# National BioResource Project (NBRP) methods

## for freezing zebrafish sperm and *in vitro* fertilization

These methods were established by National BioResource Project (zebrafish) based on a method originally developed by Utsumi et al. at Okazaki Institute for Integrative Biology (Utsumi, H., Koshida, S., and Takada, S., unpublished). Please acknowledge to National BioResource Project (zebrafish) when you describe this method in literature.

## freezing zebrafish sperm

### *Equipment*

- 2 ml cryotubes (IWAKI, 2782-002)
- 15 ml tubes (CORNING, 430766)
- 50ml tubes (CORNING, 430291)
- Lifelon tubes (Polyvinyl chloride, 8 mm inner diameter. 11 mm outer diameter, Kokugo)
- Styrofoam container with lid (inner W160 x D230 x H150 mm, outer W190 x D265 x H205 mm)
- Stainless-steel tube racks for 50 ml tubes
- Liquid nitrogen
- 0.5 ml tubes
- Pestle homogenizer (for 0.5 ml tubes)
- 20 ul glass capillaries (minicaps 20 ul, Hirschmann Laborgerate)
- Forceps
- Scissors
- Syringe needles
- Dissection plate (made of SYLGARD, Dow Corning Toray, Co. Ltd.)
- Dissection microscope

## *Reagents*

- Tricaine solution (4.2ml Tricaine stock solution/100ml distilled water) [(1)]

  Tricaine stock solution: Dissolve 400 mg of tricaine powder (SIGMA, A5040) in 98 ml distilled water. Adjust the pH of the solution to 7.0 by adding ~2 ml of 1M Tris (pH 9) followed by adding distilled water to make total volume up to 100 ml.

- Cryopreservation solution (10 % DMF-FBS) [(2)]

  Add 90 μl of FBS (standard fetal bovine serum, Hyclone) and 10 μl of DMF (N,N-dimethylformamide, Nacalai tesque) in 0.5 ml tubes and chill the tube on ice.

## *Adult male fish*

Keep male fish separate from female fish a week before testis isolation.

## *Method*

Room temperature should be kept around 20 °C.

1. Set 50 ml tubes in a tube rack and tie up the tubes to be immobilized (Figure 1).

(Figure 1)

2. Cut the lifelon tubes into 4 cm length and insert these into the 15 ml tubes.

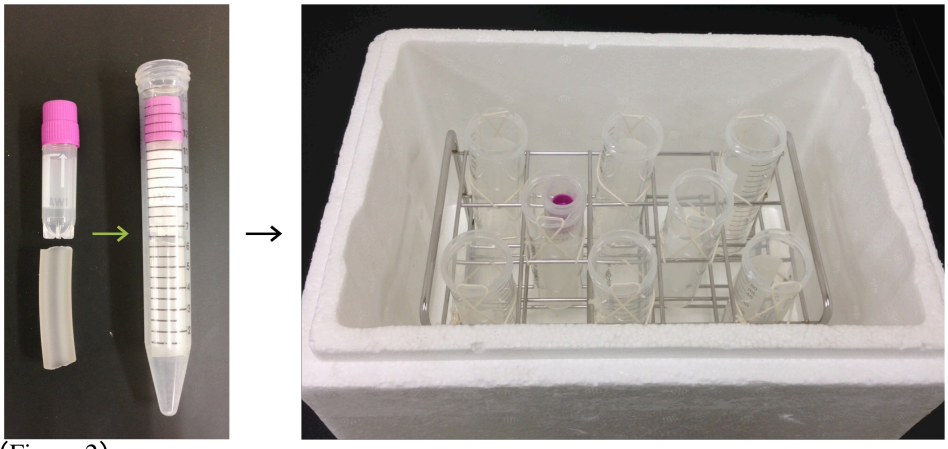
3. Place the 50 ml tubes and tube rack in the styrofoam container, and then, pour liquid nitrogen into the container. Inside of the tubes should be free from liquid nitrogen. Put a lid with a weight (~2 kg) on the container and pre-chill the 50 ml tubes for more than 20 min.

4. Anesthetize the fish by bath application of tricaine solution.

5. Dry briefly the anesthetized fish with paper towel and put on a dissection plate. Cut the abdomen along with midline and open it. Fish is pinned to the plate to keep open the abdomen using syringe needles, and then, dissect the testis with scissors and forceps. As sperm can be activated by contact with water, forceps should be washed with 10% DMF-FBS to minimize sperm activation. Transfer the isolated testis into the prepared pre-chilled 10% DMF-FBS in 0.5 ml tubes and homogenize the testis with pestle. Homogenates should be kept on ice.

6. Pipette 10 ul of the testis homogenates and fill the homogenates inside glass capillary. This procedure results in obtaining 9 capillaries/specimen. Put these capillaries in 2 ml tubes. The capillary end where the homogenate has been injected should be kept upside.

7. Insert the lifelon tube followed by 2 ml tube holding samples softly into a 15 ml tube, and then, insert the 15 ml tube softly into the pre-chilled 50 ml tube prepared in step 3 (Figure 2). Close the lid of the styrofoam container tightly by putting a weight (~2 kg) on top of the lid. Make sure that the cold nitrogen gas does not leak from the container.



(Figure 2)

8. Freeze the homogenate-containing capillaries for 20 min in the vapor phase of liquid nitrogen, and store the 2 ml tubes in liquid nitrogen or -150 °C freezer until use.

**Note:** As sperm cells are able to move even in cold 10% DMF-FBS, freezing steps from 4 to 7 should be completed promptly. Keep the lid of styrofoam container closed during 20 min of the homogenate freezing process.

## *in vitro* fertilization

### *Equipment*

- Cryopreserved sperm vials
- 9 cm Petri dish
- Small plastic teaspoon
- Micropipette

### *Reagents*

- Hank's solution (0.137 M NaCl, 5.4 mM KCl, 0.25 mM $Na_2HPO_4$, 0.44 mM $KH_2PO_4$, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$)
- Tricaine solution (described above)
- Methylene blue water (500 ml system water added with a small drop of concentrated methylene blue solution)

### *Adult female fish*

Keep female fish separate from male fish a week before egg collection.

### *Method*

1. Anesthetize the fish by bath application of Tricaine solution.
2. Wipe the fish gently with a paper towel.

3. Rinse the fish with Hank's solution and gently place the fish on a paper towel to dry briefly. As eggs are activated by contact with water that may interrupt fertilization, the area around genital papilla should be kept dry.

4. Place the fish on a 9 cm dish and squeeze eggs from the fish by sweeping the belly by a finger from pectoral fin to the tail. The finger used for sweeping should be moistened with Hank's solution for smooth movement, and thereby preventing the fish from injure. If the fish has eggs, these will be easily squeezed by gentle pressure. Do not continue to squeeze when you cannot obtain any eggs by gentle pressure.

5. Carefully collect the eggs from the body of the fish with a plastic teaspoon. Pick out one capillary from the sperm vial in liquid nitrogen. Wait for a while until the sperm homogenate dissolves. Pipette the homogenate and expel on the collected eggs.

6. Add 800 ul methylene blue water to the mixture of sperm and eggs to activate sperm cells, and thereby promote fertilization.

7. Let the sperm and eggs keep together until the egg membrane swells. Add methylene blue water to the level up to half the volume of the dish. Incubate the dish at 28 °C.

8. Several hours later, confirm fertilization and transfer the fertilized eggs to fresh methylene blue water.

Note: Step 5-6 should be completed promptly to prevent the eggs from drying.

***References***

(1) Westerfield, M. (2000) "The zebrafish book" 4th ed., Univ. of Oregon Press, Eugene.

(2) Aoki, K., Okamoto, M., Tatsumi, K., and Ishikawa, Y. Zoological Science (1997) 14: 641-644. Cryopreservation of medaka spermatozoa.