# Controlled- Rate Embryo Freezing and Thawing

## *1.0* Equipment

***1.1*** BioCool

**1.2** Dissecting microscope

***1.3*** Timers

***1.4*** $LN_2$ dewar

***1.5*** Heat sealer

***1.6*** Long forceps

***1.7*** P200 pipette

**1.8** P1000 pipette

***1.9*** Safety Glasses

***1.10*** Cell tram

***1.11*** EZ Grip

**1.12** Metal rod

**1.13** Perspex rack

**1.14** Ruler

**1.15** Brady TLS Thermal Labelling System

**1.16** Heated stage

**1.17** Incubator 5% CO2, 95% Air

**1.18** Heating Blocks

**1.19** Scissors

***1.20*** Vac seal bowl

## *2.0* Supplies

**2.1** 95% Methanol

**2.2** Cotton buds

**2.3** $LN_2$

**2.4** 35:3004 Culture Dishes

**2.5** 1-200µl pipette tips

**2.6** 100-1000µl pipette tips

**2.7** Gloves

**2.8** FFP2 masks

**2.9** EZ-Tip 135µm/170µm pipette tips

**2.10** 0.25ml French straws

**2.11** 1ml syringe

**2.12** Permanent marker pen

**2.13** Brady labels

**2.14** Brady ribbon ink

**2.15** 1.5M ProH solution

**2.16** 1M Sucrose solution

**2.17** M2

**2.18** 1% Hyaluronidase

**2.19** Tissues

**2.20** Room temperature tap water

## 3.0 Controlled-rate embryo freezing

### 3.1 Straw preparation

3.1.1 Print the labels for the straws.

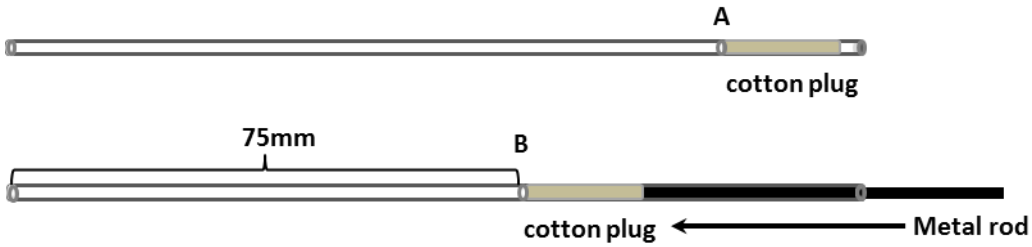
3.1.2 Prepare enough 0.25ml French straws so that the embryos can be frozen in batches of 30 per straw for 2-cell stage. If freezing down 8-cell, morulae or blastocysts only put a maximum of 25 embryos in a straw.

3.1.3 Decant a small amount of 1.5M ProH solution into an embryo culture dish. Label the lid and the base of the dish with the letter "P".

3.1.4 Decant a small amount of 1M sucrose solution into an embryo culture dish. Label the lid and the base of the dish with the letter "S".

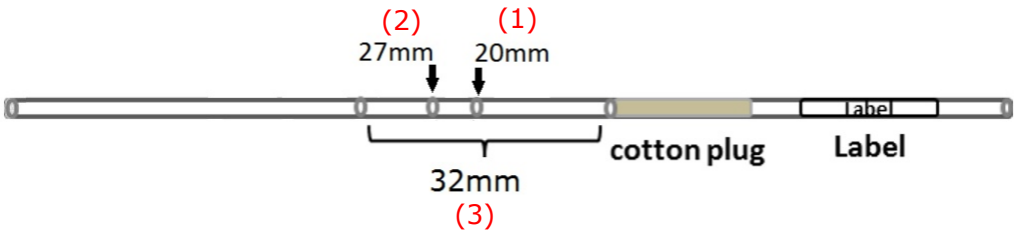
3.1.5 Push the cotton/polyvinyl alcohol plug into the straw, from position A to position B, using a metal rod with a stop (Picture 1).

**Picture 1**




3.1.6 Place the straw(s) on the Perspex rack marked out for slow/controlled-rate freeze. Using a permanent marker pen, make three calibration marks using the guidelines on the rack to obtain the correct distances. The calibration marks should be placed at (1) 20mm, (2) 27mm and (3) 32mm intervals from the cotton PVA plug (Picture 2).

**Picture 2**




3.1.7 Place the label at the plug-end of the straw, leaving enough room to attach a 1ml syringe on the same end.

3.1.8 Attach a 1ml syringe to a straw and use it to aspirate the 1.0M sucrose solution from the dish marked "S", until the meniscus reaches mark 3.

3.1.9 Aspirate air, to move the sucrose meniscus from mark 3 to mark 2. Dab the end of the straw on a piece of tissue to remove any excess sucrose left on the end of the straw before aspirating the ProH.

3.1.10 Aspirate the 1.5M ProH solution from the dish marked "P" until the sucrose meniscus moves from mark 2 to mark 1.

3.1.11 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. Now remove the syringe.

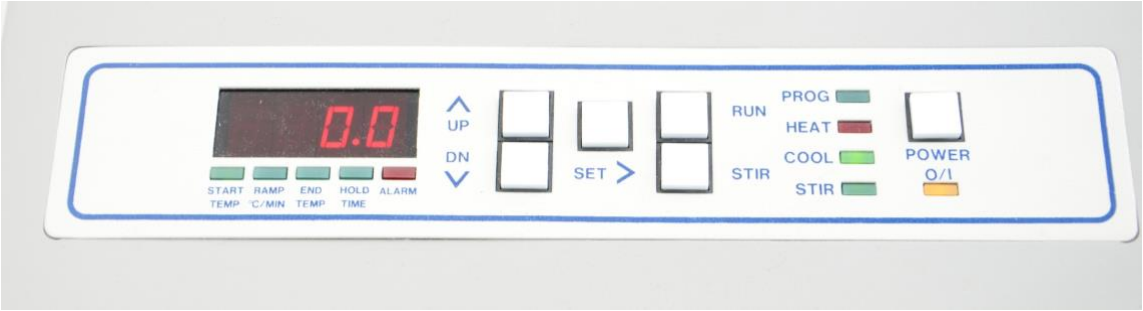
3.1.12 Repeat steps 3.1.5 – 3.1.11 for each straw.

## 3.2 Preparing the freezing machine

3.2.1 Wearing gloves, remove the lid/freezing rack to check that the reservoir is filled to within 2.5cm of the top with 95% methanol. The level should be just below or on the ledge. If the level is low, top the machine up.

3.2.2 Turn on the BioCool (Picture 3).

**Picture 3**




3.2.3 When the machine is turned on it will display "**SP C**" followed by the start temperature "**-7°C**".

**NOTE:** The BioCool should be programmed to:

- Start at -7°C
- Drop 0.5°C/MIN
- Stop at -30°C
- Hold at -30°C for 90mins

3.2.4 If the programme is correct, press the "**Run**" button to get it to the start temperature of -7°C. Ensure the **START TEMP** light is illuminated.

**NOTE: The machine takes approximately 15 mins to reach the start temperature. Make sure this time is taken into account when preparing the embryos for freezing.**

3.2.5 The display will flash "**-7°C**" when temperature has stabilised. DO NOT put the straws into the BioCool until the temperature display is flashing.

## 3.3 Loading Embryos into Straws

3.3.1 Using an embryo handling device wash the embryos destined for freezing through 2 wash drops of M2 (approx.150ul) in an embryo culture dish. A separate dish will be required for each stock, including control stock.

3.3.2 Place a drop (approx. 150µl) of the 1.5M Propylene glycol (ProH) cryoprotectant, into the centre of an embryo

culture dish. One ProH dish will be required per stock, including controls.

3.3.3 Once all the embryos have been washed through M2, transfer the embryos from one stock, currently in an M2 wash drop, into the centre of the corresponding ProH drop. Then start a countdown timer set for 5 minutes.

3.3.4 Repeat step 3.3.3 for each stock.

3.3.5 When the embryos for the last stock have been transferred to their ProH drop, start a 5 minute timer and another timer for 15 minutes.

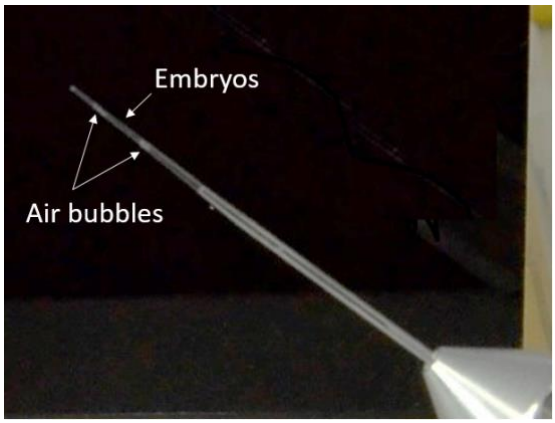
3.3.6 Carefully examine all of the embryos and reject any which show signs of damage or degeneration.

3.3.7 The embryos cannot be loaded into the straws until the 5 minute timer for that stock sounds.

3.3.8 When the first 5 minute timer goes off, check the embryos to ensure they have not degenerated during the equilibration time, then using an EZ Grip load the embryo handling pipette with ProH and introduce 1 air bubble into the end of the pipette.

3.3.9 Aspirate 30 embryos into the pipette in the smallest amount of ProH possible and introduce a second air bubble into the end of the pipette (Picture 4).

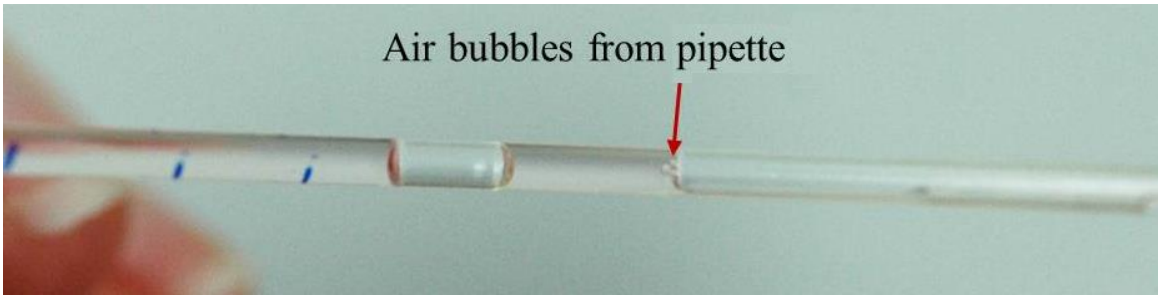
**Picture 4**




3.3.10 Place the tip of the pipette through the open end of the appropriately labelled straw and into the ProH fraction.

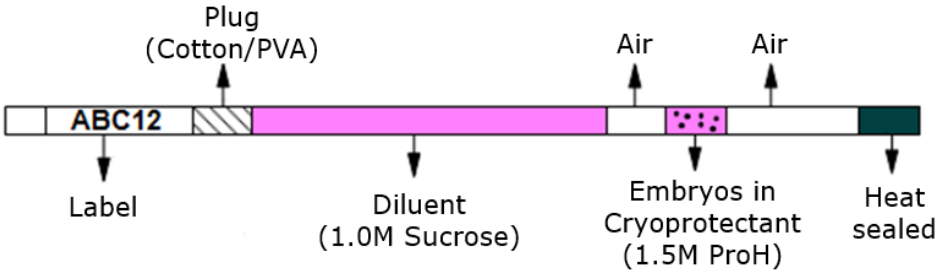
3.3.11 Gently expel the embryos and the air bubbles into the ProH fraction in the straw. As soon as the second bubble is in, cease expelling and carefully remove the pipette from the straw (Picture 5).

**Picture 5**




3.3.12 After the embryos are placed in the ProH fraction, seal the open end of the straw with a heat sealer (Picture 6).

**Picture 6**




3.3.13 Repeat steps 3.3.8 – 3.3.12 for each straw.

**NOTE:** When you get to the last two straws, divide the number of embryos equally between them. For example, instead of putting 30 embryos in the penultimate straw and 10 embryos in the last straw put 20 in each straw.

3.3.14 Wait until the 15 minute timer goes off before loading the straws into the BioCool freezing machine.

## 3.4 Loading straws into the BioCool

3.4.1 Only once all of the embryos have equilibrated in ProH for a minimum of 15 mins, and when the display on the BioCool is flashing "**-7°C**", can the straws be loaded into the BioCool.

3.4.2 Make sure the straw holding rack is in place on the BioCool.

3.4.3 One at a time, carefully insert the straws into the straw holder on the BioCool. If resistance is felt when inserting the straw into the straw holder do not force the straw as it could cause the straw to snap. This resistance can be caused by the sealed end of the straw being too wide. If this is the case trim the edges of the seal with a pair of scissors, ensuring that the seal is not removed in the process (Picture 7).

**Picture 7**

3.4.4 After the last straw has been placed in the holder, start a countdown timer set for 5 mins.

3.4.5 When the timer goes off, seed the straws by dipping a cotton wool bud in $LN_2$. Lift the straw holding rack up just enough to dab each straw with the cooled cotton bud at the top of the sucrose fraction, just below the plug. This will create a small white dot on the straw. Try to keep as much of the straw in the BioCool as possible and the

length of time the straws are out of the BioCool to a minimum (Picture 8).

**Picture 8**

3.4.6 Repeat the seeding procedure for each straw.

3.4.7 After the last straw is seeded, set the timer for another 5 mins.

3.4.8 At the end of the 5 mins check the straws have seeded. Ice crystals will have formed from the point of seeding down the length of the straw. This should be visible by the sucrose and ProH in the straw looking frosted as opposed to clear.

3.4.9 If the straws have not seeded, repeat steps 3.4.5 – 3.4.8.

3.4.10 Having successfully seeded the straws, press the "Run" button on the BioCool. The "Run" light should come on, if not, press again.

3.4.11 Make sure the temperature is starting to drop on the BioCool to ensure the programme is running.

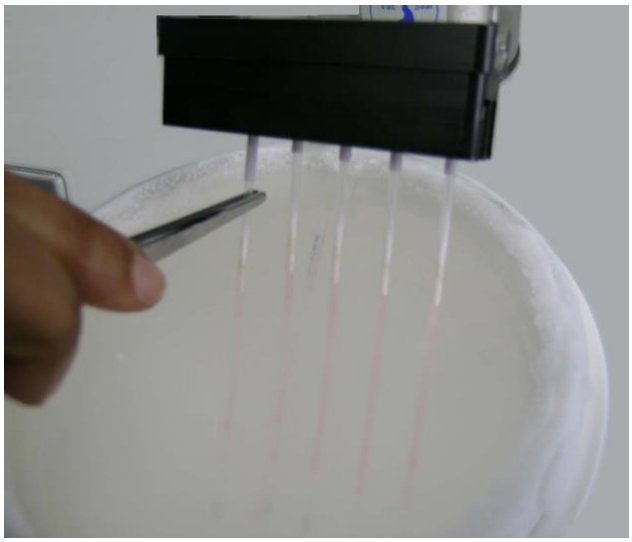
### 3.5 Removing the straws from the BioCool

3.5.1 Once the machine reaches -30°C and the "End Temp" and "Hold" lights come on, the straws can be removed and plunged into $LN_2$.

3.5.2 To remove the straws, take one rack out of the BioCool at a time and quickly submerge the straws in $LN_2$ in a small $LN_2$ carrying dewar.

3.5.3 Hold onto the straw rack and use forceps to remove the straws from the rack by pulling them downwards (Picture 9).

**Picture 9**



**NOTE**: Ensure the straw is submerged as much as possible in $LN_2$, particularly the ProH fraction, containing the embryos.

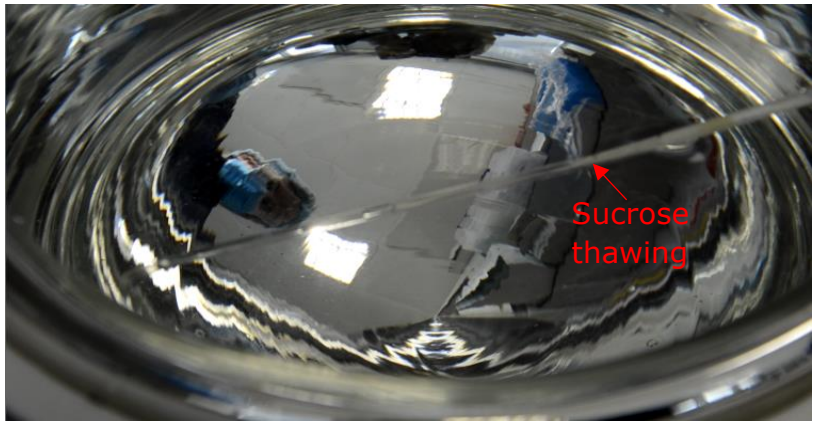
## 4.0 Thawing controlled-rate frozen embryos

4.1.1 Transfer the required straw(s) from the current location to a 1L Dewar containing liquid nitrogen.

4.1.2 Using forceps pick up the straw near the plug and hold in the air for 30 seconds (use a timer).

4.1.3 Plunge the straw into a vac seal bowl containing tap water at room temperature until the contents have completely thawed (picture 10).

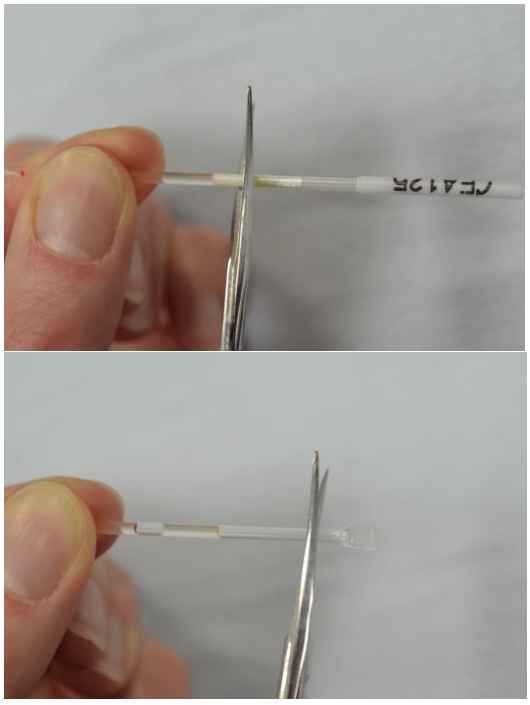
**Picture 10**




4.1.4 Wipe the outside of the straw gently with a tissue to remove any excess water.

4.1.5 Cut through the cotton plug just below the PVA glue mark and then cut off the sealed end of the straw. This will leave about half the cotton plug in place to act as a plunger (Picture 11).

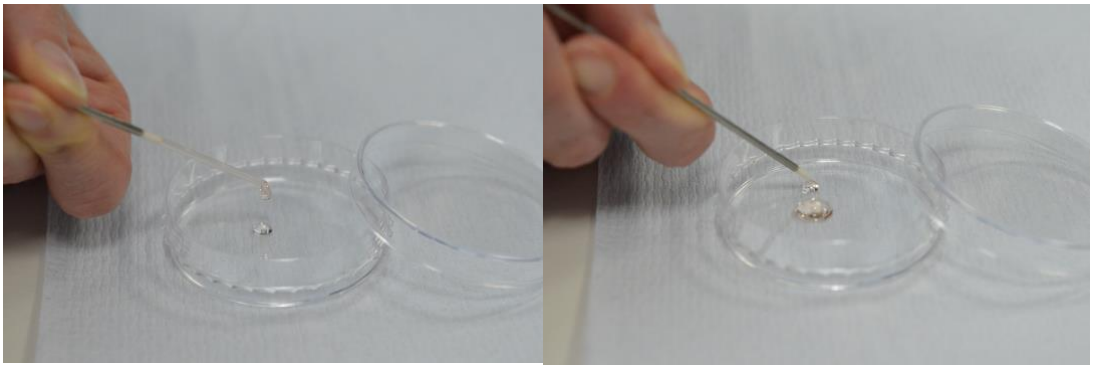
**Picture 11**



4.1.6 Use a metal rod to push the cotton plug and expel the contents of the straw in a single drop in an embryo culture dish. Do not allow the tip of the straw to touch the drop as this will cause the embryos to stick to the straw (Picture 12).

4.1.7 To remove any drops from the edge of the straw, touch the straw to one side of the main drop.

**Picture 12**



4.1.8 Allow the embryos to equilibrate for 5 minutes at room temperature, during this period they will shrink considerably.

4.1.9 Using an embryo handling device transfer the embryos to a 200µl drop of M2 in an embryo culture dish and allow them sit in the drop at room temperature for 5 minutes. During this time they will rapidly take up water and assume normal appearance.

4.1.10 After 5 minutes in the first M2 drop, change the tip on the embryo handling device and transfer the embryos into a fresh 200µl drop of M2. Allow the embryos to sit at room temperature for another 5 minutes.

4.1.11 After 5 minutes in the second M2 drop score the embryos; Move all Slightly Damaged, Abnormal, and Lysed embryos back to first drop of M2. Refer to Appendix for category definitions.

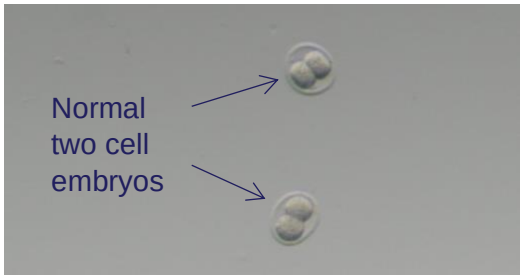
4.1.12 Note, when thawing 4 cell and above embryos, as long as 50% of the blastomeres are alive, the embryos can be transferred. These embryos are not suitable for re-freezing or culturing however.

4.1.13 The embryos are now ready for use.

## 5.0 Appendix

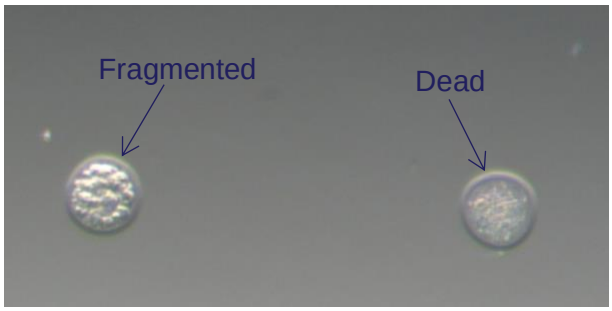
When scoring embryos they should be placed into one of four categories:

**Normal** – Embryos that have thawed well and all blastomeres are intact.




**Slightly Damaged** – This applies to embryos at stage 4-cell and above. If >50% of the blastomeres survived, the embryo can still be used for transfer. If <50%, they should not be used.

**Abnormal** – This includes embryos of an abnormal shape, or embryos which are dead.




**Lysed** – When the zona pellucida has ruptured and the contents of the embryo has leaked out.