

## Western Blotting Protocol

### Sample preparation:

1. Place cells in a microcentrifuge tube and centrifuge to collect cell pellet.
2. Lyse the cell pellet with 100  $\mu$ l lysis buffer on ice for 30 min (For  $1 \times 10^6$  cells, lyse with 100  $\mu$ l lysis buffer).
3. Centrifuge at 14,000 rpm (16,000  $\times$  g) for 10 min at 4°C.
4. Transfer the supernatant to a new tube and discard the pellet. Remove 20  $\mu$ l supernatant and mix with 20  $\mu$ l of 2x sample buffer.
5. Boil for 5 min. Cool at RT for 5 min. Microcentrifuge for 5 min.
6. Load up 40  $\mu$ l of sample to each well of a 1.5 mm thick gel\*.
7. Set gel running conditions according to the manufacturer's instructions. Transfer the proteins to a nitrocellulose or PVDF membrane with variable power settings according to the manufacturer's instructions.

\*Guidelines for choosing gel percentages are based on protein size to be detected: 4-5% gel, >200 kD; 7.5% gel, 120-200 kD; 8-10% gel, 40-120 kD; 13% gel, 15-40 kD; 15% gel, < 20 kD.

### Membrane Blocking:

1. Remove the blotted membrane from the transfer apparatus and immediately place in blocking buffer consisting of 5% nonfat dry milk/TBS-T\*\*.
2. Incubate the blot for 1 hr at room temperature, or overnight at 4°C with agitation.

### Antibody Incubation:

1. Dilute the primary antibody to the recommended concentration/dilution in 5% nonfat dry milk/TBS-T\*\* (usually at 1  $\mu$ g/ml). Place the membrane in the primary antibody solution and incubate 2 hrs at room temperature, or overnight at 4°C with agitation.
2. Wash three times for 5 min each with Wash buffer (TBS containing 0.1% Tween-20).
3. Incubate the membrane for 30 min at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody, diluted to 1:1000 in 5% nonfat dry milk/ TBS-T\*\*.
4. Wash 4 times for 10 min each with TBS containing 0.1% Tween-20, and once for 2 min with PBS.

### Protein Detection:

1. Incubate membrane (protein side up) with 10 ml ECL (enhanced chemiluminescence substrate) for 1-2 min. The final volume required is 0.125 ml/cm<sup>2</sup>.

2. Drain off excess detection reagent, wrap up the blots and gently smooth out any air bubbles.
3. Place the wrapped blots, protein side up, in an X-ray film cassette and expose to x-ray film. Exposures can vary from 5 seconds to 60 min.

### **Tips:**

#### **High background**

1. Transfer buffers may have become contaminated. Contamination can be transferred to the blots from electrophoresis and related equipment used in blot preparation.
2. Post-antibody washes may not have been performed for a sufficient period of time or were not performed in a high enough volume.
3. The blocking and incubation agents used were not freshly prepared or were too dilute.

#### **No signal or poor signal**

1. Transfer efficiency may have been poor. Check protein transfer by staining the gel and/or membrane.
2. Incorrect storage of antibodies or ECL western blotting detection reagents may result in a loss of signal.
3. Insufficient protein may have been loaded on the gel. Depending on the location of the target protein, membrane or nuclear preparations may be required (instead of whole cell lysates).
4. Film exposure time may have been too short.

### **Solutions and Reagents:**

#### **1X Cell Lysis Buffer:**

20 mM Tris-HCl, pH 7.5

150 mM NaCl

1% NP-40

2 mM EDTA

1 µg/ml leupeptin

1 µg/ml aprotinin

1 mM Na<sub>3</sub>PO<sub>4</sub>

1 mM PMSF

5 mM NaF

3 mM Na<sub>4</sub>P<sub>2</sub>O<sub>4</sub>

#### **5X SDS sample Buffer:**

312.5 mM Tris-HCl (pH 6.8)

10% SDS (w/v)  
250 mM DTT  
50% Glycerol  
0.05% Bromophenol Blue (w/v)  
Use at 1X

**10X SDS Running Buffer**

Dissolve 144 g of Glycine, 30 g of Tris base and 10 g SDS in 800 ml of distilled H<sub>2</sub>O.

Add distilled H<sub>2</sub>O to 1 liter

Use at 1X

**Transfer Buffer:**

2.25 g Tris base  
10.5 g Glycine  
1 g SDS  
200 ml Methanol  
Add distilled water to 1.0 L

**10X TBS-T (Tris-buffered saline containing Tween-20):**

Dissolve 80 g of NaCl, 2 g of KCl, 30 g of Tris base and 10 ml Tween-20 in 800 ml of distilled H<sub>2</sub>O.

Adjust the pH to 7.4 with HCl. Add distilled H<sub>2</sub>O to 1 liter.

Use at 1X (containing 0.1% Tween-20).

**Blocking Buffer:**

1X TBS-T with 5% nonfat dry milk

**Wash Buffer:**

1X TBS-T

**Primary and Secondary Antibody Dilution Buffer:**

1X TBS-T with 5% nonfat dry milk

\*\*If phosphorylation-specific antibodies are used, the membrane blocking buffer and antibody dilution buffer should not contain milk.

**Alternate Blocking Buffer:**

1X TBS-T with 4% Bovine Serum Albumin (BSA)

**Alternate Primary and Secondary Antibody Dilution Buffer:**

1X TBS-T with 4% Bovine Serum Albumin (BSA)

**Blotting Membrane:**

Nitrocellulose or PVDF membrane