# Chapter 4

# Cryopreservation and In Vitro Fertilization at the Zebrafish International Resource Center

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## Summary

In recent decades, laboratories throughout the world generated several thousand mutant, transgenic, and wild-type zebrafish lines and more lines continue to be produced. At the same time, relatively little effort has been expended to develop reliable, high-throughput, standardized, long-term cryopreservation storage methods, even though laboratories and the research community as a whole struggle to maintain the large number of lines alive. Safe and reliable methods for maintaining these valuable genetic resources are vital for future biomedical research.

Cryopreservation is the most efficient method for large-scale, long-term storage of important genetic materials. It extends the time offspring can be produced from individual fish, reduces the need to maintain live populations, and can prevent catastrophic loss of irreplaceable research lines. Cryopreservation is also the most cost-effective alternative for maintaining genetic resources because it reduces costs for animal and facility maintenance, personnel, and space. In addition, it provides novel opportunities to develop new types of research using large numbers of lines. For example, several genetic strategies, such as TILLING—or enhancer and gene trapping—depend on the use of cryopreservation to bypass generations of live organisms until a strain is revived for research.

This chapter describes and discusses the current cryopreservation method used at the Zebrafish International Resource Center. This method is derived from the initial protocol developed for zebrafish over 20 years ago that has recently been refined.

**Key words:** Danio rerio, Cryoprotectant, Germplasm, Liquid nitrogen repository, Sperm, Sperm library, In-vitro fertilization.

## 1. Introduction

In recent years, large- and small-scale genetic screens around the world have generated thousands of novel zebrafish strains *(1, 2)*. In addition, a number of new technologies became available,

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such as generation of transgenic reporter lines, targeted knockout of genes *(3, 4)*, TILLING (Targeting Induced Local Lesions In Genomes) *(5)*, viral and transposon based insertions *(1, 2, 6)*, and enhancer or gene trap strategies *(7, 8)*. Several mutagenesis programs and consortia will use these new technologies to generate, identify, and characterize thousands of genes in the near future (http://www.zf-models.org/). Thus, further increase in the number and variety of zebrafish strains is certain.

In spite of the significant cost to generate these lines, little effort has been made to safely preserve them for future generations of researchers. Typically, the capacity of laboratories to maintain strains is limited by personnel and facility space. Many lines are in danger of becoming extinct without a concerted effort to bank them.

Currently, cryopreservation is the preferred method for preserving samples from endangered species and for storing rare or important genetic materials to maintain genetic diversity. Cryopreservation is the best alternative to live stocks for several reasons *(1)* Facility space is efficiently used; *(2)* the effective reproductive time of males is extended; *(3)* valuable resources do not become extinct if a line cannot be maintained live; *(4)* reverse genetic mutageneses such as TILLING, are more feasible because live fish do not need to be maintained; *(5)* cryopreservation is highly cost-effective because it saves funds needed for electricity, water, filtration, tank cleaning, repair, food, and personnel.

However, the cryopreservation protocols currently used by the zebrafish research community can be relatively difficult to learn and laboratories cannot adapt them easily. Moreover, success rates and fertilization rates vary considerably among laboratories, most likely because a number of different protocols have been developed *(5, 9)* as modifications of the original procedure by Walker and Streisinger *(10)*, which was based on the original cryopreservation method of Harvey et al. *(11)*. These modifications have largely been tested empirically by determining whether or not they affect or improve overall post-thaw fertilization rates. Without a strong understanding of the biophysical principles that govern the physiology of frozen cells, it is unclear whether the community currently uses the most practical, reliable, and efficient method today.

Cryopreservation generates several conditions that can lead to severe cell damage *(12)*. These include solution damage, intracellular and extracellular ice formation, and dehydration. When tissues are cooled slowly, water moves out of cells and ice forms in the extracellular space. The movement of water out of cells can lead to cellular dehydration, and too much extracellular ice can lead to mechanical damage by crushing. Although some organisms and tissues can tolerate extracellular ice, intracellular ice crystals almost always severely damage cells. Solutes that are excluded

from the ice crystal structure during freezing will affect the balance of osmotic pressure. The stresses associated with dehydration and intracellular ice crystallization are particularly damaging to cells because intracellular structure, organelles, and molecules are severely disrupted *(12, 13)*.

The risks of cellular damage can be greatly reduced by adding a cryoprotectant to the freezing solution, which lowers the freezing temperature and increases the viscosity of the liquid. Instead of crystallizing, the solution forms amorphous ice (vitrification). Vitrification of water can also be achieved by extremely rapid cooling, in the absence of a cryoprotectant *(14, 15)*. Cryoprotectants and solutes in general increase the viscosity and decrease the freezing temperature. To achieve these effects inside the cell, cryoprotectants must readily penetrate the cell membrane. Unfortunately, common cryoprotectants such as methanol, glycerol, dimethylsulfoxide (DMSO), or *N*,*N*-dimethyl acetamide (DMA) are often toxic in high concentrations. Therefore, one of the challenges to develop an effective cryopreservation method is limiting the damage produced by the cryoprotectant itself.

Recently, three modifications of the original Harvey method *(11)* have been reported *(5, 9, 16)*. The first study tested ethylene glycol, DMSO, glycerol, and DMA as potential cryoprotective agents to replace methanol that has cytotoxic effects and is known to mutate DNA *(9)*. The second modification aimed at procedural optimization and led to a more streamlined protocol that can be more easily adapted to various laboratory conditions *(5)*. However, both methods produce only modest improvements on Harvey's protocol in terms of post-thaw fertilization rates, materials, and procedure (**Table 1**, *see* **Note 1**). Recently, another variation that omits the use of powdered milk as an anticoagulant of sperm tails has been developed in a study where four cryoprotectants: DMSO, DMA, methanol, and glycerol were tested side by side *(16)*.

These three methods may work sufficiently well for some research laboratories, however there is insufficient information or rigor to allow them to be used in a robust way for high-throughput applications. Moreover, some protocols still include the use of additives such as powdered milk, and storage containers such as glass capillary tubes and cryovials, that are inefficient and hamper necessary quality controls such as motility estimation and cell density measurements. In addition, the use of uncrushed, crushed, or powdered dry ice for freezing presents significant challenges for standardization of freezing rates within or among the different protocols.

A goal of the Zebrafish International Resource Center (ZIRC) is to serve as a repository for zebrafish lines and to preserve as many of these invaluable resources as possible for the research community. Therefore, ZIRC aims to develop robust cryopreservation

**Table 1**
**Overview of Published Zebrafish Cryopreservation Methods**

| | Study | | | |
|---|---|---|---|---|
| **Factor** | **Harvey et al. *(11)*** | **Morris et al. *(9)*** | **Draper et al. *(5)*** | **Yang et al. *(16)*** |
| Cryoprotectant | 10% methanol | 10% DMA | 8% methanol | 8% methanol |
| Extender | Ginsburg | BSMIS | Ginsburg | HBSS |
| Collection | Squeezing | Dissection | Squeezing | Dissection |
| Container | Capillary | Capillary | Cryovial | Straw |
| Dilution | HBSS | BSMIS | HBSS | HBSS |
| Freezing method | Dry ice | Dry ice | Dry ice | Controlled-rate freezer |
| Freeze time | 30 min | 30 min | 20 min | – |
| Freeze rate | 16°C/min | ? | ? | 10°C/min |
| Motility | 43 ± 12% | 12 ± 6% | ? | 78 ± 10% |
| Thawing | Air | 37°C | 33°C, 8 s | 40°C, 5 s |
| Fertility | 51 ± 36%[a] | 14 ± 10% | 28 ± 18% | 33 ± 20% |

A cross-comparison of key steps and agents used in zebrafish cryopreservation methods
*DMA*, *N,N*-dimethyl acetamide; *HBSS*, Hanks' balanced salt solution; *BSMIS*, buffered sperm motility-inhibiting solution
[a]Reported as % hatching, which may differ from % fertility. *See* **Note 1** for discussion of the issues in comparing different protocols and their outcomes

protocols for its own purposes and for laboratories in the research community to ensure that resources can be stored for future generations of researchers. Here, we present and discuss the cryopreservation protocol developed by Draper and Moens *(5)*, which was adapted from the cryopreservation method by Harvey et al. *(11)*. The ZIRC currently uses this method because it has the highest throughput capability and therefore fits ZIRC's need to cryopreserve large numbers of zebrafish strains.

## 2. Materials

### 2.1. Sperm Freezing Solutions

1. *Tricaine anesthetic stock solution.* 400 mg Tricaine powder, 97.9 mL $ddH_2O$, add 2.1 mL 1 M Tris–HCl (pH 9.0). Mix components in an amber glass bottle with a screw cap and

adjust to pH 7.0 with 1 M Tris-HCl (pH 9.0) (*see* **Note 2**). Store refrigerated. To anesthetize fish, add 4.2 mL Tricaine stock solution to 100 mL fish water in a crystallizing dish (*see* **Note 3**).

2. *10X Ginsburg Fish Ringer's (the salt mixture used in the cryopreservation solution) (17).* To 400 mL $ddH_2O$ add in order, 32.5 g NaCl, 1.25 g KCl, 1.75 g $CaCl_2 \cdot 2H_2O$, adjust with $ddH_2O$ to 500 mL. To avoid precipitation of its components, it is important to add each reagent in this order and to allow each reagent to dissolve completely before adding the next. Autoclave and refrigerate. 10X Ginsberg stock solution can be stored in the refrigerator and used as needed.
3. *10X Sodium bicarbonate ($NaHCO_3$) (the buffering component in the cryopreservation solution).* 50 mL $ddH_2O$, 0.10 g $NaHCO_3$. Must be prepared fresh each day of cryopreservation.
4. *1X Ginsberg Fish Ringer's mix.* (To make 25 mL) 2.5 mL 10X Ginsberg Fish Ringer's, 2.5 mL 10X $NaHCO_3$, 20 mL $ddH_2O$. The 1X Ginsberg Fish Ringer's is the final working solution that is prepared before each freeze event.
5. *Cryopreservation solution without methanol (A).* 10 mL 1X Ginsburg Fish Ringer's (at room temp.), 1.5 g Powdered Skim Milk (Carnation® instant milk; *see* **Note 4**).
6. *Cryopreservation solution with methanol (B).* 9 mL 1X Ginsburg Fish Ringer's (at room temp.), 1 mL methanol (Acetone free, absolute), 1.5 g powdered skim milk (Carnation® instant milk). To prevent precipitation of components, it is important to mix Ginsberg Fish Ringer's with methanol before adding powdered skim milk. For comment on the choice of methanol (*see* **Note 5**). Clearly label cryopreservation media *with* and *without* methanol (*see* **Note 6**).

### 2.2. Sperm Freezing Materials

1. *Powdered dry ice from liquid $CO_2$.* To obtain more reproducible freezing rates, we maximize contact between dry ice and the freezing vessels (**Subheading 2.2, items 12** and **13**, and **Subheading 3.1.3, steps 9, 10**). To this end, we use powdered, not crushed dry ice. To produce powdered dry ice, attach a fire extinguisher cone to the liquid output (**Fig. 1a**) of a $CO_2$ tank that has a liquid siphon. It is useful to angle the cone 90° downward. Position a Styrofoam box or cooler directly under the cone to catch the dry ice. For safety purposes, wear a face shield, ear protection, and cryogloves. Open the liquid $CO_2$ output valve quickly so that it does not surge and clog the output valve. Compact the powdered dry ice in the cooler as it is produced (*see* **Note 7**). When the box is filled with powdered dry ice (a depth of 20 cm or more), turn off the liquid $CO_2$ output valve.

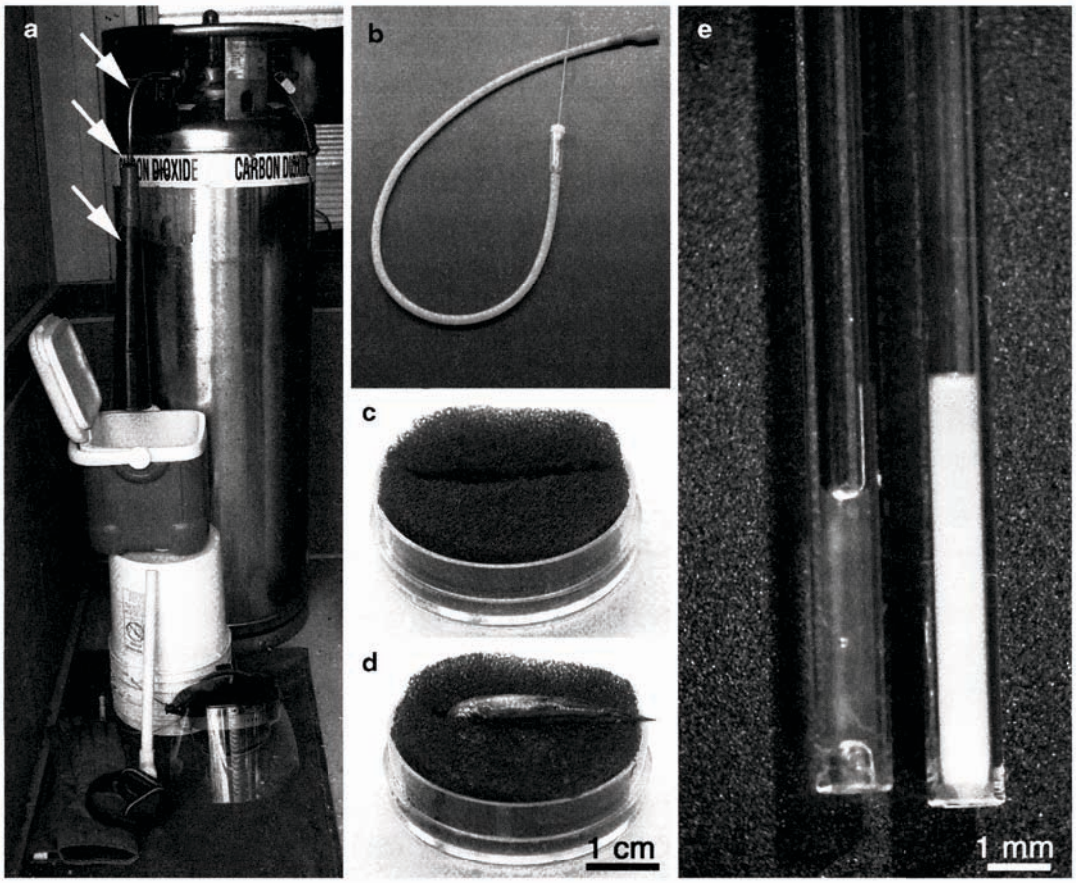


Fig. 1. Zebrafish sperm cryopreservation tools. (**a**) Tools to make powdered dry ice from liquid $CO_2$. A fire extinguisher cone is attached to the liquid output of a liquid $CO_2$ tank (*arrows*). At the bottom: cooler, safety gloves, goggles, ear protection, isolation pad, and PVC tubing to compact dry ice powder. (**b**) The aspirator tube assembly. The aspirator is a soft rubber tube adaptor that has a mouthpiece on one end and a capillary holder on the other. (**c**) Sponge fish holder. A 35 × 10 mm Petri dish with a sponge that is cut to hold males. (**d**) The sponge needs to be moistened before fish are placed in it. (**e**) Examples of empirically determined sperm quality. On the *right*, milky, opaque milt indicates high density of sperm cells whereas lower quality sperm (*left*) is watery and more transparent. The observed difference in opacity correlates only loosely with sperm cell density counts using a hemocytometer and does not correlate well with post-thaw fertilization rates. *Scale bars*: (**c**, **d**) 1 cm; (**e**) 1 mm.

2. 10 µL disposable capillary micropipets (Drummond Scientific Cat. No. 2-000-010; includes one aspirator tube assembly).
3. Aspirator tube assembly (**Fig. 1b**; Sigma-Aldrich cat. no. A-5177).
4. Plastic spoon to remove fish from MESAB (*see* **Note 8**).
5. Sponge fish holder (to hold male while squeezing; **Fig. 1c, d**).
6. 2 glass crystallizing dishes (one for tricaine anesthetic, the other for rinse and recovery; **Fig. 2a**, 6/7).

7. 0.5 mL microcentrifuge tubes for cryopreservation solution (mark tubes whether they will be with or without methanol) in 0.5 mL microcentrifuge rack. (Prepare the correct number of tubes according to the number of fish lines used. Divide the tubes into one row without methanol and one with methanol; **Fig. 2b**, 17/18).
8. Dissecting microscope with incident stage lighting.
9. Forceps (Millipore cat. no. XX6200006).
10. Watch glasses (Pyrex cat. no. 9985-75).

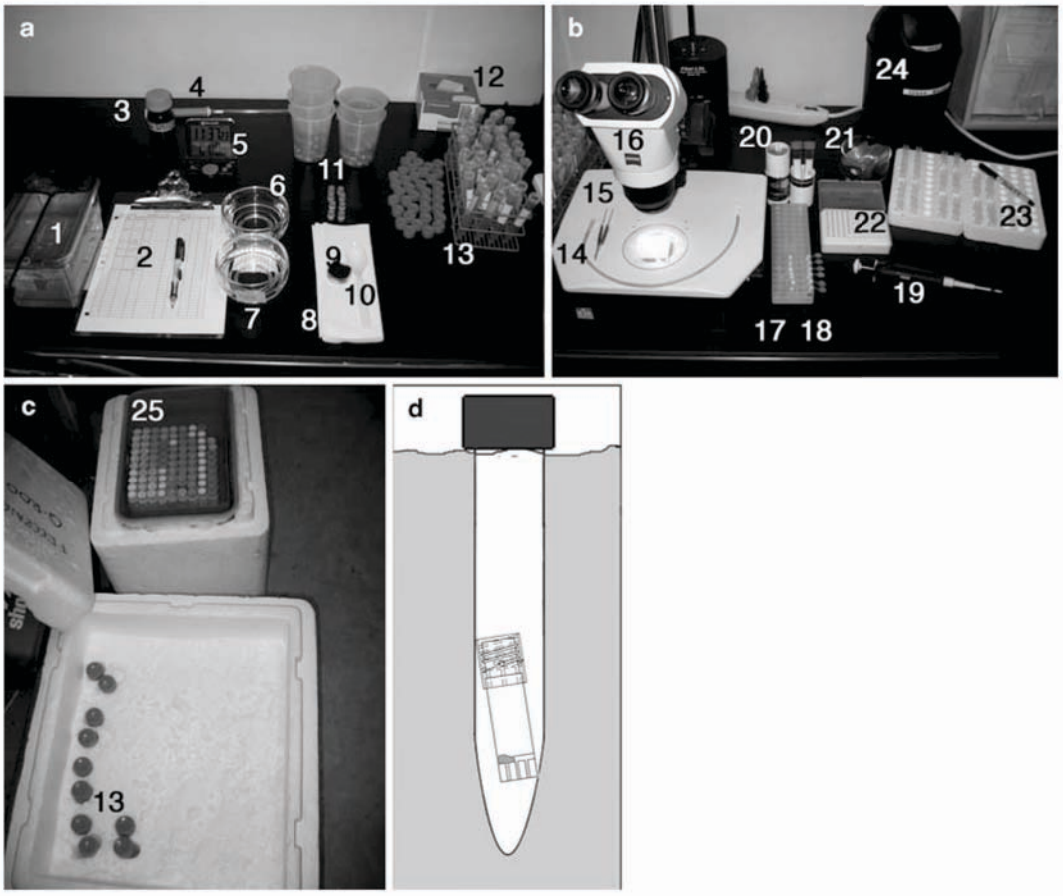


Fig. 2. Preparation of the cryopreservation workspace. (**a**) Assistant's side, (**b**) squeezer's side, (**c**) box with powdered dry ice and Falcon tubes (*foreground*), Styrofoam container with liquid nitrogen fiberglass tray insert and color-coded screwcap cryovials. (**a**) On the assistant's side 1. fish tank; 2. data records sheet; 3. Tricaine stock solution; 4. Pasteur Pipet; 5. timer; 6. recovery dish; 7. anesthetic dish; 8. Stack of paper towels; 9. Sponge fish holder (*see also* **Fig. 1 c, d**); 10. spoon; 11. screwcaps for cryovials (in cups and on table); 12. paper wipes; 13. rack with 15 mL Falcon tubes and lids. (**b**) On the squeezer's side: 14. aspirator tube assembly; 15. forceps; 16. dissection scope; 17. row of tubes containing cryoprotectant solution without methanol; 18. row of tubes containing cryoprotectant solution with Methanol; 19. micropipetr; 20. 20 μL glass capillaries, prelabeled at 1.67 cm; 21. watchglasses; 22. micropipet tips; 23. pre-labeled empty cryovials; 24. waste container for glass and sharp objects. (**c**) 25. Container with powdered dry ice and inserted Falcon Tubes on the *left*; 26. liquid nitrogen and immersed color-coded cryovials. (**d**) Profile of 15 mL Falcon Tube containing a 2 mL cryovial with sample, submerged in dry ice.

11. 10 × 10 cryoboxes (Taylor-Wharton cat. no.R24K-9C44).
12. 15 mL conical tubes (Falcon cat. no. 352099).
13. 2 mL cryogenic vials (Corning cat. no. 430488).
14. Cryogenic vial color-coded cap inserts (Corning cat. no. 430499).
15. Nalgene scissor-type forceps (NNI cat. no. 6320 0010).
16. 10 µL single channel pipetter and tips (preset to 10 µL).
17. Cryogloves.
18. Black marker (water insoluble).
19. Timer.
20. Paper towels.
21. Liquid nitrogen or vapor freezer.
22. 4-L Liquid nitrogen Dewar flask.
23. Styrofoam cooler retrofitted with fiberglass tray insert (US Plastic Corp. cat. no. 49267, **Fig. 2c**, 25).

### 2.3. Solutions for Sperm Thawing

1. *Hank's Stock Solution 1.* 8.0 g NaCl, 0.4 g KCl in 100 mL $ddH_2O$. Store refrigerated.
2. *Hank's stock solution 2.* 0.358 g $Na_2HPO_4$ anhydrous, 0.60 g $KH_2PO_4$ in 100 mL $ddH_2O$. Store refrigerated.
3. *Hank's stock solution 4.* 0.72 g $CaCl_2$ in 50 mL $ddH_2O$. Store refrigerated.
4. *Hanks' stock solution 5.* 0.601 g $MgSO_4$ in 50 mL $ddH_2O$. Store refrigerated.
5. *Hank's stock solution 6.* 0.35 g $NaHCO_3$ in 10.0 mL $ddH_2O$. Must be prepared fresh for each cryopreservation session.
6. *Hank's premix.* 10.0 mL Hank's stock solution 1, 1.0 mL Hank's stock solution 2, 1.0 mL Hank's stock solution 4, 86.0 mL $ddH_2O$, 1.0 mL Hanks' sock solution 5. Must be combined in this order to avoid salt precipitation. Store refrigerated.
7. *Hank's final.* 9.9 mL Hank's premix, 0.1 mL Hank's stock solution 6.

### 2.4. Materials for Sperm Thawing

1. Assembled water bath.
2. 100 µL single channel pipetter and tips (set on 70 µL).
3. 35 × 10-mm Petri dishes.
4. Paper towels.
5. 2 Metal spatulas (Fisherbrand cat. #14-373-25A).
6. 2 Crystallizing dishes (for Tricaine and rinsing).
7. Plastic spoon.

8. Black marker.
9. Scissor-type forceps.
10. 2 L glass flask filled with fish water.
11. Glass measuring pipets (1 and 5 mL).
12. 1 Gallon recovery tank.
13. Pre-sorted healthy females the afternoon before squeezing (selected by looking for opaque area around urogenital opening). Use no more than 15 fish per 1 gallon tank; do not feed again until after squeezing.
14. List of samples to be thawed and their locations.

## 3. Methods

### 3.1. Sperm Cryopreservation

This procedure is generally carried out in teams of two but can be done alone under certain circumstances (*see* **Note 9**). An ideal workspace includes a ca. 1.5 × 1 m countertop (**Fig. 2a, b**) and two chairs with wheels.

#### 3.1.1. Preparations in Advance

1. Prepare solutions (**Subheading 2.1, items 1** and **2**) and collect materials (**Subheading 2.2, items 2–22**).
2. Mark capillary micropipets at 1.67 cm (3.33 µL) to normalize sperm volume.
3. With black marker, pre-label 2 mL Corning vials with numbers and appropriate stock information. Place vials in order on the vial storage rack (supplied with vials). Prepare two vials for each male because samples will be split in half and aliquoted into two cryovials. Insert color-coded caps into vial lids, alternate colors between fish stocks. Remove the lids and sort into plastic cups, set aside.
4. It is convenient to prepare a sheet to record the freezing information. The records sheet can be filled in ahead of time with stock and vial information and which vial cap color represents each stock. Later, locations of each vial can be recorded here and time can be kept for when samples are to be moved in and out of dry ice. This information will help maintain organization during freezing and can later be transferred to a database that tracks freezing data and sample locations.
5. Sort males into tanks the afternoon before they are to be squeezed (use no more than 20 fish per 1 gallon tank, do not feed until after squeezing).

*3.1.2. Preparations on the Morning of Cryopreservation*

1. Make fresh sperm freezing solutions (**Subheading 2.1, items 3–6**) and place on orbital mixer for 20 min.
2. Mix 4.2 mL Tricaine solution with 100 mL fish water in a crystallizing dish.
3. Add fish water to a second crystallizing dish to rinse fish before squeezing and to hold fish recovering from anesthesia and squeezing.
4. Place fish tanks with males and nets on a cart near workspace.
5. Fill styrofoam container with approximately 20 cm of dry ice (**Fig. 2c**).
6. Fill 4-L Dewar flask with liquid nitrogen.
7. Set aside lid and place cryobox in an insulated fiberglass tray. Fill with liquid nitrogen until the level is three-fourth way up the cryobox. Keep lid on cooler. Refill as needed while freezing.
8. Fill 0.5 mL centrifuge tubes on left with cryopreservation solution **without** methanol and label clearly (**Fig. 2b**, 17).
9. Fill 0.5 mL centrifuge tubes on right with cryopreservation solution **with** methanol and label clearly (**Fig. 2b**, 18).
10. Dampen sponge in fish holder with fish water.

*3.1.3. Cryopreservation Procedure*

1. Assemble the aspirator tube. Place the marked glass capillary in the rubber adapter on one end of the aspirator tube assembly (**Fig. 1b**).
2. Anesthetize the male fish. Place 1–2 males in a crystallizing dish that contains Tricaine diluted in fish water. Once gill movement has slowed, remove fish and rinse briefly in second crystallizing dish containing fish water (*see* **Notes 2** and **3**).
3. Dry the fish. Place fish on stack of paper towels and use a spoon to roll and blot the fish. Pay special attention to dry the region around the urogenital opening between the anal fins (*see* **Note 10**). Place fish belly up into foam fish holder (**Fig. 1c, d**)
4. Position the fish. Place male under the dissecting scope and expose the urogenital opening. Gently spread the anal fins apart with the end of the capillary tube.
5. Collect the sperm. Expel sperm by gently stroking the sides of the fish from posterior to anterior with smooth forceps (Millipore). Place capillary tube near the urogenital opening and collect sperm with gentle suction as it is expelled. Note the sample quantity and quality on the record sheet (*see* **Fig. 1e, Notes 11** and **12**). Avoid feces that might be expelled along with the sperm. Return fish to crystallizing dish without

Tricaine for recovery. An advantage of this method is that it permits pooling sperm from different males (*see* **Notes 13** and **14**).

6. Normalize the cryoprotectant concentration. If sperm volume reaches or exceeds the normalizing mark on the capillary tube (1.67 cm or greater), proceed directly to **step 7**. If the sperm volume does **not** reach the normalizing mark, add Cryopreservation solution **without** methanol up to the mark to normalize the volume.
7. Add the cryoprotectant to the sperm. Pull up Cryopreservation solution **with** methanol to the orange mark on capillary (total volume is 20 μL). Expel sperm and cryoprotectant mixture onto a clean watch glass (avoid bubbles). Use pipettor (set to 10 μL) to mix by stirring and pipeting up and down 1–2 times. Be quick; **steps 7–10** should be completed in 30 s (*see* **Note 15**).
8. Place the sperm into the cryovials. Pipet 10 μL of sperm/cryoprotectant medium into the bottom of a labeled 2 mL cryovial. Pipet the remaining 10 μL into a second cryovial, labeled with the same information (*see* **Note 16**).
9. Transfer the cryovials into Falcon tubes. Close cryovials with preselected color cap and drop each one into the bottom of a 15 mL Falcon tube (at room temperature). Cap Falcon tubes.
10. Freeze the sperm. Insert the cryovial-containing Falcon tubes immediately into the powdered dry ice. The tubes in the dry ice should be deep enough so that only the cap protrudes (**Fig. 2c, d**). Start a timer that runs continuously and record on the record sheet the time each pair of samples is placed in the dry ice. Organize the tubes in dry ice so that you keep track of the samples while continuing to freeze and add more. Keep each sample on dry ice for 20 min (*see* **Note 17**).
11. Place the cryovials into liquid nitrogen. After the sperm samples have been frozen on dry ice for 20 min, transfer them two at a time to a cryobox in an insulated fiberglass tray that is partially filled with liquid nitrogen. Keep track of the location of each sample in the box using the record sheet. Store the sperm in the fiberglass tray until all of the samples can be transferred to the liquid nitrogen freezer at once. Refill the tray as needed with liquid nitrogen from the Dewer flask. Wear cryogloves and protective glasses when handling liquid nitrogen and when placing the cryobox in the liquid nitrogen freezer for long-term storage. *See* **Note 18** for comments about sperm quality control and its impact on variability of post-thaw fertilization rates.

### 3.2. In Vitro Fertilization with Cryopreserved Sperm

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

#### 3.2.1. Preparations in Advance

1. Prepare solutions (**Subheading 2.3, items 1–4** and **6**) and collect materials (**Subheading 2.4, items 1–14**).

#### 3.2.2. Preparations on the Morning of Thawing

Start working as soon as the lights are turned on in the fish room.

1. Set a water bath to 33°C.
2. Mix 4.2 mL tricaine solution and 100 mL fish water in a crystallizing dish.
3. Fill second crystallizing dish with fish water for rinsing.
4. Fill 1-gallon recovery tank with fish water.
5. Make Hank's final and place on ice (prepare fresh solutions (**Subheading 2.3, items 5** and **7**))
6. Put females and nets on cart and position near workspace.
7. Fill 4-L Dewar flask with liquid nitrogen.
8. Fill fiberglass tray (insulated in Styrofoam cooler) with liquid nitrogen.
9. Identify vials by location in freezer and put in tray with liquid nitrogen.

#### 3.2.3. In Vitro Fertilization with Cryopreserved Sperm

1. Anesthetize females. Place females in a finger bowl containing tricaine diluted in fish water. Once gill movement has slowed, remove fish and dip in fish water to rinse off excess tricaine.
2. Dry the fish. Blot female dry on a paper towel (excess water on eggs will activate sperm prematurely and decrease or prevent optimal fertilization rates).
3. Squeeze the females. Squeeze eggs from females into 35-mm plastic Petri dish. Dampen fingers in the recovery dish and blot on paper towel. Place two fingers on the dorsal side of the fish to support the back. With one finger of the other hand, express the eggs by gently pressing from anterior to posterior along the ventral side (**Fig. 3a**). Start just behind the pectoral fins and move slowly and gently toward the tail (*see* **Note 19**).
4. Collect the eggs. If "good" eggs are obtained (*see* **Note 20**), use the metal spatula to gently move eggs away from the fish body (**Fig. 3b**). For recovery, slide the fish out of the dish into a 1-gallon tank with clean fish water.
5. Combine the eggs from several females. If possible, try to obtain several clutches of eggs (*see* **Note 21**) and combine them in one dish using the metal spatula. Do not use "bad" or "mixed" eggs (Fig. 3d). If you cannot get three "good"

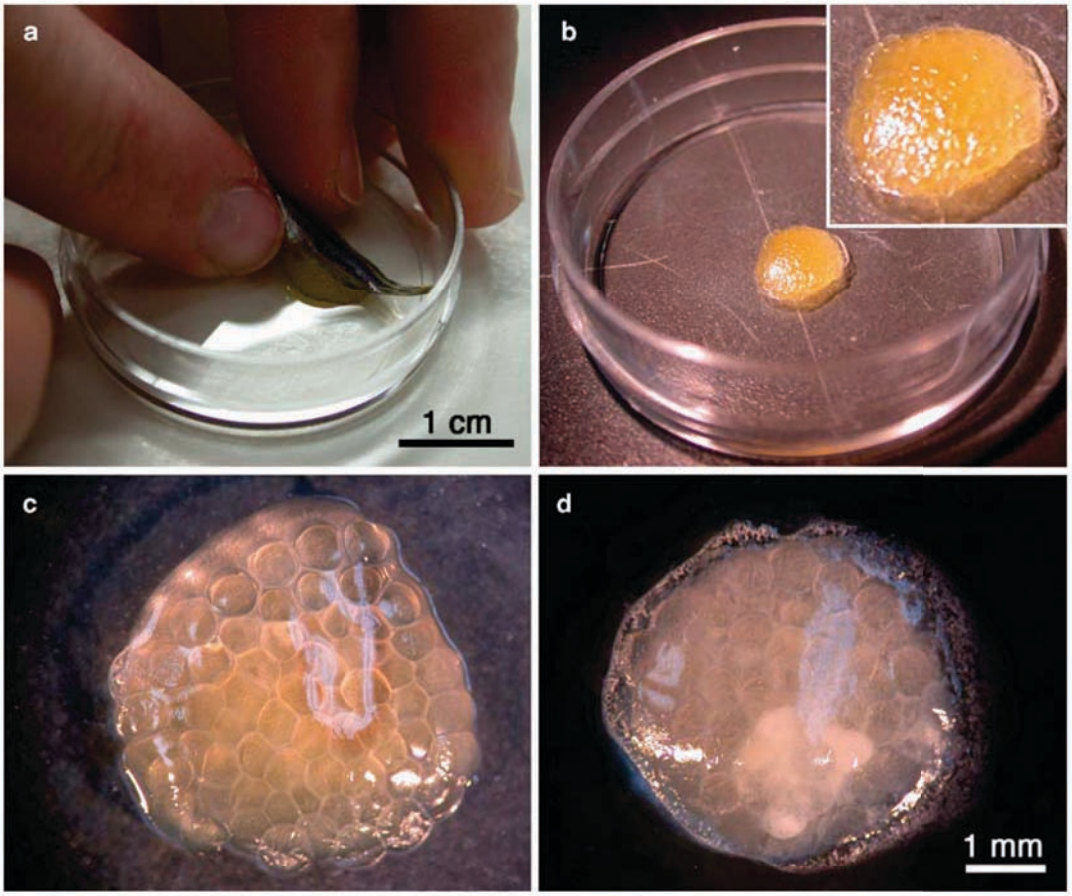


Fig. 3. Visual assessment of egg quality. (**a**) Anesthetized female is squeezed gently to release eggs into 35 mm Petri dish. (**b**, **c**) "Good" quality batches of eggs. Eggs are yellowish in incident light and appear translucent with transmitted light. (**d**) Lower quality batch of eggs. Note the opaque, white eggs intermingled with normal looking ones. Scale bars: (**a**) 1 cm; (**b–d**) 1 mm

clutches within 4 minutes of your first clutch, proceed to **step 6** (one "good" clutch might be sufficient **Fig. 3c**). Keep dish covered in a humidity chamber while sperm are thawed (*see* **Note 22**).

6. Thaw the sperm. Remove sperm vial from liquid nitrogen and remove cap. Make sure no liquid nitrogen is inside the vial by tipping the vial upside down. Immerse vial about half way into a 33°C water bath for 8–10 s.
7. Add Hank's final solution. Quickly add 70 mL Hank's Final to vial and mix by pipeting up and down. Immediately add the mixture to the eggs and stir gently with pipet tip.
8. Activate the sperm for fertilization. Without delay, activate sperm and eggs by adding 750 µL fish water. Swirl the dish to

mix eggs and sperm. Incubate for about 5 min at room temperature.

9. After 5 min, fill the dish with fish water and maintain it at 28°C. Later, transfer fertilized embryos to 100 × 20 mm Petri dishes. After 2–3 h, count fertilized and unfertilized embryos. To determine the fertilization rate, infertile eggs need to be counted as well (*see* **Note 23**). Distribute fertilized embryos to 50 per dish (100 × 20 mm) before submitting them to the nursery.
10. Take good care of the larvae! For the first 5 d, change the water daily and remove dead embryos. *See* **Note 24** for further comments about variability in post-thawing fertilization rates.

## 4. Notes

1. Until the present, there has been no attempt at standardization of protocols and terms. Developing standards to compare protocols and results among laboratories is difficult because of inconsistent use of terminologies. In 2005, the ZIRC, in collaboration with the Smithsonian Institution and the Louisiana State University Agricultural Center, hosted a symposium on the cryopreservation of zebrafish lines at which several examples of these types of discrepancies came to light.

   One problem is that common cryobiological terms are used differently in reference to laboratory standards and therefore, can be misleading. For example, one laboratory may pool 300–1000 unfertilized eggs from several females to ensure the successful recovery of a line, whereas another lab may use 50–100 eggs. The first laboratory reports 100% success in recovering a line (fertilization rates may be as low at 5%), whereas the second lab reports only 30% success, even though their fertilization rates are higher when fertilization per egg is compared. In addition, fertilization rates are also difficult to compare in the absence of standardized concentrations of frozen sperm samples.

   Several other relatively poorly understood parameters may contribute to variability in post-thaw fertilization rates such as the quality of eggs, conditioning of males, the quantity and "turbidity" of sperm samples (**Fig. 1e**) at the time of freezing, and even the definition of successful fertilization—which can range anywhere from sperm–egg penetration to first cell

division to development until 24 h, hatching, or inflation of the swim bladder.

In addition to terminology differences, a number of technical differences were identified within the cryopreservation method itself depending on the goals of individual labs and the extent to which they have modified the protocol by Walker and Streisinger *(9, 10, 18)*. For instance, different types of storage vessels and liquid nitrogen freezers are used, either of which could affect post-thaw success rates.

2. Tricaine (3-amino benzoic acid ethyl ester, also called ethyl m-aminobenzoate, MESAB, and MS-222) is available in powdered form from Sigma (cat. no. A-5040), or as Finquel (part no. C-FINQ-UE) from Argent Chemical Laboratories, Inc. Purchase the smallest amount possible and store the Tricaine powder at –20°C because the compound deteriorates over time.
3. Add more drops of Tricaine stock to the anesthetic solution when anesthesia of fish takes longer than 3–4 min. Tricaine is absorbed and diluted by water dripping from nets as more fish are anesthetized. Keep two males in anesthetic at all times to maintain a steady pace when performing the procedure. During anesthesia, fish may occasionally bleed from their gills. Because pH is buffered in the Tricaine solution, we exclude pH fluctuations as a cause for gill bleeding and speculate that stress and high blood pressure might be contributing factors. Fish that bleed from the gills do not typically recover from anesthesia.
4. Powdered skim milk was used in the original protocol to avoid clumping of sperm cell tails *(11)*. Unfortunately, powdered milk has disadvantages because important quality parameters such as cell motility and density cannot be assessed before the cells are frozen and because this particular brand of powdered milk is not readily available everywhere. We recently reported that powdered milk can be omitted *(16)*. However, these results were obtained from sperm collected from dissected testes that were frozen with a controlled rate freezer at cell concentrations ranging from $10^7$ to $10^8$ cells/mL.
5. Currently, few alternative cryoprotectants other than methanol have been tested for their suitability to cryopreserve zebrafish sperm. Alternative cryoprotectants that have been tested include DMA, ethylene glycol, DMSO, glycerol, and ethanol *(9, 16)*. Currently, methanol appears to be the most reliable cryoprotectant. Glycerol is least suitable for this purpose *(9, 16)*.

6. Label each solution clearly to distinguish between cryopreservation medium **with** and **without** methanol. After preparing the freezing solutions, mix the solutions well on a vortex to obtain a fluid consistency and to avoid clumping of powdered milk. Rock both solutions gently on an orbital mixer for 20 min. Before use, aliquot the solutions into labeled 0.5 mL centrifuge tubes while avoiding surface bubbles. Use one set of cryopreservation media tubes for each fish line to avoid cross contamination of samples.
7. To compact the dry ice, do not use metallic instruments or tools because of the risk of electrical shock. When dry ice particles exit the liquid $CO_2$ dewar, they crystallize and generate electric potential due to friction in the fire extinguisher cone. PVC tubing is suitable. It is also helpful to use an isolating pad (*see* **Fig. 1a**).
8. To drain fluids more easily from the plastic spoon, drill 3–4, 2 mm holes into the tip of the spoon.
9. We find that it is helpful to work in teams of two: one assisting and one squeezing. The assistant anesthetizes fish, dries them before squeezing, and ensures recovery after the sperm samples have been obtained (**steps 1**, **2**, **4** and recovery of fish in **step 5**). The assistant is also the record keeper.

   The other person squeezes males, mixes sperm with cryoprotectant, aliquots it into two cryovials, and hands the vials to the assistant to be capped and placed on dry ice (**steps 5–10**). The assistant then notes on the record sheet the time the vials are placed on dry ice and tracks the time that samples need to be moved from dry ice to liquid nitrogen. Either of the team members can carry out **step 11** (vials into liquid Nitrogen). However, it is usually the person assisting, who can keep better track of the time samples remain in dry ice.

   It is possible for one person to perform the procedure when, for example, males are used for preparation of genomic DNA samples and are not recovered from anesthesia. In this situation, it is not necessary to track fish recovery after squeezing and therefore assistance may not be required (*see* **Note 11**).
10. Sperm motility is completely blocked at 300 mOsmol/kg. One percent of sperm are motile around 288 mOsmol/kg. Decreasing the osmotic pressure of the solution further will activate more sperm *(16)*. Because water will dilute seminal fluid and activate sperm, it is important to dry the urogenital area as thoroughly as possible.

11. The preferred method for cryopreservation at the ZIRC is to expel sperm and revive the donor for future needs rather than dissecting testes. At ZIRC, a large amount of work goes toward identifying carriers for genetically distinct lines to provide as many lines as possible to the research community. After cryopreservation, revived males can be used again for additional freezing of germplasm, for in-house breeding of the line, to provide as adults, or to be used to generate embryos that are supplied to researchers. Because the ZIRC uses fish for multiple purposes, it is necessary to give males a sufficient recovery period after cryopreservation, typically 1 mo before they are anesthetized and squeezed again for cryopreservation. This helps to reduce stress and maximize a healthy reproductive lifespan. If the males are not needed for anything other than cryopreservation, it is possible to give them a shorter resting period of 2 weeks.
12. The amount and quality of sperm varies from fish to fish. Good sperm is white and opaque. Poor sperm appears watery. We keep track of sperm sample quality and assign numbers for quantity (empirical: milky, opaque sample; **Fig. 1e**, *right*).

    In general, the minimal requirements to keep a sample are either 0.5 µL of good sperm (3–4 mm in capillary), or 1.5 µL of poor sperm (**Fig. 1e**, left; or 6–7 mm in capillary).
13. Several samples can be pooled when collecting sperm into a glass capillary. This can be achieved by adding sperm from several males up to the normalizing mark on the capillary. In this case, the cryoprotectant is then added directly, omitting **Subheading 3.1, step 6**.
14. A general advantage of this protocol is that it is relatively easy to pool several samples and thus, to increase the total volume of sperm in individual samples. Additionally, pooling of samples is beneficial for post-thaw fertilization rates (Carmichael and Varga, personal observation; Bruce Draper, personal communication) because sperm from a fertile male may compensate for sperm from less fertile males in the mix.
15. Speed is important! The time between adding the cryoprotectant and placing sperm vials in dry ice (**steps 7–10**) should not exceed 30 s. Methanol permeates sperm cells rapidly *(11)*.
16. The use of standard laboratory pipets and 2 mL cryovials makes it easy to learn and perform the Draper–Moens protocol. However, with the Draper–Moens protocol, the small sample size stored in cryovials represents a disadvantage. With this method, the storage space in the freezer is

not efficiently used compared with the Walker–Streisinger Method *(10)* for example, where up to 40 capillaries with frozen sperm samples can be stored in one 5 mL cryovial. In contrast, the advantage to storing individual samples in 2 mL vials is that each sample can be labeled and handled independently. The use of 2 mL screw-cap cryovials is also beneficial for maintaining good safety standards, such as preventing the spread of pathogens among frozen samples.

17. It is crucial to observe the 20-min time limit. If samples are kept for less than 20 min on dry ice, intracellular crystallization may damage the cells as a result of incomplete freezing by the time they are submerged into liquid nitrogen. In contrast, if they remain on dry ice for longer than 20 min, they risk osmotic pressure imbalance and cellular dehydration. Unfortunately, the exact freezing rate obtained with these methods has not been accurately determined. Preliminary measurements indicate an asymptotic freezing curve with a relatively abrupt change in freezing rate 2 min after the sample has been placed into dry ice. Initially, the freezing rate is approximately 30°C/min(during the first 1–2 min) followed by a much slower freezing rate of approximately 2–3°C/min (3–10 min; Carmichael and Varga, unpublished observations).

    Other methods will produce different freezing rates that need to be tested for post-thaw fertility rates. Good results have been obtained in a recent series of tests with a controlled rate freezer at a constant freezing rate of 10°C/min *(16)*.

18. The relatively small sample sizes and the current practice of adding powdered skim milk prevent assessment of important sperm quality control parameters such as cell motility, morphology, density, and total cell count before the sperm is frozen. In addition, the current protocol specifies that the time between adding cryoprotectant to sperm and placing the sperm on dry ice is limited to 30 s or less, which also reduces the opportunity to assess sample quality. More research is necessary, and our recent studies suggest that exposure time to the cryoprotectant may be extended up to 10 min without major impact on sperm motility and cell survival *(16)*. Without assessment of these important sperm quality control parameters, it is difficult to predict resultant post-thaw fertilization rates. As a result, little is understood about the integrity of individual samples, and post-thaw fertilization rates continue to be highly variable. Yang and colleagues also found that a final dilution of ~$10^8$ cells/mL yielded the most optimal post-thaw fertilization rates, making the use of powdered milk unnecessary because coagulation of sperm

tails was less likely at this density. In the existing cryopreservation protocols, the cryoprotectant solutions are standardized at the expense of varying sperm densities. To maximize post fertilization success for individual samples, both cell density and final dilutions need to be calculated to obtain optimal results.

19. Only gentle pressure is necessary; if the female can produce eggs, they will be released readily. If gentle pressure fails to release eggs, do not continue to squeeze harder because this might injure the fish.

20. "Good" eggs have a yellowish, translucent color (**Fig. 3b, c**), whereas eggs that have remained in the female too long are white and watery (**Fig. 3d**). To maximize the likelihood of obtaining "good" eggs, collect them during the first 2 h after the light has been turned on in the fish facility. The characteristics of eggs are as follows: "good" – slightly yellowish, granular-looking eggs, translucent, not watery (**Fig. 3b, c**); "bad" – eggs already broken down, white-ish (**Fig. 3d**), watery, or with the consistency of baby cereal or cottage cheese (not shown); "mixed" – some potentially good eggs mixed with bad eggs (**Fig. 3d**).

21. Ideally, 3–4 clutches of eggs should be obtained. Additional clutches should be collected within 4 min of the first clutch to prevent the eggs from drying out (*see* **Note 22**). The number of eggs collected for post-thaw in vitro fertilization should take into account the typical survival rate in the nursery (if necessary) and the fertilization rates of previous test thaws (*see* **Note 23**). Combine the clutches using the metal spatula. Allow females to rest one month before they are anesthetized and squeezed again for in vitro fertilization.

22. To prevent the eggs from drying out, while obtaining additional eggs we prepare a humidity chamber for short-term storage by placing a moist paper tissue into a 100 × 20 mm Petri dish. Place the 35 × 10 mm dish containing the eggs upside down in the chamber.

23. Post thaw fertility rates may be highly variable because of differences in sperm quality and quantity among individual fish (*see also* **Note 3**). To test whether frozen samples can be recovered, we perform "test thaws" for each frozen line. We set a threshold of at least 5% post thaw fertility, which is calculated by counting all fertile embryos divided by the total number of infertile plus fertile embryos (no. fertile embryos/no. total eggs). If the first sample does not pass the threshold, we thaw one or more additional samples, up to three samples per line. If all samples fail the test, new samples will be frozen and the process of test thawing will be repeated.

In the absence of quality controls such as cell counts and motility assessment before freezing, test thawing allows us to be confident that frozen lines can be recovered.

24. Several factors in addition to those already mentioned (*see* **Notes 18** and **23**) may also contribute to the variable outcome of post-thaw fertilization, including (but not limited to): skill level and proficiency of the individual researcher, materials used in different laboratories or between different freezing events, male variability, variations in sperm sample volume and quality, varying freezing rates, or thaw rates, and egg quality during in vitro fertilization. To predict sample reliability accurately and, therefore, the probability of recovering valuable research lines, current techniques including thawing and *in vitro* fertilization need to be further improved and standardized. Currently, the Draper–Moens method *(5)* on which this protocol is based is the most successful and efficient protocol and yields relatively consistent post-thaw fertility rates (averaging around 20% at the ZIRC; varying between 0 and 80%). With this method, 100% recovery of lines can be ensured by freezing a sufficient number of samples and by pooling samples for lines with low fertility rates.

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