Reproductive Engineering Techniques in Mice

Technical Manual

Naomi Nakagata

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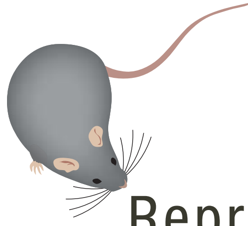

Reproductive Engineering Techniques **in Mice**

Technical Manual

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Preface

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

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

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

Naomi Nakagata

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* 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

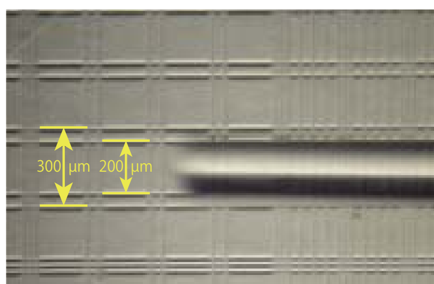
1. Heat the center of a glass capillary pipette with the upper part of the flame of an alcohol lamp. When the center of the glass capillary pipette has softened sufficiently, remove it from the flame and quickly pull it at both ends.
2. Split the glass capillary pipette in two by placing the center of 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.

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.

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

[When the hemocytometer is in focus]



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]

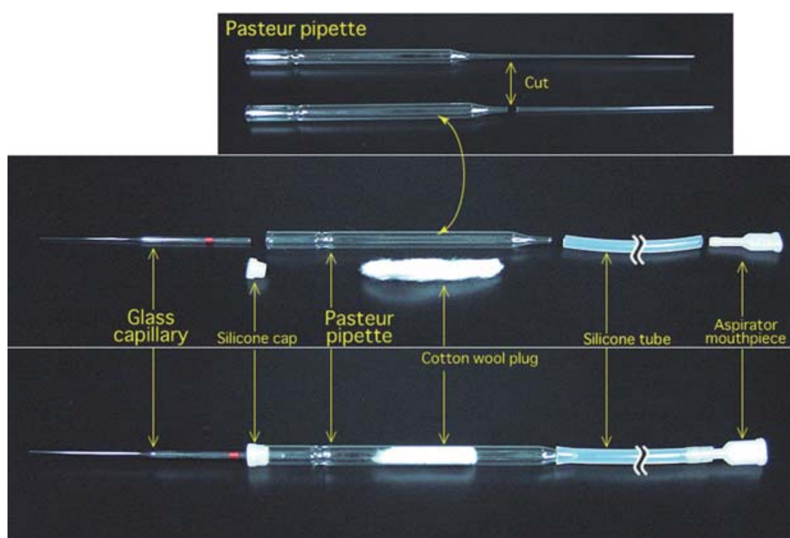
[The polished end of a glass capillary pipette]

[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)

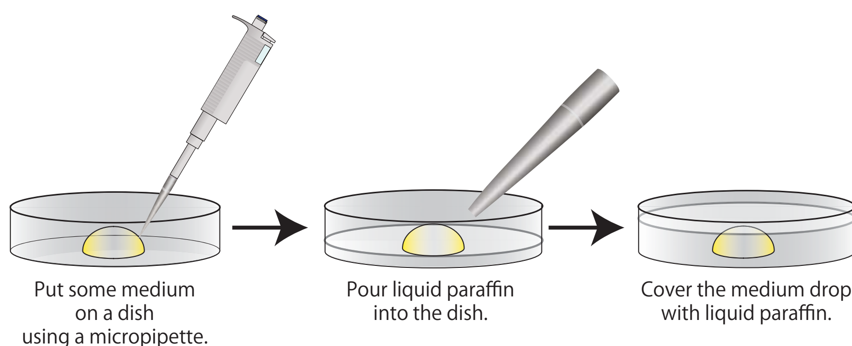
Procedures

Superovulation

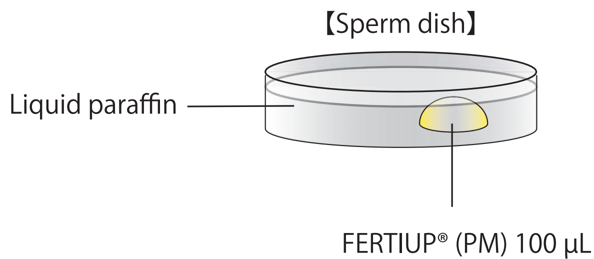
1. Induce superovulation by injecting 7.5 IU of pregnant mare's serum gonadotropin (PMSG) i. p. into each mature female mouse (8-12 weeks old). (PMSG is usually administered during the light cycle, between the hours of 14:00 and 18:00).
2. Follow this up 48-52 hours later with a 7.5 IU i. p. injection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

Preparation of Dishes

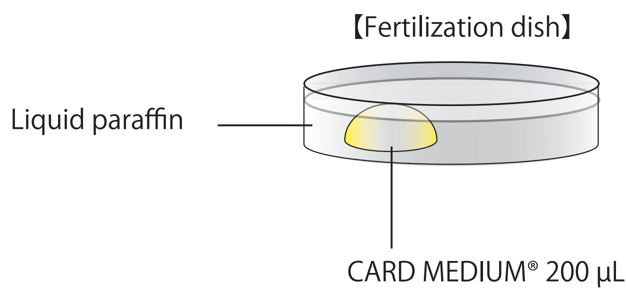
1. Prepare dishes as instructed below and keep them in an incubator (37℃, 5% CO_2 in air) to allow them to gas-equilibrate.



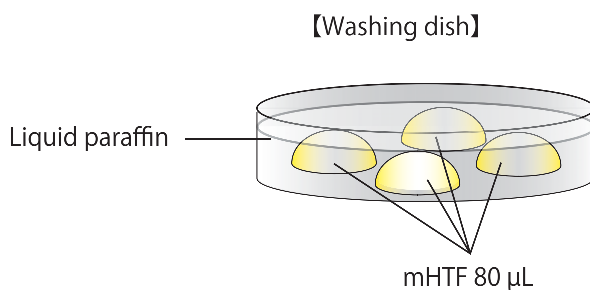
a. Sperm dish
Put 1 drop (100 µL / drop) of FERTIUP® (PM) into a dish and cover it with liquid paraffin 30 minutes before collecting sperm, and place the dish in an incubator.



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



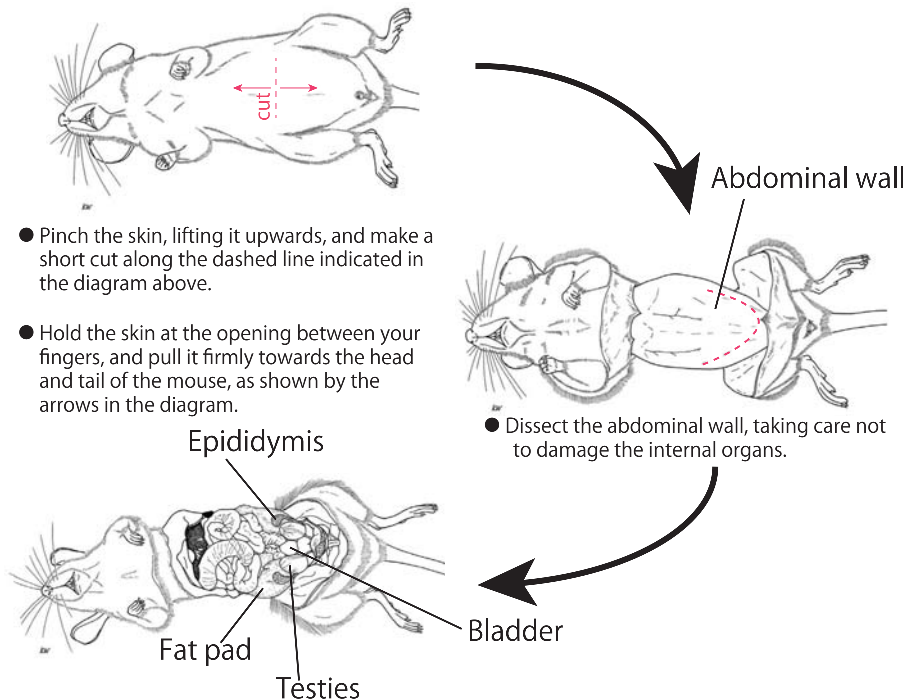
Note

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.

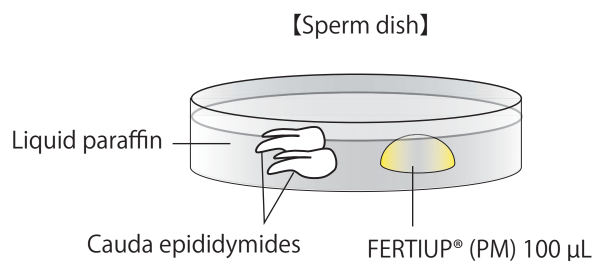
Collection of Spermatozoa

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.

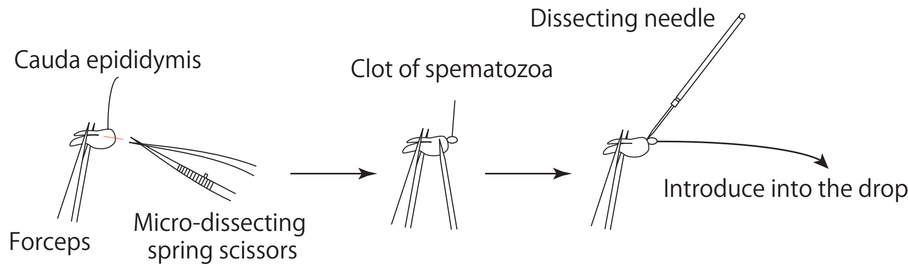


3. Place the removed cauda epididymides in a sperm dish containing liquid paraffin.

【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).

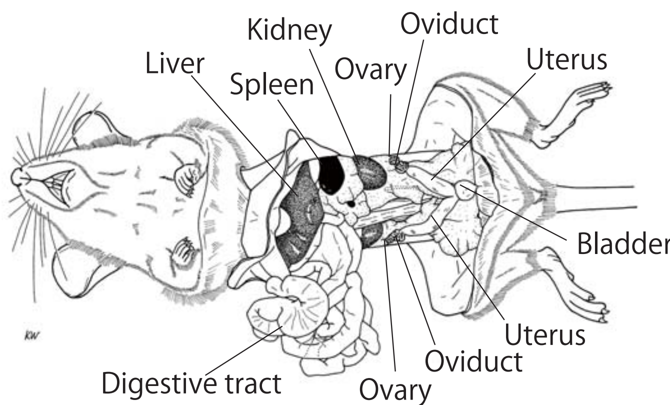


[Releasing Spermatozoa] No. 02-02

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

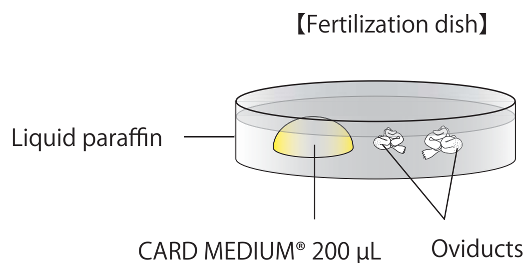
1. Sacrifice a superovulating mature female mouse (8-12 weeks old) approximately 15-17 hours after administering hCG.
2. Dissect the mouse to expose the abdominal cavity.
3. Move the digestive tract from inside the abdomen and expose the uteruses, oviducts and ovaries.
4. Remove the uteruses, oviducts 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】

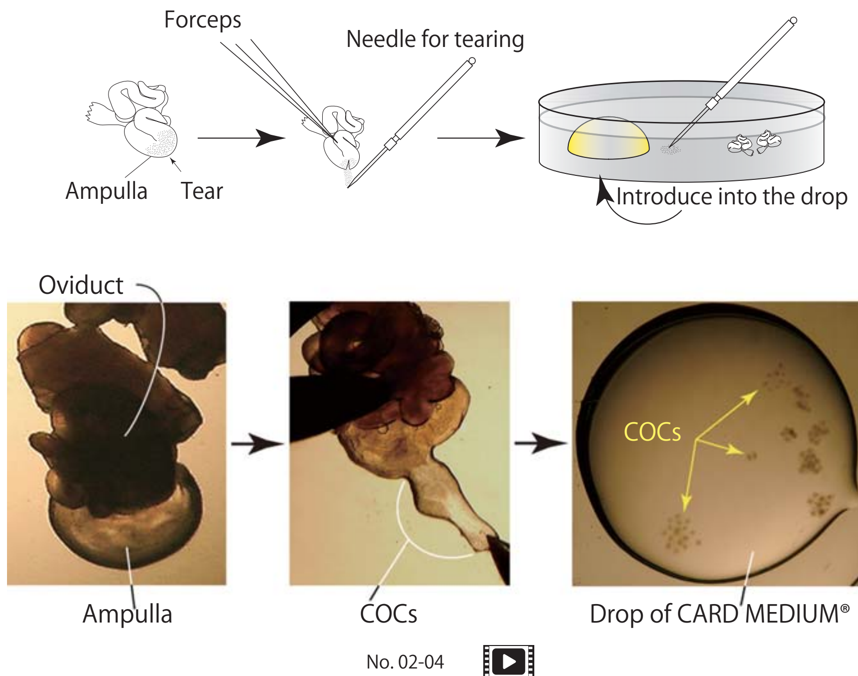


Note

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®]

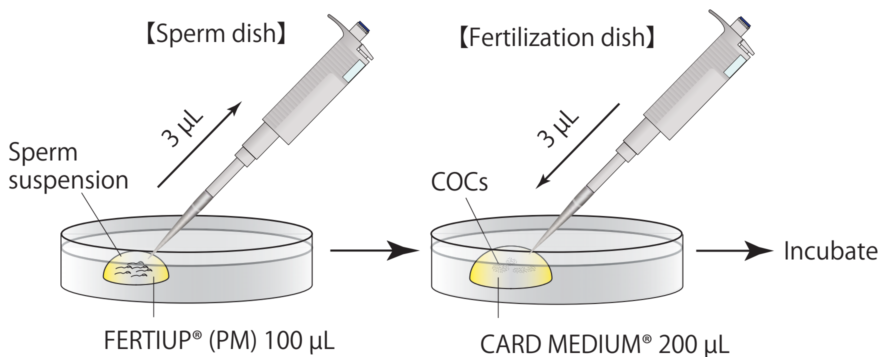


No. 02-04

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).

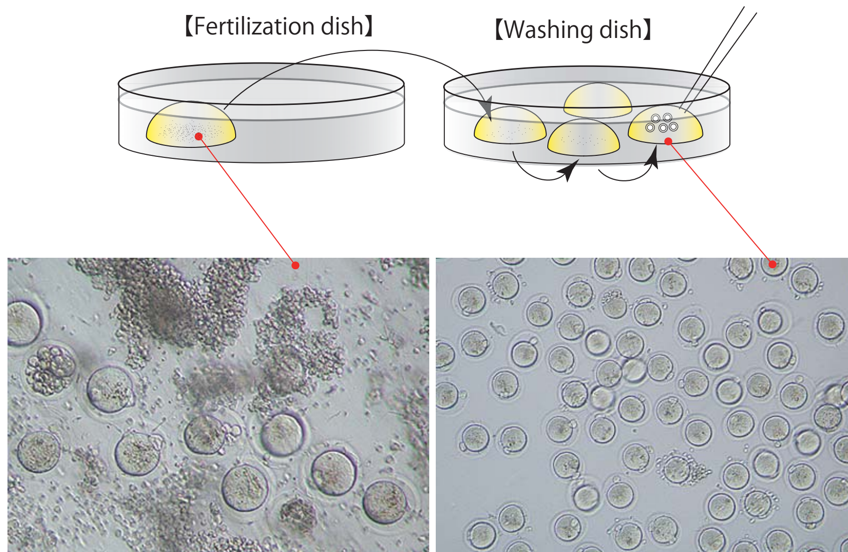


Note

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.

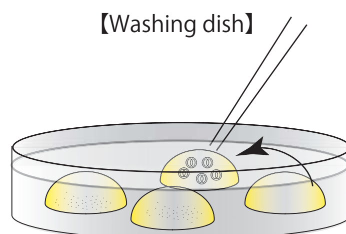
3. 3 hours after insemination, wash the oocytes 3 times in fresh mHTF (80 µL) in a washing dish, avoiding the transfer of CARD MEDIUM®.



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.)



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.

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).

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

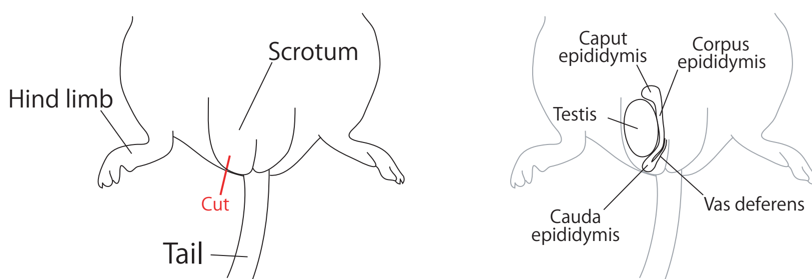
1. Male mouse (over 12 weeks old)
2. Anesthetic
3. Hot plate (37℃)
4. Fine scissors
5. Pair of watchmaker's #5 forceps
6. Wound clip (Autoclip 9 mm; Clay Adams 427631) and clip applicator (Mik-Ron Autoclip Applier; Clay Adams 427630)
7. Temperature data logger (Thermochron iButton Cat. No. DS1921G; Maxim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

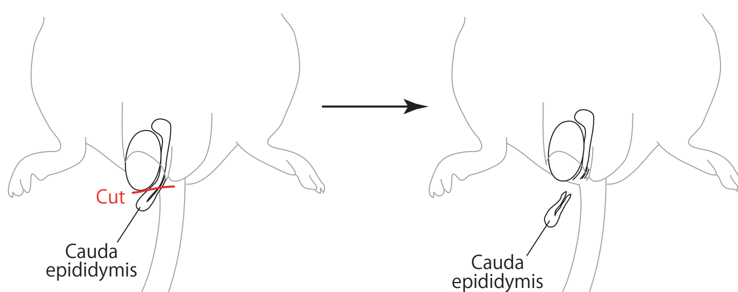
Procedures

Collection of Cauda Epididymis

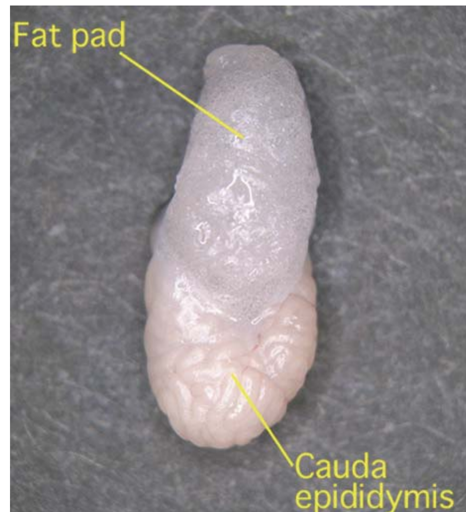
1. Anesthetize a male mouse.
2. Make a small incision in the scrotum of the mouse and expel a cauda epididymis.



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



[Collected Cauda Epididymis]

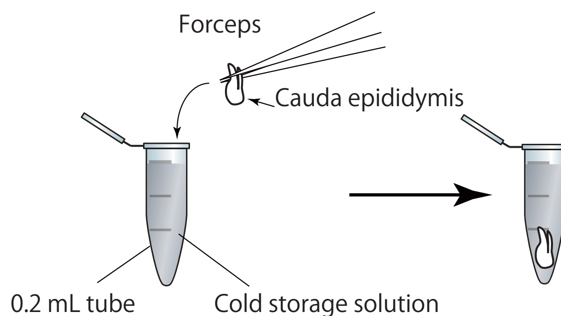


4. Push the testis back into the abdomen and close the wound using a wound clip.
5. Keep the mouse warm on a 37℃ warming plate until the mouse recovers from the effects of the anesthesia.

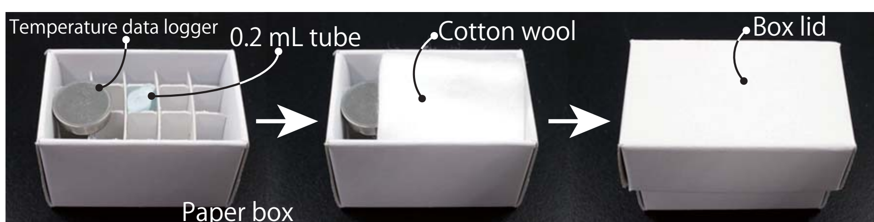
Packing and Transport of Cauda Epididymis

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.



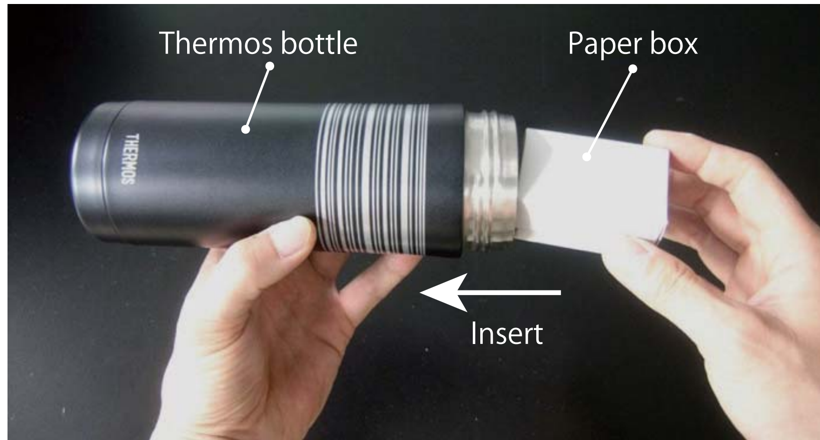
2. Place the tube containing the cauda epididymis, a temperature data logger and a piece of cotton wool in the paper box.



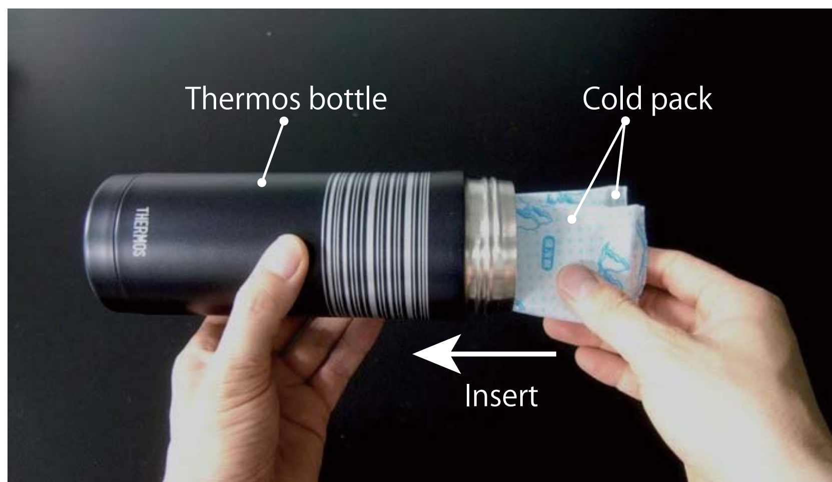
Comment

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.

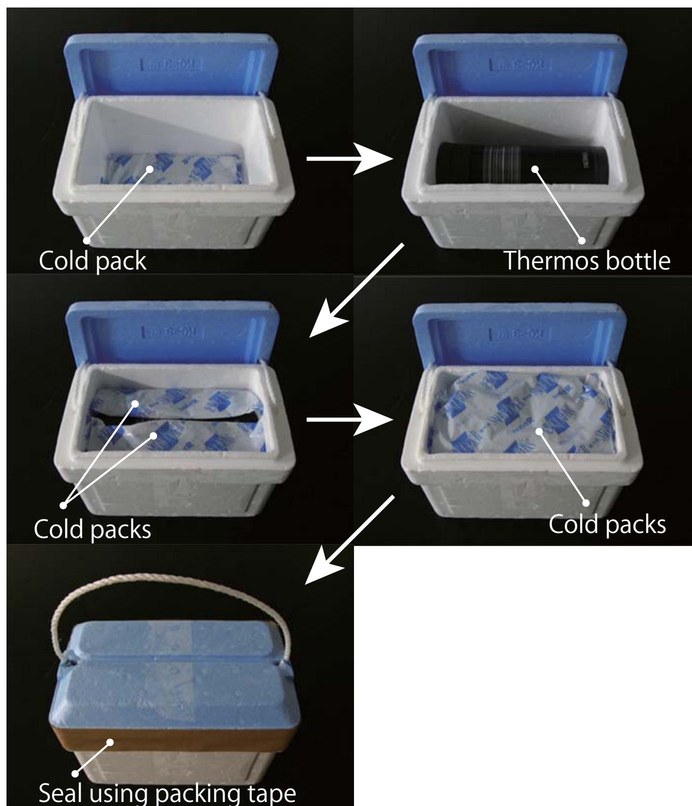
6. Place a cold pack (large) at the bottom of a foam transport box, then put the thermos bottle on top of it.
7. Pack one cold pack (large) on either side of the bottle, then set a further pack (large) on top and close the lid.
8. Seal the lid of the foam transport box using packing tape.

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.

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.

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.

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

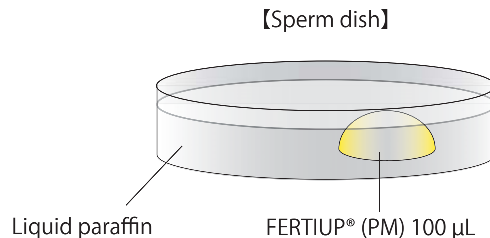
Materials and Equipment

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

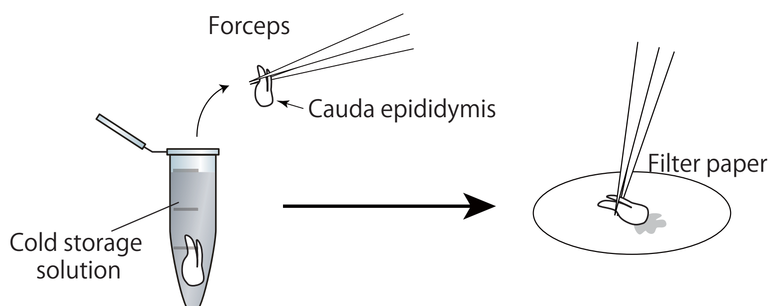
Collection of Cauda Epididymis

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)

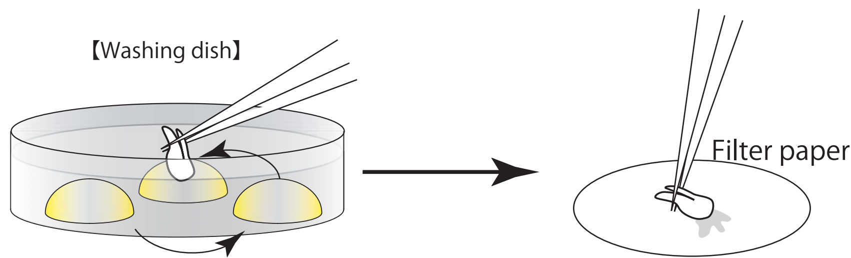


2. Remove the 0.2 mL tube containing the sample from the foam transport box.

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.

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.

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.

3-1 Cryopreservation of Mouse Spermatozoa

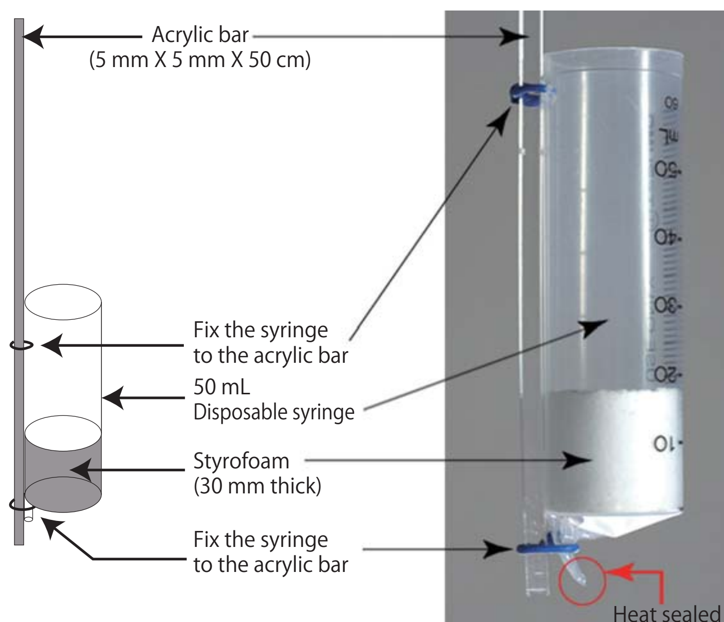
Materials and Equipment

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

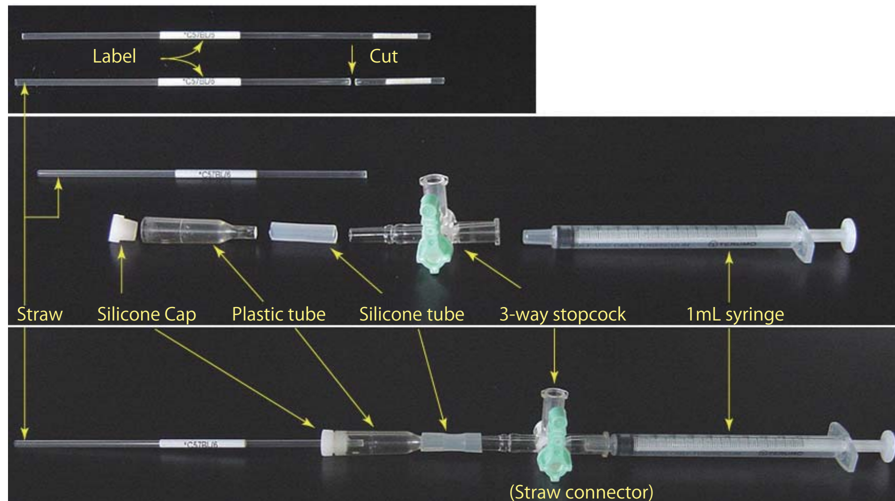
1. Insert a piece of styrofoam tightly into the bottom of the syringe.
2. Heat seal the mouth of the syringe tip.
3. Fix the syringe to the acrylic bar.



Preparing a Straw Connector

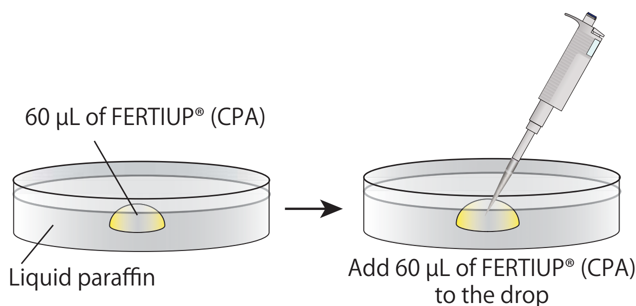
1. Using a 1 mL syringe, a 3-way stopcock, a piece of silicone tube, a plastic tube and a silicone cap, make a straw connector as shown in the diagram below.
2. To use the straw connector, cut away the cotton plug from a straw, then attach the straw to the silicone cap at the end of the connector.

[Connecting the Straw connector and Straw]

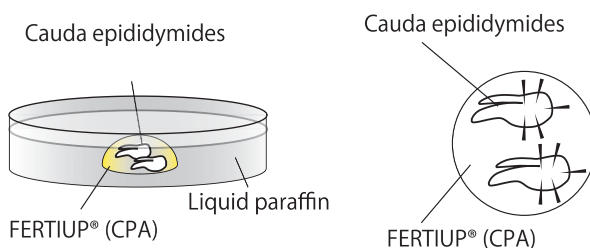


Preparing Sperm Suspension

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℃ 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.



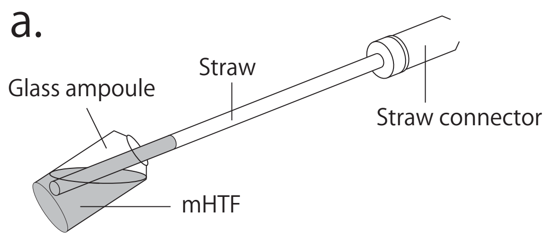
6. Place the dish on a hot plate at 37℃ for 3 minutes. During this time, rotate the dish every minute to disperse sperm from the organs in the FERTIUP® (CPA).

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

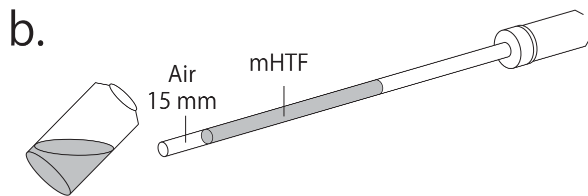
Preparing Freezing Straw Containing Sperm Suspension

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.

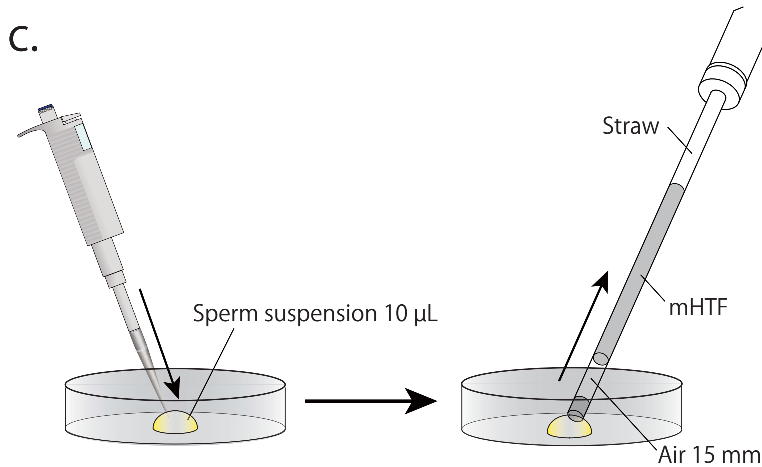
a.



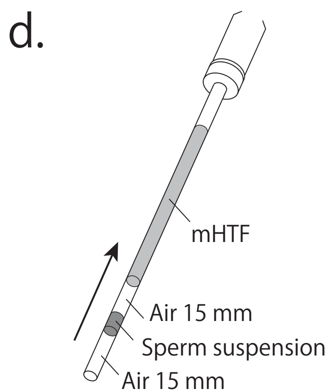
b.



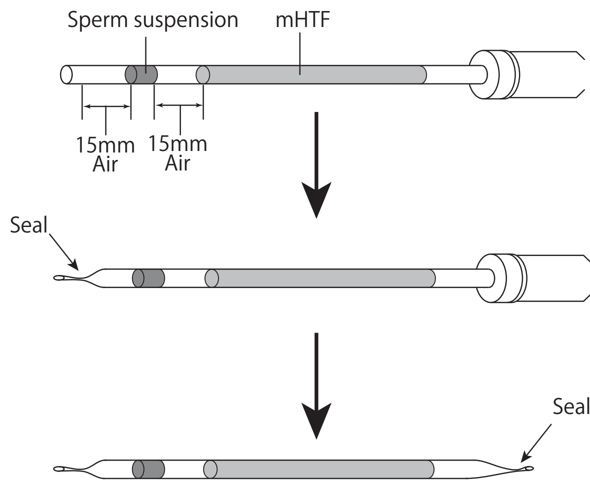
c.



d.



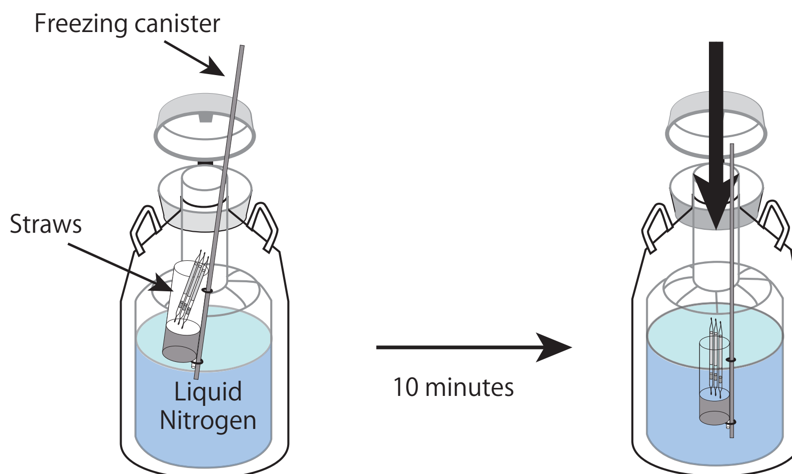
3. Seal both sides of the straw using an impulse sealer.



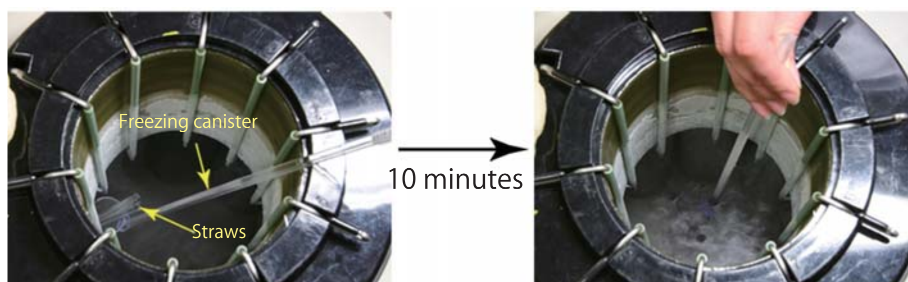
4. Create 10 samples per mouse in the same manner as described above.

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]

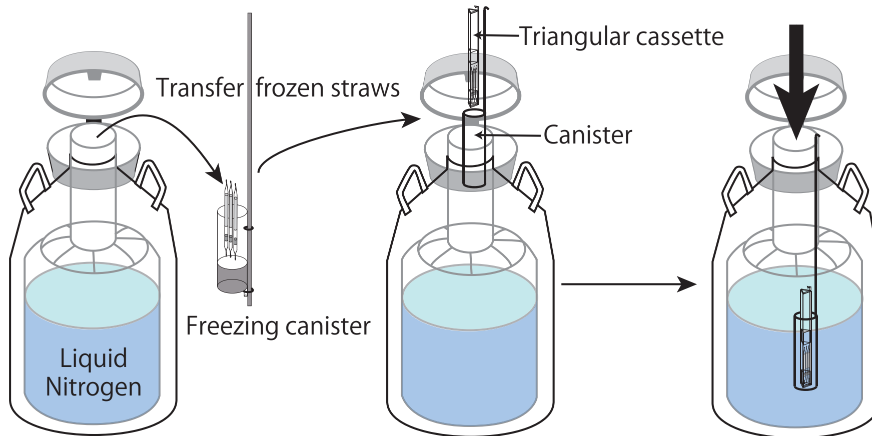


[Freezing the Straws] No. 05-02

Comment

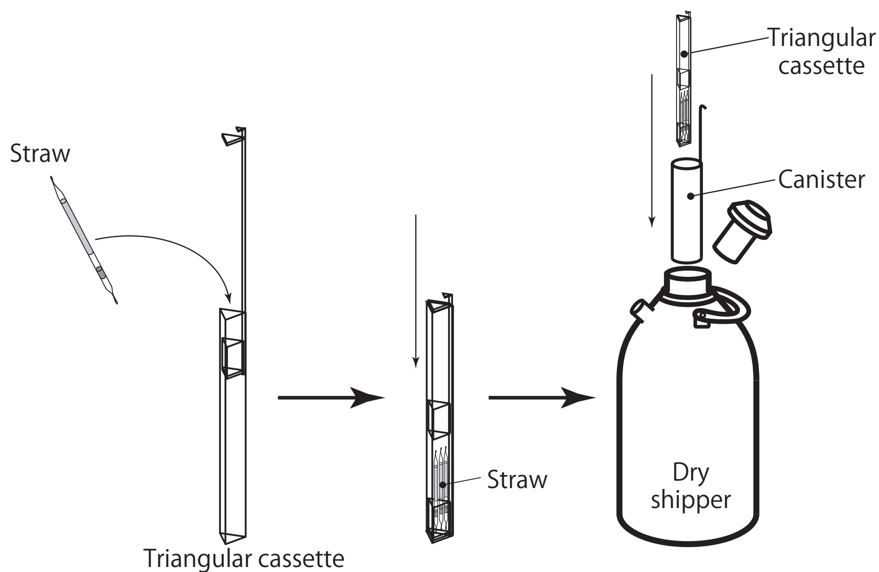
Loading 100 μL of mHTF into the straw prevents the straw from floating on the surface of liquid nitrogen.
This is because the mHTF acts as a weight that forces the straw to sink into the liquid nitrogen.

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

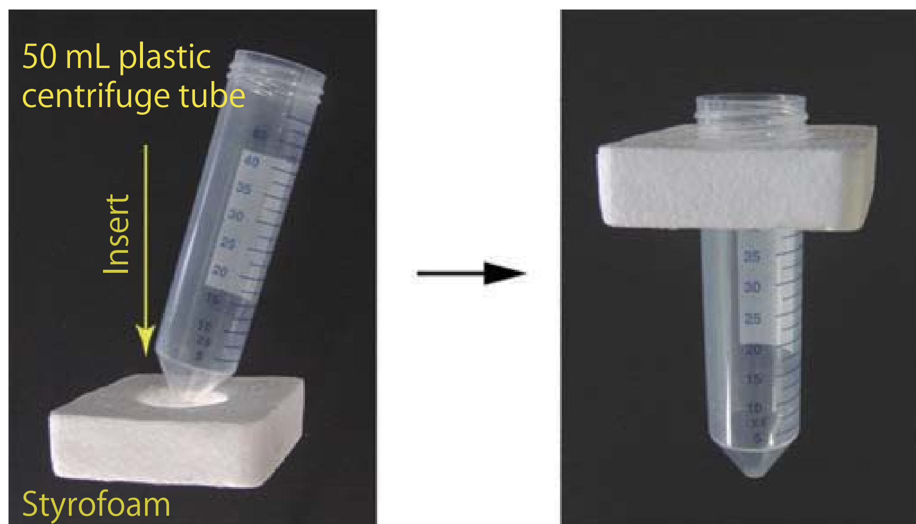
Materials and Equipment

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

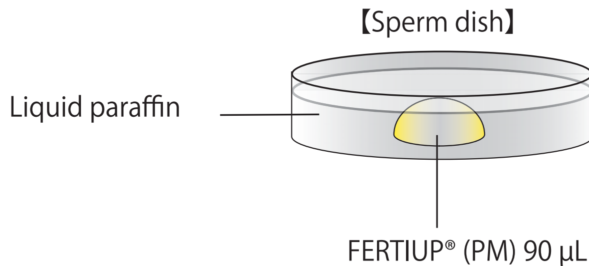
1. Using some styrofoam and a 50 mL plastic centrifuge tube, make the float as shown in the diagram below.



Preparation for Thawing

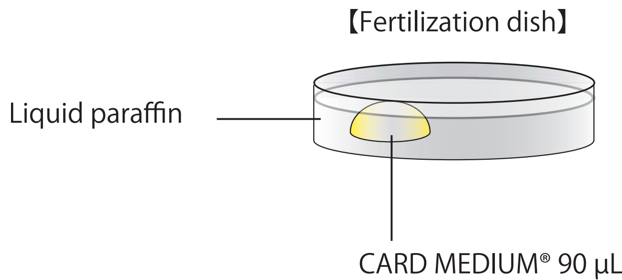
1. Prepare a water bath maintained at 37℃.
2. Pour water (37℃) into the 50 mL plastic centrifuge tube part of the styrofoam/centrifuge tube assembly, and float it in a water bath.
3. Put 1 drop (90 μL / drop) of FERTIUP®(PM) into a dish and cover it with liquid paraffin 30 minutes before thawing a frozen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

【Sperm dish】



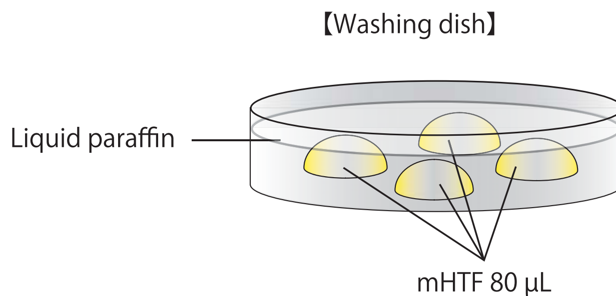
4. Put 1 drop (90 μL / drop) of CARD MEDIUM® into a dish and cover it with liquid paraffin 10 minutes before collecting of oocytes, and place the dish in an incubator (37℃, 5% CO_2 in air).

【Fertilization dish】



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.

【Washing dish】

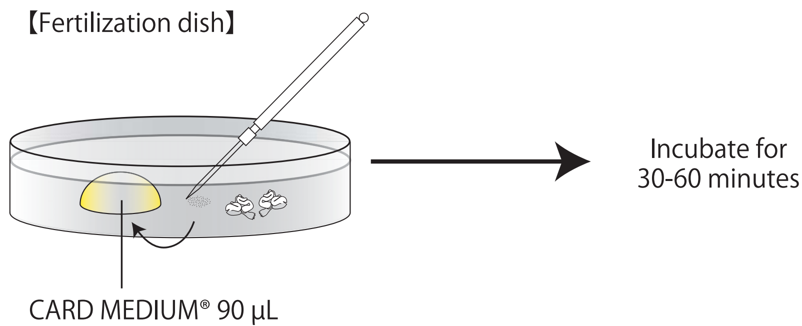


Note

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

Collection of Oocytes

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



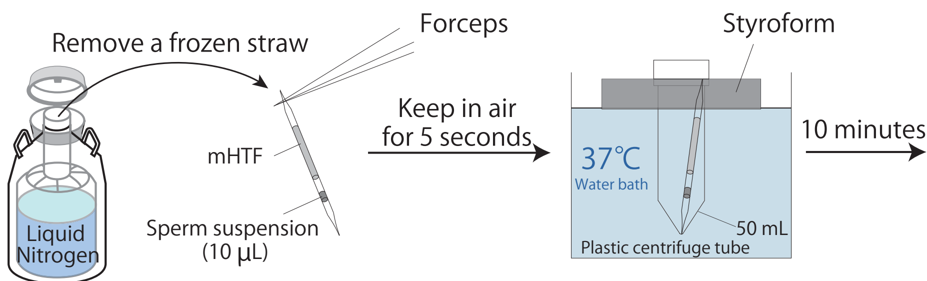
Note

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

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

Thawing the Mouse Spermatozoa

1. Remove a frozen straw from the liquid nitrogen and hold it in the air for 5 seconds.
2. After completing step 1, immediately immerse the frozen straw in the styrofoam/ centrifuge tube assembly (in a water bath maintained at 37℃) for 10 minutes.
3. 10 minutes after immersion, remove the straw from the styrofoam/centrifuge tube assembly.
4. Use fine tissues to wipe any water from the straw.



Note

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

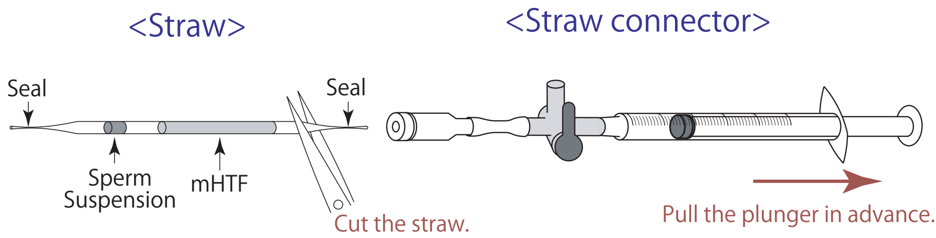
Furthermore, frozen-thawed mouse spermatozoa are sensitive to environmental changes.

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

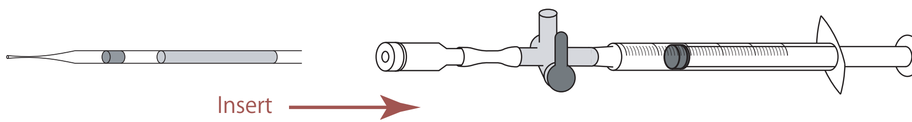
[Thawing of Mouse Spermatozoa] No. 06-01

Transferring and Preincubating the Thawed Sperm Suspension

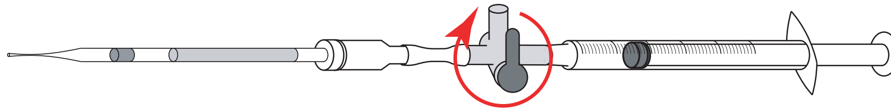
1. Pull the plunger out of the syringe in the straw connector, and cut the straw between the mHTF and the seal.



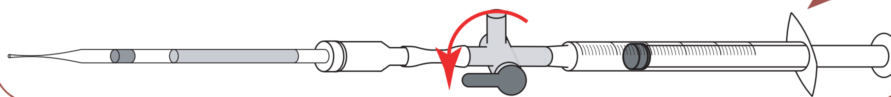
2. Insert the cut straw into the straw connector.



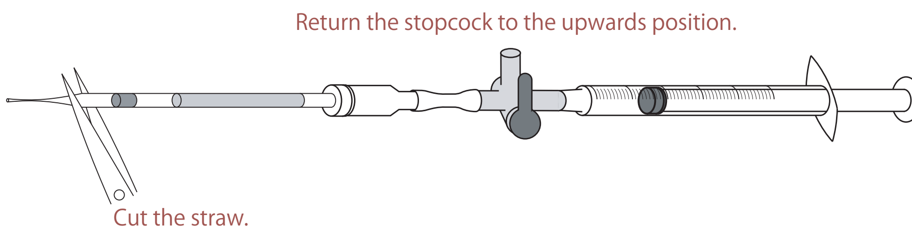
3. Because the insertion of the straw into the straw connector creates pressure inside the straw, turn the stopcock in order to release the pressure.



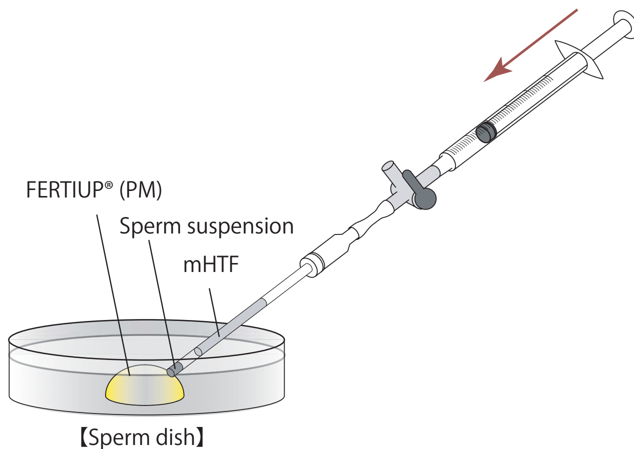
If you have forgotten to pull the pulunger out of the syringe in advance, you can do so by turning the stopcock to the left.



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



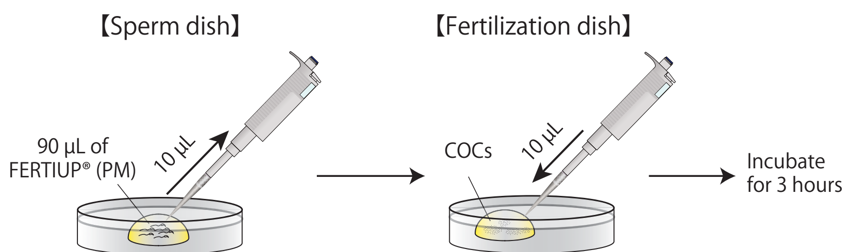
5. Push the plunger to transfer only the sperm suspension into the drop of FERTIUP®(PM) (sperm dish), and place the dish in an incubator (37℃ , 5% CO_2 in air) for 30 minutes.



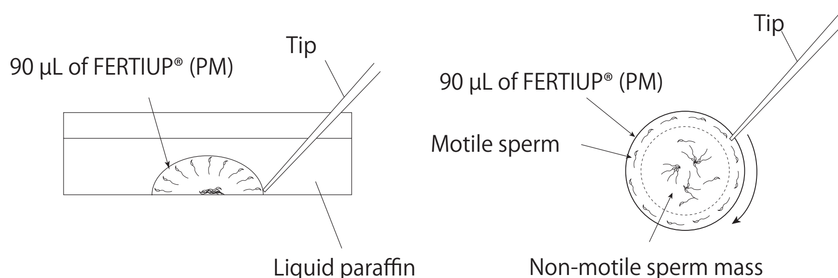
[Transfer of the Thawed Sperm Suspension] No. 06-02

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

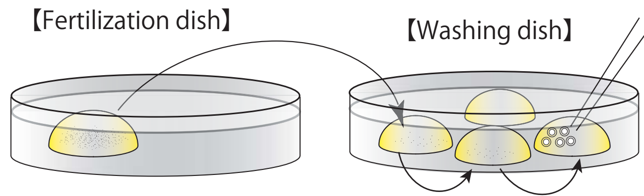
Spermatozoa with high motility have a tendency to gather near the edge of the drop.

Comment

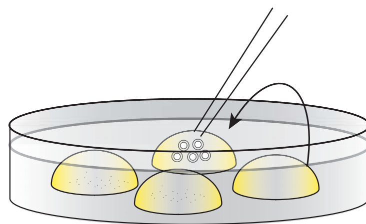
It is possible to aspirate 10 µL of sperm suspension 3-4 times per drop.

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.)



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

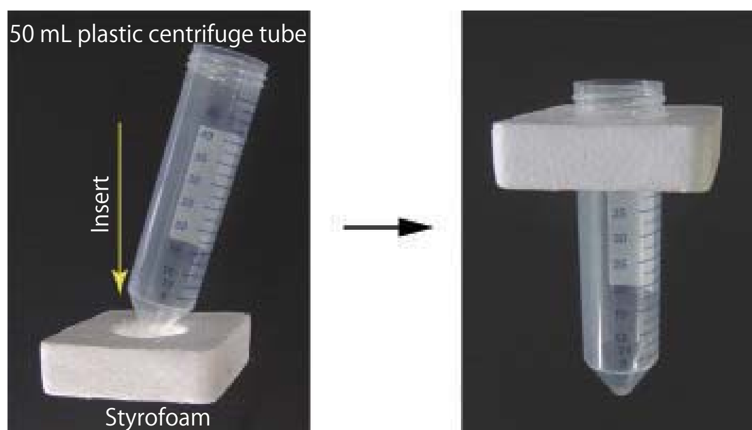
Materials and Equipment

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

Procedures

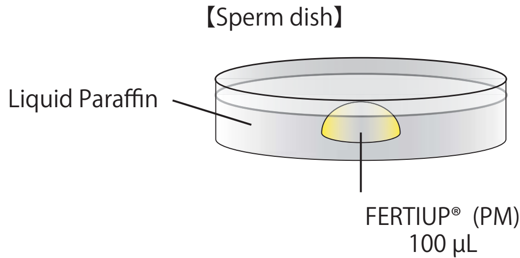
Preparation of the Float for Thawing

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

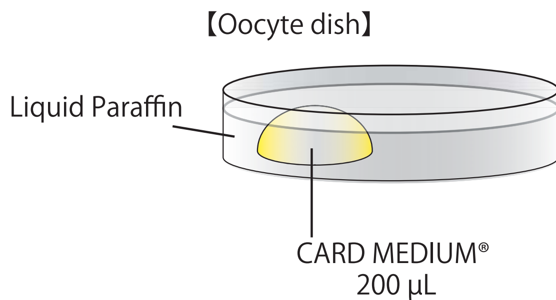


Preparation for Thawing

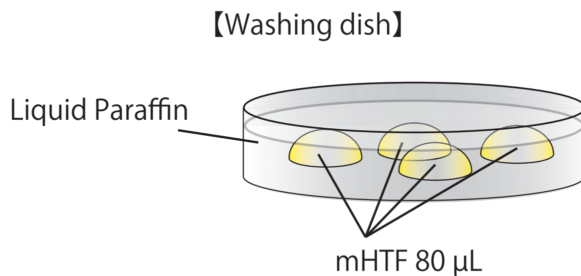
1. Prepare a water bath to 37℃.
2. Pour water (37℃) into the 50 mL plastic centrifuge tube section of the styrofoam/centrifuge tube assembly, and float it in a water bath.
3. 30 minutes before thawing a frozen sperm sample, put 1 drop (100 µL/drop) of FERTIUP® (PM) into a dish and cover it with liquid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



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

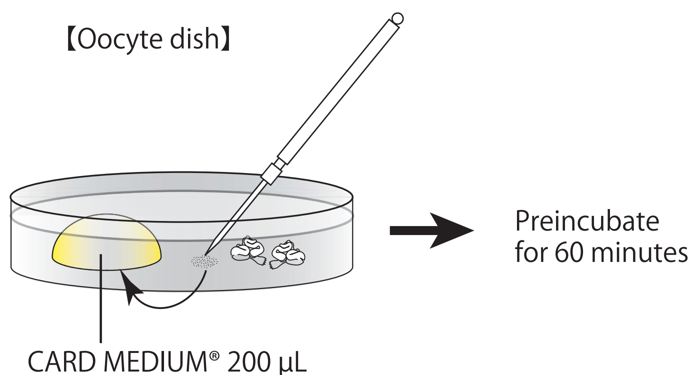


5. Put 4 drops (80 µL / drop) of mHTF into a dish and cover them with liquid paraffin. Place the dish in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.



Collection and Preincubation of Oocytes

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



Note

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

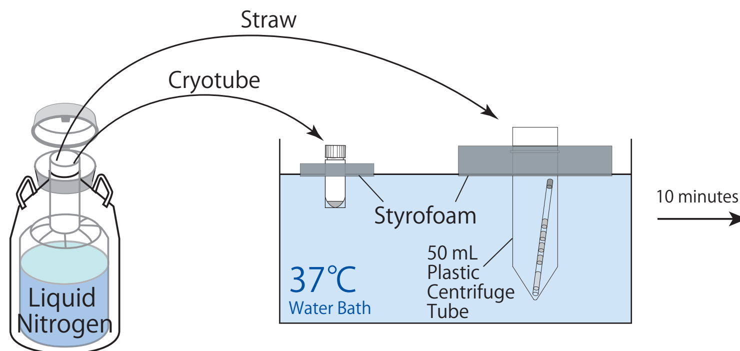
Note

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

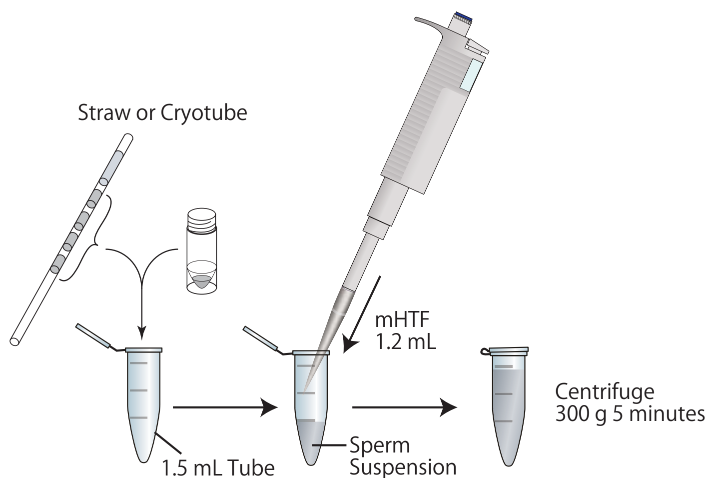
Moreover, when carrying out this process alone, do not sacrifice multiple mice at once; instead, sacrifice one mouse and swiftly remove its oviducts before moving on to the next mouse.

Thawing the Mouse Spermatozoa

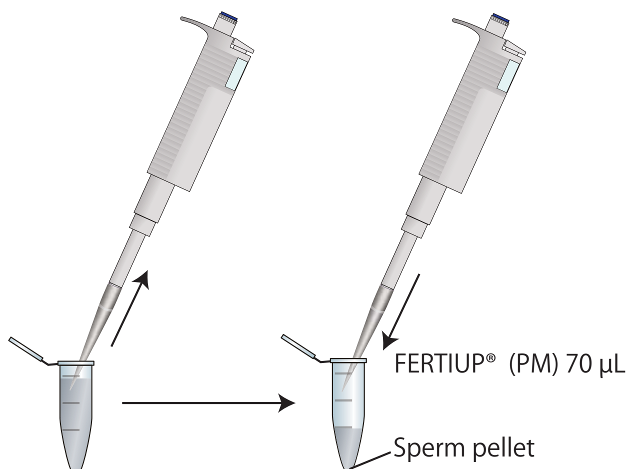
1. Remove a frozen sperm sample from the liquid nitrogen. If the sperm sample is stored in a cryotube, open the cap and discard any liquid nitrogen in the tube. Immerse the sample in a water bath maintained at 37℃ (using a styrofoam or the styrofoam/centrifuge tube assembly) for 10 minutes.



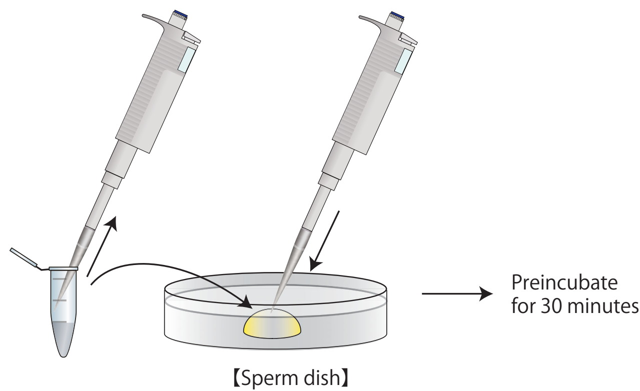
2. Transfer the sperm suspension from the cryotube or the straw into a 1.5 mL tube. Slowly add 1.2 mL of mHTF kept at 37℃ to the tube, and centrifuge it at 300 g at room temperature for 5 minutes.



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

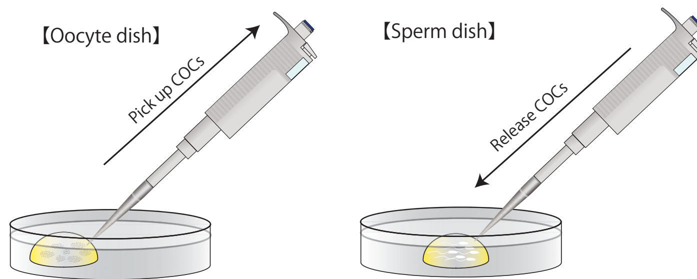


4. After pipetting gently, transfer all of the contents in the tube into the 100 µL drop of FERTIUP® (PM) (Sperm dish). Place the dish in an incubator (37℃, 5% CO_2 in air) for 30 minutes.

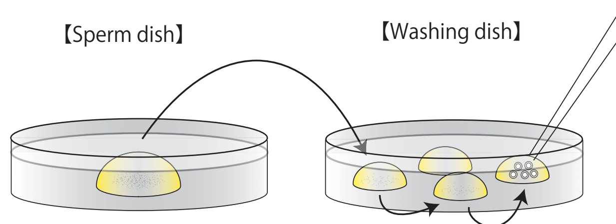


Insemination

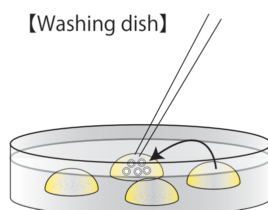
1. Using a tip, suck up the preincubated COCs with a minimum amount of medium from the drop of CARD MEDIUM® (Oocyte dish). Then, release them into the drop of sperm suspension (Sperm dish), and incubate it in an incubator (37℃, 5% CO_2 in air).



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



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



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

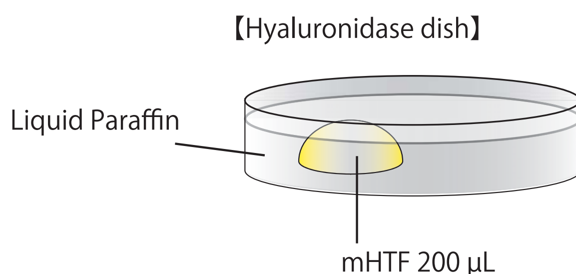
1. Plastic dishes (35 mm X 10 mm Cat. No. 430588; CORNING)
2. mHTF
3. Liquid paraffin
4. Hyaluronidase in mHTF (Hyaluronidase, Cat. No. H-3506, Sigma)
5. The *Saturn 3* laser system (Research Instruments Ltd, Cornwall, UK)
6. Humidified incubator (37℃, 5% CO_2 in air)

Procedures

Preparation of Sperm and Dishes

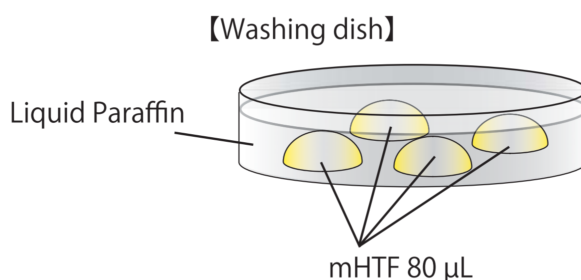
1. For IVF, sperm must be prepared in the methods described at the chapters of *In Vitro* Fertilization on page 8, *In Vitro* Fertilization using Epidydimal Sperm Transported at Cold Temperature on page 18 and *In Vitro* Fertilization using Cryopreserved Spermatozoa on page 28.
2. Put a 200 µL drop of 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.

【Hyaluronidase dish】



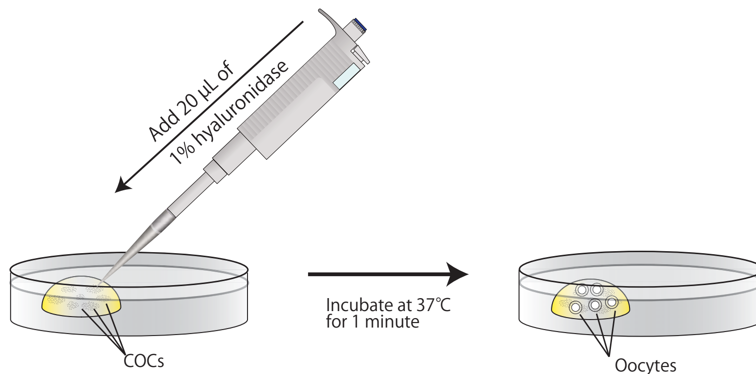
3. Put 4 drops (80 µL/drop) of mHTF into a dish. Cover them with liquid paraffin and place them in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.

【Washing dish】

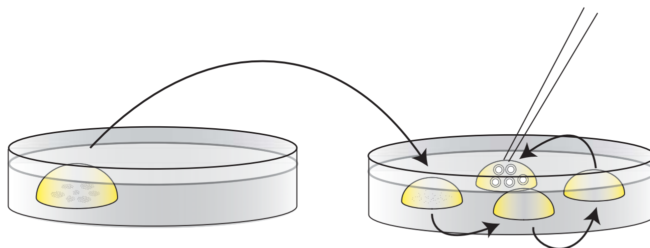


Preparation of Denuded Oocytes

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.

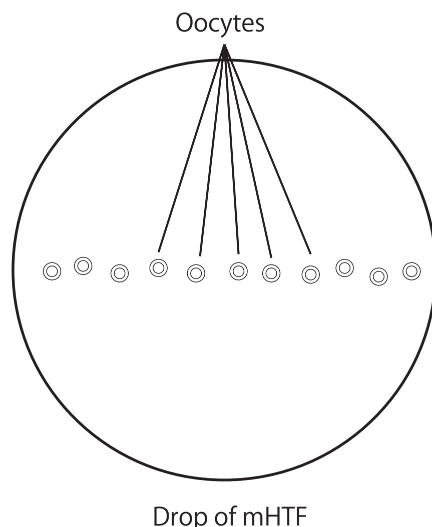


3. Promptly collect and transfer the oocytes into a 80 µL drop of mHTF (Washing dish), and in turn wash them in the drops in the washing dish.



Dissection of the Zona Pellucida using a Laser

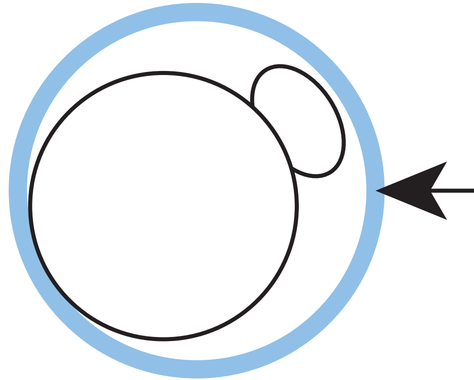
1. Put a 100 µL drop of mHTF into a dish. Cover it with liquid paraffin and place it in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.
2. Transfer fifty denuded oocytes into the drop of 100 µL mHTF.
3. Arrange the oocytes in a line along the bottom of the plastic dish.



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 Epidydimal 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.

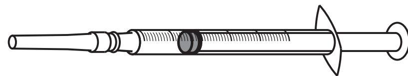
Materials and Equipment

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

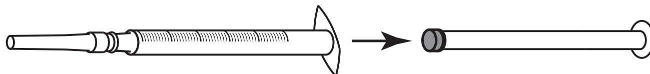
Procedures

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.

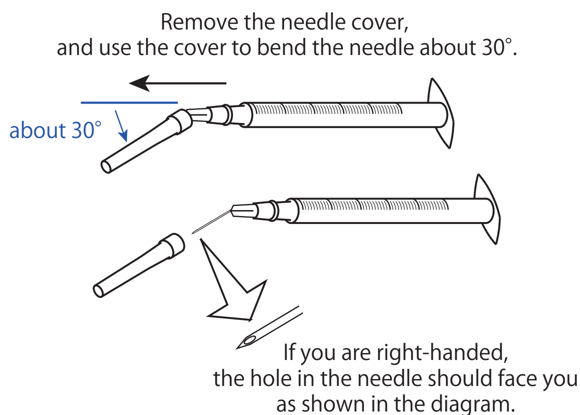


Take a 1 mL syringe with a 30 G needle.



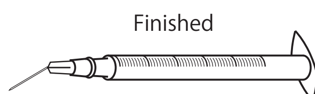
Remove and discard the plunger.

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



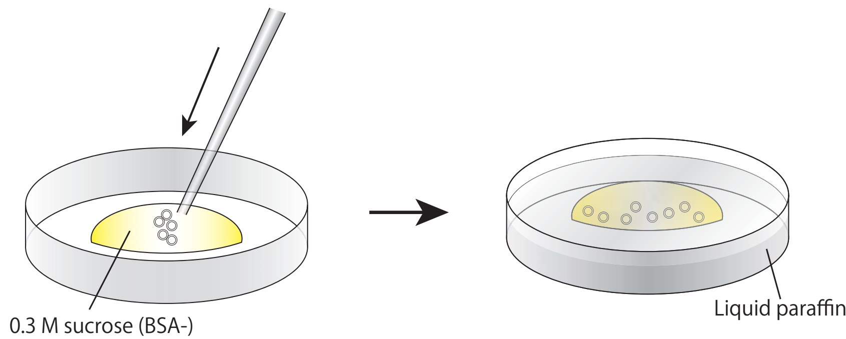
If you are right-handed,
the hole in the needle should face you
as shown in the diagram.

Finished

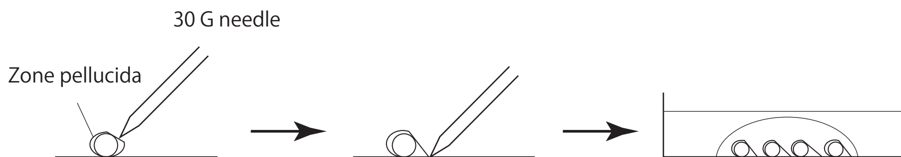


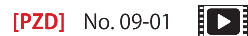
PZD

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.

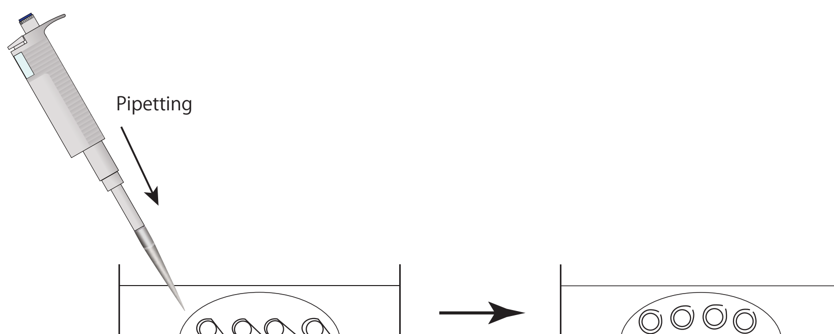


4. Under a stereomicroscope, partially dissect the zona pellucida (PZD) of the oocytes using a single downward motion with a 30 gauge needle.



[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.

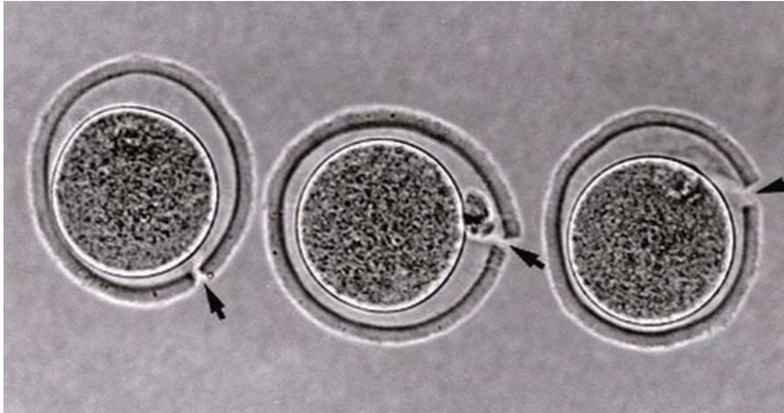
Comment

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.

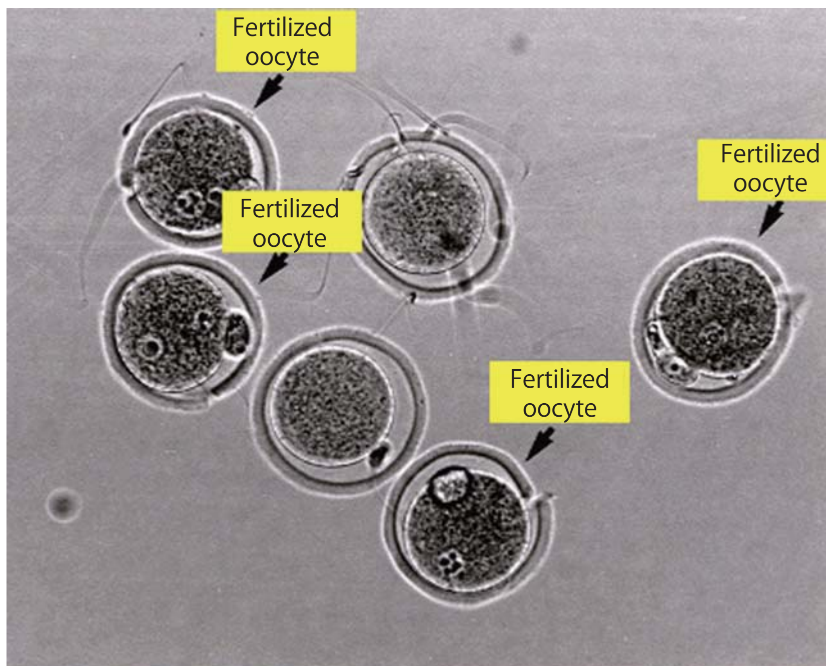
[Micrograph : PZD Oocytes]



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 Epidydimal 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

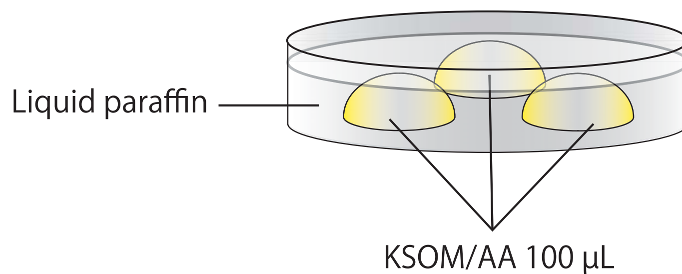
Superovulation and Selection of Plugged Females

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

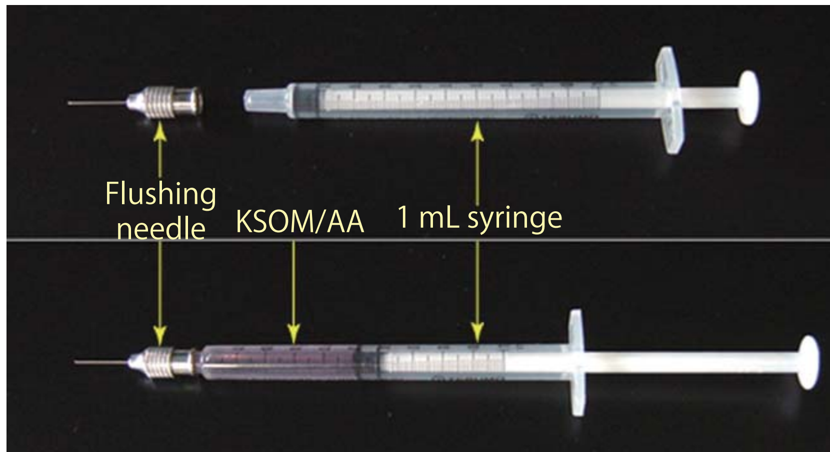
1. Put 3 drops (100 µL / drop) of KSOM/AA into a dish and cover them with liquid paraffin. Place the dish in an incubator (37℃, 5% CO_2 in air) for at least 30 minutes.

【Washing dish】



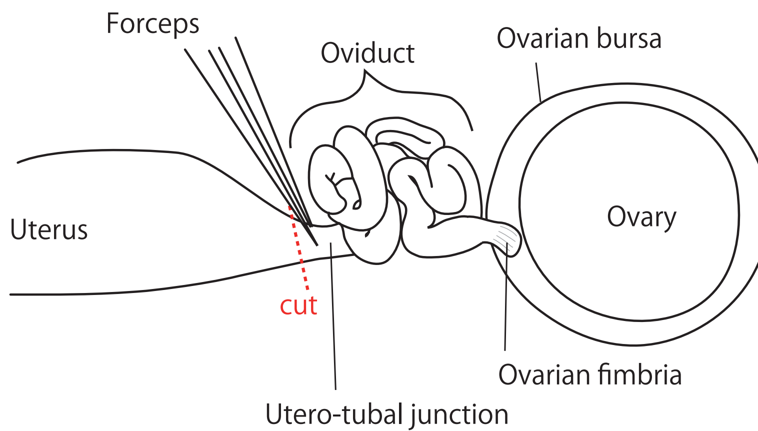
Preparation of Flushing Needle

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.

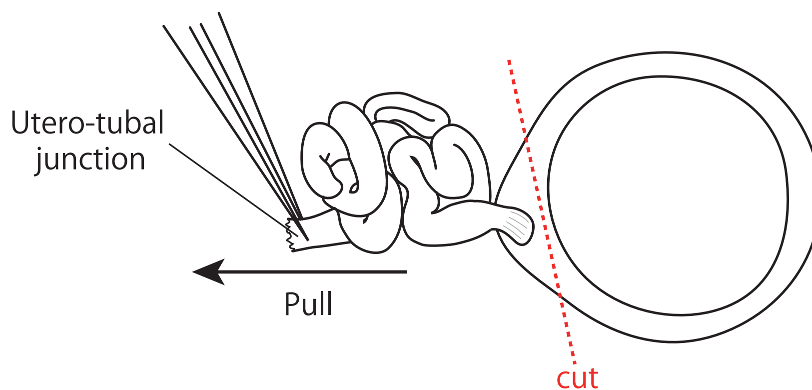


Collecting Embryos

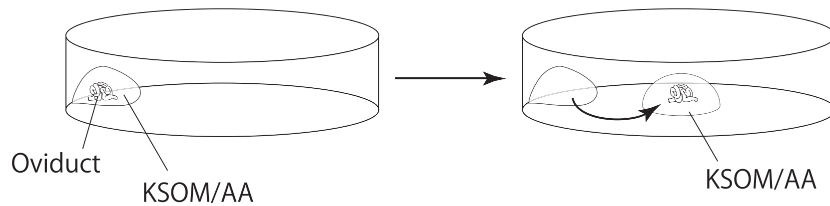
1. One day after a vaginal plug has been observed, remove the uteruses, oviducts and ovaries from the females, and place them on sterile filter paper.
(Please refer to the chapter of *In Vitro* Fertilization on page 9.)
2. Pinch the utero-tubal junction and make a cut on the uterus side of it.



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.

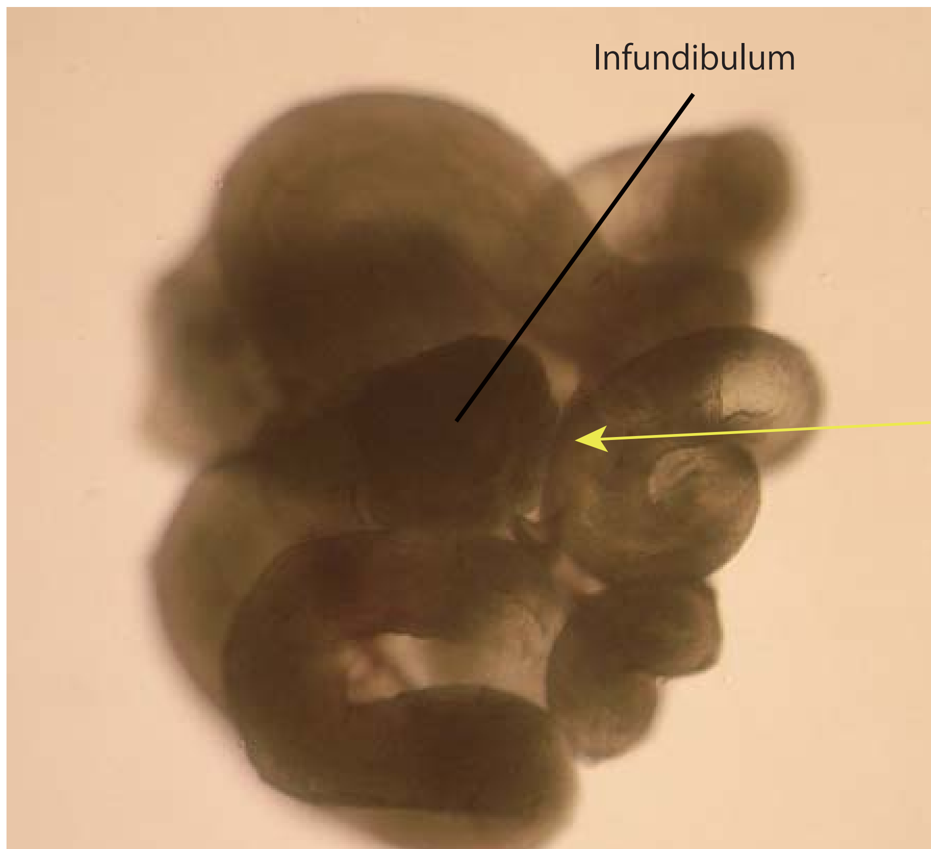


5. Look closely at the oviduct to find the infundibulum, which is a tube of a larger diameter than the oviduct.
6. Set the oviduct so that the flushing needle can be easily inserted.

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

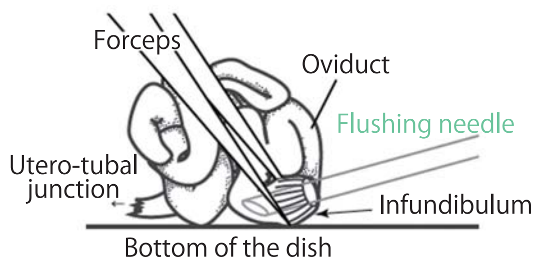
If you are right-handed, position the oviduct as shown in the diagram on the left. It is easy to insert the needle into the infundibulum from this position (as indicated by the yellow arrow).

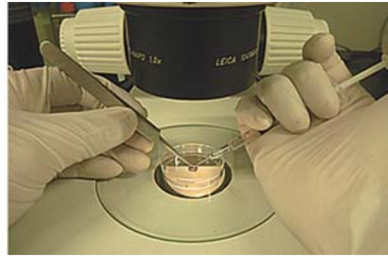
7. Hold the infundibulum against the bottom of the dish using a pair of forceps, and insert the needle into it.

Note

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

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

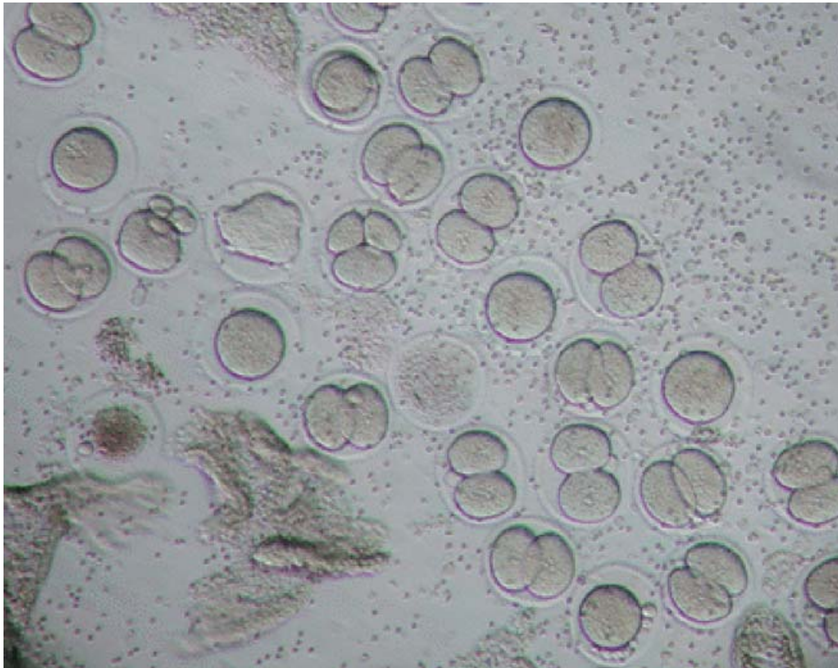




[Flushing] No. 10-01

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

[Micrograph : After Flushing of Oviduct]



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.

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.