Rescue protocols to recover sperm frozen using the classical Nakagata method or Ostermeier's method with Monothioglycerol supplemented Cryoprotective Agent.

Sperm concentrations in samples frozen using the classical Nakagata method (18% Raffinose, 3% Skimmed milk CPA) or the method published by Ostermeier *et al*., (2008) (18% Raffinose, 3% Skimmed milk supplemented with 477µM monothioglycerol (MTG) are typically stored in larger volumes of cryoprotective agent than our current samples. The concentration of sperm in these samples is therefore lower than that of samples stored using the gCPA protocol. This protocol provides adjustments to the MBCD + GSH IVF protocol which can be used to optimise results using these legacy samples.

A. IVF procedure

1. Prepare IVF dishes and GSH solution as described in the standard 'frozen' IVF protocol.

2. Harvest oocytes as described in standard IVF protocol.

B. Thawing sperm samples frozen in straws using the Ostermeier, Monothioglycerol method

1. Remove the required straws from the storage tanks and place in a flask of liquid nitrogen.

2. Quickly transfer the straw into a 37°C water bath and leave for 10 minutes.

Note: To ensure warming of the frozen sperm, completely immerse the part of the straw containing the sperm in the water bath. Furthermore, frozen-thawed mouse sperm are sensitive to environmental changes. If the straw is not kept in the water bath long enough (10 minutes), the motility of the cryopreserved sperm will be reduced.

3. Carefully dry the straw with a paper towel (vigorous rubbing can cause friction and increase temperature inside straw).

4. Cut the heat-sealed end of the straw, nearest to the sperm. Then cut through the centre of the cotton plug (Fig 2).

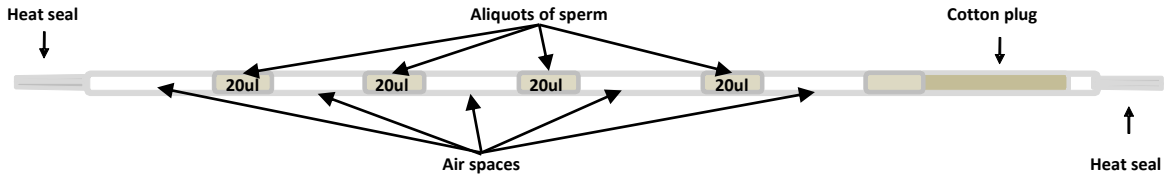

Fig 2.

5. Use a metal rod to expel all the sperm suspension into the centre of a 353004 60mm Petri Dish.

6. Using a wide bore pipette tip, add 30µl sperm suspension from the dish into the centre of the drop of 90µl TYH+MBCD pre-incubation medium (Fig 3).

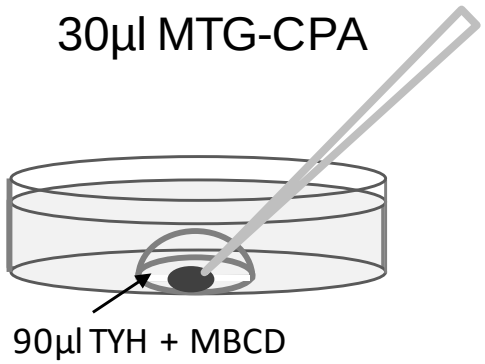

Fig 3.

Note: Do not disturb the dishes containing the frozen/thawed sperm until the sperm are moving rapidly within the medium. If the dishes are disturbed before the sperm starts to move they will not recover full motility.

7. Pre-incubate the frozen/thawed sperm in TYH+MBCD medium for 30min at 37ºC in the CO_2 incubator.

8. Proceed with the IVF, adding 10-20µl sperm to each fertilisation drop as described for frozen/thawed sperm in the standard protocol.

C. <u>Thawing sperm samples frozen in vials according to the classical Nakagata method.</u>

1. Using forceps hold the cryotube in air for 30 seconds. If liquid nitrogen is present in the cryotube, wait for it to evaporate and escape by rolling the cryotube around on the bench.

2. Take special care that the cryotube is not filled with liquid nitrogen before plunging into the water bath (such tubes may explode).

3. Thaw the sperm sample rapidly by placing the vial in a 37°C water bath and keeping it there for 10 minutes.

4. Once thawed, using a wide bore tip, pipette 30µl sperm suspension from the vial into the centre of the drop of 90µl TYH+MBCD pre-incubation medium (see Fig 3, above).

5. Sperm motility will initially appear poor but after ~10 minutes the motility will return to normal levels in ~50% of the spermatozoa.

Note: Do not disturb the dishes containing the frozen/thawed sperm until the sperm are moving rapidly within the medium. If the dishes are disturbed before the sperm starts to move they will not recover full motility.

6. Pre-incubate the frozen/thawed sperm in TYH+MBCD medium for 30min at 37ºC in the CO_2 incubator.

7. Proceed with the IVF, adding 10-20µl sperm to each fertilisation drop as described for frozen/thawed sperm in the standard protocol.