

Cryopreservation of Mouse Spermatozoa in *CARD FERTIUP*

Department of Reproductive Engineering, Center for Animal Resources and Development, Kumamoto University, Japan
Senior Editor: Naomi Nakagata



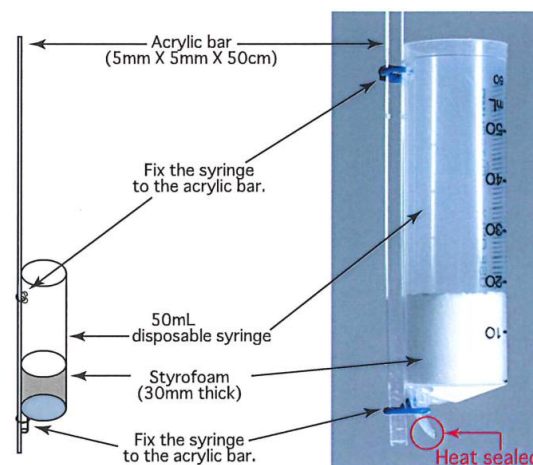
– Materials and Equipment –

1. Male mice (12-70 weeks old)
2. Micro-spring scissors (5 mm blade)
3. Pair of watchmaker's #5 forceps
4. CARD FERTIUP (Cryoprotectant: CPR; KYUDO CO., LTD.)
5. CARD mHTF
6. Plastic dish (35 mm X 10 mm Cat. No.430588; CORNING)
7. Pipette tips
8. 0.25 mL PETG cotton-plugged Sperm Straws (01026; Cryo Bio System, France)
9. Micropipettes
10. Straw connector
11. Impulse sealer
12. Freezing canister
13. Cryobiological container
14. Hot plate

– Procedure –

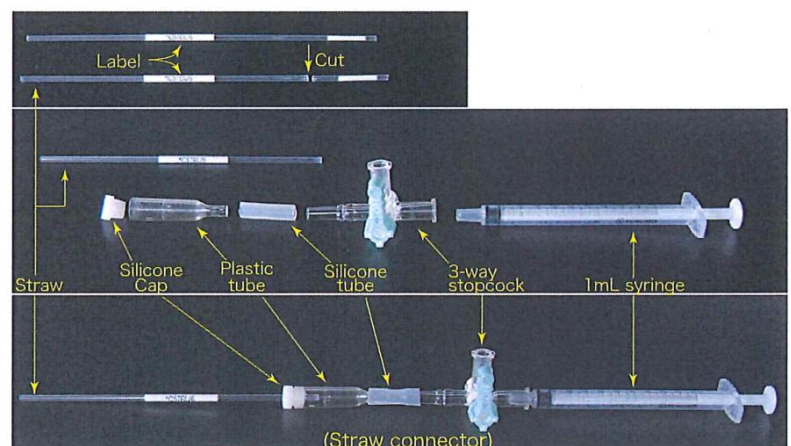
●● Preparation the Freezing Canister ●●

- 1 Insert a piece of styrofoam tightly into the bottom of the syringe.
- 2 Heat seal the mouth of the syringe tip.
- 3 Fix the syringe to the acrylic bar.



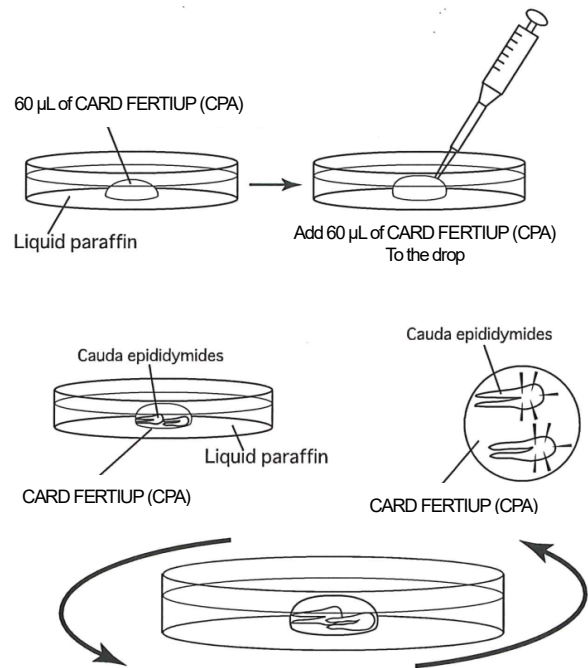
●● Preparing a Straw Connector ●●

- 1 Using a 1 mL syringe, a 3-way stopcock, a piece of silicone tube, a plastic tube and a silicone cap, make a straw connector.
- 2 To use the straw connector, cut away the cotton plug from a straw (0.25 mL French Straw), then attach the straw to the silicone cap at the end of the connector.



●● Preparing Sperm Suspension ●●

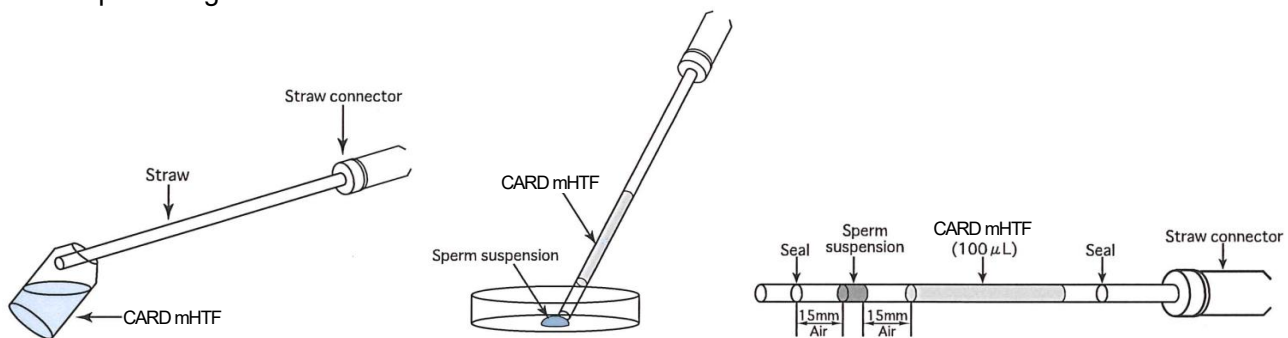
- 1 Make a drop of 60 μL of CARD FERTIUP (CPA) on a 35 mm plastic dish and cover it with liquid paraffin.
- 2 Add a 60 μL aliquot of the same solution to the drop (final volume: 120 μL) to make a tall, semispherical drop.
- 3 Sacrifice a male mouse (>12 weeks old) via cervical dislocation and remove the two cauda epididymides aseptically.
- 4 Place the cauda epididymides on a piece of filter paper and completely remove any fat and blood under a microscope.
- 5 Transfer the cauda epididymides into the drop of CARD FERTIUP (CPA) and use a pair of watchmaker's #5 forceps and micro-spring scissors to make 5 or 6 incisions in the epididymides.
- 6 Place the dish on a hot plate at 37°C for 3 minutes. During this time, rotate the dish every minute to disperse sperm from the organs in the CARD FERTIUP (CPA).



●● Preparing Freezing Straw Containing Sperm Suspension ●●

- 1 Connect a straw to a straw connector.
- 2 Carefully aspirate the contents into the straw in following order:
 - a. 100 μL of CARD mHTF,
 - b. 15 mm of air,
 - c. 10 μL of the sperm suspension,
 - d. Another 15 mm of air.
- 3 Seal both sides of the straw using an impulse sealer.

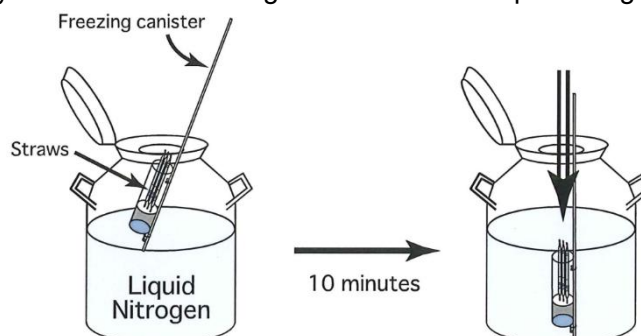
Comment: Loading 100 μL of CARD mHTF into the straw prevents the straw from floating on the surface of liquid nitrogen. This is because the CARD mHTF acts as a weight that forces the straw to sink into the liquid nitrogen.



- 4 Create 10 samples per mouse in the same manner as described above.

●● Sperm Freezing using a Cryobiological Container ●●

- 1 Put the samples into a freezing canister and float them on liquid nitrogen in a cryobiological container.
- 2 After 10 minutes, quickly immerse the freezing canister into the liquid nitrogen.



In Vitro Fertilization using Spermatozoa Cryopreserved in *CARD FERTIUP*

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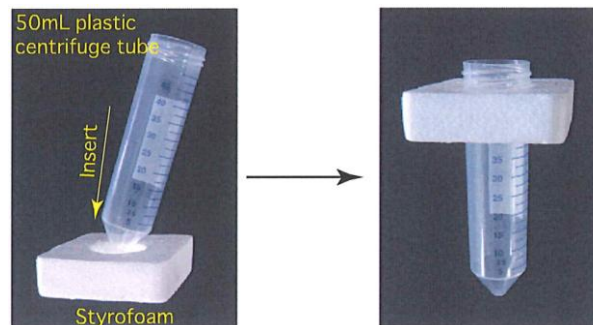
– Materials and Equipment –

1. Female mice superovulated with CARD HyperOva or PMSG and hCG
2. CARD FERTIUP (Preincubation medium: PM; KYUDO CO., LTD.)
3. CARD MEDIUM (KYUDO CO., LTD.)
4. CARD mHTF
5. Pipette tips (Pipette Tip Cat. No.114 Quality Scientific Plastics)
6. Plastic dish (35 mm X 10 mm Cat. No.430588; CORNING)
7. Straw connector
8. Water bath maintained at 37°C
9. Float for thawing
10. Micropipettes
11. Humidified incubator (37°C, 5% CO₂, 95% air)

– Procedure –

●● Preparation of the Float for Thawing ●●

1 Using some styrofoam and a 50 mL plastic centrifuge tube, make the float as shown in the diagram below.



●● Preparation for Thawing ●●

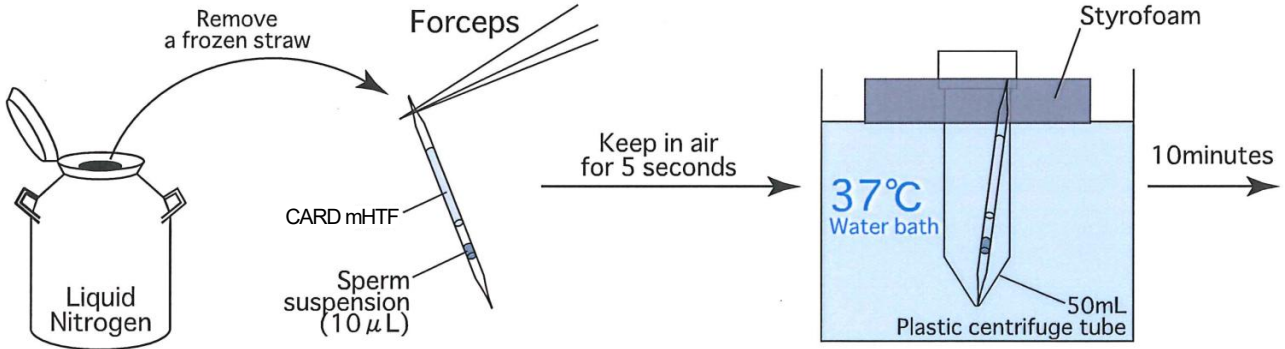
- 1 Prepare a water bath maintained at 37°C.
- 2 Pour water (37°C) into the 50mL plastic centrifuge tube part of the styrofoam/centrifuge tube assembly, and float it in a water bath.
- 3 Put 1 drop (90 μ L / drop) of CARD FERTIUP (PM) into a dish and cover it with liquid paraffin 30 minutes before thawing a frozen straw, and place the dish in an incubator (37°C, 5% CO₂ in air).

●● Thawing the Mouse Spermatozoa ●●

- 1 Remove a frozen straw from the liquid nitrogen and hold it in the air for 5 seconds.
- 2 After completing step 1, immediately immerse the frozen straw in the styrofoam/centrifuge tube assembly (in a water bath maintained at 37°C) for 10 minutes.

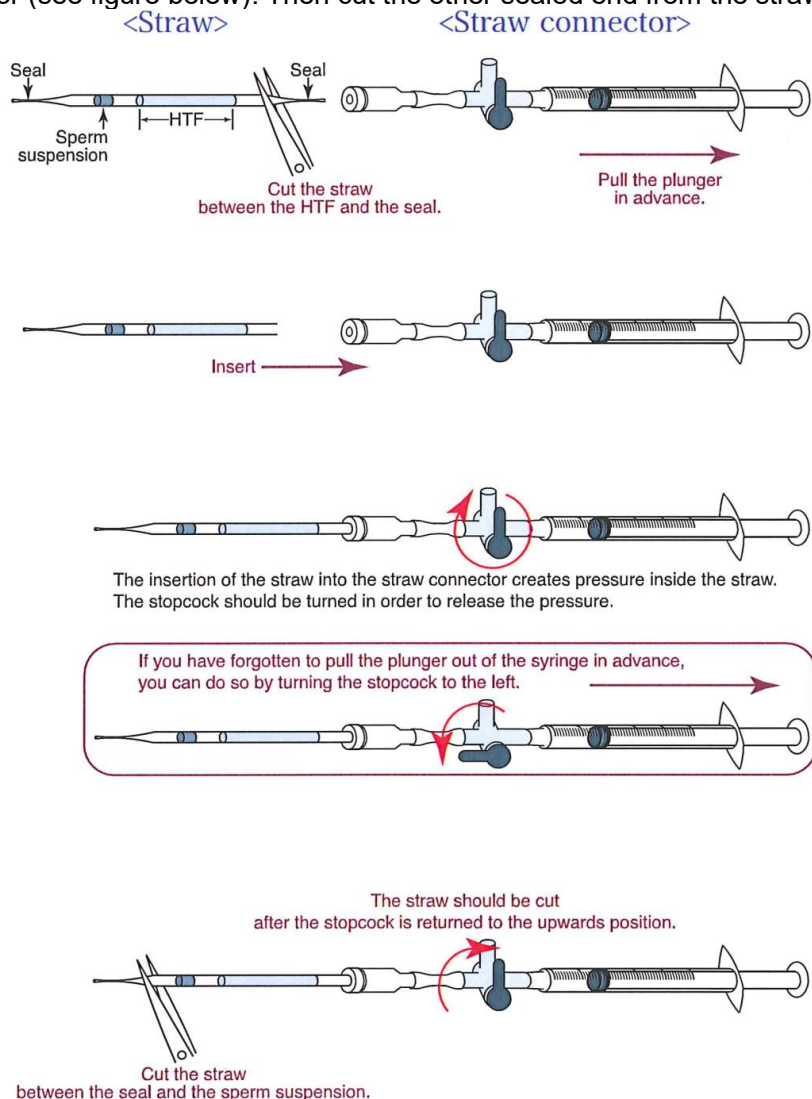
Note: To ensure warming of the frozen sperm, completely immerse the part of the straw containing the sperm in the water bath. Furthermore, frozen-thawed mouse spermatozoa are sensitive to environmental changes. If the straw is not kept in the water bath long enough (10 minutes), the motility of the cryopreserved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

- 3 10 minutes after immersion, remove the straw from the styrofoam/centrifuge tube assembly.
- 4 Use fine tissues to wipe any water from the straw.



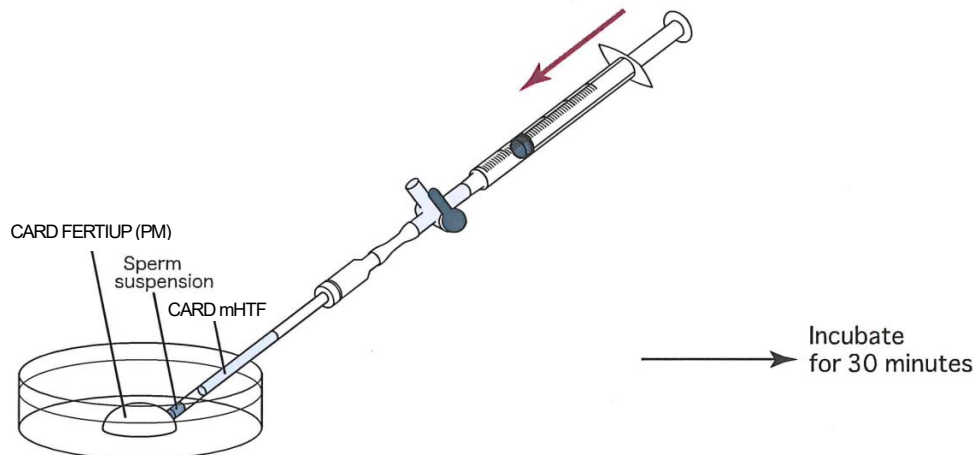
●● Transferring and Preincubating the Thawed Sperm Suspension ●●

- 1 Cut the sealed end of the straw furthest from the sperm and attach the cut end to the straw connector (see figure below). Then cut the other sealed end from the straw.



2 Push the plunger to transfer only the sperm suspension into the drop of CARD FERTIUP (PM), and place the dish in an incubator (37°C, 5% CO₂ in air) for 30 minutes.

Note: Do not disturb the dishes containing cryopreserved spermatozoa until they are moving sufficiently within the medium. If the dishes are disturbed before the spermatozoa start to move, then they will not recover full motility.



●● Preparation of Dishes for Fertilization and Washing ●●

- 1 Put 1 drop (90 μ L / drop) of CARD MEDIUM into a dish and cover it with liquid paraffin 10 minutes before collecting of oocytes, and place the dish in an incubator (37°C, 5% CO₂ in air).
- 2 Put 4 drops (80 μ L / drop) of CARD mHTF into a dish and cover them with liquid paraffin. Place the dish in an incubator (37°C, 5% CO₂ in air) for at least 30 minutes.

●● Insemination ●●

- 1 Sacrifice female mice 15-17 hours after an hCG injection and remove the oviducts.
- 2 Using fine, sharp needles, release up to 4-6 cumulus-oocytes-complexes (COCs) masses into each drop of CARD MEDIUM (90 μ L).

Note: Be sure to carry out all operations, from sacrificing the female and removing her oviducts to introducing the COCs into a drop of CARD MEDIUM, in the shortest time possible (within 30 seconds). Moreover, when carrying out this process alone, do not sacrifice multiple mice at once; instead, sacrifice one mouse and swiftly remove its oviducts before moving on to the next mouse.

- 3 Using a wedge-shaped pipette tip (Pipette Tip Cat. No.114 Quality Scientific Plastics), aspirate 10 μ L of the preincubated sperm suspension from the edge of the drop.

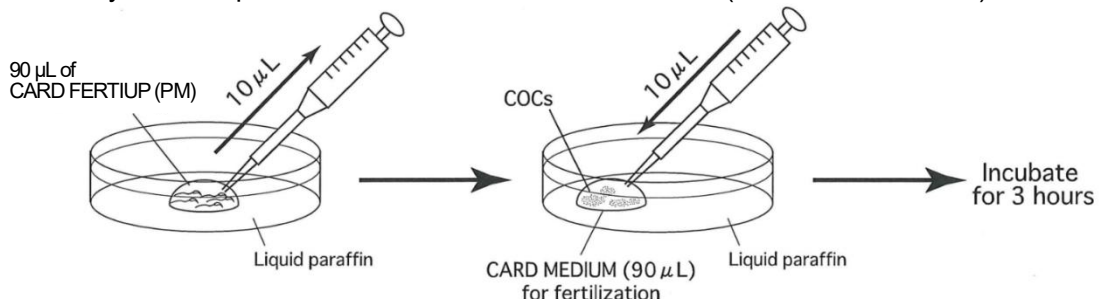
Comment: Spermatozoa with high motility have a tendency to gather near the edge of the drop.

Comment: It is possible to aspirate 10 μ L of sperm suspension 3-4 times per drop.

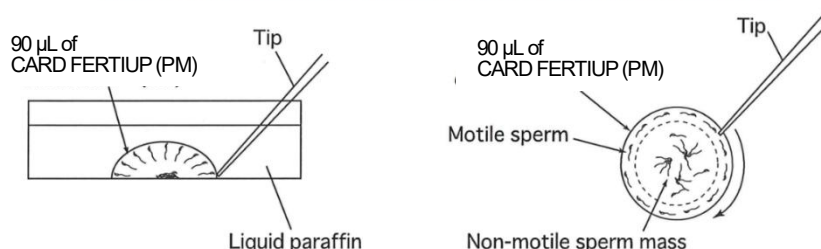
- 4 Add 10 μ L of sperm to each drop of fertilizing CARD MEDIUM containing the COCs.

Note: Perform the pipette operation mentioned in steps 1 and 2 as gently as possible.

- 5 Incubate the oocytes and spermatozoa for 3 hours in an incubator (37°C, 5% CO₂ in air).

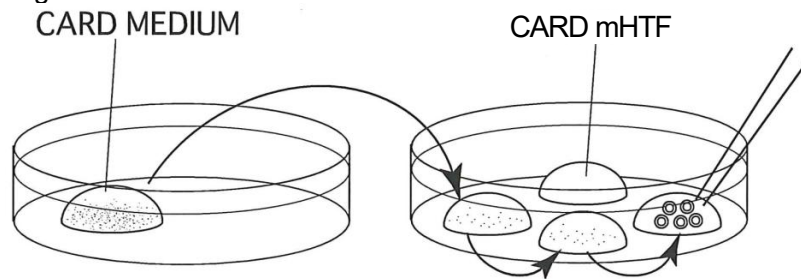


[Aspirating the Sperm Suspension from the Edge of the Drop]



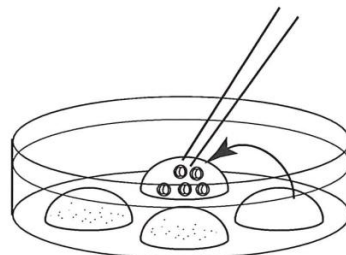
6 After incubating for 3 hours, wash the oocytes 3 times in fresh CARD mHTF (80 μ L) in a washing dish, avoiding transfer of CARD MEDIUM.

Comment: If many spermatozoa are attached to the zona pellucida of the oocytes, they can be removed by pipetting 20 μ L (using a 20 μ L pipette and a tip) 20-30 times in the fertilization dish before washing.



7 6 hours after insemination, observe them in the third drop of CARD mHTF and remove any parthenogenetic oocytes which have only one pronucleus.

8 After overnight culture of the oocytes, transfer the obtained 2-cell stage embryos only to the fourth drop of CARD mHTF. These embryos can now be vitrified or transferred.



– References –

For information on cryopreservation techniques for mouse spermatozoa, or on techniques used in thawing, *in vitro* fertilization and obtaining pups through embryo transfer, please visit

<https://card.medic.kumamoto-u.ac.jp/card/japanese/manual/index.html>

References

- 1) Takeo T., Hoshii T., Kondo Y., Toyodome H., Arima H., Yamamura KI., Irie T., and Nakagata N. 2008. Methyl-beta-cyclodextrin improves fertilizing ability of C57BL/6 mouse sperm after freezing and thawing by facilitating cholesterol efflux from the cells. *Biol Reprod.* 78(3): 546-51.
- 2) Takeo T., and Nakagata N. 2010. Combination medium of cryoprotective agents containing L-glutamine and methyl- β -cyclodextrin in a preincubation medium yields a high fertilization rate for cryopreserved C57BL/6J mouse sperm. *Lab Anim.* 44(2): 132-7.

– Note –

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