



## Immunoprecipitation-Western Blotting Protocol

### Solutions and Reagents

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

**Cell Lysis Buffer (1X):** 20 mM Hepes (pH 8.0), 250 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.2 % NP-40, 0.5 mM DTT, 2.5 mM sodium pyrophosphate, 1 mM Glycerolphosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 µg/ml Leupeptin, 1 µg/ml Pepstatin, 0.3 µM Aprotinin, 15 µM E-64

**Protein A Sepharose:** Wash Protein A Sepharose twice with Cell Lysis Buffer. Resuspend the resin in 1 volume of Cell Lysis Buffer (50 % bead slurry). This can be stored for 2 weeks at 4°C.

**3X SDS Sample Buffer:** 187.5 mM Tris-HCl (pH 6.8 at 25°C), 6 % w/v SDS, 30 % glycerol, 150 mM DTT, 0.03 % w/v bromophenol blue,

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

**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):** For 1 liter of 10X TBS, use 24.2 g Tris base and 80 g NaCl. Adjust pH to 7.6 with HCl (use at 1X).

**Primary Antibody Dilution Buffer:** 1X TBS, 0.05 % Tween-20 with 5 % nonfat dry milk. For 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g nonfat dry milk and mix well. While stirring, add 10 µl Tween-20 (100 %).

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

**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 PVD membranes, which we recommend. However nitrocellulose membranes may also be used.

### **Preparing Cell Lysates**

1. Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
2. To harvest cells under non-denaturing conditions, remove media and rinse cells once with ice-cold PBS.
3. Remove PBS and add 0.5 ml 1X ice-cold Cell Lysis Buffer plus 1 mM PMSF to each plate (10 cm<sup>2</sup>) and incubate the plate on ice for 5 minutes.
4. Scrape cells off the plate and transfer to microcentrifuge tubes. Keep on ice.
5. Sonicate 4 times for 5 seconds each on ice.
6. Microcentrifuge for 10 minutes at 4°C, and transfer the supernatant to a new tube. The supernatant is the cell lysate. If necessary, lysate can be stored at -80°C.

### **Immunoprecipitation**

1. Take 200 µl cell lysate and add primary antibody; incubate with gentle rocking overnight at 4°C.
2. Add Protein A Sepharose (20 µL of 50 % bead slurry). Incubate with gentle rocking for 1–3 hours at 4°C.
3. Microcentrifuge for 30 seconds at 4°C. Wash pellet 2 times with 500 µL of 1X Cell Lysis Buffer. Keep on ice during washes.
4. Resuspend the pellet with 20 µL 3X SDS Sample Buffer. Vortex then, microcentrifuge for 30 seconds.

5. Heat the sample to 95–100°C for 2–5 minutes.
6. Load the sample (15–30  $\mu$ L) on SDS-PAGE gel (12–15 %).
7. Analyze sample by Western blotting.

### **Protein Blotting**

1. Electrotransfer to PVDF membrane.

### **Membrane Blocking and Antibody Incubations**

*Note: Volumes are for 10 cm x 10 cm (100 cm<sup>2</sup>) 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.