



Sample Cryopreservation Procedures

CRYOPRESERVATION – DMSO/Pentastarch

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

1.0 PRINCIPLE:

The marrow or peripheral blood stem cells are collected, cryopreserved and stored while the patient undergoes myeloablative therapy. The frozen cells are then thawed, treated and reinfused in order to repopulate the ablated bone marrow. The cells should be frozen using a programmable (controlled rate) freezer. If this type of freezer is not available, the cells may be placed in a mechanical freezer at -80°C overnight and then transferred to liquid nitrogen storage.

2.0 SPECIMEN:

- 2.1 Autologous bone marrow collected in the operating room
- 2.2 Umbilical cord blood
- 2.3 Peripheral blood stem cells or peripheral blood leukocytes collected by apheresis.

If necessary, the cell suspension is first concentrated by manual or automated technique (see appropriate procedure.)

3.0 SUPPLIES AND EQUIPMENT:

- 3.1 Laminar flow hood
- 3.2 Sebra heat sealer
- 3.3 Tube stripper
- 3.4 Autoclave
- 3.5 Programmable liquid nitrogen freezer
- 3.6 Freezing racks
- 3.7 Freezing canisters
- 3.8 Liquid nitrogen freezer or
- 3.9 Mechanical (-80°C) freezer
(For 500 ml of Pentastarch solution)
- 3.10 Wide mouth bottle (500ml) - Nalgene #2105-0016
- 3.11 Autoclave tape
- 3.12 540 mg Dextrose - Anhydrous (d-glucose) powder - Fisher #D16-500
- 3.13 60g Pentastarch powder - (McGaw) (IND required)
- 3.14 240 ml Plasmalyte-A - Baxter # 2B2543
- 3.15 160 ml Human Serum Albumin (25%)
- 3.16 50 ml Cryoserv DMSO - Research Industries, Tera Pharmaceuticals, Inc.
- 3.17 Cryocyte freezing container (250ml: Baxter#4R5461 and 1000ml: #4R5463)
- 3.18 Dual access coupler (Fenwal Sepacell Laboratory Adapter Set 4C2459 or equivalent)
- 3.19 Plasma transfer sets with spike and needle adapter - Fenwal 4C2240
- 3.20 Nylon three-way stopcocks - Baxter Pharmaseal K75 or Medex Inc.#MX511
- 3.21 Blood bag spike injection site - Baxter #1C8333
- 3.22 60 ml luer lock syringes
- 3.23 Micropin - Burrin #MP-1000
- 3.24 Cryovials - 1.8 ml
- 3.25 Recombinant human DNase (Dornase Alfa Pulmozyme) - Genentech #1004 (see 5.1)

4.0 PROCEDURE:

- 4.1 Preparation of Cryoprotectant
 - 4.1.1 Add Plasmalyte-A, dextrose and Pentastarch to 500ml wide mouth bottle.
 - 4.1.2 Place autoclave tape on bottle and autoclave for 20 minutes.
 - 4.1.2.1 Press "OFF/ON" button to turn on autoclave and to fill jacket with steam.
 - 4.1.2.2 Loosen bottle cap and place bottle in autoclave.

- 4.1.2.3 Close door and turn wheel to lock.
 - 4.1.2.4 Select "SLOW EXHAUST" cycle when jacket has filled (gauge on upper right front panel will register 15 lbs. pressure).
 - 4.1.2.5 Press "START" button. The cycle will run automatically with indicator lights at top of control panel lighting for each step of the cycle. The sequence control knob will also rotate indicating each part of the cycle.
 - 4.1.2.6 When the buzzer sounds and the "COMPLETE" light is illuminated, press "STOP" button.
 - 4.1.2.7 Open door and remove bottle.
 - 4.1.2.8 Make sure that stripes on autoclave tape have turned black.
 - 4.1.2.9 Press "OFF/ON" button.
- 4.1.3 Tighten lids and transport bottles to Cellular Engineering Laboratory. Place in laminar flow hood and allow cooling to room temperature.
- 4.1.4 Equally divide mixture into 4 sterile 250ml Cryocyte freezing bags (»72.5 ml/bag) or 2 sterile 1000ml Cryocyte freezing bags (»145 ml/bag).
- 4.1.4.1 Remove plunger from 60ml syringe and place barrel in small clamp on ring stand under hood.
 - 4.1.4.2 Attach needle adapter of Cryocyte bag to syringe.
 - 4.1.4.3 Using aseptic technique, pour sterile Pentastarch solution through syringe barrel and into freezing bag. As barrel empties, refill until sufficient volume of solution has entered bag.
 - 4.1.4.4 Close roller clamp on tubing and seal off access port.
 - 4.1.4.5 Label bags:
Pentastarch Solution (250 ml bag / 1000 ml bag:) Plasmalyte-A – 60 ml / 120 ml
 Dextrose – 135 mg / 270 mg
 Pentastarch – 15g / 30g
- 4.1.5 Store bags in freezer at –20°C. or colder.
- 4.1.6 Remove an aliquot for Sterility check. Order in HIS computer and transport to Receiving and Accessioning or directly to microbiology laboratory.
- 4.2 Cryopreservation of Bone Marrow / Peripheral Blood Stem Cells
- 4.2.1 On morning of marrow harvest or peripheral stem cell collection, thaw appropriate number of bags of Pentastarch solution. These may be thawed at room temperature or in a 37°C water-bath or incubator.
 - 4.2.2 When solution has thawed to room temperature add appropriate volume of 25% human serum albumin (125ml bag – 40 ml, 250ml bag – 80 ml) to each bag.
 - 4.2.3 Draw appropriate volume of DMSO (125ml bag –12.5 ml, 250ml bag –25 ml) into a 60 ml syringe. Before attaching syringe to Cryocyte bag, draw approx. 10 cc's of air into the syringe. Attach the syringe to the cryocyte bag and withdraw 20 to 30 ml of solution and immediately shake the syringe several times. Quickly inject content of the syringe back into the bag while mixing it at the same time.
 - 4.2.3.1 This step should be done fast to prevent formation of thick and heavy precipitate, which could clog up syringe and tubing (precipitate dissolves by mixing the solution for 2-3 minutes). Close the roller clamp and heat-seal tubing.
 - 4.2.4 Label bags with lot numbers and expiration dates of both DMSO and HSA, which were added to the pentastarch solution. Initial and date each bag. Assign a four-day expiration date from date prepared.
 - 4.2.5 Log in unit of bone marrow or peripheral blood stem cells and assign a unit number. The unit number for PBSC may also be assigned prior to pheresis. Recall Lotus 1-2-3 worksheet (CELLPROC.).
 - 4.2.6 Before processing, remove any necessary samples. (see appropriate procedures)
 - 4.2.7 Concentrate the marrow or peripheral stem cells by

- 4.2.8 manual or automated method (see appropriate procedures).
Label appropriate number of Cryocyte freezing bags with labels containing:
- 4.2.8.1 Unit number
 - 4.2.8.2 Date and time of collection and expiration
 - 4.2.8.3 Patient's name and Medical Record number
 - 4.2.8.4 Total volume
 - 4.2.8.5 Patient's ABO and Rh
 - 4.2.8.6 Reagents and anticoagulants used.
 - 4.2.8.7 Type of product: BM or PBSC

Note: There should be no more than 1.5×10^{10} nucleated cells per freezing bag. Because paper labels can fall off of the bags at extremely low freezing temperatures, also write patient name, medical record number, unit number and date directly on corner of freezing bag.

- 4.2.9 Close all clamps on the Cryocyte bags.
- 4.2.10 Label three cryovials with patient name, medical record number, specimen number, specimen type, and date.
- 4.2.11 After processing is completed, remove any necessary samples (see appropriate procedures).
- 4.2.12 Add 100mg/ml of human recombinant DNase to the cell suspension (if required by protocol).
- 4.2.13 After appropriate volume of DNase is added, remove samples for cryovials (3.0 ml) and sterility check (1.0 ml).
- 4.2.14 Prepare a syringe to transfer the concentrated cell suspension to the freezing bags.
 - 4.2.14.1 Place a 3-way stopcock on the 60cc syringe.
 - 4.2.14.2 Attach needle adapter end of plasma transfer set to female luer connector of the stopcock.
 - 4.2.14.3 Attach female luer connector from freezing bag to the male luer connector of the stopcock.

Perform Remaining Freezing Steps On Ice:

- 4.2.15 Aseptically spike bag containing cell suspension with coupler end of transfer set.
- 4.2.16 Open roller clamp to freezing bag.
- 4.2.17 Open stopcock between cell suspension bag and syringe.
- 4.2.18 Withdraw 25 ml of well-mixed bone marrow or PBSC concentrate into syringe.
- 4.2.19 Open stopcock to freezing bag and expel contents of syringe into the bag.
 - 4.2.19.1 Withdraw air from bag into syringe, turn syringe so that it is pointing down, and push the cell suspension back into the bag. This clears marrow or PBSC from the tubing.
- 4.2.20 Close the roller clamp and detach tubing with luer connector.
- 4.2.21 Attach the female luer connector of the 2nd freezing bag to the male luer connector of the stopcock and repeat steps 4.2.16 through 4.2.21. Continue this process until each freezing bag contains 25 ml of cell suspension.
- 4.2.22 PREPARE CONTROLLED RATE FREEZER (Note: if freezing in mechanical freezer, proceed to step 4.2.35).
 - 4.2.22.1 Confirm that the controlled rate freezer and controller are plugged in and that there is sufficient liquid nitrogen for the freezing procedure.
- 4.2.23 For CRYOMED FREEZER (refer to the Operator's Manual for detailed instructions and information concerning operation of this instrument).
 - 4.2.23.1 Turn the power-switch on (located on back panel). All the indicators on the front panel should light up. After approximately 4 seconds, the unit will display the parameters for the particular section of a particular function.

4.2.23.2 Verify the calibration of the strip chart recorder by using the CHAM and SAMP keys in the TC SCANNER cluster. Press the CHAM keypad to force the chart recorder pen to mark the zero degree level. If necessary, adjust the zero knob on the recorder. Press the SAMP keypad to force the strip recorder to mark -180. Then press the SCAN keypad to alternate between sample and chamber temperature.

4.2.23.3 Press keypads "1" and "." (decimal point) to select Freezing Bag program. Make sure program is "1." Section "1". Press RUN so that unit will prechill to 4°C and hold.

4.2.23.4 Place freezing press in chamber.

4.2.24 For PLANER FREEZER:

(refer to the Operator's Manual for detailed instructions and information concerning operation of this instrument).

4.2.24.1 Turn on power switch on the chamber unit no more than 5 minutes prior to beginning freezing program.

4.2.24.1.1 If Using MR3 Temperature Controller

4.2.24.1.1.1

Select desired freezing program by pressing RUN/HOLD key. Use >FORWARD and <BACKWARD arrow keys or the numeric keys to select correct program number.

4.2.24.1.1.2 Press ENTER.

4.2.24.1.1.3 Press RUN. Program will ramp to the start temperature.

4.2.24.1.1.4 When the start temperature is attained the freezer will go into the HOLD mode and maintain the temperature. An alarm will sound and the display will instruct the operator to insert the samples. The alarm can be stopped from sounding by pressing CLEAR.

4.2.24.1.2 If Using MR-Win Computer Program:

4.2.24.1.2.1 Disable internal printer.

4.2.24.1.2.2 Turn on computer attached to freezer and enter Windows environment.

4.2.24.1.2.3 Select the MR3 -WIN icon.

4.2.24.1.2.4 Choose "Program".

4.2.24.1.2.5 Choose "Select"

4.2.24.1.2.6 Select appropriate program.

4.2.24.1.2.7 Choose "OK"

4.2.24.1.2.8 Choose "Add".

4.2.24.1.2.9 Type in the sample id number

4.2.24.1.2.10 Choose "Configuration".

4.2.24.1.2.11 Type in user's name.

4.2.24.1.2.12 Select "Run".

4.2.25 Prepare a fresh syringe to transfer the freezing solution into the freezing bags.

4.2.25.1 Place a 3-way stopcock on the 60cc syringe.

4.2.25.2 Spike bag of freezing solution with a plasma transfer set.

4.2.25.3 Attach needle adapter end of transfer set to stopcock.

4.2.26 Attach one of the cryocyte bag connectors to remaining port of stopcock. Keep marrow and freezing solution on ice throughout procedure.

4.2.27 Open stopcock between freezing solution into syringe.

4.2.28 Withdraw 25 ml of freezing solution into syringe.

4.2.29 Open stopcock to freezing bag and slowly expel contents of syringe into bag, swirling bag to mix as the solution is added.

4.2.30 Withdraw air from bag into syringe, turn syringe so that it is

pointing down, and push enough air back into bag to clear the tubing.
Open the stopcock to the freezing solution bag and expel remainder of air.

- 4.2.31 Close the roller clamp and detach the tubing with female luer connector from the stopcock.
- 4.2.32 Attach the female luer connector of the 2nd freezing bag to the male luer connector of the stopcock and repeat steps 4.2.27 through 4.2.32. Continue this process until each freezing bag contains 50 ml of cell suspension/freeze solution.
- 4.2.33 On ice, slowly add 3 ml of freezing solution to the sample for cryovials. Place approximately 2.0 ml of cell suspension - freeze solution in each cryovial. Vials should be frozen in controlled rate freezer along with bone marrow or peripheral blood stem cells.
- 4.2.34 Seal tubing of freezing bags twice and cut as close to bag as possible. Test for leakage by turning bag upside down and squeezing gently. If tubing leaks and cannot be resealed, transfer contents of bag to a new freezing container (perform sterility check).
- 4.2.35 Controlled Rate Freezing (for mechanical freezer, go to step 4.2.36):
- 4.2.35.1 For Cryomed freezer:

- 4.2.35.1.1 Place bags between freezing plates so that ports extend over edge.
- 4.2.35.1.2 Place thermocoupler between plastic of two bags. Do not let thermocouple touch metal.
- 4.2.35.1.3 Close door and press RUN. The Cryomed will perform the selected program automatically.
- 4.2.35.1.4 Monitor the temperature recorder throughout the freezing process.
- 4.2.35.1.5 Label freezing chart with patient's name, medical record number, date and unit number.
- 4.2.35.1.6 Remove the press from the freezer and immediately transfer bags to pre-chilled freezing canisters and into liquid nitrogen storage.
- 4.2.35.1.7 Press WARM keypad to return chamber to ambient temperature.

4.2.35.2 For Planer freezer:

- 4.2.35.2.1 Place freezing bags in freezing cassettes. Remove cover from freezing chamber and slide cassettes into freezing rack.
- 4.2.35.2.2 Place temperature probe in a cassette containing a freezing bag.

IF Using MR3 Temperature Controller:

- 4.2.35.2.2.1 Press the RUN/HOLD button. The Planer will execute the freezing program automatically.
- 4.2.35.2.2.2 At the end of the run the alarm will sound and the display will show that the program is finished. The program will hold at the final temperature until the RUN key is pressed.

IF Using MR-Win Computer Program:

- 4.2.35.2.3.1 Select "RUN".
- 4.2.35.2.3.2 In an emergency the program can be aborted at any stage by pressing the RESET key. Press ENTER to confirm that you wish to ABORT. (If you do not wish to abort press CLEAR.)
- 4.2.35.2.3.3 When program is completed, remove the bags from the freezer and immediately transfer to pre-chilled freezing canisters and into liquid nitrogen storage. Transfer vials to vial storage freezer.
- 4.2.35.2.3.4 Press RUN key. Chamber will automatically warm to 20°C. Display will read "READY TO RESTART". Press RUN to return to MAIN MENU.
- 4.2.35.2.3.5 Turn off power switch on chamber unit.

When all freezing is completed for the day, close valve from the liquid nitrogen source to controlled rate freezer(s).

4.2.36 Mechanical Freezer (omit this step if using controlled rate freezer, above):

- 4.2.36.1 Place each freezing bag into a pre-chilled freezing canister labeled with patient's name, harvest number and date. Canisters can be cooled on ice or in -20°C freezer.
- 4.2.36.2 Place canisters in -70°C to -80°C alarmed freezer in horizontal position for 12 to 24 hours.
- 4.2.36.3 Freeze vials (see appropriate procedure).
- 4.2.36.4 Transfer canisters into appropriate racks in liquid nitrogen freezer.
- 4.2.36.5 Transfer vials to vials storage freezer.

4.2.37 Fill out patient's cryopreservation card and file.

4.2.38 Enter data in database.

5.0 PROCEDURE NOTES:

5.1 Some protocols require the use of DNase to prevent clumping due to release of free DNA from cells damaged during processing. When using the recombinant product, a concentration of 100ug/ml of the cell suspension has been shown to be appropriate in some situations. The actual amount used should be determined by experimentation when the protocols are designed.

6.0 REFERENCES:

- 6.1 Stiff, P., et al: Blood (1987), 70:974-978
- 6.2 Stiff, P., et al: Cryobiology (1983), 20:16-17
- 6.3 Operator's Manual: Cryomed Controllable Rate Freezer
- 6.4 Operator's Manual: Planer Kryo 10 Controlled Ra

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