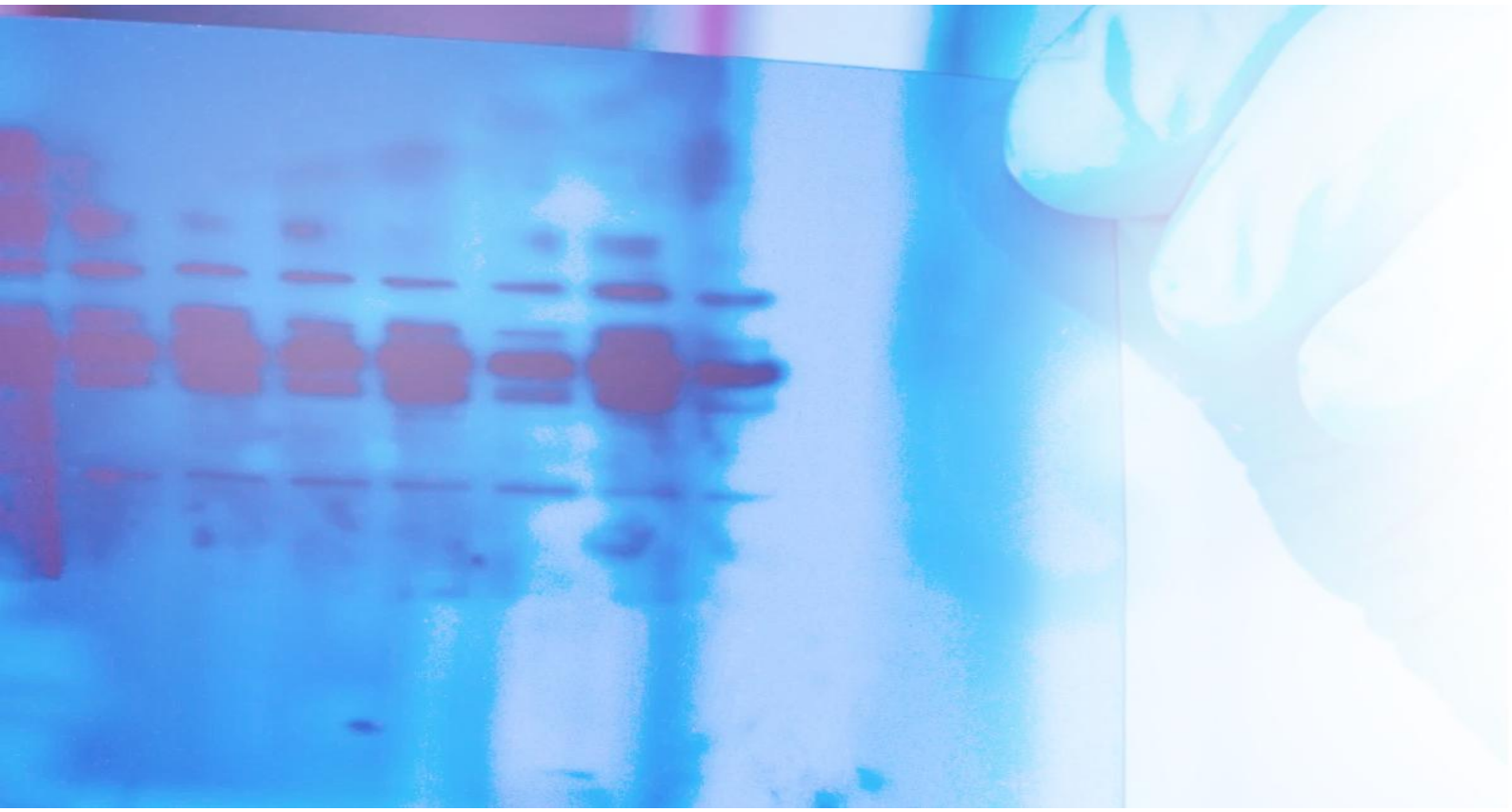
BT LAB
Bioassay Technology Laboratory

WB Guide

A guide to Western Blotting



Introduction

Western blotting is a widely used analytical technique in molecular biology and immunogenetics to detect specific proteins in a sample of tissue homogenate or extract.

Western blot technique uses three elements to achieve its task of separating a specific protein from a complex: separation by size, transfer of protein to a solid support, and marking target protein using a primary and secondary antibody to visualize. A synthetic or animal-derived antibody (known as the primary antibody) is created that recognizes and binds to a specific target protein. The electrophoresis membrane is washed in a solution containing the primary antibody, before excess antibody is washed off. A secondary antibody is added which recognizes and binds to the primary antibody. The secondary antibody is visualized through various methods such as staining, immunofluorescence, and radioactivity, allowing indirect detection of the specific target protein.

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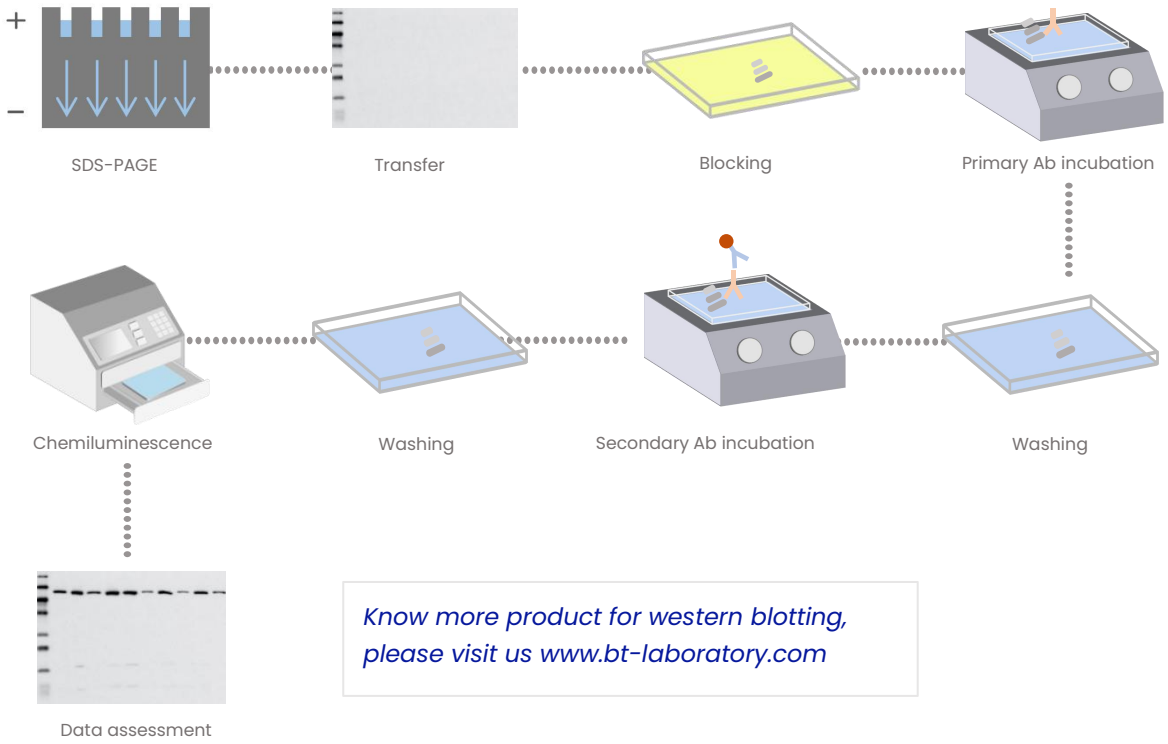

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Lysis buffers

Lysis buffers is a buffer solution used for the purpose of breaking open cells for use in molecular biology experiments that analyze the labile macromolecules of the cells.

Lysis buffer recipes

Buffer	Components
Tris-HCl	20mM Tris-HCl, pH 7.5
RIPA	150 mM NaCl 1.0% NP-40 or Triton X-100 0.5% sodium deoxycholate 0.1% SDS 50 mM Tris, pH 8.0
NP-40	150mM NaCl 1% NP-40 50mM Tris, pH 8.0
Tris-Triton X-100	10 mM Tris, pH 7.4 100 mM NaCl 1 mM EDTA 1 mM EGTA 1% Triton X-100 10% glycerol 0.1% SDS 0.5% deoxycholate

Protease and Phosphatase Inhibitors

Inhibitor	Protease/phosphatase inhibited	Final concentration in lysis buffer
Aprotinin	Trypsin, chymotrypsin, plasmin	2 µg/mL
Leupeptin	Lysosomal	1-10 µg/mL
Pepstatin A	Aspartic proteases	1 µg/mL
PMSF	Serine, cysteine proteases	1 mM
Sodium fluoride	Serine/threonine phosphatases	5–10 mM
EDTA	Metalloproteases that require Mg^{2+} and Mn^{2+}	5 mM
EGTA	Metalloproteases that require Ca^{2+}	1 mM
B-glycerophosphate	Serine & threonine phosphatases	1-2 mM

Sample preparation

Lysate preparation from cell culture

1. Wash the cells with ice-cold PBS.
2. Drain the PBS, then add ice-cold lysis buffer (1 ml per 107 cells/100 mm2 dish/150 cm2 flask; 0.5ml per 5x106 cells/60 mm2 dish/75 cm2 flask).
3. Scrape adherent cells off the dish with a cold plastic cell scraper, then gently transfer the cell suspension into a pre-cooled micro centrifuge tube.
4. Agitate cells for 30 minutes at 4°C.
5. Centrifuge at 16,000 x g for 20 minutes in a 4°C pre-cooled micro centrifuge.
6. Gently remove the tubes from the micro centrifuge and place on ice. Transfer the supernatant to a fresh tube kept on ice, and discard the pellet.

Lysate Preparation from tissues

1. Dissect the tissue of interest with clean tools, on ice preferably, and as quickly as possible to prevent degradation by proteases.
2. Place the tissue in round-bottom micro centrifuge tubes or Eppendorf tubes and immerse in liquid nitrogen to “snap freeze”. Store samples at -80°C for later use or keep on ice for immediate homogenization.
3. For a ~5 mg piece of tissue, add ~300 ml lysis buffer rapidly to the tube, homogenize with an electric homogenizer, rinse the blade twice with another 2 X 300 ml lysis buffer, then maintain constant agitation for 2 hours at 4°C. Volumes of lysis buffer must be determined in relation to the amount of tissue present. Protein extract should not be too dilute, to avoid the need to load large volumes per gel lane. The minimum protein concentration is 0.1 mg/ml; optimal concentration is 1-5 mg/ml.
4. Centrifuge for 20 minutes at 16,000 rpm at 4°C in a micro centrifuge. Gently remove the tubes from the centrifuge and place on ice. Transfer the supernatant to a fresh tube kept on ice; discard the pellet.

Determination of protein concentration

Perform a protein quantification assay such as a Bradford Protein Assay or BAC assay to determine the concentration of the lysate. Protein samples can be frozen at -20°C or -80 °C for later use or prepared for gel loading for immediate use.

Loading buffer recipe

Loading Buffer	Components
2X Laemmli buffer	4% SDS 5% 2-mercaptoethanol 20% glycerol 0.004% bromophenol blue 0.125 M Tris HCl Adjust pH to 6.8

Loading and running buffer conditions

Condition	Sample loading buffer	Gel running buffer

Reduced & denatured	SDS + β-ME or DTT	SDS
Reduced & native	β-ME or DTT, No SDS	No SDS
Denatured	No β-ME or DTT	SDS
Native	No β-ME or DTT	No SDS

Electrophoresis

After sample preparation, samples in loading buffer must be loaded onto a gel. Proteins in the sample are separated from each other based on their size by SDS-PAGE gel electrophoresis. Electrophoresis is performed with a negative pole (cathode) on one end of the gel and a positive pole (anode) on the opposite end of the gel. The negatively charged SDS bound to proteins causes migration of protein complexes towards the positive pole (anode) during electrophoresis, allowing proteins to be separated by size. In general, the larger the protein, the slower it migrates through the gel. Acrylamide gels can be prepared at different concentrations. As a general rule, low molecular weight proteins are best resolved on high percentage gels, whereas large proteins require lower percentage gels for sufficient resolution.

SDS-PAGE Protocol

1. Prepare or purchase a pre-made gel of appropriate polyacrylamide percentage to best resolve your protein of interest based on molecular weight.

Protein size	Gel percentage
4-40 kDa	Up to 20%
12-45 kDa	15%
10-70 kDa	12.5%
15-100 kDa	10%
50-200 kDa	8%
>200 kDa	4-6%

2. Load samples containing equal amounts of protein (10-50 µg protein from cell lysate or 10-100ng purified protein) prepared in sample buffer into SDS-PAGE wells. Include a molecular weight marker in one of the lanes.
3. Fill the electrophoresis apparatus with 1X running buffer (25 mM Tris base
   192 mM glycine, 0.1 % SDS, Adjust pH to 8.3) as instructed by the manufacturer.
4. Run the gel as recommended by the manufacturer. Voltage may vary depending on research needs. 1 hour at 100V is a standard guideline.

Protein transfer

1. Prepare the PVDF membrane by wetting it in methanol for 30 seconds and then soaking it briefly in distilled water followed by 1X transfer buffer. Handle the membrane carefully, ideally with rounded tweezers to avoid scratching or puncturing the surface.
2. Soak filter papers and sponges in the transfer buffer for 10 mins prior to assembly of the transfer sandwich.
3. Once electrophoresis is complete, remove the gel from the electrophoresis apparatus and equilibrate it by soaking it in transfer buffer for 10 mins.
4. Prepare the sandwich according to the illustration below. Sequentially assemble the layers of the sandwich. Gently remove any air bubbles with a roller or pipette. Bubbles between the gel and the membrane will inhibit the transfer of proteins to the membrane.
5. Place the sandwich into a transfer cassette and perform semi-dry or wet (also known as tank) transfer according to the manufacturer's instructions of the blotting apparatus. For wet transfer, the gel side of the cassette holder should face the cathode (-) while the membrane side should face the anode (+). For semi-dry transfer, the gel side should face the cathode plate (-), while the membrane side should face the anode plate (+).

Membrane blocking

1. Block the membrane for 1 h at room temperature or overnight at 4°C using blocking buffer.
2. Incubate the membrane with appropriate dilutions of primary antibody in blocking buffer. We recommend overnight incubation at 4°C; other conditions can be optimized.
3. Wash the membrane in three washes of TBST, 5 minutes each.
4. Incubate the membrane with the recommended dilution of conjugated secondary antibody in blocking buffer at room temperature for 1 hour.
5. Wash the membrane in three washes of TBST, 5 min each.
6. For signal development, follow the kit manufacturer's recommendations. Remove excess reagent and cover the membrane in transparent plastic wrap.
7. Acquire image using darkroom development techniques for chemiluminescence, or normal image scanning methods for colorimetric detection.

Primary antibody: After blocking, the membrane is incubated in a solution containing the primary antibody. The primary antibody recognizes and binds the epitope or the specific amino-acid sequence of the protein of interest.

Secondary antibody: After washing to remove unbound primary antibody, secondary antibody is added. Secondary antibody recognizes the primary antibody. Secondary antibodies used for western blotting are typically conjugated with an enzyme; the most commonly used enzymes are Horse Radish Peroxidase (HRP) and Alkaline Phosphatase (AP).

Note: Some primary antibodies are directly conjugated to HRP, eliminating the need for the secondary antibody incubation steps. In this case, it is possible to proceed to detection after the primary antibody incubation and subsequent rinses. If elimination of the secondary antibody step is desired, Novus offers HRP conjugated primary antibodies and Lightning-Link Antibody Labeling Kits, which can be used to conjugate an unlabeled primary antibody to HRP or other desired conjugates.

Immunoblotting

1. After transfer, carefully disassemble the transfer stack and rinse the membrane briefly in distilled water or 1X TBST(20 mM Tris base, 150 mM NaCl, 0.1% Tween 20).
2. Gently mark MW ladder bands with a pencil for size detection. If all blue molecular weight markers were used, this step can be omitted as the bands of all blue markers will be visible after detection when used in conjugation with the Blue Marker Antibody.
3. If desired, stain the membrane with Ponceau red (a reversible protein stain) for 30 seconds to visualize protein bands to confirm that protein transfer was successful. Rinsing the membrane briefly with distilled water after Ponceau staining will reveal protein bands. Wash away Ponceau red with several washes in 1x TBST until membrane is clear. Additionally, Coomassie staining of the gel after transfer can help assure that proteins were completely transferred from the gel to the membrane (minimal or no protein staining should be visible on a coomassie-stained gel after successful complete transfer).
4. Incubate the membrane in blocking solution(1X TBST, 5% non-fat dry milk or 5% BSA) for 1 hour at room temperature or overnight at 4˚C with constant rocking.
5. Dilute the primary antibody to working concentration in 1X TBST with 1% milk or BSA (depending on what was chosen for blocking).
6. Incubate the membrane in primary antibody solution for 1-2 hours at room temperature or overnight at 4˚C with gentle rocking. The incubation time may require optimization. However, overnight incubation at 4˚C is generally recommended to reduce non-specific background signal.
7. Wash the membrane with 1X TBST three times for 10 minutes each with gentle rocking.
8. Incubate the membrane in the diluted secondary antibody (in 1X TBST and may include 1% milk or BSA) for 1 hour at room temperature with gentle rocking.
9. Wash the membrane in 1X TBST three times for 10 minutes each with gentle rocking.

Detection

1. Prepare the ECL substrate just prior to use according to the manufacturer's instructions.
2. Incubate the membrane in the substrate according to manufacturer's directions. Typical incubation times are 1-5 minutes.
3. Carefully remove the membrane from the detection reagent and sandwich it between layers of plastic (e.g. a sheet protector or plastic wrap).
4. Expose the membrane to autoradiography film in a dark room or image with a chemiluminescent imaging system, such as a ChemiDoc.
5. The developed film or image can be lined up in the correct orientation over the blot in order to mark the MW ladder positions if the Blue Marker Antibody is not used.

Tips for Western blotting

- Do not deem an antibody to be unspecific until you have efficiently reduced background
- Do not let the membrane dry at any point during the blotting process.
- Adding up to 0.05% SDS in the transfer buffer can improve transfer efficiency in some cases.
- Avoid touching the membrane with your fingers, use tweezers instead. Oils and proteins on fingers will block efficient transfer and create dirty blots.
- Make sure the paper and membrane are cut to the same size as the gel. Large overhangs may prevent a

current from passing through the membrane in semi-dry transfers.

- Milk is not recommended for studies of phospho-proteins because milk contains casein which is a phospho-protein, causing high background.
- If target MW is larger than 100kDa, wet transfer at 4°C overnight is suggested in place of a semi-dry method; moreover, we recommend adding 0.1% SDS to the wet transfer buffer to facilitate transfer.
- Agitation of the primary antibody is recommended to enable adequate homogenous covering of the membrane and prevent uneven binding.
- Use multiple exposure lengths to identify the optimal exposure time. An initial 10 second exposure will indicate the necessary exposure time.

Troubleshooting

High background

Possible case	Solution
The primary antibody concentration may be too high	Titrate the antibody to the optimal concentration. Incubate for longer but in more dilute antibody (a slow but targeted binding is best).
The incubation temperature may be too high	Incubate membrane at 4°C.
The secondary antibody may be binding non-specifically or reacting with the blocking reagent	Run a secondary control without the primary antibody.
Cross-reactivity between the blocking agent and primary or secondary antibody	Add a mild detergent such as Tween 20 to the incubation and washing buffer.
The washing of unbound antibodies may be insufficient	Increase the number and time of washes.
The membrane has dried out.	Care should be taken to prevent the membrane from drying out during incubation

Weak or No signal

Possible case	Solution
The primary antibody and the secondary antibody are not compatible	Use a secondary antibody that was raised against the species in which the primary was raised (eg if the primary is raised in rabbit, use an anti-rabbit secondary).
Not enough primary or secondary antibody is bound to the protein of interest	Use a higher concentration of antibody or incubate longer (eg overnight) at 4°C.
There is cross-reactivity between the blocking agent and the primary or secondary antibody	Use a mild detergent such as Tween 20 or switch blocking reagent (ie commonly used blocking reagents are milk, BSA, serum or gelatin).
The primary antibody does not recognize the protein in the species being tested	Check the datasheet or perform a BLASTp alignment to see whether your antibody should react with the target protein. Run the recommended positive control.
There is insufficient antigen	Load at least 20–30 µg protein per lane, use protease inhibitors and run the recommended positive control.
The protein of interest is not abundantly present in the	Use an enrichment step to maximize the signal (eg prepare nuclear

tissue	lysates for a nuclear protein).
There is a poor transfer of protein to membrane	Check the transfer with a reversible stain such as Ponceau S. If proteins have not transferred effectively, check the transfer was not performed in the wrong direction. If using PVDF membrane, make sure that you pre-soak the membrane in methanol and then in transfer buffer.
Excessive washing of the membrane	Reduce the number or duration of washing steps.
Overuse of the primary antibody	Use fresh antibody as the effective concentration is lowered upon each use.
The detection kit is old and the substrate is inactive	Use fresh substrate.

Splotchy background

Possible case	Solution
Uneven antibody distribution	Ensure the membrane is agitated evenly by placing on a rocker or shaker during incubation.
Insufficient amount of incubation buffer	Ensure enough solution is present during each incubation period to fully submerge the membrane and allow it to float freely in the solution.
Air bubbles present during transfer	Gently remove all air bubbles from transfer "sandwich" before transfer is started. This can be done by using a roller or clean glass rod/Pasteur pipette.
Membrane problems	Ensure membrane is wetted thoroughly according to the manufacturer's protocol.
	Handle membranes with extreme care.
	Use forceps or wear gloves when handling membrane.
Aggregation of HRP conjugate	If using HRP, filter conjugate to remove HRP aggregates. Use a fresh sample of HRP conjugate.
Contamination of reagents	Filter buffers before use to remove contaminant. Make fresh buffers and re-run Western Transfer.

Non-specific bands

Possible case	Solution
Antibody concentration too high	Reduce concentrations of antibodies, particularly of primary antibody.
Too much protein loaded on gel	Reduce the amount of sample loaded on gel.
Air bubbles present during transfer	Gently remove all air bubbles from transfer "sandwich" before transfer is started. This can be done by using a roller or clean glass rod/Pasteur pipette.
Signal from chemiluminescent substrate too strong	Reduce the length of time the blot is exposed to film.
	Reduce the concentration of the substrate.

	Shorten incubation time of membrane with substrate.
	Completely remove substrate after incubation period.
	Decrease the concentration of antibodies, particularly HRP- and AP-conjugated antibodies.

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