

INFRAFONTIER-I3 - Cryopreservation training course

Hosted by the Frozen Embryo and Sperm Archive, MRC Harwell

Cryopreservation of mouse sperm using L-glutamine supplemented CPA

This protocol is based on the work published by Takeo et al., (2011).

A. Sperm collection and freezing

1. For each male to be frozen label ten 250 μ l plastic semen straws, 1 x Kleenex tissue and 1 x 35mm Falcon 351008 petri dish with the appropriate sample code. Using a permanent marker pen, mark each straw at a distance of 4.5cm and 2.3cm (Fig. 1) from the end furthest from the cotton/PVA plug. Label the straws with the appropriate sample code near the end of the cotton/PVA plug.



Fig. 1

2. Prepare the cooling chamber e.g. a 47 litre LN₂ Dewar (Fig. 2a) filled with LN₂. A column of liquid nitrogen vapour at least 10 to 15 cm high (h1) is required to generate the optimal cooling rate for sperm freezing. In our dewar, a depth of approximately 20-25cm (h2) of liquid nitrogen achieves this (Fig 2).

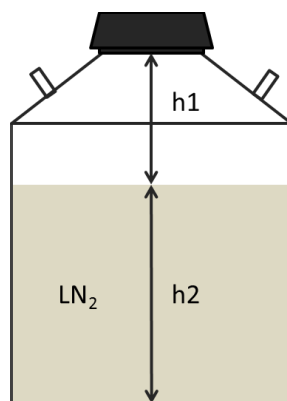


Fig. 2 Cooling chamber. A 10-15cm column of LN₂ vapour (h1) is achieved by having a depth of 20 to 25 cm LN₂ (h2).

3. Prepare the floating sperm freezing apparatus. This can be made from a 50ml syringe attached to a perspex or metal rod. It is important to seal the needle hub (Fig. 3).

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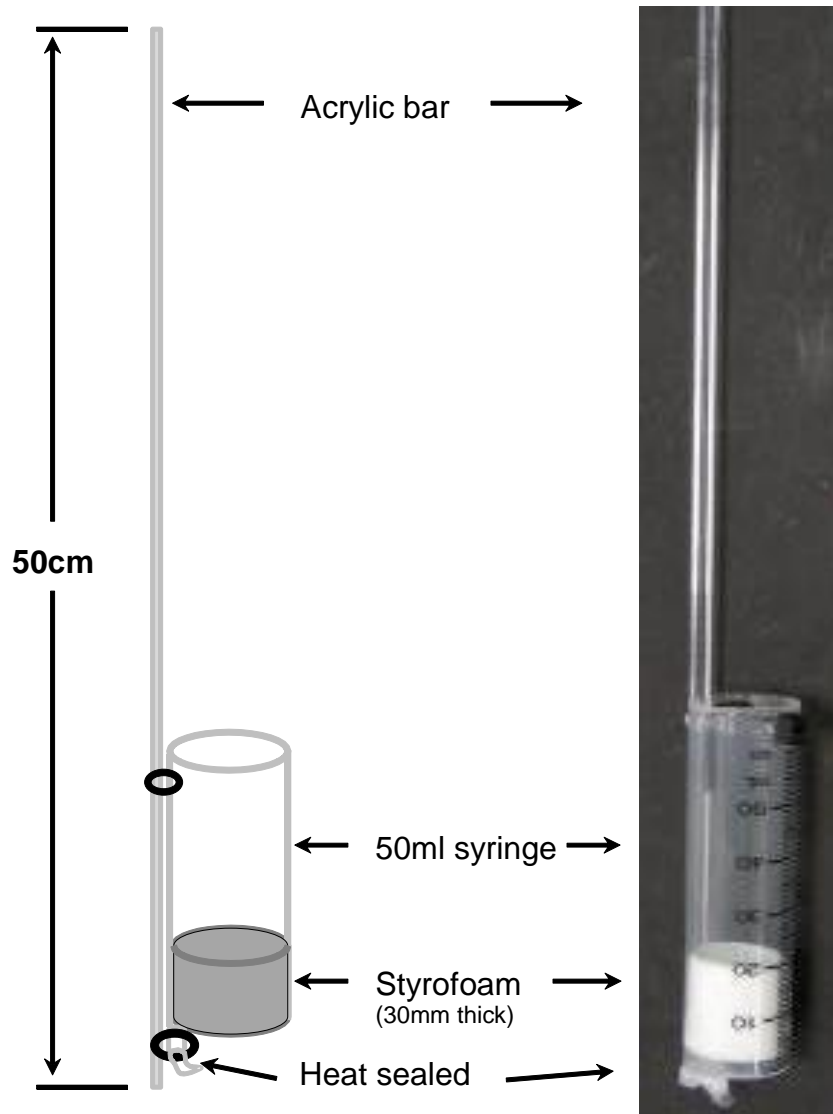


Fig. 3 Floating sperm freezing apparatus

4. Place a 60 μ l aliquot of the sperm cryoprotective agent (gCPA containing 100mM L-glutamine) into the base of a 35 mm culture dish and cover with mineral oil.
5. A second 60 μ l drop of gCPA should be added into the drop (final volume: 120 μ l) to make a tall, semi-spherical drop (Fig. 4). Prepare a separate dish for each male to be sperm frozen.

Note: The optimal volume of gCPA is 120 μ l per two epididymides. If you need to freeze down one epididymis, use a 60 μ l drop.

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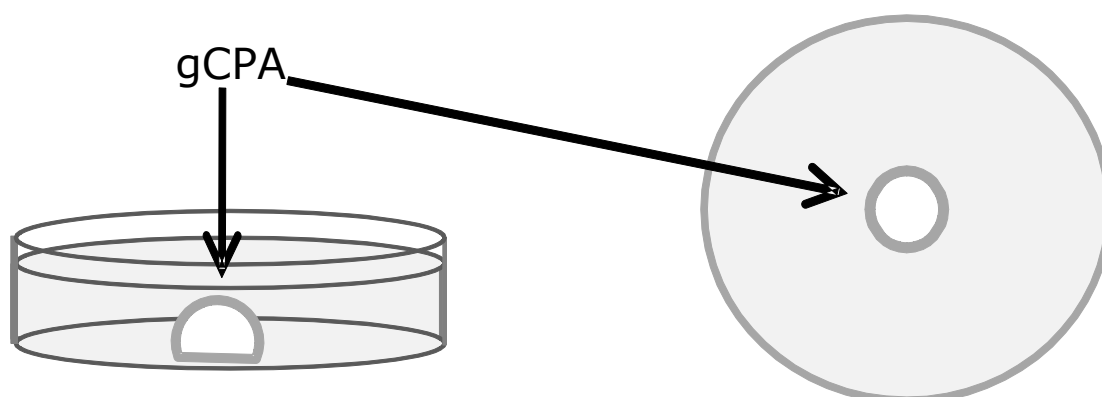


Fig. 4

6. Sacrifice the male and swab the abdomen with 70% ethanol.
7. Dissect the cauda epididymides and clean off all adipose and vascular tissue. This is best achieved by placing the organs on a tissue and examining them under a dissecting microscope lit from above.
8. Transfer the cauda epididymides into a 120 μ l drop of gCPA.
9. Make five to six cuts across the cauda epididymides using a pair of fine scissors or a similar sharp bladed tool and place the dish on a 37°C hot plate (Fig. 5). Swirl the dish gently every minute for 3 minutes to help disperse the sperm.

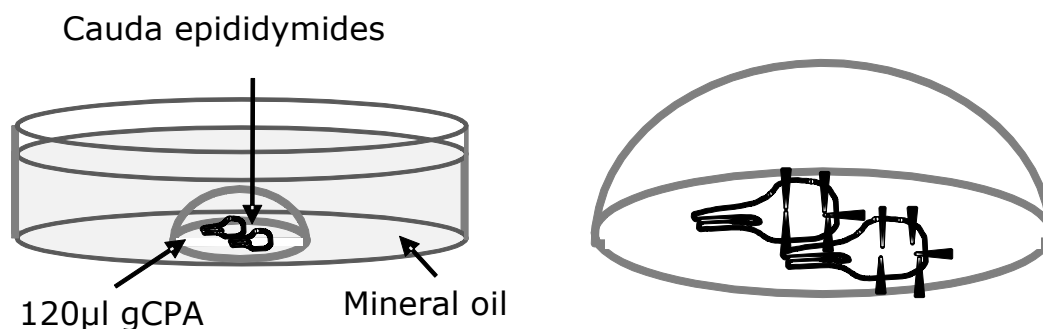


Fig. 5

10. While the sperm is equilibrating, prepare the straws (250 μ l plastic semen straws, Planer; FZA201) for freezing, as follows:
 - 10.1. Attach a 1ml syringe to the labelled end of the straw. Using the syringe, aspirate HTF (high calcium Human Tubal Fluid) solution until the meniscus reaches 4.5cm marker.

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- 10.2. Then aspirate air until the end of the HTF column is drawn up to the 2.3cm mark. Lie the syringe and straw assembly on the bench until required.
11. After 3 minutes, the sperm suspension should be divided into 10 x 10 μ l aliquots on a culture dish (Fig. 6). Then aspirate one drop into each of the 10 straws.

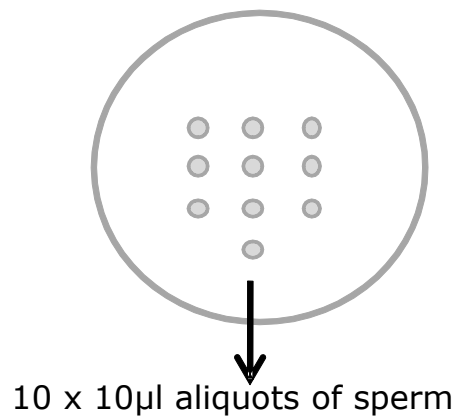


Fig. 6

12. Aspirate air until the HTF meniscus reaches the polyvinyl alcohol section, half way along the cotton plug. This will seal the labelled end of the straw.
13. Seal both ends of the straw (Fig. 7) using a double impulse heat sealer or similar device.

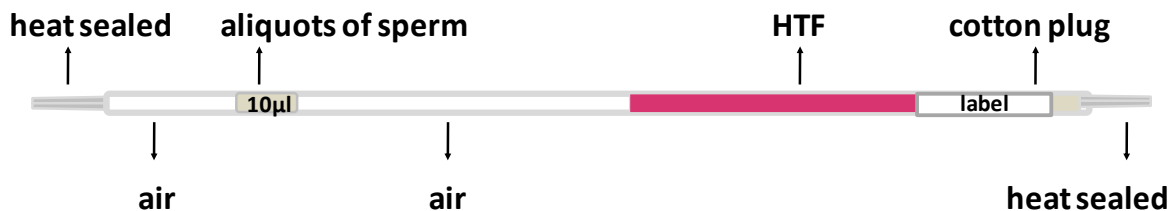


Fig. 7

14. Load the sealed straws into a floating sperm freezing device and then place them into the pre-prepared cooling chamber (Fig. 8) for 10 minutes. After 10 minutes plunge the straws directly into liquid nitrogen.

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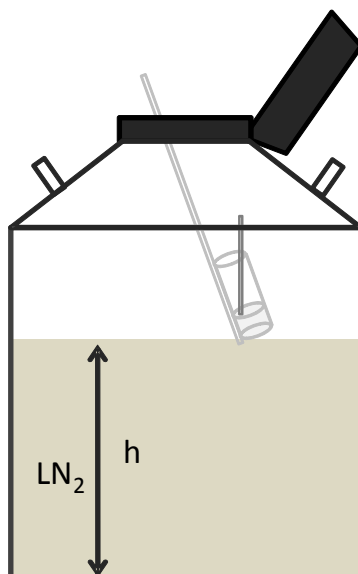


Fig. 8

15. Whilst minimising their exposure to air, transfer the straws into their long term storage locations.

Sperm Thawing Procedure

A. Preparation of sperm dispersal dishes

1. Pipette 90 μ l TYH + 0.75mM MBCD into the centre of a 35mm Petri Dish (Falcon 351008) (Fig.9)

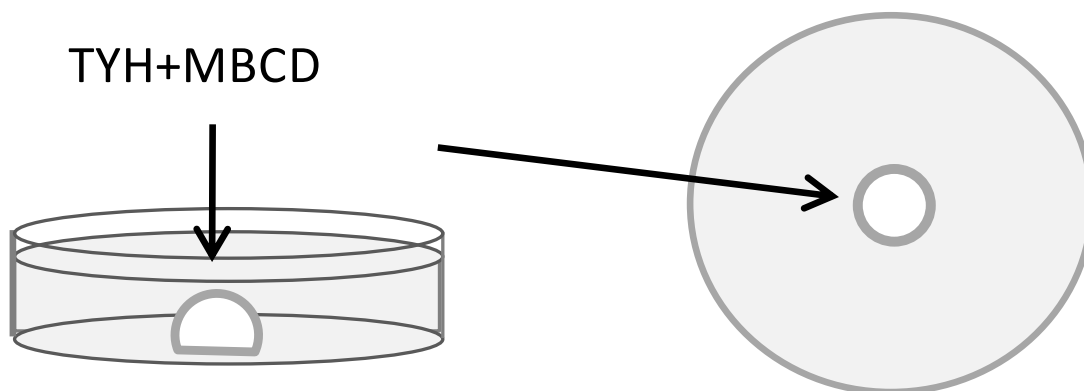


Fig. 9

2. Overlay with mineral oil and equilibrate 10-20min at 37°C, in 5% CO₂ in air.

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B. Thawing the frozen sperm samples

1. Remove the required straws from the storage tanks and place in a flask of liquid nitrogen.
2. Quickly transfer a 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 paper towel. Cut the heat-sealed end of straw and use a straw pusher to expel **only** the 10µl sperm suspension into the centre of the drop of 90µl TYH+MBCD pre-incubation medium (Fig. 10). Do not expel any of the HTF media contained within the straw.

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 start to move they will not recover full motility

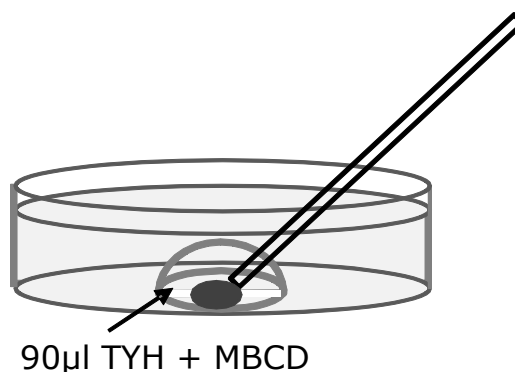


Fig. 10

4. Pre-incubate the frozen/thawed sperm in TYH+MBCD medium for 30min at 37°C in the CO₂ incubator.

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F. Experimental results

The following results show the fertilisation rates of frozen/thawed sperm harvested from six C57BL/6NTac males. In each trial the sperm from one of each pair of cauda epididymides harvested from two males was combined and then frozen using different protocols, namely:

a) the protocol described by Ostermeier et al (2008) (18% raffinose, 3% skimmed milk, supplemented with 447 μ M Monothioglycerol [MTG]) in conjunction with the standard MRC-Harwell IVF procedure but in the 'absence' of MBCD and GSH.

b) the protocol described above (18% raffinose, 3% skimmed milk, supplemented with 100mM L-Glutamine [gCPA]) in conjunction with the MBCD+GSH *in vitro* fertilisation procedure described elsewhere in these course notes.

12 x C57BL/6NTac females were used for each group within each of the 3 individual trials.

Mean fertilisation for C57BL/6NTac USA frozen sperm

Group	Trial 1	Trial 2	Trial 3	Mean (%)
MTG	38.5	45.7	29.3	37.8
MBCD+ GSH	82.8	93.2	61.0	79.0

