

Appendix A10-1

Protocol A10-1 Establishment of culture cells from medaka embryos

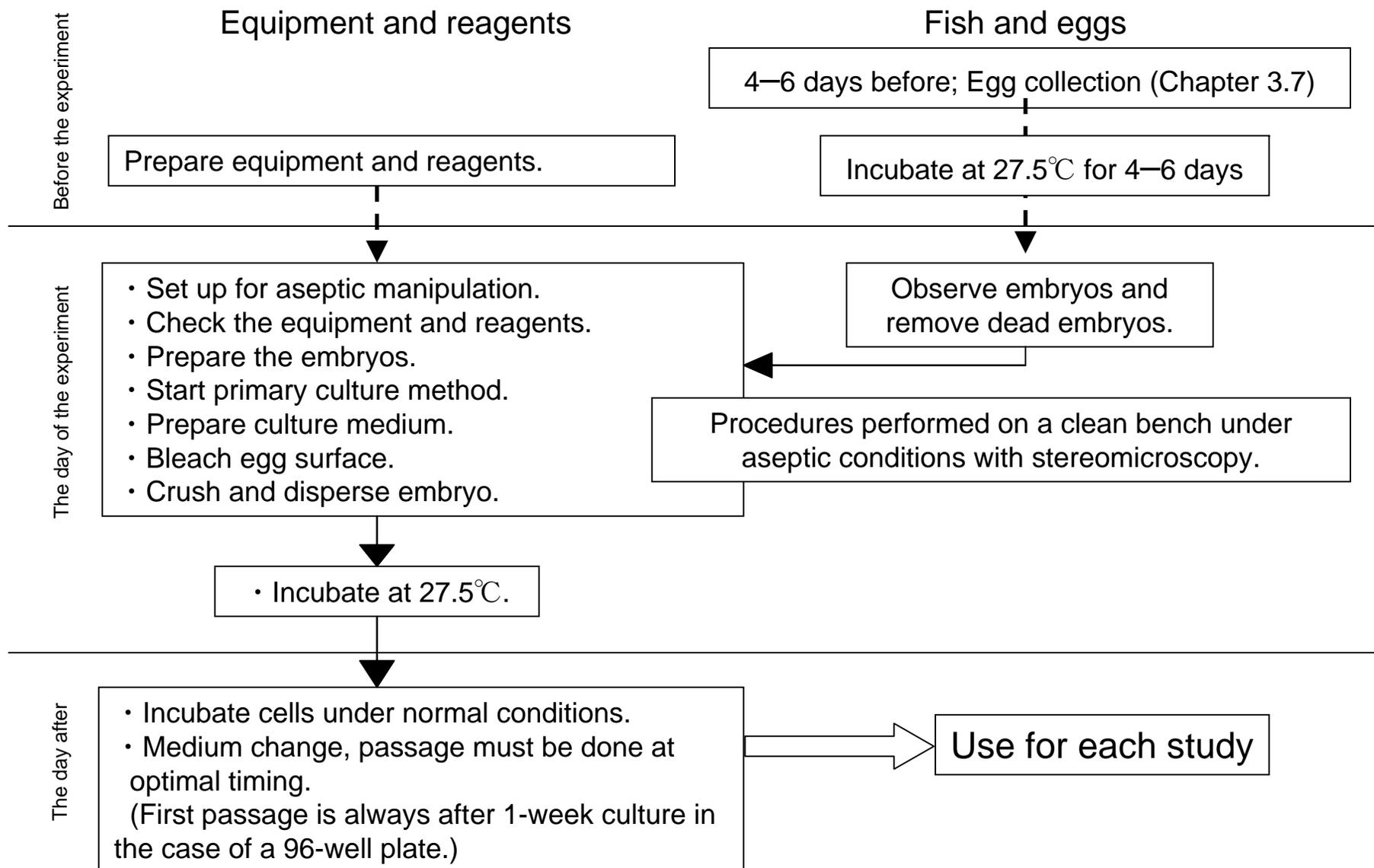
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1. Overview

In vivo studies using culture cells are very popular in biological research. As with mammalian cells, fish culture cells are commercially available or they can be established by yourself from not only wild-type fish but also from mutant or transgenic fish. You can precisely compare the wild type and homozygotic or heterozygotic mutants with the same genetic background by making cell lines from respective litter embryos. Meanwhile, comparative cellular analysis using fish and mammalian culture cells may provide more information on the functions of genes.

In this section, we will describe the establishment of culture cell lines from medaka embryos.

2. Flowchart of primary cell culture from medaka embryos



3. Equipment and reagents

Instruments

- Clean bench with basic tools (burner, aspiration tools, pipetter, pipettes, and tips)
- Stereo microscope (use in a clean bench)
- Inverted microscope (for cell observation)
- Incubator (without CO₂)
- tweezers (× 2)
- Chamber (to maintain humidity in the dishes)

Disposable labware

- Dishes (96- and 12-well flat bottom cluster plates, and 10 cm Petri dishes)
- 1 mL plastic syringes and 22-gauge injection needles (× number of embryos)

- 24-well flat bottom cluster plate is suitable for 1st passage. After the 2nd passage, select dishes suitable to your study.

Reagents

- PBS
- Culture medium
L15 medium with glutamine containing 20% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 10 mM HEPES (pH 7.2–7.5)
- Dakin's solution (bleaching solution: final 0.4% sodium hypochlorite)
(Preparation procedure)
 - 1) 21.6 mL of sodium hypochlorite (10%), add DW up to 200 mL
 - 2) 3 mL of 1N HCl, add DW up to 200 mL
 - 3) 4 g of NaHCO₃, add DW up to 60 mL
 - 4) Mix solutions from steps 1–3, add DW up to 450 mL
 - 5) Adjust to pH 8.8 using NaOH, add DW up to 500 mL
- 70% EtOH

Embryos

- 4–6 days post-fertilization (dpf) -stage embryos

4. Procedure for primary culture from 4–6 dpf embryos



Equipment and reagents required

(A) Eggs (4–6 dpf embryos)

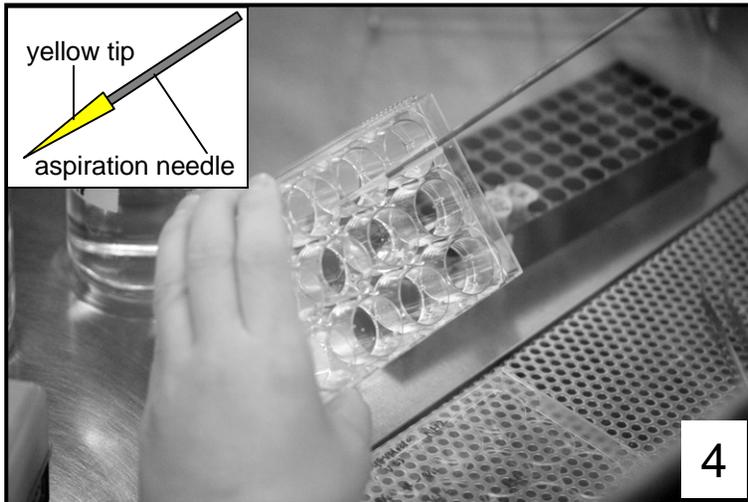
(B) 1 mL disposable syringe and 22 G needle

(Prepare the same numbers of syringes and needles for the embryos to avoid cross contamination)

- Stereo microscope
- Pipettes, pipetter, tips, and aspiration needle
- tweezers (× 2)
- PBS, culture medium
- Tools for sterilization

Special reagents for this method.

- Bleaching solution, 70% EtOH



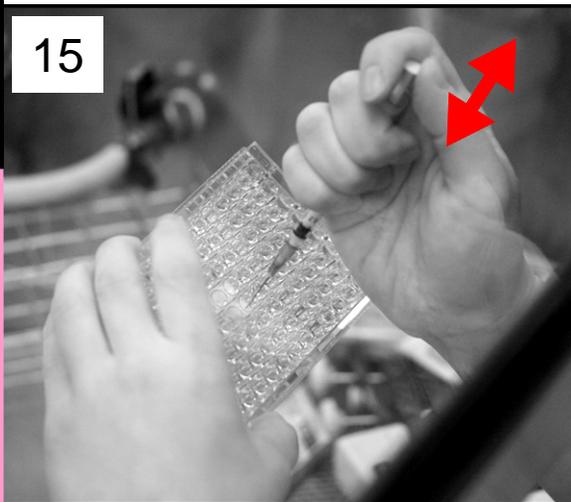
Protocol

0. Set up a clean bench and clean your hands.
(Start aseptic manipulation.)
1. Add 1 mL PBS into a well (12-well plate).
2. Transfer eggs into PBS using tweezers.
3. Rinse the eggs.
4. Remove PBS by suction (with yellow tip).
5. Add 1 mL bleach solution and start counting down.
6. After 40 seconds, quickly remove the solution, and rinse with 1 mL of PBS.

continue...



7. After removing PBS, soak in 70% EtOH for a few seconds, quickly remove the solution and rinse with 1 mL of PBS.
 8. Add 150 μ L culture medium into a new well (96-well plate, 1 well/1 embryo).
 9. Add 10 mL (or more) PBS to a new P10 Petri dish.
 10. Transfer embryos to a P10 dish using sterilized tweezers.
 11. Move to a stereo microscope.
 12. Sterilize two pairs of tweezers by flame.
 13. Remove the chorion carefully with tweezers (if possible, remove yolk sac and blood), then pick up the embryo and transfer it to a medium-filled 96-well plate with tweezers.
 14. Disperse embryonic cells by pipetting 5–10 times using a syringe with a 22 G needle.
 15. Incubate at 27.5°C in the chamber.
— the next day —
 16. Check the cell on the next day (remove the medium when you discover contamination.)
 17. Check the cell again within a week for passage.
- Refer to “Notes” for passage.



5. Notes

About the protocol

- The reason for using a yellow tip over the aspiration needle is to avoid drawing in the eggs.
- Avoid excess bleaching and EtOH treatment, and keep the restricted time.
- From our experience, a P10 Petri dish can be used for 10–15 embryos in the chorion-removing step without cross contamination.
- Do not mind bubbling in the step for dispersing embryos by pipetting; however, direct contact between the bubbles and the lid may cause contamination.

About the timing of passage

- Always allow the primary culture cells to proliferate to fully confluent cells in a 96-well plate for a week, then execute the passage to a 24-well plate using all the harvested cells.
- Do not execute the passage before a fully confluent culture. Low-confluency passage results in the termination of the cells. (If there is a bias in the cell density, you must execute the passage to a well of the same size and wait for fully confluent cells.)

About serum concentration

- A safe concentration of serum (FBS) in the medium is 20%.

Some lines may be able to grow healthily in 10% serum conditions, so that you can maintain these established lines using 10% serum. Meanwhile, some lines cannot live in 10% serum conditions even if the reduction of the serum concentration is gradual.

Therefore, you must test the required serum concentration for each cell line.