

pH3 Antibody Staining Protocol for Zebrafish

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[Abstract] Phosphorylation of histone H3 is tightly correlated with chromosome condensation during both mitosis and meiosis. Staining for phosphor histone H3 is a good indicator for the proliferation of cells of interest. This protocol provides a method to stain p histone H3 in zebrafish embryos.

Materials and Reagents

1. PFA
2. Phosphate buffered saline (PBS)
3. Acetone
4. Alcohol
5. PBS-Tween 20
6. H₂O₂
7. DMSO
8. Maleic Acid
9. p-Histone H3 antibody, 200 µg/ml (Cell Signaling Technology, catalog number: 9701)
10. Blocking reagent (Roche Diagnostics, catalog number: 1096176)
11. Diaminobenzidine (DAB) (Sigma-Aldrich, catalog number: D-5905)
12. Peroxidase-conjugated Affinity Goat anti rabbit Ig (Promega Corporation, catalog number: W4011)
13. Block solution (see Recipes)
14. DAB solution (see Recipes)
15. 10x MAB buffer (see Recipes)

Equipment

1. Shaker
2. Parafilm
3. Cold-room

Procedure

A. DAY 1:

1. Fix embryos in 4% PFA. Remove PFA before the staining.
2. 2x PBS (~5 min) rinses to remove remaining PFA. Incubate samples in -20 °C Acetone for 7 min.
3. Dump acetone (alcohol/acetone waste) and wash samples in water.
4. Do 2 x 5 min washes with PBST. Place the embryos on shaker at ~ 40 rpm for more effective washing.
5. While samples are washing, prepare block solution. Use 500 µl~1 ml/sample.
6. After 2nd wash, place samples into Block for at least 30 min on the shaker (nonspecific blocking step).
7. Dilute primary antibody (p-Histone H3 antibody, 200 µg/ml) 1/750 into the Block. (*Note: Primary antibody can be reused up to 2 times within the same week!*)
8. Shake samples O/N in the cold-room (at 4 °C). Seal tube with parafilm prevent evaporation of liquid.

B. DAY 2:

1. Do 4 x 5 min washes with PBST.
2. Dilute secondary antibody (Peroxidase-conjugated Affinity Goat anti rabbit Ig) at 1/300 into fresh block.
3. Shake secondary antibody for 2 h at room temperature (RT).
4. Remove and discard secondary antibody solution. Do 4 x 15 min washes with PBST.
5. While samples are washing, prepare DAB solution.
Note: Combine PBS and two tablets in a 50 ml conical vial. Wrap the mixture in tin foil, as the sample is light sensitive.
6. Add 24 µl hydrogen peroxide (30%) to DAB mixture (this activates the DAB).
7. Vortex solution, then transfer to samples (12 µl H₂O₂ for each tablet=15 ml of DAB mixture).
8. Remove and discard DAB (special waste stream).
9. Check if embryos have visible staining.
10. Wash samples in PBST to remove residual DAB.
11. Place samples into PFA for storage.

Note: Once samples are placed into PFA, samples must undergo acetone treatment if restained.

Recipes

1. Block solution: to make 100 ml of Block
 - 70 ml of PBST
 - 20 ml of block reagent (10% in Maleic acid buffer, MAB)
 - 10 ml of lamb serum
 - 1 ml of DMSO
2. 10x MAB buffer (1 L)
 - Maleic acid: 116.1 g
 - NaCl: 87.66 g
 - Water to about 800 ml
3. Start adding NaOH pellets while stirring the solution. The ppt will not solubilize until the pH reaches about 5.5 to 6.0. At this point, start adding the pellets VERY carefully as it is easy for the pH to overshoot. When the pH reaches 7.5, top up with water to the correct volume and then autoclave if desired.
4. DAB solution (developer)
 - 30 ml of DAB
 - 30 ml PBS
 - 2 DAB tablets (toxic)

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