Sperm freezing using CPA that has been supplemented with L-Glutamine

1.0 Equipment

1.1 Dissecting microscope

1.2 Cold Light Source

1.3 Dissecting Scissors

1.4 Dissecting Forceps

1.5 Watchmakers forceps

1.6 Spring bow Scissors

1.7 Hot block held at 37°C

1.8 Heated stage held at 37°C

1.9 35 litre liquid nitrogen dewar

1.10 1ml syringes

1.11 Marker pen with permanent ink

1.12 P20/P200/P1000 Gilson pipettes

1.13 Timers

1.14 Heat Sealer

1.15 Floater device

1.16 Cold Packs

1.17 Silver Box

1.18 Ruler

1.19 Sperm freezing Perspex rack

1.20 Powerpette

1.21 Label printer

1.22 LabelMark software

2.0 Supplies

2.1 0.25ml French Straws

2.2 Brady labels

2.3 Brady ink cartridge

2.4 200µl/1000µl pipette tips

2.5 60mm petri dish (10060)

2.6 Paper napkin

2.7 Note paper

2.8 Blue roll

2.9 Silicone fluid

2.10 70% Alcohol

2.11 Human tubal fluid (hTF)

2.12 L-Glutamine cryo-protectant agent (CPA)

2.13 Liquid nitrogen

2.14 Lifor

3.0 Procedure

3.1 Straw preparation

3.1.1 Ten straws are required for each male being frozen.

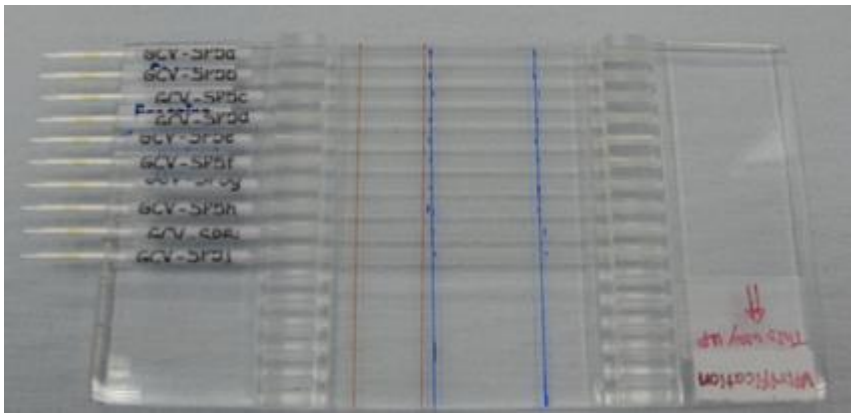
3.1.2 Using a permanent marker pen, mark each straw at a distance 4.5cm and 2.3cm from the end furthest from the cotton/PVA plug (Picture 1). (The sperm freezing Perspex template block with the appropriate guidelines can be used

to achieve this, allowing multiple straws to be marked up at once).

Picture 1

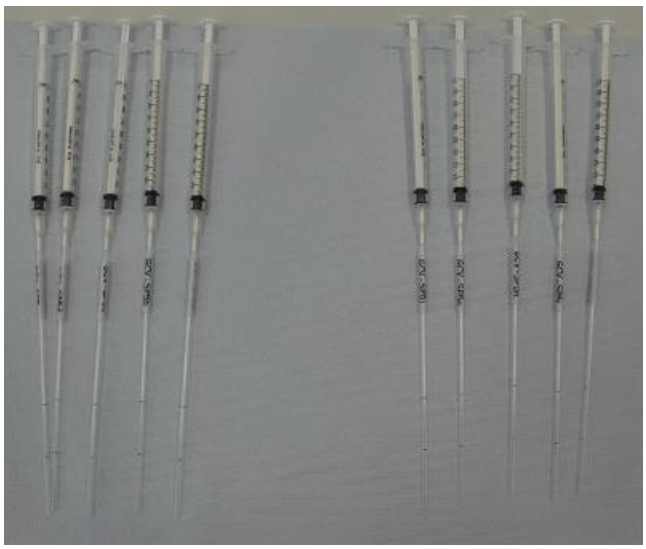
3.1.3 Label the straws with the appropriate sample code near the end of the cotton/PVA plug but not covering it (Picture 2).

Picture 2



3.1.4 Attach a 1ml syringe to the labelled end of each straw (Picture 3).

Picture 3



3.2 Preparing Tissues and Paper Squares

3.2.1 To prepare the tissues and paper squares:

For 1x male:

1x large tissue plus 1 x small square paper per male labelled with appropriate code and numerical designation of the mouse

OR

Multiple males:

1x large white tissue per male labelled with appropriate code and numerical designation of the mouse, **ONLY** 2x small square paper labelled with the appropriate code for the stock as the cauda epididymides from each male will be divided between the 2 equally (Picture 4).

Picture 4

3.3 CPA Dish Preparation

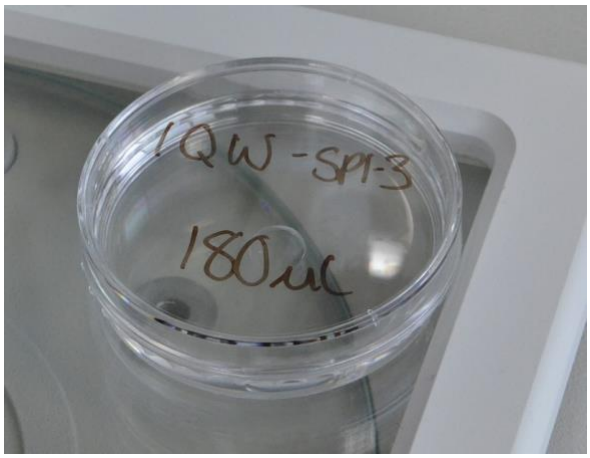
3.3.1 **For 1x male:**

1 x 60mm (10060) petri dish with the appropriate sample code and total volume of CPA in the dish written on the lid in permanent marker pen

OR Multiple males:

2 x 60mm (10060) petri dish with the appropriate sample code and total volume of CPA in the dish written on the lid in permanent marker pen (Picture 5).

Picture 5



3.3.2 See Table 1 for the volume of CPA to use per number of males in each dish.

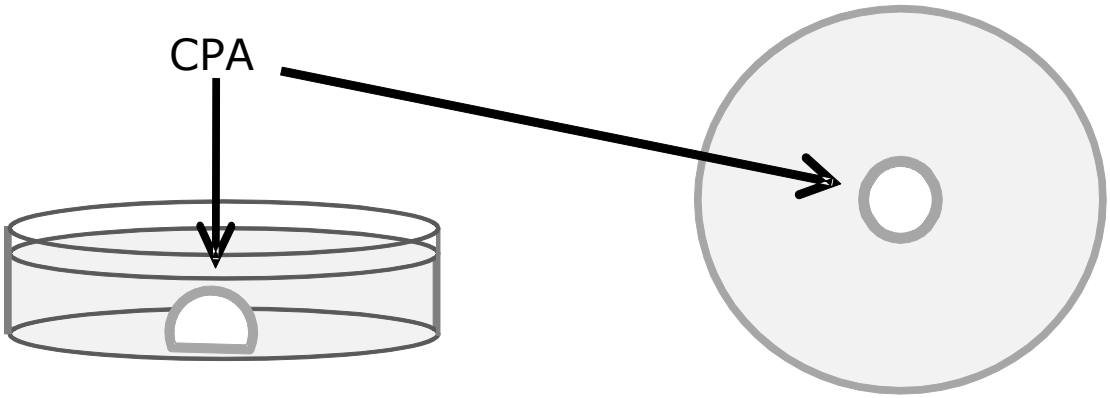
Table 1

No. males	No. cauda per dish	No. of dishes	Vol. CPA		Vol. CPA	Total vol. CPA	
1	2	1	60µl	Overlay with silicone fluid/oil	60µl	120µl	Overlay with silicone fluid/oil
2	4	1	120µl		120µl	240µl	
3	3	2	90µl		90µl	180µl	
4	4	2	120µl		120µl	240µl	
5	5	2	150µl		150µl	300µl	

3.3.3 To make the dishes, aliquot half the total required amount of CPA into the centre of the dish and overlay with silicone fluid.

3.3.4 Add a second aliquot of the remaining volume of CPA required into the first drop to achieve the total volume and overlay with silicone fluid. Preparing the dish in this way ensures a tall, semi-spherical drop which is high enough to ensure the cauda epididymides are submerged fully within the CPA (Picture 6).

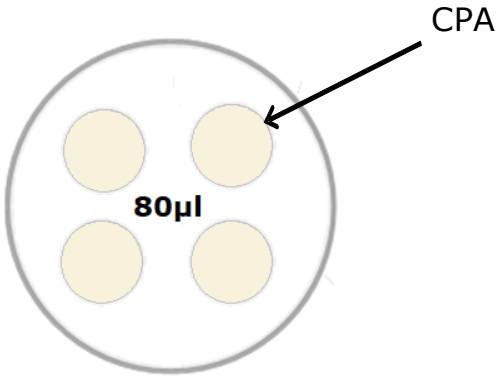
Picture 6



NOTE: the volume of CPA required is 60µl per epididymis. The CPA dishes should be placed on a hot block/heated stage at 37^{0}C for a minimum of 10 minutes before use and should be kept at this temperature constantly until no longer required.

3.3.5 Prepare a 60mm (10060) petri dish with 4 x 80µl drops of CPA to create a wash dish. If the males are pooled only one dish has to be made per stock (Picture 7).

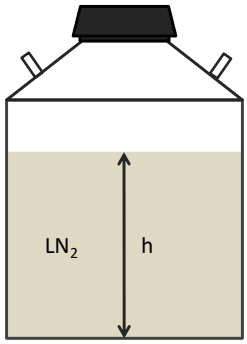
Picture 7



3.4 Preparation of the cooling chamber

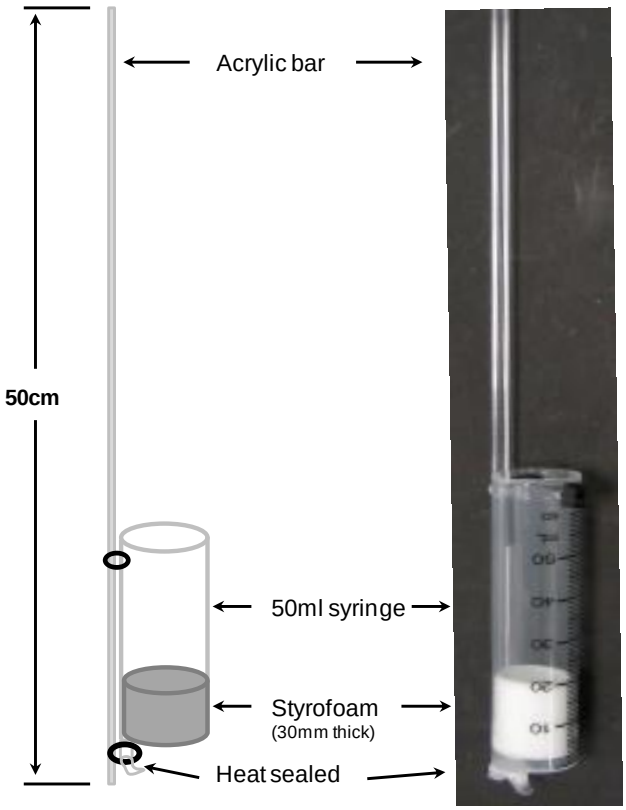
3.4.1 Prepare the cooling chamber e.g. a 35 litre LN_2 Dewar filled with LN_2 to a depth of 20-25cm (Picture 8).

Picture 8



3.4.2 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 (Picture 9).

Picture 9

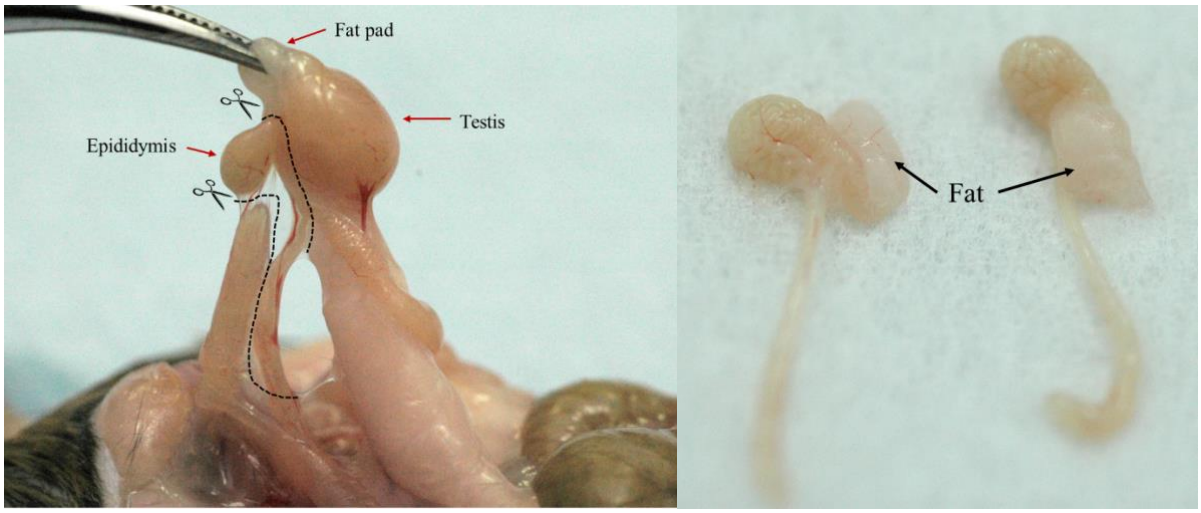


3.5 Dissecting Epididymides (N.A. for epididymides in Lifor)

3.5.1 Cull the male and swab the abdomen with 70% alcohol.

3.5.2 Dissect the cauda epididymides and place on labelled piece of square paper (Picture 10).

Picture 10



3.6 Sperm preparation

3.6.1 If epididymides are in Lifor, remove from the Lifor using watchmakers and dab gently on the appropriately labelled napkin to remove any excess Lifor. Place epididymides on labelled piece of square paper.

3.6.2 Examine the cauda epididymides on the square paper under a dissecting microscope, lit from above with a cold light source. Use a pair of watchmakers and spring bow scissors to remove any blood and adipose tissue (Picture 10).

3.6.3 Once the blood and adipose tissue has been removed, wash the epididymides in the prepared dish containing the 4 CPA drops. Work in a clockwise direction to ensure the epididymides have been washed in each drop of CPA. Dab on clean tissue to remove excess CPA.

3.6.4 Transfer the cauda epididymides into the drop of CPA in the corresponding dish on the heated stage.

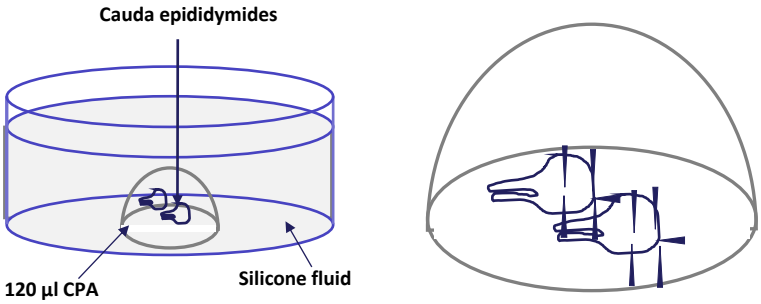
3.6.5 Make five to six cuts across the cauda epididymides (Picture 11). It is important not to cut pieces off the cauda – the aim is to make large incisions, not cut completely.

3.6.6 For fresh males incubate on a heated stage for 3 min **without the lid on** and swirl the dish every minute (at 2mins, 1min and 0mins) for 15 secs.

3.6.7 For epididymides in Lifor the time in CPA depends on the length of time the epididymides were held in Lifor. If the epididymides have been in Lifor under 24 hours; incubate on a heated stage for 3 min **without the lid on** and swirl the dish every 1 minute (at 2mins, 1mins and 0mins) for 30 secs.

3.6.8 If the epididymides are being frozen after more than 24 hours in lifor; incubate on a heated stage for 15 min **without the lid on** and swirl the dish every 5 minutes (at 10mins, 5mins and 0mins) for 30 secs.

Picture 11



3.6.9 While the sperm is equilibrating, prepare the straws for freezing, as follows:

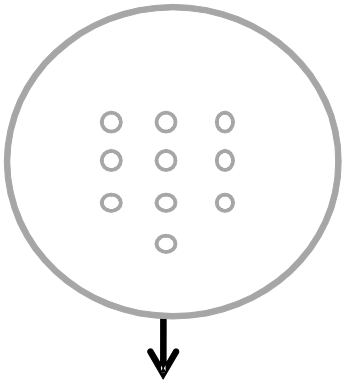
- Using the syringes attached to the straws, aspirate hTF media until the meniscus reaches the 4.5cm calibration mark (Picture 12).

- Then aspirate 2.3cm air, so the bottom of the hTF media is in-line with the 2.3cm calibration mark (Picture 12). Lay the syringe and straw assembly on a sheet of blue roll until required.

Picture 12

3.6.10 After the allotted incubation time, the sperm suspension should be pipetted into 10µl aliquots on the inside of the dish lid (Picture 13). Wipe the pipette tip on the edge of the CPA dish to remove excess silicone fluid.

Picture 13



3.6.11 The number of aliquots is determined by the number of epididymides in the dish (Table 2).

Table 2

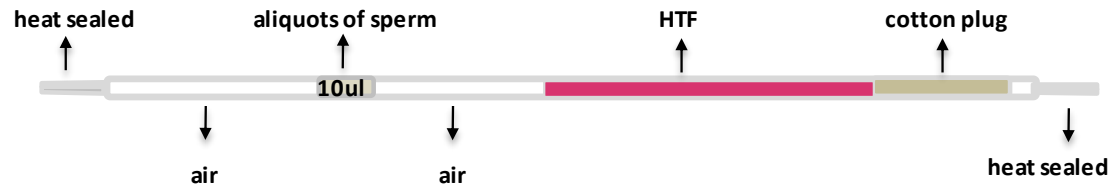
No. males	No. of dishes	No. epididymides per dish	Number of aliquots per epididymis	Total number of aliquots	Total vol. CPA
1	1	2	5	10	120µl
2	1	4	5	20	240µl
3	2	3	5	15	180µl
4	2	4	5	20	240µl
5	2	5	5	25	300µl

3.6.12 For each straw, place the straw at a 45° angle to aspirate one 10µl sperm aliquot. After aspirating the sperm aliquot continue to aspirate air until the hTF meniscus reaches the glue section, half way along the cotton plug. This will seal the labelled end of the straw.

3.6.13 Remove the syringe and place the straw back on the blue roll. Repeat step 3.5.12 for each straw.

3.6.14 Seal both ends of the straw using a heat sealer (Picture 14).

Picture 14

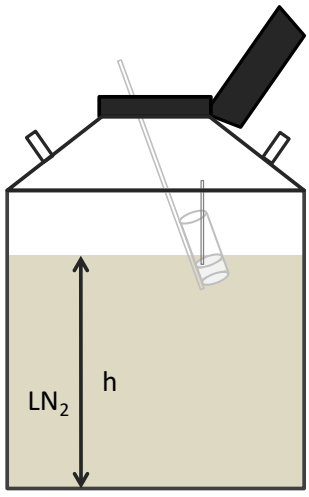


3.7 Freezing process

3.7.1 Load the sealed straws into a floating sperm freezing device and then place them into the pre-prepared cooling chamber and set a timer for 10 minutes (Picture 15).

3.7.2 After 10 minutes plunge the straws directly into liquid nitrogen.

Picture 15



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

3.8 Sperm Analysis

3.8.1 Once the samples have been placed to equilibrate in the cooling chamber, the pre-freeze progressive motility of the sperm can be determined.

3.8.2 Remove all tissue from the CPA drop. Add 200µl hTF to the remaining sperm in the drop and pipette slowly twice to dilute and mix the sperm sample.

3.8.3 Aspirate the 200µl hTF and place in the silicone oil at the side of the dish. The sperm left in the drop will still be densely populated and potentially stuck to the bottom of the dish, making it difficult to visualise the motility.

3.8.4 Replace pipette tip and add another 200µl hTF to the drop, pipetting slowly twice to mix the sperm sample.

3.8.5 Aspirate roughly 150µl of hTF and place in the silicone oil at the side of the dish.

3.8.6 Focus the magnification to the sperm on the bottom of the dish (Picture 16).

3.8.7 Visually analyse the movement of sperm in one area and estimate what proportion of sperm are moving progressively compared to non-progressive (twitching or immotile). Motility categories are: 0-30%, 30-60% & 60%+

Picture 16