Derivation of Germ-Free Stickleback Fish

Items needed (IACUC approval needed):

Petri dishes
Kim wipes
Dissecting scissors
Razor
1L and 250 ml 0.22 µm vacuum filter systems, PES membrane
Two 50 ml 0.22 µm filters
Gloves
Sterile pediatric pipettes
Autoclaved 50 ml beakers covered with foil tops
Laminar flow hood
Individually wrapped sterile 25 ml and 2 ml pipettes
Tissue culture flasks – 150 cm^2 with 0.22 µm PTFE membranes on caps

Reagents needed:

MS222 (AKA tricaine) buffered with sodium bicarbonate
Stickleback embryo medium (SBEM)
70% ethanol
PVP-I
Bleach

Drug stocks (all stored at -20°C)

- 100mg/ml Ampicillin
- 50mg/ml Kanamycin
- 8mg/ml Amphotericin

1. Collect embryos as per approved protocol with the following modifications:
    a. Squeeze eggs from females into sterile petri dishes
    b. Clean the surface of the dissection area, scissors and forceps with 70% ethanol
    c. Euthanize the male in freshly made buffered MS222 made with SBEM
    d. Spray the outside of the euthanized male with 70% ethanol prior to dissection, and wipe body with Kimwipe
    e. Dissect the testes
    f. When testes have been collected, use clean, new razor to cut up the testes
    g. Fertilize eggs in filter sterilized antibiotic SBEM
    h. Incubate eggs at 20°C until eggs reach 2-8 cell stage, ~2 hours post fertilization
    i. Remove non-viable embryos from fertilized eggs, and rinse viable embryos with fresh antibiotic SBEM 2X

2. About 6 hours post fertilization, transfer viable embryos into 50 ml beakers
    a. Wear gloves
    b. Transfer viable embryos into sterile, autoclaved 50ml beaker or conical tube containing ~40 ml filter sterilized stickleback medium

3. Clean embryos in laminar flow hood wearing gloves
    a. Clean the following with 70% ethanol and transfer into the hood to minimize number of times entering and exiting the hood (per 100 embryos cleaned):
        i. 3 sterile 50 ml beakers with foil tops
        ii. Individually wrapped, sterile transfer pipettes or sterile, individually wrapped 2 ml pipettes
        iii. 1 L filter sterilized stickleback medium
        iv. 0.003% bleach
        v. 0.2-0.4% PVP-I (some flasks may be contaminated with 0.2% PVP-I, but more fish are likely to die in the 0.4% PVP-I)
        vi. 10 sterile flasks – 250 or 500 cm^2 sterile TC flasks (in hood, add 48ml sterile stickleback medium per 250 cm^2 flask or 98 ml per 500 cm^2 prior to adding embryos)
        vii. 25 ml or 50 ml pipettes, sterile, individually wrapped
        viii. Pipette aid
        ix. Embryos in 50 ml covered beaker
        x. Large beaker for collecting liquid waste
    b. Transfer embryos to clean beaker
        i. Pour off all but ~10 ml SBEM carefully into waste container
        ii. Transfer remaining 10 ml SBEM with eggs into clean 50 ml beaker
        iii. Add ~20 ml sterile SBEM to old beaker to get remaining eggs
        iv. Transfer remaining eggs to beaker
        v. Bring volume in new beaker up to 50 ml with sterile SBEM
    c. Rinse embryos 3X with 50 ml filter sterilized SBEM
    d. Immerse embryos in ~50 ml 0.2-0.4% PVP-I solution for 10 minutes
    e. Rinse embryos with sterile SBEM 1X
    f. Transfer embryos to fresh beaker as in step b
    g. Rinse embryos in sterile SBEM additional 2X
    h. Immerse embryos in 0.003% bleach for 10 minutes.
    i. Rinse embryos in sterile SBEM 1X
    j. Transfer embryos to fresh beaker as in step b.
    k. Rinse embryos additional 2X in sterile SBEM
    l. Transfer 20-40 embryos to flasks containing sterile SBEM using sterile, individually wrapped pipettes

4. Incubate embryos in light-controlled 18-20°C in incubator

5. At 9 dpf, upon yolk absorption, test water for contamination:
    a. Record the number of fish dead or not moving in response to external stimuli. In tissue culture hood, remove dead or not responsive (sick) fish. Euthanize sick fish with buffered MS222, as per IACUC approved euthanization protocol.
    b. To determine contamination of water:
        i. Plate 50ul to 100ul of water on TSA plates and incubate at 20°C

ii. Perform PCR of 16S ribosomal RNA gene from bacteria using standard 27F and 1492R primers of potential microbes collected on 0.22 µm filter

RECIPES:

Stickleback embryo medium (SBEM)

- 4 g Instant Ocean (Aquqarium Systems)
- 1 L dH_20
- 0.25 g Sodium bicarbonate

Drug stocks (all stored at -20°C)

- 100 mg/ml Ampicillin
- 50 mg/ml Kanamycin
- 8 mg/ml Amphotericin

Make the following up fresh each time:

Antibiotic Stickleback Medium

- 250 µl Ampicillin 100 mg/ml (100 µg/ml final)
- 25 µl Kanamycin 50 mg/ml (5 µg/ml final)
- 7.8 µl Amphotericin B 8 mg/ml (250 ng/ml final)
- 250 ml Stickleback medium
- Filter sterilize

0.003% Bleach solution

- 125 µl 6.0% bleach solution
- 250 ml SM
- Filter sterilize

0.2% PVP-I solution (Polyvinylpyrrolidone-iodine [0.01% free iodine] Western Chemical Inc.)

- 5 ml 10% PVP-I stock
- 245 ml SM
- Filter sterilize

0.4% Tricaine (3-amino benzoic acidethylester)

- 400 mg Tricaine
- 97.9 ml SBEM
- Adjust to pH 7.0 with sodium bicarbonate
- This can be made ahead of time and stored for long periods at -20°C or for up to 2 weeks at 4°C