

PROTOCOLS, CONFIDENTIAL

Student: Brian Lohman

Host Lab: Dr. Michael Bell

Dates of Visit: 19 May – 30 Sept.

Title: Trypsin Cleared Alizarin and Alcan Double Stain

Rationale: To visualize bone and cartilage in whole specimens.

Fixing Protocol

1. Euthanize with MS-222
2. Fix with paraformaldehyde for 2 hours
3. Wash with 100% methanol
4. Place in 100% methanol for storage

Double Stain Protocol

1. Remove entrails of larger fish
2. Remove eyes of all fish
3. Immerse in trypsin solution for 12 hours
 - a. 0.1% trypsin in 30% saturated sodium borate
4. Wash for 10 minutes with 100mM Tris pH 7.5/10mM MgCl₂
5. Alcian stain with 0.02% Alcian/10mM MgCl₂ for 30 minutes rocking
6. Wash with 80% EtOH/100mM Tris pH 7.5/10mM MgCl₂ for 10 minutes rocking
7. Wash with 50% EtOH/100mM Tris pH 7.5/10mM MgCl₂ for 10 minutes rocking
8. Wash with 25% EtOH/100mM Tris pH 7.5/10mM MgCl₂ for 10 minutes rocking
9. Bleach for 3 to 12 hours to clear melanophores
 - a. 15% - 3% hydrogen peroxide
 - b. 85% - 1% potassium hydroxide
10. Wash twice with 25% glycerol 75% 0.1 potassium hydroxide for 10 minutes
11. Alizarin stain with 0.01% Alizarin stain for 30 minutes rocking
12. Destain with 50% glycerol 50% 0.1 potassium hydroxide rocking 10 minutes
13. Transfer to 75% glycerol 25% 0.1 potassium hydroxide
 - a. At least 2 hours but until specimen has equilibrated (sinks)
14. Transfer to 100% glycerol with thymol for storage

References:

G. Dingerkus and L.D. Uhler. *Stain Technology* 52: 229 (1977)

M.B. Walker and C.B Kimmel. *Biotechnic and Histochemistry* 82: 23 (2007)

Bleach solution is used by the Parichy Lab and published in the Zebrafish book, 5th edition. The full protocol can be found at:

<https://wiki.zfin.org/display/prot/Clearing+And+Staining+For+Larval+Fish+Cartilage+And+Bone>