

## CHAPTER 6

# SAMPLE TESTING AND CHARACTERIZATION

## Chapter 6

### SAMPLE TESTING AND CHARACTERIZATION

#### 6.1 HLA Typing

##### 6.1.1 Preliminary

###### *Principle*

Molecular HLA typing will be performed for every cord blood unit stored in the cord blood bank. The minimum level of typing will be low resolution (a level similar to serological HLA typing) for HLA-A and -B and intermediate resolution (corresponding to five or less alleles for most samples) to high resolution (corresponding to a single allele) for DRB1. The alleles which can be detected are listed in Appendix E. The types are defined according to the WHO Nomenclature Committee for Factors of the HLA System with the original list obtained as of December 1996. These will be updated semi-annually.

###### *Specimen*

The specimens for HLA typing will be frozen aliquots from the granulocyte/red cell-enriched pellets that remain after preparation of the cord blood unit. Cord blood banks will freeze 1 ml aliquots of the granulocyte/red cell-enriched pellet. Each week the cord blood bank will ship to each HLA Reference Laboratory (Dr. Terasaki's laboratory at the University of California - Los Angeles, Dr. Lee Ann Baxter-Lowe's laboratory at the University of California - San Francisco, and Dr. Jennifer Ng's laboratory at the Navy Medical Research Institute-NMRI) one vial per cord blood unit which has completed quarantine release requirements.

###### *Materials and Reagents*

Appropriate HLA typing reagents as described in the detailed protocols of each Laboratory including oligonucleotide sequences of PCR and sequencing primers.

Note: A list of these reagents will be maintained by the Laboratory in compliance with ASHI regulations. Reagents may be revised during the project as techniques and knowledge of HLA polymorphism improves. Historic records regarding reagents are maintained in accordance with ASHI regulations.

###### *Procedure*

1. Locate HLA Request Log included with each shipment. Unpack shipments of specimens stored on dry ice, confirming that the contents of the shipment correspond to the HLA Request Log. Consult with the Cord Blood Bank regarding any discrepancies between the log and contents of the shipment.
2. Log each sample into the Laboratory inventory using bar codes provided by the Cord Blood

Bank. An example of a log in sheet is provided in Appendix E.

3. Type samples according to the appropriate SOPs within the Laboratory. The final data will be interpreted with respect to a list of recognized HLA alleles that is maintained by the Medical Coordinating Center (MCC). This list will be updated semi-annually to include additional alleles that satisfy the following criteria:
  - a. Recognized by the WHO Nomenclature Committee
  - b. Sufficient length of sequence available to perform typing

HLA typing protocols comply with ASHI regulations. These reagents and protocols may be updated during the project as techniques and knowledge of HLA polymorphism improve. Historic records regarding protocols and reagents are maintained in accordance with ASHI regulations.

4. Laboratories will store remaining frozen specimens (cord blood, blood, or and DNA) until further directions are received from the MCC, not to exceed the concluding date of the project.

#### *Data Reporting*

The last working day of each week the Laboratory will send to the MCC a report and an electronic data file containing the following information for typings completed during the week:

- a. Specimen identification, including bar code label number
- b. Assigned type
- c. HLA alleles that are potentially present in the specimen
- d. Special notation for samples with unusual linkage
- e. Typing method
- f. Data required by the MCC to update typing assignments as knowledge of HLA polymorphism improves

Electronic data may be sent via diskette or Internet.

#### **6.1.2 Confirmatory**

##### *Principle*

Confirmatory molecular HLA typing will be performed for every cord blood unit that is a potential candidate for transplant. The minimum level of typing resolution will be low (a level similar to serological HLA typing) for HLA-A and -B and high resolution (corresponding to a single allele for most samples) for DRB1. The alleles that will be detected are listed in Appendix E. The types are defined according to the WHO Nomenclature Committee for Factors of the HLA System with the original list obtained as of December 1996. These will be updated semi-annually. Supplemental typing of HLA-C and -DQB1 will be performed as clinically indicated for selection of cord blood units for transplant.

## *Specimen*

The specimens for HLA typing will be frozen aliquots from the granulocyte/red cell-enriched pellets that remain after preparation of the cord blood unit. The MCC will request confirmatory typing of samples that satisfy the following criteria:

- ! satisfy the minimal match criteria for a transplant candidate
- ! no prior confirmatory typing at a level of resolution that is required for evaluation of the cord blood unit for the transplant candidate

Note: This may include retyping to detect alleles that were not detectable using reagents that were available at the time of prior confirmatory typing and/or HLA-C and -DQB1 typing as clinically indicated.

The NMRI laboratory will perform confirmatory typing for all CBUs which were not preliminary typed at their lab. The University of South Carolina will perform the typing for the remainder of the CBUs.

## *Materials and Reagents*

See *Materials and Reagents* in Section 6.1.1

## *Procedure*

See *Procedure* in Section 6.1.1

## *Data Reporting*

See *Data Reporting* in Section 6.1.1

## *Quality Control*

The MCC will compare the original typing and confirmatory typing. Concordance or discrepancy will be recorded. Concordant typings will be forwarded to the requesting Transplant Center. The HLA Laboratories will be contacted regarding discrepant typings. If review of available data and/or retyping resolves the discrepancy, the revised results will be sent to the MCC along with an explanation for the cause of the incorrect typing. These data will be reviewed by the MCC and Histocompatibility Subcommittee on a semi-annual basis to identify causes of error that can be corrected. If the discrepancy cannot be resolved by the two laboratories, the specimens will be sent to the third laboratory. The results of typing of the three samples will be forwarded to the MCC. All data will be reviewed by the MCC and Histocompatibility Subcommittee to resolve the typing discrepancy and to identify causes of error that can be corrected.

### 6.1.3 Maternal Samples

#### *Principle*

Molecular HLA typing will be performed for maternal samples when a CBU has been identified as a match for a potential recipient and for 200 CBUs collected by each CBB from ethnic minorities. The minimum level of typing will be low resolution (similar to serological HLA typing) for HLA-A and -B and DRB1, unless higher level resolution is useful to establish HLA haplotypes. Alleles which can be detected are listed in Appendix E. The types are defined according to the WHO Nomenclature Committee for Factors of the HLA System with the original list obtained as of December 1996. These will be updated semi-annually.

#### *Specimen*

Cord blood banks will freeze 1-ml aliquots of maternal blood samples. As requested, the cord blood bank will ship to a designated Laboratory (Dr. Terasaki's laboratory at the University of California - Los Angeles, Dr. Lee Ann Baxter-Lowe's laboratory at the University of California - San Francisco, or Dr. Jennifer Ng's laboratory at the Navy Medical Research Institute-NMRI) one vial per maternal blood sample requested. The Coordinating Center will also provide typing of the associated cord blood unit for use in determining the appropriate level of resolution and deducing haplotypes.

#### *Materials and Reagents*

See *Materials and Reagents* in Section 6.1.1

#### *Procedure*

See *Procedure* in Section 6.1.1

#### *Data Reporting*

Each Laboratory will send to the Coordinating Center a report containing the following information for:

- a. Specimen identification
- b. Assigned types
- c. Typing method
- d. Haplotypes (or indication that haplotypes cannot be deduced from maternal typing)

### 6.1.4 Retrospective

#### *Principle*

Retrospective molecular HLA typing will be performed for donor cord blood units and recipients. The typing will be high resolution (corresponding to a single allele for most samples) for HLA-A, -B, -C, DRB1, and DQB1. The types are defined according to the WHO Nomenclature Committee for Factors

of the HLA System with the original list obtained as of December 1996. These will be updated semi-annually. Supplemental typing of HLA-DQA, DPB, and DPA may be determined at a later date.

### *Specimen*

The specimens for HLA typing will be:

- ! frozen aliquots from the granulocyte/red cell-enriched pellets that remain after preparation of the cord blood unit.

Cord blood banks will freeze 1-ml aliquots of the granulocyte/red cell-enriched pellet. As requested, the cord blood bank will ship to a designated Laboratory (Dr. Terasaki's laboratory at the University of California - Los Angeles or Dr. Lee Ann Baxter-Lowe's laboratory at the University of California - San Francisco) one vial per cord blood unit requested for retrospective typing by the Coordinating Center.

- ! blood samples from the recipient.

### *Materials and Reagents*

See *Materials and Reagents* in Section 6.1.1

### *Procedure*

See *Procedure* in Section 6.1.1

### *Data Reporting*

Reporting of retrospective typings completed during each month will be reported on the last working day of each month. Each Laboratory will send to the Coordinating Center a report containing the information specified in *Data Reporting* in Section 6.1.1.

### **6.1.5 Shipment of HLA Samples**

Each shipment should contain bar code labeled specimens, packing lists with bar code labels, and a minimum of 10 specimens. Specimens should be shipped on dry ice using a local courier or an overnight shipper as appropriate.

For preliminary typing, specimens should be sent after the CBU has cleared quarantine or with the Cord Blood Bank Director's approval.

## 6.2 SCREENING FOR GENETIC DISEASES

### *Principle*

Transmission of heritable disorders through hematopoietic stem cells is a risk associated with umbilical cord blood transplantation. Attempts to minimize this risk will be accomplished by 1) restricting collections of cord blood to those mother/infant pairs that have uncomplicated pregnancies and deliveries, normal physical examinations, and family histories unremarkable for genetic diseases; 2) obtaining results of state screening programs for thalassemia and sickle cell disease.

Viable mononuclear cells, plasma and DNA from the umbilical cord blood graft will be stored for future genetic disease screening. Specimens will be available for genetic disease screening: 1) to evaluate the donor for presence of a specific genetic disease, 2) to determine carrier status for a specific genetic disease, or 3) to evaluate new strategies for genetic disease screening as they become available.

### *Specimens*

0.5 ml aliquots of leukocyte enriched umbilical cord blood cells ( $\geq 3 \times 10^6$ /vial) from the leukocyte pellet prior to cryopreservation; aliquots of plasma (1 mL/vial) from the leukocyte poor plasma fraction; aliquots of the granulocyte/red cell-enriched pellet.

### *Procedure*

An umbilical cord blood unit that has been identified for a patient with a specific enzyme deficiency should be screened for the same enzyme deficiency found in the potential transplant recipient. When feasible, the sample should be screened prior to transplantation. Cryopreserved nucleated cells, plasma and/or DNA will be made available for this purpose. Samples may be sent to the following laboratories:

DISEASE	ENZYME ASSAY	SAMPLE
Hurler syndrome	a-L-iduronidase	Cells
Maroteaux-Lamy syndrome	aryl sulfatase B	Cells
Adrenoleukodystrophy	co-A ligase	Plasma
Metachromatic Leukodystrophy	aryl sulfatase A	Cells
Globoid Cell Leukodystrophy	galactocerebrosidase	Cells
Mannosidosis	mannosidase	Cells
Fucosidosis	fucosidase	Cells
Wolman syndrome	acid lipase	Cells
Lesch-Nyhan syndrome	HGPRT	Cells
Type III Gaucher disease	glucocerebrosidase	Cells

All listed diseases can be evaluated using DNA based methods should cells or plasma not be available.

### *Quality Control*

1. The quality control assurance tests will be performed by the reference laboratories.
2. Bar code labels on the plasma or cell specimens shipped to the reference laboratories will be verified with the bar code labels on the TEST RESULTS DATASHEETS that will have accompanied the specimen. Test results will be handwritten by the reference laboratory.

### *Notes*

The Medical Director of the CBB will be notified of positive test results. Donor units identified as indicative of complete enzyme deficiency will be discarded and the donor/donor's mother will be notified of positive test result. Donor units identified as indicative of partial enzyme deficiency (carrier status), will not be discarded. Use of units with partial enzyme deficiency are only contraindicated for patients being treated for the same enzyme deficiency. Donor/donor's mother will not be notified of a positive test results unless: 1) the donor is a female carrier of co-A lipase deficiency (adrenoleukodystrophy) or 2) the donor is a carrier of glucocerebrosidase deficiency (Gaucher disease).

## 6.3 MATERNAL INFECTIOUS DISEASE SCREENING

### *Principle*

Maternal serum samples will be tested for the following infectious disease markers:

- ! Cytomegalovirus (CMV) IgM antibody
- ! Anti-HBc (antibody to hepatitis B core antigen)
- ! Anti-HCV (antibody to hepatitis C virus)
- ! HbsAg (hepatitis B surface antigen)
- ! HIV-1/2
- ! HIV p-24 antigen
- ! HTLV-I/II
- ! Syphilis

Each cord blood bank must determine if the facility performing their infectious disease testing can utilize frozen, batched samples, or if the samples must be stored and shipped at 4°C within a certain time span. For facilities performing tests manually, frozen samples may be batched for shipment to the testing facility.

### *Specimen*

Cryovials containing maternal serum, stored frozen at or below -20°C, or at 4°C, depending on the requirements of the testing facility.

### *Equipment*

Electronic Bar Code Scanner  
Computer

### *Materials*

Serum samples  
Infectious Disease Sample log  
Small biohazard specimen bags  
Small zipper-locked bags  
Temperature indicator strips

### *Procedure*

1. A set of samples will be removed from the freezer. The bar code number will be scanned into the Maternal Sample database and infectious disease log (if applicable), indicating that the samples have been sent for testing.
2. Each set of samples will be placed in an individual biohazard specimen bag.

3. Batched, frozen samples will be packed in dry ice in a styrofoam shipping container and sent to the testing facility. Samples stored and shipped at 4°C will be packed in a styrofoam container with sufficient frozen cold packs to maintain the temperature during transport. A temperature indicator strip will be sealed in a small zipper-locked bag and placed on top of the sample shipment.

#### *Quality Control*

1. One set of maternal samples at a time will be prepared for shipping.
2. The scanned number will be compared by visual inspection to the number on the serum tubes.
3. Samples will be handled and transferred as quickly as possible from storage to the shipping container, to maintain the appropriate sample temperature.
4. Sample tubes will be packed in sufficient dry ice or frozen cold packs to maintain the sample temperature until they arrive at the testing laboratory. Testing facilities will be instructed to note the temperature visible on the temperature indicator strip at the time of sample arrival.

## 6.4 COLONY-FORMING ASSAYS: CFU-GM, CFU-GEMM AND BFU-E

### *Principle*

One surrogate measure of the hematopoietic cell number and engraftment potential of a sample of cord blood is its content of hematopoietic colony forming cells. These cells are enumerated in a culture-based assay that allows growth of a population of immature hematopoietic cells into colonies of mature hematopoietic cells over approximately two weeks.

This assay involves the growth of colony forming units-granulocyte-macrophage (CFU-GM), colony forming units-granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM), and burst forming units erythroid (BFU-E) under specified conditions. By counting the number of these elements formed at 14-16 days the concentration of progenitor cells that are present in the cord blood sample can be calculated. Each colony or burst results from one proliferating progenitor cell. Using methylcellulose, a semisolid media, the progeny of each precursor remain localized so that a visible colony is distinguishable.

### *Specimen*

Umbilical cord blood that has been red-cell and volume-depleted according to the Processing CBU SOP. Sample to be provided with nucleated cell count and adhesive bar code labels in the Colony Assay 'In-Box' or institutional equivalent in accordance with the Processing CBU SOP.

### *Equipment*

Bar Code Scanner  
Inverted Microscope  
CO<sub>2</sub> Incubator  
Automatic Micro Pipetter  
Vortex Mixer

### *Reagents*

Methocult Medium	StemCell Technologies (aliquoted and stored according to Procedure Note)
Iscove's Modified Dulbecco's Medium	GIBCO
Fetal Calf Serum (FCS) (Pre-tested and Filtered)	StemCell Technologies, HCC-6100
Sterile Water	

### *Supplies*

3 ml syringes (2)	Hospital Supply Room
17 gauge x 1½ inch blunt-ended needles <u>or</u> as recommended by StemCell Technologies	

24-well tissue culture plate	
Sterile Pipette Tips	Fisher Cat #21-197-8H
Study bar code labels	
Laboratory Worksheet	Laboratory Stationary
Graft Characterization Form	Cord Blood Bank Stationary

### *Procedure*

Note: Procedures 1-12 are to be performed in the tissue culture hood under sterile conditions and using sterile technique.

1. Prepare the laboratory worksheet by placing an adhesive bar code label on the sheet. Scan this label and the label on the sample tube to confirm identity.
2. Record the identity of the responsible technologist, the date and the time, the lot numbers of the MethoCult medium and Iscove's medium, and the cell concentration of the sample (provided by the processing technologist) on the worksheet.
3. Label the tissue culture dish with an adhesive bar code label and the date.
4. Thaw an aliquot of MethoCult medium. Dilute cells to 0.5 ml in 2% FCS (2 ml FCS per 98 ml Iscove's medium). Use the following numbers of nucleated cells for each umbilical cord blood unit:  
  
Umbilical cord blood:             $0.5 \times 10^5$ /tube or  $1.25 \times 10^4$ /well
5. Add the 0.5 ml of cell suspension containing  $0.5 \times 10^5$  cells to the MethoCult medium tube.
6. Mix each tube by vortexing twice for 3 seconds each time.
7. Allow the tubes to sit at room temperature for approximately 5 minutes. This allows MethoCult to drain down the side of the tube.
8. Label rows of a 24-well tissue culture plate with the bar code label, date, and cell concentration.
9. Using a blunt ended needle and a 3 ml syringe, draw up 1.5 ml of mix. **Avoid forming bubbles.**
10. Place .5 ml into each of 3 wells of a tissue culture plate. Replace lids and swirl gently to cover the bottom of the dish.
11. Fill the fourth well of a tissue culture plate with 1.5 ml sterile water. Tissue culture plates may be used for different patient samples. If cells are placed in position A-C on one row with sterile water in D, cells should be placed in B-D on the next row, with sterile water in A. Sterile water should also be placed in wells A-D of the first and sixth rows. This will allow the sterile water and humidity to be evenly distributed on the plate. (See diagram below.)

	1	2	3	4	5	6	
A	⊗	⊗	○	⊗	○	⊗	⊗ = sterile water
B	⊗	○	○	○	○	⊗	
C	⊗	○	○	○	○	⊗	
D	⊗	○	⊗	○	⊗	⊗	

- Place the tissue culture plate into a 37°C incubator with 5% CO<sub>2</sub> for 14-16 days.
- At the end of culture, remove the tissue culture plate from the incubator. Recover the laboratory worksheet and scan the label on the datasheet and the plate to confirm identity. Record the date and the identity of the technologist responsible for scoring on the datasheet.
- Colony growth is enumerated using an inverted microscope with scoring according to the following criteria (and using the handbook provided by Stem Cell Technologies).

- CFU-GM:
- colorless, sometimes granular or foamy cells
  - ≥ 30 cells/colony
  - cells often disbursed from a center
  - each focal center is counted as a distinct colony
  - macrophage colonies may be dispersed and have no well defined center, but are still counted as one colony

- CFU-GEMM:
- “fried egg” appearance
  - compact, spherical hemoglobinized area at center or at one side of flat lawn of nonhemoglobinized translucent cells (granulocytes, macrophages, and/or megakaryocytes)
  - 40 or more cells
  - can be mistaken for pure erythroid colonies if not examined under high power

- BFU-E:
- bright red
  - ≥ 50 cells/burst
  - cells in each portion of a burst are tightly packed
  - a multicentric burst is counted as a single entity
  - bursts generally appear to look as though they would fit back together (e.g. “continental drift”)
  - cells from different individual centers of a burst that are closest to the center of mass of the whole BFU-E tend to be in the same focal plane as those from adjoining centers

If the colony count is greater than 100 colonies per plate, score the plate > 100.

- Record the counts for each colony type on the datasheet. Using the mean count from each

triplicate plate, calculate the total CFU-GM x 10<sup>5</sup>, CFU-GEMM x 10<sup>5</sup>, and BFU-E x 10<sup>5</sup>.

16. Open the Graft Characterization database and scan in the bar code identifier. Transcribe the calculated total CFU-GM, CFU-GEMM, and BFU-E from the worksheet into the database and onto the form.

#### *Quality Control*

1. The colony count for each plate should be within 15% of the others in the triplicate.
2. Each week the laboratory supervisor will select colony assay plates for blinded counting by each qualified lab staff member to determine reproducibility. Any staff person whose counts are outside 15% of the average will undergo retraining.
3. Each month the laboratory supervisor will select a cord blood sample for parallel assay by each qualified lab staff member to determine reproducibility. Any staff person whose assays are outside 15% of the average will undergo retraining.

#### *Procedure Notes*

1. Preparation of MethoCult medium: Each bottle comes from the manufacturer in a volume of 100 ml. Thaw the medium by soaking in a 32-35°C waterbath for 30-45 minutes and protected from light. Label sixty-six 17 x 20 mm snap cap tubes with the medium's lot number, expiration date, date aliquoted, volume, and technician's initials.
2. Using a 3 ml syringe with a blunt needle, aspirate 1.5 ml medium into each snap cap tube. Place the tubes in the -20°C freezer to use.
3. Volume required (ml) = 
$$\frac{\text{Cell Number Required}}{\text{Cells/ml}}$$

#### *References*

Colony Assays of Hematopoietic Cells Using Methylcellulose Medium-An Introductory Manual, Stem Cell Technologies, Vancouver, Canada, 1992.

Areman E, Deeg HJ, Sacher R. Peripheral Blood Stem Cell Processing Protocol. In: *Bone Marrow and Stem Cell Processing: Manual of Current Techniques*. F.A. Davis Company, Philadelphia, 1992.

## 6.5 FLOW CYTOMETRY FOR PRE-FREEZE GRAFT CHARACTERIZATION

### *Principle/Purpose*

Flow cytometry uses immunostaining to differentiate antigens on immature hematopoietic cells (HPC). These antigens are a potential predictor of time to engraftment. Flow cytometry allows rapid acquisition (separation and identification) of a significant number of these low frequency antigens/cells. This procedure briefly outlines the steps needed to stain different aliquots of cells, to pass them through the flow cytometer (acquisition), and to analyze the resulting computer acquisition files.

### *Specimen*

Umbilical cord blood that has been red cell- and volume-depleted according to the Processing CBU SOP. Sample to be provided with cell count in the Flow Cytometry 'In-Box' or institutional equivalent in accordance with the CBU Separation and Sample Preparation SOP.

### *Equipment*

Repeating Pipetter

Automatic Muro Pipetter (or equivalent)

20, 200, and 1000  $\mu$ l Pipetters

Flow Cytometer and Analysis Section with CD34 Analysis Template (Procount)

Becton-Dickinson (or equivalent)

Electronic Bar Code Scanner

12x75 mm Test Tube Holder

Refrigerator

### *Reagents*

<b>Tube</b>	<b>Marker</b>	<b>Stains</b>	<b>Supplier</b>
1	Procount (or equivalent)	Nucleic Acid Dye/34/45	Becton-Dickinson
2	Procount Control (or equivalent)	Nucleic Acid Dye/Gamma 1/45	Becton-Dickinson
3	34+/61+ (optional)	CD61 FITC / CD34 PE / CD45 (Per-CP or equivalent)	Becton-Dickinson
4	34+/90+ (optional)	CD90 FITC / CD34 PE / CD45 (Per-CP or equivalent)	Becton-Dickinson
5	34+/38- (optional)	CD38 FITC / CD34 PE / CD45 (Per-CP or equivalent)	Becton-Dickinson
6	34+/Control (optional)	IgG1 FITC Control / CD34 PE / CD45 (Per-CP or equivalent)	Becton-Dickinson
7	19+/16+ & 56+	CD3 FITC / CD16 & 56 PE / CD19 APC / CD45 Per-CP (or equivalent)	Becton-Dickinson
8	3+/8+/4+	CD3 FITC / CD8 PE / CD4 APC / CD45 Per-CP (or equivalent)	Becton-Dickinson

FACS Lysis Buffer  
FACS Buffer  
PBS Buffer

Becton-Dickinson  
Procedure Note 1

### *Supplies*

TruCount Tubes (or equivalent)  
12x75 mm Polystyrene Tubes  
Eppendorf 1.7 ml Microcentrifuge Tubes (or equivalent)  
10 ml Pipettes  
200  $\mu$ l Pipette Tips  
1000  $\mu$ l Pipette Tips  
Automatic Pipetter Tips  
Graft Characterization Form

### *Procedures*

1. Check the sample ID.
  - a. Prepare the Graft Characterization Form by placing an adhesive bar code label on the sheet. Scan this label and the label on the sample tube to confirm identity. If identity is not confirmed, i.e. identical, do not proceed until discrepancy is resolved.
  - b. Record the identity of the Responsible Technologist, the date and the time, the lot number and expiration date of the wet reagents (antibodies, buffer, lysis solution, and fixative), and the volume and cell concentration of the sample (provided by the Processing Technologist) on the worksheet and, if applicable, in the computer database.
2. Immunostain the cells.
  - a. Obtain the 400  $\mu$ l of post-processed cells.

Note to ProCOUNT users: If automated cell count exceeds cell concentration of  $45 \times 10^3 / \mu$ l, dilute sample and record dilution factor on the Graft Characterization Form.
  - b. Label 2 TruCount tubes with the CBU number and numbers 1 and 2.
  - c. Label additional clear polystyrene 12x75 mm tubes with the CBU number and tube number.
  - d. If CD34+ subset analysis performed, aliquot 50  $\mu$ l of cells to tubes 3-6.
  - e. Add 10  $\mu$ l of stain from each antibody pre-mix (3-6) to each corresponding sample tube (3-6), and incubate for 20 minutes in the dark at 4°C.

- f. Add 20  $\mu\text{l}$  of ProCount antibody to tube 1, add 20  $\mu\text{l}$  of ProCount Control antibody stain to tube 2, and 50  $\mu\text{l}$  of cells to both. Add 10  $\mu\text{l}$  of Multi-Test antibody stain (or equivalent) and 50  $\mu\text{l}$  of cells to tubes 7 and 8. Incubate tubes in the dark at room temperature for 20 minutes.
- g. Add 1 ml of FACS lysing solution to tubes 3-7 and incubate in the dark at room temperature for 5 minutes (or institutional equivalent).
- h. If CD34+ subset analysis performed, wash cells in tubes 3-6 with 1 ml of FACS buffer each, decant supernatant, add 250  $\mu\text{l}$  of FACS lysis solution to each tube, and store at 4°C.
- i. Add 450  $\mu\text{l}$  of FACS lysis buffer to tubes 1, 2, 7, and 8, and incubate for 30 minutes in the dark at room temperature (or institutional equivalent).
- j. Wrap tubes 1-8 in aluminum foil, mark the bundle of tubes with the date of collection and the bar coded unit number, and store in the dark at 4°C.
- k. Perform acquisition of samples within 96 hours.

### 3. Acquisition:

- a. Acquire sample data according to manufacturer's instructions for Becton Dickinson Flow Cytometer (or equivalent).
- b. To enhance the reliability for the determination of CD34+ cell subpopulations, efforts should be made to acquire at least 1,000 CD34+ events for tubes 3, 4, 5, and 6.
- c. For Procount (or equivalent) tubes, acquire a minimum of 300 CD34+ cells. For Multi-Test (or equivalent) tubes, acquire a minimum of 10,000 lymphocytes (45 bright low-side scatter).

### 4. Analysis:

- a. Access the ProCount file (or equivalent).
- b. Initiate appropriate program for tubes 1 and 2 to analyze the acquired data.
- c. As appropriate, record the number of dye positive events, the number of nucleated cells per microliter, total number of CD45+ events, number of CD45+ cells per microliter, percent of lymphocytes of CD45+ cells, number of CD34+ events, the number of reference beads, and total number of beads added to each TruCount tube. The data system will calculate the % of CD45+ events, the number of CD34+ cells per microliter, and the total CD34+ cells in the collection.
- d. If CD34+ subset analysis is performed, analyze tubes 3-6 using Analysis of CD34+ Sub-

Pops template (or equivalent).

- e. Adjust all gates to include the appropriate cell populations, adjust marker M1 to include 0.50% of control cells exhibiting the most fluorescence, and adjust M2 to include 85% of control cells of least fluorescence.

Note: Adjustment may be necessary for the M1 marker of tube 3 (CD61+CD34+) if the 0.50% setting excludes part of a clearly defined positive population.

- f. Copy markers M1 and M2, paste them into the sample histogram, adjust the markers to include the appropriate cell populations if necessary, and for tubes 3-6 record the percentage of CD 34++ cells that fall within the M1 or the M2 boundary as it applies to the stains exhibited.
- g. Record the number of CD34+ events acquired and the percent of CD34+ events expressing the second marker. The data system will calculate the number of CD34 subset cells per microliter and the total number of CD34 subsets in the collection.
- h. Analyze tube 8 using the T-cell Analysis template (or equivalent).
- i. Adjust all gates and quadrant settings to encompass all appropriate cell populations.
- j. Record the total number of CD45+ events acquired (if applicable), the number of lymphocytes, and the number of CD3+ events acquired. The data system will calculate the percent of CD45+ events, the number of CD3+ cells per microliter, and the total CD3+ cells in the collection.
- k. Record the percentage of CD4+, CD8+, and CD4+/CD8+ cells. The data system will calculate the total CD4+, CD8+, and CD4-/CD8- cells in the collection.
- l. Analyze tube 7 using the B-cell and Natural Killer Cell Analysis template (or equivalent) and select file sample tube 7 for all plots in the screen set up.
- m. Adjust all gates and quadrant settings to include the appropriate cell populations.
- n. In the lymphocyte gate, calculate and record the percent of cells that are CD19+/CD16-CD56-, the percent of cells that are CD19-/CD16+/56+, and the percent of cells that are CD19+/CD16+CD56+. The data system will calculate the total CD19+ and CD16+56+ in the population.

### *Quality Control*

- 1. At regular intervals as defined by institutional procedures, the Laboratory Supervisor will select previously assayed data files for blinded counting by each qualified lab staff member to determine accuracy. Any staff person whose counts are outside 10% of the average will undergo re-training.

2. At regular intervals as defined by institutional procedures, the Laboratory Supervisor will select a cord blood sample to be tested in at least 3 parallel assays by each qualified lab staff member to determine reproducibility. Any staff person whose assays are outside 10% of the average will undergo re-training.
3. At regular intervals as defined by COBLT participating cord blood banks, a cord blood sample will be selected for parallel assay by COBLT cord blood banks to determine reproducibility between facilities. Any staff person whose assays are outside 10% of the average will undergo re-training.
4. All training is administered according to institutional training procedures.

#### *Procedure Notes*

1. Prepare FACS buffer by adding 5 g of bovine serum albumin to 500 ml of phosphate buffered saline pH7.4. This buffer is stored at 4°C in a bottle labeled with contents, data of preparation, the identity of the responsible technologist, and the expiration date (one month following preparation).
2. Prepare the FACS lysing buffer by adding 45 ml of purified water to 5 ml of 10x Becton Dickinson FACS Lysing Solution. Store at 4°C in an aluminum foil covered 50 ml conical tube labeled with contents, date of preparation, the identity of the responsible technologist, and the expiration date (one month following preparation).

#### *References*

Becton Dickinson. ProCount Progenitor Cell Enumeration Kit Instruction Manual. Becton Dickinson. San Jose, CA; 1996.

Rose N, Friedman H, Fahey J, ed. Manual of Clinical Laboratory Immunology. Preparation, Staining, and Analysis by Flow Cytometry of Peripheral Blood Leukocytes. 3rd ed. Washington DC American Society for Microbiology; 1986: 226-235.

Shapiro HM. Practical Flow Cytometry. Goals and Methods in Data Analysis. 3rd ed. New York, New York. Wiley-Liss, Inc.; 1995: 179-182.