

WESTERN BLOTTING (W)

Introduction

In a Western blot/immunoblot, (denatured) proteins are separated using gel electrophoresis on the basis of size/mass. After separation the proteins are transferred from the gel onto a membrane (typically nitrocellulose or Polyvinylidene Fluoride (PVDF)), where they can be identified through its reaction with a specifically labeled antibody or antigen.

Since most gel electrophoresis procedures result in denaturation of the antigen, only polyclonal and monoclonal antibodies that recognize the denatured form of an antigen can be utilized in immunoblotting. Through spatial resolution this method provides molecular weight information on individual proteins and separates isoforms from processed products. After proteins have been transferred onto a suitable membrane, they can be stained for visualisation and directly identified by immunodetection.

Materials/ reagents

- **Sample: cells/tissue with the proteins/antigens of interest or recombinant or purified protein**
- **(Labeled) primary antibody**
- **(Labeled) secondary antibody**
- **Developing solution/substrate**
- **Wet blot Transfer Buffer:**
 - 25mM Tris
 - 190 mM glycine
 - 20% methanol
 - pH adjusted to 8.0
- **Semi-dry Transfer Buffer:**
 - 39 mM Glycine
 - 48 mM Tris base
 - 0.037% SDS
 - 20% Methanol
 - pH 8.3.
- **Wash buffer:**
 - 0.05% Tween 20
 - Phosphate Buffered Saline (PBS)
- **Blocking buffer**
 - A:**
 - 5% non-fat dry milk
 - 10 mM Tris pH 7.5
 - 100 mM sodium chloride
 - 0.1% Tween 20
 - B:**
 - 3% Bovine serum albumin (Fraction V)
 - PBS
 - 0.05% Tween 20
 - Keep at 4°C to prevent bacterial contamination
- **Stripping buffer:**
 - 0.2 M glycine pH 2.8
 - 0.5 M sodium chloride
- **Neutralizing buffer:**
 - 0.5 M Tris
 - 1.5 M sodium chloride
- **Ponceau S**
 - **A:** 0,1 % (w/v) Ponceau S in 5 % acetic acid.
 - **B:** 2 % (w/v) Ponceau S in 30 % TCA and 30 % sulfosalicylic acid

Equipment

Transfer:

- Polyvinylidene Fluoride (PVDF) membrane or other membrane
- Filter paper
- Western blotting apparatus

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Procedure **Tissue preparation and gel electrophoresis (SDS-PAGE)**
See protocol [SDS-PAGE](#).

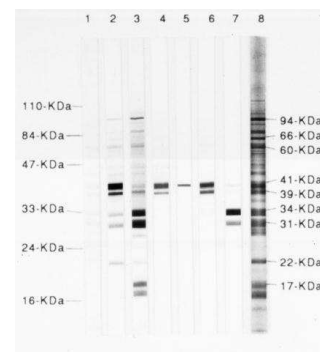
Transfer

In order to make the proteins accessible to antibody detection, they are moved from the gel to an appropriate membrane (nylone, nitrocellulose or PVDF etc). The membrane is placed face-to-face with the gel, and current is applied to large plates on either side. The charged proteins move from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection.

Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Transfer can be done in wet or semi-dry conditions. Semi-dry blotting is faster and easier, and requires a lot less buffer volumes than wet blotting.

Small Proteins (<20 kDa) are transferred with better reproducibility in semi-dry conditions. Big proteins, however, do not transfer well in semi-dry blotting. If a semi-dry transfer system is used for large proteins add SDS to help transfer.

Wet conditions are recommended for high molecular weight proteins. Tank blots transfer high molecular weight proteins (>60 kDa) better than semi-dry. Small proteins may be blotted through the membrane and lost for detection. Use the power conditions recommended by the manufacturer.



Wet transfer

1. Soak the gel(s) and the membrane(s) in [wet blot transfer buffer](#) for a minimum of 15 minutes to remove electrophoresis salts and detergents.
2. For each gel, saturate two sponges and two pre-cut filter papers in transfer buffer.
3. Assemble sandwich in the following order:

Sponge	-
Filter paper	
Gel	
Membrane	
Filter paper	
Sponge	+

4. Repeat with a second cassette for the second gel, if needed.
5. Insert the cassette into the electrode module. Be sure that the direction of the transfer is from gel towards membrane. The gray side of the cassette should be next to the gray side of the electrode module (i.e., the cathode side).
6. Place a stir bar and a cooling unit (stored at -20°C) in the buffer tank. Place the electrode module in the buffer tank.
7. Fill the tank with wet blot transfer buffer to the bottom edge of the top row of holes on the cassette. Place the buffer tank on a magnetic stir plate and stir at medium speed.
8. Attach electrodes, the time and current depends on the protein size:

Protein size	
< 20 kDa	Transfer proteins from gel to membrane at 20 mA constant current for 45 mins in transfer buffer
<120 kDa	Transfer proteins from gel to membrane at 20 mA constant current for 1 hour in transfer buffer
>120 kDa*	Transfer proteins from to membrane at 20 mA constant current for 90 minutes in transfer buffer + SDS
>250 kDa*	Transfer proteins from to membrane at 20 mA constant current for 1 hour and 45 minutes in transfer buffer + SDS

Note: to ensure complete transfer of large molecular weight proteins, 0.05% SDS can be added to the transfer buffer.

Semi-Dry Transfer

1. Cut 6-10 sheets of filter paper same size as the gel.
2. Cut membrane to the size of the gel.
3. Membrane activation for PVDF: soak in methanol for 15 seconds and then transfer to a container filled with distilled water for 5 minutes.
4. Soak membrane in [semi-dry transfer buffer](#) for 10 minutes while preparing transfer sandwich.

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5. Soak filter paper in transfer buffer for several minutes to 10 minutes to avoid trapping of air bubble.
6. Assemble sandwich in the following order:

3-5 sheets filter paper -
Gel
Membrane
3-5 sheets filter paper +

7. Use a roller to roll over the sandwich gently to remove trapped air bubbles.
8. Apply few ml transfer buffer on top of the sandwich to avoid drying out during membrane transformation.
9. Transfer at 100 V for 40-60 minutes.

Blocking

1. Remove the blot from the transfer apparatus or staining tray and immediately place into [blocking buffer](#).
2. Incubate the blot for 30 minutes at 37°C, 1 hour at room temperature, or overnight at 4°C.
3. Remove the blocking solution. Rinse membranes in wash buffer once.
4. Membranes may be stored in [wash buffer](#) at 4°C for up to 1 week.

Ponceau S

[Ponceau S](#) (sodium salt) may be used to prepare a stain for rapid reversible detection of protein bands on nitrocellulose or PVDF membranes.

Probing with antibodies

Incubation with primary antibody

Incubation Buffer: Dilute the antibody in blocking buffer at the suggested dilution. If the datasheet does not have a recommended dilution try a range of dilutions (1:50-1:3000) and optimize the dilution according to the results. Too much antibody will result in non-specific bands.

It is traditional in certain laboratories to incubate the antibody in blocking buffer, while other laboratories incubate the antibody in TBST without a blocking agent. The results are variable from antibody to antibody and a difference may be found that makes a difference to either use no blocking agent in the antibody buffer or the same agent as the blocking buffer.

Incubation Time: The time can vary between a few hours and overnight (rarely more than 18 hours), and is dependent on the binding affinity of the antibody for the protein and the abundance of protein. A more dilute antibody is recommended for a prolonged incubation to ensure specific binding.

Incubation Temperature: Room temperature or at 4°C. If incubating in blocking buffer, it is imperative to incubate at 4°C or contamination will occur and thus destruction of the protein overnight (especially phospho groups).

Agitation of the antibody is recommended to enable adequate homogenous covering of the membrane and prevent uneven binding.

1. Dilute the antibody in the corresponding [blocking buffer](#).
2. Decant the blocking buffer from the blot.
3. Add the antibody solution.
4. Incubate with agitation for 30 minutes at 37°C, one hour at room temperature, or overnight at 4°C.
5. Wash 3 times for 10 minutes with wash buffer.

Incubation with secondary antibody

Incubation Buffer: Dilute the antibody in blocking buffer at the suggested dilution, if the datasheet does not have a recommended dilution try a range of dilutions (1:1000- 1:20,000) and optimize the dilution according to the results. Too much antibody will result in non-specific bands.

As with the primary antibody you may choose to incubate the secondary antibody in blocking buffer or not.

Incubation Time: 1-2 hours.

Incubation Temperature: Room temperature or at 4°C.

Agitation of the antibody is recommended to enable adequate homogenous covering of the membrane and prevent uneven binding.

1. Dilute the secondary antibody in the corresponding blocking buffer.
2. Incubate with secondary antibody diluted in Blocking buffer for 45 minutes at room temp.
3. Wash 3 times for 10 minutes with Wash buffer.

Stripping

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Stripping is used when more than one protein is investigated on the same blot, or the same protein with different antibodies. There are different ways to strip for different membranes, one method is:

1. Strip the membrane for at most 5 minutes in [stripping buffer](#).
2. Neutralize in [neutralizing buffer](#).

Analysis

The detection system used is dependent on the systems present:

Colorimetric detection

The colorimetric detection method depends on incubation of the Western blot with a substrate that reacts with the reporter enzyme (such as peroxidase) that is conjugated to the secondary antibody. This converts the soluble dye into an insoluble form of a different color that precipitates next to the enzyme and thereby stains the nitrocellulose membrane. Development of the blot is then stopped by washing away the soluble dye. Protein levels are evaluated through densitometry (how intense the stain is) or spectrophotometry.

Chemiluminescence

Chemiluminescent detection method depends on incubation of the Western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by photographic film, and more recently by CCD cameras which capture a digital image of the Western blot. The image is analysed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density. More advanced software allows further data analysis such as molecular weight analysis if appropriate standards are used. So-called "enhanced chemiluminescent" (ECL) detection is considered to be among the most sensitive detection methods for blotting analysis.

Safety

- Under no circumstances shall Hbt be liable for any damage arising from the use of this protocol. User should be trained and be familiar with the test procedure.
- Samples of tissue, serum or blood origin should be handled to guidelines for prevention of transmission of blood borne diseases.
- Some enzyme substrates are considered hazardous, due to potential carcinogenicity. Handle with care and refer to Material Safety Data Sheets for proper handling precautions.
- Wear appropriate protective clothing, gloves, and eyewear to avoid any accidental contact with reagents.
- Acrylamide present in SDS-PAGE gels is a potent cumulative neurotoxin: wear gloves at all times!
- Use extreme caution, is a known carcinogen.
- Reagents which contain preservatives may be toxic if ingested, inhaled, or in contact with skin.

Notes

- Please note that this is a general protocol. Optimal dilutions for the primary and secondary antibodies, cells preparation, controls, as well as incubation times will need to be determined empirically and may require extensive titration. Ideally, one would use the primary antibody as recommended in the product data sheet.
- This protocol is to be used for research purposes only.
- When making buffer(s) fresh every time, there is no need to add sodium azide to the buffer.
- The appropriate negative and positive controls should be included in every trial.
- Use gloves when manipulating filter papers, gels and membranes. Oil from hands blocks the transfer.
- Nitrocellulose membranes and PVDF membranes are chosen for their non-specific protein binding properties (i.e. binds all proteins equally well).
- Nitrocellulose membranes are cheaper than PVDF, but are far more fragile and do not stand up well to repeated problings. PVDF membranes require careful pre-treatment: cut the membrane to the appropriate size then soak it in methanol for 1-2 min. Incubate in ice cold transfer buffer for 5 min. The gel needs to equilibrate for 3-5 min in ice cold transfer buffer. Failure to do so will cause shrinking while transferring and an unsightly pattern of transfer.
- Since extra negative charges are needed to reach 1 A in a wet transfer system, adjust the pH of the transfer buffer to approximately pH 8.0 using NaOH.

Technical assistance

For technical assistance and more information please contact Hycult Biotech directly at support@hycultbiotech.com