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Technical Report

A simple and reliable protocol for cryopreservation of Medaka (*Oryzias latipes*) spermatozoa

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Cryoconservation of sperm is a very suitable strategy for the preservation of important strains or mutants. Although this procedure is routinely used in aqua culture for a variety of different species (Rana and Mc Andrew, 1989, Steyn and van Vuren, 1987, Wheeler and Thorgaard, 1991), an efficient protocol that allow reliable sperm cryoconservation of small freshwater fish without a need to kill precious males are hardly found. Poorly defined media like milk powder, egg extracts or fetal calf serum used as cryoprotectants (Aoki *et al.*, 1997, Chao *et al.*, 1987, Wheeler and Thorgaard, 1991) may be the cause for variable fertilization rates. Here we describe a rapid and reliable protocol that uses sucrose and DMSO as cryoprotectants (Holtz, 1993). Our protocol allows to efficiently freeze and recover sperm obtained from living fish.

In brief sperm is obtained from the males by gently squeezing their testis with a pair of forceps. The sperm is subsequently mixed with freezing medium in a glass capillary. Sperm containing capillaries are brought to successively lower temperatures and are eventually stored in liquid nitrogen. Successful recovery of the preserved stock after several month of storage in liquid nitrogen is routinely achieved by *in vitro* fertilization.

Sperm freeze

Successfully breeding male fish are separated from their females one day prior to the sperm squeezing. Fish are anesthetized for about five minutes in a 0.17 mg/ml buffered (pH 7) tricaine solution set up with tank water. 50 μl of freshly prepared freezing medium (0.6 M Sucrose and 10%DMSO, Sigma) is put into a preecooled depression slide. Anesthetized fish are put into a prewetted foam bed and traces of liquid and feces are removed from the anal region with a paper tissue under the dissecting microscope. Using a pair of Millipore forceps the sperm is carefully squeezed out of the fish by gently striping from the middle of the belly close to the air bladder towards the anus. The expelled sperm is aspirated into a microcapillary where the level of sperm in the is measured with a ruler. Sperm is mixed by aspirating two volumes of ice cold freezing from the depression slide and the capillary is put into a prelabelled cryo-tube. The tube and capillary are transferred to a 15 ml falcon tube that has been precooled on dry ice. Here sperm is incubated for 20 minutes before the cryo tube is screwed with its cap and directly transferred into liquid nitrogen. Any warming the tube is carefully avoided as once thawed, the sperm can not be refrozen.

In vitro fertilization

For *in vitro* fertilization females that produce many good eggs are isolated a day prior to the fertilization and kept single to prevent them from spontaneous spawning. Ripe oozytes are isolated from the ovary of donor female after decapitation and transferred into a petri dish containing 1*x* Yamamoto ringer (Yamamoto, 1975). The follicle layer is removed manually with a pair of sharp forceps. For the fertilization 100 μl 1*x* Yamamoto ringer is put into a depression slide, one sperm capillary is rapidly thawed between the fingers, the sperm is expelled into the ringer and carefully mixed. As much as possible ringer solution is removed from the oocytes before they are overlaid with this sperm suspension. After incubation at room temperature for 2–5 minutes successful fertilization is scored by the occurrence of the cortical reaction (concentration of oil droplets at the vegetal pole). Unfertilized oocytes will stay opaque. Fertilized eggs are transferred into a petri dish containing hatching solution (Kirchen and

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West, 1976) and incubated at 28°C. On average one capillary of sperm is sufficient to fertilize more than sixty oozytes. The fertilization rates observed routinely were higher than 85%.

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A detailed protocol is available upon request.

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