Protocol for Western blotting

1. Preparation of lysate from 60 mm×15 mm cell culture plate:

- (1) Aspirate the growth medium from the cells, and wash the cells with PBS.
- (2) Aspirate the PBS, then add 600µL trypsin EDTA solution.
- (3) Incubation for several minutes at 37 °C and check the culture with inverted microscope to be sure that the cells are rounded up which indicates they are detached from the surface.
- (4) Add 2 mL fresh complete medium, then pipet all the adherent cells into cell suspension and pipet up and down.
- (5) Transfer the cell suspension into a pre-cooled 15 mL centrifuge tube and centrifuge at 1200 rpm for 5 minutes.
- (6) Aspirate the filtrate, add 1mL PBS and resuspend the cell, transfer the cell suspension into a pre-cooled 1.5 mL microcentrifuge tube.
- (7) Centrifuge at 1200 rpm for 5 minutes at 4 °C, then aspirate the filtrate.
- (8) Add 35 μL RIPA Lysis Buffer containing protease inhibitor cocktail and phospho-stop , and mix or vortex briefly to resuspend the cells completely
- (9) Incubate on ice for 15 minutes. Vortex briefly to resuspend and lyse residual cells.
- (10) Centrifuge at 13200 rpm for 15 minutes at 4 $^{\circ}$ C.
- (11) Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice, and discard the pellet.

2. Protein Quantification:

We use Beyotime BCA Protein Assay Kit.

- (1) Dilute $10\mu l$ BSA Standard (5 mg/mL)to $100\mu L$.
- (2) Prepare Working Reagent by mixing 50 parts of BCATM Reagent A with 1 part of BCATM Reagent B (50:1, Reagent A:B).
- (3) a.Pipette 0、1、2、4、6、8、12、16、20 μ L of standard into a 96 microplate well and dilute each standard to 20 μ L.
 - b.Pipette 20 µl of each diluted sample replicate into the microplate well.
 - c. Add 200 µl of the Working Reagent to each well and mix plate thoroughly by pipette.
 - d.Cover plate and incubate at 37 $^{\circ}$ C for 30 minutes.
- (4) Measure the absorbance at 595 nm on a plate reader.
- (5) Prepare a standard curve by plotting the average Blank-corrected 595 nm measurement for each BSA standard vs. its concentration in $\mu g/ml$. Use the standard curve to determine the protein concentration of each unknown sample.

3. Reduce and denature samples:

Boil each cell lysate in LDS sample buffer(4×) at 95 °C for 5 minutes. Lysates can be aliquoted

and stored at $-20 \, \text{C}$ for future use.

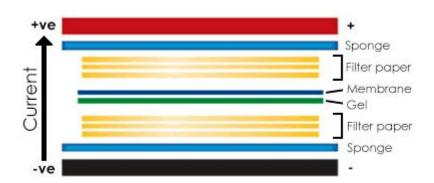
4. Loading and running the gel:

Load $60-80~\mu g$ of total protein from cell lysate into the wells of the SDS-PAGE gel(10% Gel percentage), along with pre-stained molecular weight marker.

5. Run the gel for 55 minutes at 180 V.

6. Transfer proteins from the gel to membrane:

- (1) a.Prepare transfer buffer(1L): 3.03 g Trizma base (25 mM), 14.4 g Glycine (192 mM),200 mL methanol(20%),800 ml water;
 - b. Prepare PBST(1L):PBS(1L), 0.5ml Tween 20 (0.05%)
- (2) Cut a piece of PVDF membrane according to the size of gel and wet in methanol.for 2 minutes.
- (3) Transfer the membrane to the transfer buffer.
- (4) Assemble transfer stack as follows:



(5) Attach the electrodes. Set the power supply to 350 mA (constant current) for 1h 20 min at 4 $^{\circ}$ C.

7.Blocking:

- (1) Prepare Blocking buffer:Add 2.5g non-fat powerdered milk (5%) to 50 mL PBST buffer, mix thoroughly.
- (2) Remove the membrane to a small container and stain in Ponceau stain reagent for several minutes, then wash for short time with PBST to check the transfer.
- (3) Cut the membrane into several stripes according to the size of target protein.
- (4) Compeletely.destain in PBST.
- (5) Incubate the membrane in 5% milk for 1 h at room temperature.

8. Primary antibody incubation:

Incubate the membrane with appropriate dilutions of primary antibody in blocking buffer, 4°C

overnight at recommended concentration.

9. Secondary antibody incubation:

- (1) Wash the membrane in 5 washes of PBST, 6 min each.
- (2) Incubate the membrane with the recommended dilution of conjugated secondary antibody in blocking buffer at room temperature for 1 h.

10. ECL detection:

We use Pierce™ ECL Western Blotting Substrate.

- (1) Wash the membrane in 3 washes of PBST,5 min each.
- (2) Prepare the substrate working solution by mixing equal parts of Detection Reagents 1 and 2. Use 0.125 mL Working Solution per cm² of membrane.
- (3) Incubate blot with working solution for 1 minute at RT.
- (4) Remove blot from working solution and place it in a clear plastic wrap. Use an absorbent tissue to remove excess liquid.
- (5) Place the membrane in a chemiluminescence imaging system with the protein side facing up and carefully press out any bubbles from between the chemiluminescence imaging system and the membrane.
- (6) Cover he chemiluminescence imaging systemand detect.