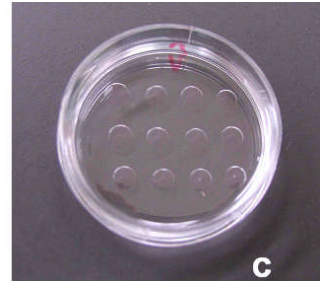
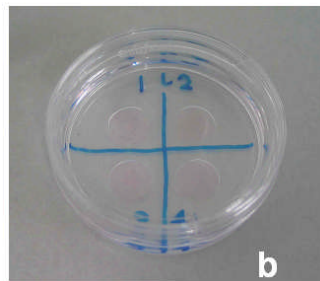
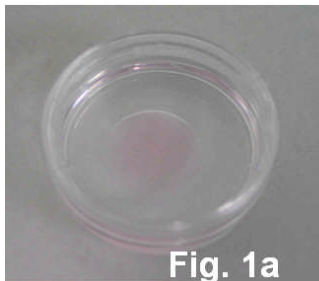


## IVF with frozen sperm

### 1. Preparation of culture dishes

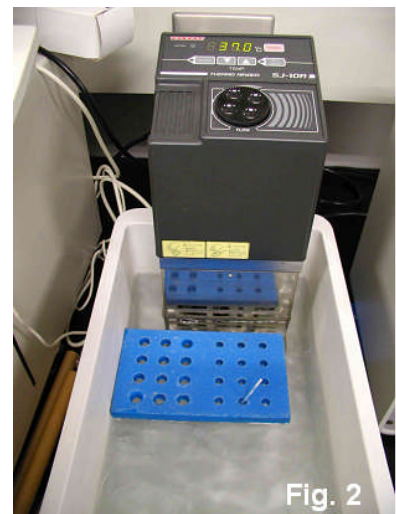
On the day of experiment, drops of medium are prepared as follows, and kept in a CO<sub>2</sub> incubator (37°C, 5% CO<sub>2</sub>) to equilibrate for at least half an hour.

- 1) For sperm preincubation: a 35 mm plastic dish containing a 450  $\mu$ L drop of mHTF medium (about 15 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 insemination: a 35 mm plastic dish containing four 80  $\mu$ L drops of HTF medium overlain with oil (Fig. 1b).
- 3) For embryo culture: a 35 mm plastic dish containing drops of embryo culture medium (e.g., CZB, KSOM/aa medium) overlaid with oil (Fig. 1c).



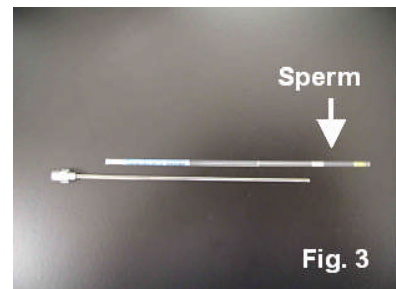
### 2. Recovery of frozen sperm

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



### 3. Preincubation of the recovered sperm

- 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 medium (PB1)-only area (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 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.



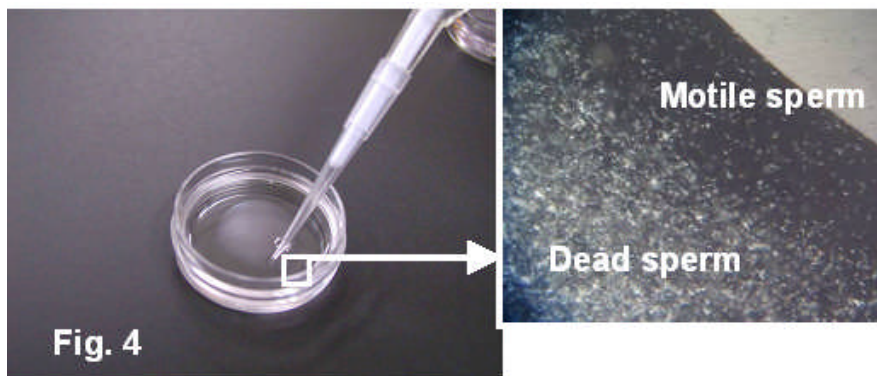
- 2) Transfer the entire volume (~10  $\mu$ L) of sperm solution to the drop of mHTF medium in the culture

dish (Fig. 1a).

3) Incubate it (37 °C, 5% CO<sub>2</sub>) for 60 min.

#### 4. Insemination

- 1) Collect mature MII oocytes from females that have been induced to superovulate, and place them into the 80 µL drops of HTF medium (Fig. 1b).
- 2) Add 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). The final concentration is 300–500 sperm/µL. It's depends on the motility of sperm.



#### 5. Transfer the oocytes to the culture medium

- 1) At 4–6 h after insemination, pick up the morphologically normal oocytes and wash them twice in fresh medium.
- 2) Transfer these oocytes into culture drops of fresh culture medium and culture them until embryo transfer (Fig. 1c).

Note: We recommend using mHTF for the first IVF trial. If the fertilization rate is low (e.g., < 20%), please consider use of methyl-β-cyclodextrin in BSA-free PVA-containing HTF (see the medium composition attached) to facilitate sperm capacitation\*.

\*Taguma K et al. *Exp Anim*, 58: 395-401, 2009; Takeo et al. *Biol. Reprod.* 78: 546-551, 2008

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Composition of media for IVF and embryo culture

	mHTF		PVA–HTF*	mCZB	KSOM
	mM	mg/100 ml	mg/100 ml	mg/100 ml	mg/100 ml
NaCl	101.6	581.7	581.7	476.5	555
KCl	4.7	35	35	35.8	18.6
KH <sub>2</sub> PO <sub>4</sub>	0.4	5	5	16.3	4.8
MgSO <sub>4</sub> 7H <sub>2</sub> O	0.2	5	5	29.6	5
NaHCO <sub>3</sub>	25	210	210	211	210
CaCl <sub>2</sub> 2H <sub>2</sub> O	2	30	30	25	25.1
EDTA 2Na	-	-	-	3.7	0.4
Polyvinyl alcohol	-	-	10	10	-
Phenol red	-	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% syrup)	23.3	400 ul	400 ul	370 ul	174 ul
Glutamine	-	-	-	14.6	14.6
Hypotaurine	-	11	11	-	-
Penicillin G	-	10	10	6	6.3
Streptomycin sulfate	-	-	-	5	5
Bovine serum albumin	-	300	-	300	100

\*Add 0.4 mM Methyl- $\beta$ -cyclodextrin before use