Skeletal Prep Protocol
(Modified from Wallin et al., Development, 120: 1109-1121, 1994)
Protocol obtained from Steve Rodda of McMahon Lab

N.B.: Alcian Blue stains cartilage (blue) and Alizarin Red stains ossified bone (red).

Solutions to make:

1. **100% EtOH**

2. **100% Acetone**

3. **0.3% Alcian Blue:** 0.3g Alcian Blue 8GX (Sigma cat. #A5268)
   100ml 70% EtOH

4. **0.1% Alizarin Red[1]:** 0.1g Alizarin Red S (Sigma cat. #A5533)
   100ml 95% EtOH

5. **Alcian Blue/Alizarin Red Stain:**

	For 500ml:
1 volume 0.3% Alcian Blue	25ml
1 volume 0.1% Alizarin Red	25ml
1 volume Glacial Acetic Acid	25ml
17 volumes 70% EtOH	425ml

6. **1% (w/v) KOH:** 10g KOH
   990ml ddH_2O

Protocol:

1. For embryos >15dpc, skin and eviscerate animals[2].
2. Fix embryos in 100% EtOH for 24 hrs at RT on a rocker[3].
3. Transfer embryos to 100% Acetone for 24hrs at RT on a rocker.
4. Stain embryos in Alcian Blue/Alizarin Red stain for 3-4 days at 37°C on a rocker.
5. Rinse embryos 1X in ddH_2O.
6. Transfer embryos to 1% KOH and incubate at RT on a rocker for a minimum of 3hrs[4].
7. Transfer embryos to fresh 1% KOH and incubate O/N at RT on a rocker. (N.B.: depending on intensity of stain and amount of tissue remaining on embryos, additional KOH washes may be necessary).
8. Transfer embryos through the following series of glycerol/KOH washes:
   20% glycerol/1% KOH (24hrs at RT on rocker)
   50% glycerol/1% KOH (24hrs at RT on rocker)
   80% glycerol/1% KOH (24hrs at RT on rocker)
9. Store and photograph embryos in 80% glycerol/1%KOH solution at RT[5].

Notes:

[1]Alizarin Red S will not dissolve completely in 95% EtOH. It is a good idea to transfer some of the undissolved Alizarin Red S when preparing the Alcian Blue/Alizarin Red Stain.
[2]Use unfixed animals preferably. Protocol does work with 4% PFA fixed embryos, but not Bouin's fixed embryos.
[3]Store embryos in 20ml glass scintillation vials with plastic-lined caps.
[4]Aluminum-line caps will react with 1%KOH, and solution will leak out of vials!!
[5]For long term storage, transfer embryos to 80% glycerol (No KOH!), since dye will continue to leach out of skeletons in KOH solution.