THE CRYOTEC METHOD

Manual

For Oocytes, Embryos And Blastocyst Vitrification

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VITRIFICATION

PART 1 - Materials Required

- **Cryotech Vitrification Kit**
  Equilibration Solution (ES): 1 vial of 1.0 ml
  Vitrification Solution (VS): 2 vials of 1.0ml
  4 Cryotecs
  3 Vitri Plates with 3 wells each
- Microscope (Turn off the heating plate)
- Stop watch (with count up function)
- Tweezers
- Micro pipette for 300µl
- Cooling Rack
- Liquid Nitrogen

Note: Solutions to be used within 30 days of opening the vials. Sterile aseptic conditions of dispensing should be maintained.

PART 2 – Preparation for Vitrification

1. Bring ES and VS vials to room temperature (25~27°C) at least 1 hour before vitrification.

2. Write information of oocyte/embryo on the handle of Cryotec, and set on Vitri Plate (Fig.1).

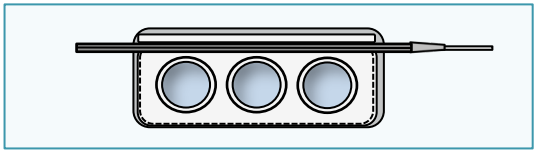

Fig 1. Vitri Plate with Cryotec

3. Fill 90% of the cooling rack with fresh liquid nitrogen

4. Take the culture dish containing oocyte/embryo out from the incubator. Check the quality of the oocyte, embryo or blastocyst.

Note:

Use a right size pasteur pipette.

- 140-150 μm for oocyte and cleavage stage embryo.
- 160~200 μm for blastocyst.

For oocyte vitrification take the cumulus cells off.

Best timing of vitrification of blastocyst: the size (diameter) should be between160~220 μm for perfect survival after vitirfication.

PART 3 – Equilibration (12 – 15 min)

1. *Write ES, VS1 and VS2 on the lid of the Vitri plate.*
   Fill the wells of Vitri Plate with 300µl ES, 300µl VS1 and VS2, respectively (Fig. 2). Put the lid on the Vitri Plate immediately.

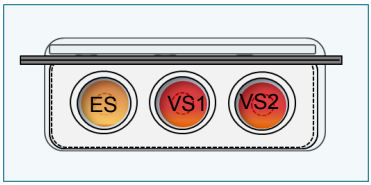

Fig. 2. Preparation of Vitri Plate

2. Aspirate the oocyte/embryo at the middle of the fine part of a pasteur pipette.

3. Put the oocyte/embryo with small amount of medium on the surface of ES well. Start the stopwatch. As oocyte /embryo sink to the bottom, it will shrink (Fig. 3).

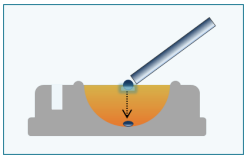

Fig. 3. ES Equilibration

4. Put the lid and wait for the recovery of the shrinkage. When the oocyte/embryo volume is completely recovered, it is the end of this step. If you can't confirm the complete recovery, the limit time of this step is 15 min for oocyte and blastocyst, and 12 min for 4-8 cells embryo.

Note: Oocyte vitrification is complete when the width of perivitelline space becomes equal to the width before immersing in ES.

PART 4 – Vitrification Step in VS1 (30 – 40 sec)

1. Aspirate the oocyte/embryo with **ES** till the middle of the pipette. Transfer the oocyte/embryo at the middle depth of **VS1** with **ES** (Step 1).

2. Expel the remaining **ES** outside the well (Step 2/1) and aspirate fresh **VS1** from the edge of the wall (Step 2/2**)**. Oocyte/embryo floats immediately to the surface of **VS1** (Step 2/3).

3. Aspirate oocyte/embryo at the top inside of pipette. Transfer it again to the bottom of **VS1** (Step 3).

4. The oocyte/embryo floats slowly to the middle depth and stops (Step 4).

5. Expel **the** remaining **VS1** outside the well, and aspirate fresh **VS2**. Aspirate oocyte/embryo at the top inside of the pipette.

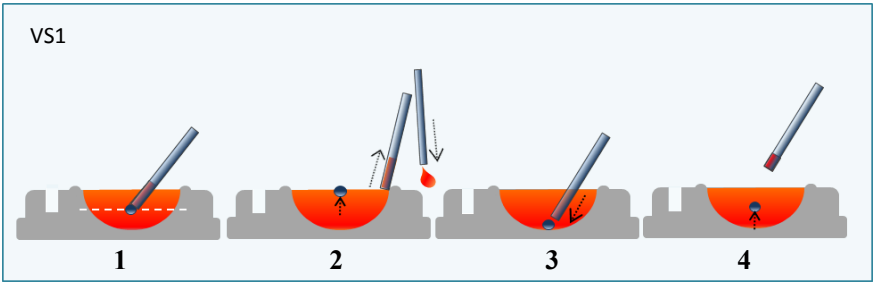

Fig. 4. VS 1 washing of oocyte/embryo (Step 1 – 4)

PART 4 – Vitrification Step in VS2 (10 – 20 sec)

1. Transfer the oocyte/embryo to the middle depth of **VS2** (Step 5).

2. Expel the remaining **VS** outside the well (Step 6/1) and aspirate fresh **VS2** from the edge of the wall (Step 6/2).

3. Expel **VS2** arround the oocyte/embryo and mix the solution around oocyte/embryo to exchange the remaining previous solution (Step 7). Expel and wash inside of the pipette with fresh **VS2**.

4. Take oocyte/embryo at the top of pipette (Step 8)

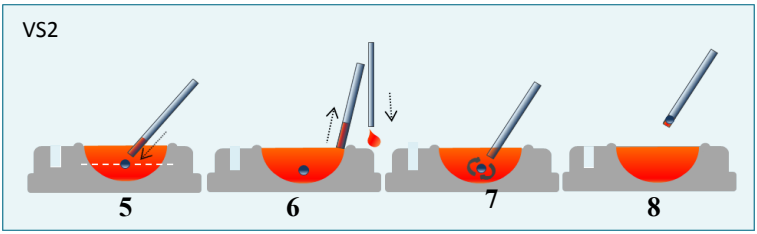

Fig. 5. VS2 washing of oocyte/embryo (Step 5 – 8)

PART 5 – Loading Cryotec

1. Place the oocyte/embryo near the end of Cryotec sheet with minimal volume of **VS2**. (1 oocyte/embryo per droplet is recommended) (Fig. 6). Immediately submerge the Cryotec into fresh liquid nitrogen.
2. Put the straw cap on Cryotec in the liquid nitrogen.

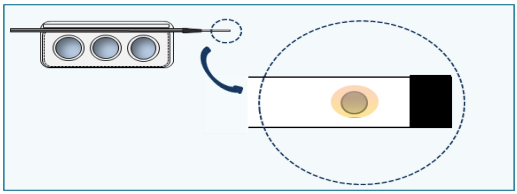

Fig. 6. Oocyte/embryo on the Cryotec

Note: 1 cryotec stores upto 4 oocytes/embryos

Warming

PART 1 - Materials Required

- **Cryotech Warming Kit**
  Warming Solution (TS): 1 vial of 1.8 ml
  Diluent Solution (DS): 1 vial of 0.5 ml
  Washing Solution (WS): 1 vial of 1ml
  1 Warm Plate with 4 wells
- Microscope (Turn off the heating plate)
- Stop watch (with count up function)
- Tweezers
- Micro pipette for 300µl
- Cooling Rack
- Liquid Nitrogen

PART 2 – Preparation for Warming

1. Place the Warm Plate and TS vial (with cap) in the incubator at 37°C >3 hours before warming (overnight storage is recommended). Bring DS and WS vials to room temperature (25~27°C) at least 1 hour before warming.

2. Retrieve the cane with the specific cryotec, quickly immerse the cane in cooling rack filled with fresh liquid nitrogen.

3. When you are absolutely ready, take the warm Plate and TS out of the incubator, and fill the rectangular well with 1.8 ml of TS (Fig. 7, Step 1/1).

PART 3 – Step 1 Warming (1 min)

1. Quickly (within 1 sec) put the Cryotec into TS well. Start the stopwatch for 1 min (Fig. 7, Step 1/2).

2. Oocyte/embryo separates from the Cryotec sheet by itself, and begins to float. Confirm the oocyte/embryo existence in TS well. Do not touch the oocyte/embryo before 1 minute. While waiting, fill the DS well with 300 µl of Dilution Solution (Fig.7, Step 2/1).

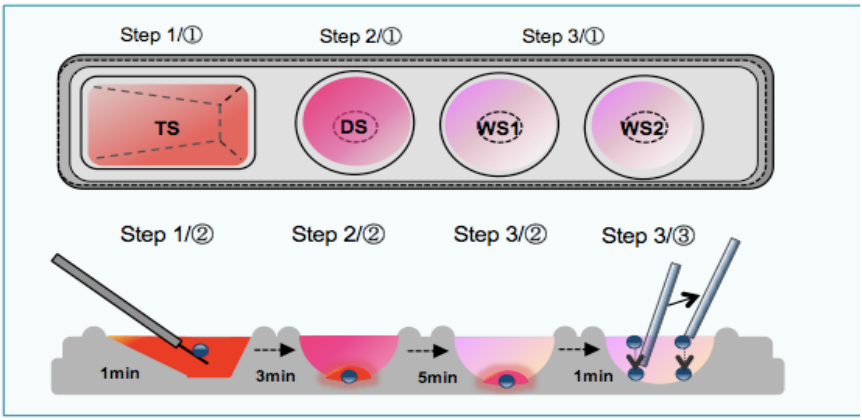

Fig. 7. Procedure for warming (Step 1-3)

PART 3 – Step 2 Dilution (3 min)

1. At the end of 1 min. aspirate the oocyte/embryo and 3mm long of TS into the pipette (Fig. 8.1).

2. Transfer TS to the bottom center of DS (Fig. 8.2), and expel the oocyte/embryo slowly at the bottom of TS layer in DS well (Fig. 8.3). This is for most gradual displacement from TS to DS. Wait for 3 min (Fig. 7, Step 2/2). While waiting, fill the WS1 and WS2 well with 300 µl of Washing Solution (Fig. 7, Step 3/1).

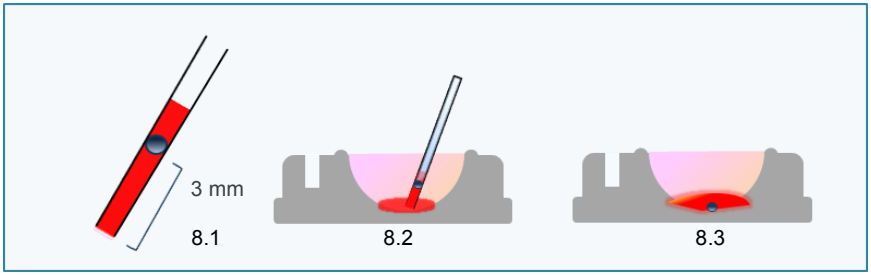

Fig. 8. Gradual replacement of solutions (TS→DS, DS→WS1)

PART 3 – Step 3 Washing

Washing 1 (5 min)

1. Aspirate the oocyte/embryo and 3 mm long of DS into the pipette (Fig. 8.1).

2. Transfer DS to the bottom center of WS1 (Fig. 8.2), and expel the oocyte/embryo slowly at the bottom of DS layer in WS1 well (Fig. 8.3). This is for most gradual displacement from DS to WS1. Wait for 5 min (Fig. 7, Step3/2).

3. Give a survival judgment at the end of this step depending on the recovery of the shrunken oocyte/embryo.

Washing 2 (1 min)

1. Aspirate the oocyte/embryo with minimal volume of WS1.

2. Put the oocyte/embryo on the surface of the WS2 well (Fig. 7, Step3/3). When oocyte/embryo sinks to bottom, aspirate and put on the surface. It will again sink to the bottom, thus washing is done twice.

3. Put the oocyte/embryo in the droplet of the culture media for the recovery for ICSI and ET.

Note: After the warming, 2 to 4 hours culture for oocyte, and 3 hours for embryo is recommended

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