# IVF with frozen sperm

## 1. Preparation of culture dishes

On the day of experiment, drops of medium are prepared as follows, and kept in a $CO_2$ incubator (37 °C, 5% $CO_2$ in air) to equilibrate for at least half an hour.

1) For sperm preincubation: a 35 mm plastic dish containing a 400 μL drop of PVA-HTF-0.4mM MBCD medium* (about 20 mm in diameter, with the edge widened with the tip of a pipette) overlaid with silicone or mineral oil (Fig. 1a).
2) For oocyte collection and fertilization: a 35 mm plastic dish containing four 80 μL drops of mHTF-1.25 mM reduced glutathione (GSH) medium** overlaid with oil (Fig. 1b).
3) For embryo culture: a 35 mm plastic dish containing twelve 10 μL drops of CZB medium containing glucose (or KSOM a/a medium) overlaid with oil (Fig. 1c).

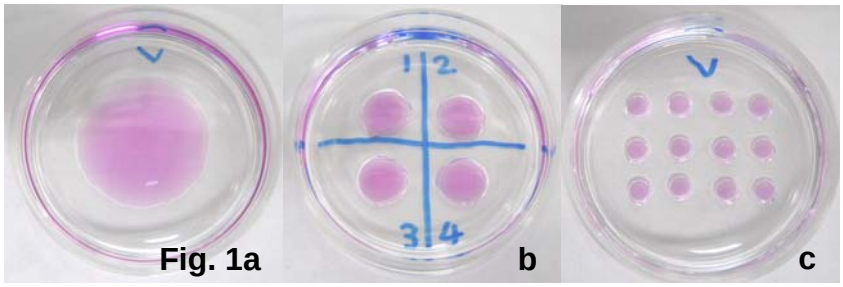


Fig. 1a b c

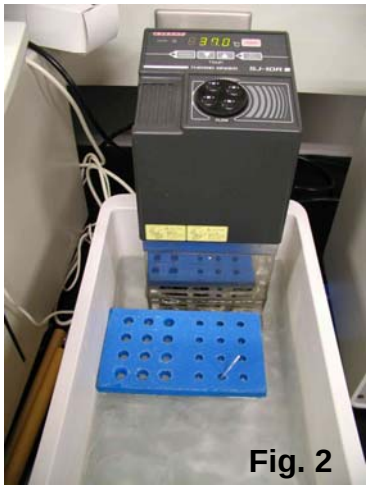


Fig. 2

## 2. Recovery of frozen sperm

1) Retrieve a straw containing the frozen sperm from the liquid nitrogen cryogloves and a face mask to avoid accidental injury.
2) After holding the straw in the air for 10 s, immerse it in a water bath at 37 °C for 15 min (Fig. 2).

## 3. Preincubation of the recovered sperm

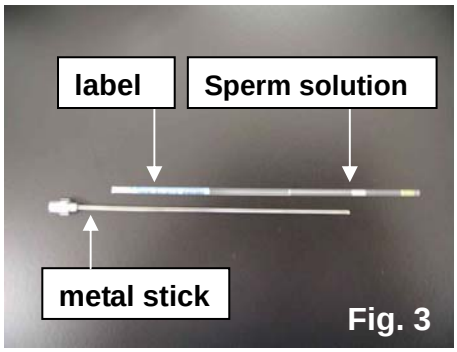


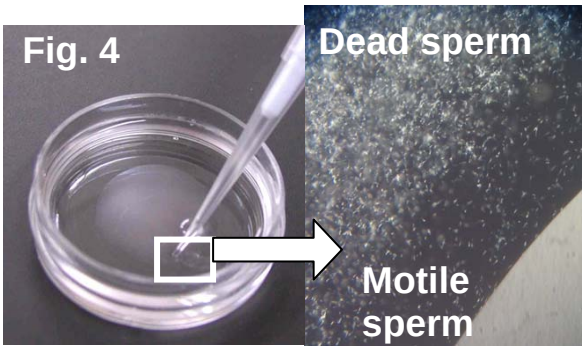
Fig. 3

1) Wipe the surface of the straw with a paper towel and expel the sperm solution onto the bottom of a plastic dish. The sperm solution takes up about 5 mm of the length of the straw and is enclosed in the end opposite the label. It is more opaque than the PB1 solution (Fig. 3).
Cut the straw with scissors between the edge and the sperm solution, and retrieve only the sperm solution by pushing it out with a metal stick (plunger) inserted from the opposite end or with a micropipette. Care must be taken not to mix the sperm solution with the PB1 solution, which is also enclosed in the straw as a weight balancer.
1) Transfer the entire volume (~10 μL) of sperm solution to the drop of PVA-HTF-0.4mM MBCD medium in the culture dish ( Fig. 1a).
2) Incubate it (37 °C, 5% $CO_2$) for 45-60 min.

## 4. Insemination

1) Collect mature MII oocytes from females who have been induced to superovulate, and place them into the 80 μL drops of mHTF-GSH medium ( Fig. 1b).

2) Add about 20 μL of the thawed sperm suspension to a drop of HTF medium containing the oocytes. It is critically important to collect motile sperm from the periphery of a drop with a thin pipette ( Fig. 4). When the motility of sperm is less, or the number of oocytes are more than 30, add about 30-40 μL of the sperm suspension.




3) Check the condition of the oocytes enclosed by cumulus cells after about 20-30 min from insemination. If the amount of sperm solution in the drop of fertilization dish is not enough, add 10 - 20 μl of sperm solution from sperm preincubation dish.

## 5. Transfer the oocytes to the culture medium

(a) At 4–6 h after insemination, pick up the morphologically normal oocytes and wash them in two drops of PB1 or M2 medium.

(b) Transfer these oocytes into culture drops of fresh CZB or KSOM a/a medium (Fig. 1c).

## 6. Assessment of fertilization and embryo transfer

(a) On the day after IVF, count the numbers of two-cell embryos and the morphologically normal unfertilized oocytes. We usually calculate the fertilization rate as: no. two-cell embryos/(no. two-cell embryos + morphologically normal unfertilized oocytes).

(b) The two-cell embryos are transferred into the oviducts (usually about 8-10 embryos/oviduct) of pseudopregnant females on the day 0.5 (the day following sterile mating).

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## Composition of media for IVF and embryo culture

| | mHTF | | PVA− mHTF* | mCZB | KSOM *** |
|---|---|---|---|---|---|
| | mM | mg /100 ml | mg /100 ml | mg /100 ml | mg /100 ml |
| NaCl | 101.6 | 581.7 | 581.7mg | 476.5mg | 555mg |
| KCl | 4.7 | 35 | 35 | 35.8 | 18.6 |
| $KH_2PO_4$ | 0.4 | 5 | 5 | 16.3 | 4.8 |
| $MgSO_4 \cdot 7H_2O$ | 0.2 | 5 | 5 | 29.6 | 5 |
| $NaHCO_3$ | 25 | 210 | 210 | 211 | 210 |
| $CaCl_2 \cdot 2H_2O$ | 2 | 30 | 30 | 25 | 25.1 |
| EDTA・2Na | - | - | - | 3.7 | 0.4 |
| Polyvinyl alcohol | - | - | 10 | 10 | - |
| Phenol red (Na塩) | - | 0.2 | 0.2 | 0.2 | 0.2 |
| Glucose | 2.8 | 50 | 50 | 100 | 3.6 |
| Na pyruvate | 0.3 | 3.6 | 3.6 | 3.5 | 2.2 |
| Na lactate（60%ｼﾛｯﾌﾟ） | 23.3 | 400μl | 400μl | 370μl | 174μl |
| Glutamine | - | - | - | 14.6 | 14.6 |
| Hypotaurine | - | 11 | 11 | - | - |
| Penicillin G・K salt | - | 10 | 10 | 6 | 6.3 |
| Streptmycin sulfate | - | - | - | 5 | 5 |
| Bovine serum albumin | - | 300 | - | 300 | 100 |

* : Add 0.4 mM Methyl-β-cyclodextrin (MBCD) before use.

*** : Add the essential and non essential amino acids before use.

### Preparation methods of sperm preincubation and fertilization media

1. Sperm preincuvation medium: PVA-HTF-0.4mM MBCD medium*

(1) Prepare 50 mM MBCD (stock solution )

Add 66.7 mg of MBCD (Methyl-β-cyclodextrin; Sigma C-4555-1g) to 1 mL of PVA-mHTF

(2) Prepare PVA-HTF-0.4mM MBCD medium

On the morning of experiment, add 8 μL of 50 mM MBCD to 992 μL of PVA-mHTF

2. Fertilization medium: mHTF-1.25 mM GSH medium**

(1) Prepare 25 mM GSH (stock solution)

Weigh about 3 mg of GSH, and add 130 μL mHTF/ mg of GSH

[Glutathione; Sigma G6013-5G), 25 mM GSH : 7.683 mg/mL＝1 mg/130 μL]

(2) Prepare mHTF-1.25 mM GSH medium

On the morning of experiment, add 25 μL of 25 mM GSH to 475μL of mHTF

### References

Hasegawa et al. J. Reprod. Dev., 58:156-161, 2012; Takeo et al. Biol. Reprod., 85:1066-1072, 2011.