Derivation of Gnotobiotic Stickleback Fish

Kat Milligan-McClellan[1]

[1]University of Connecticut

Dr. Napaaqtuk Lab
Tech. support email: **kathryn.milligan-myhre@uconn.edu**

Kat Milligan-McClellan
University of Connecticut

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Protocol status: Working
We use this protocol and it's working

Created: Jan 09, 2019

ABSTRACT

This protocol is used to generate gnotobiotic stickleback fish. Based on the zebrafish protocols, this was optimized for stickleback. See the paper https://doi.org/10.1242/dmm.021881 for the first description of the use of this protocol. This was created by Kat Milligan-McClellan with extensive help from Erika Mittge at the University of Oregon.

GUIDELINES

Use a biosafety cabinet class II to ensure sterility of fish. If this protocol is performed without a biosafety cabinet, sterility drops considerably.

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MATERIALS

Tricaine methanesulfonate MS222 **Sigma Aldrich**

Petri dishes, untreated, 100mm, sterile
Petri dishes, untreated, 20mm or 60mm, sterile
Kim wipes or paper towels
Sharp pointed dissecting scissors similar to this
Fine point forceps similar to this
Razor
1L and 250ml 0.22µm vacuum filter systems, PES membrane
Two 50ml 0.22µm filters
Gloves
Sterile pediatric pipettes
50ml beakers covered with foil tops and autoclaved
Biosafety cabinet class II
Individually wrapped sterile 25ml and 2ml pipettes
Tissue culture flasks – 25cm^2, 75cm^2, 150cm^2 or 300cm^2 with 0.2 µm PTFE membranes on caps
Jars with paper towels inside and on the bottom of the jars (to prevent scissors and forceps from being damaged)
Incubator or temperature controlled room for raising fish

Media:
Use double distilled (ddH$_2$0) or higher purity water (reverse osmosis or ultrapure water AKA Milli-Q or NANA-pur), for all reagents except the 70% ethanol and 10% bleach. Use a minimum of distilled water for the 70% ethanol and 10% bleach.

70% ethanol

- 736ml of 95% ethanol
- Bring to 1L with dH$_2$O
- Store in air tight container

10% bleach

- 10ml bleach
- 90ml dH$_2$0

Stickleback medium

- 4g Instant Ocean (goes into solution best if add a little at a time while stirring)
- 1L ddH$_2$0
- 0.25g sodium bicarbonate

- Can be stored at room temperature

10% PVP-I stock solution (Polyvinylpyrrolidone-iodine)

- 0.5g 0.01% free iodine, Western Chemical Inc)
- Bring to 50ml with ddH_20
- store at room temperature in foil covered, tightly sealed container

0.4% Tricaine, AKA MS222

- 400mg Tricaine ** Tricaine must be pharmaceutical-grade.
- 97.9ml stickleback medium
- Adjust to pH 7.0 with sodium bicarbonate
- Filter sterilize with 0.22um vaccuum filter
  This can be made ahead of time and stored for weeks at -20°C or for up to 1 week at 4°C

Make these fresh every time:

Antibiotic stickleback medium, filter sterlized

- 250ul Ampicillin stock of 100mg/ml (100ug/ml final)
- 25ul Kanamycin stock of 50mg/ml (5ug/ml final)
- 7.8ul Amphotericin B stock of 8mg/ml (250ng/ml final)
- 250ml stickleback medium
- filter sterilize with 250ml 0.22um vacuum filter system, PES membrane

0.003% Bleach solution

- 125ul 6.0% bleach solution
- 250ml stickleback medium
- Filter sterilize with 250ml 0.22um vacuum filter system, PES membrane

0.2% PVP-I solution (Polyvinylpyrrolidone-iodine (0.01% free iodine, Western Chemical Inc)

- 5ml 10% PVP-I stock
- 245ml stickleback medium
- Filter sterilize with 250ml 0.22um vacuum filter system, PES membrane

BEFORE START INSTRUCTIONS

- Tools for dissections should be rinsed in 10% bleach and soaked in 70% ethanol prior to use
- Gloves should be worn throughout the protocol to prevent contamination from skin microbiome
- Any person handling fish should be trained on proper fish care BEFORE starting this protocol and should receive IACUC or other training (see ethics statement below)
- $25cm^2$ flasks can hold about 10 eggs; $75cm^2$ flasks can hold about 20 eggs and 50ml of water; $150cm^2$ flasks can hold about 40 eggs and 100ml of water; $300cm^2$ flasks can hold about 80 eggs and 200ml of water

Fertilize eggs

1 Put on gloves

2 Gently squeeze eggs from females into sterile 20 or 60mm petri dishes. Place the cover on the eggs and transfer to the dissection area.

3 Clean the surface of the dissection area with 70% ethanol

4 Sterilize dissecting tools (scissors and forceps) with a brief rinse in 10% bleach followed by rinse in 70% ethanol

5 Euthanize the male in freshly made, filter sterilized tricaine/MS222 buffered with sodium bicarbonate

6 Spray the outside of the euthanized male with 70% ethanol prior to dissection, and wipe body with Kimwipe or paper towel

7 Dissect the testes out of the male fish by cutting from next to the anal vent to jaw, removing or pushing the intestine to the side, and using the sterile forceps to remove the testes. These are generally black but may be silver. Transfer the testes to the top of a sterile petri dish

8 When testes have been collected, use a clean, new razor to macerate the testes using lengthwise cuts and cross cuts

9 Add 5-10 drops of filter-sterilized antibiotic stickleback medium to the macerated testes with a sterile transfer pipette. Pippette up and down to fully mix the testes. The solution should be cloudy. Transfer 3-5 drops of the testes to each clutch of stickleback eggs. 1 set of testes is typically enough to fertilize up to 3 clutches of eggs.

10 Incubate eggs with sperm for 15 minutes at room temperature.

11 Add filter-sterilized antibiotic stickleback medium to the fertilized eggs to cover the eggs.

Prepare eggs for cleaning

12 Incubate fertilized eggs at 20-23°C until eggs reach 2-8 cell stage, ~2 hours post fertilization

13 Transfer fertilized eggs to sterile 100mm petri dishes and rinse in ~100ml sterile antibiotic stickleback medium

14 Remove non-viable embryos from fertilized eggs, remove egg goo with a sterile transfer pipette, separate eggs gently with sterile tweezers or paint brushes, and rinse viable embryos with fresh antibiotic stickleback medium 2X

15 About 6 hours post fertilization, transfer viable embryos into sterile autoclaved 50ml beakers with foil tops, about 100 eggs per beaker

16 add ~40ml filter sterilized stickleback medium to the eggs in the beaker

Prepare the hood

17 Clean the following with 70% ethanol and transfer the following items into the hood to minimize number of times entering and exiting the hood (per 100 embryos cleaned):

- 3 sterile 50ml beakers with foil tops
- Individually wrapped, sterile transfer pipettes or sterile, individually wrapped 2 ml pipettes
- 1 L filter sterilized stickleback medium
- 0.003% bleach
- 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)
- Large beaker for collecting liquid waste

18 Sterilize the items above by exposing them to UV light for a minimum of 10 minutes.

19 Turn UV light off, then bring in embryos. Spray outside of beaker with 70% EtOH prior to bringing embryos into the hood. MAKE SURE NO ETHANOL GETS INTO THE BEAKER WITH THE EGGS

Clean surface of chorions

20 Transfer embryos to clean autoclaved, foil covered beaker

21 Pour off all but ~10ml stickleback medium carefully into waste container

22 Transfer remaining 10ml stickleback medium with eggs into clean 50 ml beaker

23 Add ~20ml sterile stickleback medium to old beaker to get remaining eggs

24 Transfer remaining eggs to beaker

25 Bring volume in new beaker up to 50ml with sterile stickleback medium

26 Rinse embryos 3X with 50ml filter sterilized stickleback medium

27 Immerse embryos in ~50ml 0.2-0.4% PVP-I solution for 10 minutes

28 Rinse embryos with sterile stickleback medium 1X

29 Transfer embryos to fresh beaker as in step 21-25

30 Rinse embryos in sterile stickleback medium additional 2X

31 Immerse embryos in 50ml 0.003% bleach for 10 minutes.

32 Rinse embryos in sterile stickleback medium 1X

33 Transfer embryos to fresh beaker as in steps 21-25.

34 Rinse embryos additional 2X in sterile stickleback medium

35 Add sterile stickleback medium to tissue culture flasks.

- 25cm^2 flasks add 19ml of stickleback medium (assumes about 1ml of medium will be used to transfer eggs)
- 75cm^2 flasks add 48ml of stickleback medium (assumes 2ml of medium in transfer)
- 150cm^2 flasks add 96ml of water (assumes about 4ml of medium in transfer)
- 300cm^2 flasks add 194ml of water (assumes about 6ml of medium in transfer)

36 Transfer embryos to flasks containing sterile stickleback medium using sterile, individually wrapped pipettes. Tightly close flasks after transfer

- For 25cm^2 flasks add up to 10 eggs
- 75cm^2 flasks add up to 20 eggs
- 150cm^2 flasks add up to 40 eggs
- 300cm^2 flasks add up to 80 eggs

Post-cleaning fish husbandry

37 Move flasks to incubator in upright position to ensure eggs do not get stuck in the neck of the flask in the transfer

38 Incubate embryos in light-controlled 18-20°C in incubator, laying flasks flat. Make sure eggs do not go into the neck of the flask.

39 Check for contamination of water by microscopy throughout the experiment. Using an inverted light microscope, examine the inside of the bottom of the flask for cocci, rods, or filamentous microbes, which may be moving or stationary. Presence of microbes indicates contamination

40 Daily, record the number of fish dead or not moving in response to external stimuli. Always transfer flasks in upright position and ensure no eggs are in the neck of the flask.

41 Fish can remain in germ free flasks until 14 days post fertilization without food. At that point they will require food.

42 To determine contamination of water by culture, plate 50ul to 100ul of water on TSA plates and incubate at 20°C. Presence of microbes indicates contamination.

43 To determine contamination of flask by microscopy, visualize bottom of flask using inverted light microscopy under a minimum of 40X magnification. Presence of microbes on inside of bottom of flask indicates contamination.

44 To determine contamination of flask by 16S PCR, collect water on 0.22um filter, extract DNA from filter, and perform PCR of 16S ribosomal RNA gene using standard 27F and 1492R primers. Presence of a band of about 1400 bp from the PCR product on gel electrophoresis indicates contamination.