THE NEST
OF BLUE SKY FERTILITY

The Nest **Thawing Protocol**

with Kitazato kit, Reproplate and Cryotop straws

Materials/Supplies

- Kitazato Thawing Media (Below media is good for up to thawing 4 straws).

  No.1 Thawing Solution (TS): 2 X 4ml vial

  No.2 Diluent Solution (DS): 1 X4ml vial

  No.3 Washing Solution (WS): 1 X 4ml vial

- Repro Plate or Oosafe 6-well dish.
- Petri Dishes (35mm, Falcon 351008 or equivalent)
- Cooling Rack (Ref. 84010 or equivalent): Blue Styrofoam box for liquid nitrogen.
- Stripper tips (170-200um).
- Heated dual stage with Stereomicroscopes.
- Stopwatch or Timer (with count up function will be ideal).
- Liquid Nitrogen.
- Tweezers.
- Micro pipette: 100-1000uL
- Egg/embryo culture media with 10 and 20% protein

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Preparation for Oocyte thawing

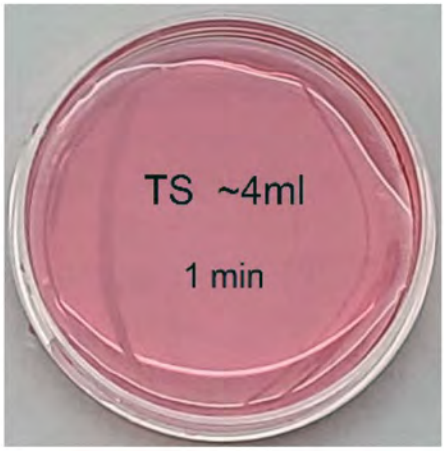
1. Warm TS vial (sealed) with a petri dish in an incubator or warm chamber to 37-38°C (>1.5hours).

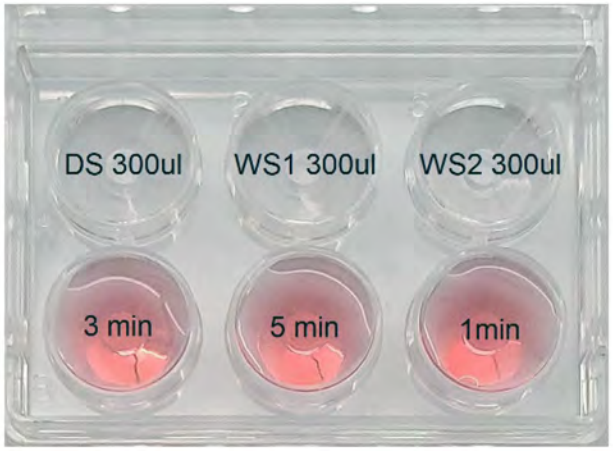
2. Take out DS and WS from refrigerator to warm at room temperature (25~ 27°C).

3. Retrieve the cane which has the specific Cryotop, quickly immerse the cane in a Cooling Rack filled with fresh liquid nitrogen. Retrieve the specific Cryotop from the cane in the liquid nitrogen. Check the information of the donor on the label of Crotop.

4. Write DS, WS1 and WS2 on the lid of a Repro Plate. Gently invert each vial of DS and WS twice to mix contents. Drop 300uL each for DS, WS1 and WS2 on the Repro Plate with micro pipette. Place it on the micro- scope stage and lid it.

5. Remove TS vial and the Petri Dish from the incubator and place the PetriDish on the microscope stage. Gently invert the vial of TS twice to mix contents and pour the full contents into the petri dish.





- Pour whole (4ml) TS medium into the petri dish right before egg thawing.
- Alternatively, pour whole TS medium into the petri dish and put it in a warm chamber.
- The petri dish with 4 ml of TS media is good for two times of thawing.

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Thawing (Warming) procedure

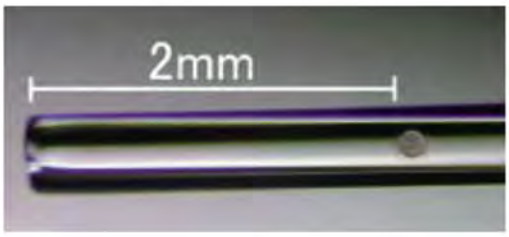
1. Carefully twist and remove the straw cap from the Cryotop in liquid nitrogen. Prop it against the corner of the Cooling Rack.

2. Be ready to use stripper tip(s). Set up the stopwatch(with count up function will be ideal). Check the time with the stopwatch for the following steps.

3. Quickly immerse Cryotop sheet into TS on the microscope stage. It should be within 1 second (Don't do this too quickly. It will create air bubbles). Find the Oocyte(s) adjusting the focus on the black mark area of the straw tip. The egg(s) will come off from the straw, otherwise aspirate it after blowing small amount of TS medium on the eggs. One minute after immersing into TS, gently aspirate the Oocyte(s) with the stripper tip and move to DS drop (Don't carry over big volume from TS to DS. Aspirate only 2mm from the end tip).

4. Blow out only TS into the BOTTOM center of DS slowly, then gently place the Oocyte(s) on the bottom of the TS layer. Leave it for 3 minutes. This is for mostly gradual displacement from TS to DS.

5. 3 minutes later, after immersing into DS, gently aspirate the Oocyte(s) in DS with the stripper tip. Also, aspirate DS until theOocyte (s) reaches 2mm from the tip of the stripper tip.



6. Blow out only DS into the BOTTOM center of WS1 slowly, then gently place the Oocyte(s) on the bottom there. Leave it for 5 minutes.

7. 5 minutes later, after immersing into WS1, aspirate the Oocyte(s) with minimal volume of WS1 with striper tip and transfer it to the TOP center of WS2. After the Oocvte(s) free-falls to the bottom of WS2, do the same work again in WS2 (See below illustration) .le You can perform WS2 wash step at 37°C heated stage.

8. Transfer the oocyte(s) to a culture dish containing the culture medium with 20% SPS. Incubate the oocvte(s) in a 37°C incubator to complete recoverv. ICSI can be performed in 2-3 hours.

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Additional instructions:
Please do not deviate from this protocol.

1. Temperature of TS medium

a. Temperature of TS medium at the time of egg thawing is very important. Keep TS media in a warm chamber with temperature 37-38°C until thawing.

b. Pour whole amount (4ml) into the 35mm falcon petridish right before egg thawing

c. If there is any delay, you can store the 35mm falcon petridish containing TS solution in the 37-38°C warm chamber or incubator without COz gas.

2. Dishes for TS step

a. Dish for egg thawing (TS step): We recommend to use petridish (35mm, 4ml TS solution). We can use up to 2 times the same solution. After using TS solution once, please keep the petridish in 37-38°C warm chamber or incubator without COz gas for 10-20 minutes before reusing it.

b. Some embryologists like to use Reprolife warm plate or Inner-well dish(organ culture dish) for TS step (see below pictures). If vou want to use those dishes, we recommend to make 2 ml of TS media aliquots and warm aliquoted TS in the warm chamber or incubator with no CO2 as at least for 1 hours. Pour whole amount(2ml) in the prewarmed Reprolife warm dish or inner-well dish right before eag thaw
(Use only one time and discard it.)

3. Cryotop straw: Finding the eggs

The frozen eggs are located on the same side of Identification marks.