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We use this protocol and it's working

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Stickleback Crossing Protocol

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Holly Valentine: Adapted from M. Currey, University of Oregon

Stickleback Stock Center

Holly Valentine

ABSTRACT

Included are the steps to remove eggs, dissect testes, and create crosses of stickleback.

MATERIALS

Solutions:

Make solution of Ginzberg's Ringers, store at 4° C.

1. Mix solids into 750 ml of npH2O
   - 6.6g NaCl
   - 0.25g KCl
   - 0.3g CaCl2
   - 0.2g NaHCO3
1. Bring to 1 liter total volume with npH2O.

2. Make testes solution (100ml)

- Add 100µl of Gentamycin and 100µl of Anti-biotic/mycotic to 100ml of Ginzburg's Ringers solution.
- Store at 4° C.

3. Stock MS-222 Solution (4g/L)
400 mg tricaine powder
97.9 ml DD water
~2.1 ml 1 M Tris (pH 9).

- Adjust pH to ~7.
- Aliquot 7.5 ml per 15 ml sterile tube
- Store in -20°C.

Euthanasia Solution (300 mg/L)

7.5ml of stock MS-222 solution
100 ml of fish water or SBEM

- Store at 4°C for up to 6 months

Anesthesia solution (168 mg/L)

4.2 ml of stock MS-222 solution
100 ml of fish water or SBEM

- **Make fresh for each use**

Stickleback Crossing
(Created 4/1/08 by M. Currey)
Materials Needed:

- 25, 45 and 90mm disposable sterile petri dishes (standard)
- Sterile Ginzburg's Fish Ringers solution containing antibiotic and antimycotics for testes (see Testes Solution recipe)

Gently pushing eggs from the female into a petri dish.

Materials:

- MS222 Tricaine Methanesulfonate (see Mesab recipe)
- 100% ethanol
- Fine scissors and forceps
- Wide-blade entomology forceps
- Sterile Embryo medium (6ml of Instant Ocean into 1L npH2O)
- Squeeze wash bottles
- Stress coat (standard from pet store)
- Sterile flat razor blades
- Sterile disposable large bore transfer pipettes (VWR cat# 691)
- Dissecting microscope

Squeezing eggs from Female

1 Squeeze Female: Wearing gloves, gently squeeze gravid female. Gravid females have extended abdomens and their eggs have “dropped”. If eggs do not emerge with very slight pressure the female is not ready. Squeeze eggs with motion from pectoral girdle posterior to the cloaca into a 25 mm sterile petri dish. The small size of petri dish allows the sperm to be concentrated on the eggs.

Dissect Testes from Male

2 Euthanize male by emersion in MS-222.

3 Clean a large, 30 cm by 60 cm or similar size, glass sheet, fine scissors, and forceps with 70% ethanol.

3.1 When male no longer responds to stimuli and opercular movement has ceased, remove and rinse fish with clean water and sever spinal cord behind skull to ensure euthanization.

3.2 Use scissors to make incision just posterior to the cloaca and cut anteriorly to the pelvic girdle making an incision along the mid-line of the fish. Make another cut to each side of the fish from the cloaca dorsally approximately 15 mm so that the body cavity is easily accessed. Incisions should be made just deep enough to cut the skin and body wall muscle while being wary to not cut into the stomach and intestines that are located just below the skin surface. Locate paired testes and vas deferens.

3.3 Testes are variable in shape and coloration, but are usually long and pigmented, usually having the same pigmentation as the skin surface, and sit in the dorsal part of the coelom near the kidneys. The vas deferens are threadlike and are usually as long as the testis to which it connects. Use fine forceps to grab vas deferens, sever near the cloaca, and remove one (or often) both testes. Doing so keeps the testes intact, allowing them to contain viable sperm for up to 1 month at 4°C in Testes Solution.

Storing of Testes:

4 If only a single cross is to be made with a male, proceed to step 5. Otherwise, place testes into a sterile 15 ml screw top conical tube containing 10 ml cold Testes Solution. Store testes at 4°C for up to 4 weeks. To store testes for extended periods, change out medium once a week.

Prepare Testis:

5 Remove one testis and place on the inside lid of the 25 mm petri dish into which a females eggs have been squeezed. Using a sterile razor, slice off a small piece of testis (a large testis can be used to fertilize approximately 5 clutches). Make cuts as perpendicular as possible to the major axis of the testis and perform multiple cuts from the same end. This allows as much of the sperm to remain packaged and inactivated at the center of the testis. Use blade to macerate testis. Under a dissecting scope swimming sperm should cause a 'sparkling' refraction. Add approximately 0.25 ml of sterile embryo medium to testis prep with disposable pipette, mix, and then add to the eggs one drop at a time, using same disposable pipette, dispersing drops between petri dishes so that all eggs are covered with sperm mixture. Add an additional 0.25ml to the dish to transfer remaining sperm from dish to eggs, one drop at a time.

Fertilization (time=0)

6 Fertilization of most eggs will occur almost instantaneously. However, incubation of the sperm with the eggs for about 5-10 minutes increases chances of a successful fertilization. At this point cover embryos with SBEM. Record fertilization time (this is the time that the initial drops of sperm were added) on the surface of the petri dish. Place in incubator at 20°C, or incubate at room temperature (about 23°C) for 2-4 hours for rapid development.

Separation of Embryos and Second Cleaning (time = 2 hr)

7 The animal pole of the embryo is established at the site of sperm entry, and at 20°C the first cell syncitium is usually visible within 45 minutes. The first cell division usually occurs approximately 25 minutes later, and the two cell stage is fully visible approximately 1.5 hours post fertilization (hpf). During this time, the chorion thickens and attaches to the petri dish as well as to other embryos. At or after 2 hpf, use the wide blade entomological forceps to detach the embryos from one another, as well as to dislodge embryos from the bottom of the petri dish. Remove all unfertilized eggs to limit mold contamination, and record the total number of eggs and embryos. Rinse embryos 3 – 4 X with SBEM, then distribute 30-50 embryos to 90 mm petri dishes with about 100 ml fresh SBEM. Enter cross information into database, print labels for dishes, and place embryos into incubator at 20°C.

Raise Embryos (check daily)

8 Embryos will develop at approximately 2.5 times zebrafish time [see http://zfin.org/zf_info/zfbook/stages/index.html], and hatch at approximately 7-9 dpf. 48 hpf stickleback embryos have completed most major morphogenetic processes, and the melanic pigment cells start to migrate. A beating heart can be seen after ~72 hr. Check petri dishes daily and remove those that have arrested development. Change SBEM every 3rd day to minimize death or developmental delays.