Cryodropper Cryopreservation and Thaw of Mouse Sperm

Animals:

Male mice for sperm collection (proven fertility, mated 4-7 days prior to collection)

Equipment:

Cryodropper for Sperm Vitrification (red line: ParaTechs 80020)
Cryovial (2ml; Corning #430659 or 4 ml; Corning # 430662) or other suitable LN_2 storage option
Stereomicroscope with transmitted and reflected illumination source with 20x and 40x magnification
Impulse heat sealer (American International Electric AIE-105T)
CO_2 incubator at 37˚C with 5% CO_2
Slide warmer or 37˚C incubator
Water bath @ 37˚C (500ml beaker of water in a 37˚C incubator works well)
Gel loading tips (ex: USA Scientific 1022-0600)
Pipettors and tips; ex: 1ml, 200ul, 20ul, 2ul
Eppendorf rack
Portable LN_2 vapor phase freezer with Styrofoam raft (see photo)
Sharpie for labeling
Kimwipes
Tissue culture dishes; 35mm
Timer
Forceps (Watchmakers #5)
Scissors, dissection and small angled
LN_2 vapor phase storage dewar

Media and Reagents Required:

Paraffin Oil, (Sigma-Aldrich 18512), equilibrated for 30 minutes at 37˚C with 5% CO_2
FERTIUP cryoprotectant (CPA) (Cosmobio KYD-001) (Can be made in-house as well, Behringer et al., pg. 674-675)
FERTIUP preincubation medium (PM) (Cosmobio KYD-002) (Can be made in-house as well, Behringer et al., pg. 614)

Sperm Freezing

1. Prepare 2 male mice (>8 weeks old with proven fertility) by mating (plug positive) 4-7 days prior to sperm cryopreservation.

2. Prepare 1 sperm dish per mouse by depositing a 60µl drop of CARD CPA on a 35mm dish. Cover the drop with paraffin oil. Add a second 60µl aliquot of CPA to the first drop to make a tall, semi-spherical drop of CPA. Equilibrate at 37˚C (not in CO_2).

3. Label each Cryodropper. Color code or label the bulb portion.

4. Prepare Cryodroppers: prepare 90µl drops of CARD PM medium in a dish, 1 drop per Cryodropper. Let equilibrate at 37°C with 5% CO_2 for 10 min. Load medium into the Cryodropper by squeezing the bulb, inserting the open end into the drop, and releasing the pressure on the bulb. Hold the Cryodropper by the open end and gently flick the medium into the bulb portion. Gently squeeze the bulb to remove any medium remaining in the sperm loading area and wipe the liquid off with a kimwipe. Store open end up in an Eppendorf rack at 37°C with 5% CO_2 until sample loading.

5. Set up portable LN_2 vapor phase freezer with Styrofoam raft.

6. Label and precool the cryogenic storage vials. Up to 4 Cryodroppers will fit per vial.

7. Euthanize the mice. This varies by institution, please follow all applicable regulations.

8. Remove the cauda epididymides. Place them on a kimwipe and, under a microscope, completely remove all fat and blood.

9. Transfer one epididymis from each male to each sperm dish. This keeps the sperm from two males mixed in the sperm dishes. Note: the following steps must be performed quickly; under 30 minutes total from sperm release to freezing.

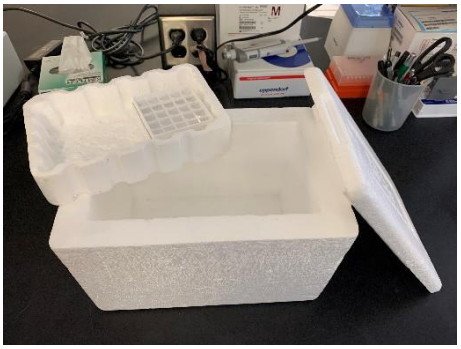
10. Using watchmaker's forceps and small angled scissors, make at least 6 incisions in each epididymis.

11. Place dish on slide warmer at 37˚C for 3 minutes. Rotate dish every minute to disperse sperm from tissue. Gently squeeze remaining sperm from the tissue as the tissue is removed from the medium.

12. Loading Cryodroppers: Using a gel loading pipette tip, carefully load the straw portion with 10µl sperm suspension in the center. Seal with a heat sealer. Gentle pressure applied to the bulb of the Cryodropper can be used to test the seal. If the device is not sealed, repeat.

13. Put loaded Cryodroppers directly on Styrofoam float in LN_2 vapor for 10 minutes.

14. Transfer Cryodroppers to vials. The vials are then transferred to a LN_2 dewar and stored in the vapor phase.



Portable LN_2 vapor phase freezer with Styrofoam raft

Sperm Thaw

1. The Cryodropper containing vial is removed from the storage vessel and kept in LN_2 vapor until ready for thaw.

2. Using precooled forceps, the Cryodropper is immersed in a water bath at 37˚C until the media in the dropper is thawed (@10 sec). Incubate for an additional 10 minutes at 37˚C.

3. Remove device from water bath and gently dry with a kimwipe.

4. Using scissors, cut the tip of the Cryodropper off and transfer sperm drop to a fresh petri dish.

5. Gently shake CARD PM medium to end of device with single flick of wrist. Too much force and the medium will be lost.

6. Apply medium to sperm drop. Cover the sperm drop with Paraffin equilibrated in the CO_2 incubator for at least 30 minutes prior to sperm thaw.

7. Incubate in the CO_2 incubator at 37˚C to capacitate for 45 minutes.

8. Sperm sample is ready for use for *in vitro* fertilization.

References:

Sperm cryopreservation and thaw adapted for use with Cryodroppers by B. Stone from the following:

1. **Behringer R, Gertsenstein M, Nagy KV, Nagy A.** 2014. Manipulating the Mouse Embryo: A Laboratory Manual, 4th ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press

2. **Nakagata N.** 2015. Reproductive Engineering Techniques in Mice, 3rd ed. Cosmo Bio Co., Ltd. Tokyo, Japan