

**IVF recovery procedure using frozen sperm samples  
(CPA prepared with L-Glutamine), incorporating methyl-  
β-cyclodextrin and reduced glutathione**

## **1.0 Equipment**

- 1.1** Incubator (5% CO<sub>2</sub>, 37°C)
- 1.2** Dissecting microscope
- 1.3** Angle poised lamp
- 1.4** Cold light source
- 1.5** Heated stage
- 1.6** Dissecting scissors
- 1.7** Dissecting forceps
- 1.8** Watchmakers forceps
- 1.9** P20 Gilson pipette
- 1.10** P100 Gilson pipette
- 1.11** P200 Gilson pipette
- 1.12** 10ml Gilson pipette
- 1.13** Falcon tube rack
- 1.14** Electronic powerpette
- 1.15** General purpose LN2 forceps
- 1.16** 1L liquid nitrogen transport dewar
- 1.17** Metal rod
- 1.18** Scissors
- 1.19** 37°C water bath
- 1.20** Positive displacement pipette

**1.21** Cell Tram**2.0 Supplies:**

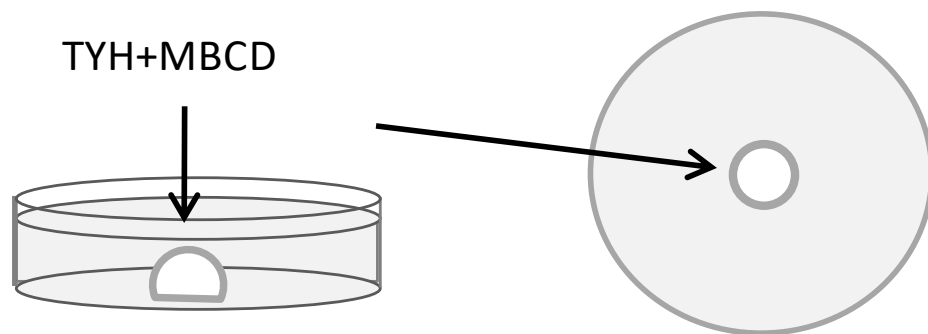
- 2.1** TYH + MBCD medium
- 2.2** Human tubal fluid (hTF)
- 2.3** Silicone fluid
- 2.4** Reduced glutathione (GSH)
- 2.5** 14ml Falcon Tubes
- 2.6** 0.2µm syringe filters
- 2.7** 10ml syringe
- 2.8** 60mm embryo culture dishes (35:3004)
- 2.9** 10ml serological pipettes
- 2.10** 200µl pipette tips
- 2.11** Wedge shaped pipette tips
- 2.12** 1000µl pipette tips
- 2.13** 10ml Diamond Pipette tips
- 2.14** Tissues
- 2.15** 70% alcohol
- 2.16** Permanent marker pen
- 2.17** Liquid nitrogen
- 2.18** EZ-Tip 135µm/170 µm pipette tips
- 2.19** Napkins

### 3.0 Procedure

#### 3.1 Preparation of sperm dispersal dishes

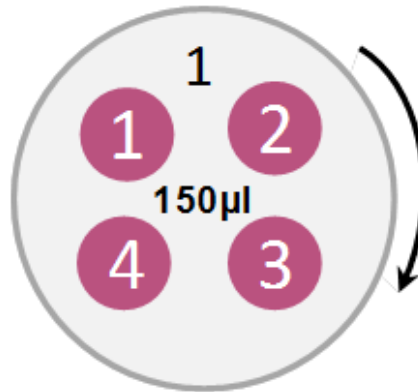
- 3.1.1 Prepare one dispersal dish IVF (plus one dish for control sperm sample, if applicable, and a spare dish in case a sample is not viable and another sample is required).
- 3.1.2 Pipette 90µl TYH + MBCD into the centre of an embryo culture dish (Picture 1). Overlay with silicone fluid. Equilibrate the dishes in a 5% CO<sub>2</sub> incubator set at 37°C for at least 15 minutes, or overnight, before use.

**Picture 1**



#### 3.2 Preparation of wash/culture dishes

- 3.2.1 Prepare one wash dish per three female mice to be used.
- 3.2.2 Pipette 4 x 150µl drops of hTF into an embryo culture (Picture 2). Overlay with silicone fluid. Equilibrate the dishes in a 5% CO<sub>2</sub> incubator set at 37°C for at least 15 minutes, or overnight, before use.

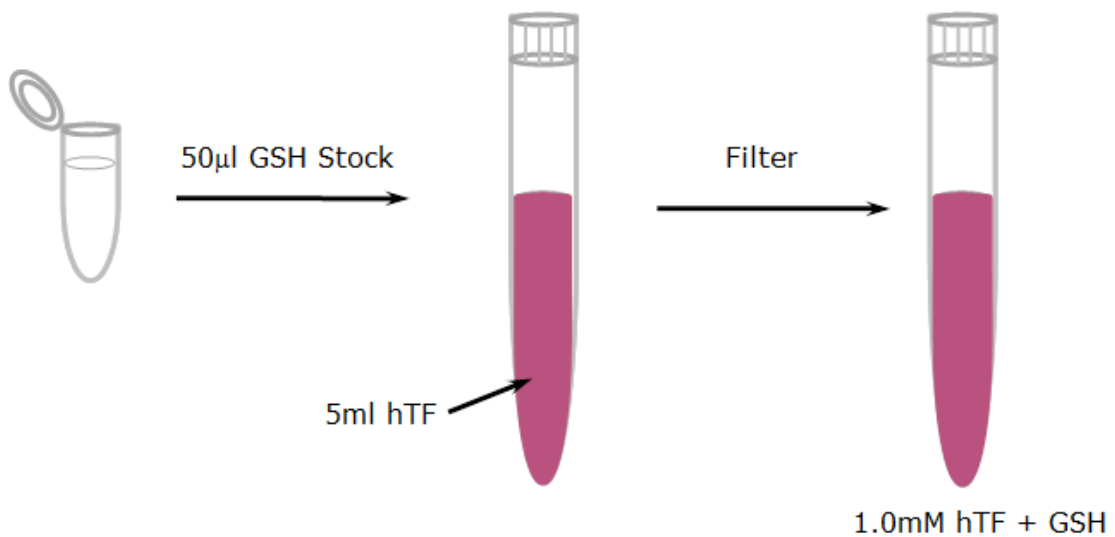
**Picture 2**

### 3.3 Preparation of fertilisation medium containing reduced glutathione

- 3.3.1 Take 1ml hTF medium and add it to a tube containing 30.7mg reduced glutathione (GSH). Close the lid, mix the medium and the powder in the tube (Picture 3).

**Picture 3**

- 3.3.2 Take 50µl of the GSH solution and add it to 5ml hTF medium and mix them together gently. The final concentration is 1.0mM GSH (Picture 4).

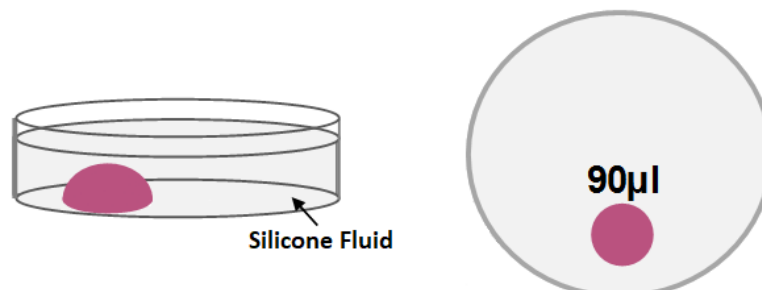
**Picture 4**

### 3.4 Preparation of fertilisation dish containing 1mM reduced glutathione (GSH)

- 3.4.1 Prepare one fertilisation dish per three female mice to be used.

**NOTE:** Offset the fertilisation drop to provide sufficient space for harvesting oocytes.

- 3.4.2 Pipette a **90µl** drop of the **1mM** GSH solution into an embryo culture dish (Picture 5). Overlay with silicone fluid. Equilibrate the dishes in an incubator.

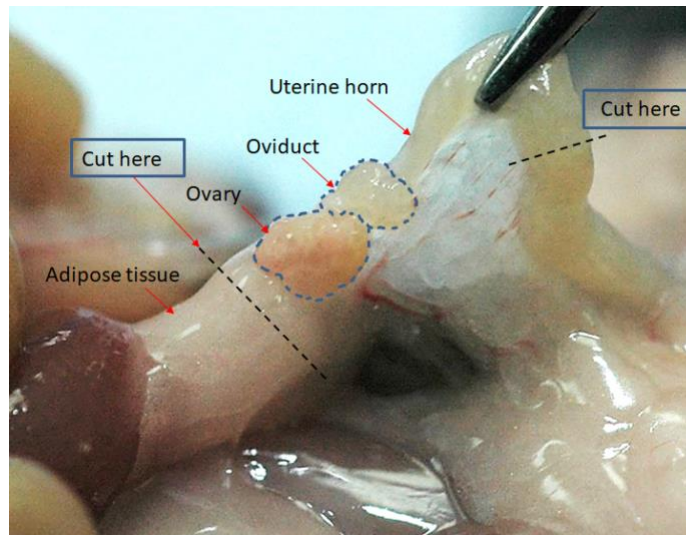
**Picture 5**

### 3.5 Oocyte harvesting

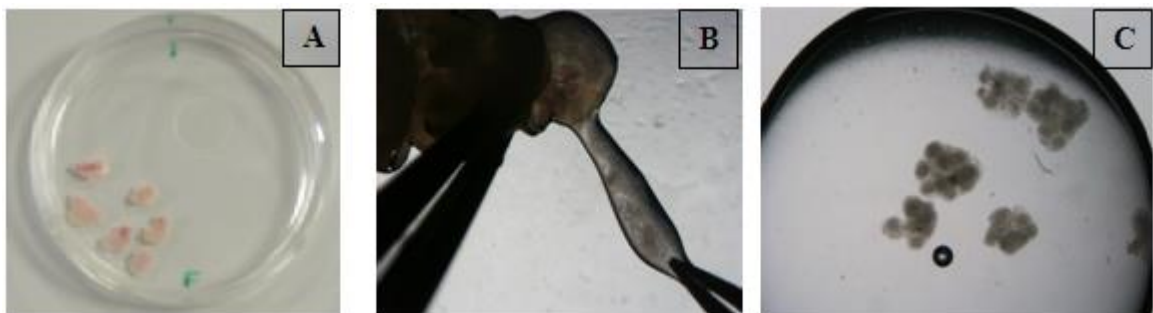
- 3.5.1 Dissect the oviducts (Picture 6) from three superovulated female mice and transfer them into the

silicone fluid overlaying the pre-incubated fertilisation drop (Picture 7A). A deviation of the number of females per dish is acceptable in certain circumstances and must be recorded.

**Picture 6**



**Picture 7**



- 3.5.2 Under the microscope, hold an oviduct down with watchmakers forceps.
- 3.5.3 Gently tear the swollen ampulla with a second pair of watchmakers forceps to release the cumulus masses into the silicone fluid (Picture 7B).
- 3.5.4 Using the watchmakers forceps, drag the cumulus masses through the silicone fluid and into the fertilisation drop (Picture 7C).
- 3.5.5 Remove the oviduct from the dish and discard with the cadavers.

3.5.6 Repeat steps 3.5.2 – 3.5.5 for each oviduct.

### 3.6 Preparation of sperm samples

**NOTE:** It may be necessary to refer to the thawing protocol supplied by the originator when handling imported sperm.

3.6.1 Once all the cumulus masses have been harvested remove the required straws from the storage tanks and place in a flask of liquid nitrogen.

3.6.2 Quickly transfer straw into a tube in a 37°C water bath and leave for 10 minutes (Picture 8).

**Picture 8**



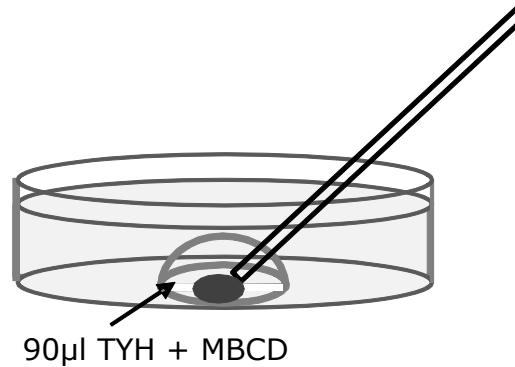
3.6.3 Remove the straw from the water bath and dry with a tissue.

3.6.4 Cut through the centre of the cotton plug, and then cut the heat-sealed end of the straw, nearest to the sperm.

3.6.5 Keeping the sperm dispersal dish in the incubator, use a metal rod to expel the **10µl** sperm suspension into the

centre of the drop. (Picture 9). **Do not expel any of the HTF media contained within the straw.**

**Picture 9**

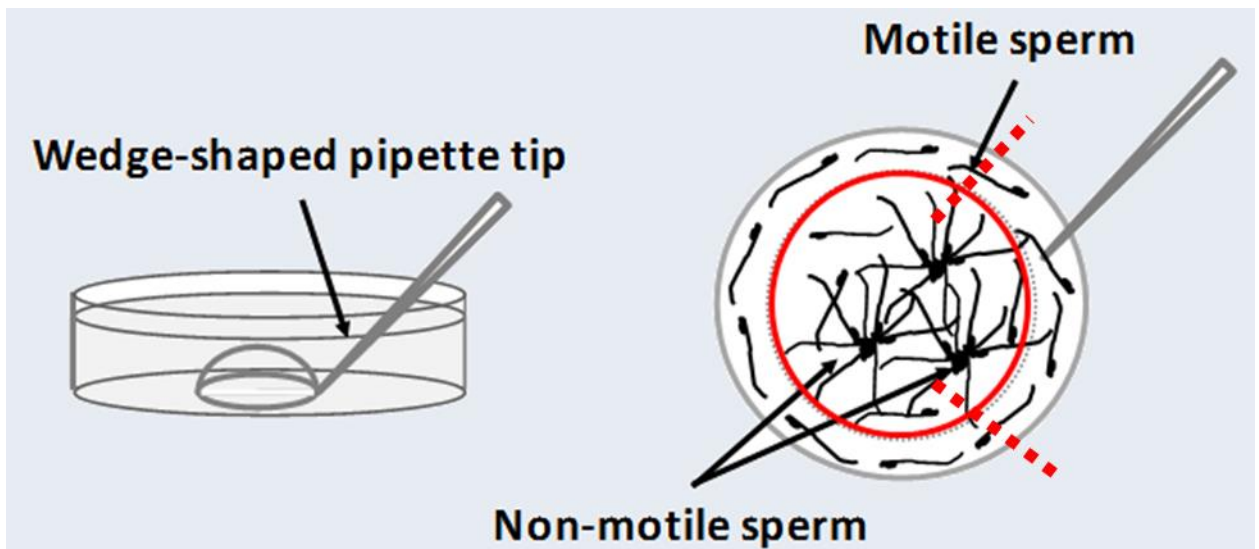


- 3.6.6 Pre-incubate the frozen/thawed sperm in TYH+MBCD medium for a total of **30 minutes**.

### **3.7 Fertilisation**

- 3.7.1 **The cumulus masses should be incubated in the fertilisation drop for a minimum of 30mins prior to sperm being added to the dish.**
- 3.7.2 After 25mins of pre-incubation, rotate the sperm dispersal dish 5 times whilst it is still in the incubator.
- 3.7.3 Incubate the sperm for a further 5mins.
- 3.7.4 Remove the sperm dispersal dish from the incubator along with the corresponding fertilisation dishes; place on heated stage under the dissecting microscope.
- 3.7.5 Looking down the microscope, using a pipette fitted with a wedge-shaped pipette tip, gently aspirate **10-20µl** of pre-incubated sperm from the peripheral part of the sperm dispersal drop (Picture 10). Try to avoid aspirating any sperm debris.



**Picture 10**

**NOTE:** it is recommended to aspirate sperm in between the '1 o'clock and 5 o'clock' position to ensure the correct concentration of sperm is collected. Rotate the dish 90° and repeat for each subsequent dish.

**NOTE:** pipette the sperm slowly to prevent shearing forces from damaging the sperm.

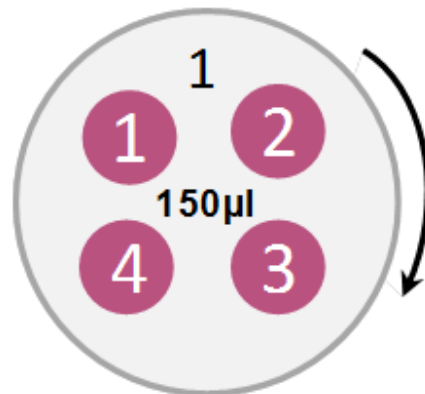
**NOTE:** sperm held in Lifer may be of a lower quality and may require more sperm adding to the fertilisation dish.

- 3.7.6 Add sperm to the centre of the fertilisation drop containing the cumulus masses. Return the dishes to the incubator.
- 3.7.7 If there is concern over the quality of the sperm; concentration and/or progressive motility, remove the one fertilisation dish per IVF from the incubator after 10mins and assess the sperm motility and concentration again. It may be necessary to add more of the sperm suspension if the sperm quality is poor.
- 3.7.8 Incubate the dishes for approximately 3-4hrs to allow fertilisation to occur.

### 3.8 Washing Zygotes

- 3.8.1 After 3-4 hours incubation, use an embryo handling device to aspirate all of the presumptive zygotes from the fertilisation drop and transfer into drop 1 of the corresponding wash dish (Picture 11).

**Picture 11**



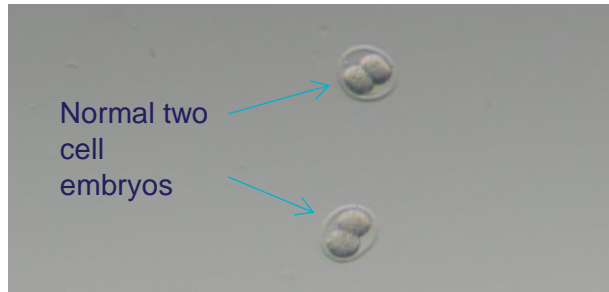
- 3.8.2 Leaving the degenerating zygotes in drop 1, move the good quality presumptive zygotes through drops 2 and 3.
- 3.8.3 Move the presumptive zygotes into drop 4 and culture them overnight. Record numbers of the degenerating zygotes.
- 3.8.4 Change embryo handling device tip between IVF's and for control dishes.

### 3.9 Scoring the IVF

- 3.9.1 After culturing the presumptive zygotes overnight, use an embryo handling device to move any unfertilised or degenerating cells to drop 3. Leave the 2-cell embryos in drop 4.

#### 4.0 Appendix 1

Normal one cell embryo

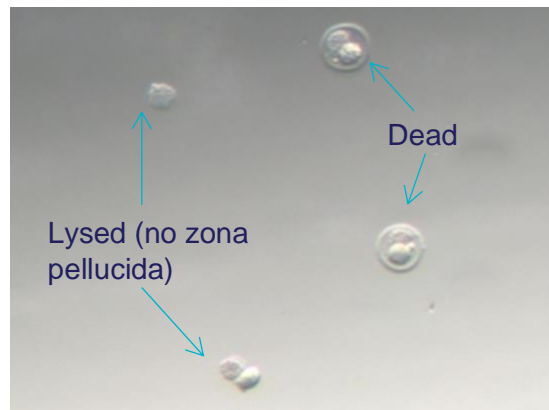


Normal two cell embryos



Fragmented

Dead



Lysed (no zona pellucida)

Dead