



THE CRYOTEC METHOD

[Complete Manual for Ensuring 100% Survival of Oocytes and Embryos]



Our goal is to provide all patients throughout the world with a reliable 100% survival rate through the use of the world's most effective vitrification technique.

All of the necessary materials and methods to achieve this can be found in these two packages alone:

“Cryotech® Vitrification Kit 101”

“Cryotech® Warming Kit 102”

We hope that all infertility patients around the globe can share in the joy of these packages filled with the miracle of science.



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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.

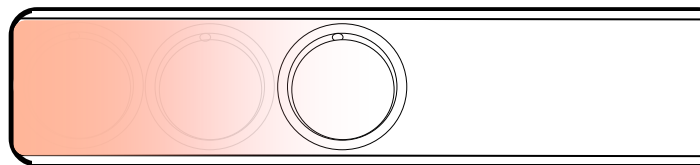
High magnification: For observing the oocyte/embryo (x45-55).

- This maximum magnification allows you to check areas in detail.

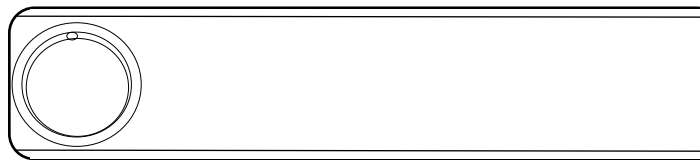
[The Position of the Oocyte/Embryo During Aspiration]

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

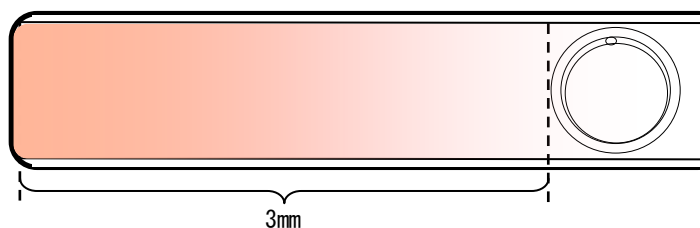
1. Aspirating a small amount of the solution (roughly double the diameter of the oocyte) after the oocyte/embryo (standard procedure).



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

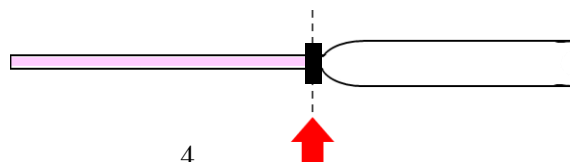


- 2.3. Aspiring 3mm of solution after the oocyte/embryo (dilution via TS and DS).



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.



Vitrification Protocol

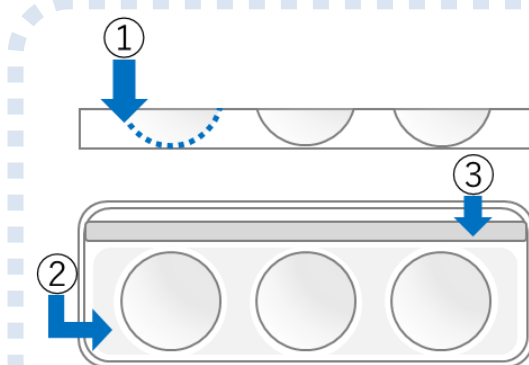
The purpose of cryopreservation via vitrification

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.

【Materials】

- **Cryotech® Vitrification Kit 101**
 - Equilibration Solution (ES): 1.0 ml/vial×1
 - Vitrification Solution (VS): 1.0ml/vial×2
 - 4 Cryotecs
 - 3 Vitri-plates with 3 wells each (and lid)
- **Microscope (with heating stage disabled)**
- **Timer (with count-up function)**
- **Tweezers**
- **Scissors**
- **Micro-pipette (-300µl)**
- **Pasteur pipette (with mouth piece) or Stripper (with stripper tips)**
- **Cooling rack**

Vitri Plate Characteristics



The vitrification 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 (carrier 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°C and 27°C.
 2. Bring out the ES and VS vials to room temperature (26±1°C: 25-27°C) at least 1 hour before performing this protocol.
- (CHECK: Examine the vials by inspecting visually and -touching with your fingers before using them to confirm the presence of any abnormalities, such as unusual colorations or temperatures higher/lower than expected.)**
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, 160-250µm.
 4. In the event that the diameter of the blastocyst to be vitrified is greater than 220µm, conduct the following pre-shrinking process:
 - Immerse the blastocyst in hypertonic solution (DS:WS = 2:3) until it reaches the proper size. This is the most effective and non-invasive contraction method.

[STEP 1]

Equilibration ES (8-15 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.

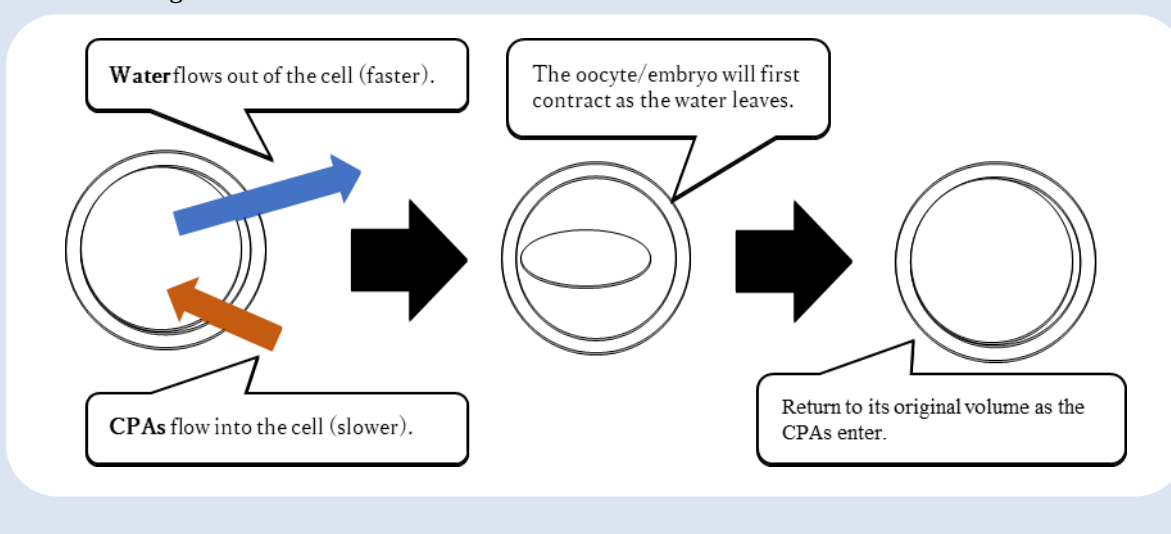


	Figure	Procedure
1		1. Fill well 1 of the Vitri Plate with 300_μl of ES at room temperature (①). 2. Fill each of the other wells with 300_μl of VS at room temperature (② and ③).

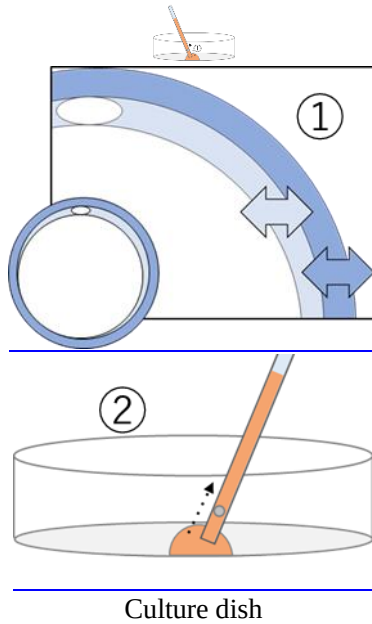
CHECK: Avoid forming bubbles when dispensing.

Attach the chip to the 300_μl micropipette. Aspirate 300_μl of solution into the pipette and expel it inside of the vial. Then aspirate again the solution into the pipette and slowly dispense it. This will avoid the unnecessary formation of bubbles.

CHECK: If bubbles form, let the solution sit.

If bubbles do form in the solution, these will disappear if you simply leave it for a while. Do not attempt to remove the bubbles with the micropipette, as this will change the amount of solution within the well.

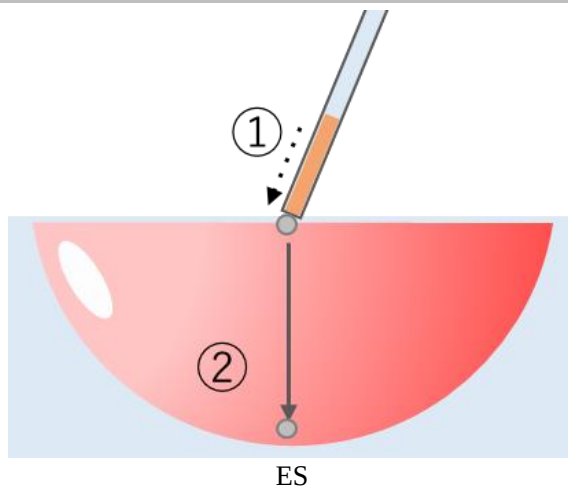
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1. Inspect and note the details of the oocyte/embryo at high magnification, paying careful attention to the size of the zona pellucida relative to the perivitelline space (in oocytes) or the cavity (in blastocysts) in order to confirm that the oocyte/embryo has fully recovered its original volume during equilibration. (①)
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).

CHECK: Note that once the oocyte/embryo is placed in the ES, its volume will begin shrinking immediately. Please confirm the shrinkage; if it doesn't shrink, the oocyte/embryo is not viable.

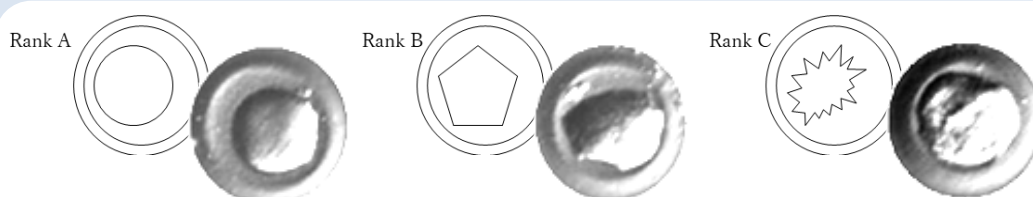
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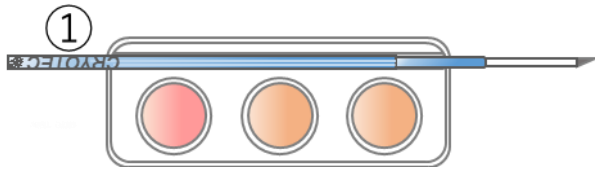
1. Place the oocyte/embryo and culture medium on the surface of the ES, directly in the center (①).
2. Use the timer to begin counting up.
3. The oocyte/embryo will begin to shrink and sink to the bottom of the well (this should occur within 30 seconds) (②). Within 90 seconds, the oocyte/embryo should have reached its smallest size.
4. Continue waiting and observe as the oocyte/embryo fully recovers its original volume.

Evaluating oocyte/embryo quality based on shape during shrinkage

You can gauge the quality of the oocyte/embryo by the membrane permeability of its cell. A high-quality oocyte/embryo will maintain its spherical shape while shrinking (Rank A). If there is variation in the membrane's permeability, irregular edges will form (Rank B), and as the quality decreases it will display an extremely non-uniform shape (Rank C). Please note that these grades do NOT affect survival rate; however, because this represents the quality of the oocyte/embryo itself, it may indicate a difference in fertility, developmental ability, or pregnancy rate at a later stage.



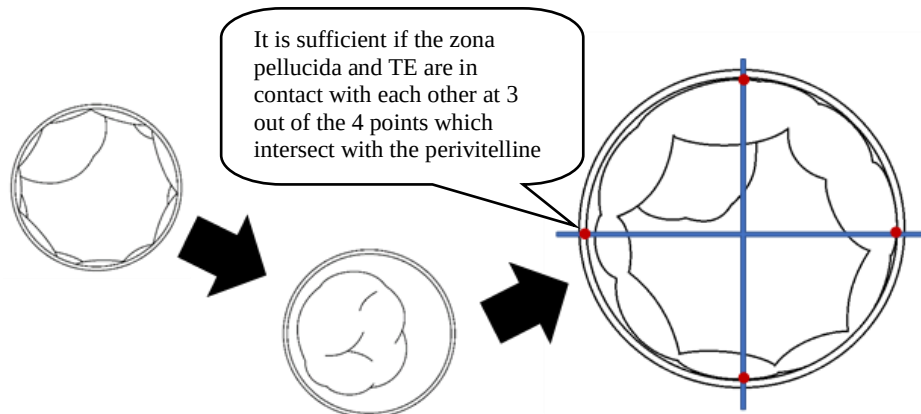
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1. While waiting for the oocyte/embryo to recover its volume, remove the straw containing the Cryotec from the package and prepare the Cryotec. Fill in the oocyte/embryo information on the back side (opposite of the logo) and place the Cryotec in the groove on the Vitri Plate, making sure to keep the logo facing up (①).
2. Prepare the fresh liquid nitrogen in the cooling rack.
3. Once the oocyte/embryo has fully returned to its original size, the **ES** equilibration is complete.

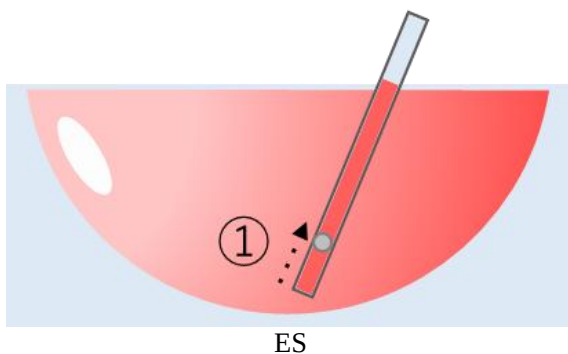
Determining equilibration completion

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 (160µm - 220 µm in diameter) – 15 minutes; 4-8 cell stage embryos - 12 minutes. It has been conclusively determined that sufficient equilibration occurs after 15 or 12 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.

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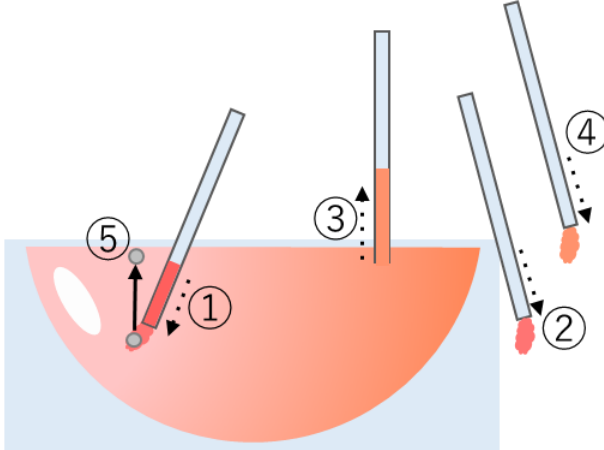


1. Aspirate the oocyte/embryo with a small amount of ES (about the size of 2 oocytes) (①).

[STEP 2]

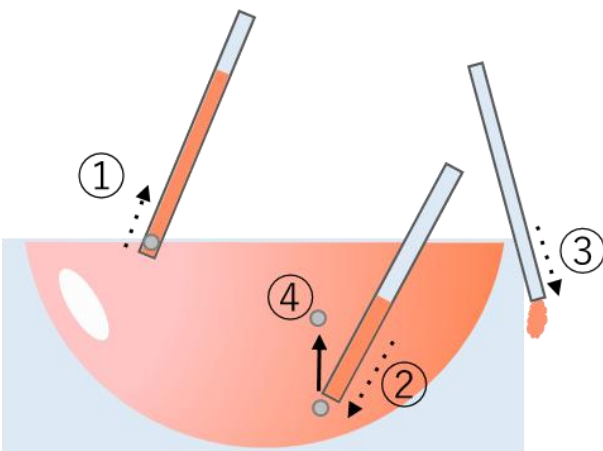
Equilibration in VS1 (30-60 seconds)

The purpose of VS1 is to replace all extracellular ES with VS. This step is complete when the densities of the cell and VS1 are equal.

	Figure	Procedure
1	 VS1	1. Deposit the oocyte/embryo into the middle depth of the left side of the VS1 well (①). 2. Expel all remaining ES within the pipette into the well's draining groove (②). 3. Aspirate fresh VS1 from the edge of the well (③) and quickly expel it (④). 4. The oocyte/embryo will quickly float to the surface of VS1 (⑤). (Be sure to immediately focus on the surface of VS1 as soon as you place the oocyte/embryo.)

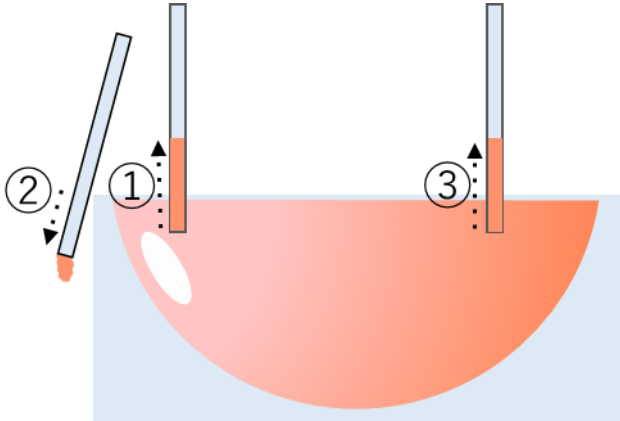
CHECK:

Because the released oocyte/embryo will quickly float to the surface, your microscope's focus should be set on the liquid's surface, allowing you to confirm the presence of the oocyte/embryo.

2	 VS1	1. Aspirate fresh VS1 from the edge of the well, then aspirate the oocyte/embryo into the tip of the pipette (①). 2. Deposit the oocyte/embryo into the bottom of the right side of the VS1 well (②). (Make sure to deposit ONLY the oocyte/embryo.) 3. Expel all remaining VS1 within the pipette into the well's draining groove (③). 4. The oocyte/embryo should float slowly to middle depth and completely stop. This confirms the completion of VS1 equilibration (④).
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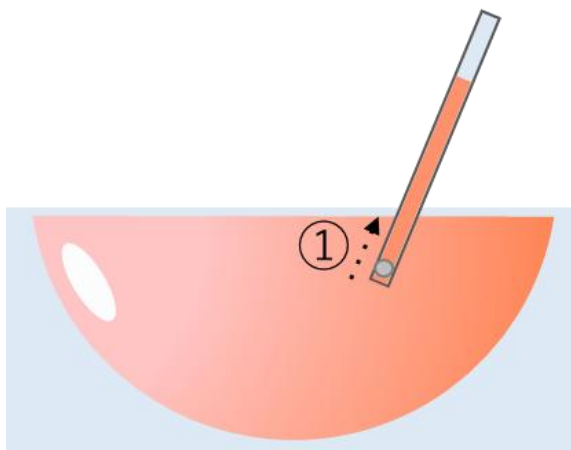
CHECK:

Use high magnification on the microscope to confirm that the oocyte/embryo has completely stopped in the middle of the solution. Make sure that the oocyte/embryo remains in focus for a while, confirming that it has stopped floating.

3	 VS2	1. Aspirate and expel 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)
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CHECK:

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

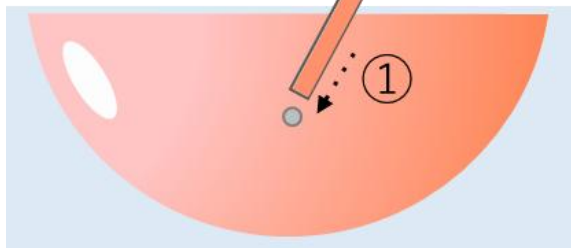
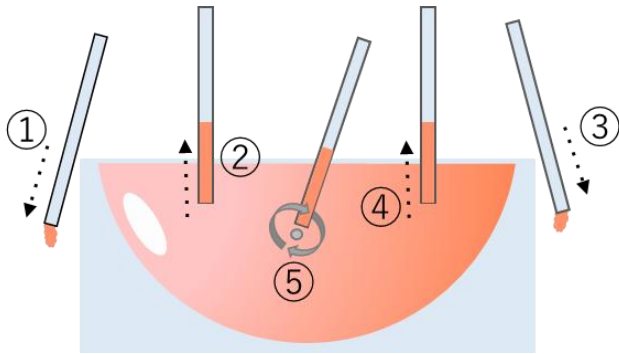
4	 VS1	1. Aspirate the oocyte/embryo within VS1 into the tip of the pipette (①).
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[STEP 3]

Shrinkage in VS2 (10-20 seconds)

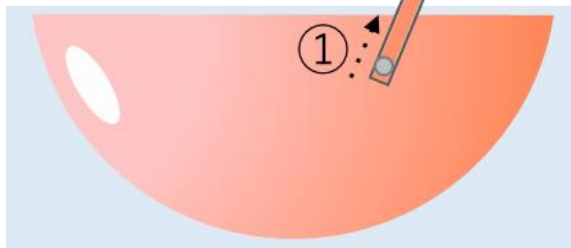
The purpose of VS2 is to confirm that ES has been completely replaced by VS. This is confirmed in two ways:

1) The oocyte/embryo **shrink completely** (into a moon/kidney/bean shape), and 2) the oocyte/embryo **is not floating**. (It means that there is no more ES inside)

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

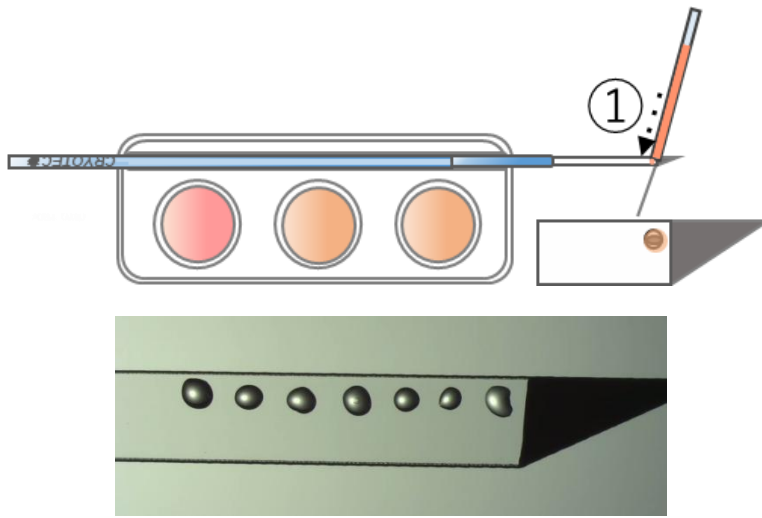
CHECK:

If you can observe the oocyte/embryo's shrinkage by naturally, there is no need to stir it.

3	 VS2	Aspirate the oocyte/embryo into the tip of the pipette (①).
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Placing the Oocyte/Embryo

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

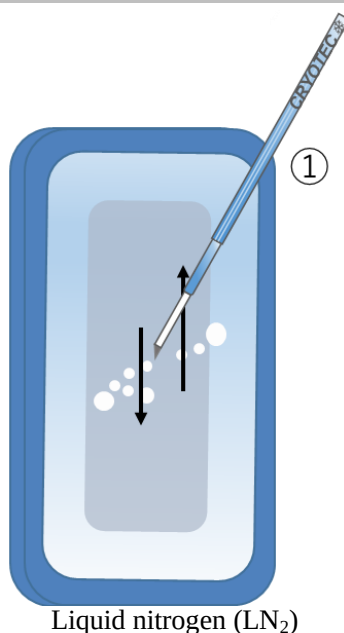
CHECK: 1 oocyte = 1 drop. DO NOT REDUCE the volume of the droplet.

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 Cryotech 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

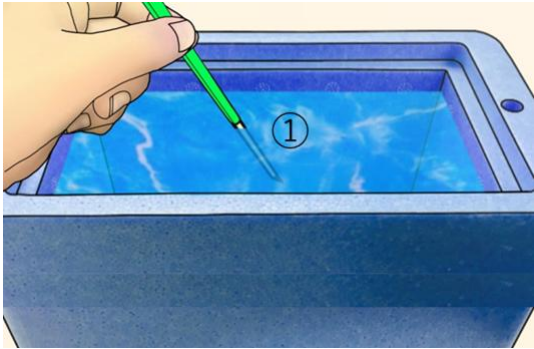
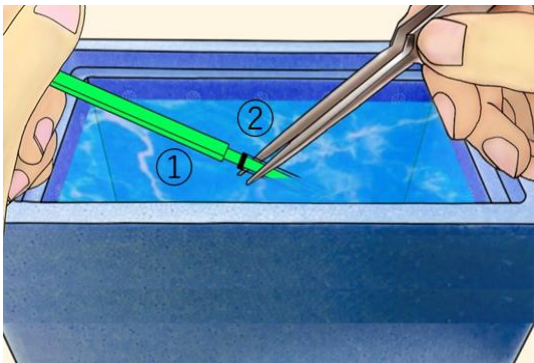
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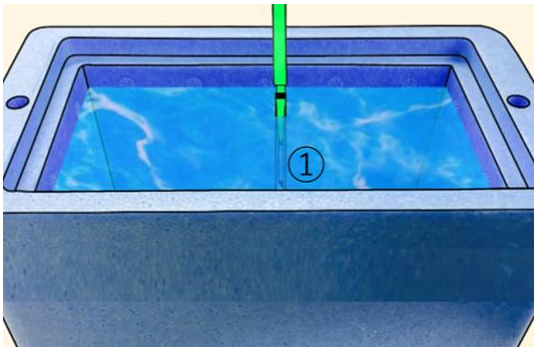
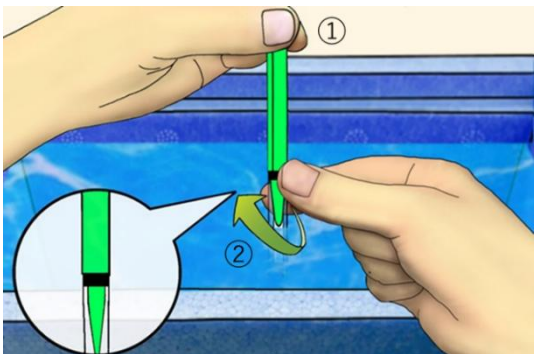
Liquid nitrogen (LN₂)

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 (①).</u>
2		1. Place the cap in the liquid nitrogen. After a few seconds, make sure there are no bubbles remaining in the cap, then gently place the cap over the Cryotec (making sure to keep it under the surface of the liquid nitrogen). 2. <u>Keep the tip of the sheet in liquid nitrogen (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) (②).</u>

CHECK: DO NOT HOLD the cover cap with tweezers by the middle part since it could break.

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 tighten the cap using your fingers (②).

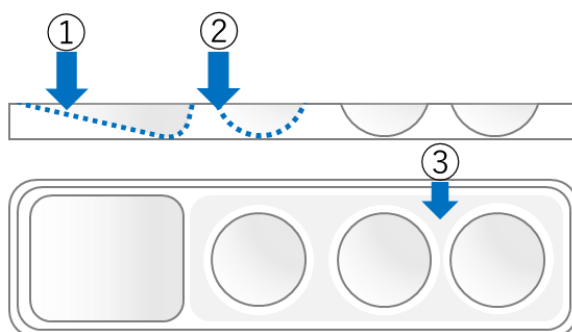
3. Warming Protocol

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 °C to -20 °C, which is the range in which ice crystals are most likely to form.

[Materials]

- **Cryotech® Warming Kit 102**
 - Thawing Solution (TS) 1.8 ml/vial×1
 - Diluent Solution (DS) 0.5 ml/vial×1
 - Washing Solution (WS) 1.0 ml/vial×1
 - 1 Warm Plate with 4 wells
- **Microscope (with heating stage disabled)**
- **Timer (with count-up function)**
- **Tweezers**
- **Micro-pipette (-300µl)**
- **Pasteur pipette (with mouth piece) or Stripper (with stripper tips)**
- **Cooling rack**



Special thawing plate characteristics

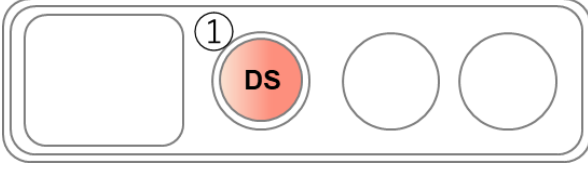
Our Warm Plate, designed exclusively for its use with the Cryotec Method, features ① a rectangular wells inclined for TS use. It also includes ② hemispherical wells and ③ space for expelling solution, similar to the our Vitri Plate. The slope of the TS well is designed to allow stable placement of the 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. Use an incubator to heat the Warm Plate and TS vials (with caps closed) to 37 °C at least 2 hours prior to beginning the thawing process (warming overnight is acceptable).
 - When using a CO₂ incubator, make sure that the TS vial cap is tightly secured before introducing it into the incubator (as above, overnight warming is acceptable).
2. Keep DS and WS at room temperature (26±1°C: 25-27°C) for at least 2 hours prior to thawing.
3. Prepare the liquid nitrogen in the cooling rack. Remove the Cryotec from the liquid nitrogen 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.

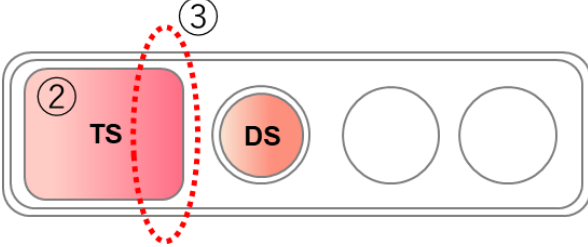
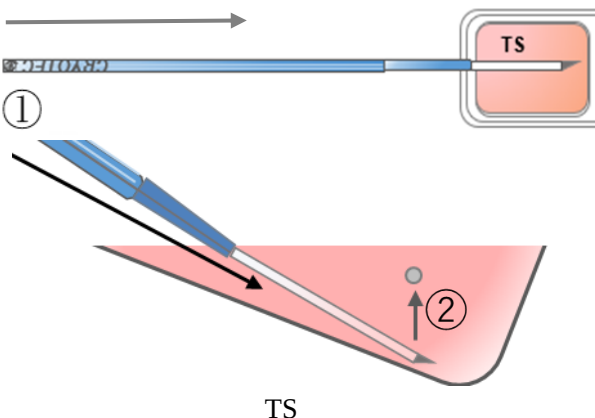
[STEP 4]

Warming in TS (1 minute)

	Figure	Procedure
1		1. Ensuring the Warm Plate has reached 37 °C, remove it from the incubator and place 300µl of room temperature DS into the second well (the first round well) (①).

CHECK:

In order to ensure the maximum temperature of 37°C, it would be best if all room-temperature solutions were placed immediately before they're required (since they would reduce the overall temperature of the Warm Plate). However, because this step only takes 1 minute, there isn't enough time to do this, which is why DS is dispensed in advance.

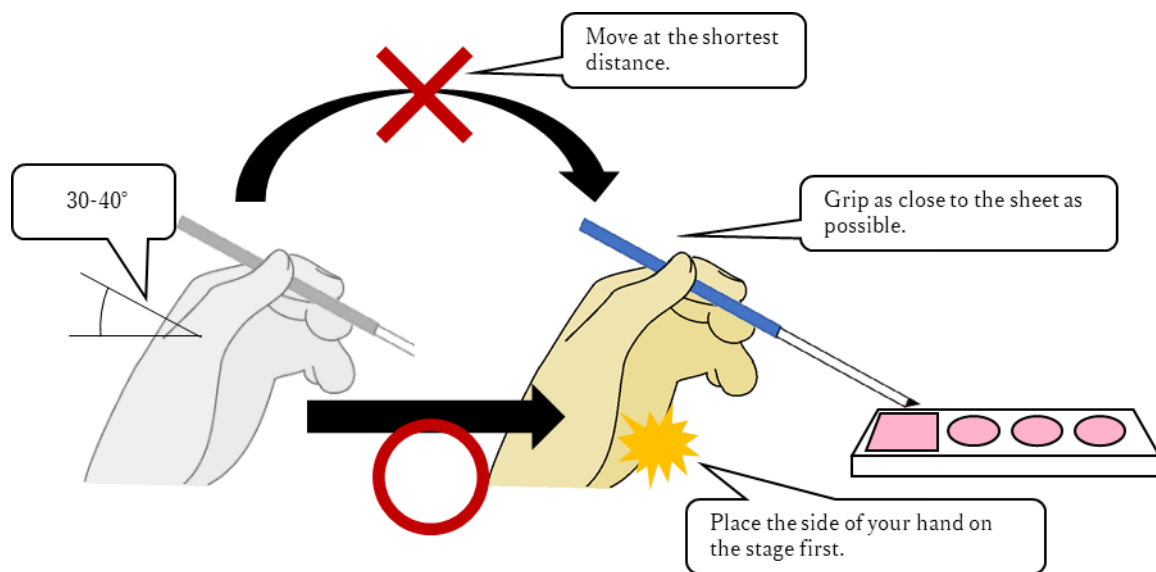
2		1. Remove the TS vial (1.8 ml) which was previously warmed to 37°C from the incubator and pour the entire contents into the TS well (rectangular well) (②). 2. Set the focus of the microscope to the bottom of the TS well (specifically, the position where the Cryotec sheet will rest when inserted; the dotted line on the left) (③).
3		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. The oocyte/embryo begins to float naturally away from the Cryotec sheet during warming (②).

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.

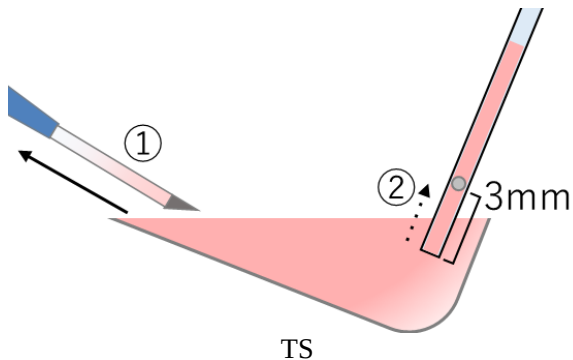
Tips for inserting the Cryotec into the TS well

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 (3 minutes)

	Figure	Procedure
1		1. Let the Cryotec sit for 1 full minute, then slowly remove it from the TS well (①). 2. Aspirate the oocyte/embryo within the TS, then slowly aspirate 3-mm of TS into the pipette (②).

CHECK:

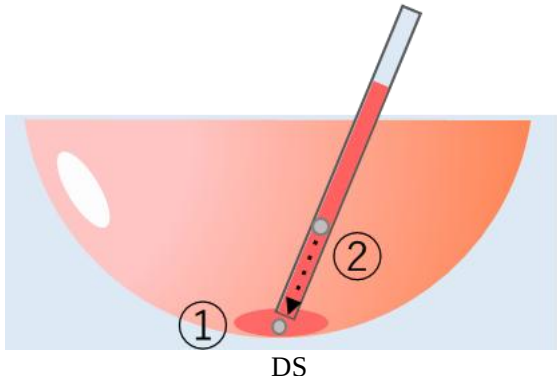
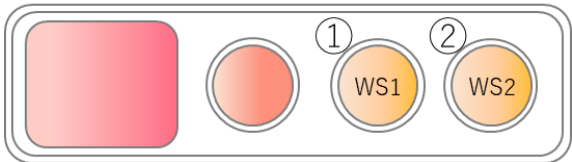
If the oocyte/embryo has still not separated from the sheet after a full minute, place the Pasteur pipette beneath it and apply gentle pressure to separate it. Be sure not to come into direct contact with the oocyte/embryo.

CHECK: If the oocyte/embryo disappears

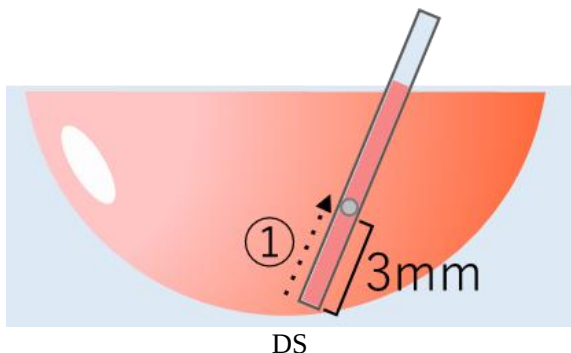
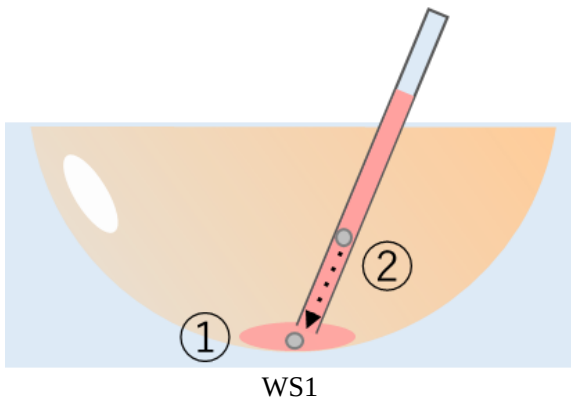
If you successfully place the oocyte/embryo on the Cryotec sheet during the vitrification process, the oocyte/embryo is in the TS well. Our TS solution has minimal toxicity, so you will have time to patiently search for the oocyte/embryo.

CHECK:

You can measure out 3mm using the indicator on the Warm Plate lid.

2		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 3 minutes.
3		1. While waiting for those 3 minutes, place 300-μl of WS into wells WS1 and WS2 (① and ②).

Dilution in WS1 (5 minutes)

	Figure	Procedure
1	 DS	1. Aspirate the oocyte/embryo within the DS, then slowly aspirate 3-mm 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 5 minutes. 4. After 5 minutes, compare the current form of the oocyte/embryo with the previous form you memorized. If you can confirm that the volume of the oocyte/embryo has fully recovered, or is close to it, this indicates that it is alive.

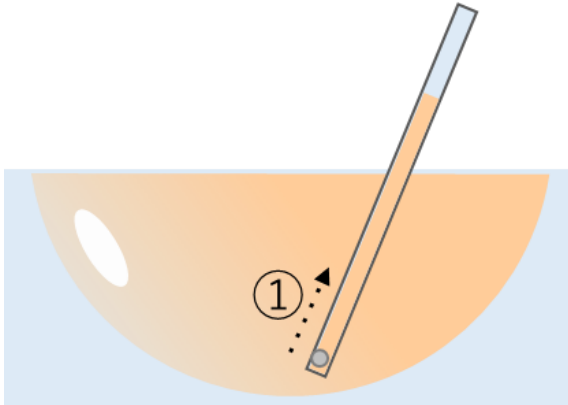
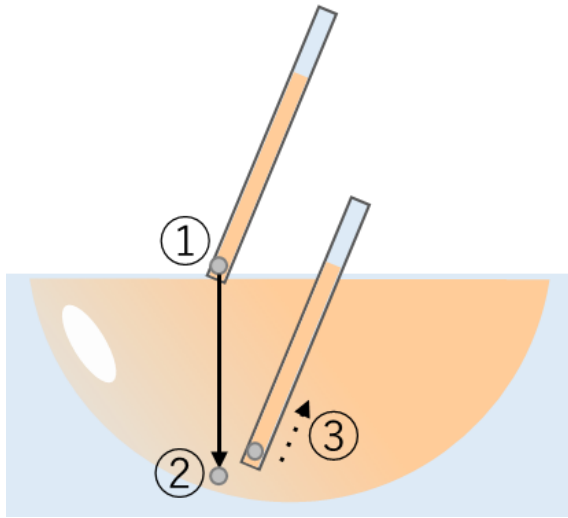
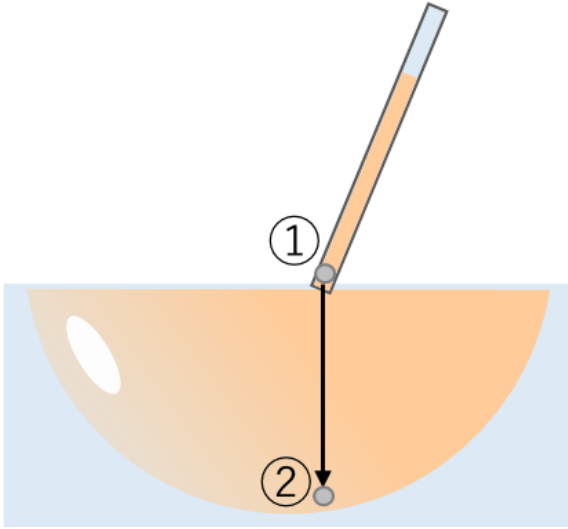
CHECK:

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 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:

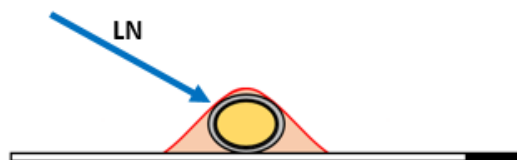
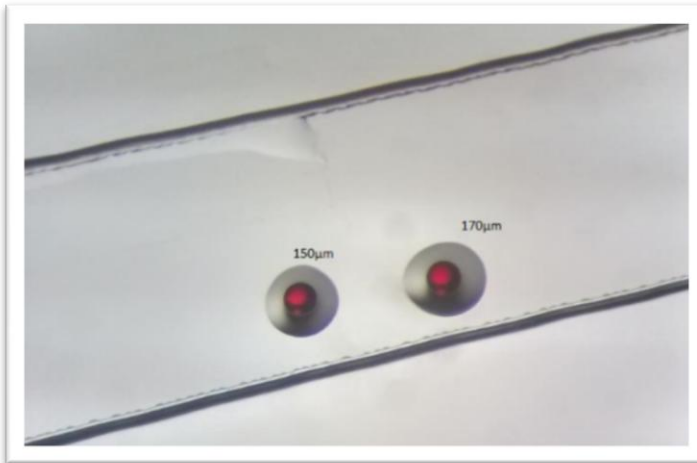
- For a 2-cell stage embryo, 1 blastomere
- For a 4-cell stage embryo, 2 blastomeres
- For an 8-cell stage embryo, 3 blastomeres

Washing in WS2 (1 minute)

	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 a small amount of WS2 and the oocyte/embryo into 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 reach 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.

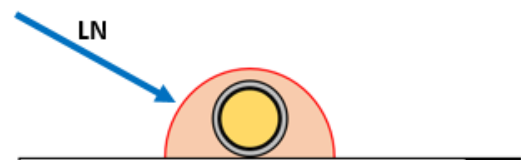
CHECK:

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



A. Minimal volume

- ✗ Oocyte/embryo loses its spherical shape
- ✗ Direct contact with LN
- ✗ Sample gets stuck to Cryotec



B. Without minimizing volume

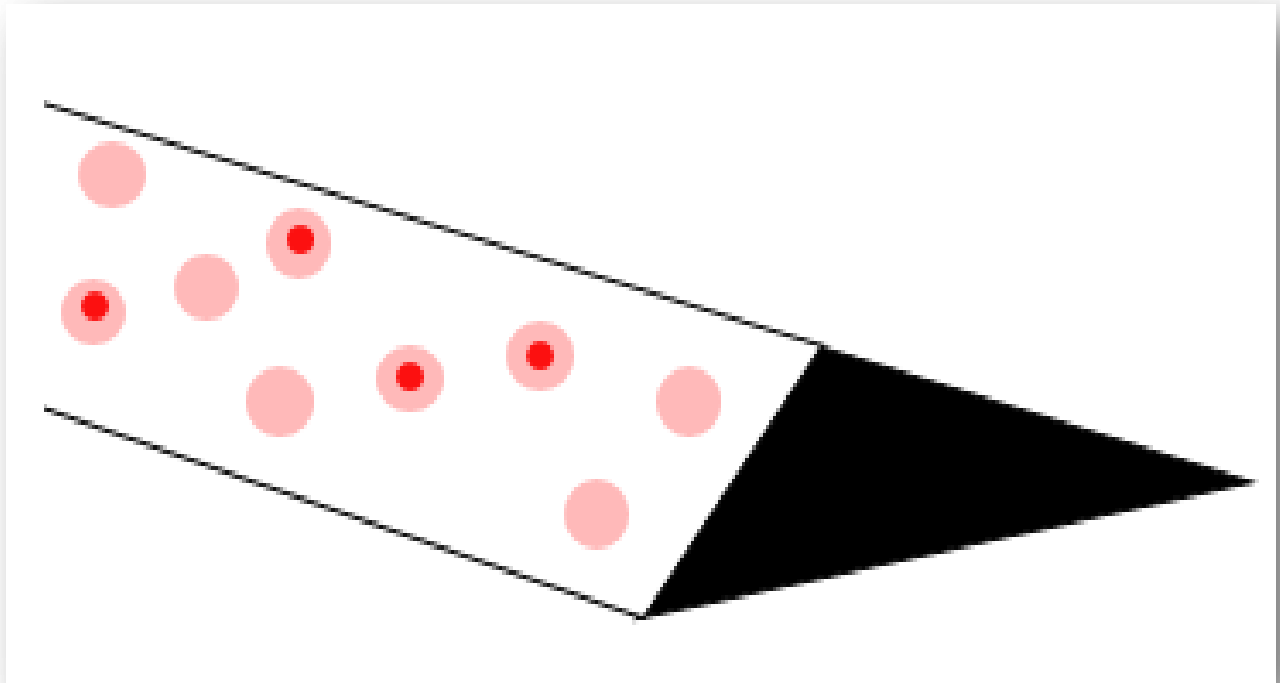
- ✓ Oocyte/embryo keeps its spherical shape
- ✓ Indirect contact with LN
- ✓ Sample comes off itself from the Cryotec



A. Horizontal pipette. It results in droplets of bigger volume and the shape is not spherical.



B. Pipette perpendicular to the sheet. It results in round droplets and with the optimal volume for vitrification.



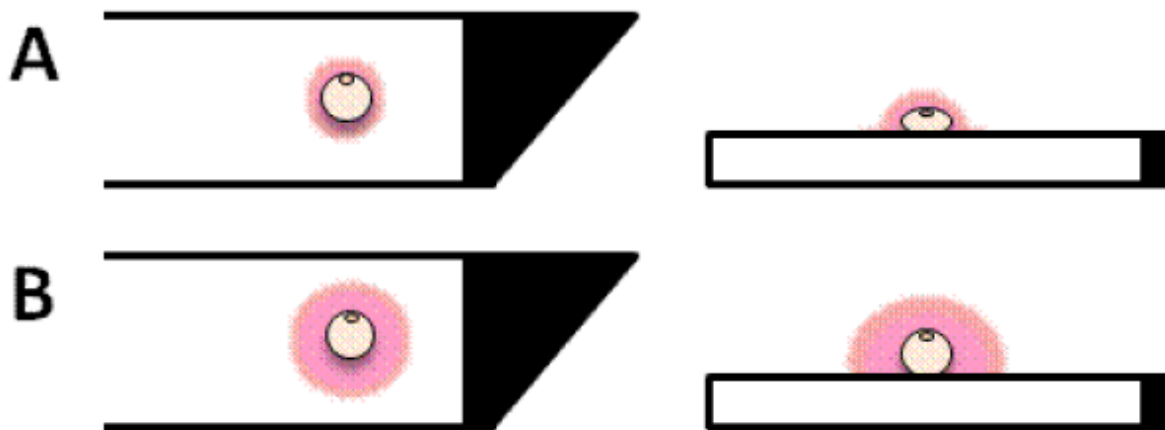


Fig 1

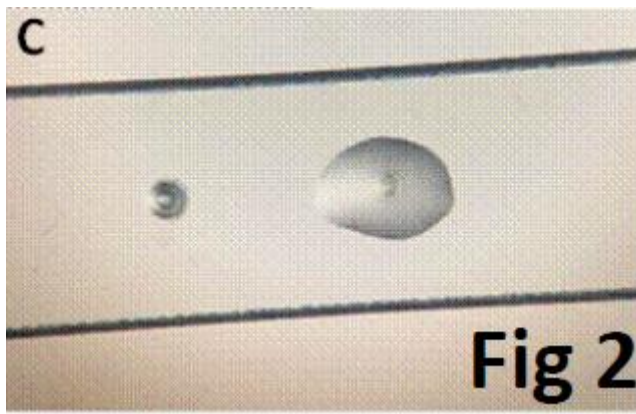
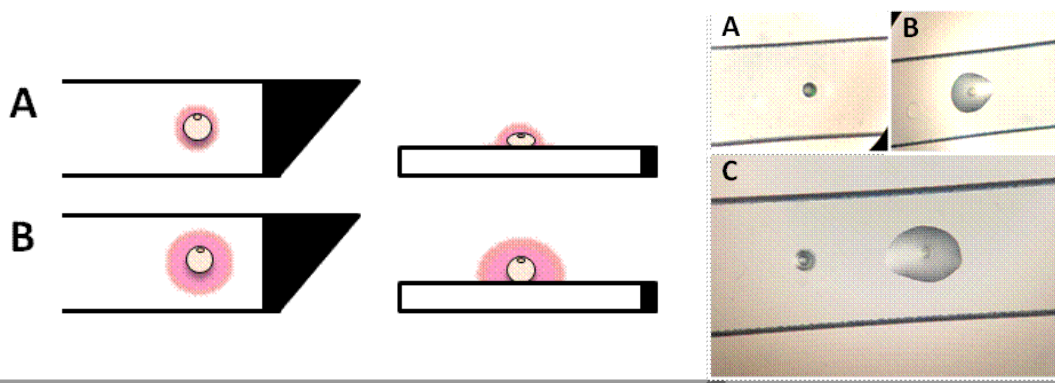


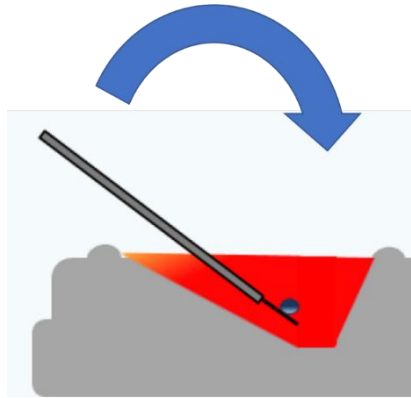
Fig 2



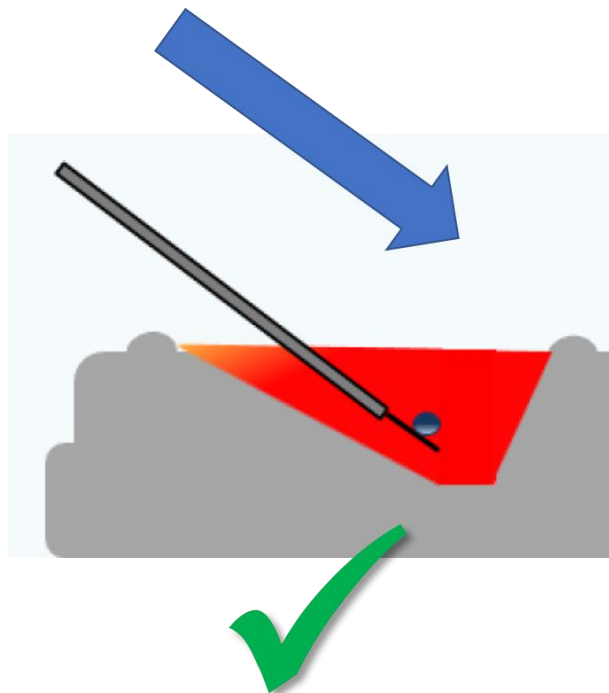
1. Regarding the bubbles after plunging into TS

This happens when we introduce the Cryotec doing a movement perpendicular to the surface of the TS. By doing this movement, the sheet surface exposed is too big and it will bring air inside the TS which will cause the bubble formation. To avoid this, we need to change the movement we do when we introduce the Cryotec into TS: please introduce it in a diagonal movement, following the angle of the TS well in the warming plate. By doing this movement, the exposed surface of the sheet will be much smaller, and it will not carry air into the TS.

Please refer to the following images:



We tend to do this movement when we are in rush, and the fast plunging into the TS in a perpendicular angle will bring a lot of air and make bubbles. And as you mention, the bubbles will attach to the oocyte/embryo and it will float and carry it away and make us lose it. In the case this happens, please search in the surface of the TS, and also search in the shallow part of the well, since the capillarity will attract the bubbles (with the sample) to the nearest wall.



To fix this problem, introduce the Cryotec in a diagonal movement to avoid introducing air into the TS. And please remember to keep an eye on your oocyte/embryo for one minute after introducing the Cryotec into TS because they like to run away!!