

## Alcian Blue – Alizarin Red Staining of Mouse Skeleton

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**[Abstract]** Our lab has used the Alcian blue-Alizarin red staining method (Hanken and Wassersug, 1981) with certain modifications to characterize skeleton deformities in mice lacking Pek/Perk, encoding a translational control eIF2alpha kinase (Zhang *et al.*, 2002). Our protocol to conduct this experiment is described here.

### **Materials and Reagents**

1. Neural buffered formalin (Sigma-Aldrich, catalog number: HT5014)
2. Alcian blue 8GX (Sigma-Aldrich, catalog number: A5268)
3. Trypsin (Sigma-Aldrich, catalog number: T1426)
4. Saturated sodium borate (Sigma-Aldrich, catalog number: S9640)
5. Alizarin red (Sigma-Aldrich, catalog number: A3882)
6. Thymol (EM Life Science, catalog number: TX0615-1)
7. Ethanol
8. Glacial acetic acid
9. Potassium hydroxide
10. Glycerol
11. KOH
12. Trypsin solution (see Recipes)

### **Equipment**

1. Conical tube

### **Procedure**

A. Adults:

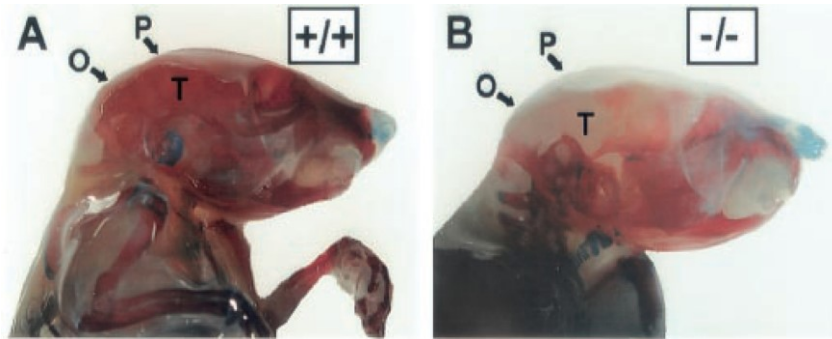
1. Fix mouse skeleton in 10% neutral buffered formalin for at least 24 h.

2. Rinse the sample in ddH<sub>2</sub>O O/N (1 h for embryos) with gentle shaking, and post-fix it in 70% ethanol.  
*Note: At this point, samples can be stored in 70% ethanol for a long period.*
3. Remove skins and internal organs carefully from the sample.  
*Note: Remove all skins, even those on the small toes.*
4. Stain the sample with 0.02% Alcian blue 8GX (prepared in ethanol/ glacial acetic acid, 7:3) for 1 to 2 days.  
*Note: Cartilage tissues will be stained blue.*
5. Wash the sample with plain ethanol/ glacial acetic acid (7:3) for 1 h.
6. Soak the sample in 100% ethanol O/N, and then in ddH<sub>2</sub>O for 1 to 2 days.
7. Treat the sample with 1.0% trypsin (prepared in water solution containing 30% saturated sodium borate) O/N.
8. Should limp and blue cartilage be readily observed at this point, proceed to stain the sample with Alizarin red (prepared in 0.5% KOH) O/N.  
*Note: Add enough (no specific amount) saturated Alizarin red until the solution appears dark purple. Mineralized bones will be stained red.*
9. Treat the sample with a gradient series of 0.5% KOH/ glycerol (*i.e.*, 2:1, 1:1, 1:2 and 100% glycerol, 2 days for each step), and store it in glycerol with a crystal of thymol.

#### B. Embryos:

1. A similar protocol can be used to stain embryos. To do this, fix embryos in 90% ethanol for at least 1 week.
2. Treat the sample with 0.01% Alcian blue 8GX for 3 days, and then perform rehydration through a gradient series of ethanol (70% ethanol, 2 to 3 h, twice; 40% ethanol, 2 to 3 h; 15% ethanol, 2 to 3 h; ddH<sub>2</sub>O, until the sample sinks to the bottom of a conical tube). Treat the sample further with fresh 1% KOH for 1 to 2 days until it becomes clear.
3. Treat the sample with 0.001% Alizarin red for 2 to 3 days until the bone becomes purple.
4. Rinse the sample ~ 3 times in 1% KOH, several hours each time.
5. Treat the sample through a gradient series of glycerol-KOH (20% glycerol/ 1% KOH, 24 h; 50% glycerol/ 1% KOH, 24 h; 80% glycerol/ 1% KOH, 24 h; 100% glycerol, 24 h x 2).

**Representative data**



**Figure 1.** This figure is adapted from the original (Zhang *et al.*, 2002). Shown here is Alizarin Red (mineralized bone) and Alcian Blue (cartilage) skeletal staining of 18-day-old wild-type A and *Perk*<sup>-/-</sup> mutant mice B. The mineralization of the flat bones of the skull (P, parietal; O, occipital; T, temporal) is greatly reduced in the *Perk*<sup>-/-</sup> mutant mouse.

**Recipes**

1. Trypsin solution
  - 1.0% trypsin
  - 30% saturated sodium borate
  - H<sub>2</sub>O

**Acknowledgments**

This protocol was adapted from previously described work by Hanken and Wassersug (Hanken and Wassersug, 1981). PZ was supported by a research assistantship in the Cavener lab at the Pennsylvania State University.

**References**

1. Hanken, J. and Wassersug, R. (1981). The visible skeleton. *Funct Photogr* 16(4): 22-26.
2. Zhang, P., McGrath, B., Li, S., Frank, A., Zambito, F., Reinert, J., Gannon, M., Ma, K., McNaughton, K. and Cavener, D. R. (2002). [The PERK eukaryotic initiation factor 2 alpha kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas.](#) *Mol Cell Biol* 22(11): 3864-3874.