Western Blot Protocol

Western blotting (WB) is the most widely performed immunoassay and is the best initial validation technique used to identify proteins of interest within a tissue homogenate or cell extract. In WB, proteins are first separated based on size (molecular weight) via gel electrophoresis. The resulting gel is then placed next to a membrane, made of nitrocellulose or polyvinylidene fluoride (PVDF), and the application of an electrical current induces the proteins to transfer from the gel to the membrane. The membrane can then be probed with antibodies that are specific for a target protein of interest. Lastly, the membrane is exposed to secondary antibodies and detection reagents enabling the visualization of the protein of interest.

PhosphoSolutions' ultimate guide to doing western blots is broken down into 5 sections, containing a detailed list of steps, buffers, and specific materials needed within each section. Additionally, helpful technical tips are offered throughout the protocol to provide insight about various factors that should be considered when performing western blotting. Our main goal is to help researchers obtain publishable and reproducible results.

Let's Begin…. Happy Blotting!

Sections

Sample Preparation:

Before you Begin:

- ✓ **Prepare sample:**
  Lysate: Thaw if frozen, then sonicate lysate in recommended lysis buffer to break up any residual debris and for easy pipetting without clogging.
  Organs and Cells: Refer to 'Lysate Preparation: Organs and Tissue Culture Cells' protocol
- ✓ **Heat Block:** set to 95˚C

Materials Required:

- ✓ **4X Sample Buffer:** 0.125 M TRIS, 8%(w/v) SDS, 40%(v/v) Glycerol, 20%(v/v) β-Mercaptoethanol (BME), pH 6.8 w/HCl
- ✓ **Screw cap microfuge tubes**
- ✓ **Lysis Buffer:** 1%(w/v) SDS, 10 mM TRIS, 1 mM EDTA, pH 8.0
- ✓ **Vortexer**
- ✓ **Mini-centrifuge**

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1. Prepare lysate samples in screw cap microfuge tubes with appropriate amount of 4X sample buffer.
   Tech Tips:
   a. Use fresh BME in 4X sample buffer to ensure samples are properly reduced and denatured.
   b. Mix additional 4X sample buffer with lysis buffer to adjust final volumes for protein samples and to fill empty lane(s). Adjust the volume of the molecular weight marker so each lane runs proportional down the gel.
2. Vortex samples and boil for 5 minutes at 95°C.
3. Allow samples to cool. Then vortex and spin down samples in mini-centrifuge to collect all the lysate before loading onto gel.

SDS PAGE:

Materials Required:

- ✓ **Lysate sample**
- ✓ **Lysis Buffer:** 1%(w/v) SDS, 10 mM TRIS, 1 mM EDTA, pH 8.0
- ✓ **4X Sample Buffer:** 0.125 M TRIS, 8%(w/v) SDS, 40%(v/v) Glycerol, 20%(v/v) β-Mercaptoethanol (BME), pH 6.8 w/HCl
- ✓ **1X Running Buffer:** 25 mM TRIS, 80 mM Glycine, 35 mM SDS, do not adjust pH
- ✓ **Electrophoresis Apparatus and power source**
- ✓ **Gel loading pipette tips**
- ✓ **Protein Marker**
- ✓ **Deionized water (dH_2O)**
- ✓ **4X Lower Gel Buffer:** 1.5 M TRIS, pH 8.8 w/ HCl
- ✓ **4X Upper Gel Buffer:** 0.5 M TRIS, pH 6.8 w/ HCl
- ✓ **10% APS:** 10%(w/v) Ammonium Per Sulfate dissolved in dH_2O
- ✓ **10% SDS:** 10%(w/v) Sodium Dodecyl Sulfate dissolved in dH_2O
- ✓ **30% Acrylamide/Bis Solution (37.5:1)**
- ✓ **TEMED:** N,N,N',N' - tetramethylethylene diamine

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1. Prepare appropriate percentage SDS gel based on the molecular weight of the protein of interest. (Gel recipes and recommendations are listed below).

   Tech Tips:

   a. Add TEMED and 10% APS as last ingredients of gel recipe since they are responsible for starting the polymerization process.
   b. When testing multiple antibodies using the same lysate, try using a stacking layer with one large trough instead of multiple lanes to maximize the number of strips available for testing.
2. Assemble gels in the electrophoresis apparatus and add 1X running buffer to recommended fill level.
3. Using gel loading pipette tips, load cooled samples and protein marker onto gel. For a multi-lane gel load ~5-50 µg of protein per lane. For trough gel load ~0.5-1.0 mg of protein.

   Tech Tip:

   a. When using multi-lane gels, load equal volumes of sample to each lane to prevent lateral band spreading. In any empty lanes, load a 'blank buffer' consisting of lysis buffer containing 4X sample buffer at the same volume as samples. Molecular weight ladder also must be brought up to same volume with the 'blank buffer' in multi-lane gels.
4. Run gels per electrophoresis apparatus' manufacturer's recommendations.

   Tech Tips:

   a. Allow gel to run until the dye front has passed through the gel. The run time for the gel will vary depending on the percentage of the gel. A 7.5% gel will run the fastest, a 15% the slowest. Check gel frequently.
   b. If gel front begins to exhibit a "smile", reduce voltage.

Western Blot Protocol

Lower (Separating) gel (~10 mL)				
MW of Protein of Interest	**50-250 kDa**	**30-100 kDa**	**15-30 kDa**	**5-15 kDa**
Gel Percentage	**7.5%**	**10%**	**12%**	**15%**
30% Acrylamide	2.5 mL	3.3 mL	4.0 mL	5.0 mL
4X Lower Buffer	2.5 mL	2.5 mL	2.5 mL	2.5 mL
dH_2O	4.8 mL	4.0 mL	3.3 mL	2.3 mL
10% SDS	100 uL	100 uL	100 uL	100 uL
10% APS	100 uL	100 uL	100 uL	100 uL
TEMED	6 uL	6 uL	6 uL	6 uL

Upper (Stacking) gel (~3 mL)	
Gel Percentage	**4%**
30% Acrylamide	400 uL
4X Stacking Buffer	750 uL
dH_2O	1.79 mL
10% SDS	30 uL
10% APS	30 uL
TEMED	3 uL

4X Lower Gel Buffer (1 L)	
Trizma®Base	181.71 g
Dissolve Trizma®Base completely in ~800 mL dH_2O. Adjust to pH 8.8 using concentrated HCl. QS to 1000 mL with dH_2O.	

4X Stacking Gel Buffer (1 L)	
Trizma®Base	60.56 g
Dissolve Trizma®Base completely in ~800 mL dH_2O. Adjust to pH 6.8 using concentrated HCl. QS to 1000 mL with dH_2O.	

1X Running Buffer (1 L)	
Trizma®Base	3.05 g
Glycine	6 g
SDS	1 g (or 10 uL of 10% SDS)
Dissolve all above completely in ~800 mL dH_2O. QS to 1000 mL with dH_2O.	

Protein Transfer:

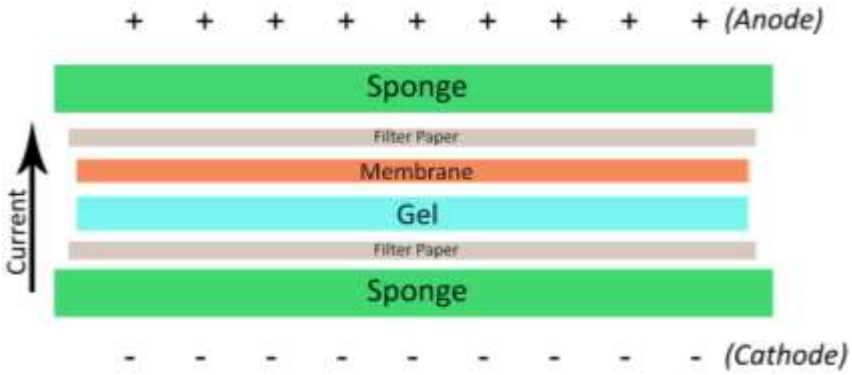
Before you begin:

- ✓ **Prepare SDS PAGE gel:** Soak gel in transfer buffer for 10 minutes to equilibrate after removing from electrophoresis apparatus.
- ✓ **Cut Whatman filter paper:** Cut 2 pieces of filter paper slightly larger than membrane to keep gel and membrane held together and to prevent any separation or slippage.

Materials Required:

- ✓ **Transfer Buffer:** 25 mM TRIS, 190 mM Glycine, 10% Methanol, do not adjust pH
- ✓ **Transfer apparatus and power source**
- ✓ **100% Methanol**
- ✓ **Membrane:** PVDF or Nitrocellulose
- ✓ **Coomassie Blue:** 0.1%(m/v) Brilliant Blue R-250, 40% Methanol, 10% Acetic Acid, 50% dH_2O
- ✓ **Gel Destain:** 10% Acetic Acid, 90% dH_2O
- ✓ **Ponceau S stain:** 0.2% Ponceau S stain, 3% Trichloroacetic Acid, 3% Sulfosalicylic Acid, 94% dH_2O
- ✓ **Plastic bag**

1. Select either polyvinylidene difluoride (PVDF) or nitrocellulose membrane as the solid support for protein transfer. If using PVDF be sure to activate the membrane by soaking it in methanol for 1 minute prior to incubating it in transfer buffer for at least 15 minutes.
2. Assemble transfer 'sandwich' system sequentially based on manufacturer's recommendation.



Tech Tip:

a. Carefully remove any air bubbles present between the gel and the membrane with a roller, as bubbles will prevent proteins from transferring from the gel to the membrane.

3. Set the voltage, amperage, and time based on manufacturers recommendations.

Tech Tip:

a. Larger molecular weight (~150-250 kDa) proteins will take a longer time to transfer than smaller molecular weight (~25-100 kDa) proteins. Adjust transfer times based on

manufacturer's recommendations and place an additional membrane in transfer to collect any protein that has potentially blown through the primary membrane.

4. After transfer, rinse membrane thoroughly in dH_2O to remove any remaining transfer buffer on the membrane and air dry the membrane until completely deactivated (solid white).

Tech Tips:

a. After transfer, rinse gel with dH_2O and incubate gel in Coomassie Blue to verify transfer efficiency. Gently rock and soak gel for 30 minutes. Discard Coomassie Blue and soak in gel destain overnight rocking at room temperature. A Kimwipe can be placed in the corner of the box to speed up the destaining process.

b. Stain your membrane with Ponceau S after transfer to confirm successful protein transfer and to determine the exact alignment of lanes and placement of the proteins on the gel. Collect excess Ponceau S for reuse and make sure to thoroughly rinse membranes in dH_2O after staining to remove any remaining excess Ponceau S on the membrane and so protein bands are visible. Ponceau S staining is reversible and will not interfere with antibody labeling of the membrane.

5. Mark molecular weight markers and lanes on the membrane using a gel pen. The markers will fade over time or wash away in any buffer. For long term storage, seal protein blot in a plastic bag and store in dark or dimly lit area.

Transfer Buffer (1 L)	
Trizma® Base	3.025 g
Glycine	14.075 g
100% Methanol	100 mL
Dissolve Trizma base and glycine completely in ~700 mL dH_2O. Add the Methanol and then QS to 1000 mL with dH_2O.	

Immunolabeling:

Before you begin:

- ✓ **Prepare membrane:** Label and cut (if needed) membrane for immunolabeling.
  PVDF membrane: activate with 100% methanol for 30 seconds, and then rinse with dH_2O.
  Nitrocellulose: DO NOT SOAK NITROCELLULOSE MEMBRANE IN METHANOL.

Materials required:

- ✓ **Wash Buffer (1X TTBS):** 14 mM NaCl, 2 mM TRIS, 0.1%(w/v) Tween 20, pH 7.6
- ✓ **Blocking Buffer:** 5%(w/v) Non-Fat Dry Milk (NFDM) or 3%(w/v) Bovine Serum Albumin (BSA) in 1X TTBS
- ✓ **Incubation Buffer:** 1%(w/v) NFDM or 1%(w/v) BSA in 1X TTBS
- ✓ **Primary Antibody**
- ✓ **Secondary Antibody:** There are a variety of detection methods used, though antibodies conjugated with HRP are the most common and will be described in this protocol.
- ✓ **100% Methanol**

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1. Cut and label desired number lanes from membrane. If using a large trough gel for a single lysate, cut and label individual strips.
2. If using PVDF, membrane must be activated by immersing it in methanol for 30 seconds.
3. Block membrane in 5% nonfat dried milk (NFDM) in 1X TTBS for 30 minutes while rocking at room temperature to saturate any free binding sites on the membrane.
   Tech Tip:
   a. If experiencing significant background noise, try using 3% BSA in 1X TTBS as the blocking buffer.
4. Using the manufacturer's recommended dilution, dilute primary antibody in 1% NFDM in 1X TTBS, making sure final volume will completely cover membrane during incubation.
   Tech Tips:
   a. Optimal primary antibody dilutions should be determined experimentally using a dilution curve.
   b. If experiencing significant background noise, try using 1% BSA in 1X TTBS to dilute primary antibody.
5. Incubate membrane in primary antibody overnight while rocking at 4°C.
6. Discard primary antibody solution and wash membrane using 1X TTBS 3 x 5 minutes at room temperature.
7. Incubate membrane in HRP conjugated secondary antibody at a dilution of 1:10,000-1:30,000 in 1% NFDM in 1X TTBS while rocking for 1 hour at room temperature.

*Make sure to select appropriate secondary antibody depending on the primary antibody's host species.

8. Discard secondary antibody solution and wash membrane using 1X TTBS 3 x 5 minutes.

Tech Tip:

a. When working with a new antibody it is recommended that both BSA and NFDM be tested to optimize signal strength and quality.

Imaging & Data Analysis:

Before you begin:

- ✓ **Prepare Imaging System:** Warm up camera in machine and open software program for imaging.

OR

- ✓ **Prepare Dark Room and Film:** Warm up dark light and layout all equipment used to easily find in minimal light

Materials Required:

- ✓ **Imaging system or film and dark room**
- ✓ **ECL detection substrate**
- ✓ **Conical tube-** for mixing ECL substrate

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1. Prepare chemiluminescent imaging system. If using film, prepare film and box in dark room.
2. Using the manufacturer's recommended instructions, prepare ECL detection substrate in 15 mL conical tube. Determine optimal volume to cover membrane completely.
3. Incubate membrane in substrate for time indicated by ECL manufacturer. (Time may vary depending on sensitivity of substrate used).
4. ECL detect with imaging system or with film.

   Tech Tip:

   a. If testing an antibody for the first time, it is recommended that multiple exposures are captured at varying lengths of time to optimize signal quality.
5. Quantitate western blot data using imaging system's software or a preferred standalone software if available.

* Multiple bands within a western blot raises a critical flag concerning an antibody's specificity. Any antibody that produces multiple banding in western blot should not be used in IHC unless additional testing can be performed to validate the specificity of the antibody.