Gene Transfection & Western Blotting Protocol

Schedule

Day 1	Transfection
Day 2	Changing of medium
Day 3	Cell lysis Immunoprecipitation Preparation of SDS polyacrylamide gel
Day 4	Electrophoresis Blotting
Day 5	Blocking Reaction with Antibody Washing and Detection

Materials

Materials used on Day 1

Calcium Phosphate Transfection Kit: Invitrogen Catalogue No. K2780-01

Falcon tube (Catalogue No. 35-2054: 12 x 75 mm, 5-mL tube)

Cells: 293 cells (human embryonic kidney cells), near confluent in φ60-mm dish

Plasmid: cMyc-tagged human LH receptor/pcDNA3.1, 1 μg/μL, purified using Qiagen Plasmid Maxi Kit

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): Bio-Rad, Catalogue No. 161-0158

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

Materials used on Day 4

6x sample buffer (= 6x sample buffer with reducing reagents):
12% SDS, 40% glycerol, 30% β-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.
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Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8

Bio-Rad Catalogue 162-0176, immuno-blot PVDF Membrane

Materials used on Day 5

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%, 0.2% Tween-20

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

ECL Plus: ECL Plus Western Blotting Detection Reagents, Amersham Biosciences, Product Code RPN2132

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

Gene Transfection and Western Blotting Protocol

Day 1 Transfection (Calcium-phosphate-mediated transfection)

↓ Prepare solution A and B

Solution A: 150 μL

10 μg plasmid: x μL (1.0 μg/μL plasmid → x = 10)

sterile H_2O: 150 - 18 – x μL (x = 10 → 122 μL)

2 M $CaCl_2$: 18 μL (0.24 M $CaCl_2$ in Solution A)

Solution B: 150 μL

2x HBS (Hepes Buffered Saline): 150 μL

↓ Mix Solution A and Solution B

Using a Pasteur pipette, slowly add solution A dropwise to solution B while bubbling air through solution B with another pipette. Continue until solution A is depleted. This is a slow process that should be performed over 1 – 2 minutes.

Autopipetter

Pasteur pipette (Provides air to make bubbles in Solution B.)

Pipetteman pipette (Used to slowly add Solution A.)

Solution B in Falcon tube

↓ Incubate at room temperature (R.T.) for 15 ~ 30 minutes.

↓ Remove the spent medium of the 293 cells in the φ60-mm dish and add 5 mL of fresh medium.

↓ Add the mixture of Solution A and B dropwise to the media in the dish.

↓ Incubate overnight at 37C in a humidified CO_2 incubator.

Day 2 Changing of medium

Gently change all of the medium.

Day 3 Cell Lysis

(1) Cell harvest and immunoprecipitation

(2) Preparation of running gel

(1) Cell harvest and immunoprecipitation

Step 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 x a (mL)

Lysis Buffer: 0.75 x a + 1.5 x b

a: number of dishes

b: number of immunoprecipitation samples

Step 2: Harvest the cells
1) Wash 293 cell surfaces with 0.75 mL of 0GB/PI.
2) Pour 0.75 mL of 0GB/PI onto the cells and harvest the cells. Transfer the cell suspension to a micro-centrifuge tube.
3) Repeat 2). again.
4) Centrifuge the tube at 15,000 rpm for 10 minutes at 4C.

Step 3: Prepare cell lysate
1) Discard the supernatant produced during steps 2-4, add 0.75 mL of LB/PI to the sediment, and place on ice for 30 minutes. During the incubation for 30 minutes on ice, the cells and LB/PI should be mixed using the vortex mixer for a few seconds at 5, 10, 15 and 30 minutes after the start of incubation.
2) Centrifuge the tube at 15,000 rpm for 10 minutes at 4C and transfer the supernatant to a new tube.

Step 4: 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.

Step 5: Immunoprecipitation
Mix cell lysate (Step 3-2) and washed AC (Step 4-4) overnight using a rotator at 4C

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

Number of gels	1	2
30% Acrylamide/Bis solution (mL)	2.5	5
Running gel buffer (mL)	2.5	5
10% SDS (mL)	0.1	0.2
H2O (mL)	4.9	9.8
Total (mL)	10	20

↓ Mix (Do not make bubbles).

↓ Add the following per 10-mL of gel mixture.

10% APS, 50μL
TEMED, 5μl

↓ Pour into gel maker.

↓ Pour water over the surface of the gel and leave overnight at RT.

Day 4 SDS-PAGE and Western Blotting

(1) 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 x b
LB + NaCl: 1.5 x b
b: number of immunoprecipitation samples

2) Prepare 1x sample buffer
Dilute 6x sample buffer to 1x using H_2O.
Required amount of 1x sample buffer (μL): 40 x c
c: number of samples

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
Methanol (mL)	200	400
Total (mL)	1000	2000

(Add methanol after mixing 10x transfer buffer and H_2O, then fill to 1000 or 2000 mL using H_2O.)
Cool the buffer at 4ºC.

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.

(2) Prepare acrylamide gel

Stacking gel

Number of gels	1	2
30% Acrylamide/Bis solution (mL)	0.33	0.65
Stacking gel buffer (mL)	0.63	1.25
10% SDS (mL)	0.05	0.1
H_2O (mL)	1.53	3.05
Total (mL)	2.53	5.05

↓ Mix (Do not make bubbles).
↓ Add the following per 2.53 mL of gel mixture.
10% APS, 50 μL
TEMED, 1.25 μL
↓ 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 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 r.t. for 1 hour and at 95 for 5 minutes.
6) Spin for a short time and transfer the supernatant to a new tube.

(4) 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 1 hour.

(5) 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.

−
gel holder cassette
fiber pad
filter paper
gel
PVDF membrane
filter paper
fiber pad
gel holder cassette
+

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

Day 5 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 membrane: blocking buffer, 5 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.