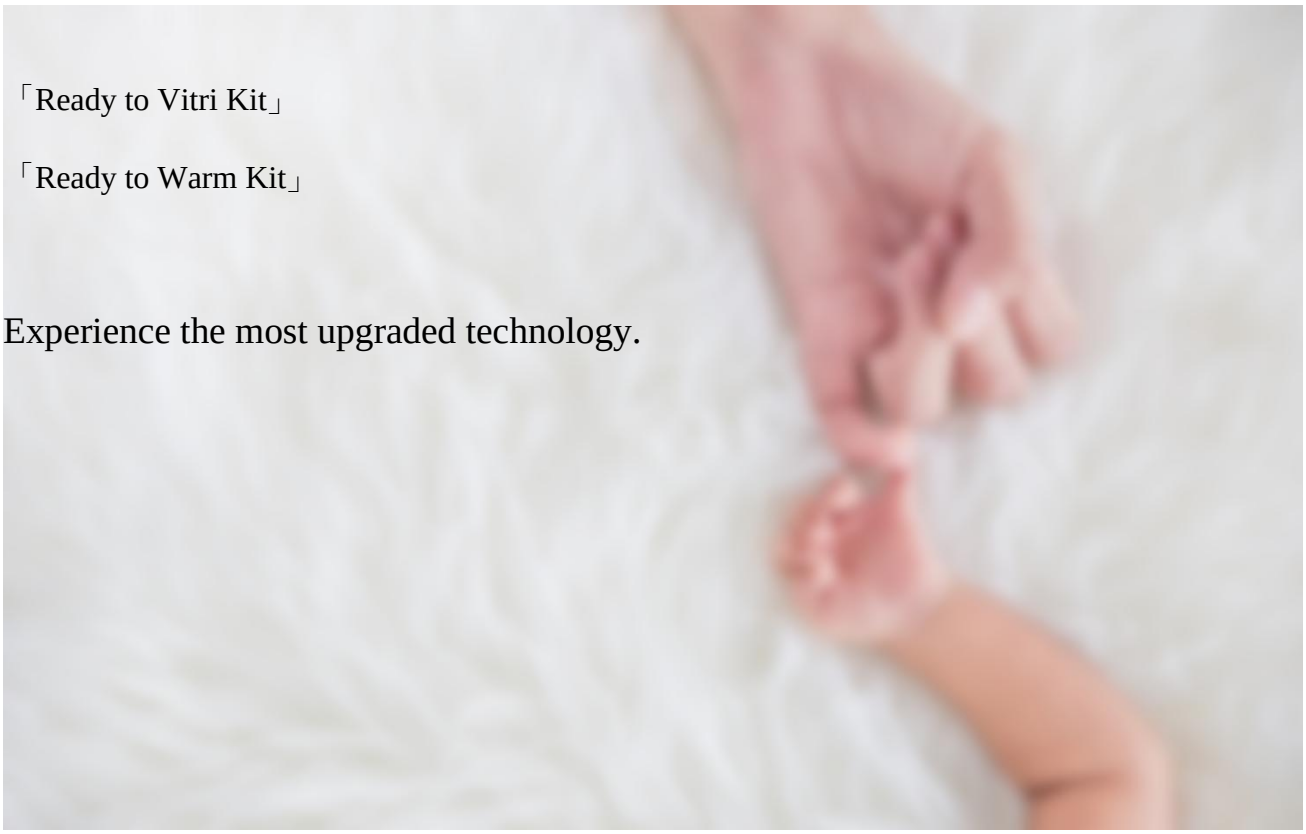
THE CRYOTEC METHOD
Ready to Use

REPROLIFE

To everyone who looks after the patients every day

Reprolife deeply cares of patients and their precious lives, and we do it in a non-stressful way.



「Ready to Vitri Kit」

「Ready to Warm Kit」

Experience the most upgraded technology.

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1. Basic Operation

[Microscope Magnification]

The Cryotec Method uses only two magnification levels of the microscope for a simple operation.

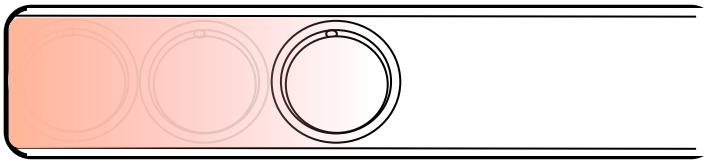
Low magnification: For manipulating the oocyte/embryo (x12-15).
- This magnification allows you to easily view the entire oocyte/embryo at once.

High magnification: For observing the oocyte/embryo (x45-55).
- This maximum magnification allows you to check areas in detail.

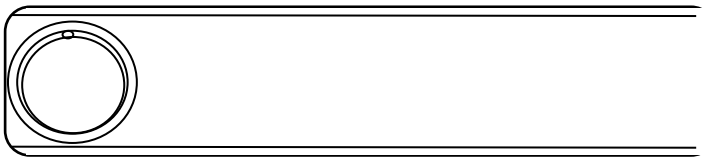
【The Position of the Oocyte/Embryo During Aspiration】

Using Cryotec Method protocols, there are only three positions for the oocyte/embryo that you will have to be familiar with.

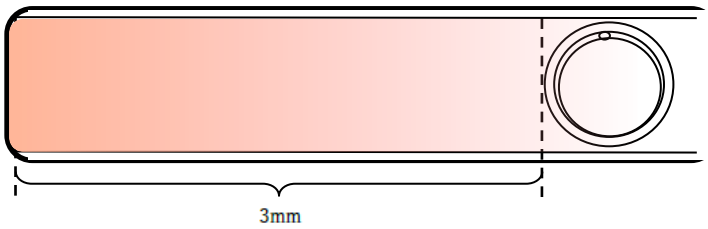
①Aspirating a small amount of the solution (roughly double the diameter of the oocyte) after the oocyte/embryo (standard procedure).



②Placing the oocyte/embryo itself at the tip of the pipette (equilibration via vitrification).

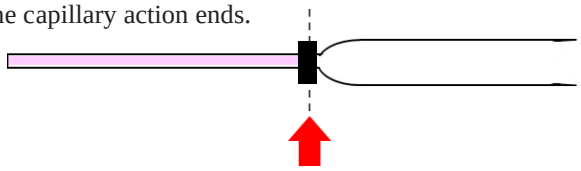


③Aspirating 3mm of solution after the oocyte/embryo (dilution via TS and DS).



Tip!

All actions involving the pipette and oocyte/embryo can be made significantly easier by using capillary action (a natural occurrence) to aspirate 1mm of solution into the pipette beforehand. To make things even easier, you can make a mark on the pipette to indicate the threshold at which the capillary action ends.



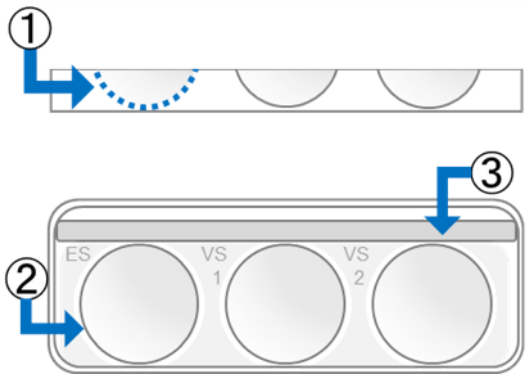
2. Vitrification Protocol

The purpose of cryopreservation via vitrification

Using cryopreservation via vitrification prevents any change or deterioration in the quality of the oocyte/embryo, ensuring that it preserves the same state it was in before freezing.

【Preparation】

- Ready to Vitri Kit
- Freezing device (Cryotec)
- Stereo microscope (Turn off the heating plate)
- Timer (with count-up function)
- Tweezers
- Scissors
- Handling tool
- Cooling rack
- Liquid Nitrogen



RtU Vitri Plate Characteristics

The RtU Vitri plate, specially designed for use with the Cryotec Method, has 1) rounded wells, 2) space for depositing solution expelled during washing, and 3) a groove for securely holding the Cryotec (Freezing device).

Because each well is hemispherical, they are less likely to create "blind spots" due to shadows in the microscope. This makes it much more difficult to lose track of the oocyte/embryo.

[Vitrification Preparation]

1. Maintain the room temperature between 25℃ and 27℃.
2. Ready to Vitri Kit should be at room temperature (26±1℃: 25-27℃) for at least 30 minutes.

Check! Before use, be sure to confirm the plate solutions visually and check for any abnormalities in color or temperature.

3. Prepare the manipulation pipette with an inner diameter that matches the diameter of the oocyte/embryo to be vitrified:
   - For oocytes/embryos, 140-150μm.
   - For blastocysts, 160-220μm.

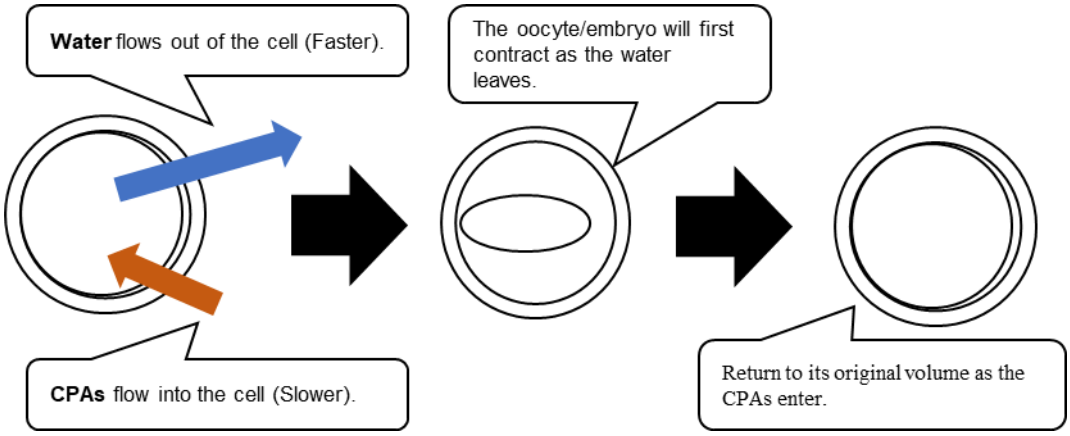
[STEP 1]

Equilibration in ES（7-13 minutes：at room temperature）

The purpose of this step is to introduce cryoprotectant agents (CPAs) into the cell, the successful completion of which is indicated by the complete recovery of the cell's volume.

The mechanism behind cell shrinkage and recovery

Because the inside of the cell is a culture medium (with an osmotic pressure of approximately 300) and the extracellular space is filled with ES (with an osmotic pressure of approximately 2,400), water within the cell will flow out of it due to this difference between intra- and extracellular osmotic pressures. At the same time, because CPAs can penetrate the cell membrane, they flow into the cell. Because the intra- and extracellular osmotic pressures naturally attempt to equilibrate, these reactions occur simultaneously. However, the rate at which water flows out of the cell is slightly faster than that of CPAs flowing into the cell. This means that the oocyte/embryo will first shrink as the water leaves, then return to its original volume as the CPAs enter.



How to remove the film

In order to remove the film, firmly hold the plate body with one hand. Be careful not to touch the inside of the plate with your hands. Carefully peel it off little by little.

Check! To avoid the solution coming out, please make sure to not make pressure in the area over the well when removing the film.

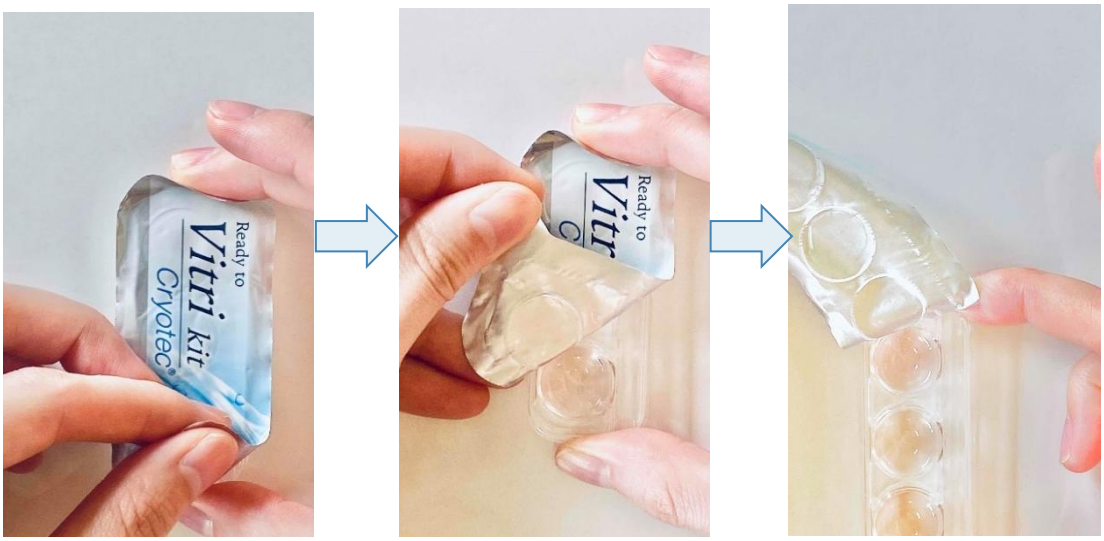
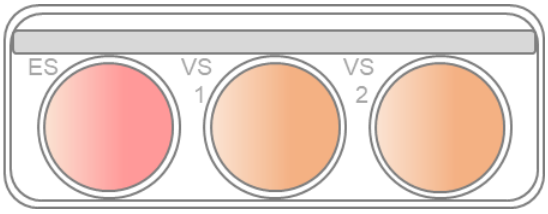
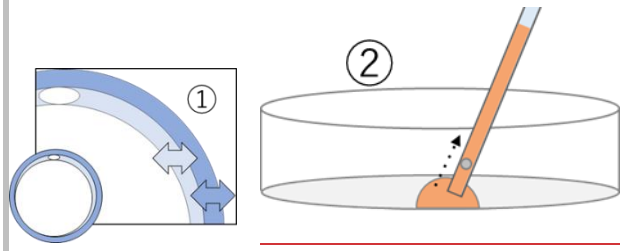
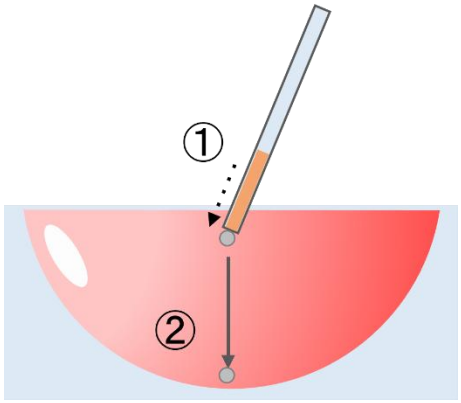
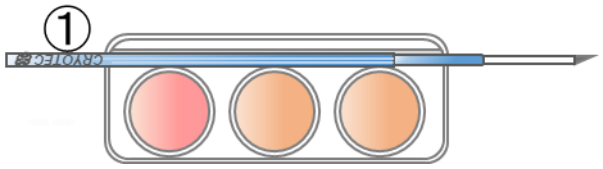
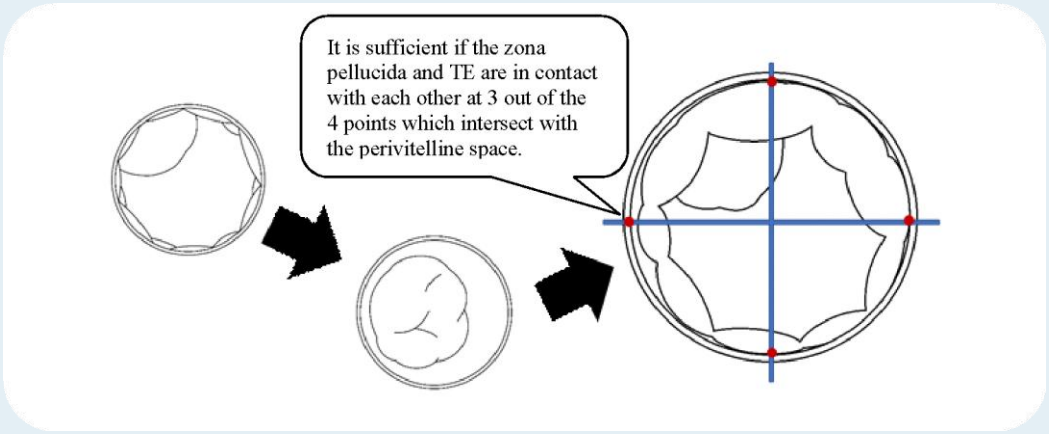


	Figure	Procedure
1		1. Carefully remove the film of the RtU Vitri plate after bringing it to room temperature.
2	 Culture dish	1. Inspect and note the details of the oocyte/embryo at high magnification, paying careful attention to the size of the zona pellucida relative to the perivitelline space (in oocytes) or the cavity (in blastocysts) (①) in order to confirm that the oocyte/embryo has fully recovered its original volume during equilibration. 2. Aspirate the oocyte/embryo into the tip of the pipette along with a small amount of culture medium (roughly the size of 2 oocytes) (②).
3	 ES	1. Place the oocyte/embryo and culture medium which you aspirated in step 1-2-2 into the **ES**, directly in the center (①). 2. Start the timer to begin counting up. 3. The oocyte/embryo sinks slowly while shrinking (②). 4. Wait for full recovery of oocyte/embryo to its original volume or move to VS after the maximum equilibration time (13 minutes for oocytes and blastocysts and 10 minutes for cleaved embryos).
4		1. During the recovery waiting time, remove the Cryotec from the plastic protection. Write the oocyte/embryo information on the back of the handle (opposite of the logo side) and set it in the groove of the vitrification plate with the Cryotec logo facing up (①). 2. Prepare fresh liquid nitrogen in the cooling rack. 3. Once the oocyte/embryo has fully returned to its original size, or when the maximum time is elapsed, the ES equilibration is complete.

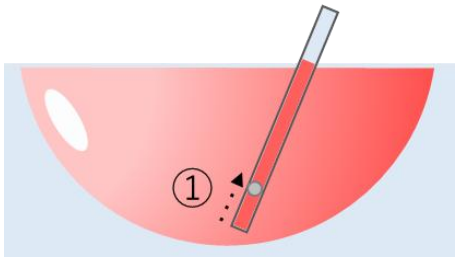
Check! You are able to use provided lid, while Cryotec is being setting in the groove.
Check! Please make sure there is no damage on the Cryotec in advance.

Determining equilibration completion

If volume recovery cannot be confirmed, or if you are unsure in your judgment of complete recovery, the maximum completion times for ES equilibration are as follows: Oocytes and blastocysts – 13 minutes; 4-8 cell stage embryos – 10 minutes. It has been conclusively determined that sufficient equilibration occurs after 13 or 10 minutes, respectively.



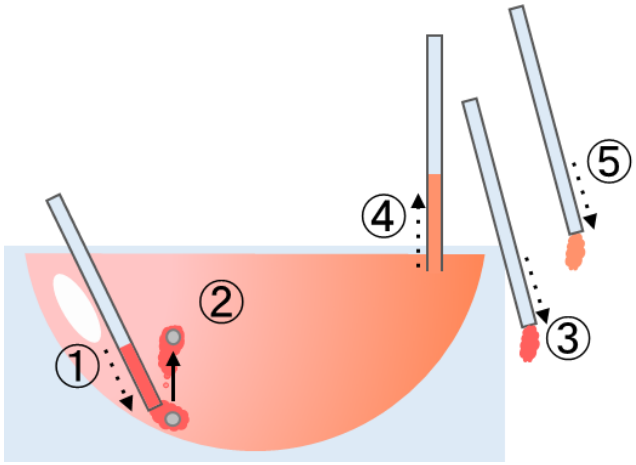
It can sometimes be difficult to determine whether a blastocyst's volume has completely recovered due to the blastocyst cavity. Therefore, in the case of blastocysts, inspect the contact between the zona pellucida and TE as shown in the figure above and determine recovery accordingly. Viewing the blastocyst from the top, imagine a cross overlaid across the cell; if the zona pellucida and TE are in contact with each other at 3 out of the 4 points which intersect with the perivitelline space, the blastocyte has sufficiently recovered.

5	 ES	1. Aspirate the oocyte/embryo with a small amount of ES (about the size of 2 oocytes) (①).

[STEP 2]

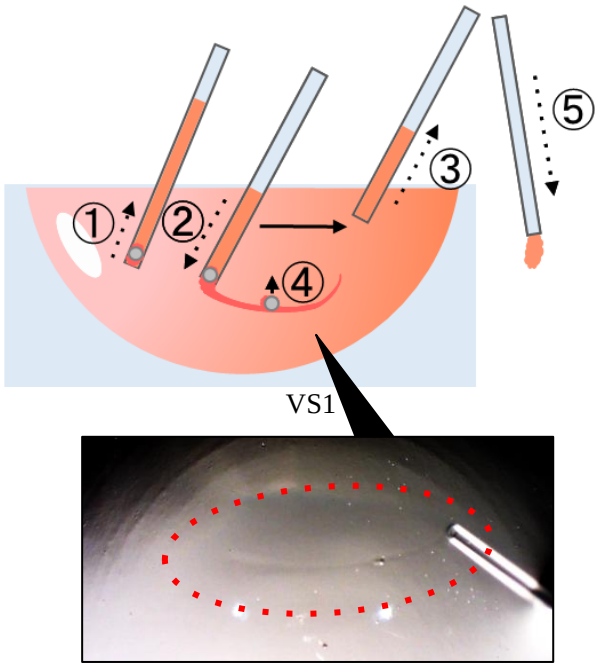
Equilibration in VS1 (30-60 seconds)

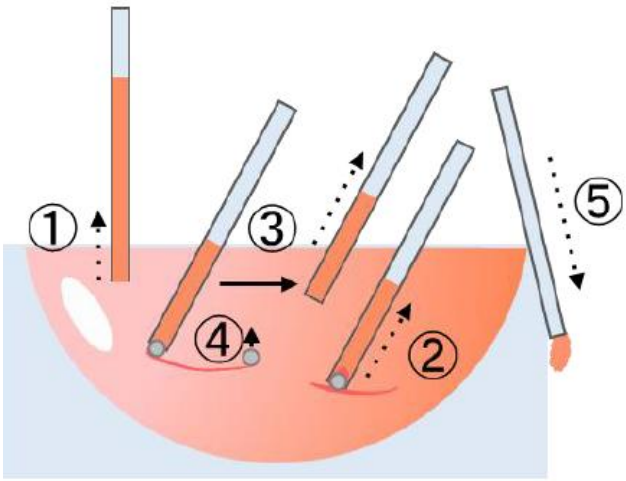
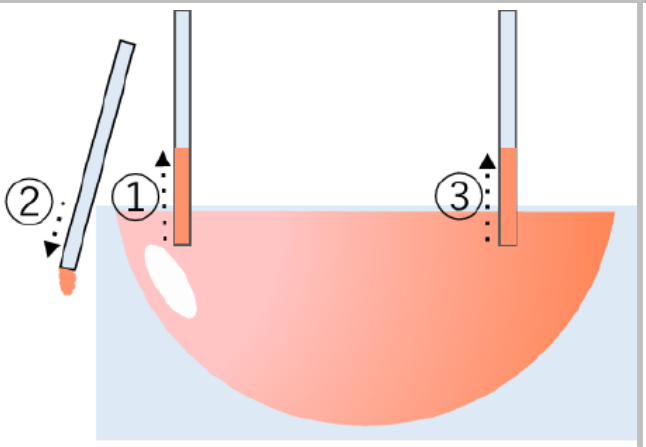
The purpose of VS1 is to replace all extracellular ES with VS. The oocyte/embryo is moved horizontally and vertically, and the end of this step determined to be complete by matching the weight of the cell and VS1.

	Figure	Procedure
1	 VS1	1. Release the oocyte/embryo into the bottom of the VS1 well (①), then slowly take out the pipette. 2. The oocyte/embryo is surrounded by ES, so they will slightly rise with it (②). 3. After observing the oocyte/embryo floating, all ES remaining inside the pipette is discarded outside of the well (③). Aspirate fresh VS1 from the edge of the well and discard it promptly outside of the well (④ and ⑤)

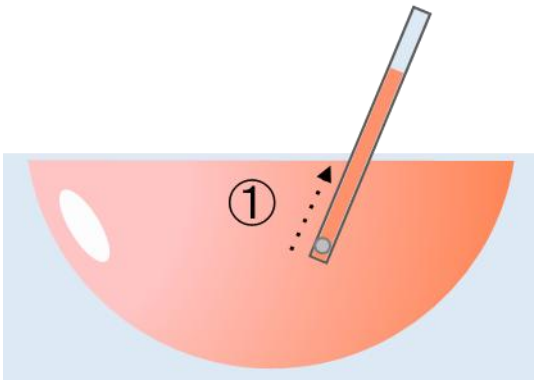
Check!

Due to the high viscosity of the solutions, the embryos barely rise and are easy to observe.

2	 VS1	1. After aspirating fresh VS1 from the edge of the well, aspirate the oocyte/embryo into the tip of the pipette (①). 2. In order to completely remove the remaining ES, the pipette is inserted in the middle depth of the VS1 and the oocyte/embryo is released by a horizontal movement while releasing media. Release more media after the oocyte/embryo is out while keeping the same motion, then take out the pipette (② and ③). 3. The oocyte/embryo naturally floats and slightly separates from the released medium (④). 4. Rinse the pipette (⑤).

3	 VS1	1. Repeat the previous procedure to promote further dehydration. After aspirating fresh VS1 from the edge of the well (①), pick up the oocyte/embryo into the tip of the pipette (②). 2. Similar to 2-2, insert the pipette into a clean area in the middle depth of the VS1 well. Release the media while moving horizontally, and release the oocyte/embryo during the movement. (③). 3. Observe the oocyte/embryo at maximum magnification. The solution exchange from ES to VS can be confirmed complete by confirming shrinkage and remaining at the same focus (④). 4. Pipette rinsing: Expel the entire amount of VS1 remaining inside the pipette into the space out of the well. (⑤).
4	 VS2	1. Aspirate and expel out of the well fresh VS2 from the edge of the VS2 well (① and ②). 2. Aspirate fresh VS2 once again from the edge of the VS2 well (③)

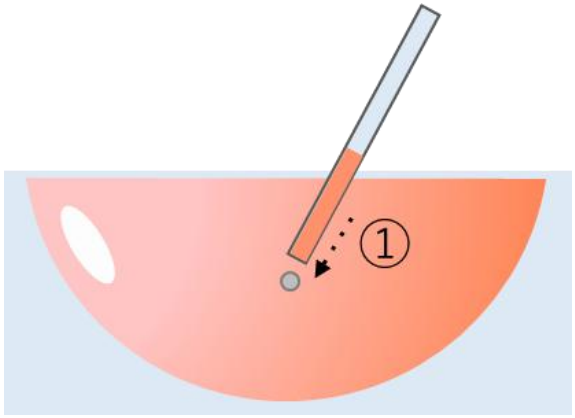
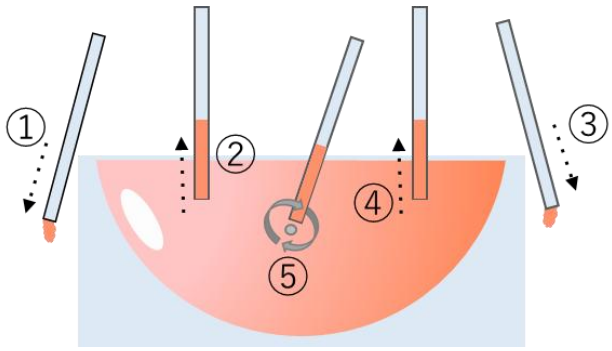
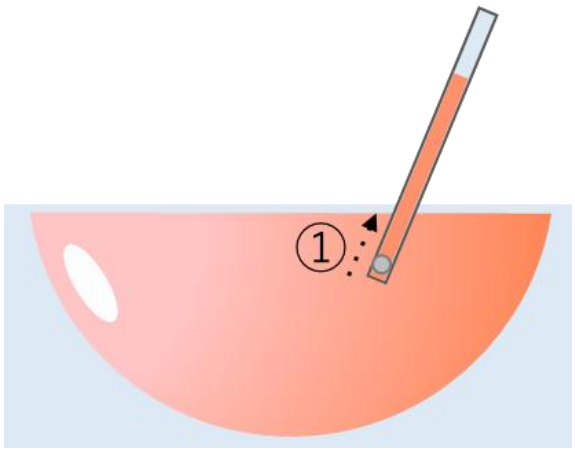
CHECK:
The purpose of aspirating fresh VS2 before aspirating the oocyte/embryo from VS1 is to prevent bringing VS1 into VS2.

5	 VS1	1. Aspirate the oocyte/embryo from VS1 into the tip of the pipette (①).

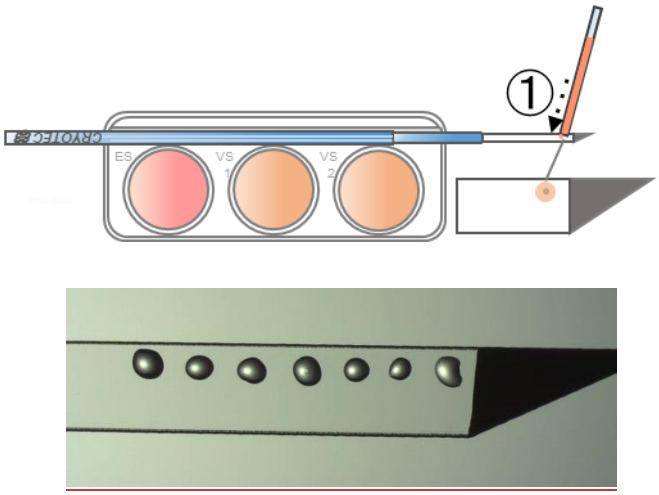
[STEP 3]

Shrinkage in VS2 (10-20 seconds)

The purpose of VS2 is to confirm that ES has been completely replaced by VS, which is determined by the cells shrinking.

	Figure	Procedure
1	 VS2	1. Place the oocyte/embryo into the middle depth of **VS2** (①).
2	 VS2	1. Expel all remaining VS1 within the pipette into the well's draining groove (①). Aspirate then expel fresh VS2 from the edge of the well (② and ③). 2. Aspirate fresh VS2 once again (④). 3. Stir the area around the oocyte/embryo 5 times and observe the oocyte/embryo from multiple angles to confirm complete shrinkage (⑤).
3	 VS2	1. Aspirate the oocyte/embryo into the tip of the pipette (①).

Placing the Oocyte/Embryo

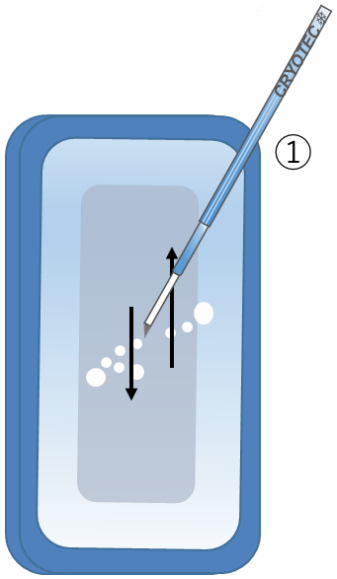
4		1. Deposit the oocyte/embryo along with a small amount of VS2 near the marker (black triangle) on the Cryotec sheet (①).

CHECK: 1 oocyte = 1 drop. DO NOT REDUCE the volume of the droplet.

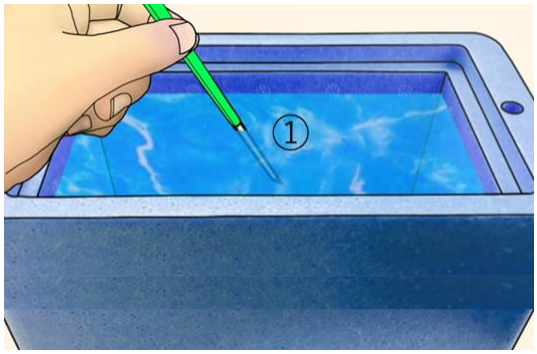
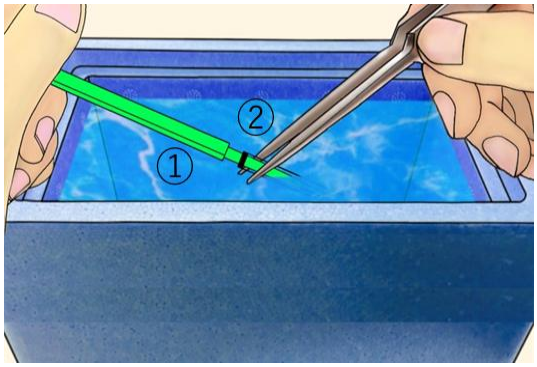
Do not attempt to reduce the size of the droplet

Conventional methods would attempt to minimize the size of the droplet in order to increase the cooling rate. However, since the freezing reagent used in the Cryotec method has higher vitrification ability than these conventional methods, there is no need to do this. In attempting to minimize the droplet, you may apply pressure to the oocyte/embryo due to surface tension. This may damage the oocyte/embryo, or cause it to stick to the sheet, making it difficult to remove during thawing. This can also increase the time required for TS, which may damage the oocyte/embryo. To reiterate, our method does NOT require you to minimize the droplet. If the droplet is overly large, or if two or more oocytes/embryos are placed into a single droplet, simply re-aspirate it back into the pipette and make a new droplet at a different location on the sheet.

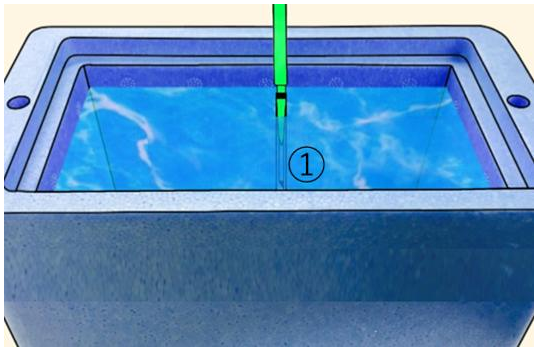
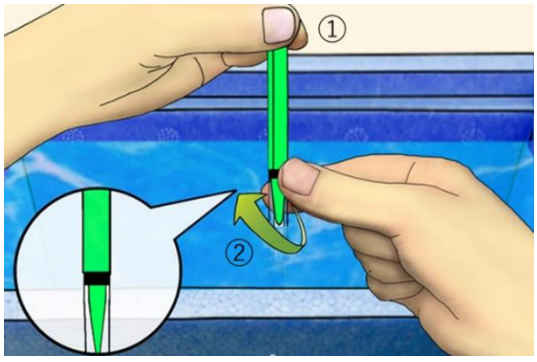
Ultra-Rapid Freezing

5	 Liquid nitrogen (LN_2)	1. After confirming the presence of the oocyte/embryo in the droplet on the sheet, immediately place the Cryotec into the liquid nitrogen and gently shake it until bubbles are no longer appearing. This will raise the cooling rate (ultra-rapid cooling) (①).

Attaching the Cap

	Figure	Procedure
1		1. <u>Keep the sheet inside the liquid nitrogen</u> (①).
2		1. Place the cap in the liquid nitrogen. After a few seconds, make sure there are no bubbles coming out of the cap. 2. <u>Keep the tip of the sheet in liquid nitrogen</u> (①), and with the help of your tweezers place it close to the opening of the cover cap (use the black mark as a guidance). Insert the cap all the way on the Cryotec (②).

CHECK: DO NOT HOLD the cover cap with tweezers by the middle part since it could break and try to close the cover cap by gripping it tightly with tweezers, as the cover cap can be damaged. Broken or damaged cover caps and Cryotecs should not be used.

3		1. After using the tweezers to place the cap, hold the Cryotec upright (①).
4		1. While continuing to keep the tip of the sheet in the liquid nitrogen (①), lift the Cryotec partially up into the air, then firmly twist tight the cap using the fingers (②).

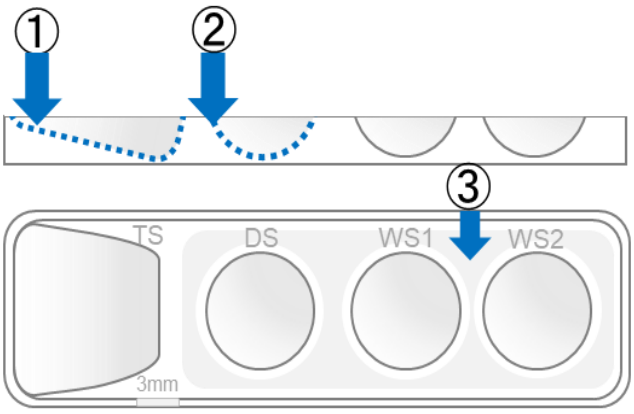
3. Warming Protocol

Temperatures at the risk of forming ice crystals

The thawing process is the most likely stage at which ice crystals may form. Even with an increased cooling rate during vitrification, ice crystals may still form if the heating rate is too low. It is extremely important to move quickly from -80 ℃ to -20 ℃, which is the range in which ice crystals are most likely to form.

【Preparation】

- Ready to Warm Kit
- Stereo microscope (Turn off the heating plate)
- Timer (with count-up function)
- Tweezers
- Handling tool
- Cooling rack
- Liquid Nitrogen



RtU Warm plate characteristics

Our RtU Warm Plate, designed exclusively for its use with the RtU Cryotec Method, features

① a trapezoidal well with a slope for TS use. It also includes

② hemispherical wells and

③ space for expelling solution, similar to the RtU Vitri Plate. The slope of the TS well is designed to allow stable placement of the Cryotec sheet. The hemispherical wells allow easier making of a liquid layer of the solution during dilution and makes it possible to replace gradually the osmotic pressure.

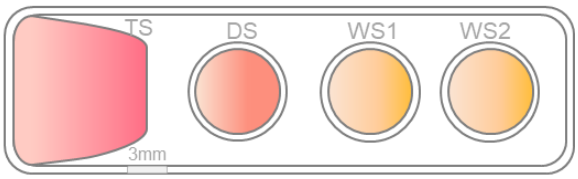
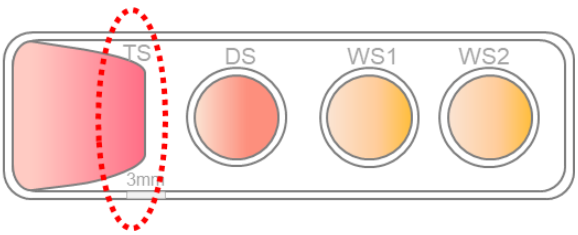
[Warming Preparations]

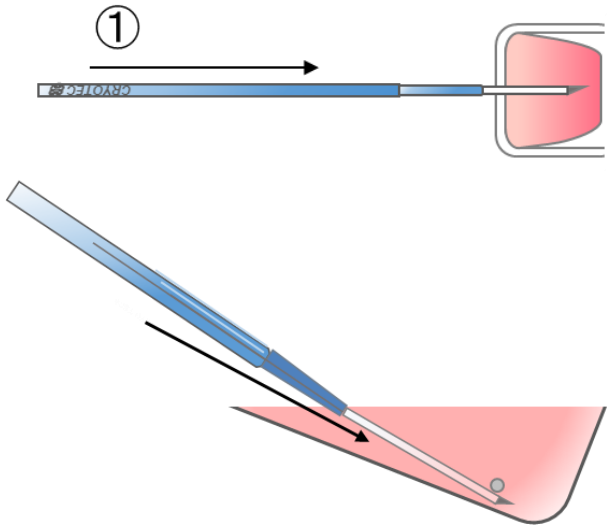
1. Warm the RtU Warm plate in a 37°C incubator, at least 30 minutes before the procedure (overnight warming is acceptable).
2. Prepare the liquid nitrogen in the cooling rack.
3. Remove the Cryotec from the storage tank and place it in the cooling rack. Inside the liquid nitrogen remove the cap and place the Cryotec against the inside wall of the cooling rack.

CHECK! Please do not grip the cover cap strongly with tweezers when removing the cover cap. The cover cap and Cryotec sheet can be damaged.

[STEP 4]
Warming in TS (1 minute)

How to remove the film
In order to remove the film, firmly hold the plate body with one hand. First, pull the film a little to let the air out of TS well. Be careful not to touch the inside of the plate with your hands. Carefully peel it off little by little.
Check! To avoid the solution coming out, please make sure to not make pressure in the area over the well when removing the film.

	Figure	Procedure
1		1. Carefully remove the film of the RtU Warm plate after being warmed to 37°C in an incubator.
2		1. Set the focus of the microscope to the bottom of the TS well (specifically, the position where the Cryotec sheet will rest when inserted; the dotted line on the left)

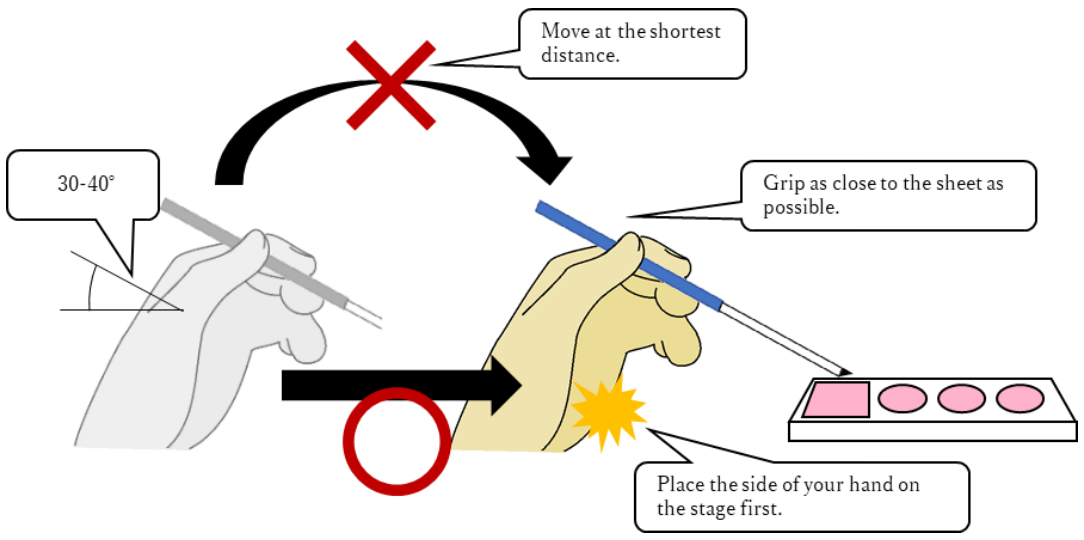
3	 TS	1. Immediately insert Cryotec from liquid nitrogen into the TS (within 1 second) (①). 2. Start the timer and stay still for **1 minute** without moving Cryotec. 3. Ensure that the oocyte/embryo spontaneously leaves the Cryotec sheet and begins to float during warming process. ※ If the oocyte/embryo begins to float away from the Cryotec sheet spontaneously, it should be moved to the bottom to prevent it from reaching the surface. ※If the oocyte/embryo does not come off spontaneously from the Cryotec, gently move the sheet sidewards, or carefully aspirate the oocyte/embryo directly from the sheet.

CHECK: Be sure to let it sit for a full minute!

Immerse the Cryotec in the TS and let it sit undisturbed for a full minute. If you move it, it can upset the temperature balance of the entire solution due to the extremely low temperature of the Cryotec. In addition, if the oocyte/embryo has come loose from the sheet, any movement may displace it and cause you to lose sight of it. If you are unable to see the oocyte/embryo on the Cryotec sheet, remain patient and do not attempt to move the sheet.

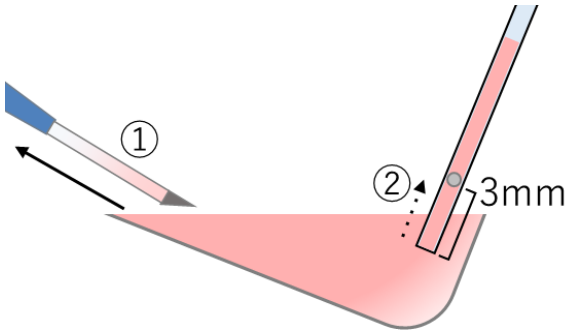
Tips for inserting the Cryotec into the TS well

Prepare the pipette using your right hand and hold the Cryotec in your left hand, keeping your grip as close to the sheet as possible. Immediately insert it at an angle of 30 to 40 °, using the curve of the TS well to guide you. This may cause your hand to unconsciously arc, so it's important to make an effort to move your hand in a short, straight line. You can also place the side of your hand on the stage first, which will keep it more stable as you insert the Cryotec. Be sure to move your hand quickly from the liquid nitrogen to the stage of the microscope, then carefully and gently insert the Cryotec sheet into the TS well. Move as fast as possible between the liquid nitrogen and TS using the curve of the TS well, to avoid making any bubbles on the sheet. If any air bubbles form on the sheet, it may make it more difficult to find the oocyte/embryo, or the oocyte/embryo may stick to a bubble and move within the solution. Please exercise all necessary caution.



[STEP 5]

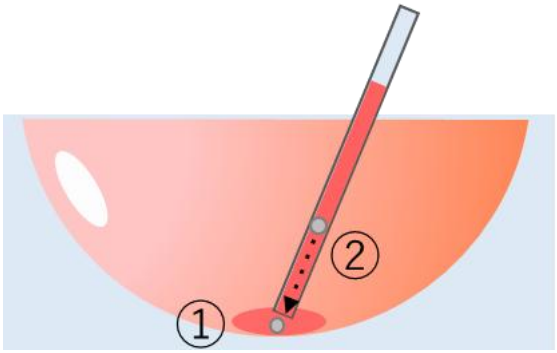
Dilution in DS (2 minutes)

	Figure	Procedure
1	 TS	1. After 1 minute in TS, slowly remove the Cryotec. (①). 2. Aspirate the oocyte/embryo from the TS, then slowly aspirate 3mm of TS into the pipette (②).

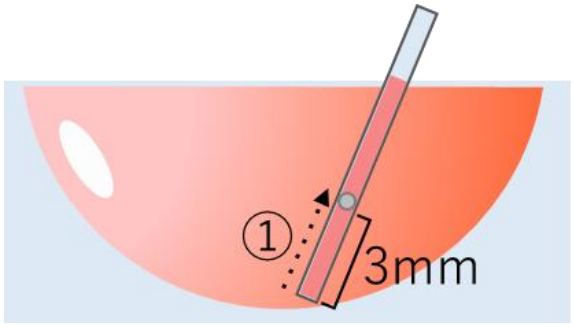
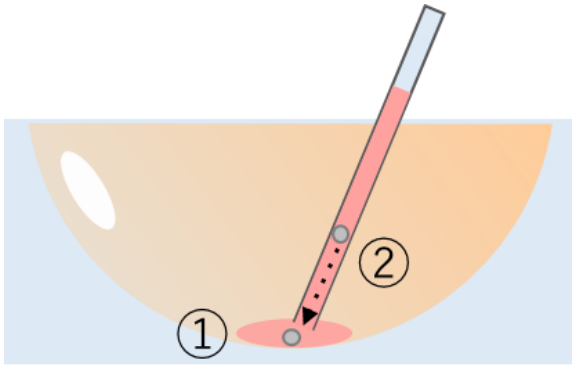
CHECK:
If the oocyte/embryo has still not separated from the sheet after a full minute, place the Pasteur pipette beneath it and apply gentle pressure to separate it. Be sure not to come into direct contact with the oocyte/embryo.

CHECK: If the oocyte/embryo disappears
If you successfully place the oocyte/embryo on the Cryotec sheet during the vitrification process, the oocyte/embryo is in the TS well. Our TS solution has minimal toxicity, so you will have time to patiently search for the oocyte/embryo.

Check!
3mm can be measured by the 3mm indicator on the main body of the RtU Warm plate (lower right of TS well) and on the lid.

2	 DS	1. Insert the tip of the pipette into the bottom center of the DS well and slowly expel the TS to form a TS layer at the bottom (①). 2. Place the oocyte/embryo gently on the bottom of the TS layer (②). 3. Turn off the microscope light and wait for 2 minutes.

Dilution in WS1（3 minutes）

	Figure	Procedure
1	 DS	1. Aspirate the oocyte/embryo within the DS, then slowly aspirate 3mm of DS into the pipette. (①).
2	 WS1	1. Insert the tip of the pipette into the bottom center of the WS1 well and slowly expel the DS to form a DS layer at the bottom (①). 2. Place the oocyte/embryo gently on the bottom of the DS layer (②). 3. Use high magnification to carefully inspect and memorize the detailed form of the oocyte/embryo. Turn off the microscope light and wait for 3 minutes. 4. After 3 minutes, compare the current shape of the oocyte/embryo with the previous shape you memorized. If you can confirm that the volume of the oocyte/embryo has partially or fully recovered, this indicates that it is alive.

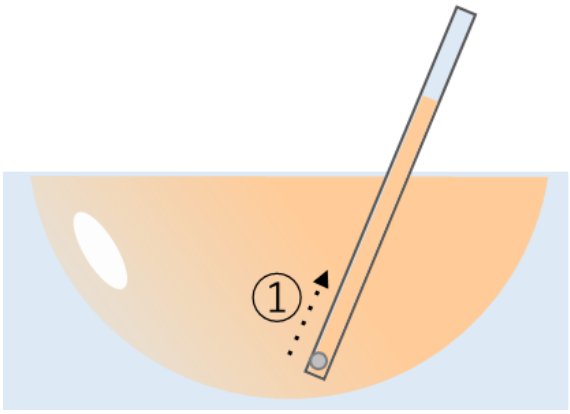
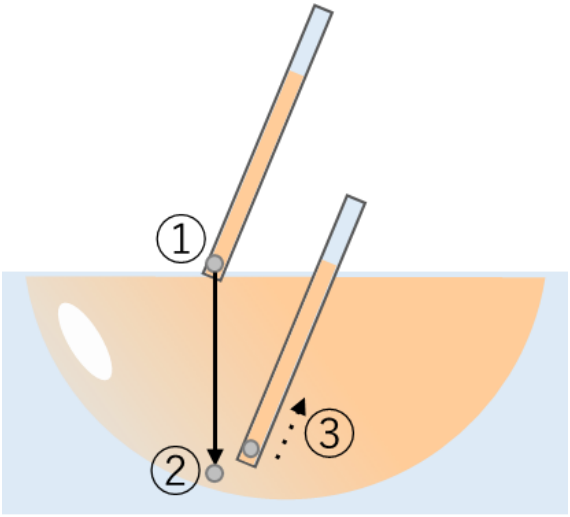
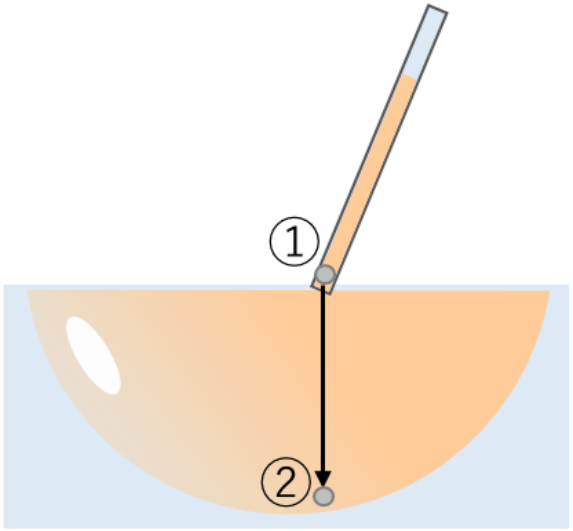
CHECK:

It's important to confirm during this step whether or not the oocyte/embryo was damaged during the vitrification/thawing process. An oocyte/embryo which has survived will undergo a normal membrane reaction and subsequent volume recovery.

The reaction during this stage is due to condensation: The oocyte/embryo becomes isotonic as it enters WS (300) from DS (900). In other words, while the volume of a fully intact oocyte/embryo will have recovered completely, a lower quality oocyte/embryo will need more time to do so. In the case of a dead or damaged oocyte/embryo, normal membranous reaction will not occur at all. As such, you will see no change in volume whatsoever. In the case of blastocysts, once the blastocyst cavity begins to form/expand, or the blastocyst cavity is completely re-expanded, it can be considered to have survived. In general, it has been shown that human embryos are likely to become children if more than 30% of blastomeres survive:

- For a 2-cell stage embryo, more than 1 blastomere
- For a 4-cell stage embryo, more than 2 blastomeres
- For an 8-cell stage embryo, more than 3 blastomeres

Washing in WS2 (30 seconds)

	Figure	Procedure
1	 WS1	1. Aspirate a small amount of WS1 and the oocyte/embryo into the pipette (①).
2	 WS2	1. Place the oocyte/embryo on the left side surface of the WS2 well (①). 2. The oocyte/embryo will slowly sink to the bottom of the well (②). 3. After the oocyte/embryo reaches the bottom of the well, aspirate the oocyte/embryo at the tip of the pipette (③).
3	 WS2	1. Place the oocyte/embryo on the right-side surface of the WS2 well (①). 2. The oocyte/embryo will slowly sink to the bottom of the well once again (②). 3. Once the oocyte/embryo reaches the bottom of the well, the washing step is completed. 4. Move the oocyte/embryo to the culture dish for performing a recovery culture until ICSI or embryo transfer.

CHECK:
We recommend culturing the oocyte for 2 hours for ICSI and the blastocyst for at least 1 hour before embryo transfer.