INFRAFRONTIER-I3 - Cryopreservation training course

Hosted by the Frozen Embryo and Sperm Archive, MRC - Harwell

Harvest and cryopreservation of *in vivo* derived mouse embryos (E2.5)

A. Superovulation of embryo donors

1. To synchronise oestrus and induce superovulation, administer 0.1ml (5iu) PMS by i.p. injection during the afternoon of day -3 before ovulation (see scheme below).
2. Administer 0.1ml (2.5iu) hCG by i.p. injection during the afternoon of day -1.
3. Mate the females immediately after the hCG injection (maximum of two females per stud).
4. Check the females for the presence of a copulatory plug the following morning (day 0.5).

PMS (Day -3)	hCG and mate (Day -1)	Plug check (Day +0.5)	Embryo harvest and freeze (Day 2.5)
Wednesday	Friday	Saturday	Monday
Thursday	Saturday	Sunday	Tuesday
Friday	Sunday	Monday	Wednesday
Saturday	Monday	Tuesday	Thursday
Sunday	Tuesday	Wednesday	Friday

B. Collection of embryos

1. Sacrifice the mice required for embryo harvesting on day 2.5.
2. Place each mouse on its back and wet the abdominal fur with 70% alcohol.
3. Tear the abdominal skin and cut through the body wall to locate the reproductive tract.
4. Using forceps and fine scissors, carefully remove both oviducts. Try to remove no more than 1-2mm of uterine horn.
5. Place each pair of oviducts into a drop of M2 (approximately 20µl) in a 60mm (PAA10060X) petri dish. Up to 4 pairs of oviducts can be placed in each dish. The dishes should be marked so that the animal from which the oviducts were collected can be identified.

Fill a 1ml sterile syringe with M2. Fit a square ended flushing needle and then expel any air bubbles from the assembly. The flushing needle should be approximately 30 or 34 gauge, depending on the diameter of the infundibulum.

6. View the oviducts on low level magnification and with the aid of fine forceps, locate the infundibulum. Pin down the oviduct using one pair of forceps, and gently insert the flushing needle into the infundibulum.

7. While pinning the oviduct down with the needle carefully flush the lumen with 100-200µl M2. The embryos should be seen emerging from the open end of the uterus.

8. Remove the first oviduct and repeat for the second oviduct in the drop. Continue until each oviduct has been flushed. Try to keep the media flushed through the oviducts separate for each mouse.

C. Washing and scoring embryos

1. The embryos harvested from all females of one strain will be pooled together and washed through the same dishes. **Embryos from different strains must be pooled and washed through separate dishes.** Label one 60mm (PAA10060X) dish and one 35mm Falcon 351008 dish for each stock. To the 60mm dish add two drops of M2, each approximately 250µl in volume. Add one drop of M2, of similar volume, to the 35mm dish.

2. Using a mouth pipette (aspirator assembly, Sigma Chemical Co., Cat. No. A5177) fill a glass embryo handling pipette with M2 from the first drop in the large dish. Carefully introduce 3 or 4 air bubbles into the end of the pipette. This will help to control the capillarity of the M2 medium.

3. Search through the first flush drop and transfer all of the embryos found close to the edge of the first drop in the large dish.

4. Examine each embryo for damaged or degenerated cells and move the good quality embryos to the centre of the dish. Only transfer those embryos which a) have an intact zona pellucida, b) are free of adherent debris, and c) do not possess any damaged blastomeres.

5. Count and record the number of embryos harvested from each mouse, separately counting good quality and poor quality embryos.

6. Repeat steps 3 to 5 for each mouse per stock, pooling all of the good quality embryos in the centre of the first drop in the large dish. **NB: Embryos from different strains must be pooled and washed through separate dishes.**

7. Fill a clean pipette with M2 from the second drop in the large dish, as described in step 2 above.

8. Move all of the good quality embryos from the first drop to the second drop in the large dish, in the smallest amount of medium possible, checking that none have been left behind or are stuck in the pipette.

9. Fill a clean pipette with M2 from the small dish (drop 3) and transfer the good quality embryos into the small dish.

10. Leave the embryos in the small dish, at room temperature until all stocks are ready to freeze.

D. Preparation and loading embryos into straws for freezing

NB: this procedure can also be used for 2-cell embryos produced by IVF.

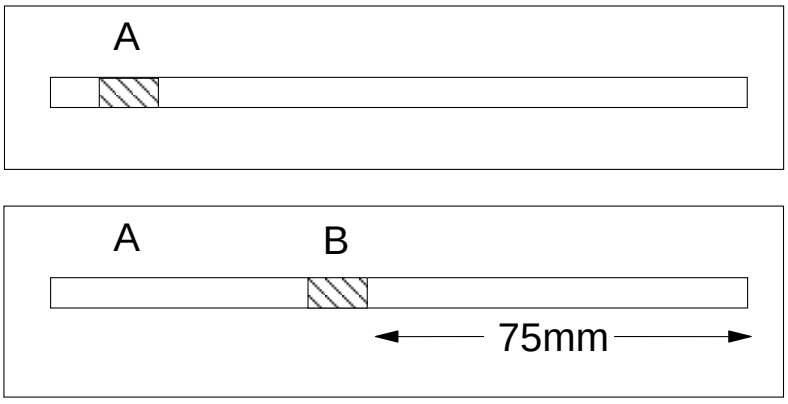
1. Fill the liquid nitrogen dewar which supplies the programmable freezer to a depth of at least 25cm liquid nitrogen, and insert the heater element ensuring it is securely attached to the handles of the dewar.

2. Close the pressure relief valve on the side of the pressure gauge, switch on the programmable freezer and activate the heating element so that the pressure in the vessel rises.

3. Prepare and label 133mm straws (0.25ml French straws, Planer Plc, Cat. No. FZA201) for freezing, as follows:

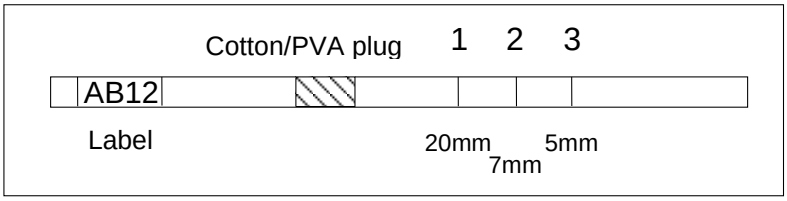
   3.1. Prepare enough straws so that the embryos can be frozen in batches of 20-25 per straw.

   3.2. Push the cotton/polyvinyl alcohol plug into the straw, from position A to position B, using a metal rod with a stop.



   3.3. Place the straw(s) on a perspex support. Using a permanent marker pen, make three three calibration marks using the guidelines on the plate to obtain

the correct distances. The calibration marks should be placed at 20mm, 7mm and 5mm intervals from the cotton PVA plug, see below:



3.4. Label the straws with a unique code to identify each stock and batch of embryos. We use adhesive Brady labels to identify each straw, using an alphabetical code to identify each stock. Other options are available. Place the labels close to position A, but not at the end of the straw (see diagram above).

3.5. Attach a 1ml syringe to the labelled end of the straw. Using the syringe, aspirate a 1.0M sucrose solution (in M2 medium) until the meniscus reaches Mark 3.

3.6. Aspirate air, to move the sucrose meniscus from Mark 3 to Mark 2.

3.7. Aspirate 1.5M ProH until the sucrose meniscus moves from Mark 2 to Mark 1.

3.8. Aspirate air until the sucrose meniscus reaches the polyvinyl alcohol, half way along the cotton plug. This will seal the labelled end of the straw.

4. Start the programmable freezer, to cool the machine to start temperature (0˚C).

5. Place a drop (approx. 150µl) of the 1.5M Propylene glycol (ProH) cryoprotectant, into the centre of a 35mm Falcon 351008 Petri dish. One ProH dish will be required per stock.

6. Fill a clean glass pipette with ProH and introduce 3-4 air bubbles into the end of the pipette as described above.

7. Transfer the embryos from one stock into the centre of the ProH drop. Start a countdown timer set for 5 minutes.

8. When the embryos for the last stock have been transferred to their ProH drop start two timers, one set for 5 minutes and a second set for 15 minutes.

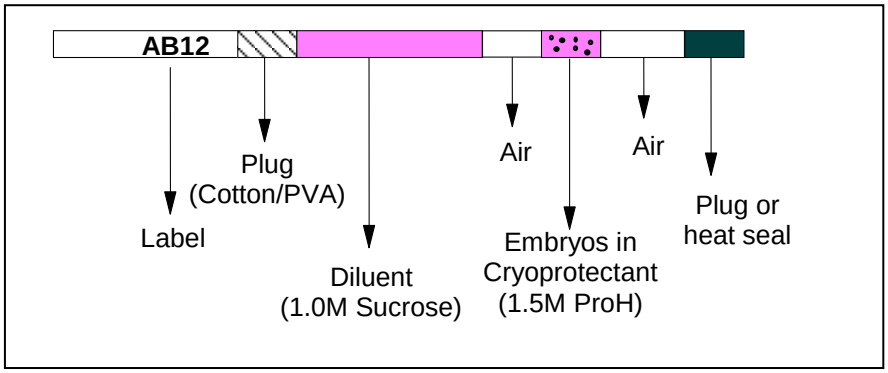
9. When the first 5 minute timer goes off, load the embryo handling pipette with ProH and introduce 2 air bubbles into the end of the pipette. Carefully examine all of the embryos for that stock and reject any which show signs of damage or degeneration.

10. Aspirate 20-25 embryos into the pipette in the smallest amount of ProH possible and introduce another air bubble into the end of the pipette.

11. Pass the tip of the pipette through the open end of the straw into the ProH fraction in the straw.

12. Gently blow the embryos and the air bubbles into the ProH fraction in the straw.

13. Remove the glass pipette and seal the open end of the straw with Cristaseal, PVA or a heat sealer (see below).



14. Repeat steps 10 to 13 until all of the embryos have been loaded into straws.

15. Wait until the 15 minute timer rings before proceeding to load the straws into the freezing machine.

E. Cryopreservation of embryos using a controlled-rate freezing machine

1. Place the straws (sealed end first) into a straw holder and insert this into the controlled rate freezing machine (now at the start temperature of 0°C). Run the programme, which will cool the straws to -7°C and hold them at that temperature for 5 minutes to equilibrate.

2. Fill a small dewar with liquid nitrogen and cool a cotton swab in the liquid nitrogen.

3. After the straws have equilibrated, gently remove a straw holder until the sucrose fraction of the straw is just accessible. Seed the straws by drawing the cooled cotton swab across the sucrose fraction. Repeat for all straws. Return the straw holders into the machine.

4. Leave the straws in the machine for 5 minutes then inspect the straws individually, to ensure that ice crystals have formed in the ProH fraction. Work quickly to ensure the straws do not warm up. If seeding has failed in any straws, repeat step 3.

5. When all of the straws have seeded, run the next step of the programme, which will decrease the temperature from -7°C to -30°C at a rate of 0.3°C per minute (taking approximately 1hour 15mins).

6. As the machine approaches -30°C, fill a small dewar with liquid nitrogen which will be used for plunging the straws.

7. When the machine temperature has fallen below -30°C, quickly remove the straw holders and plunge them into the liquid nitrogen dewar. Using the straw ejector key, remove the straws from their holders. At all times ensure the ProH fraction of the straw is completely submerged in the liquid nitrogen.

8. Transfer the straws to a secure liquid nitrogen dewar or equivalent.