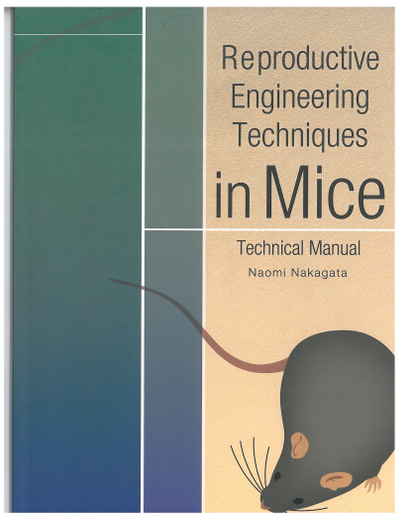
# Reproductive Engineering Techniques **in Mice**




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

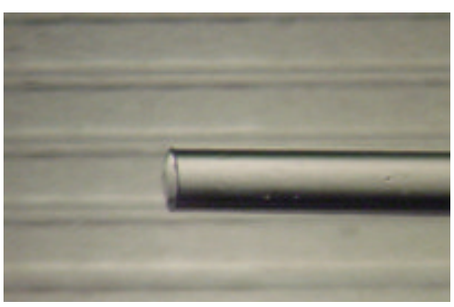
### Cleaning and Sterilizing Glass Capillary Pipettes

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.

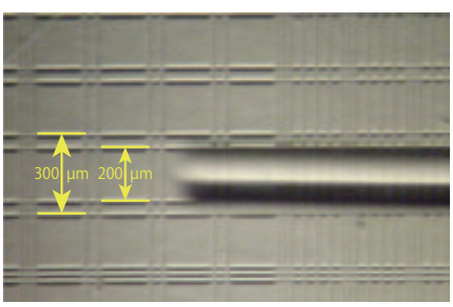
### Making Pipettes for Embryo Handling

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.

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



**[When the hemocytometer is in focus]**



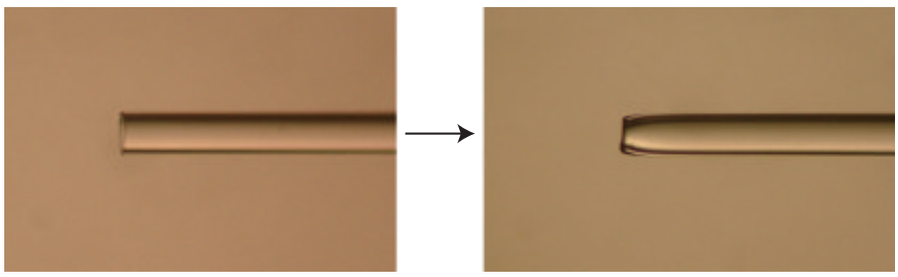

**[Making Pipettes for Embryo Handling]** No. 01-01

**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]

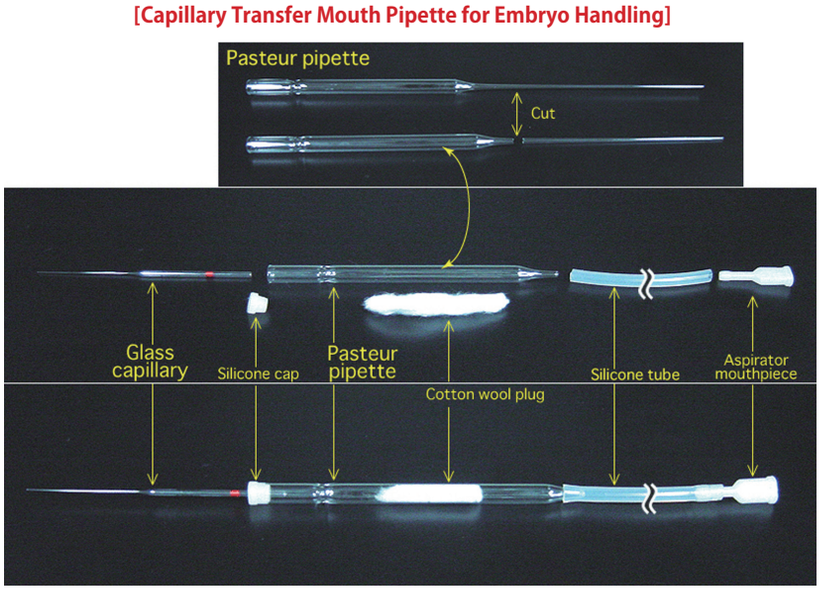


[Polishing the Tip of the Glass Capillary Pipette] No. 01-02

## 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)

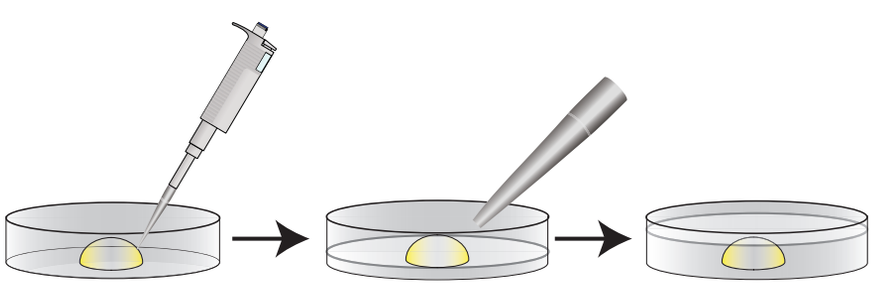
## Procedures

### Superovulation

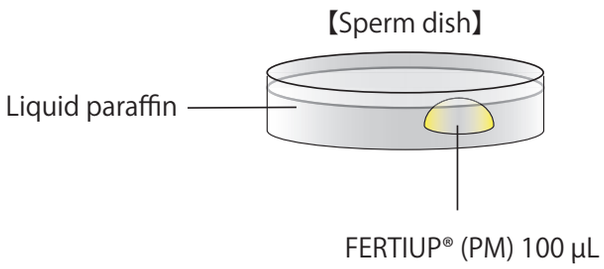
1. Induce superovulation by injecting 7.5 IU of pregnant mare's serum gonadotropin (PMSG) i. p. into each mature female mouse (8-12 weeks old). (PMSG is usually administered during the light cycle, between the hours of 14:00 and 18:00).
2. Follow this up 48-52 hours later with a 7.5 IU i. p. injection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

### Preparation of Dishes

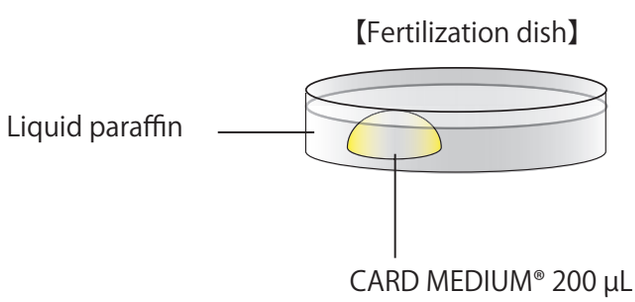
1. Prepare dishes as instructed below and keep them in an incubator (37℃, 5% $CO_2$ in air) to allow them to gas-equilibrate.



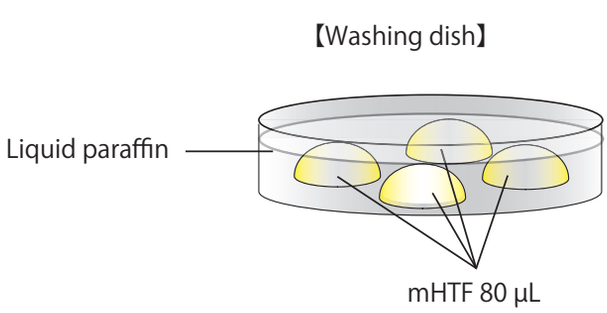
a. Sperm dish
Put 1 drop (100 µL / drop) of FERTIUP® (PM) into a dish and cover it with liquid paraffin 30 minutes before collecting sperm, and place the dish in an incubator.




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



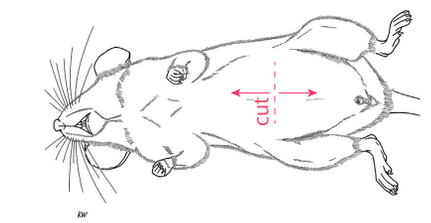

**Note**

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.

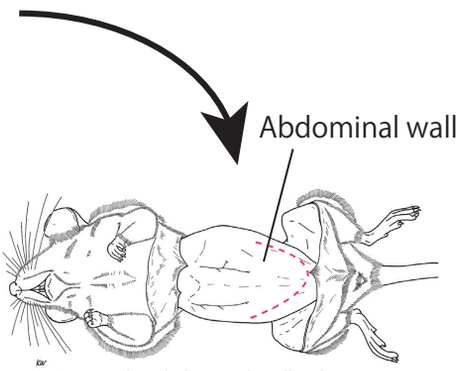
Please refer to the CARD MEDIUM® instruction manual.

## Collection of Spermatozoa

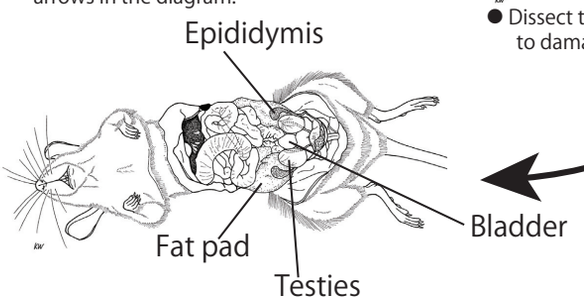
1. Sacrifice 1 or 2 mature male mice (3 to 6 months old) and remove their cauda epididymides, avoiding as much fat, blood and tissue fluid as possible.
2. Place the tissue on sterile filter paper to blot away any blood and fluid.




- 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.

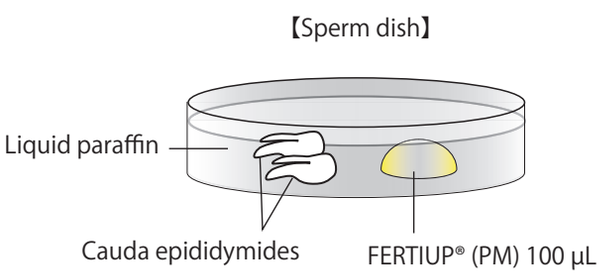



- Dissect the abdominal wall, taking care not to damage the internal organs.

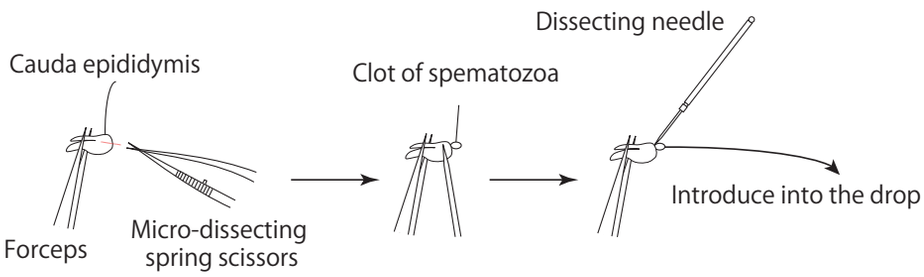



**[Removing the Cauda Epididymides]** No. 02-01

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




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).

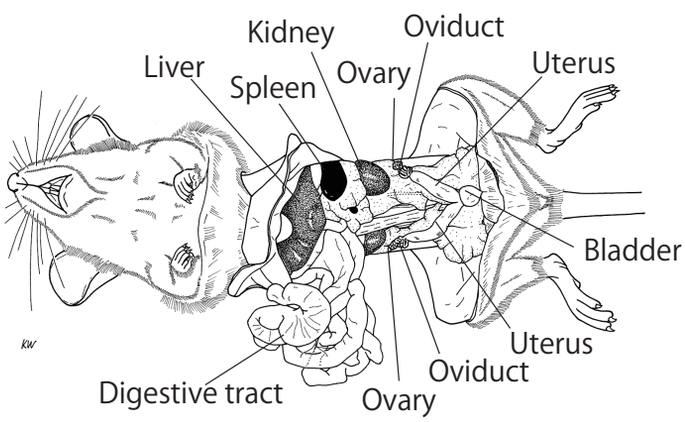



**[Releasing Spermatozoa]** No. 02-02

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

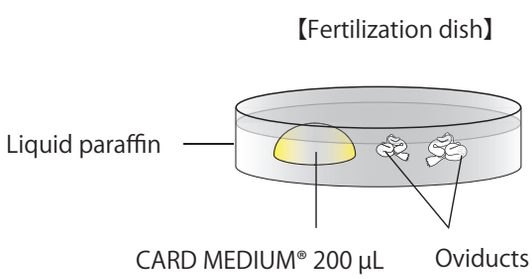
## Collection of Oocytes

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.

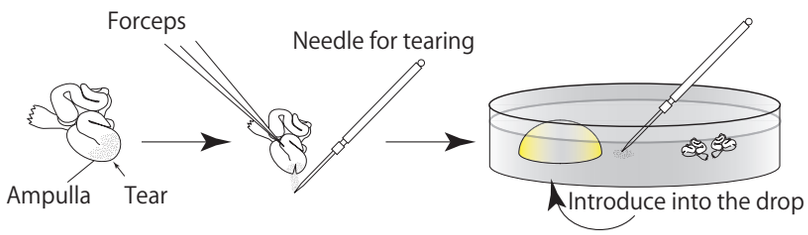


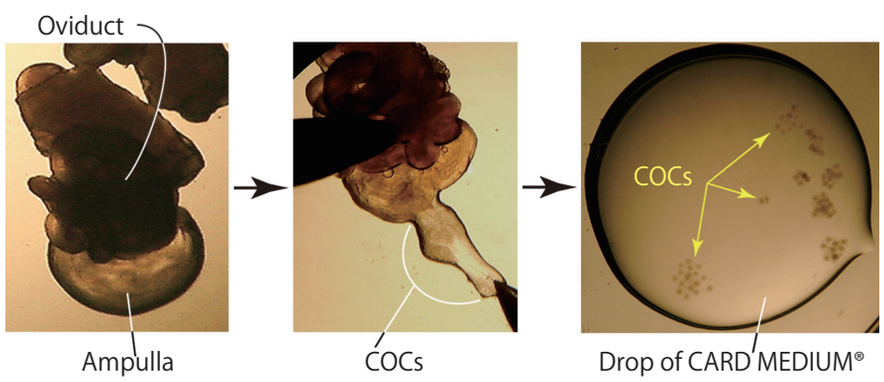

**Note**

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®]**



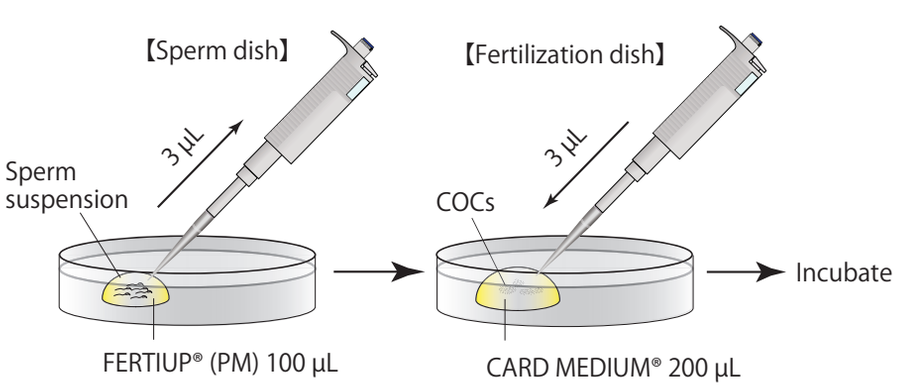




No. 02-04

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).

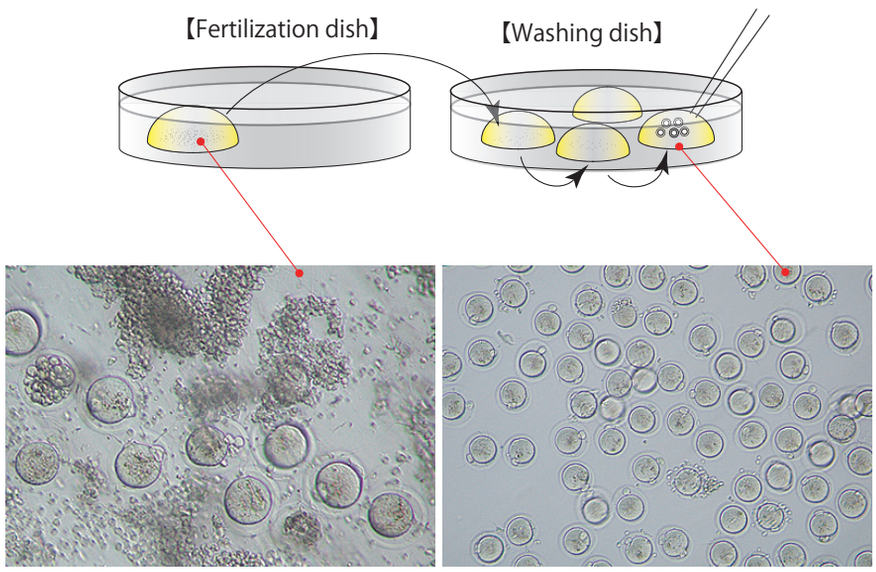



**Note**

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).

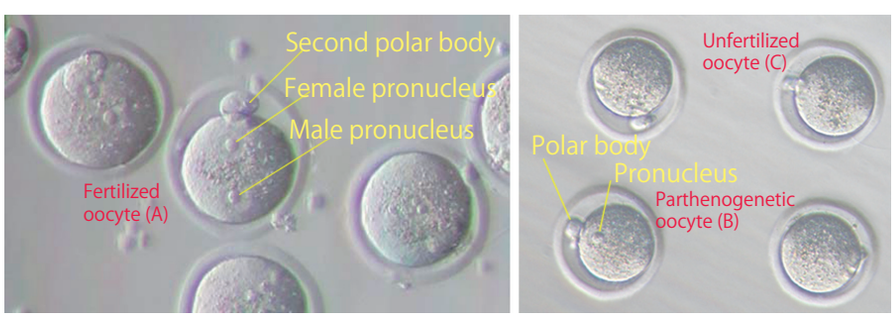
Moreover, when carrying out this process alone, do not sacrifice multiple mice at once; instead, sacrifice one mouse and swiftly remove its oviducts before moving on to the next mouse.

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®.

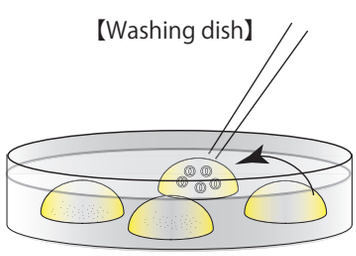



4. 6 hours after insemination, observe the oocytes in the third drop of mHTF and remove any parthenogenetic oocytes which have only one pronucleus.

**[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