



Sperm freezing protocol using gametes harvested from epididymides after transportation at refrigerated temperatures (4-8°C)

This protocol is similar with the mouse sperm cryopreservation protocol. The only major change is the equilibration time of sperm in gCPA during cryopreservation, which is extended from 3min to 15min. This ensures more sperm is collected from the epididymides prior to freezing. This protocol is based on the work published in Takeo et al., (2012 & 2014).

A. Receiving the refrigerated package

1. On arrival the recipient should remove the aluminium lined box from the polystyrene container and place it in a refrigerator (4°C) if the sperm freezing is not going to be performed immediately.
2. Shortly before performing the sperm freezing, open the aluminium lined box and retrieve the Eppendorf tube which contains the epididymides.
3. Allow epididymides to warm to room temperature by placing the Eppendorf tube on the bench for 30min before collecting the sperm.

B. 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 Falcon 353004 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 LN₂ Dewar (Fig. 2) filled with LN₂ to a depth of 20-25 cm.

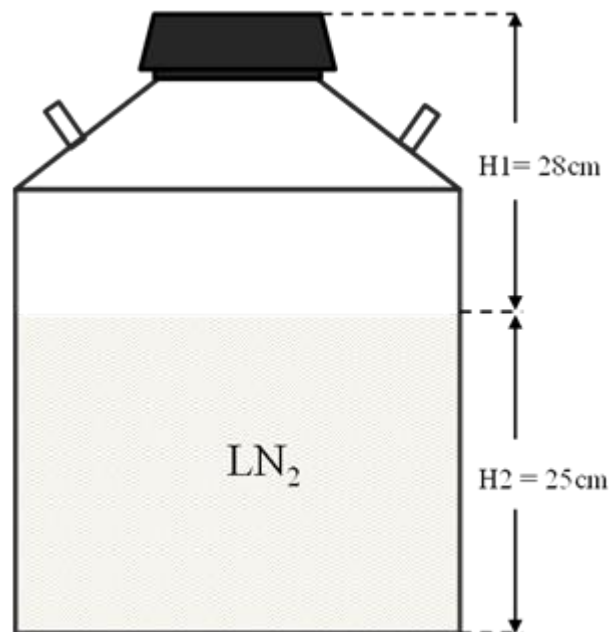


Fig. 2 Cooling chamber. Depth of LN₂ (H2) = 25 cm.

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)

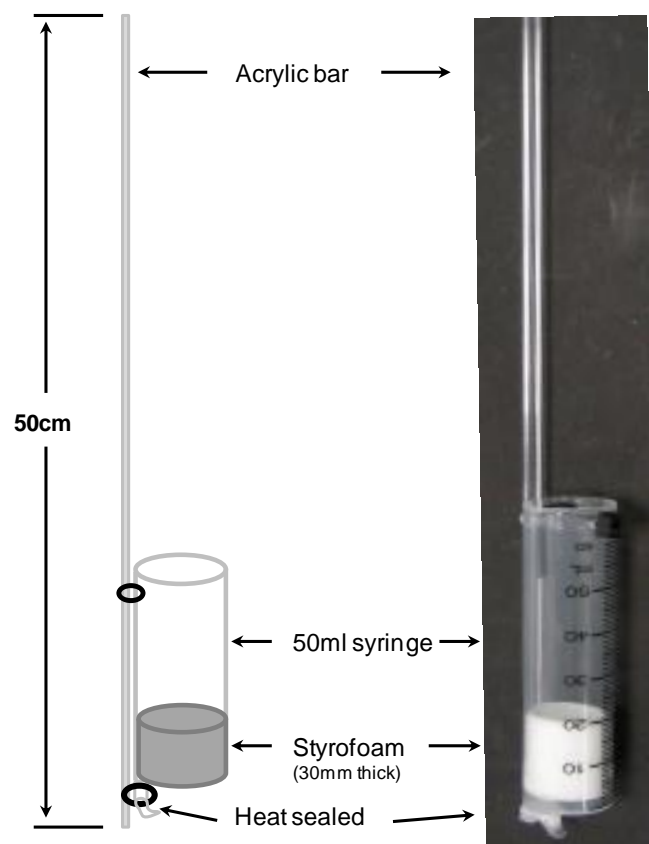


Fig. 3 Floating sperm freezing apparatus

6. Place a 60 μ l 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 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). A separate dish should 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 60 μ l drop per epididymis.

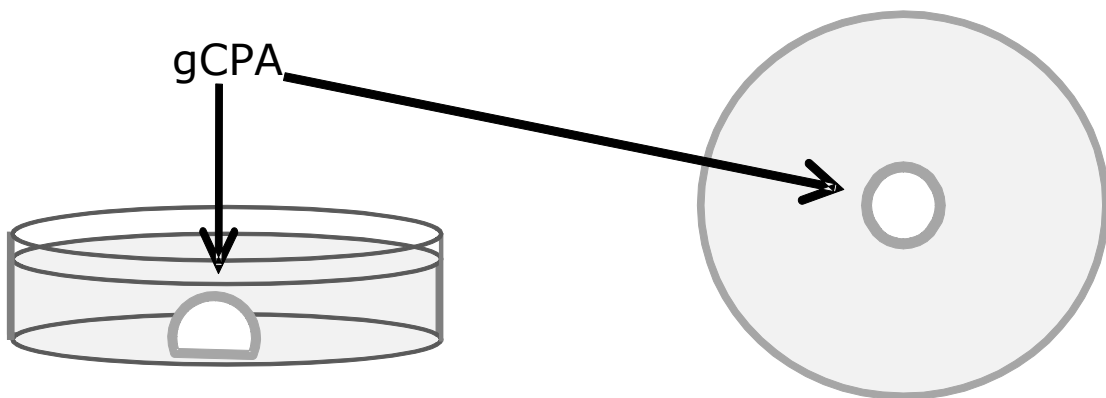


Fig. 4

8. The cauda epididymides should be wiped free of any Lifer solution using paper tissue.
9. Wash each cauda epididymis through 4 x drops of gCPA (Fig. 5). Steps 10 & 11 are very important, if you skip these two steps sperm

motility may be compromised.

NOTE: To make a wash dish, put 4 x 80 μ l drops gCPA into a 60mm petri dish (Falcon 353004). There is no need to overlay with silicone fluid or mineral oil.

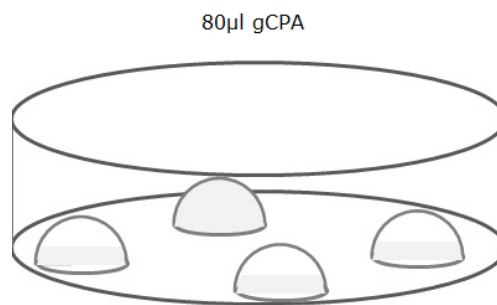


Fig. 5

10. Clean off all adipose and vascular tissue. This is best achieved by placing the organs on a paper tissue and examining them under a dissecting microscope lit from above.

11. Transfer the cauda epididymides into a 120 μ l 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 $^{\circ}$ C hot plate (Fig. 6). Keep the dishes on 37 $^{\circ}$ C hot plate for 15min avoiding the light from the microscope.

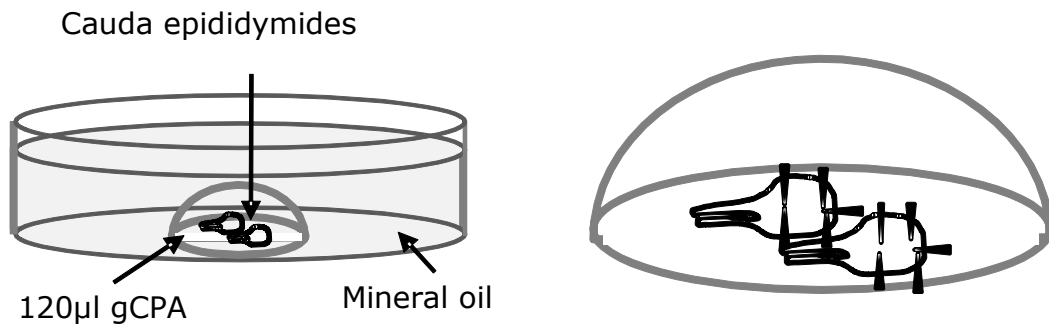


Fig. 6

13. After every 4 and half minutes, 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 15 minutes, the sperm suspension should be divided into 10 x 10µl aliquots on a culture dish lid (Fig. 7). Then aspirate one drop into each of the 10 straws.

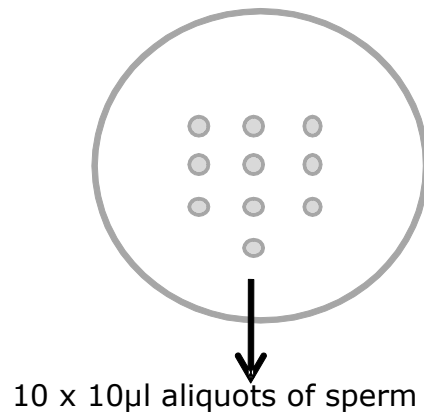


Fig. 7

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. 8) using a double impulse heat sealer or similar device.

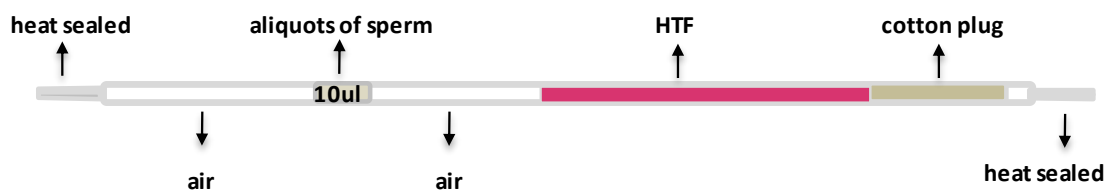


Fig. 8

18. Load the sealed straws into a floating sperm freezing device and then rest the device on the surface of the LN₂ in the pre-prepared cooling chamber (Fig. 9) for 10 minutes. After 10 minutes plunge the straws directly into the liquid nitrogen.

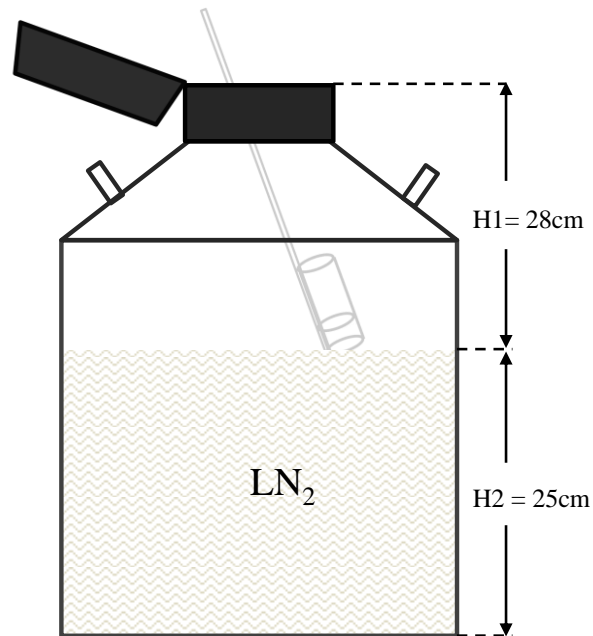


Fig. 9

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