

## Mouse Embryo Cryopreservation – PROH method

This slow freezing method uses PROH as a cell permeating cryoprotectant, and it mainly includes the following steps: 1) Embryo equilibration in 1.5 M PROH; 2) Loading equilibrated embryos into straws; 3) Cooling embryos from room temperature to  $-7.5\text{ }^{\circ}\text{C}$ ; 4) Seeding at  $-7.5\text{ }^{\circ}\text{C}$  to induce ice formation outside cells; 5) Slow cooling at  $0.3\text{ }^{\circ}\text{C}/\text{min}$  to  $-30\text{ }^{\circ}\text{C}$ ; 6) Plunging straws into liquid nitrogen and storage in liquid nitrogen ; 7) Thawing embryos in solution containing sucrose for step-wise removal of PROH and restoration to physiological environment.

### A. Preparation of Freezing and Thawing Solutions

#### 1.5 M PROH (1,2-Propanediol, Sigma P-4347) in M2 medium

- 1) Pipette 8.8 ml M2 medium into a sterile 15 ml Falcon tube.
- 2) Accurately pipette and add 1.2 ml PROH (1.2432 g). PROH is viscous, and pipette tip should stay inside PROH until suction is stopped, and then excess PROH must be removed from the pipette outside before expelling PROH into M2. After expelling the initial amount of PROH, allow it to collect at the end of pipette before expelling the remaining PROH into the solution. Repeat for a few times until there is no collection of PROH at the end of pipette, and then rinse any residual PROH into the solution.
- 3) Mix by gently rocking for 10 min at room temperature on a rocker.
- 4) Filter PROH solution through a  $0.2\text{ }\mu\text{m}$  filter into a sterile 15 ml Falcon tube.
- 5) Store at  $4^{\circ}\text{C}$  and use within 1 week.

#### 1.0 M Sucrose (Sigma 1888) in M2 medium

- 1) Add 7 ml of M2 medium to a sterile 15 ml Falcon tube.
- 2) Weigh and add 3.423 g sucrose to the M2 medium.
- 3) Dissolve sucrose by gently rocking on a rocker at room temperature until completely dissolved.
- 4) Add more M2 medium to the tube until final volume is 10 ml.
- 5) Filter the solution through a  $0.2\text{ }\mu\text{m}$  filter into a sterile 15 ml Falcon tube.
- 6) Store at  $4\text{ }^{\circ}\text{C}$  and use within 1 week.

#### 0.5 M Sucrose (Sigma 1888) in M2 medium for thawing embryos

- 1) Add 7 ml of M2 medium to a sterile 15 ml Falcon tube.
- 2) Weigh and add 1.7115 g sucrose to the M2 medium.

- 3) Dissolve sucrose by gently rocking on a rocker at room temperature until completely dissolved.
- 4) Add more M2 medium to the tube until final volume is 10 ml.
- 5) Filter the solution through a 0.2  $\mu\text{m}$  filter into a sterile 15 ml Falcon tube.
- 6) Aliquot and store at  $-20\text{ }^{\circ}\text{C}$  and use within 2 months.

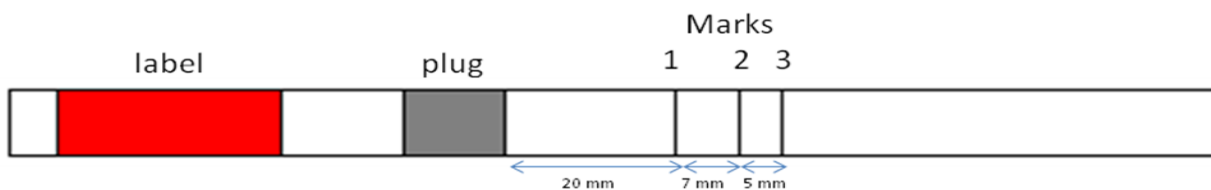
## B. Cryopreservation of Embryos

### 1. Turn on and start BioCool freezer

- 1) Turn on a BioCool IV freezer by pressing the POWER button.
- 2) Press "RUN" to start cooling the 100% ethanol in cooling bath to  $-7.5\text{ }^{\circ}\text{C}$  (will take  $\sim 15$  min). Make sure the stirrer is activated.
- 3) When the bath temperature has reached and stabilized at  $-7.5\text{ }^{\circ}\text{C}$ , the program will pause at this temperature.

### 2. Mark cryo straws

- 1) Calculate the number of straws needed for embryo cryo, then print straw labels, and write down the straw ID of each straw on an EC Record form.
- 2) Wearing gloves, disinfect pre-marked tray and a metal rod (with a stopper) using 70% ethanol, and then wipe dry completely.
- 3) Take out cryostraws (0.25 ml, IMV Technologies) from a straw bag or container, and then close the bag or container tightly.
- 4) Push the plug into each straw using a metal rod so that 56 mm remain on the left end including the plug and 77 mm remain on the right end.
- 5) Place the straws in the disinfected pre-marked tray, and make 3 marks on each straw: Mark 1, 20 mm from plug; Mark 2, 7 mm from Mark 1; and Mark 3, 5 mm from Mark 2. *Do not let non-sterile things touch the right ends of the straws before embryo loading.*



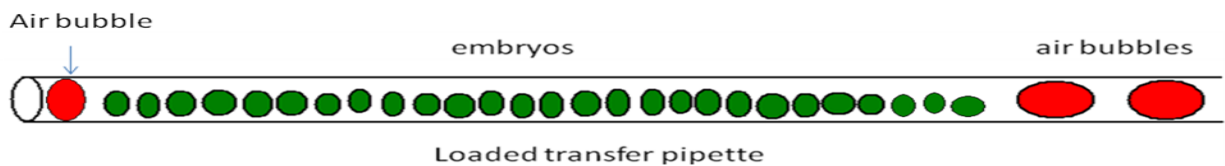
### 3. Load straws with freezing solutions

- 1) To a sterile and labeled dish, pipette 0.5-1 ml of 1.0 M sucrose.
- 2) To another sterile and labeled dish, pipette 0.5-1 ml 1.5 M PROH.
- 3) Attach the left end of a straw to a 1 ml Monoject syringe (Atlantic Healthcare, Portland).
- 4) Draw up 1.0 M sucrose to Mark 3, and then aspirate air until sucrose reaches Mark 2.

- 5) Wipe off sucrose from the straw end outside, and aspirate 1.5 M PROH until sucrose reaches Mark 1.
- 6) Wipe off the end of straw, and then aspirate air until sucrose saturates the PVA powder of the plug, which will seal the straw.
- 7) Repeat steps 3-6 to load the rest straws with freezing solutions.
- 8) Place the straws on a piece of paper towel and label the straws by wrapping each straw at the place between the plug and left end.
- 9) Mark half of the straws using a cryo pen for easy splitting storage in LN<sub>2</sub>.

#### 4. Equilibrate embryos with 1.5 M PROH and load embryos into straws

- 1) Make two 200- $\mu$ l drops of M2 medium for washing embryos in a labeled dish.
- 2) Make two 300- $\mu$ l drop of 1.5 M PROH in another labeled dish.
- 3) Wash good 2-cell embryos through the two drops of M2 medium.
- 4) Transfer the washed embryos with minimum of M2 into the first 1.5 M PROH drop. Set timer for 15-20 min.
- 5) Once embryos have settled to the drop bottom, wash the pipette using fresh 1.5 M PROH, and then transfer the embryos into the second 1.5 M PROH drop.
- 6) Suck some 1.5 M PROH into the washed transfer pipette, and then two air bubbles, 20-25 embryos, and one small air bubble to the end of pipette (shown below).



- 7) At low magnification under a dissecting microscope, carefully insert the transfer pipette tip into the right straw end, move the pipette tip carefully into the PROH column, and then expel all embryos into **PROH section** of straw. Embryos in the PROH column are visible under dissecting microscope.
- 8) Place each straw gently in the horizontal position at **room temperature** to allow embryos to reach equilibration.
- 9) Repeat steps 6-8 to finish loading the rest of the embryos into the remaining straws.
- 10) Seal the right end (non-plug end) or both ends of each straw with an impulse heat sealer. Be certain that an air-tight seal has been made at the each end, especially the right end.
- 11) Carefully take the straws to the BioCool freezer that is waiting at -7.5 °C. Do not “ping” or drop straws.



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*Note: Once embryos have been loaded into a straw, it is extremely important that the straw be handled very gently to avoid dislodging the embryos from the fluid column!*

## 5. Embryo freezing

- 1) After 15-20 min total exposure to 1.5 M PROH (dish and straw combined), CAREFULLY put straws into the rack of pre-cooled BioCool freezer at  $-7.5^{\circ}\text{C}$  with the plug ends facing up. Make sure the parts of the straw plug and solution columns are completely immersed in ethanol, but the plug end opening of each straw is in the air.
- 2) Cover the cooling bath with lid and hold for 5 min.
- 3) Seed the straws by submerging a cotton swab in  $\text{LN}_2$  and touching it to straw just below the plug in the sucrose section. ***Be certain not to let  $\text{LN}_2$  touch the straw at the column containing the embryos.*** Small ice crystal at the seeding area should become visible.
- 4) Keep the straws at  $-7.5^{\circ}\text{C}$  for additional 5 min.
- 5) After the 5 minute timer has alarmed, check straws to make sure they seeded by quickly and carefully pulling straws upward. Ice crystal should have formed completely in both the sucrose and the PROH sections. ***Keep the PROH section submerged in cooling alcohol at ALL times.***
- 6) Once all straws are frozen, press "RUN" button to initiate freezing at  $-0.3^{\circ}\text{C}/\text{min}$  to  $-30^{\circ}\text{C}$ , which will take about 80 min.
- 7) When BioCool freezer reaches  $-30^{\circ}\text{C}$  (end temperature) and bath temperature has stabilized, hold the straws for additional 5 min at this temperature. Again, measure the temperature of the ethanol using a thermocouple to ensure that the BioCool has indeed reached  $-30^{\circ}\text{C}$ .
- 8) Quickly transfer (one by one) and plunge all of the straws directly into the small Dewar of  $\text{LN}_2$  using precooled forceps.
- 9) Store embryos straws in liquid nitrogen.

## C. Thawing Embryos

1. Disinfect a pair of scissors with 70% ethanol, and wipe dry.
2. Prepare a beaker of clean  $\text{DH}_2\text{O}$  at room temperature (RT).
3. Prepare a Falcon tube containing  $\sim 10$  ml of sterile  $\text{DH}_2\text{O}$  at RT.

4. Wear PPE (safety glasses, etc.), grasp the straw plug end using forceps and hold in air for 40 seconds (critical time!), and then gently and carefully immerse the straw into the DH<sub>2</sub>O at RT in the beaker prepared above until ice disappears (~10 s).
5. Gently remove the straw from water and wipe it dry.
6. Holding the straw firmly and horizontally, gently cut off the heat seal at the non-plug end or the heat seals at both ends (if the plug end is also heat sealed) of the straw using the sterile scissors, and then gently cut through middle of the cotton/PVA plug leaving half the cotton plug in place to act as a plunger.
7. Holding the straw vertically and using a metal rod, push the plug (DO NOT push the plug out of straw) to expel the **entire** liquid contents of the straw into an empty Falcon dish at **room temperature (RT)**. Do not allow the tip of the straw to touch the expelled media as the embryos will stick to the straw.
8. Cover the dish, and incubate the embryos for 5 min at RT (22-25 °C). Embryos will shrink considerably as PROH and water leave the cells under the osmotic influence of 1.0 M sucrose.
9. During waiting, prepare a dish containing two 200- $\mu$ l drops of 0.5 M sucrose in M2 medium at RT, and two 200- $\mu$ l drops of M2 medium at RT, and cover the dish.
10. After 5 min of incubation at RT in the PROH-sucrose mixture, transfer the embryos to the first 200- $\mu$ l drop of 0.5 M sucrose in M2 medium at RT, and then transfer the embryos to the second 200- $\mu$ l drop of 0.5 M sucrose in M2 medium. Incubate embryos in 0.5 M sucrose at RT for total 5 min.
11. Wash the embryos through the first 200- $\mu$ l drop of M2 medium at RT, and then incubate the embryos in the second 200- $\mu$ l drop of M2 medium at RT for 5 min. Now the embryos have normal appearance and are ready for embryo transfer or culture.

### Reference

Renard JP and Babinet C (1984) High survival of mouse embryos after rapid freezing and thawing inside plastic straws with 1-2 propanediol as cryoprotectant. *J Exp Zool* 230: 443-448.