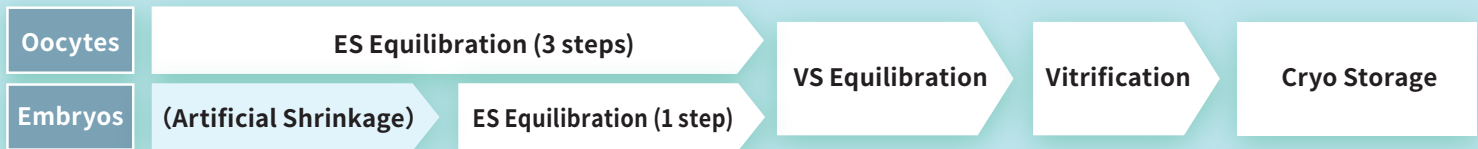


Vitrification Protocol for Oocytes / Embryos

KITAZATO®

Vitrification Procedure



PART 1 Materials Required

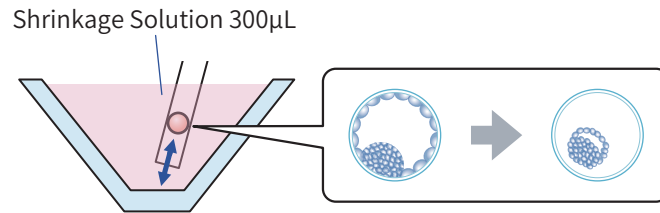
1. Vitrification Media VT601 / VT621 [Ref. 91101 / 91207]
 - No.0 BS (Basic Solution) 1.5mL vial ×1*¹
 - No.1 ES (Equilibration Solution) 1.5mL vial ×1
 - No.2 VS (Vitrification Solution) 1.5mL vial ×2
 - ※Equilibrate all the solutions to room temperature before use
 2. Shrinkage Solution VT525-4 [Ref. 91366]*²
 3. Cryotop (1 Cryotop is for maximum 4 oocytes/embryos)
 4. Oocyte Cryo Plate [Ref. 83061] (for oocytes) / Repro Plate (for embryos)
 5. Pipette for handling oocytes / embryos (hereinafter referred to as “pipette”)
 6. Timer (with count up function)
 7. Liquid nitrogen
 8. Cooling Rack [Ref. 84014] for liquid nitrogen container
 9. Forceps
 10. Micro pipettes: 20μL (for oocytes), 300μL
 11. Cryo storage equipment (cane, etc.)
 12. Stereo microscope (stage must be turned off)
- *¹ For oocytes vitrification
*² For preparation of blastocysts vitrification

TIPS

- Use pipette that has a suitable internal diameter for oocytes or embryos for better handling. (For oocytes: about 120-140μm, for cleavage stage embryos: about 150-180μm, and 180-250μm for blastocysts.)
- All vitrification procedures are performed at room temperature.

PART 2 Preparation before Blastocysts Vitrification

In the case of blastocysts reaching the expanded stage (Grade 4 or higher according to the Gardner classification), there is an increased risk of insufficient replacement of water with cryoprotectant. Therefore, we recommend performing shrinkage artificially using Shrinkage Solution (VT525-4) prior to ES equilibration.



Preparation

Bring Shrinkage Solution to room temperature and dispense 300 µL on Repro Plate, cover with a lid and leave it before use.

ES equilibration procedure will follow when shrinkage is confirmed, so preparation for ES equilibration on P.5 (Prepare one ES well and two VS wells, respectively 300µL) should be made at the same time.

STEP 1

Blastocysts Shrinkage

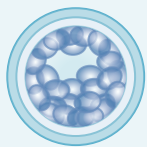
Transfer the blastocysts into the Shrinkage Solution and conduct pipetting to stimulate shrinkage . After confirming the shrinkage of the blastocysts, accelerate ES equilibration on P.5.

※ Proceed to ES equilibration after 2 minutes if the shrinkage of the blastocysts cannot be confirmed.

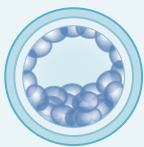
Information

Applicability Guide for Shrinkage Solution

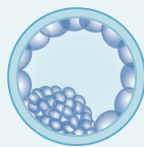
Developmental stages of blastocyst



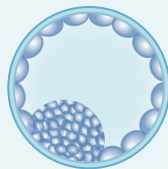
Grade 1
Early blastocyst



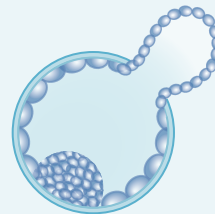
Grade 2
blastocyst



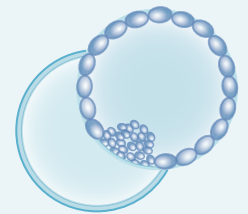
Grade 3
Full blastocyst



Grade 4
Expanded blastocyst



Grade 5
Hatching blastocyst



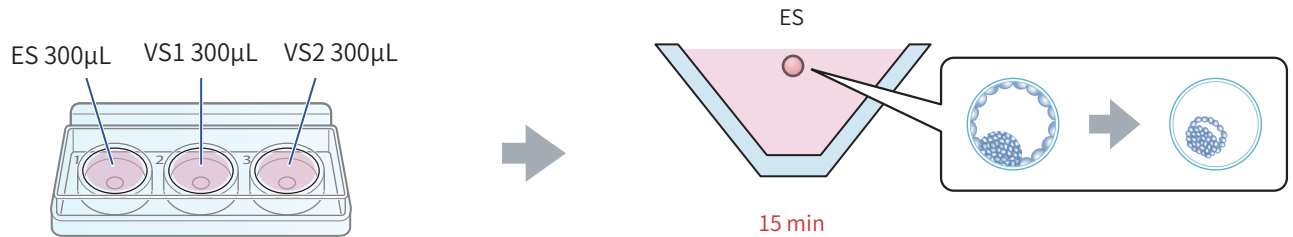
Grade 6
Hatched blastocyst

Shrinkage with a Shrinkage Solution (VT525-4) is recommended from and after the expanding blastocyst stage

PART 3 ES Equilibration

ES equilibration procedure is different for oocytes and embryos. Refer to this page for embryos and P.6 for oocytes. (The subsequent procedures are the same between the two.)

Embryos ES Equilibration



Preparation

Prepare one ES well and two VS wells, respectively 300µL on a Repro Plate. Cover the Repro Plate until ready to use.

STEP 1

Transfer of Embryos to ES

Aspirate the embryos near the tip of the pipette and gently place it with minimal volume of culture medium to the surface center of ES to avoid dilution.

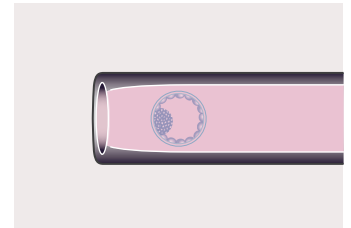


Fig.1

STEP 2

ES Equilibration

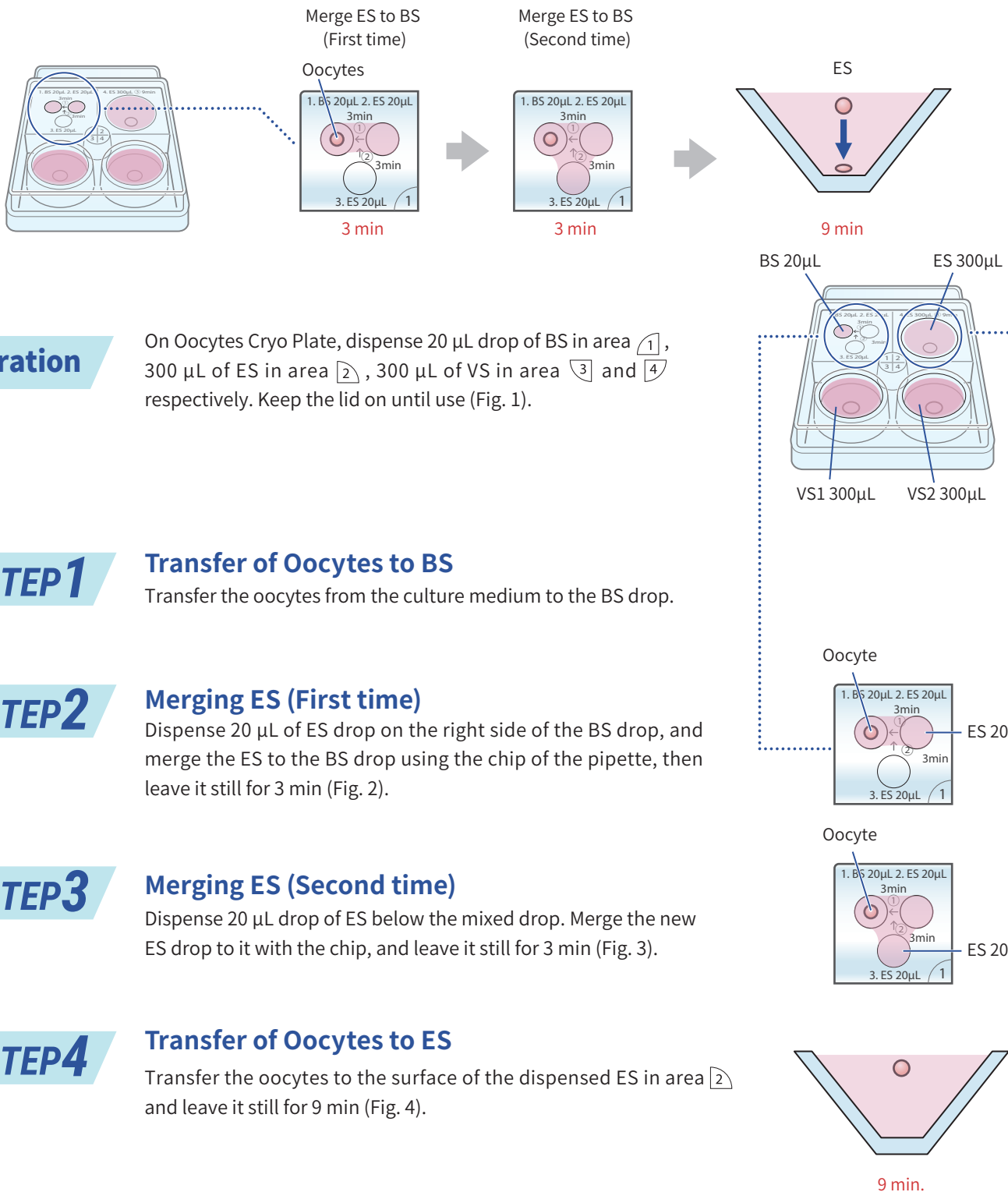
Set the timer and leave the embryos still for 15 min. The embryos placed on the surface will sink as they shrink in about 30 seconds.

to P.7 →

Vitrification Protocol

The ES equilibration procedure is different for oocytes and embryos. Refer to this page for oocytes and P.5 for embryos. (The subsequent procedures are the same between the two.)

Oocytes ES Equilibration



Preparation

On Oocytes Cryo Plate, dispense 20 µL drop of BS in area ①, 300 µL of ES in area ②, 300 µL of VS in area ③ and ④ respectively. Keep the lid on until use (Fig. 1).

STEP 1

Transfer of Oocytes to BS

Transfer the oocytes from the culture medium to the BS drop.

STEP 2

Merging ES (First time)

Dispense 20 µL of ES drop on the right side of the BS drop, and merge the ES to the BS drop using the chip of the pipette, then leave it still for 3 min (Fig. 2).

STEP 3

Merging ES (Second time)

Dispense 20 µL drop of ES below the mixed drop. Merge the new ES drop to it with the chip, and leave it still for 3 min (Fig. 3).

STEP 4

Transfer of Oocytes to ES

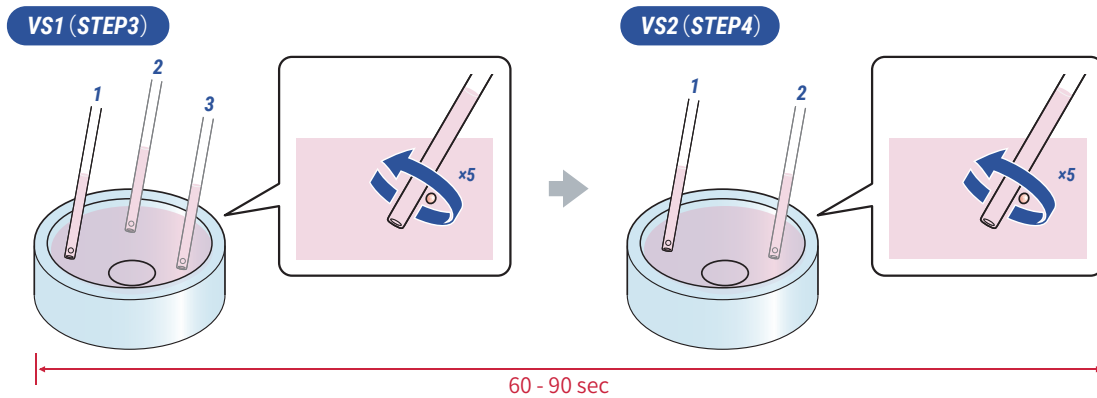
Transfer the oocytes to the surface of the dispensed ES in area ② and leave it still for 9 min (Fig. 4).

TIP

- Although it may be difficult to confirm the recovery of oocytes depending on the angle of observation, STEP4 is completed within 9 minutes even in such cases.
- Merging the drops allows gentle osmolality change, so leave the oocytes untouched.

PART 4 VS Equilibration

From this page onwards, the procedures for oocytes and embryos are the same. Prepare Cryotop and liquid nitrogen before starting the following steps.



STEP 1

Transfer of Oocytes/Embryos to VS1

After completion of ES equilibration, aspirate the oocytes/embryos with minimal volume of ES at the tip of the pipette (Fig. 1). Transfer the aspirated oocytes /embryos to the surface of VS1 and set the timer.

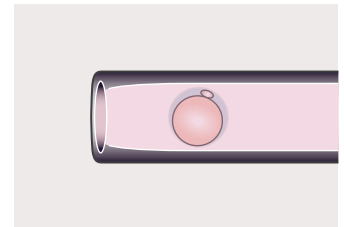


Fig.1

STEP 2

Washing Pipette

Expel the remaining ES in the pipette to the outside the well, and wash the pipette by aspirating and expelling sufficient volume of VS (Fig. 2 and 3).

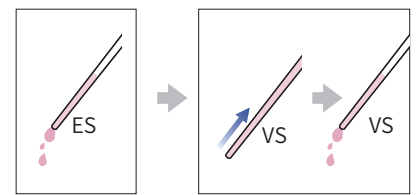


Fig.2

Fig.3

STEP 3

VS1 Equilibration

Aspirate the oocytes/embryos and perform steps the below ① and ② in three positions in the VS1.

- ① Expel the oocytes/embryos from the pipette to the VS.
- ② Stir around the oocytes/embryos 5 times gently for 5 seconds.

The oocytes/embryos are equilibrated and dehydrated by performing steps ① and ② in three different positions.

STEP 4

VS2 Equilibration

Wash the pipette with VS2 as explained in STEP2 (Fig. 3).

Perform ① and ② of STEP3 twice at different positions in the VS2.

Confirm the completion of VS equilibration by the below two criteria before loading oocytes/embryos on Cryotop.

- The oocytes/embryos are shrunk.
- The oocytes/embryos does not rise to the surface. (They stay in focus under microscope.)

If the above two points are not confirmed, perform ① and ② again in no rush.

TIPS

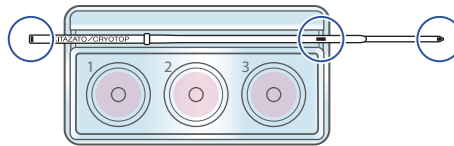
- VS equilibration is performed efficiently by washing the pipette well with VS.
- Referential operation time in VS1 and VS2 together is 60-90 seconds to ensure dehydration. It is recommended to keep oocytes/embryos immersed in VS for at least 60-90 seconds.

PART 5 Vitrification • Cryopreservation

Preparation

Prepare Cryotop with its black identification mark facing up.

Fill up the container with liquid nitrogen as close to its maximum capacity as possible. This is preparation to quickly plunge Cryotop into it.



STEP 1

Loading Oocytes/Embryos on Cryotop

Focus on the black tip mark of Cryotop (Fig. 1), expel the VS horizontally near the black mark, and place the oocytes/embryoed (Fig. 2).

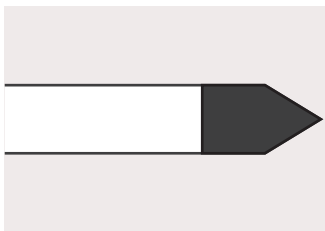


Fig.1

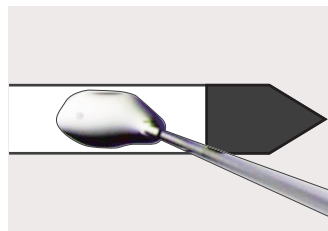


Fig.2

STEP 2

Removal of Excess VS on Cryotop

Aspirate excess VS on the Cryotop from the extended drop edge, preferably from distal point from the oocytes or embryos (Fig. 3). The optimal liquid volume is such that the drop shadow is minimized or disappeared (Fig. 4).

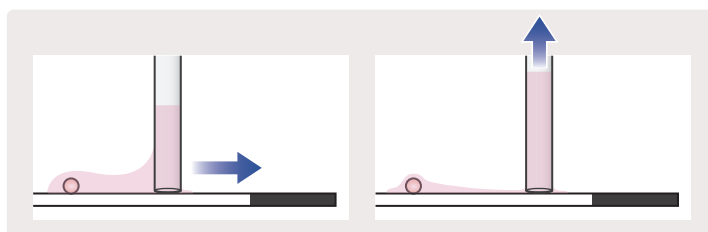


Fig.3

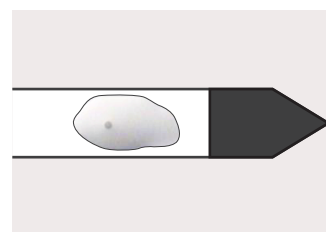


Fig.4

TIPS

- When aspirating oocytes or embryos from the VS2, make sure to hold them at the tip of the pipette.
- Make a droplet on the Cryotop by spreading the VS sideways. It prevents you from aspirating oocytes/embryos while removing excel VS.

STEP 3

Confirmation of Oocytes/Embryos

Remove the pipette from the Cryotop and confirm if the oocytes/embryos are on the Cryotop.

STEP4

Rapid Cooling / Vitrification

Plunge the Cryotop into liquid nitrogen within 1 second for vitrification.

STEP5

Capping Cryotop

Keep the tip of the Cryotop in liquid nitrogen to cap it with the cover straw using forceps (Fig. 1).

Use your fingers to hold the upper part of the cover straw and twist to cap it tight in air (Fig. 2 and 3).

Tip of the Cryotop must be kept submerged in liquid nitrogen .

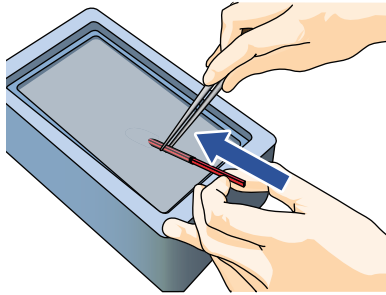


Fig.1

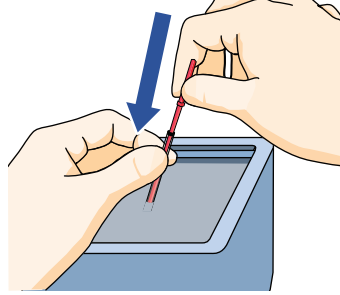


Fig.2

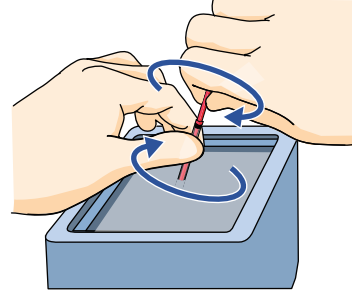


Fig.3

STEP6

Make sure the Cryotop is securely capped. Use cane to store in a liquid nitrogen tank.

TIP

Fill up the container with liquid nitrogen to enhance rapid temperature transition of Cryotop.

Information

Number of Oocytes/Embryos for one piece of Cryotop

Oocytes : Maximum 4 per Cryotop.

Embryos : Depends on the number of embryos to be used after warming.

For example, if single embryo transfer is planned, store one embryo on one piece of Cryotop.

Warming Procedure



PART 1 Materials Required

1. Thawing Media VT602 / VT622 [Ref. 91121 / 91208]
 - No.1 TS (Thawing Solution): 4mL vial x 2
 - No.2 DS (Diluent Solution): 4mL vial x 1
 - No.3 WS (Washing Solution): 4mL vial x 1
2. 35mm dish
 - ※Prewarm TS and 35mm dish to 37°C and equilibrate DS and WS to room temperature before use.
3. Repro Plate
4. Pipette for handling oocytes / embryos (hereinafter referred to as “pipette”)
5. Timer (with count up function)
6. Liquid nitrogen
7. Cooling Rack [Ref. 84014] for liquid nitrogen container
8. Forceps
9. Micro pipette: 300μL
10. Stereo microscope (Stage must be turned off).

CAUTION

- For optimal handling, please use a pipette with an appropriate internal diameter for oocytes (approximately 140-180μm), or embryos (approximately 180-250μm).
- All the warming procedures except for immersion in TS are performed at room temperature.

PART 2 Preparation

STEP 1

Warming TS

Prepare Thawing Media (VT602 / VT622).

Prewarm the TS vials capped and 35mm dishes to 37°C for the number of patients.

Required time: >30 min in an incubator and >5 min in a water bath (40°C). Do not warm more than 8 hours to maintain the quality of the product.

STEP 2

Preparation of Cryotop

Remove the Cryotop from the storage tank and quickly transfer it to a container filled with liquid nitrogen.

Remove the cover straw keeping the Cryotop tip submerged in liquid nitrogen. Place the Cryotop upright in the corner to keep the closest proximity to the TS (Fig. 1 and 2).

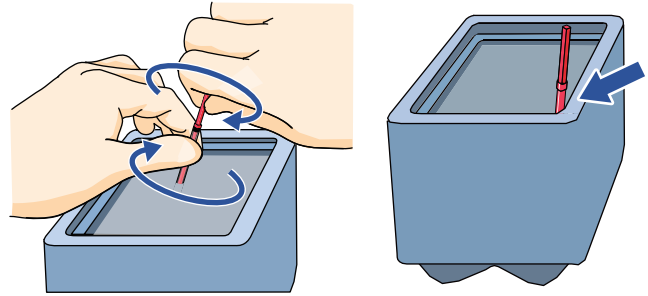


Fig.1

TIP

Fill up the container with liquid nitrogen, so that Cryotop can be transferred into TS in a short distance for warming.

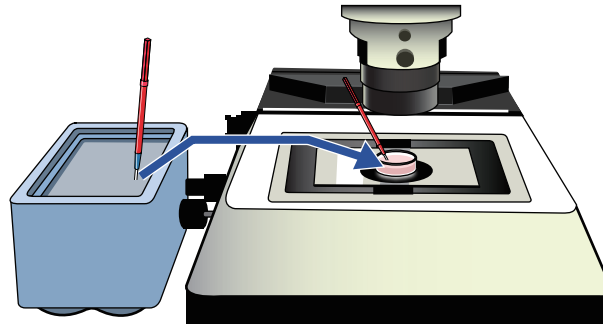


Fig.2

STEP 3

Preparation of the Dish

Prepare one DS well and two WS wells on Repro Plate, respectively 300 μ L and cover with a lid.

After preparing the DS and WS, take out the warmed TS vial and 35 mm dish from the incubator. Pour the entire volume into the dish (Fig. 3).

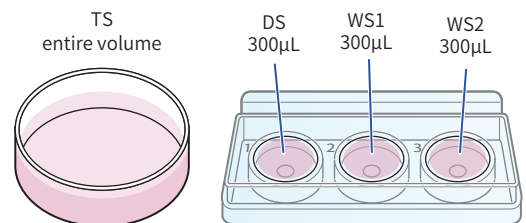


Fig.3

TIP

Invert the TS vial five times to mix its contents for its high concentration of cryoprotectants. The entire volume of TS must be prepared in the dish.

STEP 4

Setting of a Stereo Microscope

Focus on the center of the TS using the pipette in advance (Fig. 4), and set the magnification of the microscope to the minimum (widest field of view).

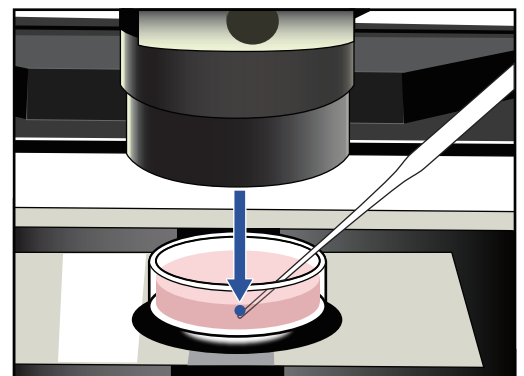
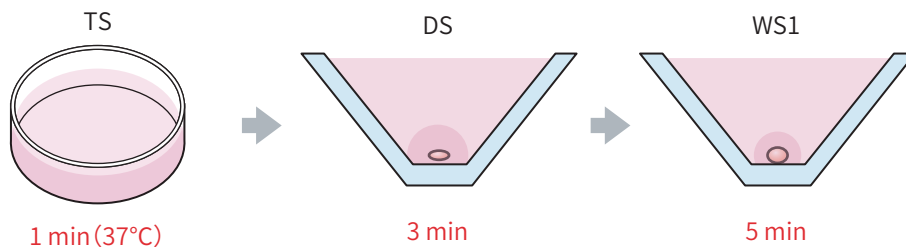


Fig.4

PART 3 Immersion in TS, DS and WS1



STEP 1

Immersion in TS

Start the timer. (It is easier to check time with a count-up function).

Within 1 second, immerse the Cryotop sheet into TS.

Adjust the microscope focus on the tip of the sheet for oocytes/embryos. Continue observing even after the oocytes/embryos naturally detach themselves from the sheet. Don't touch it for one minute.

TIP

If the oocytes / embryos remain attached to the sheet after 1 minute in TS, gently expel some of the TS over them or give vibration to the cryotop by flicking its tip with a pipette, and let them detach. In either method, proceed with care to avoid damaging the oocytes / embryos, regardless of the elapsed time in TS.

STEP 2

Immersion in DS

After immersion in TS, aspirate the oocytes/embryos and additional TS for approximately 2 mm with the pipette (Fig. 1).

Put the pipette tip in the center of the DS well bottom and place the oocytes/embryos in the TS layer expelled in the DS. Leave it for 3 min (Fig.2).

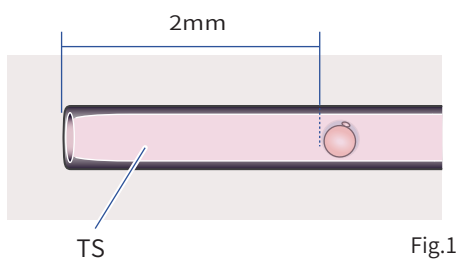


Fig.1

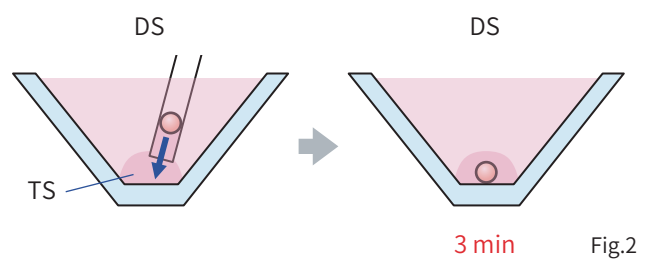


Fig.2

STEP 3

Immersion in WS1

Wash the pipette with DS.

Aspirate the oocytes/embryos and additional DS for approximately 2 mm with the pipette. Put the pipette tip in the WS1 well bottom and place the oocytes/embryos in the DS layer expelled in the WS1. Leave it for 5min.

TIPS

- Gradual osmolality change is important in the dilution process to ensure the previous solution mix gently with a new one.
- The “2mm” aspiration can be approximate. The purpose here is to bring in the previous solution to the next. The width of Cryotop sheet is 1mm, hence a guide when making a 2mm guesstimate.

PART 4 Washing

STEP 1

Washing in WS2

Wash the pipette with WS2.

Aspirate the oocytes/embryos at the tip of the pipette (Fig. 1) and transfer them to the surface of WS2. It is to minimize the volume of the WS1 carried into the WS2.

After the oocytes / embryos settle to the bottom, aspirate and transfer them to the surface of the WS2 and wait for them to settle to the bottom (Fig. 2). No need to check the time here.

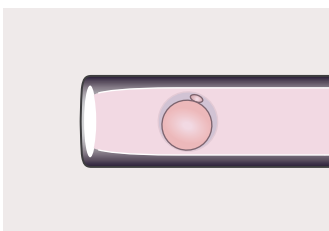
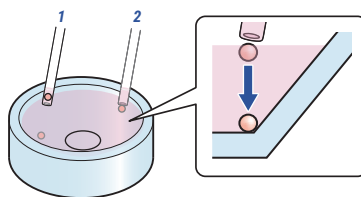


Fig.1



WS2

Fig.2

STEP 2

Recovery Culture

Perform recovery culture of oocytes/embryos after washing.

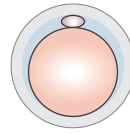
The estimated recovery culture time is as below:

Oocytes: 2 to 3 hours

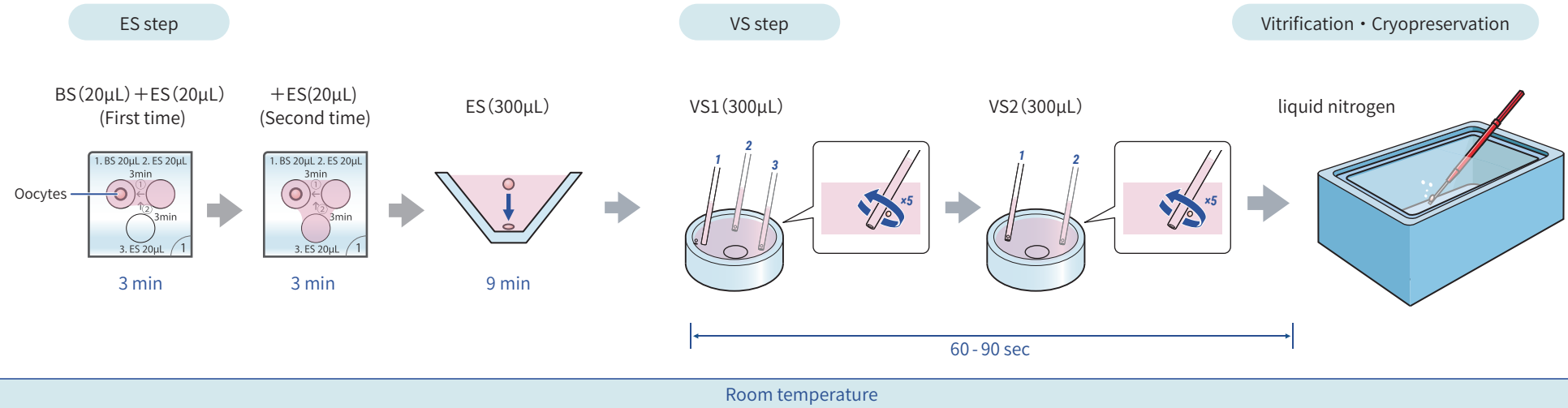
Embryos: 30 min to 2 hours

The recovery culture for embryos is performed to confirm the embryos's viability. Once survival is confirmed, proceed to the next procedure regardless of time. It takes 2 to 3 hours for oocytes spindles to recover. Start ICSI or other following procedures after confirming recovery of spindle or after 2 to 3 hours of recovery culture.

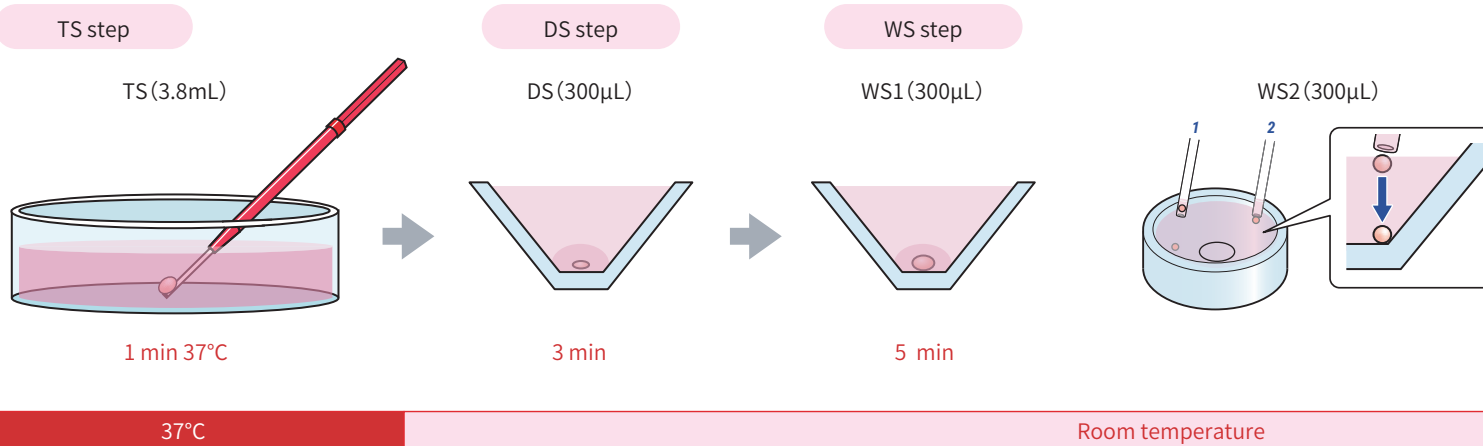
Oocytes Protocol



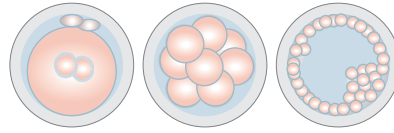
Vitrification



Warming



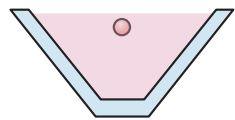
Embryos Protocol



Vitrification

ES step

ES (300 μ L)

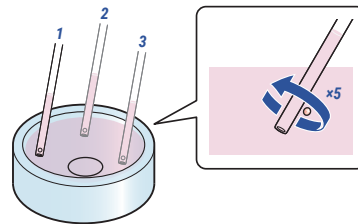


15 min

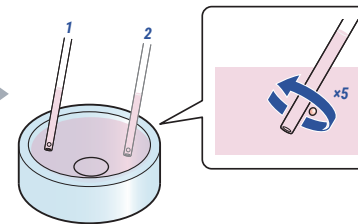


VS step

VS1 (300 μ L)

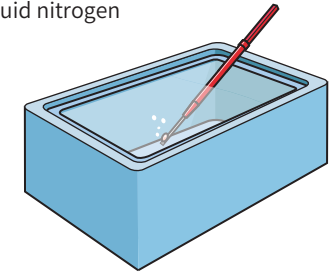


VS2 (300 μ L)



Vitrification • Cryopreservation

liquid nitrogen



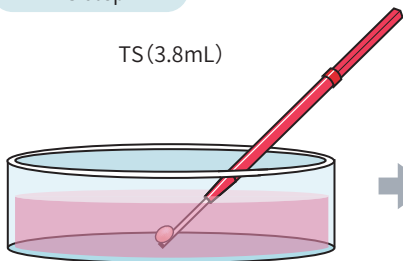
60 - 90 sec

Room temperature

Warming

TS step

TS (3.8mL)

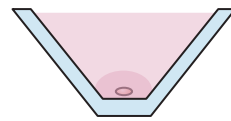


1 min 37°C



DS step

DS (300 μ L)

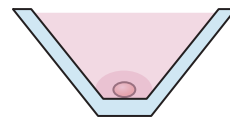


3 min



WS step

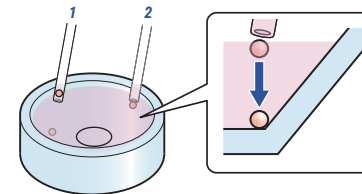
WS1 (300 μ L)



5 min



WS2 (300 μ L)



37°C

Room temperature

Quality Results for Life