

CHOW LAB WESTERN BLOT PROTOCOL

A. Preparation of Tissue Lysate

1. Anaesthetize and decapitate the mouse, take out the adrenal glands, cut the glands into pieces.
2. Collect tissue and lyse with 100 μ l RIPA buffer(with protein inhibitor). For $\frac{1}{2}$ gland, use 100 μ l buffer.
3. Homogenize around 30sec, till the tissue is very small.
4. Spin at 13,000 rpm (16,000 g) in an Eppendorf tube for 12 min.
5. Transfer the supernatant to a new tube and discard the pellet.
6. Determine the protein concentration (see Protein Quantification Assay)

B. Running Bis-Tris Gel (Invitrogen #NP0322BOX)

1. Take 12 μ l sample and mix with 4 μ l 4x Sample Buffer (Invitrogen #NP0007), add 1.6 μ l 10x Reducing Agent (Invitrogen #NP0004, - 4 °C)
2. Heat sample at >70°C 10 min
3. Load 5 μ l Protein Standards (BIO-RAD #161-0374, -20 °C)
4. Prepare 1x SDS running buffer (Invitrogen #NP0002, rt), fill the upper chamber 200ml, for reduced samples add 500 μ l antioxidant (Invitrogen #NP0005, - 4 °C) into the upper chamber, lower chamber 800ml.
5. Run the gel with 200 V constant voltage, 50 min. expected start current is 100-125 mA and end is 60-80 mA.

C. Preparation of Membrane

1. Use or cut a piece of PVDF (polyvinylidene difluoride) membrane (BIO-RAD #162-0174).
2. Wet for a few seconds in 100% methanol on a rocker at room temp till the membrane is translucent.
3. Transfer the wetted membrane into 1x Blotting buffer until it is equilibrated (After the membrane is equilibrated it is easy to be submerged into the buffer.) and ready to use.

Note: do not allow the membrane to dry (white spots will form where the membrane is dry)

D. Membrane Transfer

1. Prepare 1 liter of 1x transfer buffer, for reduced samples add 1 ml of antioxidant.
2. Assemble "sandwich", Pre-wet the sponges, filter papers (slightly bigger than gel) in 1x Blotting buffer. Make sure there is no air bubbles.
(-) Sponge - filter paper - gel - membrane - filter paper - sponge (+)
3. Transfer for 1 hr at 30 V constant voltage, expected start current is 170 mA and end current is 110 mA.
4. When finished, immerse membrane in Blocking buffer and block overnight.

E. Antibodies and Detection

1. Rinse in TNT buffer for 3 times, each time 10 min.
2. Incubate with primary antibody diluted in TNT buffer for 60 min at room temp.

3. Wash 3 x 10 min with TNT buffer.
4. Incubate with secondary antibody diluted in Blocking buffer for 45 min at room temperature.
5. Wash 3 x 10 min with TNT buffer, and rinse 3 x 10 min in PBS (no Tween-20).
6. Scan in appropriate channels (700 nm for Cy5.5 anti-body, 800 nm for IRDye800 antibody).

Step 4 - 6 use Odyssey Infrared Imaging System (Dr. Mircheff's lab)

Buffers for Westerns

25x Protein Inhibitor Solution, store at -20°C
1 tablet dissolved in 2 ml ddH₂O Roche Cat#1-697-498

RIPA buffer, store at -4°C
Tris-HCl 50 mM
NaCl 150 mM
EDTA 1 mM
Triton X-100 1%
SDS 0.1%
NP40 1%
Adjust PH 7.4 with HCl
Before use add Protein Inhibitor Solution

10x Transfer Buffer: 1 L
Trizma base 30.3 g (= 0.25 M)
Glycine 144 g (= 1.92 M)
pH should be 8.3; do not adjust

To make 1 L of 1x Blotting buffer:
200 ml Methanol
100 ml 10x Blotting buffer
700 ml water

TNT Buffer
Tween-20 0.5%
NaCl 0.15 M
Tris 25 mM
PH 7.4