

A High-throughput sperm freezing protocol for zebrafish

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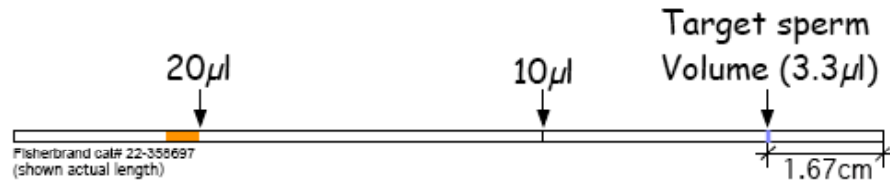
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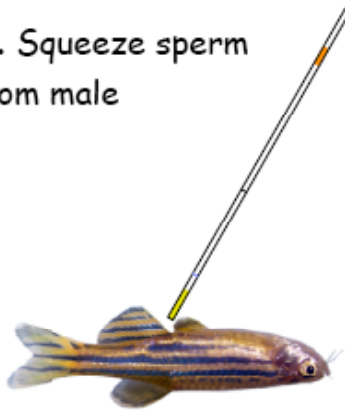
High-throughput zebrafish Sperm Freeze

J. Stout, R. Hernandez, B. Draper and C. Moens

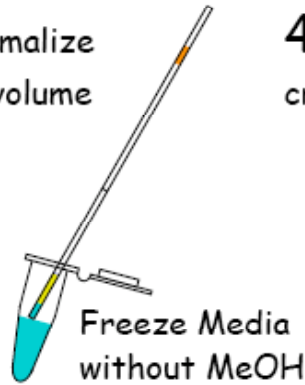
1. Mark capillary tubes with target volume



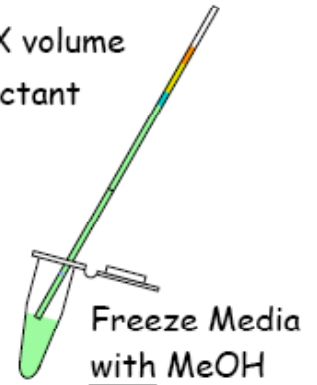
2. Squeeze sperm from male



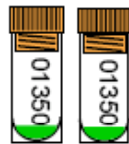
3. Normalize sperm volume



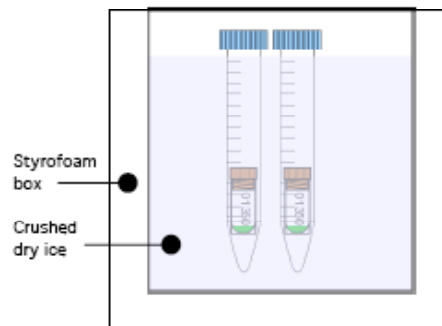
4. Add 5X volume cryoprotectant



5. Mix on clean watch glass and aliquot into 2 cryovials.



6. Place cryovials in 15 ml conical tubes, cap and ram into crushed dry ice.



7. After 20 min on dry ice, transfer cryovials to liquid nitrogen.

A high-throughput method for zebrafish sperm cryopreservation

Below is a method for zebrafish sperm cryopreservation that is an adaptation of the Harvey method (Harvey et al., 1982). We have introduced two changes to the original protocol that both streamline the procedure and increase sample uniformity. First, we normalize all sperm volumes using freezing media that does not contain the cryoprotectant (i.e. methanol). Second, cryopreserved sperm are stored in cryovials instead of capillary tubes. The rates of sperm freezing and thawing ($\Delta^{\circ}\text{C}/\text{time}$) are probably the two most critical variables to control in this procedure. For this reason, do not substitute different tubes for those specified. Working in teams of 2 it is possible to freeze the sperm of 100 males/team in ~2 hrs.

Sperm Freezing Solutions

Ginsberg Fish Ringers (Make fresh every 3 days ;Ginsburg, 1963)

NOTE: ORDER OF ADDITION IS IMPORTANT TO PREVENT PRECIPITATION

In 450 ml sterile ddH₂O dissolve:

NaCl	3.25 g
KCl	0.125 g
CaCl ₂ •2 H ₂ O	0.175 g

Then add:

NaHCO ₃	0.10 g
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Bring final volume to 500 ml with sterile ddH₂O and store at 4°C.

Freezing Medium (Make fresh daily)

A. WITHOUT Methanol

Ginsburg Fish Ringers	10 ml (room temp.)
Powdered Skim Milk	1.5 gm

B. WITH Methanol

NOTE: ORDER OF ADDITION IS IMPORTANT TO PREVENT PRECIPITATION

Ginsburg Fish Ringers	9 ml (room temp.)
Methanol	1 ml
Powdered Skim Milk	1.5 gm

After assembling freezing media, mix well for 20 min. on orbital shaker or rocker. Before using, aliquot into 1 ml eppendorf tubes, avoiding surface bubbles.

Materials needed for sperm freezing

10µl disposable pipettes (Fisherbrand cat# 22-358697)
250ml beakers for fish water containing MESAB/Tricaine
watch glasses (Pyrex cat# 9985-75)
P20 pipetman (or equivalent) and tips
Sponge fish holder (to hold male while squeezing)
Dissecting microscope with above stage lighting
Forceps (e.g. Millipore type)
2.0ml cryogenic vials (Corning cat# 430488)
10X10 cryoboxes (Nalgene cat#03-337-7AA)
Styrofoam container filled with ~ 15 cm of finely crushed dry ice
15 ml conical tubes (Falcon # 352099)
Dewar flask containing liquid nitrogen
Long tongs (e.g. CMS Fisher Health Care cat# 10-316B)
Cryogloves
Recovery tank for males
One eppendorf tube filled with freezing media without methanol
One eppendorf tube filled with freezing media with methanol
Cryofreezer (e.g. Taylor-Warton K-series)

Sperm Freezing

1. **MARK CAPILLARY TUBES:** Prior to beginning, mark the 10µl capillary tubes with a lab pen at the level of 3.33 µl (i.e. 16.7 mm from bottom; see Fig. 1).
2. **ANESTHETIZE MALE:** Place male(s) in beaker containing MESAB/tricaine diluted in fish water (recipe in "The Zebrafish Book")
3. **DRY FISH:** Once anesthetized, gently blot fish dry on paper towel, paying special attention to dry ventral side. Water activates sperm so it is important to thoroughly dry around the cloacae. Position fish on sponge holder, ventral side up (Fig. 2). If region around anal fin is still moist, gently dry with a kimwipe. Take care to not squeeze sperm prematurely!
4. Place marked capillary tube in rubber mouth pipette adapter that is included with capillary tubes. The adaptor is a rubber hose that has a mouth piece on one end and a capillary holder on the other.
5. Place male under the scope and expose the urogenital opening by carefully spreading the anal fins apart using the end of the capillary tube.
6. **COLLECT SPERM:** Expel sperm by gently stroking the sides of the fish with smooth forceps (e.g. Millipore), or by gently squeezing the sides of the fish between your index finger and thumb. Collect sperm in capillary tube as it is expelled using gentle suction (Fig. 2). Avoid feces that may be expelled with sperm.

7. **NORMALIZE SPERM VOLUME TO 3.3 μ l:** If the volume of sperm reaches, or exceeds pen mark on capillary tube (i.e. 16.7 mm or greater), then proceed to step 8. If sperm volume does not reach target volume, then normalize volume to pen mark using Freeze Medium WITHOUT Methanol (Fig. 3). Minimum amount of sperm that is acceptable varies with quality of sperm. Good sperm is white and opaque. Poor sperm looks watery. In general we accept as minimums: 1 μ l good sperm (or 1/3 target) and 2 μ l poor sperm (or 2/3 target). Volume is now normalized to 3.3 μ l.
 8. **ADD CRYOPROTECTANT TO SPERM :** Suck up Freeze Medium WITH Methanol to the orange mark (approx. total volume is now 20 μ l; Fig. 4). Expel sperm and cryoprotectant mixture onto clean area of a watch glass, paying special attention to not introduce bubbles. Gently mix by pipetting up and down ~ 4 times (avoid introducing bubbles).
 9. **PLACE 10 μ l SPERM INTO EACH OF 2 CRYOVIALS:** Pipette 10 μ l of the sperm:cryoprotectant solution into the bottom of two separate cryovials that have been labeled with relevant information (Fig. 5). Cap vials and drop them into the bottom of room temperature 15 ml Falcon tubes- one cryovial/tube. Cap Falcon tube.
 10. **FREEZE SPERM FOR 20 MIN. ON DRY ICE:** Immediately insert the pair of cryovial-containing Falcon tubes into crushed dry ice. The tubes should be inserted into the dry ice deep enough that only their caps show (Fig. 6). In order to keep track of tubes in dry ice, number pairs of tubes from 1-20 (on caps) and record the time they go into the dry ice. A simple method for making finely crushed dry ice is to wrap it in a towel and pulverize it with a hammer.
 11. **PLACE CRYOVIALS INTO LIQUID NITROGEN:** After the sperm has been frozen on dry ice for 20 min., transfer them to a liquid nitrogen-containing dewar flask. Sperm is stored here until time to place into liquid nitrogen freezer. When placing in the freezer boxes, place freezer box in a bath of liquid nitrogen so that samples do not heat up. Use long metal tongs to recover vials from dewar flask and handle with cryogloves.
 12. **Store cryovials long term in a cryogenic liquid nitrogen freezer.** To maintain viability of sperm, it is important that vials are stored immersed in liquid nitrogen. In our experience, vials stored in the vapor phase lose viability over time.
- Speed is important!** The time between adding the cryoprotectant to placing sperm vials in dry ice (i.e. steps 6-10) should be no more than 30 sec.

***In vitro* Fertilization using Cryopreserved Sperm**

We find that this works best with two people. One person squeezes eggs from females while the other person thaws the sperm.

1. Set a water bath to 33°C.
2. Remove cryovial from liquid nitrogen freezer and transfer to liquid nitrogen-containing dewar flask until ready to thaw.
3. Squeeze eggs from females into 35mm plastic petri dish. If possible, try to obtain 3 clutches of eggs and combine into one dish. If you can not get three good clutches within 1 min of your first clutch, then proceed to step 4 (one good clutch is sufficient). Definition of good clutch: >150 uniformly nice looking eggs (i.e. yellowish, with no white debris indicative of degradation). Keep dish covered while sperm is thawed.
4. Remove vial from liquid nitrogen and remove cap. Quickly immerse vial ~1/2 way into 33°C water bath for 8-10 sec.
5. Quickly add 70µl room temperature Hanks to vial and mix by pipetting up and down. Immediately add to eggs and mix gently with pipette tip.
6. Without delay, activate sperm and eggs by adding 750µl fish water. Swirl to mix. Incubate 5 min at room temperature.
7. After 5 min, fill dish with fish water and incubate dish at 28°C. After 2-3 hrs. count and transfer fertile embryos to 100mm dishes (50/dish). Count infertile eggs so that fertilization frequency can be determined.
8. Take good care of the larvae! For first 5 days, change water daily and remove dead embryos. Put only larvae that have swim bladders into nursery on day 5.

References

Ginsburg, A. S. (1963) Sperm-egg association and its relationship to activation of the egg in salmonid fishes. *J. Embryol. Exp. Morphol.* **11**: 13-33.

Harvey, B., R. N. Kelley and M. J. Ashwood-Smith (1982) Cryopreservation of zebra fish spermatozoa using methanol. *Can. J. Zoology.* **60**: 1867-1870.

Results:

The Moens lab built a library of 8,600 frozen sperm samples from ENU-mutagenized F1 male zebrafish for TILLING between 2001 and 2004. Each year, selected sperm vials were thawed to recover mutations of interest. We recorded an average of 30% fertility with a standard deviation of 16%, a maximum of 62% (one sample) and a minimum of 0% (one sample). As shown in the chart below, percent fertility has not changed significantly over the years since the library was made.

