CRYOPRESERVATION OF MOUSE SPERMATOZOA

Note:
Method from Peter H. Glenister & Claire E. Thornton (1998) (MRC Mammalian Genetics Unit, Harwell, OX11 0RD, UK) adapted from original descriptions of N. Nakagata (University of Tokyo, Japan) and J. Sztein (The Jackson Laboratory, USA). The following protocol is part of a one-day intensive course on "Sperm cryopreservation" given at MRC in Harwell (UK) by Peter H. Glenister and Claire E. Thornton.

Internet links:
http://www.mgu.har.mrc.ac.uk/sperm.html
sperm@har.mrc.ac.uk

References:
Thornton C.E., Brown S.D.M, Glenister P.H. (1999). Large numbers of mice established by in vitro fertilization with cryopreserved spermatozoa: implications and applications for genetic resource banks, mutagenesis screens, and mouse backcrosses. *Mammalian Genome* 10: 987-992.

Nakagata N. (1994). Cryopreservation of embryos and gametes in mice. *Exp. Anim.* 43: 11-18.

Marschall S., Hrabé de Angelis, M. (1999) Cryopreservation of mouse spermatozoa: double your mouse space. *Trends in Genetics* 15(4): 128-131.

Materials:

Cryoprotectant solution
1.8 ml Nunc cryotubes
Cryotube rack
Eppendorf centrifuge 5415C and 1.5 ml Eppendorf tubes
35 mm cell-culture Petri dishes (e.g. Falcon 3001)
Deep polystyrene box with lid suitable for holding liquid nitrogen
Small Dewar flask of liquid nitrogen
CO_2 incubator (37^{o}C, 5% CO_2)
Hot block at 37^{o}C
Sexually mature male mice at least 8 weeks old (preferably not recently mated)

Cryoprotective Agent (CPA):

18% raffinose (Sigma R-7630), 3% skim milk (Difco Betalab 0032-17)

Place 9 ml of Sigma water (Sigma W-1503) in a screw top 15 ml Falcon tube (#2097) and equilibrate to 60 ^{o}C in a water bath. Add 1.8 g of raffinose and dissolve by gentle inversion. Add 0.3 g of skim milk and dissolve by gentle inversion. Make up to 10 ml if necessary. Aliquot into Eppendorf tubes and centrifugue at 14,000 rpm for 10 min. Tip off supernatant and Millipore filter (0.2-0.45 μm) into cryotubes or Eppendorf tubes. Store at -20 ^{o}C (1.1 ml aliquots).

Cryopreservation Method:

1).- Prepare the cooling apparatus. Place a platform, (for example, the insert from a Gilson yellow tip box), into the polystyrene box. This acts as a support for the cryotube rack. Carefully pour liquid nitrogen into the polystyrene box to just cover the platform. Place a cryotube rack on top of the platform so that it is suspended in liquid nitrogen vapour. Replace the lid of the polystyrene box and allow it to fill with vapour. Replenish the liquid nitrogen as necessary during the freezing session, but do not allow the level to rise above the platform.

2).- Thaw one aliquot of cryoprotectant solution for each male mouse and bring to 37 ^{o}C in the incubator or hot block. Mix by inversion if there is any precipitation.

3).- Pipette 1.1 ml CPA into a small Falcon 35mm dish (#3001) on the hot block at 37 ^{o}C. Dissect the vas deferens and cauda epididymes from the mouse and clean off all fat and blood. This is best achieved by placing the organs on a tissue and examining them under a microscope. Using watchmaker's forceps, mice the caudae and squeeze the sperm gently out of the vas deferens. To disperse the sperm, tap and shake the dish gently for about 30 sec. Place in a CO_2 incubator at 37 ^{o}C for 10 minutes, resting the sperm dish at an angle on the lid.

4).- Keeping the dish at an angle, remove the epididymal and vas tissue from the suspension by scraping them to one side of the dish. Aliquot 100 µl into each of 10 cryotubes (or 200 µl aliquots in 5 cryotubes). Replace the screw cap and tighten to seal the cryotube.

5).- Place the cryotubes into the pre-cooled freezing apparatus (polystyrene box) and leave for 10 minutes.

6).- Remove and plunge into liquid nitrogen. Store in liquid nitrogen refrigerator until required.

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THAWING MOUSE SPERMATOZOA AND IN VITRO FERTILISATION (IVF)

Materials:

Aliquot(s) of frozen sperm
Water bath at 37 ^{o}C
Eppendorf centrifuge 5415C and 1.5 ml Eppendorf tubes
CO_2 incubator (37^{o}C, 5% CO_2)
Superovulated female mice
Medium for IVF
Mineral oil (Sigma M-8410)
35 mm cell-culture Petri dishes (e.g. Falcon 3001)
60 mm cell-culture Petri dishes (e.g. Falcon 3004)
Medium M2 containing 0.4% w/v BSA
+0.5 d.p.c. pseudopregnant female mice

MEM medium for IVF:

We use Sigma MEM (Sigma M-4655) with additions as follows per 100 ml:

2.5 mg	Na Pyruvate (Sigma P-2256)
0.38 mg	EDTA (Sigma E-5134)
7 mg	Penicillin (Sigma P-4687)
5 mg	Streptomycin (Sigma S-1277)
300 mg	BSA (Sigma A-3311)

Dissolve the BSA without shaking. Filter sterilise ~10 ml aliquots into Falcon tubes (#2001). The medium can be frozen at -20 ^{o}C. If frozen medium is used, re-filter on thawing.

Note: Medium M16 can be used as well for IVF.

Thawing method and IVF:

1).- The day before IVF, prepare an appropriate number of IVF dishes and pre-incubate overnight to equilibrate. A set of dishes for IVF consists of:

A 35 mm Petri-dish containing ~3 ml IVF medium for oocyte collection.
A fertilisation dish (35 mm) containing a 200 µl drop of IVF medium overlaid with mineral oil.
A wash dish (60 mm) containing four 100 µl drops of IVF medium overlaid with mineral oil.
A culture dish (60 mm) containing four 100 µl drops of IVF medium overalid with mineral oil.

2).- Using forceps, hold the cryotube in air for 30 sec, and then thaw rapidly by placing in a 37^{o}C water bath. Take special care that the tube has not filled with liquid nitrogen before plunging into the water bath (such tubes may explode!). If liquid nitrogen is present in the tube, wait for it to evaporate and escape first.

3).- When the sample is thawed, pipette into an Eppendorf tube. Centrifugue at 3,000 rpm for 4 minutes. Discard the supernatant and resuspend the pellet in 55 µl of IVF medium (pre-incubated at 37 ^{o}C). Flick the tube to lift the pellet off the bottom and then incubate at 37 ^{o}C, 5% CO_2 for 10 minutes to allow resuspension of sperm. Take 10 µl aliquots and pipette gently into the fertilisation dishes. There should be enough sperm for 5 x 200 µl fertilisation drops. Do not pipette the sperm up and down in the drop.

4).- Dissect oviducts from 5 superovulated female mice per fertilisation drop. Place the oviducts into pre-incubated IVF medium, pin down with forceps, and tear out the cumulus masses. Using a 1 ml Gilson set at 100 ml, transfer the 10 cumulus masses into one of the fertilisation drops being careful to transfer as little IVF medium as possible. Repeat for each fertilisation dish. Incubate at 37^{o}C, 5% CO_2 for ~5 hours (3-7 hours).

5).- Pick up the eggs from the fertilisation drops and wash through 4 x 100 µl drops of pre-incubated IVF medium (one wash dish for each fertilisation dish). Transfer the washed eggs to the culture dish.

6).- Incubate overnight. Next day transfer 2-cell embryos to a holding dish of M2 medium at room temperature and then to the oviducts of +0.5 d.p.c. pseudopregnant foster mothers (5-8 per oviduct).

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Hints and tips:

The timing of the hCG hormone injection and the collection of the ovulated eggs is important. If "old" eggs are used many become resistant to fertilisation and may undergo parthenogenic activation. We find that hCG administered approx. 18 hours followed by the addition of eggs to the sperm suspension before approx. 8 hours the following day, works for our particular strain of mice. Other strains may well react differently.

MEM is certainly not the only medium that can be used for IVF. Other possibilities are HTF and T6, recipes for which are well documented. Different media may work better for different strains.

Dividing a 100 µl sperm sample between 5 fertilisation dishes or a 200 µl sperm sample between 10 fertilisation dishes *may* maximise the yield of fertilised eggs. It also created more work, uses a lot of mice and potentially reduces the concetration of sperm capable of fertilisation. This saturation approach is only recommended for "good" strains of males and where large numbers of offsprings are required. We have successfully used the whole of one sperm sample from a "poor" strain in one 500 µl fertilisation drop. In this case the number of offspring was much reduced but still allowed re-establishment of the stock.

Results obtained at MGU, Harwell:

The following results have been obtained using frozen/thawed sperm from (C3H/HeH x BALB/c) F1 to fertilise fresh (C3H/HeH x 101/H) F1 eggs *in vitro*.

Sperm volume	No. fertilisation drops	No. liveborn
50 µl	1	35
50 µl	1	28
50 µl	1	36
50 µl	2	82
50 µl	3	65
50 µl	5	108
100 µl	10	177

Sperm volume refers to the volume of thawed sperm recovered after centrifugation and resuspension in IVF medium. Nothe that where the number of fertilisation drops is >1 the sperm suspension, after centrifugation, was divided equally between the drops. As we normally freeze 10 x 100 µl of 5 x 200 µl aliquots per male we can predict for this particular cross, that is possible to recover approx. 1000 liveborn offspring from the frozen sperm of one male.

References

Okuyama M, Isogai S, Saga M, Hamada H, Ogawa S (1990) *J. Fert. Implant.* (Tokyo), **7**, 116.

Nakagata N, Takeshima T (1992) Theriogenology, 37, 1283.

Sztein JM, Farley JS, Young AF, Mobraaten LE (1997) Cryobiology, 35, 46.

Huffstadt U, Balling R (personal communication, 1997)

Sztein JM, Farley JS, Young AF (personal communication, 1997)

Wood MJ, Whittingham DG (personal communication, 1997)