

CLEARING AND STAINING FOR LARVAL FISH¹

1. Fixation

- a. Fix fish in 4% paraformaldehyde in PBS for 2x overnight at 4°C.

2. Dehydration

For larvae less than 20 mm SL:

- a. Wash 2x 5 minutes in PBS.
- b. Dehydrate in 50% EtOH / 50% nano water for 24 hours.
- c. Dehydrate in 100% EtOH for 24 hours.

For larvae more than 20 mm SL:

- a. Wash 2x 5 minutes in PBS.
- b. Dehydrate in 50% EtOH / 50% nano water for 48 hours.
- c. Dehydrate in 100% EtOH for 48 hours with one intermediate solution change.

3. Staining cartilage

- a. Prepare cartilage staining solution. For 100 ml final volume:
70 ml 100% EtOH, 30 ml acetic acid, 20 mg Alcian blue.
- b. Transfer specimens to staining dish.
- c. Incubate in cartilage staining solution for 24 hours at room temperature with mild agitation.

4. Neutralization

- a. Wash in saturated sodium borate solution for 9-12 hours.

5. Bleaching (optional)

- a. Prepare bleaching solution. For 100 ml final volume:
15 ml 3% hydrogen peroxide, 85 ml 1% potassium hydroxide.
- b. Incubate in bleaching solution for:
20 minutes – specimens less than 20 mm SL
40 minutes – specimens more than 20 mm SL

6. Trypsin digestion

- a. Prepare digestion solution. For 100 ml final solution:
35 ml saturated sodium borate, 65 ml nano water, and 1 g trypsin.
- b. Incubate in solution at room temperature with mild agitation until specimens are 60% clear.

7. Staining bone

- a. Prepare bone staining solution: 1% potassium hydroxide with 0.1 g/100 ml Alizarin red.
- b. Incubate in bone staining solution for 24 hours at room temperature with mild agitation.

8. Destaining

For specimens less than 20 mm SL:

- a. Incubate in trypsin digestion solution for 40-48 hours.

For specimens more than 20 ml SL:

- a. Incubate in trypsin digestion solution until specimen is clear and solution remains unstained. Change to fresh solution every 2 days.

9. Preservation

- a. Transfer to 30% glycerol / 70% of 1% potassium hydroxide. Incubate 2-3 days at room temperature with mild agitation.
- b. Transfer to 60% glycerol / 40% of 1% potassium hydroxide. Incubate 2-3 days at room temperature with mild agitation.
- c. Transfer to 100% glycerol with thymol.

¹ adapted from: Potthoff, T. (1984). "Clearing and staining techniques." **In Ontogeny and systematics of fishes (based on an international symposium dedicated to the memory of Elbert Halvor Ahlstrom)**. H.G. Moser, W.J. Richards, D.M. Cohen, M.P. Fahay, A.W. Kendall, Jr. and S.L. Richardson, eds. (Lawrence, KS: Special Publication 1, American Society of Ichthyologists and Herpetologists, Allen Press), p. 35–37.