



Cell Lysis and Western Immunoblotting Protocol

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1. Solutions and Reagents

- **10X Phosphate Buffered Saline (PBS):**
To prepare 1 L of 10X PBS buffer: 80g NaCl, 2g KCl, 14.4 g Na₂HPO₄, 2.4g KH₂PO₄, adjust pH to 7.4.
- **1X Cell Lysis Buffer with protease/phosphatase inhibitors:**
20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na₃VO₄, 10mM NaF, 1mM PMSF, 1µg/ml leupeptin, and 0.3µM aprotinin, 130µM bestatin, 14µM E-64.
- **2X SDS Sample Buffer:**
125mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 100mM DTT, 0.02% Bromophenol Blue
- **Transfer Buffer:**
25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
- **10X Tris Buffered Saline (TBS):**
To prepare 1 L of 10X TBS: 24.2 g Tris base, 80 g NaCl; adjust pH to 7.6 with HCl (use at 1X)
- **Blocking Buffer:**
1X TBS, 0.1% Tween-20 with 5% w/v BSA; for 150 ml, add 15 ml 10X TBS to 135 ml water, mix. Add 7.5 g BSA and mix well. While stirring, add 0.15 ml Tween-20 (100%)
- **Wash Buffer/TBST:**
1X TBS, 0.1% Tween-20(100%)
- **HRP-conjugate secondary antibody**
- **Chemiluminescence reagents and film for detection**

2. Cell Lysis Protocol

Westerns are performed using cell lysates from harvested cells.

- a) Treat cells by specific regulator for desired time, wash cells with 1X PBS.
- b) Detach adherent cells using a cell scraper or centrifuge suspended cells, and resuspend in 1X Cell Lysis Buffer with inhibitors.
- c) Remove a small volume (50 µl). To perform a protein assay, determine the protein concentration for each cell lysate.
- d) To the remaining volume of cell lysate, add an equal volume of 2X SDS Sample Buffer.
- e) Boil each cell lysate at 100 °C for 5 min.
- f) Centrifuge at 14,000 rpm in a microcentrifuge for 5 min.
- g) Load equal amounts (10–20 µg) cell lysate onto SDS-PAGE gels using loading tips, along with molecular weight markers.
- h) Run the gel and transfer to nitrocellulose for western immunoblotting.

3. Western Immunoblotting Protocol

Membrane Blocking

1. Block membrane by incubating 1 hour at room temperature or overnight at 4°C with shaking in Blocking

Solution (5% BSA in TBST (50mM Tris, 100mM NaCl, 0.1% Tween-20, pH 7.4)).

Incubation with Primary Antibody

2. Dilute primary antibody at the appropriate dilution in Blocking Solution.
3. Incubate the membrane with diluted primary antibody for 1 hour at 37°C, or 2 hours at room temperature, or overnight at 4°C with agitation.
4. Remove antibody solution. Wash the membrane 3 times for 5-10 minutes each time at room temperature in TBST with shaking.

Incubation with Secondary Antibody

5. Incubate membrane with diluted HRP-conjugate secondary antibody (according to manufacturer's instructions) in Blocking Solution for 1 hour at room temperature with shaking.
6. Repeat Step 4.
7. Wash membrane with TBST for 2-5 minutes before proceeding Chemiluminescent Reaction.

Chemiluminescent Reaction

8. Prepare and use the Chemiluminescent substrate according to the manufacturer's instructions.
9. Immediately wrap the membrane and expose to X-ray films for 10 second to 1 hour period. The exposure time may vary according to the amount of antibody and antigen.

4. Peptide Competition Protocol

Before proceeding Western Immunoblotting, add specific Blocking Peptide (Refer to [SAB inc.](#) peptide catalog#) to the diluted primary antibody in a molar ratio of 10:1 (peptide to antibody) and incubate the mixture at 4°C for overnight or at room temperature for 2 hours.