Protocol for Western Blotting

Materials

Materials used on Day 3

・Protease inhibitor stock:

1 µg/µL pepstatin A in DMSO

200 µM leupeptin in OG Buffer

200 mM PMSF: Freshly made. Ex) 34.8 mg PMSF in 1 mL isopropanol or DMSO.

・OG buffer: 150 mM NaCl, 20 mM HEPES, 1mM EDTA, pH 7.4

・OG buffer with protease inhibitors (OGB/PI): OG buffer containing 0.5% (v/v) of protease inhibitor stock

*For example, add 5 µL of protease inhibitor stock to 1 mL of OG buffer.

・Lysis buffer (LB): 0.5% Nonidet P-40, 200 mM NaCl, 20 mM HEPES, 1 mM EDTA, pH 7.4

・Lysis buffer with protease inhibitors (LB/PI): lysis buffer containing 0.5% (v/v) of protease inhibitor stock

*For example, add 5 µL of protease inhibitor stock to 1 mL of lysis buffer.

・Agarose-conjugated anti-cMyc antibody: Santa Cruz, Catalogue No. sc-40AC.

・30% Acrylamide/Bis solution, 37.5:1 (2.6% C)

・Running gel buffer: 1.5 M Tris-HCl, pH 8.8

Materials used on Day 5

- **6x sample buffer** (= 6x sample buffer with reducing reagents):

  12% SDS, 40% glycerol, 30% ß-mercaptoethanol, 300 mM DTT, 120 mM EDTA, 1 mg/mL bromophenol blue, 375 mM Tris-HCl, pH 6.8.

- **Pre-stained standard**: Invitrogen Catalogue No. LC5925
- **10x running buffer**: 250 mM Tris-HCl, 1920 mM glycin, 1% SDS
- **10x transfer buffer**: 250 mM Tris-HCl, 1920 mM glycin
- **10x PBS** (0.1 M sodium phosphate): 13.8 g $NaH_2PO_4 \cdot 4H_2O$ in 1 L solution, pH adjusted to 7.2 using NaOH.
- **Stacking gel buffer**: 0.5 M Tris-HCl, pH 6.8
- **immuno-blot PVDF Membrane.** Bio-Rad Catalogue 162-0176,

Materials used on Day 6

TBS: 20 mM Tris-HCl, 500 mM NaCl, pH 7.5

T-TBS: 0.2% Tween-20 in TBS

Blocking buffer: PBS with 10% glycerol, 5% milk, 0.2% Tween-20

Antibody: horseradish peroxidase conjugate anti c-Myc mouse monoclonal IgG (Santa Cruz Biochemistry, sc-40 HRP)

Chemiluminescent HRP Substrate: MILLIPORE , Immobilon Western.

Film: Hyperfilm ECL 18x24, Amersham Biosciences, Product Code RPN2103K

Procedure

Day 3 Cell Lysis and immunoprecipitation

(1) Prepare the buffers

Add 0.5% (v/v) of protease inhibitor stock to 0G buffer or lysis buffer to make 0GB/PI or LB/PI, respectively.

Volume of buffer (mL)

0G Buffer: 3.0 ml / dish

Lysis Buffer: 0.75 ml / dish ,

(2) Preparation of lysate

1) Wash 293 cell surfaces twice with 1 mL PBS and aspirate.

2) Pour 1 mL of LB/PI onto the cells and incubate for 5min on the ice.

3) Transfer LB/PI to a micro-centrifuge tube.

4) Centrifuge the tube at 15,000 rpm for 10 minutes at 4C and transfer the supernatant to a new tube.

(3) Wash agarose-conjugated anti-cMyc antibody (AC) for use in immunoprecipitation

1) Pour 30 μL of AC into a new tube.

2) Add 0.75 mL of LB/PI.

3) Spin for a short time at 15,000 rpm, discard the supernatant, and add 0.75 mL of LB/PI.

4) Spin for a short time at 15,000 rpm and discard the supernatant.

(4) Immunoprecipitation

Mix cell lysate (Step (2)-2) and washed AC (Step (3)-4) overnight using a rotator at 4C

Day 4 SDS-PAGE and Western Blotting

(1) Preparation of running gel (7.5% acrylamide)

Number of gels	1	2
30% Acrylamide/Bis solution (mL)	3	6
6Running gel buffer (mL)	3	6
50% glycerol	2.4	4.8
dH2O	3.5	7.0
10% SDS	120 μ	240 μ
10% APS	50 μ	100 μ
TEMED	10 μ	20 μ
Total	12 ml	24 ml

・Pour into gel maker.

・Pour 80% Etoh over the surface of the gel and leave for 30min at RT.

(2) Prepare Stacking gel

Number of gels	1	2
30% Acrylamide/Bis solution (mL)	0.65	1.3
Stacking gel buffer (mL)	1.25	2.5
dH2O(mL)	3.05	6.1
10% SDS(μl)	50	100
10% APS(μl)	50	100
TEMED(μl)	5	10
total	5.1	10.2

- Drain the water from the top of the running gel.
- Pour the stacking gel mixture on top of the running gel.
- Put the comb in place.

(3) Prepare reagents

1) Prepare LB/PI with NaCl

1-1) LB + NaCl: Add 58 mg of NaCl (powder) per 1 mL of LB

1-2) Add 5 µL of protease-inhibitor (PI) stock per 1 mL of the following buffer.

Volume of buffer (mL)

Lysis Buffer (LB): 3.0 ml / sample

LB + NaCl: 1.5 ml / sample

2) Prepare 1x sample buffer

Dilute sample buffer stock using H_2O.

3) Prepare 1x running buffer

Number of tanks	1	2
10x running buffer (mL)	50	100
H2O (mL)	450	900
Total (mL)	500	1000

4) Prepare transfer buffer

Number of membranes	1	2
10x transfer buffer (mL)	100	200
H2O (mL)	700	1400
Ethanol (mL)	200	400
Total (mL)	1000	2000

(Add Ethanol after mixing 10x transfer buffer and H_2O, then fill to 1000 or 2000 mL using H_2O.)

Cool the buffer at 4°C.

5) Prepare blocking buffer

Prepare 100 - 150 mL of blocking buffer for 1 membrane

For 100 mL of buffer:

10x PBS: 10 mL + Glycerol: 10 mL + NaCl: 0.9 g

+ H_2O: 80 mL

→ Mix → Add 5 g of skim milk

→ Mix → Add 0.2 mL of Tween 20. → Fill to 100 mL using DDW.

(4) Prepare the sample

1) Spin the cell lysate and agarose mixture for a short time and discard the supernatant.
2) Wash the AC with **LB/PI** twice: Add 0.75 mL of LB/PI, mix using the vortex mixer, spin for a short time, and discard the supernatant. Repeat.
3) Wash the AC with **LB/PI containing NaCl** twice. Completely aspirate the supernatant.
4) Add 40 µL of 1x sample buffer and mix using the vortex mixer.
5) Incubate at 95C for 5 minutes.
6) Spin for a short time and transfer the supernatant to a new tube.

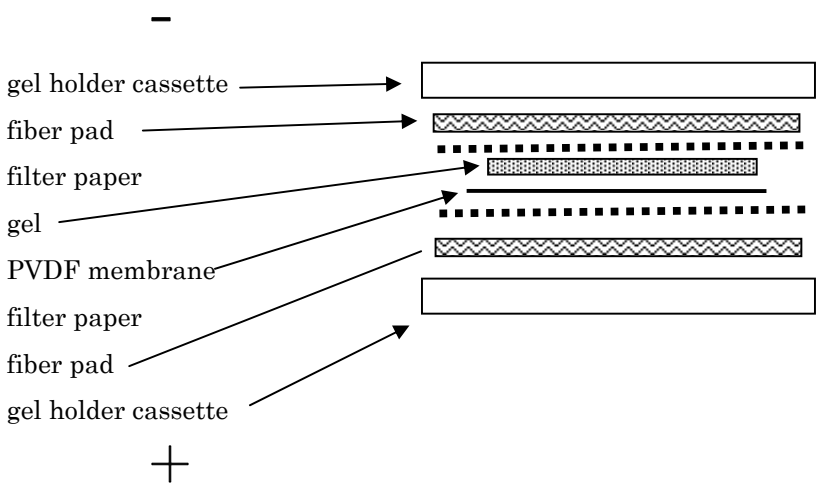
(5) SDS-PAGE

1) Set the gel with the running buffer and apply the marker and the samples.
2) Electrophoresis: at 200 V (constant voltage), for approximately 50 min.

(6) Western Blotting

1) Cut PVDF membrane into 7.5 x 10 cm_2, soak in methanol, and then soak in transfer buffer.
2) Disassemble the gel, cut the corner of the gel to mark the orientation, and soak the gel in transfer buffer for 5 minutes.

3) Assemble the gel and the membrane.



4) Blot overnight with 30 V (constant voltage) at 4C.

(or 2 hours with 80V)

Day 6 Blocking, reaction with antibody, and detection

1) Soak the membrane in TBS for 5 minutes.

2) Soak the membrane in blocking buffer for 2 hours.

3) React with antibody (for one or two membranes: blocking buffer, 10 mL; Santa Cruz anti cMyc-HRP mouse monoclonal IgG, 10 µL) for 2 hours.

4) Prepare T-TBS (0.2% Tween-20 in TBS):

For one membrane,

TBS: 50 mL

Tween-20: 100µL

Wash the membrane with blocking buffer 5 times for 5 minutes each.

5) Wash with T-TBS and TBS.

Soak in T-TBS for 10 minutes. → Soak in TBS for 5 minutes. → Soak in T-TBS for 10 minutes. → Soak in TBS for 5 minutes.

6) Mix ECL-plus A solution (5 mL) and B solution (125 µL), and pour over the membrane. Leave at r.t. for 5 minutes.

7) Drain ECL-plus, wrap the membrane, and expose the film.

8) Develop the film.

Reference

Nakamura K et al. Mol Endocrinol 18: 1461-1470, 2004.