Procedure Title: Preparation of Hematopoietic Stem Cells for Cryopreservation Without Tumor Purging

Procedure #: HSC.B521.01

I. Principle

Peripheral blood stem cells (PBSC) are harvested by apheresis and can be cryopreserved, thawed after chemotherapy treatment, and infused into the patient to restore marrow function. In this procedure, peripheral blood stem cells are treated with a cryopreservation solution containing 10% dimethyl sulfoxide (DMSO). This protects the cells from the deleterious effects of ice crystal formation during cryopreservation.

II. Reagents, Supplies, Equipment

Dimethyl sulfoxide (DMSO)
Human serum albumin
Sterile syringes (1mL, 5mL, 10mL, 30mL, 60mL)
Cryocyte freezing bag
Intra Via Container
Solution transfer set
Sterile needle
Alcohol wipes
Povidine swabs
Laminar flow hood
Light microscope
Digital balance
Floor centrifuge
Hemacytometer
Sterile tubing welder
Sebra tube sealer
Refrigerator
Kryorack cold platform
Labtop vial cooler
Plasma expressor

III. Procedure

A. Evaluate the integrity of the specimen and the container. Document any observations that could affect the quality of the product on the Cryopreservation of PBSC Worksheet.

B. Remove samples from the apheresis unit for testing

1. Attach a sampling site coupler to an available port in the apheresis bag.

2. Attach a needle to a sterile syringe of appropriate size to contain the entire volume of cell suspension.

3. Wipe the port septum first with povidone iodine, then with alcohol wipe.

4. Enter the unit with the needle through the port.

5. Invert the bag several times to mix the unit contents thoroughly before drawing up sample.

6. Remove the volume of sample needed. The following samples are required for any process:

a. Bacterial and fungal sterility to a sterile 15ml capped centrifuge tube (1ml).

b. CD34 testing to a sterile capped test tube (0.5ml)

c. White blood cell count (at least 0.2ml)

d. If the patient diagnosis id Neuroblastoma:

Remove a minimum of 30×10^6 cells for Immunocytology

C. Determine the initial volume of PBSC by weight or by measuring with a syringe. Record the volume on the Cryopreservation of PBSC Worksheet.

D. Determine the total number of white blood cells in the initial product

1. Prepare a dilution of the PBSC in Turk=s solution.

2. Determine the white blood cell concentration using a hemacytometer.

3. Calculate the total number of white blood cells in the initial product.

4. Record all calculation steps on the Cryopreservation of PBSC Worksheet.

E. The following guidelines need to be considered in the determination of the final cell concentration and volume using the total number of white blood cells in the initial product:

1. Pre- cooled freezing media (prepared by adding 20% v/v DMSO to patient=s autologous plasma) is added to pre- cooled cell suspension in a 1:1 ratio.

2. Cryopreserve at an optimal concentration (up to 200 x 10^6/ml). Exceed 200 x 10^6/ml with the approval of the laboratory director or designee.

3. Cell suspension should be cryopreserved in equal, 30 to 90ml portions per cryocyte freezing bag.

4. It is optimal to use the patient=s autologous plasma to prepare the cryopreservative solution and to resuspend the post centrifugation cell pellet if centrifugation is necessary. Therefore, the total volume of plasma available needs to be measured and considered when determining volume for cryopreservation. If sufficient plasma to freeze at less than 200 x 10^6/ml is not available, sterile human serum albumin can be used to make up the volume.

5. The final volume of cell suspension with cryopreservative solution should include both the volume to be cryopreserved plus the volume to be removed for testing or vial storage. At least 8 ml should be added to the volume to be cryopreserved to determine the final volume to prepare.

6. A minimum of two cryocyte bags need to be cryopreserved. Therefore, a minimum of 60 ml of cells and freezing media must be prepared. (30ml x 2 bags).

F. From the guidelines listed in section E, calculate the final cell concentration, the total volume for cryopreservation, and the number of cryocyte freezing bags to be frozen. See Example 1 in the Calculations section of this procedure.

G. If centrifugation is necessary follow instructions below. If centrifugation is not necessary, determine the volume of cell suspension by weight or by measuring with syringe, record the volume of cell suspension on the Cryopreservation of PBSC worksheet and proceed to step I.1.

1. Obtain a transfer pack of appropriate size to contain the volume of cells in the apheresis bag. Weigh the empty transfer pack.

2. Transfer the cell suspension from the apheresis bag to the transfer pack. Re-weigh the transfer pack with cells. Calculate the volume of cell suspension.

3. Centrifuge the unit at 400 x g for 10 minutes.

4. Tare an empty transfer pack for expressing plasma into.

5. After centrifugation, express excess plasma from cell pellet using a plasma expressor in the laminar flow hood.

6. Re-weigh the plasma bag. Calculate the volume of plasma available.

   (Note: If an extra source of patient plasma was provided from the apheresis collection, the plasma can be measured and then the bag and plasma can be weighed and used as a tare. Plasma can then be expressed into this bag from the centrifuged sample.)

H. If centrifugation was necessary in Section G, add plasma back to the cell suspension bag to dilute for cryopreservation.

1. Determine the volume of plasma to add to the cell suspension bag to achieve one-half of the final volume for cryopreservation.

2. Attach a needle to a sterile syringe.

3. Enter the plasma bag and aspirate the volume needed to add to the cell suspension. Dispense into the cell suspension bag.

4. Place the cell suspension bag into a refrigerator (2^{o} to 8^{o}C).

I. Prepare the cryopreservation solution

For centrifuged samples determine the total volume of cryopreservation solution (one- half the final volume for cryopreservation). The cryopreservation solution is prepared by adding DMSO to autologous plasma to make a solution that is 20% DMSO v/v.

If the sample was not centrifuged, calculate the amount of DMSO to make the final volume of cells and autologous plasma 10% DMSO v/v.

Dilute the DMSO with an appropriate volume of autologous plasma following the steps below.

1. Attach a sterile syringe to a needle

2. Add pre determined volume of autologous plasma to an empty transfer pack or viaflex bag of appropriate size to contain the total volume of cryopreservation solution

(Note: If sufficient plasma volume is not available, add 25% human serum albumin to make up the volume.)

3. Wipe all 10ml DMSO ampules needed, first with a povidine iodine wipe, then with an alcohol wipe. Carefully snap the glass ampule off. (if a 75 ml DMSO bottle is used wipe the septum with povidine iodine then with alcohol).

4. Attach a sterile syringe to a needle.

5. Transfer DMSO to the cryopreservation solution bag. (Tip: tilt ampule to remove as much volume as possible if 10 ml DMSO ampule is used).

6. Place the cryopreservation solution bag in the refrigerator for a minimum of 20 minutes.

J. Prepare bags, labels, and cassettes. Refer to procedure HSC E301.

(Note: Place all labeled cryocyte freezing bags and empty aluminum cassettes in the refrigerator until needed.)

K. Add cryopreservation solution to the cell suspension bag

1. Remove a blue Kryorack cold platform and place in the laminar flow hood.

2. Place the pre- cooled cell suspension bag and cryopreservation solution bag on the cold platform. Keep the cell bag on the cold platform throughout subsequent processing steps.

3. Attach a sterile syringe to a needle.

4. Gently moving the cell suspension bag back and forth across the cold platform, transfer the calculated volume of cryopreservation solution slowly to the cell suspension bag.

L. Remove samples for testing and vial storage

Note: Cryovials must be kept cold until cryopreservation. A labtop cooler device can be used.

1. Attach a sterile syringe to a needle.

2. Mix the cell suspension well by inverting the bag several times. Remove the total volume of sample needed.

3. Aliquot samples. The following samples are required for any process:

   a. Bacterial and fungal sterility to a sterile 15ml capped centrifuge tube (1ml).

   b. White blood cell count (at least 0.1ml)

   c. A minimum of six cryovials for future quality control testing (1ml per cryovial).

M. Transfer the cell mixture for cryopreservation to cryocyte freezing bags.

1. Attach a sterile syringe to a needle for the transfer.

2. Transfer the pre- determined volume of cell mixture to each cryocyte freezing bag. Remove the air bubbles from each bag.

N. Prepare the controlled rate freezer to maintain 4^{o}C. Refer to procedure HSC.D331.

O. Prepare the freezing bags for cryopreservation. Refer to procedure HSC.C101

P. Cryopreserve the cells using the controlled rate freezer. Refer to procedure HSC.D331.

Q. Transfer the each cassette and vial to its assigned storage space after the completion of the freezing program.

R. Shut down the controlled rate freezer. Refer to procedure HSC.D331.

S. Determine the total number of white blood cells in the final product

1. Prepare a dilution of the PBSC after addition of cryopreservative solution in Turk=s solution.

2. Determine the white blood cell concentration using a hemacytometer.

3. Calculate the total number of white blood cells in the final product.

4. Since no white blood cells should be lost during the processing steps of this procedure, average the white blood cell counts before and after processing.

5. Record all calculation steps on the Cryopreservation of PBSC Worksheet.

T. Submit samples for sterility testing to the CHLA Microbiology Department.

VIII. Calculations

Example 1

Determining the cell concentration and total volume for cryopreservation

Total cells= 30×10^9 cells
110 ml
Concentration= 272×10^6 cells/ml

O*ptimal to freeze at 200 x 10^6 cells/ml therefore centrifugation necessary*

Determine the total final volume

Minimum volume of cell suspension with cryopreservative solution needed to give a cell concentration not to exceed 200×10^6/ml
[30×10^9 cells / 200×10^6/ml]= 150ml or greater

Volume of cells for testing and vial storage = 8 ml

Total final volume chosen = **168ml/**
Chosen to yield 2 bags at 80 ml per bag
A *known amount of 8ml will be removed for testing + vials*

Determine cell concentration

30×10^9 cells / 168ml = **178.6×10^6 cells/ml**

Adding plasma back to the cells and preparing the freezing media

Volume of cells after centrifugation and plasma expression = **30ml**
Volume of plasma after plasma expression= **98ml**

Need to add 54ml plasma to 30ml of cells =84ml
Need to add 17ml DMSO to 44ml of plasma

and 23ml 25% human serum albumin = 84ml

Total volume= **168ml/**
Note: Only 98ml plasma available so 23ml 25% HS albumin added

160ml will be available to cryopreserve for future transplant. Two bags at 80ml per bag will be cryopreserved. 8ml will be used for testing and vial storage.

IV. References

1.Schwartzberg L., Birch R., Blanco R., Wittlin F., Muscato J.,Tauer K., Hazelton B., West W:
Rapid and sustained Hematopoietic reconstitution by peripheral blood stem cell infusion alone following high- dose chemotherapy. *Bone Marrow Transplantation* 1993. 11:369-374.

2.Lakay L., Bostrom B., Smith J., Moss T., Ramsey N:
Clinical collection and use of peripheral blood stem cells in pediatric patients. *Transplantation* 1989. 47:316-618.