
Protocol Section:	Embryo Cryopreservation	Policy No:	P-CMMR1-ECA-00
Protocol Subject:	Equilibrium Freezing for Cleavage Stage Embryos: Collection, freezing and thawing procedures for ENU and Non-ENU Mice <i>Protocol A</i>	Effective Date:	
		Date Reviewed:	24 October 2004
		Date Revised:	

This procedure was first described by Songsasen et al (1995, Cryobiology)

1.0 Material and Solutions

- 1.1 Controlled Rate Freezer [ThermoForma Cryomed 7452]
- 1.2 1/4 ml plastic insemination straws [IMV International]
- 1.3 1.0 litre dewar
- 1.4 Heat Sealer: [Impulse Sealer]
- 1.5 Brady labels [LAT-16-361]
- 1.6 Disposable, sterile plastic ware:
Filters, 0.22um pore size, [Millex]
Screw cap tubes, [Falcon]
Pipettes [Fisher]
1ml Syringes, [BD]
Petri dishes, 35mm and 60mm [Fisher]
- 1.7 Mouth pipette
- 1.8 Cryomarker pens
- 1.9 Chemicals:
Cryoprotectant (CPA): 1.5M Ethylene Glycol prepared in PBS solution
Dulbecco's phosphate buffered saline (PBS)

2.0 Media Preparation

- 2.1 Prepare 50 ml of 1.5 M Ethylene Glycol (EG) in PBS by measuring 4.16ml EG into 50 ml centrifuge tube. Make to 50 ml mark with PBS solution and dissolve. Filter sterilize into sterile screw cap tubes in ~5 ml volumes. Store at -75°C for > 6 months.
- 2.2 Prepare fresh PBS using Dulbecco's PBS (Gibco 21600-069).
- 2.3 Prepare 2 straw loading dishes of CPA in 35mm Petri dishes, about 3ml each.

3.0 Method - FREEZING

- 3.1 Mark straws 7cm from open end of straw
- 3.2 Push cotton plug down to the mark
- 3.3 Attach a Brady label to each straw
- 3.3 Attach a 1ml syringe to each straw from the plug end
- 3.4 Aspirate 2 columns of 1.5M CPA separated with an air bubble. Fill each straw and set aside ready for loading embryos.
- 3.5 Switch on the controlled rate freezer and allow to wait at -7°C.
- 3.6 Transfer the collected embryos to a dish of 1.5M CPA. Set a timer to record the length of time embryos have been exposed to CPA.
- 3.7 After the embryos have settled to the bottom of the dish, pipette sets of 20 into each straw.

Protocol Section:	Embryo Cryopreservation	Policy No:	P-CMMR1-ECA-00
Protocol Subject:	Equilibrium Freezing for Cleavage Stage Embryos: Collection, freezing and thawing procedures for ENU and Non-ENU Mice Protocol A	Effective Date:	
		Date Reviewed:	24 October 2004
		Date Revised:	

Being careful to exclude any bubbles. Total exposure time of embryos to the CPA should not exceed 15 minutes.

- 3.8 Once the straws are loaded, aspirate another column of CPA into each straw leaving a 0.5cm space before reaching the cotton plug. Take the straw out from the CPA and draw up the contents until the liquid reaches the cotton plug. Remove the syringe careful not to bump the straw and create more bubbles.
- 3.9 Heat seal the end of all the straws and load the straws into the controlled rate freezer. Embryos may be held like this for up to an hour.
- 3.10 When all straws have been loaded into the controlled rate freezer, they may be seeded. Using a cotton swab with its end flatten dip it into a small amount of liquid nitrogen and touch each straw at the upper column of CPA and the lower column being careful to avoid touching the column which contains the embryos. An ice crystal will form and spread to the rest of the straw gradually.
- 3.11 Verify by sight that all straws have been seeded, and then allow them to hold at -7°C for 10 minutes.
- 3.12 The freezer is pre-programmed to cool the straws from -7°C to -35°C at a rate of 0.3°C/min.
- 3.13 Once reaching -35°C, the ramp speed will increase to 10°C/min to a temperature of -140°C. Straws are held at this temperature for 15 minutes.
- 3.14 Plunge the straws directly into a dewar of liquid nitrogen as fast as possible.
- 3.15 From there the straws may be sorted and transferred to the appropriate storage vessel.

4.0 THAWING

- 4.1 Prepare a 1.0 litre dewar of liquid nitrogen
- 4.2 Prepare a 35°C and a 22°C water bath
- 4.3 Remove the appropriate goblet from the liquid nitrogen vessel and locate the straws required for freezing. Transfer them to the dewar rapidly.
- 4.4 Each straw must be thawed individually.
- 4.5 Remove a straw from the dewar and hold in the air for 5-6 seconds
- 4.6 Place it in the 35°C water bath for 10-15 seconds
- 4.7 Place it in the 22°C water bath for 1 minute
- 4.8 Dry the outside of the straw and wipe with alcohol to sterilize. Cut off the heat seals leaving the cotton plug in place.
- 4.9 Expel the contents into a marked Petri dish and count and record the number of embryos recovered from each straw.
- 4.10 Transfer viable embryos into fresh sterile PBS rinsing them 3 times to clear away residual CPA.
- 4.11 Embryos may be placed into KSOM for culture or directly transferred into pseudopregnant females.

Issued by Lab Manager: _____ **Date:** _____

Approved by Facility Management: _____ **Date:** _____