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We use this protocol and it's working

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Slow freeze (cryopreservation) protocol for human ovarian tissue

Elizabeth L Tsui[1], Monica M Laronda[1], Hannah McDowell[1]

[1]Ann & Robert H Lurie Children's Hospital of Chicago

Elizabeth L Tsui
Ann & Robert H Lurie Children's Hospital of Chicago, Northwe...

ABSTRACT

Purpose: This protocol describes the procedure for cryopreservation of organ donor derived human ovarian tissue via slow freeze with a semi-automated controlled rate freezer in a research setting.

GUIDELINES

****CRITICAL****Personnel who process and handle human specimens must adhere to Office of Research Safety guidelines at their institution. They must be up to date on their original and refresher courses for the following training (or equivalent):
1. Biosafety Certification
2. Bloodborne Pathogens Certification
3. Training in Filling and Maintaining Liquid Nitrogen Tanks (as appropriate for LN2 use)
4. Working with Formaldehyde Certification (as appropriate for fixation)
5. CITI training (highly encouraged)

MATERIALS

All materials are listed as Type (Vendor, Catalog Number)

- OFC Holding Media (Sage/Origio, #ART-8040)
  - Alternative: Leibovitz-15, L-15 (Caisson Labs, LVL02-6X500ML or equivalent)
- OFC Freezing media (Sage/Origio #ART-8050)
  - Alternative: 1.5M ethylene glycol in MOPS Buffered Holding Solution with 0.1M sucrose or equivalent
- 2 mL cryovial (TPP, 89020 or equivalent)
- Refrigerator
- Liquid nitrogen (LN2) storage tank
- Dewar or nearby source of LN2
- Control rate freezer, suitable for vials, (Biogenics #CL8800i or equivalent)
- Cryolabels for vials e.g., CryoDTermoTM - DYMO-Compatible Cryogenic labels (GA International, EG1F-081GA or equivalent).
  - Ensure that these are suitable for liquid nitrogen storage
- Long forceps (Electron Microscopy Sciences, 72918 or equivalent)
- Long cotton swab (Medline, MDS202055 or equivalent)

SAFETY WARNINGS

Personnel will wear appropriate personal protective equipment. Areas are disinfected with the Dymon **Do-it-all germicidal foaming cleaner** (or equivalent) according to manufacturer's instructions.

Equipment set up

15m

1 The controlled rate freezer has three components: computer, temperature controller - measures temperature of the tube rack, and cryobath (with internal tube rack suitable for cryovials) 15m

Set up programmable freezer by opening the relevant freezer software and programming the controlled rate freezer as follows:

1. Hold at 4 °C for 00:05:00
2. Cool at 2 °C per minute from +4 °C to -7 °C
3. **ALARM** "Seed Now." Press "pause" and allow for manual seeding (described below) before continuing with the protocol (usually indicated by pressing "run").
4. Hold for 00:10:00 at -7 °C (Allowing for ice crystal formation)
5. Cool at -0.3 °C per minute from -7 °C to -35 °C
6. **Alarm** "Plunge after -40 °C

Note

It may be useful to save this program in your controlled rate freezer's computer (if possible).

2 Start the protocol using the controlled rate freezer software.

3 Fill the cryobath with liquid nitrogen and gently lower in the internal tube rack to allow the internal tube rack to get to the protocol's starting temperature (4 °C).

Ensure that the cryobath has a minimum of 2 inches of liquid nitrogen at all times throughout the protocol to allow for appropriate cooling. Keep a back up dewar filled with liquid nitrogen to aid with filling.

Safety information

Use appropriate personal protective equipment when using liquid nitrogen.

Preparing the tissue for slow freeze

30m

4 For adequate penetration of cryoprotectant, tissue pieces used for slow freeze should be no larger than 5 mm in any dimension. Prior to transfer to cryovials, tissue must be kept in appropriate holding media buffered for air, such as OFC Holding Media or Leibovitz-15, and at 2-4 °C to prevent the tissue from drying out.

All following steps for this section should be performed at 2 °C to 4 °C . Maintain all steps at this temperature and reduce warming of tissues by handling cryovials with long forceps.

5 Prepare and label 2.0 mL cryovials with 1 mL 4 °C of cooled Freezing Media. Ensure that labels do not obscure the meniscus.

6 Place the tissue pieces processed for slow freeze in the appropriate cryovials and verify that tissue sinks into the Freezing Media.

A maximum of 4 tissue pieces processed for slow freeze can be stored in each vial, but ensure that anatomic position is accurately noted, and that there is at least 2x volume of Freezing Media to tissue volume.

7 Allow tissue pieces to equilibrate in cryovial with 4 °C cold Freezing Media for 00:30:00 30m
.

Note

If not done already, this time can be used to set up the controlled rate freezer as described above.

Freeze program 35m

8 When the internal tube rack has reached 4 °C **and** the tissue in labeled cryovials have equilibrated for 00:30:00 at 4 °C , add cryovials to internal tube rack, and begin the freeze protocol. 30m

Note

If the freezer was set up in advance, ensure that enough liquid nitrogen remains in the cryobath prior to beginning the freeze protocol.

9 Allow freeze program to proceed as described above.

10 **Seeding cryovials.**

At -7 °C , the freezer will alarm to alert the user to "SEED NOW."

To "seed" cryovials means to generate a small piece of ice within the tube in order to allow for full ice crystal formation throughout the frozen vial.

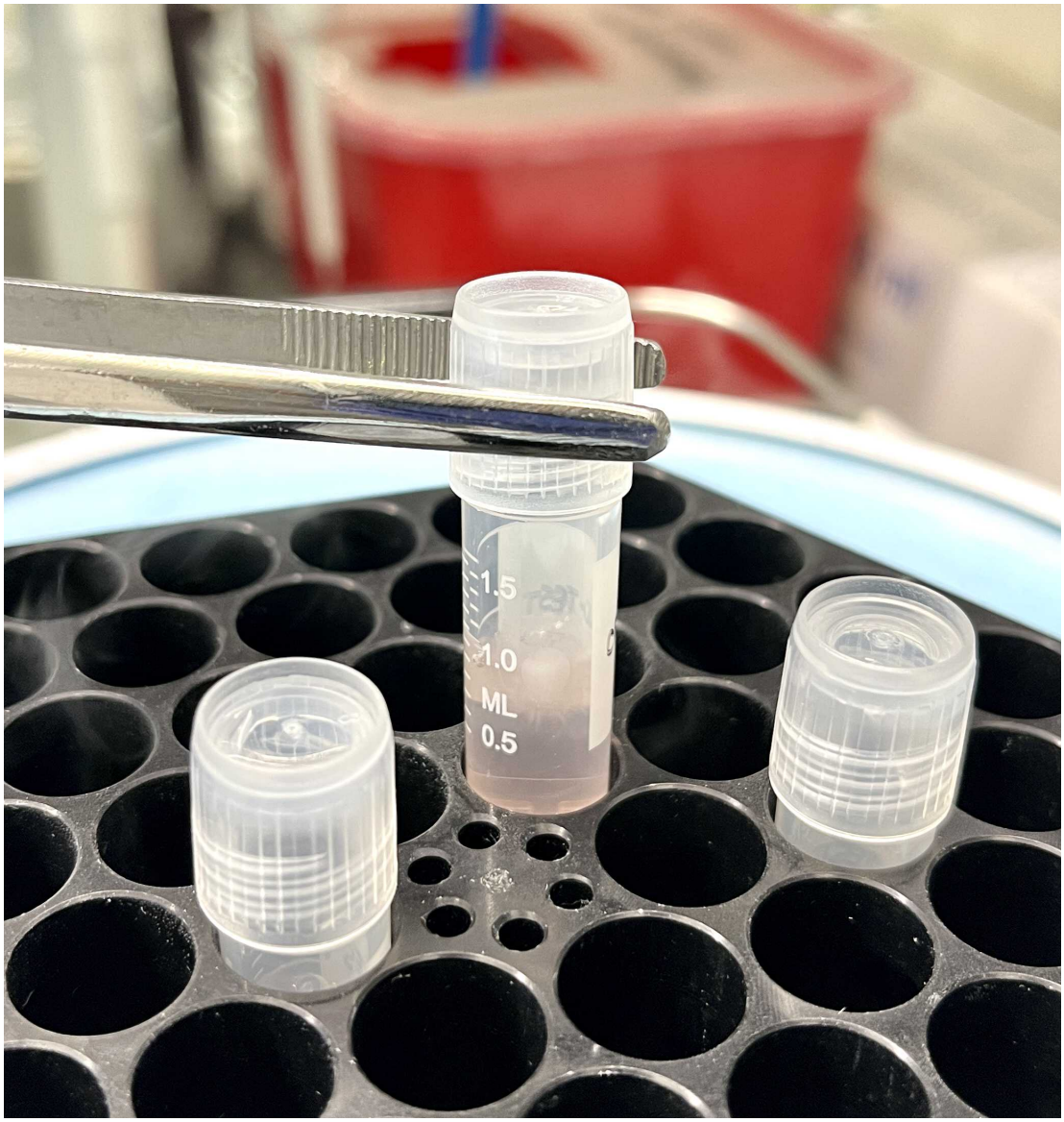
10.1 Press "Pause" on the program to allow for time to seed. This should occur within 00:05:00 5m

10.2 Dip a long cotton swab into the liquid nitrogen, hold for a few seconds, then remove and press the cold swab to the side of cryovial at the meniscus.

If the seed is successful, you will see a small ice crystal bloom at the meniscus.

> Note
>
> Sometimes, the cotton swab needs to be removed to see the seed.

> Note
>
> When seeding multiple vials, it may be necessary to repeat the cooling of the long cotton swab, which can be accomplished by dipping the long cotton swab back into the liquid nitrogen for a few seconds.



Successful "seed." Note the ice bloom/starburst at the 1.0mL mark.

10.3 Repeat for all remaining cryovials.

10.4 When finished, press "Run" to resume freeze program.

11 Allow for the remainder of the freeze program to proceed.

Note

Approximately every 00:20:00 , ensure that the liquid nitrogen level within the cryobath is at or above 2 inches. If necessary, refill the cryobath with additional liquid nitrogen.

12 When the protocol is complete, the freezer will alarm, and the internal tube rack's temperature will freefall from -35 °C . Once the tube rack has reached -40 °C or colder, use long forceps to remove each cryovial and submerge each cryovial in liquid nitrogen by plunging liquid nitrogen.

Repeat for all remaining cryovials. From this point on, the cryovials should be kept in liquid nitrogen storage.

Long-term storage

13 After all cryovials are plunged into liquid nitrogen, transfer to liquid nitrogen storage in a pre-cooled storage container. Cryovials should be stored in the liquid phase of the liquid nitrogen storage tank (or colder than -150 °C) for long-term storage.

14 Allow controlled rate freezer to come to room temperature and dry prior to storage or next use.