Cryodropper Vitrification and Thaw of Mouse 2-cell Embryos

Equipment:

Cryodropper for Embryo Vitrification (black line: ParaTechs 80010)
Cryovial (2ml; Corning #430659 or 4 ml; Corning # 430662) or other suitable LN_2 storage option
Stereomicroscope with transmitted and reflected illumination source with 20x and 40x magnification
Impulse heat sealer (American International Electric AIE-105T)
Water bath @ 37˚C (500ml beaker of water in a 37˚C incubator works well)
Portable LN_2 vapor phase freezer with Styrofoam raft (see photo)
CO_2 incubator at 37˚C with 5% CO_2
Slide warmer or 37˚C incubator
Pipettors and tips; ex: 1ml, 200ul, 20ul, 2ul
Tissue culture dishes; 35mm, 60mm
Embryo handling pipette assembly (ParaTechs #90010)
Embryo pipettes
Eppendorf tubes, racks
Fine tip Sharpie for labeling
Kimwipes
Timer
Forceps
Scissors
LN_2 vapor phase storage dewar
0.22µm filter units for media sterilization (ex: Millipore SCGVUORE and SLGP033RS)
Refrigerator/freezer for media storage

Media and Reagents Required:

M2 medium (Millipore MR-015-D)
KSOM^{AA} medium (Millipore MR-121-D)
Ficol PM70 (Sigma-Aldrich F2878)
Sucrose (Sigma-Aldrich S1888)
Ethylene Glycol (Sigma-Aldrich 102466)
Dimethyl sulfoxide (DMSO) (Sigma-Aldrich D2650)
PBS pH7.4 (Gibco 10010023)
Paraffin Oil (Sigma-Aldrich 18512)
M2 with 0.5 M sucrose
M2 with 0.25M sucrose
FS medium
Pre-VS medium
VS medium

Vitrification of embryos

1. Embryos are kept in M2 at 37°C until cryopreservation.

2. Make vitrification and pre-vitrification media.

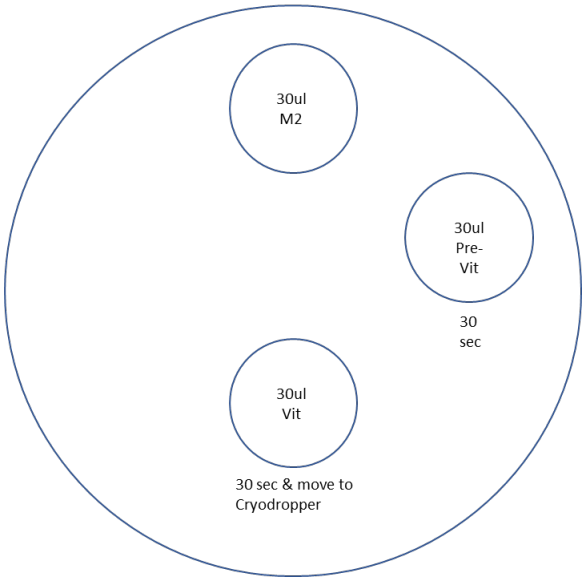
3. Set up portable LN_2 vapor phase freezer with Styrofoam raft.

4. Label each Cryodropper as desired. Color code or label the bulb portion.

5. Preload 100µl M2 with 0.5M Sucrose in the embryo Cryodropper bulb. Place the 100µl drop on a petri dish lid. Load medium into the Cryodropper by squeezing the bulb, inserting the open end into the drop, and releasing the pressure on the bulb. Hold the Cryodropper by the open end and gently flick the media into the bulb portion. Gently squeeze the bulb to remove any media remaining in the embryo loading area and wipe the liquid off with a kimwipe. Preload <5µl vitrification media into the center of the straw area by gently positioning a drop using the same method as before or with a gel loading tip. Store open end up in an Eppendorf rack.

6. On a cover of a 35mm dish, position a drop of M2 (20-30µL per drop), a drop of pre-vitrification solution, and a drop of vitrification solution in order, clockwise.

Vitrification Layout



7. Label and precool the cryogenic storage vial. Up to 4 Cryodroppers will fit per vial.

8. Using an embryo handling pipette, embryos are first transferred to the drop of M2 in the 35mm dish.

9. Prefill the embryo pipette with a small amount of the pre-vitrification solution. Under the dissecting microscope, transfer embryos from the M2 drop to the drop of pre-vitrification solution.

10. Incubate 30 seconds.

11. Prefill the embryo pipette with a small amount of vitrification solution. Transfer embryos from the pre-vitrification solution drop to the vitrification solution drop.

12. Incubate 30 seconds.

13. Collect the embryos and load them into the vitrification solution preloaded in the Cryodropper. It is helpful to focus the microscope on the embryos in the pipette.

14. Seal Cryodropper at the end with a heat sealer. Gentle pressure applied to the bulb of the Cryodropper can be used to test the seal. If the device is not sealed, repeat.

15. Using forceps, plunge the Cryodropper into LN_2 until the media in bulb freezes (@10 seconds).

16. Store on a raft in LN_2 vapor until all samples are processed.

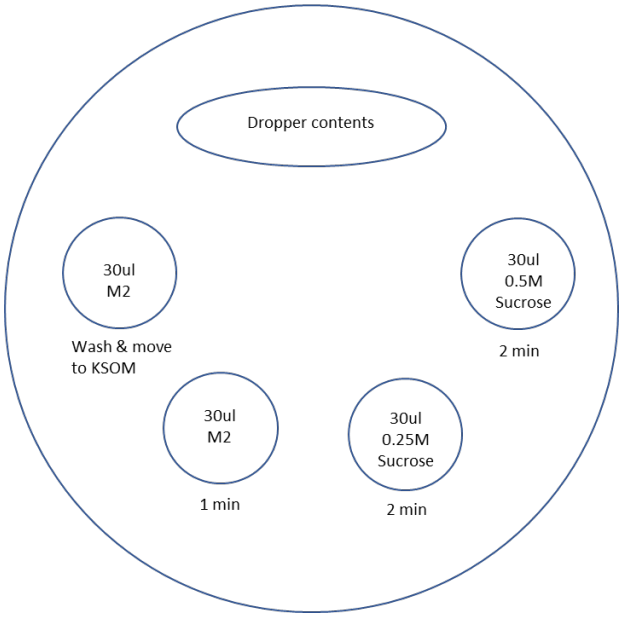
17. Transfer Cryodroppers to cryovials.

18. The vials are then transferred to a LN_2 dewar and stored in the vapor phase.

Thawing of vitrified embryos

1. Prepare a thaw dish for each Cryodropper as follows: a 60mm petri dish lid with space across the top (for ~100µl of M2 with 0.5 M sucrose from Cryodropper), and 20-30µl drops of each clockwise: M2 with 0.5M sucrose, M2 with 0.25M sucrose, and M2 (2 drops). Prepare incubation dish as follows: 60mm dish with 100µl KSOM elongated drop through center, 30µl KSOM drop on either side for washing embryos prior to culture. Cover with paraffin and equilibrate at 37°C with 5% CO_2 for at least 30 min.

Recovery Layout



2. The Cryodropper containing vial is removed from the storage vessel and kept in LN_2 vapor until ready for thaw.

3. Using precooled forceps, the Cryodropper is immersed in a water bath at 37˚C until the media in the dropper is thawed (@10 sec). Using a kimwipe, dry the outside of the Cryodropper.

4. The sealed tip of the Cryodropper is cut off and the embryos are deposited onto the culture dish, avoiding bubbles. The medium in the Cryodropper bulb is gently flicked down to the straw and expelled from the Cryodropper into the embryo drop. The embryos are immediately collected from the drop using an embryo pipette prefilled with M2 with 0.5M sucrose.

5. The embryos are transferred to a new drop of M2 with 0.5M sucrose followed by an incubation of 2 min.

6. Using an embryo pipette prefilled with M2 with 0.25M sucrose, the embryos are transferred to the drop of M2 with 0.25M sucrose and are incubated for another 2 min.

7. Using an embryo pipette prefilled with M2, the embryos are transferred to the first drop of M2 and are incubated for 1 min.

8. The embryos are then washed through the last drop of M2, through the 2 drops of KSOM, and are then transferred to the long drop of KSOM under oil.

9. Embryos are cultured at 37°C with 5% CO_2 (up to 2 days for late morula or blastocyst stage).

Protocol adapted by B. Stone from Wai Hung Tsang and King L. Chow *BioTechniques Protocol Guide 2010* (p. 55) doi 10.2144/000113258.

Media Recipes:

Recipes					
Medium	Component	Amount	Total Volume	%	Notes
FS	Ficol PM70	3.0g	10ml		in PBS (pH 7.4), filter sterilize
	Sucrose	1.7g			
Pre-VS	Ethylene glycol	10µl	100µl	10%	prepare fresh
	DMSO	10µl		10%	
	M2	80µl		80%	
VS	Ethylene glycol	15µl	100µl	15%	prepare fresh
	DMSO	15µl		15%	
	FS	60µl		60%	
	M2	10µl		10%	
M2 with 0.5M sucrose	sucrose	34.0g	200ml		prepare in M2, filter sterilize, store in 1.5ml aliquots at -20˚C
	M2	~200ml			
M2 with 0.25M sucrose	sucrose	1.7g	20ml		prepare in M2, filter sterilize, store in 100µl aliquots at -20˚C
	M2	~20ml			

Portable LN_2 vapor phase freezer with Styrofoam raft: