Billy Hinchen with help from Tassos Pavlopoulos

Western Blotting for *Parhyale hawaiensis* embryos

Solutions

Homogenisation Buffer

1X PBS + Protease Inhibitors (these are at 10X, use at 1X)	15ul
Sample Buffer + B-mercaptoethanol	7.5ul

- Made as follows:
  - 100ul 3X sample buffer
  - 18.3ul B-mercaptoethanol

Mix this fresh each time.

5X Running Buffer		[1X buffer]
Tris	15.14g	25mM
Glycine	95.84g	250mM

Make up to1L with dH2O. Add SDS to 0.1%

Transfer Buffer

Tris	3.03g
Glycine	14.4g
Methanol	200ml (optional)

Make up to 1L with dH2O

Washing Buffer (PBST)
1X PBS + 0.1% Tween-20

Blocking Buffer
PBST + powdered semi-skimmed milk (add 5g to 100ml PBST)

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Protocol

PART A – Prepare Protein Samples

1. Place at least 100 embryos in 1.5ml eppendorf tube and remove as much ASW as possible
2. Add the 22.5ul homogenisation buffer prepared fresh.
3. Homogenise
4. Boil for 5 minutes
5. Spin at max ('SPAM') for 1 minute

Sample may be stored at -20ºC at this point.

PART B – Prepare gel and samples

1. Prepare a 5ml 10% Resolving gel solution
    a. the size is dependent upon the size of the protein of interest

dH2O	1.9ml
30% acrylamide mix	1.7ml
1.5M Tris (pH 8.8)	1.3ml
10% SDS	0.05ml
10% APS (ammonium persulphate)	0.002ml

Approximately 3ml is added between the glass plates.

2. Add ~300ul isobutanol along the top of this
3. Allow to set for ~15 minutes
4. Discard the isobutanol and rinse with dH2O
5. Prepare no more than 2ml stacking gel solution

dH2O	1.4ml
30% acrylamide mix	0.33ml
1mM Tris (pH 6.8)	0.25ml
10% SDS	0.02ml
10% ApS	0.02ml
TEMED	0.002ml

6. Fill the remaining space between the glass plates and add the comb
7. Boil samples for 5 minutes
8. SPAM for 10 minutes

PART C – Load and run gel

1. Load the plates in the bath, filling the middle space with 1X Running Buffer, ensure there is no leakage
2. Clean the wells by squirting some of the running buffer over them using a syringe
3. Fill the remaining outer sections of the bath with running buffer just to the point at which the bottom of the gel is covered
4. Load 10ul of sample per well, not forgetting to include ladders of your choice
5. Run at 50V until the samples reach the resolving gel and then progressively increase the current to 200V. You can stop when the bromophenol blue in the sample buffer runs out of the gel.

PART D – Transfer to membrane

1. Carefully separate the two glass plates and cut off and discard the stacking gel layer. Measure the width and height of remaining gel. Mark the top left corner (but cutting it off) to maintain a sense of correct orientation.
2. Incubate the gel in transfer buffer in a sufficiently large petri dish on a rotating platform for 15 minutes.
3. Cut 2 pieces of Whatman filter paper and 1 piece of membrane (be careful not to touch the membrane with you're your bare hands) to slightly larger than the gel. Mark the top left corner of the filer paper and membrane in the same manner as the gel.
4. Fill 2 large petri dishes, one with MeOH, the other with dH_2O in the fume hood.
5. Wash the nylon membrane for 10 minutes in the MeOH, then 5 minutes in the dH_2O, and then a further 10m minutes in transfer buffer. Be sure completely submerge the membrane in the washing solutions.
6. Assemble the electroblotting cassette as follows:
   -ve: Foam | Filer paper | Gel | Membrane | Filer paper | Foam :+ve

This should be assembled in the transfer buffer petri dish, ensuring to wet and smooth down (with the side of a 15ml falcon tube) each successive layer.

7. Once assembled, run in the gel tank, with cooling block, at 100V for 1 hour.
8. Carefully remove the membrane from the cassette and briefly wash first in MeOH (10 seconds) then dH_2O (5 minutes) as described in step D4.
9. Wash the membrane in Ponceau S Solution: do this in a Petri dish as described for the washes but use just enough to cover the membrane. Mark the now visible bands of the ladder using a pencil.
10. Wash the membrane in MeOH then dH_2O as described above.

PART E – Expose membrane to antibody

Washes/blocks are at room temperature on a rotating platform unless otherwise stated.

1. Block the membrane in blocking solution (do this in a petri dish), and leave for 1 hour.

2. Dilute primary antibody of interest to the desired concentration (typically 10o times more dilute than the concentration used for an antibody staining, e.g. if you used the primary at 1:500 for an antibody stain, use 1:5000 here) in blocking solution.
3. Seal (via heating) the membrane between 2 sheets of plastic on just 3 sides, add the antibody-block solution, and seal the final side. Cut off one corner of the small pack and remove all bubble present before resealing.
4. Leave over night at 4C on a rotating platform
5. Remove the membrane from its packet and wash 4 x 15 minutes in PBST
6. Apply your secondary antibody in a newly made plastic packet as described in step E3. Your secondary should similarly be a 100 time more dilute than you would normally use in an antibody stain.
7. Leave for 1 hour
8. Remove and wash 4 x 15 minutes in PBST

PART F – Develop/detect signal

1. Mix the chemiluminescence solutions A and B at a ratio of 1:40. You will need 0.1ml of mixed solution per cm^2 of membrane.
2. Soak the membrane, protein side up, in the solution for 5 minutes.
3. Discard excess solution, place the membrane between 2 sheets of clingfilm, and place into a metal x-ray cassette.
4. Under red light expose a new x-ray film to the membrane for up to 30 minutes (the time is dependent upon the required strength of signal), then develop. If you are not remaining in the dark room under red light for the entire time be sure to leave the membrane and x-ray film securely in the metal cassette.