
Cell Lysis and Western Immunoblotting Protocol

1. Solutions and Reagents

1.1.1 10X Cell Lysis Buffer (Cat #: 3500-1)

200 mM Tris-HCl (pH 7.5)
1.5M NaCl
10 mM Na₂EDTA
10 mM EGTA
10% Triton
25 mM sodium pyrophosphate
10 mM β-glycerophosphate
10 mM Na₃VO₄ (activated)
10 µg/ml leupeptin

1.1.2 Add Phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1mM.
(Stock solution 100mM PMSF)

NOTE: Add fresh before each use

1.1.3 Add protease and phosphatase inhibitors.

1X protease inhibitor mixture consists of 2 mM AEBSF, 1 mM EDTA, 130 µM bestatin, 14 µM E-64, and 0.3 µM aprotinin,

1.2. 2X Laemmli Sample Buffer: 62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, 710 mM beta-mercaptoethanol

1.3. TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20) – TBST is Tris Base Saline buffer with 0.1% Tween-20

1.4. 5% NFDM: Use 25 g non-fat dried milk in 500 ml TBST

1.5. 5% bovine serum albumin (BSA): Use 25 g in 500 ml TBST

1.6. Goat anti-rabbit HRP secondary antibody (*Epitomics Cat#3050-1 recommended*)

1.7. Chemiluminescence reagents; such as ECL materials and film for detection

2. Cell Lysis and Western Immunoblotting Protocol

Westerns are performed using cell lysates from harvested cells.

2.1. Adherent cells

2.1.1 Grow cells to ~90% confluency.

2.1.2 Wash cells twice with TBS to remove media

2.1.3 Add the appropriate volume of 1x Cell lysis buffer (ex: 3ml to a T175 flask)

2.1.4 Transfer cell lysates to Eppendorf tubes and sonicate for 10–15 seconds.

2.1.5 Spin at 14,000 rpm, 4 °C for 10 minutes.

2.2. Suspension cells

2.2.1 Pipette cells gently into a conical tube and centrifuge 10 min. at 1000 rpm

2.2.2 Wash cells twice with TBS

2.2.3 Add the appropriate volume of 1x Cell lysis buffer and transfer to Eppendorf tubes (ex: 1ml for 1×10^7 cells)

2.2.4 Sonicate for 10–15 seconds.

2.2.5 Spin at 14,000 rpm, 4 °C for 10 minutes.

2.3. Remove a small volume (50 μ l) of supernatant. To perform a protein assay, determine the protein concentration for each cell lysate.

2.4. To the remaining volume of cell lysate, add an equal volume of 2X Laemmli Sample Buffer (for a final concentration of 1 mg/ml).

2.5. Boil each cell lysate in sample buffer at 100 °C for 5 min and aliquot. Use a 26-gauge needle to shear released chromatin. Store lysates at -20 °C. Note: Aliquot cell lysates (50–100 μ l) in order to avoid repeat freeze/thaw cycles.

2.6. Defrost tubes containing cell lysate at 37 °C. Centrifuge at 14,000 rpm in a microcentrifuge for 5 min.

2.7. Load equal amounts (10–20 μ g) cell lysate onto SDS-PAGE gels using gel loading tips, along with molecular weight markers.

2.8. Run gels and transfer for western immunoblotting.

3. Antibody Characterization through Western Immunoblotting

3.1. Block nitrocellulose for 1 hour at room temperature or overnight at 4 °C using 5% NFDm.

3.2. Incubate nitrocellulose with appropriate dilutions of primary antibody in 5% BSA overnight at 4 °C or for 2 hours at room temperature

3.3. Wash nitrocellulose with three 5-min washes using TBST.

3.4. Incubate nitrocellulose with 1:1000 (1:500 if stronger signal is desired) dilutions of goat anti-rabbit HRP labeled secondary antibody (***Epitomics cat#3050-1***) in 5% NFDm in TBST overnight at 4°C or room temperature for 1 hour. (Overnight at 4°C is recommended for best results).

3.5. Wash nitrocellulose in 3 washes of TBST, then rinse in TBS prior to addition of chemiluminescence reagents.

3.6. Remove excess chemiluminescence reagent and sandwich nitrocellulose blot in any type of transparent plastic wrap (saran wrap, transparent sheet protector, etc.).

3.7. Acquire image using darkroom development techniques.