Sample preparation

1. Protein extraction

1) Experimental instruments and reagents:

Homogenizer, RIPA buffer, PMSF (proteinase inhibitor), phosphatase inhibitor, scissors, tweezers, alcohol cotton, PBS, pipette, centrifugal machine.

RIPA preparation—Add 1μL PMSF and phosphatase inhibitor in each 100μL RIPA. Prepare when needed. Calculated the total volume of RIPA buffer before treating the samples.

2) For tissue sample:Sterilize scissors and tweezers with alcohol cotton, wash the tissue with PBS, add appropriate amount of tissue into homogenizer, cut the tissue into pieces and add enough RIPA buffer, then homogenize in ice water bath for 1-4 minutes. After homogenizing, transfer the solution to an EP tube and centrifuge at 12000rpm for 5 minutes, take supernatant.

3) For cell sample:

A) Adherent cells:Take out and wash the culture medium with PBS. Add appropriate amount of RIPA buffer (150μL RIPA buffer per 5X10^6 cells), Crack on ice for 5-10 minutes, pipette several times until cell desquamate. Then pipette cracking solution into EP tube. Centrifuge at 12000rpm for 5 minutes, take supernatant. For adherent cells in large bottle, use pancreatin to elute them and RIPA buffer afterwards. The lysis method refers to suspension cell processing.

B)Suspension cell:Add cells in an EP tube and centrifuge at 2000-3000 rpm for 5 minutes, remove the supernatant medium. Add 100-150μLRIPA bufferto each 20μLcells, pipette several times until cells disrupt completely.Centrifuge at 12000rpm for 5 minutes, take supernatant.

The protein can be stored for one week in 4℃, for 2 months in -20℃, and for 6 months in

-80℃.

2. Measurement of protein concentration

1) Experimental instruments and reagents:

ELISA microplate, Microplate reader, centrifuge, 1 mg/mL BSA, BCA reagent, PBS.

2) Standard curve:use total volume 20μL of BSA and PBS to make the standard curve, operate according to the follows.

Dilute BSA protein standard into serial concentrations: 0, 0.2, 0.4, 0.6, 0.8, 1.0 mg/mL (0, 4μL, 8μL, 12μL, 16μL, 20μL BSA, add up to 20μL with PBS), set duplicates for each gradient.

3) Dilute sample to test the concentration

Ratio of 10 times: take 18μL PBS and 2μL sample supernatant, set triplicates for each sample.

Ratio of 20 times: take 19μL PBS and 1μL sample supernatant, set triplicates for each sample.

The total volume is 20μL.

4) BCA reagent preparation:Prepare fresh solution before use, avoid of being contaminated by protein. (Detailed steps reference instructions）

5) Incubation:Add 200μL BCA to standard and sample wells, incubate at 37℃for 15-20 minutes.

Read the OD value at 568nm with microplate reader. Average the standard OD value, minus the blank OD value, then make the standard curve and standard curve equation. Average the sample OD value, calculate according to the equation to get the protein concentration.Use the calculation to get the loading volume of samples. Recommend total protein amount of each sample is 50ug.

Note:All OD value must minus the blank OD value before calculating.

3. Sample denaturation

Mix sample supernatant and 5× SDS buffer according to the ratio 4:1, incubate in boiling water bath for 10 minutes. Samples with high concentration can be diluted with PBS before boiling, loading volume of samples should be near 10ul.

Store the processed sample at -20℃(validity for 6 months), -80℃ for long time.

WB Experiment Procedure

1. Gel preparation

Molecular weight of protein（kDa）	Concentration of separation gel（%）
>140	6%
100-140	8%
30-100	10%
10-30	12%
<10	15%

1) Prepare separation gel according to the molecular weight of protein. Shake slowly to mix completely, avoid oxygen in when shaking hard. Pour the mixed gel in between two glass panes, inject anhydrous ethyl alcohol above it to avoid oxygen in to affect polymerization.

2) Remain horizontal, incubate at 37℃ for 1 hour till it polymerizes completely.

3) Prepare the stacking gel according to proportion. Shake slowly to mix completely, pour out the anhydrous ethyl alcohol above separation gel, and absorb the anhydrous ethyl alcohol above solidified separation gel with filter paper. Pour spacer gel gently, insert comb carefully and avoid bubbles.

4) Incubate at 37℃ for 1 hour till it polymerizes completely, then take out the comb.

2. Adding samples

Add electrophoretic buffer to electrophoresis tank, the buffer solution must be above sample wells, the bottom of gel must be immersed by buffer solution. Level within glass panes should be above level outside. Add samples according to the calculated loading volume and add pre-stained protein marker, make sure the total protein in every well is between 30-50μg, the total loading volume of samples should be less than 30μL.

Make sure that samples are added in short time, and avoid of samples splash.

3. Electrophoresis

Cover the lid of electrophoresis tank, mind the positive and negative, and choose the appropriate voltage to do electrophoresis. Usually in inconsecutive system, the voltage of stacking gel (80V is suggested) is lower than the voltage of separation gel (110-150V is suggested). Sample will be at the same level when into the separation gel.
Electrophoresis time is about 2-3 hours till bromophenol blue reaches the bottom of gel.

4. Wet transfer

1) Take out the gel after electrophoresis, rinse in the cold electrophoretic buffer solution for few seconds. According to the sandwich mode, open the Electrical transfer folder, stack up a sponge mat soaked by transferring buffer at both sides. Then place the qualitative filter paper soaked by transferring buffer at both sides. Place the gel flat on the negative filter paper, and then place flat the PVDF membrane
(the PVDF is soaked in methyl alcohol for 5minutes,
then saturated in transferring buffer) on the gel. Wipe off the bubbles, and fold the folder. Note that wipe off all the bubbles.

2) Load the transferring buffer in transfer tank, insert the folder. Put the tank in ice water, make sure PVDF membrane is near the positive pole, amino acid and protein with electronegative will move to the positive pole.

3) Choose the constant current, the electric current of each tank is suggested to be 150-200mA. Adjust the time according to molecular weight of the protein.

4) After transferring, take out the transferred PVDF membrane, rinse the membrane with washing buffer at room temperature.

5. Blocking

Take out the rinsed membrane, put it into the blocking buffer (5% skim milk powder or 5% BSA). Incubate on the shaker at room temperature for 2 hours.

6. Incubation of antibody

The indirect method is recommended. Add unlabeled primary antibody, the antibody binds to the antigen protein. Then add the enzyme labeled secondary antibody to test.

1) Add diluted primary antibody to blocked membrane, incubate over night at 4℃.

2) Wash the membrane with washing bufferfor 10 minutes and repeat 3 times.

3) After adding the diluted secondary antibody, incubate at room temperature for 2 hours. Wash the membrane for 10 minutes and repeat 3 times.

7. Detection

The detection method depends on the label at secondary antibody. ECL and DAB are usually used.

1）ECL method

Use HRP to catalyze chemiluminescent compound, an intermediate is formed, record the luminescence with machine. Also we can use X-ray film to fixate the images and use DAB as colour developing reagent.

A) ECL preparation: mix the substrate A and B as the ratio 1:1. Prepare when needed.

B) Cover the blotting membrane with mixed substrate for 1-5 minutes, observe the fluorescence in dark.

C) Record the result with machine or in dark room with autoradiography film or chemiluminiscence imaging system.

D) For autoradiography film, expose for several seconds to minutes according to the fluorescence intensity. The exposed film will be soaked in developing liquid till strips appear, then rinse in the fixing solution. Wash the film with water and hang up to dry.

2）DAB Detection

A) Add 1mL water and a drop of chromogenic reagent A, B, C, mix fully.

B) Chromogenic reaction: put appropriate amount of DAB flat on blotting membrane, observe in room temperature, obvious brown protein strip will appear.

8. Data analysis

Analyze the bands with Bandscan software