



## Sample Cryopreservation Procedures CRYOPRESERVATION – DMSO (Neat)

ISHAGE/ISCT 2001 – TECHNICAL BREAKFAST 1 & 16  
STORAGE and CRYOPRESERVATION of CELL PRODUCTS

### 1.0 PRINCIPLE:

The bone marrow cells are aliquoted into four (4) cryobags at approximately 40 to 60 mL each and chilled on ice for 30 minutes. Dimethylsulfoxide (DMSO) is added to each bag to a final concentration of 10% (v/v). The cryobags are then placed in aluminum canisters and frozen at a controlled rate, so that compensation occurs for the eutectic point transitions (changes in the molecular structure of ice). Frozen Bone Marrow is stored in the liquid nitrogen storage tank in the liquid phase. The expiration date is set at ten (10) years from collection date.

### 2.0 SPECIMEN:

2.1. Bone marrow: cells may be frozen after Buffy Coat, Washed Density Gradient preparation, or T cell depletion as per required protocol. The cells should be depleted of erythrocytes and resuspended in appropriate medium containing a protein source (i.e., Lactated Ringer's + Plasma Protein Fraction) prior to freezing.

### 3.0 REAGENTS AND EQUIPMENT:

Reagent lot # and equipment certification/maintenance records should be recorded in the Lot # notebook and the Equipment Record notebook, respectively.

- 3.1 Lactated Ringer's + PPF (LR+PPF) refer to SOP III-1.10
- 3.2 Dimethylsulfoxide (DMSO)
- 3.3 Cryocyte Bags (Nexell # 4R9952)
- 3.4 Syringes, 3 mL, 20 mL, 60 mL
- 3.5 Needles 16 Ga 1"
- 3.6 Towel
- 3.7 Canisters (aluminum) - hold and protect the cryocyte bags
- 3.8 Ice Bath/Tray
- 3.9 Tubing Sealer; Sebra model 2100 or equivalent
- 3.10 Biosafety Hood (class IIA/B3) Forma Scientific or equivalent
- 3.11 Programmable Freezer - (Cryomed 1010)
- 3.12 Liquid Nitrogen (LN2) - low pressure (22) PSI dewar
- 3.13 Cryoplus III Liquid Nitrogen Storage Tank (Cryomed/Forma)
- 3.14 Needle Recapping Device (OnGard Systems Ultimate Recapper or equivalent)
- 3.15 Low Temperature Freezer (-20° C)
- 3.16 Patient Addressograph labels
- 3.17 Plasma Expressor (Baxter)
- 3.18 Isolator Microbial tubes (Wampole)
- 3.19 Iodine "Pops" Antiseptic applicators -10% PVP Iodine (Medi-Flex 26-02-86)
- 3.20 Alcohol Preps-70% Isopropyl Alcohol (Kendall-Webcol 6818)
- 3.21 Protective Tape (Fisher Scientific 11-867C)
- 3.22 Scissors
- 3.23 Sampling Site Couplers (Fenwal 4C-2405)
- 3.24 Hemastats
- 3.25 Sterile Tubing Welder (SCD) Model 312 (Haemonetics)
- 3.26 Transfer Pack 600 mL (Fenwal 4R-2024)
- 3.27 Scale, High Capacity -(Satorius LC3400P or equivalent)

### 4.0 PROCEDURE:

- 4.1 PREPARATIVE STEPS - PERFORM PRIOR TO CRYOPRESERVATION
  - 4.1.1 LABELING THE CRYOCYTE BAGS and the CANISTERS
    - 4.1.1.1 Using a permanent marker (Sharpie), write on each cryocyte bag the following information:

NAME OF INTENDED RECIPIENT  
IDENTIFICATION NUMBER: (hospital # or social security #)  
DATE & ACCESSION #  
CELL PRODUCT (EX: allogeneic BM-T CELL DEPLETED, autologous BM- PURGED, or BACKUP)  
if autologous - "FOR AUTOLOGOUS USE ONLY"  
SEQUENCE #: Ex: 1 OF 4

- 4.1.1.2 Set aside, the cryocyte bags, until needed.
- 4.1.1.3 Label a cryocyte bag as Thermocoupler Bag, set aside.
- 4.1.1.4 Label four (4) cryovials with Name, Cell Source, Date, Accession #.
- 4.1.1.5 Aluminum canisters may be labeled with a patient Addressograph label, with the necessary information in 4.1.1.1 added.
- 4.1.1.6 Apply protective tape over label to seal LN<sub>2</sub> out.
- 4.1.17 Place the canisters into the Low Temperature Freezer until needed.
- 4.1.18 Record the lot numbers of the DMSO and cryocyte bags on the "Bone Marrow Cryopreservation" sheet (form II-8).

## 4.2 CRYOPRESERVATION PROCEDURE

4.2.1 Weigh the bag containing the bone marrow cells. Subtract out the weigh of the bag (600 mL transfer pack = 32g).

4.2.1.1 If the volume in the bag is greater than ( > ) 250 mL, the volume must be reduced.  
**Go To Steps 4. 3.1 - 4. 3.8**

4.2.1.2 If the volume in the bag is equal to or less than ( < ) 250 mL, place the bag inside the biological safety cabinet. Spike the bag (if not already done) with a sampling site coupler.

4.2.2 Using a syringe-needle setup, remove 1.0 mL and prepare a 1:10 dilution using Lactated Ringer's + PPF, aliquot into cryovials as follows: 0.5 mL for counts, four (4) - 1.0 mL aliquots for freezing, 1.5 mL for microbiology testing.

4.2.2.1 Prepare Isolator Microbial Tube using Iodine "Pop" and Alcohol prep before injecting 1.5 mL into the tube. Appropriately label the vial and send to the Microbiology lab.

4.2.3 Place the cryovials for freezing into a icebath.

4.2.4 Using 60 mL syringe-needle setup, withdraw 60 mL of the cell suspension in each syringe. The fourth syringe usually contains <60 mL.

4.2.5 Using the Needle Recapping device carefully recap and remove the needle from a syringe (discard needle into an appropriate sharps container) and attach the syringe to Cryocyte bag #1. Dispense the cell suspension into the bag, leaving the syringe attached.

4.2.6 Repeat step 4.2.5 with the other three (3) syringes and Cryocyte bags.

4.2.7 Record the volume in each bag on the "Bone Marrow Cryopreservation Flow Sheet"- (form II-8).

4.2.8 Dispense into the Thermocoupler bag, a volume equal to the largest of the four (4) bone marrow bags, of LR+PPF.

4.2.9 Calculate the amount of Dimethylsulfoxide (DMSO) to be added to each bag:

- 4.2.9.1 Volume in bag x 10% (0.01) = Volume of DMSO
- 4.2.9.2 Record the volume of DMSO to add on the "Bone Marrow Cryopreservation Flow Sheet" (form II-8).
- 4.2.9.3 Total Bag Volume = Volume (cells) + Volume (DMSO). Record total bag volume on the "Bone Marrow Cryopreservation Flow Sheet" (form II-8).
- 4.2.9.4 Using a permanent marker, gently record the Total Bag Volume onto the surface of each Cryocyte bag.
- 4.2.10 Place the Cryocyte bags into the icebath with the cryovials. Incubate on ice for approximately 30 minutes.
- 4.2.11 Set an alarm for 30 minutes and another for 20 minutes.
- 4.2.12 Upon the 20 minute alarm obtain a fresh bottle of DMSO and aliquot the appropriate volume of DMSO into five (5) - 20 mL syringes and 0.5 mL of DMSO into a 3.0 mL syringe.
- 4.2.13 Start the programmable freezer to allow it to chill to 4oC. (Refer to the Programmable Freezer Operation Procedure).
- 4.2.14 Upon the 30 minute alarm remove the empty 60 mL syringe from each of the Cryocyte bags and attach a 20 mL syringe containing the correct volume of DMSO.
- 4.2.15 Add 0.1 mL of DMSO to each of the cryovials. Invert the cryovials several times to mix the suspension. Replace the vials into the ice bath.
- 4.2.16 Starting with the Thermocoupler bag, sequentially and quickly, dispense the DMSO in each syringe into the bag while agitating the cells. Using the syringe's plunger, aspirate off the air inside each bag and seal the line, three (3) times with the Sebra heat sealer.
- 4.2.17 Using a pair of scissors, cut the middle heat seal and discard the attached line and syringe. Quickly, dry each cryocyte bag and place inside the appropriate pre-chilled canisters, i.e., place Bag #1 into canister #1. The Thermocoupler bag does not require a canister.
- 4.2.18 When cutting the line be careful not to leave any bone marrow in the sealed area. Bone marrow in the open end of the tubing after sealing must be avoided because it may contaminate the liquid nitrogen in the storage tank.
- 4.2.19 Place the canisters and cryovials into the freezing chamber.
- 4.2.20 Place the Thermocoupler bag inside the bag press and insert the Thermocoupler into one of the sampling ports.
- 4.2.21 Close the chamber door and press the RUN button. Record START time on the "Bone Marrow Cryopreservation Flow Sheet".
- 4.2.22 The following controlled freezing steps will occur:

	FROM	TO	RATE	COMMENT
Step 1.2	4oC	-6oC	-1oC/min	chamber temp.

Step 1.3	-6oC	-6oC	-----	Hold temp for 10 min.
Step 1.4	-6oC	-50oC	-25oC/min	chamber temp. is lowered to absorb the heat of fusion release which occurs at about -10oC to -20oC
Step 1.5	-50oC	-14oC	+15oC/min	allow chamber to approximate sample temperature.
Step 1.6	-14oC	-60oC	-1oC/min	sample temp.
Step 1.7	-60oC	-90oC	-5oC/min	sample temp.
Step 1.8	END OF FREEZE. ALARM!!!			

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- 4.2.23 Press RUN button to silence the alarm.  
Record END time on the "Bone Marrow Cryopreservation Flow Sheet"(form II-8).
- 4.2.24 Open door, remove the bone marrow canisters and the cryovials, place into liquid nitrogen storage bank.  
Record Tank # and Frame # on the "Bone Marrow Cryopreservation Flow Sheet"(form II-8).
- 4.2.25 Remove chart recording and label it with an accession number and a label indicating the intended recipient's name and hospital number. The freezing chart will become a part of the permanent processing record.

#### 4.3 VOLUME REDUCTION PROCESS

- 4.3.1 If the bag containing the bone marrow has a sampling site coupler in one of the ports, the cells must be transferred to a new 600 mL transfer pack prior to centrifugation.
- 4.3.2 Attach a second 600 mL transfer pack to the bag containing the cells using the Sterile Tubing Welder (SCD 312) do not break the weld open.
- 4.3.3 Centrifuge the bag containing the bone marrow cells at 400G for 10 min.
- 4.3.4 Carefully remove the bag from the centrifuge bucket and hang it on the plasma expressor.
- 4.3.5 Place the empty transfer pack on the scale, tare the scale.
- 4.3.6 Open the weld connecting the bags and gently apply pressure using the plasma expressor. Remove the necessary supernate to obtain a final bag volume of < 250 mL, hemastat the line.
- 4.3.7 Heat seal the line and resuspend the cells.
- 4.3.8 GO BACK To Step 4.2.1

#### 5.0 PROCEDURE NOTES:

- 5.1 DMSO is potentially toxic to the cells at temperatures above 0°C, therefore upon addition of the DMSO, the subsequent steps must be performed with deliberation and speed.
- 5.2 Liquid Nitrogen is EXTREMELY COLD and can cause SEVERE burns or damage to skin and eyes.  
USE CRYOPROTECTIVE GLOVES AND PROTECTIVE EYE/FACEHIELD.
- 5.3 Bone marrows are given a 10 year storage period and considered expired thereafter.
- 5.4 Do not fill the Cryocyte bag to more than 80 mL.  
Excessive volume may preclude the use of the protective canisters and increase the chance for bag breakage.
- 5.5 Frozen Cryocyte bags/canisters are fragile/brittle, therefore, handle with care.

#### 6.0 LIMITATIONS OF PROCEDURE: N/A

## 7.0 QUALITY CONTROL:

- 7.1 The thawing and infusion process includes the evaluation of the cells' viability using both the trypan blue dye exclusion test and the Hematopoetic Progenitor Assay: Colony Forming Units (CFU-GM/BFU-E/CFU-GEMM).
- 7.2 A sample cryovial may be thawed to check the viability using either the trypan blue exclusion and/or Hematopoetic Progenitor Assay: Colony Forming Units (CFU-GM/BFU-E/CFU-GEMM).
- 7.3 The freezing chart should not show an unusual or unexpected changes in the temperatures. The appropriate heat of fusion curve should stay below 0°C.

## 8.0 REFERENCES:

- 8.1 N.C. Gorin, "Cryopreservation and Storage of Stem Cells", Bone Marrow and StemCell Processing: A Manual of Current Techniques, Eds., Areman, E., Deeg, H.J., Sacher, R.A., F.A. Davis Co., 1992, pp. 292-308.
- 8.2 W.E. Janssen, C. Lee, "Cryopreservation of Bone Marrow in Standardized Medium", Bone Marrow and Stem Cell Processing: A Manual of Current Techniques, Eds., Areman, E., Deeg, H.J., Sacher, R.A., F.A. Davis Co., 1992, pp. 317-319.

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