Western Blots Of Zebrafish Embryos

(Source: S. O'Shea and M. Westerfield from Zebrafish Book 5th Edition)

Dechorionating & deyolking

1. Remove embryos from their chorions in batches of ~100 by placing in 1 mg/ml of pronase and swirling occasionally (5-10 minutes for 24 hpf embryos, 10-20 minutes for 3 day embryos). Finish dechorionation by gentle trituration using a Pasteur pipette. The chorions float and can be decanted. Rinse three times in cold Ringer's solution.

2. Transfer the embryos to cold Ringer's with EDTA and PMSF. Remove yolks by triturating with a glass pipette that has been drawn out to have a tip diameter approximately the size of the yolk.

3. Transfer the dechorionated, deyolked embryos to fresh, cold Ringer's solution and rinse twice.

4. Embryos can be frozen in liquid nitrogen and stored at 70°C at this point by transferring to a microcentrifuge tube and removing as much liquid as possible.

Solutions:

- Pronase:
  5 mg/ml pronase diluted to 1 mg/ml in Embryo Medium

- PMSF:
  Stock - 100 mM phenylmethylsulfonylfluoride in isopropanol. Immediately before use, add 30 l of stock/10 ml Ringer's (final conc. 0.3 mM PMSF).

- EDTA:
  Stock - 10 mM EDTA, pH 7.0. Add 1 ml of stock/10 ml Ringer's (final conc. 1 mM EDTA)

Preparation of gel sample

1. Remove from freezer and thaw the frozen, dechorionated, deyolked fish.
2. Microfuge for 1-2 minutes to pellet.
3. Remove excess liquid.
4. Add 150-200 l SDS sample buffer (for example, ~50-100 3 day embryos or 100-150 24 hpf embryos; this will yield enough for a 1.5 cm curtain or four 0.4 cm lanes of about 25-30 l each).
5. Homogenize with microfuge pestle until uniform in consistency.
6. Repeat step 5 until sample is no longer stringy.
7. Boil 5 minutes in a water bath.
8. Microfuge, 1-2 minutes.
9. Transfer supernatant to a new microfuge tube. Discard pellet or add more sample buffer and homogenize again if significant pellet remains.
10. Freeze at -70°C or run immediately on gel.

Solutions:

- SDS sample buffer:
  0.63 ml 1M Tris-HCl, pH 6.8
  1.0 ml glycerol
  0.5 ml -mercaptoethanol
  1.75 ml 20% SDS
  6.12 ml H2O
  (10 ml total)
  Store at -20°C in aliquots.

For cytoskeleton/extracellular matrix preps:

Add an extraction step between steps 3 and 4 (above).

1. Homogenize dechorionated, deyolked embryos in extraction buffer.
2. Incubate overnight at 4°C.
3. Centrifuge 20 minutes at 5000 x g.
4. Remove supernatant.
Solutions:

**Protein extraction buffer:**

10 mM Tris, pH 7.4  
2% Triton X 100  
1 mM PMSF  
1 mM aprotinin  
1 mM leupeptin  
1 mM trypsin inhibitor

5. Load, run and transfer gel

**Immunoblotting (using PVDF blotting paper):**

1. After the antigen is blotted, immerse the membrane at a 45° angle, into the blocking solution. Gently agitate for 60 minutes at room temperature or overnight at 4°C.
2. Decant the blocking solution and add TTBS to the membrane. Wash for 10 minutes with gentle agitation at room temperature.
3. Decant the TTBS and add the primary antibody diluted in 1% dried milk in TTBS. Incubate 4 hours at room temperature (or overnight at 4°C) with gentle agitation.
4. Remove the unbound 1° antibody by washing twice for 5 minutes each in TTBS.
5. Add alkaline-phosphatase conjugated 2° antibody solution (diluted 1:3000 in 1% dried milk in TTBS). Incubate 1-2 hours at room temperature with gentle agitation.
6. Wash membrane twice for 5 minutes each in TTBS and then, just before color development, once for 5 minutes in TBS to remove the Tween-20.
7. Immerse membrane in color development solution. Proteins present at 100 ng or greater will immediately become visible as purple bands. Lower amounts will take longer, but should be visible within 30 minutes. Staining can continue up to 4 hours.
8. Rinse membrane four times for 5 minutes each in dH2O.

Solutions:

**Blocking solution:**

3% dried milk in TBS

**TBS:**

20 mM Tris, pH 7.5  
500 mM NaCl

**TTBS:**

20 mM Tris, pH 7.5  
500 mM NaCl  
0.05% Tween-20

**Western Blot Color Development Solution:**

66 l nitroblue tetrazolium (NBT) stock  
33 l 5-bromo-4-chloro-3-indolyl galactopyranoside (BCIP) stock  
10 ml color development buffer  
Both NBT and BCIP stocks are 50 mg/ml in dimethylformamide. NBT stock is made by suspending 50 mg NBT in 700 ml dimethylformamide. Vortex. Add 300 l distilled water to dissolve. Store both stocks at 4°C in dark.

**Western Blot Color Development Buffer:**

102 mg MgCl2  
4.2 g NaHCO3  
500 ml d2O