家畜受精卵・移植技術

Embryo Transfer Technology
for Domestic Animal
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Manual for Ovum Pick-Up and In Vitro Fertilization

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Introduction

In vitro fertilization (IVF) technology is a technique that involves ovum (immature ovum) pick-up from ovaries; this manual describes ovum pick-up from domestic animal ovaries. Before transplantation, the ovum is matured under artificial conditions during which fertilization and further development occur. IVF technology in cattle is significant for the following reasons: it allows a large quantity of embryos to be produced at low cost, selective breeding of high quality beef cattle with graded carcasses is made possible, and it provides the foundation for advanced technology such as nuclear transplantation. IVF also shortens the generation interval during breeding improvement of dairy cattle. The number of cows that received a transplanted IVF embryo reached 9,525 in 2005 (according to the Livestock Industry Promotion Division, Ministry of Agriculture, Forestry and Fisheries of Japan).

There are two common methods of immature bovine ovum pick-up: one involves ovaries derived from carcasses in the slaughterhouse; the other is a method developed by Callsen et. al.[1)] and Pieterse et. al.[2)] in which a follicular ovum is obtained from the ovaries of living cattle using ultrasonic diagnostic equipment (transvaginal ultrasound-guided follicular aspiration, hereinafter referred to as OPU). The method developed by Callsen et. al.[1)] involves an ovarian puncture on the surface of the body, while that developed by Pieterse et. al.[2)] is based on the aspiration removal technique used with human eggs. The latter is currently the most commonly adopted method and the detailed procedure will be explained later in this manual. The procedure can be outlined as follows: the 7.5 MHz ultrasonic diagnostic equipment used in OPU needs a probe mounted with the aspiration needle attached to the suction pump. When the probe is inserted into the vagina, the needle punctures a follicle in the ovaries via the vaginal wall and aspirates the ovum together with follicular fluid. This approach, in which the ova obtained by OPU undergo IVF to create embryos (OPU-IVF), is considered a suitable technique for the efficient production of numerous embryos from a single individual donor cow or for donor cows that cannot produce a normal embryo because of superovulation treatment.

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In Vitro Fertilization (IVF)

This technology involves picking up an oocyte (immature oocyte) from a cattle carcass in order to maturate, fertilize and develop it to the stage at which it becomes transferable to recipient cow. The difference in OPU-IVF lies in the method of oocyte collection. The procedures that occur after the maturation culture stage are basically similar to each other.

Table 1. IVF schedule

	Procedure	Description/remarks
Day -2	Collection of ovaries ↓ (Preservation of the ovaries)*	Stored and transported in physiological saline (25°C) (within 8 hours) * Preserve the ovary in physiological saline (20°C) for approx. 20 hours until the BSE analysis result becomes available.
Day -1	Aspiration of oocytes Searching of oocytes ↓ Maturation culture	Lactated Ringer's solution + 1% CS TCM-199 + 5% CS at 38.5°C, in 5% CO_2 in air, for 20 hours
Day 0	Sperm treatment ↓ Insemination ↓ Embryo culture	Percoll separation (2100 rpm, 10 min) Sperm washing solution (BO + 10 mM Hypotaurine + 4 u/ml heparin) Sperm diluting solution (BO + 20 mg/ml BSA) Oocyte washing solution (BO + 10 mg/ml BSA) Sperm concentration (3×10^6/ml) 5 h at 38.5°C, 5% CO_2 in air CR1aa + 5% CS 9 days at 38.5°C, 5% CO_2 in air
Day 2	Observation on initial development	Initial embryo development observed, cumulus cells denuded
Day 3 - 6	Observation on morphology	Petri dishes shaken every 24 h and the morphology of the embryos observed
Day 7 - 9	Inspection of blastocyst development	Inspection of blastocyst development during Day 7 to Day 9 Transfer/freezing of blastocysts

1. Ovary collection and transport

(1) Preparation

1) Prepare a physiological saline solution, sterilize the solution in an autoclave and store it at room temperature. Otherwise use commercially available physiological saline solution.
2) Disinfect the thermos bottle by filling it with boiling water.
3) Add 1 ml antibiotics (Gentamicin) to 1 liter physiological saline solution kept at 25°C.
4) Bring all the necessary items to the slaughterhouse. The items include the thermos bottle filled with normal saline solution, surgical scissors, pincettes, beakers, sterile paper towels, sterile latex gloves, a sterile metal sieve, a lab coat and long boots.

(2) Collect ovaries and transport

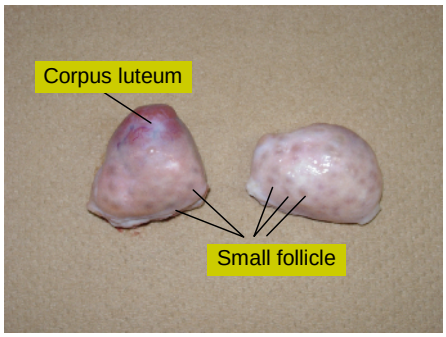
1) Transfer the collected ovaries to the metal sieve to wash the blood, etc., off with physiological saline solution which has been prepared in addition to the above mentioned solution.
2) Immerse the ovaries in the physiological saline solution in the thermos bottle for transport back to the lab.

(3) Regarding BSE analysis

In a case where oocyte collection cannot be carried out until the BSE analysis result becomes available, preserve the ovaries overnight in a cool incubator set at 20°C.

2. Oocyte collection and search

Typically, a single ovary contains an average of 20 to 30 small follicles (with a diameter of 2 to 6 mm). The oocytes found in these small follicles are in the germinal vesicle (GV) stage, which is the stage before maturation. The immature oocytes that are picked up are subjected to IVF after the developmental culture stage.



Pic. 1. Ovaries derived from a carcass in slaughterhous

There are two common methods of oocyte collection: the aspiration method and the slit method. With the first method, the oocytes are aspirated from follicles together with follicular fluid by means of an injection syringe. In the second method, the ovarian cortex is sliced with a surgical scalpel or a razor to obtain the oocyte from the fractured follicles.

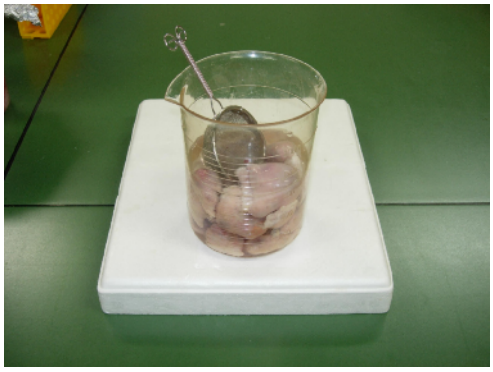
While the slice method can pick up a greater number of oocyte from a ovary than the aspiration method, the latter takes less time compared to the former and also yields a slightly higher developmental rate. This manual gives an explanation of the aspiration method.

(1) Preparation

1) Prepare lactated Ringer's solution containing 1% CS on the day of collection and store it at room temperature.
2) Prepare the following items:
   Sterile paper towels, sterile latex gloves, pincette, injection syringe (5 ml), injection needle (19G needle tip with an obtuse angle), thermostat bath (set at 30°C), petri dish (with the bottom lined in a reticular pattern at 1 cm intervals), Pasteur pipette, beaker, surgical scalpel or razor.

(2) Aspiration which immature

1) Put on the sterile latex gloves and wash the collected ovaries several times with the physiological saline solution. Then preserve them in a beaker at room temperature.
2) Take the ovaries out of the beaker with the pincette or a tea strainer etc. Trim away any unnecessary tissue (oviduct, fat etc.) with the surgical scissors and wipe the blood and the physiological saline solution off of the ovaries with the sterile paper towel.
3) Grasp the ovary (Fig. 2) and hold the 5 ml injection syringe with the 19G needle (Fig. 1).



Pic. 2. Ovaries after washing

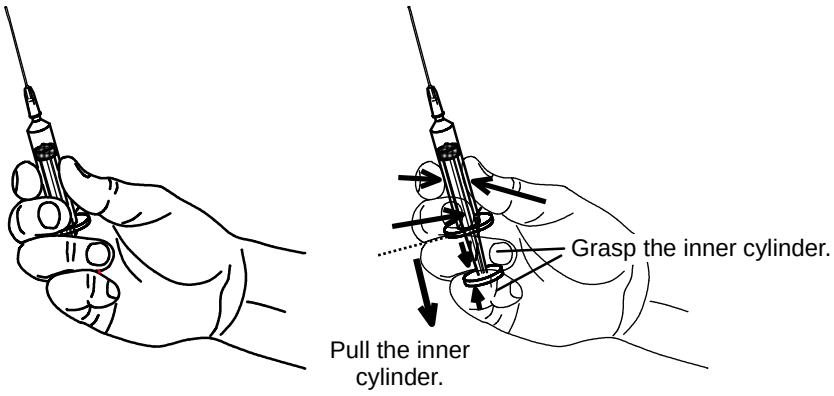


Fig. 1. How to hold the injection syringe

4) Insert the needle through the ovarian cortex and aspirate the oocytes together with the follicular fluid from the small follicles that have a diameter of 2 to 6 mm (Fig. 3). Try to aspirate the oocytes as much as possible during the first insertion instead of removing the needle halfway through.

   * **The injection syringe used for ovum aspiration should be sterilized only after it has been cleansed to remove oil inside the syringe.**

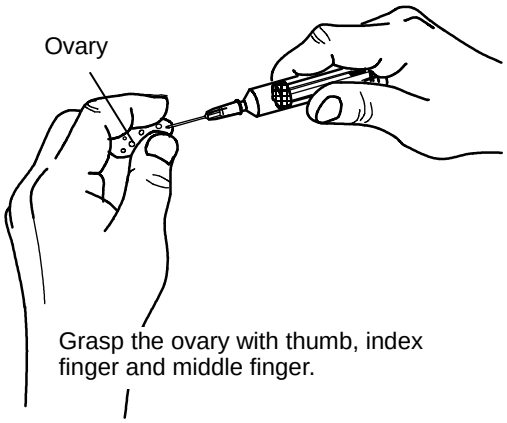


Fig. 2. How to grasp the ovary

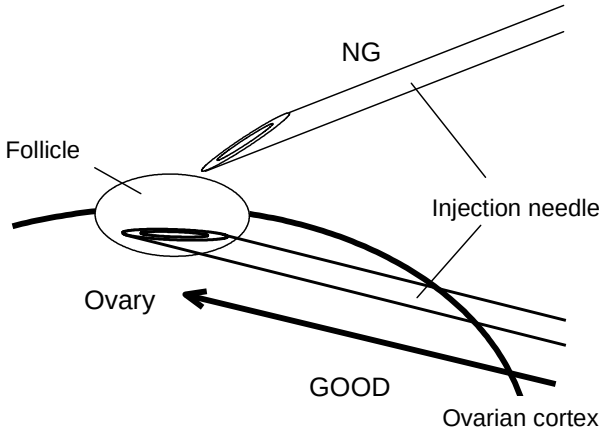


Fig. 3. How to puncture the follicle

5) Once the aspiration of the ovary is complete, slowly inject the aspirated fluid into a 50 ml centrifuge tube. Rapid injection could cause isolation of cumulus cells.

6) Once processing of all the ovaries is complete, remove the supernatant in the centrifuge tube by means of an aspirator and add lactated Ringer's solution supplemented with 1% CS (Fig. 4).

7) Remove the supernatant as in 6) and add lactated Ringer's solution supplemented with 1% CS (Fig. 4).

8) When the supernatant becomes clear enough to observe under a microscope, transfer the sediment and the supernatant to the 90 mm petri dish. Then rinse the inside of the centrifuge tube out with lactated Ringer's solution supplemented with 1% CS to make sure no aspirated oocytes remain in the tube (Fig. 5).

9) When the number of collected ovaries is minimal, the aspirated fluid can be directly transferred to the 60 mm petri dish. If the fluid is not clear enough for microscopic observation even after precipitation of oocytes and cells, clear the supernatant with lactated Ringer's solution supplemented with 1% CS as described in steps 6) to 8).

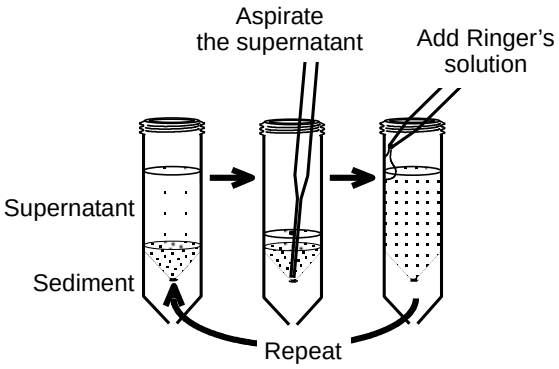


Fig. 4. Cleansing the aspirated supernatant

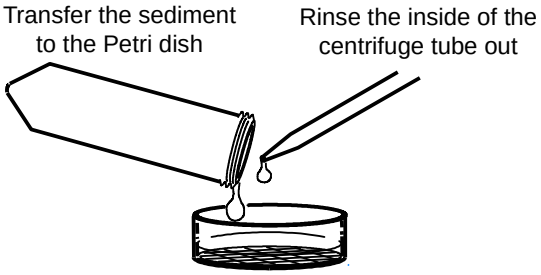


Fig. 5. Transfer to the Petri dish

(3) Oocyte search

1) Place a 90 mm petri dish carefully under the microscope.
2) Search for oocytes in each grid in the order depicted in the following figure.

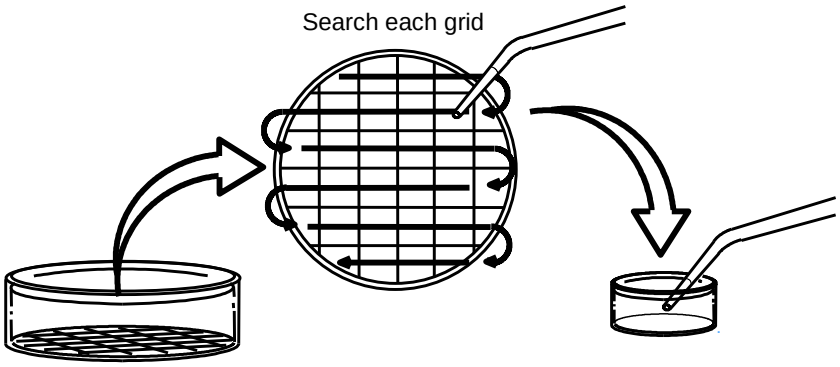
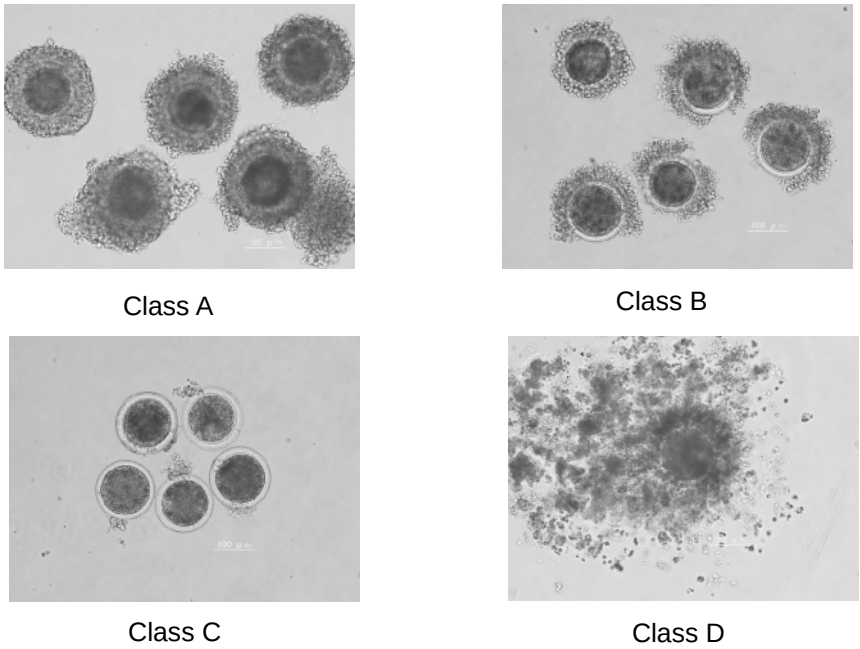


Fig. 6. Ovum Search

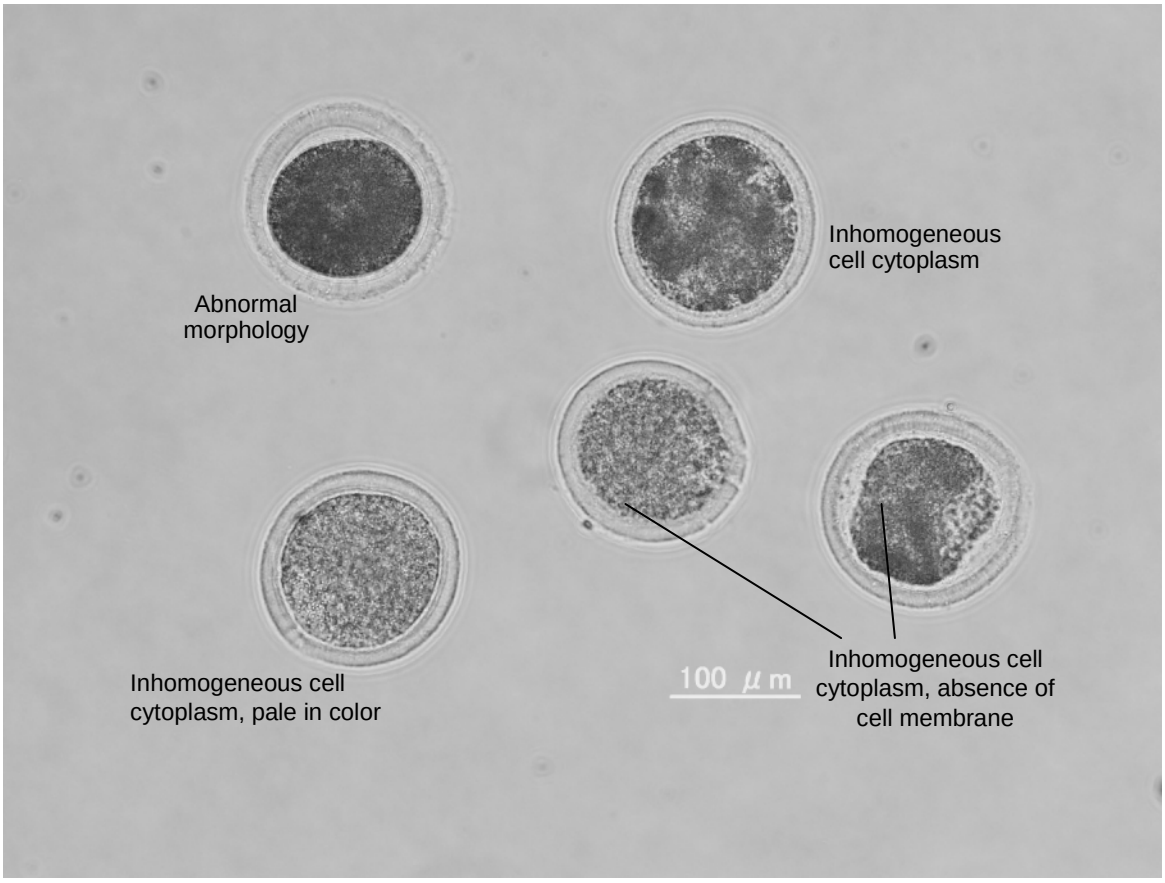
3) Aspirate the oocytes with a modified Pasteur pipette and transfer to a 35 mm Petri dish containing lactated Ringer's solution supplemented with 1% CS.
4) Classify the collected oocytes as shown below.



Pic. 3. Oocyte classification

Class A: Cumulus cells are attached in 3 or more layers or over the entire zona pellucida.
Class B: Cumulus cells are attached in 2 or less layers or over more than 1/3 of the zona pellucida.
Class C: A completely bare ovum or an ovum with less cumulus cells than Class B.
Class D: Cumulus cells are swollen or degenerated in a web-like pattern.

While oocytes classified as A and B are usually used for the culture, the lesser oocytes classified as C and D are also cultured if IVF is to be used for the reproduction cattle (in this case, there is a requirement that as many blastocysts as possible be developed). In addition, the development rate of the oocytes classified as Class D is no less than that of Class A or B oocytes provided they have normal cell cytoplasm. Conversely, oocytes with cumulus cells in the shape classified as Class A or B will not develop into a blastocyst if they have abnormal cell cytoplasm (degenerated oocyte: pale in color, unclear cell membrane, etc.).



Pic. 4. Degenerated oocytes

3. Maturation culture

(1) Preparation

1) Prepare a culture medium (TCM-199 containing 5% CS) the day before or on the day of collection.
2) Make a 300 μl spot with a micro pipette in the 35 mm petri dish (Falcon: 3001 or 1008).
3) Add liquid paraffin from the side of the dish to cover the drop without disturbing its shape.
4) Add 300 μl of culture medium to the drop to to make 600 μl drop.
5) Confirm that the drop is completely covered with the paraffin. If some part is uncovered, add a few more drops of liquid paraffin.
6) Incubate for more than 2h in a CO_2 incubator before use (equilibration).
7) Dispense remaining culture medium into 35 mm Perti dishes, 2.5 ml in each dish, and cover with liquid paraffin (2 ml) for washing oocytes before culture.

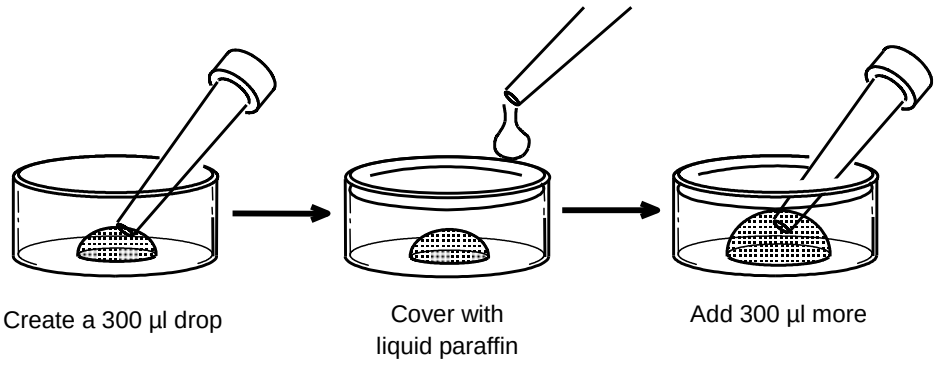


Fig. 7. Method of forming drop (600 μl)

* When there are not many ova to culture, make 4 or 5 drops of 100 μl in the 35 mm petri dish.
To make a drop of 100 μl, make a spot with a microdroplet (approx. 10 μl) first. Then cover it with liquid paraffin (4.5 ml) and add 90 μl of culture solution to the spot to make a total of 100 μl.

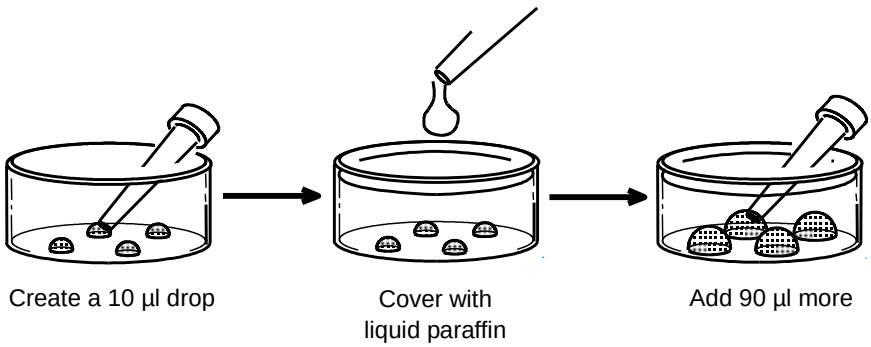


Fig. 8. Method of forming drop (100 μl)

* In both methods, do not allow the tip of the micropipette to touch the bottom of the Petri dish.

(2) Maturation culture

1) Wash the selected immature oocytes once in a 35mm Petri dish containing lactated Ringer's solution supplemented with 1% CS.
2) Wash the oocytes well in two 35 mm Petri dish of containing TCM-199 + 5% CS.
3) Transfer the oocytes into the microdrops prepared for maturation culture at the rates shown below.

    600 μl drop: 80 oocytes

    100 μl drop: 10 to 20 oocytes

4) Culture for 20 hours in a CO_2 incubator in air containing 5% CO_2 at 38.5°C.

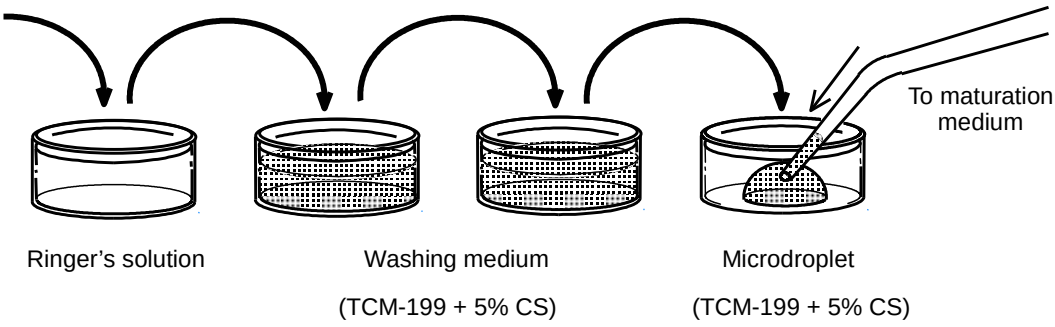


Fig. 9. Transfer of oocytes to maturation medium

* About washing procedure

In the IVF technique, “washing” means removing traces of the previous medium completely from the culture environment, when transferring the embryos (oocytes) to a new culture medium. If we take the transfer of oocytes to the maturation medium as an example, this means washing the oocytes with the washing medium (TCM-199 + 5%CS) to replace their surrounding environment with TCM-199 + 5%CS so that no trace of the m-PBS + 3%CS is brought into the maturation medium (and at the same time removing the cumulus and granulosa cells, etc that are shed by the oocytes), before starting the maturation culture.

Example: Transfer of oocytes to maturation medium

1) Keep oocytes in the lactated Ringer’s solution containing 1% CS.
2) Aspirate TCM-199 + 5%CS, which is also the culture medium used in the next step, with a Pasteur pipette from a washing Petri dish.
3) Spray the washing medium from the pipette on to the oocytes in the m-PBS + 3%CS solution.
4) Aspirate the Ringer’s solution diluted with the rinse solution (TCM-199 containing 5% CS) as well as the ova. At this moment, the environment of the ova is not yet completely replaced with the TCM-199 containing 5% CS solution.
5) In step 4), make sure to aspirate as many ova as possible together with a limited amount of solution. This is in order to transfer as many ova as possible without transferring the lactated Ringer’s solution containing 1% CS into the rinse solution.
6) Transfer the oocytes into the washing Petri dish.

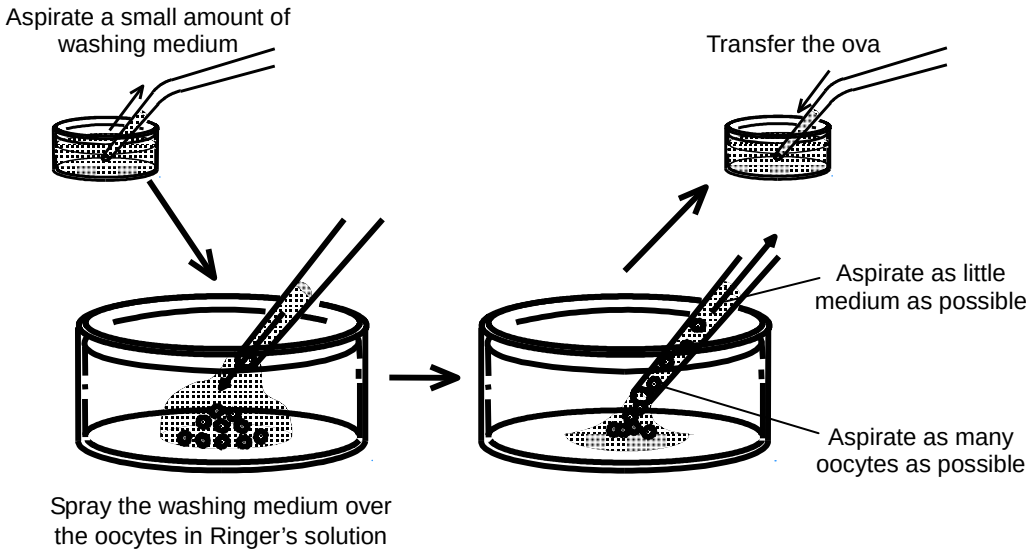


Fig. 10. Washing oocytes

7) Repeat the same procedure in a petri dish used for rinsing. Aspirate the culture medium at a different place than where the oocytes are present. Using the medium, squirt the oocytes with enough force to roll them in order to ensure that the culture medium around the oocytes is replaced.

8) Aspirate the scattered oocytes to gather them up and transfer them to a different place in the same petri dish.

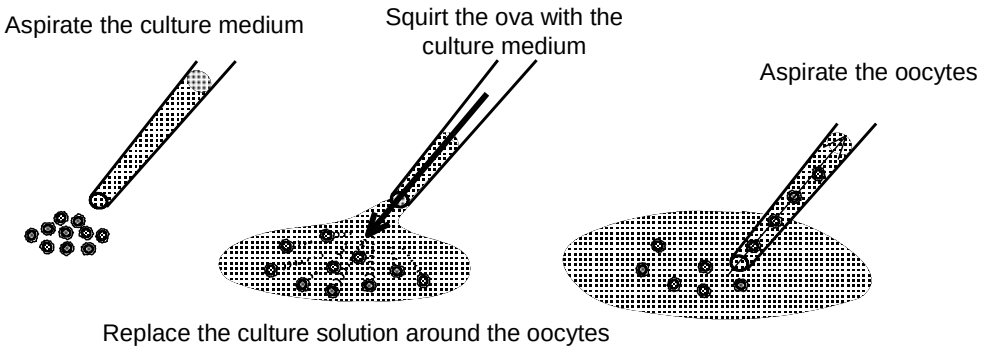


Fig.11. Washing the oocytes in a petri dish

9) Then repeat similar operations in the washing medium, i.e., carry out the steps <7> to <8> with the Petri dishes A, B, C and D, finally transferring the oocytes into the maturation medium. Do this in such a way that the environment in the Petri dish D of the second set is only TCM-199 + 5%CS without any trace of m-PBS + 1%CS (Fig. 12).

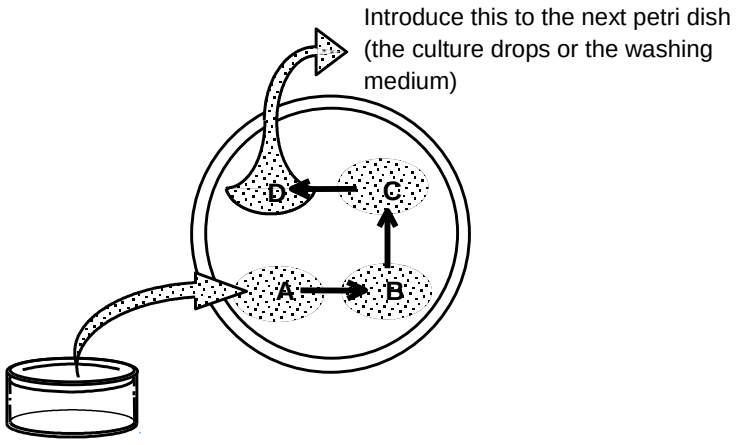


Fig.12. Transfer of the oocytes in Petri dish

***** This is a basic procedure used in techniques other than IVF as well. So, the concept and the technique need to be understood well.

4. Sperm treatment

(1) Preparation

1) Prepare the following culture solutions on the day of collection.
   a) 90% Percoll solution
      - Put 3 ml of this solution into a centrifuge tube for pre-culturing in a CO_2 incubator.
   b) Sperm washing solution (BO solution + 10 mM Hypotaurine + 4 u/ml heparin)
      - Put this solution in a bottle for pre-culturing in a CO_2 incubator.
   c) Sperm diluting solution (BO solution + 20 mg/ml BSA)
      - Put this solution in a centrifuging tube for pre-culturing in a CO_2 incubator.
   d) Oocyte washing solution (BO solution + 10 mg/ml BSA)
      - Create 4 spots of 5 μl oocyte washing solution in a 35 mm petri dish (NUNC 153066) and cover these with liquid paraffin for pre-culturing in a CO_2 incubator.
      - Dispense remaining the solution into 35 mm Petri dishes, 2.5 to 3 ml for pre-culturing in a CO_2 incubator.
   e) 3% NaCl solution (Put 4.95 ml of this solution in a test tube and store at room temperature.)

2) Prepare the following items:
   1 or 2 straws of frozen semen, a hemocytometer with two chambers, a centrifuge, narrow-bottomed centrifuge tubes, an aspirator, a straw cutter, measuring pipette, alcohol-soaked cotton wool, micropipettes, a constant thermostatic chamber, a counter, a vortex mixer.

* **Keep the narrow-bottomed centrifuge tubes and measuring pipettes at 37°C on a warming plate or in a constant temperature chamber.**

(2) Thawing frozen semen and selecting surviving sperm

*** To avoid the risk of lowered sperm activity because of temperature change, carry out all steps listed below while keeping the narrow-bottomed centrifuge tubes warm.**

1) Thaw 1-2 straws of frozen semen in warm water at 37°C and place on the top of the 90% Percoll solution.
2) Centrifuge the tubes at 800 G (2100 rpm) for 10 minutes.
3) Aspirate out the supernatant with the aspirator in order to withdraw as much of the percoll solution as possible.

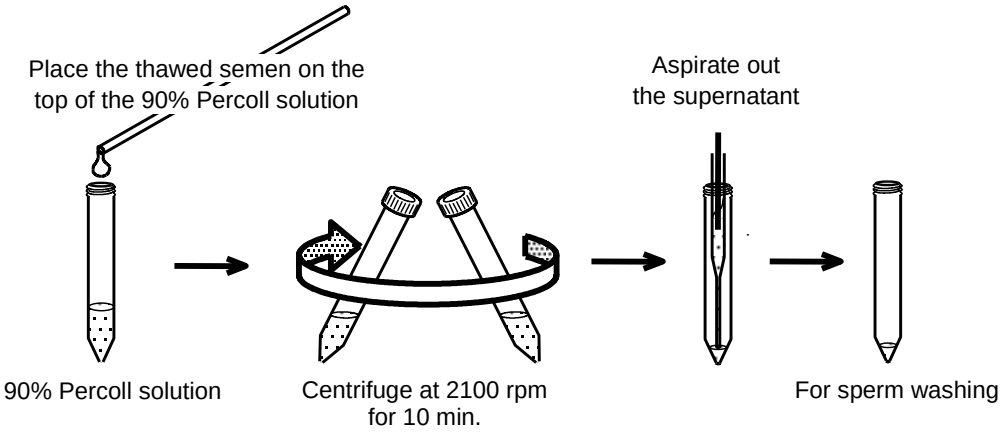


Fig. 13. Thawing frozen semen and selecting surviving sperm

(3) Sperm washing

1) Add 6 ml of the sperm washing solution into the centrifuge tube containing semen and mix well by pipetting.
2) Centrifuge the tubes at 485 G (1800 rpm) for 5 minuets.
3) Aspirate out the supernatant.
4) Re-suspend the sperms in about 500 µl of sperm washing solution.

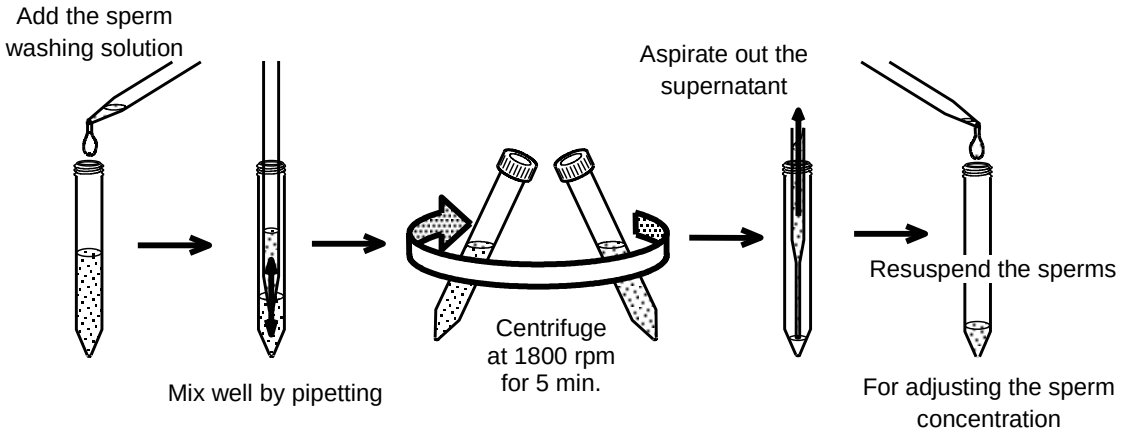


Fig. 14. Sperm washing by centrifugation

(4) Sperm counting

1) Add 50 µl of the sperm suspension to 4.95 ml of 3%NaCl solution (100 times dilution).
2) Mix on uniformly with the **vortex mixer**.
3) Count sperms in the solution using the hemocytometer (2 chambers). Count all the sperms in a counting chamber and take the mean of the two chambers. Take a 5 × 5 square as one counting area. Count the sperms that cross the top and left boundaries of the square and do not count those that cross the bottom and right boundaries.

4) Use 2 counting chambers to obtain average counts. For the calculation that follows, the average sperm count should be used.

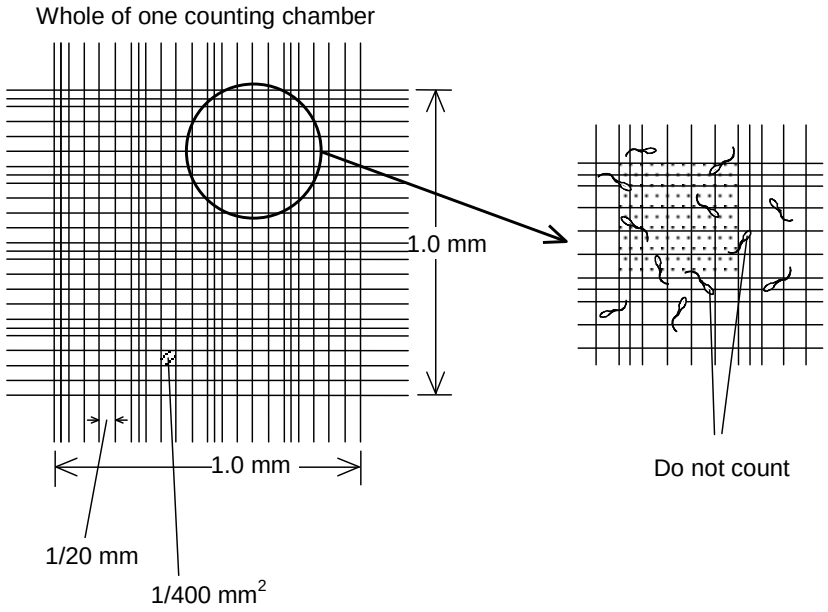


Fig. 15. Counting chamber of the hemocytometer showing sperms in it.

(5) Preparation of a sperm concentration

Before proceeding, measure the volume of the sperm suspension (Sus) using a measuring pipette.

1) **First dilution**: prepare a sperm concentration of 6×10^6/ml.
Calculate the sperm concentration of the suspension based on the average sperm count (Avg) and the suspension volume (Sus). Then add the sperm washing solution to make a total concentration of 6×10^6/ml.

Volume of sperm washing solution to be added to the sperm suspension (ml)
= (Avg/6) × Sus – Sus

Avg = Sperm count (mean of 2 counts) Sus = Volume of sperm suspension (ml)

Using the calculation result, add the sperm washing solution to the sperm suspension to make **First dilution**.

2) **Second dilution**: prepare a sperm concentration of 3×10^6/ml (the final concentration).
Add an amount of sperm diluting solution equal to the volume of the sperm suspension after the first dilution, to adjust the sperm count to 3×10^6/ml (1/2 the concentration of the first diluent).

Amount of sperm diluting solution to be added (ml)
= volume to the first dilution (ml)

Using the calculation result, add the sperm diluting solution to the first dilution to make **the second dilution**.

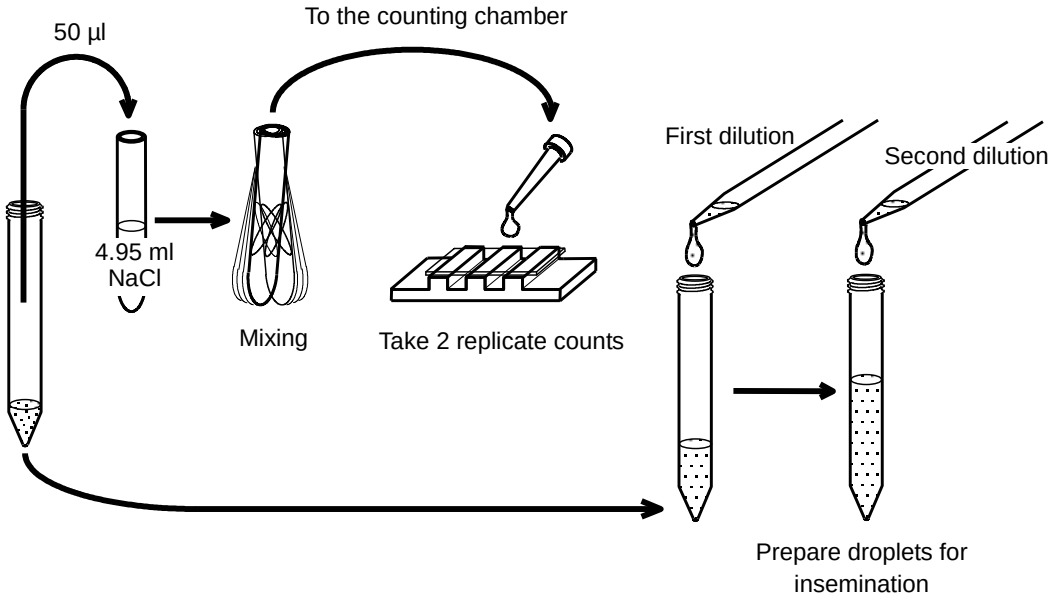


Fig. 16. Counting the sperms and diluting the sperum suspension

Finally, the concentrations of the various reagents and sperm used during the first and second dilutions are tabulated as follows.

Table 2. Concentration of reagents and BSA

Substance	At the time of preparation		After first dilution	After second dilution (to be used for the insemination medium)
	Sperm washing solution	Sperm diluent		
BSA	-	20 mg/ml	-	10 mg/ml
Hypotaurine	10 mM	-	10 mM	5 mM
Heparin	10 u/ml	-	10 u/ml	5 u/ml
Sperm	-	-	6×10^6 cell/ml	3×10^6 cell/ml

*** During sperm processing, the optimal final sperm concentration and heparin concentration will vary among breeding bulls. Preliminary testing is required to determine optimal concentrations. In a case where this method yielded a low success rate in the early division of the fertilized ovum, some countermeasures can be taken such as extending the semination time, increasing the sperm concentration, etc. These measures may improve the success rate.**

5. In vitro insemination

(1) Co-culturing of mature oocytes and sperms

1) Add 95 μl of the sperm suspension with its sperm concentration adjusted to 3×10^6/ml to each drop in the Petri dishes prepared for fertilization (having 5 μl drops of oocyte washing solution) and were being equilibrated in a CO_2 incubator until then, to make fertilization drops of volume 100 μl each. Prepare these Petri dishes just before in vitro insemination and store in a CO_2 incubator.
2) Wash the maturation-cultured oocytes twice with oocyte washing solution.
3) After checking the sperm activity, transfer washed oocytes into the fertilization drops (20 oocytes per drop). As far as possible, transfer the oocytes only, as oocyte washing solution carried onto the fertilization drop lowers the sperm concentration.
4) Incubate at 38.5°C for 5 h in an incubator, in air containing 5% CO_2.

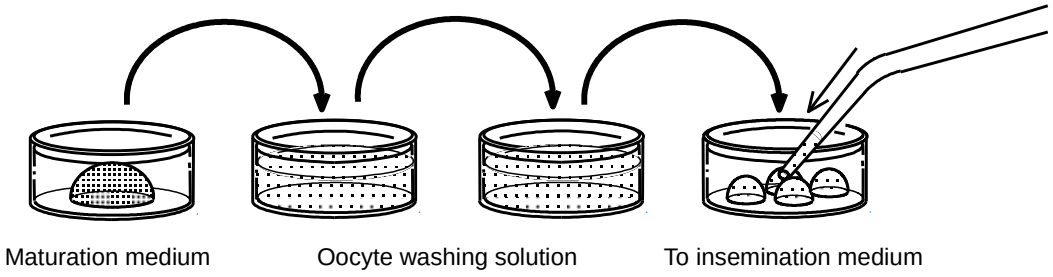
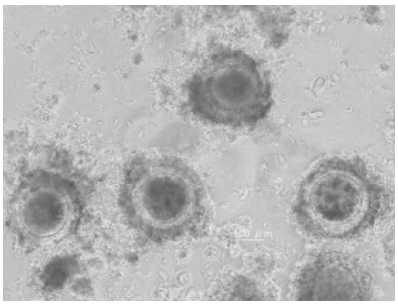


Fig. 17. Transfer of mature oocytes to insemination medium.

Pic. 5. Oocyte during insemination



6. Developmental culture

(1) The culture medium

Culture medium: CRlaa + 5%CS (+0.25 mg/ml linoleic acid-albumin)*

Culturing method: Co-culture or without co-culture (no difference in the percent of embryo development)

At 38.5°C in air containing 5%CO_2

80 oocytes/600 µl, 5 µl/oocyte

*** Compared to TCM-199 + 5%CS, the CRlaa + 5%CS medium is known to have better the development rate to blastocysts, but the embryos have lower cyrotolerance (Imai et.al.[3]). To counter this disadvantage, 0.25 mg/ml of linoleic acid-albumin is added to the CRlaa + 5%CS medium. This improves the cyrotolerance to the level of the TCM-199 + 5%CS medium without lowering the development rate to blastocysts (Imai et. al.[4]).**

(2) Washing the oocytes after insemination

After the in vitro insemination step, the cumulus cells and sperms will be attached to the oocytes. If these are allowed to be carried over to the embryo culture medium, there is an increased risk of polyspermy, etc. Therefore, these cells need to be removed to some extent while washing the oocytes.

1) Wash the embryo with culture medium.
2) Remove the oocytes from the fertilization drops and wash them in two steps with the washing medium by pipetting. Prepare a pipette with a slightly larger bore diameter than the oocyte and pipette in such a way that the oocytes get denuded of cumulus cells by rubbing against each other. The use of too narrow a pipette for this purpose will lead to a lower cleavage rate of the embryos and sometimes burst the embryos.

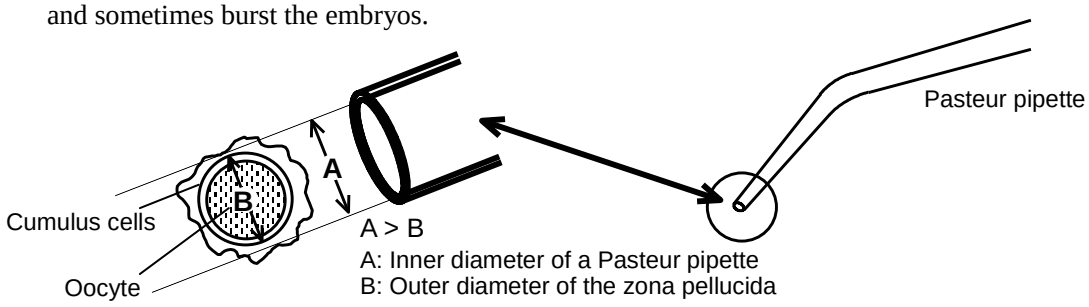


Fig. 18. Preparation of pipette for cumulus cell-denuding oocytes

3) Transfer the oocytes to embryo culture droplet.
4) Culture up to Day 9 for embryo development, taking the day of fertilization as Day 0.

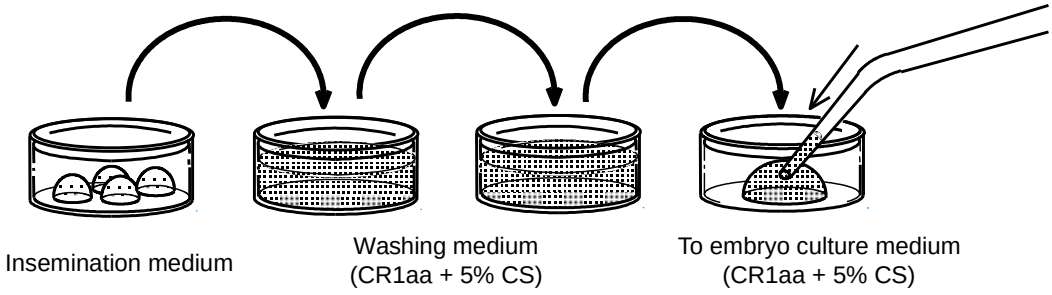


Fig. 19. Transfer of oocytes to embryo culture medium

7. Operations 48h after start of embryo culture

(1) Separating the embryos from cumulus cells

1) Tap the side of the Petri dish and detach, by pipetting, the embryo sitting at the bottom of the dish surrounded by cumulus cells.
2) Denude the cumulus cells attached to the zona pellucida around the embryo using a Pasteur pipette with a slightly larger bore diameter than the embryo.
3) If CRlaa is the culture medium, remove the cumulus cells from the Petri dish bottom with a Pasteur pipette to the extent possible.

*** In all the above operations (particularly <3>) take extra care not to damage the bottom of the Petri dish.**

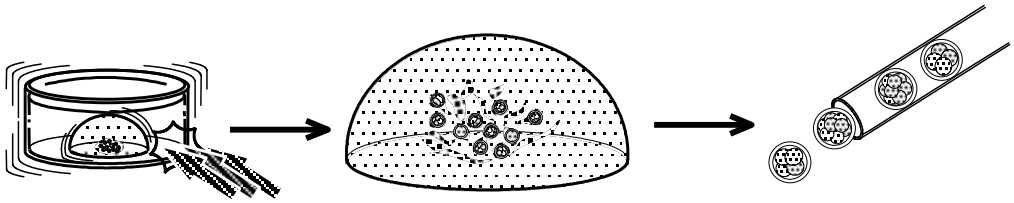
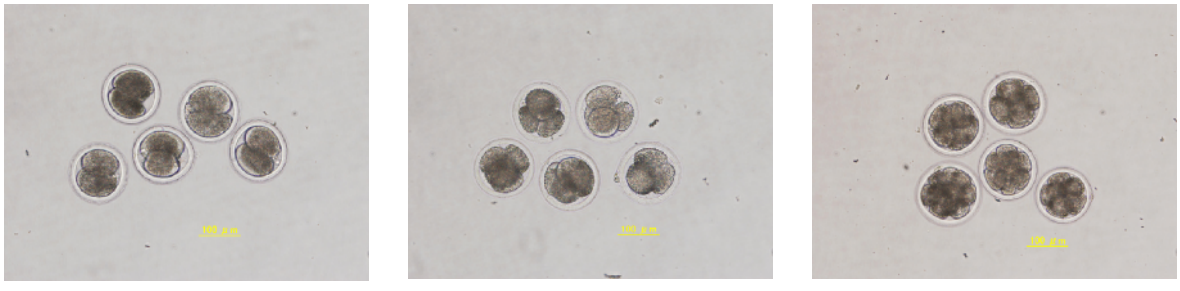


Fig. 20. Denuding embryoes of cumulus cells

(2) Observation of initial embryo development

Classify the oocytes/embryos into 4 categories (i.e., “degenerated oocyte”, “1-cell stage oocyte (unfertilized)”, “2-4 cell stage embryo” and “embryo with more than 4 cells”, according to the stage of development (number of cleavages).

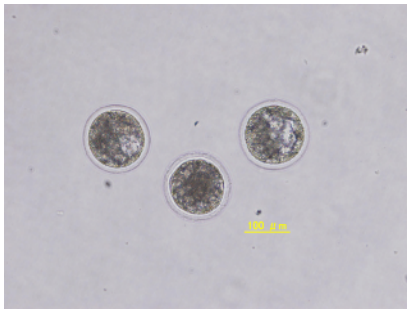
*** Complete this observation as fast as possible (taking 3-4 minutes per dish). Continue the culturing after this initial observation of embryo development.**



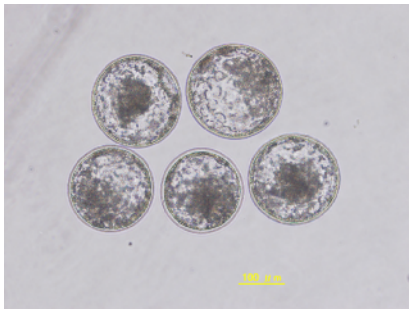
Pic. 6. Embryos during initial development observation

8. Continuation of embryo culture

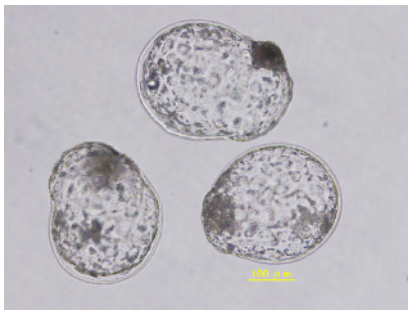
- Tap sides of the Petri dish after every 24h during the culturing to prevent the embryo sticking to the cumulus cells at the bottom and getting engulfed and pressed down.
- Make observations on blastocyst development during Day 7 to Day 9 (day of fertilization = Day 0)



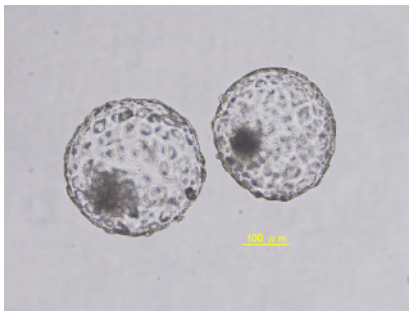
Blastocysts



Expanded blastocysts



Hatching blastocysts



Hatched blastocysts

Pic. 7. Observation on blastocyst development

9. Freezing IVF blastocysts

(1) Cryopreservation medium

1.5 M ethylene glycol + 0.1 M sucrose + m-PBS containing 20% FCS

1) Pipette the freezing solution into a 4-well plate, etc. (750 μl to 1 ml).
2) Prepare the following items:
   Straw for embryo cryopreservation, sealing powder, straw rack for a program freezer, pincette or forceps, etc.
3) Start the program freezer and maintain it at a temperature of -7°C (in pause mode).

(2) Loading and equilibration of the embryos solution in the straw

1) Place the embryos into the cryopreservation medium.
2) After 1-2 minutes, load the embryos in a 0.25 ml straw and seal the straw. This method requires the creation of liquid layers and air layers in the straw as shown in Fig. 22.

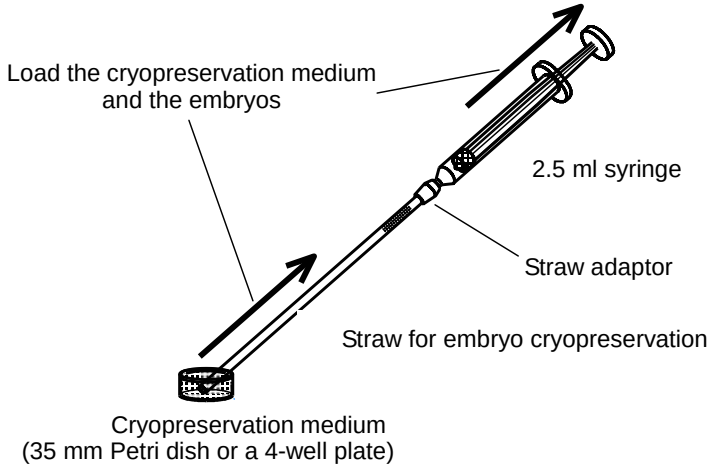


Fig. 21. Handling method for loading the medium into the straw

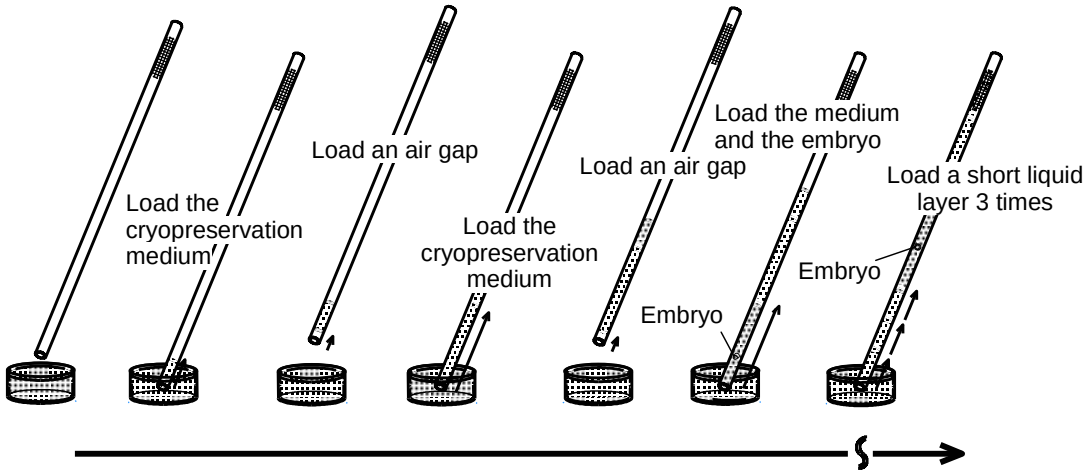


Fig. 22. Method for loading liquid layers and air gaps in the straw

3) Seal the tip of the straw with the sealing powder.
4) Check if the embryo is located in the middle of the liquid layer under a stereomicroscope.
5) If they are not located in the middle, let the straw stand so that the embryos can relocate.

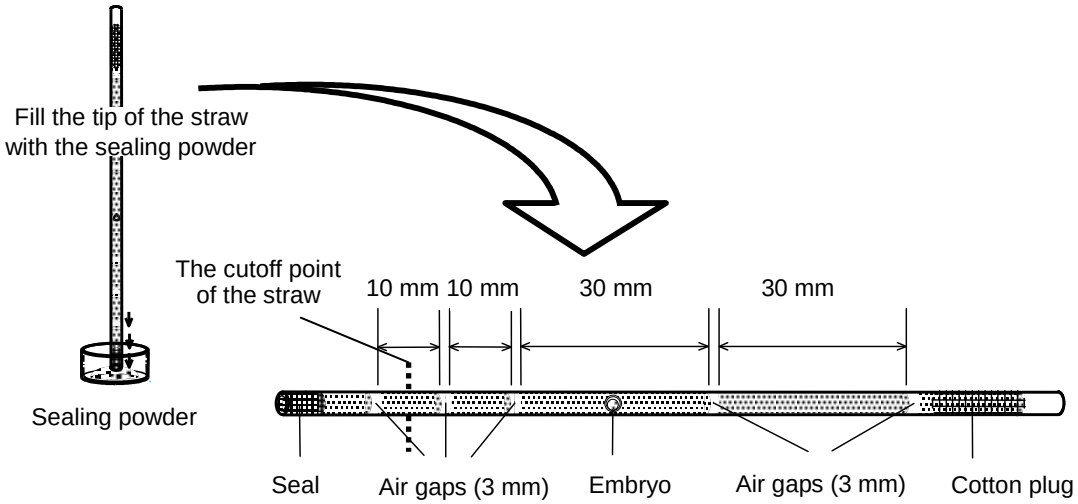


Fig. 23. An example of loading an embryo in a straw

6) Set and equilibrate the straw in the straw rack for approx. 15 min.

*** Place the embryos in the cryopreservation medium. Equilibrate for a total of more than 15 minutes. This 15 minutes is the duration from the time when the blastocysts are placed in the cryopreservation medium to the time when the straw is placed in a programmable freezer at -7°C. In other words, equilibration occurs in the straw for about 10 out of the 15 minutes.**

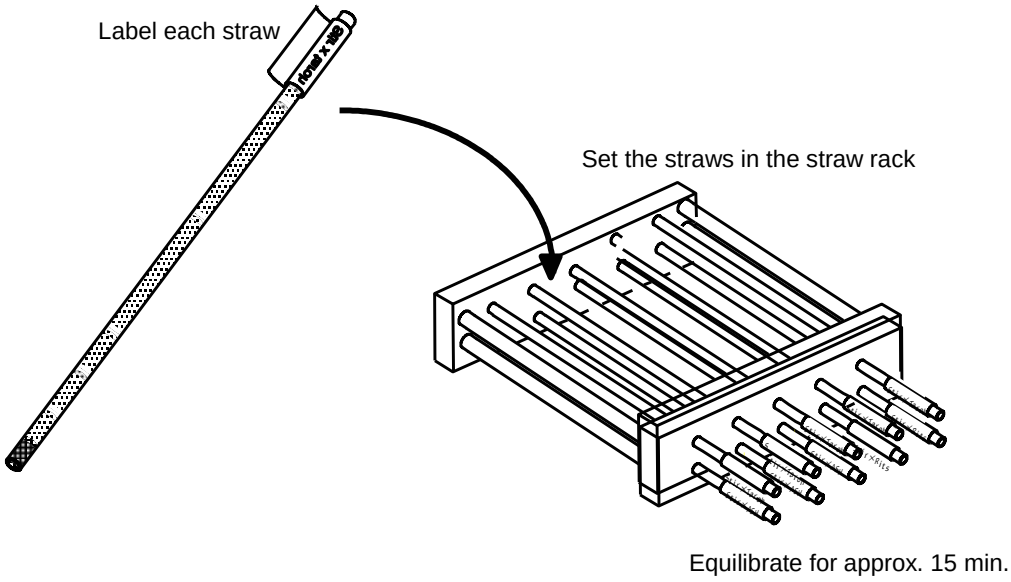


Fig. 24. Setting the straw in the straw rack

(3) Setting the straw rack to the program freezer and ice-seeding

1) After equilibration, put the straw rack in the alcohol bath of the program freezer and start the cooling program set at -7°C.
2) Two minutes after, ice-seed the straw with the pincette frozen in liquid nitrogen as shown in Fig. 25.
3) Within the 15 min allotted by the program freezer for -7°C cooling, check if the embryo layer is ice-seeded.

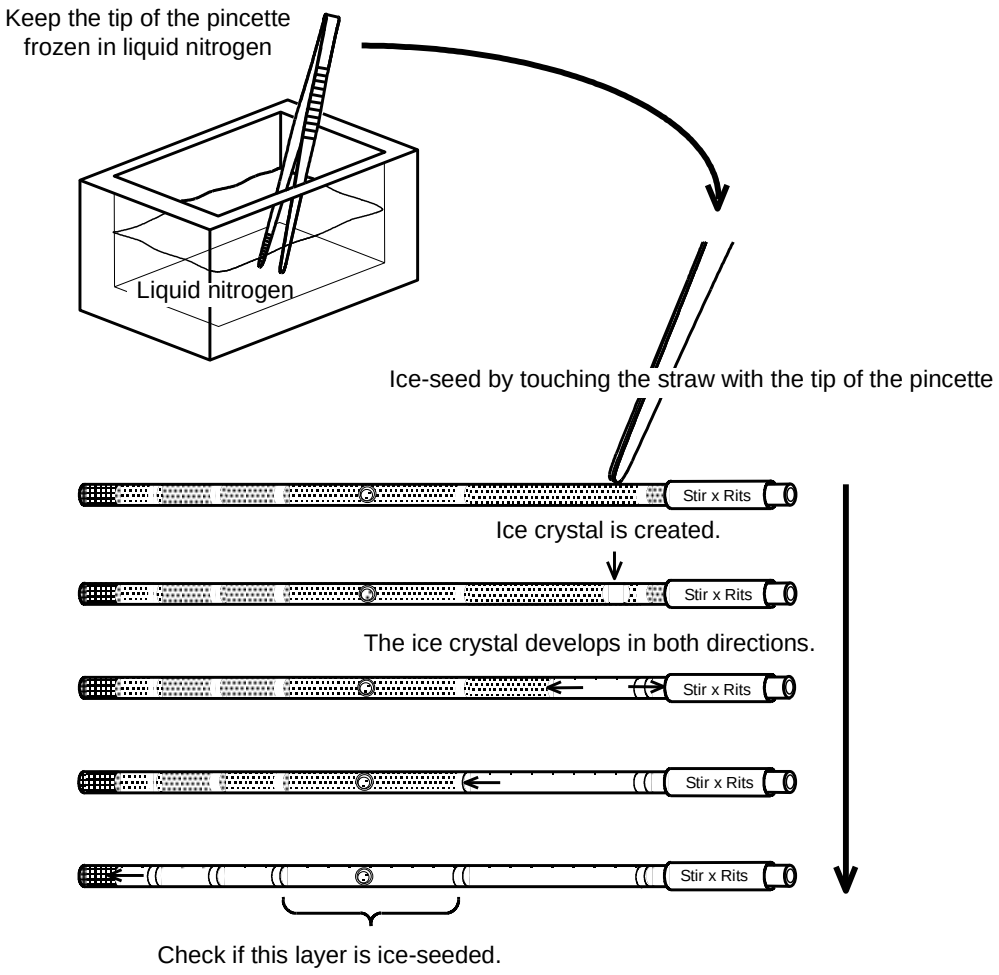


Fig. 25. Ice-seeding process

4) If the ice-seeding proceeds slowly, pause the program (maintain at a temperature of -7°C) until the embryo layer is ice-seeded.

5) Once the embryo layer is ice-seeded, resume the program and wait until the program freezer reaches -30°C.

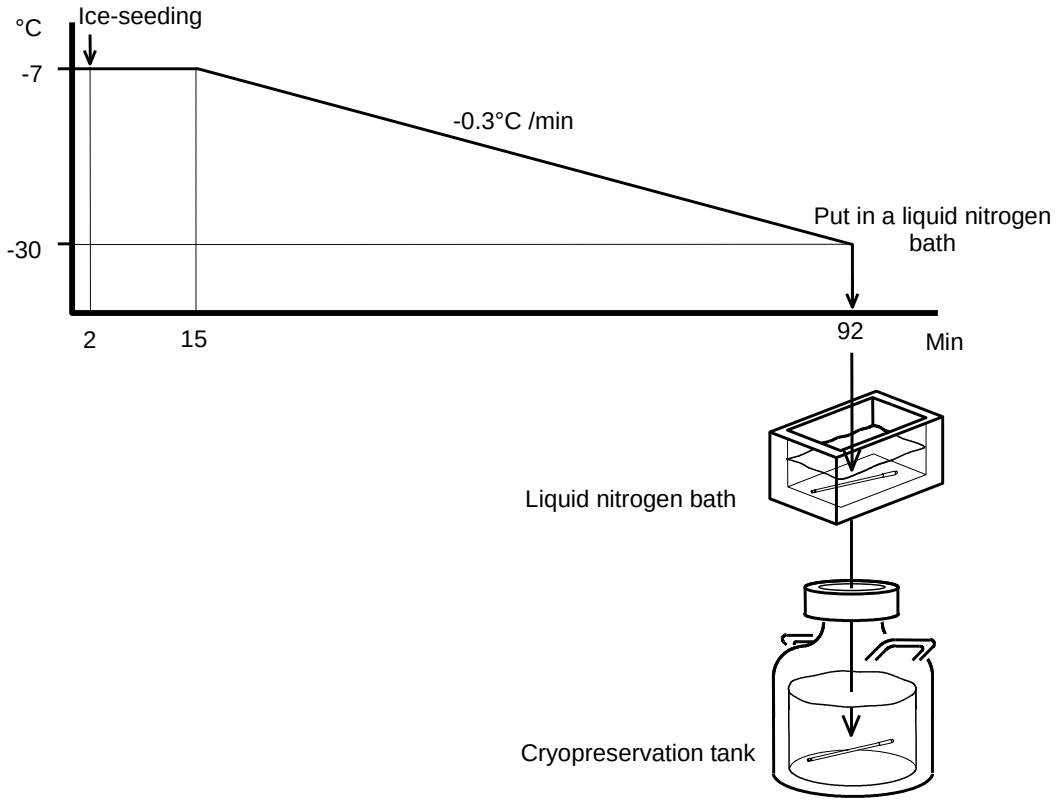


Fig. 26. Cooling curve

6) Once the program freezer has reached -30°C, put the straw in a Dewar bottle or a styrofoam container, etc. containing liquid nitrogen.

7) Store the straw in the cryopreservation tank.

10. Thawing cryopreserved embryos

Method of thawing embryos for transfer

(1) Preparation

1) Prepare the following items:

Embryo transfer device, straw cutter, warm water at 30°C, alcohol-soaked cotton

(2) Thawing and embryo transfer

1) Remove straw from liquid nitrogen and hold in air for 10 seconds. Then dip in warm water at 30°C for thawing.
2) When the crystals have disappeared, remove the straw from the warm water, wipe the surface with alcohol-soaked cotton wool, cut the straw at the dotted line in Fig. 23 (see page 21) with a straw cutter and transfer it into an embryo transfer device.

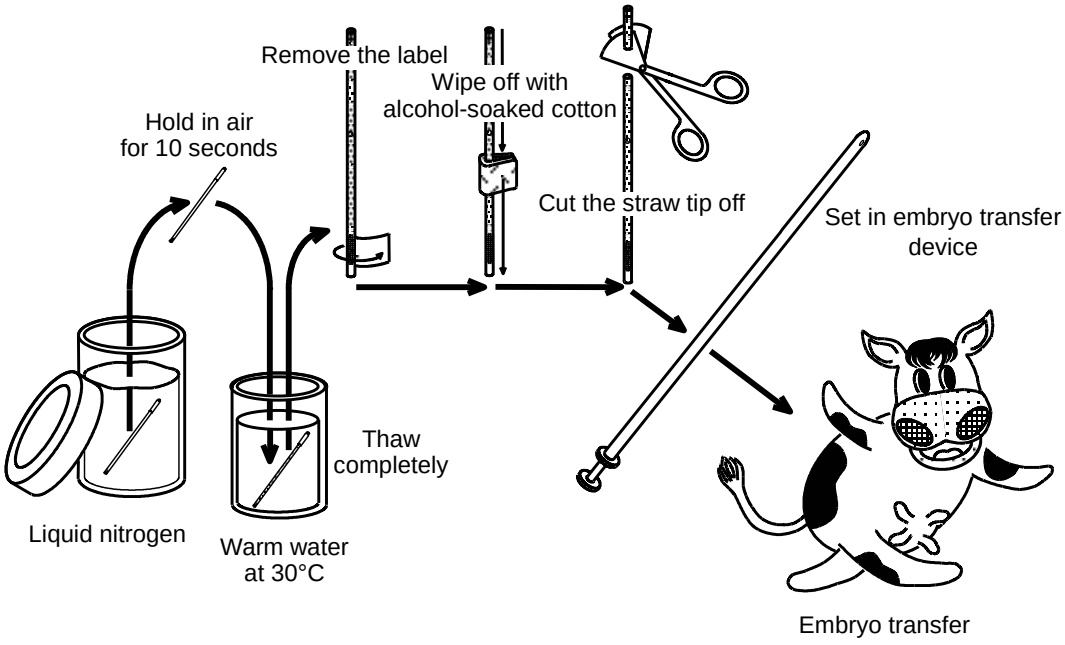


Fig. 27. Thawing embryo for transfer

Observations on survival of cryopreserved embryos

(1) Preparation

1) Prepare m-PBS containing 20% FCS and pipette into a 4-well plate. Maintain the temperature of the 4-well dish at 38.5°C in an incubator.
2) Prepare 20% FCS + 0.1 mM βME (β-mercaptoethanol) + TCM-199. Also prepare petri dishes to be used for washing and cultivation and pre-culture them in a CO_2 incubator.
3) Maintain the temperature of a 90 mm petri dish at 38.5°C with a warming plate.

(2) Thawing and culture

1) Remove straw from liquid nitrogen and hold in air for 10 seconds. Then dip in warm water at 30°C for thawing (use the same procedure as that for embryo transplantation).
2) When the crystals have disappeared, remove the straw from the warm water, wipe the surface with alcohol-soaked cotton wool, cut the straw at the dotted line in Fig. 23 (see page 21) with a straw cutter and transfer it into the pre-warmed 90 mm petri dish.
3) Examine the embryo under a microscope and then transfer it into m-PBS containing 20% FCS for 10 min until equilibrium is reached.
4) Wash the embryo with a solution of 20% FCS + 0.1 mM βME + TCM-199 and culture it in the CO_2 incubator.

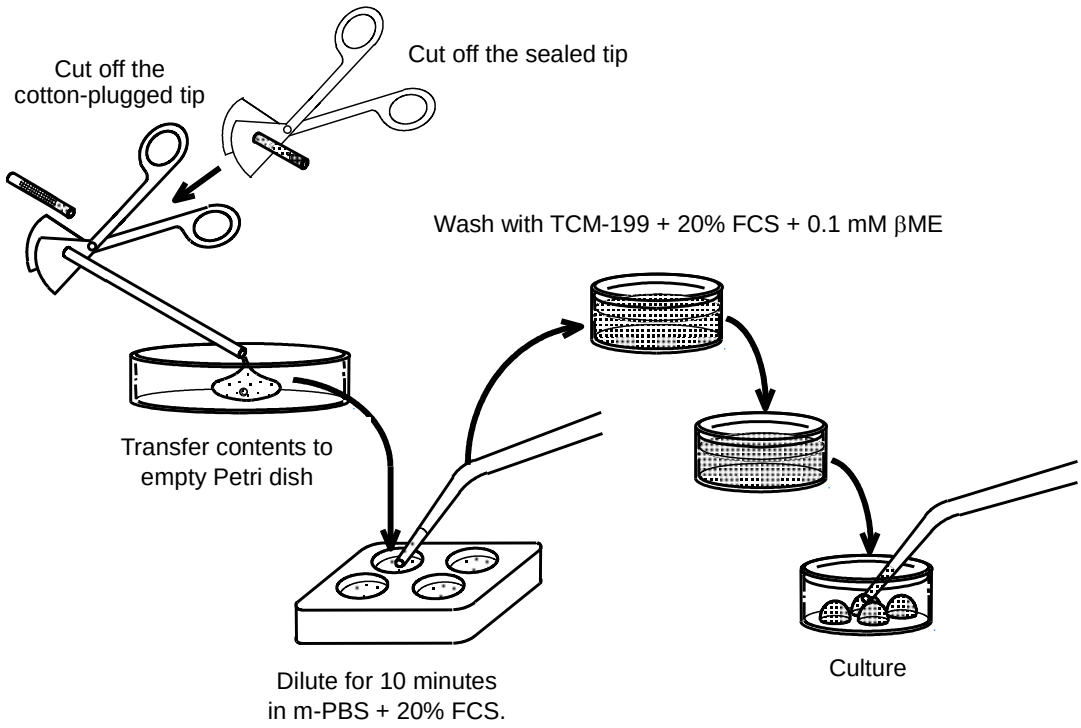


Fig. 28. Thawing, dilution of cryoprotectant and method of embryo culture

5) Check embryo development every 24h and look for the stages listed below.

Table 3. Developmental stages of the embryos to be observed at different times from start of culturing

Culturing time	24h	48h	72h	96h
Developmental stage	Expanded blastocysts	Hatching blastocysts	Hatched blastocysts	Hatched blastocysts

Transvaginal Ultrasound-Guided Follicular Aspiration (OPU)

This technology involves aspiration of oocytes from the ovaries of living cattle. Ultrasound diagnostic equipment is required for this procedure in order to observe small follicles that cannot be distinguished with the naked eye or by rectal palpation. Since the operation of the ultrasound diagnostic equipment can affect the outcome, this manual provides an explanation of the basic operational techniques for such equipment.

[Ovarian observation using ultrasound diagnostic equipment]

1. Ovarian observation using a linear rectal probe

- Conceptual impression of an ovarian observation

  During the ovarian observation, the probe is held over the ovary. The ovarian image produced by the probe can be seen as a cross-section view; the same view would be obtained if the ovary was sliced by the probe using an action similar to that of a “kitchen knife.” The centerline of the probe can therefore be seen as the blade of the knife, which “slices” the ovaries and any other tissues and organs. This concept can be applied to a probe regardless of its type: linear or convex.

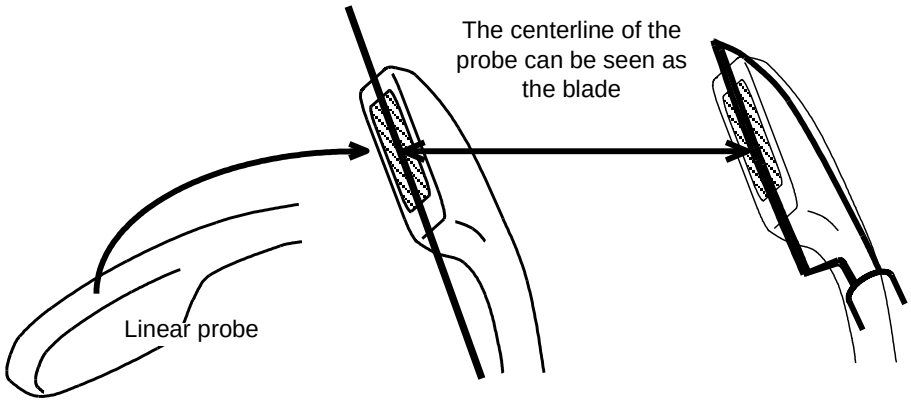
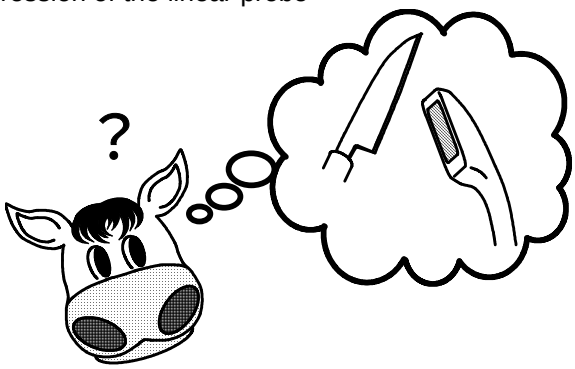


Fig. 29. Conceptual impression of the linear probe



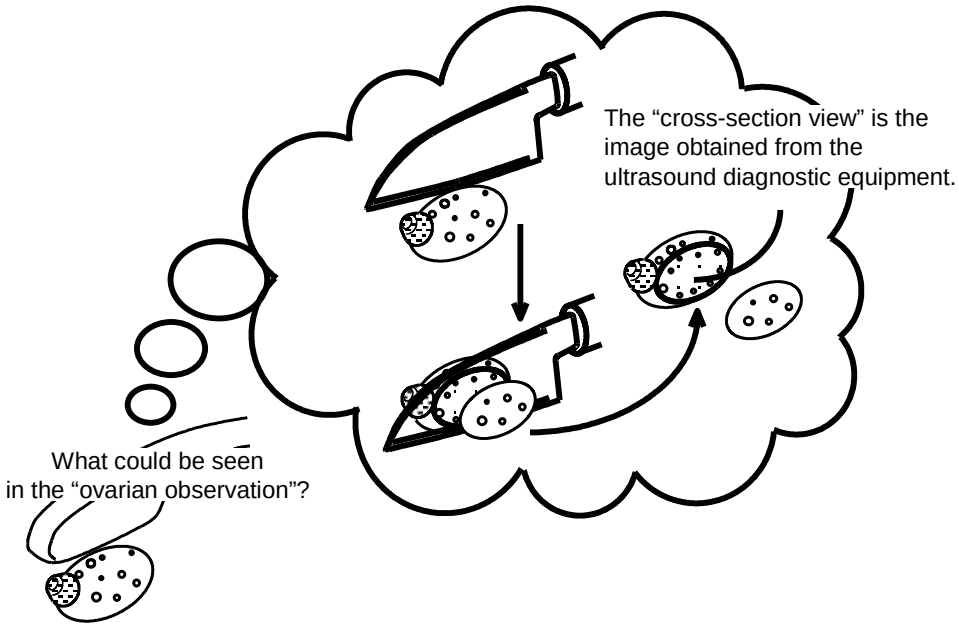


Fig. 30. Conceptual impression of slicing the ovary

When the ovary is sliced on the edge, a small cross-section image results. When sliced through the center, a larger image is obtained. The same also applies to the ovarian tissues such as the corpus luteum and follicles: the image generated from the edge is small but is larger from the center.

The ovary consists of the surface, the ovarian cortex and the inner core or the ovarian medulla. Since follicles can be found in the cortex, no follicles will be found in the middle of the cross-section if the ovary is sliced through the center. When the edge is sliced, evenly spread follicles will be seen even though the area of the cross-section image is rather small.

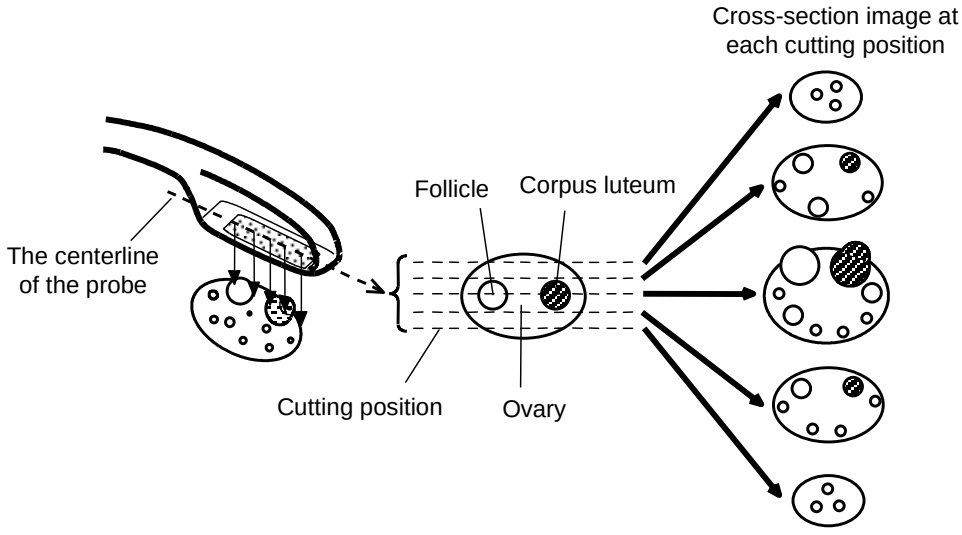


Fig. 31. Images of the cross-section at each cutting point

The cutting method presented in Fig. 31 involves a particular movement of the probe in relation to the position of the ovary, which is illustrated on the left in Fig. 32. Another method is to have the probe revolve around the cord as shown on the right in Fig. 32. The application of one or both of these methods is dependent upon the individual situation and the purpose the images will serve.

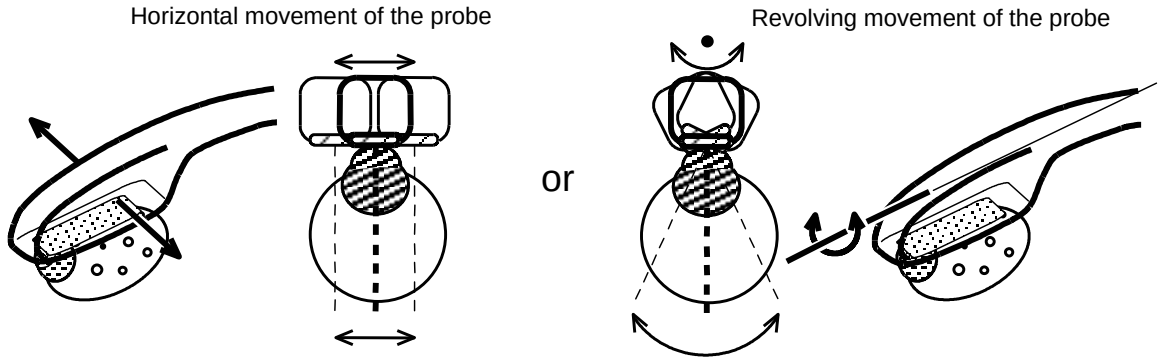


Fig. 32. Cutting methods with the probe

2. Ovarian observation using a convex transvaginal probe

The current transvaginal probes used for OPU are mostly of the convex type. While the type of probe differs, the concept of the generation of a “cross-section view” remains the same. In the case of a convex probe, however, the generated image is in sector form as shown in Fig. 33. The image display can be set in 4 different ways. These images are vertically and horizontally inverted, according to the position of the probe. The probe operator can choose one of the settings for display during operation. All of the relevant techniques explained in this manual are based on the ultrasound image illustrated in the bottom left corner in Fig. 33.

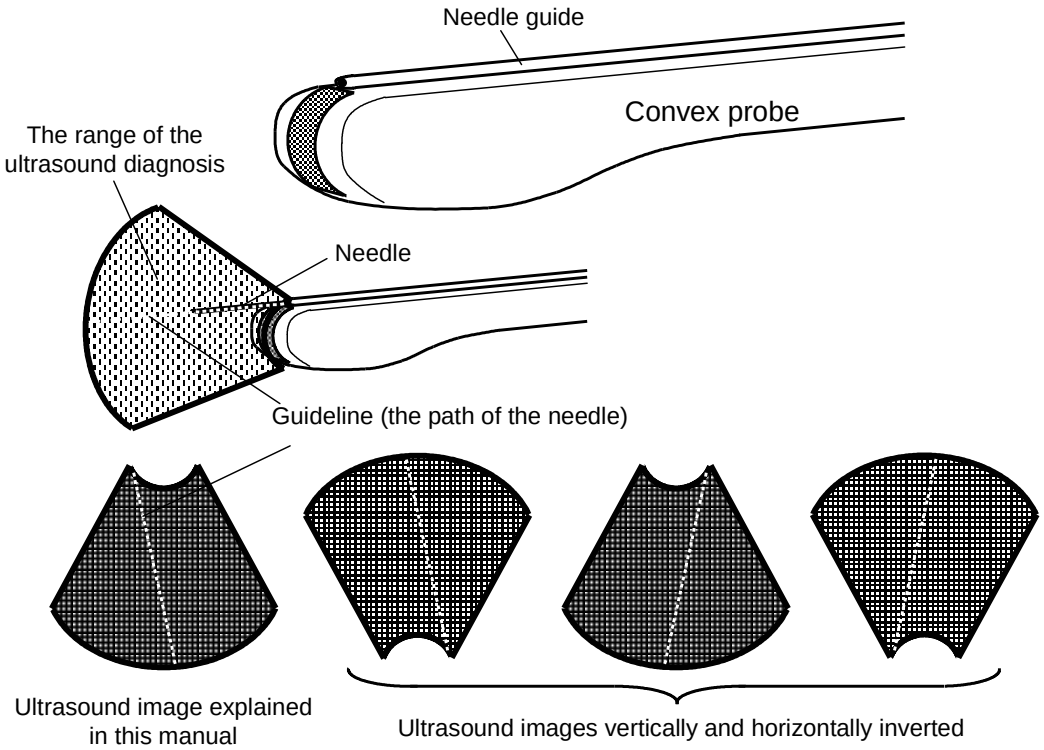


Fig. 33. Convex probe and the obtained ultrasound images

- Images obtained during ovarian examination - how they are displayed and how they can be displayed.

When the positional relationship between the probe and the ovary is as shown in the position of ovary A and B figures below, the image obtained corresponds to the ultrasound image A and B underneath these figures. If a transvaginal probe is used in OPU, etc. (the aspiration needle is used), a “guideline” should be displayed on the ultrasound image as it marks the path of the oocyte collection needle.

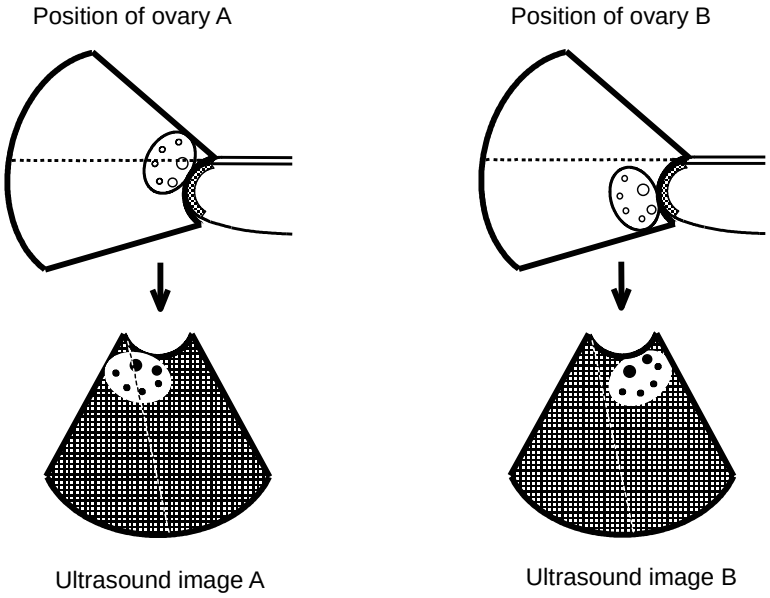


Fig. 34. Ovarian position and its ultrasound image

In regard to the orientation of an oval ovary, there are 3 prominent patterns for holding the ovary against the probe. The ultrasound images obtained with each pattern are shown in Fig. 35.

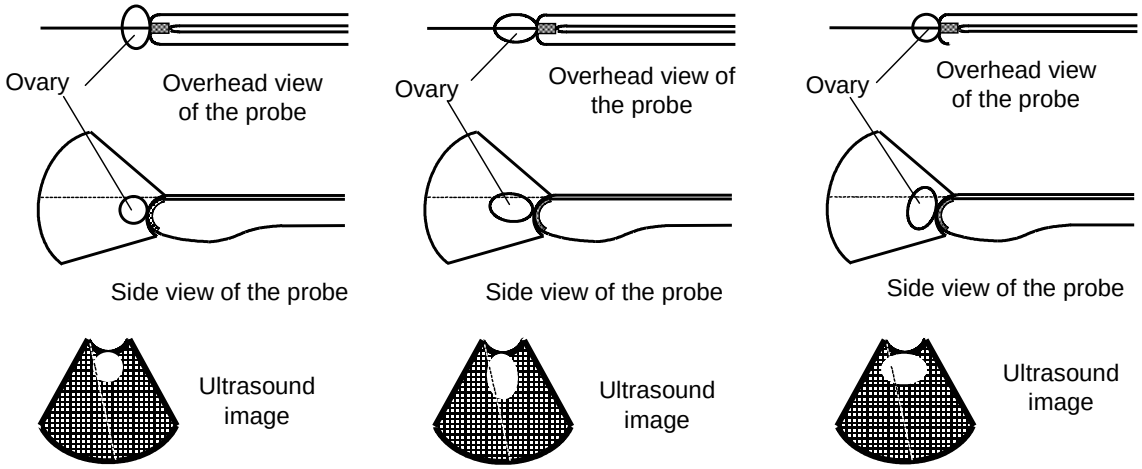


Fig. 35. Ovarian orientation and corresponding ultrasound

- Ovary holding method

In order to obtain the ovarian images presented above, the ovary needs to be held onto the probe in the same way as in rectal palpation. There are 2 main methods of ovary holding.

Holding method A

This is a relatively easy method used to hold the ovary onto the probe. Bring the ovary closer to the probe while grasping it with a hand. In order to count the follicles, move the ovary left and right as shown in Fig. 36. For aspiration of the oocyte, also move the ovary up and down during the operation.

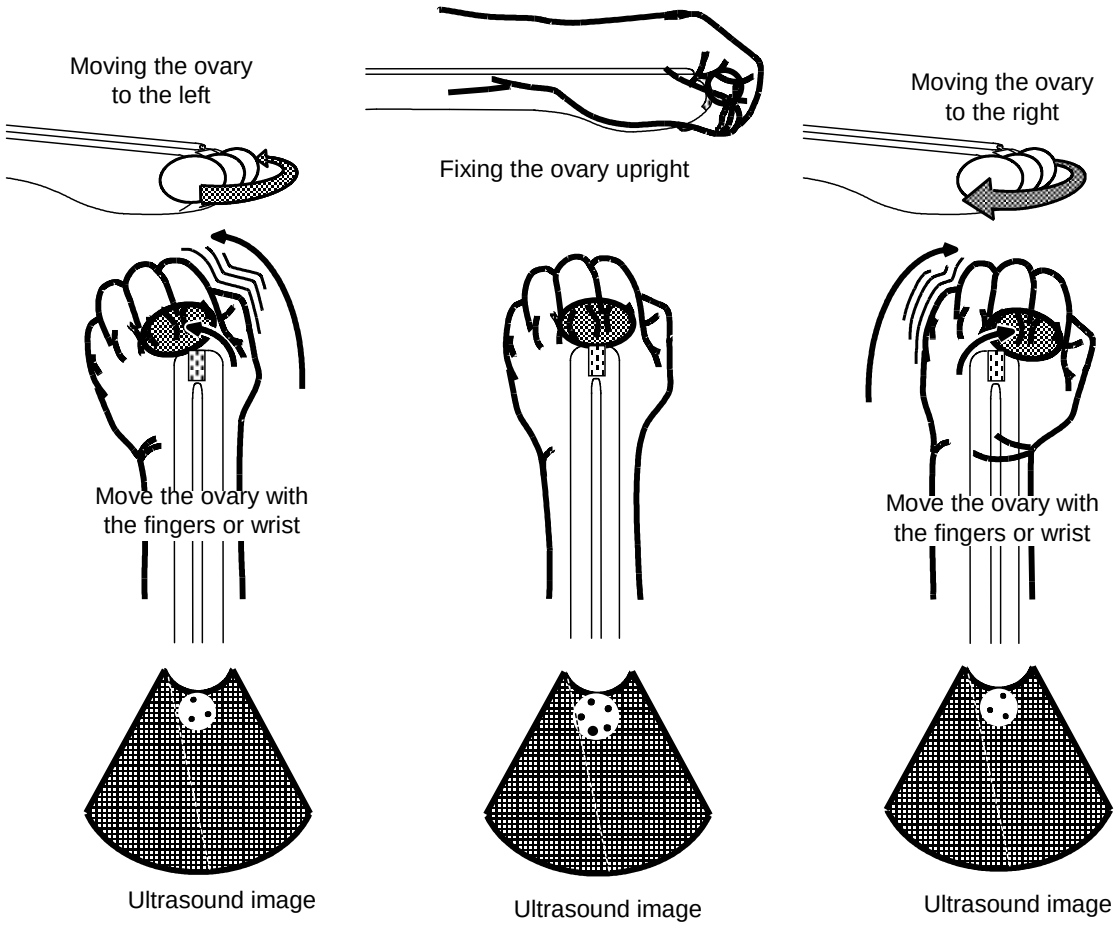


Fig. 36. Holding method A

Holding method B

In the above-described method A, the ovarian image that is displayed is in the form of a circle. In contrast, method B displays the image as an oval; in this image, not only is the area of the cross-section larger, but there are also more follicles displayed. This is advantageous for aspiration. Depending on the location of the follicles, some can be difficult to aspirate using only method A, therefore, a combination of methods A and B is required.

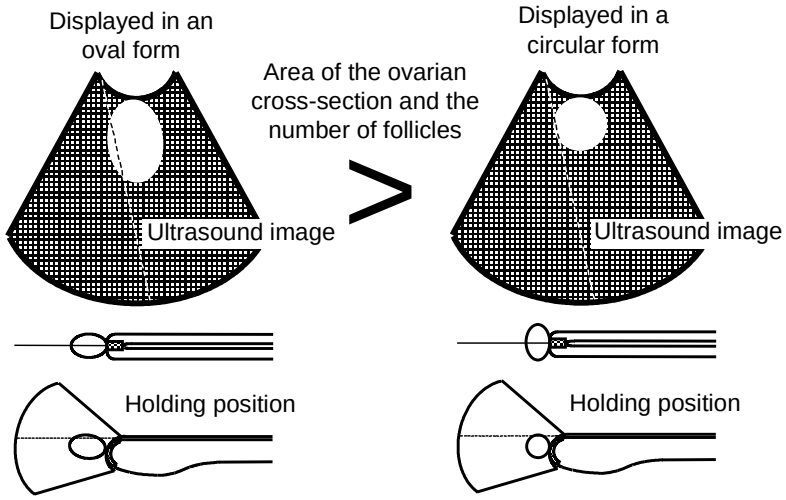


Fig. 37. Considerations for the holding method based on the displayed image

While the ovary is “grasped” in the hand in method A, the ovary needs to be held as it is “picked up” with the hand in method B, which displays the image as an oval.

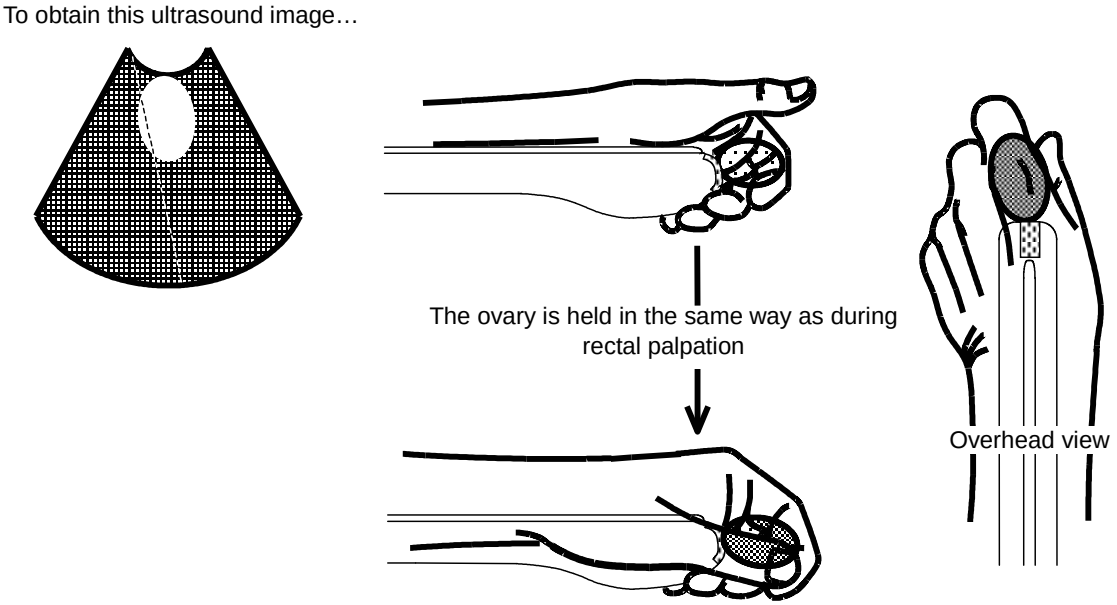


Fig. 38. Holding method B

Comparison of holding methods A and B

In method A, the ovary is held onto the probe as it is grasped in the hand. There are therefore many contact points on the ovary, which makes it relatively easy to hold its position by putting some pressure on it. In method B, the ovary is held onto the probe as it is picked up. The vectors of the contact points are therefore mostly towards the center of the ovary, which makes it easier to move it in the hand. One method is not better than the other, and they should be applied depending upon the situation and the purpose.

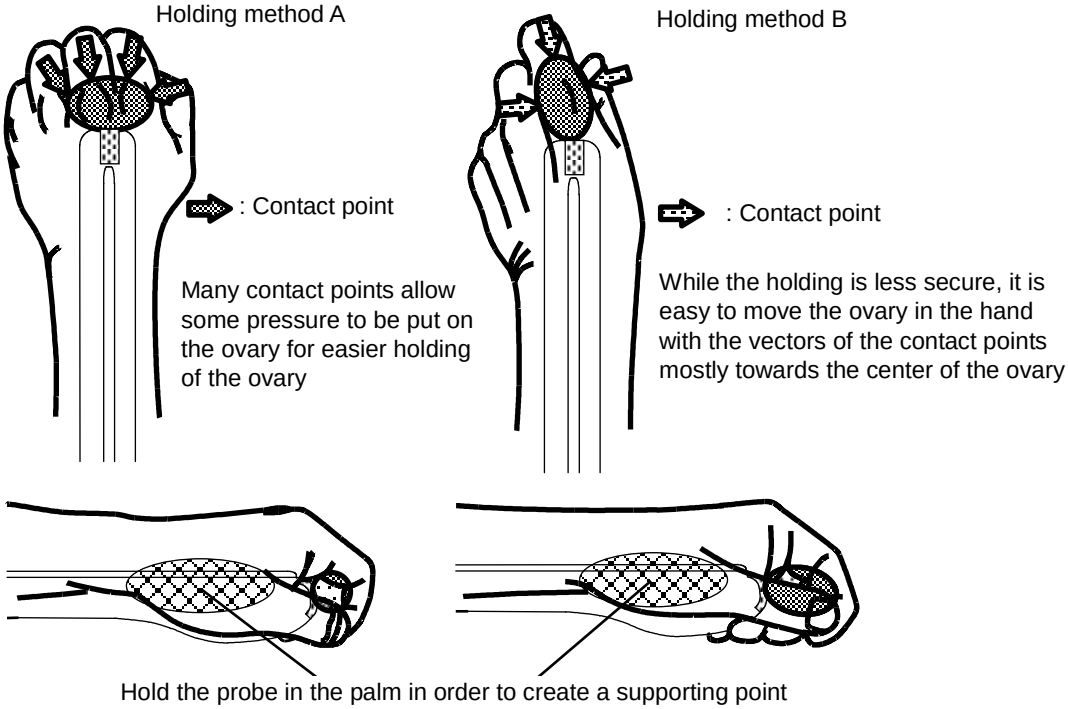


Fig. 39. Comparison of holding methods A and B

With the rectal probe, the ovary remains in the same position and the probe is moved to obtain the image. With the transvaginal probe, this occurs the other way around. The ovary is grasped in the same manner as during the rectal palpation and is brought into contact with the probe via the vaginal wall. To slice the ovary, move the ovary left and right or up and down. The probe inserted into the vagina should basically remain still except for the forward and back movements during insertion and withdrawal.

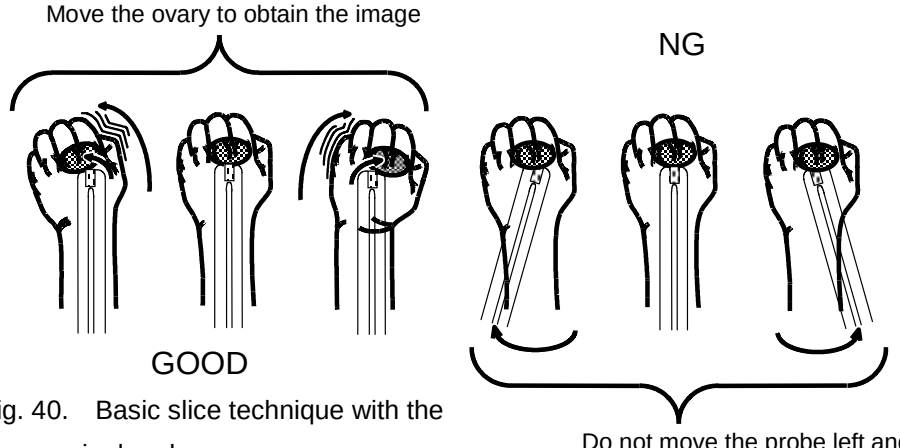


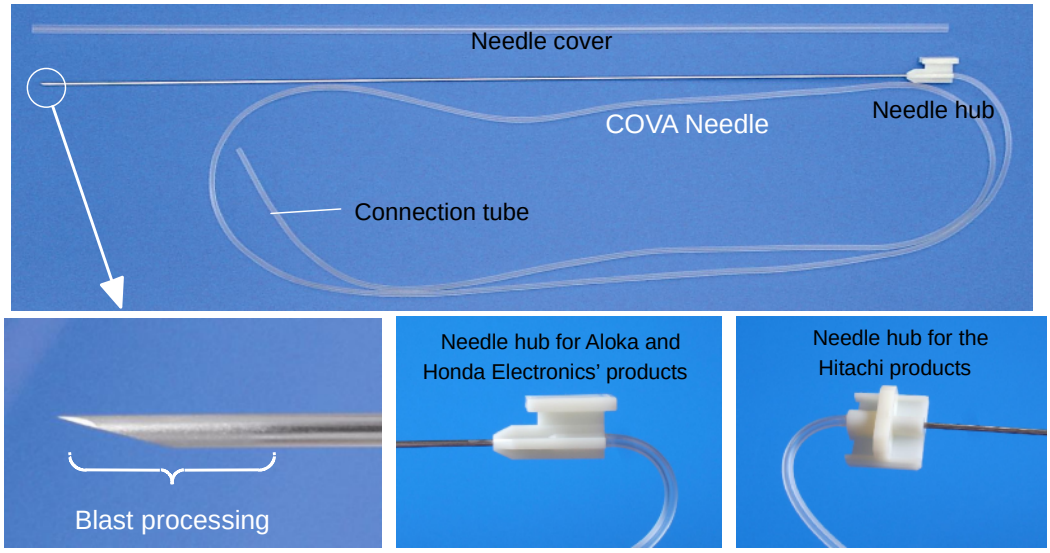
Fig. 40. Basic slice technique with the transvaginal probe

[Oocyte aspiration]

1. Types of oocyte collection needles and methods for aspiration of follicular fluid

Currently, the most common types of ovum collection needles and methods for aspiration of follicular fluid can be categorized into four main types. The oocyte collection needles are distinguished according to their shape: one with a length of ±500 mm (long needle) and one with a 50 to 100 mm length (short needle). There are two methods used for aspiration of follicular fluid: a one-way method and a two-way method. It is important that the method to be employed is carefully considered before purchasing required items, such as the oocyte collection needle, probe, needle guide and accessories, as these items are currently rather expensive and cannot be used interchangeably for both methods.

For aspiration, the two-way method is more advantageous than the one-way method due to the possible damage that may occur to the collected oocytes with the one-way method as well as the actual performance of the aspiration (blood coagulation can occur in the needle, etc.). However, the two-way method requires rather expensive equipment and a needle. The National Livestock Breeding Center participated in the development of a COVA needle (Misawa Medical Industry Co., Ltd.) to be used with the one-way method[5)] and the center has now put this needle into use (Pic. 8). Features of the needle include: a needle tip with an obtuse angle, which allows for efficient follicular fluid aspiration, treatment of the needle by a blast processing up to the needle tip, which also makes the needle tip visible in the ultrasound image, and silicon coating for the needle, which ensures a better puncture.



(Pictures provided by Misawa Medical Industry Co., Ltd.)

Pic. 8. Overview of the COVA needle

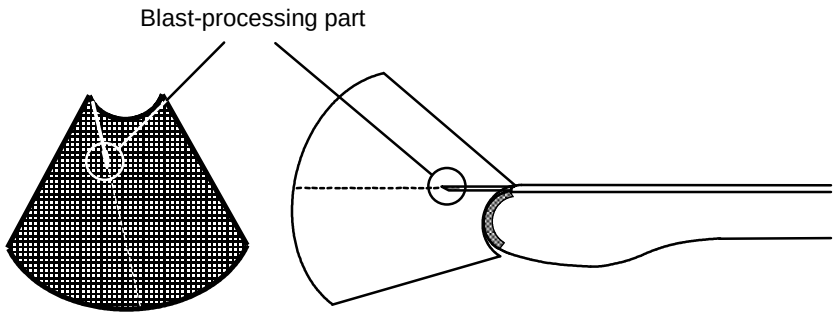


Fig. 41. Impression of an ultrasound image showing the blasted needle tip

2. Puncturing the vaginal wall

Once the probe is located in the vagina, the ovum collection needle must first penetrate the peritoneal cavity via a puncture in the vaginal wall in order for follicular aspiration to occur. To achieve this, ensure that the probe, the vaginal wall and the ovary are in close contact and insert the oocyte collection needle in a way that ensures it is oriented towards the center of the ovary. Then, in order to change the position of the ovary, withdraw the needle from the ovary while leaving the needle in the peritoneal cavity (this means that the needle continues to penetrate the vaginal wall).

In doing this, the needle should remain in the peritoneal cavity until approximately 10 follicles have been aspirated.

3. Changing the position of the follicle

Move the ovary to place the follicle in line with the guideline for the aspiration because the needle tip runs through the guideline displayed in the image. Insert the aspiration needle and aspirate the oocyte together with the follicular fluid. Fig. 43 indicates a situation where the follicle was originally located beneath the probe and is then brought up in line with the guideline. If the ovary is located above the probe, adjust the position of the follicle while moving the ovary down.

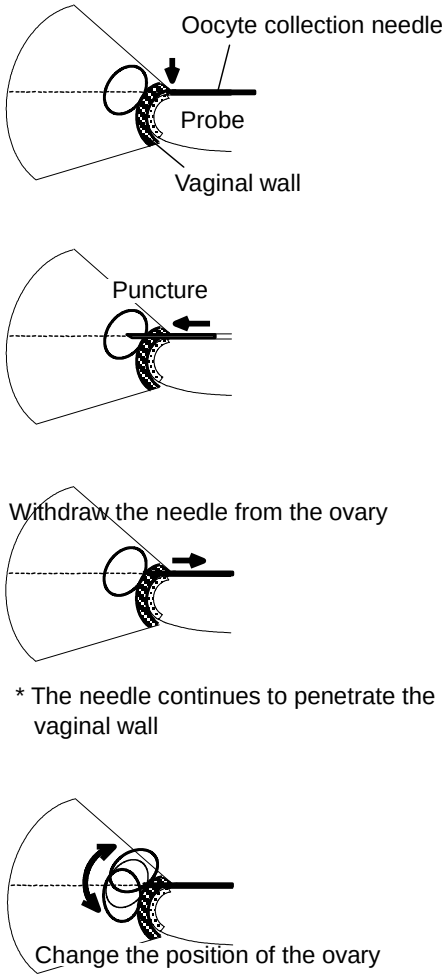


Fig. 42. Puncture in the vaginal wall

4. Aspiration pressure

Too low an aspiration pressure reduces the oocyte collection rate. Too high a pressure, on the other hand, denudes the cumulus cells and the number of denuded oocytes increases. In general, an aspiration pressure of 35 to 100 mmHg or an aspiration rate of 20 to 36 ml/min should be applied. It is, however, necessary to determine the most appropriate aspiration pressure for the aspiration equipment using ovaries obtained from a slaughterhouse, etc., since the flow of the aspirated fluid is dependent on the aspiration needle and the width of the tube connected to it[6), 7)]. The fact that some aspiration equipment cannot aspirate any oocyte at all using the above-mentioned pressure emphasizes the importance of such preliminary testing. Ideally, apply an aspiration pressure that yields a collection rate of more than 90%. At the National Livestock Breeding Center, for example, the FV4 manufactured by Fujihira Industry Co., Ltd. is used with an aspiration pressure of 100 to 120 mmHg (18 to 22 ml/min aspiration rate) for the OPU operation.

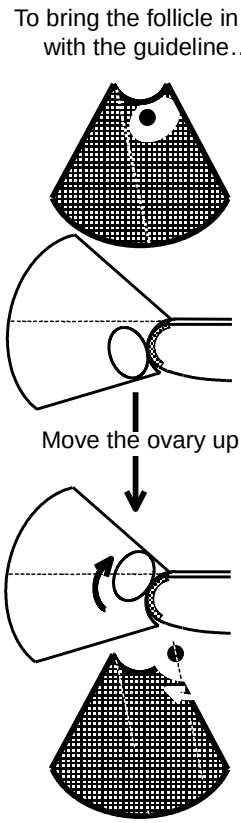
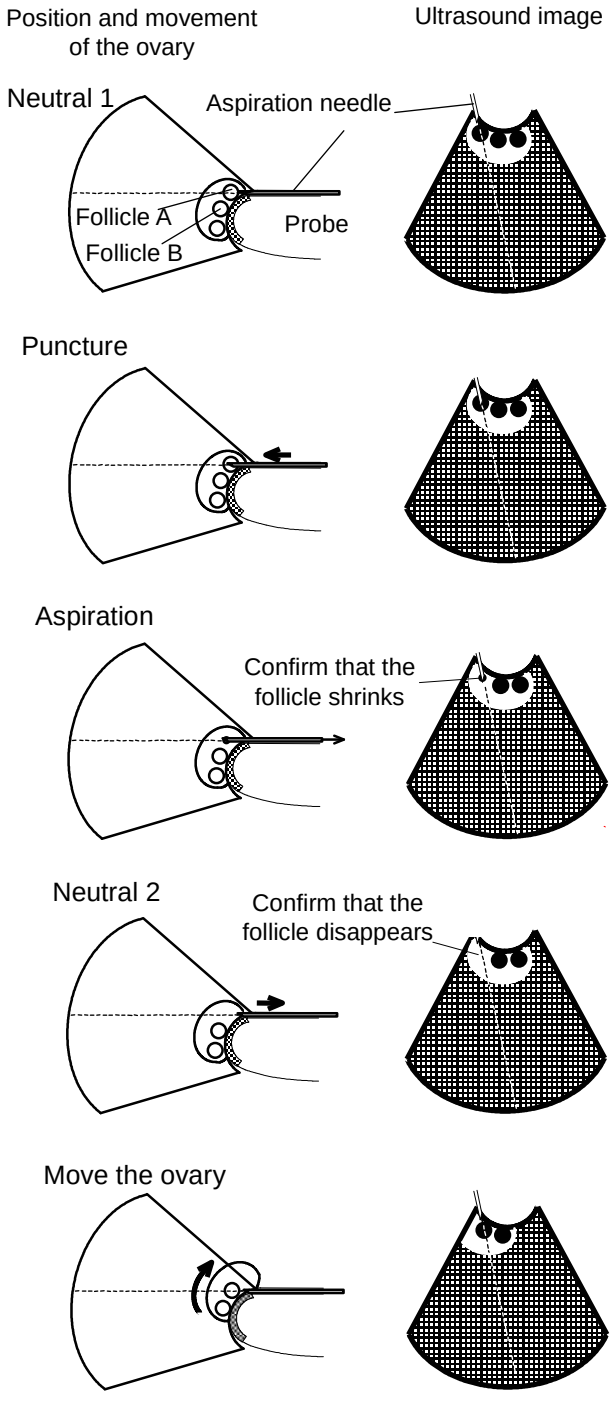


Fig. 43. Changing the position of the ovary while watching the ultrasound image

5. Consecutive oocyte aspiration

Multiple follicles can be found in one ovary, and all of these must be aspirated. With one puncture through the vaginal wall, multiple follicles should be aspirated in order to shorten the duration of the operation and also to reduce damage to the donor cow. Therefore, it is necessary to repeat the procedure of moving the ovary, puncturing it with the oocyte collection needle and aspirating the oocyte.

By way of illustration, the following is an explanation of the handling technique to be used when follicle A in the ovary is aspirated first and is followed by follicle B.



Neutral 1

Bring follicle A in line with the guideline.

Puncture

Insert the aspiration needle into follicle A. Make sure the image of the needle tip can be seen inside the follicle.

Aspiration

Since the aspiration pump is set to negative pressure, the aspiration of the oocyte and follicular fluid will occur naturally. During this procedure, confirm that the aspiration is causing the follicle to shrink.

Neutral 2

Withdraw the aspiration needle upon confirmation that the aspiration has caused the follicle to disappear.

Move the ovary

Bring follicle B in line with the guideline

→ Repeat the procedure from Neutral 1

Fig. 44. Consecutive oocyte aspiration

6. Aspiration of multiple follicles

When aspirating multiple follicles, the question is which follicle to begin with.

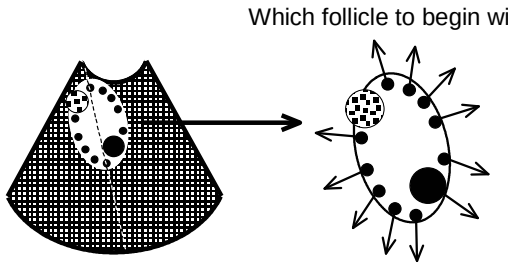


Fig. 45. Aspiration of multiple follicles

If there are small- and medium-sized follicles as well as a large follicle as shown in Fig. 45, the process can proceed as follows (Fig. 46).

1) First aspirate the small- and medium-sized follicles located above the dotted line that divides the ultrasound image of the ovary in half horizontally (probe side).
2) Turn the ovary 180°.
3) Aspirate the remainder of the small- and medium-sized follicles and then the large follicle.

* **The greater the distance between the probe and the follicles to be aspirated, the greater the chance that the oocyte collection needle will miss the follicle. Therefore it is mandatory to keep the range of the needle (from the needle guide to the needle tip) as short as possible.**

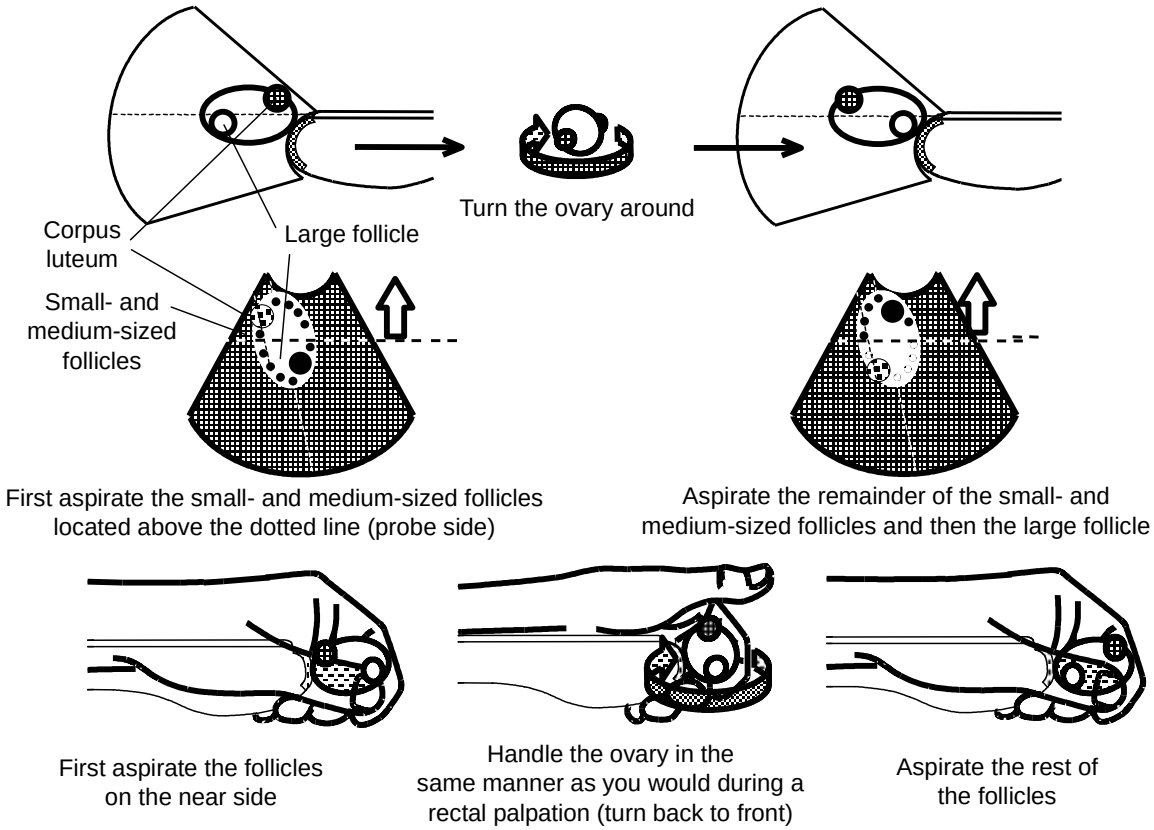


Fig. 46. Oocyte aspiration while turning the ovary around

When the follicles on the same cross-section are to be aspirated as described above, it is crucial to monitor the side-to-side movement on the ultrasound monitor. This movement is a true representation of the up-and-down movement of the ovary held on to the probe. Therefore, the OPU operator should be aware that the monitor displays a 90 degree-rotated image of the hand movement.

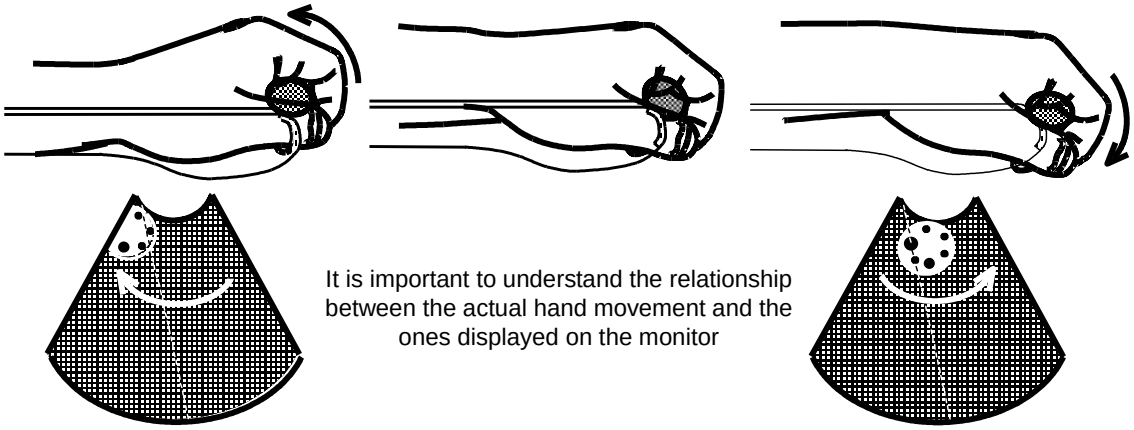
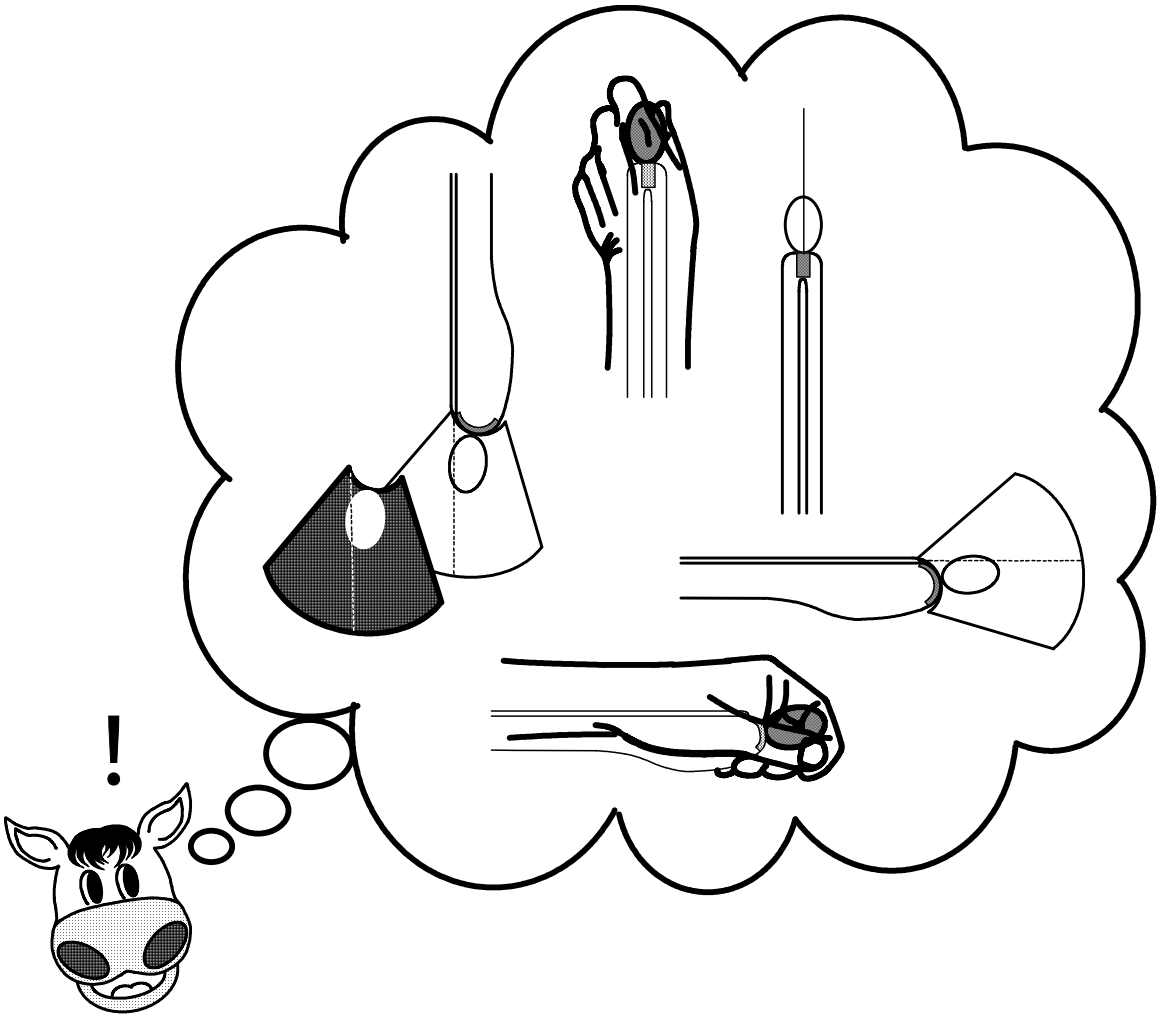


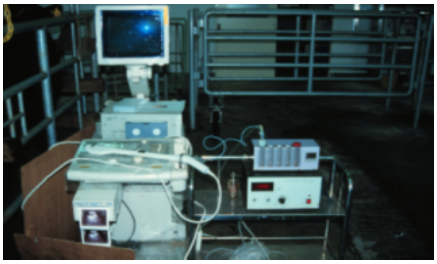
Fig. 47. Hand movement and how it is displayed on the monitor



[Practical side of OPU]

1. Appliance and equipment

Ultrasound diagnostic equipment: SSD-1200 (ALOKA)
Electronic convex probe for veterinary use:
UST-9109P-7.5 (ALOKA)
Oocyte collection needle guide: (FHK)
Oocyte collection needle: COVA Needle (Misawa Medical Industry Co., Ltd)
Aspirator: model FV4 (FHK)
Thermostatic device: model FV5 (FHK)
Oocyte collection bottle: 50 ml centrifuge tube



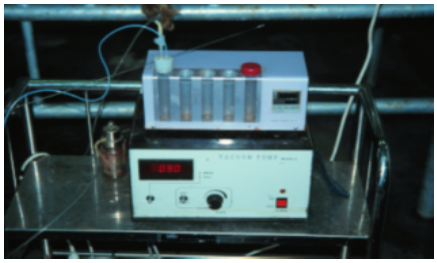
Pic. 9. Ultrasound diagnostic equipment and peripheral devices

In addition to the list above, the following appliances and equipment are also commonly used: ultrasound diagnostic equipment and probe by HITACHI and Honda Electronics and oocyte collection needles and aspirator by FHK and COOK.

Pic. 10. Transvaginal probe

2. Oocyte preservation solution

Using 1% CS-containing lactated Ringer's solution (the same solution as used for ovum aspiration from a slaughterhouse-derived ovary) as a basic culture solution, 2 types of ovum preservation solution should be prepared: an oocyte aspiration solution for OPU and an oocyte search solution for searching for collected oocytes. The difference between these 2 solutions is whether or not heparin has been added to prevent blood coagulation. For OPU a heparinized oocyte aspiration solution is used (page 49).



Pic. 11. Aspirator and thermostatic device

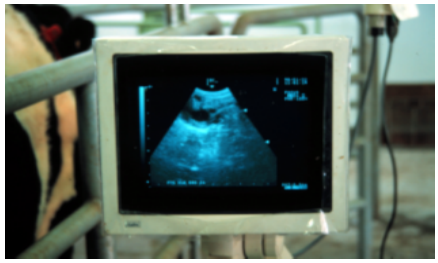
3. Procedure for OPU

1) Stabilize the position of the Donor cow, remove feces from the rectum and administer caudal epidural anesthesia to the cow. Clean and disinfect the tail area and the vulva first with Isodine® solution (10% povidone-iodine), then with Osban® solution (Benzalkonium Chloride) and finally with ethanol cotton. For a difficult or stressed cow, a sedative (such as Selactar®) can be administered.

Pic. 12. Probe insertion in the vagina

2) Insert a probe mounted with an oocyte collection needle guide and lead it to the vaginal wall at the top of the cervix.

3) Insert a hand into the rectum and manually lead the ovary into the vicinity of the probe tip. Then display the ovary on the monitor of the ultrasound diagnostic equipment (Holding method A).



Pic. 13. Ultrasound diagnostic image

4) While watching the ultrasound image, check the number of follicles larger than 2 mm in the ovary (estimated number of follicles). To achieve this, move the ovary slowly from one end (the point at which the ovarian image disappears from the monitor) to the other while counting the follicles; these appear successively on the monitor. Make sure to move the ovary slowly in order to avoid miscounting.

5) Aspirate approx. 5 ml of an oocyte aspiration solution into an oocyte collection bottle. During this procedure, the oocyte collection needle and the connecting tube should be filled with the solution. Keep the solution still by closing the tube (Fig. 48), by using the stopper of the infusion tube, or by bending the connecting tube, etc.

6) Insert the oocyte collection needle into the needle guide. Use this needle to puncture the vaginal wall.

7) Turn the aspirator on. Keep the aspirator on when the needle tip has penetrated the vaginal wall and is in the peritoneal cavity. This will maintain negative pressure inside the needle, the connecting tube, and the oocyte collection bottle.

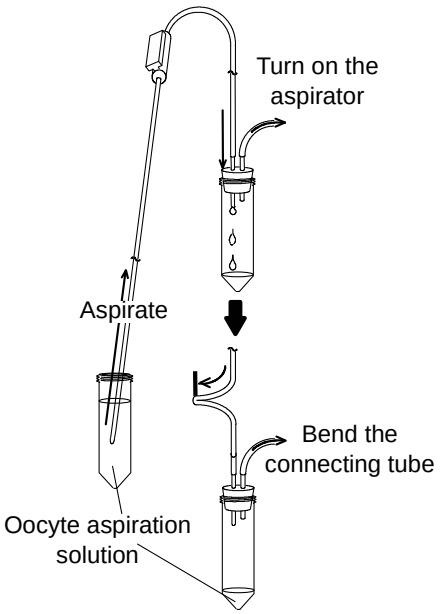


Fig. 48. Aspiration and containment of the aspiration solution

8) Move the ovary in order to position the follicle in line with the guideline displayed on the ultrasound image.

9) Slide the oocyte collection needle to insert it into the follicle and aspirate the follicular fluid.

10) The OPU assistant will need to keep an eye on the flow of the aspiration solution in the connecting tube while watching the ultrasound image. If the size of the follicle does not change after being punctured or if the solution in the tube is not flowing, there could possibly be a blockage in the needle or the tube caused by blood coagulation, etc. If this occurs, increase the aspiration pressure and proceed with the collection from the follicle. If this does not resolve the situation, forcibly irrigate the oocyte collection needle with the oocyte aspiration solution using an injection syringe with a 19G needle (a 19G needle is small enough to fit into a 17G oocyte collection needle as it is smaller than the inner diameter of the 17G needle).

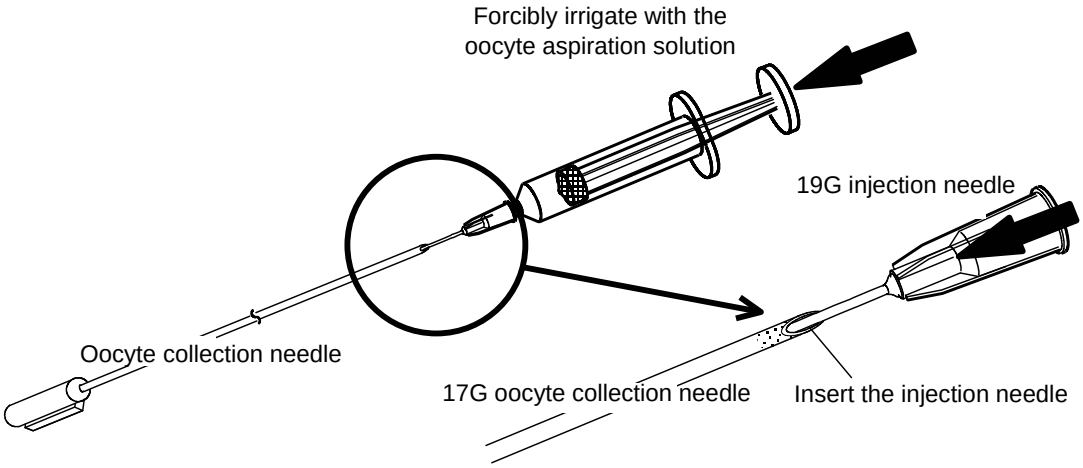


Fig. 49. How to deal with blood coagulation

11) When the aspiration is complete and approx. 10 oocytes have been collected, withdraw the oocyte collection needle from the needle guide. In order to avoid blood coagulation, irrigate the inside of the oocyte collection needle and the connecting tube with the oocyte aspiration solution. Then resume oocyte aspiration following the same procedure. The assistant needs to slightly agitate the oocyte collection bottle to mix the blood and the oocyte aspiration solution.

12) Once all the follicles (oocytes) have been aspirated, once again irrigate the needle and the tube with the oocyte aspiration solution.

4. Filtration of the collected fluid

1) The collected fluid will be red as a result of blood being mixed in with the oocytes. This makes searching for the oocytes under a microscope difficult. Therefore, transfer the collected fluid to an Emcon filter to filter the blood.

2) Continue to pour oocyte search solution (or oocyte aspiration solution) into the filter until the red color of the blood disappears and the collected fluid becomes transparent (Fig. 50).

3) If it appears that a blood clot (tubifex-like) has formed in the needle or in the tube, mix and break up the clot with a graduated pipette to make the solution transparent.

4) During the process of breaking up a large clot, the pipetting action could become too powerful and may denude the cumulus cells off the oocyte present in the filter. In such a case, transfer the large clot to a container such as beaker, centrifuge tube, etc. for further pipetting (Fig. 51, 52).

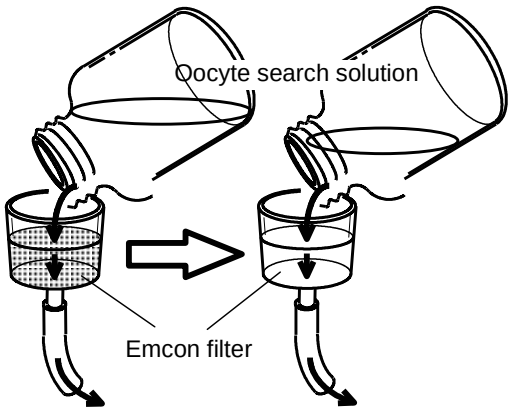
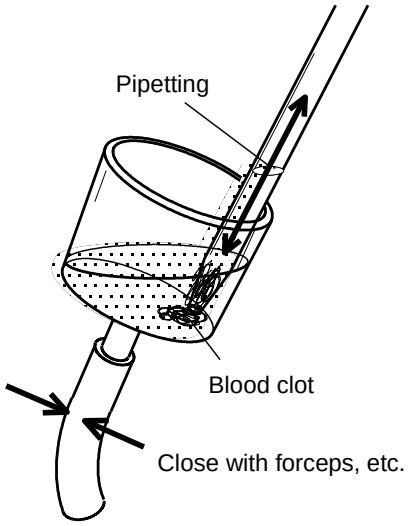


Fig. 50. Filtrating the collected fluid



Fig, 51. Pipetting clotted blood in the filter

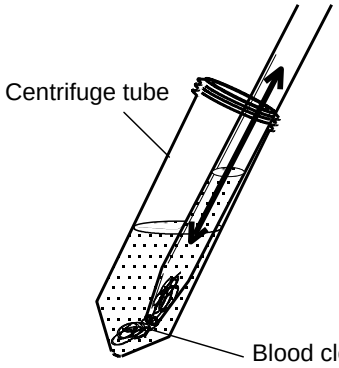


Fig. 52. Pipetting clotted blood in a centrifuge tube

5) Decant the fluid obtained from the pipetting in step 4) into the Emcon filter. There is a possibility that filtering the solution one time will not be enough to make it transparent. If this is the case, repeat the filtration process several more times. Transfer the clotted blood to a 90 mm petri dish in order to search for the oocyte once the solution becomes transparent.

6) Once the solution becomes transparent, repeat the following process 2 or 3 times: slightly agitate the fluid in the filter → transfer the fluid to the 90 mm petri dish → add more oocyte search solution to the filter. The reason for this process is to avoid the denuding of the cumulus cells from the oocytes. This could happen when any oocytes remaining on the filter surface are collected by pipetting.

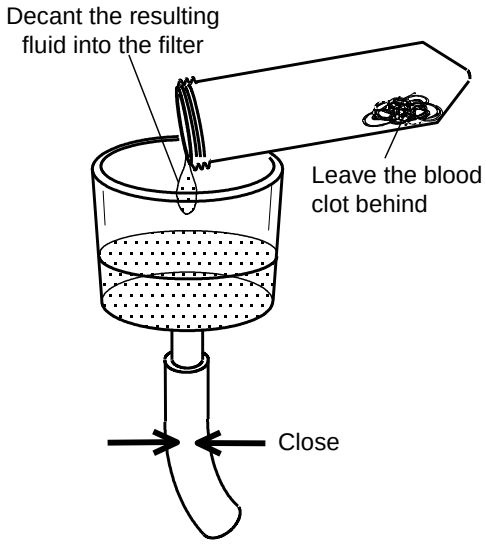


Fig. 53. Collecting the solution into the

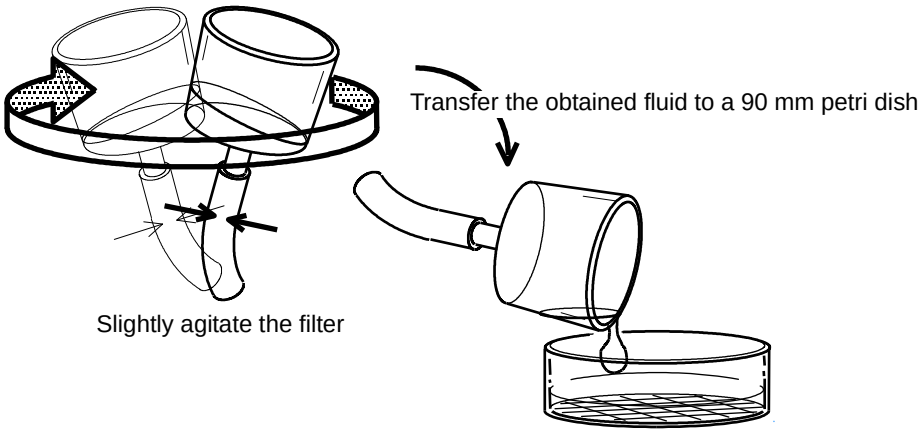


Fig. 54. Oocyte collection through filter agitation

7) With a graduated pipette, remove the mucosal fluid as well as any cells adhering to the filter surface and once again transfer these to a Petri dish. Repeat this procedure until nothing remains on the filter surface.

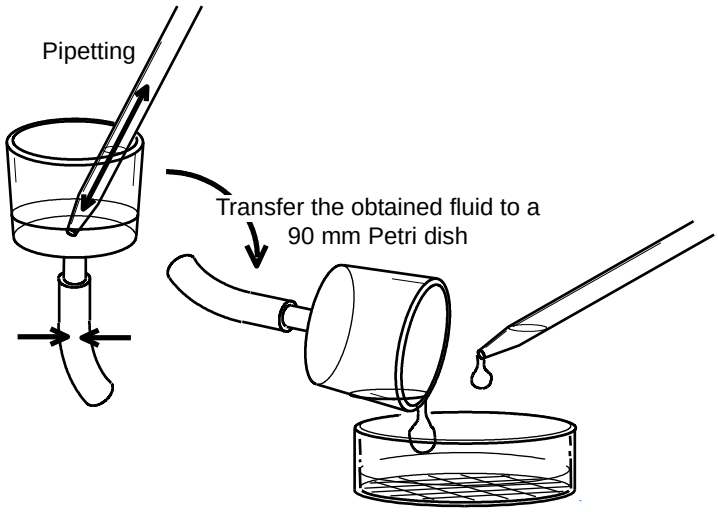


Fig. 55. Oocyte collection by pipetting in the filter

8) Search for the collected oocyte under a stereomicroscope.

*** Since the collection of oocyte via pipetting tends to cause denude of cumulus cells, the agitation collection method should be used as a first choice. If the pipetting method is used, pipetting should be done gently and with as few repetitions as possible. If the oocyte has lost some of their cumulus cells, their subsequent maturation culture will be affected.**

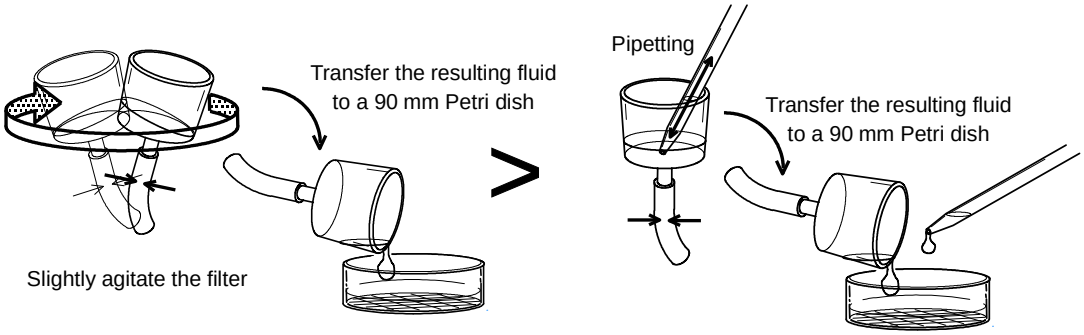


Fig. 56. Comparison of the agitation method and the pipetting method

5. Oocyte search

1) Basically, the same procedure is used as that used in the search for oocyte aspirated from carcass-derived ovaries. However, the following method is applied during OPU as it is highly likely that the collected fluid will contain clotted blood.

2) Since the collected oocytes are often covered with clotted blood, the oocytes need to be searched for in such a way that the clot is broken up with a glass rod (or with a searching rod fabricated by heating and rounding the tip of a Pasteur pipette, etc.)

3) After the Petri dish has been searched once, break up the clots and stir. Then begin the second round of searching. Even technicians who are familiar with searching for oocytes in carcass-derived ovaries could overlook some oocytes. It is therefore recommended that the oocytes search be conducted more than 3 times.

4) If there are multiple clots or if the clots are diffuse on the bottom of the petri dish, transfer the clots to another Petri dish (a 90 mm or 60 mm Petri dish) for an individual oocyte search (Fig. 57).

5) In both cases, the oocyte search solution could turn red again during the agitation of the clots or the clots could be too entwined to break up. In such cases, transfer the clots to the Emcon filter again for collection via a pipette.

6) Repeat the oocyte search using the same procedure as above.

7) Use the utmost effort to avoid making a mistake during the search, even with one ovum, as the estimated transmission ability of the donor cows assigned to OPU is generally high.

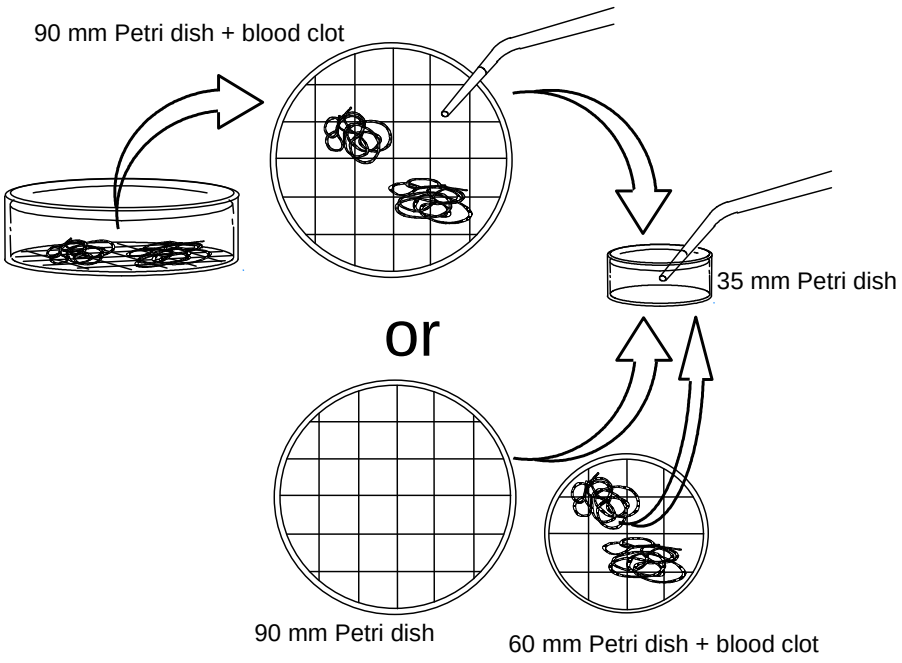


Fig. 57. Oocyte search in the presence of a blood clot

Preparation of Solutions

[Ovum pick up – maturation culture]

⊙ Physiological saline (1 liter): For transporting ovaries

Sodium chloride	NaCl	9.0 g

1) Stir and dissolve NaCl (9.0 g) in ultrapure water and make up the volume to 1 liters.
2) Cover the mouth of a glass bottle containing the solution with aluminum foil and parchment paper, seal tightly with a hemp thread.
3) Sterilize the solution in an autoclave (121°C, 15 minutes).

⊙ Lactated Ringer's solution containing 1% CS (1 liter): For searching collected oocytes

Lactated Ringer's solution	1000	ml
Calf serum (CS)	10 - 10.1	ml
Antibiotics (PC-SM) (See page 27.)	1000	μl

1) Put 10 - 10.1 ml of CS into 1000 ml of commercial lactated Ringer's solution, add 1000 μl of antibiotics, and mix by inverting.
2) Refrigerate the solution if it is to be preserved (for about 1 week).

⊙ 10 U/ml heparinized lactated Ringer's solution containing 1% CS (1 liter): liquid used for ovum pick up (OPU)

Lactated Ringer's solution containing 1% CS	100	ml
Heparin（Novo Heparin 1000U/ml）	1	ml

- Add 0.1 ml of Novo Heparin to 100 ml of the above-mentioned lactated Ringer's solution. The volume to be prepared will depend on the number of animals to be treated.

⊙ 25 mM Hepes buffered TCM-199 + 5% CS (100 ml): For maturation culturing

25 mM Hepes buffered TCM-199	95.0	ml
CS	5.0	ml
Antibiotics (PC-SM)	100	µl

1) Place 95.0 ml of 25 mM Hepes buffered TCM-199 and 5.0 ml CS in a measuring cylinder, add 100 µl antibiotic, cover with Parafilm and mix by inverting the cylinder several times.
2) Sterilize the medium by filtration using a 0.22 µm filter and refrigerate (for about 1 week).

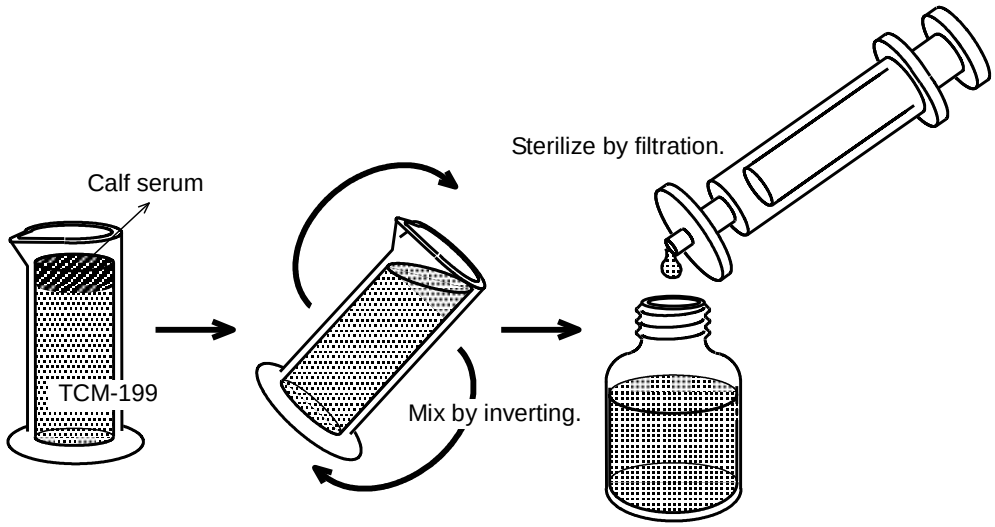


Fig. 58. Preparation of 25 mM Hepes buffered TCM-199 + 5% CS

[Sperm treatment – insemination]

1. Preparation of Percoll solution

After frozen sperm has been thawed, 90% Percoll solution is used to select surviving sperm. Here, a 10X concentration of sperm TL is prepared for diluting with Percoll liquid (100% Percoll).

⊙ 10X concentration sperm TL (100 ml): Percoll diluting solution

Sodium chloride	NaCl	4.675	g
Potassium chloride	KCl	0.23	g
Sodium dihydrogen phosphate (anhydrous)	NaH_2PO_4	0.035	g
Hepes		2.38	g

1) Pour about 50 ml of ultrapure water into a beaker and dissolve all contents above.
2) Add NaOH to the solution to take the pH to 7.3; measure using a pH meter.
   * Because of its very low pH level, the original solution is prepared to make nearly half of the intended volume and is then adjusted to take the pH level to 7.3 by adding NaOH.
3) Transfer the solution to a graduated cylinder and add ultrapure water to make a total volume of 100 ml.

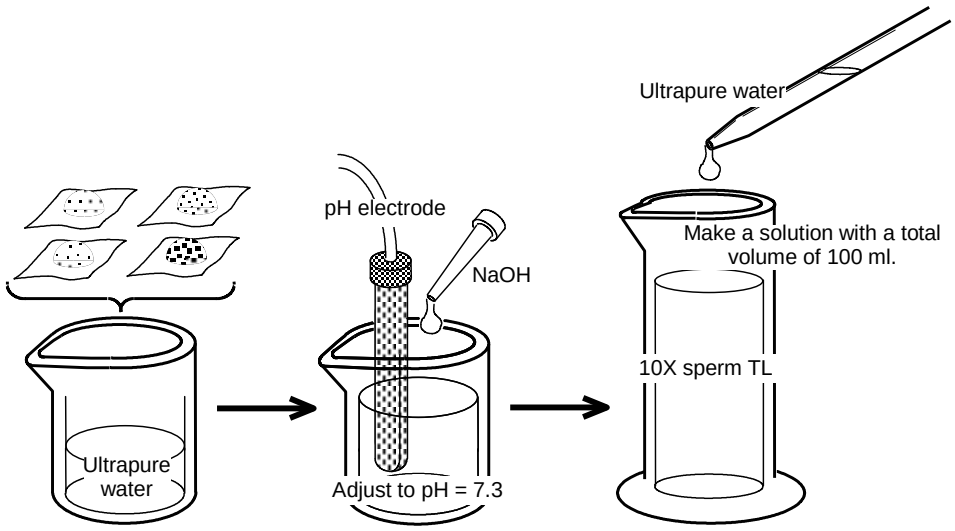


Fig. 59. Preparation of 10X concentration sperm TL

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⊙ 90% Percoll solution (50 ml): Solution for selecting surviving sperm

a)	Percoll		45	ml
b)	10X sperm TL		5	ml
c)	1M calcium chloride aqueous solution	$CaCl_2$	98.5	µl
d)	0.1M magnesium chloride aqueous solution (hexahydrate)	$MgCl_2 \cdot 6H_2O$	197	µl
e)	DL-lactic acid (syrup)		184	µl
f)	Sodium bicarbonate	$NaHCO_3$	0.1045	g
g)	0.5% phenol red		10	µl
	* c) 1M $CaCl_2$: 1.1098 g /10 ml			
	1M $CaCl_2 \cdot 2H_2O$: 1.4701 g /10 ml			
	d) 0.1M $MgCl_2 \cdot 6H_2O$: 0.2985 g /10 ml			

1) Mix chemicals a) and b) to make a 50 ml solution.
2) Add chemicals c) to g) to the solution and mix by inverting.
3) Sterilize the solution by filtration using a 0.22 µm filter and refrigerate.

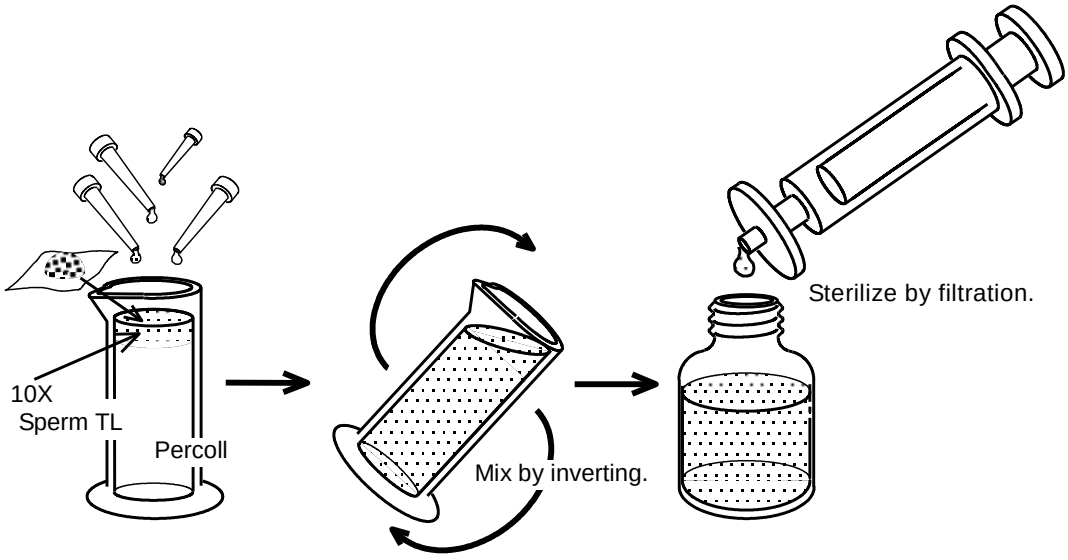


Fig. 60. Preparation of 90% Percoll solution

2. Preparation of Brackett and Oliphant (BO) solution

Keep a stock solution (BO-A solution and BO-B solution) ready and prepare as needed.

⊙ BO-A solution (500 ml): Stock solution for BO solution preparation

	Sodium chloride	NaCl	4.3092	g
	Potassium chloride	KCl	0.1974	g
*	Calcium chloride dihydrate	$CaCl_2 \cdot 2H_2O$	0.2171	g
	(Calcium chloride anhydride:	$CaCl_2$ 0.1639 g)		
	Sodium dihydrogen phosphate dihydrate	$NaH_2PO_4 \cdot 2H_2O$	0.0840	g
	(Sodium dihydrogen phosphate anhydride:	NaH_2PO_4 0.0646 g)		
*	Magnesium chloride hexahydrate	$MgCl_2 \cdot 6H_2O$	0.0697	g
	0.5% phenol red		100	µl

1) Dissolve the above components in ultrapure water sequentially and make up the volume to 500 ml using volumetric flask.
2) Sterilize the solution by filtration using a 0.22 µm filter and refrigerate (for about 1 month).

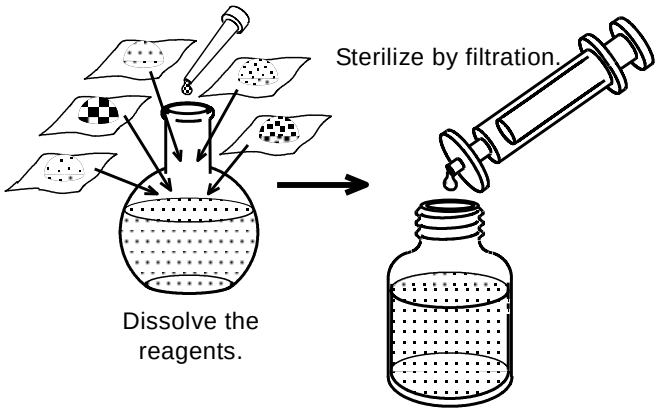


Fig. 61. Preparation of BO-A solution

*** Calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$) and magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) are highly hygroscopic. So, weigh and prepare in the following method:**

1) Weigh $CaCl_2 \cdot 2H_2O$ or $MgCl_2 \cdot 6H_2O$ in a weighing dish.
2) Dissolve the reagent in ultrapure water in the weighing dish and pour the solution into a volumetric flask.
3) Repeat step 2) several times.

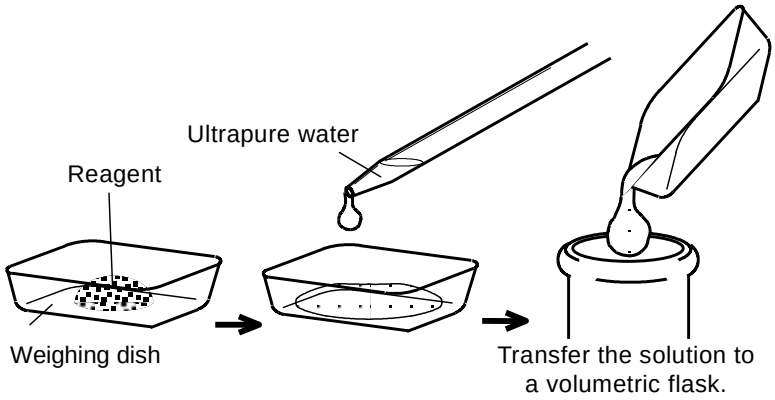


Fig. 62. Weighing in a weighing dish

If the volume of each reagent is too small, or if it is so hyposcopic that the scale of measurement increases within a few seconds, the reagent can be weighed and used in the following method:

1) Weigh a 10-fold weight of each reagent in a weighing dish.
2) Dissolve the reagent by adding drops of ultrapure water.
3) Measure the solution using a measuring pipette.
4) Add a 1/10 volume of the solution to the solution to be prepared (in a volumetric flask).

*** In step 1), the volume may be 2 to 5 fold. In step 3), a graduated cylinder or similar container may be used. If this method is used for the BO-A solution, each reagent can be weighed by adding about 10 ml of ultrapure water to measure with a measuring pipette.**

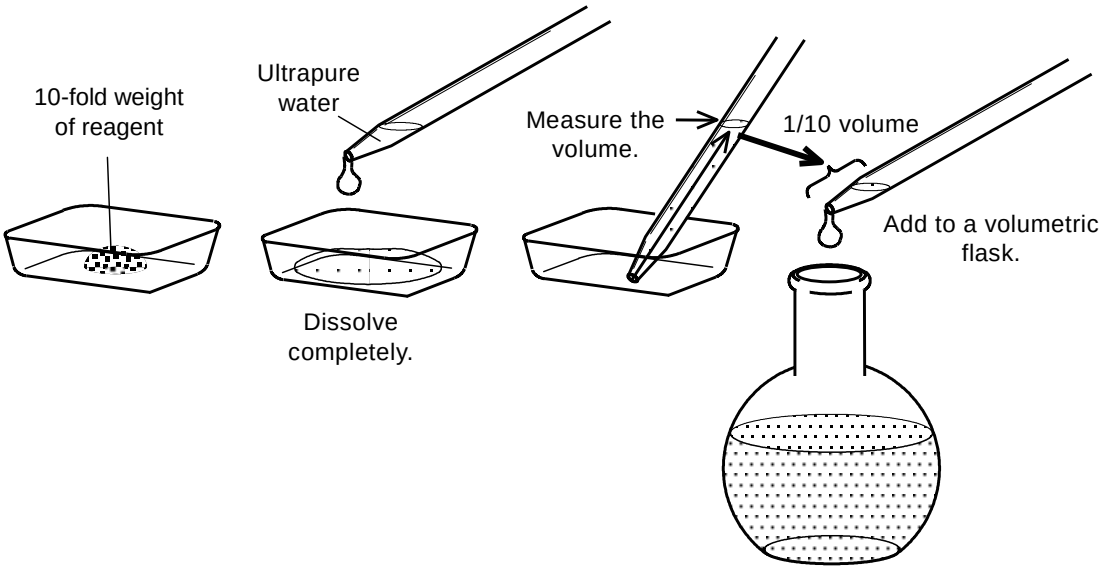


Fig. 63. Measurement method for using a 10-fold weight of reagent

⊙ BO-B solution (200 ml): Stock solution for BO solution preparation

Sodium bicarbonate	$NaHCO_3$	2.5873	g
0.5% phenol red		40	µl

1) Dissolve the above components in ultrapure water and make up the volume to 200 ml using volumetric flask.
2) Pass CO_2 gas directly through the solution until its color changes to neutral between light red and light yellow.
3) Sterilize the solution by filtration using a 0.22 µm filter and refrigerate (for about 1 month).

Phenol red is an indicator of the hydrogen ion concentration (pH). It gives a light yellow to light red color in the pH range 6.4-8.2. Blowing in of CO_2 gas makes the solution acidic and changes the color from light red to yellow.

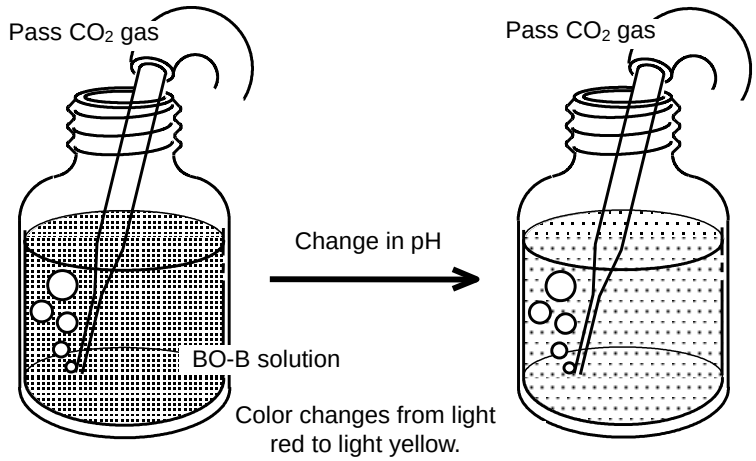


Fig. 64. Passing CO_2 gas into BO-B solution

*** Although the solution's pH is adjusted during the preparation of the stock solution by passing CO_2 gas, the color can be changed back to the original color during chilled storage (changes along with the air in the container). Thus aeration with CO_2 gas should be performed again during the preparation of the BO solution mentioned below.**

⊙ BO solution (100 ml): Basal medium for preparing sperm washing solution, sperm diluting solution and oocyte washing solution.

Prepare on the day of use or the previous day.

Sodium pyruvate	0.01375	g
BO-A solution	76	ml (dilutor)
BO-B solution	24	ml (dilutor)
Antibiotics (PC-SM) (See page 27.)	100	µl

1) Measure out 0.01375 g of sodium pyruvate and place in a 100 ml measuring cylinder.
2) Make up volume to 76 ml with BO-A solution and fully dissolve the sodium pyruvate.
3) Pass CO_2 into BO-B solution until it turns light yellow.
4) Add 24 ml of BO-B solution to the solution prepared in <3> to make up the volume to 100 ml.
5) Add 100 µl of antibiotics.
6) Mix the solution by inverting the container several times and then prepare the sperm treatment solutions (sperm washing solution, sperm diluting solution and oocyte washing solution) as detailed below (page 58, Figure 65).

3. Preparation of sperm treatment solutions (sperm washing solution, sperm diluting solution, and oocyte washing solution)

The following culture solutions are prepared with BO medium 100 ml.

⊙ Sperm washing solution (10 ml): For sperm washing after Percoll treatment

BO medium + 10 mM hypotaurine + 10 u/ml heparin

BO medium	10	ml
Hypotaurine	0.01091	g
Heparin (Novo Heparin 1000U/ml)	100	μl

1) Dissolve the above components sequentially and mix the solution by inverting it.
2) Sterilize the solution by filtration using a 0.22 μm filter. Then gas-eqilibrate until used by leaving a CO_2 incubator with the cap of the container slightly loosened.

⊙ Sperm diluting solution (10 ml): For use during the second dilution

BO solution + 20 mg/ml BSA

BO solution	10	ml
BSA (crystallized and lyophilized)	0.2	g

1) Take the BO solution in a beaker or similar container, add BSA and leave standing to dissolve the BSA.
2) Sterilize the solution by filtration using a 0.22 μm filter. Then gas-eqilibrate until used by leaving a CO_2 incubator with the cap of the container slightly loosened.

⊙ Oocyte washing solution (50 ml): For washing mature oocytes

BO solution + 10 mg/ml BSA

BO solution	50	ml
BSA (crystallized and lyophilized)	0.5	g

1) Take the BO solution in a beaker or similar container, add BSA and leave standing to dissolve the BSA.
2) Sterilize the solution by filtration using a 0.22 μm filter.
3) Prepare fertilization drops in 35 mm Petri dishes, dispense the remainder 35 mm Petri dishes, and gas-equilibrate both in a CO_2 incubators.

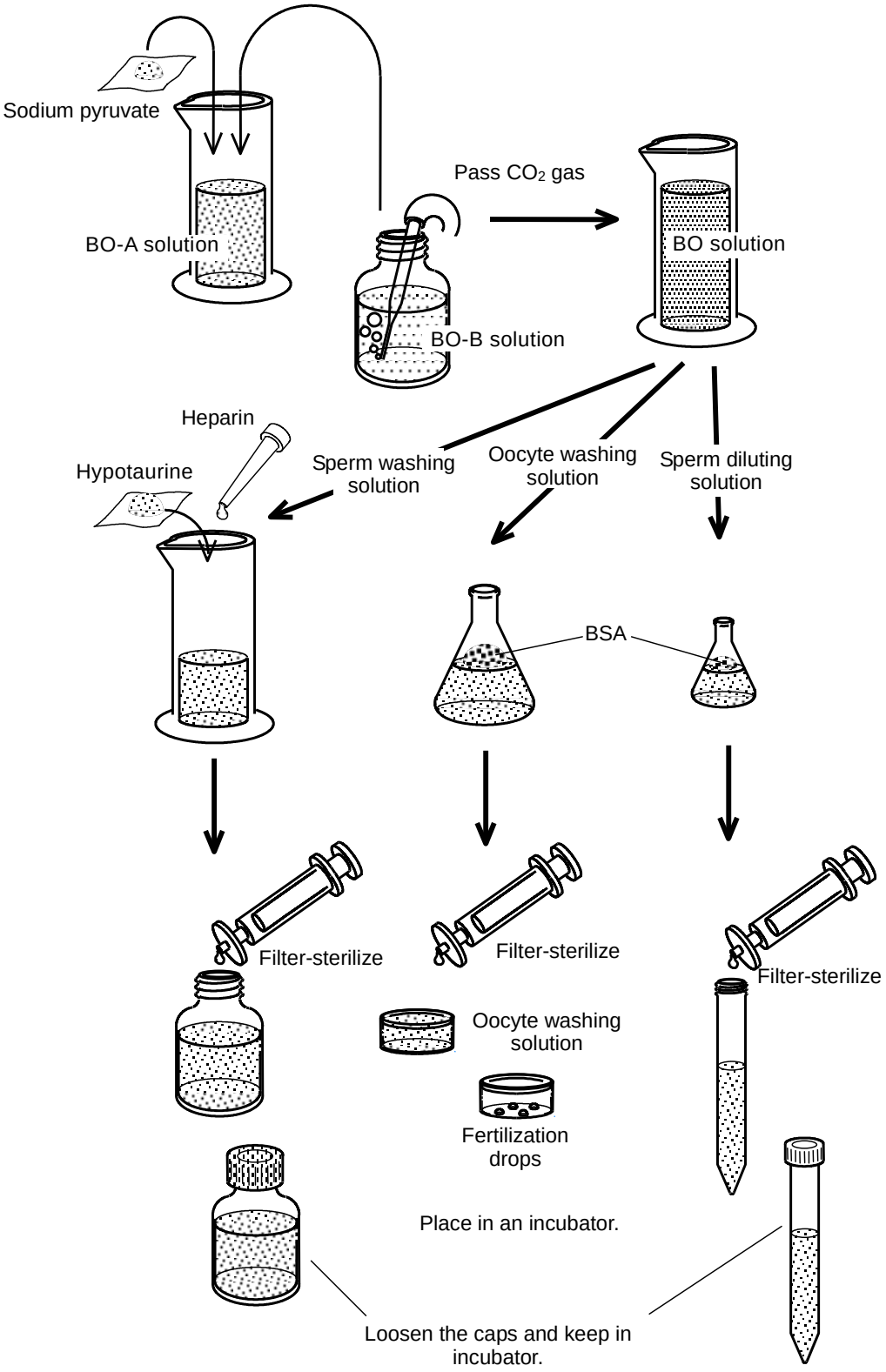


Fig. 65. Preparation of sperm treatment solutions

[In vitro culture]

1. Preparation of CR1aa: Basal culture medium for embryo culturing (use after adding CS and LAA)

   The stock solution (CR1-A solution and CR1-B solution) is prepared in advance.

⊙ CR1-A solution (760 ml): Stock solution for CR1aa preparation

Sodium chloride	NaCl	6.7031	g
Potassium chloride	KCl	0.2311	g
Sodium pyruvate		0.0440	g
Sodium bicarbonate	$NaHCO_3$	2.2011	g
0.5% phenol red		2.0	ml

1) Dissolve the above components in ultrapure water and make up the volume to 760 ml using measuring cylinder.
2) Sterilize the solution by filtration using a 0.22 μm filter and refrigerate (for about 1 month).

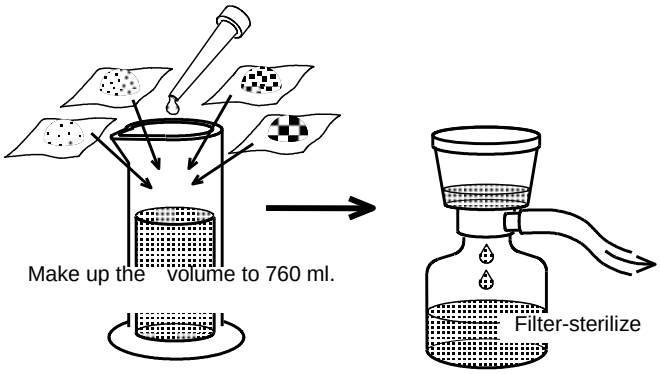


Fig. 66. Preparation of CR1-A solution

⊙ CR1-B solution (200 ml): Stock solution for CR1aa preparation

L(+)-Lactic acid hemicalcium salt	0.5996 g

1) Dissolve the above reagent in ultrapure water and make up the volume to 200 ml using volumetric flask.
2) Sterilize the solution by filtration using a 0.22 μm filter and refrigerate (for about 1 month).

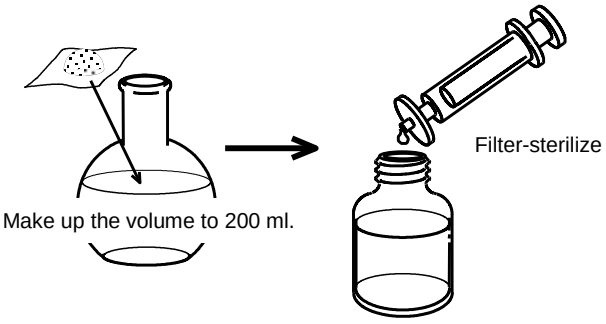


Fig. 67. Preparation of CR1-B solution

⊙ CR1aa (100 ml): Basal culture medium for embryo culturing (use after adding CS and LAA)

a)	CR1-A solution	76	ml
b)	CR1-B solution	20	ml
c)	BME essential amino acids (X50)	2	ml
d)	MEM nonessential amino acids (X100)	1	ml
e)	L-glutamic acid (Dissolve 1 ampule (20 mg) in ultrapure water (10 ml))	1	ml
f)	BSA (fatty acid-free)	0.3	g
g)	Antibiotics (PC-SM) (See page 27)	100	µl

1) Add each solution a) to e) to a container such as beaker and mix the solution using a measuring pipette.
2) Add BSA (fatty acid-free) and leave standing to dissolve.
3) Add antibiotics (PC-SM) and mix.
4) If the medium used has serum added, follow the preparation method for CR1aa + 5% CS.
5) If the medium is used without serum, sterilize the solution by filtration using a 0.22 µm filter and refrigerate (for about 1 week).

⊙ CR1aa + 5% CS (105.26 ml): In vitro culture solution

CR1aa	100	ml
CS	5.26	ml

1) Add 5.26 ml of CS to 100 ml of CR1aa, and mix by inverting.
2) Sterilize the solution by filtration using a 0.22 μm filter and refrigerate (for about 1 week).

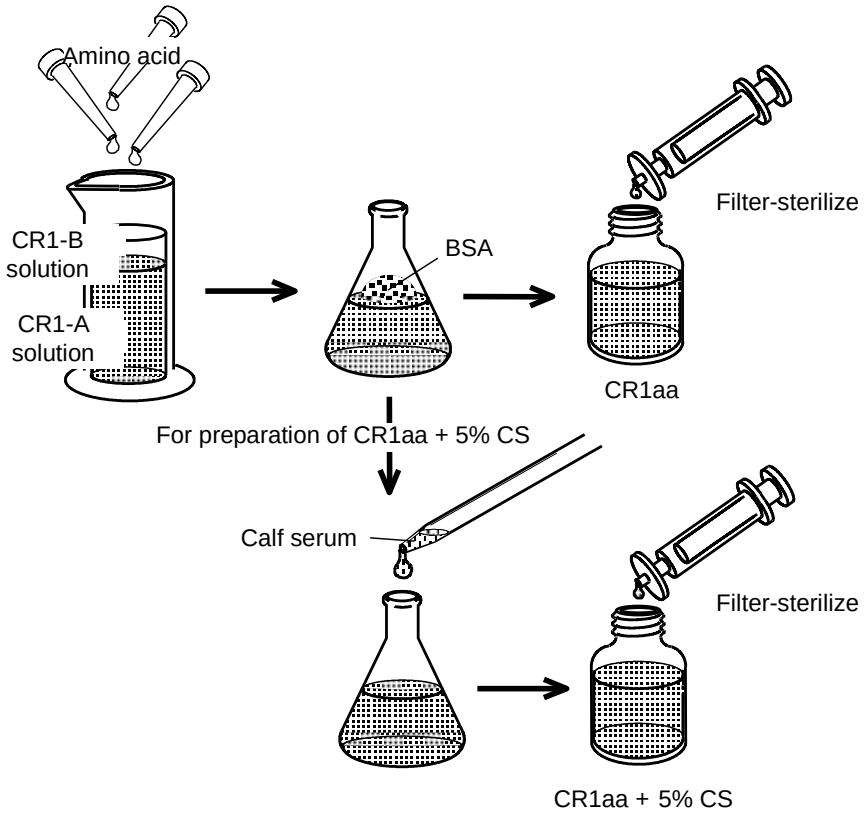


Fig. 68. Preparation of CR1aa and CR1aa + 5% CS

2. Linoleic acid albumin (LAA): For improving the cryotorelance of embryos when CR1aa + 5% CS is used as the embryo culture medium

⊙ 50 mg/ml LAA stock solution (10 ml): Stock solution to be added to CR1aa containing 5% CS

Add 10 ml of CR1-A solution to a bottle containing LAA (500 mg), and dissolve completely (for about 1 month).

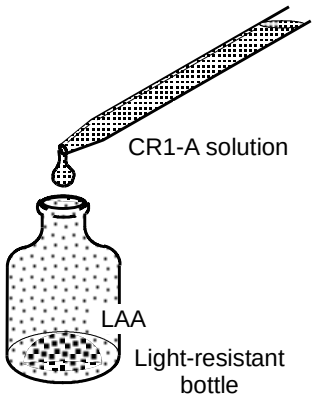


Fig. 69. Preparation of LAA stock solution

⊙ 0.25 mg/ml LAA + CR1aa containing 5% CS (10 ml): In vitro culture solution with enhanced freezing tolerance

1) Remove 50 µl from 10 ml CR1aa containing 5% CS using a micropipette.
2) Add 50 µl of LAA stock solution and mix by inverting.
3) Sterilize the solution by filtration using a 0.22 µm filter and refrigerate (for about 1 week).

*** If used infrequently, LAA may be weighed out and added directly to the culture medium to make its concentration in the culture medium 0.25 mg/ml.**

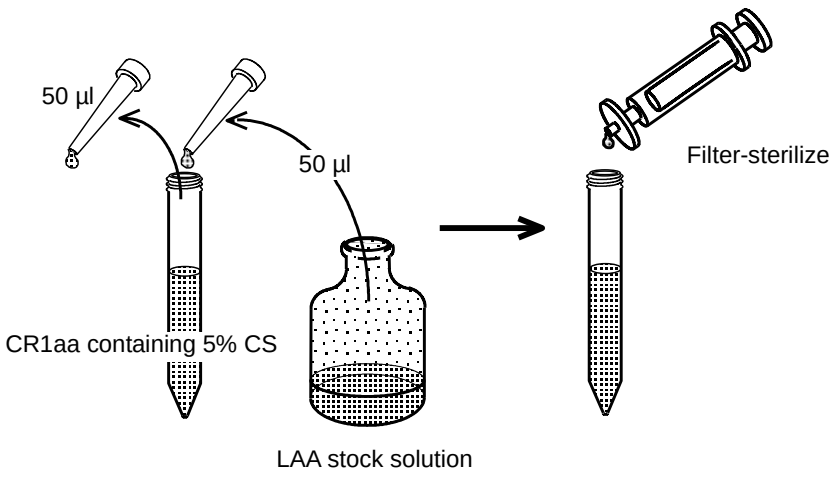


Fig. 70. Preparation of 0.25 mg/ml LAA + CR1aa + 5% CS

[Freezing and thawing of in vitro fertilized embryo]

1. Preparation of modified Dulbecco's PBS

Prepare D-PBS by mixing PBS-A solution and PBS-B solution, and add sodium pyruvate and glucose to the solution. Although modified D-PBS generally contains BSA, this manual suggests that it not be added in order to extend the shelf life of the solution.

⊙ PBS-A solution (approx. 700 ml): D-PBS(-)

Sodium chloride	NaCl	8.0	g
Potassium chloride	KCl	0.2	g
Sodium hydrogen phosphate (anhydrous)	Na_2HPO_4	1.15	g
Potassium dihydrogen phosphate (anhydrous)	KH_2PO_4	0.2	g

- Dissolve the above components ultraqure water and make up the volume to approx. 700 ml using a liter volumetric flask.
- PBS(-) readymade powder and tablets are commercially available.

  *** A magnetic stirrer should be used for dissolving.**

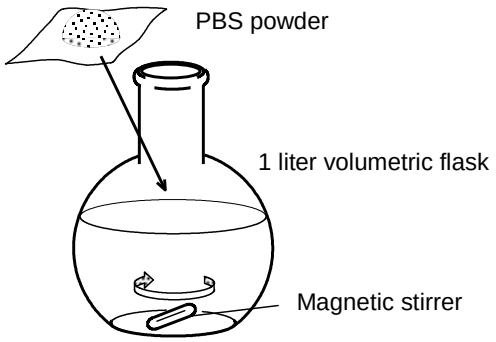


Fig. 71. Preparation of PBS-A solution

⊙ PBS-B solution: Dilution of metal salts stock solution

The stock solution of metal salts should be prepared in advance and should be diluted to make PBS-B solution; this is then mixed with PBS-A solution to make Dulbecco's PBS.

1) Stock solution of metal salts (100 ml)

Calcium chloride anhydride	$CaCl_2$	1.0 g
(Calcium chloride dihydrate:	$CaCl_2 \cdot 2H_2O$ 1.1395 g)	
Magnesium chloride (hexahydrate)	$MgCl_2 \cdot 6H_2O$	1.0 g

1) Dissolve the above components in ultrapure water and make up the volume to 100 ml.
2) Sterilize the solution by filtration using a 0.22 μm filter and refrigerate (for about 1 month).

2) PBS-B solution (approx. 100 ml)

Dilute 10 ml stock solution of metal salts with approx. 100 ml of ultrapure water to make PBS-B liquid.

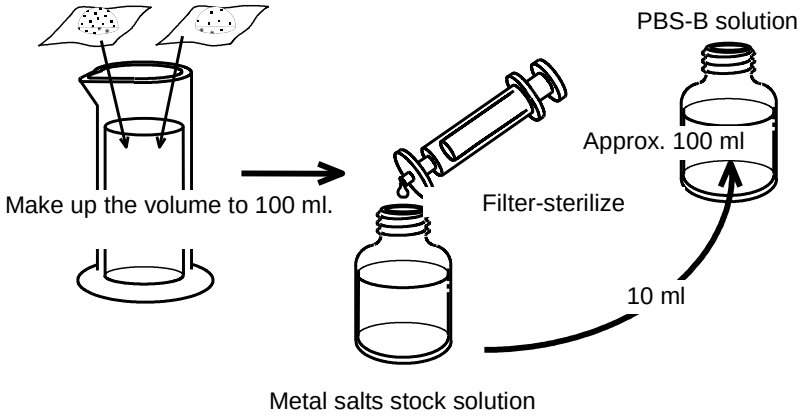


Fig. 72. Preparation of PBS-B solution

⊙ Modified Dulbecco's PBS: D-PBS + sodium pyruvate + glucose (1 liter):

Basal culture medium for use after thawing and implantation

PBS-A solution	Approx. 700	ml
PBS-B solution	Approx. 100	ml
Glucose	1.0	g
Sodium pyruvate	0.036	g

1) Add solution B to solution A while stirring, slowly, in 4-5 portions, to prepare D-PBS(+).
2) Add glucose and sodium pyruvate to approx. 800 ml of D-PBS(+), dissolve completely, and make up the volume to 1 liter.
3) Sterilize the solution by filtration using a 0.22 μm filter and refrigerate (for about 1 month).

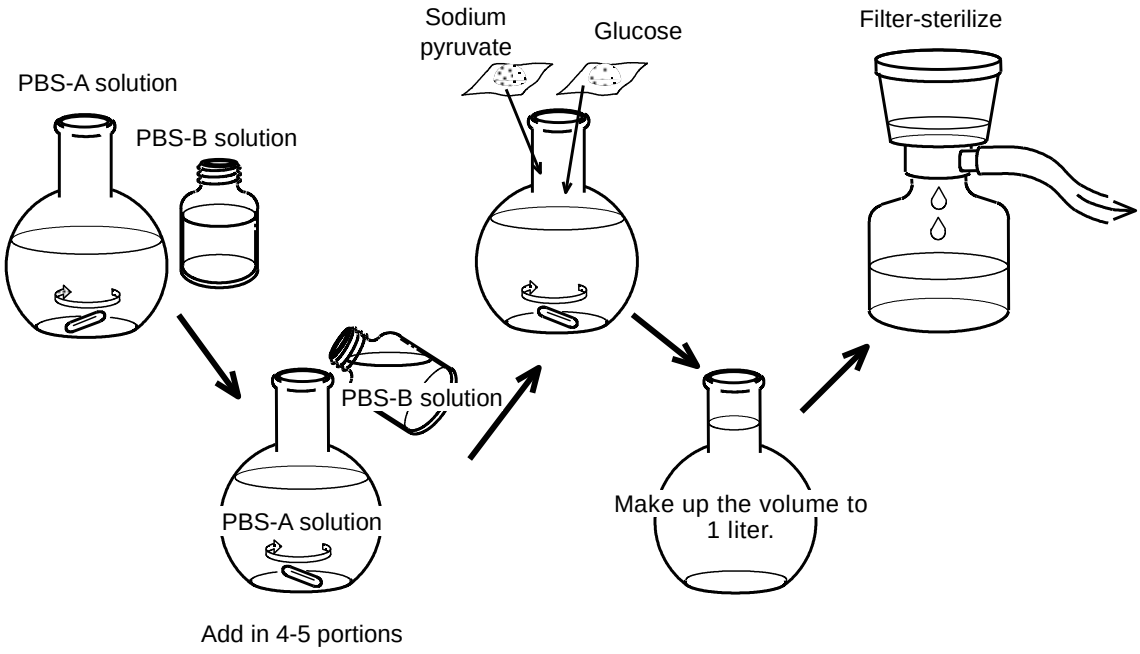


Fig. 73. Preparation of modified Dulbecco's PBS

2. Preparation of cryopreservation medium

⊙ Modified D-PBS + 20% FCS (10 ml): Basal medium used for cryopreservation of IVF embryos

a)	Modified D-PBS (m-PBS)	8	ml
b)	FCS	2	ml
c)	BSA (fraction V)	0.04	g
d)	Antibiotics (PC-SM)	10	µl

1) Add each liquid a) and b) to a container such as a beaker and mix the solution using a measuring pipette.
2) Add BSA (fraction V) and leave standing to allow it to dissolve.
3) Add antibiotics (PC-SM) and mix.
4) If preparing the cryopreservation medium, follow the preparation method for 1.5M EG + 0.1M Suc + m-PBS + 20% FCS.
5) If no cryopreservation medium is to be prepared, sterilize the solution by filtration using a 0.22 µm filter and refrigerate (for about 1 week).

⊙ 1.5M ethylene glycol + 0.1M sucrose + m-PBS + 20% FCS (10 ml): Cryopreservation medium

Sucrose (Suc)	0.3423	g
Ethylene glycol (EG)	0.83	ml
m-PBS containing 20% FCS	10	ml (dilutor)

1) Place Suc in a measuring cylinder.
2) Add 5 – 6 ml of m-PBS + 20% FCS and dissolve Suc completely.
3) Add 0.83 ml of EG to the solution.
4) Add PBS + 20% FCS and make up the volume to 10 ml.
5) Seal the measuring cylinder with Parafilm and mix contents by inverting several times.
6) Sterilize the solution by filtration using a 0.22 µm filter and refrigerate (for about 1 week).

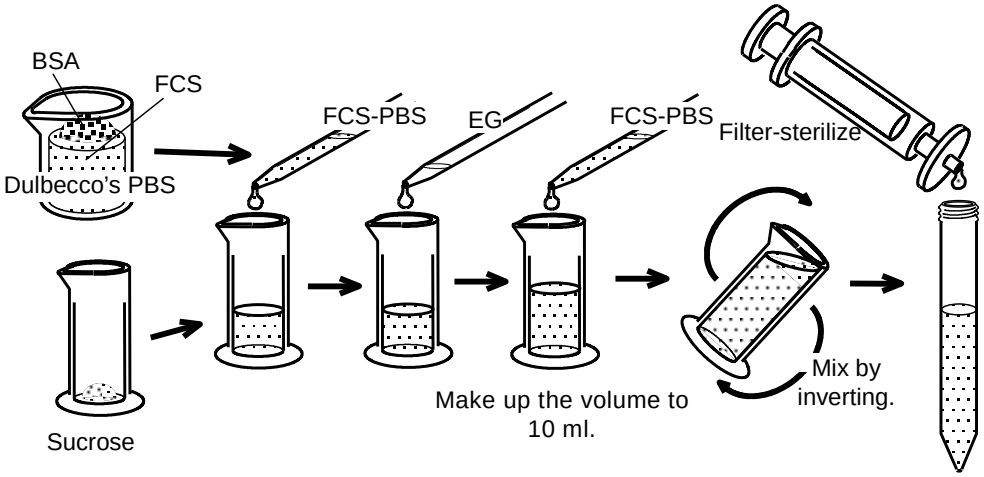


Fig. 74. Preparation of modified D-PBS + 20% FCS,
and 1.5M ethylene glycol + 0.1M sucrose + m-PBS + 20% FCS

3. Preparation of culture solution to be used after thawing

Prepare the 20% FCS + 0.1 mM β-mercaptoethanol + TCM-199 solution. Because the mount of BME added is very small, only 0.1 mM, first prepare a stock solution of 100 times the concentration.

⊙ β-mercaptoethanol stock solution (10 ml)

TCM-199	10	ml
β-mercaptoethanol (βME)	7	μl

1) Place 10 ml of TCM-199 in a test tube.
2) Remove 7 μl of TCM-199 from the test tube using a micropipette.
3) Add 7 μl of βME.
4) Seal the test tube with Parafilm and mix contents by inverting several times.
5) Sterilize the solution by filtration using a 0.22 μm filter and refrigerate (for about 1 week). Alternatively, place in micro tubes or similar and freezing.

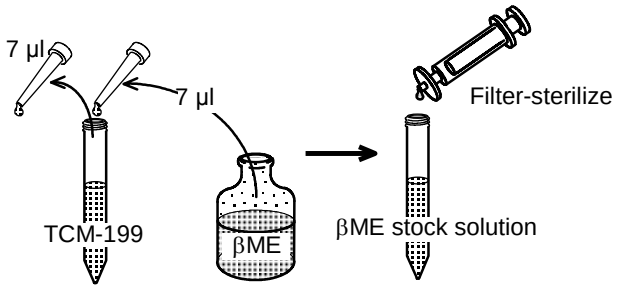


Fig. 75. Preparation of β-mercaptoethanol stock solution

⊙ 20% FCS + 0.1 mM βME + TCM-199 (10 ml): Culture medium to be used for identifying survivors after thawing

TCM-199	7.9	ml
βME stock solution	100	µl
FCS	2.0	ml
Antibiotics (PC-SM)	10.0	µl

1) Place 7.9 ml of TCM-199 in a test tube.
2) Add 100 µl of βME stock solution using a micropipette.
3) Add 2.0 ml of FCS.
4) Add 10 µl of antibiotics and seal the test tube with Parafilm; mix the solution by inverting several times.
5) Sterilize the solution by filtration using a 0.22 µm filter and refrigerate (for about 1 week).

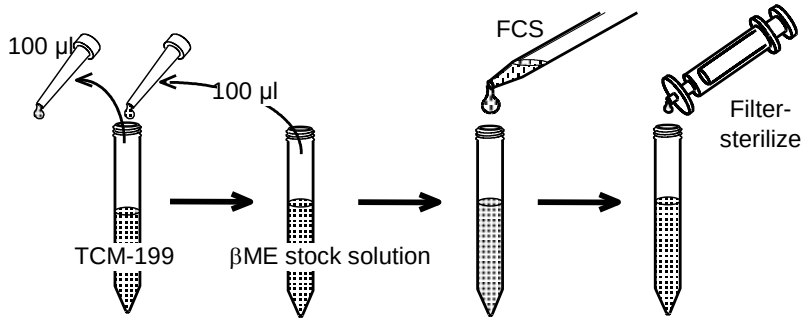


Fig. 76. Preparation of 20% FCS + 0.1mM βME + TCM-199

[General processes for culture solutions]

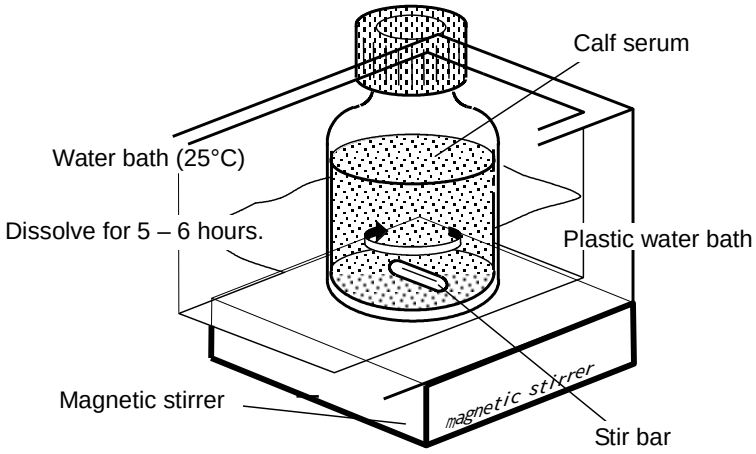
1. Inactivation of calf serum (CS)

⊙ Thawing of CS

1) Completely thaw the frozen CS container in a 25°C water bath.
2) Maintain the temperature for 5-6 h after the serum thaws to a liquid to allow all the components in the CS to dissolve fully.

*** At this stage, CS should be stirred in order to ensure efficient dissolving of solutes. One of the following approaches can be used: (This step should also be performed during the inactivation process.)**

a) Shake the container by hand every few minutes.
b) Use a mechanical shaker.
c) Stir the container using a combination of a water bath and a magnetic stirrer (Fig. 77).



Set a magnetic stirrer under a plastic water bath.

Fig. 77. Thawing of CS Approach c)

⊙ Inactivation of CS

The inactivation process should be started after the CS has thawed; it should take place at 56°C for 30 minutes. The time for increasing the temperature from 25°C (for thawing) to 56°C (for inactivation) is not included in the inactivation time. As with the thawing methods, there are a variety of ways to inactivate CS, however, this manual only explains two approaches below:

Method a): Gradually increase the temperature to 56°C using the water bath program

1) Start the inactivation process when the CS is at 25°C after it has been warmed for 5 - 6 hours.
2) Adjust the setting of the water bath to 56°C to make the temperature increase gradually.
3) When it reaches 56°C, start the inactivation process (for 30 minutes).
4) When finished, take out the container of CS, cool it down to nearly room temperature, and sterilize it by filtration using a bottle filter unit or similar (0.22 μm).
5) Depending on the volume used at one time, place the solution in several containers (ensure they are made of a material appropriate for cryopreservation: e.g., use a polypropylene tube or a centrifuge tube), and cryopreserve.

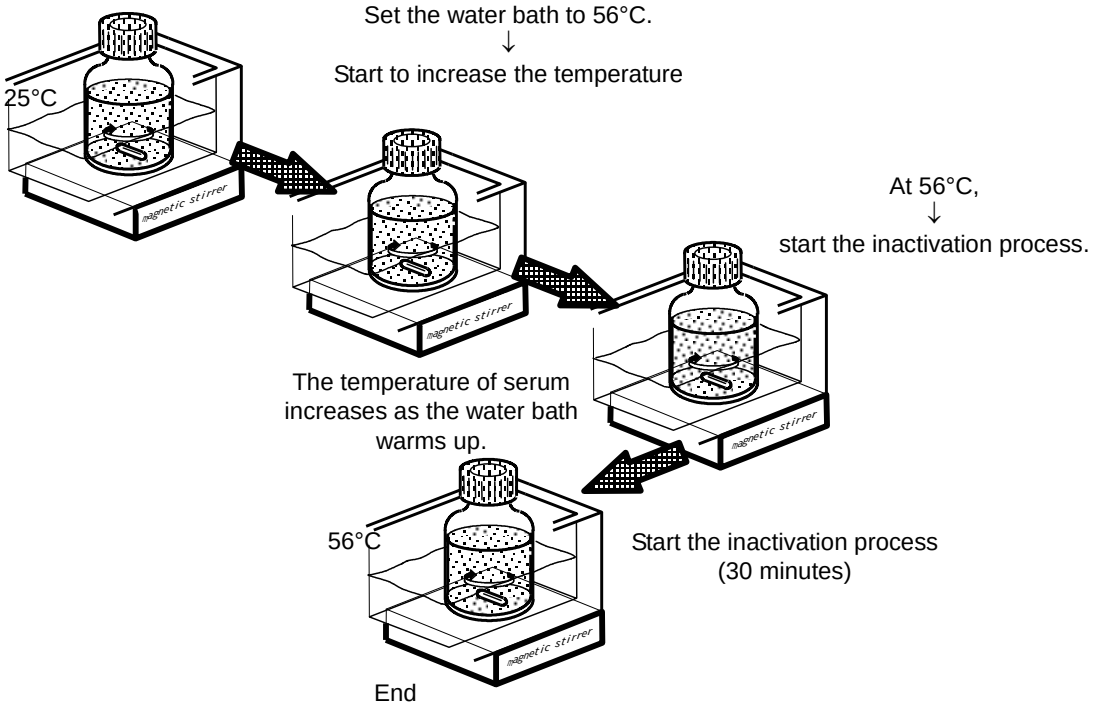


Fig. 78. Inactivation of CS Method a)

Method b): Place the container in a 56°C water bath to warm it up

Before proceeding with the actual procedure, use dummy samples to determine how long it takes for solution in a container at 25°C to reach 56°C.

1) Pour water into an empty CS bottle, place a thermometer in the water, and seal the bottle with Parafilm (dummy sample).
2) Keep the dummy sample at 25°C using a polystyrene foam container or similar.
3) Set the temperature of the water bath to 56°C.
4) Place the dummy sample in the water bath, and start the timer.
5) When the thermometer of the dummy sample reaches 56°C, record the time.
6) Repeat these steps several times and calculate the mean time, which is defined as the "time to warm up." National Livestock Breading Center uses 500 ml bottles and the time to warm up has been established as 15 minutes.

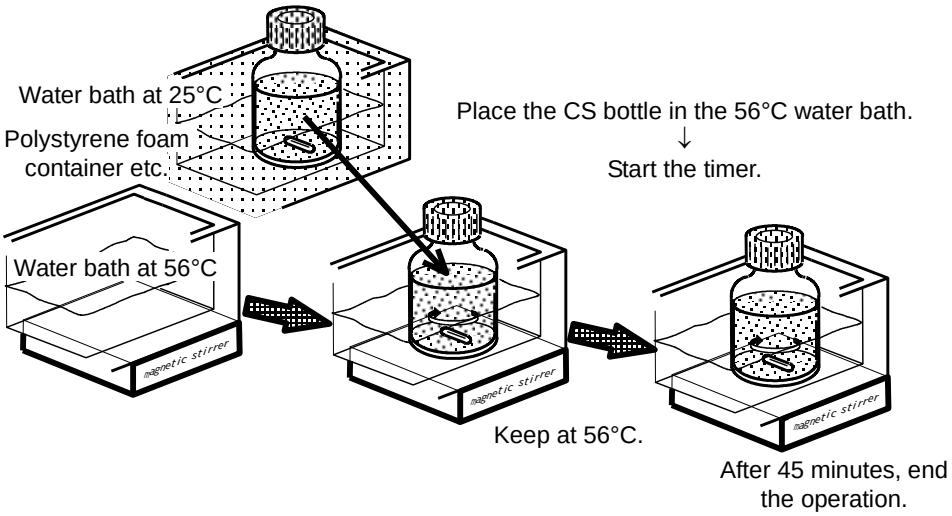


Fig. 79. Inactivation of CS Method b)

When the time to warm up has been obtained, inactivate CS under the same conditions (including the room temperature).

After thawing CS (see Fig. 79),

1) Keep the CS bottle at 25°C using a polystyrene foam container or similar.
2) Set the temperature of the water bath to 56°C.
3) Place the CS container in the water bath and start the timer.
4) When the time to warm up and inactivation time has passed, end the inactivation process, and take out the CS container to allow it to cool down to near room temperature. Sterilize by filtration using a bottle filter unit etc. (0.22 μm).
5) Depending on the volume used at one time, the solution can be placed in several containers (ensure that these are made of a material that is appropriate for cryopreservation: e.g. use a polypropylene tube or a centrifuge tube), for cryopreservation.

*** National Livestock Breading Center finishes the inactivation process 45 minutes (time to warm up (15 minutes) and inactivation time (30 minutes)) after starting the timer.**

2. Antibiotics: add to the culture medium and the cryopreservation medium to prevent bacterial propagation

⊙ Penicillin-Streptomycin (PC-SM) Mixture

Penicillin (PC)		
Crystalline Penicillin G Potassium, Meiji	100,000	unit
Streptomycin (SM)		
Streptomycin Sulfate 1 g "Meiji" For Injection	0.1	g
m-PBS	1	ml

1) Weigh out 0.1 g of SM and add to the flacons containing 100,000 unit PC.
2) Cover with Parafilm and keep refrigerated until use.
3) When ready for use, dissolve in 1 ml m-PBS and mix well.

*** This solution expires after one week of refrigeration.**

PC-SM should be added to the solutions in a way in which PC-SM is diluted 1000-fold.

Example: PC-SM 1 μl/1ml of a solution

The final concentrations of PC and SM are as follows:

PC: 100 U/ml

SM: 100 μg/ml

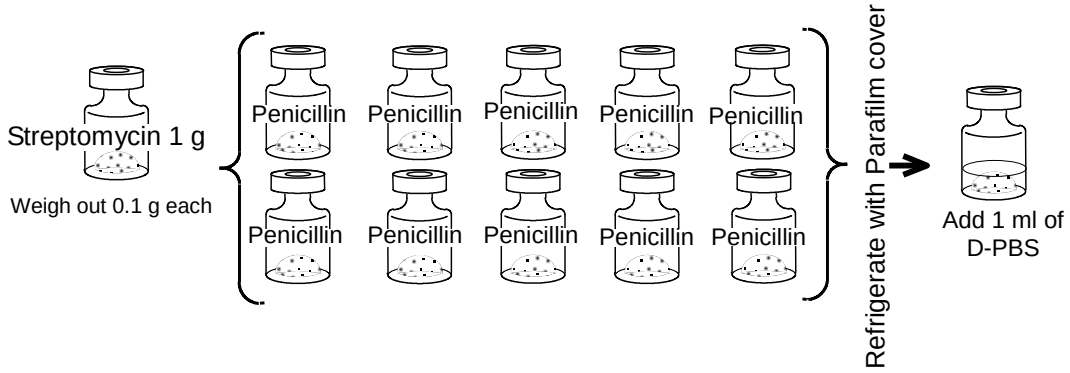


Fig. 80. Preparation of Penicillin-Streptomycin

List of Culture Solutions and Reagents available at The National Livestock Breeding Center

	Product name	Manufacturer	Catalogue No.	Content
Culture solution	TCM-199	GIBCO	12340-030	500 ml
	D-PBS	GIBCO	14287-080	500 ml
	CS	GIBCO	16170-0787	500 ml
	FCS	Hyclone	SH30088.03	500 ml
Reagent	BME essential amino acids	SIGMA	B-6766	100 ml
	MEM non-essential amino acids	GIBCO	11140-050	100 ml
	L-glutamic acid,lyophilized	GIBCO	12419-016	10 ml
	D-PBS(-) (Tablet)	TAKARA BIO INC	T900	200 tablets
	BSA (Fatty-acid free)	SIGMA	A7030	10 g
	BSA (Fraction v)	SIGMA	A9647	50 g
	Hypotaurin	SIGMA	H1384-1G	1 g
	Heparin (Novo heparin 1000U/ml)	Leo Pharmaceutical Products		5 ml
	2-Mercaptoethanol	SIGMA	M7522	100 ml
	Linoleic acid-albumin	SIGMA	L8384	500 mg
	Lactic acid (Hemicalcium salt)	SIGMA	L2000	50 g
	Phenol Red solution (0.5%)	SIGMA	P0290	100 ml
	Sucrose, Ultra Pure	RESERCH ORGANICS (Funakoshi Co., Ltd.)	0928S	500 g
	Sodium pyruvate	Wako Pure Chemical Industries	199-03062	25 g
	Ethylene glycol	Wako Pure Chemical Industries	058-00986	500 g
	Sodium chloride (NaCl)	Wako Pure Chemical Industries	191-01665	500 g
	Potassium chloride (KCl)	Wako Pure Chemical Industries	163-03545	500 g
	Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	Wako Pure Chemical Industries	031-00435	500 g
	Sodium dihydrogenphosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$)	Wako Pure Chemical Industries	192-02815	500 g
	Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	Wako Pure Chemical Industries.	135-00162	500 g
	Sodium bicarbonate ($NaHCO_3$)	Wako Pure Chemical Industries	191-01305	500 g
	Liquid paraffin	Nacalai Tesque	261-14	500 ml

Product name	Manufacturer	Catalogue No.	Content
Percoll™	Amersham Biosciences	17-0891-01	1 L
DL-Lactic Acid Sodium Salt	SIGMA	L7900	100 ml
HEPES > 99.5%	SIGMA	H4034-25G	25 g
Sodium Hydroxide Solution (1 mol/L)	Sigma Aldrich Japan	28-3010-5	500 ml
Sodium Hydroxide Solution (0.1 mol/L)	Sigma Aldrich Japan	28-3040-5	500 ml
Penicillin G Potassium 200,000 Units For Injection	Meiji Seika Kaisha		10 vials
Streptomycin Sulfate 1 g “Meiji” (titer)	Meiji Seika Kaisha		10 vials

List of Instruments and Equipment available at The National Livestock Breeding Center

	Instrument/equipment	Gauge	Manufacturer	Catalogue No.
Petri dish	Petri dish for cultivation	35 mm	Falcon	3001
				1008
	Petri dish for washing and semination	35 mm	Nunc	153066
	Petri dish for ovum search	90×15 mm		Not specified
	4 well multidish		Nunc	176740
Syringe	Syringe for ovum aspiration	5 ml		
	Syringe for culture solution preparation	10 ml	Aldrich	Z11687-4
		20 ml	Aldrich	Z11688-2
		50 ml	Aldrich	Z11840-0
Filter	Filter for filter sterilization	0.22 μm	Sartorius	Minisart, 16534
	Filter unit	0.2 μm	Nalgene	153-0020 (for culture solution)
		0.2 μm	Nalgene	162-0020 (for serum)
Pasteur pipette		150 mm	Hilgenberg	3150101
Centrifuge tube		15 ml		
		50 ml		
Culture flask for storing culture solution		25 cm^2		
		250 cm^2		
Measuring pipette		2 ml		
		5 ml		
		10 ml		
		25 ml		
OPU ovum collection needle	**COVA Needle**		**Misawa Medical Industry Co., Ltd.**	

*** Disposable articles such as centrifuge tubes, culture flasks and measuring pipettes have a fairly consistent quality and there is, therefore, no need to choose one from a specific manufacturer. With regard to the articles used for cultivation such as petri dishes, however, it is necessary that the ones provided by the above specified manufacturers are used as the quality of these articles could affect the cultivation result.**

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Postscript from the Author

The OPU-IVF technique, when used according to a carefully planned schedule, allows for more efficient production of embryos than consecutive superovulation treatments. Further, with regard to the transfer of developed blastocysts, the rate of conception for fresh embryos can be expected to be the same as that of an in-vivo derived embryo. There are, however, few published manuals outlining the procedures involved in the OPU technique.

This manual uses illustrations to provide a clear explanation of the basic techniques involved in interpreting ultrasound images as well as handling the ovaries during the OPU-IVF process. It would give me great satisfaction to see this manual become a good reference for technicians who are just making a start with using the IVF process in cattle and I hope it contributes to the development of this technique, the use of which will only continue to increase in the future.

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