Western blotting Protocol

Solutions and Reagents

Note: Prepare solutions with Milli-Q or equivalently purified water.

Transfer Buffer: 25 mM Tris base, 192 mM glycine, 20 % methanol (pH 8.5)

SDS Sample Buffer (1X): 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2 % w/v SDS, 10 % glycerol, 50 mM DTT, 0.01 % w/v bromophenol blue or phenol red

Blocking Buffer: 1X TBS, 0.1 % Tween-20 with 5 % w/v nonfat dry milk; for 150 ml, add 15 ml 10X TBS to 135 ml water, mix. Add 7.5 g nonfat dry milk and mix well. While stirring, add 0.15 ml Tween-20 (100 %).

10X TBS (Tris-buffered saline): To prepare 1 liter of 10X TBS: 24.2 g Tris base, 80 g NaCl; adjust pH to 7.6 with HCl (use at 1X).

Primary Antibody Dilution Buffer: 1X TBS, 0.1 % Tween-20 with 5 % blocking agent; for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g BSA and mix well. While stirring, add 20 µl Tween-20 (100 %).

Chemiluminescent HRP Detection: secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP), ECL™ chemiluminescent reagent (GE Healthcare)

Wash Buffer TBS/T: 1X TBS, 0.1 % Tween-20

Blotting Membrane: This protocol has been optimized for PVDF membranes, which we recommend. However nitrocellulose membranes may also be used.

Protein Blotting

1. Treat cells by adding fresh media containing regulator for desired time

2. Aspirate media from cultures; wash cells with 1X PBS; aspirate.

3. Lyse cells by adding 1X SDS Sample Buffer (100 µl per well of 6-well plate or 500 µl per plate of 10 cm^2 plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.

4. Sonicate for 10–15 seconds to shear DNA and reduce sample viscosity.

5. Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.

6. Microcentrifuge for 5 minutes.

7. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm).

8. Electrotransfer to PVDF membrane.

Blocking and Antibody Incubations

Note: Volumes are for 10 cm x 10 cm (100 cm2) of membrane; for different sized membranes, adjust volumes accordingly.

1. After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.

2. Incubate membrane in 20 ml of blocking buffer for 1 hour at room temperature or overnight at 4°C.

3. Wash three times for 5 minutes each with 15 ml of TBS/T.

4. Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation 1-2 hours at room temperature.

5. Wash three times for 5 minutes each with 15 ml of TBS/T.

6. Incubate membrane with HRP-conjugated secondary antibody (at the appropriate dilution; usually 1: 3,000-6,000) in 10 ml of blocking buffer with gentle agitation for 1 hour at room temperature.

7. Wash three times for 5 minutes each with 15 ml of TBS/T.

Detection of Proteins

1. Incubate membrane with 4 ml ECL™ with gentle agitation for 1 minute at room temperature.

2. Drain membrane of excess developing solution, do not let dry, wrap in plastic wrap and expose to x-ray film. An initial ten seconds exposure should indicate the proper exposure time.