Cryopreservation protocol for Ciona intestinalis *sperm*

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In *Ciona* research, the use of genetic resources has raised the interest in standard procedures for sperm freezing. Cryopreservation of *Ciona intestinalis* sperm represents a fundamental tool for the storage and distribution of transgenic and mutant lines of this important model system. Sperm cryopreservation was investigated in *Ciona* with the aim to develop a procedure for routine use that provides repeatable good post-thaw fertility (capable of bulk fertilisation and subsequent development of normal larvae).

Note: Since for unknown reasons some batches of cryopreserved sperm are not able to fertilize eggs, it is a good idea to freeze as many aliquots (pailettes) as possible.

Reagents: DMSO, 0.22 µM Millipore filtered natural sea water (MFSW), EtOH, liquid N2.

Equipment: Pasteur pipettes, 2 ml epps vials, 50ml Falcon tubes, cryopreservation paillettes, metallic grid with a 6 cm-high polystyrene outer frame, 15 cm Petri dishes, sterile forceps (flame sterilized with alcohol), Bunsen burner, 0.22 µM Millipore Filter, cryo-container.

1. Collect sperm with a sterile Pasteur pipette and transfer it into a pre-chilled 2 ml vial on ice.
2. Dilute sperm 1:5/1:10 (depending on sperm dryness) adding pre-chilled 0.22 µM Millipore-filtered natural sea water (MFSW).
3. Add 1:10 DMSO to diluted sperm, wait 15 min and then transfer readily the solution to cryo-paillettes on ice.
4. Chill sperm by leaving cryo-paillettes for 15 min on a metallic grid (with an outer 6 cm-high polystirene frame) floating on liquid N2.
5. Adapt sperm to pre-freezing conditions by keeping cryo-paillettes into liquid N2 for 10 min.
6. Store cryo-paillettes in liquid N2 into a cryo-container.
7. To use cryopreserved sperm for fertilization, remove a cryo-paillette from liquid N2, hold it in hands for 30 sec, cut it with sterile forceps and let sperm to drop in 15 cm Petri dish containing ~300 eggs collected from at least 2 individuals.

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