Sperm freezing protocol based on the CARD protocol using gCPA that has been supplemented with L-Glutamine.

A. Sperm collection and freezing

1. For each male to be frozen label ten 250µl plastic semen straws, 1 x Kleenex tissue and 1 x 60mm (10060) petri dish with the appropriate sample code.

2. Using a permanent marker pen, mark each straw at a distance approximately 4.5cm and 2.3cm (Fig. 1) from the end furthest from the cotton/PVA plug.

3. Label the straws with the appropriate sample code near the end of the cotton/PVA plug.

Fig. 1

4. Prepare the cooling chamber e.g. a 47 litre LN_2 Dewar (Fig. 2) filled with LN_2 to a depth of 20-25cm.

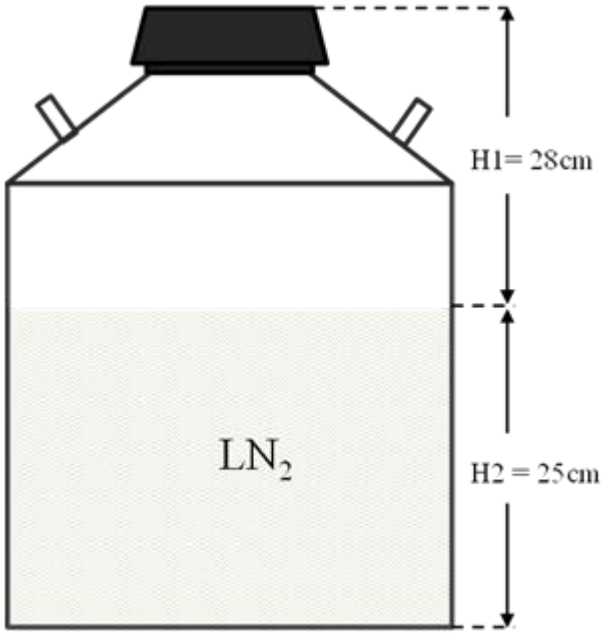

Fig. 2 Cooling chamber. Depth of LN_2 (H2) = 25 cm.

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

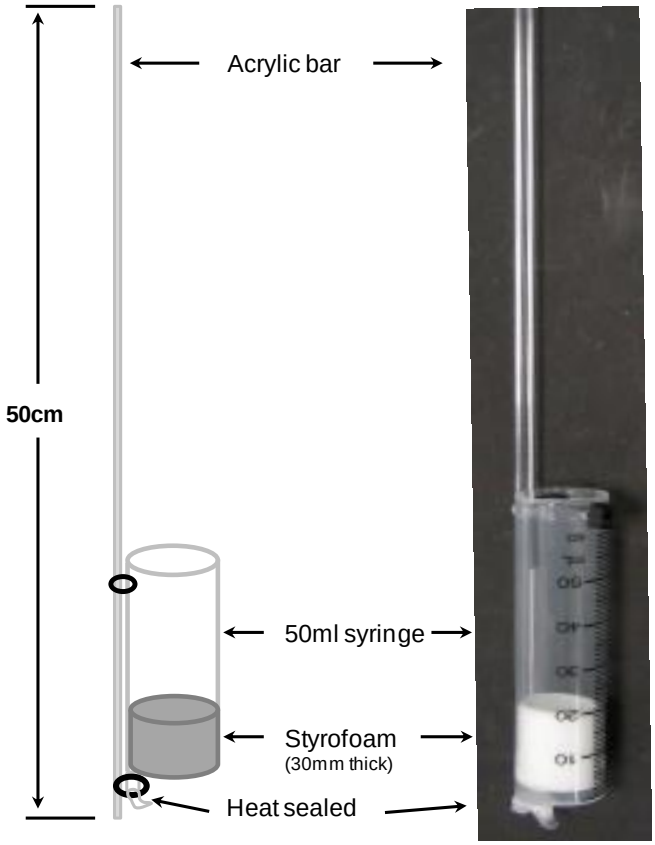

Fig. 3 Floating sperm freezing apparatus

6. Place a 60µl aliquot of the sperm cryoprotective agent (gCPA containing 100mM L-glutamine) into the base of a 60 mm culture dish and cover with silicone fluid or mineral oil.

7. A second 60µl drop of gCPA should be added into the drop (final volume: 120µl) to make a tall, semi-spherical drop (Fig. 4). A separate dish may be prepared for each male to be sperm frozen. Alternatively, the sperm from up to 5 males may be pooled together. To do this, it may be convenient to prepare two 60mm

dishes and place one epididymis from each male into each drop.

NOTE: Use a 60µl drop per epididymis.

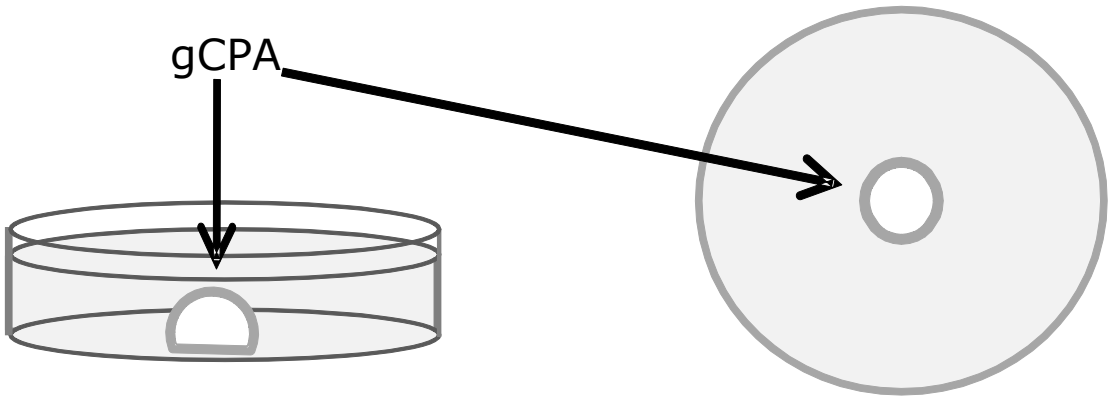

Fig. 4

8. The selected male should be at least 10 weeks old, and not have been used for mating for at least 3 days before sperm collection.
9. Cull the male and dissect the cauda epididymides.
10. Clean off all adipose and vascular tissue. This is best achieved by placing the organs on notelet paper and examining them under a dissecting microscope lit from above.
11. Transfer the cauda epididymides into a 120µl drop of gCPA.

12. Make six to seven 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). Keep the dishes on a 37°C hot plate for 3min avoiding the light from the microscope.

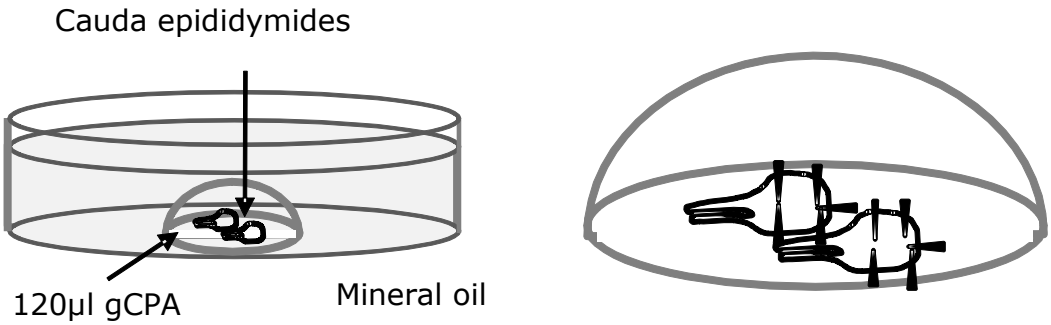

Fig. 5

13. At 2min, 1min and 0min on the countdown timer, gently swirl the dish for 20 seconds to help disperse the sperm.

14. While the sperm is equilibrating, prepare the straws (250µl plastic semen straws, Planer; FZA201) for freezing, as follows:

    a) Attach a 1ml syringe to the labelled end of the straw. Using the syringe, aspirate HTF solution until the meniscus reaches 4.5cm marker.

    b) Then aspirate 2.3cm air and lay the syringe and straw assembly on the bench until required.

15. After 3 minutes, the sperm suspension should be divided into 10 x 10µl aliquots on a culture dish lid (Fig. 6). Then aspirate one drop into each of the 10 straws.

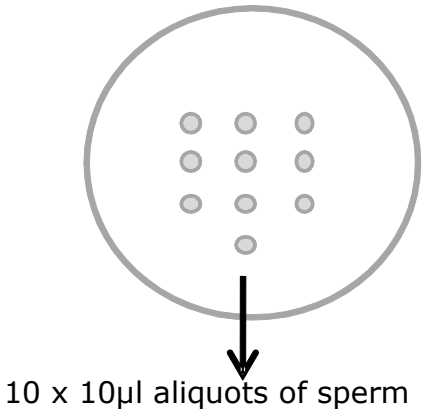

Fig. 6

16. 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.

17. Seal both ends of the straw (Fig. 7) using a double impulse heat sealer or similar device.

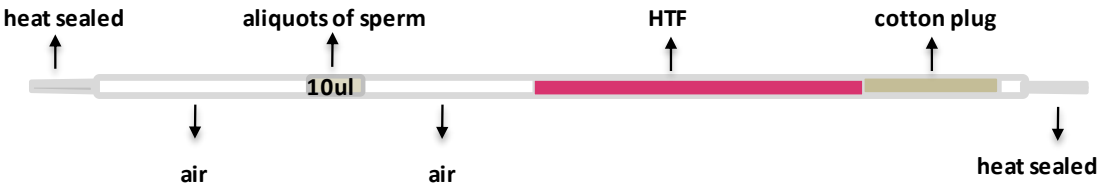

Fig. 7

18. Load the sealed straws into a floating sperm freezing device and then rest the device on the surface of the LN_2 in the pre-prepared cooling chamber (Fig. 8) for 10 minutes. After 10 minutes plunge the straws directly into the liquid nitrogen.

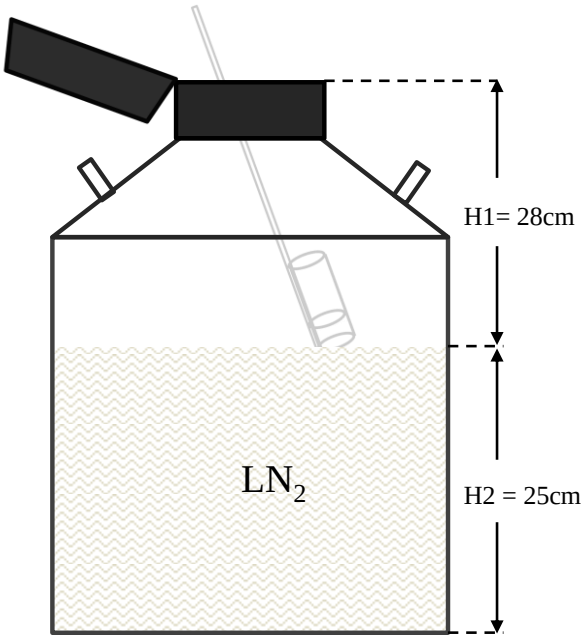

Fig. 8

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