Protocol

In Vitro Fertilization of the African Turquoise Killifish *Nothobranchius furzeri*

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The ability to perform in vitro fertilization, together with sperm cryopreservation, greatly facilitates the long-term laboratory maintenance of wild-type and transgenic model organisms and helps prevent genetic drift. It is also useful in cases where reproduction may be compromised. In this protocol, we present a method for in vitro fertilization of the African Turquoise killifish *Nothobranchius furzeri* that is compatible with the use of fresh or cryopreserved sperm.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Buffered sperm motility–inhibiting solution (BSMIS), 1× <R>

Cryopreserved sperm (optional; see Step 5)

See Protocol: ***Sperm Cryopreservation of the African Turquoise Killifish*** **Nothobranchius furzeri** *(Dolfi et al. 2023).*

Double-distilled deionized H_2O

Embryo water (0.003-g/L methylene blue, Sigma-Aldrich M9140, in aquarium H_2O)

Fetal bovine serum (FBS, Gibco 10270-106)

Nothobranchius furzeri females aged 9–11-wk (appropriate age for strains in the GRZ-AD background; adjust accordingly for strains with different developmental times/life spans)

N. furzeri males aged 9–11-wk (optional; see Step 5)

Tricaine solution <R>

Prepare at 0.5 mg/mL for anesthesia; 1.5 mg/mL for euthanasia. Please consult your local animal use legal guidelines.

For this protocol, we did not adjust the pH of the 1.5-g/L tricaine (which in our hands is around pH 4 when made with system water). However, we believe that the pH of the tricaine can range from 4 to 7 and this will not impact the success rate of IVF.

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Equipment

Forceps
Glass slide (optional; see Step 11)
Incubator for raising embryos preset to 28°C
Microcentrifuge tubes (1.5-mL, for fresh sperm only)
Paper towels
Petri dish (10-cm)
Plastic Pasteur pipettes (2-mL)
Scissors (optional; see Step 5)
Stereomicroscope
Water bath preset to 30°C

METHOD

Plan your experiment, considering that one female typically provides sufficient eggs for one to two tubes of in vitro fertilization (IVF) with frozen or fresh sperm. Sperm quality is often highly variable; therefore, we recommend performing IVF with sperm from different batches and to include "backup" tubes of sperm, in case quality control indicates low-quality sperm (see Steps 6 and 8). Eggs cannot be frozen; therefore, we also recommend that experiments are planned in such a way as to use all collected eggs. We recommend performing this protocol with the help of another person.

1. Prepare 0.25× BSMIS by diluting 1 volume of BSMIS 1× in 3 volumes of double-distilled deionized H_2O. Prepare a sufficient volume for 120 µL to be used for each sperm aliquot.
2. Euthanize a female by transferring it to a tank containing room-temperature, 1.5 mg/mL tricaine solution until it stops breathing and opercular movements (repetitive "flapping" of the operculum/gills) are absent. With more time in tricaine, these movements will first become slow and regular, then slow and irregular, until ceasing completely. Larger females typically take longer to euthanize (2 min or slightly more for the largest females).

   Based on our experience, both mated and unmated females are suitable for egg collection. A potential advantage of mated females is that their fertility status is known. Fertility rates decrease in older females (Api et al. 2018); therefore, for the N. furzeri *GRZ-AD strain, we recommend using females at 9–11 wk of age.*
3. Carefully remove the euthanized fish from the water and dry it with a paper towel (Fig. 1A).
4. While wearing latex gloves, lay the female on an open palm, gently apply pressure to the abdomen with a finger, and move it down toward the anal fin until eggs come out (Fig. 1B).
5. Prepare sperm.

   Either fresh or frozen sperm can be used (Dolfi et al. 2021). The average fertilization rates with frozen sperm (~25%) are very slightly lower than those with fresh sperm (closer to 30%) but there is a lot of variability with both preparations. Both are less effective than natural fertilization.

For IVF Using Frozen Sperm

i. As the first egg emerges (Step 4), have your partner place a cryovial containing a 60-µL aliquot of frozen sperm for 30 sec to 1 min in the 30°C water bath until completely thawed. For even distribution of heat, gently shake the tube in the water bath (by hand, with moderate vigor—as if ringing a hand bell) (Fig. 1C).

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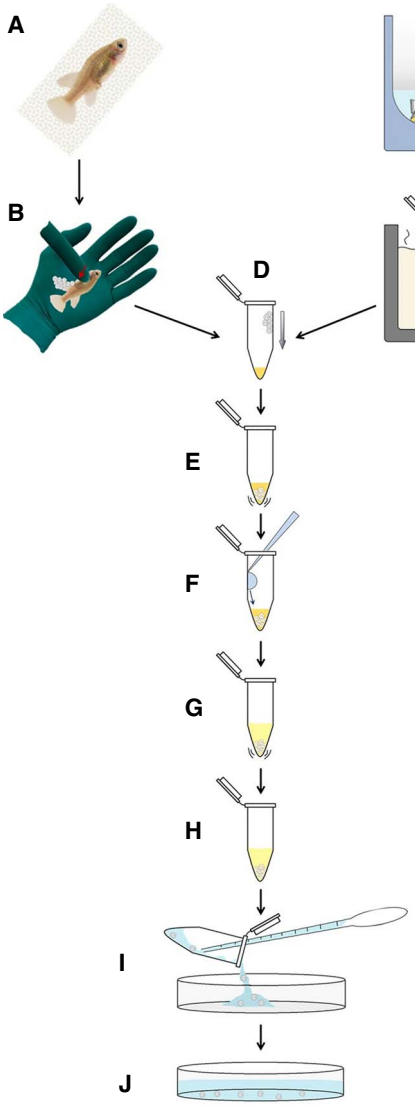

FIGURE 1. Step-by-step procedure for in vitro fertilization of the African turquoise killifish. A female is anesthetized or euthanized and dried with a paper towel (*A*). Eggs are extracted by applying gentle pressure to the abdomen (*B*) while frozen sperm is simultaneously thawed in a warm water bath by a partner (*C*). The eggs are then added to the sperm (*D*) and mixed through gentle shaking or finger flicking (*E*). Activating 0.25× BSMIS solution is added to the mixture (*F*), and the tube is again gently shaken/flicked (*G*). The tube is left upright on a rack for 10 min at room temperature to allow fertilization to occur (*H*). The eggs are then washed into a Petri dish using embryo water (*I*), washed twice with embryo water, and left to develop in an incubator at 28°C (*J*). (Reproduced from Dolfi et al. 2021.)

For IVF Using Fresh Sperm

ii. Prior to collecting the eggs, place 600 µL of FBS into a microcentrifuge tube.

iii. Anesthetize your male of choice (ideally between 9- and 11-wk of age, for optimum gonad size and fertility) by placing it in room temperature 0.5-mg/mL tricaine solution. Keep it in tricaine until opercular movements are absent (as described in Step 2).

iv. Dry the male very carefully with a paper towel.

v. Decapitate the fish with scissors. Make an incision in the abdomen and extract the gonads using forceps.

vi. Tightly grasping the gonads with forceps, shake them vigorously in the FBS for 1 min or until the FBS is opaque.

Sperm in FBS is very stable; therefore, fresh sperm can be harvested up to 15 min before collecting the eggs.

IVF success largely hinges on sperm quality; therefore we recommend performing the sperm quality check outlined in Step 11 before IVF, particularly if eggs/females are in short supply. If a quality check is performed for frozen sperm, we recommend that it is done as quickly as possible to minimize the time between sperm thawing and its use for fertilization (ideally sperm should be used immediately after thawing).

6. Use forceps to pick up all the eggs at once and position them at the bottom of the tube containing the sperm. Put no more than 35 eggs in the tube (Fig. 1D).
7. Close the tube cap and gently shake by hand or carefully finger flick the tube for 10–20 sec to distribute the sperm evenly between the eggs (Fig. 1E).
8. Add 120 µL of room-temperature 0.25× BSMIS to the edge of the tube and let it slide to the bottom (Fig. 1F).
9. Close the tube cap and gently mix the solution for 20–30 sec, again through careful shaking by hand or flicking the tube with your finger (Fig. 1G).
10. Allow fertilization to occur by opening the tube and leaving it upright on a rack for 10 min at room temperature (Fig. 1H).
11. While waiting for fertilization to occur, perform a sperm quality check. Take 15 µL of the egg–sperm mixture and place it on a glass slide or Petri dish. Check sperm activation by observing the edges of the drop where the light contrast is highest under a stereomicroscope.

    At least a proportion of the sperm should be motile, showing directional movement (consult Dolfi et al. 2021 for representative movement trajectories). Ideally 40%+ of the sperm should be moving, but lower percentages do not necessarily eliminate the possibility of successful IVF.
12. Wash the fertilized embryos from the tube into a 10-cm Petri dish using room-temperature embryo water (Fig. 1I).
13. Change the water twice; slowly pour out the old water into a waste container, keeping the angle such that the embryos remain in the dish. Fill the dish with fresh embryo water (just past the halfway mark), swirl for a few seconds, and discard the water again. Add fresh embryo water and place in an incubator at 28°C (Fig. 1J).
14. Proceed with embryo husbandry and hatching as with eggs collected from regular breeding, such as described in Dodzian et al. (2018).

RECIPES

Buffered Sperm-Motility Inhibiting Solution (BSMIS), 1×

75 mM NaCl
70 mM KCl
2 mM $CaCl_2$
1 mM $MgSO_4$
20 mM Tris-Cl, pH 8.0

BSMIS is usually prepared fresh for every sperm freezing session. We note, however, that BSMIS that had been stored for several months at 4°C did work in our hands the few times we used it. We store the component stock solutions for up to a year at room temperature, without a noticeable decline in potency (for our purposes). You may wish to store them at lower temperatures to increase shelf life.

Tricaine Solution

Dissolve ethyl 3-aminobenzoate methanesulfonate (tricaine; Sigma-Aldrich E10521) in tank water to achieve a concentration of 0.5 mg/mL.

Store for no longer than 2–3 mo at 4°C. We note that our recommendation is largely based on the fact that the solution is usually used up by then rather than because we have observed any loss of potency after that period.

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