Western Blot

I. Preparation of Solutions and Reagents

1. 1× PBS Buffer: Prepare by dissolving PBS powder in double-distilled water (ddH_2O).
2. 1× TBST Buffer: First, dissolve TBS powder in ddH_2O to prepare TBS buffer. Then add Tween-20 at a ratio of 1 mL Tween-20 per 1 L TBS buffer. Stir until completely dissolved.
3. 5× Electrophoresis Buffer: Weigh 15.1 g Tris, 94 g glycine, and 5 g SDS. Add ddH_2O and stir until all solutes are fully dissolved. Adjust the final volume to 1 L with ddH_2O and mix well. Store at room temperature. Dilute to 1× with ddH_2O before use.
4. 1× Transfer Buffer: Weigh 3 g Tris and 14.4 g glycine, add to 800 mL ddH_2O, and shake until dissolved. Add 200 mL methanol and mix thoroughly. Store at 4 °C.
5. Blocking Buffer (5% BSA): Weigh 0.5 g BSA powder and dissolve it in 10 mL PBST. Vortex thoroughly to mix. Prepare freshly before use.
6. 5× SDS Sample Loading Buffer: Dissolve 0.606 g Tris in 5 mL ddH_2O, then adjust the pH to 6.8 using 1 M HCl. Add 1 g SDS and stir until completely dissolved. Add 5 mL glycerol and 0.002 g bromophenol blue, mix thoroughly, and adjust the volume to 10 mL with ddH_2O. Aliquot into 1 mL per tube and store at –20 °C. Before use, add 100 µL β-mercaptoethanol to each tube.

II. Experimental Procedure

A. Protein Sample Preparation

1. Protein Extraction from Adherent Cells:

(1) Remove the old culture medium. Wash the cells with PBS, then aspirate the waste liquid. Repeat the wash once.

(2) Add 50 µL of cell lysis buffer (containing protease inhibitors) and 50 µL of 1× SDS loading buffer to each well. Let stand for 1–2 minutes.

(3) Use a cell scraper to detach the adherent cells, and collect the cell suspension into a 1.5 mL microcentrifuge tube (keep on ice).

(4) Lyse the cells on ice for 20 minutes, vortexing every 5 minutes for 30 seconds each time.

(5) Heat the tubes in a metal bath at 100 °C for 10 minutes.

(6) Allow the tubes to cool, then centrifuge at 13,000 rpm for 12 minutes at 4 °C. Collect the supernatant and store at –20 °C.

2. Protein Extraction from Suspension Cells:

(1) Transfer the cell suspension to a 1.5 mL microcentrifuge (EP) tube. Centrifuge at 4000 rpm for 3 minutes. Discard the supernatant.

(2) Add 1 mL PBS to wash the cells. Centrifuge at 4000 rpm, 4 °C for 3 minutes, and discard the supernatant. Repeat the wash once more.

(3) Add 100 µL of cell lysis buffer (containing protease inhibitors). Lyse the cells on ice for 30 minutes, vortexing briefly every 5 minutes.

(4) Disrupt the cells using an ultrasonic cell disruptor. Keep the probe below the liquid surface; press and hold the button for 3 seconds per pulse, repeating 15–20 times per tube.

(5) Centrifuge at 13,000 rpm, 4 °C for 13 minutes. Collect the supernatant and add one-fourth volume of 5× SDS loading buffer. Mix thoroughly by pipetting.

(6) Place the EP tube in a metal heating block and boil at 100 °C for 10 minutes. Allow the tube to cool, then transfer to –20 °C for storage.

B. SDS-PAGE Gel Preparation

1. The lower layer of the gel is the separating gel, and the upper layer is the stacking gel. First, prepare the separating gel solution and pour it into the gel cassette. Add an equal volume of anhydrous ethanol or ddH_2O on top to level the surface of the separating gel solution. Let it stand for 30–40 minutes to polymerize.
2. After the separating gel has solidified, pour off the anhydrous ethanol or ddH_2O on top, add the stacking gel solution, and insert the comb. Let it stand for 15–20 minutes until the stacking gel solidifies, completing the gel preparation. If the prepared gel is not used immediately, it can be stored submerged in ddH_2O at 4 °C for 1–2 weeks.

Note: Before preparing the gel, select an appropriate resolving gel concentration based on the molecular weight of the target protein. The stacking gel is typically prepared at a concentration of 5%. The separation range corresponding to each resolving gel concentration, as well as the gel formulations, are as follows:

Separating Gel Concentration	Protein Molecular Weight Range (kDa)
6%	50-300
8%	30-200
10%	20-100
12%	12-60
15%	5-40

F1. The appropriate molecular weight range of proteins for separation using different concentrations of separating (resolving) gels.

Composition	6%	8%	10%	12%	15%
30% Acr-Bis (mL)	2.0	2.7	3.3	4.0	5.0
1.5 M Tris-HCl (pH 8.8) (mL)	2.5	2.5	2.5	2.5	2.5
10% SDS (μL)	100	100	100	100	100
10% APS (μL)	100	100	100	100	100
TEMED (μL)	10	10	10	10	6
ddH2O (mL)	5.3	4.6	4.0	3.3	2.3

F2. Formulas for Separating Gels of Different Concentrations (10 mL Total Volume)

Composition	Volume
30% Acr-Bis (mL)	0.67
1.0 M Tris-HCl (pH 6.8) (mL)	0.5
10% SDS (μL)	50
10% APS (μL)	50
TEMED (μL)	5
ddH2O (mL)	3.73

F3. 5% Stacking Gel Recipe (4 mL Total Volume)

C. Electrophoresis

Load 10–40 μg of protein sample per well. Run at a constant voltage of 80 V for 30 minutes. When the bromophenol blue dye front enters the separating gel, increase the voltage to 120 V and continue running for approximately 1 hour.

D. Transfer

Soak the PVDF membrane in methanol for about 5 minutes, then transfer it to the transfer buffer to equilibrate. Assemble the transfer "sandwich" with the black side of the transfer cassette facing down, placing the components in this order: sponge, filter paper, gel, membrane, filter paper, sponge. Place the assembled cassette into the transfer tank, add an ice pack, and fill the tank with transfer buffer until the cassette is just submerged. Place the tank on ice. Run the transfer at a constant current of 200 mA for about 80 minutes.

E. Blocking

Place the membrane with the gel-contacting side facing up and immerse it in blocking buffer. Incubate on a shaker at room temperature for 1 hour. Wash the membrane 3 times with TBST buffer, 5 minutes each time.

F. Primary Antibody Incubation

Prepare the primary antibody dilution solution (refer to the product website for recommended antibody dilution). Place the membrane in the primary antibody dilution and incubate on a shaker overnight at 4 °C, or for 2.5 hours at room temperature. Wash the membrane 3 times with TBST buffer, 5 minutes each time.

G. Secondary Antibody Incubation

Select an HRP-conjugated secondary antibody that recognizes the host species of the primary antibody. Prepare the secondary antibody dilution at the appropriate concentration (refer to the product website for recommended dilution). Incubate the membrane in the secondary antibody dilution on a shaker at room temperature for 1 hour. Wash the membrane 3 times with TBST buffer, 5 minutes each time.

H. Detection

Prepare the ECL chemiluminescent detection working solution. Add the working solution dropwise onto the membrane surface and detect the protein bands using a chemiluminescence imager.

III. Experimental Notes

1. Transfer conditions should be adjusted according to the molecular weight of the target protein. A constant current of 200 mA for 80 minutes is suitable for proteins with a molecular weight between 30–150 kDa. For high molecular weight proteins, increase the current or extend the transfer time; for low molecular weight proteins, reduce the current or shorten the transfer time.
2. When the target protein is too large or too small, commonly used protein molecular weight markers and internal controls (such as β-actin or GAPDH) may not transfer well under standard conditions.
3. It is recommended to select appropriate markers and internal control proteins in advance (e.g., Histone H3 or α-Tubulin).

Choose PVDF membranes with appropriate pore sizes based on the molecular weight of the target protein:

For proteins < 20 kDa: a 0.1 μm membrane

For proteins 20–50 kDa: a 0.22 μm membrane

For proteins > 50 kDa: a 0.45 μm membrane

IV. Recommended Experimental Products

A. Cell Lysis Buffer

RIPA Lysis Buffer (Strong), C0045

RIPA Lysis Buffer (Medium), C0046

RIPA Lysis Buffer (Weak), C0047

B. Protease Inhibitor

Protease Inhibitor Cocktail (EDTA-Free, 100× in DMSO), C0001

Protease Inhibitor Cocktail, MS-safe (10×), C0055

Protease and Phosphatase Inhibitor Cocktail, MS-safe (10×), C0057

C. Antibody: https://www.targetmol.com/all-antibodies

D. BCA Protein Quantification Kit, C0050

E. Tween 20, T9526

F. Bovine Serum Albumin (BSA), T5664