Western Immunoblotting Protocol

Blocking

1. Block membrane by incubating 1 hour at room temperature or overnight at 4℃ with shaking in Blocking Solution (5% BSA or nonfat milk in TBST (50mM Tris, 100mM NaCl, 0.05% Tween-20, pH 7.6)). Note: Use 5% BSA in Blocking Solution for phosphor specific antibodies.

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 37^0C, or 2 hours at room temperature, or overnight at 4^0C with agitation.
4. Remove antibody solution. Wash the membrane 3 times for 5-10 minutes each time at room temperature in TBST (50mM Tris, 100mM NaCl, 0.05% Tween-20, pH 7.6) with shaking. Note: Increase the concentration of Tween-20 to 0.1% reduces the background and increases the specificity, but it will reduce the sensitivity.

Incubation with Second Antibody

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

Chemiluminescent Reaction

8. Prepare and use the Chemiluminescent substrate (for AP or HRP) 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 mount of antibody and antigen.

Peptide Competition

Before proceeding Western Immunoblotting, add Blocking Peptide to the diluted primary antibody in a molar ratio of 10:1 (peptide to antibody) and incubate the mixture at 4^0C for overnight or at room temperature for 2 hours.