Western Blotting / Immunoblotting (WB / IB)

Cell Lysate Preparation:

A. Reagents and Materials

Phosphate Buffered Saline; 1X PBS, pH 7.4

SDS-Loading Buffer: 0.1M Tris-HCl, pH6.8, 0.1M DTT, 2%SDS, 20% glycerol, 0.02% bromophenol blue

Cell Detaching Trypsin Buffer: 0.25% trypsin in 0.53M EDTA solution

Cell Lysis Buffer (1% NP40, 0.5% DOC, 1mM EDTA , 65mM Tris-HCl pH=6.4, 1mM PMSF, 1ug/ml Apoprotin, 1ug/ml Leupeptin, 1ug/ml Pepstatin)

BCA Protein Assay Kit

B. Equipment

Ultrasonicator

C. Protocol

Cell culture and harvest

a) Cell subculture

i) Suspension cells: spin down cells and re-suspend pellet in RPMI 1640 with 10% FBS to make the final concentration around 10^5 cells/ml, and incubate at 37℃, 5%CO2.

ii) Adherent cells: digest adherent cells in Cell Detaching Trypsin Buffer. Then spin down cells and re-suspend the pellet in DMEM with 10% FBS to make the final concentration around 10^5 cells/ml, and incubate at 37℃, 5%CO2 .

b) Incubate cells for about three days and count the cells. When the concentration reach 10^6 cells/ml, detach cells, spin down and wash twice with 1XPBS .

c) Add cell lysis buffer to cell extracts (1ml lysis buffer /5×10^7 cells). Vortex the mixture for 5min and store at -20℃.

Preparation of tissue extracts

a) Thaw the frozen, raw tissue sample by vortexing and repeat the freeze-thaw cycle twice.

b) Disrupt tissue with ultrasonicator. Keep the entire operation on ice.

Measure concentration of cell samples

a) Transfer contents to a microcentrifuge tube. Centrifuge at 12,000 rpm for 15 minutes. Then collect supernatant into an appropriately labeled tube (for the detection of membrane bound proteins, use the insoluble, cell pellet). Dilute the supernatant lysate and the whole lysate without centrifugation at 1:4, 1:8 and 1:16 with 1XPBS. Measure protein concentration using cell lysis-compatible protein assay (BCA protein assay).

b) Mix the cell lysate with SDS loading buffer to make the desired final concentration. Incubate the lysates at 100°C for 10min.

SDS-PAGE

A. Reagents and Materials

Stacking gel buffer: 1M Tris-HCl, pH6.8, 0.4% SDS

Separating gel buffer: 1.5M Tris-HCl, pH8.8, 0.4% SDS

Acrylamide stock solution: 29% acrylamide plus 1.0% bis-acrylamide

10% ammonium persulfate

TEMED(N,N,N',N'-tetramethylene-ethylenediamine)

Electrophoresis buffer: 0.025M Tris Base, 0.192M Glycine, 0.1% SDS

Coomassie gel stain solution: 0.2% Coomassie blue R-250, 30% Methanol, 10% acetic acid.

Coomassie gel destain solution: 30% Methanol, 10% acetic acid.

B. Equipment

Minigel apparatus: Bio-Rad Mini-Protean 3 Dodeca Cell

Power supply: Bio-Rad PowerPac HC

Gradient gel former: Bio-Rad Model 485 Gradient Former

C. Protocol

Assemble multi-casting chamber according to the manufacturer's instructions. All following operations related with apparatus should follow manufacture's instructions.

Make separating gel as following formula in a suitable beaker. Ammonium persulfate and TEMED should be added before pouring gel.

	Separating Gel (total 10 ml)				Stacking Gel (2ml、6ml)	
Separating/ stacking concentration	8%	10%	12%	15%	5%	5%
Separating gel buffer	2.5	2.5	2.5	2.5	—	—
stacking gel buffer	—	—	—	—	0.25	0.75
Acrylamide stock solution	2.6	3.3	4	5	0.33	1
H2O	4.8	4.1	3.4	2.4	1.4	4.2
10% ammonium persulfate	0.1	0.08	0.08	0.08	0.02	0.06
TEMED	0.06	0.04	0.04	0.04	0.002	0.006

Pour the separating gel

After adding ammonium persulfate and TEMED immediately mix the gel solution gently and carefully introduce solution into gel casting chamber. Stop the pouring when gel solution reach to about 6 cm height and layer about 0.5 ml isopropanol on top of the separating gel solution to keep gel surface flat. Allow gel to polymerize within 10-30 minutes.

Pour the stacking gel

When the gel has polymerized a distinct interface will appear between the separating gel and the isopropanol. Drain isopropanol and wash the surface with distill water. After adding ammonium persulfate and TEMED immediately mix the gel solution gently and carefully introduce solution onto separating ge until solution reach top of front plate. Carefully insert comb into gel until bottom of teeth reach top of front plate. Allow gel to polymerize within 10-30 minutes.

Loading samples

After stacking gel has polymerized, remove comb carefully and place the gel into electrophoresis chamber. Add electrophoresis buffer to inner and outer reservoir. Load samples and appropriate protein markers into wells (20-25ug total protein per sample).

Running gel

Cover the lid and attach electrode plugs to proper electrode. Turn on power supply to 80V and run about 30 minutes. Change voltage to 150V when samples form single narrow line and enter the separating gel. Stop electrophoresis when dye front migrate to the bottom of the gel. It takes about 40~60 minutes.

Processing Gel for different uses

a) Staining gel with Coomassie Blue. If separated proteins are needed to be seen in the gel directly, stain gel with Coomassie gel stain solution with constant rocking for 40 minutes and then destain with Coomassie gel destain solution until background staining disappears.
b) Immunoblotting: If specific protein is needed to be detected by antibody, process gel as described in the protocol of Gel Transfer and Western Blot.

Gel Transfer

A. Reagents and Materials
Transfer buffer 10X stock solution: 0.25M Tris, 1.92M Glycine. Add methanol to 10% after dilution to 1X buffer and just before use.
Blocking buffer: 5.0% non-fat dry milk in 1 X PBS, pH=7.4.
PVDF membrane
B. Equipment
Electroblotting apparatus: Bio-Rad Trans-Blot Cell
Power supply: Bio -Rad PowerPac H
C. Protocol
This protocol is the following steps of SDS-PAGE.

Electrotransfer

Soak membrane sequentially in 100% methanol for 30 seconds and into transfer buffer for at least 1 minutes. Arrange gel-membrane sandwich as described in manufacturer’s instruction. Place the transfer sandwich unit into buffer tank, fill with pre-cooled transfer buffer and attach the electrodes. Set the power supply to 50mA and transfer overnight.

Blocking

Transfer PVDF membrane to blocking buffer. Rock the blocking membrane on shaker for 1 hours at RT or keep it at 4℃ overnight.

Immunoblotting

A. Reagents and Materials

TBST: solution: 0.02M Tris, 150 mM NaCl, 0.05% Tween-20.

Antibody dilution buffer: Add 5% non-fat dry milk to TBST just before use.

Developing solution/Fixative solution for X-ray film

Membrane washing buffer: TBST

Primary antibody;

Secondary antibody, eg. Caprine Anti-rabbit IgG（SAA544Rb19）

Horseradish Peroxidase Substrate

X-ray film

B. Equipment

Rotary shaker

C. Protocol

This protocol is to be followed after Gel Transfer..

Primary antibodies incubation

a) Dilute the primary antibody in antibody dilution buffer to suitable antibody concentration, then incubate for 1hour at room temperature with agitation to enable adequate homogeneous covering of the membrane and prevent uneven binding. Overnight at 4℃ with agitation is also acceptable.

b) Wash membrane: Pour off primary antibody solution and wash membrane 3 times for 5 minutes each time in Membrane washing buffer.

Secondary antibodies incubation

a) Dilute secondary antibody in antibody dilution buffer (1:5,000, Caprine Anti-rabbit IgG) and incubate with shaking for 1 hour at room temperature.

b) Wash membrane: Pour off secondary antibody solution and wash membrane 3 times for 5 minutes each time in Membrane washing buffer.

Development

Mix the two solutions of HRP substrate reagent in appropriate ratio immediately prior to adding to the membrane. Incubate with the membrane for 1 minute. Drain and remove excess reagent and place the membrane in plastic wrap to ensure a dry surface for film exposure.

Exposure in darkroom

Exposure the membranes to X-ray film in cassette for 1 minute. Manual film development is used to control the incubation time of the x-ray film in the developing solution and fixative solution. Normally, about 2-5 min for development and 5 min for fixation. Dry the film after thoroughly washing with water.