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# Western Blot

Western blotting is a widely used method for the identification and quantification of proteins in biological samples. It combines electrophoretic separation, transfer to a membrane and antibody-based detection. The sequence of methods is as follows:

1. Electrophoresis (SDS-PAGE): The proteins are separated according to their size.
2. Transfer: The separated proteins are transferred to a membrane (e.g. PVDF or nitrocellulose).
3. Blocking: Non-specific binding sites on the membrane are saturated with a blocking solution.
4. Antibody incubation: A primary antibody binds specifically to the target protein, a secondary antibody with a detection marker (e.g. enzyme or fluorescence) enables visualization.
5. Detection: The proteins are visualized by chemiluminescent, fluorescent or colour-bound signals.

Western blot is frequently used in biomedical research, diagnostics and biotechnology, e.g. to analyze protein expression, post-translational modifications or disease signatures.

Figure 1: Western Transfer and Protein Detection

## A. ANTIBODIES

- ReliaTech´s Antigen specific probing antibody (primary antibody; e.g. human antibody)
- Secondary antibody (e.g. anti-human antibody) conjugated to detection marker (e.g. Alkaline Phosphatase)

## B. RECOMMENDED MATERIALS

- SDS-PAGE apparatus and accessories
- Submerged Western Transfer Cassette and accessories

- A nitrocellulose membrane, approximately the size of the gel, presoaked in Western Transfer Buffer for five minutes

  *The membrane should be handled with gloves and clean forceps to avoid contamination with extraneous proteins*

- Western Transfer Buffer (TOWBIN buffer)
  *25 mM Tris, 192 mM glycine, 20% (v/v) methanol (pH 8.3)*

  | | |
  |---|---|
  | *Tris base* | *3.03 g* |
  | *Glycine* | *14.4 g* |
  | *$dH_2O$* | *500 mL* |
  | *Methanol* | *200 mL* |

  *Adjust volume to 1 L with $dH_2O$*
  *The pH will range from pH 8.1 to 8.5 depending on the quality of the Tris, glycine, methanol, and $dH_2O$*

- Commercially available detection kit (e.g. alkaline phosphatase conjugate kit)

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## 1. TRANSFER PROTOCOL

1. Load the protein sample onto a 4-20% Tris-Glycine polyacrylamide gel and run until desired resolution is achieved.

   *The electrophoresis can be followed by using pre-stained molecular weight markers.*

2. Set up the Submerged Western Transfer Cassette as described below and in Figure 2:

a. Submerge the open Transfer Cassette cathode plate onto the tray pre-filled with Western Transfer Buffer.
b. Place a sponge support pad onto the cassette and remove the air bubbles by gently rolling a Pasteur pipette over the pad.
c. Place a piece of blotting paper onto the sponge support pad.
d. Remove the gel from the electrophoresis plates, cut off approximately the bottom 3mm of the gel so that the membrane can be laid flat against the gel, and place the gel over the blotting paper. Expel air bubbles as before.
e. Carefully place the pre-soaked nitrocellulose membrane onto the gel and expel air bubbles. Ensure that the membrane remains directly over the gel before proceeding.
f. Place a second piece of blotting paper onto the nitrocellulose membrane and remove air bubbles.
g. Place a sponge support pad onto the second piece of blotting paper and remove air bubbles.
h. Gently close the cassette by placing the anode plate over the exposed pad.

3. Carefully place the assembled cassette into the transfer tank containing Western Transfer Buffer up to the pre-fill level and adjust the buffer level, as needed, after the addition of the cassette.
4. Connect the assembled apparatus to an electrophoresis power supply and for approximately 1.5 hours at a constant current of 400 mA.

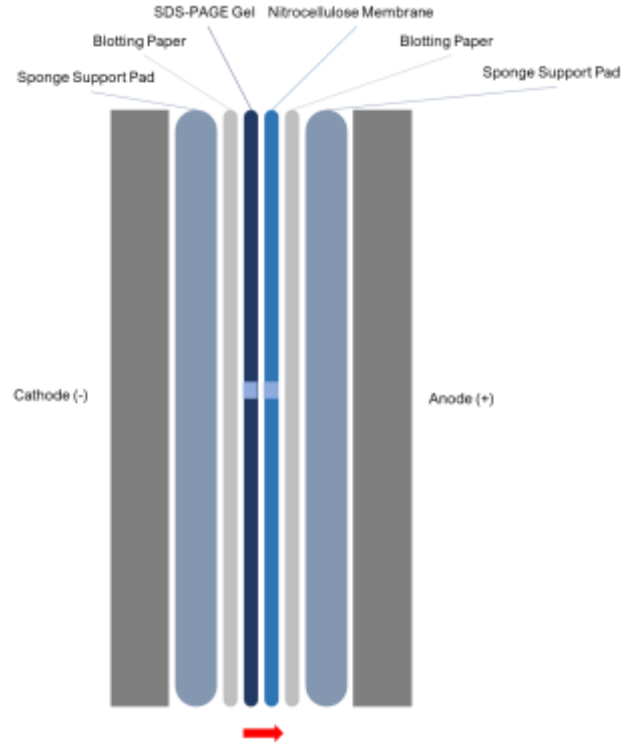


Figure 2: Western Transfer Cassette

## 2. IMMUNOSTAINING PROTOCOL

1. After the transfer is complete, incubate the membrane in blocking solution (3% Nonfat Dry Milk in $dH_2O$) for 30 minutes with gentle agitation on an orbital shaker.
2. Wash the membrane three times with TBST (TBS, pH 7.2 with 0.1 % TWEEN-20) in a clean tray on an orbital shaker; each wash lasting 5-10 minutes.
3. Dilute the probing (primary) antibody in TBST to a volume of 50 mL (approximate final concentration of 0.2 µg/mL) and incubate the membrane in the antibody solution for 1-4 hours at room temperature.

   *The optimum incubation time depends on the antibody/antigen binding affinity and must be pre-determined for each antibody*

4. Wash the membrane three times as in step 2.
5. Dilute the secondary antibody in TBST according to the manufacturer´s specification. Incubate the membrane in a clean tray containing 50 mL of diluted secondary antibody for 1 hour at room temperature on an orbital shaker.
6. Wash the membrane three times as in step 2.
7. Colour development requires the use of a commercially available alkaline phosphatase conjugate substrate kit (for this example). Follow the manufacturer´s instructions.
8. After the bands become clearly visible, stop the colour by placing the membrane in a tray containing $dH_2O$ for at least ten minutes.