MOUSE EMBRYO CRYOPRESERVATION FACILITY at CNB-CSIC

http://www.cnb.csic.es/~criocnb
criocnb@cnb.csic.es

Responsible Technician: **Julia Fernández (jfdez@cnb.csic.es)**
Support Technician: **Óscar J. Sánchez (ojsanchez@cnb.csic.es)**
Responsible Scientist, coordinator: **Lluís Montoliu (montoliu@cnb.csic.es)**

Ultrarapid embryofreezing protocol

(adapted from Kristina Vintersten, TCF EMBL 1996)
According to the method of Jillian Shaw,
Human Reproduction (1988),Vol. 3, pp 905-908 and
Reprod. Fert. Dev. (1991), Vol. 3, pp 621-626
and personal communication

Solutions:

1. Cryoprotectant

Sucrose-Grade I	860 mg (Sigma #S-5390)
Bovine serum albumin (BSA)	80 mg (Sigma #A-3311, embryo tested)
M2 medium	6.5 ml (Sigma #M-7167)
DMSO	3.3 ml (Sigma #D-2650)

Dissolve the first three ingredients and filter through a 0.2 µm Millipore filter. Add 3.3 ml Dimethyl Sulfoxide while agitating the solution on ice (this prevents the solution from getting too hot which may precipitate the BSA). Adjust the volume to 10 ml (if necessary with sterile M2).

Aliquot into sterile 1.5 ml Eppendorf tubes and store at -20 ºC (for 2-3 month).

2. Thawing media

Sucrose Solution: 860 mg (Sigma #S-5390) in 10 ml M2 (0.25 M Sucrose)
M2 medium (Sigma #M-7167)
M16 medium (Sigma #D-2650)

Prepare freshly and filter through a 0.2 µm Millipore filter.

Procedure:

A. Snap Freeze:

1. Collect 8 - cell to compacted morula stage embryos in a minimal volume of M2 using drawn out glass capillaries.

2. Transfer embryos into the cryoprotectant solution inside an artificial insemination straw (91 mm, IMV, France, #006426) (30-40 ml cryoprotectant solution).

3. Heat seal the end of the straw.

4. Incubate straw on ice for 3 minutes.

5. Immerse the straw directly into liquid nitrogen.

B. Thawing of embryos:

1. Remove straw from liquid nitrogen.

2. Immediately immerse the straw in a 37 ºC waterbath and incubate for 3 seconds or until the ice has melted (ice crystals are very shortly formed, the cryoprotectant turns from milky back to clear).

3. Remove the straw and dry with tissue-paper.

4. Cut off carefully the heat sealed end and expel the cryoprotectant solution into 2 ml of the 0.25M sucrose solution in a clear glass dish (DISMADEL, Spain) or standard tissue-culture p35 plastic dish using a 1 ml syringe with tubing connected to the end of the straw (alternatively use a 'Capillette', BRAND or similar device)

5. Incubate for 10 minutes at RT.

6. Transfer embryos into a second dish with M2 medium and incubate for 10 minutes at RT.

7. Wash the embryos very gently through several droplets of M2.

8. Culture overnight in M16 (or KSOM, Millipore-Chemicon #MR-121-D), at 37 ºC, 5% CO_2.

9. Transfer the blastocysts into the uterus of recipient mice using M2

Additional "hints and tricks" (original from Kristina Vintersten)

The freezing medium contains the following: 6.5 ml M2 medium, 80 mg Bovine serum albumin, 860 mg sucrose, 3.3 ml dimethyl sulphoxide. Adding the extra BSA is a modification from the original protocol (Shaw et al) that we have made. I find that our recovery rates are significantly higher by doing so. Sucrose is used for dehydrating the embryos.

I thaw the embryos in three steps: Qiuck thawing in 37 ºC degrees waterbath (3 seconds), 10 minutes in M2 + the same amount of sucrose as we add to the freezing media. This is clever, since the embryos can get rid of all the DMSO before they start to rehydrate. Another 10 minutes in pure M2 media = rehydration. This way you will be able to use so low concentration of DMSO that it will not be toxic for your embryos, and still be able to prevent ice crystals from forming while the embryos are frozen. You will get a crystalformation for about 3 seconds at thawing, but this seems to be no problem for the embryos to survive.

1) While making up the cryoprotectant, make sure to keep the tube on ice. Add the DMSO dropwise, and mix gently between each 10 drops. If the solution turns cloudy, you have been to fast, and the solution is useless! You can test the cryoprotectant before actual use: Fill 40microliter in the end of a straw, heatseal and plunge in liquid nitrogen. Pick it up again, and check that no icecrystals have formed (the solution should stay clear). Now put it in 37 ºC degrees waterbath and watch it carefully. You should see that it suddenly turns white, and then clear again after about 3 seconds.

2) When loading the straws, be very careful not to get any water (from the ice) into the tip of the straw. This would destroy the cryoprotectant and kill the embryos. For this reason, I have constructed a cooling block to use instead of ice. If you decide to go for this protocol, I might be able to get you one of these blocks too.

3) When sealing the straws: you will only seal ONE end! = the end where you have put the cryoprotectant/embryos. The other end will stay open. The liquid nitrogen can go in and out of the open end of the straw, this is no problem. And most important: there is no risk att all that a straw will explode for you (like cryovials can do).

4)The straws I use are normal bovine insemination straws, "French type mini-straws 25 ml. But when ordering them, make sure you get TRANSPARENT colours! Loading under a normal stereomicroscope will allow you to se through the straw wall, and watch how the embryos go in the cryoprotectant. This is important, because you have to make sure that only an absolutely minimal amount of M2 is going in with the embryos. Otherwise the M2 might destroy the cryoprotectant!

5) The glass dishes for thawing have a rounded inner wall. If you disperse the embryos very carefully on the edge, they will stay at a higher level in the dish. DMSO as being heavy, will sink down to the bottom, getting away from the embryos quicker. Using these dishes is also a modification of the original protocol we have made, and it does make a difference, especially for "difficult" embryos/lines.