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## INTRODUCTION

Western blotting uses antibodies to identify individual proteins within a cell or tissue lysate. Antibodies bind to highly specific sequences of amino acids, known as epitopes. Because amino acid sequences vary from protein to protein, western blotting analysis can be used to identify and quantify a single protein in a lysate that contains thousands of different proteins. First, proteins are separated from each other based on their size by SDS-PAGE gel electrophoresis. Next, the proteins are transferred from the gel to a membrane by application of an electrical current. The membrane can then be processed with primary antibodies specific for target proteins of interest. Next, secondary antibodies bound to enzymes are applied and finally a substrate that reacts with the secondary antibody-bound enzyme is added for detection of the antibody/protein complex.

## BUFFERS AND REAGENTS

### Lysis Buffer

#### **NP-40**

150 mM NaCl  
1% NP-40 or Triton X-100  
50 mM Tris pH 8.0

#### **RIPA**

150 mM NaCl  
1% NP-40 or Triton X-100  
0.5% sodium deoxycholate  
0.1% SDS  
50 mM Tris, pH 8.0  
Tris-HCl  
20 mM Tris-Hcl, pH 7.5

## Loading Buffer

### **2X Laemmli buffer**

4% SDS  
5 % 2-mercaptoethanol  
20% glycerol  
0.004% bromophenol blue  
0.125 M Tris HCl, pH 6.8

## Running Buffer

### **1X running buffer**

25 mM Tris base  
192 mM glycine  
0.1% SDS  
Adjust to pH 8.3

## Transfer Buffer

### **1X transfer buffer (wet)**

25 mM Tris base  
192 mM glycine  
20 % methanol  
Adjust to pH to 8.3

### **1X transfer buffer (semi-dry)**

48 mM Tris base  
39 mM glycine  
20 % methanol  
Adjust pH to 8.3

## Blocking Buffer

### **Blocking solution**

1X TBST  
5% non-fat dry milk OR 5% BSA

## LYSATE PREPARATION

1. Wash cell culture dish on ice with ice-cold PBS.
2. Aspirate PBS and add ice-cold lysis buffer (1 mL per confluent 10<sup>7</sup> cells/100mm dish/150 cm<sup>2</sup> flask). See the table below for lysis buffer recommendations based on the subcellular location of the protein of interest.

| Subcellular Location   | Recommended Buffer |
|------------------------|--------------------|
| Whole Cell Lysate      | NP-40              |
| Nucleus                | RIPA               |
| Mitochondria           | RIPA               |
| Cytoplasm              | Tris-HCl           |
| Membrane-bound Protein | RIPA               |

3. Using a cell scraper, scrape adherent cells off the dish and transfer the cell suspension into a microcentrifuge tube. If required, the cells can be trypsinized and washed with PBS prior to resuspension in lysis buffer.
4. Agitate cells for 30 minutes at 4 °C.
5. Centrifuge cell lysate mixture at 4 °C. The time and centrifugation force vary for each cell type, but a general guideline is 20 minutes at 12,000 rpm.
6. Transfer the supernatant (lysate) to a fresh tube on ice.

## SAMPLE PREPARATION

1. Determine the protein concentration of each cell lysate.
2. Determine how much protein to load (Recommended: 10-50 µg/lane) and add an equal volume 2X Laemmli buffer.
3. Reduce and denature the samples by boiling the lysates in sample buffer at 95-100 °C for 5 minutes. This step should be only be skipped if the antibody datasheet recommends non-reducing or non-denaturing conditions.

## SDS-PAGE

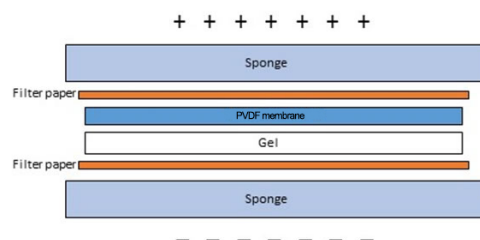
1. Prepare or purchase a pre-made gel of appropriate polyacrylamide percentage to best resolve your protein of interest based on molecular weight.

| Protein Size | Gel Percentage |
|--------------|----------------|
| 4–40 kDa     | 20%            |
| 12–45 kDa    | 15%            |
| 10–70 kDa    | 12.5%          |
| 15–100 kDa   | 10%            |
| 25–100 kDa   | 8%             |

2. Load samples containing equal amounts of protein (10-50 µg/lane protein from cell lysate or 10-100 ng/lane 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 as instructed by the manufacturer.
4. Run the gel as recommended by the manufacturer; 1-2 hours at 100 V is standard, but time and voltage may require optimization.

## PROTEIN TRANSFER

1. Prepare or purchase a pre-made gel of appropriate polyacrylamide percentage to best resolve your protein of interest based on molecular weight.
2. Soak filter papers and sponges in the transfer buffer for 10 minutes prior to assembly of the transfer “sandwich”.
3. After electrophoresis, remove the gel from the electrophoresis apparatus and equilibrate it by soaking in transfer buffer for 10 minutes.
4. Prepare the sandwich. 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 transfer according to the manufacturer's instructions of the blotting apparatus.

## IMMUNOBLOTTING

1. After transfer, rinse the membrane briefly in distilled water or 1X TBST.
2. Gently mark molecular weight 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 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.
4. Incubate membrane in blocking solution for 1 hour at room temperature or overnight at 4 °C with constant rocking.
5. Optional step: Rinse the membrane for 5 minutes in 1X TBST.
6. Dilute the primary antibody to working concentration in 1X TBST with 1% milk or BSA (use whatever was chosen for blocking).
7. Optional: To visualize the molecular weight markers in addition to the protein of interest, add 1 µg/mL Blue Marker Antibody to the primary antibody solution.
8. Incubate the membrane in primary antibody solution for 1 hour at room temperature or overnight at 4 °C with gentle rocking. This time may require optimization. In most cases, overnight incubation at 4 °C increases signal strength and reduces background signal relative to 1 hour incubation at room temperature.
9. Wash the membrane with 1X TBST three times for 10 minutes each with gentle rocking.

**Tip:** Increase the number of washes to 5-6 if high background occurs.

10. Incubate the membrane in the appropriate diluted secondary antibody (in 1X TBST and may include 1% milk or BSA) for 1 hour at room temperature with gentle rocking. HRP conjugated secondary antibodies are most common for western blot analysis.
11. Wash the membrane in 1X TBST three times for 10 minutes each with gentle rocking.

**Tip:** Do not let the membrane dry at any point during the blotting process.

### 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.

**Tip:** More sensitive substrates may require shorter incubation times or even dilution to achieve optimal signal and avoid overexposure.

3. Carefully remove the membrane from the detection reagent and sandwich it between layers of plastic (i.e. 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.

**Tip:** Clip the top right corner of your film as a guide for film orientation in a dark room.

**Tip:** Use multiple exposure lengths to identify the most optimal exposure time. An initial 10 second exposure will indicate the necessary exposure time.

5. To mark the molecular weight in cases where the Blue Marker Antibody is not used, lineup the developed film over the blot to visualize the ladder.

### NOTE

Following target protein detection, a second antibody can be used to reprobe the same blot for a second protein. This second antibody is often specific for a loading control protein. To strip and reprobe your blot, please read our protocol on western blot stripping and reprobing. In addition, our Loading Control Guide can be used to assist you when choosing the optimal loading control to standardize expression of your target protein.