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

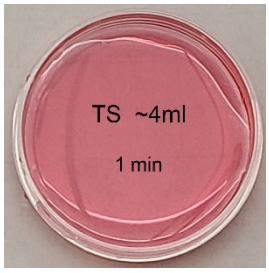
- Repro Plate or Oosafe 6-well dish.
- Petri Dishes (35mm, Falcon 351008 or equivalent)
- Cooling Rack (Ref. 84010 or equivalent): Blue Styrofoam box for liquid nitrogen.
- Stripper tips (170-200um).
- Heated dual stage with Stereomicroscopes.
- Stopwatch or Timer (with count up function will be ideal).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

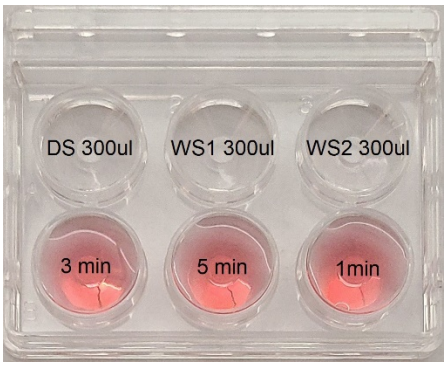
Preparation for Oocyte thawing

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 Cryotop.

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 300µL 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.

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.

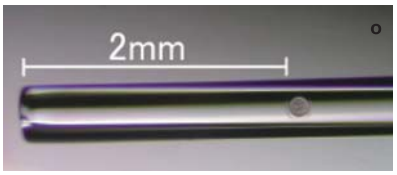
2. Be ready to use stripper tip(s). Set up the stopwatch(with count up function will be ideal). Check the time with the stopwatch for the following steps.

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 Oocyte(s) free-falls to the bottom of **WS2,** do the same work again in **WS2** (See below illustration).

   ☞ 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 oocyte(s) in a 37°C incubator to complete recovery. 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 CO_2 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 CO_2 gas for 10-20minutes 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 you 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 CO_2 gas at least for 1 hours. Pour whole amount(2ml) in the prewarmed Reprolife warm dish or inner-well dish right before egg thawing. 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'.