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Oocyte Vitrification Protocol with Vit Kit - Freeze NX

The following protocol is for use of Vit Kit - Freeze NX (PN 90188) with your vitrification/cryostorage device of choice. Please familiarize yourself with proper handling of the device. Vit Kit - Freeze NX contains Equilibration NX - ES (ES), Vitrification NX - VS (VS), and Washing NX - WS (WS).

Procedures must be performed at room temperature (20–27°C). It is advised to minimize exposure of specimen to light during equilibration in ES and VS solutions. Retrieved oocytes should be denuded with hyaluronidase to confirm they are MII.

INITIAL PREPARATION

1. Bring the quantity to be used of ES, VS, and WS to room temperature (20–27°C).

   Avoid bringing the entire vials of ES, VS, and WS to room temperature repeatedly when a partial of the solution is needed each time. It is better to aliquot the quantity to be used and return the vials to 2–8°C right after aliquoting.

2. Fill a liquid nitrogen reservoir with liquid nitrogen (LN_2) – sufficient to achieve a depth of 4 inches or to completely submerge cryotube on cane – and place close to microscope. Attach a cryotube or goblet (uncapped) to the bottom clamp of a cryocane and submerge in the liquid nitrogen in preparation for storage of the vitrified specimens.
3. Determine the number of specimens to be vitrified.
4. Label each sterile Petri dish (bottom) and vitrification device with necessary information.
5. Carefully examine the vitrification device before starting procedure.
6. Gently invert each vial of ES and VS to mix contents before use.

OOCYTE VITRIFICATION PROTOCOL

7. Aseptically dispense 20 µL drop of WS, ES1, and ES2 in close proximity and ES3 on an open dish or inverted lid of sterile Petri dish as shown in Figure 1, and place the dish on the microscope stage:
   - one (1) 20 µL drop of WS
   - three (3) 20 µL drops (60 µL total) of ES (ES1, ES2, ES3)
8. Remove the culture dish containing MII oocytes from the incubator and check the quality of the specimens under microscope. Where possible, select only the best quality MII stage oocyte(s).

Minimize the exposure of the specimen(s) to light during equilibration in WS, ES, and VS drops.

9. Transfer the oocyte (up to 2 at a time) with minimal volume of medium from the culture dish (in incubator) into the 20 µL drop of WS for 1 minute.
10. Merge the drop of ES1 to WS (see Figure 1, arrow 1) with the tip of the transfer pipette and allow spontaneous mixing of the two solutions to occur for 2 minutes.
11. Then merge the drop of ES2 (arrow 2) to the middle of the previously merged drops and leave for 2 minutes.
12. Transfer the oocyte(s) with minimal volume of solution from merged drop to ES3 drop for 6–10 minutes.

    Equilibration of oocyte(s) in ES3 is complete when the thickness of the zona pellucida and perivitelline space is equal. The oocyte(s) will settle to the bottom of the drop within 3 minutes.

13. During the equilibration time in ES3, aseptically dispense one (1) 50 µL drop of VS prior to complete equilibration and prepare the vitrification device of choice for loading. (Figure 2).

The following steps should be completed in 80–110 seconds.

Exposure of specimens to VS should be limited to prevent cytotoxicity. Specimen(s) tend to float in VS, so adjust the focus through the microscope to maintain continuous visualization during exposure and keep the tip of the transfer pipette nearby to assure rapid transfer between drops.

14. Rinse and fill the transfer pipette tip with VS immediately before equilibration in ES is complete and draw up the specimen(s) with minimal volume of ES into the pipette tip and transfer into the drop of VS for 60 seconds. Unload oocytes to the bottom of VS. While unloading, oocytes will float to the top of VS. To ensure complete rinse with VS, gently move the oocytes back to the bottom center of VS by pipetting.

    During this process, oocytes will be dehydrated and float back again.

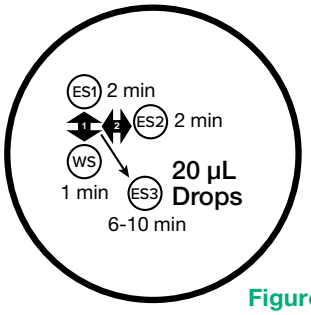

Figure 1

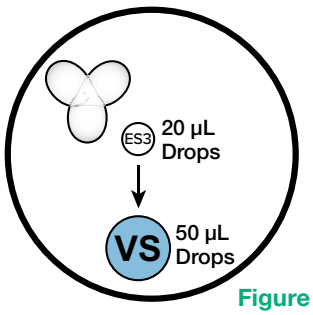

Figure 2

Oocyte Vitrification Protocol with Vit Kit - Freeze NX

15. **Load and seal the vitrification device as directed by manufacturer**
16. Place the vitrified specimen(s) on the vitrification device of choice into the submerged LN_2 filled cryotube or goblet (on the cryocane) (Figure 3). Cap the cryotube or goblet, or attach upside down with another uncapped cryotube in order to secure the vitrified device in liquid nitrogen.
17. Move the LN_2 reservoir close to the LN_2 cryotank and transfer the cryocane with contents to the cryotank for long-term storage.

For additional details on the use of these products, each laboratory should consult its own laboratory procedures and protocols which have been specifically developed and optimized for your individual medical program.

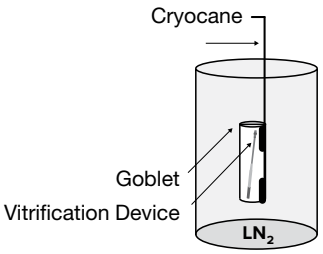

Figure 3

TIPS FOR OOCYTE VITRIFICATION

- Washing NX (WS) is only needed for oocyte vitrification.
- While waiting for exposure time in ES and VS, cover the dish and move away from the light source.
- Oocytes will migrate during the process. This is normal and oocytes can be left alone during migration.
  - After the merging of WS and ES1, oocytes may migrate to the outer edge of the WS drop.
  - Before merging the WS/ES1 drop and ES2, if you move the oocytes to the center of the ES/WS1 and merge ES2 to that center juncture, the oocytes may migrate from ES2.
- Consider dispensing the ES3 drop later, such as during Step 10 or 11, to minimize any risk of evaporation of ES3.
- Consider dispensing the VS drop later, such as towards the end of ES exposure, to minimize any risk of evaporation of VS.
- Consider moving the oocyte around in VS until it is no longer floating (it may also appear similar to a crescent or boomerang shape). This is an indication the oocyte is fully equilibrated and there is no carryover of ES in the specimen. This process might look like:
  - Release oocyte at the 12 o'clock position into the VS drop and start the 50–60 second timer. Oocyte will float to the top of VS.
  - After 10 seconds, gently move the oocyte to the 6 o'clock position in the VS drop and let sit for ~20 seconds.
  - After 20 seconds, gently move the oocyte to the 9 o'clock position in the VS drop until the end of the countdown.
- The timing for exposure to VS is CRITICAL.
  - Maintain microscopic visualization of specimen(s) by adjusting focus as needed, during rapid exposure to VS (specimens will float in the drop).
  - Keep transfer pipette tip close to drop for quick manipulations.
  - Load, seal, and plunge the vitrification device within **80 seconds, not to exceed 110 seconds** after initial exposure to VS. For best performance, the time from initial VS exposure to plunging in LN_2 should be in the range of 75–80 seconds.

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