IVF recovery procedure using freshly harvested sperm, incorporating methyl-β-cyclodextrin and reduced glutathione

1.0 Equipment

1.1 Incubator 5% CO2, 95% Air

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 Miniature scissors

1.10 P20 Gilson pipette

1.11 P100 Gilson pipette

1.12 P200 Gilson pipette

1.13 10ml Gilson pipette

1.14 Falcon tube rack

1.15 Electronic powerpette

1.16 Permanent marker pen

1.17 Positive displacement pipette

1.18 Cell Tram

1.19 Vertical laminar airflow cabinet (LAF)

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 60mm Petri dishes (10060)

2.10 10ml serological pipettes

2.11 Wedge shaped pipette tips

2.12 200µl pipette tips

2.13 1000µl pipette tips

2.14 10ml Diamond Pipette tips

2.15 EZ-Tip 135µm/170 µm pipette tips

2.16 Blue roll

2.17 Tissues

2.18 Napkins

2.19 Square Paper

2.20 70% alcohol

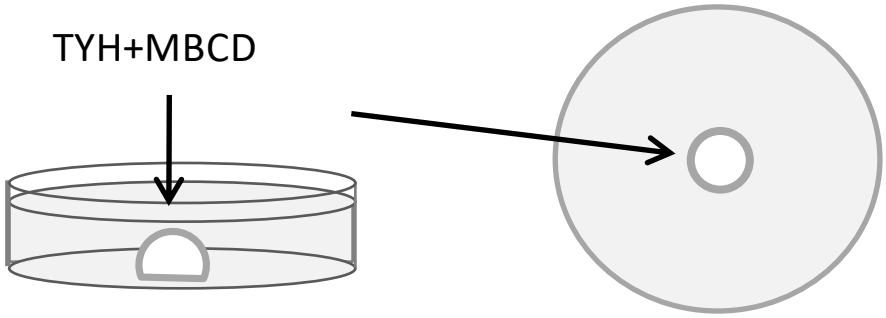
3.0 Procedure

3.1 Preparation of sperm dispersal dishes

3.1.1 Prepare one dispersal dish per IVF (plus one dish for control sperm sample, if applicable, and a spare dish in case the sperm required for the IVF 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_2 incubator set at 37°C for at least 15 minutes, or overnight, before use.

Picture 1

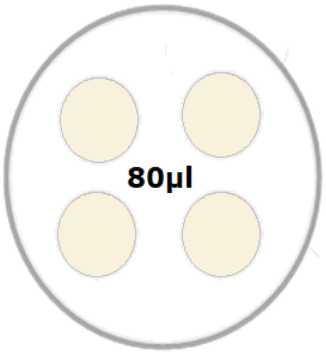


3.2 Preparation of wash dish for epididymides held in Lifor

3.2.1 This dish is only required for cauda epididymides held in Lifor. One dish is required for every male being used for IVF.

3.2.2 Pipette 4 x 80µl drops of TYH + MBCD into an embryo culture dish (Picture 2). **Do not** overlay with silicone fluid.

Picture 2



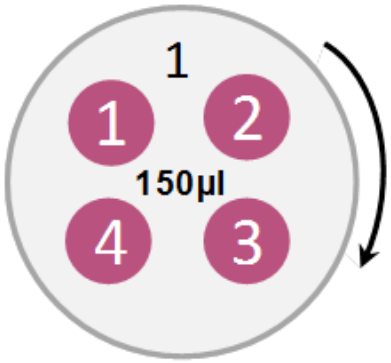
80µl MBCD x 4 drops

3.3 Preparation of wash/culture dishes

3.3.1 Prepare one wash dish per three female mice to be used.

3.3.2 Pipette 4 x 150µl drops of hTF into an embryo culture dish (Picture 3). Overlay with silicone fluid. Equilibrate the dishes in a 5% CO_2 incubator set at 37°C for at least 15 minutes, or overnight, before use.

Picture 3



3.4 Preparation of fertilisation medium containing reduced glutathione

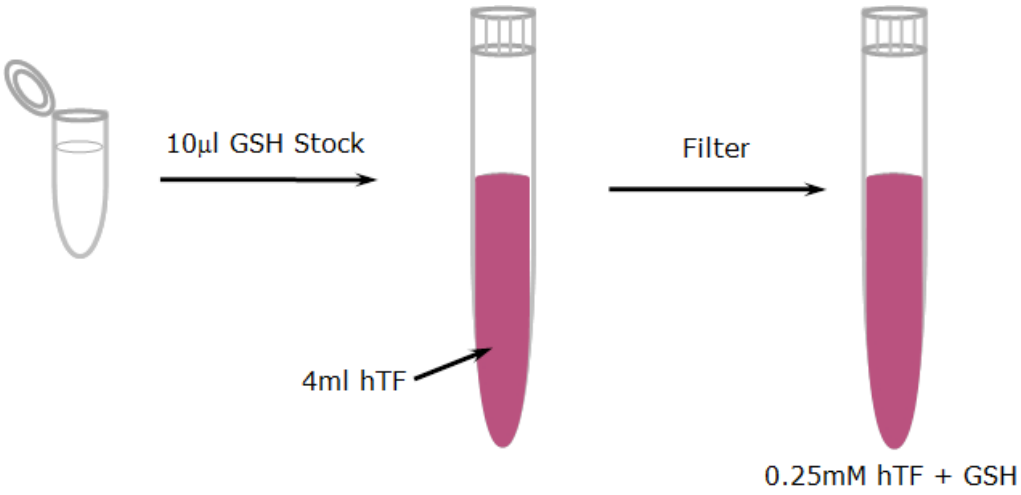
3.4.1 Take 1ml hTF medium and add it to a tube containing 30.7mg reduced glutathione (GSH). Close the lid, mix the medium and the powder in the tube (Picture 4).

Picture 4

3.4.2 Take 10µl of the GSH solution and add it to 4ml hTF medium and mix them together gently. The final concentration is 0.25mM GSH (Picture 5).

3.4.3 Before use, filter the solution using 0.22µm syringe end filter.

Picture 5



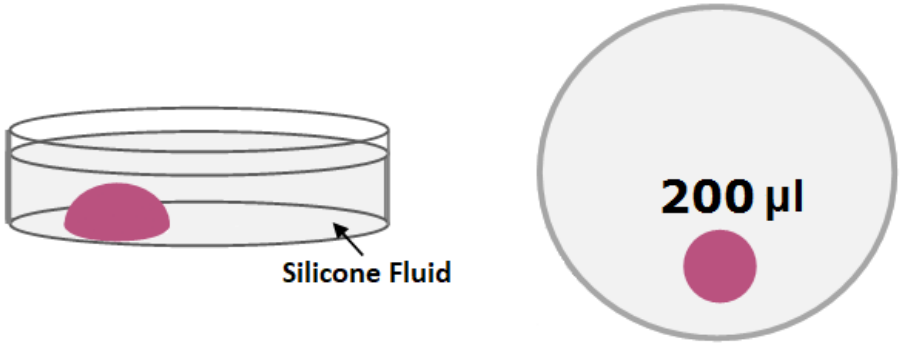
3.5 Preparation of fertilisation dish containing 0.25mM reduced glutathione (GSH)

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

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

3.5.2 Pipette a **200µl** drop of the **0.25mM** GSH solution into an embryo culture dish (Picture 6). Overlay with silicone fluid. Equilibrate the dishes in a 5% CO_2 incubator set at 37°C for at least 15 minutes before use.

Picture 6



3.6 Harvesting Sperm from epididymides held in Lifor

3.6.1 Remove the cauda epididymides from the Lifor and dab gently onto a napkin to remove any excess Lifor.

3.6.2 Place both cauda epididymides on a piece of square paper.

3.6.3 Under a dissecting microscope lit from above, remove all adipose and vascular tissue from the cauda epididymides, using clean watchmakers forceps and miniature scissors.

3.6.4 Wash the epididymides through each of the 4 x MBCD drops to ensure all the Lifor has been removed. Working in a clockwise direction.

3.6.5 Follow steps 3.7.5 – 3.7.10.

3.7 Preparation of sperm samples

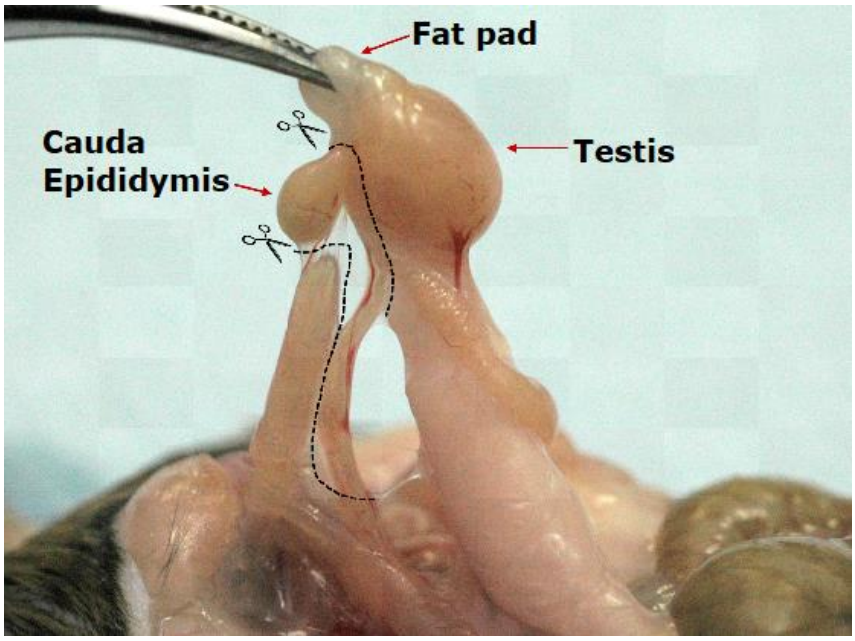
3.7.1 The selected male should be at least 10 weeks old, and not been used for mating for at least 3 days before sperm collection.

3.7.2 Cull the male and place on a paper napkin. Wet the fur on the abdomen with 70% alcohol.

3.7.3 Using forceps to hold the cauda epididymis, dissect from the body along the incision lines visible in Picture 7.

3.7.4 Place both cauda epididymides on a piece of square paper.

Picture 7

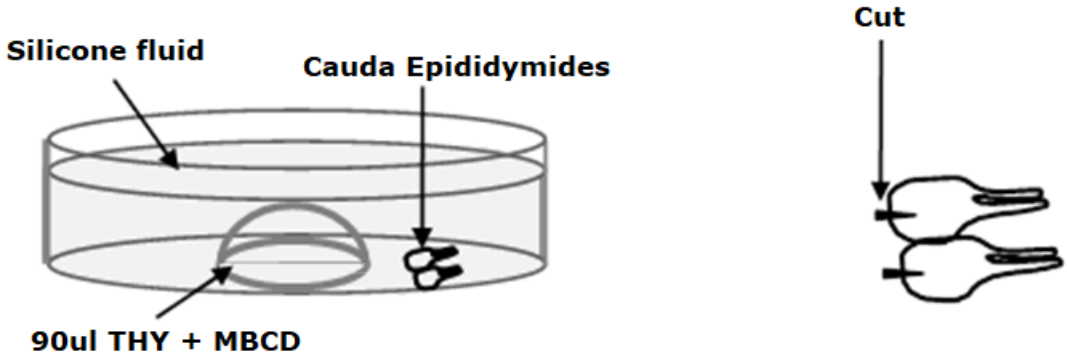


3.7.5 Under a dissecting microscope lit from above, remove all adipose and vascular tissue from the cauda epididymides, using clean watchmakers forceps and miniature scissors.

3.7.6 Place the cleaned cauda epipidymides into the silicone fluid **next to** the sperm dispersal drop.

3.7.7 Pinch the base of the cauda epididymis using watchmakers forceps then cut the apex of the cauda epididymis using miniature scissors (Picture 8).

Picture 8



3.7.8 Using a second pair watchmaker forceps, gently tease out a small 'ball' of the sperm from the cauda epididymis, and drag it into the sperm dispersal drop. Remove the epididymis from the dish.

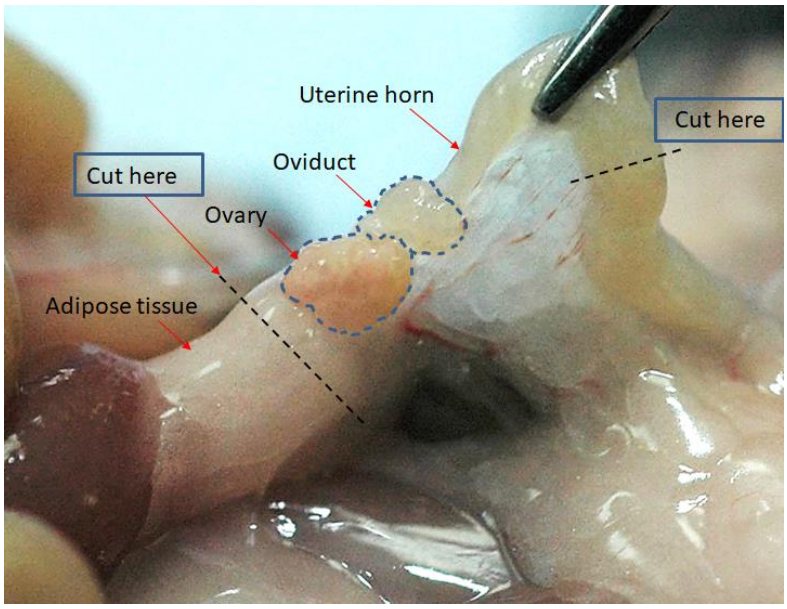
3.7.9 Repeat step 3.7.8 for the second cauda epididymis.

3.7.10 Allow the sperm to capacitate in the TYH + MBCD drop for 60 minutes in an incubator.

3.8 Oocyte harvesting

3.8.1 Dissect the oviducts (Picture 9) from three superovulated female mice and transfer them into the silicone fluid overlaying the pre-incubated fertilisation drop (Picture 10A). A deviation of the number of females per dish is acceptable in certain circumstances and must be recorded.

Picture 9



3.8.2 Under the microscope, hold an oviduct down with watchmakers forceps.

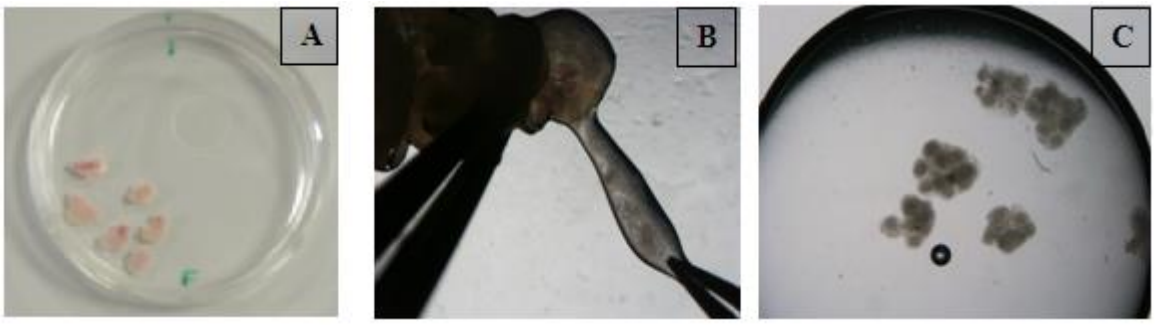
3.8.3 Gently tear the swollen ampulla with a second pair of watchmakers forceps to release the cumulus masses into the silicone fluid (Picture 10B).

3.8.4 Using the watchmakers forceps, drag the cumulus masses through the silicone fluid and into the fertilisation drop (Picture 10C).

3.8.5 Remove the oviduct from the dish and discard with the cadavers.

3.8.6 Repeat steps 3.8.2 to 3.8.5 for each oviduct.

Picture 10



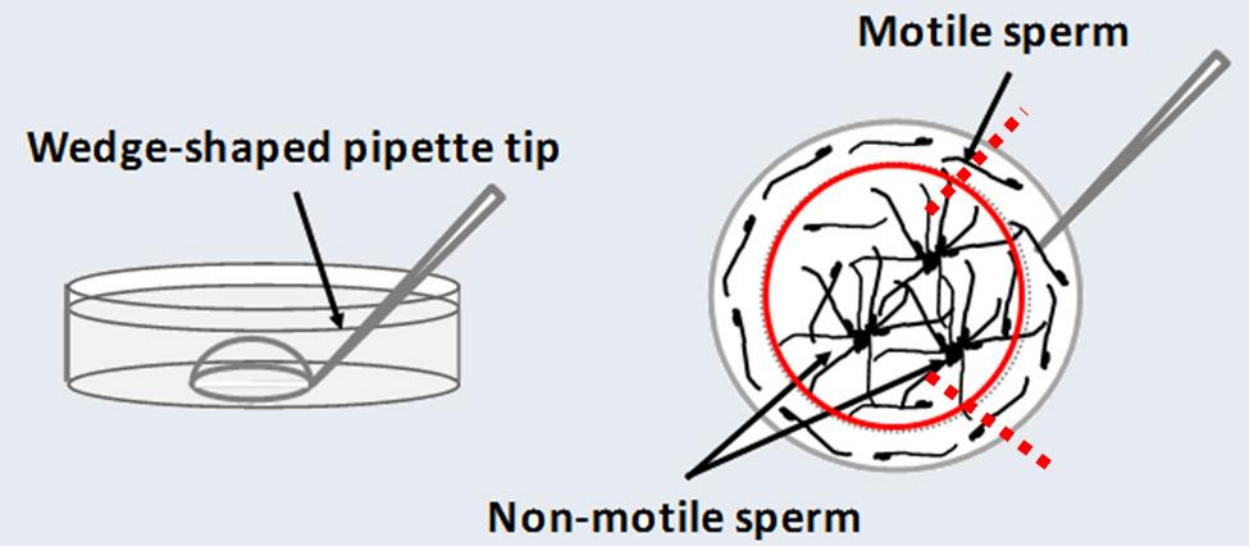
3.9 Fertilisation

3.9.1 **The cumulus masses should be incubated in the fertilisation drop for a minimum of 30mins prior to sperm being added to the dish.**

3.9.2 After the sperm has capacitated for 60 minutes, remove the sperm dispersal dish from the incubator along with the corresponding fertilisation dishes; place on heated stage under the dissecting microscope.

3.9.3 Looking down the microscope, using a pipette with a wedge-shaped tip, gently aspirate **1-5µl** of pre-incubated sperm from the peripheral part of the sperm dispersal drop (Picture 11). Try to avoid aspirating any sperm debris.

Picture 11



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 Lifor may be of a lower quality and may require more sperm adding to the fertilisation dish.

3.9.4 Add sperm to the centre of the fertilisation drop containing the cumulus masses. Return the dishes to the incubator.

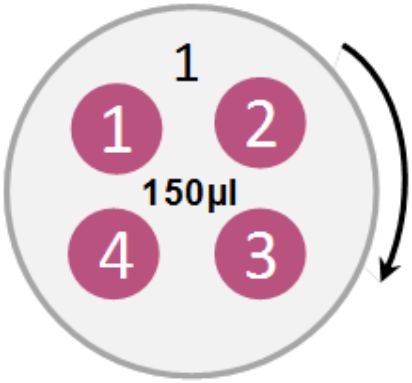
3.9.5 If there is concern over the quality of the sperm; concentration and/or progressive motility, remove the one fertilisation dish per IVF from the incubator after 10mins and assess the sperm motility and concentration again. It may be necessary to add more of the sperm suspension if the sperm quality is poor.

3.9.6 Incubate the dishes for approximately 3-4hrs to allow fertilisation to occur.

3.10 Washing zygotes

3.10.1 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 12).

Picture 12



3.10.2 Leaving the degenerating zygotes in drop 1, move the good quality presumptive zygotes through drops 2 and 3.

3.10.3 Move the presumptive zygotes into drop 4 and culture them overnight.

3.11 Scoring the IVF

3.11.1 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