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℃ 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℃,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℃.

(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 10μl BSA Standard (5 mg/mL)to 100μL.

(2) Prepare Working Reagent by mixing 50 parts of BCA™ Reagent A with 1 part of BCA™ Reagent B (50:1, Reagent A:B).

(3) a.Pipette 0、1、2、4、6、8、12、16、20 μL of standard into a 96 microplate well and dilute each standard to 20 μL.

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℃ 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 μg/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℃ for 5 minutes. Lysates can be aliquoted

and stored at -20 ℃ for future use.

4. Loading and running the gel:

Load 60–80 μg 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 ℃.

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 0.125mL Working Solution per cm^2 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.