

## **IVF recovery procedure using freshly harvested and frozen sperm, incorporating methyl- $\beta$ -cyclodextrin and reduced glutathione**

This protocol is based on the work published by Takeo et al., (2011). The reagents can be prepared according to the protocols provided here or purchased separately or as part of a mouse in vitro fertilisation kit from Cosmo Bio Co., Ltd ([www.cosmobio.co.jp](http://www.cosmobio.co.jp))

### **A. Preparation of sperm dispersal dishes**

NB: This medium is referred to as Fertiup®: PM, and can be purchased separately or as part of a mouse IVF kit from Cosmo Bio Co., Ltd ([www.cosmobio.co.jp](http://www.cosmobio.co.jp))

1. Pipette 90 $\mu$ l TYH + 0.75mM MBCD into the centre of a 60mm Petri Dish (Falcon 353004) (Fig.1)

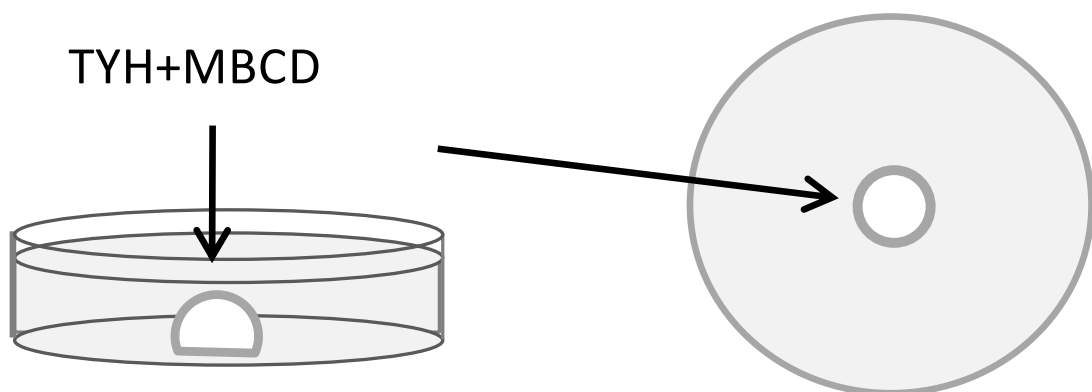


Fig. 1

2. Overlay with mineral oil or silicone fluid and equilibrate for 10-20min, or overnight, at 37°C, in 5% CO<sub>2</sub> incubator.

**B. Preparation of fertilisation medium containing reduced glutathione (GSH – Sigma: G4251)**

NB: This medium is referred to as CARD medium and can be purchased separately or as part of a mouse IVF kit from Cosmo Bio Co., Ltd ([www.cosmobio.co.jp](http://www.cosmobio.co.jp)).

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 (Fig. 2).

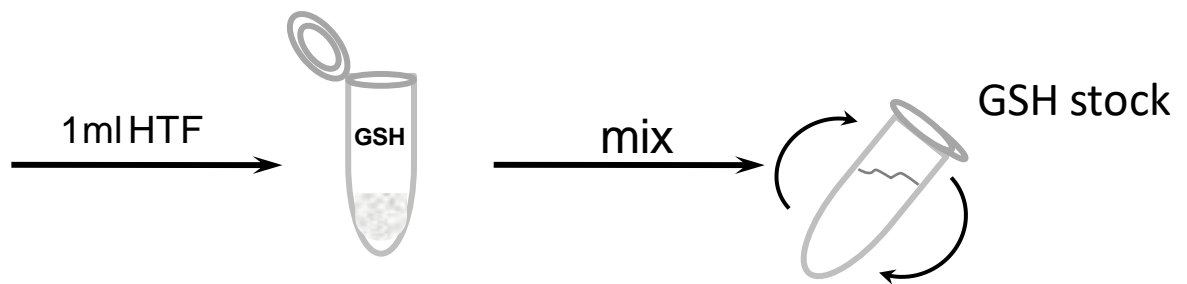


Fig. 2

2. **For frozen/thawed sperm samples**, take **50µl** of the GSH solution and add it to **5ml** HTF medium and mix them together gently (Final concentration **1mM** GSH, Fig. 3).
3. **For freshly harvested sperm samples**, take **10µl** of the GSH solution and add it to **4ml** HTF medium and mix them together gently (Final concentration **0.25mM** GSH, Fig 3).

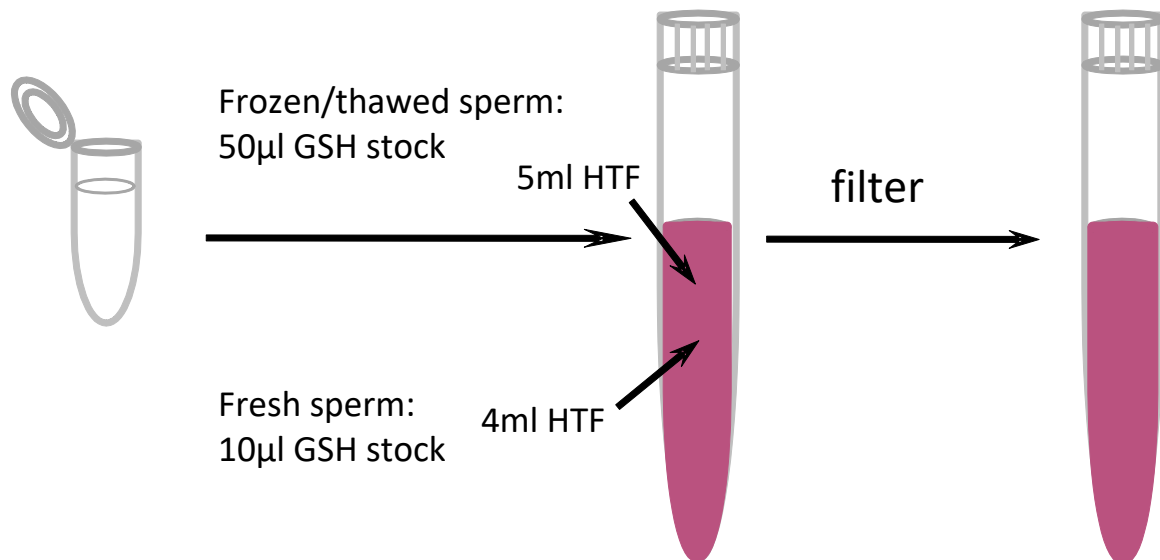


Fig. 3

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

### C. Preparation of fertilisation dishes

1. Make a drop of the fertilisation medium, containing the correct concentration of GSH, in a 60mm Petri Dish (Falcon 353004) (Fig. 4), overlay with mineral oil or silicone fluid. The volume of the drop should be **200µl** if using a freshly harvested sperm sample or **90µl** if using a frozen sperm sample.
2. Ideally, the fertilisation dishes should be prepared on the morning of the IVF session. If this is not possible, store the dishes at 4°C overnight, then allow the dishes to warm up to room temperature on the bench for 30min, before equilibrating for 10-20min at 37°C, in 5% CO<sub>2</sub> incubator.

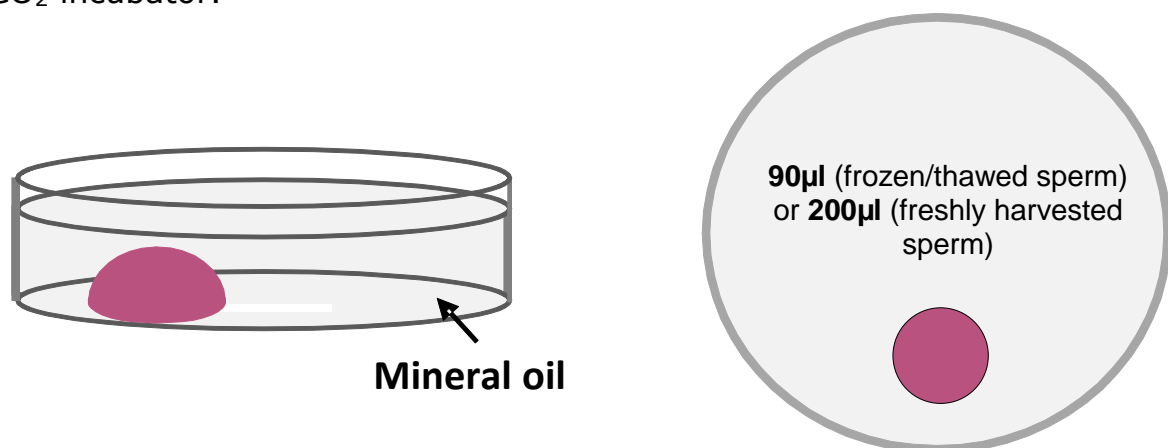


Fig. 4

**Note:** Offset the drop slightly from the centre to allow room to harvest cumulus masses.

#### **D. Preparation of freshly harvested sperm samples**

1. The selected male should be at least 10 weeks old, and not have been used for mating for at least 3 days before sperm collection.
2. Cull the male and dissect the cauda epididymides.
3. Clean off all adipose and vascular tissue. This is best achieved by placing the organs on a tissue and examining them under a dissecting microscope lit from above.
4. Place the cauda epididymides into the oil next to the sperm dispersal drop and nick the apex of the cauda epididymides using miniature scissors. Using watchmakers forceps gently tease out a small 'ball' of the sperm from the cauda epididymides and drag it into the sperm dispersal drop (Fig. 5).

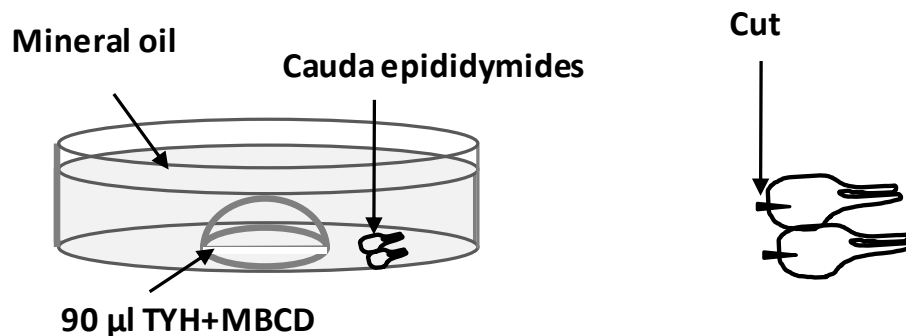


Fig. 5

5. Remove the tissue from the dish. Allow the sperm to disperse throughout the medium for 60 minutes at 37°C in the CO<sub>2</sub> incubator.

#### **E. Oocyte harvesting**

1. Dissect the oviducts from three superovulated female mice (for superovulation method, see Appendix A) and transfer them into the mineral oil or silicone fluid overlaying the pre-incubated fertilisation drop.
2. Under a dissecting microscope, hold each oviduct down with forceps and gently tear the swollen ampulla with a second pair of forceps to release the cumulus masses into the oil. Using the forceps, drag the clutches through the oil and into the fertilisation drop. Then remove the oviduct from the dish.

3. Repeat steps 1-2 for each fertilisation dish in succession. Aim to take no more than 5 minutes from collecting the oviducts to returning the fertilisation drop (including oocytes) to the incubator.
4. **The oocytes should be exposed to the GSH in the fertilisation drop for at least 30mins before adding the sperm suspension.**

#### **F. Thawing the frozen sperm samples**

1. Remove the required straw(s) from the storage tanks and place in a flask of liquid nitrogen.
2. Quickly transfer a straw into a 37°C water bath and leave for 10 minutes.

**Note:** To ensure warming of the frozen sperm, completely immerse the part of the straw containing the sperm in the water bath. Furthermore, frozen-thawed mouse sperm are sensitive to environmental changes. If the straw is not kept in the water bath long enough (10 minutes), the motility of the cryopreserved sperm will be reduced.

3. Carefully dry the straw with a tissue. Cut the heat-sealed end of the straw and the labelled end of the straw in the middle of the cotton plug. Use a straw pusher to expel **only** the 10µl sperm suspension into the centre of the drop of 90µl TYH+MBCD pre-incubation medium (Fig. 6). Do not expel any of the HTF media contained within the straw.

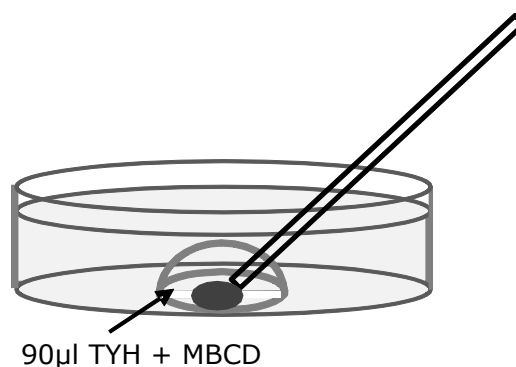


Fig. 6

**Note:** Do not disturb the dishes containing the frozen/thawed sperm until the sperm are moving rapidly within the medium. If the dishes are disturbed before the sperm start to move they will not recover full motility.

4. Pre-incubate the frozen/thawed sperm in TYH+MBCD medium for 30min at 37°C in the CO<sub>2</sub> incubator. After 25min, rotate the dish 5 times to encourage the motile sperm towards the peripheral part of the pre-incubation drop.

### **G. *In vitro* fertilisation**

1. Add the sperm to the fertilisation drop:

**Frozen/thawed sperm** must be equilibrated in the TYH + MBCD medium for **30 minutes** in a 37°C CO<sub>2</sub> incubator before adding to the fertilisation drop. Take a **10-20µl** aliquot of the sperm suspension from the peripheral part of the pre-incubation drop. This region will contain the most motile sperm. Try to avoid aspirating any sperm debris (Fig. 7).

**Freshly harvested sperm** should be allowed to disperse in the TYH + MBCD medium for approximately **60 minutes** in a 37°C CO<sub>2</sub> incubator before adding to the fertilisation drop. Add **3-5µl** freshly harvested sperm to the fertilisation drop, once again, avoiding aspirating any sperm debris (Fig 7).

**Note:** This step should be performed gently under a light microscope and is best achieved using a wedge-shaped 10µl pipette tip. Following this procedure it is easy to collect good quality motile sperm without picking up dead sperm or cell debris.

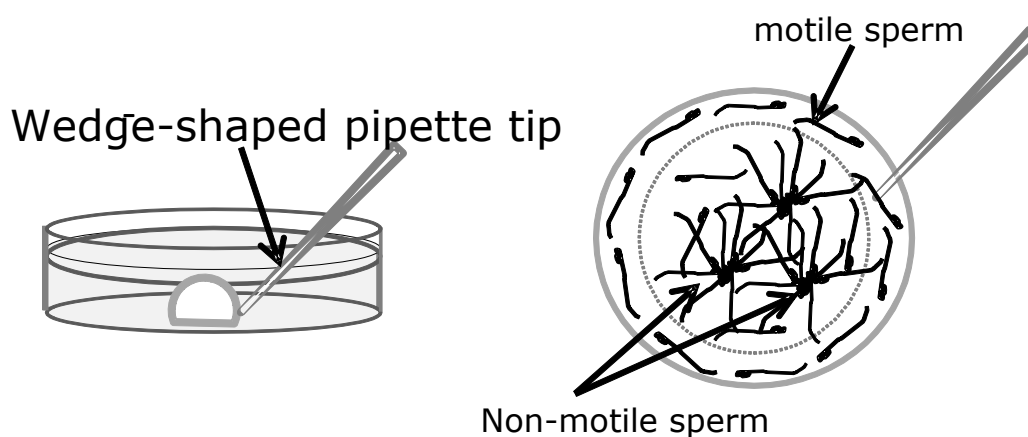


Fig. 7

2. When the sperm suspension has been added to all of the fertilisation drops return the dishes to the incubator. One dish can be removed from the incubator following 10mins incubation to assess the sperm motility and concentration again. It is also possible to observe whether cumulus cells are being removed. If the motility and concentration of sperm is poor and few cumulus cells are being removed, it may be necessary to add more of the sperm suspension to each fertilisation drop. Return the dish(es) to the incubator.
3. Incubate the dishes at 37°C, in 5% CO<sub>2</sub> incubator for approximately 3-4hrs to allow fertilisation to occur.

## **H. Washing and culturing the fertilised oocytes**

1. Prepare the wash drops by placing 4 x 150µl drops of HTF (without GSH) in a 60mm culture dish (Falcon 353004). Overlay with mineral oil or silicone fluid (Fig. 8). Equilibrate for 10-20min, or overnight, at 37°C, in 5% CO<sub>2</sub> incubator.

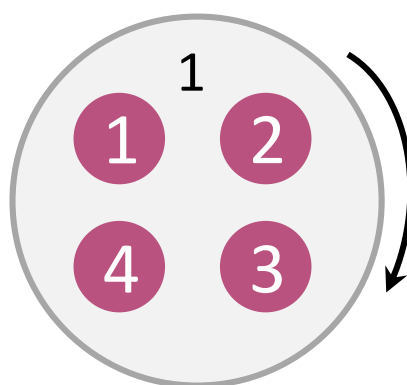


Fig. 8

2. Collect all oocytes from the fertilisation drop and take the oocytes through three washes (drops 1, 2 & 3) to remove the cell debris, degenerating oocytes and dead sperm. Degenerating oocytes can be left in drop 1.
3. Move the presumptive zygotes into drop 4 and culture them overnight.



## **I. Scoring the IVF**

1. Next morning, separate the 2-cell embryos from the unfertilised or degenerating oocytes. Place all the 2-cell embryos in drop 4 and the 1-cell or degenerated oocytes/embryos in drop 3.
2. Wash the embryos through two drops of M2.
3. Either transfer the 2-cell embryos to the oviducts of 0.5 day pseudopregnant foster mothers, or:
4. Prepare the 2-cell embryos for cryopreservation according to a standard protocol for *in vivo* derived embryos, or:
5. Culture the embryos in KSOM, plus amino acids.

## **Appendix A - Timetable of events for IVF**

<b>Day -3</b> (e.g. Saturday)	<b>Day -1</b> (e.g. Monday)	<b>Day 0</b> (e.g. Tuesday)	<b>Day 1</b> (e.g. Wednesday)
	Prepare sperm pre- incubation and wash dishes for IVF.	07:00 Prepare GSH and fertilisation dishes for IVF.	Morning: score the IVF success (2-cell vs others).
Superovulate females by injecting 0.1ml (5iu) PMS at 16.00.	Induce ovulation in the females by injecting 0.1ml (5iu) hCG at 16.00.	Harvest fresh sperm sample. Pre-incubate for for 60mins.	Prepare the 2-cell embryos for cryopreservation, embryo transfer or culture.
		Harvest oocytes into fertilisation media.	
		Thaw cryopreserved sperm sample. Pre- incubate for 30mins.	
		Add sperm suspension to fertilisation drops containing oocytes.	
		12:30 Wash the presumptive zygotes and culture overnight.	

This timetable assumes that the mice are exposed to 12 hours of darkness between 19:00 and 07:00.