

Protocol D - Cryopreservation of sperm [Practical guide of protocols: sperm quality]

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Protocol D

Cryopreservation of sperm

I - Introduction

Cryopreservation of gametes and embryos is a key issue in the development of reproductive technologies providing the possibility to preserve genetics from rare or valuable breeds (gene banking), giving insurance against death, infertility or illness and enabling semen and embryos to be marketed or to be exported. Long-term preservation of sperm is commonplace in cattle breeding and is being adopted in aquaculture. Creation of sperm banks to stock genetic potential of valuable males for selection programmes, or banking for routine sperm handling, are being adopted in some fish farms.

As has been noticed by different researchers (Billard and Zhang, 2001), the progressive interest in the application of cryopreservation to aquaculture has revealed how useful this method could be in the management of fish reproduction, and moreso when it is combined with other reproductive technologies, often inapplicable in mammals, such as androgenesis or sex reversal. Some of the applications, in use or under development, are:

- (i) Storage of sperm for handling routine in those species requiring artificial insemination (salmonids, turbot).
- (ii) Crossbreeding programming independently of the maturation period or availability of breeders.
- (iii) Utilization of all the sperm from species or individuals with a large production.
- (iv) Increase of the fertile life of the individual (especially interesting for males of salmon, protandric species).
- (v) Transport of gametes or embryos between farms instead of breeders.
- (vi) Marketing of well-characterized and standard quality sperm (genetics as a business).
- (vii) Hybridization between species with different maturation periods.
- (viii) Reduction of the synchronization treatments.
- (ix) Year-round supply of gametes.

The creation of sperm banks of the selected stock to prevent outbreaks, catastrophes and genetic drift is essential to develop genetic selection programmes in commercial aquaculture, but it is also necessary for conservation of strains or species in danger of extinction, until the environmental conditions are recovered (1275 fish species threatened according to the International Union for Conservation of Nature, www.iucnredlist.org). Reproductive technologies are being developed to recover a population just from frozen sperm, applying further hybridization programmes of using androgenetic procedures (Babiak *et al.*, 2006; Grunina *et al.*, 2006). Biotechnology, ecotoxicology and basic sciences could also benefit from cryobiology through the preservation of gametes or embryos from model species or strains as well as from sperm or embryos genetically modified with research or commercial production purposes (polyploids, transgenic).

Sperm cryopreservation procedures have been developed for many aquacultured species (Billard and Zhang, 2001; for review) including different salmonids (Lahnsteiner *et al.*, 1995, Cabrita *et al.*, 2001), carp (Linhart *et al.*, 2000), sturgeons (Grunina *et al.*, 2006), turbot (Dreanno *et al.*, 1997), sea bass (Fauvel *et al.*, 1998) or seabream (Cabrita *et al.*, 2005).

II - Purpose

Sperm freezing causes cellular damage and reduces sperm quality and fertility post-thaw, so careful protocols have to be developed to minimize fertility loss. In this Protocol, sea bass sperm is cryopreserved in liquid nitrogen (LN) using two different containers (0.25 ml straws and 1.5 ml cryotubes), according to the method developed by Fauvel *et al.* (1998) with minor modifications. After freezing/thawing sperm quality is assessed and compared with that of fresh sperm. Motility is evaluated following Protocol *D* and the percentage of viable and non-viable cells is established using fluorescent probes according to the method described by Cabrita *et al.* (2005).

III - Procedure

(i) Collect milt by abdominal massage from anaesthetized males after cleaning and drying the genital pole area (see Protocol *E*). Sperm will be collected with a syringe (without needle), and samples with whitish and dense appearance will be used for freezing, discarding those contaminated with faeces and urine.

(ii) Evaluate sperm motility and concentration (Protocol *E*) and discard samples having less than 1×10^9 spz ml^{-1} as well as less than 80% motile sperm cells.

(iii) Dilute sperm samples in cryoprotectant (CPT) solution (1:3; sperm: CPT solution). The extender may be prepared in advance. Add 10 mg ml^{-1} BSA and 10% DMSO before use.

(iv) Load 0.25 ml french straws and 2 ml cryovials with the diluted sample using a 1000 μl micropipette. Close the cryovials and seal the straws with a sealing machine or PVP powder, avoiding heating during handling.

(v) Fill a 15 cm interior height styrofoam box with LN and place the straws in a 6 cm high tray floating on LN surface. Close the box and keep the samples on the tray for 12 min. Then plunge them into the LN.

(vi) In another similar box, place the cryovials floating in a 2 cm high tray for 10 min before being dropped into LN.

(vii) Wait at least 10-15 min before proceeding with the thawing process. Thaw straws in a 35°C water bath for 10 sec, and cryovials in a 50°C water bath for approx. 60 sec.

(viii) Immediately after thawing evaluate sperm motility according to Protocol *D*.

(ix) Cell viability is assessed using the fluorescent probes PI/SYBR14. Add 2.5 μl of IP working solution and 0.5 μl of SYBR14 working solution prepared in advance to 5 μl of fresh or 15 μl of frozen/thawed sperm in eppendorf tubes. Eppendorf tubes are incubated in the dark for 5 min. Subsequently, add 500 μl of a non-activating medium (Protocol *E*) and smear a drop (20 μl) on a slide.

(x) Observe under a fluorescence microscope. Dead cells are red labelled by PI and viable ones green labelled by SYBR14.

Note: Beakers, tubes and straws containing sperm must be handled without touching them under the sample level to avoid sperm overheating throughout this procedure.

IV - Materials and equipment

- Materials for sperm sampling (see Protocol *E*)
- Plastic tray with crushed ice
- 5-10 ml glass tubes

- 10, 200 and 1000µl micropipettes and tips
- 100-250 ml glass flasks
- Liquid nitrogen containers and supply
- 0.25 ml french straws
- 2 ml cryovials
- Styrofoam box (15 cm interior height)
- Forceps (longer than 15 cm)
- Timer
- Water bath and thermometer
- 1.5-ml eppendorf tubes
- Glass slides for fluorescence microscopy
- Fluorescence microscope
- Gloves and lab coat

V - Reagents and solutions

- 10 mg ml⁻¹ BSA
- 10% DMSO
- Extender for cryopreservation: KHCO₃, 10.1 mg. ml⁻¹; reduced glutathione, 1.99 mg. ml⁻¹ and sucrose, 42.78 mg. ml⁻¹ in distilled water. Check and adjust the osmolarity to 310 mOsm kg⁻¹. The extender may be prepared in advance and, immediately before use, add 10mg ml⁻¹ BSA and 10% DMSO
- Non-activating solution (see Protocol E)
- LIVE/DEAD sperm viability Kit (Molecular Probes).
- Liquid nitrogen must be handled with care, using appropriate gloves and forceps and avoiding contact with the skin or clothes. Aspiration of nitrogen vapours may cause asphyxiation. Work in an aerated place or in a room equipped with grid ventilation at ground level.

VI - Results and discussion

A general schematic flow chart of cryopreservation of sperm in fish is shown in Fig. D.1. In this procedure sperm is frozen in two different containers. The use of straws (0.25 ml straws) allows the homogeneous freezing of the sample, giving optimal conditions for sperm preservation. Nevertheless, preservation of small volumes is not practical for sperm handling on farm. The use of larger containers (2 ml cryotubes) is more advantageous from this point of view, but it could increase cell damage during the freezing/thawing process. Sperm frozen in straws should recover an initial motility similar to that of fresh sperm at thawing. However, significant differences in motility could be observed at further post-activation times, due to the motility decreases being faster in thawed samples. Sperm frozen in cryotubes can probably display a slight decrease in motility, even immediately after thawing. There is no reference about spermatozoa viability after freezing/thawing, but a significant reduction in the percentage of viable cells at thawing is also expected, especially with the use of cryovials, indicating that a higher sperm/egg ratio is required for further fertilization with thawed sperm. Sperm viability can be assessed using fluorescent probes (Fig. D.2).

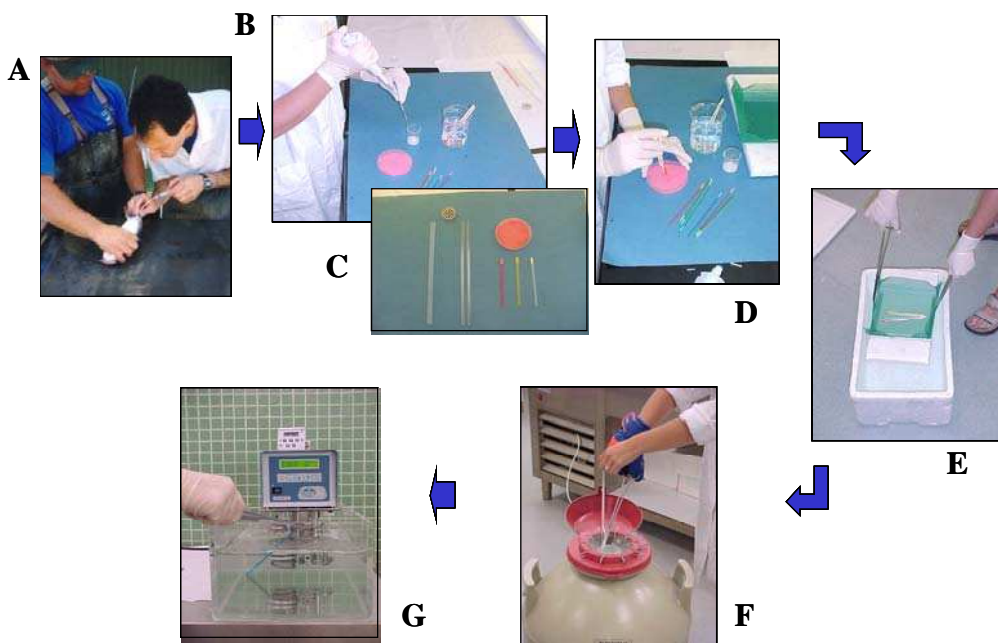


Fig. D.1. Process of freezing/thawing. (A) Sperm extraction. (B) Sperm dilution with the freezing extender and loading in the straws. (C) An example of different straws, sealing powder and beads. (D) Sealing of straws using PVP powder. (E) Placing a floating tray with the straws in a Styrofoam box containing liquid nitrogen. (F) Storage of a canister containing the straws in a liquid nitrogen tank. (G) Thawing a straw in a water bath.

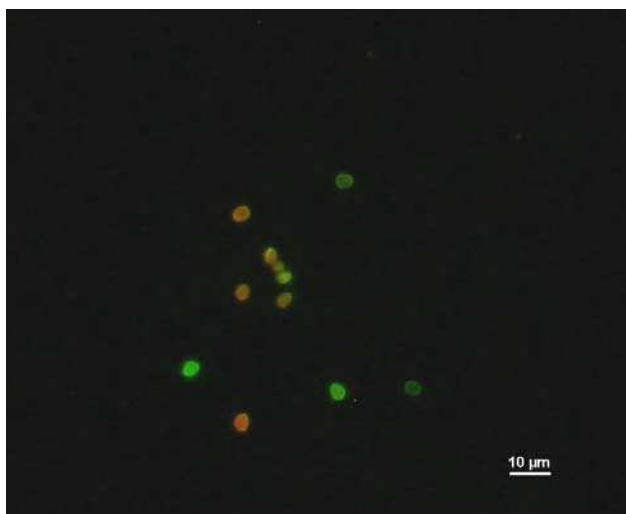


Fig. D.2. Staining of viable and non-viable sperm cells (Rainbow trout) using fluorescent probes. Dead cells are red labelled by PI and viable ones green labelled by SYBR14.

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