

Cryopreservation of Hematopoietic Stem/Progenitor Cells for Therapeutic Use

Suzanne M. Watt, Eric Austin, and Sue Armitage

Summary

To date, more than 25,000 hematopoietic transplants have been carried out across Europe for hematological disorders, the majority being for hematological malignancies. At least 70% of these are autologous transplants, the remaining 30% being allogeneic, which are sourced from related (70% of the allogeneic) or unrelated donors. Peripheral blood mobilized with granulocyte colony stimulating factor is the major source of stem cells for transplantation, being used in approx 95% of autologous transplants and in approx 65% of allogeneic transplants. Other cell sources used for transplantation are bone marrow and umbilical cord blood. One crucial advance in the treatment of these disorders has been the development of the ability to cryopreserve hematopoietic stem cells for future transplantation. For bone marrow and mobilized peripheral blood, the majority of cryopreserved harvests come from autologous collections that are stored prior to a planned infusion following further treatment of the patient or at the time of a subsequent relapse. Other autologous harvests are stored as backup or “rainy day” harvests, the former specifically being intended to rescue patients who develop graft failure following an allogeneic transplant or who may require this transplant at a later date. Allogeneic bone marrow and mobilized peripheral blood are less often cryopreserved than autologous harvests. This is in contrast to umbilical cord blood that may be banked for directed or sibling (related) hematopoietic stem cell transplants, for allogeneic unrelated donations, and for autologous donations. Allogeneic unrelated donations are of particular use for providing a source of hematopoietic stem cells for ethnic minorities, patients with rare human leukocyte antigen types, or where the patient urgently requires a transplant and cannot wait for the weeks to months required to prepare a bone marrow donor. There are currently more than 200,000 banked umbilical cord blood units registered with the Bone Marrow Donors Worldwide registry. In this chapter, we describe several protocols that we have used to cryopreserve these different sources of hematopoietic stem/progenitor cells, keeping in mind that the protocols may vary among transplant processing centers.

Key Words: Hematopoietic stem cells; hematopoietic progenitor cells; HPC-A; HPC-M; cord blood; cryopreservation; cryoprotectant; controlled-rate freezer; DMSO.

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1. Introduction

Cryopreservation of hematopoietic stem/progenitor cells (HSC/HPC) for therapeutic use has been carried out for more than 30 yr. Three types of HSC/HPC harvests are now routinely used for transplantation for hematological disorders and each may be cryopreserved with, or without, preprocessing. These are bone marrow (BM), mobilized peripheral blood (MPB; most often following granulocyte colony stimulating factor [G-CSF] administration), and umbilical cord blood (UCB). The latter is sourced at birth and may be collected *in utero* or *ex utero* (1,2). The HSC/HPC may be autologous (cells to be transplanted back in the donor) or allogeneic (cells to be transplanted in recipients other than the donor) donations, preference being given to autologous transplants where these are deemed possible.

Over the past decade, there have been considerable changes in the indications for usage of these different cell sources for transplantation (3,4). In Europe, there has been a 600% increase in BM and MPB HSC/HPC transplants from just over 4200 in 1990 to 25,207 in 2003. Most transplants (95%) were for malignant diseases. In 1990, most HSC/HPC transplants used autologous or allogeneic BM donations. This changed first to MPB for autologous transplants, and by 2003, 97% of autologous HSC transplants were MPB derived. For allogeneic HSC transplants the pace of change was slower, but by 2003 65% of all allogeneic HSC transplants were MPB derived, with BM remaining a significant source of HSC/HPC in some European and Asian countries. UCB, a rich source of HSC/HPC is an established alternative for bone marrow (particularly in children), when a matched unrelated allogeneic bone marrow donor is not available. The sourcing of these stem cells for patients from ethnic minorities or with rare tissue (human leukocyte antigen [HLA]) types is a particular challenge, and it is for these patients that UCB may provide a unique source of matched stem cells. Banked UCB units are also important for patients needing immediate unrelated allogeneic transplantation. Currently, more than 200,000 banked UCB units are available for transplantation via the Bone Marrow Donors Worldwide registry (www.bmdw.org) or Netcord (www.NETCORD.org). There has recently been renewed interest in the use of UCB for HSC transplantation with the development of: (1) multiple UCB transplants into a single recipient, (2) nonmyeloablative transplants, (3) improvements in hematological reconstitution by transplantation of UCB with other sources of CD34+ HSCs, T-cell subsets, ex vivo expanded UCB cells, or mesenchymal stem cells, and (4) genetic selection of UCB units (1,2). Thus, cryopreservation of hematopoietic stem cells for therapeutic use takes place mainly for autologous MPB and for banked UCB units.

Optimal cryopreservation and recovery of viable HSC/HPC (as determined by engraftment potential) from each of the sources described depends on a

number of parameters, including (1) transit times and storage temperatures following harvest, (2) preprocessing prior to cryostorage, (3) the selected cryoprotectant, (4) cooling and thawing rates, temperatures, and protocols, and (5) longer term storage temperatures. As well as optimizing HSC/HPC viability and function, the aim is to reduce cryoprotectant toxicity and osmotic stress. In 1959, dimethyl sulfoxide (DMSO) was introduced as a cryoprotectant for HSC/PPC (5) and is the cryoprotectant used in the protocols. For therapeutic use of HSC/HPC, cryopreservation is performed as soon after harvesting as possible (6) with DMSO concentrations of 1–2 M, a controlled-rate freezer (CRF) cooling rate of 1°C/min, storage and/or transport below –135°C, preferably between –150 and –196°C, and rapid thawing at 37°C with slow dilution of DMSO (7–11). The theory and experimental evidence and other practices that have been developed for optimizing cryostorage and thawing of these cells are detailed in a number of publications (7–25) and elsewhere. An established protocol for cryostorage of MPB HSC/HPC collected by apheresis (termed HPC-A in JACIE guidelines) and BM HSC/HPC (termed HPC-M in Joint Accreditation Committee of ISCT Europe and EBMT (JACIE) guidelines) donations is described first, and this is followed by a description of the cryostorage of UCB from related (directed donations) or unrelated donors (*see Notes 1–21*).

2. Materials

2.1. Cryopreservation of HSC/HPC From Mobilized Peripheral Blood and Bone Marrow

1. Cryocyte freezing bags of varying capacities (Miltenyi Biotec., cat. no. Baxter R995).
2. Cryosure DMSO (Wak-Chemie Medical GmbH).
3. 4.5% (w/v) human albumin solution (HAS) if autologous plasma is not available.
4. Transfer set with luer adaptor (Baxter Biotec., cat. no. EMC2240).
5. Sample site coupler (Baxter Biotec., cat. no. EMC1401).
6. Plasma transfer set with two couplers (Baxter Biotec., cat. no. EMC2243).
7. Air darts (Baxter Biotec., cat. no. EMC0413).
8. Syringes (2.5, 5, 20, and 50 mL).
9. Needles (23 and 19 gage).
10. Cryovials (NalgeNunc Int.).
11. Aerobic/anaerobic BactAlert bottles (BioMerieux Corp.).
12. Overbags for double bagging (Synpac Ltd.).
13. Laminar air flow (LAF) cabinet.
14. Sterile connecting device (SCD) (Terumo Medical Corp.).
15. Dielectric line sealer Baxter Biotec.
16. Vacuum packer/sealer (Multivac).
17. Melting ice packs-sealed into plastic bags.

18. Programmable liquid nitrogen controlled rate freezer (CRF) (e.g., Kryo 10) (Planer plc, UK).
19. Freezing cassettes (Planer plc).
20. Ice bucket to hold melting ice packs.
21. Environmental monitoring plates (Biomerieux Corp.).
22. Yellow bin bag or plastic box for clinical waste.
23. Sharps bin.
24. Laminated harvest calculation sheet and processing sheets.
25. Labels (*see Note 3*).
26. Automated cell counter.
27. Top pan balance (accuracy to 0.1 g).
28. Scissors.
29. Particle counter.
30. Isopropyl alcohol (IPA) wipes.
31. Sterile biocide (e.g., Klercide, Shield Medicare Ltd.).
32. Cryovot liquid nitrogen vapor phase storage vessel.

2.2. Umbilical Cord Blood Cryopreservation

1. Harvest bag.
2. Cord blood collection: either whole blood or red cell and plasma depleted product.
3. Yellow bin bag for clinical waste.
4. Sharps bin.
5. Syringes, luer-lock (1, 5, 10, 20, and 50 mL).
6. Cryovials (2 and 4.5 mL).
7. Needles (19 gage).
8. "2991" coupler (Gambro BCT).
9. Spike-to-luer transfer set (Gambro BCT).
10. Luer/spike interconnector (Gambro BCT).
11. Three-way stopcock (Discofix).
12. 600-mL transfer bag.
13. Sample site coupler.
14. Preinjection swabs for disinfection of sampling sites.
15. 10% Dextran (Gentran 40, Baxter Corp.).
16. Double-bagged ice block.
17. DMSO.
18. 50% (w/v) DMSO diluted in Dextran-40 (Pall Corp. Europe).
19. Cryocyte or appropriate freezing bags.
20. Labels for freezing bags (*see Note 3*).
21. 1 tube cane (Planer plc).
22. Programmable CRF (e.g., Kryo 10, Planer plc, Sunbury, UK).
23. Liquid nitrogen storage vessel (Planer plc).
24. BioArchive automated, controlled rate, liquid nitrogen freezer (intended for cryopreservation and storage of HPC) (ThermoGenesis Corp.).
25. BioArchive controlled rate freezing module (ThermoGenesis Corp.).

26. Syringe pump (Invac.)
27. Dielectric hand held line sealer (RFS Services).
28. Overwrap bags for double bagging (Thermo Genesis Corp./Synpac Ltd.).
29. SCD (Terumo Medical Corp.).
30. Mixer.
31. Syringe pump (Invac).
32. Vacuum sealer (Fuji/Multivac).
33. Freezing cassettes for BioArchive (Thermo Genesis Corp.).
34. Jig for PALL freezing bag (Thermo Genesis Corp.).
35. PALL freezing bags compatible with BioArchive (Pall Corp. Europe).
36. Environmental monitoring plates for contact, settle, and finger dabs (Biomerieux Corp.).
37. Aerobic/anaerobic culture bottles, e.g., BacTAlert (Biomerieux Corp.).

3. Methods

Details are given of protocols for the cryopreservation of HSC/HPC from MPB and BM (*see Subheading 3.1.*) and UCB cryopreservation (*see Subheading 3.2.*).

3.1. Cryopreservation of HSC/HPC From Mobilized Peripheral Blood and Bone Marrow

Bone marrow (HPC-M) or mobilized peripheral blood (HPC-A) HSC/HPC are harvested from patients prior to autologous transplant for backup or “rainy day” purposes (specifically being intended to rescue patients who develop graft failure following an allogeneic transplant or who may require this transplant at a later date). Less frequently, cells for allogeneic use are cryopreserved prior to transplantation. The following protocol describes the cryopreservation of these harvests using a CRF, with DMSO (in autologous plasma or HAS if no plasma is available) as a cryoprotectant. Donor T lymphocytes or therapeutic T cells (termed TC-T in JACIE guidelines; *see Note 4*) may also be harvested from the blood and given to the patient as part of a low-intensity conditioning transplant procedure or to induce a graft vs tumor reaction. The TC-T are cryopreserved in a similar manner (*see Notes 1, 2, and 6*). Harvests are then stored longer term in liquid nitrogen (vapor phase) storage facilities at a temperature of less than -135°C . Sterile techniques are required for all procedures. In the United Kingdom, this process is carried out in a good manufacturing practice (GMP)-grade clean room environment as an open process, although closed processes are under investigation. The clean rooms are environmentally monitored for bacterial and fungal contamination. All BM and all blood products are treated as potentially infectious (*see Note 13*). Samples for microbiology testing are taken to check contamination of the product during processing. Because HSC/HPC occur in the CD34 subset, samples are also taken for both nucleated and CD34 cell counts and viability (as determined by dye exclusion

using 7-amino-actinomycin. D. (7-AAD) and flow cytometry, or using the colony forming unit (CFU) assay (22,26). This allows the efficiency of cell manipulations and the robustness of the cryopreservation to be evaluated, while at the same time determining the cell or CD34+ cell dose per kg of patient or recipient body weight (26,27). Cells are best processed the same day and as soon after harvesting as possible (6), but when unavoidable may be stored overnight at 4°C (see **Note 12**). The BM or MPB harvests may be volume reduced or red cell depleted (6), prior to cryostorage in order to reduce the numbers of cryocyte bags stored or the volume of incompatible (i.e., Rh and ABO mismatched) red cells in the graft. In such cases, the buffy coat, reduced from over 1 L to a volume of 100–150 mL, is then cryopreserved using similar techniques to those described next.

3.1.1. Preparations Prior to Processing Samples

1. Relevant forms and labels are used and completed to record and track products for processing, testing, and cryostorage. The harvest should be correctly labeled according to JACIE or other accreditation guidelines (see **Notes 3–8** and **11**). A unique donation barcode number (ISBT128 standards) is assigned to the harvest and is subsequently attached to the relevant harvest record form and cryobag labels. The time of collection and receipt into the processing facility should be noted. The temperature on arrival is taken and should be below 8°C if the time from collection is greater than 1 h.
2. The total nucleated cell count is obtained using a hematology analyser. The sample for this can be taken from the harvest bag by either:
 - a. Removing a section of the bag line. The line is first “stripped” into the bag a minimum of five times. A double heat seal is then made in the line isolating approx 0.5 mL of cell suspension. The seal furthest away from the bag is then cut removing the section of line containing the cell sample.
 - b. Taking the cell collection into the clean room, inserting a sample site coupler into the collection bag through one of the sample ports, and removing approx 0.5 mL of sample using a syringe and 23-gage needle. This operation is carried out in the laminar air flow (LAF) cabinet.
3. Prior to analysis on the hematology analyser, the cell sample is diluted 1 in 5 and 1 in 10 using phosphate buffered saline either alone or supplemented with 0.5% (w/v) HAS (see **Notes 12** and **13**).
4. Processing of harvests and their cryopreservation are currently carried out in the clean room as open processes, although closed processes may be developed. The number of cryocyte bags required for freezing is determined prior to entering the clean room. Each “50 cryocyte bag” can hold a maximum of 20 mL, a “250 cryocyte bag” can hold a minimum of 30 mL and a maximum of 70 mL, a “500 cryocyte bag” can hold a minimum of 70 mL and maximum of 100 mL, and a “750 cryocyte bag” can hold a minimum of 100 mL and maximum of 130 mL. The “50 and 250 cryocyte bags” are usually used for TC-T preparations only.

5. Prime the CRF liquid nitrogen Dewar prior to entering the clean room.
6. Prior to entering the clean room and sterile area preparation, all items for transfer into the clean rooms are wiped down with IPA wipes.
7. Staff should proceed through clean room lobbies, where they gown in clean room garments. On entering change areas, a particle counter, a hand sealer, and charged battery pack are collected, wiped with an IPA impregnated wipe, and carried in to the clean room. All of the cell processing is performed in a LAF hood producing a Grade A environment and within a clean room giving a Grade B background (*see Notes 3, 5, and 6*).
8. The LAF hood is wiped down with Klercide (or an equivalent), left for 2–5 min, and then wiped down again with sterile 70% (v/v) IPA. Two settle agar plates are placed to the left- and right-hand sides of the workspace within the hood for environmental monitoring.
9. The harvest and plasma bag details are checked in order to match the paperwork details and the clean room processing sheet is signed to verify this. All bags are weighed using a top pan balance to confirm volumes (assuming approx 1 g/mL).
10. The paperwork is prepared on the bench away from the hood. By the end of the processing, the clean room operation record sheet will be filled in with the details of the procedure being performed, batch numbers of consumables used, operator details, cleaning details, and environmental monitoring details, whereas the stem cell processing sheet will be completed with details of the processing volumes, total cell and CD34 positive cell counts, and other relevant information.

3.1.2. Preparation and Addition of Diluent

The required amount of diluent, either as plasma (*see Subheading 3.1.2.1.*) or HAS (*see Subheading 3.1.2.2.*) is added in the clean room within the LAF hood as described next.

3.1.2.1. AUTOLOGOUS PLASMA

1. Sterile connect (*see Note 14*) together the plasma and cell collection bags using either a sterile connecting device (use a pair of “Spencer wells” to clamp the line prior to opening the sealed join) or by a plasma transfer set with two couplers, one coupler inserted into each bag. It is important to ensure the wheel clamp is closed when inserting the spike couplers.
2. Place the bag of cells onto a balance and tare its weight to zero.
3. Open the wheel clamp or unlock the Spencer wells.
4. Allow the diluent to flow into the bag of cells until the required volume has been added and then reclamp the line.
5. Heat seal the tubing in three places and break the middle seal to separate the two bags.
6. Thoroughly mix the cells and diluent by gentle hand agitation.

3.1.2.2. 4.5% (w/v) HAS

1. Remove the metal cap from a bottle of 4.5% (w/v) HAS, disinfect with an IPA wipe, and place bottle inside LAF.
2. Insert an air dart into the bottle.
3. Remove a volume of 4.5% (w/v) HAS using a 50-mL syringe with 23-gage needle.
4. Insert sample site coupler into cell bag port.
5. Wipe injection site of sample site coupler with sterile alcohol wipe.
6. Inject required volume into cell bag.
7. Thoroughly mix the cells and diluent.

3.1.3. Preparation of Cryoprotectant

The cryoprotectant used here is 20% (v/v) DMSO diluted in either autologous plasma or 4.5% (w/v) HAS. The 20% (v/v) DMSO solution allows equal volumes to be added to the cell suspension resulting in a final concentration of 10% (v/v) DMSO (*see Notes 15–17*).

3.1.3.1. WITH AUTOLOGOUS PLASMA

1. Prior to adding DMSO, the plasma must be cooled to below 8°C. This is achieved by placing the plasma bag between two bags of melting ice.
2. Sterile connect the plasma bag to a cryocyte bag either using a sterile connecting device or with a plasma transfer set with two couplers.
3. Place the cryocyte bag on a balance and tare its weight to zero.
4. Open the connecting device and transfer the required volume of plasma to the cryocyte bag (assuming 1 g/mL).
5. Heat seal the tubing in three places and break the middle seal to separate the two bags.
6. Place the cryocyte bag containing plasma on an ice bag prior to adding DMSO.

3.1.3.2. WITH 4.5% (w/v) HAS

1. Disinfect rubber septum on bottle of 4.5% (w/v) HAS and place inside LAF hood as in **Subheading 3.1.2.2., step 1**.
2. Insert an air dart into the bottle.
3. Remove a volume of 4.5% (w/v) HAS using a 50-mL syringe with 23-gage needle.
4. Wipe injection site of sample site with sterile IPA wipe.
5. Inject 4.5% (w/v) HAS into the cryocyte bag.
6. Repeat **steps 3–5** until the required volume of 4.5% (w/v) HAS is achieved.
7. Place the cryocyte bag containing 4.5% (w/v) HAS on an ice bag prior to adding DMSO.

3.1.3.3. ADDING DMSO

1. Calculate the required volume of DMSO (1 vol DMSO to 4 vol HAS or plasma).
2. Remove the metal cap from the bottle of DMSO, disinfect the rubber septum with an IPA wipe, and place the bottle inside the LAF.

3. Insert an air dart into the bottle of DMSO.
4. Remove a volume of DMSO using a 20- or 50-mL syringe with a 23- or 19-gage needle together with 3 mL of air.
5. Inject DMSO very gradually into a cryocyte bag containing plasma or HAS (on bag of melting ice), ensuring the diluent is gently agitated (*see Note 18*).
6. Repeat **steps 3 and 4** until required the volume of DMSO has been added.
7. Use the air in the syringe to empty the coupler tubing of DMSO.
8. Record the start and finish times for the addition of the DMSO on the calculation sheet.
9. Leave the cryocyte bag containing cryoprotectant on a bag of melting ice for at least 10 min prior to adding to cells.

3.1.4. Adding Cryoprotectant to Cells

Do not add leukocytes to the cryoprotectant. Aim to keep the time from adding the cryoprotectant to putting the cryocyte bags on to freeze to less than 20 min. Monitor the weight of the cryoprotectant added using the top pan balance.

It is important to maintain the cells at around 4–8°C during the addition of the cryoprotectant to prevent an increase in temperature owing to the DMSO that will result in toxicity (*see Notes 15–18*).

The procedure is as follows:

1. Sterile connect together the cell bag and bag of cryoprotectant (*see Note 14*).
2. Place the cell bag on a balance and tare the measured weight to zero.
3. Open the clamp between the two bags and allow the required weight of preservative to be added to the cell bag. The mixing rate should be about 10 mL/min with constant mixing.
4. When all the cryoprotectant has been added seal the tubing in three places and break the middle seal.
5. Place the bag of cells with cryoprotectant between two ice bags for 10 min prior to distributing into cryocyte bags.
6. Prepare cryocyte bags by sealing off and removing one dark blue and one light blue luer port (*see Notes 18 and 19*).
7. Close the clamp on the remaining port line.
8. Insert a transfer set with spike coupler and luer adapter into a port of the bag of cells.
9. Connect the bag of cells and cryocyte bag via luer connectors.
10. Place the cryocyte bag on a balance and tare as before.
11. Open the line clamp and transfer the required volume of cells into the cryocyte bag.
12. Express excess air in the cryocyte bag back into the cell bag.
13. Seal off the line as close as possible to the cryocyte bag.
14. Close off the line clamp and remove luer connector and remaining line from the cell bag.
15. Repeat **steps 9–14** for each cryocyte bag until all bags have been filled.
16. Take samples from the final cryocyte bag for archiving (into cryovials) and microbiology testing (Bact Alert bottles or equivalent) before heat sealing the line.

17. A control cryocyte bag for the CRF (*see Note 20*) is set up as in **steps 9–16, Subheading 3.1.3.1., steps 1–6, Subheading 3.1.3.2., steps 1–7, and Subheading 3.1.3.3., steps 1–8** but by adding equal volumes of plasma or 4.5 % (w/v) HAS and cryoprotectant to a cryocyte bag.
18. The cryocyte bags are labeled and placed into an outer bag and vacuum sealed using the Multivac vacuum sealer at 225 mBar. The bags are kept on ice until frozen in the CRF.
19. The laminar hood surface is monitored after the procedure on left- and right-hand sides of the work area by contact plates (10 s contact per plate). Left and right glove prints of the operator are made by dabbing the surface of an agar settle plate with the fingers of each hand. The clean room is decontaminated by thoroughly spraying with Klercide and wiping down/mopping the floor with a dedicated mop, the head of which is changed daily. The empty collection bags are retained for 24 h in the fridge.

3.1.5. Freezing Cells

This operation should ideally be carried out by another member of staff, who can receive the cryocyte bags through the hatch immediately after they are filled, so that the time prior to freezing is minimized.

The bags of cells are frozen in a CRF (*see steps 1–10 and Note 20*) to ensure that the optimum freezing parameters resulting in maximum cell viability are achieved. One such cell freezer is the Kryo 10 produced by Planer plc. This freezer can be programmed manually within the MR3 controller or from a computer linked to the controller using the KSK10 software. The software allows a full audit trail for each individual freezing procedure including details of the products frozen together with a graph of the freezing run itself. The Kryo 10 freezer has two temperature probes, one for measuring the temperature within the freezer chamber and the second that is inserted into a control bag monitoring the temperature of a simulated product throughout the freezing run.

1. Fill the liquid nitrogen Dewar to 12-cm depth with liquid nitrogen.
2. Attach the heater and vapor withdrawal tube to the Dewar ensuring all clips are in place.
3. Turn on the CRF and press rocker switch on heater control box. The heater will stay on until a pressure of approx 5 lb/sqin is attained.
4. Select program to be used either within MR3 control box or download from computer software. An example program is shown in **Table 1**.
5. Place cryocyte bags into precooled stainless steel cassettes and keep on ice until freezer is ready for loading.
6. When freezer indicates the loading temperature has been reached, place freezing cassettes into the rack inside the chamber ensuring the cassettes are parallel to the direction of the fan.
7. Insert bag probe into dummy bag and place lid onto freezing chamber.
8. Press run to start the freezing cycle.

Table 1
Example of Freezing Profile Used on the KRYO10 Controlled Rate Freezer

Start temperature	6°C
Ramp 1	Hold for 12 min
Ramp 2	Rate -2°C per min to -5°C (Chamber)
Ramp 3	Rate -1°C per min to -40°C (Chamber)
Ramp 4	Rate -5°C per min to -160°C (Chamber)
No seeding	Trigger on temperature

Validation was carried out in-house.

9. Once the freezing run has completed remove cassettes and open carefully and place frozen bags into a liquid nitrogen vapor-phase storage vessel at -180°C.
10. Archive sample cryovials must be frozen within the same run as the bags and preferably stored with the frozen harvests.

3.1.6. Environmental and Product Monitoring/Testing

1. To complete the process, the agar plates (including those with a set of glove prints) used for environmental monitoring are incubated at 35–37°C and 22°C for 3 and 7 d, respectively, to check for bacterial and fungal/yeast growth. Any positive plates and Bactalert bottles inoculated with the final processed product are sent for identification to the relevant microbiology laboratories.
2. As part of quality monitoring, CD34 with viability (using 7-AAD, flow cytometry, and CFU) assays are routinely set up on each harvest before, 1 wk after cryopreservation, and at the time of issue.

3.1.7. Retention of Records

It is important to ensure that all records relating to the harvest and cryopreservation are completed fully. The record forms must be filled in at all relevant stages to ensure that all actions and instructions are traceable. Records must be stored for 30 yr according to EU legislation (*see Note 10*).

3.2. UCB Cryopreservation

This section describes two procedures for cryopreserving donated UCB. The first is a method used for an intended recipient or is a directed UCB donation. These directed UCB units are usually collected from mothers who have a child with a condition that may be treated with an HSC/HPC transplant or where there is a family history of a genetic disease that may benefit from HSC/HPC transplantation. This procedure is compared with one for cryopreserving altruistic unrelated allogeneic UCB donations for an unrelated UCB bank (*see Notes 9 and 10*).

There are two techniques for collecting UCB, (1) *in utero*—following the birth of the baby, prior to delivery of the placenta, and (2) *ex utero*—following delivery of the placenta. Generally *in utero* collections are performed by the midwife and this is the technique favored for directed collections. *Ex utero* techniques are predominantly performed by trained cord blood bank staff collecting for unrelated UCB banks (see **Note 21**). At the time of collection, the midwives would have been fully informed and supplied with a collection kit from the stem cell processing laboratory. Following stringent cleaning of the umbilical cord, a needle is inserted into the umbilical vein and the blood drains out of the placenta under gravity into a purpose designed collection bag containing an adequate volume of anticoagulant to prevent the blood clotting. A 2- to 3-cm cord biopsy is collected as an alternative source for DNA extraction that can be used for testing and HLA typing. Directed UCB units are stored without volume reduction in the vapor phase of liquid nitrogen in cryovats. Where the cord blood banks are planning to bank thousands of donations, storage space is crucial. Therefore, it is often the practice for collections for the unrelated bank to be red cell and plasma depleted using such systems as the Sepax system (Biosafe SA www.biosafe.ch) to reduce the UCB collection to a standard volume. This reduces storage requirements of the bank and provides a standard product for cryopreservation. The UCB donations are cryopreserved and stored in either liquid or vapor-phase nitrogen. Many cord blood banks use a BioArchive system that provides automatic controlled rate freezing, liquid nitrogen storage, and retrieval facilities using robotics in a single machine. Details of these preprocessing procedures are not described here but may be found elsewhere (**1,28**). The directed UCB unit and cord biopsy once collected are cooled and delivered by courier immediately with appropriate paperwork to the processing center for cryopreservation. All collections for the unrelated UCB Bank are stored at $22 \pm 2^\circ\text{C}$ and processed within 24 h of collection.

3.2.1. Receipt of Harvest and Preprocessing Requirements

The objectives of the protocols below are to ensure that the UCB units are processed and cryopreserved in a uniform and aseptic manner, tested for microbiological contamination, and assessed for nucleated cell and CD34 content and viability prior to and following cryostorage. The cryostorage of UCB units described here is carried out in a LAF cabinet in a clean room as in **Subheading 3.1**. pending the introduction of closed systems where this may not be required (**29**). For directed UCB units, procedures are similar to those for BM and MPB as detailed in **Subheading 3.1**. Specific modifications to these processes for archiving unrelated allogeneic reduced volume UCB units in a BioArchive are also described.

UCB should be treated as potentially infectious. In handling it, the same precautions should be observed as with MPB and BM donations. The virology status of the mother donating the UCB unit must be confirmed. Although collections and storage are usually restricted to mothers who have had mandatory virology negative clearance, occasionally a request may be granted for the collection and storage of a directed UCB unit from a mother who is virology positive. Potentially, virology positive UCB harvests are quarantined in a designated cryovot until the status is confirmed. Unrelated collections are accepted only from mothers who have consented to be tested for HIV and hepatitis C virus (HCV) during their pregnancy.

1. Appropriate forms and labels are completed to record and track the donations from collection, processing, testing, freezing, storage, and issue. All UCB donations, associated samples, products, and records must be labeled according to appropriate standards (i.e., FACT-Netcord; *see Note 9*). All collections are assigned a unique barcode number (ISBT 128 standard) that is subsequently used to identify all related samples, paperwork, and the final cryopreserved product. Labels for directed donations include the mother's name and date of birth and clearly identify the donation as restricted for either a named person or a family. All unrelated donations are identified with their unique ISBT 128 donation number.
2. For the directed donations, the Kryo 10 CRF Dewar should be prepared ready for use before entering the clean room and set up using the appropriate freezing program as described in **Subheading 3.1.** (*see Notes 5, 6, 8, 9, and 11*). Following the automatic nightly maintenance period including liquid nitrogen filling of the system, the Bio Archive defaults to a ready status for freezing.
3. Any part of the processing or cryopreservation that is not a closed process must be carried out in a clean room. Procedures for entering and preparing the clean rooms are as described in **Subheading 3.1.1.**

3.2.2. Cell Analyses

A sample of UCB taken from the collection or from the bleed-line (as described in **Subheading 3.1.1.**) is used to perform a full blood count using a hematology analyser. The nucleated cell dose of a donation is used to assess the suitability for transplant. For directed UCB units, CD34 and viability assays (7-AAD, flow cytometry, and CFU) are performed on the starting material and on aliquots of the cryopreserved harvest stored in cryovials either 1 wk after cryopreservation or at the time of issue. The unrelated UCB units are assessed for nucleated cell content pre- and postvolume reduction, and the process product is assayed for CD34+ cell content and dye exclusion viability with 7-AAD. At the time of issue for transplant an integral sample attached to the frozen unit is removed and assayed for functional and dye exclusion viability (CFU and 7-AAD) and nucleated and CD34+ cell content as an indicator of the state of the cryopreserved cells at the time of transplant. The UCB samples are tested for ABO

and Rh blood group antigens, for specific viruses and bacterial and fungal contaminants, HLA typed, and a hemoglobinopathy screen is performed (**1,28**). Short tandem repeat analysis on a sample removed from the bleed-line of the freezing bag is used to confirm identity of the donation at issue for transplantation (**30,31**).

3.2.3. Preparation and Addition of the Cryoprotectant

The cryoprotectant stock used is 50% (w/v) DMSO in Dextran-40 (purchased ready prepared) or 20% (v/v) DMSO made up in 10% (w/v) Dextran-40 in 0.9% (w/v) saline. The final concentration of DMSO after addition to the harvest is 10% (v/v) DMSO (*see* **Notes 15–17**). Fifty percent (v/v) DMSO solution is generally used for cryopreserving cord blood units for the unrelated bank, a strategy implemented to minimize liquid nitrogen storage requirements.

For the directed UCB unit:

1. Insert a sample site coupler into a port on the harvest bag (*see* **Note 18**).
2. Remove a 4-mL sample using a 5-mL syringe and place into a labeled 4.5-mL cryotube to be used for the test (**Subheading 3.2.2.**).
3. Place the collection bag on the flat bed mixer to mix thoroughly. Ensure collection details, process volume, and batch details of reagents are recorded before proceeding.
4. Pre-cool the cryoprotectant and diluent on ice and add the DMSO slowly as in **Subheading 3.1.** and as follows:
 - a. Calculate the volume of cryoprotectant needed to freeze the UCB volume. The volume of DMSO to be added to the Dextran will give a concentration of 20% (v/v) DMSO in 8% (w/v) Dextran-40 in saline.
 - b. Insert a “2991” coupler into the port of a 600-mL transfer bag.
 - c. Withdraw the required volume of 10% (w/v) Dextran-40 in 0.9% (w/v) saline into 50-mL syringes and transfer into the 600-mL bag via the coupler. Place on the cold block.
 - d. Remove the required volume of DMSO using a 50-mL syringe with a 19G needle together with 3 mL of air.
 - e. Remove the needle and connect to the coupler on the Dextran-40 bag. Add the DMSO at a rate of 2.5 mL per 15 s (i.e., 10 mL/min) to the transfer bag containing Dextran-40 (on a cold block) ensuring the bag is gently agitated.
 - f. Use the air in the syringe to empty the coupler tubing of DMSO.
 - g. Record the start and finish times for the addition of the DMSO on the calculation sheet.
 - h. Once all the DMSO is added, close the coupler and seal the coupler tubing with three seals.

Allow the solution to cool on the cold block for a further 5 min. If necessary, the cryoprotectant solution can be stored between 2 and 8°C for up to 24 h. Ensure that the bag is in a secondary container and placed in a controlled and monitored cold room or refrigerator.

3.2.4. Cryopreservation Preparation

For open processes in the clean room, all items are assembled in the Class 2 LAF safety cabinet after wiping with IPA as in **Subheading 3.1**. Once the procedure has started it must be completed without interruption. As with BM and MPB, it is recommended that only one donation be processed at a time in an individual Class 2 cabinet. Record all data during processing and cryostorage. Environmental and product monitoring are essentially as described in **Subheading 3.1**.

For the directed donations:

1. Calculate the number of cryocyte bags required as in **Subheading 3.1**.
2. Prepared cryocyte bag labels (with time, bag volume, and number of bags) are completed and placed into the label pocket of each bag. Using the line sealer, place three spot seals across the edge of each pocket.
3. Check that all roller clamps are closed and the freezing bag are joined using the light blue and dark blue luer connectors.
4. Unused lines are clamped or sealed and removed.
5. Four cryovials are labeled with the baby's surname and cryopreservation date.

For the unrelated donations one freezing bag is required:

1. Prepare printed labels with the unique barcoded and eye readable donation number, proper name of product, i.e., red cell and plasma-depleted cryopreserved cord blood, name, and additives including anticoagulant and cryoprotectant, recommended storage temperature, and name of cord blood bank. Affix to the freezing bag.
2. Prepare the freezing canister with the barcoded donation label inside and affix barcoded label on the allocated area on the outside, which is scanned by the BioArchive machine in order to function.

3.2.5. Adding the Cryoprotectant to the Cells

3.2.5.1. PROCEDURE FOR DIRECTED UCB DONATION

1. Place the flat bed mixer in the LAF cabinet.
2. Prepare the following transfer assembly for addition of cryoprotectant to the harvest, taking the red port on the three-way stopcock (*see Note 18*) nominally as NORTH Attach:
 - a. A 50-mL syringe to the SOUTH port.
 - b. A transfer set to the EAST port.
 - c. A luer/spike interconnector to the NORTH (**Fig. 1**).
 - d. Ensure the stopcock is shut.
3. Insert the spike (EAST) on the transfer assembly into the spare port on the cryoprotectant bag. Insert the interconnector (NORTH) into the harvest bag and leave on the mixer to rotate. Leave the cryoprotectant bag on the cold block.
4. Add volume of diluted cryoprotectant equal to that of the cells to the harvest bag via the 50-mL syringe.

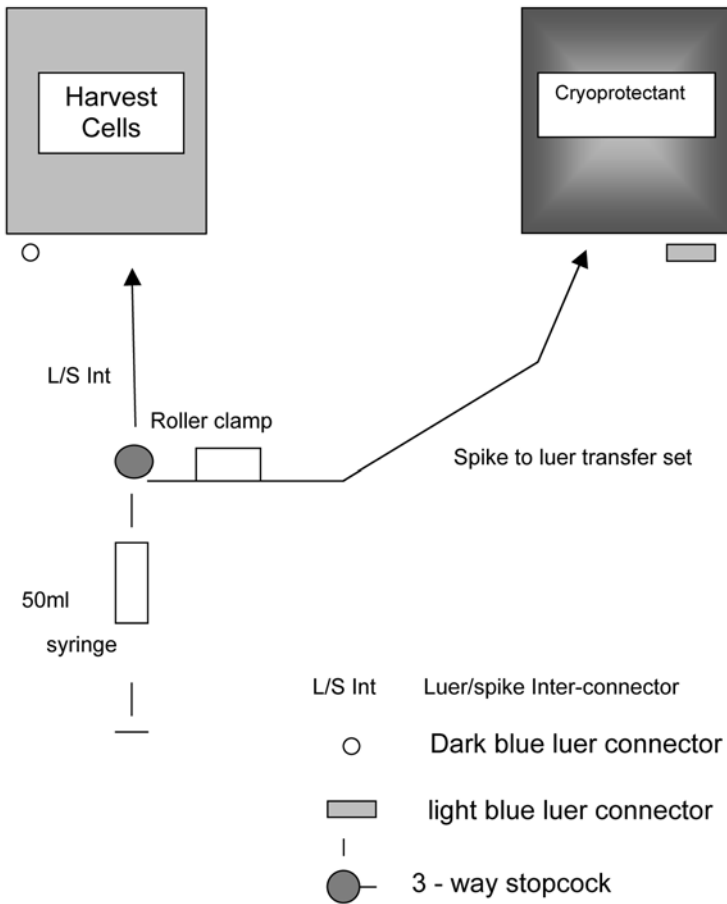


Fig. 1. Addition of cryoprotectant to harvest cells. The protocol is described in **Subheading 3.2.5.1.** and allows optimal transfer of the cryoprotectant to the harvested cells.

- a. Adjust the stopcock to allow the cryoprotectant to be pulled into the syringe.
- b. Ensure all air is pushed back into the cryoprotectant bag before readjusting the stopcock and adding the contents of the syringe through the interconnector into the harvest bag.
- c. The cryoprotectant must be added to the harvest cells slowly (5 mL every 30 s, i.e., 10 mL/min).
- d. Record the start and finish times on the calculation sheet.
5. On completion of the cryoprotectant transfer, close the stopcock and move the roller clamp on the transfer setup to the stopcock and close. Carefully detach the transfer set from the EAST port of the stopcock and attach the cryocyte bag to

the spare male luer connector. Discard the cryoprotectant bag and attached transfer set into a sharps bin.

6. Place the cell and cryoprotectant solution on the mixer on the cold block.
7. With a 50-mL syringe/tap assembly, opening roller clamps, and using Spencer wells as necessary, transfer the appropriate amount of cells/cryoprotectant into the first bag. Remove any residual air from the cryocyte bag and clamp.
8. Fill the remaining bags in the same manner, retaining 4 mL in the syringe for the cryotubes and blood culture bottles.
9. Close the stopcock and seal off the cryocyte bags with three seals close to the ports. Remove the bags at the third seal. Detach the syringe from the assembly and attach a 19-gage needle.
10. Dispense 0.5-mL aliquots into the four pre-labeled cryotubes. Inoculate two BactAlert culture bottles.
11. Check the cryotubes are correctly labeled and put them into the storage cane.
12. Perform right and left glove prints as in **Subheading 3.1.4**.
13. Place each cryocyte bag into an individual vacuum-pack bag and seal in the Multivac C100 vacuum packer at 250 mbar for 1.8 s. Seal the bags so that they fit the cryocyte bags firmly.
14. Keep the cryocyte bags and cryotube cane on the cold pack, and freeze without delay.

3.2.5.2. CONCENTRATED UCB SAMPLES

The following describes the procedure for UCB that has been reduced to a standard volume of 21 mL and have been cooled to 4°C. The concentrated cells are automatically transferred to the freezing bag on completion of the volume–reduction process when a system such as the Sepax process and the Pall cryobags specified are used.

1. Place a cold pack with the cooled UCB cell concentrate secured on top to a mixer. Attach a luer connector to the freezing bag bleed-line. Switch on the mixer.
2. Attach a syringe filled with 5.5 mL 50% (w/v) DMSO diluted in Dextran-40 to the luer connector. Push 0.5 mL of DMSO into the line and break the seal (*see Notes 15–17*).
3. Install the syringe on a syringe pump. Switch on the pump, confirm the syringe size, and select an infusion rate of 20 mL/h allowing the DMSO to be added over 15 min. Press start to commence infusion. During infusion the edges of the pack must be manually manipulated frequently to ensure thorough mixing.
4. A warning alarm on the pump sounds when 4% of the DMSO volume in the syringe remains, and the pump continues infusing. On completion the pump automatically stops and sounds an alarm.
5. Remove the syringe from the pump and slowly transfer the remainder of the DMSO to the freezing bag, continuing to mix.
6. Remove air from the freezing bag using the syringe, ensuring all cells and DMSO are returned to the freezing bag; heat-seal the bleed-line just above the injection port.

7. Remove samples from the freezing bag for archiving (into cryovials) and bacterial and fungal screening (BacTAlert bottles or equivalent).
8. Place the freezing bag in the jig for the Pall cryobags (this ensures that the bag is not over-filled) and heat-seal as follows: one immediately below the injection port, one in the middle of the bleed-line segment, one on the bleed-line as close as possible to the freezing bag, and one on each of the links between the major and minor bag compartments.
9. Visually check all heat-seals and pressure test to ensure the integrity of the seals.
10. Weigh the final freezing bag and place in an overwrap bag and vacuum seal as close to the freezing bag as possible, check the seal.

3.2.6. Freezing Cells, Testing, and Records

Directed donations are frozen using the CRF as detailed in **Subheading 3.1**. Once the freezer program is completed the frozen bags and sample tubes are transferred to the storage vessel. Any material of unknown virology status must be stored in a quarantine tank until the virology status is negative. The cryovials are stored in the same vessel as the cryobags until such time as all the harvest has been reinfused or discarded. All records relating to the collection and cryopreservation, including a copy of the freezing profile printout, must be completed, ensuring all actions and procedures are traceable. Reports and records must be stored securely for 30 yr according to EU legislation (*see Note 8*).

For the volume reduced unrelated UCB units to be banked and stored in the BioArchive, the following procedure is followed:

1. Place each freezing bag in the appropriately labeled BioArchive metal freezing canister.
2. Transfer to the CRF module of the BioArchive system and initiate freezing. Select the port to be used and insert the CRF module. Once the donation number has been read from the canister, verify the port, select the freezing profile, and scan the barcode of the unit from the paperwork. Following barcode verification of the unit with the sample barcode “wanded” by the operator, the freeze program is initiated (*see Table 2* for an example).
3. On completion of the freeze process, the BioArchive system automatically moves the canister containing the frozen unit to a permanent storage location in the Dewar. The location is recorded in the BioArchive system database and on the freeze profile printout. Status information throughout the process is displayed on the computer monitor.
4. Cryovials are frozen separately in this procedure and are placed in the next available position in a separate Dewar.
5. Environmental monitoring is carried out as in **Subheading 3.1**.
6. Records must be fully completed and stored for 30 yr (*see Notes 8* and **21** for additional information on UCB bank programs).

Table 2
Example of a Freezing Profile Used for Unrelated UCB Volume Reduced Units

Prefreeze rate	10°C/min
Freeze start temp	-1°C
Freeze power	100%
Freeze exit temp	-11°C
Postfreeze rate	-2°C/min
Target temp	-50°C

Validation was carried out in-house.

4. Notes

1. There have been a number of excellent review papers mainly in the journal *Cryobiology* published by Academic Press (ISSN 0011-2240).
2. Websites of interest include the Society for Low Temperature Biology (www.sltb.info) and the Society for Cryobiology (www.societyforcryobiology.org).
3. Relevant national and international standards/guidelines are found (where not provided here in detail) in the most recent versions of or on relevant websites as in **Notes 4–10**.
4. JACIE guidelines are standards for blood and marrow progenitor cell processing, collection, and transplantation. From the Joint Accreditation Committee of ISCT-Europe and EBMT (JACIE) (www.jacie.org; www.ebmt.org).
5. Department of Health. A code of practice for tissue banks providing tissues of human origin for therapeutic purposes (<http://www.dh.gov.uk>; www.mhra.gov.uk; www.hta.gov.uk).
6. UKBTS/NIBSC (Red Book): guidelines for the blood transfusion services in the United Kingdom.
7. Department of Health. Guidance on the microbiological safety of human organs, tissues, and cells used in transplantation. Advisory committee on the microbiological safety of blood and tissues for transplantation (MSBT) (<http://www.dh.gov.uk>).
8. The EU directive for tissues and cells (<http://www.hfea.gov.uk>; www.dh.gov.uk). In the United Kingdom, mandatory licensing of cell processing and storage facilities for clinical transplantation became the remit of the Human Tissue Authority in April 2006 (www.hta.gov.uk).
9. FACT-Netcord international standards for cord blood collection, processing, testing, banking, selection, and release (<https://office.de.netcord.org>; www.unmc.edu; www.ebmt.org).
10. UKBTS/NIBSC cord blood donor selection guidelines (<http://www.transfusionguidelines.org.uk>).
11. Rules and guidance for pharmaceutical manufacturers and distributors (The “Orange Guide”) Author: Medicines and Health Products Regulatory Agency. Published by The Stationary Office, London UK.

12. If the apheresis collection has a total nucleated cell count greater than $200 \times 10^9/L$, it requires diluting back to this cell concentration. If the collection is to be processed immediately the dilution can be done prior to adding the cryoprotectant, however, if the cell collection is to be stored overnight at $4^\circ C$, the collection will need diluting prior to storage. The diluent can be autologous plasma collected during the leukapheresis procedure or 4.5% (w/v) HAS.
13. It is important to ensure that the predonation virology screening (maximum 30 d prior to donation) has been done and a hardcopy of the results has been obtained. Untested donations may be processed, but must be stored in quarantine tanks until product testing is completed. In cases of clinical need, known mandatory marker positive donations may be processed while ensuring their complete separation from other donations. All waste materials used in processing viral marker positive donations must be autoclaved prior to disposal. Staff handling hepatitis B virus positive donations must have up-to-date immunization against hepatitis B virus.
14. A problem with using a sterile connecting device within a clean room is that of the excessively high particle numbers that are generated. To obviate this we place the sterile connecting device in a small cabinet (Powdercap™) that houses a Hepa filter allowing total removal of the particles.
15. Neat DMSO can dissolve various types of plastic, therefore it is important to ensure that any plastic material coming into contact with neat DMSO is resistant. At the time of writing, there are no commercially available sample site couplers fully validated that are resistant to DMSO, although closed systems are under development. Therefore, we have found that it is best to use a cryocyte bag to make up the cryoprotectant. The volume of cryoprotectant required is equal to the volume of cell suspension plus 10 mL with an additional 40 mL for a control bag.
16. The addition of DMSO to water causes an exothermic reaction. The heat release is such that plasma proteins may be denatured. This is avoided by precooling the diluent and adding the DMSO to the diluent slowly.
17. Although the method described here uses 10% (v/v) DMSO as the cryoprotectant other methods use 5% (v/v) DMSO (**13,15,24,32,33**) either alone or in combination with 6% (w/v) hydroxyethyl starch (**34,35**). In a randomized phase III trial, Rowley et al. (**21**) compared 10% (v/v) DMSO vs 5% (v/v) DMSO in combination with 6% (w/v) hydroxymethyl starch that indicated that engraftment times for patients receiving cells frozen using the combination cryoprotectant was 1 d faster than for those frozen with DMSO alone. An advantage of using the lower concentration of DMSO is that it reduces the exposure to the patient. The exposure to DMSO can also be lowered by freezing the cells at a higher concentration. This has been shown to have little effect on engraftment (**36**).
18. Some methods use syringes and three-way taps to prepare cryoprotectant, add cryoprotectant to cells, and aliquot into freezing bags. A useful tap system, Discifix, is produced by Braun with up to five three-way taps linked together in series.
19. When adding cells to cryocyte bags it is important that the volume of cells put into each bag does not exceed the manufacturer's limits both maximum and minimum

(see **Subheading 3.1.1., step 4**). If these values are not adhered to, the viability of the cells may be compromised during the freezing procedure.

20. It is not essential to use a CRF. Some groups freeze cells by placing directly into a -80°C mechanical freezer (**32–34**).
21. **Reference 37** describes requirements for an effective national cord blood stem cell bank program being developed in the United States and contains additional information on UCB banking.

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