Western Blot Notes

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1 Reagents and buffers

RIPA buffer, 10mL

- 10mL Thermo Scientific RIPA Lysis and Extraction Buffer (Cat. 89900)
- 100μL 100X Halt Protease Inhibitor (Cat. 87786)
- 100μL 100X 0.5M EDTA solution (Cat. 87786, included with the protease inhibitor)

10X running buffer, 1L *(250mM Tris, 1.92M glycine, 1%(w/v) SDS, pH8.3)*

- 30.30g tris base
- 144.10g glycine
- 10.00g sodium dodecyl sulphate (SDS)
- 1L water

Mix 100mL with 900mL water to produce 1L of final 1X working solution.

1X Trans-Blot Turbo transfer buffer, 1L

- 200mL 5X Trans-Blot Turbo transfer buffer (part of Bio-Rad cat. 1704272)
- 200mL absolute ethanol
- 600mL water

5X tris-buffered saline (TBS), 1L *(100mM tris, 750mM NaCl, pH7.6)*

- 12g tris base
- 44g NaCl

Dissolve in roughly 500mL water, adjust pH to 7.6 with HCL. Top up to 1L with water.

1X TBS with Tween©20 (TBST), 1L *(20mM tris, 150mM NaCl, 0.1%(w/v) Tween©20, pH7.5)*

- 200mL 5X TBS
- 800mL water
- 1mL Tween©20

Blocking buffer/secondary antibody buffer, 100mL

- 5g skim milk
- 100mL TBST

Primary antibody buffer, 100mL

- 5g bovine serum albumin
- 95mL TBST
- 5mL 1%(w/v) NaN_3

2 Sample preparation

2.1 Cell cultures

Refer to the procedures in the Bio-Rad general western blotting protocol here.

2.2 Tissues and organs

1. Add approximately double the volume of RIPA buffer to the volume of the tissue, or just enough to immerse the tissue, and a little bit more. The less you add, the higher the final

sample protein concentration.

2. As there is no available sonicator in UTAS Newnham, blend the samples with a rotary homogeniser until no visible chunks are left. There are two rotary homogenisers available, one with a replaceable plastic tip for livers, and another with a permanent metal tip for tougher tissues like muscle.

3. Clean the tip with absolute ethanol, 70% ethanol, and water (in this particular order) between samples.

3 Protein quantification

Refer to the BCA assay protocol here.

4 SDS-PAGE

1. Normalise sample protein concentrations and add Laemmli sample buffer.

2. Boil samples at 95°C for 5 minutes.

3. Prepare gels and assemble the Mini-PROTEAN tank. You can buy the gels by following this link. **Note:** There are four available gel slots in the tank; two outer slots and two inner ones. Use the inner slots when running one or two gels as the voltage distribution across these slots are more even and thus result in straighter bands.

4. Load your samples using the fine pipette tips.

5. Run the gels at 150V until desired separation is achieved. Usually it takes around 45 minutes for the dye front to completely leach out the gel bottom. In the meantime, begin preparing for the next section (transfer/blotting).

6. Pry open the gel cassette **CAREFULLY** with the green prying tool in order to release the actual gel.

5 Blotting

Refer to the kit instructions (Bio-Rad cat. 1704272) here.

Note: The transfer machine can be quite stubborn at times, refusing to recognise the cassettes. You will know when it displays the error message "NO LOAD DETECTED". When this happens, reload the cassette into the bay and try again. You can also try cleaning the metal contacts on the cassette, or swapping cassettes from one bay to the other. Also, for 10 minute high-molecular weight transfers, the cassettes get quite hot so you might want to pre-chill the

cassettes in the cold room. After the transfer is completed, move the membranes into TBST immediately to prevent them from drying out.

6 Checking transfer quality (optional)

1. Flood the membranes with Ponceau S Solution (Sigma cat. P7170) for about 5 minutes.
2. Rinse with distilled water until background is clear.
3. Take a few pictures with your phone camera or something.
4. Rinse with TBST a couple of times until the stain is mostly removed.

7 Primary antibody incubation

1. Flood the membranes with blocking buffer for one hour.
2. Tip out the blocking buffer, then rinse with TBST twice or until solution is clear. This is to prevent contaminating the primary antibody with skim milk.
3. Incubate membranes overnight (at least 16 hours for most antibodies) in primary antibody solution in the cold room (4°C).

8 Imaging

1. Tip the primary antibody solution into its original container for reuse.
2. Rinse the membranes three times (five minutes each time) with TBST.
3. In the meantime, prepare your secondary antibody solution in a 1:5000 dilution. For example, mix 2μL secondary antibody with 10mL blocking buffer to make 10mL of secondary antibody solution. You need approximately 7mL to adequately flood one of those 200mL Sistema blue containers from Coles, but just round it up to 10mL for convenience.
4. Incubate the membranes in the prepared secondary antibody solution at room temperature for one hour.
5. Repeat step 2.
6. Prepare 1mL of chemiluminescent substrate (ECL solution) by mixing 500μL Stable Peroxide solution with 500μ Luminol/Enhancer solution (both found in Thermo cat. 34094) in a foil-covered 15mL falcon tube. 1mL is adequate for two full-sized membranes. Prepare more later if necessary.

7. Turn on the Fujifilm LAS-3000 Imager during the last round of membrane rinsing, as it takes some time to cool down to the required sub-zero temperature.

8. Choose your first membrane(s)/antibody to be imaged. Place into a separate container and flood with the prepared ECL solution. Ensure all surfaces are covered in the ECL using a pasteur pipette.

9. Place the membrane into the imager, and ensure no giant bubbles are present under the membrane.

10. Close the imager door, and start the imaging process.

11. Repeat steps 8-10 for as many membranes/antibodies you have.

Antibody catalogue (Building C, Newnham Campus)

All primary antibodies here should be diluted 1:2000 in primary antibody buffer before use unless otherwise stated.

1. Anti-rabbit IgG secondary antibody **dilute 1:5000*
2. Anti-mouse IgG secondary antibody **dilute 1:5000*
3. AMPKα
4. Phospho-AMPKα (Thr172)
5. AS160
6. Phospho-AS160 (Thr642)
7. Akt
8. Phospho-Akt (Ser473)
9. Phospho-Akt (Thr308)
10. ATF-6
11. Cleaved Caspase-3 (Asp175)
12. CREB
13. Phospho-CREB (Ser133)
14. CHOP
15. eIF2α
16. Phospho-eIF2α (Ser51)
17. Erk1/2
18. Phospho-Erk1/2 (Thr202/Tyr204)
19. GAPDH **dilute 1:5000*
20. GFP
21. GSK-3β
22. Phospho-GSK-3β (Ser9)
23. Glut4
24. Insulin Receptor β
25. Phospho-IGF-I Receptor β (Tyr1131)/Insulin Receptor β (Tyr1146)
26. mTOR
27. Phospho-mTOR (Ser2448)
28. PTP1B
29. p38 MAPK
30. Phospho-p38 MAPK (Thr180/Tyr182)
31. p70 S6 Kinase
32. Phospho-p70 S6 Kinase (Thr421/Ser424)
33. SHP-2
34. Phospho-SHP-2 (Tyr580)
35. Phospho-Tyrosine (HRP Conjugated)
36. ZIP7/SLC39A7
37. 4E-BP1