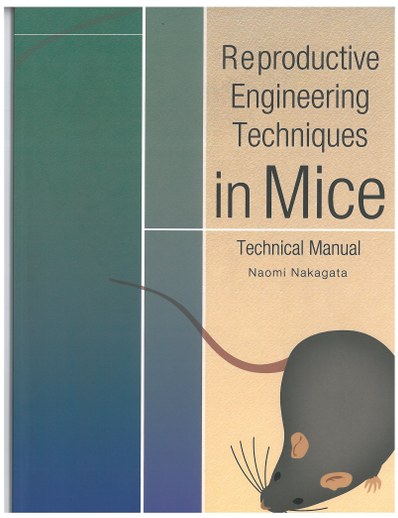
Reproductive Engineering Techniques **in Mice**



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

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2-1 Collection and Transport at Cold Temperature of Cauda Epididymis

Materials and Equipment

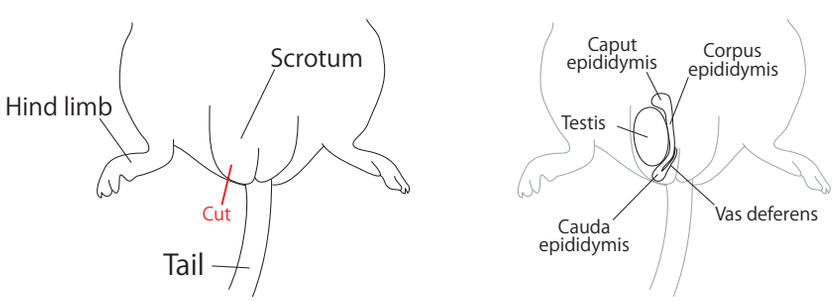
1. Male mouse (over 12 weeks old)
2. Anesthetic
3. Hot plate (37℃)
4. Fine scissors
5. Pair of watchmaker's #5 forceps
6. Wound clip (Autoclip 9 mm; Clay Adams 427631) and clip applicator (Mik-Ron Autoclip Applier; Clay Adams 427630)
7. Temperature data logger (Thermochron iButton Cat. No. DS1921G; Maxim Integrated Products)
8. Cold storage solution for cauda epididymides (Cat. No. KYD-007-EX, Cosmo Bio Co., Ltd.)
9. CARD Cold Transport Kit (Cat. No. KYD-006-EX, Cosmo Bio Co., Ltd.)
   - Thermos bottle (Cat. No. JMK-501; Thermos K.K.)
   - Paper box (in which a 0.2 mL tube can stand)
   - Cotton wool
   - Cold packs (small and large)
   - Polystyrene foam transport box (Cat. No. KC-3, KARUX)

   Both the CARD cold temperature transport kit and the preservation solution must be precooled to 4-8℃ before use.

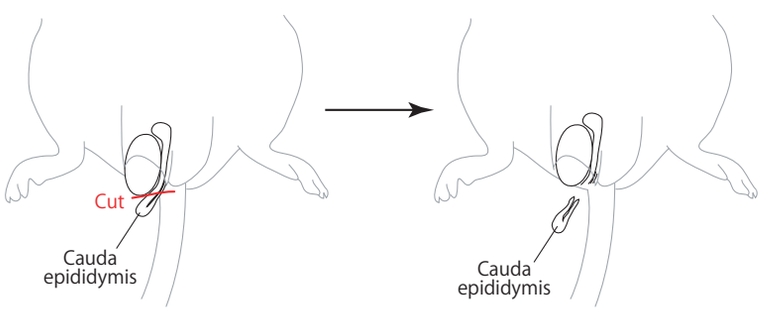
Procedures

Collection of Cauda Epididymis

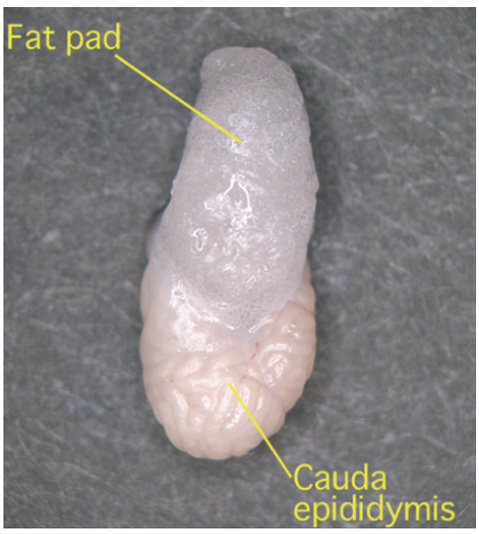
1. Anesthetize a male mouse.
2. Make a small incision in the scrotum of the mouse and expel a cauda epididymis.



3. Cut the vas deferens and the corpus epididymis, and collect the cauda epididymis.



[Collected Cauda Epididymis]



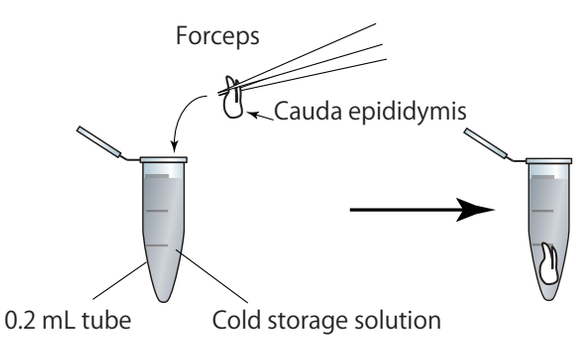
[Removing one Cauda Epididymis from an Anesthetized Male] No. 03-01

4. Push the testis back into the abdomen and close the wound using a wound clip.
5. Keep the mouse warm on a 37℃ warming plate until the mouse recovers from the effects of the anesthesia.

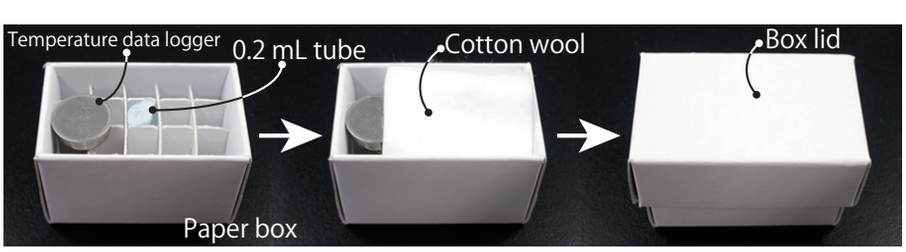
Packing and Transport of Cauda Epididymis

The items to be used when packing the cauda epididymis should be kept at 4-8 ℃ until just before use. Moreover, packing procedures should be completed as quickly as possible to prevent the cauda epididymis and the packing items from warming up.

1. Put the removed cauda epididymis into the 0.2 mL tube containing cold storage solution.



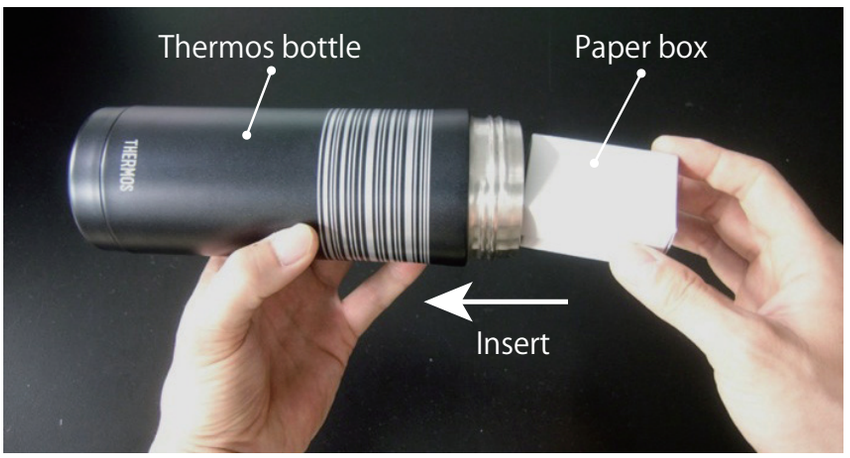
2. Place the tube containing the cauda epididymis, a temperature data logger and a piece of cotton wool in the paper box.



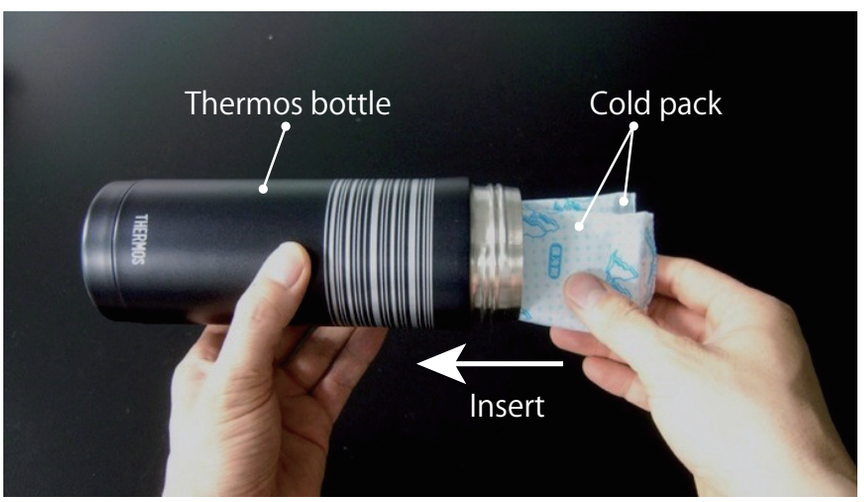
Comment

1 week after the operation, the male mouse can be used for mating with a female mouse.

3. Insert the paper box containing the cauda epididymis into a thermos bottle.



4. Insert two cold packs (small) into the thermos bottle.



5. Close the bottle cap.

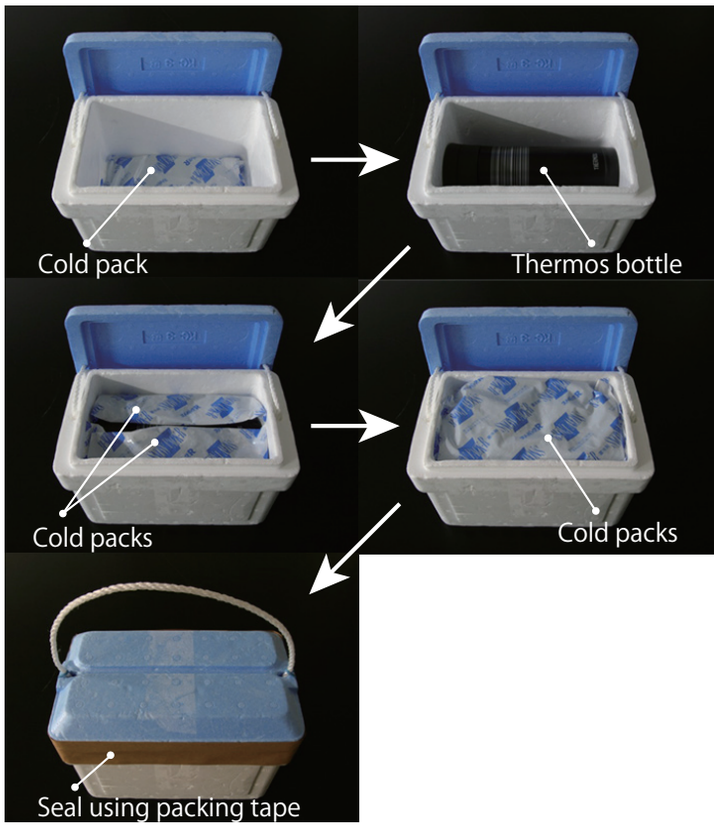
6. Place a cold pack (large) at the bottom of a foam transport box, then put the thermos bottle on top of it.
7. Pack one cold pack (large) on either side of the bottle, then set a further pack (large) on top and close the lid.
8. Seal the lid of the foam transport box using packing tape.

Note

Take care not to place the paper box upside down.

Note

It is only possible to place the thermos bottle in the center of the foam transport box and not the actual bottom, because the length of the thermos bottle is the same as that of the inner length of the foam transport box.
This is to protect the thermos bottle during shipping.



9. Keep the foam transport box in the refrigerator until a courier comes to pick it up.
10. Send the samples via a regular courier service.

References

1. Takeo T., Tsutsumi A., Omaru T., Fukumoto K., Haruguchi Y., Kondo T., Nakamuta Y., Takeshita Y., Matsunaga H., Tsuchiyama S., Sakoh K., Nakao S., Yoshimoto H., Shimizu N., and Nakagata N. 2012. Establishment of a transport system for mouse epididymal sperm at refrigerated temperatures. *Cryobiology*. **65**(3): 163-168.

Note

The sample must be transferred at a refrigerated temperature. Please ask the courier service directly about conditions during transport.

Comment

Epididymal sperm at cold temperature maintain fertilizing ability for up to 72 hours.

2-2 *In Vitro* Fertilization using Epididymal Sperm Transported at Cold Temperature

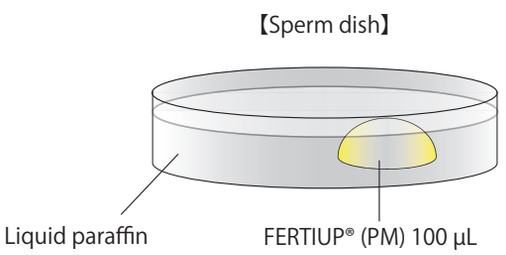
Materials and Equipment

1. Cauda epididymis transported at cold temperature
2. FERTIUP® (Preincubation medium: PM, Cat. No. KYD-002-EX, Cosmo Bio Co., Ltd.)
3. mHTF
4. Liquid paraffin
5. Micropipettes
6. Plastic dishes (35 mm X 10 mm Cat. No. 430588; CORNING)
7. Fine scissors
8. Pair of watchmaker's #5 forceps
9. Filter paper
10. Humidified incubator (37℃, 5% CO_2 in air)

Procedures

Collection of Cauda Epididymis

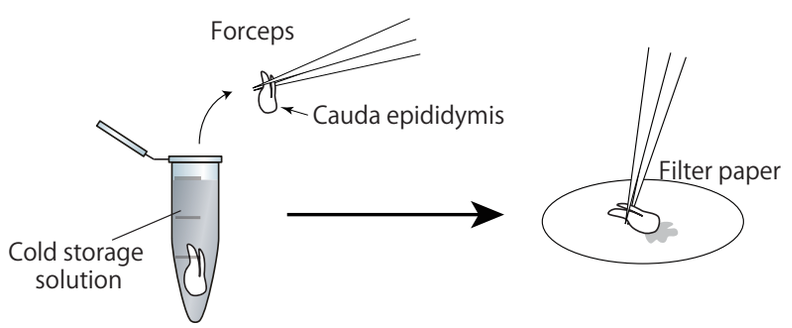
1. Put 1 drop (100 µL / drop) of FERTIUP® (PM) into a dish and cover it with liquid paraffin 30 minutes before collection of epididymal sperm transported at cold temperature, and place the dish in an incubator (37℃, 5% CO_2 in air)



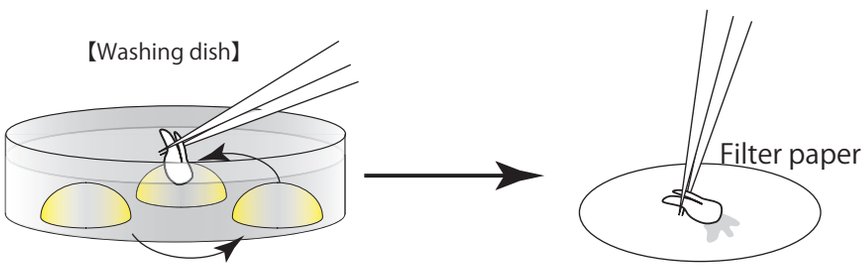
2. Remove the 0.2 mL tube containing the sample from the foam transport box.

[Removing the sample] No. 04-01

3. Open the tube, pick up the cauda epididymis and wipe away any cold storage solution using filter paper.



4. Wash the cauda epididymis in each of the three drops of mHTF in a washing dish. After washing, wipe away any excess mHTF using filter paper.



5. Place the cauda epididymis in the sperm dish containing liquid paraffin. Epididymal spermatozoa transported at cold temperature can be utilized for *in vitro* fertilization in the same manner as fresh spermatozoa.
Please refer to the chapter of *In Vitro* Fertilization on page 6.

Comment

To make a washing dish, put 3 drops (about 100 µL / drop) of mHTF into a dish without liquid paraffin just before use.

Note

If you find it difficult to release sperm from the cauda epididymis, make one more incision in cauda epididymis to release more sperm.

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.

References

1. Takeo T., Tsutsumi A., Omaru T., Fukumoto K., Haruguchi Y., Kondo T., Nakamuta Y., Takeshita Y., Matsunaga H., Tsuchiyama S., Sakoh K., Nakao S., Yoshimoto H., Shimizu N., and Nakagata N. 2012. Establishment of a transport system for mouse epididymal sperm at refrigerated temperatures. *Cryobiology*. **65**(3): 163-168.

3-1 Cryopreservation of Mouse Spermatozoa

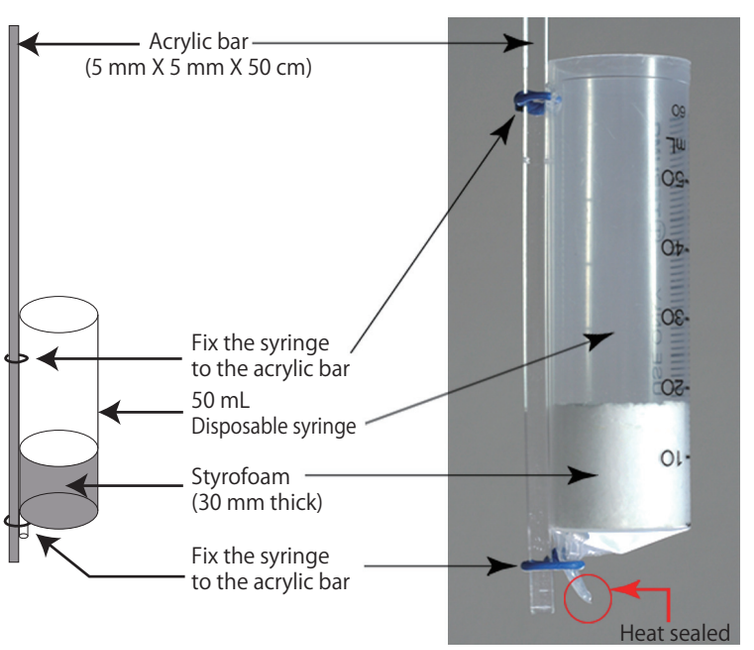
Materials and Equipment

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

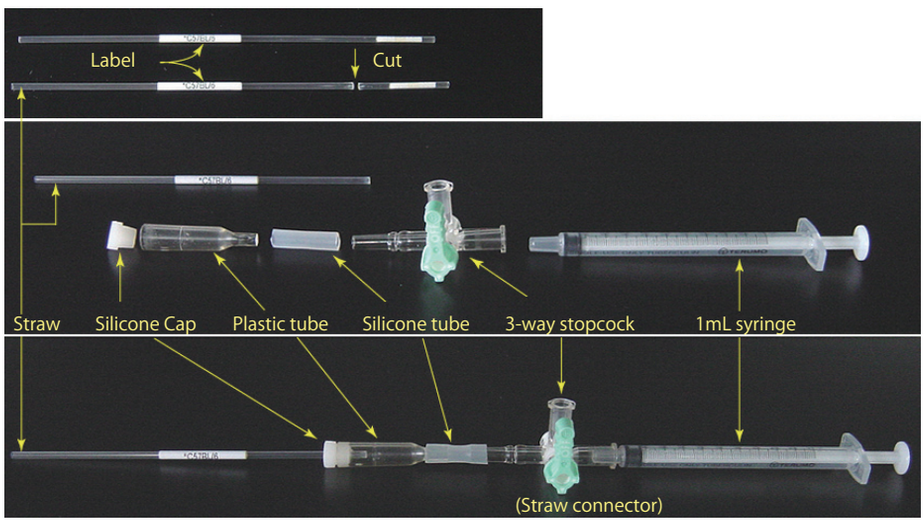
1. Insert a piece of styrofoam tightly into the bottom of the syringe.
2. Heat seal the mouth of the syringe tip.
3. Fix the syringe to the acrylic bar.



Preparing a Straw Connector

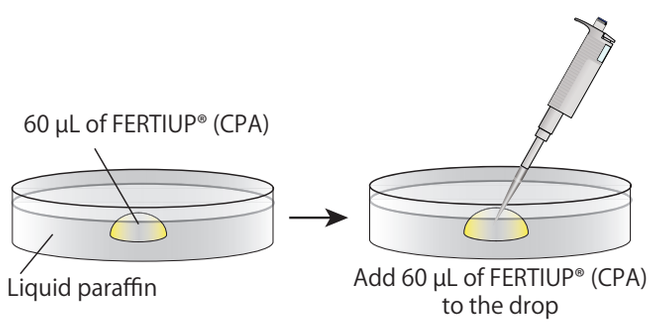
1. Using a 1 mL syringe, a 3-way stopcock, a piece of silicone tube, a plastic tube and a silicone cap, make a straw connector as shown in the diagram below.
2. To use the straw connector, cut away the cotton plug from a straw, then attach the straw to the silicone cap at the end of the connector.

[Connecting the Straw connector and Straw]

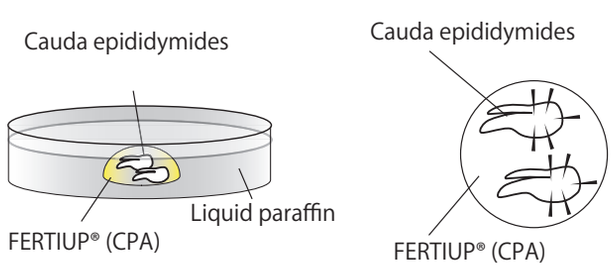


Preparing Sperm Suspension

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.



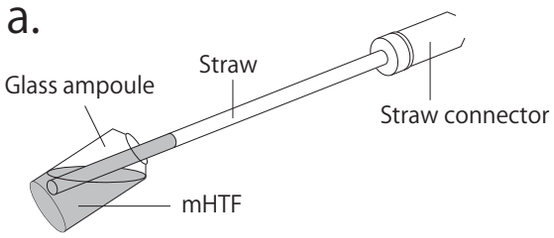
6. Place the dish on a hot plate at 37℃ for 3 minutes. During this time, rotate the dish every minute to disperse sperm from the organs in the FERTIUP® (CPA).

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

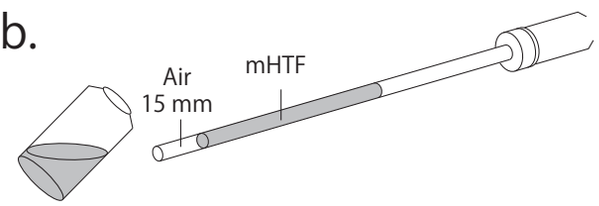
Preparing Freezing Straw Containing Sperm Suspension

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.

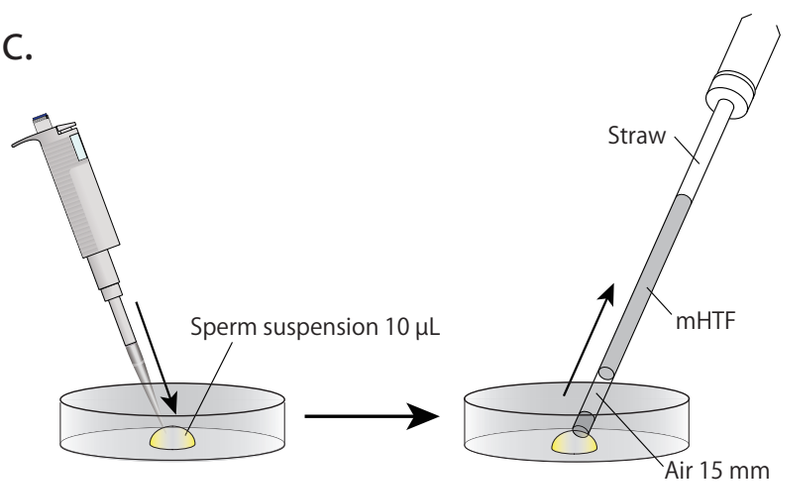
a.



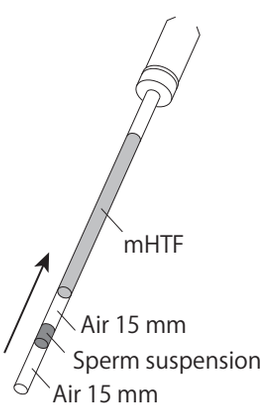
b.



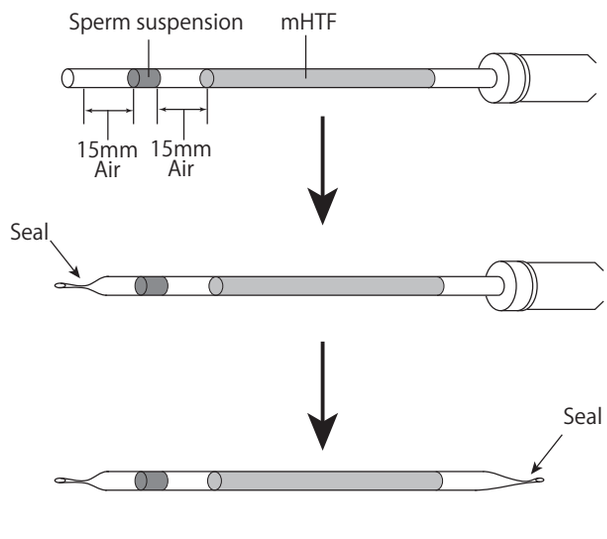
c.



d.



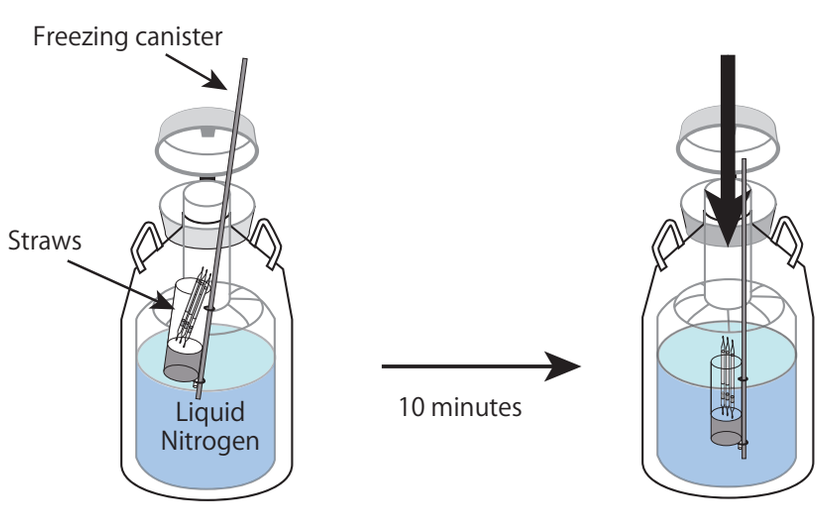
3. Seal both sides of the straw using an impulse sealer.



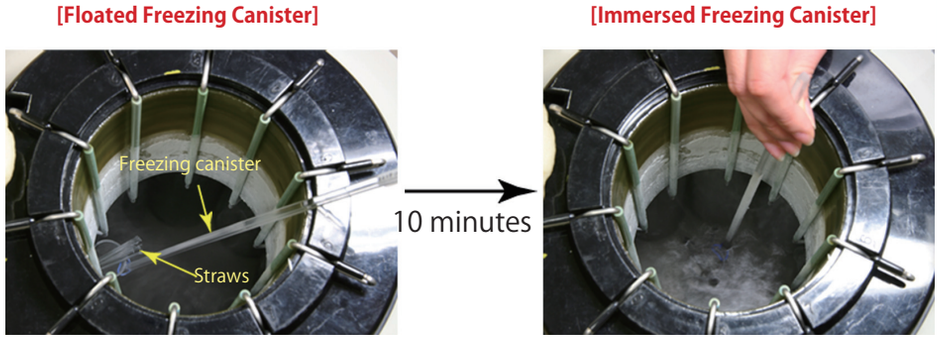
4. Create 10 samples per mouse in the same manner as described above.

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]

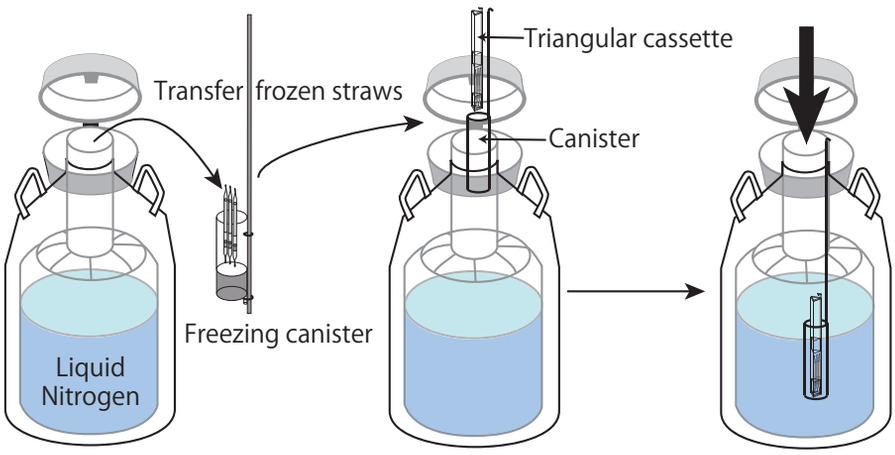


[Freezing the Straws] No. 05-02

Comment

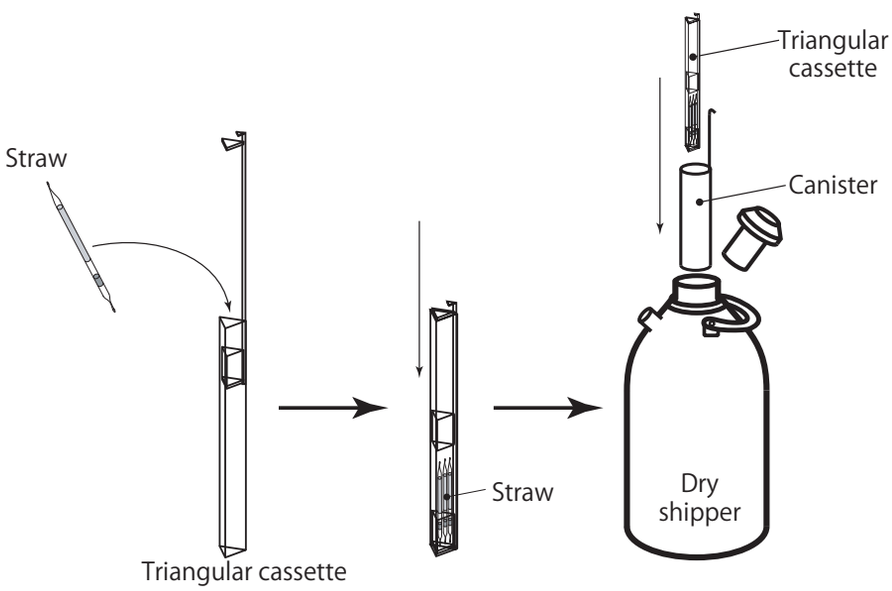
Loading 100 μL of mHTF into the straw prevents the straw from floating on the surface of liquid nitrogen.
This is because the mHTF acts as a weight that forces the straw to sink into the liquid nitrogen.

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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