ROCKLAND

Western Blotting (WB) Protocol

I. Reagents Required

Reagent	Preparation
UltraPure Sterile Water (MB-009-1000)	N/A
1X RIPA Lysis Buffer (MB-030-0050)	Add 1X protease and phosphatase inhibitors just before use. Keep on ice.
2X SDS-PAGE Sample Buffer without DTT or β-ME (MB-018)	N/A
10X PBS pH 7.2 (MB-008)	Make 1X solution by diluting with UltraPure Sterile Water. Keep on ice.
10X SDS-PAGE Running Gel Buffer (MB-017) orTris-Glycine (MB-029-1000)	Make 1X solution by diluting with UltraPure Sterile Water.
Opal Prestained Protein Standard 10–245kDa (MB-210-0500) or 10-180kDa (MB-209-0500)	N/A
BlockOut® Universal Blocking Buffer for Westernblot (MB-073)	N/A
Blotto A Pre-Mixed (B552-0500)	N/A
Blotto B Pre-Mixed (B553-0500)	N/A
Bovine Serum Albumin Fraction V (BSA-50)	Make BSA Blocking Buffer by diluting 5% (w/v) BSA in 1X TTBS.
10X TTBS pH 7.5 (MB-013)	Make 1X solution by diluting with UltraPure Sterile Water.
1X TBS pH 7.8 (MB-069-1000)	N/A
Anti-Rabbit IgG (H&L) GOAT Antibody Peroxidase Conjugated (611-103-122)	Use at 1:50,000
Anti-Mouse IgG (H&L) RABBIT Antibody Peroxidase Conjugated (610-1319-0500)	Use at 1:40,000
Anti-Goat IgG (H&L) DONKEY Antibody Peroxidase Conjugated (605-703-125)	Use at 1:40,000
Chemiluminescent FemtoMax™ (FEMTOMAX-110)	Super Sensitive HRP Substrate for Microwell/Membrane.Mix the two components in a 1:1 ratio just before use.

II. Procedure for Cell Lysis

1. Grow cells to optimal confluency in appropriate growth medium.
2. Remove growth medium, gently rinse cells with ice-cold 1X PBS. Discard PBS.
3. Add 0.5 mL of ice-cold 1X RIPA Lysis Buffer or 2X SDS-PAGE Sample Buffer per 1 x 10^7 cells (approximately 0.5 mL for a sub-confluent 100 mm plate or 75 cm^2 flask, 0.7 mL for a 150 cm^2 flask). Incubate 5 minutes on ice. Alternatively, non-stimulated cells can be trypsinized and washed with ice-cold PBS before lysis.
4. Dislodge cells using a cell scraper and transfer to a tube. Keep on ice.
5. Disrupt cells and shear DNA by sonication using two, 7-second, 50W pulses with a 20-second interval per ~0.5-mL sample.
6. Clarify by high-speed centrifugation for 10–15 minutes at ~15,000 × g, 4°C.
7. Transfer the resulting supernatants (whole cell lysates), which contain the protein to be examined, to fresh, pre-cooled microcentrifuge tubes.
8. If 1X RIPA Lysis Buffer was used, take a 10 µL aliquot of each extract and determine protein concentration. The protein concentration of each sample should be determined so that the amounts of proteins from the different samples can be compared. When resuspended in 2X SDS-PAGE Sample Buffer, determining protein concentration is difficult due to the presence of interfering compounds with most colorimetric protein assays.
9. Snap-freeze the supernatant in labeled, chilled tubes; store at −70°C until needed for electrophoresis, transfer, and detection steps. Extracts prepared in this manner may be stored for months at −70°C without appreciable degradation of target when avoiding repeating freeze/thaw cycles.

III. Procedure for SDS-PAGE Sample Preparation & Separation

1. Determine the best gel to use according to the molecular weight (MW) of the protein of interest:
    a) Use 4-8% gels to separate proteins 100 to 500 kDa in size.
    b) Use 4-20% gels to separate proteins 10 to 200 kDa in size.
2. If using a lysate already in Sample Buffer, thaw lysate and transfer 25 µL of lysate to a clean pre-labeled microcentrifuge tube. Add reducing agent (β-ME, DTT or TCEP) as needed and mix well by pipetting.
3. Transfer any other protein samples to clean pre-labeled microcentrifuge tubes and bring to optimal concentration with UltraPure Sterile Water. Add an equal volume of 2X Sample Buffer and reducing agent as needed.
4. Place all samples into a heating block (set to 95°C) or water bath. Heat samples for 5 minutes.
5. Clarify by high-speed centrifugation for 3 minutes to pellet any debris.
6. Prepare gel, electrophoresis equipment and fill with 1X SDS-PAGE Running Gel Buffer as required.
7. Start by loading 5 µL of the Protein Standards and continue loading 10–35 µg of lysate, or 50–100 ng of purified protein, per lane. Use gel-loading tips to help to avoid cross-contamination between lanes. Use a clean tip for each sample.
8. Allow the gel to electrophorese for 45–90 minutes (120–150V). Stop the run immediately after the dye front migrates out from the bottom of the gel.

IV. Procedure for Protein Transfer & Immunoblotting

9. Transfer proteins onto 0.2 µm or 0.45 µm nitrocellulose or PVDF membrane as recommended for the specific product. Use forceps and wear gloves.
10. Block membrane using recommended Blocking Buffer for a minimum of 30 minutes at room temperature, with gentle mixing using an orbital shaker.
11. Rinse nitrocellulose membrane with UltraPure Sterile Water and incubate overnight at 4°C with gentle mixing in 5–10 mL Blocking Buffer containing primary antibody at the recommended dilutions:
    a) Antiserum: 1:500
    b) IgG Fraction, Protein A/G and affinity-purified antibodies: 0.5–1 µg/mL
12. Wash the blot 2 times for 5 minutes each with 5–10 mL of TTBS and then rinse with 1X TBS.
13. Incubate blot 30 minutes at room temperature with gentle mixing in 5–10 mL of blocking buffer containing HRP-conjugated secondary antibody at the recommended dilutions.
14. Wash the blot 2 times for 5 minutes each with 5–10 mL of TTBS. Rinse with 1X TBS.
15. Add 2–5 mL of chemiluminescent HRP substrate and proceed to protein detection with the system of preference.