HiPer® Western Blotting Teaching Kit

Product Code: HTI009

Number of experiments that can be performed: 5/20

Duration of Experiment: ~ 2 days

Day 1: 6-8 hours (SDS- PAGE and Electroblotting)
Day 2: 3 hours (Immunodetection, Observation and Results)

Storage Instructions:

- The kit is stable for 6 months from the date of receipt
- Store 5X Sample Loading Buffer, Prestained Protein Ladder, Primary and Secondary Antibody at -20°C
- Store Protein sample, 30% Acrylamide- Bisacrylamide Solution, 2.5X Tris-SDS Buffer (pH 8.8), 5X Tris-SDS Buffer (pH 6.8), 10X Transfer Buffer, 10X Blocking Buffer, 10X Assay Buffer, 10X Wash Buffer and 5X Tris-Glycine-SDS Gel Running Buffer at 2-8°C
- Other reagents can be stored at room temperature (15-25°C)

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Index

Aim:

To learn the technique of Western Blotting for the detection of a specific protein.

Introduction:

Western blotting or protein immunoblotting is a very sensitive and analytical method that involves detection of a specific protein in a complex mixture. Protein samples are first separated using SDS Polyacrylamide gel electrophoresis (SDS-PAGE) followed by the immobilization of proteins on nitrocellulose or PVDF membranes. The transfer of proteins from the gel to the membrane is done electrophoretically. The transferred protein is detected using specific primary antibody and secondary enzyme labeled antibody and substrate. This method utilizes the principle of antigen-antibody interaction for identification of specific antigens by monoclonal or polyclonal antibodies.

Principle:

Western blotting or immunoblotting is a method used for identifying a specific protein in a complex mixture along with determination of its molecular weight. Protein samples are first electrophoresed on SDS-PAGE. In this process proteins migrate through the gel and they are separated according to their size and charge. These separated proteins are electrotransferred onto nitrocellulose/PVDF membrane for further analysis. To detect the protein (antigen) blotted on the membrane it is incubated with an antibody (primary) specific for the protein of interest. The membrane is then incubated with a second antibody (secondary) which is specific for the first antibody. The secondary antibodies are covalently attached to an enzyme, e.g. alkaline phosphatase or horseradish peroxidase. These enzymes form a coloured precipitate upon reacting with a chromogenic substrate. As a result a visible band can be seen on the membrane where the primary antibody is bound to the protein. The entire procedure can be divided into following steps:

SDS-PAGE:
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.

Western blotting:
Immunoblotting or Western blotting is the electro transfer of resolved proteins from the polyacrylamide gel to the nitrocellulose/PVDF membrane in presence of a specific buffer called transfer buffer. For this transfer procedure, the gel is placed on the membrane and both of them are sandwiched between two filter papers as shown in Figure 1:

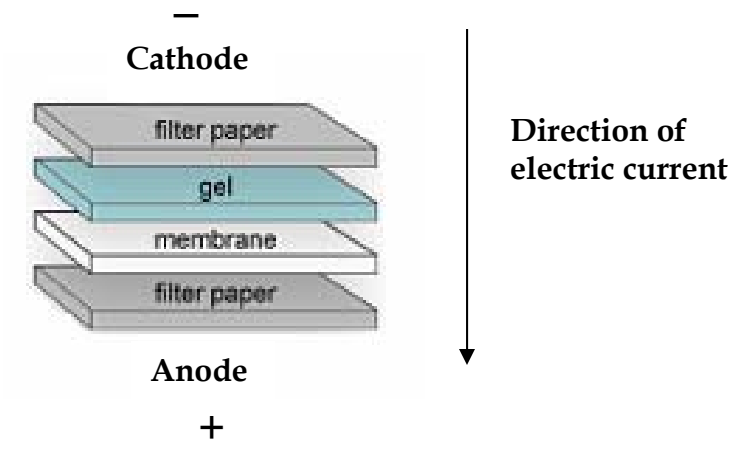


Fig1: Arrangement of the gel and membrane for electrotransfer

This set is placed between two sponge pads and then placed in a plastic cassette. The entire set is then placed inside a gel tank filled with cold transfer buffer. The resolved proteins are transferred to the corresponding positions on the membrane after the electrotransfer. The protein of interest is immunodetected on the membrane.

Immunodetection:
After electrotransfer, proteins bound to the membrane are detected immunologically. This process is known as Immunodetection or Immunoblotting. A suitable blocking reagent (non-fat dry milk/BSA) is used to block the unoccupied sites on the membrane. Then the membrane is probed with a primary antibody specific to the protein of interest. The primary antibody binds to the protein (antigen) and an antigen (Ag)-antibody (Ab) complex is formed on the membrane. The membrane is washed to remove excess unbound primary antibody. It is then treated with an enzyme-labeled (Alkaline phosphatase/Horseradish peroxidase) secondary antibody which attaches to the primary antibody of the Ag-Ab complex. Finally, the membrane is incubated in a solution containing phosphatase or peroxidase substrate which results in a visible coloured band on the membrane where the Ag-Ab complex is formed. As a result the molecular weight of the protein of interest can be determined.

Kit Contents:

This kit can be used for the rapid detection of a protein of interest in a given sample.

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	20 expts	
1	ML037	Acrylamide-Bisacrylamide Solution 30% (29:1)	44 ml	145 ml	2-8°C
2	ML039	2.5X Tris-SDS Buffer (pH 8.8)	36 ml	120 ml	2-8°C
3	ML040	5X Tris-SDS Buffer (pH 6.8)	10 ml	32 ml	2-8°C
4	MBT092	Prestained Protein Ladder	0.030 ml	0.110 ml	-20°C
5	ML041	5X Tris-Glycine-SDS Gel Running Buffer	200 ml	2 x 400 ml	2-8°C
6	TKC037	5X Sample Loading Buffer	0.085 ml	0.33 ml	-20°C
7	DS0064	Staining solution	125 ml	500 ml	RT
8	DS0065	Destaining solution	125 ml	500 ml	RT
9	MB003	Ammonium persulphate (APS)	0.15 g	4 x 0.15 g	RT

10	MB026	Tetramethylethylenediamine (TEMED)	0.2 ml	0.7 ml	2-8°C
11	MB002	Agarose	0.3 g	1.15 g	RT
12	TKC179	Protein Sample	0.24 ml	0.84 ml	-20°C
13	ML043	10X Transfer Buffer	200 ml	2 X 400 ml	2-8°C
14	TKC297	Blocking Agent	1.2 g	4.2 g	2-8°C
15	TKC145	Diluent Buffer	120 ml	420 ml	2-8°C
16	TKC146	10X Assay Buffer	25 ml	85 ml	2-8°C
17	TKC180	10X Wash Buffer	60 ml	210 ml	2-8°C
18	TKC147	Primary antibody	0.02 ml	0.08 ml	-20°C
19	TKC148	Secondary antibody	0.01 ml	0.04 ml	-20°C
20	TKC143	TMB/H_2O_2	20 ml	80 ml	2-8°C
21	TKC149	Nitrocellulose membrane with filter paper	5 Nos.	20 Nos.	RT

Materials Required But Not Provided:

Glass wares: Conical flask, Measuring cylinder, Beaker, Petri dish, staining tray
Reagents: Methanol, Distilled water
Other requirements: Protein Electrophoresis apparatus, Gel rocker, Micropipettes, Tips, Microwave/Burner/Hotplate

Storage:

HiPer® Western blotting Teaching Kit is stable for 6 months from the date of receipt without showing any reduction in performance. On receipt, store the Protein marker, 5X Sample Loading Buffer, Protein sample, Primary antibody and Secondary antibody 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, Blocking agent and 10X Transfer Buffer should be stored at 2-8°C. Other reagents can be stored at room temperature (15-25°C).

Important Instructions:

1. Read the entire procedure carefully before starting the experiment.
2. **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.
3. **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.
4. Thaw all refrigerated samples before use.
5. Clean the entire apparatus with detergent and then with distilled water*. Ensure that the plates are free of detergent.
6. **Preparation of 1X Assay Buffer:** To prepare 50 ml of 1X Assay Buffer, take 5 ml of 10X Assay Buffer and add 45 ml of sterile distilled water*.
7. **Preparation of 1X Transfer Buffer:** To prepare 1000 ml of 1X Transfer Buffer, take 100 ml of 10X Transfer Buffer, add 200 ml of methanol and 700 ml of sterile distilled water*. Store at 2-8°C. Mix well before use.
8. **Preparation of Blocking Buffer:** To prepare 20 ml of Blocking Buffer, take 0.2 g of Blocking Agent and add 20 ml of Diluent Buffer.
9. **Preparation of 1X Wash Buffer:** To prepare 1000 ml of 1X Wash Buffer, take 100 ml of 10X Wash Buffer and add 900 ml of sterile distilled water*.

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

Procedure:

Day 1: SDS- PAGE

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 40 µl of protein sample in a tube and add 8 µl of 5X Sample Loading Buffer to it. Boil the tube containing protein sample at 100°C in a boiling water bath. Do not boil the tube containing Prestained Protein Ladder.

8. Load samples in alternative wells as follows:

   Lane 1: Prestained Protein Ladder – 5 µl
   Lane 3: Protein Sample – 20 µl
   Lane 5: Protein Sample – 20 µl

9. Connect the power cord to the electrophoretic power supply according to the conventions: Red-Anode and Black- Cathode. Electrophorese at 120 volts and 90 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 blotting and staining destaining procedure.

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

11. To the gel pieces of lane no. 1 and 3 add 20 ml of water and proceed for staining destaining procedure.

12. Cut the Gel along lane no. 4. Transfer lane no. 5 i.e. protein sample in 10 ml of cold Transfer buffer. Incubate at Room Temperature for 10 minutes and proceed with electroblotting.

Staining and Destaining of Gel:

1. After removing water, add 50 ml of Staining Solution in the tray containing gel, till the bands are visible. Sometimes the gel may have to be kept overnight in the staining solution for visualization of the bands.
2. Remove gel from the Staining Solution. The Staining Solution can be re-used 2-3 times.
3. Wash the gel by rinsing with distilled water till a considerable amount of stain leaches out from the gel. Keep changing the distilled water for 3-4 times.
4. Add 50 ml of Destaining Solution to the gel. Destaining should be carried out with constant moderate shaking.
5. Continue destaining till clear, distinct bands are observed.
6. Remove gel from the Destaining Solution. The Destaining Solution can be re-used 2-3 times.

Electroblotting:

1. Assemble the gel with nitrocellulose membrane and filter papers as shown in figure 1. This blotting sandwich is placed within the blotting cassette. Try to avoid air bubble between gel and nitrocellulose membrane by rolling a glass tube on the membrane.

   Note: Take out the transparent sheets carefully while using the nitrocellulose membrane.

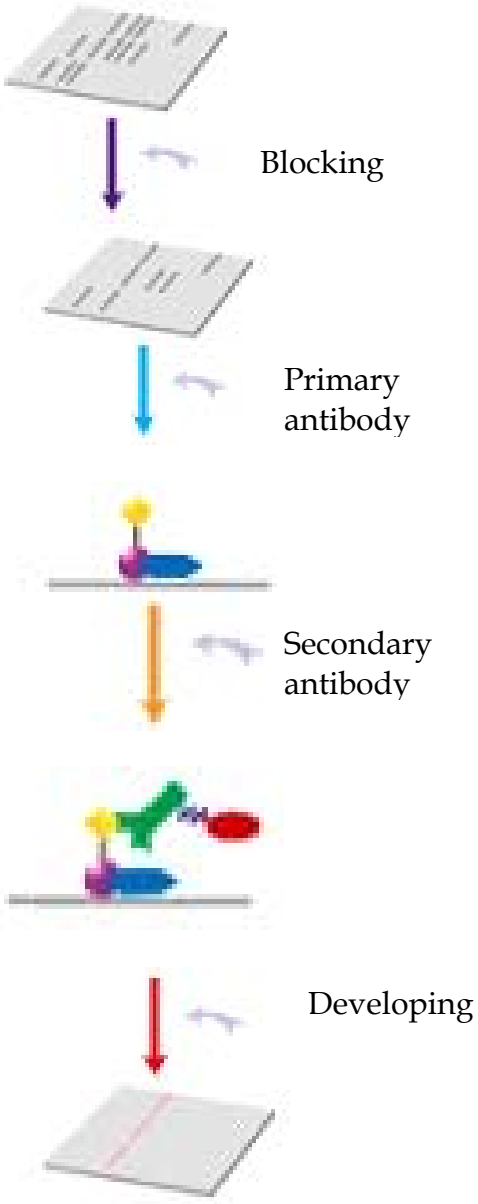
2. Insert this cassette into the gel transfer apparatus filled with cold transfer buffer and then connect the transfer unit to power supply as per conventions.
3. Electrophoreses the sample at 150V, 300 mA for 2 hours for blotting.
4. Remove the nitrocellulose membrane after electrophoresis from the blotting cassette and place the membrane (with protein side up) in 20 ml of 1X Blocking Buffer taken in petri dish.
5. Keep it overnight at 4^0C.

Day 2: Immunodetection:

1. Discard off the blocking buffer.
2. Wash the membrane with 20 ml of 1X Wash Buffer for 5 minutes. Repeat the wash once.
3. Immerse the membrane in 20 ml of 1X Assay Buffer. Add 4 µl of primary antibody solution and mix gently for an hour on a gel rocker. After that discard the primary antibody solution.

4. Wash the blot with 20 ml of 1X Wash Buffer for 5 minutes. Repeat the wash once. Discard the buffer each time.
5. Immerse the blot in 20 ml of 1X Assay Buffer. Add 2 µl of HRP labeled secondary antibody. Mix gently for an hour. Discard the HRP labeled antibody solution.
6. Do a quick washing of the blot with 20 ml of 1X Wash Buffer. Wash the blot with 20 ml of 1X Wash Buffer for 10 minutes. Repeat the wash. Discard the buffer each time.
7. Immerse the washed blot in 3 ml of TMB/H_2O_2 (substrate) solution, mix gently for 5-10 minutes, within this time coloured band will appear.
8. Remove the blot; wash with distilled water, discard and dry.
9. Compare the SDS-Polyacrylamide gel with the developed membrane.

Flow chart:



Observation and Result

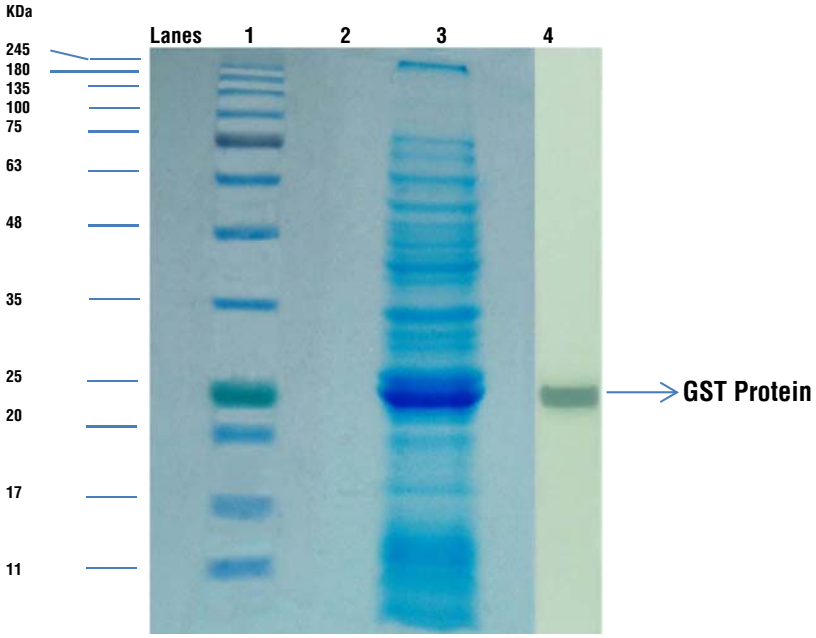


Fig 3: Gel Picture and immunoblot of the Protein sample after SDS-PAGE and Western blotting

Lane 1: Prestained Protein Ladder
Lane 3: Protein Sample
Lane 4: Immunodetection on the blotted membrane

Interpretation:

After staining and destaining of the gel several bands appear in the sample which is a crude bacterial cell lysate. After performing the Western blotting procedure a thick band can be seen on the nitrocellulose membrane which corresponds to the GST protein which is detected by anti-GST antibody. The molecular weight of GST protein is 26 kD and the position of the band corresponds to the protein size.

Troubleshooting Guide:

Sr.No	Problem	Possible Cause	Solution
1	No visible bands on the blot	Poor transfer of protein from gel to the membrane.	Increase the transfer time. Check that there is no air bubble between the gel and the membrane during electrotransfer
2	High background and additional bands on the blot	Washing/blocking is not done properly	Follow the washing/blocking steps thoroughly

3	More bands appear on the blot than expected	Protein sample got degraded	Always keep the protein sample on ice

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