

# **HiPer<sup>®</sup> Protein Silver Staining Teaching Kit**

**Product Code: HTP002**

**Number of experiments that can be performed: 5**

**Duration of Experiment: 1 day**

## **Storage Instructions:**

- The kit is stable for 12 months from the date of receipt
- Store Prestained Protein Ladder, 5X Sample Loading Buffer at -20°C
- Store Protein samples, 5X Tris-Glycine-SDS Gel Running Buffer, Acrylamide/Bis-acrylamide Solution 30% (29:1), Tris-SDS solutions and TEMED at 2-8°C
- Other kit contents can be stored at room temperature (15-25°C)



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## Aim:

To learn the technique of silver staining of proteins following SDS-PAGE.

## Introduction:

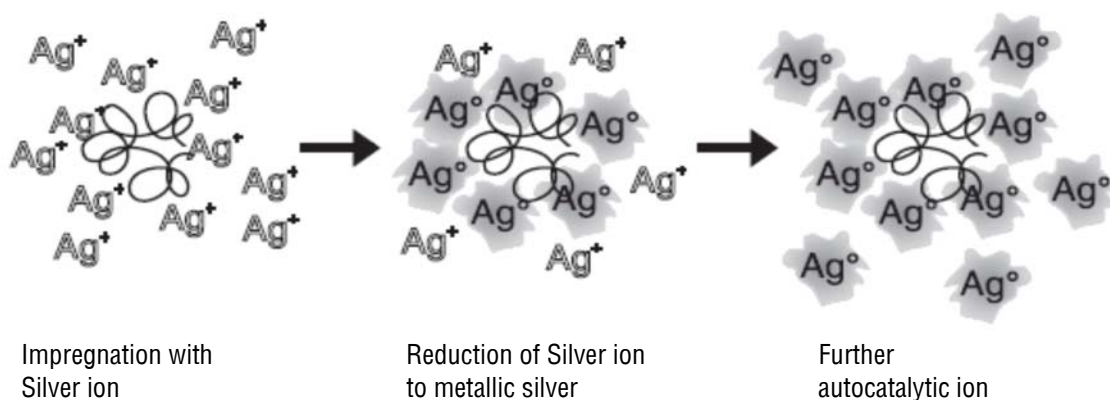
The ability of silver to develop images was discovered in the mid-17<sup>th</sup> century and this property was exploited in the development of photography, followed by its use in histological procedures. Silver staining for detection of proteins following gel electrophoresis was first reported in 1979 by Switzer et al. resulting in a major increase in the sensitivity of protein detection. More than 100 publications have subsequently appeared describing variations in silver staining methodology. This group of procedures is generally accepted to be 100 times more sensitive than methods using Coomassie Brilliant Blue R-250, being able to detect lower nanogram amounts (as low as 1ng) of protein per gel band or spot.

## Principle:

All silver staining methods depend on the reduction of ionic silver to its metallic form. Protein bands can be visualized on the gel due to the difference in oxidation/ reduction potential between the sites occupied by protein and the adjacent sites not occupied by the protein on the gel. The silver cations complex with protein amino groups, particularly the  $\epsilon$ -amino group of lysine, and with sulphur residues of cysteine and methionine. The gel is impregnated with silver nitrate in a weakly acidic (pH 6) solution. Any free silver nitrate is washed out of the gel prior to development, as precipitation of silver oxide will result in high background staining. Development is subsequently achieved by selective reduction of ionic silver by formaldehyde made alkaline by sodium carbonate. Sodium carbonate acts as a buffer to the formic acid produced by the oxidation of formaldehyde, which results in reduction of silver ion till protein bands appear in the gel. Silver stains are normally monochromatic, resulting in a dark brown image. The colors produced depend on:

1. The size of silver particles.
2. The distribution of silver particles within the gel.
3. The refractive index of the gel.

Silver staining can be used immediately after electrophoresis. Clarity is dependent on the amount of sample loaded on the gel. Compared to Coomassie staining, silver staining is a time-consuming, but more sensitive method for staining of proteins in gels. Silver staining is used to assess the purity of a protein preparation, such as antigen preparation for development of polyclonal antibodies.



**Fig 1 :** The exceptional high sensitivity of Silver staining is the result of an autocatalytic process.

### Kit Contents:

The kit can be used to perform silver staining for visualization of proteins separated by SDS-PAGE.

**Table 1: Enlists the materials provided in this kit with their quantity and recommended storage**

Sr. No.	Product Code	Materials Provided	Quantity	Storage
			5 expts	
1	ML037	Acrylamide/Bis-acrylamide Solution 30% (29:1)	40 ml	2-8°C
2	ML039	2.5X Tris-SDS Buffer (pH 8.8)	35 ml	2-8°C
3	ML040	5X Tris-SDS Buffer (pH 6.8)	9 ml	2-8°C
4	MBT092	Prestained Protein Ladder	0.030 ml	-20°C
5	ML041	5X Tris-Glycine-SDS Gel Running Buffer	200 ml	2-8°C
6	TKC037	5X Sample Loading Buffer	0.084 ml	-20°C
7	TKC044	Protein Sample 1	0.12 ml	2-8°C
8	TKC045	Protein Sample 2	0.12 ml	2-8°C
9	MB003	Ammonium Persulphate (APS)	0.15 g	R T
10	MB026	Tetramethylethylenediamine (TEMED)	0.2 ml	2-8°C
11	MB002	Agarose	0.4 g	R T
12	TKC039	Fixative solution	2 x 150 ml	RT
13	TKC040	Reducer solution	2 x 150 ml	RT
14	MB157	Silver Nitrate	6 x 0.1g	RT
15	TKC041	Developing solution	2 x 150 ml	RT
16	TKC042	Stopping solution	2 x 150 ml	RT

### Materials Required But Not Provided:

**Glass wares:** Conical flask, Measuring cylinder, Beaker

**Reagents:** Sterile distilled water, Ethanol

**Other requirements:** Incubator, Micropipettes, Vortex Mixer, Tips, Staining trays, Protein Electrophoresis Unit, Gel rocker/ shaker, Aluminum foil

### Storage:

HiPer® Silver Staining Teaching Kit is stable for 12 months from the date of receipt without showing any reduction in performance. On receipt, store the Protein marker and 5X Sample Loading Buffer at -20°C. 30% Acrylamide-Bisacrylamide Solution, 2.5X Tris-SDS Buffer (pH 8.8), 5X Tris-SDS Buffer (pH 6.8), 5X Tris-Glycine-SDS Gel Running Buffer, Protein samples and TEMED should be stored at 2-8°C. Other kit contents can be stored at room temperature.

### Important Instructions:

1. Read the entire procedure carefully before starting the experiment.
2. Refer page numbers 9-11 for SDS-PAGE.
3. **Preparation of 10% APS Solution:** Before starting the experiment, dissolve 0.15 g of Ammonium persulphate in distilled water to make a final volume of 1.5 ml. Store at 2-8°C. Use within 3 months.
4. **Preparation of 1X Tris-Glycine-SDS Gel Running Buffer:** To prepare 500 ml of 1X Tris-Glycine-SDS Gel Running buffer, take 100 ml of 5X Tris-Glycine-SDS Gel Running Buffer and add 400 ml sterile distilled water\*. Store at 2-8°C. Mix well before use. The 1X Tris-Glycine-SDS Gel Running Buffer can be reused 4-5 times.
5. Thaw all refrigerated samples before use.
6. Clean the entire apparatus with detergent and then with distilled water\*. Ensure that the plates are free of detergent.
7. **Preparation of staining solution for 1 expt:** Add 1 vial containing preweighed 0.1 g of Staining Reagent silver nitrate to 100 ml of sterile distilled water just before starting the experiment
8. **Preparation of 50% ethanol for 1 expt:** To 75 ml of 100% ethanol add 75 ml of Sterile distilled water\*
9. Wear clean gloves when handling all the materials. Take special care to touch only the same corner of the gel to prevent staining artifact. Clean glasses very carefully when preparing the gel. Place gel in clean container before staining.
10. **In between every step of silver staining protocol, it is recommended to change the tray and rinse the tray with Molecular biology grade water before adding any solution. Cover the tray with aluminium foil throughout the experiment.**

\* Molecular biology grade water is recommended (Product code: ML024).

### Procedure:

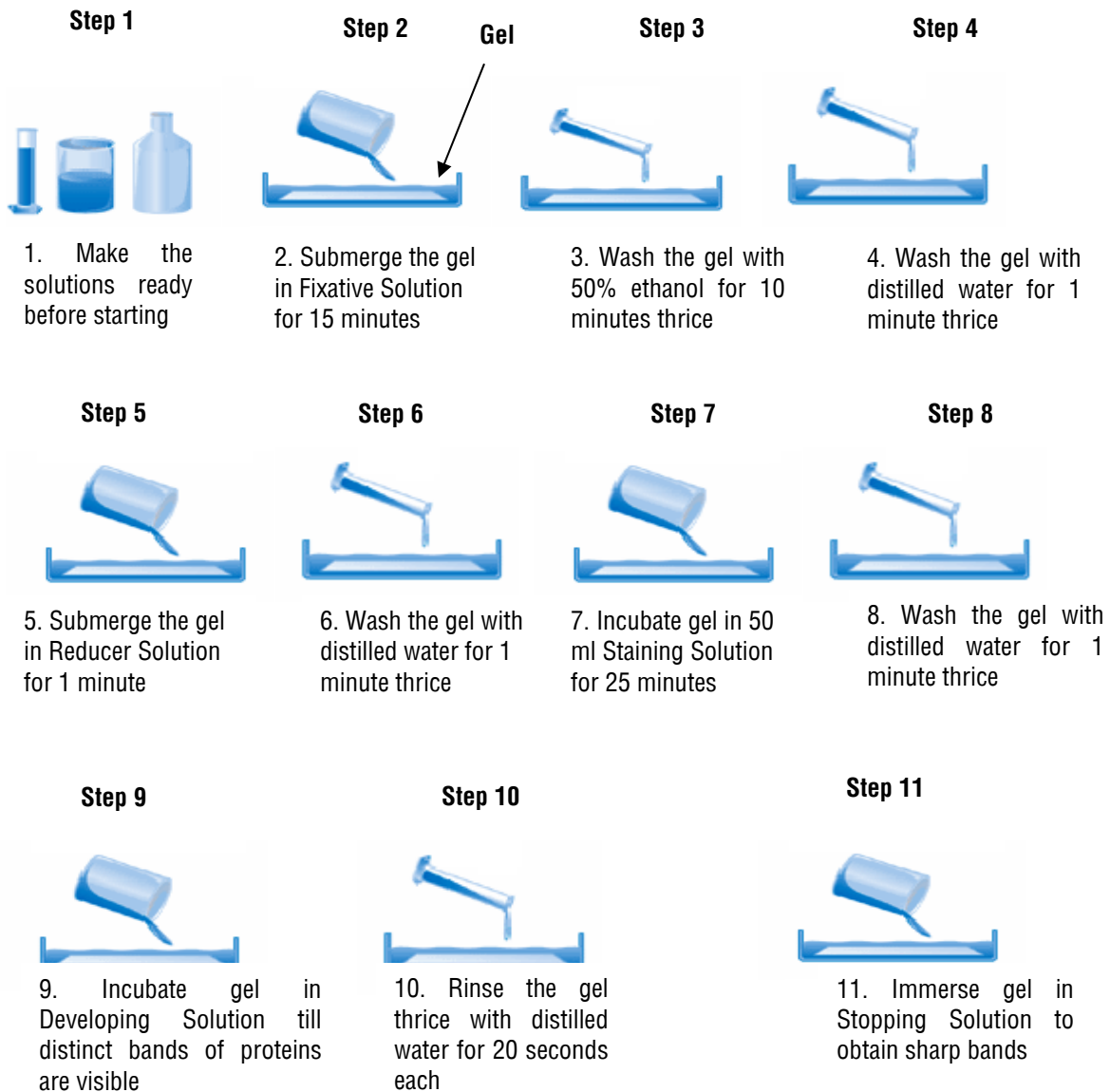
1. Prepare 12% Separating and 5% Stacking Gel. (Refer Page Numbers 9-11 for SDS-PAGE)
2. **Sample Preparation:** Take 2 tubes for protein samples. Label them respectively. Take 20 µl of each sample in the respective tube and add 5 µl of 5X Sample Loading Buffer to it. Boil the tubes containing Protein Samples at 100°C in a boiling water bath. Do not boil the tube containing Prestained Protein Ladder.
3. Load 5 µl of Prestained Protein Ladder and 20 µl of Protein samples to alternate wells in the gel. Run the gel.

4. Carefully remove the gel from in-between the glass plates into the tray. Add 50 ml of Fixative solution and incubate for 15 minutes with constant moderate shaking.
5. Discard the Fixative Solution.
6. Wash the gel by adding 50 ml of 50% ethanol for 10 minutes with constant moderate shaking.
7. Repeat the above step twice.
8. Wash the gel with 50 ml of Sterile Distilled Water\* for 1 minute with constant moderate shaking.
9. Repeat the above step twice.
10. Remove the gel and submerge it in 50 ml of Reducer solution for 1 minute with constant moderate shaking.
11. Discard the Reducer solution and wash it with 50 ml of Sterile Distilled Water\* for 1 minute each.
12. Repeat the above step twice.
13. Incubate the gel in 50 ml of freshly prepared Staining Solution for 25 minutes ensuring that the gel is completely submerged in the liquid. Incubate with constant moderate shaking.
14. Remove the gel from the Staining Solution and wash it thrice with Sterile Distilled Water\* for 1 minute each.
15. Incubate the gel in Developing Solution until color of separated protein bands develop and are distinctly visible.
16. Remove the gel from the Developing solution and rinse it thrice with Sterile Distilled Water for 20 seconds.
17. The developing reaction is then stopped by immersing the gel in Stopping Solution to obtain sharp distinct bands.

**NOTE:** The developing reaction should be arrested in time to avoid over-staining of the gel. The protein bands are observed as per their molecular size. The molecular size of the separated protein bands can be determined by comparing with a reference protein ladder.

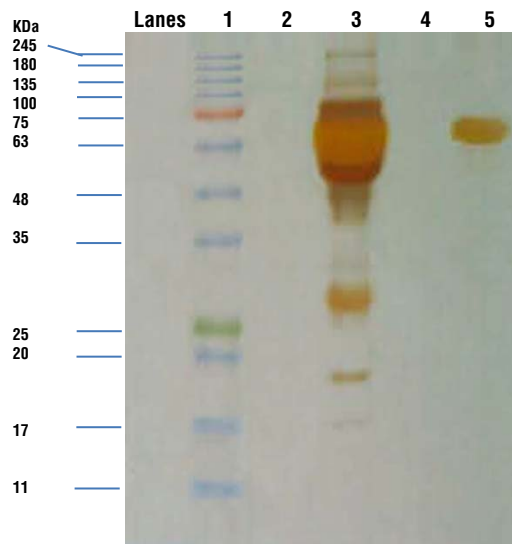
\* Molecular biology grade water is recommended (Product code: ML024).

## Flowchart:



### Observation and Result:

After completing the Silver Staining procedure, check for the bands of the protein sample. Compare with reference protein marker.



**Fig 1: Gel picture of protein samples after silver staining**

Lane 1: Prestained Protein Ladder

Lane 3: Protein Sample 1

Lane 5: Protein Sample 2

### Interpretation:

Sharp bands will be observed if the protein samples are successfully stained using Silver Staining method. After immersing the gel in stopping solution, compare the molecular weight of the samples with that of the protein marker. Protein Sample 1 contains serum hence multiple bands can be seen. Protein sample 2 contains purified protein hence 1 major band can be seen.



**Troubleshooting Guide:**

<b>Sr.No</b>	<b>Problem</b>	<b>Possible Cause</b>	<b>Solution</b>
1	Large discolored spots on the gel	Pressure on the gel during handling	Avoid crushing the gel with fingers. Keep minimal contact with the gel while performing the protocol
2	Slow or no development of bands	Developer solution is not mixed properly	Ensure the developing solution is mixed thoroughly before use
3	Low band sensitivity	Long exposure of Reducer Solution	Do not place the gel in reducer solution for more than 1 minute

## SDS-PAGE

### Aim:

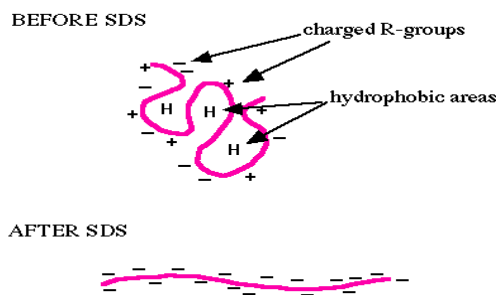
To learn the technique of SDS-PAGE.

### Introduction:

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique used in biochemistry, genetics and molecular biology to separate proteins according to their molecular weight. The electrophoretic mobility of proteins depends upon their size. The purpose of SDS-PAGE is to separate proteins according to their size. As proteins are amphoteric compounds, their net charge can therefore be determined by the pH of the medium in which they are suspended. Therefore, at a given pH and under non-denaturing conditions, the electrophoretic separation of proteins is determined by both size and charge of molecules. As proteins are high molecular weight molecules, it needs porous gels to get separated. Polyacrylamide gels are those which provide a means of separating proteins by size as they are porous. This kit enables the students to learn the technique of SDS-PAGE.

### Principle:

To separate different protein molecules of different shapes and sizes, they first have to be denatured so that the proteins no longer have any secondary, tertiary or quaternary structure. Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by “wrapping around” the polypeptide backbone. SDS denatures all the proteins to their respective primary structure. SDS confers a negative charge to the polypeptide in proportion to its length.



**Fig2: Denaturation of protein by SDS**

SDS treatment has two important features:

1. All proteins retain only their primary structure.
2. All proteins have a large amount of negative charge.

Polyacrylamide is the best gel recommended to provide such an environment. Polyacrylamide is a synthetic gel which is thermo-stable, transparent, strong and relatively chemically inert and can be prepared with a wide range of average pore sizes. It can withstand [high voltage](#) gradients and is feasible to various staining and destaining procedures and can be digested to extract separated fractions or dried for [autoradiography](#) and permanent recording. A polymer gel is formed of acrylamide monomers and the proteins are run through this gel by electrophoresis, hence this entire process is called Polyacrylamide Gel Electrophoresis (PAGE).

There are two layers of gel, namely Stacking or spacer gel, and Separating or resolving gel.

**Stacking gel** - The stacking gel contains large pores of polyacrylamide gel (generally 5%). This gel is prepared with Tris buffer of pH 6.8 which is of about 2 pH units lower than that of the electrophoresis buffer. This gel is formed over the separating gel.

**Separating Gel** - The separating gel contains small pores of polyacrylamide gel (5-30%). The Tris buffer used is of pH 8.8. In this gel, macro molecules separate according to their size.

The materials used in SDS-PAGE and their roles are as follows:

1. **Tris:** It is used as a buffer because it is an innocuous substance to most proteins. Its pKa is 8.3 at 20°C and reasonably a very satisfactory buffer in the pH range 7.0 – 9.0.
2. **Acrylamide:** This is a white crystalline powder and while dissolving in water, autopolymerisation takes place. It is a slow spontaneous process by which acrylamide molecules join together by head on tail fashion. But in presence of free radicals generating system, acrylamide monomers are activated into a free-radical state. These activated monomers polymerise quickly and form long chain of polymers. This kind of reaction is known as Vinyladdition polymerisation.
3. **Bisacrylamide (N,N'-Methylenebisacrylamide):** Bisacrylamide is the most frequently used cross linking agent for polyacrylamide gels. Chemically it has two acrylamide molecules coupled head to head fashion at their non-reactive ends.
4. **Sodium Dodecyl Sulphate (SDS):** SDS is the most common denaturing agent used to denature native proteins to individual polypeptides. 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/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 a rod like structure possessing a uniform charge density that is same net negative charge per unit length.
5. **Ammonium Persulphate (APS):** APS is an initiator for gel formation.
6. **N, N, N', N'-tetramethylethylenediamine (TEMED):** Chemical polymerisation of acrylamide gel is used for SDS-PAGE. It can be initiated by ammonium persulfate and the quaternary amine, N,N,N',N'-tetramethylethylenediamine (TEMED).

## Procedure:

1. Assemble the electrophoresis unit such that the glass plates are clamped to the unit along with the spacers placed in-between them at two vertical edges.
2. Prepare 1% agarose (0.05g in 5ml of distilled water). Boil to dissolve the agarose and pour a thin horizontal layer at the lower edge of the plates to seal the assembly. Let it solidify by allowing it to cool down for 5-10 minutes

3. **Preparation of 12% Separating Gel-** To prepare separating gel, add the components as follows:

30% Acrylamide-bisacrylamide Solution	- 6 ml
Distilled water*	- 3 ml
2.5X Tris-SDS Buffer (pH 8.8)	- 6 ml
10% APS Solution	- 125 µl
TEMED	- 18 µl

Pour the gel in-between the plates and allow it to solidify for an hour. Immediately after the gel is poured, add distilled water to level the gel.

4. After an hour pour off the water by inverting the casting assembly.

5. **Preparation of 5% Stacking Gel-** To prepare stacking gel, add the components as follows:

30% Acrylamide-bisacrylamide Solution	- 1.3 ml
Distilled water*	- 5.1 ml

5X Tris-SDS Buffer (pH 6.8)	- 1.6 ml
10% APS Solution	- 75 µl
TEMED	- 10 µl

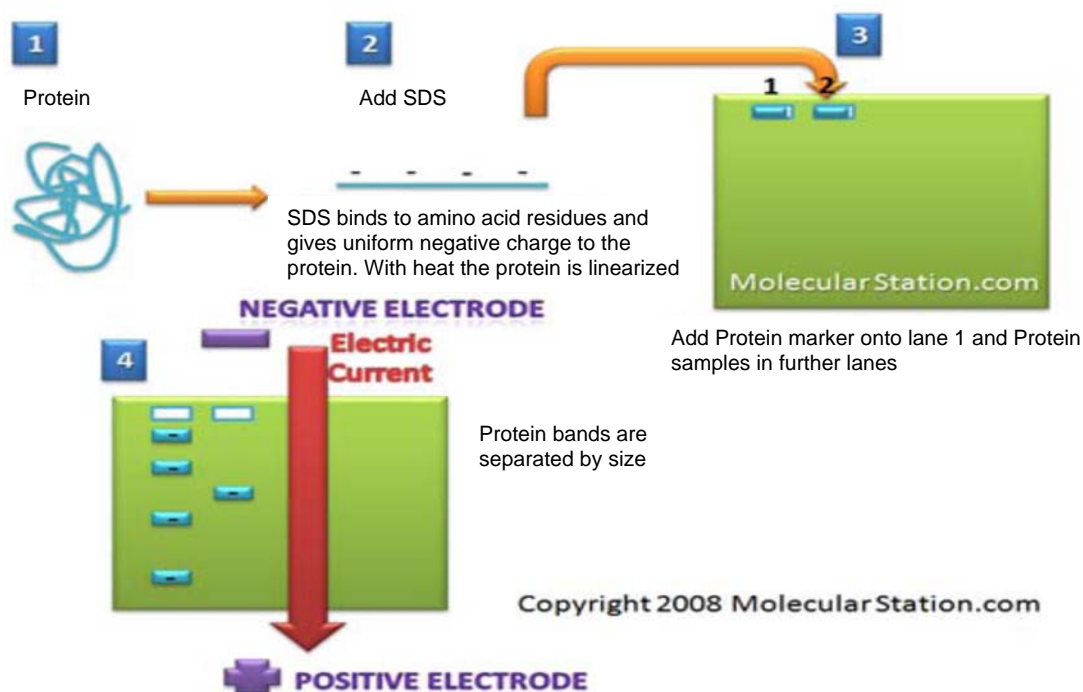
After addition of TEMED gently mix all the components by swirling the beaker. Pour the stacking gel on top of the separating gel and immediately place the comb avoiding air bubbles. Allow it to solidify for 30 minutes.

**Note:** Acrylamide is a potential neurotoxin and should be treated with great care. Always wear an face mask and use gloves.

6. Pour 1X Tris-Glycine-SDS Gel Running Buffer in the unit such that the buffer connects the two electrodes, and hence completes the flow of current. Remove the comb from the Stacking Gel carefully.
7. **Sample Preparation:** Take 3 tubes for protein samples and 1 tube for Protein Marker. Label them respectively. Take 20 µl of each sample in the respective tube and add 5 µl of 5X Sample Loading Buffer to it. Take 3 µl of Protein Marker in the respective tube and add 4 µl of 5X Sample Loading Buffer and 13 µl of 1X PBS. Boil the tubes containing Protein Samples at 100°C in a boiling water bath. Do not boil the tube containing Protein Marker.
8. Load 20 µl of the samples immediately after the heat treatment in the wells created by the comb in the Stacking Gel.
9. Connect the power cord to the electrophoretic power supply according to the conventions: Red- Anode and Black- Cathode. Electrophorese at 100 volts and 10 mA until dye front reaches 0.5 cm above the sealing gel.
10. Carefully remove the gel from in-between the plates using spatula into the plastic tray containing distilled water. Wash the gel for 1 minute. Discard the water & proceed for staining destaining procedure.

\* Molecular biology grade water is recommended (Product code: ML024).

## Flowchart:



## Technical Assistance:

At HiMedia we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance mail at [mb@himedialabs.com](mailto:mb@himedialabs.com)