**General antibody staining protocol for chicken embryos (I. Skromne, 02/23/10)**

For freshly collected embryos follow steps A-E. For embryos that have undergone In Situ Hybridization start below at step 1.

1. Collect embryos flat in appropriate buffer (1xTyrodes’, Pannett-Compton’s, Ringer’s, Hank’s, 1xPBS).
2. Fix embryos in 4% paraformaldehyde (PFA) in 1xPBS for 1-hour at room temperature or overnight at 4°C (do not over-fix). Make sure embryos remain flat, and if embryos have cavities (brain ventricles, heart), puncture organs to allow liquid flow.
3. Wash embryos three times, 15 min each wash in 1xPBT (1xPBS + 0.1% Tween-20).
4. Incubate embryos at 65°C fro 1-hour to inactivate endogenous alkaline phosphatase (do not over-bake embryos).
5. Continue with step 2 of the protocol.
6. After the color reaction of the In Situ Hybridization protocol has been completed, rinse embryos 3-times in 1xPBS and fix for 1-hour at room temperature with 4% paraformaldehyde (PFA) in 1xPBS. If necessary, flatten embryos before fixing. After embryos have been fixed, rinse them 3-times with 1xPBS.
7. Wash embryos twice, 15 min each wash in PBX (1x PBS, 1% Triton X-100).
8. Block embryos for 1 hour in blocking buffer (1x PBX containing 2mg/ml BSA and 10% goat serum).
9. Incubate embryos 36 hours (overnight + 1-day) at 4°C on a rocking platform in primary antibody made in blocking buffer. See dilutions of commonly used primary antibodies at the end of protocol.
10. At the end of incubation period wash embryos as follow.
	1. 3 quick PBX rinses. Don’t forget to save antibody for another day.
	2. 5 washes in PBX, each 1-hour long.
	3. 1 wash in blocking buffer.
11. Incubate embryos 36 hours (overnight + 1-day) at 4°C on a rocking platform in secondary antibody made in blocking buffer. See dilutions of commonly used secondary antibodies at the end of this protocol.
12. At the end of incubation period wash embryos as follow.
	1. 3 quick PBX rinses. Don’t forget to save antibody for another day.
	2. 5 washes in PBX, each 1-hour long.
13. Continue with the protocol appropriate for your secondary; i.e. fluorophore, biotin or HRP labeled secondary antibody.

**\*For biotin labeled antibodies** continue on step 9.

**\*For HRP-conjugated secondary antibody** continue on step 12 with the following modification: do the first wash for 1 hour and the second wash let go overnight at 4°C on a rocking platform.

**\*For fluorophore labeled secondary antibody** continue on step 19.

1. From the Vectastain ABC kit, mix together 40 ul solution A with 40 ul of solution B in 4 ml PBS in a glass tube, mixing gently, 30 minutes before you plan to use this solution. This amount of AB solution is enough for 1 tube; multiply these volumes accordingly for the number of tubes you are processing.
2. Incubate embryos for 1 hour in 4 ml of AB solution.
3. Wash embryos as follows. NOTE: Next washes are done in 1xPBS **without** Triton X-100.
	1. 3 quick rinses in PBS
	2. 1 overnight wash in PBS. Leave embryos on rocker at 4°C.
4. Wash two times for 1 hour in 1xPBS.
5. Prepare the following mix in a glass tube. Multiply amounts according to the number of tubes you have. NOTE: Wear gloves, as DAB is a carcinogen. Protect DAB form light.

4.90 ml of water

4.90 ml of Phosphate Buffer

 0.1 ml of DMSO

 0.125 ml of DAB stock solution (from freezer)

1. Incubate in DAB solution for 30 minutes in the dark.
2. Replace used DAB solution with fresh one.
3. Take 10 ul of a 30% hydrogen peroxide solution and add it to 1 ml distilled water. Take 10 ul of this diluted hydrogen peroxide solution and add it to the embryos.
4. Cover embryos and monitor reaction closely. This should start very quickly, within a couple of minutes. If you see a good-looking brown precipitate stop the reaction. Background can often be a problem.
5. Remove DAB solution and rinse embryos quickly with tap water.
6. Fix embryos in 4% paraformaldehyde (PFA) in 1xPBS for 30 minutes. Make sure the embryos are flat.
7. Store embryos at 4°C in 4% PFA in 1xPBS.

# Dilutions of commonly used antibodies

Primary antibodies

|  |  |  |
| --- | --- | --- |
| Epitope | Host species and Ig type | Dilution |
| Anti-Myc | Mouse IgG | 1:100 |
| Anti-Myosine heavy chain | Mouse IgG | 1:100 |
| Anti-GFP | Rabbit IgG | 1:500 |
| Anti-Phospho-Histone | Rabbit IgG | 1:200 |
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Secondary antibodies

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| --- | --- | --- | --- |
| Epitope | Conjugated to | Host species and Ig type | Dilution |
| Anti-Mouse IgG | Biotin | Donkey IgG | 1:1000 |
| Anti-Rabbit IgG | Biotin | Donkey IgG | 1:1000 |
| Anti-Mouse IgG | Alexa 488 | Goat IgG | 1:2000 |
| Anti-Rabbit IgG | Alexa 488 | Goat IgG | 1:2000 |
| Streptavidin | Alexa 546 | N.A. | 1:500 |
| Anti-Rabbit IgG | Cy5 | Goat IgG | 1:500 |
| Anti-Rabbit IgG | HRP | Horse | 1:200 |
| Anti-Mouse IgG | HRP | Horse | 1:200 |
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# Solutions

4% paraformaldehyde (PFA)

1xPBS

Distilled water

PBX = 1x PBS, 1% Triton X-100

Blocking Buffer = 1x PBX containing 2mg/ml BSA and 10% goat serum.

DAB stock solution =

Phosphate buffer

80 ml 0.1 M Na2HPO4 (26.8 g/L Na2HPO4-7H20 or 14.196 g/L of Na2HPO4 anhydrous)

20 ml 0.1 M NaH2P04 (13.8 g/L NaH2PO4-H20)

Make stock solutions of each buffer component and autoclave.

Mix indicated volumes.

Store at 4°C.