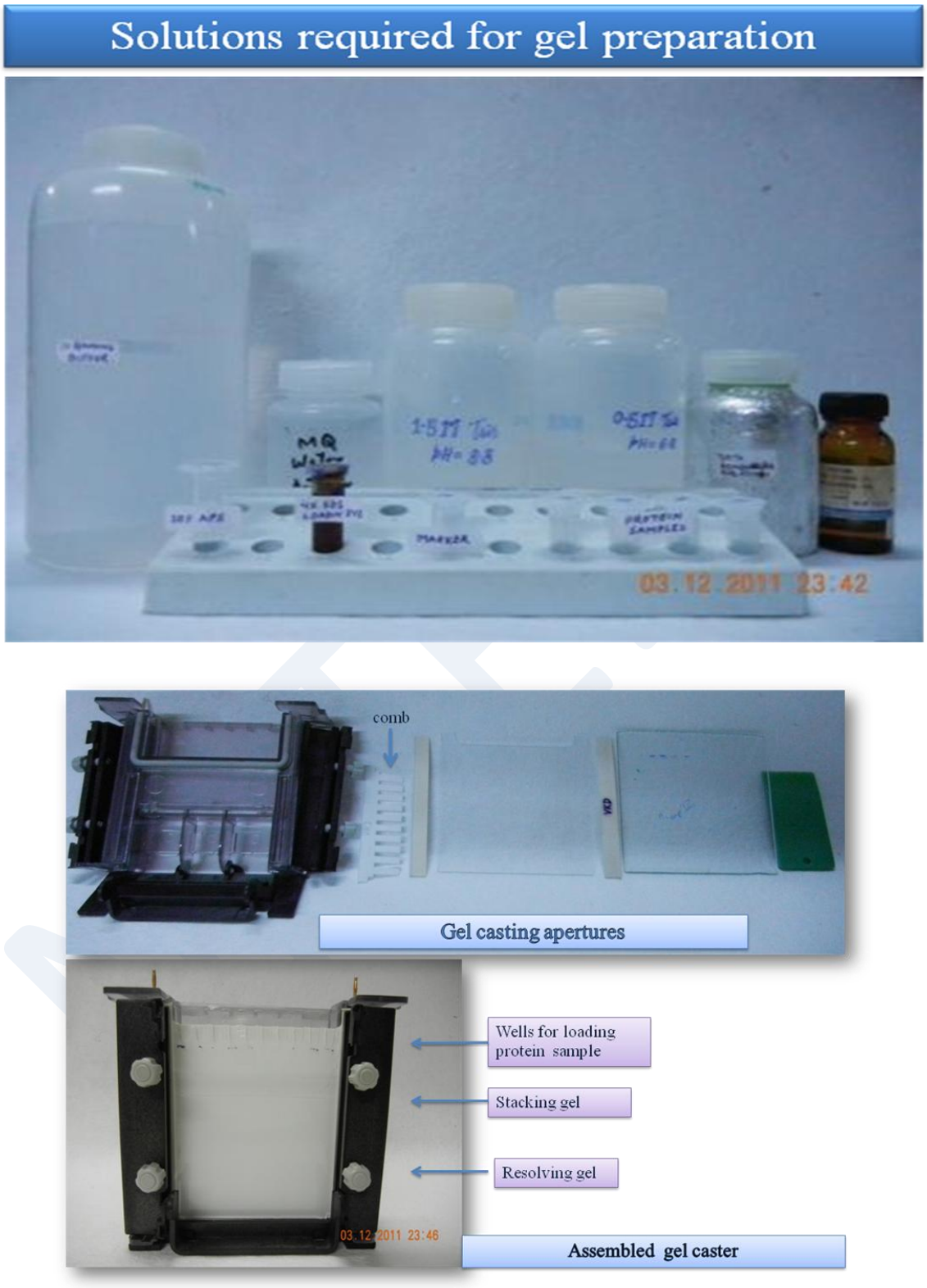


Figure 2B: Experimental set-up and requirements for a polyacrylamide gel electrophoresis

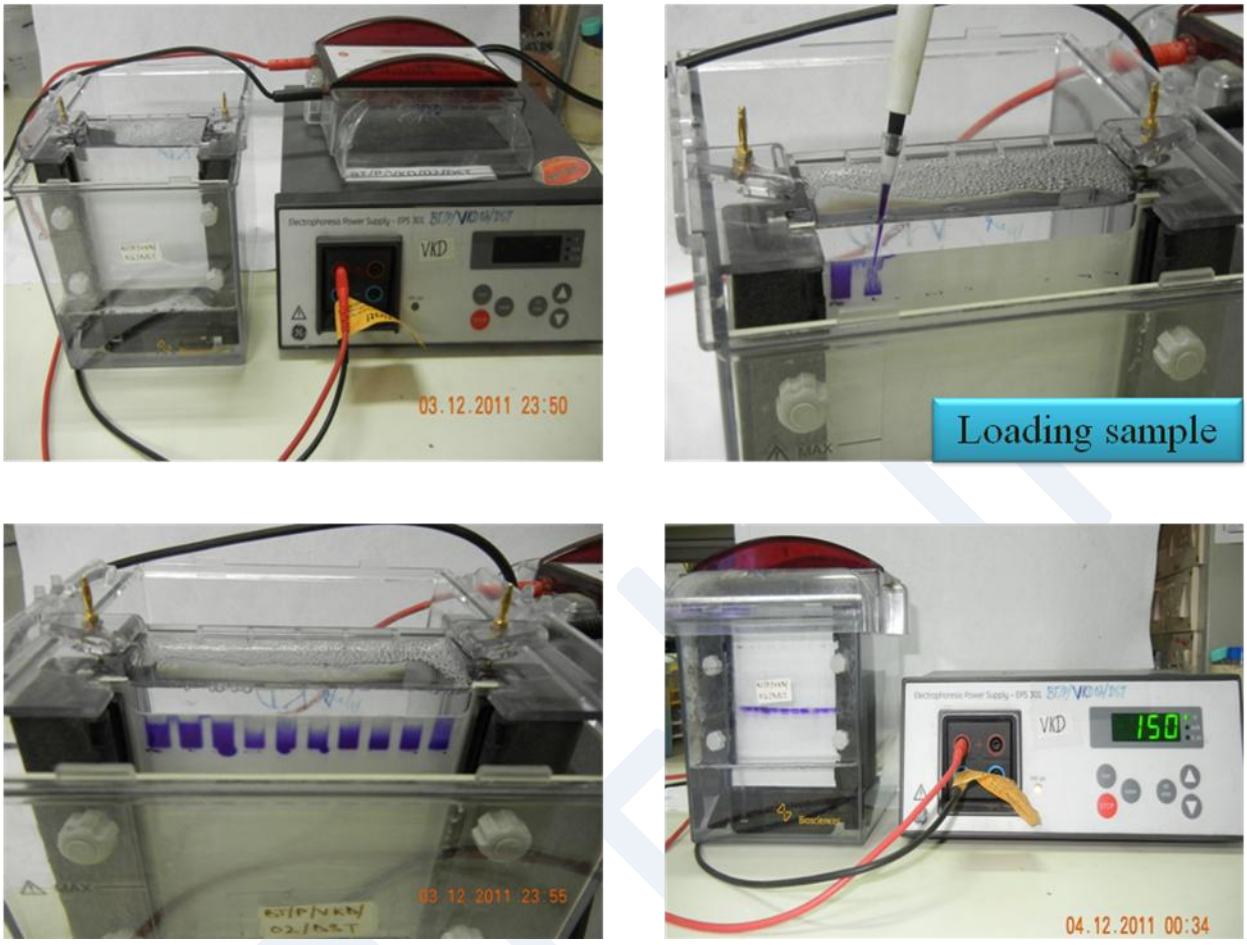


Figure 2C: Experimental set-up and procedure for a polyacrylamide gel electrophoresis

The original use of polyacrylamide gels as electrophoretic separating media involved using a single gel with a uniform pH throughout. This system has only occasional use in today's laboratory and replaced by discontinuous gel electrophoresis. Discontinuous gel electrophoresis system uses a separating gel of 2-5% of acrylamide (larger pore size) with pH 6.8 while resolving gel is at 12-20% acrylamide (small pore size) with pH 8.8. As you can see the pH of the gel is discontinuous. What are the advantages of a discontinuous gel system? The main advantage is that the proteins electrophorese quickly through the stacking gel of larger pore size and "stack" at the interface of resolving gel where they experience bigger pore size. In the resolving gel proteins are separated based on mass or size. The other advantage of the discontinuous system is protein stacking by a process call **isotachphoresis**.

The electrophoratic buffer in cathodic chamber contains chloride ions (called the leading ions) whose electrophoretic mobility is greater than the mobility of the proteins in the sample because of highest charge density. The electrophoresis buffer contains glycine ions (called the trailing ions) whose electrophoretic mobility is less than the mobility of the proteins in the sample as only a small fraction of glycine will be in anionic form giving overall small negative charge. The net result is that the faster migrating Cl^- ions leave a zone of lower conductivity between themselves and the migrating protein. The higher voltage gradient in this zone allows the proteins to move faster and to "stack" in the zone. However, as soon as protein crossed Cl^- ion zone, it will experience zone of high conductance (low voltage) and slow down. The same phenomenon will keep Glycine behind Protein. As protein is moving faster than Glycine, it will leave a zone of low conductance (high voltage). Glycine will move fast to reach zone of high voltage but as soon as reaches zone of protein it will experience relatively low voltage and slow down. In this way protein reached interface of stacking and resolving gel stacked between Glycine and Cl^- ions. The process is called isotachphoresis. After leaving the stacking gel, the protein enters the separating gel. The separating gel has a smaller pore size and higher pH compared to the stacking gel. In the separating gel, the ionization of glycine favours formation of more anion form (Henderson-Hasselbach equation) giving net charge density on glycine higher then protein. Thus, in resolving gel Cl^- ion moves

fastest, followed by Glycine. Proteins are not sandwiched between these two like stacking gel. Here proteins are separated based on mass (as charge density is identical in all protein after SDS treatment). Before loading a protein sample in polyacrylamide gel it is boiled with loading buffer which contains sodium laurlysulfate SDS ($C_{12}H_{25}SO_4Na$), anionic surfactant and reducing agents like dithiothreitol (DTT) or 2-mercaptoethanol. Most of the proteins binds SDS with constant-weight ratio (one SDS molecule per two amino acids) leading to identical charge densities. However, unless proteins are completely unfolded SDS cannot bind uniformly to protein amino acid residues as some amino acid residues may not be accessible due to folding of protein. Thus, reducing agent, which further denatures the proteins by reducing disulfide linkages and enables SDS to bind proteins uniformly. There are two more ingredients of loading buffer Bromophenol Blue and glycerol. Glycerol increases density of loading buffer so sample can settle in loading well while B romophenol Blue is a colored dye and indicates progression of electrophoresis. Electrophoresis is performed at constant current till bromophenol blue band reaches bottom of the gel.

Visualization of Band after electrophoresis:

Most of the proteins are not colored. Thus, gel after electrophoresis has to be stained using protein specific dye. Coomassie dye (detection limit 200 ng) is commonly used in laboratories for the purpose. The Coomassie dyes bind to proteins through ionic interactions between dye sulfonic acid groups and amine groups of basic amino acids as well as through Van der Waals interactions. As number of basic amino acid in a protein varies, this most commonly used dye don't stain all protein with equal affinity. Less sensitive dye include ponceau red (detection limit 250 ng) and amido black (detection limit 400 ng) . The most sensitive staining is silver staining (detection limit 1 ng). This involves soaking the gel in $AgNO_3$ which results in precipation of metallic silver (Ag^0) at the location of protein forming a colored deposit (Fig.3).

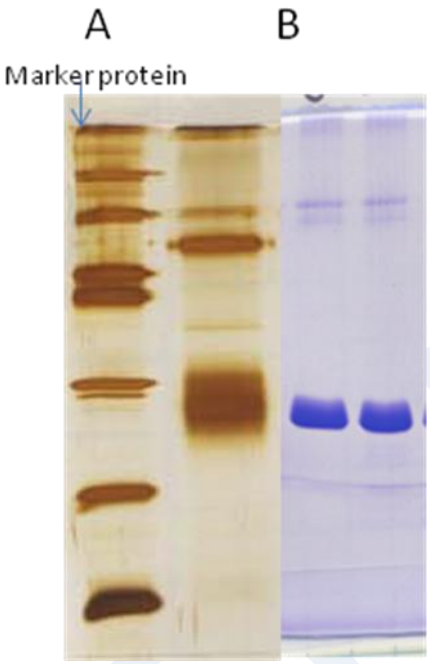


Figure 3: (A) gel stained with silver staining (B) gel stained with coomassie dye

Application of SDS-Poly acrylamide gel electrophoresis (SDS-PAGE)

1. **Assessment of purity of protein**: Single band in the sample indicates purity of sample (unless impurities also have identical mass).
2. **Molecular mass determination of unknown protein:** Standard proteins of known molecular mass (protein marker) are loaded in one of the lanes. Unknown protein is also loaded simultaneously in a separate lane. After electrophoresis, the gel is stained to visualize standard proteins as well as unknown protein. Bromophenol blue is indicated as tracker dye which shows the front of electrophoresis. Mobility of each standard protein relative to Bromophenol blue is calculated (relative mobility). A standard plot using relative mobility of known molecular mass proteins (protein marker) and log of molecular mass was plotted. Mass of unknown protein is estimated from standard plot (Fig. 4)

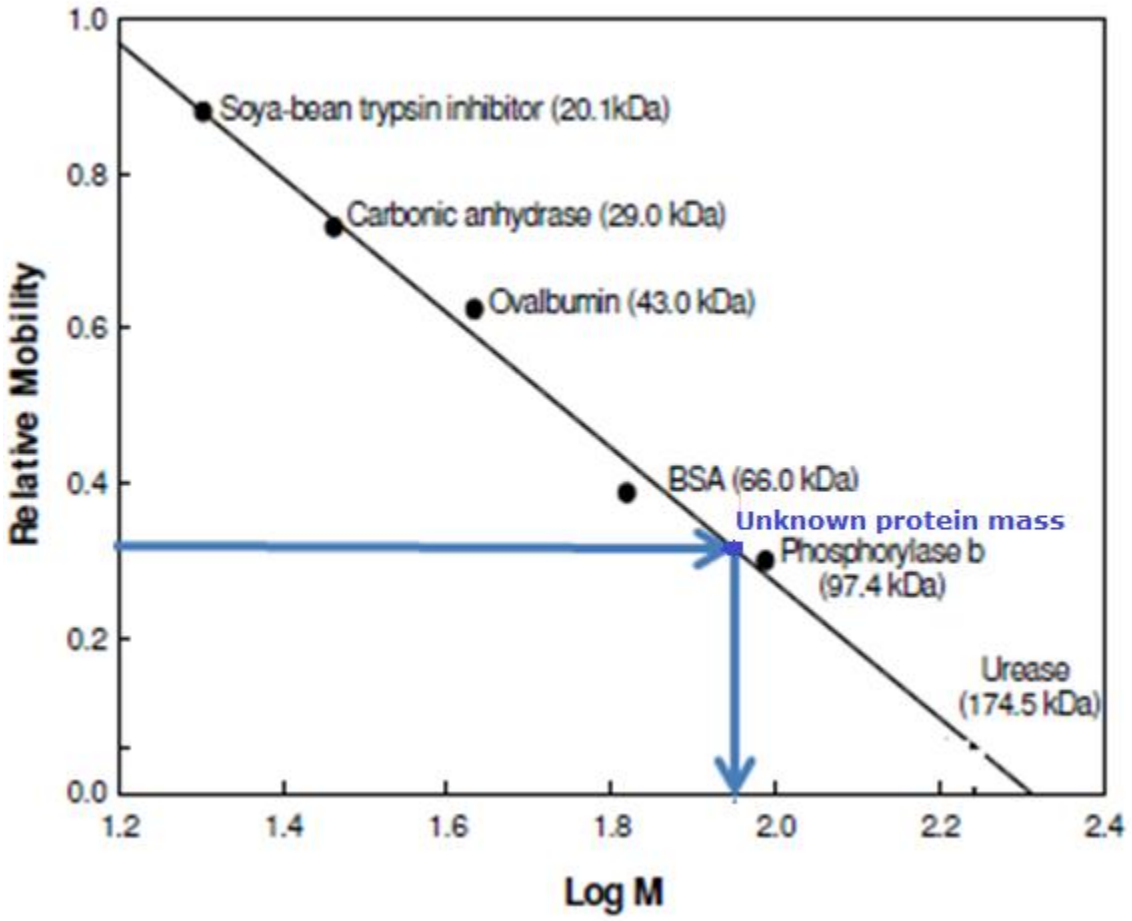


Figure 4: Standard plot for estimation of mass of unknown protein.