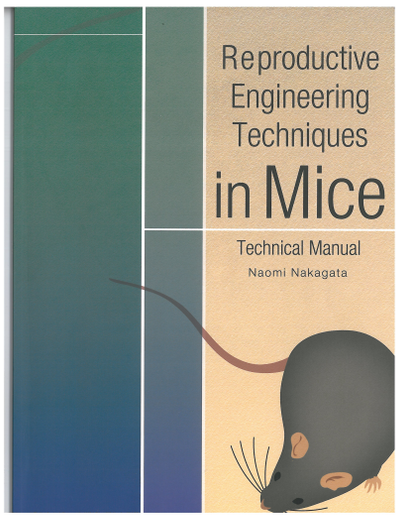
Reproductive Engineering Techniques **in Mice**



Technical Manual
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Preface

In recent years, the number of genetically engineered mice being produced has increased dramatically. Moreover, the rapid progress in the development of genome-editing (TALEN and CRISPR/Cas9) techniques for molecular biology research has been remarkable, so much so that a genetically engineered mouse strain can be produced easily in a few months. Production has been supported by reproductive engineering techniques such as *in vitro* fertilization, embryo and sperm cryopreservation, and embryo transfer techniques. Such techniques have become invaluable peripheral technologies, and their use is expanding rapidly.

This rapid expansion has led to the publication of many technical manuals relating to reproductive engineering techniques in mice hereto. However, mouse reproductive engineering techniques mainly involve delicate operations under a stereoscopic microscope, which means that a sufficiently detailed technical manual has not yet been published.

With that in mind, in this book we aim to create a mouse reproductive engineering technique manual that can be easily understood by anyone. We included a generous number of diagrams, photographs and videos in our manual, and explained each step of each reproductive engineering technique as clearly and thoroughly as we could. We sincerely hope that our manual will become the definitive guide for students, technicians, researchers and other people wishing to study mouse reproductive engineering techniques.

Naomi Nakagata

CONTENTS

* Please see page 90 for details.

3-1 Cryopreservation of Mouse Spermatozoa

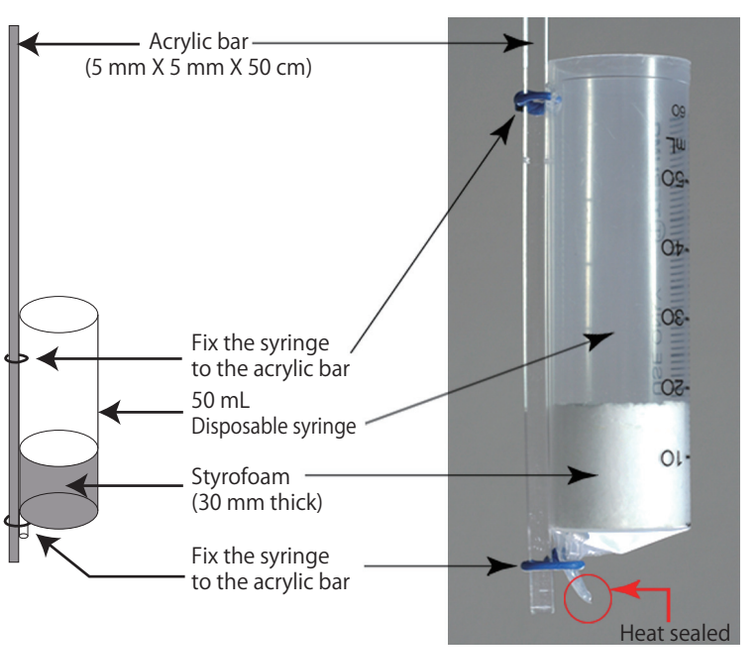
Materials and Equipment

1. Male mice (over 12 weeks old)
2. Micro-spring scissors (5 mm blade)
3. Pair of watchmaker's #5 forceps
4. FERTIUP® (Cryoprotectant: CPA, Cat. No. KYD-001-EX, Cosmo Bio Co., Ltd.)
5. mHTF
6. Liquid paraffin
7. Plastic dish (35 mm X 10 mm Cat. No. 430588; CORNING)
8. Pipette tips
9. Sperm Straws (10 Pieces x 10 Units, EOG sterilized, Cat. No. KYD-S020X10, Cosmo Bio Co., Ltd.)
10. Micropipettes
11. Straw Connector (Cat. No. KYD-S025, Cosmo Bio Co., Ltd.)
12. Impulse sealer
13. Freezing Canister (Cat. No. KYD-S018, Cosmo Bio Co., Ltd.)
14. Triangular Cassette (10 units, Cat. No. KYD-S021 or KYD-S035, Cosmo Bio Co., Ltd.)
15. Cryobiological container or Dry Shipper
16. Hot plate (37℃)

Procedures

Preparing the Freezing Canister

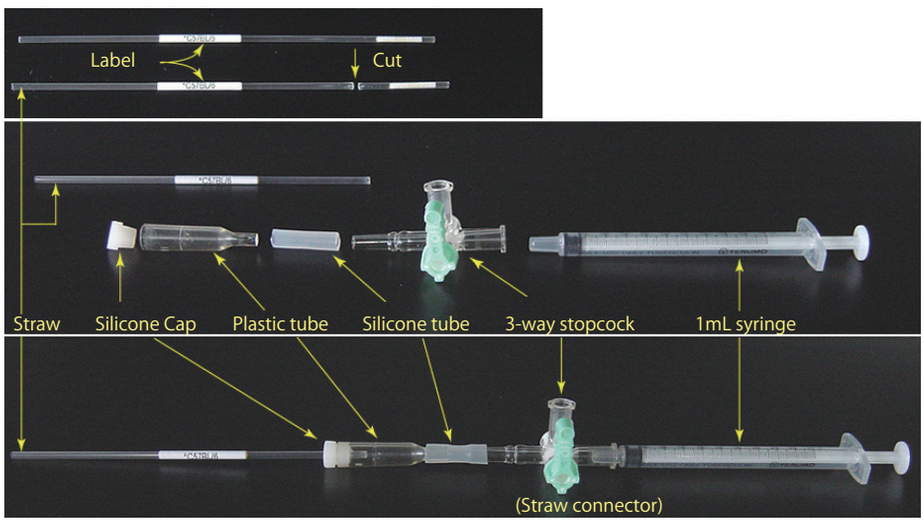
1. Insert a piece of styrofoam tightly into the bottom of the syringe.
2. Heat seal the mouth of the syringe tip.
3. Fix the syringe to the acrylic bar.



Preparing a Straw Connector

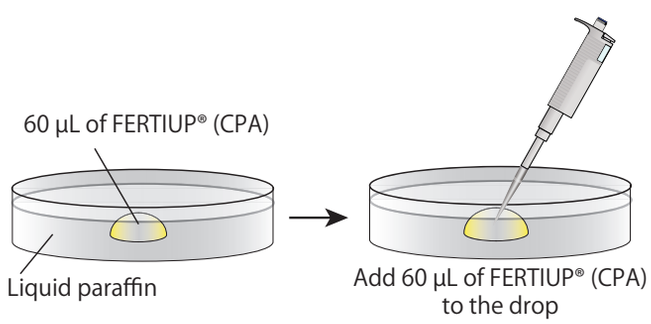
1. Using a 1 mL syringe, a 3-way stopcock, a piece of silicone tube, a plastic tube and a silicone cap, make a straw connector as shown in the diagram below.
2. To use the straw connector, cut away the cotton plug from a straw, then attach the straw to the silicone cap at the end of the connector.

[Connecting the Straw connector and Straw]

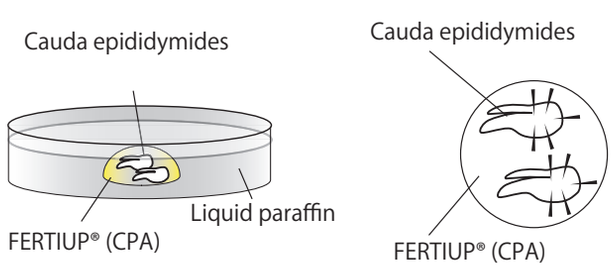


Preparing Sperm Suspension

1. Prepare a drop of 60 µL of 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. Keep the dish on a hot plate at 37°C until use.



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 FERTIUP® (CPA) and use a pair of watchmaker's #5 forceps and micro-spring scissors to make 5 or 6 incisions in the epididymides.



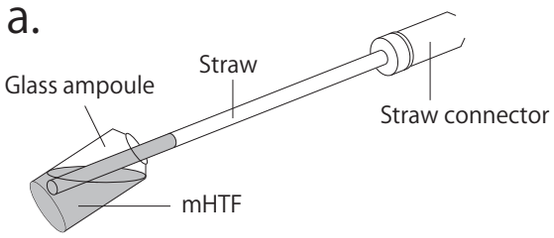
6. Place the dish on a hot plate at 37℃ for 3 minutes. During this time, rotate the dish every minute to disperse sperm from the organs in the FERTIUP® (CPA).

[Cutting the Epididymis and Preparing Sperm Suspension] No. 05-01

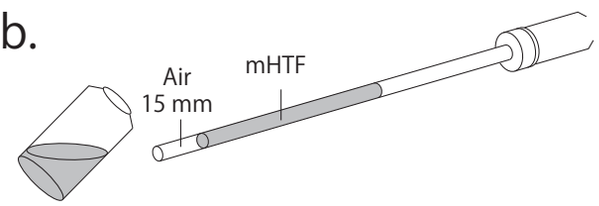
Preparing Freezing Straw Containing Sperm Suspension

1. Connect a straw to a straw connector.
2. Carefully aspirate the contents into the straw in following order:
   a. 100 µL of mHTF,
   b. 15 mm of air,
   c. 10 µL of the sperm suspension,
   d. Another 15 mm of air.

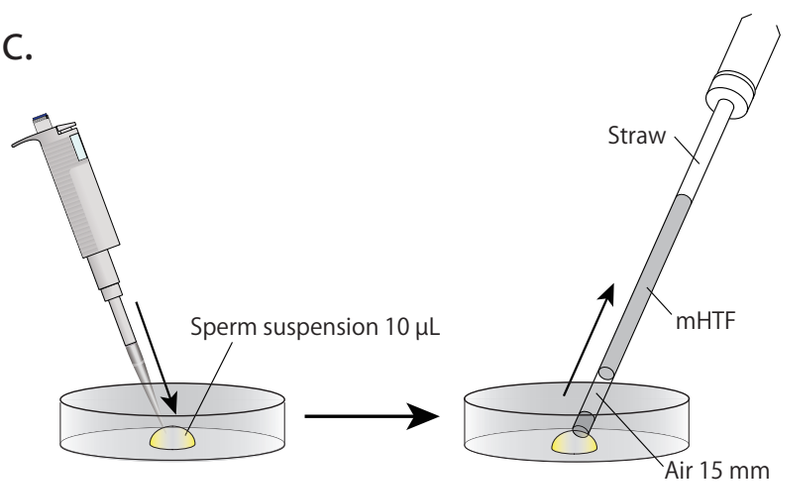
a.



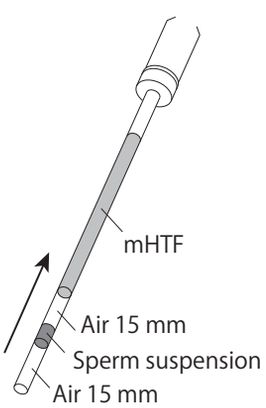
b.



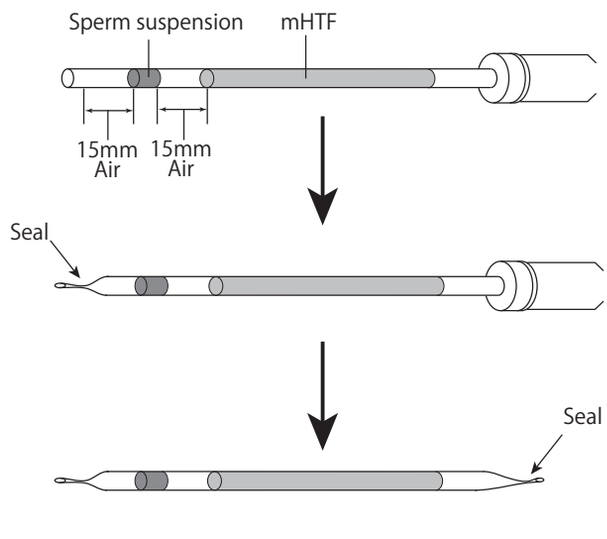
c.



d.



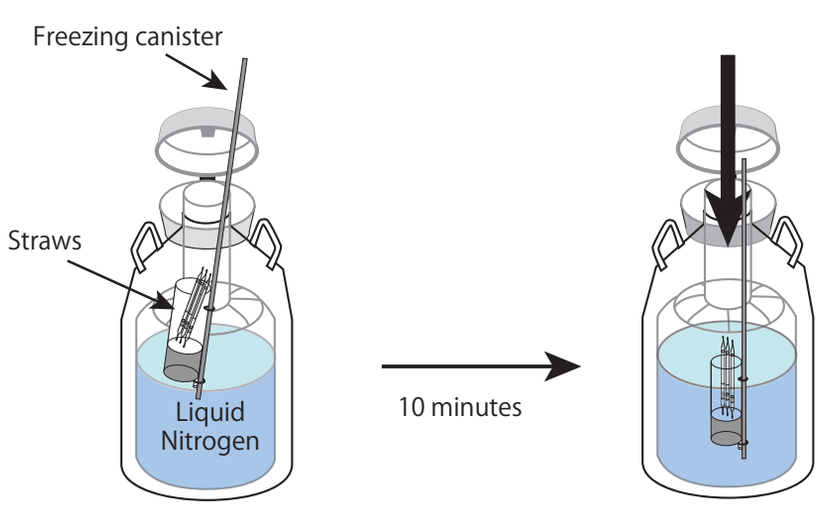
3. Seal both sides of the straw using an impulse sealer.



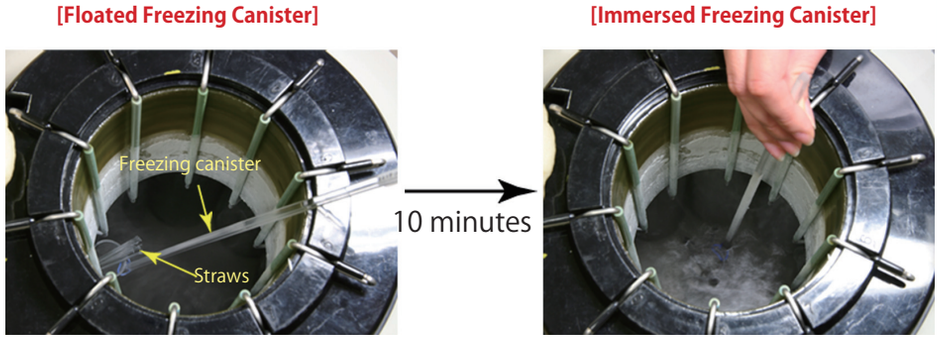
4. Create 10 samples per mouse in the same manner as described above.

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.



[Floated Freezing Canister] [Immersed Freezing Canister]

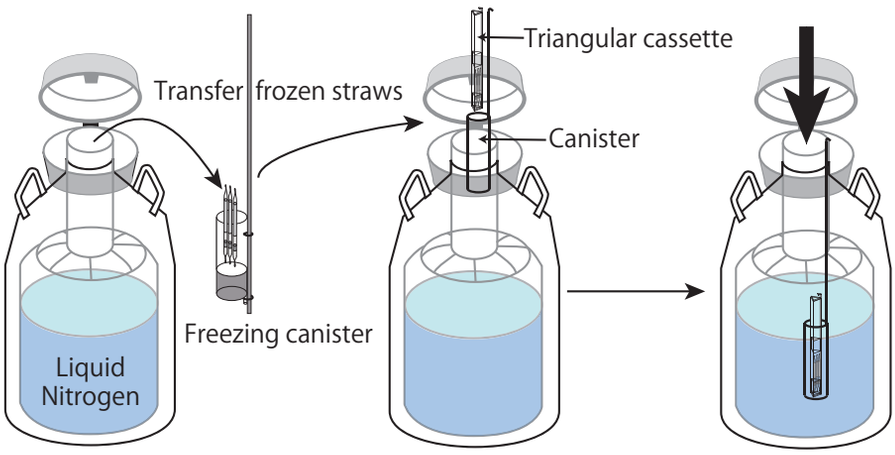


[Freezing the Straws] No. 05-02

Comment

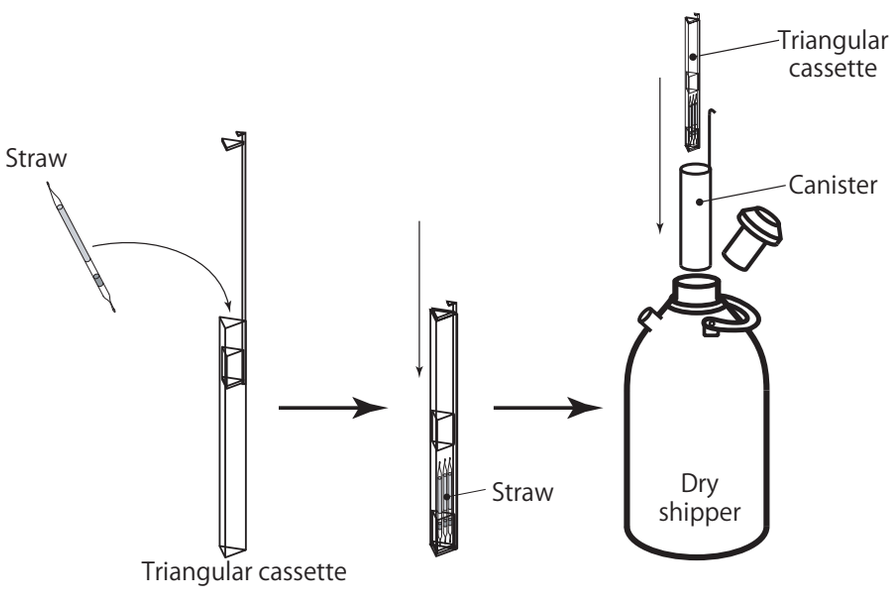
Loading 100 μL of mHTF into the straw prevents the straw from floating on the surface of liquid nitrogen.
This is because the mHTF acts as a weight that forces the straw to sink into the liquid nitrogen.

3. Take out the freezing canister filled with liquid nitrogen, and transfer the straws into a triangular cassette to store them in a liquid nitrogen tank.



Sperm Freezing using a Dry Shipper

1. Transfer the straw containing sperm suspension into a triangular cassette.
2. Set the triangular cassette in a precooled canister.
3. Return the triangular cassette to the canister in the dry shipper and leave it there for 10 minutes.



Comment

Sperm freezing using a dry shipper can be used for the transport of cryopreserved sperm.

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2. Nakagata N., Ueda S., Yamanouchi K., Okamoto K., Matsuda Y., Tsuchiya T., Nishimura M., Oda S., Koyasu K., Azuma S., and Toyoda Y. 1995. Cryopreservation of wild mouse spermatozoa. *Theriogenol*. **43**: 635-643.
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5. 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): 546-551.
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3-2 *In Vitro* Fertilization using Cryopreserved Spermatozoa

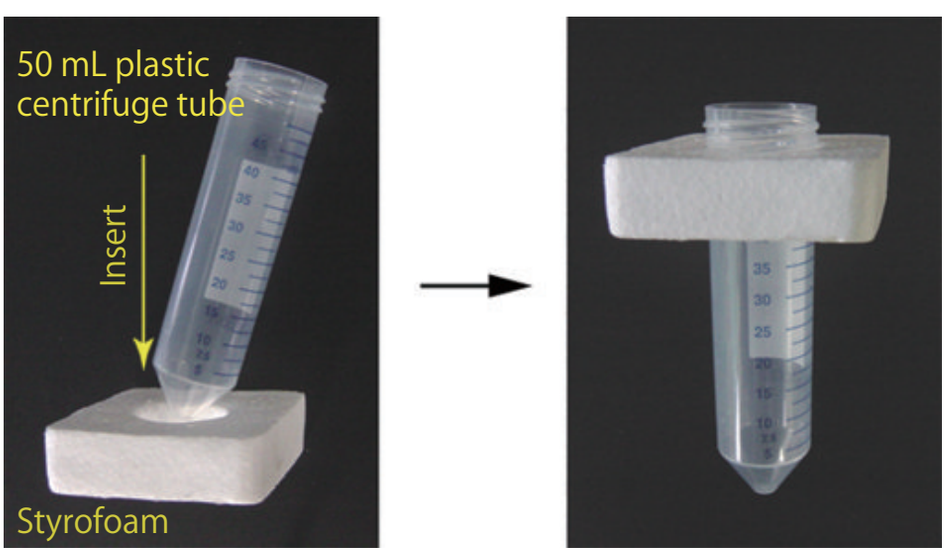
Materials and Equipment

1. Female mice superovulated with PMSG and hCG
2. FERTIUP® (Preincubation medium: PM, Cat. No. KYD-002-EX, Cosmo Bio Co., Ltd.)
3. CARD MEDIUM® (Cat. No. KYD-003-EX, Cosmo Bio Co., Ltd.)
4. mHTF
5. Liquid paraffin
6. Pipette tips (Cat. No. 3520; Thermo SCIENTIFIC)
7. Plastic dishes (35 mm X 10 mm Cat. No. 430588; CORNING)
8. Straw Connector (Cat. No. KYD-S025, Cosmo Bio Co., Ltd.) (Please refer to the chapter of Cryopreservation of Mouse Spermatozoa on page 21.)
9. Water bath maintained at 37℃
10. Float for thawing
11. Micropipettes
12. Humidified incubator (37℃, 5% CO_2 in air)

Procedures

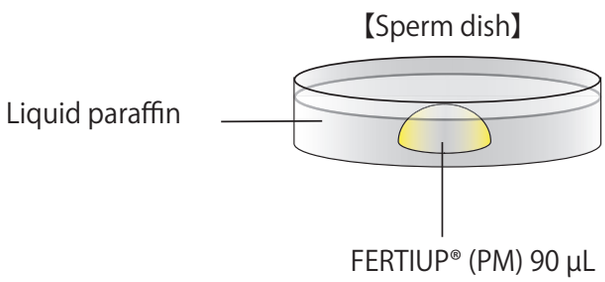
Preparation of the Float for Thawing

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

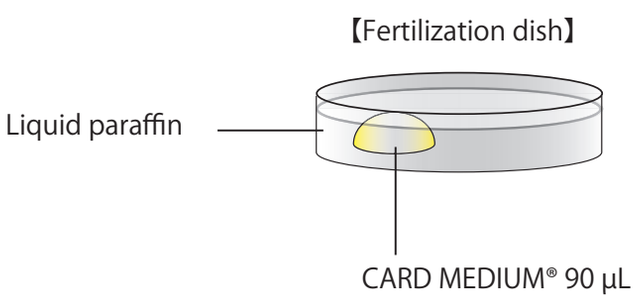


Preparation for Thawing

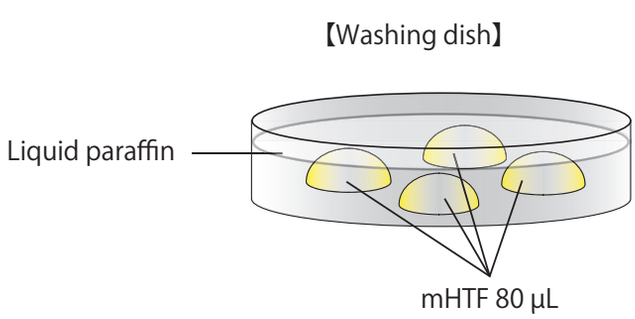
1. Prepare a water bath maintained at 37℃.
2. Pour water (37℃) into the 50 mL plastic centrifuge tube part of the styrofoam/ centrifuge tube assembly, and float it in a water bath.
3. Put 1 drop (90 µL / drop) of FERTIUP®(PM) into a dish and cover it with liquid paraffin 30 minutes before thawing a frozen straw, and place the dish in an incubator (37℃, 5% CO_2 in air).



4. Put 1 drop (90 µL / 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℃, 5% CO_2 in air).



5. Put 4 drops (80 µL / drop) of mHTF into a dish and cover them with liquid paraffin. Place the dish in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.

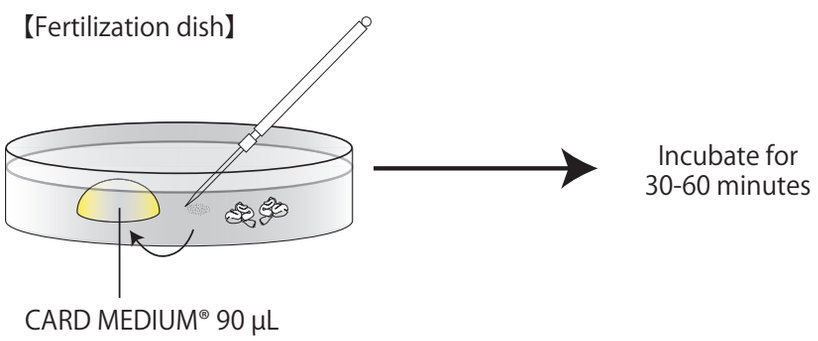


Note

There are three different methods of preparing CARD MEDIUM®, depending on whether *in vitro* fertilization will be carried out using fresh, frozen-thawed or cold-temperature transported spermatozoa.
Please refer to the CARD MEDIUM® instruction manual.

Collection of Oocytes

1. Sacrifice female mice 15-17 hours after an hCG injection and remove the oviducts. (Please refer to the chapter of *In Vitro* Fertilization on page 9.)
2. Using fine, sharp needles, release up to 4-6 cumulus-oocyte complexes (COCs) masses into each drop of CARD MEDIUM® (90 µL) (Fertilization dish).
3. Keep the fertilization dish including COCs in an incubator (37℃ , 5% CO_2 in air) for 30-60 minutes before insemination.



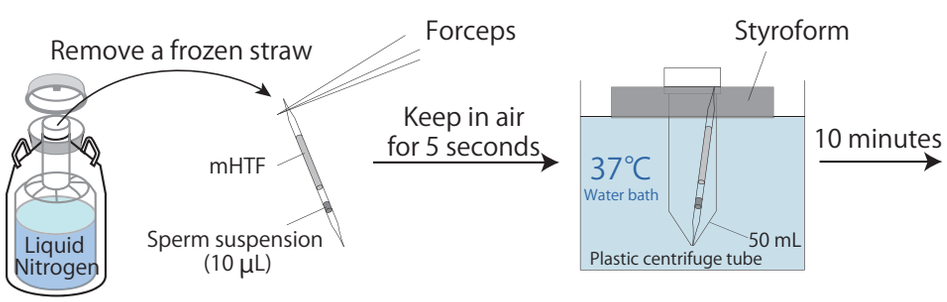
Note

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.

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℃) for 10 minutes.
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.



[Thawing of Mouse Spermatozoa] No. 06-01

Note

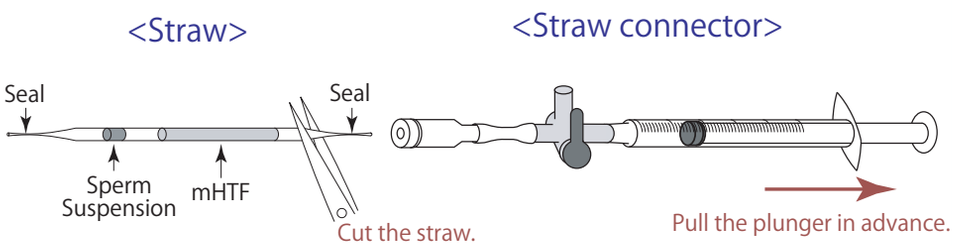
To ensure warming of the frozen sperm, completely immerse the part of the straw containing the sperm in the water bath.

Furthermore, frozen-thawed mouse spermatozoa are sensitive to environmental changes.

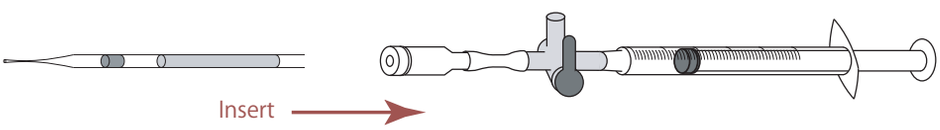
If the straw is not kept in the water bath long enough (10 minutes), the motility of the cryopreserved spermatozoa will be reduced.

Transferring and Preincubating the Thawed Sperm Suspension

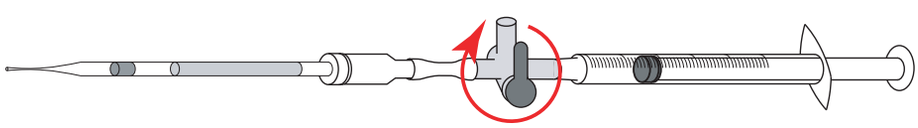
1. Pull the plunger out of the syringe in the straw connector, and cut the straw between the mHTF and the seal.

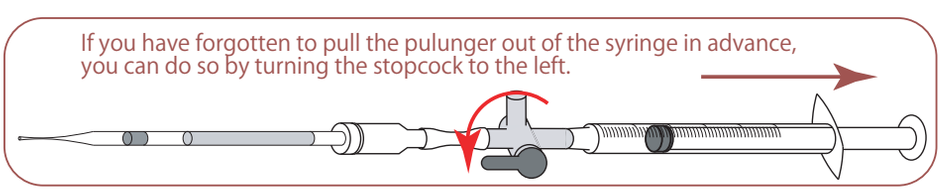


2. Insert the cut straw into the straw connector.

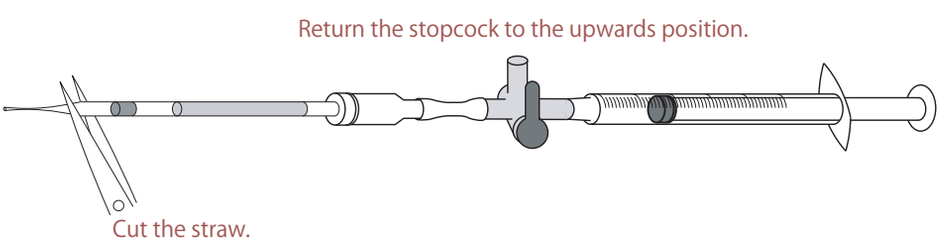


3. Because the insertion of the straw into the straw connector creates pressure inside the straw, turn the stopcock in order to release the pressure.

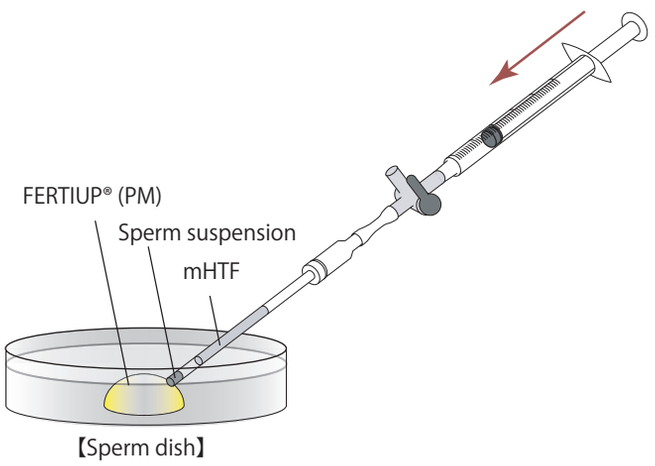




4. Return the stopcock to the upwards position, and cut the straw between the seal and the sperm suspension.



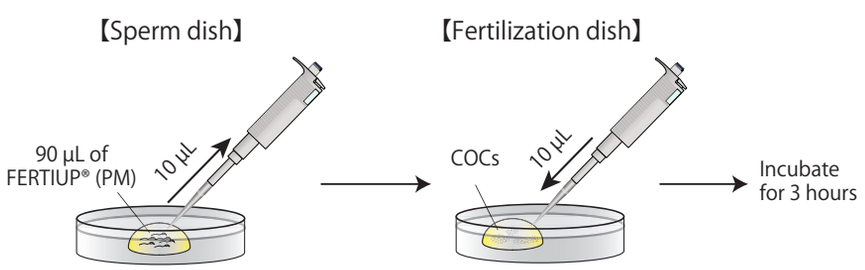
5. Push the plunger to transfer only the sperm suspension into the drop of FERTIUP®(PM) (sperm dish), and place the dish in an incubator (37℃ , 5% CO_2 in air) for 30 minutes.



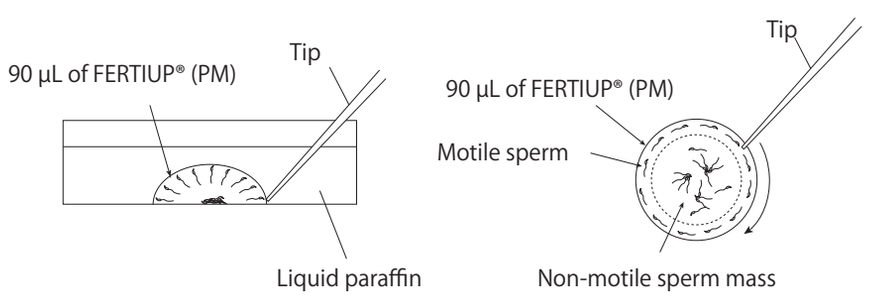
[Transfer of the Thawed Sperm Suspension] No. 06-02

Insemination

1. Using a wedge-shaped pipette tip (Cat. No. 3520; Thermo SCIENTIFIC), aspirate 10 µL of the preincubated sperm suspension from the edge of the drop.
2. Add 10 µL of sperm to each drop of fertilizing CARD MEDIUM® containing the COCs.
3. Incubate the oocytes and spermatozoa for 3 hours in an incubator (37℃ , 5% CO_2 in air).



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



[Aspiration of Preincubated Sperm Suspension and Insemination of Oocytes]
No. 06-03

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

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.

Comment

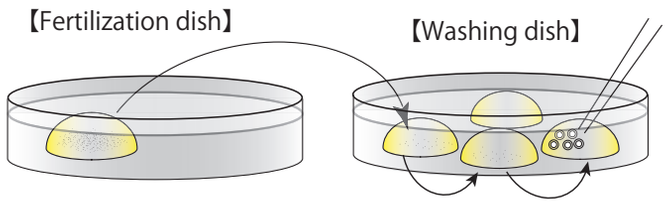
Spermatozoa with high motility have a tendency to gather near the edge of the drop.

Comment

It is possible to aspirate 10 µL of sperm suspension 3-4 times per drop.

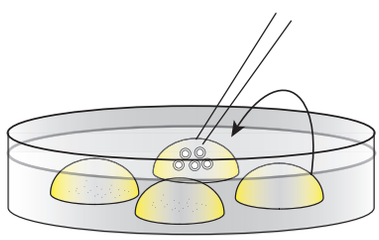
Note

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



5. 6 hours after insemination, observe them in the third drop of mHTF and remove any parthenogenetic oocytes which have only one pronucleus.
(Please refer to the chapter of *In Vitro* Fertilization on page 11.)
6. After overnight culture of the oocytes, transfer the obtained 2-cell stage embryos only to the fourth drop of mHTF. These embryos can now be vitrified or transferred.
(Please refer to the chapters of Simple Vitrification of Mouse Embryos on page 54 and Embryo Transfer into the Oviduct on page 66.)

【Washing dish】



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3-3 Rescue *In Vitro* Fertilization Method for Legacy Stock of Cryopreserved Spermatozoa

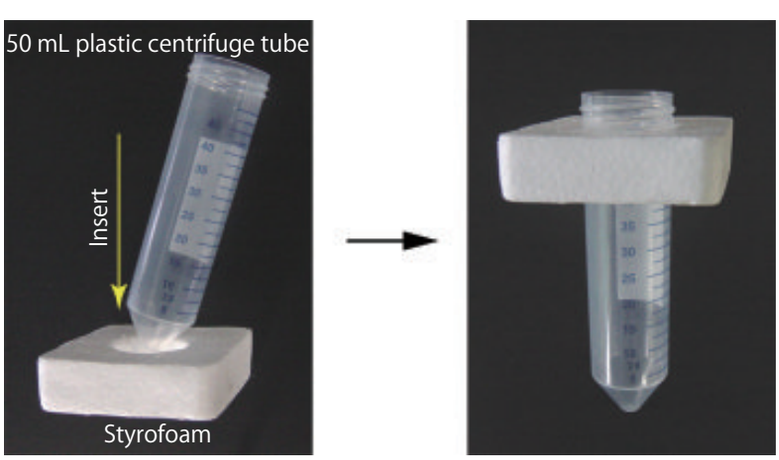
Materials and Equipment

1. Legacy stock of cryopreserved spermatozoa
2. Female mice superovulated with PMSG and hCG
3. FERTIUP® (Preincubation medium: PM, Cat. No. KYD-002-EX, Cosmo Bio Co., Ltd.)
4. CARD MEDIUM® (Cat. No. KYD-003-EX, Cosmo Bio Co., Ltd.)
5. mHTF
6. Liquid paraffin
7. Water bath maintained at 37℃
8. Float for thawing
9. 1.5 mL tube (Quality Scientific Plastics 1.5 mL Graduated Microcentrifuge Tube with Flat Top Cap, Natural Cat. No. 509-GRD-Q)
10. Centrifuge
11. Micropipettes
12. Plastic dishes (35 mm X 10 mm Cat. No. 430588; CORNING)
13. Humidified incubator (37℃, 5% CO_2 in air)

Procedures

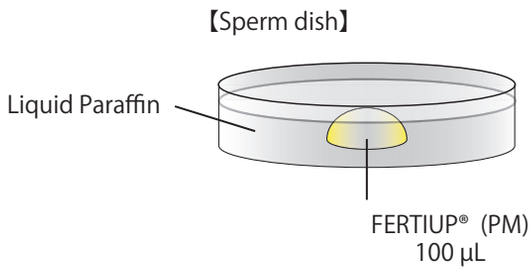
Preparation of the Float for Thawing

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

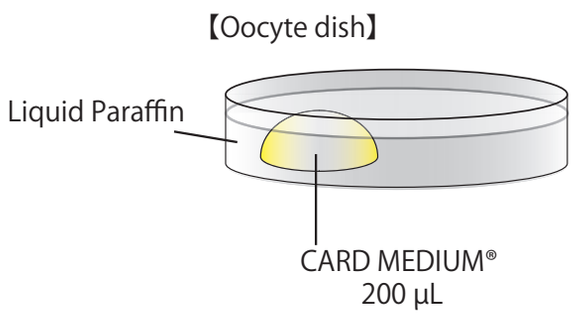


Preparation for Thawing

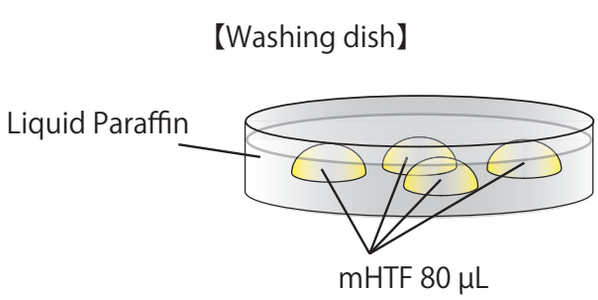
1. Prepare a water bath to 37℃ .
2. Pour water (37℃) into the 50 mL plastic centrifuge tube section of the styrofoam/centrifuge tube assembly, and float it in a water bath.
3. 30 minutes before thawing a frozen sperm sample, put 1 drop (100 µL/drop) of FERTIUP® (PM) into a dish and cover it with liquid paraffin. Place the dish in an incubator (37℃ , 5% CO_2 in air).



4. 10 minutes before collecting oocytes, put 1 drop (200 µL/drop) of CARD MEDIUM into a dish and cover it with liquid paraffin. Place the dish in an incubator (37℃ , 5% CO_2 in air).

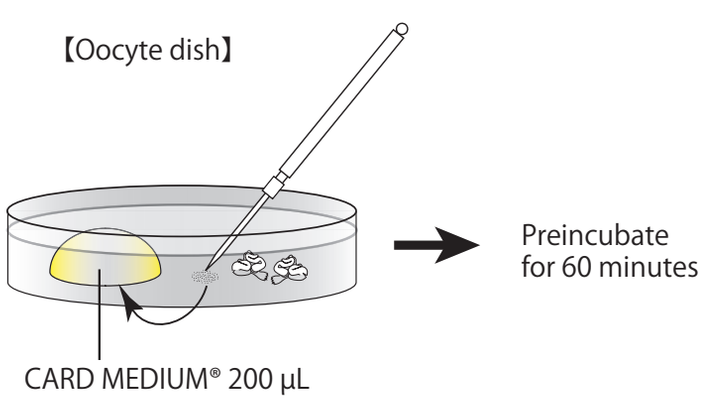


5. Put 4 drops (80 µL / drop) of mHTF into a dish and cover them with liquid paraffin. Place the dish in an incubator (37℃ , 5% CO_2 in air) for at least 30 minutes.



Collection and Preincubation of Oocytes

1. Sacrifice female mice 15-17 hours after an hCG injection and remove the oviducts. (Please refer to the chapter of *In Vitro* Fertilization on page 9.)
2. Using fine, sharp needles, release between 6 and 20 masses of cumulus-oocyte complexes (COCs) into a drop of CARD MEDIUM® (200 µL) (Oocyte dish), and preincubate the dish for 60 minutes.



Note

There are three different methods of preparing CARD MEDIUM®, depending on whether *in vitro* fertilization will be carried out using fresh, frozen-thawed or cold-temperature transported spermatozoa. Please refer to the CARD MEDIUM® instruction manual.

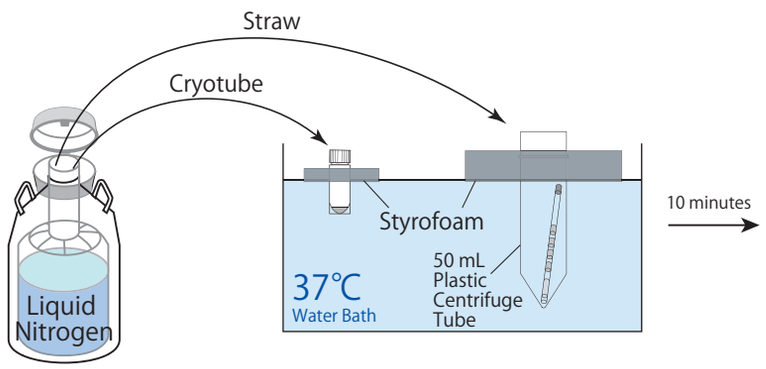
Note

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).

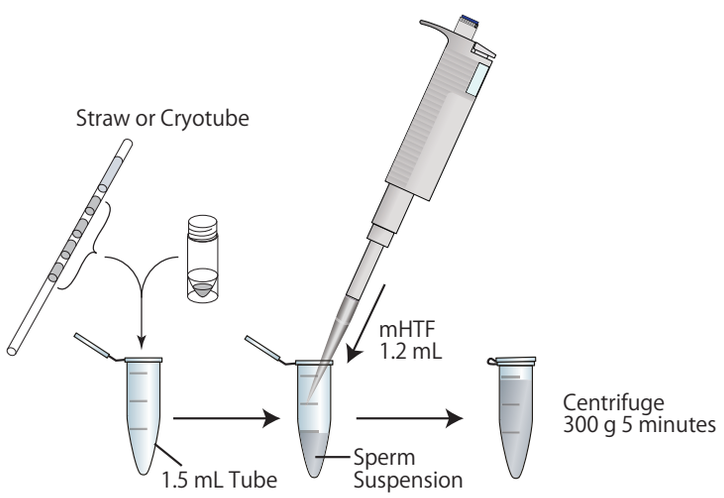
Moreover, when carrying out this process alone, do not sacrifice multiple mice at once; instead, sacrifice one mouse and swiftly remove its oviducts before moving on to the next mouse.

Thawing the Mouse Spermatozoa

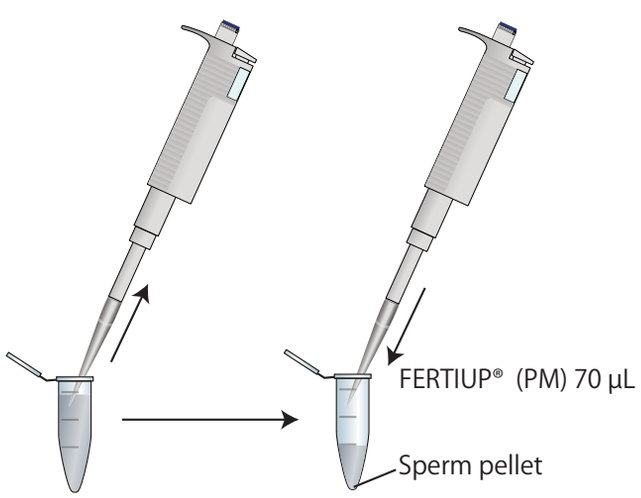
1. Remove a frozen sperm sample from the liquid nitrogen. If the sperm sample is stored in a cryotube, open the cap and discard any liquid nitrogen in the tube. Immerse the sample in a water bath maintained at 37℃ (using a styrofoam or the styrofoam/centrifuge tube assembly) for 10 minutes.



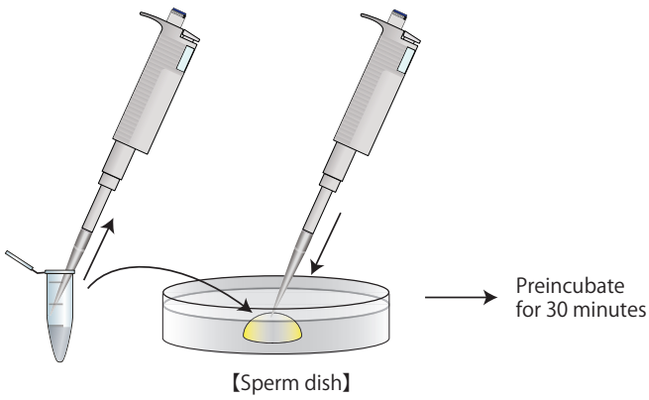
2. Transfer the sperm suspension from the cryotube or the straw into a 1.5 mL tube. Slowly add 1.2 mL of mHTF kept at 37℃ to the tube, and centrifuge it at 300 g at room temperature for 5 minutes.



3. After centrifugation, remove as much supernatant as possible, and add 70 µL of FERTIUP® (PM) kept at 37℃ into the tube (the final volume is approx. 100 µL).

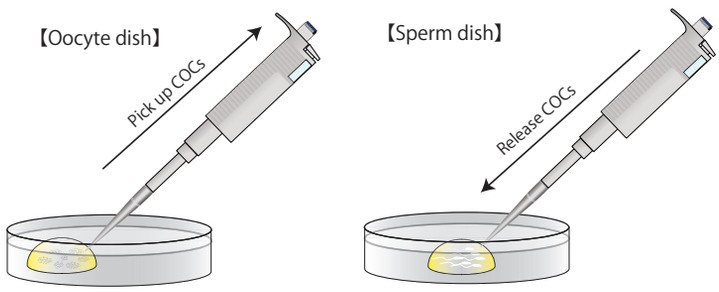


4. After pipetting gently, transfer all of the contents in the tube into the 100 µL drop of FERTIUP® (PM) (Sperm dish). Place the dish in an incubator (37℃ , 5% CO_2 in air) for 30 minutes.

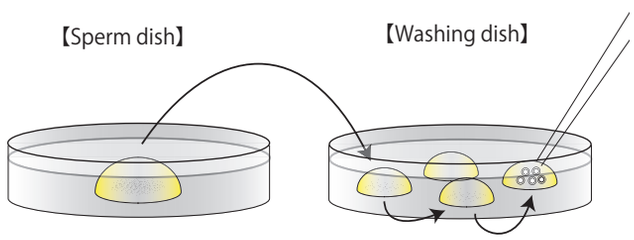


Insemination

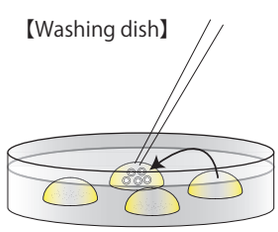
1. Using a tip, suck up the preincubated COCs with a minimum amount of medium from the drop of CARD MEDIUM® (Oocyte dish). Then, release them into the drop of sperm suspension (Sperm dish), and incubate it in an incubator (37℃, 5% CO_2 in air).



2. After incubating for 3 hours, wash the oocytes 3 times in fresh mHTF (80 µL) in a washing dish.



3. 6 hours after insemination, observe them in the third drop of mHTF and remove any parthenogenetic oocytes which have only one pronucleus.
(Please refer to the chapter of *In Vitro* Fertilization on page 11.)
4. After culturing the oocytes overnight, transfer the obtained 2-cell stage embryos only to the fourth drop of mHTF. These embryos can now be vitrified or transferred.
(Please refer to the chapters of Simple Vitrification of Mouse Embryos on page 54 and Embryo Transfer into the Oviduct on page 66.)



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