Cryopreservation of Client Depositor Semen Aspirate

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1 Introduction

Therapeutic bank patients hereafter referred to as "client depositors" can address the risks of infertility with certain medical treatments and surgeries by taking advantage of semen cryopreservation procedure [1–4].

2 Principle

Biological time ceases at the liquid nitrogen temperature of −196 °C, a fact that can be used for the long-term preservation of sperm. Human spermatozoa have morphologic properties which allow them to swell to approximately five times their iso-osmotic volume before lysing. The controlled rate addition of TEST-yolk buffer and its removal during thawing by a slow thaw and wash procedure results in preservation of sperm membrane integrity [1–4].

3 Equipment

A. Computer-assisted semen analyzer
B. Sperm counting chamber (Vitrolife)
C. Eppendorf pipette
D. Aliquot mixer
E. Vortex
F. −20 °C freezer
G. LN_2 Dewar with 11″ canisters
H. Sterile specimen container
I. Sterile 15 mL centrifuge tubes with caps
J. Sterile serological pipettes (1, 2, and 5 mL capacity)
K. Sterile corning cryovials (1 and 2 mL capacity)
L. Colored cryomarkers
M. Test tube racks (for 15 mL test tubes)
N. Cryovial racks
O. Stainless steel canes for cryovials
P. Plastic cryosleeves
Q. Cryogloves
R. Protective goggles
S. Nitrile gloves
T. 37 °C incubator
U. LN_2
V. Eosin-nigrosin stain
W. Frosted slides
X. Coverslips

4 Reagents

A. Freezing medium (TEST-yolk buffer with gentamicin sulfate. Each bottle is sterile and for one-time use. Parameters for acceptable reagent performance:
   1. Quality control consists of a pre-freeze and post-thaw done on new lot number of TEST-yolk buffer on the semen specimen of a normal donor. The control should meet the following criteria: 50 % survival, calculated by the following formula:

$$\frac{\%\,\text{postthaw motility}}{\%\,\text{prefreeze motility}}$$

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A. Agarwal et al. (eds.), *Andrological Evaluation of Male Infertility*, DOI 10.1007/978-3-319-26797-5_17

2. Results are recorded in the appropriate Quality Control Precision book. Any unacceptable results are addressed in a quality assurance report with supervisory review.
3. Storage requirements: Stored frozen at −20 °C until time of use.
4. The lot number and vial expiration date are checked and recorded on the quality control cryopreservation worksheet.

B. Sperm Washing Media: Modified HTF containing 5.0 mg/mL human serum albumin.
1. The reagent is stored at 4 °C. It should be warmed to 37 °C prior to use.
2. Quality control is performed on all new lots of media using a normal donor specimen meeting the following criteria: >50 % recovery of motility.
3. The results are recorded in the appropriate Quality Control Book.

5 Specimen

The client depositor semen aspirate(s) will be collected by the surgeon performing the epididymal aspiration. The specimen(s) must be collected into a clean sterile container with 1–5 mL of sperm washing media. The specimen(s) must be labeled with the patient's name and clinic number. The aspirate(s) may be from the left and/or right epididymis. These aspirates are treated as separate specimens. The Andrology Technologist must go to the operating room where the procedure is taking place to obtain and identify the specimen(s).

A. Unacceptable specimens: Any semen aspirate is considered acceptable. While the specimen (aspirate) of a client depositor may not meet the minimal "normal criteria" of a banked specimen from a donor who is not compromised by a disease process, present and future techniques used for in vitro fertilization may enhance a subnormal specimen.

6 Calibration

No calibration standards exist for this procedure.

7 Quality Control/Quality Assurance

Quality assurance is maintained by the quality control of the freeze media and the testing equipment that is documented in the Precision Notebook. Cryosurvival on a processed normal donor specimen is evaluated on new lot numbers of TYB media.

8 Procedure

Note: Sterile technique should be used throughout specimen processing. Gloves are mandatory for all procedures dealing with body fluids. Latex, however, can be toxic to sperm. Therefore, care should be taken to prevent contamination of the specimen with latex or talc, or preferably, not used at all. Instead, vinyl or nitrile gloves are recommended.

A. A clinic number is necessary for client vial identification and billing purposes.
B. After notification that an aspirate will be performed, the client depositor's cryobank file can be made. This consists of the following forms:
1. The *Semen Collection and Storage Agreement*. The OR nurse or doctor will have the client read through the whole agreement, including the fee schedule on the back page, and sign where the patient signature is indicated under the Director's signature as well as initial the bottom of every page. This should be done on two different storage agreements so that one can be kept in the client's file and one can be sent home with/to the client.
2. The demographics of the Sperm Bank questionnaire can be filled out by the technologist as a way of obtaining the patient/client depositor information or by the client himself.
3. A Cryopreservation Worksheet labeled with patient name, clinic number, and specimen type number. New freeze number should be assigned to the patient and used for labeling the patient vials as well as written on the Cryopreservation Worksheet. If there is more than one specimen, have a worksheet for each specimen.

C. When the laboratory receives the call that an aspirate is ready, take out one bottle of frozen TEST-yolk media and put it into the 37 °C incubator for 20 min to thaw.
D. A technologist will go to the procedure room to pick up the specimen(s). He/she will take some sterile individually wrapped Eppendorf tubes, 5–6 15 mL centrifuge tubes containing warm (37 °C) sperm wash media, and a 5–20 μL Eppendorf pipette. Once the presence of sperm in the aspirate(s) is confirmed, the labeled specimen(s) (patient name, clinic number, and specimen number) is/are identified by the surgeon, nurse, and technologist before leaving the operating room. Put the labeled specimen vial(s) into a biohazard bag and keep warm in your hand during transportation to the Andrology Laboratory.
E. Using sterile technique, measure the volume of each specimen and record on the appropriate Cryopreservation Worksheet.
F. Record in a note book the patient's name, clinic number, and freeze number.
G. Mix the specimen in the Eppendorf tube using an individually wrapped sterile pipette tip and sterile pipette,

then place 3–4 drops (~15 μL per drop) of the specimen into a labeled sterile culture plate.

H. Slowly add immersion oil so that the droplets are covered.

I. View the droplets under a high-powered inverted microscope and count the number of motile vs. nonmotile sperm in at least 20 fields. Write this information, along with the vial number and site of collection, on the blue-lined notebook paper.
Note: Sperm concentration will be recorded as number of sperm per high-powered field (HPF).

J. Repeat steps G–I until all vials have been completed.

K. Label a sterile 15 mL conical centrifuge tube with the patient's name, clinic number, freeze number, and specimen number.

L. Within 1 h of specimen collection, add an aliquot of freezing medium equal to 25 % of the aspirate volume to the centrifuge tube with a sterile pipette and individually wrapped sterile pipette tip. Do this for all specimens.

M. Gently rock the specimen(s) with the freezing media for 5 min on an aliquot mixer.

N. Repeat steps L and M three times or until the volume of freezing media added is equal to the specimen volume.

O. During the mixing steps above, use appropriately colored cryomarkers to label a 2 mL cryovial(s) and cane(s). The appropriate color of cryomarker can be found on a chart designated in the cryobanking area. This chart also indicates the appropriate color of index card to be kept on file.
Note: Examine each cryovial while labeling for any evidence of defects. Discard the vial if any defect (cracks, leakage) is found.
Example: The 1st banked specimen is labeled with the red cryomarker.
   1. Label cryovial(s) as follows with orange cap to the left:
      a. Client depositor name
      b. Medical Record Number
      c. Freeze number, e.g., F15-001A (15-represents 2015 or current year)
      d. Date
      e. Word "PESA"
      f. Initials
   2. Assign a freeze letter for each aspirate:
      a. For example, specimen aspirate 1 will be labeled F15-001A, specimen aspirate 2 will be labeled F15-001B, etc.; each aspirate will have its own Cryopreservation Worksheet.
      b. The volume added to the vial should not exceed 1.8 mL per vial.
      c. Record the freezing volume on the corresponding Cryopreservation Report.
   3. Label top of canes with client depositor's last name and current freeze number, e.g., Smith F15-001A.

P. Label additional 1.8 mL cryovial for each specimen and label the top(s) of the vial(s) with a black cryomarker. Each vial will contain a leftover aliquot of the cryodiluted specimen to be assessed for cryosurvival (post-thaw specimen) 24 h after freezing in LN_2.

Q. Distribute the well-mixed, cryodiluted semen into pre-labeled vials using a 1 or 2 mL sterile serological pipette. Add at least 0.2 mL to the post-thaw specimen's cryovial.

R. Place labeled vials into the labeled cryocane(s) with no more than two vials per cane (canes should be upside down and vials right-side up). Add cryosleeve(s) and put into a −20 °C freezer for 8 min. Do not open the freezer during this time.
Note: Exposure to freezing conditions should occur within 1.5 h of specimen collection.

S. After the 8 min incubation period, remove the canes from the −20 °C freezer and place into liquid nitrogen vapors.

T. After a minimum of 2 h incubation in the liquid nitrogen vapors, flip the canes, immersing the samples into liquid nitrogen.

U. After a minimum of 24 h in liquid nitrogen, thaw the aliquot in the designated post-thaw cryovial as follows:
   1. Using cryogloves and protective goggles, remove the cane containing the vial and snap it out. Loosen the cap and place in the 37 °C incubator for 20 min.
   2. As in step "I" above, mix the vial well and analyze using the high-powered microscope for count and motility.
   3. Record result(s) in the cryosurvival area of the Cryopreservation Worksheet and on the banking chart.
   4. Assess cryosurvival in the following formula:

$$\frac{\%\,\text{motility of pos-thaw specimen}}{\%\,\text{motility of pre-freeze specimen}}$$

V. The chart is then reviewed by the Medical Consultant prior to the release of the specimen. The physician signs the review form and it is placed in the client depositor's chart.
   A. The technologist who retrieved the aspirate from surgery and identified the patient name and clinic number should sign the Specimen ID Form.
   B. A final step for positive identification of the client depositor in the future is made by taking the client depositor's picture with the camera. Once the picture is developed, the client's name, clinic number, and freeze number are written at the bottom and are stored in the client depositor's cryobank folder.
   C. The patient is now required to have blood drawn.
      1. The blood tests performed for ID screening are: HIV 1/2, HBcAb, HTLV 1/2, HIV Ag, RPR, HbsAg, HCV, HCVNAT, WNV, CMV, and HIVNAT. The remaining 7 mL red top tube should be centrifuged and the serum removed. Aliquot the serum into one cryovial. The cryovial should

be labeled with patient name, MRN number, freeze number, date, and serum, then placed in a −50 °C freezer. These client depositor serum banks can be used for retesting or for future new tests. These serum specimens will be archived for 10 years after the date of distribution.

a. If any of the test results are positive, the Director, Andrology Laboratory and Sperm Bank, is contacted by the Blood Bank.
b. Positive test results are confirmed by retesting.
c. The Director contacts the patient's ordering physician, as well as the patient, either by phone or letter when any of the tests are positive.
d. If any of the blood results are positive, refer to the Frozen Semen Agreement. The cryopreserved specimens remain in quarantine.
e. All blood reports on client depositors from the Blood Bank are reviewed and initialed by the Supervisor or Director.

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