Mouse Sperm Cryopreservation

CPA Components

- D (+) raffinose pentahydrate.................Sigma R-7630 (conc. 18%)
- Skim milk, dehydrated...........................Difco 0032-17-3 (conc. 3%)
- Embryo tested water.............................Sigma W1503
- α-monothioglycerol (MTG)......................Sigma M6145 (final 477 μM)

Other Materials

- A 200-ml clean glass beaker for CPA only
- A 200-ml volumetric flask for CPA only
- A clean magnetic stir bar for CPA only
- A clean thermometer for CPA only
- Sterile 1.5-ml microcentrifuge tubes
- Sterile Falcon tubes (5-ml, 10-ml, 15-ml)
- Microwave oven
- Magnetic stirrer/hot plate

Preparation of Cryoprotective Agent (CPA)

1. Add 150 ml embryo tested water (Lot #_______________) into a glass beaker.
2. Heat water to 45-55 °C in a microwave oven or on a magnetic stirrer/hot plate.
3. Weigh and add 36 g raffinose (Lot #_______________).
4. Add a magnetic stir bar, and stir until solution is clear.
5. Weigh and add 6 g skim milk (Lot #_______________), and stir until skim milk is dissolved.
6. Transfer the solution to a 200-ml volumetric flask.
7. Rinse the beaker with 5 ml water, and transfer the rinse to the volumetric flask.
8. Carefully pipette more water to the flask to bring final volume up to 200 ml.
9. Close the volumetric flask with parafilm, and invert 5× to mix the solution.
10. Dispense CPA into 4× 50-ml Falcon centrifuge tubes, each with ~ 50 ml.
11. Carefully balance the two plastic beakers standing on the balance plates, and then place a 50-ml centrifuge tube adaptor into each beaker.
12. Open a 50-ml Falcon tube containing CPA, place the tube cap on the left plate of the balance, and insert the tube into the adaptor in the beaker on the same plate.
13. Repeat step 12 to insert another 50-ml Falcon tube containing CPA into the centrifuge tube adaptor on the right balance plate with its cap open and placed on the same plate.

14. Carefully balance the weights between the two sides of balance.
15. Close each tube with cap tightly, and then insert the two adaptors together with the Falcon tubes containing CPA symmetrically into the rotor of centrifuge.
16. Repeat steps 11-15 with the rest two 50-ml Falcon tubes containing CPA.
17. Secure the rotor using a wrench, and then place the rotor cover and secure it.
18. Cover the centrifuge lid, and spin at 11,995 rpm (18,500 x g) for 30 min with temperature set at 4 °C.
19. Wash glass beaker, volumetric flask, magnetic stir bar and thermometer with tap water, then rinse with DH_2O, and air dry. Keep them in a clean drawer.
20. When centrifugation is finished, open the centrifuge lid, and carefully remove each tube and stand them on a rack.
21. Carefully transfer the supernatant of each tube into a sterile bottle. Do not disturb the precipitate on the bottom.
22. Check osmolarity of the CPA. Should be 470-510 mOsm.
23. Filter the CPA through a 0.2 µm filter.
24. Aliquot CPA and store aliquots at -20°C for use within 2 months.

Preparation of 100 mM MTG (stock solution)

1. Add 4.75 ml of M2 medium to a sterile 5 ml Falcon tube.
2. Add 40 µl of MTG to the M2 medium. MTG is viscous, and pipette tip should stay in MTG for a few seconds until suction is stopped, and then excess MTG should be removed from the pipette outside before expelling MTG into M2. After expelling the initial amount of MTG, allow it to collect at the end of pipette before expelling the remaining MTG into the solution. Rinse any residual MTG into the solution.
3. Mix by inverting ≥ 5 times.
4. Filter the solution through a 0.2 µm filter into a sterile 5 ml Falcon tube.
5. Store at 4 °C for use within 1 week.

Preparation of 477 µM MTG in CPA

1. On the day of sperm freezing, thaw a tube of CPA at 37°C and mix well until all solutes dissolved.
2. Add the amount of 100 mM MTG to the thawed CPA according to the table below, and then mix well by inverting ≥ 5 times.

3. Use CPA+MTG for sperm cryo on the same day of preparation.

	CPA 1.2 ml	**CPA 3.6 ml**	**CPA 7.2 ml**	**CPA 12.0 ml**
100 mM MTG	6 µl	18 µl	36 µl	60 µl

Sperm Collection and Analysis

1. **Collection of epididymids and vasa deferentia**

    1) Print labels and label cryovials with printed labels and CryoColor code cap inserts. On the label, indicate the strain, volume of sperm or tissue, and unique identification number. Record this information on sperm cryo records.
    2) Sterilize dissecting tools by autoclave or 70% ethanol. Make sure the dissecting tools are completely dry before use.
    3) Prepare and warm (37 °C) an organ dish containing 0.5 ml (for both cauda epididymides) or 0.25 ml (for one cauda epididymis) CPA+MTG for sperm collection.
    4) Prepare 1 autoclaved microcentrifuge tube with 190 µl of M2 medium for sperm quality assessment. Stand the tube with cap closed at room temperature prior to use.
    5) Sacrifice a male mouse by cervical dislocation or CO_2 asphyxiation.
    6) Immediately aseptically dissect out one side or both sides of cauda epididymides along with the vasa deferentia and place in pre-warmed CPA-MTG.

2. **Collection of sperm and sperm analysis**

    1) Under a dissecting microscope with a warm stage at 37 °C, use a pair sterile forceps to fix a cauda epididymis, and a sterile needle to gently pierce holes around the tissue, and then gently press the tissue to release sperm. Attention should be paid to gently and quickly releasing as many sperm as possible from each cauda epididymis.
    2) Release sperm from vas deferens by "walking down" a needle along it while avoiding the accompanying blood vessel. If blood is released, suck it out using a **regular** sterile pipette tip with minimum sperm.

3) Gently swirl the dish for a few times to distribute sperm, and then place the dish on warm stage at 37 °C for sperm dispersion. *Do not leave the dish in the dissecting scope light during incubation of the sperm on warm stage.*
4) After 10 min of incubation, gently swirl the sperm collection dish a few times, then pipette 10µl from the sperm suspension, and then add the sperm into 190 µl warm M2 medium in a microcentrifuge tube prepared above. Tap the tube to mix the sperm.
5) Assess sperm concentration, total motility (% of motile sperm), % rapid cells and progressive motility using IVOS. Score the slide under a bright field or phase contrast microscope to count % sperm with abnormal sperm heads and % sperm with abnormal sperm tails (curved or bent).

Sperm Cryopreservation

1. Remove caps of the labeled cryovials standing on a rack, and place the caps in a sterile culture dish (do not place the caps on dirty table surface).
2. Using a sterile wide-bore tip, pipette 50 to 100 µl of sperm suspension to the bottom of each cryovial, then cap the cryovials. Do not drop sperm!
3. Place the loaded vials into LN2 vapor phase (approximately -120 °C) for 10 minutes with Dewar lid covered.
4. When the timer has alarmed, transfer the vials into another Dewar flask where the vials can stand in liquid nitrogen.
5. Store the samples in liquid nitrogen tanks.

Sperm Thawing:

1. Wear safety PPE (safety glasses and cryogloves, etc.), remove a sperm cryovial from LN2 storage using precooled forceps, and place it in a small Dewar flask containing LN2. Ensure that the vial is as far away from face and eyes as comfortably possible when reading a cryovial label.
2. Bring the sample to a water bath.

3. Remove the cryovial from liquid nitrogen using pre-cooled forceps, and then quickly but carefully unscrew the cap, pouring out any liquid nitrogen that may have seeped into the vial, and put the cap back onto the cryovial.
4. Thaw the sperm by floating the cryovial on a floating rack in a 37 °C water bath for 5-10 min
5. Remove the cryovial from water bath, wipe dry the cryovial, and tap or swirl the vial gently to mix the sperm before IVF.

References

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