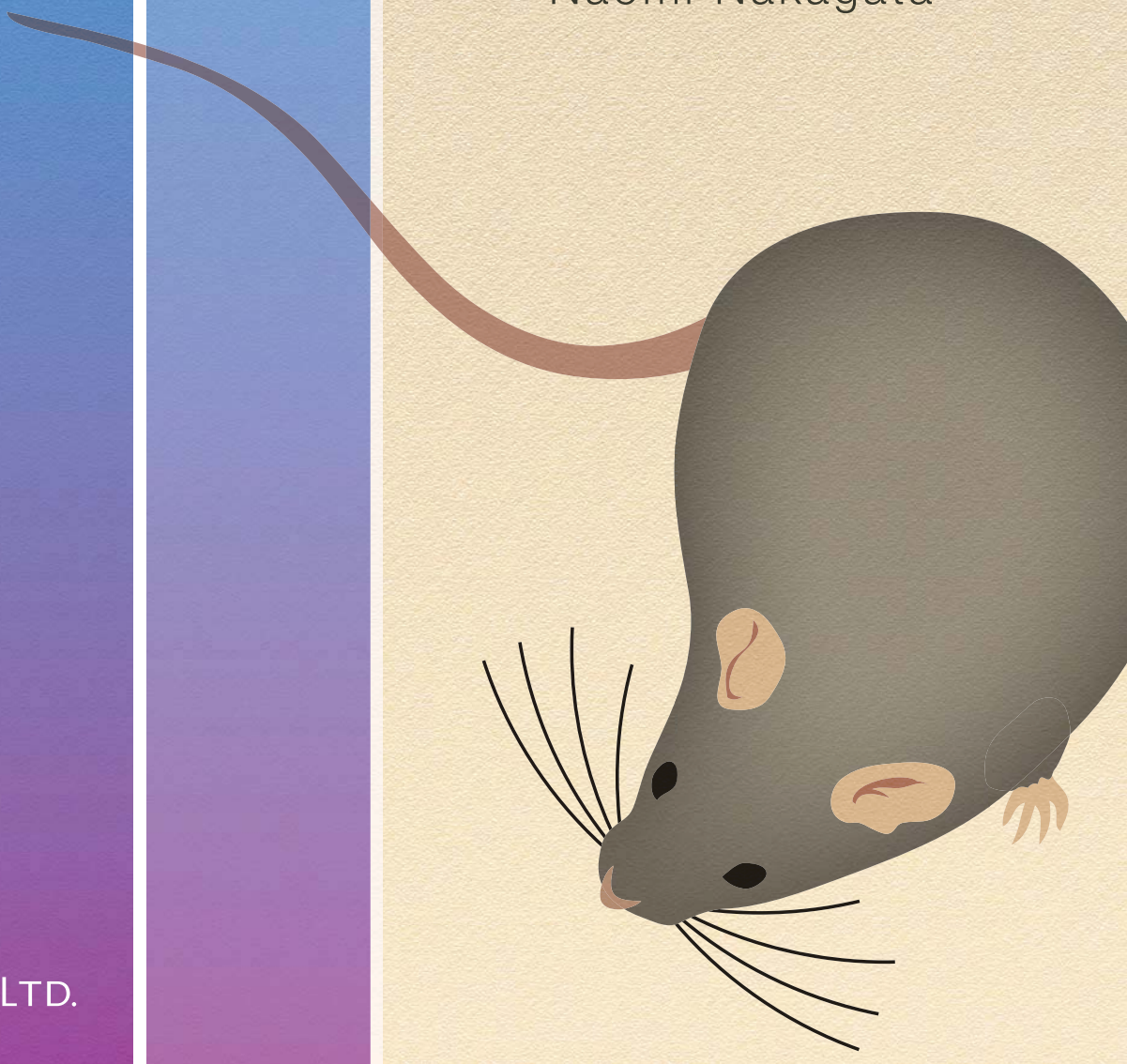
Reproductive Engineering Techniques in Mice

Technical Manual

Naomi Nakagata



Cosmo Bio Co., Ltd.

Reproductive Engineering Techniques **in Mice**

Technical Manual

By Naomi Nakagata
In cooperation with Shuuji Tsuchiyama
Division of Reproductive Engineering
Center for Animal Resources & Development (CARD)
Kumamoto University, Japan

3rd Edition July 2016
Published by COSMO BIO CO., LTD.
Toyo-Ekimae Bldg., 2-20, Toyo 2-Chome, Koto-ku, Tokyo 135-0016, Japan
Phone; +81-3-5632-9617
Cover Design COSMO BIO CO., LTD.

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.

1-1 Preparing and Assembling Pipettes for Embryo Handling

Materials and Equipment

1. Glass capillary pipettes (Calibrated Micropipettes; 2-000-200; Drummond Scientific Company, USA)
2. Alcohol lamp (or STRAIGHT HEAD LAB BURNER; Cat. No. RK4102; REKROW INDUSTORIAL INC.)
3. Ampoule cutter
4. Hemocytometer
5. Pasteur pipette
6. Cotton wool
7. Silicone tube
8. Silicone cap
9. Aspirator mouthpiece

Note

Assembled capillary transfer mouth pipettes for embryo handling is available from Cosmo Bio Co., Ltd. (Embryo manipulation instrument set, Cat. No. KYD-S036)

Procedures

Cleaning and Sterilizing Glass Capillary Pipettes

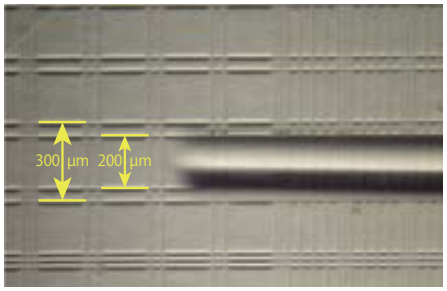
1. Immerse glass capillary pipettes in a 99:1 ratio with 70% ethanol and concentrated hydrochloric acid for over 12 hours.
2. Rinse the glass capillary pipettes under running tap water for at least 3 hours.
3. Rinse the glass capillary pipettes 4 or 5 times using distilled water.
4. Heat sterilize the glass capillary pipettes at 180℃ for at least 3 hours.

Making Pipettes for Embryo Handling

1. Heat the center of a glass capillary pipette with the upper part of the flame of an alcohol lamp. When the center of the glass capillary pipette has softened sufficiently, remove it from the flame and quickly pull it at both ends.
2. Split the glass capillary pipette in two by placing the center of the thin section into the flame again.
3. Cut the glass capillary pipettes to an appropriate length (10 cm) by scoring the thin section of the glass capillary pipette using an ampoule cutter, then breaking off the excess part.
4. Check the diameter of the edge of the capillary using a hemocytometer under a microscope.

[When the edge of a glass capillary is in focus]

[When the hemocytometer is in focus]



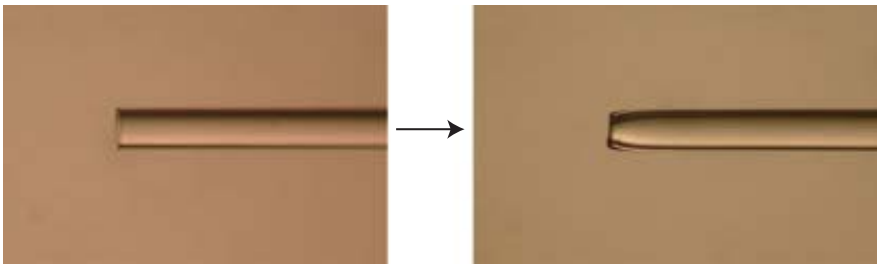
[Making Pipettes for Embryo Handling] No. 01-01

Note

The dimensions of the capillary depends on both the extent of heating and the timing with which the capillary is pulled.
With practice, you will master the technique and will be able to pull capillaries into pipettes with the required dimensions.
The outer diameter of a pulled pipette should be approximately 200-250 µm.

5. Polish and sterilize the tip of the glass capillary pipette by heating it very lightly in a flame. Take care not to overheat the tip of the glass capillary pipette as it may seal the opening.

[The end of a glass capillary pipette prior to polishing] [The polished end of a glass capillary pipette]

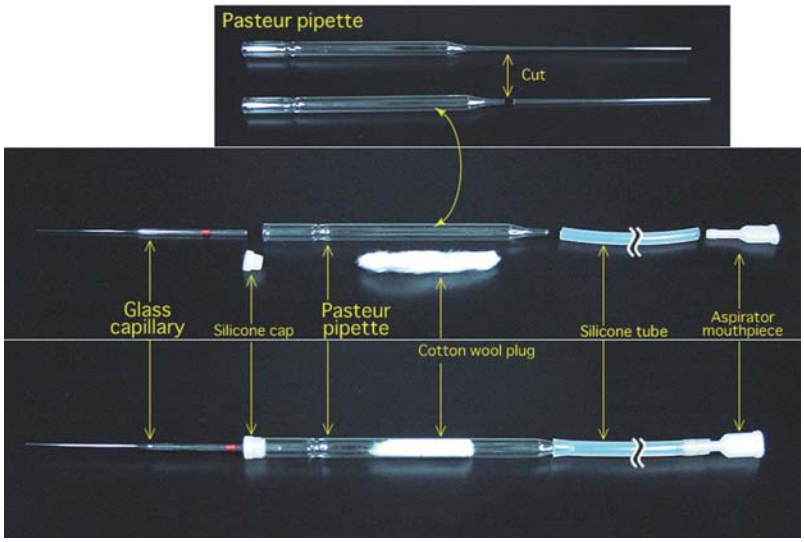


[Polishing the Tip of the Glass Capillary Pipette] No. 01-02

Assembling Capillary Transfer Mouth Pipettes for Embryo Handling

1. Cut the thin part of the Pasteur pipette with an ampoule cutter (leaving approximately 1 cm).
2. Use the flame of an alcohol lamp to polish the cut edge of the Pasteur pipette.
3. Insert a cotton wool plug into the Pasteur pipette.
4. Insert the silicone cap into the opening at the wide end of the Pasteur pipette.
5. Secure a flexible rubber tube to the opening at the thin end of the Pasteur pipette.
6. Cut the rubber tube to a length that you find easy to use, and insert an aspirator mouth piece into the end of the tube.

[Capillary Transfer Mouth Pipette for Embryo Handling]



How To Handle Embryos

1. Hold the mouthpiece of the capillary transfer mouth pipettes in your mouth.
2. Under a microscope, insert the edge of the capillary into the drop of medium. Let the medium be sucked up into the capillary; this is a natural phenomenon known as capillarity.
3. After capillarity has finished, use the mouthpiece to suck the embryos up into the capillary by breathing in, and then to release them by gently breathing out.

[How To Handle Embryos] No. 01-03

1-2 *In Vitro* Fertilization (IVF)

Materials and Equipment

1. PMSG (Pregnant Mare's Serum Gonadotropin, Cat. No. 80056-608; VWR SCIENTIFIC INC.) (37.5 IU/mL in sterile saline)
2. hCG (human Chorionic Gonadotropin, CG-10; Sigma) (37.5 IU/mL in sterile saline)
3. 1 mL disposable syringe
4. FERTIUP® (Preincubation medium: PM, Cat. No. KYD-002-EX, Cosmo Bio Co., Ltd.)
5. CARD MEDIUM® (Cat. No. KYD-003-EX, Cosmo Bio Co., Ltd.)
6. mHTF
7. Liquid paraffin
8. Micropipettes
9. Pipette tips for preparation of dishes
10. Pipette tips for insemination (Pipette Tip Cat. No.114; Quality Scientific Plastics)
11. Plastic dishes (35 mm X 10 mm Cat. No. 430588; CORNING)
12. Fine scissors
13. Pair of watchmaker's #5 forceps
14. Micro-spring scissors (5 mm blade)
15. Dissecting needle
16. Filter paper
17. Glass capillaries for embryo handling
18. Microscope
19. Humidified incubator (37℃, 5% CO_2 in air)

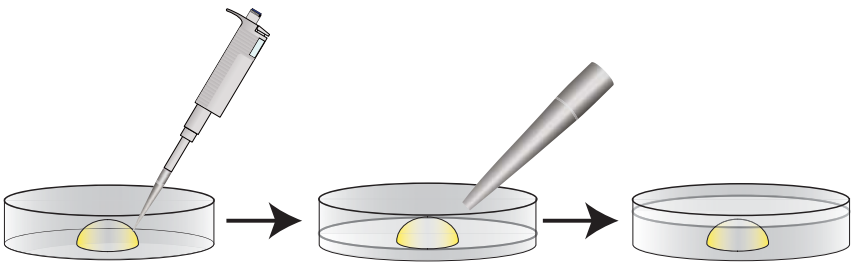
Procedures

Superovulation

1. Induce superovulation by injecting 7.5 IU of pregnant mare's serum gonadotropin (PMSG) i. p. into each mature female mouse (8-12 weeks old). (PMSG is usually administered during the light cycle, between the hours of 14:00 and 18:00).
2. Follow this up 48-52 hours later with a 7.5 IU i. p. injection of human chorionic gonadotropin (hCG).

Preparation of Dishes

1. Prepare dishes as instructed below and keep them in an incubator (37℃, 5% CO_2 in air) to allow them to gas-equilibrate.

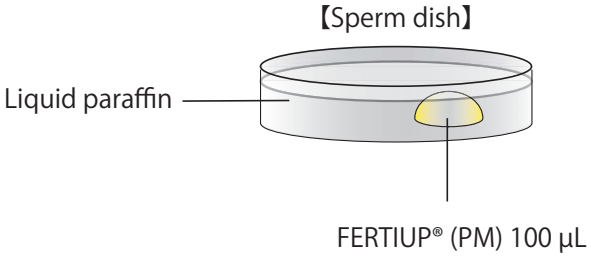


Put some medium on a dish using a micropipette.

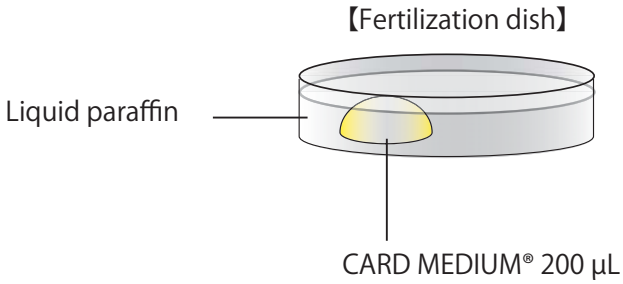
Pour liquid paraffin into the dish.

Cover the medium drop with liquid paraffin.

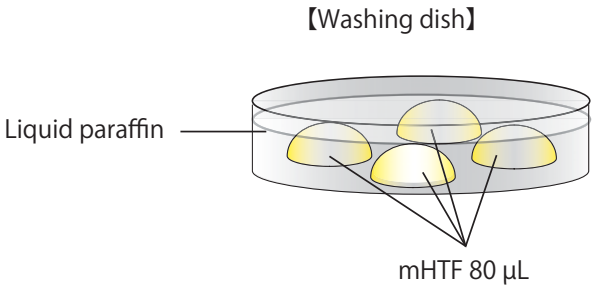
a. Sperm dish
Put 1 drop (100 µL / drop) of FERTIUP® (PM) into a dish and cover it with liquid paraffin 30 minutes before collecting sperm, and place the dish in an incubator.



b. Fertilization dish
Put 1 drop (200 µL / drop) of CARD MEDIUM® into a dish and cover it with liquid paraffin 10 minutes before collecting oocytes, and place the dish in an incubator.



c. Washing dish
Put 4 drops (80 µL / drop) of mHTF into a dish and cover them with liquid paraffin. Place the dish in an incubator for at least 30 minutes



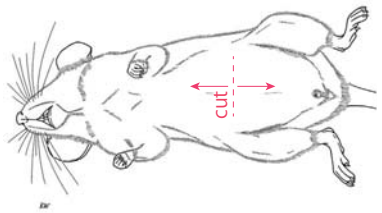
Note

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.

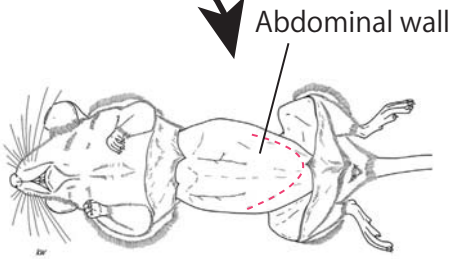
Please refer to the CARD MEDIUM® instruction manual.

Collection of Spermatozoa

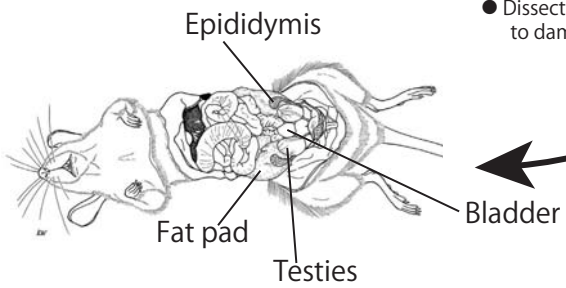
1. Sacrifice 1 or 2 mature male mice (3 to 6 months old) and remove their cauda epididymides, avoiding as much fat, blood and tissue fluid as possible.
2. Place the tissue on sterile filter paper to blot away any blood and fluid.



- Pinch the skin, lifting it upwards, and make a short cut along the dashed line indicated in the diagram above.
- Hold the skin at the opening between your fingers, and pull it firmly towards the head and tail of the mouse, as shown by the arrows in the diagram.



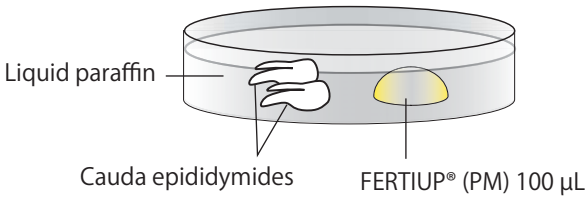
- Dissect the abdominal wall, taking care not to damage the internal organs.



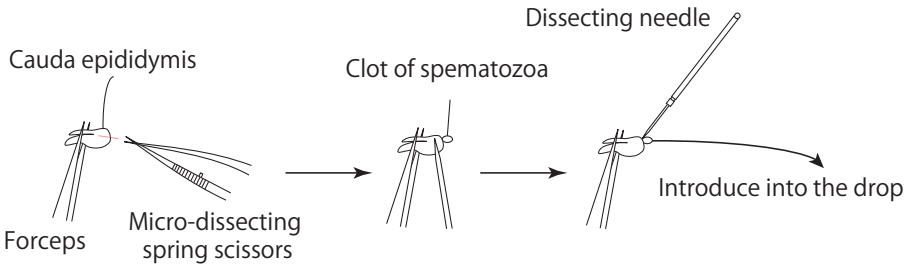
[Removing the Cauda Epididymides] No. 02-01

3. Place the removed cauda epididymides in a sperm dish containing liquid paraffin.

【Sperm dish】



4. Cut the duct of each cauda epididymis using a pair of micro-spring scissors, then use a dissecting needle to gently press the surface of the cauda epididymis and release the sperm within.
5. Use a dissecting needle to introduce the clots of spermatozoa released from the cauda epididymides into the drop of FERTIUP® (PM).

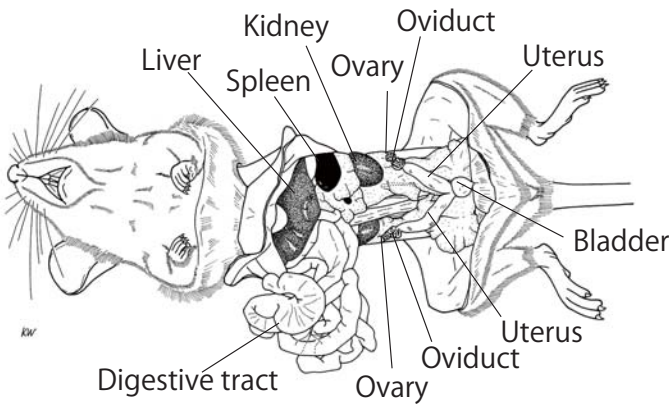


[Releasing Spermatozoa] No. 02-02

6. Allow the sperm to capacitate by placing the suspension in an incubator (37℃, 5% CO_2 in air) for 60 minutes before insemination.

Collection of Oocytes

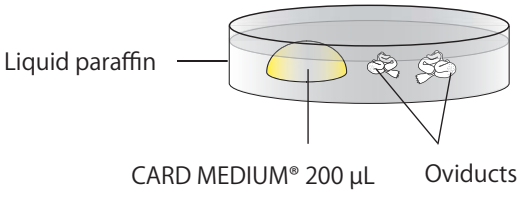
1. Sacrifice a superovulating mature female mouse (8-12 weeks old) approximately 15-17 hours after administering hCG.
2. Dissect the mouse to expose the abdominal cavity.
3. Move the digestive tract from inside the abdomen and expose the uteruses, oviducts and ovaries.
4. Remove the uteruses, oviducts and ovaries, and place them on sterile filter paper.
5. Remove the oviducts (ampullae) only, avoiding as much fat, blood and tissue fluid as possible.



[Removing the Oviduct] No. 02-03

6. Immerse the removed oviducts in liquid paraffin contained within a fertilization dish.

【Fertilization dish】

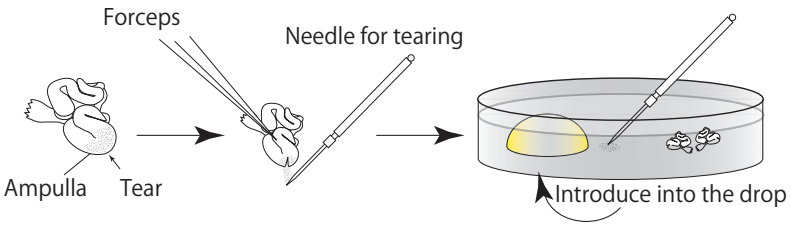


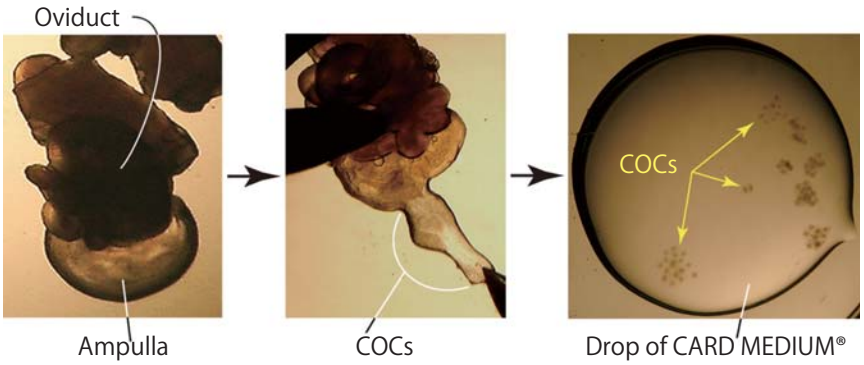
Note

The degree of fertility varies greatly depending on the spermatozoa used. Spermatozoa with high fertility levels can be observed moving in a vortex with high motility at the boundary of the incubation medium. Conversely, spermatozoa which display low motility and poor homogeneity tend to have low fertility levels.

7. Use forceps to hold the oviduct against the base of the fertilization dish, then use a dissecting needle to tear open the ampulla of the oviduct and release the cumulus-oocyte complexes (COCs) from within. Drag them into the drop of CARD MEDIUM® (200 µL).

[Introducing Cumulus-Oocyte Complex (COCs) into a drop of CARD MEDIUM®]



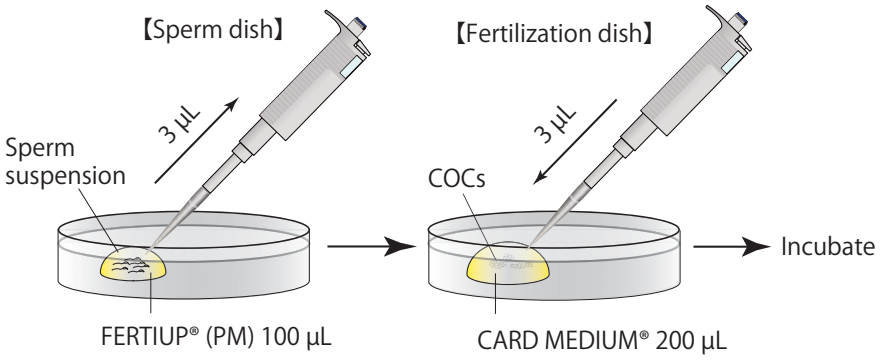


No. 02-04

8. Keep the fertilization dish including COCs in an incubator (37℃, 5% CO_2 in air) for 30-60 minutes before insemination.

Insemination

1. Use the tip of a pipette (Pipette Tip Cat. No. 114; Quality Scientific Plastics) to add appropriate amounts (usually about 3 µL) of the sperm suspension to the drop of CARD MEDIUM® containing the COCs.
2. Place the fertilization dish in an incubator (37℃, 5% CO_2 in air).

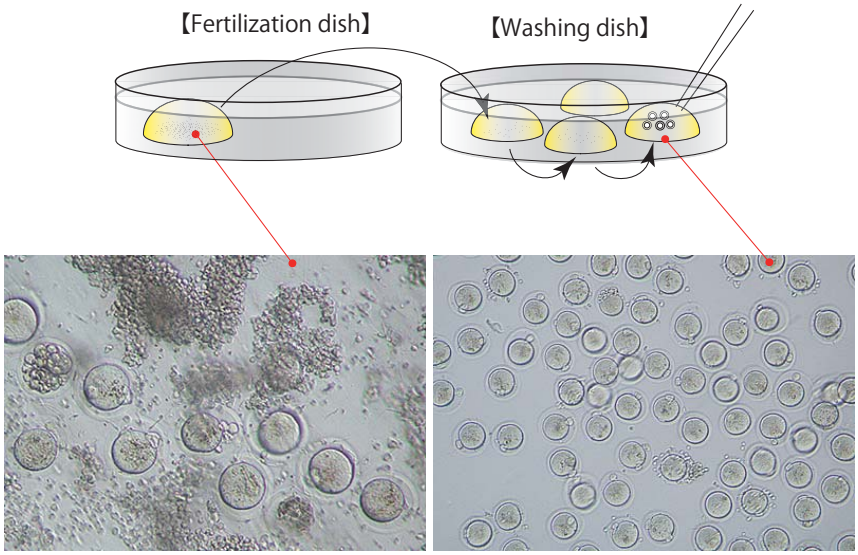


Note

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).

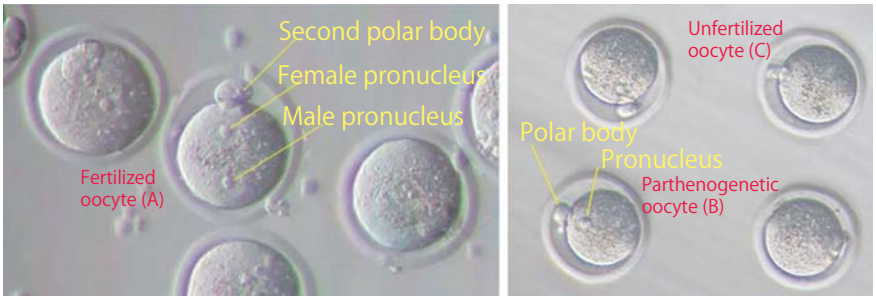
Moreover, when carrying out this process alone, do not sacrifice multiple mice at once; instead, sacrifice one mouse and swiftly remove its oviducts before moving on to the next mouse.

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

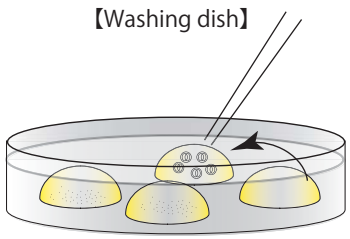


4. 6 hours after insemination, observe the oocytes in the third drop of mHTF and remove any parthenogenetic oocytes which have only one pronucleus.

[Appearance of Fertilized, Unfertilized and Parthenogenetic Oocytes]



5. After overnight culture of the oocytes, transfer the obtained 2-cell stage embryos only to the fourth drop of mHTF in a washing dish. These embryos can be vitrified, transferred to recipient females, or cultured to the blastocyst stage. (Please refer to the chapters of Simple Vitrification of Mouse Embryos on page 54 and Embryo Transfer into the Oviduct on page 66.)



Note

At this stage it is important that you identify and remove any parthenogenetic oocytes.
Please note that if you do not remove the parthenogenetic oocytes at this stage, the next day they develop to the 2-cell stage, at which point it will be impossible to distinguish the fertilized oocytes from the parthenogenetic oocytes.

Note

The fertilized oocyte has both a male and female pronucleus (A).
On the other hand, the parthenogenetic oocyte has only one pronucleus (B) and the unfertilized oocyte does not have any pronuclei (C).

References

1. Toyoda Y., Yokoyama M., and Hosi T. 1971. Studies on the fertilization of mouse eggs *in vitro*. *Jpn. J. Anim. Reprod.* **16**: 147-151.

1-3 *In Vitro* Fertilization (IVF) using Ultra-Superovulation Reagent

Materials and Equipment

1. Ultra-superovulation reagent (CARD HyperOva®)
2. Other materials are the same as those used for IVF using PMSG
   (Please refer to the chapter of *In Vitro* Fertilization on page 6.)

Procedures

Ultra-superovulation

1. Induce superovulation by injecting 0.1-0.2 mL of CARD HyperOva® i.p. into a 26-30 day old female mouse (counting the date of birth as day 0). (CARD HyperOva® is usually administered during the light cycle, between the hours of 17:00 and 18:00).
2. Follow this up 48 hours later with a 7.5IU i.p. injection of human chorionic gonadotropin (hCG).

Preparation of Dishes and Collection of Spermatozoa

1. Prepare dishes and collect spermatozoa in an identical manner to that for IVF using PMSG. (Please refer to the chapter of *In Vitro* Fertilization on page 6.)

Collection of Oocytes

When using CARD HyperOva®, the oviducts of superovulating female mice swell significantly. Please be sure to handle the oviducts directly and carefully following the method shown below so as not to break them.

1. Remove the oviducts (ampullae) from the abdominal cavity of the female mouse.
2. Touch them on sterile filter paper lightly to remove blood and tissue fluid.
3. Immerse them in liquid paraffin contained in a fertilization dish.
4. Use one drop of CARD MEDIUM® (200 µL) per female (2 oviducts).

For procedures thereafter, please refer to the chapter of *In Vitro* Fertilization on page 9.

Insemination

1. For insemination, use 6 µL of sperm suspension which has been pre-incubated in an identical manner to that for IVF using PMSG.

For the other procedures relating to insemination, please refer to the chapter of *In Vitro* Fertilization on page 10.

References

1. Takeo T., Nakagata N. 2015. Superovulation using the combined administration of inhibin antiserum and equine chorionic gonadotropin increases the number of ovulated oocytes in C57BL/6 female mice. *PLoS ONE* 10(5): e0128330. doi:10.1371/journal.pone.0128330
2. Takeo T., Nakagata N. 2016. Immunotherapy using inhibin antiserum enhanced the efficacy of equine chorionic gonadotropin on superovulation in major inbred and outbred mice strains. *Theriogenol*. doi:10.1016/j.theriogenology.2016.04.076

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

Materials and Equipment

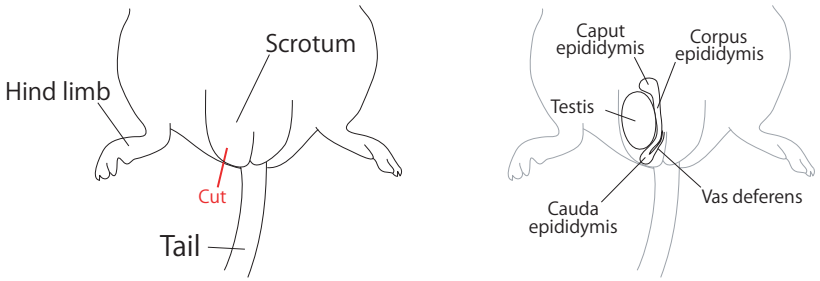
1. Male mouse (over 12 weeks old)
2. Anesthetic
3. Hot plate (37℃)
4. Fine scissors
5. Pair of watchmaker's #5 forceps
6. Wound clip (Autoclip 9 mm; Clay Adams 427631) and clip applicator (Mik-Ron Autoclip Applier; Clay Adams 427630)
7. Temperature data logger (Thermochron iButton Cat. No. DS1921G; Maxim 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.

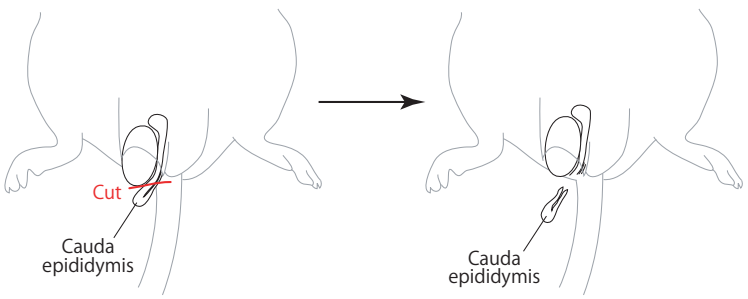
Procedures

Collection of Cauda Epididymis

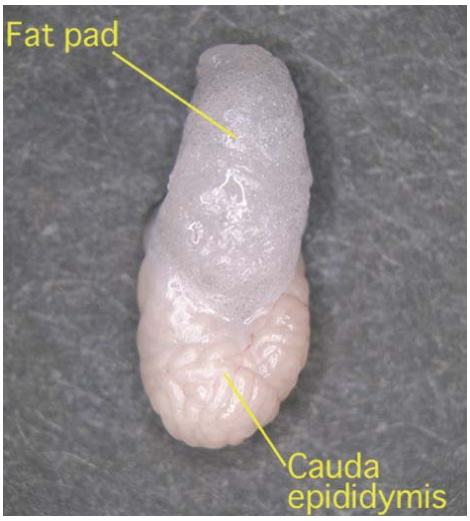
1. Anesthetize a male mouse.
2. Make a small incision in the scrotum of the mouse and expel a cauda epididymis.



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



[Collected Cauda Epididymis]



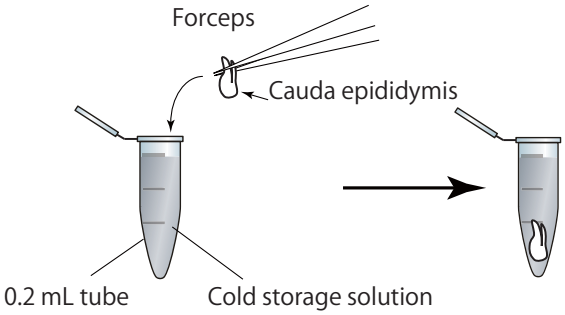
[Removing one Cauda Epididymis from an Anesthetized Male] No. 03-01

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.

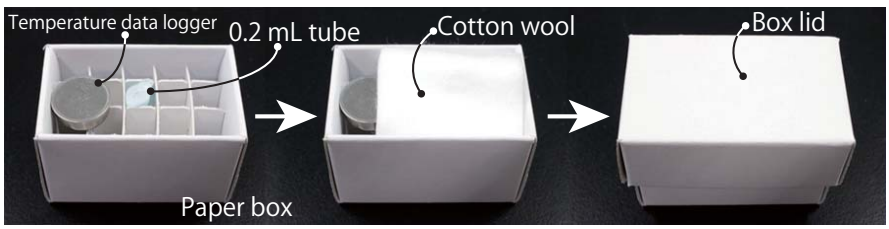
Packing and Transport of Cauda Epididymis

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.



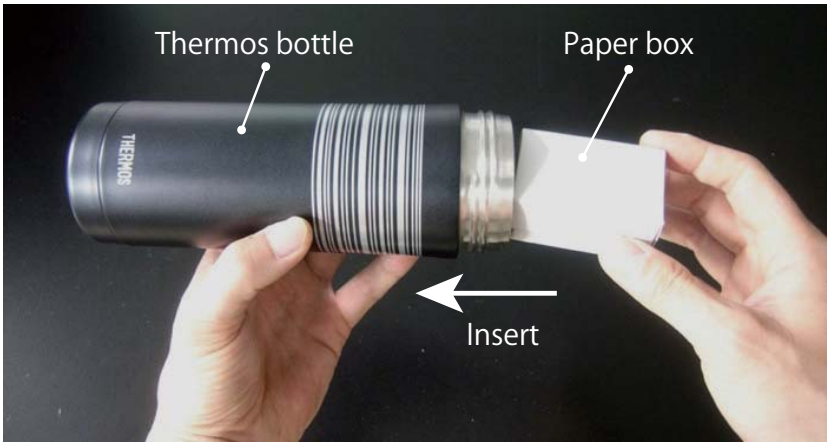
2. Place the tube containing the cauda epididymis, a temperature data logger and a piece of cotton wool in the paper box.



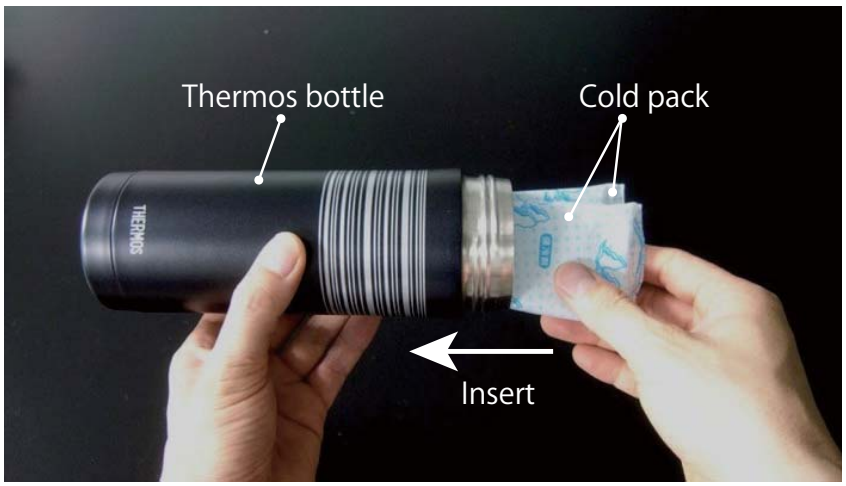
Comment

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.

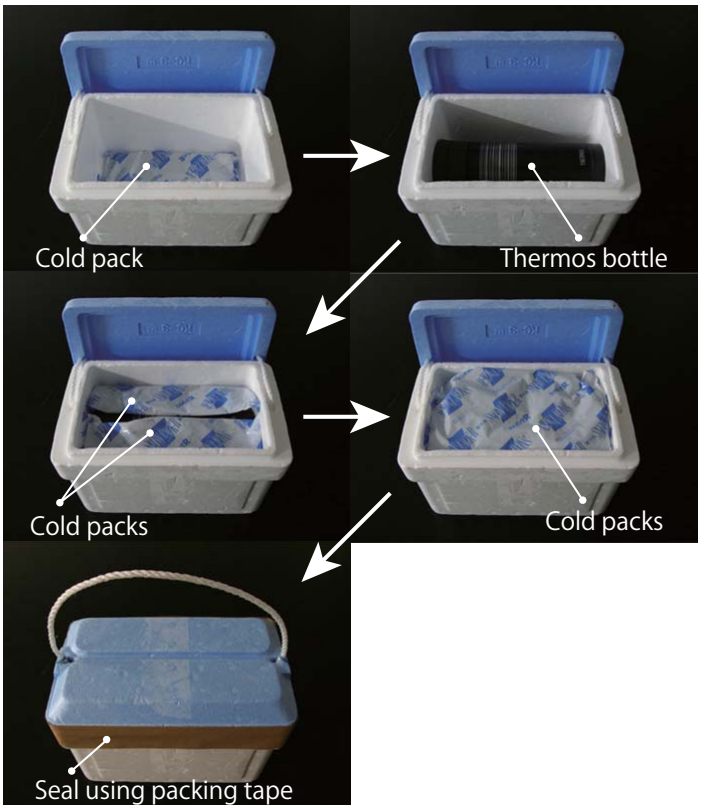
6. Place a cold pack (large) at the bottom of a foam transport box, then put the thermos bottle on top of it.
7. Pack one cold pack (large) on either side of the bottle, then set a further pack (large) on top and close the lid.
8. Seal the lid of the foam transport box using packing tape.

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

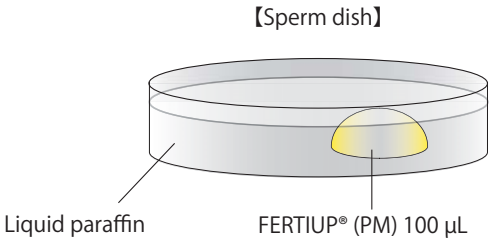
Materials and Equipment

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

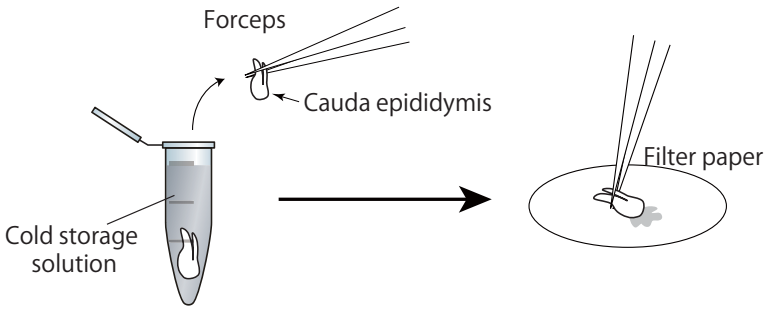
1. Put 1 drop (100 µL / drop) of FERTIUP® (PM) into a dish and cover it with liquid paraffin 30 minutes before collection of epididymal sperm transported at cold temperature, and place the dish in an incubator (37℃, 5% CO_2 in air)



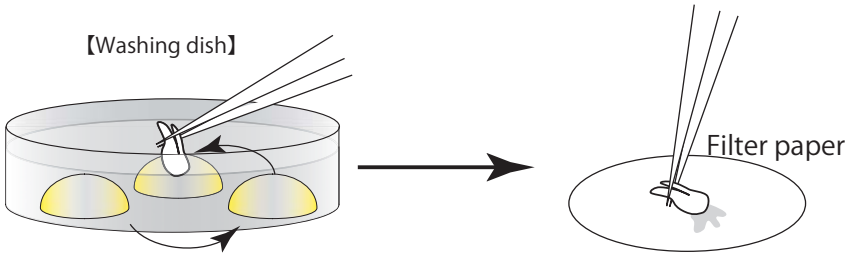
2. Remove the 0.2 mL tube containing the sample from the foam transport box.

[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

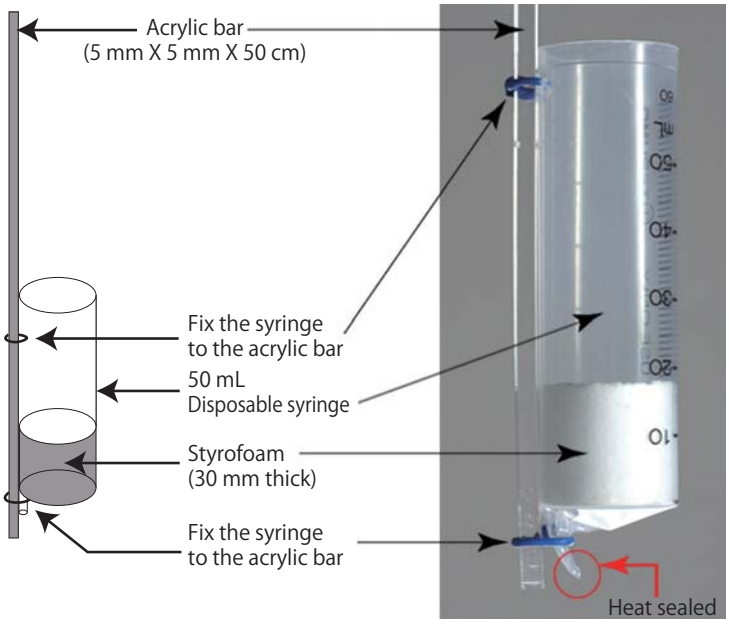
Materials and Equipment

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

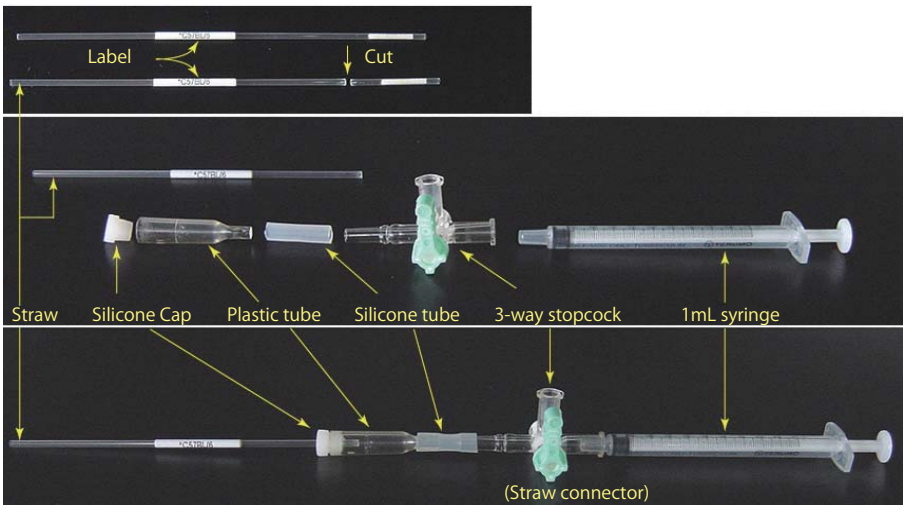
1. Insert a piece of styrofoam tightly into the bottom of the syringe.
2. Heat seal the mouth of the syringe tip.
3. Fix the syringe to the acrylic bar.



Preparing a Straw Connector

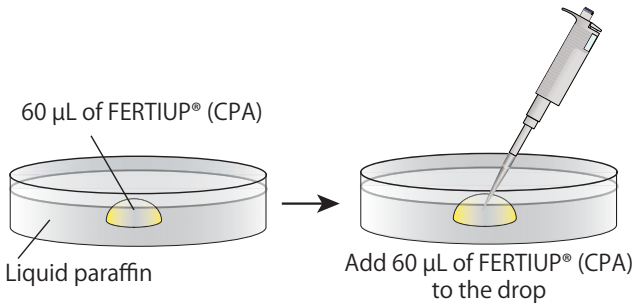
1. Using a 1 mL syringe, a 3-way stopcock, a piece of silicone tube, a plastic tube and a silicone cap, make a straw connector as shown in the diagram below.
2. To use the straw connector, cut away the cotton plug from a straw, then attach the straw to the silicone cap at the end of the connector.

[Connecting the Straw connector and Straw]

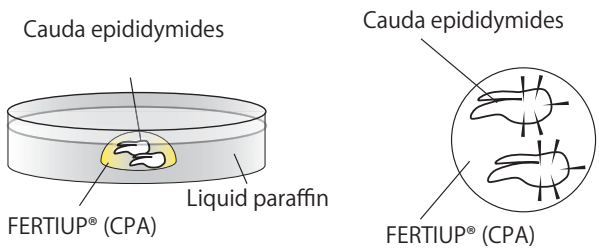


Preparing Sperm Suspension

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.



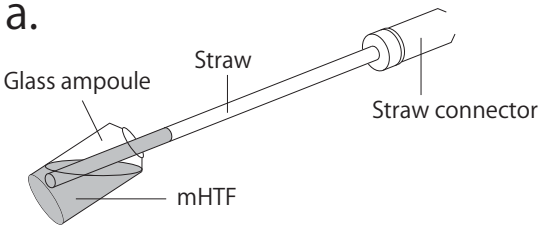
6. Place the dish on a hot plate at 37℃ for 3 minutes. During this time, rotate the dish every minute to disperse sperm from the organs in the FERTIUP® (CPA).

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

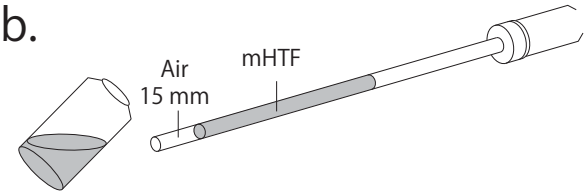
Preparing Freezing Straw Containing Sperm Suspension

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.

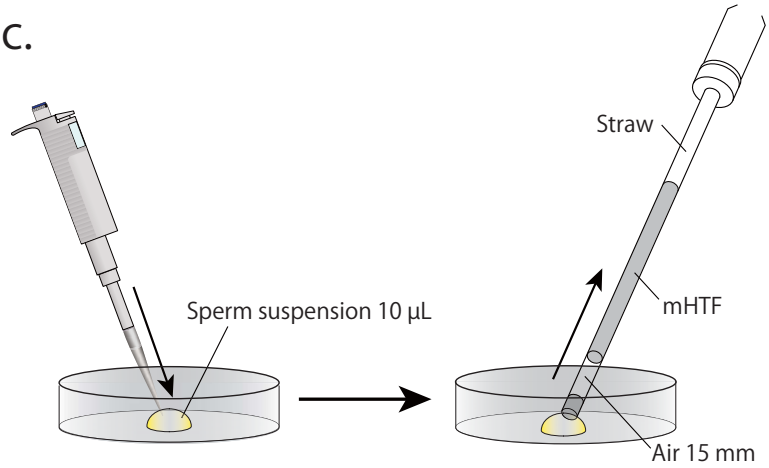
a.



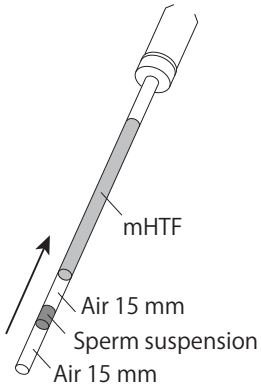
b.



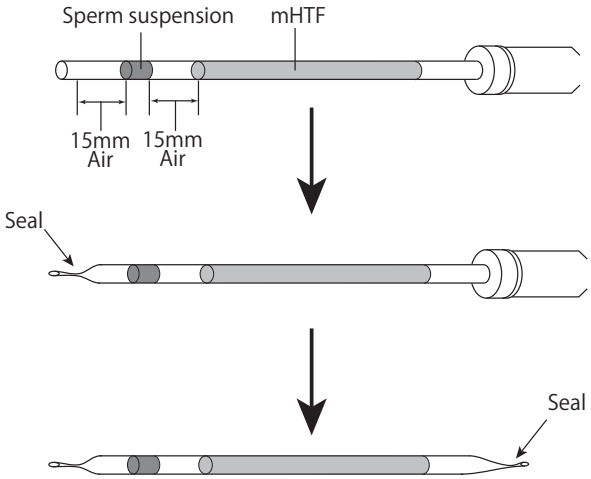
c.



d.



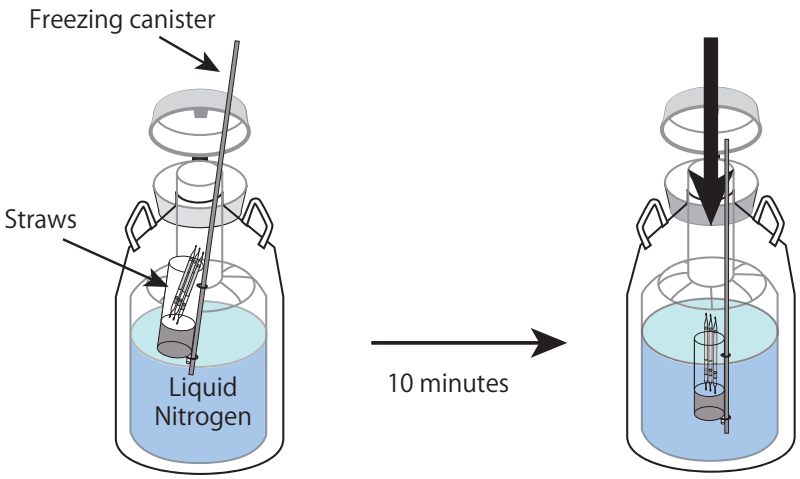
3. Seal both sides of the straw using an impulse sealer.



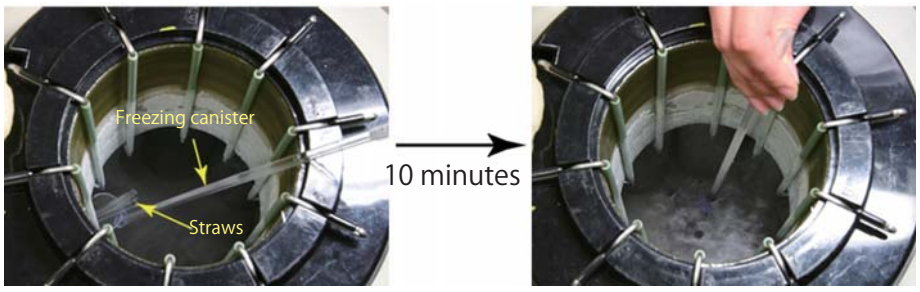
4. Create 10 samples per mouse in the same manner as described above.

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]

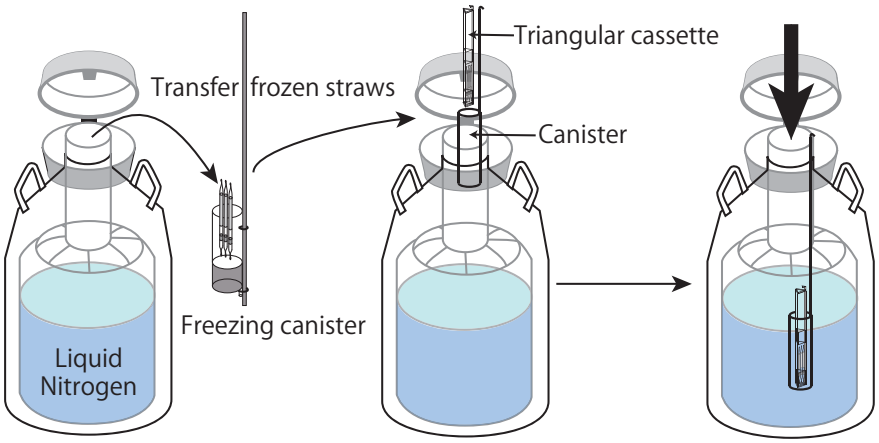


[Freezing the Straws] No. 05-02

Comment

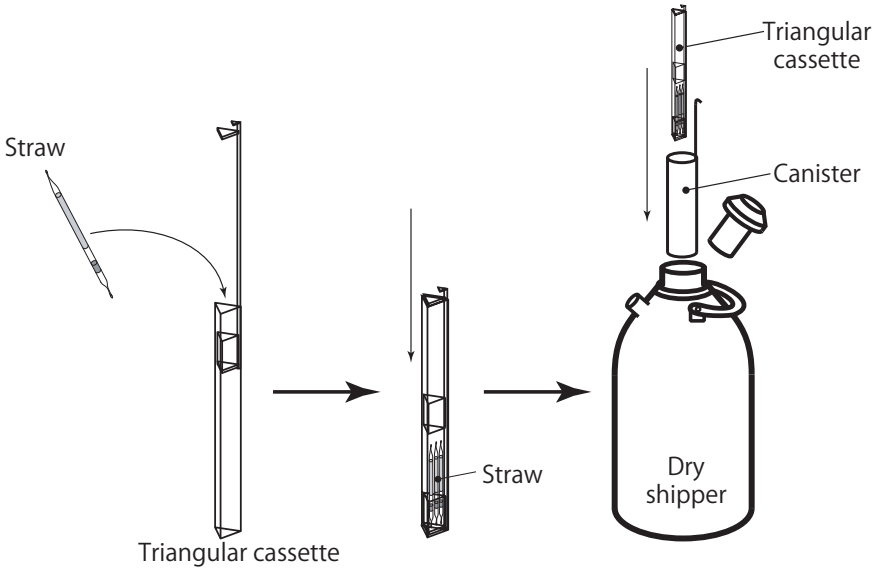
Loading 100 µL of mHTF into the straw prevents the straw from floating on the surface of liquid nitrogen.
This is because the mHTF acts as a weight that forces the straw to sink into the liquid nitrogen.

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.

References

1. Nakagata N., and Takeshima T. 1992. High fertilizing ability of mouse spermatozoa diluted slowly after cryopreservation. *Theriogenol*. **37**: 1283-1291.
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.
3. Nakagata N. 1996. Use of cryopreservation techniques of embryos and spermatozoa for production of transgenic (Tg) mice and for maintenance of Tg mouse lines. *Lab. Anim. Sci.* **46**: 236-238.
4. Okamoto M., Nakagata N., Ueda O., Kamada N., and Suzuki H. 1998. Cryopreservation of gene disrupted mouse spermatozoa. *J. Mamm. Ova. Res.* **15**: 77-80.
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.
6. Nakagawa Y., Fukumoto K., Kondo T., Koga M., Takeshita Y., Nakamuta Y., Sakaguchi M., Haruguchi Y., Tsuchiyama S., Kaneko T., and Nakagata N. 2009. Fertilization ability of C57BL/6J mouse spermatozoa frozen in a dry shipper. *Exp. Anim.* **58**(3) Suppl: 297.
7. 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): 132-137.
8. Nakagata N. 2011. Cryopreservation of mouse spermatozoa and *in vitro* fertilization. *Methods Mol Biol.* **693**: 57-73.
9. Takeo T., Nakagata N. 2011. Reduced glutathione enhances fertility of frozen/thawed C57BL /6 mouse sperm after exposure to methyl-beta-cyclodextrin. *Biol Reprod.* **85**(5): 1066-1072.

3-2 *In Vitro* Fertilization using Cryopreserved Spermatozoa

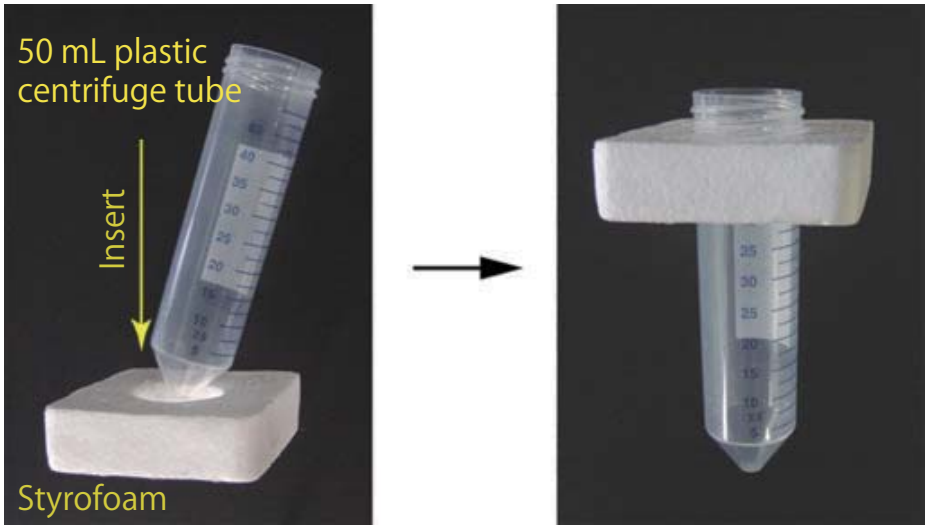
Materials and Equipment

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

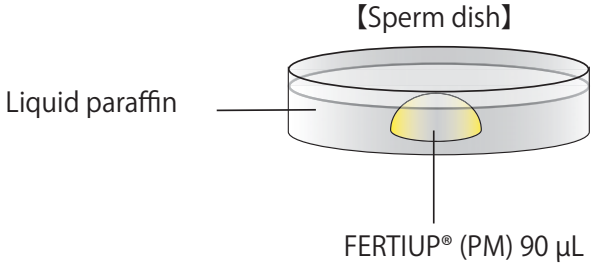
Preparation of the Float for Thawing

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

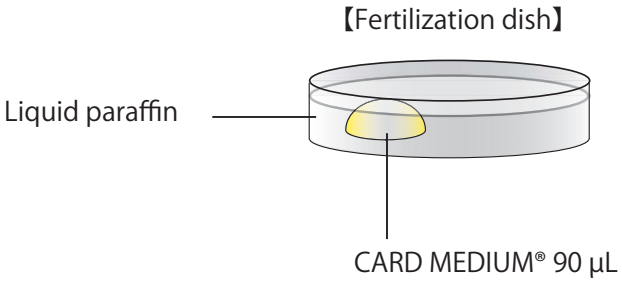


Preparation for Thawing

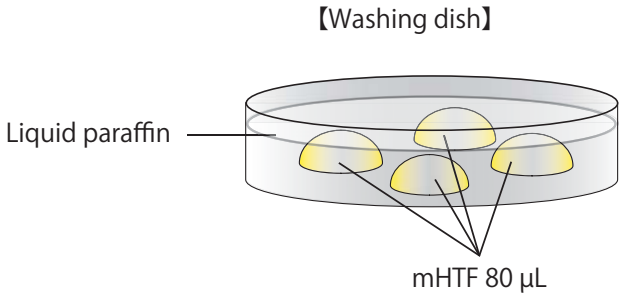
1. Prepare a water bath maintained at 37℃.
2. Pour water (37℃) into the 50 mL plastic centrifuge tube part of the styrofoam/ centrifuge tube assembly, and float it in a water bath.
3. Put 1 drop (90 µL / drop) of FERTIUP®(PM) into a dish and cover it with liquid paraffin 30 minutes before thawing a frozen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

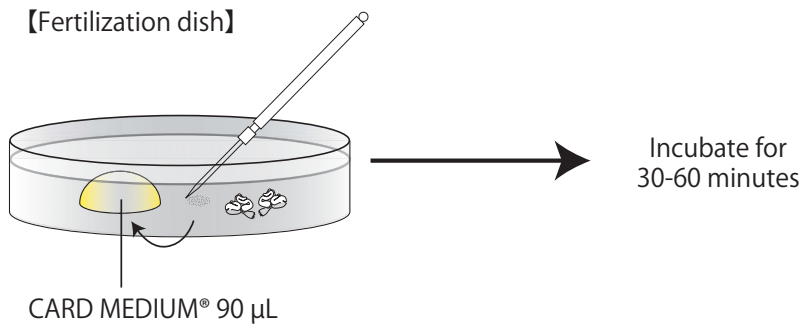


Note

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.

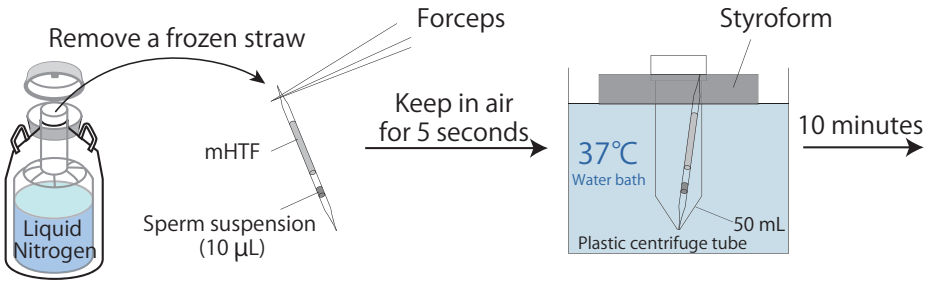
Collection of Oocytes

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.



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

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.

Note

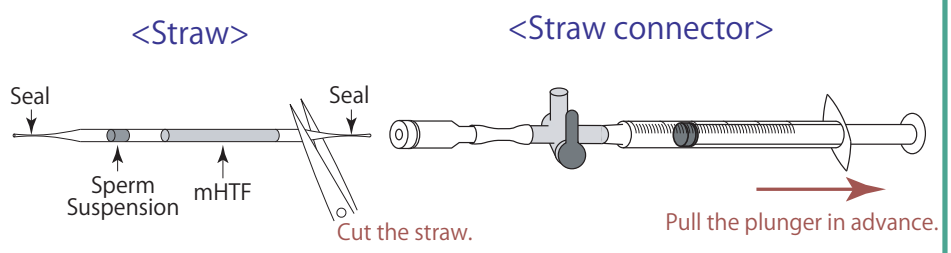
To ensure warming of the frozen sperm, completely immerse the part of the straw containing the sperm in the water bath.

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

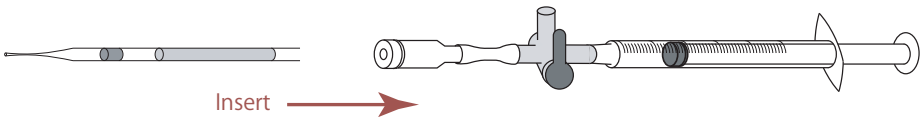
If the straw is not kept in the water bath long enough (10 minutes), the motility of the cryopreserved spermatozoa will be reduced.

Transferring and Preincubating the Thawed Sperm Suspension

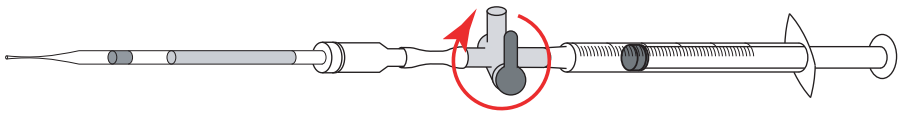
1. Pull the plunger out of the syringe in the straw connector, and cut the straw between the mHTF and the seal.



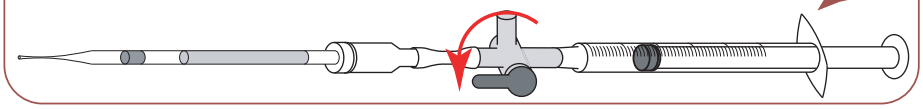
2. Insert the cut straw into the straw connector.



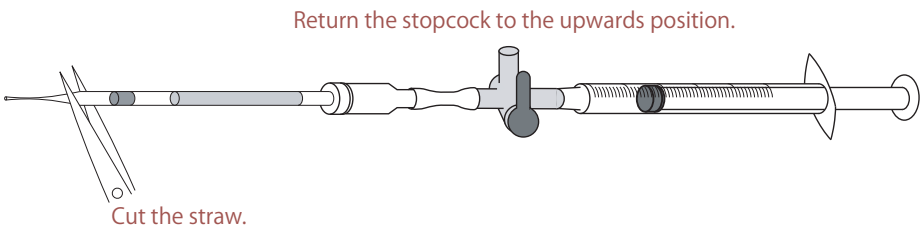
3. Because the insertion of the straw into the straw connector creates pressure inside the straw, turn the stopcock in order to release the pressure.



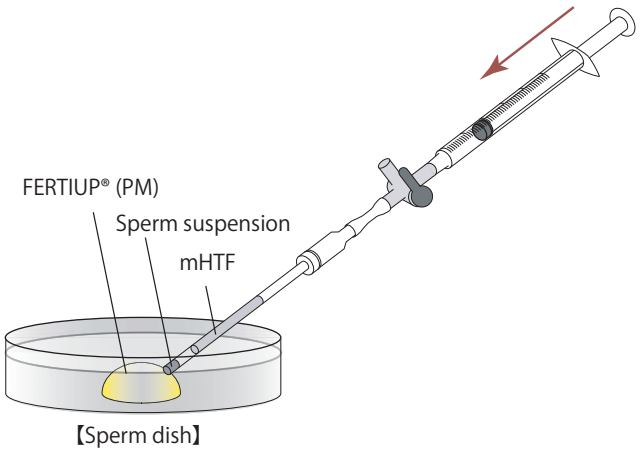
If you have forgotten to pull the pulunger out of the syringe in advance, you can do so by turning the stopcock to the left.



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



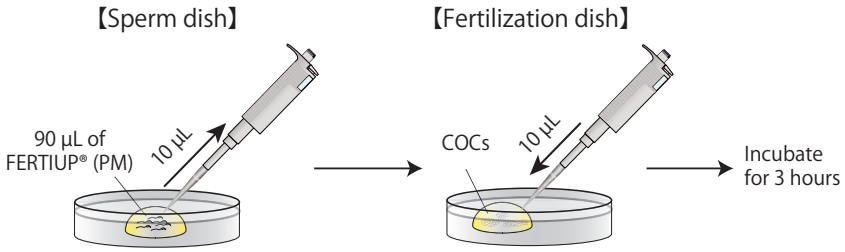
5. Push the plunger to transfer only the sperm suspension into the drop of FERTIUP®(PM) (sperm dish), and place the dish in an incubator (37℃ , 5% CO_2 in air) for 30 minutes.



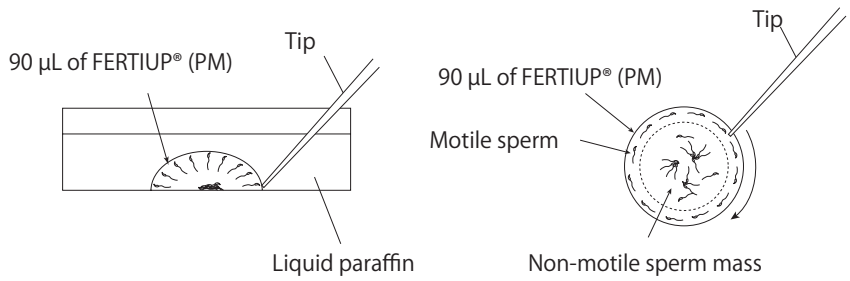
[Transfer of the Thawed Sperm Suspension] No. 06-02

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

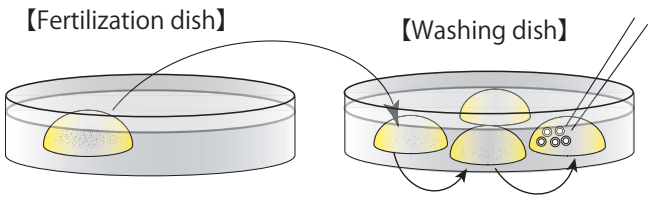
Spermatozoa with high motility have a tendency to gather near the edge of the drop.

Comment

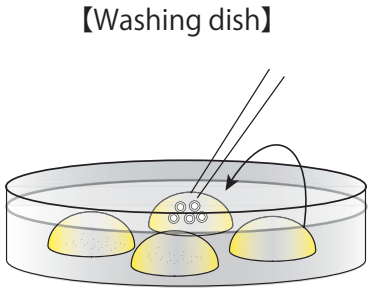
It is possible to aspirate 10 µL of sperm suspension 3-4 times per drop.

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.)



References

1. Nakagata N., and Takeshima T. 1992. High fertilizing ability of mouse spermatozoa diluted slowly after cryopreservation. *Theriogenol*. **37**: 1283-1291.
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.
3. Nakagata N. 1996. Use of cryopreservation techniques of embryos and spermatozoa for production of transgenic (Tg) mice and for maintenance of Tg mouse lines. *Lab. Anim. Sci.* **46**: 236-238.
4. Okamoto M., Nakagata N., Ueda O., Kamada N., and Suzuki H. 1998. Cryopreservation of gene disrupted mouse spermatozoa. *J. Mamm. Ova. Res.* **15**: 77-80.
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.
6. Nakagawa Y., Fukumoto K., Kondo T., Koga M., Takeshita Y., Nakamuta Y., Sakaguchi M., Haruguchi Y., Tsuchiyama S., Kaneko T., and Nakagata N. 2009. Fertilization ability of C57BL/6J mouse spermatozoa frozen in a dry shipper. *Exp. Anim.* **58**(3) Suppl: 297.
7. 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): 132-137.
8. Nakagata N. 2011. Cryopreservation of mouse spermatozoa and *in vitro* fertilization. *Methods Mol Biol.* **693**: 57-73.
9. Takeo T., Nakagata N. 2011. Reduced glutathione enhances fertility of frozen/thawed C57BL/6 mouse sperm after exposure to methyl-beta-cyclodextrin. *Biol Reprod.* **85**(5): 1066-1072.

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

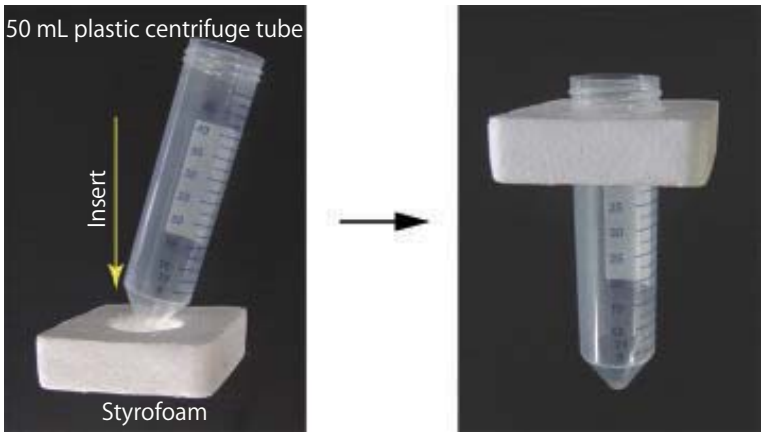
Materials and Equipment

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

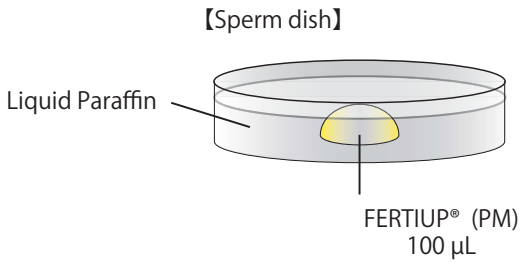
Preparation of the Float for Thawing

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

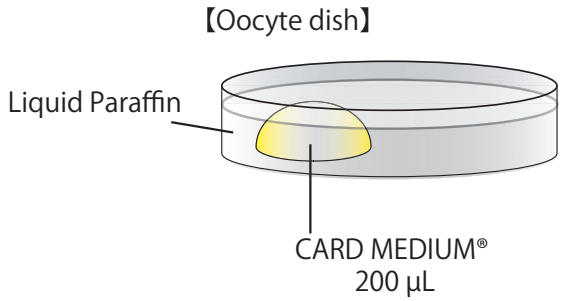


Preparation for Thawing

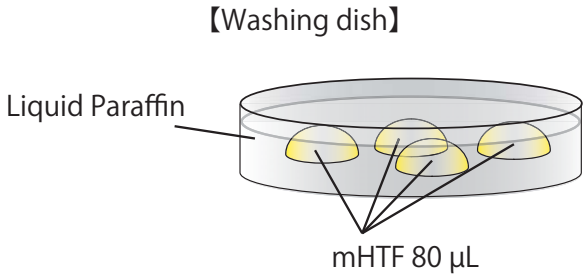
1. Prepare a water bath to 37℃.
2. Pour water (37℃) into the 50 mL plastic centrifuge tube section of the styrofoam/centrifuge tube assembly, and float it in a water bath.
3. 30 minutes before thawing a frozen sperm sample, put 1 drop (100 µL/drop) of FERTIUP® (PM) into a dish and cover it with liquid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

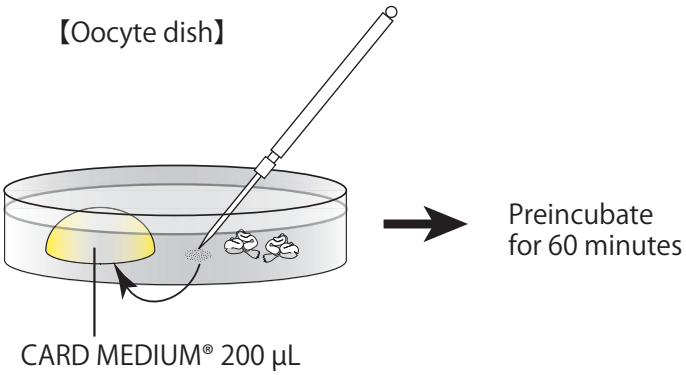


5. Put 4 drops (80 µL / drop) of mHTF into a dish and cover them with liquid paraffin. Place the dish in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.



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.

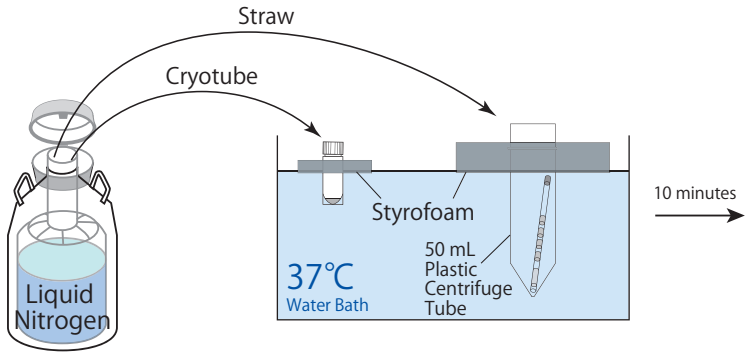
Note

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).

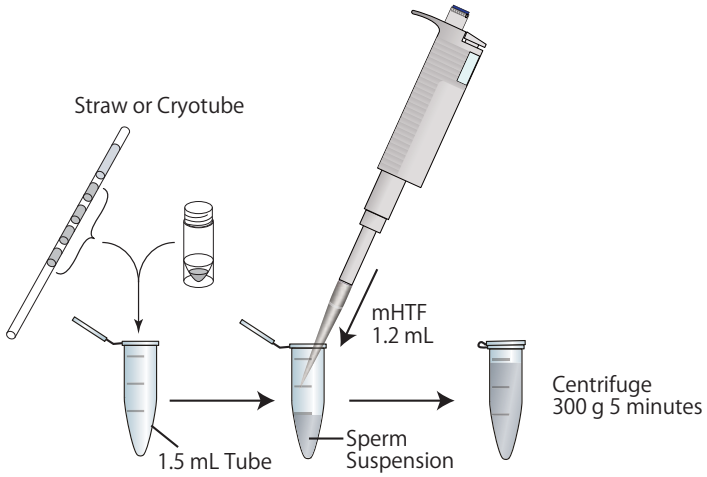
Moreover, when carrying out this process alone, do not sacrifice multiple mice at once; instead, sacrifice one mouse and swiftly remove its oviducts before moving on to the next mouse.

Thawing the Mouse Spermatozoa

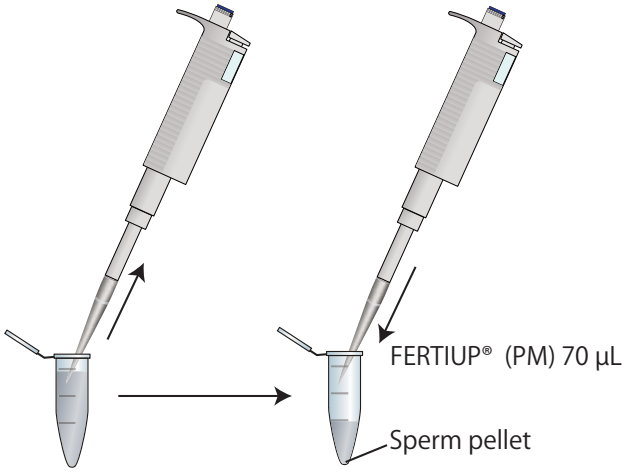
1. Remove a frozen sperm sample from the liquid nitrogen. If the sperm sample is stored in a cryotube, open the cap and discard any liquid nitrogen in the tube. Immerse the sample in a water bath maintained at 37℃ (using a styrofoam or the styrofoam/centrifuge tube assembly) for 10 minutes.



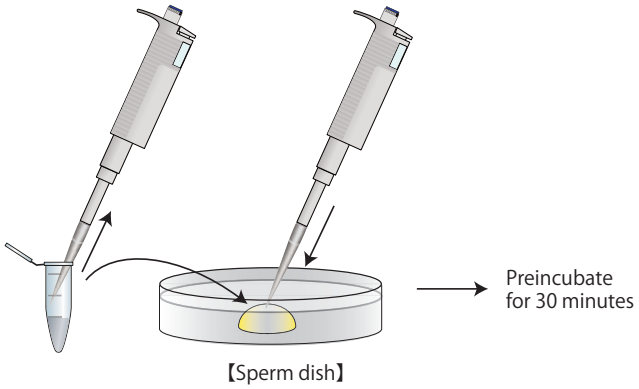
2. Transfer the sperm suspension from the cryotube or the straw into a 1.5 mL tube. Slowly add 1.2 mL of mHTF kept at 37℃ to the tube, and centrifuge it at 300 g at room temperature for 5 minutes.



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).

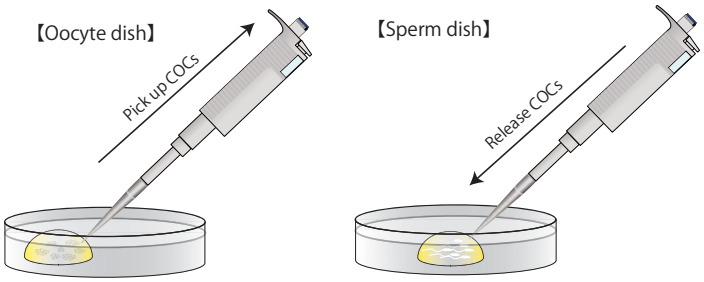


4. After pipetting gently, transfer all of the contents in the tube into the 100 µL drop of FERTIUP® (PM) (Sperm dish). Place the dish in an incubator (37℃ , 5% CO_2 in air) for 30 minutes.

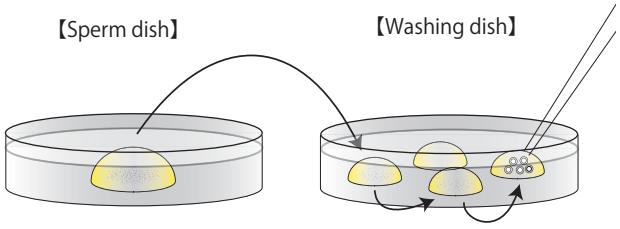


Insemination

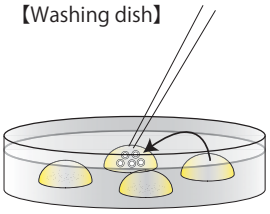
1. Using a tip, suck up the preincubated COCs with a minimum amount of medium from the drop of CARD MEDIUM® (Oocyte dish). Then, release them into the drop of sperm suspension (Sperm dish), and incubate it in an incubator (37℃, 5% CO_2 in air).



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.)



References

1. Nakagata N., Takeo T., Fukumoto K., Haruguchi Y., Kondo T., Takeshita Y., Nakamuta Y., Umeno T., and Tsuchiyama S. 2014. Rescue *in vitro* fertilization method for legacy stock of frozen mouse sperm. *J Reprod Dev.* **60**(2): 167-170.

4-1 Preparation of Laser-microdissected Oocytes

Certain strains of cryopreserved spermatozoa, such as a number of inbred strains, may possess a low fertilizing ability. In order to overcome this impediment, we use oocytes which have been laser-microdissected for *in vitro* fertilization.

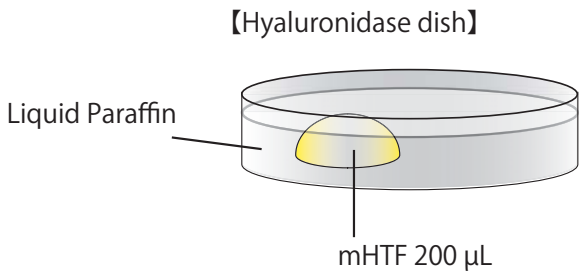
Materials and Equipment

1. Plastic dishes (35 mm X 10 mm Cat. No. 430588; CORNING)
2. mHTF
3. Liquid paraffin
4. Hyaluronidase in mHTF (Hyaluronidase, Cat. No. H-3506, Sigma)
5. The *Saturn 3* laser system (Research Instruments Ltd, Cornwall, UK)
6. Humidified incubator (37℃, 5% CO_2 in air)

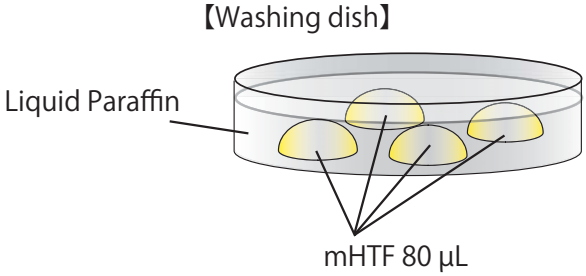
Procedures

Preparation of Sperm and Dishes

1. For IVF, sperm must be prepared in the methods described at the chapters of *In Vitro* Fertilization on page 8, *In Vitro* Fertilization using Epididymal Sperm Transported at Cold Temperature on page 18 and *In Vitro* Fertilization using Cryopreserved Spermatozoa on page 28.
2. Put a 200 µL drop of mHTF into a dish. Cover it with liquid paraffin and place them in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.

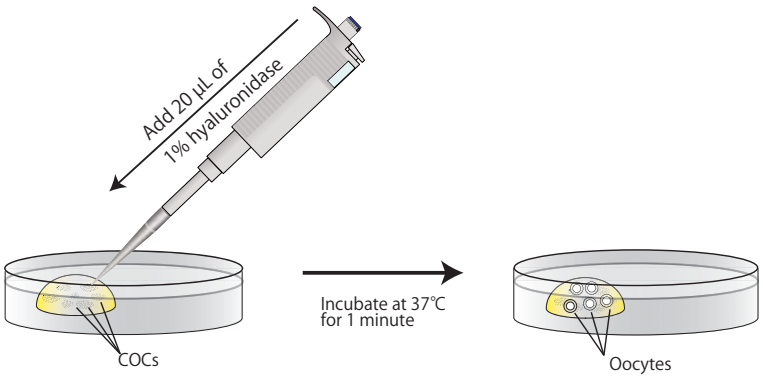


3. Put 4 drops (80 µL/drop) of mHTF into a dish. Cover them with liquid paraffin and place them in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.

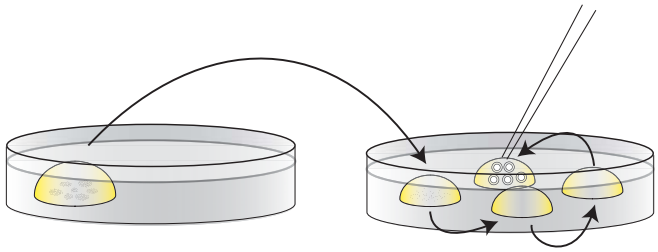


Preparation of Denuded Oocytes

1. Collect cumulus-oocyte complexes (COCs) from superovulated female mice and introduce them into a 200 µL drop of mHTF (Hyaluronidase dish).
(Please refer to the chapter of *In Vitro* Fertilization on page 6 and 9.)
2. Add 20 µL of 1% hyaluronidase to the drop of mHTF containing the COCs, and keep the dish in an incubator (37℃, 5% CO_2 in air) for 1 minute.

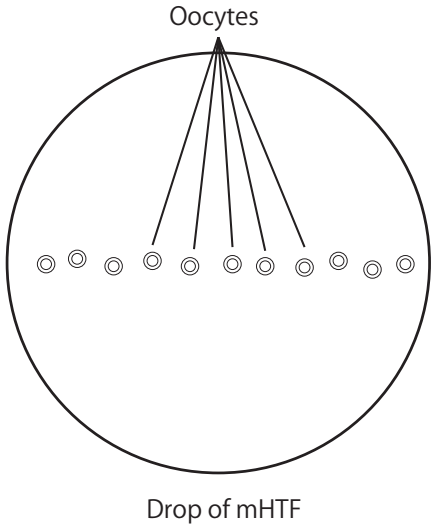


3. Promptly collect and transfer the oocytes into a 80 µL drop of mHTF (Washing dish), and in turn wash them in the drops in the washing dish.



Dissection of the Zona Pellucida using a Laser

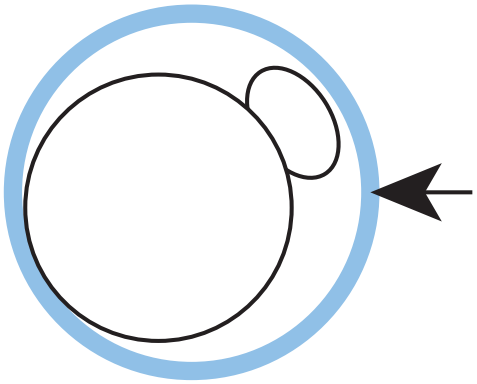
1. Put a 100 µL drop of mHTF into a dish. Cover it with liquid paraffin and place it in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.
2. Transfer fifty denuded oocytes into the drop of 100 µL mHTF.
3. Arrange the oocytes in a line along the bottom of the plastic dish.



Comment

If some cumulus cells become attached to the zona pellucida of the oocytes, they can be removed by manipulating the glass capillary.

4. Set the dish containing the oocytes on the *Saturn 3* laser system.
5. Target the zona pellucida at a spot adjacent to the first polar body and dissect it with a laser beam (see arrow).



[Dissection of Zona Pellucida using a Laser] No. 08-01

6. After dissecting the zona pellucida of all oocytes, transfer them into a drop of CARD MEDIUM® for fertilization. Place the dish in a CO_2 incubator.
(Please refer to the chapters of *In Vitro* Fertilization on page 6, *In Vitro* Fertilization using Epididymal Sperm Transported at Cold Temperature on page 18 and *In Vitro* Fertilization using Cryopreserved Spermatozoa on page 26.)

Note

To avoid injuring the plasma membrane of the oocytes, aim the laser at the area with the widest distance between the plasma membrane and the zona pellucida.

Note

The diameter of the hole is 10-12.5 µm and the pulse length is 0.55-0.60 ms.

References

1. Kaneko T., Yanagi M., Nakashima T., and Nakagata N. 2006. The improvement in fertility of cryopreserved mouse spermatozoa showing low fertility using laser-microdissected oocytes. *Reprod. Med. Biol.* **5**(4): 249-254.
2. Anzai M., Nishiwaki M., Yanagi M., Nakashima T., Kaneko T., Taguchi Y., Tokoro M., Shin SW., Mitani T., Kato H., Matsumoto K., Nakagata N., and Iritani A. 2006. Application of laser-assisted zona drilling to *in vitro* fertilization of cryopreserved mouse oocytes with spermatozoa from a subfertile transgenic mouse. *J Reprod Dev.* **52**(5): 601-606.

4-2 Partial Zona Dissection (PZD)

If you cannot utilize laser-microdissection instruments, you can dissect the zona pellucida of oocytes manually under a stereomicroscope.

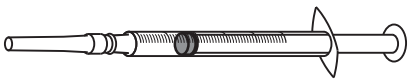
Materials and Equipment

1. Female mice superovulated with PMSG and hCG
   (Please refer to the chapter of *In Vitro* Fertilization on page 6.)
2. mHTF
3. Hyaluronidase in mHTF (Hyaluronidase, Cat. No. H-3506, Sigma)
4. 0.3 M sucrose (BSA-)
5. 0.3 M sucrose (BSA+)
6. Liquid paraffin
7. Plastic dish (35 mm X 10 mm Cat. No. 430588; CORNING)
8. Tip (10-100 µL volume)
9. Micropipette

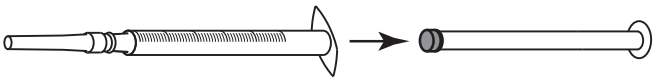
Procedures

Preparing the Needle for PZD

1. A 1mL disposable syringe with a 30 gauge needle should be altered for use in PZD as shown in the diagram below.

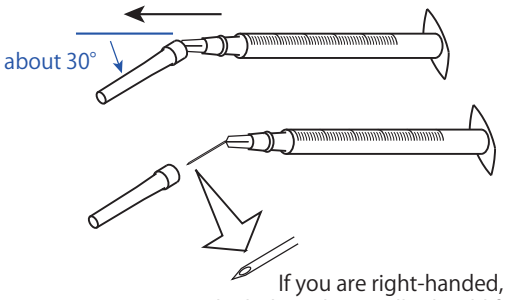


Take a 1 mL syringe with a 30 G needle.



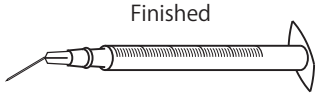
Remove and discard the plunger.

Remove the needle cover,
and use the cover to bend the needle about 30°.



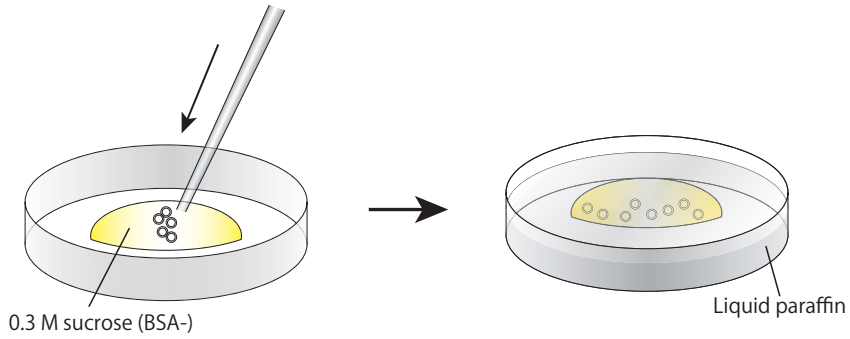
If you are right-handed,
the hole in the needle should face you
as shown in the diagram.

Finished

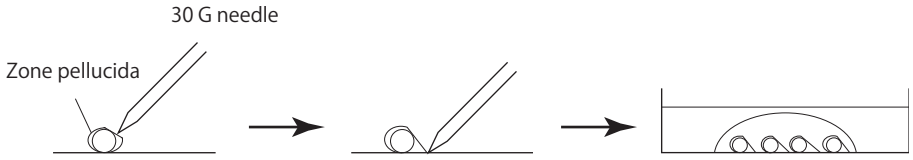


PZD

1. Collect oocytes from the oviducts of superovulated females 14-15 hours after injecting them with hCG. Denude the oocytes with hyaluronidase.
   (Please refer to the chapters of *In Vitro* Fertilization on page 9 and Preparation of Laser-microdissected Oocytes on page 37.)
2. Introduce the denuded oocytes into the upper part of a 100 µL drop of 0.3 M sucrose (BSA-) in a dish.
3. When the oocytes sink to the bottom of the dish, cover the drop with liquid paraffin.

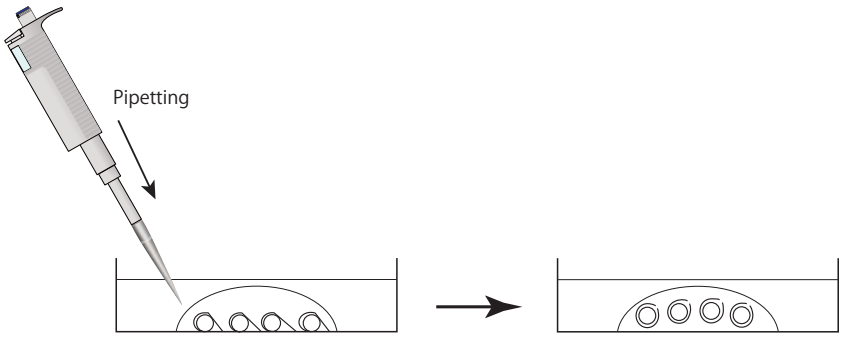


4. Under a stereomicroscope, partially dissect the zona pellucida (PZD) of the oocytes using a single downward motion with a 30 gauge needle.



[PZD] No. 09-01

5. Following PZD, neutralize the electrostatic attraction between the zona pellucida and dish surface by adding 20 µL of 0.3 M sucrose (BSA+) to the drop.
6. To remove PZD oocytes from the dish surface, spray sucrose solution on the oocytes using a micropipette.



7. Wash the PZD oocytes gently 3 times in CARD MEDIUM® to remove any leftover sucrose.

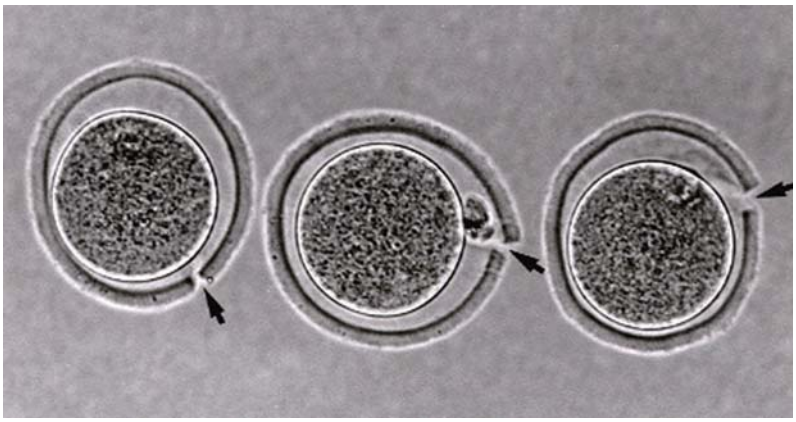
Comment

When the oocytes are placed into the 0.3 M sucrose (BSA-), the ooplasm shrinks due to osmotic forces and an electrostatic interaction occurs between the zona pellucida of the oocytes and the dish surface.
As a result, the perivitelline space widens and the oocytes become attached to the bottom of the dish.

Comment

The spray should be applied from the opposite side of the slit to prevent oocytes from escaping out of the zona pellucida.

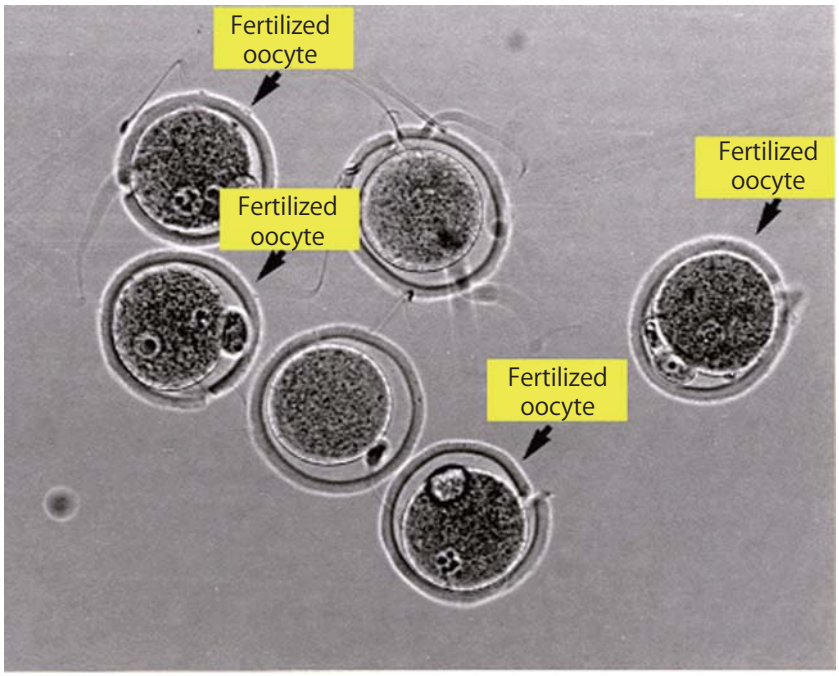
[Micrograph : PZD Oocytes]



In Vitro Fertilization and Embryo Transfer

1. Introduce the PZD oocytes into the CARD MEDIUM® containing the spermatozoa prepared previously (insemination).
   (Please refer to the chapters of *In Vitro* Fertilization on page 6, *In Vitro* Fertilization using Epididymal Sperm Transported at Cold Temperature on page 18 and *In Vitro* Fertilization using Cryopreserved Spermatozoa on page 26.)
2. Gently wash the fertilized oocytes twice in fresh mHTF 3 hours after insemination, then culture them for 3 days until they have developed to the early blastocyst stage.

[Micrograph : Fertilized Oocytes]



3. Transfer the early blastocysts into the uterine horns of a recipient on Day 3 of pseudopregnancy (Day 1 is the day on which vaginal plug is observed).
   Please refer to the chapter of Embryo Transfer into the Uterus on page 72.

References

1. Nakagata N., Okamoto M., Ueda O., and Suzuki H. 1997. The positive effect of partial zona-pellucida dissection on the *in vitro* fertilizing capacity of cryopreserved C57BL/6J transgenic mouse spermatozoa of low motility. *Biol. Reprod.* **57**: 1050-1055.

Comment

If 2-cell stage embryos are transferred to the oviducts of recipients on Day 1 of pseudopregnancy, the rate of development into live offspring will be very low.
This is because the blastomeres of the embryos escape from the zona pellucida via the peristaltic action of the oviduct when they travel through the oviduct to the uterus.

4-3 Collecting 2-Cell Stage Embryos

Materials and Equipment

1. Pair of watchmaker's #5 forceps
2. Fine scissors
3. KSOM/AA
4. Liquid paraffin
5. Plastic dish (35 mm X 10 mm Cat. No. 430588; CORNING)
6. 1 mL syringe
7. Flushing needle (30 G blunt needle)
8. Transfer pipettes

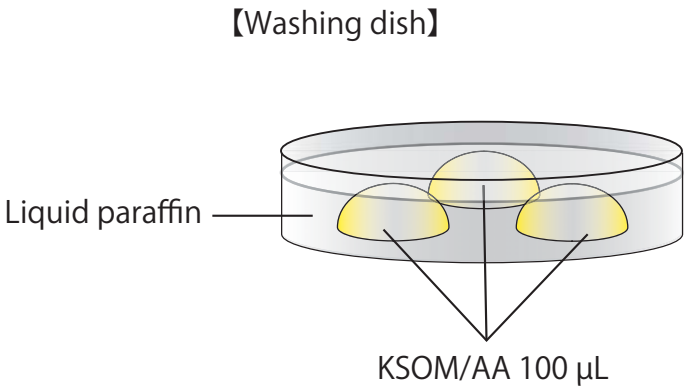
Procedures

Superovulation and Selection of Plugged Females

1. Inject females i. p. (8-12 weeks of age) with 7.5 IU of PMSG (14:00-18:00).
2. Inject females i. p. with 7.5 IU of hCG 48-52 hours after the PMSG injection. Following the hCG injection, allow females to mate with males.
3. Check for vaginal plugs the morning after mating.
(Please refer to the chapter of Embryo Transfer into the Oviduct on page 66.)

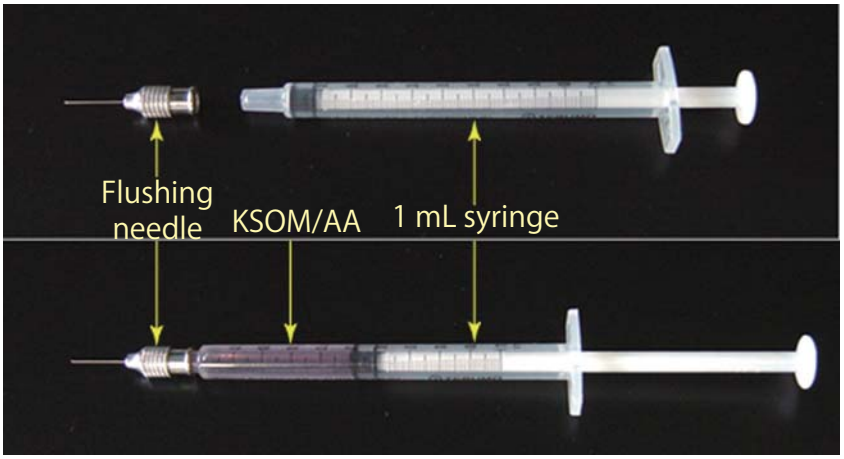
Dish Preparation

1. Put 3 drops (100 µL / drop) of KSOM/AA 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.



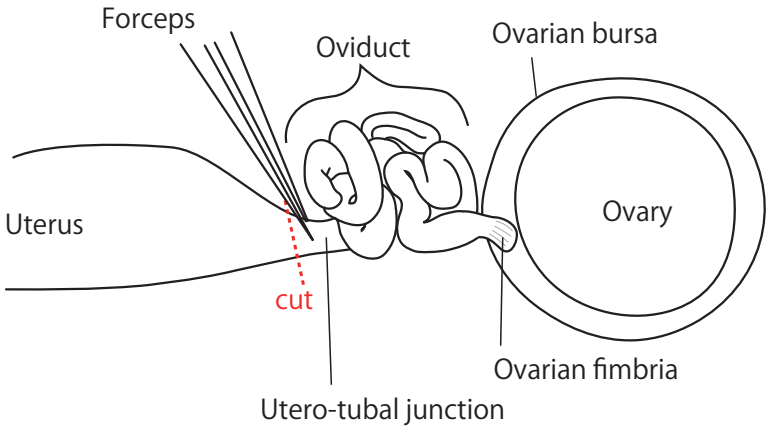
Preparation of Flushing Needle

1. Fill a syringe with KSOM/AA and connect it to a blunted flushing needle.
2. Test the syringe to ensure that it is free of air bubbles, and that the KSOM/AA is flowing smoothly.

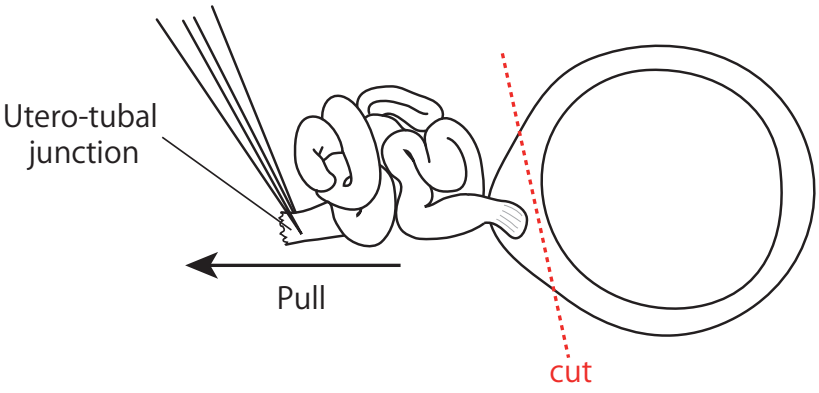


Collecting Embryos

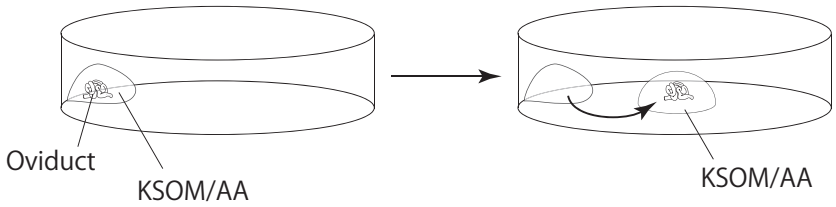
1. One day after a vaginal plug has been observed, remove the uteruses, oviducts and ovaries from the females, and place them on sterile filter paper.
(Please refer to the chapter of *In Vitro* Fertilization on page 9.)
2. Pinch the utero-tubal junction and make a cut on the uterus side of it.



3. Pull the utero-tubal junction to separate the infundibulum from the ovary, then cut the ovarian bursa.



4. After washing the oviduct in a drop of KSOM/AA, put it into another drop of KSOM/AA.

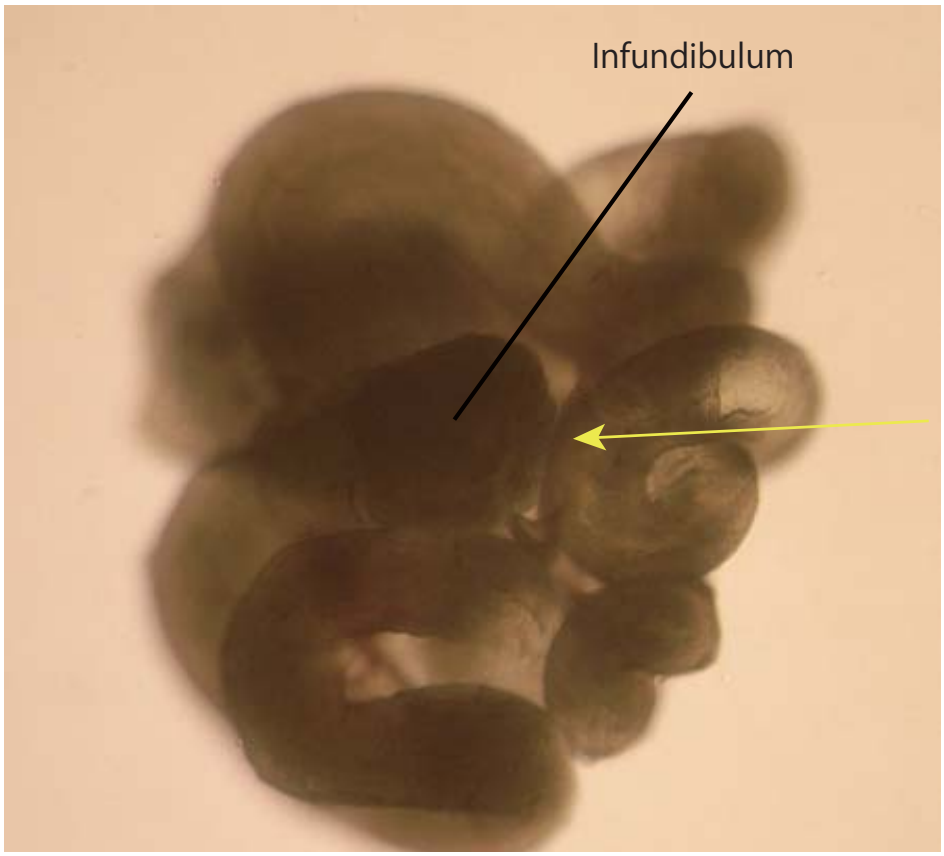


5. Look closely at the oviduct to find the infundibulum, which is a tube of a larger diameter than the oviduct.
6. Set the oviduct so that the flushing needle can be easily inserted.

Note

Because the infundibulum is usually hidden within the coils of the oviduct, you need to rotate the oviduct tenderly with forceps within the drop and look closely to find it.

[Micrograph : An Oviduct before Flushing]



Comment

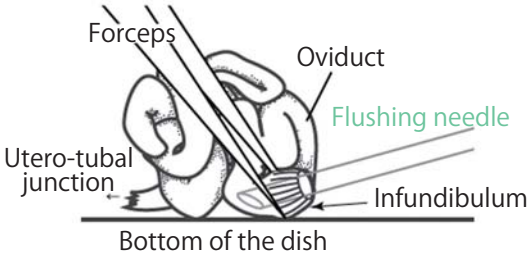
If you are right-handed, position the oviduct as shown in the diagram on the left. It is easy to insert the needle into the infundibulum from this position (as indicated by the yellow arrow).

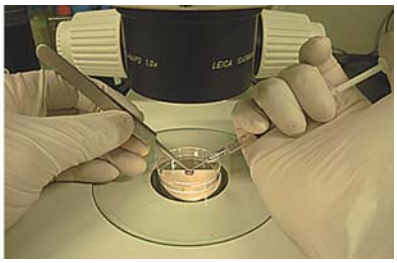
7. Hold the infundibulum against the bottom of the dish using a pair of forceps, and insert the needle into it.

Note

The infundibulum is extremely fragile, so use the forceps gently.

8. Push the plunger and slowly flush the oviduct with KSOM/AA.

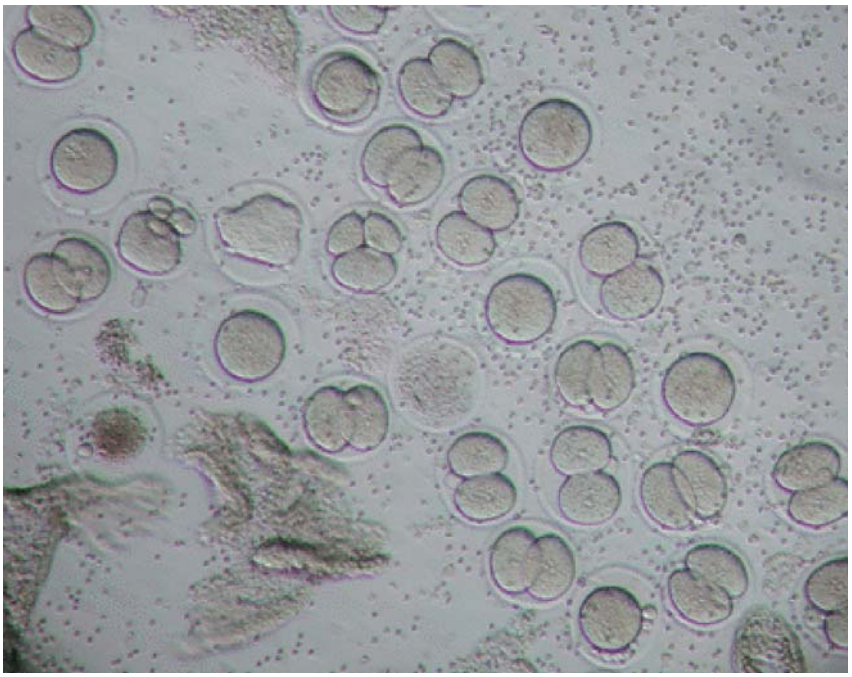




[Flushing] No. 10-01

9. Aspirate the embryos using pipettes and wash them in several drops of fresh KSOM/AA (washing dish).

[Micrograph : After Flushing of Oviduct]



Note

When flushing is successful, you should see the oviduct puffing up as you inject medium.
If you cannot find the infundibulum, do not blindly pinch the oviduct. Doing so may crush the infundibulum, which will prevent you from inserting the needle.

Note

Be sure to carry out all operations, from sacrificing the female through to flushing her oviducts, in the shortest time possible (within 5 minutes).
Moreover, when carrying out this process alone, do not sacrifice multiple mice at once; instead, sacrifice one mouse and swiftly flush its oviducts before moving on to the next mouse.

References

1. Nagy A., Gertsenstein M., Vintersten K., and Behringer R. 2003. Manipulating the Mouse Embryo, A Laboratory Manual (Third edition). *Cold Spring Harbor Laboratory Press.* ISBN 0-87969-591-9.

5-1 Transport of 2-Cell Embryos at Cold Temperature

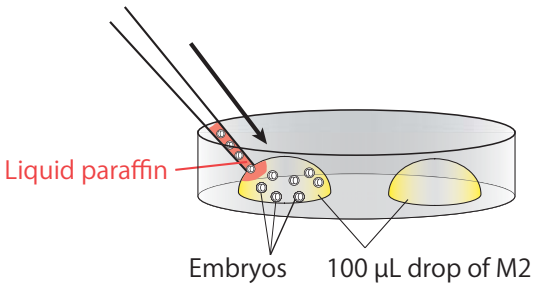
Materials and Equipment

1. 2-cell embryos (adaptable for fresh and frozen/thawed embryos)
2. Plastic dishes (35 mm X 10 mm Cat. No. 430588; CORNING)
3. Gel-loading tip (Cat. No. 010-R204S; Bio Medical Instrument)
4. M2 (Cat. No. M7167; Sigma)
5. 0.5 mL tube (Fisherbrand Flip Cap Microtubes 0.5 mL; Fisher Scientific Cat. No. FS-MCT-060 -C)
6. Transfer pipettes
7. KSOM/AA
8. Liquid paraffin
9. Temperature data logger (Thermochron iButton Cat. No. DS1921G; Maxim Integrated Products)
10. 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 0.5 mL tube can stand)
    - Cotton wool
    - Cold packs (small and large)
    - Polystyrene foam transport box (Cat. No. KC-3, KARUX)

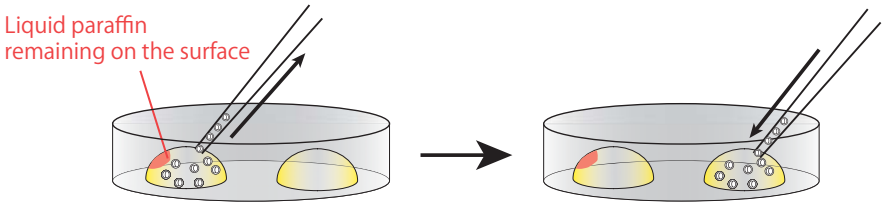
Procedures

Cold Storage of 2-cell Embryos

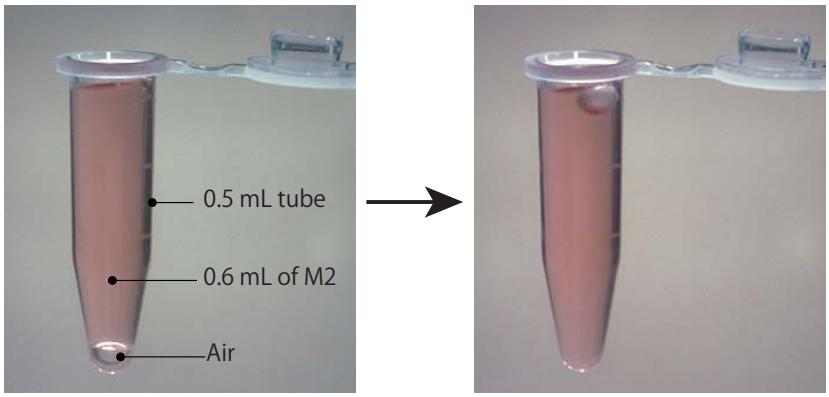
1. Place two 100 µL drops of M2 on a plastic dish.
2. Transfer the 2-cell embryos from the culture medium to the drop of M2.



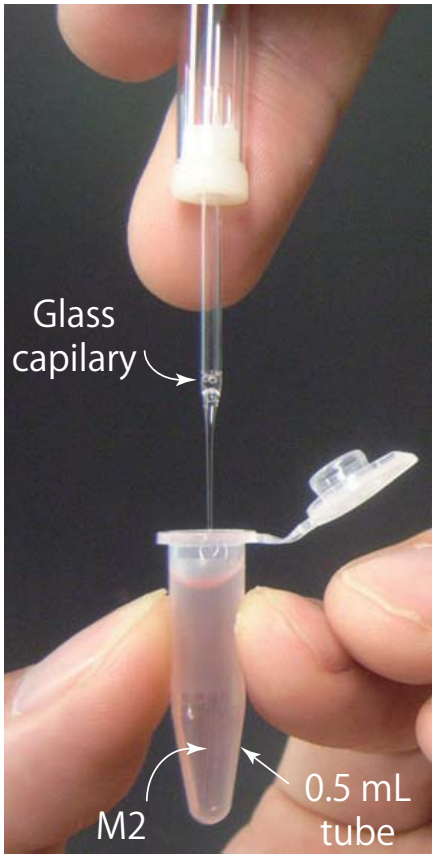
3. Exchange the capillary for embryo manipulation and aspirate the embryos into the new capillary, making sure to avoid dropping liquid paraffin onto the drop of M2. Transfer the embryos into the drop of M2 made in step 1.



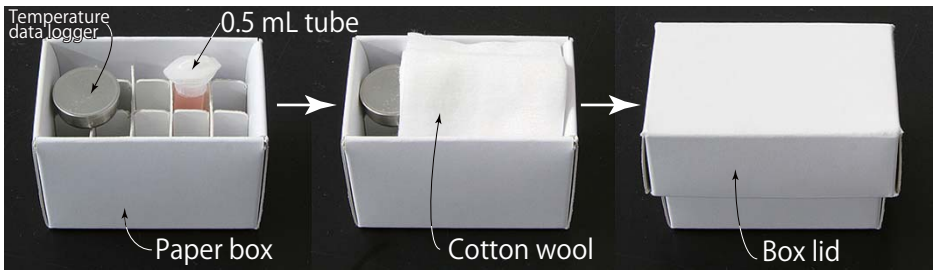
4. Fill a 0.5 mL tube with 0.6 mL of M2 at room temperature. If there is a bubble in the bottom of the tube, tap the tip of the tube to release the bubble.



5. Collect and transfer the embryos into the bottom of the tube (40 embryos/tube).



6. Place the tube containing the embryos, a temperature data logger and a piece of cotton wool in the paper box.



7. Store the paper box in the refrigerator (4-8℃).

Comment

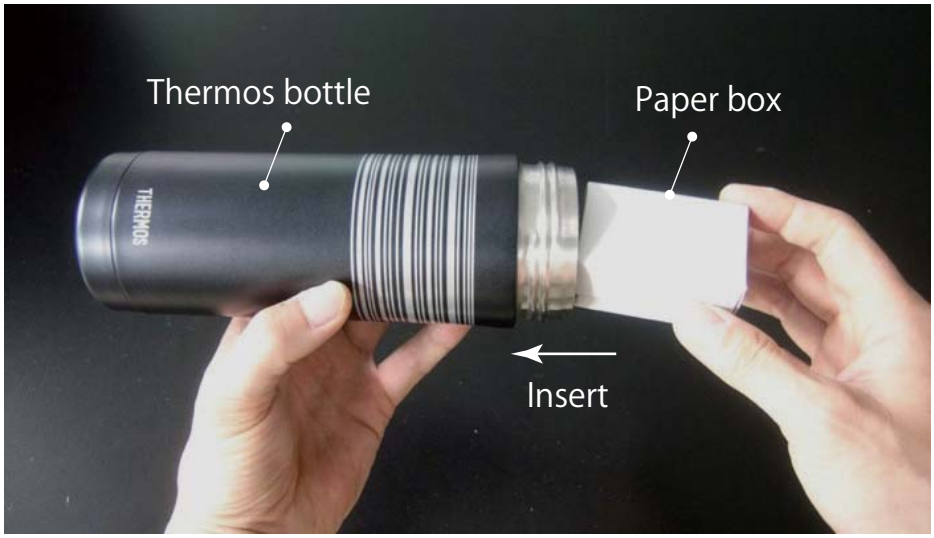
The embryos will maintain developmental ability for up to 72 hours.

Package and Transport of 2-cell Embryos

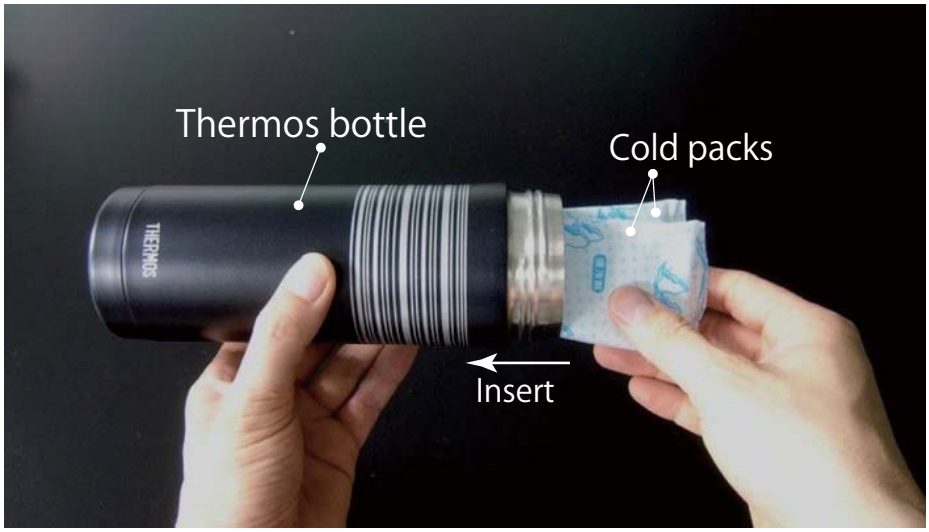
Prepare a paper box containing 2-cell embryos in the same manner as described before (Cold Storage of 2-cell Embryos).

The cold packs (large) and a foam transport box must be precooled to 4-8℃ before use. Use the cold packs (small) and a thermos bottle at room temperature.

1. Insert the paper box containing the embryos into a thermos bottle.

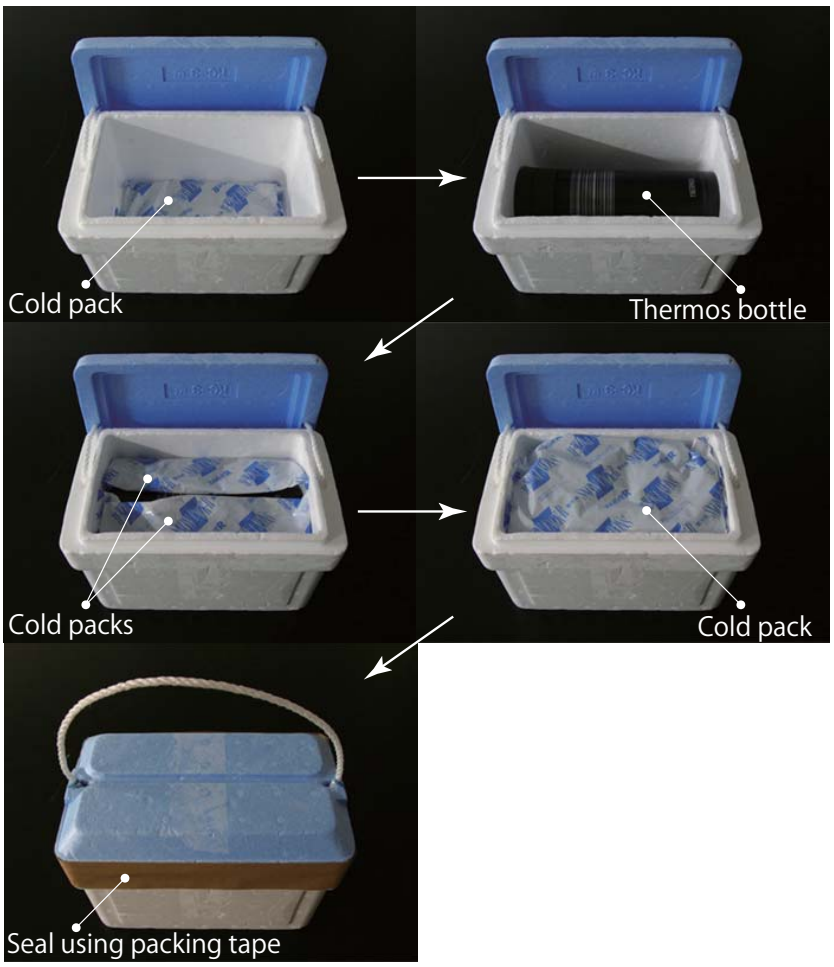


2. Insert two cold packs (small) into the thermos bottle.



3. Close the bottle cap.

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



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

Note

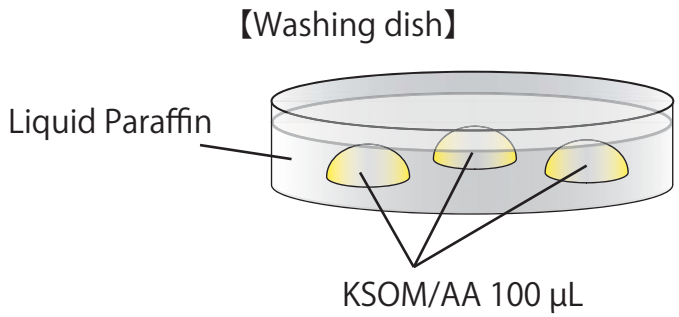
Take care not to place the paper box upside down.

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.

Collection of 2-cell Embryos from the Transport Box

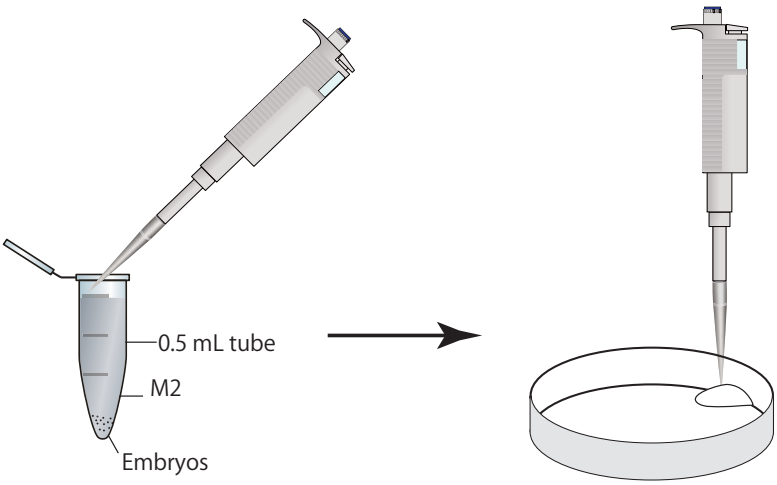
1. Put 3 drops (100 µL / drop) of KSOM/AA into a dish and cover them with liquid paraffin. Place the dish in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.



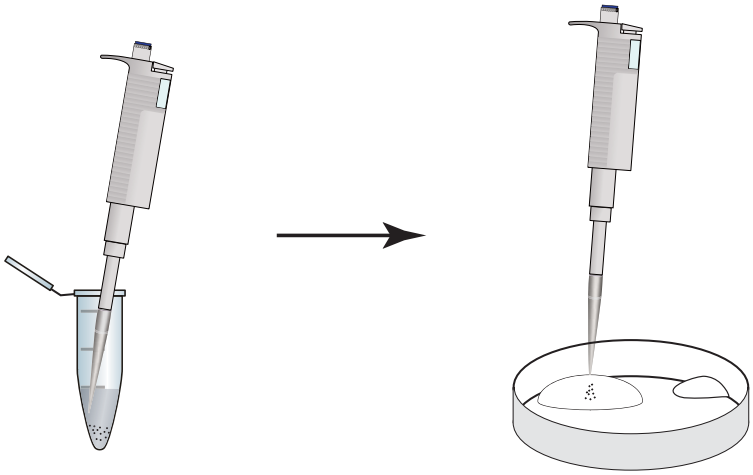
2. Retrieve the paper box containing the samples from the thermos bottle.
3. Leave the paper box at room temperature for 30 minutes.

[Removing the sample] No. 11-01

4. Open the paper box and gently remove the cotton. Once removed, pick up the tube containing the embryos and open it.
5. Collect an upper layer of 200 µL M2 from the tube using a gel-loading tip, then transfer the aliquot to the edge of the plastic dish.



6. Carefully retrieve all M2 containing the embryos from the bottom of the tube using a gel-loading tip, then transfer the aliquot to the center of the plastic dish.



Note

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

Comment

The embryos will maintain developmental ability for up to 72 hours.

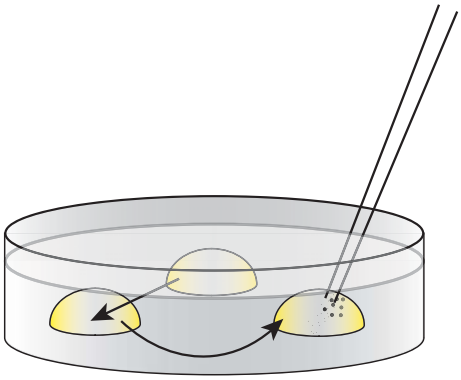
Comment

The embryos will sink to the bottom of the tube during those 30 minutes.

Note

For easy manipulation, take care to avoid aspirating air bubbles in the gel-loading tip.

7. Collect the embryos from the M2, then transfer and wash them in each of the three drops of 100 µL KSOM/AA (washing dish).



8. Transfer the embryos into the oviducts of a pseudo-pregnant mouse.

References

1. Takeo T., Kaneko T., Haruguchi Y., Fukumoto K., Machida H., Koga M., Nakagawa Y., Takeshita Y., Matsuguma T., Tsuchiyama S., Shimizu N., Hasegawa T., Goto M., Miyachi H., Anzai M., Nakatsukasa E., Nomaru K., and Nakagata N. 2009. Birth of mice from vitrified/warmed 2-cell embryos transported at a cold temperature. *Cryobiology*. **58**(2): 196-202.
2. Takeo T., Kondo T., Haruguchi Y., Fukumoto K., Nakagawa Y., Takeshita Y., Nakamuta Y., Tsuchiyama S., Shimizu N., Hasegawa T., Goto M., Miyachi H., Anzai M., Fujikawa R., Nomaru K., Kaneko T., Itagaki Y., and Nakagata N. 2010. Short-term storage and transport at cold temperatures of 2-cell mouse embryos produced by cryopreserved sperm. *J Am Assoc Lab Anim Sci.* **49**(4): 415-419.

Note

If you cannot retrieve all stored embryos, rinse the inside of the tube using the 200 µL M2 at the edge of the plastic dish.

Comment

Ideally, embryo transfer to pseudo-pregnant mice should be performed immediately upon the arrival of embryos.
(Please refer to the chapter of Embryo Transfer into the Oviduct on page 66.)

5-2 Transportation of Mouse Oviducts Containing 2-Cell Embryos at Low Temperature (0℃)

Materials and Equipment

1. 0.8 M Sucrose
2. PB1
3. KSOM/AA
4. Plastic bag
5. Thermos bottle
6. Crushed ice
7. Conical bottom cryotubes (Cat. No. 366656; NUNC)
8. Plastic dish (35 mm X 10 mm Cat. No. 430588; CORNING)

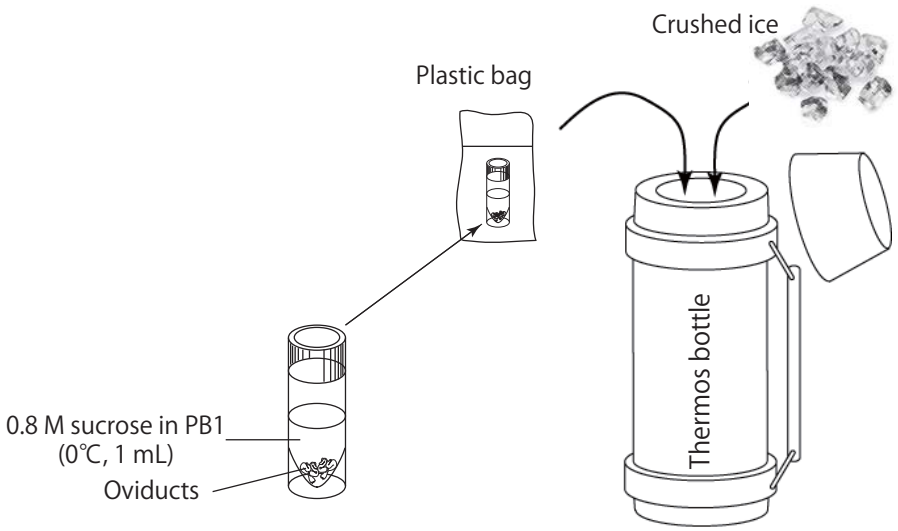
Procedures

Dissection of Oviducts from Superovulated, Plugged Females

1. Inject females i.p. (8-12 weeks old) with 7.5 IU of PMSG (14:00-18:00).
2. Inject females i.p. with 7.5 IU of hCG 48-52 hours after giving them the PMSG injection, and allow the male and female mice to mate overnight.
3. Check the females for vaginal plugs from early the next morning until noon. (Please refer to the chapter of Embryo Transfer into the Oviduct on page 66.)
4. At 44-46 hours after administering the hCG, sacrifice the plugged females.
5. Remove the oviducts of the females, and put them into a 100-200 µL drop of 0.8 M sucrose (0℃). (Please refer to the chapter of *In Vitro* Fertilization on page 9.)

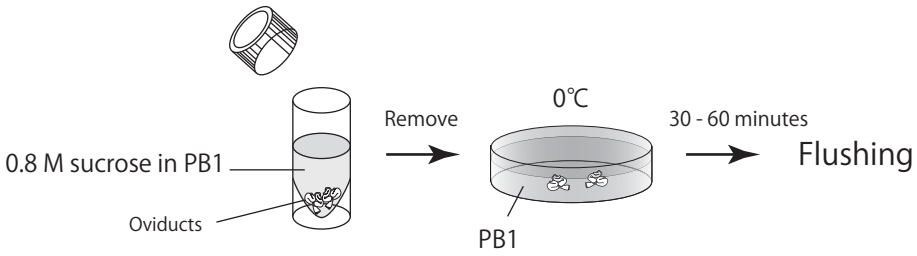
Transportation of Mouse Oviducts

1. Transfer the oviducts into a cryotube containing 1 mL of 0.8 M sucrose (0℃).
2. Enclose the tube in a plastic bag and seal it using a sealer.
3. Transfer the plastic bag to a thermos bottle containing crushed ice and transport it using a door-to-door delivery service.



Collection of Embryos

1. Remove the tube from the thermos bottle.
2. Remove the oviducts from the tube and keep them in PB1 (0℃) for 30-60 minutes.
3. Flush the oviducts with PB1 (0℃). (Please refer to the chapter of Collecting 2-Cell Stage Embryos on page 42.)
4. Wash the embryos with 3 cycles of fresh KSOM/AA (37℃).



Note

Embryos will rapidly degenerate if step 2 is skipped.

Note

The oviducts should not remain in the tube longer than 48 hours or the embryos will degenerate.
Embryos should be frozen if they will not be used immediately.
(Please refer to the chapter of Simple Vitrification of Mouse Embryos on page 54.)

References

1. Kamimura E., Nakashima T., Ogawa M., Ohwada K., and Nakagata N. 2003. Study of low-temperature (4℃) transport of mouse two-cell embryos enclosed in oviducts. *Comp. Med.* **53**: 393-396.
2. Ogawa M., Fuchiwaki M., Valdez Jr. Delgado M., Yanagita T., Ide Y., Fukumoto K., Machida H., Kawabe T., Kaneko T., Kasai M., and Nakagata N. 2005. Development after freeze-thawing of mouse embryos collected from oviducts transported at 0℃. *Exp. Anim.* **54**(3) Suppl: 242.

6-1 Simple Vitrification of Mouse Embryos

Materials and Equipment

1. 1 M DMSO
2. DAP213
3. Plastic dish (35 mm X 10 mm Cat. No. 430588; CORNING)
4. Filter unit (Millex-GV 0.22 µm Cat. No. SLGV013SL; MILLIPORE)
5. Gel loading tip (MBP Gel 200, Cat. No. 3621; Molecular BioProducts)
6. Transfer pipettes
7. Cryotubes (Cryogenic Vials Cat. No. MS-4501W; Sumitomo Bakelite, Japan is recommended. If you cannot get it, use Cat. No. 366656; NUNC.)
8. Micropipette
9. Vial canes
10. Nalgene Labtop Cooler (Cat. No. 5115-0012; NALGENE, USA)
11. Liquid nitrogen
12. Microscope
13. 0.25 M sucrose
14. KSOM/AA
15. Liquid paraffin

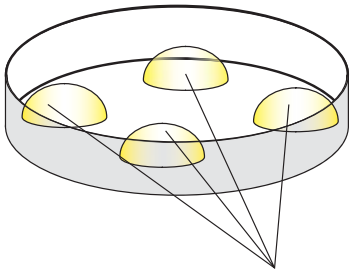
Procedures

Preparation of a Block Cooler and Cryotubes

1. A day before use, place a block cooler (Cat. No. 5115-0012; NALGENE, USA) in a freezer at -20℃.
2. About 10 minutes before commencing the vitrifying procedure, take the block cooler out of the freezer.
3. Stand some cryotubes in the block cooler. About 40 embryos / cryotube are easy to handle; in other words, when you want to vitrify 120 embryos, you need to stand 3 cryotubes in the block cooler.
4. Just before starting the procedure, check the temperature inside of the tubes is at 0℃.

Vitrification

1. Filter the 1 M DMSO and put 4 drops of it (~100 µL / drop) into a dish. One drop is to wash the embryos taken from the collection medium, while the others are to hold the washed embryos.

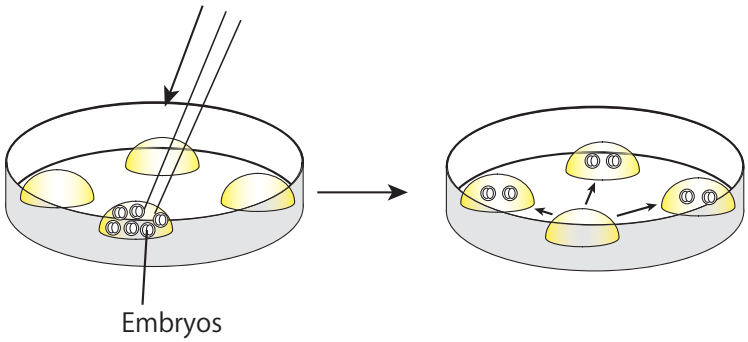


1 M DMSO 100 µL

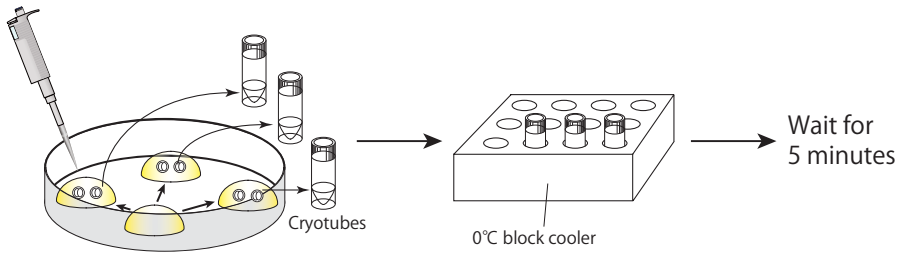
Comment

Crushed ice can be used instead of a block cooler.

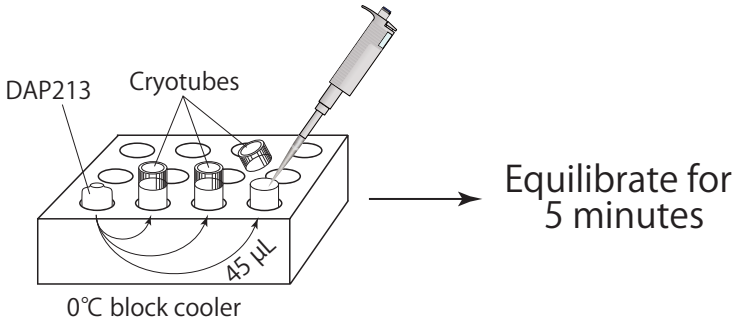
2. Place a group of embryos into one of the 4 drops to rinse them of the collection medium. Divide the rinsed embryos equally between the other drops. These aliquots will eventually be transferred to a storage vial. For example, if one were to collect 120 embryos and vitrify them in 40-embryo aliquots, the embryos would first be placed together in the rinse drop and then divided equally among the three drops.



3. Using a 20 µL pipette and a gel-loading tip, transfer the embryos contained within 5 µL of 1 M DMSO solution into a cryotube. Once transferred, put the cryotube into the block cooler at 0℃ and wait for 5 minutes.



4. Add 45 µL of cryoprotective solution (DAP213) at 0℃ into the cryotube and equilibrate for 5 minutes in the 0℃ block cooler.



Note

It is possible to keep the cryotubes in the block cooler at 0℃ for longer than 5 minutes (<20 minutes).

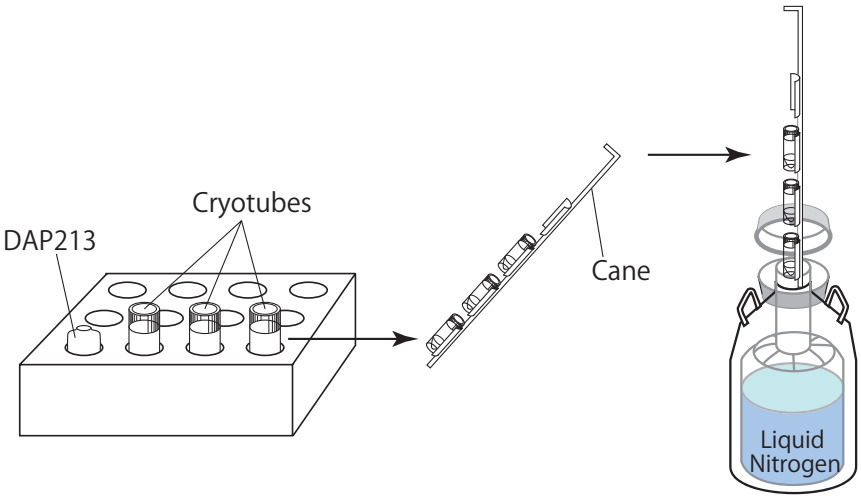
Note

If the embryos are pushed together in the center of the drop, it is easy to suck them all up in 5 µL of the 1 M DMSO solution.

Note

Do not fasten the caps too tightly after adding the DAP213, or they will be too difficult to remove quickly when samples are recovered from the freezer.

5. Quickly set the cryotubes on a cane and plunge the samples directly into liquid nitrogen.

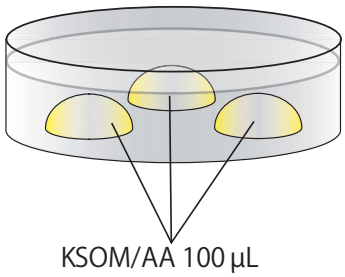


[Vitrifying Embryos] No. 13-01

Preparation for Thawing

1. Put 3 drops (100 µL/drop) of KSOM/AA 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.

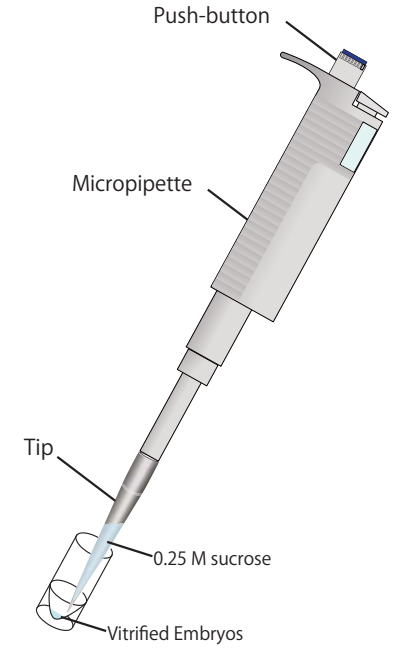
【Washing dish】



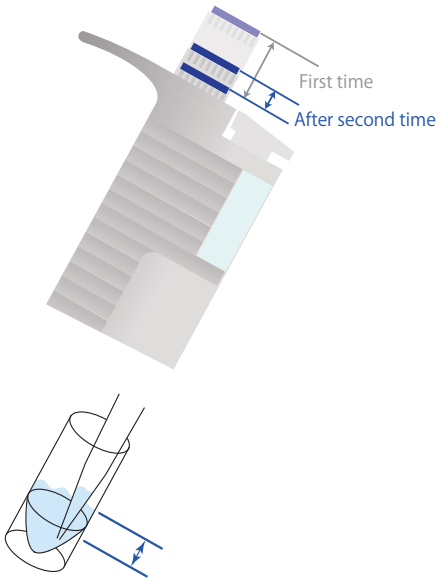
2. Warm 0.25 M sucrose in an incubator (37℃, 5% CO_2 in air) before use.

Recovering Vitrified Embryos

1. Remove the required sample from the liquid nitrogen and open the cryotube cap. Discard any liquid nitrogen in the tube and allow it to stand at room temperature for 30 seconds.
2. Add 0.9 mL of 0.25 M sucrose (preheated to 37℃) to the cryotube and warm the sample quickly via pipetting. When pipetting, take care not to generate large amounts of bubbles and to not physically damage embryos by pipetting too quickly. Once warmed, transfer the contents of the cryotube into a culture dish.



The tip must not touch the bottom of the cryotube. If it does so, the 0.25 M sucrose in the tip will freeze and you will not be able to inject the 0.25 M sucrose into the cryotube.



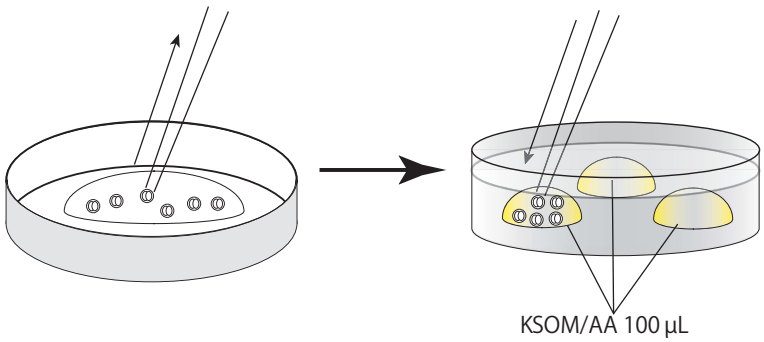
On the first press, the full volume of 0.9 mL of 0.25 M sucrose will be expelled.
On subsequent presses, around 10 amounts of 0.25 M sucrose which you have sucked up will be expelled. Pipetting smaller volumes in this way prevents bubbles from generating in the 0.25 M sucrose.

3. Place 0.4-0.5 mL of 0.25 M sucrose into the cryotube, and transfer the contents into the plastic dish. This further dilutes the cryoprotectant and ensures that all of the embryos have been transferred.

[Recovering Vitrified Embryos] No. 13-02

[Pipetting to Recover Vitrified Embryos] No. 13-03

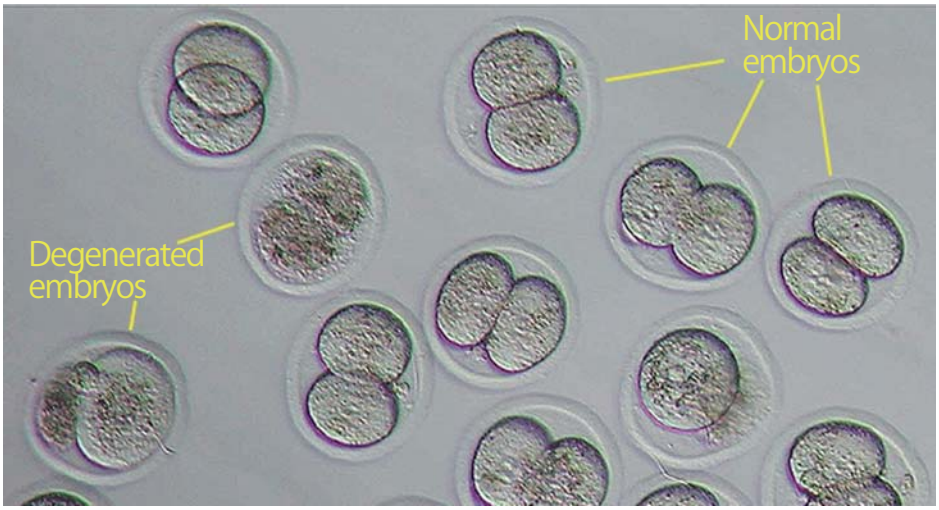
4. Aspirate the embryos from the liquid and carefully transfer them into a drop of KSOM/AA (washing dish), then keep them in an incubator (37℃, 5% CO_2 in air).



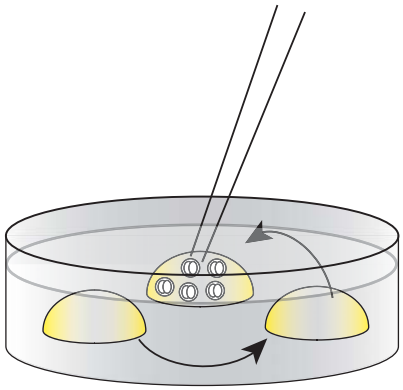
Note

It is very important to warm the sample quickly to avoid damaging the embryos due to the toxicity of the cryoprotective solution (DAP213).

1. **[Micrograph : Embryos Recovered after Vitrification]**



5. After 10 minutes, wash the embryos with 2 cycles of fresh KSOM/AA (washing dish).



References

1. Nakagata N. 1989. High survival rate of unfertilized mouse oocytes after vitrification. *J. Reprod. Fert.* **87**: 479-483.
2. Nakagata N. 1993. Production of normal young following transfer of mouse embryos obtained by *in vitro* fertilization between cryopreserved gametes. *J. Reprod. Fert.* **99**: 77-80.
3. Nakagata N. 1995. Studies on cryopreservation of embryos and gametes in mice. *Exp. Anim.* **44**: 1-8.
4. Nakao K., Nakagata N., and Katsuki M. 1997. Simple and effcient procedure for cryopreservation of mouse embryos by simple vitrification. *Exp. Anim.* **46**: 231-234.

6-2 Simple Vitrification of Mouse Oocytes

Materials and Equipment

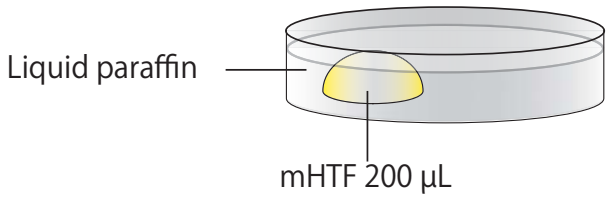
1. Female mice superovulated with PMSG and hCG
2. Plastic dishes (35 mm X 10 mm Cat. No. 430588; CORNING)
3. Liquid paraffin
4. Micropipette
5. Pipette tips
6. mHTF
7. 1% Hyaluronidase in mHTF
8. Fetal bovine serum (FBS Cat. No. 26140-087; Gibco)
9. Filter unit (Millex-GV 0.22 μm Cat. No. SLGV013SL; MILLIPORE)
10. Glass capillaries for embryo handling
11. Humidified incubator (37℃ 5% CO_2 in air)
12. Materials and equipment used for vitrification and warming of embryos (For washing warmed oocytes, mHTF drops are used.)
    (Please refer to the chapter of Simple Vitrification of Mouse Embryos on page 54.)

Procedures

Preparation of Dishes

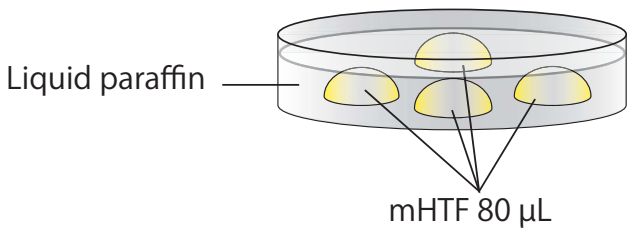
1. Put a 200 μL drop of mHTF into a dish. Cover it with liquid paraffin and place it in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.

【Oocyte dish】

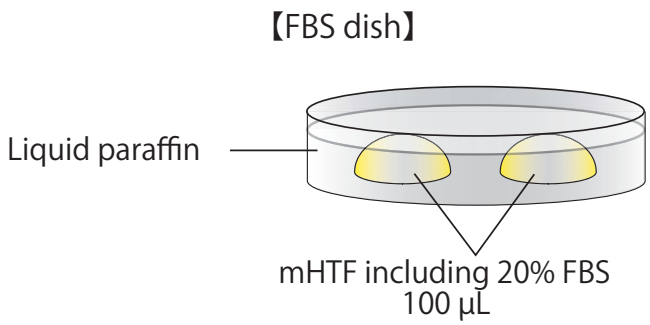


2. Put 4 drops (80 μL/drop) of mHTF into a dish. Cover them with liquid paraffin and place the dish in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.

【Washing dish】

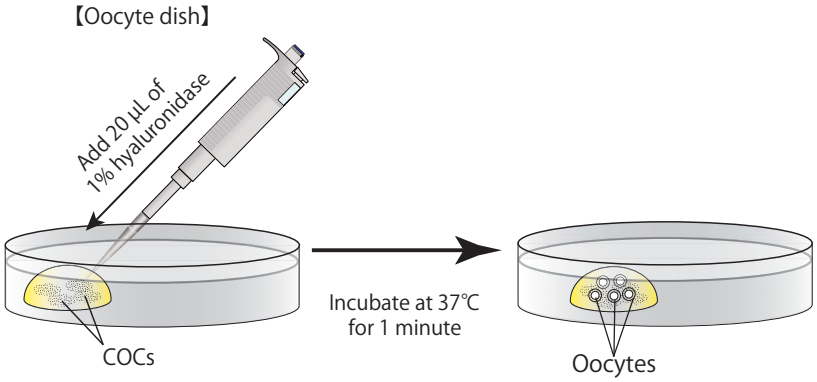


3. Prepare some mHTF including 20% FBS, and sterilize it using a filter. Put 2 drops (100 µL/ drop) of the medium into a dish. Cover them with liquid paraffin and place the dish in the incubator (37℃, 5% CO_2 in air) for at least 30 minutes.

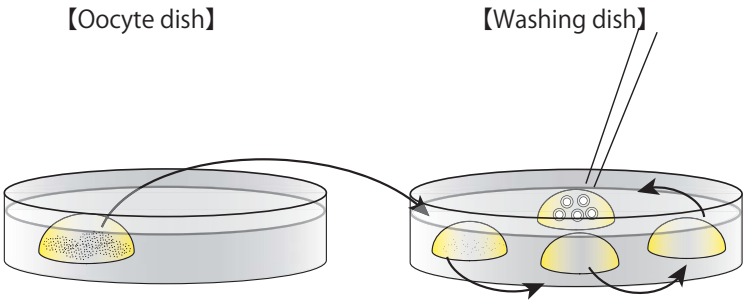


Preparation of Denuded Oocytes

1. Collect cumulus-oocyte complexes (COCs) from superovulated female mice and introduce them into a 200 µL drop of mHTF (Oocyte dish). (Please refer to the chapter of *In Vitro* Fertilization on page 9.)
2. Add 20 µL of 1% hyaluronidase to the drop of mHTF containing the COCs, and keep the dish in an incubator (37℃, 5% CO_2 in air) for 1 minute.



3. Promptly collect and transfer the oocytes into a 80 µL drop of mHTF (Washing dish), and in turn wash them in the drops in the washing dish.



Note

Be sure to carry out all operations, from sacrificing the female and removing her oviducts to introducing the COCs into a drop of mHTF (Oocyte dish), in the shortest time possible (within 30 seconds).

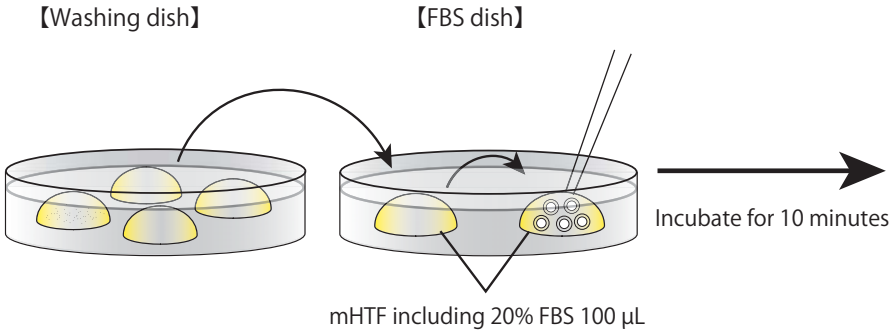
Moreover, when carrying out this process alone, do not sacrifice multiple mice at once; instead, sacrifice one mouse and swiftly remove its oviducts before moving on to the next mouse.

Comment

If some cumulus cells become attached to the zona pellucida of the oocytes, they can be removed by manipulating the glass capillary.

Culturing Oocytes in a Drop Containing FBS

1. Transfer the oocytes into the first drop in the FBS dish to rinse. Then, transfer them into the second drop to incubate (37℃, 5% CO_2 in air) for 10 minutes.



Simple Vitrification of Mouse Oocytes

1. The oocytes can be vitrified using the simple vitrification method for embryos, after removing cumulus cells and culturing them in a drop containing FBS.
   Moreover, the warming method is the same as for embryos. (Please refer to the chapter of Simple Vitrification of Mouse Embryos on page 54.)

In vitro Fertilization using Vitrified-Warmed Oocytes

1. The vitrified-warmed oocytes can be used for *in vitro* fertilization using fresh, cold temperature transported and frozen-thawed spermatozoa.
   Please refer to the chapters of *In Vitro* Fertilization on page 6, *In Vitro* Fertilization using Epididymal Sperm Transported at Cold Temperature on page 18 and *In Vitro* Fertilization using Cryopreserved Spermatozoa on page 26.

References

1. Nakagata N., Takeo T., Fukumoto K., Kondo T., Haruguchi Y., Takeshita Y., Nakamuta Y., Matsunaga H., Tsuchiyama S., Ishizuka Y., Araki K. 2013. Applications of cryopreserved unfertilized mouse oocytes for *in vitro* fertilization. *Cryobiology.* **67**(2):188-92.

Comment

FBS can prevent zona hardening in the oocyte during vitrifying and warming.

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.

6-3 Vitrification and Transplantation of Mouse Ovaries

Materials and Equipment

1. 35-mm sterile plastic tissue culture dishes
2. mWM
3. Donor: Female mouse (1 day to 30 weeks old)
4. Recipient: Four-week-old female mouse (a strain that is histocompatible with the transplanted ovary)
5. Anesthetic
6. Micro-spring scissors (5 mm blade)
7. Pair of watchmaker's #5 forceps
8. Wound clip (Autoclip 9 mm; Clay Adams Cat. No. 427631) and clip applicator (Mik-Ron Autoclip Applier; Clay Adams Cat. No. 427630)
9. Hot plate (37℃)

Procedures

Collection of Ovaries

1. Sacrifice a donor female and remove her ovaries.
   (Please refer to the chapter of *In Vitro* Fertilization on page 9.)
2. Place the ovaries in a dish containing an adequate amount of mWM.

Vitrification

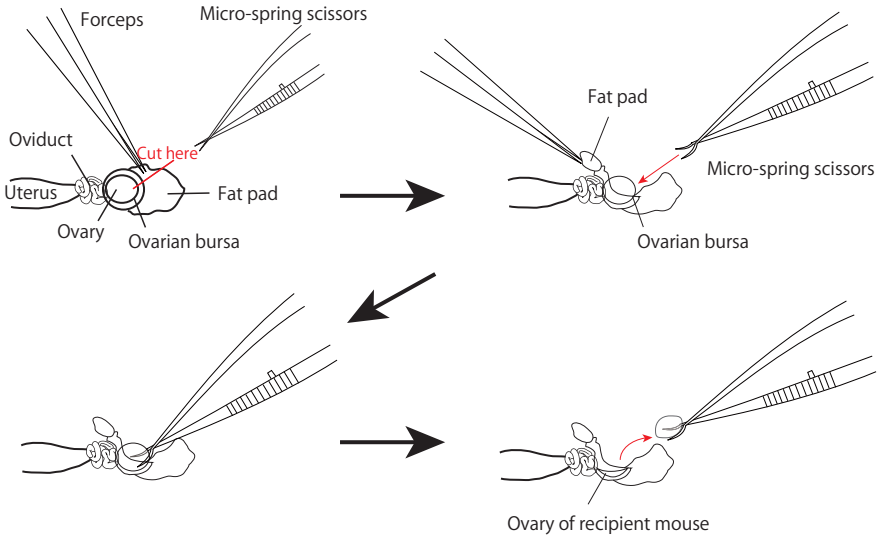
1. Ovaries can be cryopreserved following the same method used for embryos.
   (Please refer to the chapter of Simple Vitrification of Mouse Embryos on page 54.)

Transplantation

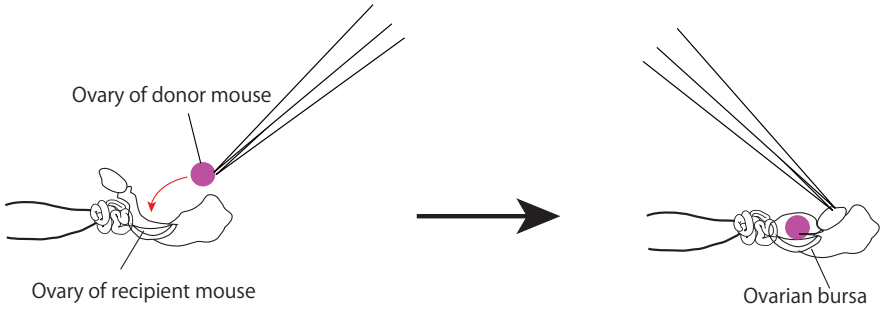
1. Anesthetize a recipient mouse.
2. Pull out the ovary, oviduct and part of the uterine horn as per the conventional procedure.
   (Please refer to the chapter of Embryo Transfer into the Oviduct on page 67.)
3. Using the micro-spring scissors, cut open approximately 1/4 of the recipient's ovarian bursa and some of the surrounding fat pad. Lift up the fat to reveal the ovary.
4. Cut off 1/2 - 2/3 of the ovary using the micro-spring scissors (curved blade).

Comment

By using micro-spring scissors with a curved blade, it becomes easier to place the donor ovary on the residual recipient ovary.



5. Insert the donor ovary into the residual ovary of the recipient, and cover it with the ovarian bursa.



[Transplantation of Mouse Ovary] No. 15-01

6. Push the ovary, oviduct, and part of the uterine horn back into the abdomen, and close the wound using wound clips.
7. Repeat the process for the ovary on the other side of the recipient mouse as described above.
8. Keep the mouse warm on a 37℃ warming plate until the mouse recovers from the effects of the anesthesia.

References

1. Migishima F., Suzuki-Migishima R., Song S.Y., Kuramochi T., Azuma S., Nishijima M., and Yokoyama M. 2003. Successful cryopreservation of mouse ovaries by vitrification. *Biol. Reprod.* **68**: 881-887.
2. Tsuchiyama S., and Nakagata N. 2009. Cryopreservation of ovaries from elderly female mice. *Exp. Anim.* **58**(3) Suppl: 248.

7-1 Vasectomy for the Creation of Sterile Males

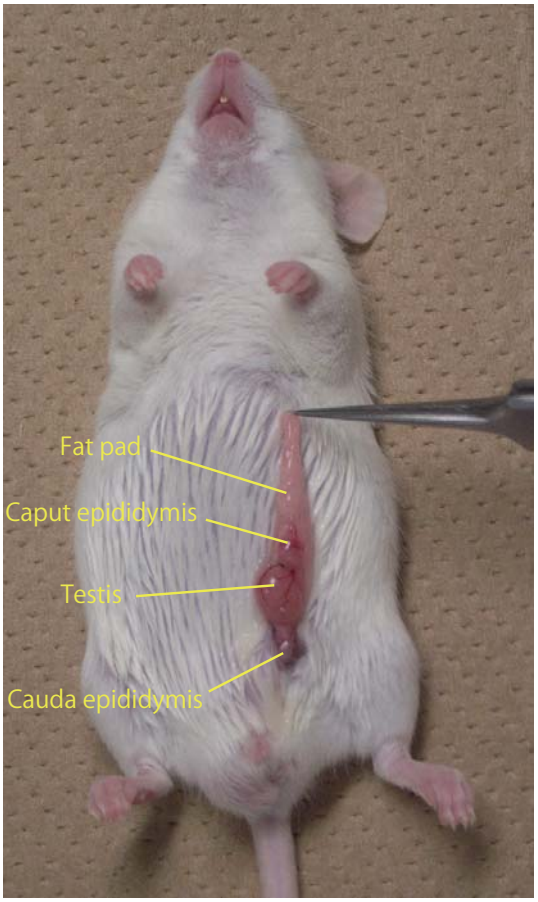
Materials and Equipment

1. Male mice (5 weeks old)
2. Anesthetic
3. Fine scissors
4. Pair of watchmaker's #5 forceps
5. Wound clip (Autoclip 9 mm; Clay Adams Cat. No. 427631) and clip applicator (Mik-Ron Autoclip Applier; Clay Adams Cat. No. 427630)
6. Hot plate (37℃)

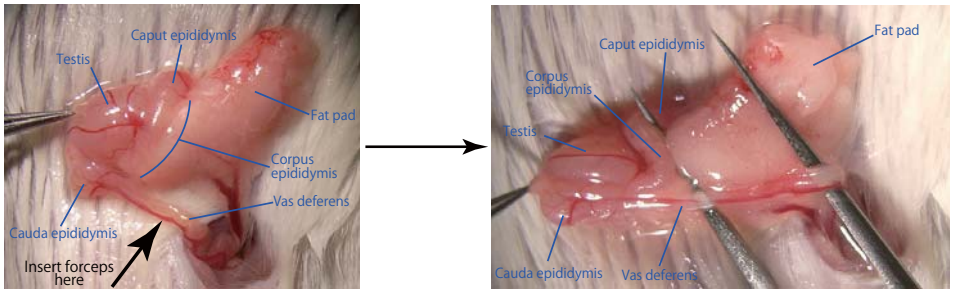
Procedures

Vasectomy

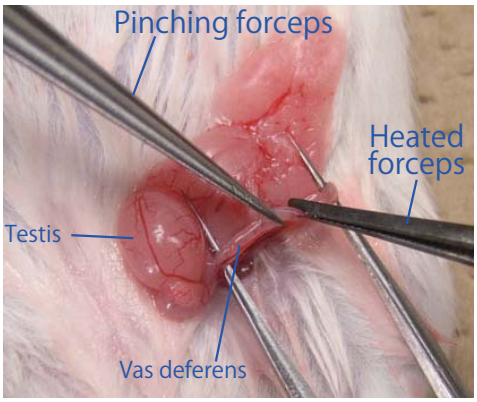
1. Anesthetize a male mouse.
2. In accordance with the conventional procedure, make a median incision. The incision should start at a point level with the top of the hind leg, and extend approximately 1 cm from this point towards the head of the mouse. After making the incision, pull out the testis, the epididymis and part of the vas deferens from the abdominal cavity.

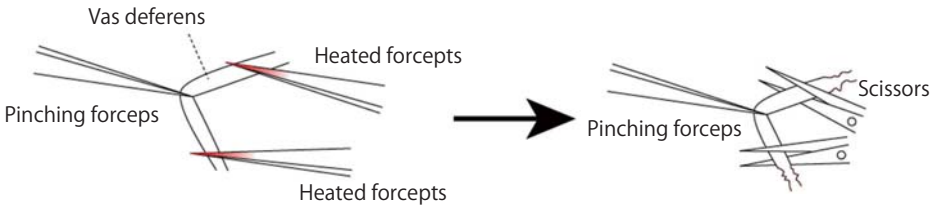


3. Pick up the vas deferens with one pair of forceps, and insert a second pair of forceps under the vas deferens to separate it from the connecting tissue.



4. Hold the vas deferens with a pair of pinching forceps, then cauterize the vas deferens at two locations using a second pair of heated forceps, as shown in the diagram below.
5. Cut the part of the vas deferens that lies between the two cauterized regions.





6. Push the testis, epididymis and part of the vas deferens back into the abdominal cavity, then close the wound using wound clips.
7. Repeat steps 2-6 for the other testis.

References

1. Nagy A., Gertsenstein M., Vintersten K., and Behringer R. 2003. Manipulating the Mouse Embryo, A Laboratory Manual (Third edition). *Cold Spring Harbor Laboratory Press.* ISBN 0-87969-591-9.

Comment

After completing the procedure, house each mouse individually. If the mice are housed in a group, they are likely to fight and some of them may be killed. The vasectomized males will be ready for use from as early as 8 weeks of age.

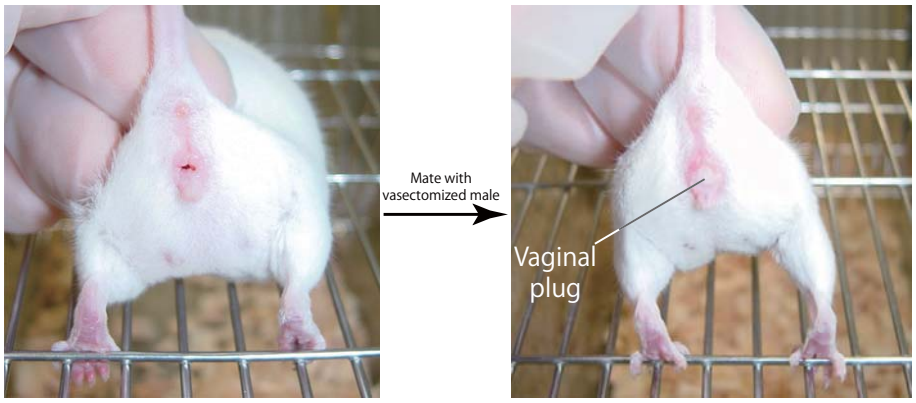
7-2 Embryo Transfer into the Oviduct

In our laboratory, we transfer 2-cell embryos through the wall of the oviduct of pseudopregnant recipients. This procedure is much easier and simpler to conduct than the conventional embryo transfer procedure, and is therefore suitable for inexperienced users.

Materials and Equipment

1. Female mice on Day 1 of pseudopregnancy (the day on which a vaginal plug is observed).

[Appearance of the vagina in proestrus] **[Vaginal plug]**

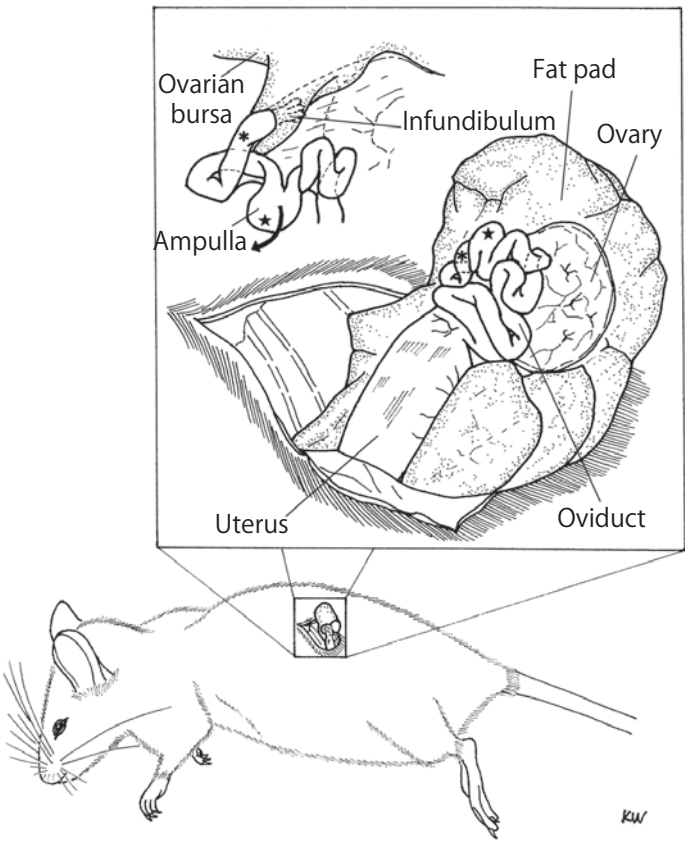


2. Anesthetic
3. Micro-spring scissors (5 mm blade)
4. Pair of watchmaker's #5 forceps
5. Serrefine clamp
6. Wound clip (Autoclip 9 mm; Clay Adams Cat. No. 427631) and clip applicator (Mik-Ron Autoclip Applier; Clay Adams Cat. No. 427630)
7. Plastic dish (35 mm X 10 mm Cat. No. 430588; CORNING)
8. Glass capillaries for embryo transfer and handling
9. Hot plate (37℃)

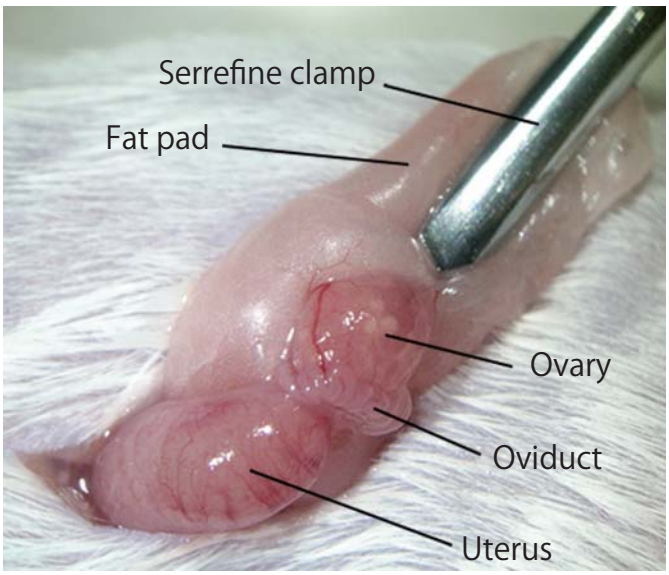
Procedures

Preparation of Mice

1. Anesthetize a female mouse.
2. Pull out the ovary, oviduct, and part of the uterine horn.



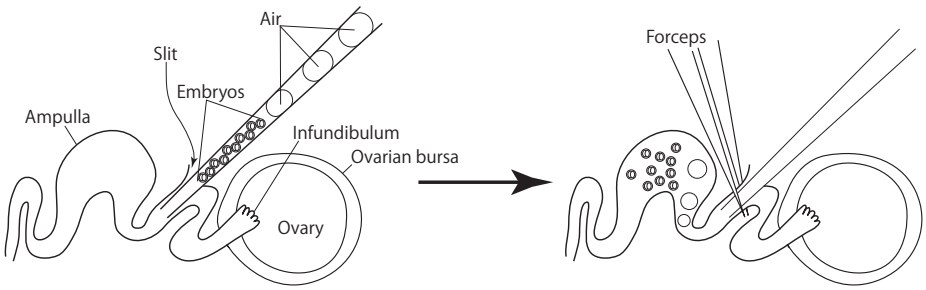
3. Clip a serrefine clamp onto the fat pad which is attached to the ovarian bursa.



Positioning of Oviduct

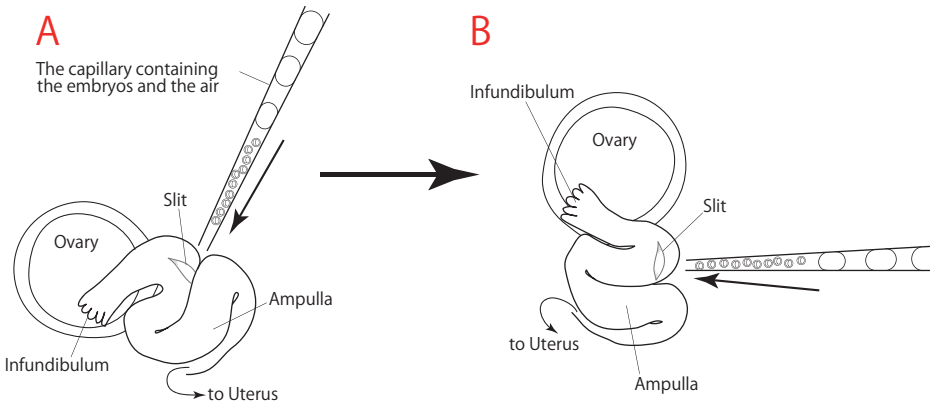
As indicated in the diagram below, embryo transfer into the oviduct is carried out by cutting the oviduct, inserting a capillary thereinto and expelling embryos towards the ampulla.

[Schematic Diagram]



Unfortunately, the oviducts of mice are small and the ducts are folded in a complicated manner, as shown in the schematic diagram of an exteriorized oviduct below (A). This makes it very difficult to insert the capillary into the oviduct towards the ampulla, because the insertion is made from above.

To make this procedure easier, position the oviduct by changing the position of the serrefine clamp and the mouse before starting the operation (B).



1. Observe the oviduct under a stereomicroscope and confirm the position of the infundibulum and ampulla using the tip of a set of forceps, or by changing the position of the serrefine clamp.
2. Position the oviduct by changing the position of the serrefine clamp and the mouse.

Note

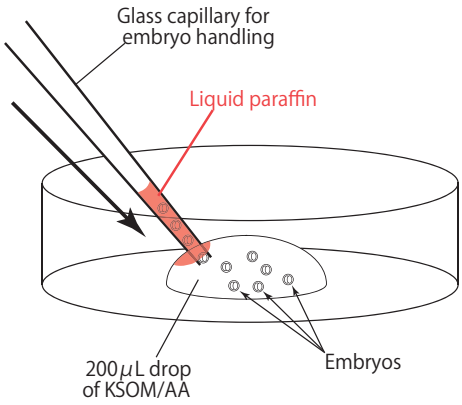
Because the folds in the oviduct vary between each mouse, look closely and adjust the position of the oviduct to make easier to work on.

Comment

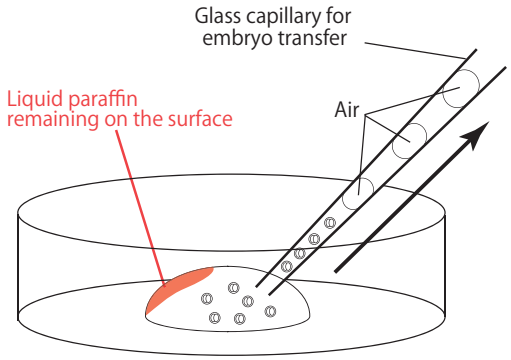
If you are left-handed, position the oviduct so that you can easily carry out the procedure with your left hand.

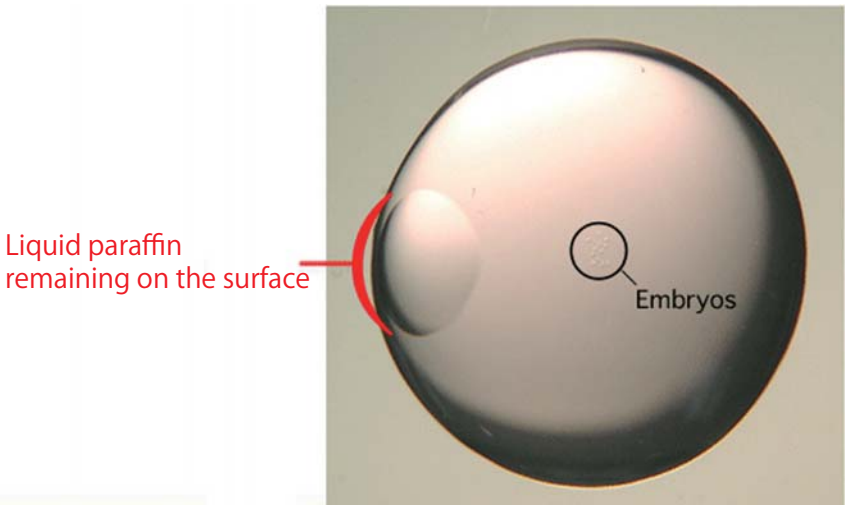
Preparation of Embryos and Glass Capillary

1. Make a 200 µL drop of KSOM/AA in a dish (without liquid paraffin), and introduce 20 embryos into the drop.



2. Aspirate air and medium in alternate intervals of 2-3 mm into a glass capillary in preparation for embryo transfer. Draw ten embryos into the glass capillary.





Comment

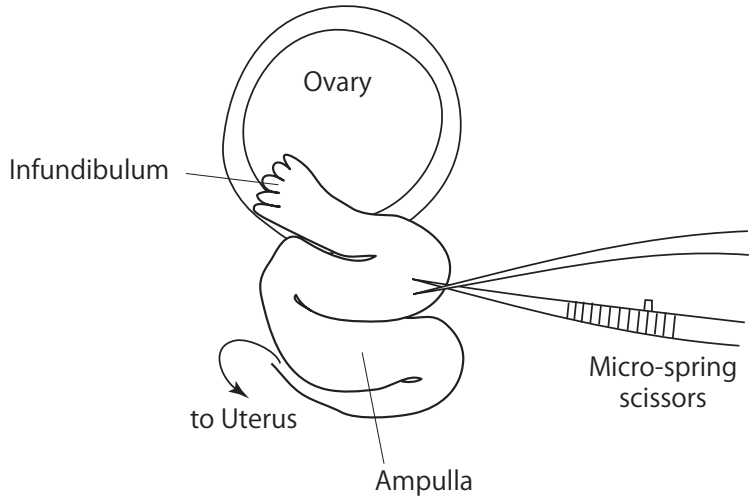
When the glass capillary is first inserted into the drop, some liquid paraffin will remain on the outer surface of the drop as shown below.

The embryos should be drawn into the glass capillary from the opposite side of the drop to avoid sucking up any liquid paraffin.

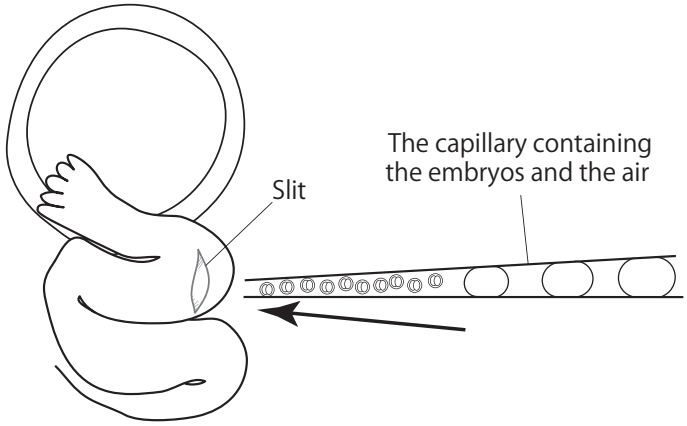
Evidence suggests that liquid paraffin which passes into the oviduct may have adverse effects on the development of the embryos into offspring.

Embryo Transfer

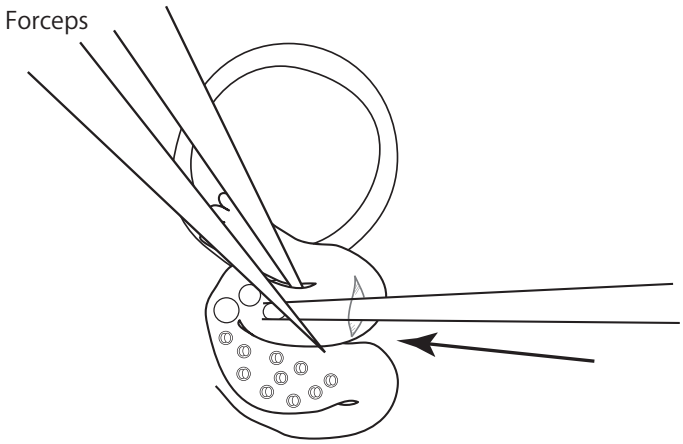
1. Using a pair of watchmaker's #5 forceps and micro-spring scissors, dissect the wall of the oviduct between the infundibulum and ampulla.



2. Insert the tip of the capillary containing the embryos into the slit, then push the capillary further into the slit towards the ampulla.



3. Use the forceps to hold the portion of the oviduct into which the capillary was inserted.
4. Expel the embryos and 2-3 of the air bubbles into the ampulla.



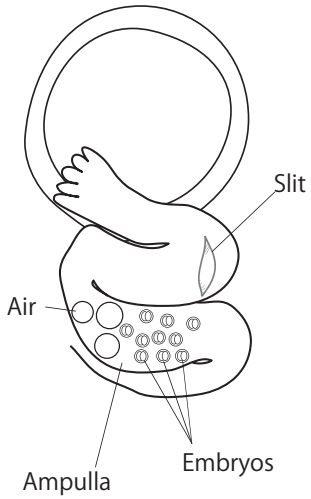
Comment

If performed successfully, you should be able to see air bubbles through the wall of the ampulla.

Note

If you cannot expel the embryos and the air bubbles into the oviduct, pull the capillary back out just a little from the slit and expel them again.

5. Withdraw the capillary gently from the slit.



[Transferring Embryos into the Oviduct] No. 17-01

6. Push the ovary, oviduct and uterine horn back into the abdomen and close the wound using wound clips.

7. Repeat the process to transfer the remaining 10 embryos into the other oviduct as described before.
8. Keep the mice warm on a 37℃ warming plate until they recover from the effects of anesthesia.

Note

Transfer the embryos after adjusting the position and direction of the oviduct. If the oviduct is aligned parallel to the capillary, then it will be easier to insert the capillary into the oviduct.

References

1. Nakagata N. 1992. Embryo transfer through the wall of the fallopian tube in mice. *Exp. Anim.* **41**: 387-388.
2. Nagy A., Gertsenstein M., Vintersten K., and Behringer R. 2003. Manipulating the Mouse Embryo, A Laboratory Manual (Third edition). *Cold Spring Harbor Laboratory Press*. ISBN 0-87969-591-9.

7-3 Embryo Transfer into the Uterus

Materials and Equipment

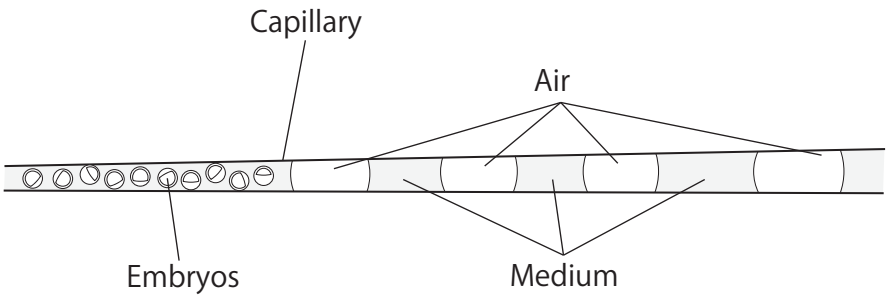
1. Female mice on Day 3 of pseudopregnancy (Day 1 is the day on which a vaginal plug is observed)
2. Anesthetic
3. Fine scissors
4. Pair of watchmaker's #5 forceps
5. Serrefine clamp
6. 27 gauge needle
7. Wound clip (Autoclip 9mm; Clay Adams Cat. No. 427631) and clip applicator (Mik-Ron Autoclip Applier; Clay Adams Cat. No. 427630)
8. Plastic dish (35 mm X 10 mm Cat. No. 430588; CORNING)
9. Glass capillary for embryo transfer
10. Hot plate (37℃)

Procedures

Embryo Transfer

Prepare recipient mice, embryos (8-cell to blastocyst stage) and a glass capillary as per the method used for embryo transfer into the oviduct.
(Please refer to the chapter of Embryo Transfer into the Oviduct on page 67.)

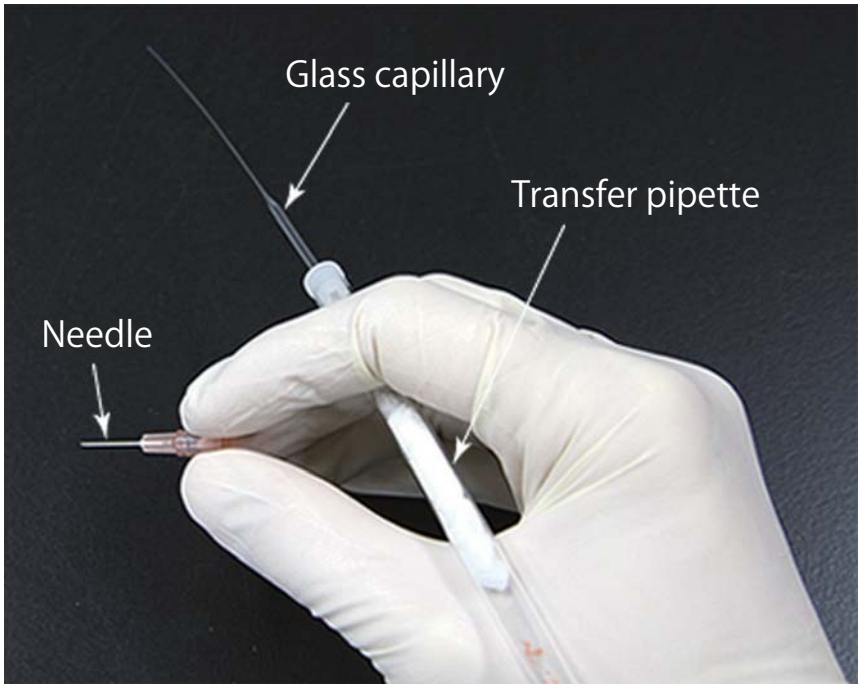
1. Pull out the ovary, oviduct and part of the uterine horn as per the conventional procedure.
2. Clip a serrefine clamp onto the fat pad which is attached to the ovarian bursa.
3. Aspirate air and medium in alternate intervals of 2-3 mm into a glass capillary in preparation for embryo transfer. Draw ten embryos into the glass capillary as indicated in the diagram below.



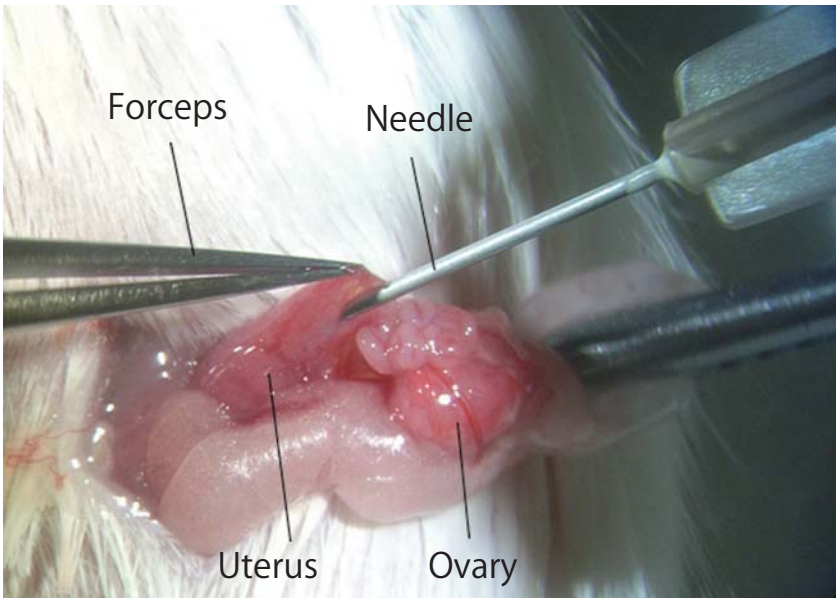
Note

When preparing the glass capillary, avoid placing the glass capillary into any liquid paraffin.

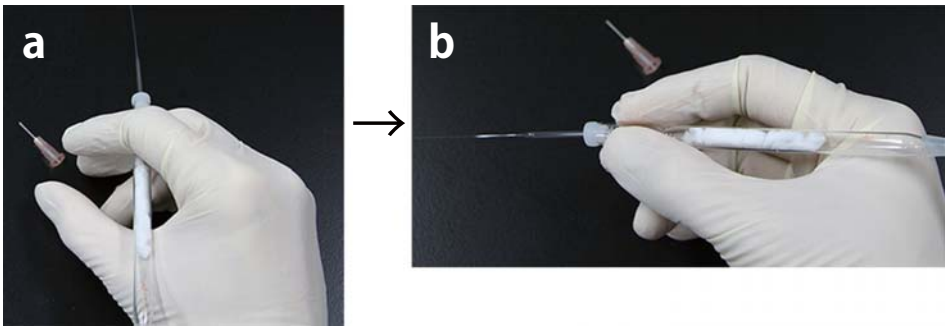
4. Hold a 27 gauge needle and the transfer pipette as shown in the picture below, and simultaneously look at both the tip of the needle and the uterus under a stereomicroscope. Be sure to look at the needle and the uterus simultaneously under the same stereomicroscope in order to confirm the position of the needle in relation to the uterus.

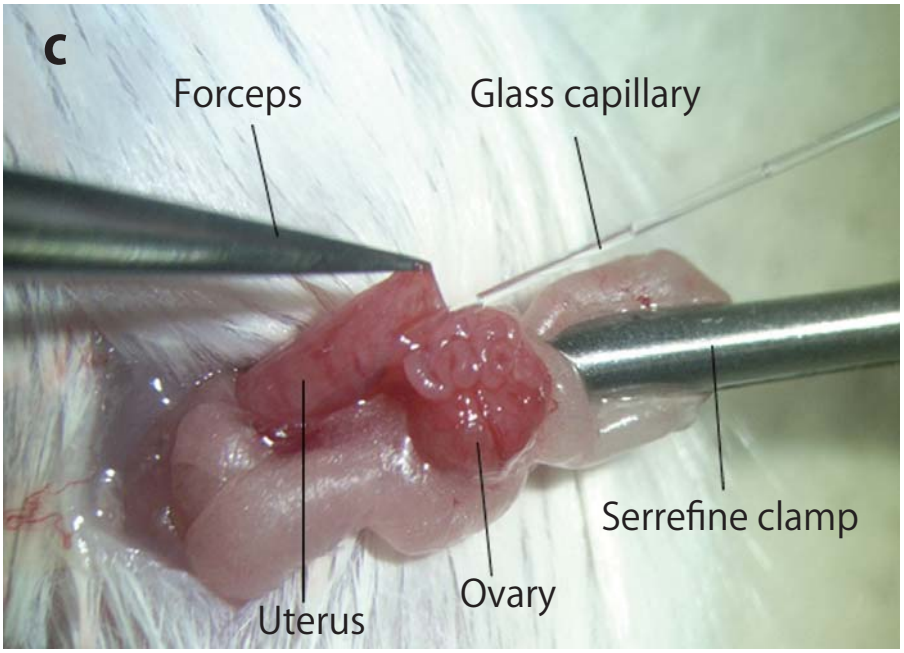


5. Gently hold the top of the uterine horn using fine forceps, and insert a 27 gauge needle into the wall of the uterus as far as the uterine cavity.



6. Release the needle and hold the transfer pipette as shown in diagrams a and b. Insert the tip of the capillary containing the embryos and air bubbles deep into the uterine cavity via the hole that you made with the needle, as shown in diagram c.





7. Expel the embryos into the uterine cavity along with 2-3 air bubbles.
8. Gently withdraw the capillary from the hole.

[Embryo Transfer into the Uterus] No. 18-01

[Performing the Operation] No. 18-02

Note

You should hold onto the top of the uterine horn and keep watching the hole made by the needle until you complete embryo transfer. If you avert your eyes from the hole before completing this procedure, it may be difficult to find the hole again.

Note

If you cannot expel the embryos and the air bubbles into the uterine cavity, pull the capillary back out just a little from the hole and expel them again.

Note

To help you keep an eye on the hole made with the needle, you should hold both the needle and the transfer pipette in your dominant hand before you start the procedure.

9. Push the ovary, oviduct and uterine horn back into the abdomen and close the wound using wound clips.

10. Repeat the process to transfer 10 embryos into the other uterine cavity as described before.
11. Keep the mice warm on a 37℃ warming plate until they recover from the effects of the anesthesia.

References

1. Nagy A., Gertsenstein M., Vintersten K., and Behringer R. 2003. Manipulating the Mouse Embryo, A Laboratory Manual (Third edition). *Cold Spring Harbor Laboratory Press.* ISBN 0-87969-591-9.

7-4 Caesarean Section and Fostering

A Caesarean section should be performed if a pregnant recipient has not given birth to babies by the estimated date of delivery.

Materials and Equipment

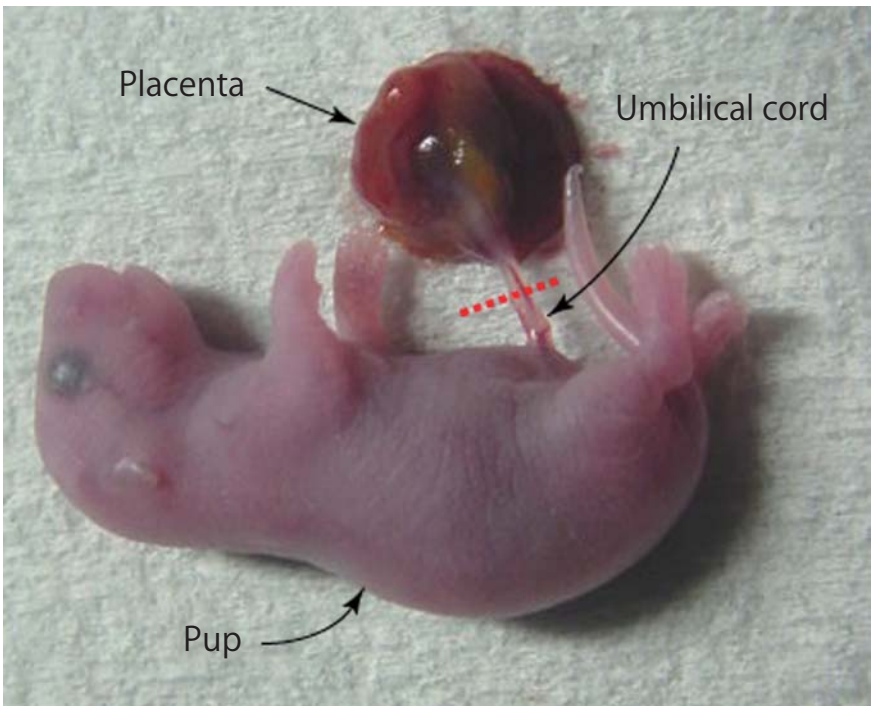
1. Foster mother (a foster mother is a female that has given birth on either the same day as, or the day preceding, the estimated date of delivery of the pregnant female)
2. Fine scissors
3. Pair of watchmaker's #5 forceps
4. Hot plate (37℃)
5. Pregnant female mouse

Procedures

Caesarean Section

1. Sacrifice the pregnant female and wipe the abdomen with a piece of cotton that has been soaked thoroughly in 70% ethanol.
2. Immediately open the abdomen and remove the uteruses containing the pups with a pair of fine scissors.
3. Place the uteruses on a paper towel, and cut through the uterine wall.
4. Quickly remove the pups from the yolk sac and amnion and cut the umbilical cord.

[Cutting the Umbilical Cord]



5. Use fine tissues to wipe the amniotic fluid, secretion, and blood from the surface of the pups' bodies.
6. Place the pups on a warming plate at 37℃, and softly pinch the tail of each pup a number of times with a pair of forceps until they start to breathe and turn sufficiently pink.

[From the Removal of the Uteruses through to the First Breaths of the Pups]
No. 19-01

Fostering

Select a foster mother whose pups have a different coat color than the caesarean sectioned pups, so that you can distinguish between them later.

1. Remove the foster mother from the cage.
2. Reduce the foster mother's litter of pups by half (for example, if the litter size of the foster mother is 10, remove 4-5 pups).
3. Mix the caesarean sectioned pups to be fostered (the same number of pups as the number of pups taken away) with the bedding, then mingle them with the remaining pups of the foster mother.
4. Put the foster mother back with the pups in the original cage.

[Fostering] No. 19-02

References

1. Nagy A., Gertsenstein M., Vintersten K., and Behringer R. 2003. Manipulating the Mouse Embryo, A Laboratory Manual (Third edition). *Cold Spring Harbor Laboratory Press.* ISBN 0-87969-591-9.

8-1 Storage of Media and Solutions in Ampoules Under Nitrogen Gas

Materials and Equipment

1. Twin jet ampoule sealer (Adelphi Manufacturing, West Sussex, UK)
2. Ampoule (sterilized via hot air sterilization (180℃, 3 hours))
3. Medium
4. Syringe and 18 gauge needle
5. Forceps
6. Nitrogen gas

Procedures

Cleaning and Sterilizing Glass Ampoules

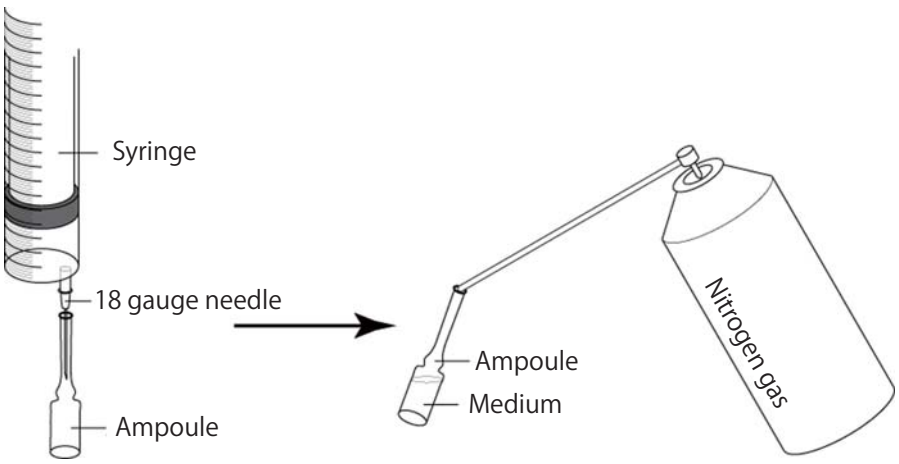
1. Rinse the glass ampoules one time using tap water.
2. Rinse the ampoules 2 times using distilled water.
3. Heat sterilize the ampoules at 180℃ for at least 3 hours.

Ignition

1. Open the gas cock and ignite the twin jet ampoule sealer.
2. Adjust the flames of the twin jet ampoule sealer so that the flame burns blue.

Sealing Ampoules

1. Add an appropriate amount of medium to each ampoule.
2. Introduce nitrogen gas into one of the ampoules and immediately seal the tip of the ampoule using the flames of the twin jet ampoule sealer. Repeat for all the remaining ampoules.



[Filling Ampoules with Medium] No. 20-01

8-2 Table of Media Composition

mHTF

Composition of mHTF

Reagent	mg/100 mL*	Vendor	Catalog Number
NaCl	593.8	Sigma	S 5886
KCl	35.0	Sigma	P 5405
$MgSO_4 \cdot 7H_2O$	4.9	Sigma	M 2773
KH_2PO_4	5.4	Sigma	P 5655
$CaCl_2$	57.0	Sigma	C 5670
$NaHCO_3$	210.0	Sigma	S 5761
Glucose	50.0	Sigma	G 6152
Na-lactate**	0.34 mL	Sigma	L 7900
Na-Pyruvate	3.7	Sigma	P 4562
Penicillin G	7.5	Sigma	P 7794
Streptomycin	5.0	Sigma	S 1277
BSA (Albmin, Bovine serum Fracition V Fatty Acid-Free)	400	MERCK/ CALBIOCEM	126575
0.5% phenol red	0.04 mL	Sigma	P 0290

*Water for embryo transfer; Sigma W1503
**Assay; 70%
mHTF is enclosed in brown ampoules and stored at 4℃.

References

1. Kito S., Hayao T., Noguchi-Kawasaki Y., Ohta Y., Hideki U., and Tateno S. 2004. Improved *in vitro* fertilization and development by use of modified human tubal fluid and applicability of pronucleate embryos for cryopreservation by rapid freezing in inbred mice. *Comp. Med.* **54**(5): 564-570.

Hyaluronidase

Composition of Hyaluronidase

Prepare 1% solution stock as indicated below. Filter-sterilize and store in 100 µL aliquots at -20℃. Before use, dilute the stock by 10 times. ex.) Add 20 µL stock solution (1%) to a 200 µL drop of mHTF containing oocytes to make a diluted solution of about 0.1%.

Reagent	mg*	Vendor	Catalog Number
Hyaluronidase	10	Sigma	H 3506

*mg/mL in mHTF

0.3 M Sucrose (BSA -)

Composition of 0.3 M sucrose (BSA-)

Reagent	mg*	Vendor	Catalog Number
Sucrose	2053.8	Sigma	S 1888

*mg/20 mL in PB1

Composition of PB1(BSA-)

Reagent	mg/100 mL*	Vendor	Catalog Number
NaCl	800.0	Sigma	S 5886
KCl	20.0	Sigma	P 5405
$CaCl_2$	12.0	Sigma	C 5670
KH_2PO_4	20.0	Sigma	P 5655
$MgCl_2 \cdot 6H_2O$	10.0	Sigma	M 2393
Na_2HPO_4	115.0	Sigma	S 5136
Na-Pyruvate	3.6	Sigma	P 4562
Glucose	100.0	Sigma	G 6152
Penicillin	7.5	Sigma	P 7794
Streptomycin	5.0	Sigma	S 1277

*Water for embryo transfer; Sigma W1503
0.3 M Sucrose (BSA-) is enclosed in brown ampoules and stored at 4℃.

0.3 M Sucrose (BSA +)

Composition of 0.3 M sucrose (BSA+)

Reagent	mg*	Vendor	Catalog Number
Sucrose	2053.8	Sigma	S 1888

*mg/20 mL in PB1

Composition of PB1(BSA+)

Reagent	mg/100 mL*	Vendor	Catalog Number
NaCl	800.0	Sigma	S 5886
KCl	20.0	Sigma	P 5405
$CaCl_2$	12.0	Sigma	C 5670
KH_2PO_4	20.0	Sigma	P 5655
$MgCl_2 \cdot 6H_2O$	10.0	Sigma	M 2393
Na_2HPO_4	115.0	Sigma	S 5136
Na-Pyruvate	3.6	Sigma	P 4562
Glucose	100.0	Sigma	G 6152
Penicillin	7.5	Sigma	P 7794
Streptomycin	5.0	Sigma	S 1277
BSA	300.0	Sigma	A 4378

*Water for embryo transfer; Sigma W1503

0.3 M Sucrose (BSA+) is enclosed in brown ampoules and stored at 4℃.

KSOM/AA

Composition of KSOM/AA

Reagent	mg/100 mL*	Vendor	Catalog Number
NaCl	555.0	Sigma	S 5886
KCl	18.5	Sigma	P 5405
KH_2PO_4	4.75	Sigma	P 5655
$MgSO_4 \cdot 7H_2O$	4.95	Sigma	M 2773
$CaCl_2 \cdot 2H_2O$	25.0	Sigma	C 7902
$NaHCO_3$	210.0	Sigma	S 5761
Glucose	3.6	Sigma	G 6152
Na-Pyruvate	2.2	Sigma	P 4562
DL-Lactic Acid sodium salt	0.174 mL	Sigma	L 1375
10 mM EDTA	100 µL	Sigma	E 6635
Streptomycin	5.0	Sigma	S 9137
Penicillin	6.3	Sigma	P 7794
0.5% phenol red	0.1 mL	Sigma	P 0290
L-Glutamine	14.6	Sigma	G 8540
MEM Essential Amino Acids solution	1.0 mL	GIBCO	11130-051
MEM Non-essential Amino acid solution	0.5 mL	Sigma	M 7145
BSA	100.0	Sigma	A 4378

*Water for embryo transfer; Sigma W1503
KSOM/AA is enclosed in brown ampoules and stored at 4℃.

References

1. Lawitts J. A., and Biggers J. D. 1993. Culture of preimplantation embryos. *Methods Enzymol.* **225**:153-164.

0.8 M Sucrose

Composition of 0.8 M sucrose

Reagent	mg*	Vendor	Catalog Number
Sucrose	5476.8	Sigma	S 1888

*mg/20 mL in PB1

Composition of PB1

Reagent	mg/100 mL*	Vendor	Catalog Number
NaCl	800.0	Sigma	S 5886
KCl	20.0	Sigma	P 5405
$CaCl_2$	12.0	Sigma	C 5670
KH_2PO_4	20.0	Sigma	P 5655
$MgCl_2 \cdot 6H_2O$	10.0	Sigma	M 2393
Na_2HPO_4	115.0	Sigma	S 5136
Na-Pyruvate	3.6	Sigma	P 4562
Glucose	100.0	Sigma	G 6152
Penicillin	7.5	Sigma	P 7794
Streptomycin	5.0	Sigma	S 1277
BSA	300.0	Sigma	A 4378

*Water for embryo transfer; Sigma W1503
0.8 M Sucrose is enclosed in brown ampoules and stored at 4℃.

PB1

Composition of PB1

Reagent	mg/100 mL*	Vendor	Catalog Number
NaCl	800.0	Sigma	S 5886
KCl	20.0	Sigma	P 5405
$CaCl_2$	12.0	Sigma	C 5670
KH_2PO_4	20.0	Sigma	P 5655
$MgCl_2 \cdot 6H_2O$	10.0	Sigma	M 2393
Na_2HPO_4	115.0	Sigma	S 5136
Na-Pyruvate	3.6	Sigma	P 4562
Glucose	100.0	Sigma	G 6152
Penicillin	7.5	Sigma	P 7794
Streptomycin	5.0	Sigma	S 1277
BSA	300.0	Sigma	A 4378

*Water for embryo transfer; Sigma W1503
PB1 is enclosed in brown ampoules and stored at 4℃.

1 M DMSO

Composition of 1 M DMSO

Reagent	mL*	Vendor	Catalog Number
DMSO	1.56	Sigma	D 2650
PB1	18.44	-	-

*Final volume: 20 mL

Composition of PB1

Reagent	mg/100 mL*	Vendor	Catalog Number
NaCl	800.0	Sigma	S 5886
KCl	20.0	Sigma	P 5405
$CaCl_2$	12.0	Sigma	C 5670
KH_2PO_4	20.0	Sigma	P 5655
$MgCl_2 \cdot 6H_2O$	10.0	Sigma	M 2393
Na_2HPO_4	115.0	Sigma	S 5136
Na-Pyruvate	3.6	Sigma	P 4562
Glucose	100.0	Sigma	G 6152
Penicillin	7.5	Sigma	P 7794
Streptomycin	5.0	Sigma	S 1277
BSA	300.0	Sigma	A 4378

*Water for embryo transfer; Sigma W1503
1 M DMSO is enclosed in brown ampoules and stored at 4℃.

DAP213

Method of preparing DAP213

1. Solution A and Solution B are first prepared and are each fully dissolved.
2. Equal volumes of A and B are then mixed to form DAP213.

Solution A

Reagent	mL*	Vendor	Catalog Number
PB1	2.3088	-	-
DMSO	3.1252	Sigma	D 2650
Propylene glycol (PG)	4.556	Sigma	134368

Caution The solution may become cloudy when DMSO is added.

Solution B

Reagent	mg*	Vendor	Catalog Number
Acetamide (AA)	1181.4	Sigma	A 0500

*mg/10 mL in PB1

Composition of PB1

Reagent	mg/100 mL*	Vendor	Catalog Number
NaCl	800.0	Sigma	S 5886
KCl	20.0	Sigma	P 5405
$CaCl_2$	12.0	Sigma	C 5670
KH_2PO_4	20.0	Sigma	P 5655
$MgCl_2 \cdot 6H_2O$	10.0	Sigma	M 2393
Na_2HPO_4	115.0	Sigma	S 5136
Na-Pyruvate	3.6	Sigma	P 4562
Glucose	100.0	Sigma	G 6152
Penicillin	7.5	Sigma	P 7794
Streptomycin	5.0	Sigma	S 1277
BSA	300.0	Sigma	A 4378

*Water for embryo transfer; Sigma W1503
DAP213 is enclosed in brown ampoules and stored at 4℃.

0.25 M Sucrose

Composition of 0.25 M sucrose

Reagent	mg*	Vendor	Catalog Number
Sucrose	1711.5	Sigma	S 1888

*mg/20 mL in PB1

Composition of PB1

Reagent	mg/100 mL*	Vendor	Catalog Number
NaCl	800.0	Sigma	S 5886
KCl	20.0	Sigma	P 5405
$CaCl_2$	12.0	Sigma	C 5670
KH_2PO_4	20.0	Sigma	P 5655
$MgCl_2 \cdot 6H_2O$	10.0	Sigma	M 2393
Na_2HPO_4	115.0	Sigma	S 5136
Na-Pyruvate	3.6	Sigma	P 4562
Glucose	100.0	Sigma	G 6152
Penicillin	7.5	Sigma	P 7794
Streptomycin	5.0	Sigma	S 1277
BSA	300.0	Sigma	A 4378

*Water for embryo transfer; Sigma W1503
0.25 M Sucrose is enclosed in brown ampoules and stored at 4℃.

mWM

Composition of mWM

Reagent	mg/100 mL*	Vendor	Catalog Number
NaCl	640.0	Sigma	S 5886
KCl	35.6	Sigma	P 5405
KH_2PO_4	16.2	Sigma	P 5655
$MgSO_4 \cdot 7H_2O$	29.4	Sigma	M 7774
$NaHCO_3$	190.0	Sigma	S 5761
Glucose	100.0	Sigma	G 6152
Na-Pyruvate	2.5	Sigma	P 4562
Ca-lactate pentahydrate	46.0	Sigma	C 8356
Streptomycin	5.0	Sigma	S 1277
Penicillin G	7.5	Sigma	P 7794
0.5% phenol red	0.2 mL	Sigma	P 0290
20 mM 2-ME	10.0 μL	Sigma	M 7522
100 mM EDTA	50.0 μL	Sigma	E 6635
BSA	300.0	Sigma	A 4378

*Water for embryo transfer; Sigma W1503
mWM is enclosed in brown ampoules and stored at 4℃.

All video files shown in this manual are provided in the supplementary USB flash drive. If you need it or have questions, please feel free to contact us.

COSMO BIO flash drive

Front Back

Contact:
Cosmo Bio Co., Ltd.
International Sales Dept.
Toyo-Ekimae Bldg., 2-20, Toyo 2-Chome, Koto-ku, Tokyo 135-0016, Japan
Tel: +81-3-56329617
Fax: +81-3-56329618
Email: export@cosmobio.co.jp
Web: www.cosmobio.com

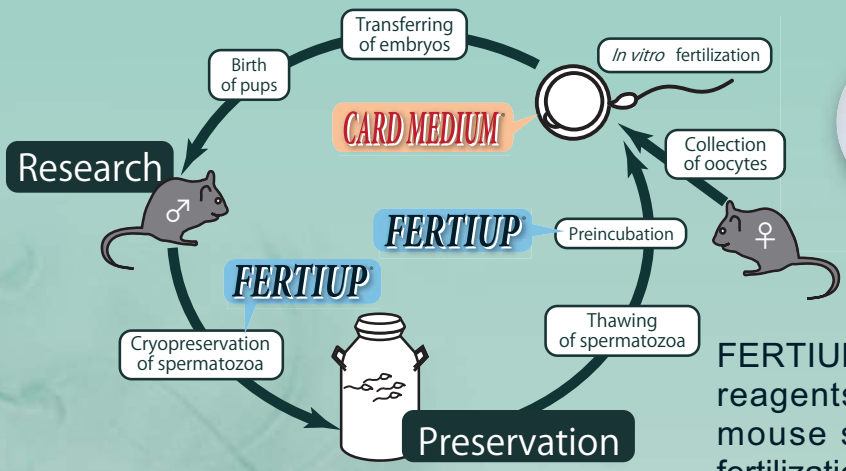
FERTIUP®

Cryoprotectant
Preincubation Medium

CARD MEDIUM®

Mouse in vitro Fertilization Medium

IVF Slump? FERTIUP® Will Get You Out!!

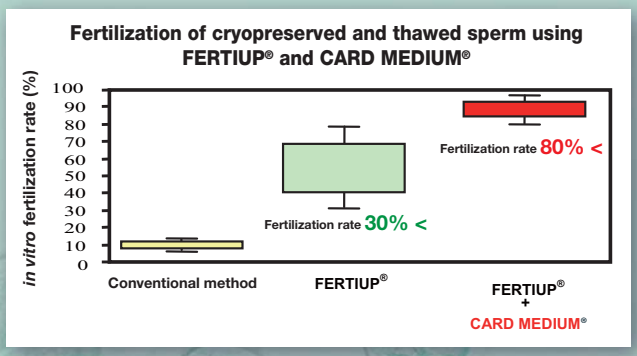


FERTIUP® MS CPA KYD-001-EX | FERTIUP® MS PM KYD-002-EX | CARD MEDIUM® KYD-003-EX

FERTIUP® and CARD MEDIUM® are valuable reagents to improve the recovery of frozen mouse spermatozoa and improve *in vitro* fertilization efficiency of laboratory mice.

Combined usage of FERTIUP® Cryoprotectant, FERTIUP® Preincubation Medium and CARD MEDIUM® Mouse Fertilization Medium offers the following benefits:

- Fertilization rates over 80%
- Improved management of transgenic mouse
- Reduction of labor, facilities and breeding costs
- Reduction of colony expansion time
- Efficient production in difficult breeding



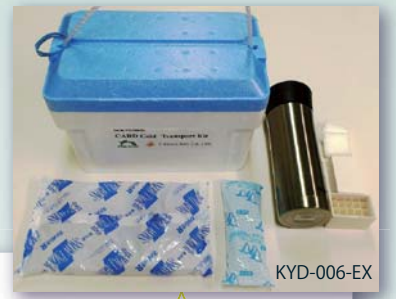
Description	Cat. No.	Quantity
FERTIUP® Cryoprotectant: CPA	KYD-001-EX	1 mL
	KYD-001-EX-X5	5 x 1 mL
	KYD-001-05-EX	0.5 mL
	KYD-001-05-EX-X5	5 x 0.5 mL
FERTIUP® Preincubation Medium: PM	KYD-002-EX	1 mL
	KYD-002-EX-X5	5 x 1 mL
	KYD-002-05-EX	0.5 mL
	KYD-002-05-EX-X5	5 x 0.5 mL
CARD MEDIUM® Kit includes 1 ampoule including medium (A), 1 vial including powder (B), a 1.5 mL plastic tube (C), a 1.5 mL plastic tube (D), a 2.5 mL disposable syringe, 1 needle, 1 filter unit (pore size: 0.22 µm)	KYD-003-EX	1 kit
FERTIUP® PM 1ML-CARD MEDIUM® set FERTIUP® Preincubation Medium: PM (1 mL) x 1 vial, CARD MEDIUM® x 1 kit	KYD-004-EX	1 set
FERTIUP® PM 0.5ML-CARD MEDIUM® set FERTIUP® Preincubation Medium: PM (0.5 mL) x 1 vial, CARD MEDIUM® x 1 kit	KYD-005-EX	1 set

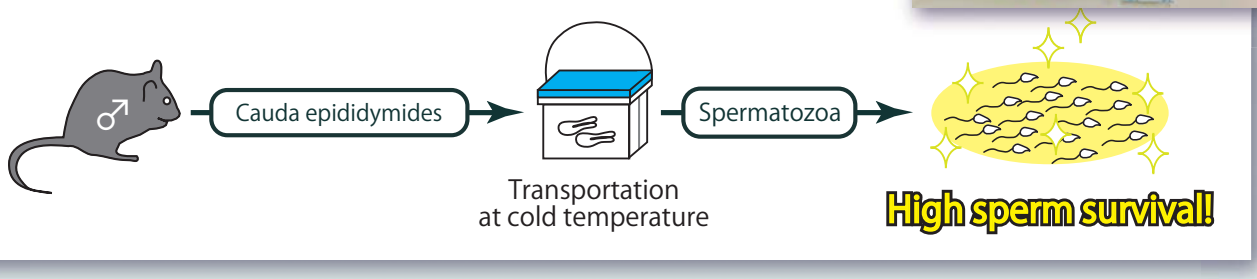
COSMO BIO CO., LTD.

CARD COLD TRANSPORT KIT

Specially designed for inexpensive, safe transportation of mouse cauda epididymides and embryos at cold temperature.

- **Reduce cost for transportation of live mice**
- **Eliminate risk of mouse fatalities or escape during transportation**
- **Prevent transmission of pathogens**
- **Valuable for the Rescue *in vitro* Fertilization Method for Legacy Stock of Cryopreserved Spermatozoa**

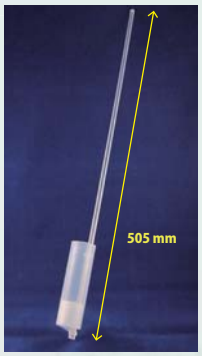

KYD-006-EX



Description	Cat. No.	Quantity
Card Cold Transport Kit	KYD-006-EX	1 set
Foam transport box (1 box), Cold packs (4 large packs, 2 small packs), Thermos bottle (1 bottle), Paper box (1 box), Shock-absorbing material (1 piece)		

FERTIUP® & CARD MEDIUM peripheral products

Description	Cat. No.	Quantity
Sperm Straws (10 Pieces x 10 Units)	KYD-S020X10	10 pc
Freezing Canister	KYD-S018	1 unit
Straw Connector (5 parts included)	KYD-S025	1PC
Triangular Cassette Short (10units)	KYD-S021	10 unit
Triangular Cassette Long (10units)	KYD-S035	10 unit
Embryo Manipulation Instrument Set	KYD-S036	1 set
Glass capillary 20PC	KYD-S037	1 set
Touch Burner APT-3	PHD-APT3-EX	10 unit

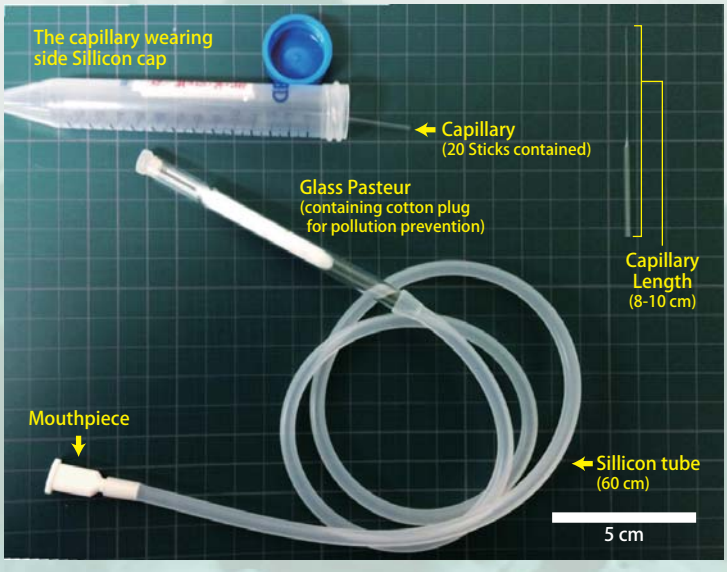


Freezing Canister
KYD-S018

Straw Connector
KYD-S025

Triangular Cassette Long
KYD-S035

Embryo Manipulation Instrument Set
KYD-S036



Triangular Cassette Short
KYD-S021

Sperm Straws
KYD-S020X10

Touch Burner APT-3
PHD-APT3-EX

COSMO BIO CO., LTD.

Search! fertiup

CARD HyperOva®

Enhanced Superovulation Reagent for Mouse

Live IVF pups from A SINGLE C57BL/6J female following superovulation with CARD HyperOva®

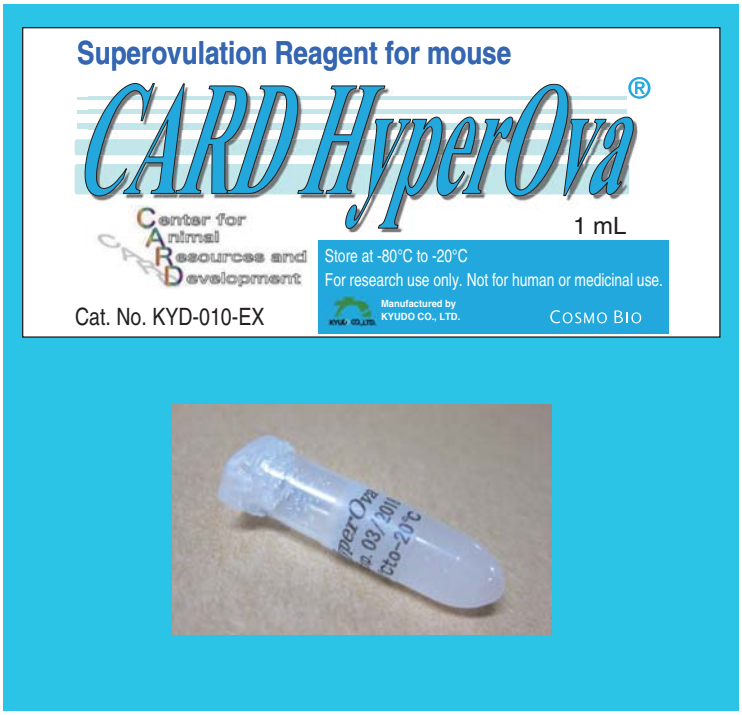
Great for IVF.

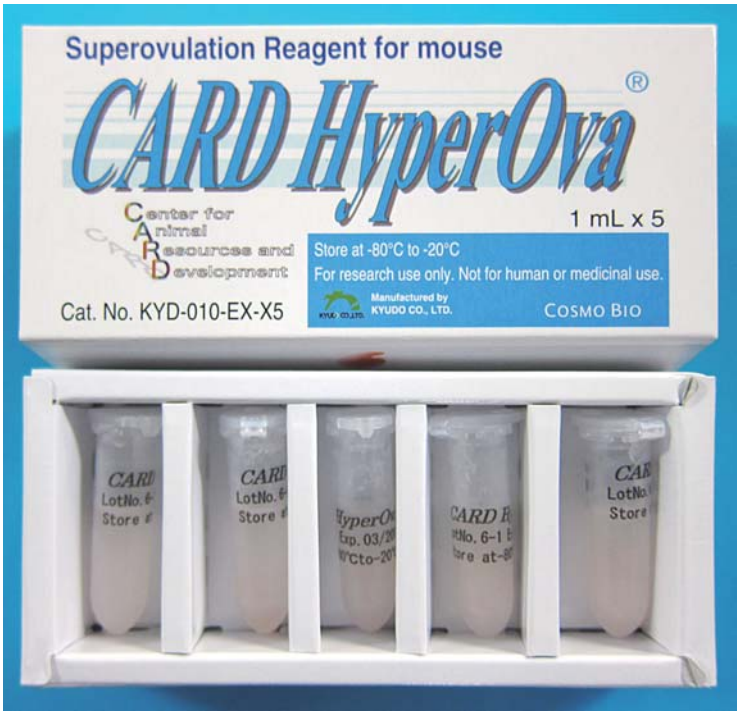
Use HyperOva® to obtain more ovulated oocytes.

Center for Animal Resources and Development

- Approximately 100 oocytes can be collected from a single C57BL/6J mouse.
- Maximize IVF efficiency by using HyperOva® in conjunction with FERTIUP® Mouse Sperm Cryopreservation Media, FERTIUP® Mouse Sperm Preincubation Media, and CARD MEDIUM® mouse fertilization Medium.

COSMO BIO CO., LTD.





Composition:

An optimized blend of anti-inhibin antibody and purified equine chorionic gonadotropin (eCG)

Superovulation Procedure:

1. Inject 0.1-0.2 mL CARD HyperOva® i.p. into a 26-30 day old female mouse (birthdate = 0). Injections are usually performed during the light cycle, between 17:00 and 18:00.
2. At 48 hours after CARD HyperOva® recipients are injected i.p. with 7.5 IU human chorionic gonadotropin (hCG) (not included).

References:

1. Takeo T., Nakagata N. 2015. Superovulation using the combined administration of inhibin antiserum and equine chorionic gonadotropin increases the number of ovulated oocytes in C57BL/6 female mice. *PLoS ONE* **10**(5): e0128330. doi:10.1371/journal.pone.0128330
2. Takeo T., Nakagata N. 2016. Immunotherapy using inhibin antiserum enhanced the efficacy of equine chorionic gonadotropin on superovulation in major inbred and outbred mice strains. *Theriogenol*. doi:10.1016/j.theriogenology.2016.04.076

Description	Cat. No.	Quantity	Storage
CARD HyperOva®	KYD-010-EX	1 mL	-20℃
	KYD-010-EX-X5	5x1 mL	-20℃

Shipping: Dry Ice

Vitrification / thaw / liquids and Medium For Mouse, Rat and Embryo Manipulation

For Mouse

Description	Application	Cat. No.	Quantity	Storage
HTF	*in vitro* fertilization	CSR-R-B071	5 mL x 10 vials	4℃
HTF	*in vitro* fertilization	CSR-R-B070	2 mL x 10 vials	4℃
mHTF	*in vitro* fertilization	KYD-008-02-EX	2 mL x 1 vial	4℃
mHTF	*in vitro* fertilization	KYD-008-02-EX-X5	2 mL x 10 vials	4℃
mHTF	*in vitro* fertilization	KYD-008-05-EX	5 mL x 1 vial	4℃
mHTF	*in vitro* fertilization	KYD-008-05-EX-X3	5 mL x 10 vials	4℃
KSOM	*in vitro* culture	CSR-R-B075	5 mL x 10 vials	4℃
KSOM	*in vitro* culture	CSR-R-B074	2 mL x 10 vials	4℃
mWM	*in vitro* culture	CSR-R-B081	5 mL x 10 vials	4℃
mWM	*in vitro* culture	CSR-R-B080	2 mL x 10 vials	4℃
0.25M sucrose	freeze-thawing	CSR-R-Y078	5 mL x 10 vials	4℃
0.25M sucrose	freeze-thawing	CSR-R-Y077	2 mL x 10 vials	4℃
1M DMSO	cryopreservation	CSR-R-T072	2 mL x 10 vials	4℃
DAP213	cryopreservation	CSR-R-T073	1 mL x 10 vials	4℃

For Rat

Description	Application	Cat. No.	Quantity	Storage
mR1ECM	*in vitro* culture	CSR-R-M174	5 mL x 10 vials	4℃
mR1ECM	*in vitro* culture	CSR-R-M191	2 mL x 10 vials	4℃

For Embryo Manipulation

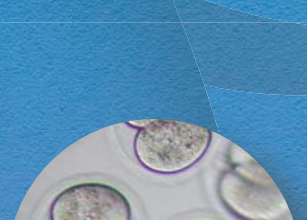
Description	Application	Cat. No.	Quantity	Storage
M2	*in vitro* manipulation	CSR-R-M084	5 mL x 10 vials	4℃
M2	*in vitro* manipulation	CSR-R-M083	2 mL x 10 vials	4℃
PB1	*in vitro* manipulation	CSR-R-P183	5 mL x 10 vials	4℃
PB1	*in vitro* manipulation	CSR-R-P185	2 mL x 10 vials	4℃
PEPeS	cryopreservation	CSR-R-P187	1 mL x 10 vials	4℃
P10	cryopreservation	CSR-R-P186	2 mL x 10 vials	4℃

Reproductive Engineering Techniques **in Mice**

Technical Manual

Naomi Nakagata

Division of Reproductive Engineering
Center for Animal Resources & Development (CARD)
Kumamoto University, Japan



COSMO BIO CO., LTD.
TOYO EKIMAE BLDG. 2-20, TOYO 2-CHOME,
KOTO-KU. TOKYO 135-0016, JAPAN
TEL : (81)3-5632-9617
FAX : (81)3-5632-9618
e-mail : export@cosmobio.co.jp
URL : www.cosmobio.com

10167

Reproductive Engineering Techniques in Mice Technical Manual
COSMO BIO CO., LTD.