Sperm collection and cryopreservation

Reagents and Solutions

1. Listing of all used chemicals with related supplier informations

<table>
<thead>
<tr>
<th>Description</th>
<th>Supplier / Cat. #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryoprotective medium (CPM), 470 – 490 mOsm</td>
<td></td>
</tr>
<tr>
<td>18% Raffinose (0.9 g 5 mL⁻¹)</td>
<td>Sigma R7630</td>
</tr>
<tr>
<td>3% Difco™ Skim Milk (0.15 g 5 mL⁻¹)</td>
<td>Becton Dickinson 232100</td>
</tr>
<tr>
<td>Embryo Transfer Water</td>
<td>Sigma W1503</td>
</tr>
<tr>
<td>Ethanol 200 Proof EtOH absolute</td>
<td>Pharmaco 111ACS200</td>
</tr>
<tr>
<td>FHM w/ phenol red</td>
<td>Fisher Scientific MR-024D</td>
</tr>
<tr>
<td>FHM w/o phenol red</td>
<td>Fisher Scientific MR-025D</td>
</tr>
<tr>
<td>Human Tubal Fluid (HTF)</td>
<td>Millipore #MR-070-D</td>
</tr>
<tr>
<td>Liquid nitrogen LN₂</td>
<td>West Air Inc.</td>
</tr>
</tbody>
</table>

2. Listing of all used equipment and materials with related supplier information

<table>
<thead>
<tr>
<th>Description</th>
<th>Supplier / Cat. #</th>
</tr>
</thead>
</table>

Mutagenetix (http://mutagenetix.utsouthwestern.edu:80)
Sperm collection and cryopreservation

- Balance FE - 123
  - Fisher

- BioCool BCIV40A controlled rate freezer
  - Kinetics Thermal System 24309

- Centrifuge Microfuge® 18
  - Beckman Coulter, Inc. 367160

- Cotton Tail® absorbent tipped applicators 1000pk
  - Fisher 23400106

- Corning 26mm syringe filters
  - Corning Incorporated 431220

- Critoseal™
  - Fisher 02-676-20

- Cryosleve, kart
  - Fisher Thermo 4000218

- Cryostore cane tabs
  - Nunc 12565255

- Cryostore canes, metal
  - Nunc 378441

- Cryostore goblets, visotubes
  - IMV technologies 006402

- Culture / Petri dish 60 x 15 & 35 x 10 mm, sterile
  - Falcon 35 3002 & 35 1008

- Falcon tube 10 mL
  - Falcon 35 2097
<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier/Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forceps Dumont #5, straight</td>
<td>Roboz RS-4976</td>
</tr>
<tr>
<td>Non-woven Gauze Non-Sterile 2 x 2</td>
<td>Fisher 22028559</td>
</tr>
<tr>
<td>Non-woven Gauze Sterile 2 x 2</td>
<td>Fisher 22028556</td>
</tr>
<tr>
<td>Haemocytometer, Neubauer-improved</td>
<td>VWR Scientific 15170-173</td>
</tr>
<tr>
<td>Insulin syringe 1cc U-100 28 G(^{1/2})</td>
<td>Becton Dickinson 329424</td>
</tr>
<tr>
<td>Iodine Prep Pads</td>
<td>Fisher 19027048</td>
</tr>
<tr>
<td>Iodine Solution</td>
<td>Fisher 19027136</td>
</tr>
<tr>
<td>Iris scissors, straight, 4.5&quot;</td>
<td>Henry Schein 100-2767</td>
</tr>
<tr>
<td>Iris scissors, straight, 4&quot;</td>
<td>Henry Schein 100-6893</td>
</tr>
<tr>
<td>Iris forceps, straight</td>
<td>Sklar 98-123</td>
</tr>
<tr>
<td>LN(_2) temporary container 350 mL</td>
<td>Pope 8600</td>
</tr>
<tr>
<td>LN(_2) storage tank CryoPlus 2</td>
<td>Thermo Electron Corporation</td>
</tr>
<tr>
<td>Metal rod</td>
<td>JacksonLaboratories</td>
</tr>
<tr>
<td>Microcentrifuge tubes 1.5 mL</td>
<td>USAScientific 14152500</td>
</tr>
<tr>
<td>Item</td>
<td>Vendor</td>
</tr>
<tr>
<td>--------------------------------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Microcentrifuge tubes 2 mL</td>
<td>USAScientific 16202700</td>
</tr>
<tr>
<td>Micro-dissecting forceps, two pairs</td>
<td>Dumont</td>
</tr>
<tr>
<td>Microscope (12x, 45x) NIKON SMZ 800</td>
<td>Micro Video Instruments</td>
</tr>
<tr>
<td>Milliex® GP Filter unit 0.22 µm</td>
<td>Millipore SLGP033RS</td>
</tr>
<tr>
<td>Monoject syringe 1 mL</td>
<td>Atlantic Healthcare SHA501400</td>
</tr>
<tr>
<td>Needles</td>
<td></td>
</tr>
<tr>
<td>26 G³⁄₈ PrecisionGlide</td>
<td>Becton Dickinson 305110</td>
</tr>
<tr>
<td>Straw (semen straw, plastic) 2.5 mL, 133 mm</td>
<td>IMV technologies 005565</td>
</tr>
<tr>
<td>Straw label / Brady label</td>
<td>Brady part LAT-17-361-2.5</td>
</tr>
<tr>
<td>Syringes</td>
<td></td>
</tr>
<tr>
<td>1 mL Tuberculin slip tip</td>
<td>Becton Dickinson 309602</td>
</tr>
<tr>
<td>5 mL Luer-Lok™ tip</td>
<td>Becton Dickinson 309603</td>
</tr>
<tr>
<td>10 mL Luer-Lok™ tip</td>
<td>Becton Dickinson 309604</td>
</tr>
<tr>
<td>Water bath Isotemp205</td>
<td>Fisher Scientific 160005100757</td>
</tr>
<tr>
<td>Wide-bore pipette tips</td>
<td>Rainin HR – 250W</td>
</tr>
</tbody>
</table>
3. Sperm Cryo Medium Preparation

1. Warm 70 mL of 100 mL of embryo water up to ~ 45°C (on the heated stirrer or in the microwave; it should be warm to the touch, condensation) in beaker or volumetric flask. Do not boil water.

2. Place on heated stirrer (~40°C). Add raffinose (18 g/100mL); dissolve until clear

3. Add skim milk (3 g/100mL), heat and stir until dissolved.

4. Bring to a little less than 100 ml with water. Use a graduated cylinder to measure or use a graduated pipette. Cover with parafilm, mix.

5. Aliquot into clean (autoclaved) centrifuge tubes. Spin at 13,000 X g (*rcf) for 15 min at room temperature, filter through 0.45 and 0.22 um filters (Corning # 431155 and # 431154 respectively) in the hood.

6. Check for Osmolality, add water gradually and record changes. Take several readings while adding water until it is stable around 480 mOsm (470-490 mOsm). If the osmolarity is higher than 490 mOsm, add water; if it is lower than 470 mOsm, start over

7. Monothyoglycerol (MTG, Sigma # M6145) –477 uM MTG in CPM:

   A: Add 10 ul of MTG to 990 ul of media to make the MTG stock. Add 5 ul of the MTG stock to 1.25 ul CPM to dilute MTG 1:100.

      Pipette slowly from MTG bottle (very viscous) remove excess MTG from the outside of pipette tip.

      B: Mix well by inverting tube or using vortex; vortexing works better.

      C: Prepare final dilution of CPM/MTG at 1:250 (e.g. 90 ml CPM with 0.361 ml MTG dilution). Measure remaining CPM with a graduated pipette or using a volumetric flask. Calculate how much diluted MTG is needed per ml of CPM.

8. Aliquot into 1.5 ml / eppendorf tube, store at – 80°C (lasts for at least 10 months, according to JaxLab).

9. On the day of use thaw CPM at the room temperature (or at +4°C overnight). If the precipitate is formed, dissolve it completely by warming to 37°C and vortexing

Method
Sperm collection and cryopreservation

Sperm collection from male mice and sperm cryopreservation

Method is adapted from references (1-4).

Sperm collection for cryopreservation is performed in the cryoprotectant medium (CPM) directly. For other purposes than cryopreservation (e.g. IVF), sperm collection is performed following the described method but other collection media must be used. Photographs of the dissection procedure of cauda epididymis and vas deferens (Figure 1) for collecting sperm are presented in Figure 2. The cryoprotectant solution, containing 18% D+raffinose and 3% skim milk in embryo-graded water, is prepared according to a method described by Nakagata (5). After dissolving the raffinose completely at 37°C using a 15 mL Falcon tube, milk powder is added and vortexed until the emulsion becomes homogenous, transferred to 2 mL tubes and centrifuged at 13’000 rcf (11’500 rpm) for 15 minutes. Supernatants are pooled and filter sterilized (0.45 m). If necessary, the CPM can be stored at +4°C for five days or at –20°C for up to three months.
A three to six-month old male is humanely euthanized by cervical dislocation, placed on its back on absorbent paper and the abdomen is generously sprayed with 70% EtOH for disinfection (Figure 2 a, b). With the 1\textsuperscript{st} pair of dissecting forceps a small fold of skin is lifted in the center of the abdomen level with the top of the legs and a small cut is made with scissors (4.5") to open the abdominal cavity (c), the skin is pulled in opposite directions towards head and tail until the abdomen is completely exposed (d). Using the 2\textsuperscript{nd} pair of forceps a small fold of the peritoneum is lifted and a small cut using 4" scissors is made to allow air to enter the abdominal cavity (e, f). The peritoneum is cut open to expose the body cavity, using caution not to cut any of the internal organs and the coils of the gut are pushed towards the head exposing the testicular fat pad (g). Pulling the testicular fat pad towards the head exposes the attached testis, vas deferens and cauda epididymis (h). The muscle tissue adjacent to the cauda epididymis is cut apart (i, j). The vas deferens is cut near the bladder end (k) and gently peeled away from the body cavity, leaving behind the major blood vessel and fat (l, m). A last cut is made just below the cauda epididymis (n). These steps are repeated for the second cauda epididymis and vas deferens.

The dissected cauda epididymis and vas deferens are placed in a 60 x 15 mm collection dish with 1 mL CPM prewarmed at 37°C. The sperm is released by slicing the cauda epididymis 5 to 7 times and “walking” down the vas deferens using two insulin syringes or forceps (o – r). After allowing the sperm to disperse for approximately 10 to 15 minutes at 37°C the tissues are removed.
Sperm collection and cryopreservation

Sperm cryopreservation is either performed using nunc cryotubes or plastic semen straws. In both cases a container with a lid and enough LN$_2$ to produce a stable vapor phase (the temperature approximately – 120°C) has to be set-up prior to freezing. Additionally an open rack to hold the cryotubes or straws in the vapor phase of LN$_2$ is needed.

For freezing sperm in cryotubes, after the collected sperm (~ 1 mL in CPM) is incubated 10 minutes at 35°C and tissues are removed, 100 L aliquots are distributed into nunc cryotubes tubes using wide-bore pipette tips and slow pipetting to avoid shearing forces. The closed tubes are immediately placed into the rack resting in the LN$_2$ vapor. Enough space is left between the tubes to ensure a full exposure to LN$_2$ vapor. After 10 minutes in the vapor phase with a descending cooling rate of approximately – 20°C to – 40°C per minute the sperm is directly transferred into the liquid nitrogen.
Sperm collection and cryopreservation

For the cryopreservation of sperm using plastic semen straws (2.5 mL, 133 mm), 100 L of the collected sperm in CPM are aspirated with a Monoject syringe applied to the labeled end of the straw. Both ends of the straw are sealed with Critoseal and placed into the rack resting in the LN₂ vapor. After 10 minutes the straws are directly immersed in the LN₂ tank for long-term storage.

Sperm thawing for ICSI--for samples before September 30, 2014

A water bath is set-up at room temperature (37°C) and an Eppendorf tube with 1mL pre-equilibrated HTF is prepared prior to thawing the straws. The straws are quickly transferred from the LN₂ storage tank to a temporary LN₂ container. Sperm frozen in the straws is rapidly thawed by transferring the straw into a 37°C water bath for approximately 15 seconds until all ice crystals are melted (visual check). The straw is dried by wiping it with a napkin carefully. The critosealed end is cut and the end re-rounded with fingers. A second cut is made between the cotton portions of the cotton plug (sealed area). A pusher (metal rod) is used to expel contents of the straw, the sperm in the cryoprotectant medium (CPM) and the diluent solution, towards the critosealed end and into the Eppendorf tube with 1mL pre-equilibrated HTF. Subsequently, the sample is centrifuged at 735 x g (max. 2000 rpm) for 3 minutes. The supernatant (CPM) is discarded and replaced with 50 L pre-equilibrated HTF and gently mixed by tapping the tube. A maximum 40 L aliquot of the sperm is taken for ICSI. The 10 L remainder is utilized for concentration and motility analysis.

Evaluation of sperm concentration and motility

Concentration and motility of sperm are determined using a Neubauer-improved haemocytometer. For counting fresh collected sperm (1 mL per male) a dilution of 1:30 for hybrid mice and 1:20 for inbred is made with FHM using 10L of sperm. Frozen-thawed and resuspended samples are diluted in a ratio of 1:10 only. The numbers of total and motile sperm are assessed twice per sample by counting sperm cells within the 25 group squares (total central large square with an area of 1 mm² and a depth of 0.1 mm resulting in a volume of 0.1 mm³ equivalent to 0.1 µL) of the hemocytometer. To ensure adequate accuracy of the method, at least 200 to 400 spermatozoa should be counted within one large square; in the case of uneven distribution of the sperm cells within the counting chamber, two or more of the corner large squares are counted and the total amount of cells is divided by the number of analyzed fields. By convention, sperm concentration is expressed as the number of spermatozoa per milliliter. Motility is defined as any movement of the sperm head (% moving), and progressive motility as the count of those spermatozoa that move in a forward, linear direction at a speed of 50 m per second (automated sperm counter).

Sperm thawing for in vitro fertilization (ICSI and IVF)--for samples after September 30, 2014

9 of 10

Mutagenetix (http://mutagenetix.utsouthwestern.edu:80)
Sperm collection and cryopreservation

A water bath is set-up at room temperature (37°C) prior to thawing the straws.

Incubate HTF media in an incubator set to 37°C and 5% CO2 for a minimum of 30 minutes

The straws are quickly transferred from the LN₂ storage tank to a temporary LN₂ container

1. Take straw from vial and put into water bath for 10 minutes
2. Record the barcode from the straw on the record sheet.
3. Wipe straw with Kimwipe
4. Cut the end with the sealing ball.
5. Expel the sperm portion into a 0.5ml microcentrifuge tube using a pipette set to 15l.
6. Add 90l HTF media to the tube
7. Incubate with the cap open for 20 minutes
8. Using a pipette set at 5l mix the sample and aliquot 5l onto the glass slide.
9. Score the sample for concentration under the 4x objective (1=low, 5=high)
10. Score the sample for motility and progressive motility under the 10x Objective
11. Score the sample for morphological defects (poor quality or low level defects)
12. Based on the score, IVF or ICSI

References