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An Easy Method for Cryopreservation of Zebrafish (*Danio rerio*) Sperm

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Abstract

We developed an easy, efficient, and cheap protocol for zebrafish sperm cryopreservation carried out on dry ice (20 min) using simple composition solution (200 mM glucose, 40 mM KCl, 30 mM Tris, pH = 8.0). The average efficiency of the present cryopreserve method was between 10% and 20% (expressed as fertilization rate). The experiments were conducted and repeated at two different locations, in different countries, yielding very similar results, showing the reproducibility and applicability of the method.
Keywords: zebrafish, *Danio rerio*, sperm, cryopreservation, dry ice

The widespread use of zebrafish (*Danio rerio*) as an animal model has led to numerous mutant strains and transgenic lines. This created the need to store these genetic resources, to prevent accidental loss, and to reduce space needed to maintain them. Cryopreservation can be a solution allowing to store genetic resources indefinitely and to recover them without compromising physiological function.[1–3] There are many zebrafish sperm cryopreservation protocols; however, they vary in basic parameters including the freezing method,[4,5] number of males to collect sperm,[5,6] and types of extenders and cryoprotectants. Herewith, we develop an optimized, cheap, and straightforward zebrafish sperm cryopreservation protocol, as an adaptation for zebrafish, of a method previously published using grayling extender.[7] Our method was consistently reproducible in different laboratories.

Wild-type zebrafish from AB lines were kept at the University of São Paulo Medical School, Brazil, whereas one other stock was maintained in Department of Aquaculture, Szent István University, Gödöllő, Hungary. Zebrafish males were separated from females 1 week before sperm collection. The fish were anesthetized before the experiments with 0.06% of tricaine methanesulfonate (MS-222).[8] The anesthetized zebrafish were gently dried with paper towel and placed belly up in the slit of a dampened foam holder. The urogenital papilla was further dried just before sperm collection to prevent contamination with water. Five microliters of sperm mixed by pooling (1 μL per male generally) was collected by stripping in 25 μL of grayling extender.[7] Immediately before freezing, 30 μL of diluted sperm (25 μL grayling extender +5 μL sperm) was toped up to the volume of 100 μL by adding 62 μL of grayling extender and 8 μL of methanol, resulting in a dilution ratio of 1:19 (sperm to extender+cryoprotectant) and 8% final concentration of methanol as cryoprotectant. Fifteen milliliters Falcon tubes were inserted into crushed dry ice before the stripping. The samples were loaded into 0.25 mL French straws that were

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placed in Falcon tubes individually. The tubes containing straws were closed with their screw caps and were held for 20 min[4] in the crushed dry ice, and then they were plunged directly into liquid nitrogen and kept until fertilization. Prior fertilization, the cryopreserved straws were thawed in a water bath at 40°C for 5 s.

Breeding tanks with a barrier separating males and females were prepared one night before fertilization. The sex ratio was 1:2 (one male to two females). In the early morning, once the light turned on, eggs were removed from at least three anesthetized females by squeezing their bellies into a dry 52 mm Petri dish. Only eggs that had a satisfactory visual appearance (round shape, yellowish color, and strong texture) were used for fertilization. The cryopreserved and thawed sperm were added to the eggs with 1 mL of system water to activate the gametes followed by gentle shaking for 2 min. Five minutes after fertilization, water was replaced on the egg batches and they were incubated at 27°C ± 1°C. The fertilization rate was determined on the following day, as the percentage of fertilized embryos related to the total number of eggs per batch. Two groups of experiments were performed, one in São Paulo while the other in Gödöllő. Twelve replicates were done at both locations.

The average fertilization rate with standard deviation was 11.42% ± 8.06% in Brazil and 19.99% ± 9.68% in Hungary (Supplementary Table S1; Supplementary Data available online at www.libertpub.com/zeb). Our lowest fertilization rate was 2.39% and highest was 35.29%. Even in some cases with low fertilization rate, we have not got 0% in any case. Consequently, all samples had a measurable efficiency, thus, the proportion of successful samples is 100%.

Nowadays, there are several methods for zebrafish sperm cryopreservation (Supplementary Table S2), but some can be challenging to reproduce.[9] Our method is simpler and cheaper than other available protocols in the literature. We have repeated the experiments 12 times at two different locations. However, we also observed variation among the pools, this disparity is normal in the available protocols and experiments as well.[10–12] The differences of diet composition may influence physiological processes such as survival, growth, and reproductive performance,[13] endocrine function,[14] differences of the sperm and egg quality, and even the definition of successful fertilization[5] can influence the disparity of results. In conclusion, this study showed a simple protocol to cryopreserve zebrafish sperm, with a good efficiency and reproducibility that can be done in any zebrafish holding facility without special equipment or devices.

Materials and Methods

See Supplementary Data for methods.

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Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Data
Supplementary Table1
Supplementary Table2

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