

Vitrification of Embryos and Thawing Vitrified Embryos

1.0 Equipment

- 1.1 Dissecting Microscope
- 1.2 Vitrification Cooling Plate (VCP)
- 1.3 Large forceps
- 1.4 Plastic sandwich box
- 1.5 200µl Gilson Pipette
- 1.6 20 µl Gilson Pipette
- 1.7 Embryo Handling Pipette
- 1.8 Perspex straw support
- 1.9 Countdown timer
- 1.10 30cm Ruler
- 1.11 Permanent marker pen
- 1.12 Metal rods
- 1.13 Heat sealer
- 1.14 Small liquid nitrogen dewar
- 1.15 Brady Label Printer
- 1.16 Incubator (5% CO₂, 37°C)
- 1.17 Heated stage (37°C)
- 1.18 Water bath (37°C)
- 1.19 Scissors

2.0 Supplies

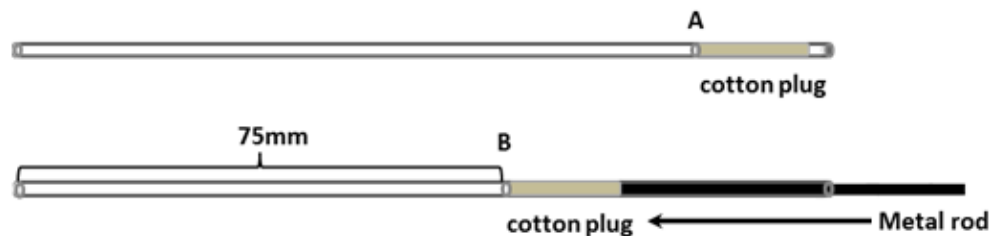
- 2.1 60mm Petri dishes (35:1007)
- 2.2 0.25ml French Straws
- 2.3 200µl pipette tips
- 2.4 Wedge shaped pipette tips
- 2.5 1.0ml Syringe
- 2.6 Single use filter (0.2µm)
- 2.7 Liquid Nitrogen
- 2.8 Ice
- 2.9 Embryo Handling Device Tips
- 2.10 Brady Labels
- 2.11 1M Dimethyl sulfoxide (DMSO) in PB1 medium
- 2.12 2M DMSO, 1M Acetamide, 3M Propylene Glycol (DAP213) in PB1 medium
- 2.13 0.25M Sucrose in PB1 medium
- 2.14 10ml luer lock syringe
- 2.15 Hyaluronidase
- 2.16 M2 media
- 2.17 KSOM media
- 2.18 Tissues

3.0 Vitrifying embryos

3.1 Preparation of straws

3.1.1 Using a metal rod, push down the cotton plug down to 75mm from the end of the straw (Picture 1).

Picture 1



3.1.2 Place the straw on the Perspex support and use a permanent marker and ruler to make two calibration marks at 17mm and 31mm intervals from the end of the straw (Picture 2).

Picture 2



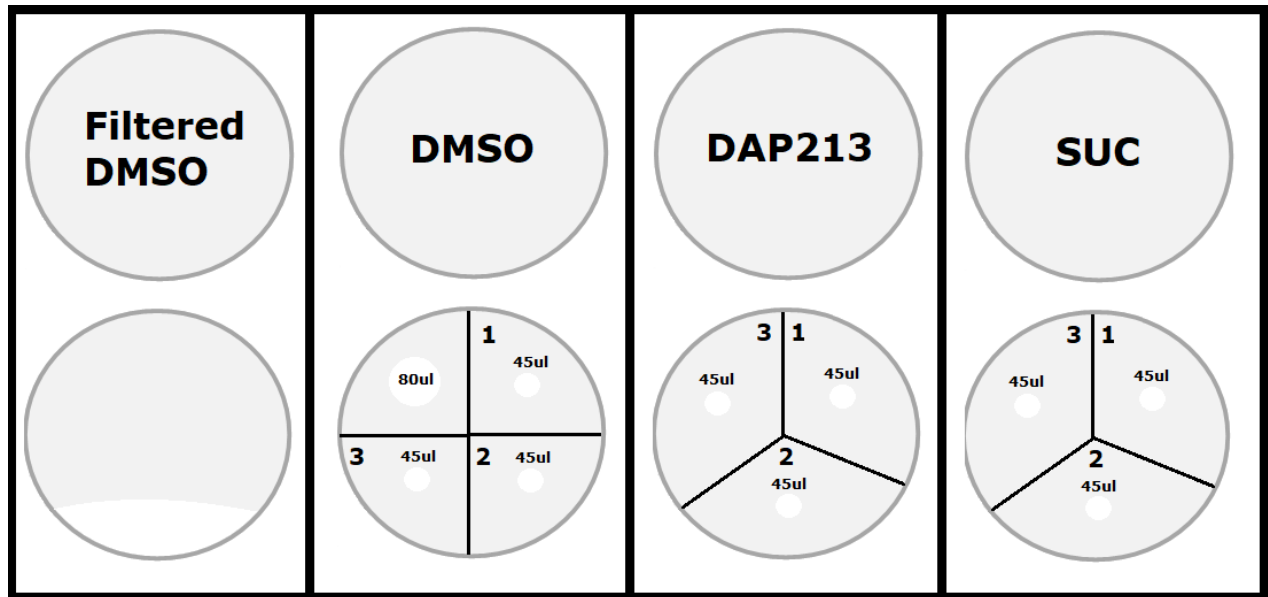
3.1.3 Label the straws and place onto the VCP.

3.2 Preparation of dishes

3.2.1 Label four embryo culture dishes as follows;

- Filtered DMSO
- DMSO
- DAP213
- SUC

3.2.2 Divide the base of the DMSO, DAP213 and Suc dishes into segments; one segment per drop/straw with the exception of the DMSO dish which requires an extra segment for the 80µL pooling drop, for example see Picture 3.

Picture 3

3.2.3 Fill a sandwich box with ice and place the vitrification cooling plate with the straws on, on top of the ice.

3.2.4 Collect a small carrying dewar full of LN₂.

3.2.5 Collect the required amount of Vitrification Solutions. Keep the 1M DMSO at room temperature and keep the DAP213 and 0.25M Sucrose cold by placing on the pre-cooled Vitrification Cooling Plate (VCP) or in ice.

3.2.6 Filter the 1M DMSO into the dish labelled "Filtered DMSO" using a single use filter (0.2µm) and a 10ml luer lock syringe. Pipette one 80µL drop of the filtered 1M DMSO solution plus the required number of 45µL drops into the separate segments of the DMSO labelled dish. Keep this dish at room temperature.

NOTE: the number of 45µL drops/segments required for all vitrification media will be the same as the number of straws.

3.2.7 Place the DAP213 and SUC dishes on the VCP. Pipette the required number of 45µl drops of 1M Sucrose

solution and DAP213 solution into the segments of the respective dishes.

3.2.8 Each DMSO drop will have a correlating DAP213 drop and Sucrose drop.

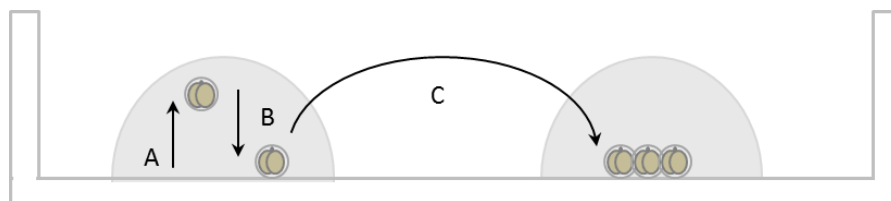
3.3 Pre-treatment of embryos in 1M DMSO solution

3.3.1 Embryos should be treated with hyaluronidase prior to vitrifying if cumulus cells are still attached.

3.3.2 Load an embryo handling pipette with the 1M DMSO solution and transfer the embryos from the culture dishes to the 80 μ L drop of 1M DMSO.

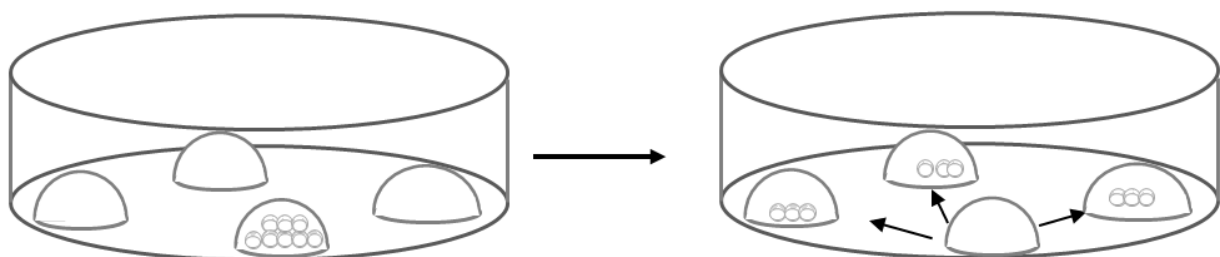
3.3.3 The embryos will first float to the top (Picture 4, step A) then sink down to the bottom of the dish (Picture 4, step B). This will take approximately 1-2 minutes.

Picture 4



3.3.4 Once the embryos have settled at the bottom of the dish (Picture 4, step C), divide the embryos equally into the remaining drops of 1M DMSO (Picture 5). The number of embryos in each drop will correspond to the number of embryos to be loaded into each straw.

Picture 5



3.3.5 Once all the embryos have been divided between the 45µL DMSO drops, place the DMSO dish onto the VCP and start a countdown timer for 5 minutes.

3.4 **Placing embryos in DAP213 solution**

3.4.1 After 5 minutes has elapsed, use a 20µl pipette with a wedge shaped tip attached, to pick up all of the embryos in the first 1M DMSO solution drop, within 5µl. Aim to aspirate 1µl of the 1M DMSO solution before picking up the embryos to avoid the embryos sticking to the inside of the pipette tip.

3.4.2 Expel the embryos from the pipette tip into the corresponding 45µl DAP213 drop by holding the pipette directly above the drop, without touching the drop itself.

3.4.3 Start a countdown timer for 5 minutes then repeat steps 3.4.1 and 3.4.2 for the remaining drops until all embryos are in DAP213.

3.5 **Loading embryos into straws**

3.5.1 Leave the embryos to partially equilibrate for the first minute of the countdown.

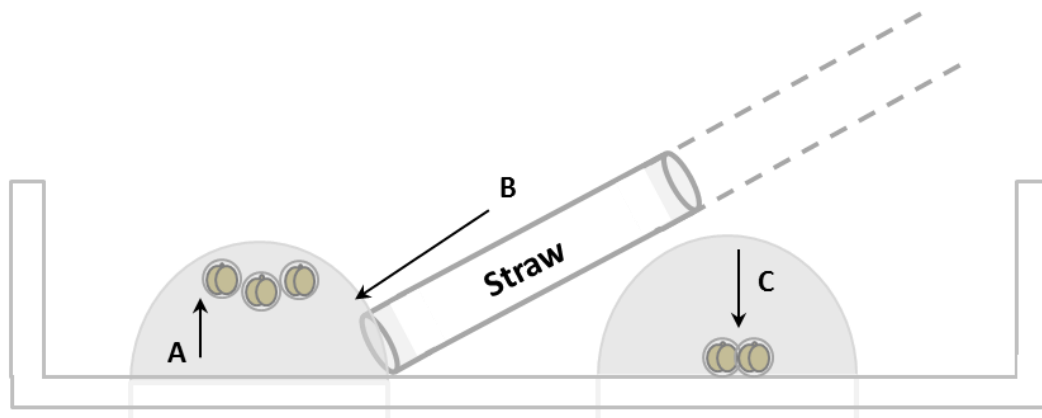
3.5.2 After 1 minute has elapsed attach a 1ml syringe to the labelled end of the first straw.

3.5.3 Aspirate 0.25M sucrose solution from the prepared droplets to the first calibration mark on the straw and aspirate air to move the meniscus of the sucrose fraction up to the second mark.

3.5.4 Aspirate the entire first drop of DAP213 containing the embryos. Ensuring the straw is held at a 45° angle and is loaded from the side of the drop (see Picture 6).

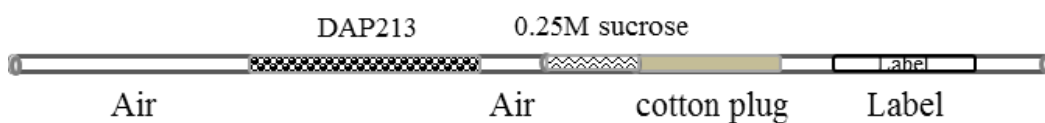
NOTE: Ensure the embryos are loaded into the straw in the same order as they are placed in the DAP213 to prevent the embryos from sinking too far down the drop and sticking to the bottom of the dish.

Picture 6



3.5.5 Aspirate air until the sucrose fraction has made contact with the PVA section of the cotton plug (Picture 7).

Picture 7



3.5.6 Remove syringe and place straw back onto the vitrification cooling plate (for at least 30 seconds).

3.5.7 Repeat 3.5.2 – 3.5.6 for all remaining straws.

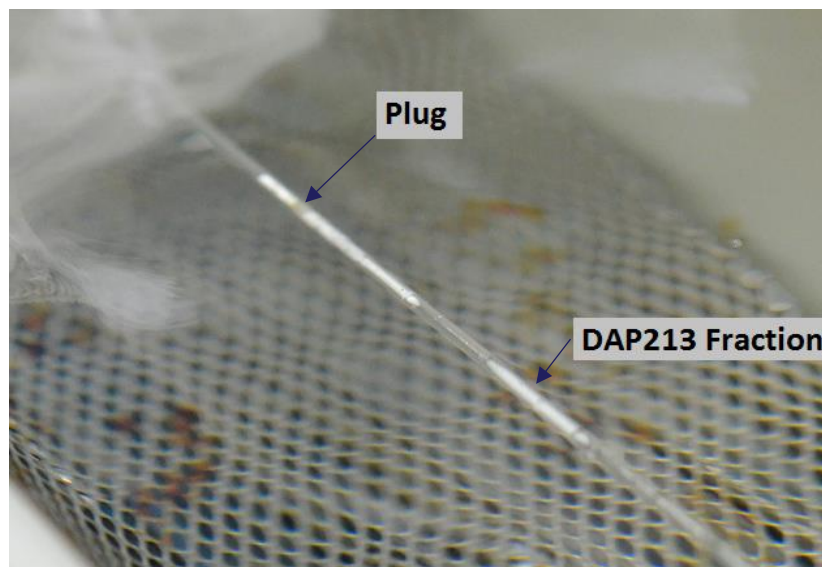
3.5.8 Heat seal the non-labelled end of each straw individually and place back onto the VCP for the remainder of the 5 min equilibration time.

3.5.9 After the 5 minutes has elapsed, plunge the straws into the small carrying dewar of liquid nitrogen.

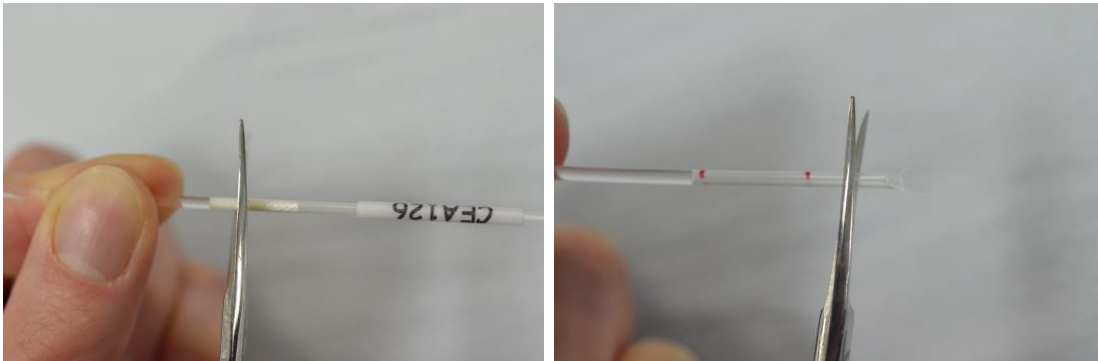
4.0 Thawing Vitrified Embryos

- 4.1.1 Place a vial containing 1ml of 0.25M sucrose in a 37°C incubator to warm for a minimum of 10 minutes.
- 4.1.2 Remove required straws from LN₂ storage and place in a small dewar of liquid nitrogen.
- 4.1.3 Place a 60mm petri dish (35:1007) on a 37°C heated stage. Transfer 1ml of pre-warmed 0.25M sucrose solution into the dish on the heated stage.
- 4.1.4 Using large forceps to remove the required straw from LN₂, immerse the whole straw in the 37°C water bath. Once the DAP213 fraction has thawed and is completely clear, remove the straw immediately from water bath. (See Picture 8).

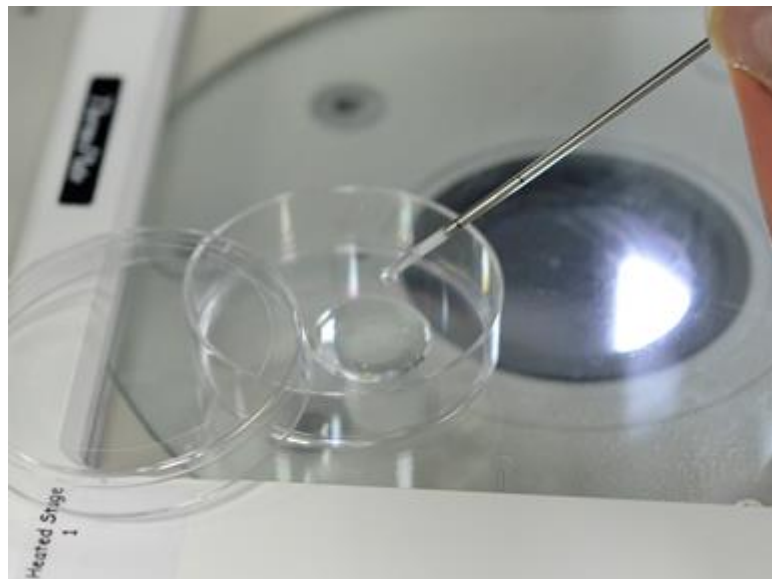
Picture 8



- 4.1.5 Wipe the straw with a tissue to dry it. Cut through the cotton plug and then cut through the sealed end (Picture 9). Hold the straw close to the ends whilst cutting to prevent the straw bending and the DAP213 section fragmenting. **Do not hold onto the DAP213 section.**

Picture 9

4.1.6 Using a metal rod, expel the whole contents of the straw into the sucrose drop ensuring the straw does not touch the surface of the drop (Picture 10).

Picture 10

4.1.7 If a droplet of media remains on the straw it should be gently dabbed into the dish, next to the sucrose drop. Make sure the main drop merges with this smaller droplet.

4.1.8 Gently swirl the dish and start a timer for 3 minutes to allow the embryos to equilibrate in the sucrose. Ensure the sucrose dish remains on the heated stage during this time.

4.1.9 After the 3 minutes has elapsed, use an embryo handling device to locate the embryos.

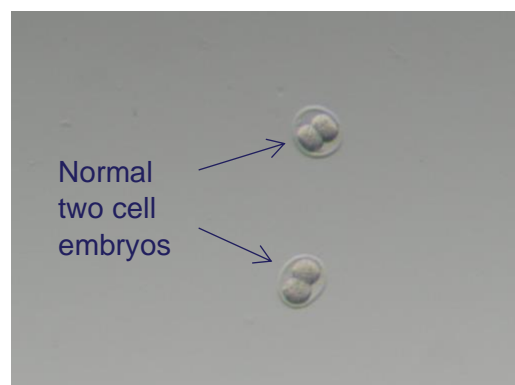
4.1.9.1 Embryos that are going to be cultured should be placed into KSOM media culture dishes and scored (Refer to Appendix to score embryos).

4.1.9.2 Embryos that are to be transferred should be placed into M2 media and scored (Refer to Appendix to score embryos).

5.0 Appendix

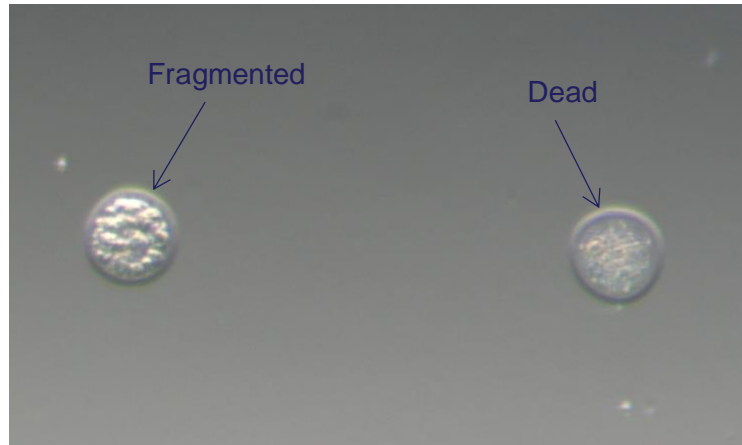
When scoring embryos they should be placed into one of four categories:

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.

