Western blot Protocol

Experimental reagents and consumables

Sample preparation reagents

1. RIPA Lysis buffer
2. Protease inhibitors
3. BCA working solution
4. BSA standard solution(5mg/mL) (RM00005)
5. 1× PBS Buffer (RM00012)
6. 5× Loading buffer (RM00001)

Gel casting reagents

1. 30% Acr-Bis (29:1) (RM00006)
2. 1 M Tris-HCl (pH6.8) (RM00003)
3. 1.5M Tris-HCl (pH8.8) (RM00002)
4. 10% SDS (RM00004)
5. 10% Ammonium persulfate (APS) (RM00007)
6. TEMED (RM00009)

Blotting Reagents and Consumables

1. 1X Tris-Glycine SDS Running Buffer (RM00010)
2. 1X Western Transfer Buffer (RM00011)
3. Filter Paper (7.5×10cm) (RM00019)
4. Nitrocellulose membrane (RM00017,RM02801,and RM00018)
5. SignalFire™ ECL Reagent (RM00020 and RM00021)
6. 1X TBST Buffer (RM00013)
7. 1X Western Blocking Buffer (RK05742)
8. skimmed milk powder (RM00014)
9. 3% Skimmed milk: 1xTBST solution containing 3% skimmed milk powder
10. Bovine Serum Albumin (BSA) (RM02802)

Antibodies

1. Primary antibody

2. Secondary Antibody Conjugated to HRP

Based on the selection of the primary antibodies, secondary antibodies such as AS014, AS003, etc., are available options.

HRP-conjugated Goat anti-Rabbit IgG (H+L) (AS014)

HRP-conjugated Goat anti-Mouse IgG (H+L) (AS003)

Experimental Procedure

1. Sample preparation

(1) Sample collection

a. Collection of Adherent Cultured Cells

After scraping or treating cells with trypsin, centrifuge at 400x g for 5 minutes, discard the supernatant. Resuspend the cell pellet in an appropriate volume of ice-cold PBS, centrifuge at 4℃, 400×g for 5minutes, discard the supernatant, and repeat the washing step once to collect the cell pellet.

b. Collection of Suspension Cultured Cells

Collect suspension cells, centrifuge at 400x g for 5 minutes at 4℃, discard the supernatant. Resuspend the cell pellet in an appropriate volume of ice-cold PBS, centrifuge at 4℃, 400×g for 5minutes, discard the supernatant, and repeat the washing step once to collect the cell pellet.

c. Collection of Tissue samples

Cut the tissue into 1-2mm small pieces on a pre-cooled surface, freeze the tissue in liquid nitrogen or a ultra-low temperature freezer for at least 30 minutes. Then rapidly grind with liquid nitrogen for 1-2 minutes to minimize protein degradation.

(2) Protein extraction

a. Lysis of cells/tissues

The cell pellets and pretreated tissue should be kept ice cold.

Add an appropriate amount of lysis buffer based on the number and type of collected cells. For regular cell pellets, add ice-cold RIPA lysis buffer at a ratio of 1 mL per 10^7 cells, mix by pipetting after adding the lysis buffer. Use

a Ultrasonic Cell Disruptor to sonicate the sample, with each sonication cycle lasting 4-5 seconds with intervals around 5 seconds until the sample appears homogeneous and non-viscous. Alternatively, place the centrifuge tube with sample on a vortex oscillator for 10 seconds every 5 minutes, and repeat this process for 20 minutes.

For tissue fragments, add 0.5 mL of ice-cold RIPA lysis buffer per 100mg of tissue to a homogenizer, grinding every 3 minutes for 5 times to crush the tissue (add protease inhibitors to the lysis buffer as needed). All processes should be conducted under low-temperature conditions.

b. Centrifugation

Transfer lysed sample to a microcentrifuge tube. Centrifuge the sample in a pre-cooled high-speed centrifuge at 13,000x g for 10 minutes at 4℃ and collect the supernatant.

c. Denaturation the proteins of the samples

Take a small volume of lysate for protein concentration measurement. Add an appropriate volume of 5x Loading Buffer to the remaining protein extraction in the centrifuge tube (final working solution is 1×). Heat at 95° C in a dry heat block for 10 minutes, then cool the liquid completely and store at -20° C.

(3) Protein Concentration Measurement (BCA Assay)

a. Preparation of BCA working solution

Prepare BCA working solution by mixing BCA Reagent A with BCA Reagent B at a ratio of 50:1 according to the number of samples needed. Mix thoroughly. The BCA working solution is stable at room temperature for up to 24 hours.

b. BSA Standard Curve Dilution

Dilute 10 μL of BSA standard (5 mg/mL) with PBS or ddH_2O to 50μL to achieve a final concentration of 1 mg/mL. The diluted protein standard can be stored long-term at -20° C.

Prepare a gradient dilution of BSA by adding 0, 1, 2, 4, 8, 12, 16, 20 μL of the diluted BSA (1 mg/mL) to individual wells of a 96-well plate, and bring the total volume of each well to 20 μL with PBS or ddH_2O.

c. Sample Concentration Determination

Add an appropriate volume of sample to a blank well of the 96-well plate and bring total volume to 20 μL with PBS or ddH_2O. Add 200 μL of BCA working solution to each well, incubate at 37° C for 20-30 minutes. Measure the OD values at 562 nm (or within 540-595 nm range) using a spectrophotometer.

Calculate the protein concentration of samples based on the BSA protein standard curve and the volume of sample used.

2. Gel Electrophoresis

(1) Assemble Gel casting stand and casting cassette

Assemble the gel casting stands and cassettes according to the user manual instructions.

(2) Gel Preparation

Choose a suitable concentration of separating gel based on the target protein size (see Table 1,2,3). Ensure no air bubbles are formed during this process.

(3) Sample Loading

Once the gel has solidified, use a pipette to vertically load the samples into the wells of the comb. It is recommended to load 25 μg of total protein per well. Ensure that the inner chamber is filled with Tris-Glycine SDS Running Buffer, while the outer chamber should have the buffer covering the bottom by 3-5 cm.

(4) Electrophoresis

After loading the samples, connect the electrophoresis power supply, ensuring that the positive and negative electrodes are connected correctly, and set appropriate electrophoresis parameters. It is recommended to use a constant voltage of 80V for the stacking gel and 120V for the separating gel. Stop the electrophoresis when the Bromophenol Blue dye front reaches the bottom of the gel and then turn off the electrophoresis power supply.

3. Membrane Transfer

(1) Preparation

a. Transfer Buffer should be pre-cooled in a -20℃ refrigerator at least 2 hours before use (i.e., after the start of electrophoresis).

b. Cut filter paper and NC membrane to appropriate sizes based on the gel area.

c. For target proteins larger than 20 kDa, use a 0.45 μm NC membrane; for proteins smaller than 20 kDa, choose a 0.2 μm NC membrane or a 0.22 μm PVDF membrane. After selection, soak the membrane in Western Transfer Buffer.

Note: For PVDF membranes, soak in methanol for approximately 1 minute until uniformly wetted without white spots before placing in Western Transfer Buffer.

(2) Transfer

Select appropriate transfer conditions based on the molecular weight of the target proteins (see Table 4).

4. Blocking

After transfer, remove the membrane and place it in an appropriate antibody incubation chamber. Incubate with Western Blocking Buffer at room temperature for 1 hour.

5. Antibody Incubation

Based on the type of primary antibody used, select one of the following specific procedures:

(1) Unconjugated primary antibody

a. Dilute the primary antibody in 3% skimmed milk according to the recommended ratio in the antibody datasheet. Incubate at room temperature for 1.5 hours or overnight at 4° C.

b. Wash with TBST Buffer four times, 5 minutes each wash.

c. Select the appropriate HRP-conjugated secondary antibody (AS014 or AS003) according to the primary antibody species source. Dilute it to the appropriate ratio and incubate at room temperature for 1 hour.

HRP-conjugated Goat anti-Rabbit IgG (H+L) (AS014), recommended at 1:2000 - 1:10000

HRP-conjugated Goat Anti-Mouse IgG (H+L) (AS003), recommended at 1:2000 - 1:10000

d. Wash with TBST Buffer four times, 5 minutes each wash.

(2) Primary antibody conjugated with HRP

a. Dilute the primary antibody in 3% skimmed milk according to the recommended ratio. Incubate at room temperature for 1.5 hours or overnight at 4° C.

b. Wash with 1x TBST (RM0001) four times, 5 minutes each wash.

6. Exposure

（1）Mix equal volumes of ECL Solution I and Solution II (RM00020 and RM00021) to prepare the chemiluminescent detection working solution. Use 1-2 mL ECL working solution per 10 cm^2 membrane.

（2）Use tweezers to carefully remove the membrane. Gently touch the lower edge of the membrane to absorbent paper to remove excess liquid. Use a pipette to evenly cover the membrane with the working solution. Incubate at room temperature for 1-2 minutes. This step can be performed on clean plastic wrap or in a plastic box.

（3）Obtain Western Blot results:

a. Traditional autoradiography

Fix the membrane in a film cassette. Expose in a darkroom for 1 minute, then immediately develop and fix to adjust exposure time for optimal signal-to-noise ratio. Alternatively, expose for 30 seconds, 1 minute, 3 minutes, and 5 minutes separately, then develop and fix together to observe results.

b. Chemiluminescent imaging system:

Place the membrane in the imaging system. Refer to the instrument manual to set appropriate parameters for optimal imaging. Adjust imaging parameters based on the biological properties of the target protein, such as abundance, to optimize the image.

Tabel 1: Gel Percentage Selection

kDa	Resolving gel concentration
X≤10	15%
10＜X≤15	13.5%
15＜X≤25	12%
25＜X≤35	11%
35＜X≤40	10%
40＜X≤55	9%
55＜X≤70	8%

70＜X≤100	7%
100＜X	6%

Note: X is the molecular weight of the target protein.

Tabel 2: Gel Preparation

	6%	7%	8%	9%	10%	11%	12%	13.5%	15%
dd H_2O（ml）	5.3	4.9	4.6	4.3	4.0	3.65	3.3	2.8	2.3
30% Acr-Bis (29:1)（ml）（RM00006）	2	2.4	2.7	3.0	3.3	3.65	4.0	4.5	5.0
1.5M Tris-HCl (pH8.8)(ml)（RM00002）	2.5								
10%SDS（mL）（RM00004）	0.1								
10% Ammonium persulfate (APS)（mL）（RM00007）	0.1								
TEMED（mL）（RM00009）	0.01								
Final volume(ml)	10								

Tabel 3: Recipe for 5% stacking gel

	5%	5%	5%	5%	5%
dd H_2O（ml）	1.37	2.1	2.7	3.4	4.1
30% Acr-Bis (29:1)（ml）（RM00006）	0.33	0.5	0.67	0.83	1.0
1 M Tris-HCl (pH6.8)	0.25	0.38	0.5	0.63	0.75

（ml）（RM00003）					
10%SDS（ml）（RM00004）	0.02	0.03	0.04	0.05	0.06
10% Ammonium persulfate (APS)（mL）（RM00007）	0.02	0.03	0.04	0.05	0.06
TEMED（mL）（RM00009）	0.002	0.003	0.004	0.005	0.006
Final volume(ml)	2	3	4	5	6

Tabel 4：Selection of Transfer Conditions

kDa	Condition（constant current）
X≤10	200mA,30min
10＜X≤15	200mA,40min
15＜X≤20	200mA，50min
20＜X≤50	200mA，1kDa/min+20min
50＜X≤100	200mA，1kDa/min+30min
100＜X≤150	250mA，1kDa/min+20min
150＜X≤180	270mA，1kDa/min,within 3h
180＜X	270mA，1kDa/min,within 4h

Note: X is the molecular weight of the target protein.