Bio Bharati Life Science Pvt. Ltd.

# Western Blot Teaching Kit

**Cat# BB-ITK050 (5 reactions)**

## Aim:

To learn the technique of Western Blotting in order to detect a specific protein in a blood or tissue sample with the help of that protein specific antibody. It involves the following experiments:

- Electrophoresis of the protein (SDS-PAGE).
- Transfer of protein onto a nitrocellulose membrane (Western Blotting).
- Immune detection of the transferred protein (Blot development).

## Principle:

Western blotting or Immunoblotting is a technique to identify a specific protein within a mixture of proteins by using antibody which is specific to the protein of interest. The technique consists of following three steps:

- **SDS PAGE:** SDS-PAGE is the most widely used method and considered as gold standard for qualitatively analysing any protein sample within a mixture of protein samples. From SDS PAGE one can check the protein purity and also determine the molecular weight of a protein by comparing with protein standard of known molecular weight. SDS or sodium dodecyl sulphate is an anionic detergent that binds strongly to proteins with a uniform negative charge, which nullify the basic charge present on that protein.

  SDS binds fairly uniformly with protein in charge to mass ratio. As a result the protein becomes fully negatively charged and they separate according to their molecular weight within a porous acrylamide bisacrylamide matrix. There will be no differential migration based on charge.

- **Immune-bolting or Western blotting:** It is the technique to transfer the separated proteins from gel to a nitrocellulose membrane with the help of transfer buffer. In this process, the gel is placed on the membrane and both of them are sandwiched between two filter papers. The gel side is placed towards the cathode and the membrane side towards anode. 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 and the protein of interest is immune detected on the membrane.
- **Immune-detection/Blot Development:** The transferred proteins bound to the surface of the nitrocellulose membrane are detected using immunological reagents. This process is known as Immune-detection. All the unoccupied space in the membrane is first blocked by non-fat skimmed milk or BSA or any other blocking agents like gelatine. The membrane is first treated with primary antibody which is specific to the protein of interest. The antigen antibody (Ag-Ab) complex formed in the membrane is then treated with an enzyme coupled secondary antibody and a suitable substrate to the enzyme, which results in a coloured band on the nitrocellulose membrane, referred to as blot development.

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**Kit Description:**

Bio Bharati Western Blotting Kit is designed to carry out 5 Western Blotting experiments.

In this kit, ready to use recombinant protein will be provided, which will be electrophoresed in duplicates with different loading volumes (10µl, 20 µl, 30 µl and 50 µl) along with a standard protein marker on a polyacrylamide gel. Following electrophoresis, the protein sample and marker will be stained to know the electrophoretic mobility of the recombinant protein, while the other electrophoresed sample will be transferred by electro blotting onto nitrocellulose membrane. The electro blotted sample will then be detected using protein specific primary antibody and secondary antibody tagged with Horse Radish Peroxidase (HRP). HRP is then detected using hydrogen peroxide as a substrate and 3, 3', 5, 5' Tetramethylbenzidine (TMB) as a chromogen. TMB acts as a hydrogen donor for the reduction of hydrogen peroxide to water by horseradish peroxidase. The TMB oxide is deposited wherever enzyme is present and appears as a blue band on the nitrocellulose membrane. Sufficient staining and de-staining solution are provided to stain and de-stain 5 duplicate gels. Electrophoresis equipment and transfer apparatus should be arranged by user.

**Duration of experiment:** Experiment is carried out over a span of two days, approximate time taken on each day is indicated below.

**Day 1**: 6-8 hours (SDS-PAGE and Electro-blotting).

**Day 2:** 3 hours (Immuno-detection, Observation and Results).

**Kit Contents:**

| Sl No | Items | Quantity for 5 reactions | Store at |
|---|---|---|---|
| 1. | SDS Separating Gel Mix | 40 ml | 4°C |
| 2. | SDS Stacking Gel Mix | 30 ml | 4°C |
| 3. | APS | 0.1 gm | 4°C |
| 4. | TEMED | 0.05 ml | 4°C |
| 5. | Gel Running Buffer 10X | 250 ml | 4°C |
| 6. | Agarose | 0.5 gm | RT |
| 7. | Pre-stained protein ladder | 30 µl | 4°C |
| 8. | 10X Transfer Buffer | 200 ml | RT |
| 9. | 10 X Wash Buffer | 30 ml | RT |
| 10. | Non-fat Skimmed milk | 7.5 gm | RT |
| 11. | Protein Sample | 1 ml | 4°C |
| 12. | Primary Antibody | 5 X 2µl | -20°C |
| 13. | Secondary Antibody | 5 X 2µl | -20°C |
| 14. | Transfer Membrane | 5 | RT |
| 15. | Filter Paper | 11 | RT |
| 16. | Developing Solution A | 2.5 ml | 4°C |
| 17. | Developing Solution B | 25 ml | 4°C |
| 18. | Developing Solution C | 20 µl | 4°C |

**Materials required but not provided:**

**Consumables**: Measuring cylinder, conical flask, Beaker, Plastic box or petri dish, Staining Tray, Forceps

**Reagents**: Distilled water, Methanol, 70% ethanol for glass plate washing

**Instrument:** Protein Electrophoresis apparatus, Micropipettes, Tips, Microwave/Burner/Hot-plate, Gel Rocker, Transfer (Western Blot) Apparatus.

**Storage:**

Bio Bharati Western Blotting Teaching kit is stable for 6 months from the date of receipt without showing any change in performance if stored properly. Once received, store SDS Separating Gel Mix, SDS Stacking Gel Mix, APS, TEMED, and Protein Sample, Developing Solution A, B and C at 4°C and rest of the items at room temperature (22°C-25°C).

**Important instructions:**

Read the instructions carefully before starting the experiment.

- Dilute all 10X buffer provided in the kit with double distilled water (Molecular Biology grade*). Dilute 1 part of 10 X buffer with 9 parts of double distilled water.
- Dilute the 10X transfer buffer to 1X with double distilled water. Add 9 parts of distilled water with 1 part of transfer buffer. Just before using, add 20 ml methanol to 80 ml 1 X transfer buffer.
- Make 1X wash buffer by mixing 1 part of 10X wash buffer with 9 parts of double distilled water.
- For making 5ml of Developing Solution, take 0.5 ml of Developing Solution A and add 4.5 ml of Developing Solution B. Mix well. Add 2 µl of Developing Solution C.
- Make sure that the glass plates are detergent free (wash the plates using 70% ethanol) before pouring the gel solution within it.
- Always wear gloves when using nitrocellulose membrane.

*Recommended to use Bio Bharati Molecular Biology grade water (Cat# BB- W0055A).

**Procedure:**

**[Day 1] Electrophoresis of the protein (SDS-PAGE)**

1. Assemble the glass plates in electrophoresis unit as per manufacturer's instruction.
2. If your gel casting chamber requires agarose for sealing at the lower edge of the plate prepare 1% agarose (0.05g in 5ml of distilled water). Dissolve the agarose by boiling 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 10% APS Solution: Before starting the experiment, dissolve 0.1 g of Ammonium per sulphate in distilled water to make a final volume of 1.0 ml. Store at 2-8°C. Try to use within 2 months.
4. Preparation of 12.5% Separating Gel: To prepare 5 ml of separating gel mix, take 5 ml SDS Separating Gel Mix, add 50µl APS and 5µl TEMED. After addition of TEMED gently mix all the components by swirling the beaker. Pour the stacking gel in between the plates and add distilled water immediately above the gel layer to solidify the gel and allow it to solidify for an hour. After an hour, pour off the water by inverting the casting assembly.

   # If your gel casting plate requires more than 5ml of separating solution, double the volume up to 10ml and change the volumes of APS and TEMED accordingly.

5. Preparation of 4% Stacking Gel: Take 5mL SDS Stacking Gel mix, add 25µl APS and 3µl TEMED. 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 to avoid generation of any air bubbles. Allow it to solidify for 30minutes.
   ***Note:*** Both the Separating and Stacking Gel solution have acrylamide which is a potent neurotoxin and should be treated with great care. Always wear face mask and gloves while handling acrylamide.
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. Carefully remove the comb from the Stacking Gel.
7. Load 5µl of pre-stained protein ladder in the first lane (lane no. 1). Load 10µl, 20µl, 30µl and 50µl of the supplied protein sample in the lanes numbered 2, 3, 4 & 5 respectively.
8. Connect the power cord to the electrophoretic power supply unit according to the conventions: Red-Anode and Black- Cathode. Electrophorese at 100-120volts until dye front reaches 1 cm above the sealing gel.
9. Carefully remove the gel from in between the glass plates using spatula while immersing the gel (sandwiched between the two glass plates) into the plastic tray containing distilled water. Wash the gel for 1minute. Discard the water & proceed for transfer.
   - **[Day 1] (continued)- Transfer of protein onto nitrocellulose membrane (Western Blotting).**
10. Assemble the gel with nitrocellulose membrane and filter papers as shown in figure below. This blotting sandwich is placed within the blotting cassette. Take utmost precaution to avoid any air bubbles between gel and nitrocellulose membrane. If there are air bubbles remove it from nitrocellulose membrane by rolling a glass tube on the nitrocellulose membrane.
    ***Note:*** Take out the two white sheets stacked at two sides of nitrocellulose membrane carefully with the help of forceps.
11. 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; red: anode, black: cathode.
12. Electrophoreses the sample for 2hours. Calculate the applied current as 1 mA/cm$^2$ of membrane.
    ***Note:*** Try to put the whole unit in a cool environment during transfer.
13. Remove the nitrocellulose membrane after electrophoresis from the blotting cassette and place the membrane (with protein side up) in 20ml of freshly prepared 1X Blocking Buffer taken in petri dish.
14. Keep it overnight at 4$^0$C.
    - **[Day 2] Immune detection of the transferred protein (Blot development).**
15. Discard the blocking buffer.
16. Wash thrice with 1X washing buffer. Keep for 5minutes for each wash.
17. Treat the membrane with Primary Antibody at 1:5000 dilution (provided with kit) for 1h at room temperature with continuous shaking.
18. Discard the antibody and repeat the Step 16.
19. Add Secondary Antibody at 1:5000 dilution (Goat Anti-Rabbit-HRP conjugate) (provided in the kit) into the membrane and keep it for 1h with continuous shaking at room temperature.
20. Discard the secondary antibody and repeat step 16.
21. Mix Developing Solutions A, B and C just before use and pour the mixed solution into the membrane and shake gently until blue colour appears.
22. Add water to stop the reaction.
    ***Note:*** Although the coloured band fades with time, the rate of colour loss can be retarded if the blots are kept in dark.
23. Compare the SDS-Polyacrylamide gel with the developed Nitrocellulose membrane.

**Observation and Result:**

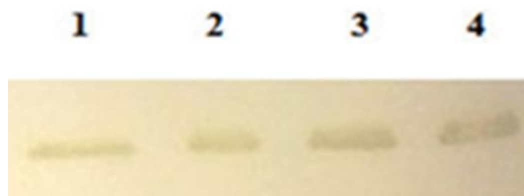


**Figure 1:** Western blot

Analysis of recombinant protein: Lane 1: 10μl, Lane 2: 20μl, Lane 3: 30μl and Lane 4: 50μl of ready to use recombinant protein were loaded respectively.

**Interpretation:**

After complete destaining of the gel, compare the samples with the protein ladder. Different volumes of the same protein sample were loaded in the gel which after transfer to nitrocellulose membrane was treated with protein specific primary antibody followed by HRP-Conjugated secondary antibody. In developed blot, the intensity also increased according to the loading volume of the protein samples.

**Technical Assistance:**

At Bio Bharati Life Science Pvt. Ltd., we always promise to deliver the best products for quality research at our customers end. All the reagents are made at our In-house R&D facility with utmost care to produce the best optimum results. We are always happy to stand beside our researcher friend at their need. For any assistance please write to us at biobharati@gmail.com or Contact Us at Telephone no.: (033)-40077640, Mobile: 09836508080.

**Trouble-shooting Guide:**

| Sl No | Problem | Reason for Problem | Solution |
|---|---|---|---|
| 1. | Poor resolution of the gel | Greater volume of sample loaded on the well | Do not overload the sample in the well. Load as per the size of your well. |
| 2. | Samples do not sink to the bottom of the well while loading | Combs removed before stacking gel properly polymerized | Allow the stacking gel to polymerize for at least 10-15 minutes before removing the combs |
| 3. | Bands on part of the slab do not move down the gel | Air bubbles generated between the plates underneath the affected lanes | Check no bubbles are present in the gel while pouring |
| 4. | No bands were seen after development | Developing solution did not work properly | Always mix all the three components of the developing solution just before use. |

*Adapted from internet*