Preparation Of Zebrafish Embryo Samples For Western Blots

(Source: M. Hille from Zebrafish Book 5th Edition)

General Information

Use 5 to 10 dechorionated embryos per gel lane. Prepare cell dissociation solution just before use. The inhibitors do not last more than a day.

Solutions:

Cell Dissociation Solution:

Use 2.5 or 5 ml as needed, but do not waste the protease inhibitors!

2.5 ml: To 2.5 ml of ice cold fish water or ice cold PBS, add ½ tablet of Roche Diagnosis Mini Completea and 0.25 mg of Sigma Soybean Trypsin Inhibitor (SBTI).b,c

5.0 ml: To 5 ml of ice cold fish water or ice cold PBS, add 1 tablet of Roche Diagnosis Mini Completea and 0.5 mg of Sigma Soybean Trypsin Inhibitor (SBTI).b,c

If you want to detect phosphorylated proteins, add orthovanadate (Na2VO4) to 10 mM from a 200, 400 or 500 mM stock made weekly.

2xSDS PAGE with reducing agent (1 mM DTT).

aThe Roche tablet is twice the recommended dose for mammalian cells. Embryos have lots of proteases, so we use a higher dosage. The Roche tablet has EGTA/EDTAb, so cells will dissociate in it. Do not add until the embryos are in a microfuge tube.

bEDTA and SBTI are great protease inhibitors. The Roche tablet has EDTA.

cAlternatively, use a cell dissociation solution with a separate protease inhibitor cocktail: 1 mM EDTA, 0.3 mM PMSF (probably useless), 0.1 mg/ml SBTI (Sigma), 70 µg/ml leupeptin (solutions cannot be frozen and thawed), and 70 µg/ml aprotinin (ICM). The Roche tablet has all of this stuff in it and maybe more. Reference: Henry et al 2001: Dev. Biol. 240, 474-487.

Procedure

Remember to work fast and keep solutions cold to decrease protease and phosphatase activities.

1. Day of isolation, get a Dewar of liquid nitrogen for quick freezing isolated cells and make the Cell Dissociation Solution.

2. Pipette out 200 µl of Cell Dissociation Solution in 1.5 ml microfuge tubes on ice.

3. Dechorionate 5 to 60 embryos.

4. Dechorionate and collect only a few groups of embryos at a time to catch the cells in a live state.

5. Drop 5-60 dechorionated embryos in each tube from the tip of a Pasteur pipette.d

6. Gently triturate the embryos with a cut pipette tip in the cell dissociation solution with a 200 µl pipetter about 4 to 8 times without making bubbles. Cut the end of the pipette tip to a size that will not shear the cells, a larger hole for cleavage stage embryos or a smaller hole for early somite stages. For late somite stage embryos, you can use the tip as is. Yolk will go into solution. Cells should remain intact.e

7. Centrifuge the microfuge tubes for 1 to 2 minutes at 4ºC at 500 rpm for cleavage stages, at 1000 rpm for early somite stages and about 2,000 rpm for late somite or prim stages. Again, the trick is not to break the cells, but collect them in the pellet.

8. To pellet cells adhering to the side-walls of the microfuge tube, rotate the tube 180º and centrifuge again for 1.0 minutes.

9. Carefully remove the supernatant with a 200 µl pipetter. You need not remove the entire supernatant at this point.

10. Add 200 µl of Cell Dissociation Solution and repeat the centrifugation in two directions.

11. Remove the supernatant.

12. Centrifuge the tubes again to pull all the solution down from the sides. Remove the last supernatant with a narrow gel loading pipette tip.

13. Add 1 to 2 µl of 2xSDS PAGE/ embryo. Because it usually takes only 5-10 embryos per lane to detect protein bands with antibodies, you can usually add 2 µl of 2xSDS PAGE/ embryo.

14. Vortex the cells and SDS Page solution, and drop into liquid nitrogen.

15. Before loading on a gel, boil the samples for 5 minutes to cleave the viscous DNA.f

Usually use 8% Tris-Glycine Denaturing Gels and 5 to 10 embryos per lane.
Use antibodies at 1/5000 or 1/2000 dilutions.
For more than 20 embryos, you should use 400 µl of cell dissociation solution. However, it is more difficult to triturate the embryos in this volume. To get rid of the majority of the yolk, you may need to wash the pellet once again with 200 µl of embryo dissociation solution and repeat the double centrifugation technique with a 180° turn. We routinely do a wash, but work fast and keep things cold.

The poke method for removing the yolk is described in Crawford et al. Mol. Biol. Cell 14, 3065-3081 (2003). I prefer the trituration method, especially after the heart starts beating, because it keeps the cells alive until cooled to 4°C and dispersed in protease inhibitors.

Cells can also be boiled before freezing in liquid nitrogen.