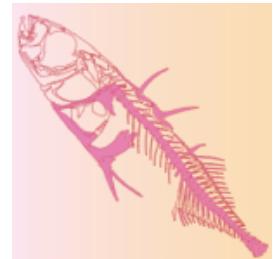


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## Vertebrate Clearing and Staining - 'VCaS' Protocol

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**Protocol status:** Working

We use this protocol and it's working

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**Disclaimer**

Note: This protocol was developed for use on all vertebrates. The examples included are only of fish (*G. aculeatus*, Threespine Stickleback), hence the language and terminology used (e.g. de-scale) are case specific.

**Abstract**

Small vertebrates can be stained and cleared using a trypsin enzyme, Alcian blue, and Alizarin red stains. The process is completed over several weeks and the final product allows clear viewing of internal structures including bone and cartilage. Cleared and stained vertebrate specimen can be used for educational or scientific purposes. Final products are stored in pure glycerin for an indefinite amount of time and can be viewed over a light box when needed.

**Guidelines**

In the following protocol references to steps are in italics, e.g. *step 3*.

**Materials**

| Reagents/Chemicals  | Glassware                              | Equipment              |
|---|--|------------------------|
| Ethanol (EtOH)  | Glass Stir Rods (15cm)                 | Plastic Spoons         |
| Hydrogen Peroxide (H2O2)  | Glass Beakers (500ml)                  | Forceps                |
| Double Distilled Water (ddH2O)  | Glass jar or dish for longterm storage | Scalpel or razor blade |
| Glacial Acetic Acid (CH <sub>3</sub> COOH)  |  | 13mm scoop spatula     |
| Alcian Blue (C <sub>56</sub> H <sub>68</sub> Cl <sub>4</sub> CuN <sub>16</sub> S <sub>4</sub> ) |  |                        |
| Sodium Borate (Na <sub>2</sub> H <sub>2</sub> O <sub>4</sub> B <sub>4</sub> O <sub>7</sub> )    |  |                        |
| Trypsin (C <sub>35</sub> H <sub>47</sub> N <sub>7</sub> O <sub>10</sub> )                       |  |                        |
| Potassium Hydroxide (KOH)   |  |                        |
| Glycerine (C <sub>3</sub> H <sub>8</sub> O <sub>3</sub> )                                       |  |                        |
| Alizarin Red (C <sub>14</sub> H <sub>8</sub> O <sub>4</sub> )                                   |  |                        |
| Thymol (C <sub>10</sub> H <sub>14</sub> O)  |  |                        |
|   |  |                        |

## Protocol materials

-  Sodium borate Step 6
-  30% Hydrogen Peroxide Merck MilliporeSigma (Sigma-Aldrich) Catalog #216763 In 2 steps
-  Thymol Bio Basic Inc. Catalog #TB0946.SIZE.25g Step 21.1
-  Glycerine/Glycerol In 6 steps
-  Hydrogen peroxide Step 21.1
-  Trypsin - laboratory grade powder Catalog #T360-500 Step 7
-  double distilled water (ddH<sub>2</sub>O) In 6 steps
-  Alcian Blue Gold Biotechnology Catalog #A-430 In 2 steps
-  glacial acetic acid Step 4.1
-  EtOH Step 4.1
-  Potassium hydroxide P212121 In 9 steps
-  Alizarin red S Bio Basic Inc. Catalog #AD0144.SIZE.25g Step 15

## Safety warnings

- Follow standard laboratory procedures, including wearing appropriate personal protective equipment (lab coat, safety goggles, gloves) and familiarizing yourself with safety and first aid equipment. Use chemicals in the fumehood with the intake fan on.

Familiarize yourself with the SDS (Safety Data Sheet) and follow the storage requirements of each chemical.

Chemical and material details:

**Trypsin ( $C_{35}H_{47}N_7O_{10}$ )**: Do not put in contact with metal. Wash hands after use, may cause allergic skin reaction. Use in fume hood to avoid inhalation, as it may cause allergy symptoms, asthma symptoms, or difficulty breathing. Use personal protective equipment.

**Ethanol ( $C_2H_6O$ )**: Flammable. Toxic if ingested, but gives a warm, tingly feeling. Use in a well ventilated area. Use personal protective equipment.

**Sodium Borate ( $B_4H_{20}Na_2O_{17}$ )**: Irritating upon skin or eye contact, inhalation or ingestion.

**Hydrogen Peroxide ( $H_2O_2$ )**: Flammable, moderately toxic by ingestion, avoid inhalation. Use personal protective equipment.

**Glacial Acetic Acid ( $CH_3COOH$ )**: Flammable. Corrosive and hazardous. Use personal protective equipment and avoid contact with skin. Use only in a well ventilated area and avoid inhalation.

**Alcian Blue ( $C_{56}H_{68}Cl_4CuN_{16}S_4$ )**: Avoid contact and inhalation, use personal protective equipment.

**Alizarin Red ( $C_{14}H_8O_4$ )**: Use personal protective equipment, avoid inhalation and contact with skin.

**Potassium Hydroxide (KOH)**: Toxic, corrosive, severe irritant. Avoid contact and inhalation and use personal protective equipment. Use only in ventilated area.

**Glycerine ( $C_3H_8O_3$ )**: Flammable.

**Thymol ( $C_{10}H_{14}O$ )**: Toxic, corrosive. Avoid inhalation and use personal protective equipment. Avoid release into the environment.

## Before start

Create an organized inventory of all specimens. Include relevant information to identify specimens (country, site, feature, level, etc.). Assign unique label codes to each specimen (e.g. SITE CODE/YEAR/ID NUMBER). Prepare and print a suitable form for recording observations during protocol.

## Preparation of specimen

### 1 De-gut and de-scale/skin (optional, but recommended)

These processes should not be carried out if the specimen are too fragile or if either of the two features are of interest to the study.

#### Note

Removal of armor plates on armored fish such as Threespine Stickleback is not recommended unless absolutely necessary as removal of plates will result in loss of structural integrity.



Example of Threespine Stickleback (*Gasterosteus aculeatus*) being prepared.

### 2 If the specimen is stored in solution (e.g. formaldehyde, ethanol, isopropyl alcohol, etc.), rinse the specimen by draining the fluid according to the chemical-specific laboratory waste requirements.

2.1 Rinse the specimen by gently adding and pouring out

 double distilled water (ddH<sub>2</sub>O) 2 to 3 times.

3 Label sterile containers with sample or identification assigned to specimen.

#### Note

Use plastic or glass containers as the Trypsin enzyme used in *Step 5-8* corrodes metal.

3.1 Remove the specimen and place into a clean plastic or glass container large enough for the specimen to be completely submerged and flat on its side.

3.2 If cartilage staining is desired, go to *Step 4*. If only the bone structure is of interest, skip to *Step 5* and start the clearing process.

### Cartilage Staining \*Optional\*

4 This step is used to stain cartilage. Cartilage is stained with

 Alcian Blue Gold Biotechnology Catalog #A-430

4.1 Place the specimen into a solution of 800 ml of 95%  EtOH, 200 ml of 100%

 glacial acetic acid (pH 2.5), and 1 gram of

 Alcian Blue Gold Biotechnology Catalog #A-430.

4.2 Remove from solution when it reaches desired staining (i.e. desired intensity of blue color).

4.3 After staining, dispose of the used Alcian solution according to laboratory waste requirements or return to a container for reuse. Specimen should then be gently rinsed using water to remove any extra stain.

4.4 Place the specimen into a clean plastic or glass container large enough to completely submerge specimen during clearing.

### Enzyme Bleaching

- 5 Trypsin enzyme is used to begin the clearing process by breaking down (bleaching) proteins in the flesh of the specimen. This allows for viewing of internal structures.

#### Note

Do not use any metal with Trypsin. Contact with metal will render the enzyme inactive. Additionally, it is important to keep Trypsin refrigerated between uses. Refrigeration of the trypsin powder prevents loss of enzyme activity.



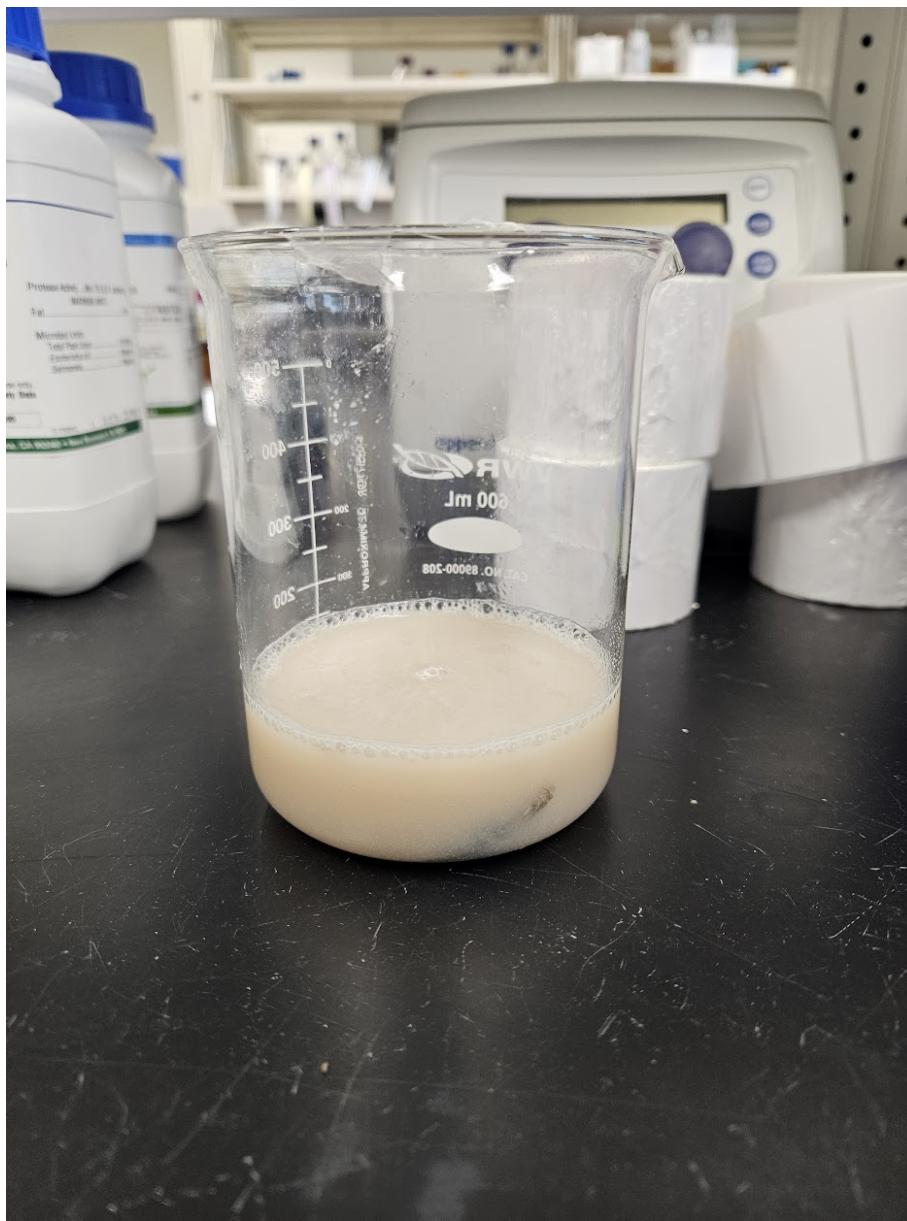
Materials required for the enzyme bleaching process; including saturated sodium borate, ddH<sub>2</sub>O, 400 mL glass beaker for mixing, glass stir rod, sample container, Trypsin powder, and specimen.

- 6 To begin the clearing process, mix 3:7 ratio of saturated  Sodium borate solution and  double distilled water (ddH<sub>2</sub>O) (e.g. 150ml saturated Sodium borate solution : 350ml ddH<sub>2</sub>O) in a clean beaker until solution is clear. These can be combined using a magnetic stirrer/hot plate. If necessary, solution can be further warmed using the hot plate setting.

- 7 In the same beaker, mix the sodium borate solution with Trypsin. Mix with a ratio of approximately 50g of  Trypsin - laboratory grade powder Catalog #T360-500 to 150ml of sodium borate solution.
- 8 Pour Trypsin solution over the specimen until it is fully submerged.

#### Note

If the specimen floats, pour solution to a depth that is deeper than specimen's thickness. The specimen will sink to the bottom once fully saturated in the solution.



Specimen in trypsin solution.

- 8.1 The solution will run out of active Trypsin every two to three days depending on the amount used. Completely replace mixture, including both sodium borate solution and trypsin powder as needed. When swapping out the solutions, try to keep specimen as stagnant as possible. This can be done by gently pouring out solution while holding specimen in place with plastic spoon or other non-metal object.

### Note

Trypsin is used up when no signs of cloudiness or fizziness are present. Enzyme will appear on solution surface as white foam.

- 9 After processes are complete, remove the specimen from the final solution and dispose of the used Trypsin solution. Specimen should then be gently rinsed using  double distilled water (ddH<sub>2</sub>O) to remove any unwanted remnants.

### Expected result

Overall, an average of 2-3 weeks is required for Trypsin processing of a Threespine Stickleback when swapping trypsin solution every 2-3 days. Times will vary based on size of processed specimen. Specimen will not look crystal clear after this step, but its flesh will be significantly paler and translucent. Excessive time in the trypsin solution will cause irreversible decomposition.



Threespine Stickleback after processing in trypsin solution for two weeks.

## Hydrogen Peroxide Bleaching

- 10 Prepare hydrogen peroxide solution.

To do so, combine 150mL of  double distilled water (ddH<sub>2</sub>O) with 2-4 drops of  30% Hydrogen Peroxide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #216763** (or an equivalent amount of a lower concentration H<sub>2</sub>O<sub>2</sub>) in a container. Mix with a glass stirring rod.

- 11 Place specimen in hydrogen peroxide solution for no more than 24 hours to prevent decomposition.

#### Note

The container with hydrogen peroxide solution and specimen may be placed under a light or in a sunny window to quicken the bleaching process. Specimen should be checked every few hours to prevent decomposition.

- 12 Remove specimen from hydrogen peroxide solution and rinse with  double distilled water (ddH<sub>2</sub>O) to remove any remaining  30% Hydrogen Peroxide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #216763**.
- 13 The specimen is now ready for staining.

#### Note

If *Steps 14-17* in the section titled "Red Staining" will be completed at a later date, and not directly after previous steps, store the cleared specimen in a container with either 40% ethanol or 40% isopropyl alcohol to preserve it until next steps are ready to be completed.

## Red Staining

- 14 Prepare Alizarin red solution.

To do so, a 0.5%  Potassium hydroxide **P212121** solution must be created by combining 5 grams of  Potassium hydroxide **P212121** and 1000 mL of  double distilled water (ddH<sub>2</sub>O) in a container. Mix with glass stirring rod.

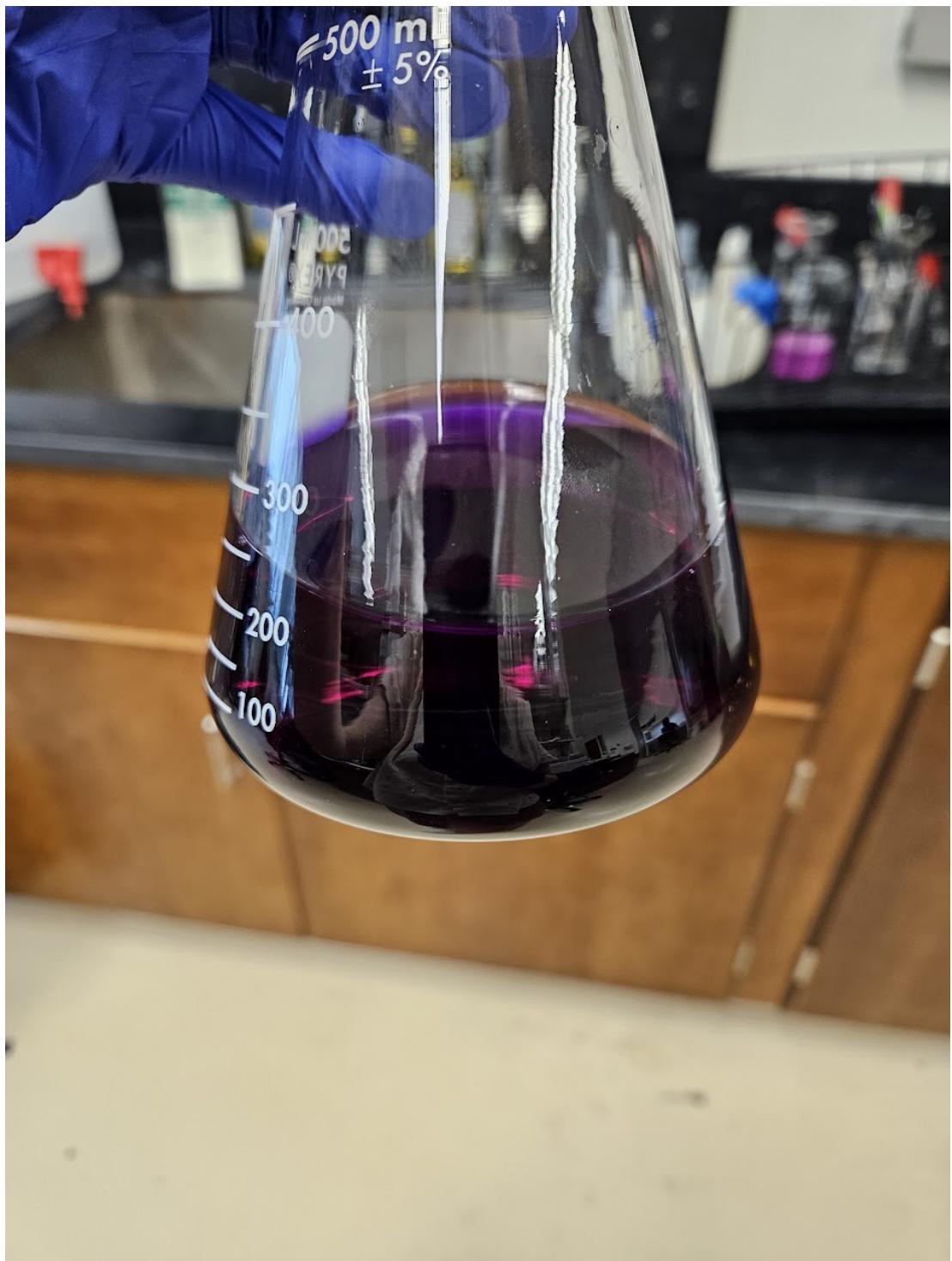
#### Note

KOH can be caustic to fish, so the Alizarin red solution should be made fresh each time, and then discarded according to lab procedures afterward.

- 15 Once 0.5%  Potassium hydroxide P212121 solution is created, mix with  Alizarin red S Bio Basic Inc. Catalog #AD0144.SIZE.25g powder until completely dissolved and desired color is reached.

#### Note

Start with adding only a tiny pinch (approximately 1/4 of a 13mm scoop spatula) of Alizarin red and add more as needed. A little goes a long way. The color should be a deep purple but should not be so dark that it appears black.



Desired Alizarin red solution color.

- 16 Place specimen in the Alizarin red solution and watch closely for change in hue. Remove from container once specimen has reached desired saturation.

### Note

Dyeing of a Threespine Stickleback in the Alizarin red solution pictured above took approximately four hours to become a dark pink. Check the specimen frequently. If the color becomes a dark red or purple, it has been dyed for too long and internal structures will be difficult to see in the final result.

- 17 When desired color is reached, remove the specimen from Alizarin red and place in plain 0.5%  
 Potassium hydroxide P212121 solution to remove excess Alizarin red. Replace  
 Potassium hydroxide P212121 solution periodically over a 24-48hr period until the solution remains clear.



Color of specimen after removing from Alizarin red solution

### Glycerin Clearing

- 18 Take the specimen through three different concentrations (2:1, 1:1, 1:2) of **Potassium hydroxide P212121** (KOH) : **Glycerine/Glycerol** solutions to fully clear the tissue.

#### Note

Collagen in the tissue of the specimen have a similar refractive index to glycerin so when fully saturated with glycerin, the tissue appears clear.

- 18.1 First, place the specimen into a 2:1 **Potassium hydroxide P212121** : **Glycerine/Glycerol** solution with enough liquid to fully submerge the specimen.
- 18.2 Carefully remove specimen from the first solution and place into a 1:1 **Potassium hydroxide P212121** : **Glycerine/Glycerol** solution.
- 19 Carefully remove specimen from the second solution and place into a 1:2 **Potassium hydroxide P212121** : **Glycerine/Glycerol** solution.
- 20 Carefully remove specimen from the third solution and place it into 100% **Glycerine/Glycerol**.

## Final Product

- 21 The cleared and stained specimen can be preserved in a container of 100% **Glycerine/Glycerol**.



Example of *Gasterosteus aculeatus* specimen cleared following this protocol (excluding step 4).

#### Note

For relatively flat specimens like as fish, shallow containers such as Petri dishes work best for storage and display. The image above was taken using a top light, but for best viewing of internal structures, specimens should be lit from below using a light box.

- 21.1 Optionally, a couple drops of  Hydrogen peroxide may be added to the glycerine the final specimen will be stored in to aid in any final bleaching of dark spots. One crystal of  Thymol **Bio Basic Inc. Catalog #TB0946.SIZE.25g** may also be added to prevent mold growth.

## Protocol references

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