National Physicians Cooperative of the Oncofertility Consortium®

Section 10. Ovarian Tissue Freezing, Thawing, Labeling, and Testing

Date Adopted	Supersedes Procedure #
10/17/2017	11/1/2010; 12/15/2009; 11/1/2009; 2/3/2009

Review Date	Print Name	Signature

Principle and/or Rationale: Ovarian tissue can be cryopreserved and stored for clinical care or research purposes

Materials and Equipment:

Equipment	Requirements	Suggested Model *	Comments
Programmable freezer suitable for slow cooling of large numbers of **VIALS**	Freezer Specs: • Freezing of vials (not straws) • Capacity of approximately 20-30 vials • Manual seed • Programmable room temp to -30°C	Freeze Control CL8800i distributed by Biogenics (Napa, CA) (See comments)	Check the ***vial*** capacity of your freezer; all vials must be frozen in the same freezing run
Liquid nitrogen storage tank	Patient ovarian tissue vials, plasma, and research tissue will be stored on site until shipment to Reprotech	Any	Patient infectious disease testing results required before shipping
Brady labeler with labels suitable for liquid nitrogen	Labels that are proven safe in liquid nitrogen	Brady TLS 2200 Labels PTL 21-427 (Biogenics, Napa, CA)	30 vials per patient is common; automatic labeler protects against errors in labeling
Dewar flask (small)	Suitable for plunging vials or cooling swabs for seeding	Any	Not required for some freezing machines
Miscellaneous instruments for dissection of ovary **(sterile)**	• Forceps • Fine, sharp scissors (some prefer curved) • Large scissors for bisecting ovary • Disposable scalpels with #10 blades • User preference	Any	**STERILE**
Pipettes, adjustable volume with **sterile** tips	Dispense volumes from 100-1000 µL	Any	**Sterile** pipette tips

Supplies:

Materials	Source	Catalogue #	Size/Unit	Comment
OFC Holding media-MOPS Diluent	Cooper/Sage	Research only	100ml bottle	Each ovary will usually require 2 bottles • 3 bottles will be required for each ovary if the research portion of tissue will be sent fresh to Northwestern
OFC Freezing media-1.5M ethylene glycol in MOPS-Diluent with 0.1M sucrose	Cooper/Sage	Research only	30ml bottle	Each ovary will usually require 1 bottle • 0.9 ml per vial frozen; full ovary 20-30 vials • no freezing media will be required for SCAFFOLD participants where tissue will only be sent fresh to Northwestern for research
Nunc CryoTube vials(1.8 ml) internally threaded	Any distributor	Nunc 377267	50/package	Internally threaded WITH gasket, self standing
CryoTube rack	Any distributor	Nunc 376589		Rack “matches” vial used; see manufacturer specs
CryoMarker, black	Any distributor	Nunc 343850	Each	
Canes, aluminum	Any distributor	Nunc 378441	50/case	
Cryo-Sleeves, clear	Any distributor	Nalgene NNI 5016-0001	100/case	Place over each cane
Large cotton swabs	Any distributor	Scopettes or other large swab	Each	For manual vial seeding
Ruler, centimeter markings	Any distributor	N/A	Each	Place under petri dish during dissection
Cane tags for storage canes				
Green, **light**	Any distributor	Nunc 378476 or Nalgene DS5020-004	100/case	Fully tested; negative
White	Any distributor	Nalgene DS5020-000		Research tissue; negative for infectious diseases
Red	Any distributor	Nunc 378406 or Nalgene DS5020-005		**Patient tissue;** positive for one or more infectious disease agents
Blue, **light**	Any distributor	Nunc 378468 or Nalgene DS5020-003		Patient; plasma
Yellow	Any distributor	Nunc 378492 or Nalgene DS5020-002		**Research tissue;** positive for one or more infectious disease agents
Gloves, exam **powder free**	Any distributor			
Specimen container, sterile or 50 ml conical tubes	Any distributor			Sterile interior
Petri dishes, 100 ml	Any distributor	Falcon 35-1029		
4-well tissue culture plates (for thawing only)		Nunc 176740		

Methods:

NOTE: All procedures should be performed using sterile technique and universal precautions in the **cold (0-5°C)**.

The dissection technique for ovarian tissue cryopreservation can be reviewed at http://oncofertility.northwestern.edu/media/dissection-human-ovary-preparation-cryopreservation

Sample Receipt, Surgical Technique and Preparation

1. Place approximately 30-40 ml of holding media in a sterile specimen container or 50 ml conical centrifuge tube, close tightly. Place in transport container. Cold temperature packs should be used to support the container in an upright position.
    - **Technical Note:** The specimen container containing media should be wrapped in a cold temperature pack and placed in an insulated travel container, such as a tall insulated coffee cup with lid or wide mouth "thermos." This container should be labeled with a biohazard label and the name and pager number of the person picking up the tissue. This container can be taken to the operating room on the day of surgery. The surgeon should place the tissue into the specimen container containing media and then place it in the insulated travel container. The tissue can then be transported to the lab within 1 hour of collection. If infectious disease testing is drawn intraoperatively, the purple top tube for plasma archive/banking can be placed in this container to ensure that the plasma sample stays with the ovarian tissue sample.
2. Specimen container should be labeled with:
    - Patient's last name
    - Patient's first name
    - Unique identification number
    - Date of surgery
    - Time of removal of ovary
    - Surgeon name
    - Hospital label can be substituted, provided time of removal is written on it
3. **Initiate sample processing within 1 hour of tissue removal.**
4. See separate procedure entitled, "**Sample Labeling,"** for procedure for labeling all vials and canes and for de-identifying research tissue.
5. Label 1.8 ml Nunc CryoTube vials according to "**Sample Labeling"** procedure.
6. Place 0.90 ml of freezing media in each of the 1.8 ml vials that will be used for ovarian tissue.
7. Place in refrigerator until use (not longer than overnight).
8. Ovary should be removed using a surgical technique that keeps the ovarian blood supply intact until the ovary is removed.
9. Once ovary is removed, pathologist or surgeon should remove any portion needed for pathologic examination ***using sterile technique***. Remainder of the ovary should be placed directly into the sterile specimen container prepared in step 1. Alternatively, the portion for pathology can be cut in the lab and the pathology sample returned for examination.
10. **Initiate sample processing within 1 hour of tissue removal.**
11. Patient/subject identification should be verified against the container label.
12. Specimen container should be transported to the laboratory in the cold **(0-5°C)** (see Technical Note above).
13. **Minimum cortical tissue sample:** the patient must have ***cortical*** tissue equivalent to 6 strips of size: 2cm x 0.5cm with a thickness of 1mm before any tissue can be allocated to research. **If the patient has less than this minimum amount of cortical tissue**, then the informed

consent dictates what will be done with the tissue: either all is frozen for the patient use or all is used for research. If this has not been indicated on the consent, then all the tissue should be frozen for patient's own use.

Tissue Preparation and Dissection

1. All tissue dissection should be performed in the cold **(0-5°C)** using sterile technique.
2. Rinse the tissue with cold **holding media**- MOPS Diluent. Dissections can be easily accomplished using a 100mm petri dish containing a small amount of holding media. Rinse tissue frequently during dissection and keep tissue in cold holding media.
3. Using blunt dissection, separate tissue planes and remove the cortical tissue from the medulla of the ovary.
    - **Technical Note**: Sharp, fine scissors are essential here; some prefer curved scissors. Forceps *without* teeth seem to work best. Rinse instruments with media before use and discard that media.
4. Medulla can be discarded, added to the research tissue or submitted to pathology.
5. The cortical tissue should be scraped free of any fatty or medullary tissues and sliced to form a flat sheet. To process tissue, place tissue in culture dish with a small amount of Holding Media. Use a sterile scalpel and/or iris scissors (or similar instruments) to cut the tissue to the proper dimensions.
6. Rinse tissue or move to clean petri dish with fresh media whenever the media becomes bloody.
7. Cortical tissue should be cut into strips with approximate dimensions of 2cm x 0.5cm with a thickness of 1mm. Tissue pieces should be thoroughly rinsed in holding media. **See attached Figure 1.**
8. The number of pieces of cortical tissue should be counted.

Addition of Cryoprotectant and Freezing Process

1. **For patient tissue:** place several (1-2) pieces of cortical tissue (2cm x 0.5cm with a thickness of 1mm) in a vial containing 0.90ml of **freezing media**-1.5M ethylene glycol in MOPS-Diluent with 0.1M sucrose (see above). If more than two pieces of tissue, label vial with number of pieces. If not marked, it is assumed that there are two pieces are in the vial. In general, the total number of vials should be 10-15 for ease of seeding.
2. Close vials, vortex gently, and equilibrate in the refrigerator (approximately 0-5.0°C) for 30 minutes.
3. Load vials in to programmable freezer at 5.0°C.
    - Cool at a rate of -2.0°C/minute from 5.0°C to -7°C.
    - Hold for 10 minutes and then seed, manually
    - Hold for an additional 10 minutes for ice crystal formation
    - Cool at a rate of -0.3°C/minute to -30°C
    - Plunge into liquid nitrogen
    - Place on canes labeled with:
        1. Patient's last name
        2. Freeze date
        3. Cane number for that patient
        4. Label cane directly on the cane
        5. When no colored cane tag is present this denotes: INFECTIOUS DISEASE STATUS UNKNOWN

- Store the samples in liquid nitrogen

Thawing Technique

1. Prepare thaw media for each vial to be thawed using a 4-well culture plate (1ml well)

	Freezing Media Volume	Holding Media Volume	Time in Each Media
1.0M EG	0.6ml	0.3ml	10 minutes
0.5M EG	0.3ml	0.6ml	10 minutes
0.0M EG	0	0.9ml	5 minutes

2. **Verify** patient name, number, and date prior to thawing the tissue.
3. Thaw the vial at room temperature for 1 minute, and then plunge into 37^{o}C water bath for 2 minutes.
4. Tip the tissue out into 60 or 100mm petri dish. If necessary to rinse vial, use freezing media.
5. For each step, ensure that tissue is completely submerged in medium.
6. Transfer the ovarian tissue to a culture dish containing 1.0M EG containing media for 10 minutes. Move the slice of tissue to a dish containing 0.5M EG containing media for 10 minutes.
7. Move the slice of tissue to media without cryoprotectant (holding medium) for 5 minutes.

Record Keeping, Reporting of Results, Infectious Disease Testing, Plasma Banking and Sample Labeling

1. All samples should be assumed infectious unless proven otherwise and universal precautions used.
2. Frozen samples will be recorded on the attached log sheet and stored alphabetically as hard copy. All thawed samples will be recorded.
3. Additional patient information will be recorded in the CD3 database.
4. A freezing report will be placed in the patient chart containing the following information:
   - Patient's last name
   - Patient's first name
   - Patient identification number (not the research number)
   - Number of vials of tissue frozen for patient own use
   - Number of vials of tissue frozen for research
   - Number of vials of plasma frozen and stored with patient tissue
   - Date patient tissue shipped to long term storage
   - Whether infectious disease tests were drawn
   - Name of person generating the report
5. All patients freezing tissue for their own use will be tested for infectious diseases according to FDA regulations for anonymous donors of reproductive tissues for leukocyte rich tissues. This testing must be performed within 7 days of tissue retrieval. Tests should be drawn on the day of surgery when it is confirmed that tissue will be frozen.
6. **Plasma archive/banking**: each patient freezing tissue for her own use must have an additional PURPLE top tube drawn for plasma banking.
   - Centrifuge sample (high speed for 10 minutes) and aliquot 1ml of plasma to each pre-labeled 1.0 ml Nunc CryoTube vials (see: **Sample Receipt, Surgical Technique and Preparation, item 2**). **Write PLASMA on each vial.**

- Place plasma samples on a cane separate from tissue samples; label cane as described below (blue cane tag).
- Plunge the samples into liquid nitrogen for storage with patient tissue.

Cane Labeling

1. General Comments
   - Cane tag and color indicates infectious disease status of the sample
   - ALL tissue should be considered infectious disease status unknown and stored as such until proven otherwise
   - All vials should be marked patient, plasma or research
   - Cane and cane tag should have patient's last name, date of freeze and cane number for that patient
   - As infectious disease results become available, an additional cane tag should be added to the cane
   - All samples of LIKE cane tag color should be stored in the same canister under liquid nitrogen
   - Samples on canes should **not** be lifted beyond the neck of the liquid nitrogen storage tank to add the cane tag
2. Cane Labeling Color Scheme:
   - **NO CANE TAG--**Infectious Disease Testing Unknown. When tissue is frozen, cane will be labeled ***without*** a cane tag on the stainless steel top.
   - **WHITE Cane Tag-**-- **Research** Tissue: Infectious Disease Status Known: all results negative/non-reactive.*
   - **GREEN Cane Tag-- Patient** Tissue: Infectious Disease Status Known: all results negative/non-reactive.*
   - **RED Cane Tag-- Patient** Tissue: Infectious Disease Status Known: one or more results positive/ reactive.
   - **YELLOW Cane Tag-- Research** Tissue: Infectious Disease Status Known: one or more results positive/ reactive.
   - **BLUE Cane Tag--** Patient **Plasma** Sample. Store with patient tissue samples.

   ***NOTE:**
   1. **CMV Results** should not affect the classification of the sample as non-infectious (if all other results are negative/non-reactive). A positive CMV result does not render a donor ineligible. Samples from patients testing positive for CMV (where all other results are negative/non-reactive) should be labeled as "Infectious Disease Status Known: all results negative/non-reactive."
   2. All frozen samples, including patient tissue, and plasma samples should be shipped together to long term storage.

Technical Notes

1. Freezing media and holding media are sterile as supplied and should ***not*** be re-filtered.
2. Do not add any other components to the media (e.g. antibiotics, protein, etc.); use them as supplied.
3. **MEDIA HANDLING:** all reagents should be labeled with date received, date opened, and discard date. All media should be discarded **one month** after opening. Expiration date is recorded on the bottle as supplied.
4. Use good tissue banking technique for all steps:
   - If instruments are autoclaved, rinse with culture media before use. Do not allow this media to come into contact with tissue.
   - Do not allow gloves to come into contact with culture media.

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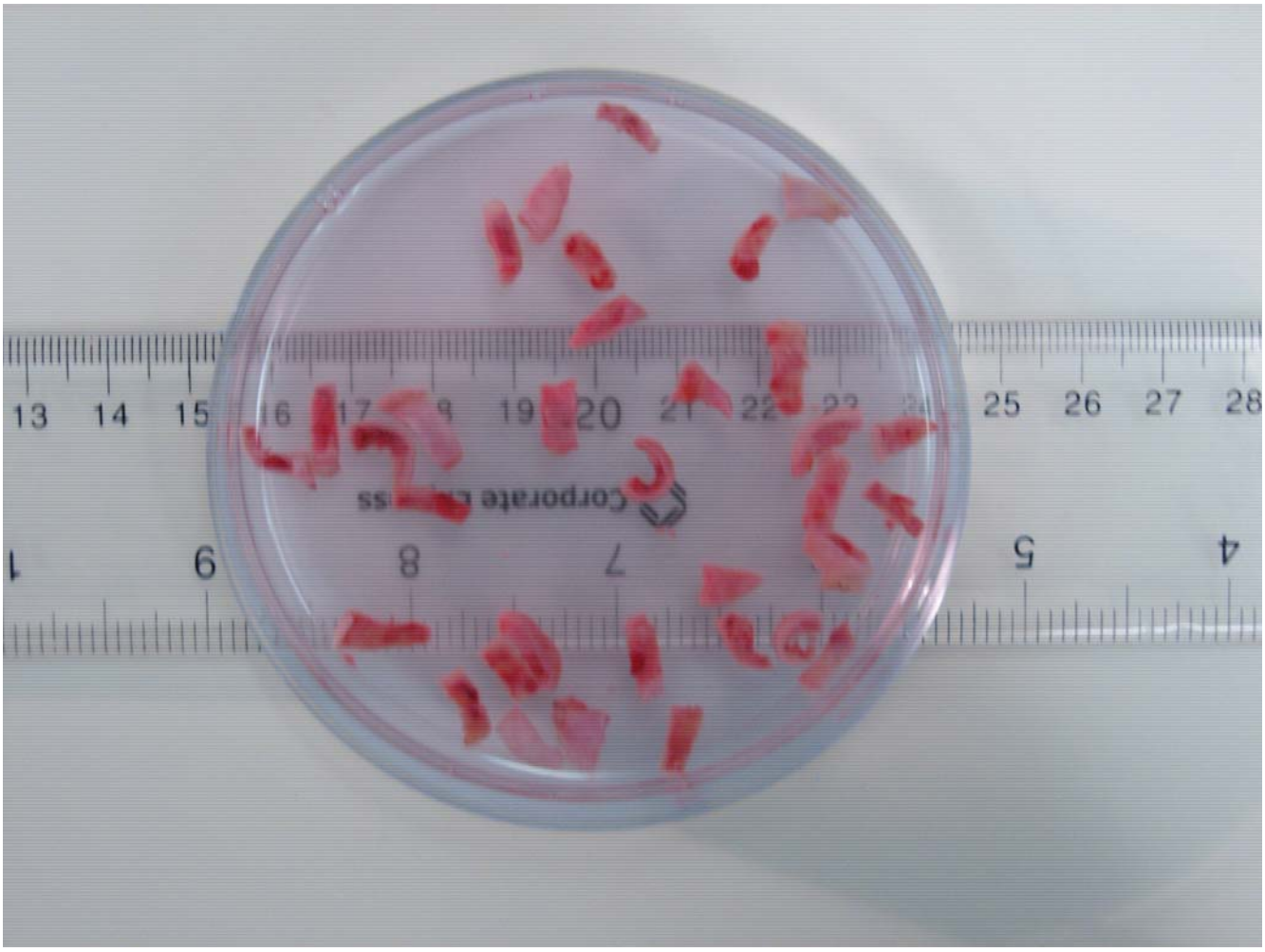
Figure 1. Illustration of Ovarian Tissue Preparation



Dissection training videos available here:

https://www.youtube.com/watch?v=yxuy-KniU24

https://www.youtube.com/watch?v=IPbNgzhzCuA