STEM CELL LABORATORY (STCL)

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DOCUMENT TITLE:
Using the BD Stem Cell Enumeration Kit for Hematopoietic Transplant Products and Mobilized Peripheral Blood

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FLOW-GEN-040
Using the BD Stem Cell Enumeration Kit for Hematopoietic Transplant Products and Mobilized Peripheral Blood

1 PURPOSE

1.1 The purpose of this process is to prepare specimens obtained from qualified Stem Cell Laboratory blood products for analysis by flow cytometry using the BD Stem Cell Enumeration assay method.

2 INTRODUCTION

2.1 The BD™ Stem Cell Enumeration (SCE) kit provides simultaneous enumeration of viable dual-positive CD45+/CD34+ hematopoietic stem cell populations in CD34 absolute counts (cells/µl) as well as the percentage of the total viable leukocyte count that is CD34+ (%CD34). This kit is cleared for in vitro diagnostic use on either a BD FACSCalibur™ flow cytometer using BD CellQuest™ or BD Cell Quest ™ Pro software or a BD FACSCanto™ II flow cytometer using BD FACSCanto™ software.

3 SCOPE AND RESPONSIBILITIES

3.1 This kit is cleared for in vitro diagnostic (IVD) use and will be used by the STCL for testing the following specimens: Fresh and thawed umbilical cord blood, fresh and thawed peripheral blood stem cells, mobilized peripheral blood, and fresh bone marrow. It is the responsibility of the STCL director, manager, and flow cytometry staff to assure that this procedure is followed.

4 DEFINITIONS/ACRONYMS

4.1 UCB – Umbilical Cord Blood

4.2 PB – Peripheral Blood

4.3 PBSC – Peripheral Blood Stem Cells

4.4 BM – Bone marrow

4.5 STCL – Stem Cell Laboratory

4.6 BD – Becton Dickinson

4.7 PBS – Phosphate Buffered Saline

4.8 BSA – Bovine Serum Albumin

4.9 MSDS – Material Safety Data Sheet

4.10 SCE – Stem Cell Enumeration

5 MATERIALS

5.1 BD Stem Cell Enumeration Kit (Becton Dickinson) includes the following:

5.1.1 Trucount tubes

5.1.2 CD45 FITC/CD34PE cocktail

5.1.3 7AAD reagent

5.1.4 Ammonium chloride lysing solution (10x)

5.2 PBS/ 1% BSA (Gibco BRL)

5.3 12x75 test tubes, Fisher brand

5.4 Trucount™ tubes (Becton Dickinson)

5.5 Flow Cytometry Worksheet

6 EQUIPMENT

6.1 Vortex mixer

6.2 BD FACSCalibur flow cytometer system

6.3 BD FACSCanto II flow cytometer system

6.4 Calibrated 10, 20, 100, 1000 microliter adjustable pipettes and tips (Rainin)

6.5 Calibrated 2000 microliter automated pipette and tips (Rainin)

6.6 Calibrated 20 ml automated pipette and tips (Rainin)

7 SAFETY

7.1 Review MSDS for sodium azide contained in BD antibody reagents.

7.2 Review MSDS for BD Trucount tubes.

7.2.1 Cobalt chloride

7.3 Use universal precautions when working with biological material.

8 PROCEDURE

8.1 **Caution:** Do not prepare more test samples than can be acquired within 1 hour of completion of staining as this is a viable cell assay.

8.2 Use the following pipetting guidelines when adding antibodies and specimen:

8.2.1 Adjust the pipette volume setting to the correct volume.

8.2.2 Keep the pipette upright and hold the Trucount tube in the hand opposite the pipetting hand while dispensing the antibody being sure to avoid touching the bead pellet with the pipette tip.

8.2.3 Use the same tip when adding the same antibody reagent to multiple testing tubes if the addition is to a clean tube.

8.2.4 Use a clean tip between different antibody vials or if the tube receiving antibody contains another agent.

8.2.5 Touch the end of the tip to an interior side of the tube just above the metal barrier to dispense the reagent so that it runs down and into the bottom of the tube. Normal pipetting may be used.

8.2.6 Use the reverse pipetting method when making specimen dilution or adding 100µl of specimen (or specimen dilution if required) to Trucount tubes. **Refer to FLOW-GEN-023 Specimen Dilution Protocol for specimen dilution guidelines.**

8.2.6.1 Hold the Trucount tube in the opposite hand to the pipetting hand.

8.2.6.2 Touch the pipette tip opposite the interior side that was used to add antibody reagent approximately 2/3rds the distance down the tube (i.e. 1/3rd from the bottom of the tube) and dispense.

8.2.6.3 If blood adheres to the side of the tube around the top, use a cotton swab to wipe clean.

8.2.7 Residual specimen may be dispensed back into the original specimen tube unless the tip was contaminated. In this case the tip with residual specimen should be disposed of.

8.2.8 Use a clean tip each time specimen is added to a Trucount tube.

8.2.9 Dispose of the dilution tube, if applicable.

8.2.10 Record the staining time information on the Flow Cytometry Worksheet based on the timer setting compared to the cytometer computer clock time.

8.3 Staining: Refer to Table 1 below to prepare process controls and test specimens.

Table 1: Use the following guidelines when preparing process controls and test samples as recommended by the manufacturer:

1. Prepare the process controls or test sample according to the following table. Prepare and run the process controls prior to staining test samples to complete the instrument setup and assure reagent performance qualification.					
Sample Type	**Tube Type**	**Reagent (µl) Normal pipetting**		**Cells (µl) Reverse pipetting**	
		CD34	**7-AAD**	**High**	**Low**
7-AAD control For Canto only	Polystyrene	20	20	100 of either	
High Control	Trucount	20	---	100	---
Low Control	Trucount	20	---	---	100
Test Sample	Trucount	20	20	100 test sample	
2. Cap the tubes, vortex gently, and incubate for 20 minutes in the dark at room temperature.					
3. Add 2 ml of 1X ammonium chloride lysing solution to each tube. ***Make 1X solution by using 2 mls of 10X ammonium chloride to 18 mls DI water from STCL spigot 1 (deionized and filtered). Double to ratio of 4 and 36 if test volume calls for more lysing solution.***					
4. Cap the tubes, vortex gently, and incubate for 10 minutes in the dark at room temperature.					
5. Immediately put the tubes on wet ice and acquire within 1 hour of lysing.					

NOTE: Store BD Trucount tubes at 2-25° C. These tubes must be used within 1 hour of removal from the foil pouch. Remaining tubes must be used within 1 month of opening the foil pouch. Carefully reseal the foil pouch immediately after removing a tube. Examine the desiccant each time the pouch is opened to determine if color has changed from blue to lavender. Discard tubes if color has changed.

8.4 Optimizing for BD SCE test acquisitions on the FACSCalibur instruments:

8.4.1 Perform instrument startup and daily quality control using BD Calibrite™ beads and FACSComp software. See SOP FLOW-GEN-014.

8.4.2 Verify that all parameters PASS prior to initiation of testing.

8.4.3 Refer to section 8.4 in procedure FLOW-GEN-038 to optimize the FACSCalibur settings in order to acquire the SCE assay controls and test samples. Save the optimized settings as BD SCE in the instrument settings folder on the FACSCalibur hard drive.

8.5 Acquiring SCE assay samples on the FACSCalibur Flow Cytometer:

8.5.1 Control cell testing must be completed, acquired on one of the FACSCalibur flow cytometers or on the FACSCanto flow cytometer,

and the results must fall within the assayed range of values for the control cell lot prior to staining test samples.

8.5.2 In the parameter description window, specify the storage location and data file name. The file naming convention for control cells is as follows: LotID (H/L) mmddyy (eg.FC012H012212). The file naming convention for test samples is as follows:

8.5.2.1 When relevant enter pt. ID using the first initial of first name and first 3 letters of last name, abbreviation for sample type, date as mmddyy.

8.5.2.2 For fresh umbilical cord blood the file name structure is UCB (A, B, C etc.) mmddyy.

8.5.2.3 Specimen designations are as follows:

8.5.2.3.1 Peripheral Blood = PB

8.5.2.3.2 Leukapheresis (Peripheral blood stem cells) = L1, L2, L3 and so on. Add letters A, B, C when dealing with selected products or products for which multiple stages in processing are monitored. Add MP for midpoint tests.

8.5.2.3.3 Peripheral blood stem cell reinfusion = LR. Add number designation if multiple reinfusions are performed.

8.5.2.3.4 Umbilical cord blood reinfusion = CBR

8.5.2.3.5 Bone Marrow=BMOR for samples taken from the OR bag or BMPO for post processed marrow.

8.5.3 Create a data storage folder on the specified drive location corresponding to the current date.

8.5.4 Enter operator name or initial in the Operator field of the parameter description window.

8.5.5 Use the following table list for entering specimen ID and patient ID information in the Parameter description window:

Specimen	Sample ID	Patient ID
Controls	Lot number	(not required)
PB	Patient history number	Last name, First name
Midpoints (leukapheresis)	Patient history number	Last name, First name
Reinfusions and fresh products	Bar code	Last name, First name
Fresh UCB	Lab ID (UCBA,B,C)	Bar code

8.5.6 Go to the Acquire menu heading and scroll to open the Acquisition and Storage window.

8.5.7 When preparing for control cell testing, verify that the acquisition will Reject Debris (R7) and the Event Count or Time stop criteria is set at 75000 of viable 45+ events or after 900 seconds.

8.5.8 For test samples change the Event count to 300,000 and leave all other parameters the same as the control setup.

8.5.9 Open the custom keywords window (from the Acquire menu).

8.5.10 In the Value fields, enter the Trucount bead count, Dilution Factor (1 if not diluted), and Sample staining volume (100 µl)

8.5.11 Click to check the SETUP box in the Acquisition Control window (also found in the browser window).

8.5.12 The flow cytometer must be placed in RUN mode prior to installing the tubes on the SIP when using the loader system, otherwise an alarm will sound.

8.5.13 Mix the test tube on the vortex at medium (see mark on mixer) speed 2-4 seconds.

8.5.14 Remove the cap and load the tube on the SIP. (If using the loader, place the tube on the carousel and replace the loader cover).

8.5.15 The carousel will circle around to the number 1 slot location automatically. NOTE: Only 1 tube at a time may be placed on the loader since samples must be kept on ice prior to acquisition.

8.5.16 If performing work without a loader, move the tube guide arm to the left and install the testing tube on the SIP making sure the tube is pushed securely onto the SIP.

8.5.17 Click Acquire in the acquisition window (optionally use the browser acquire window).

8.5.18 Observe the acquisition plots to make sure the events are showing on the screen display as they should be.

8.5.19 If no events show up on the display, an air bubble or clog may be preventing flow of test sample and the testing tube should be uninstalled while troubleshooting the problem.

8.5.20 Return to the acquisition window and proceed as follows:

8.5.21 Click on STOP, then, ABORT.

8.5.22 Remove the check from the SETUP box.

8.5.23 Click on ACQUIRE, to begin saving data.

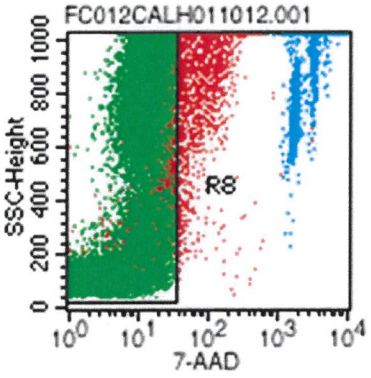
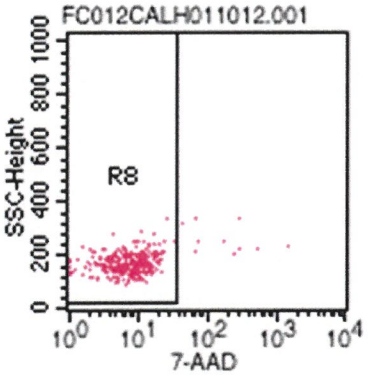
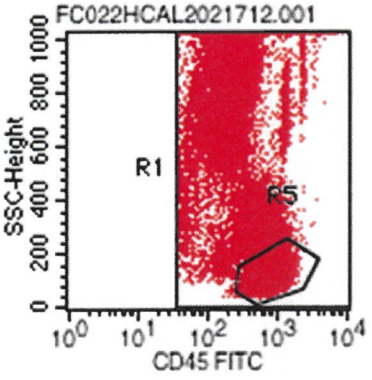
8.5.24 When the test tube has been acquired, remove the tube, replace the cap(s) and place the tube on the "To be analyzed" rack until the analysis for the batch is complete.

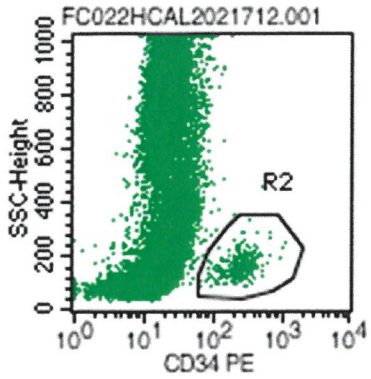
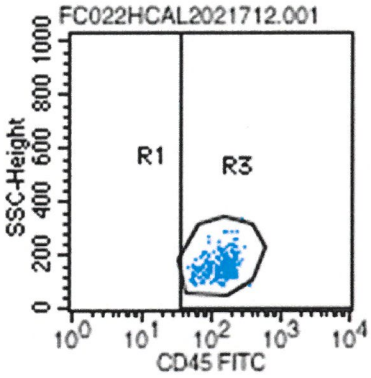
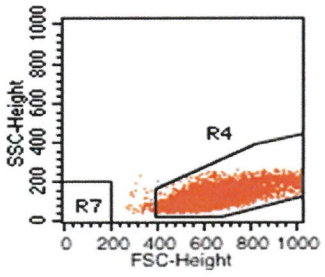
8.5.25 Place a tube with about 1 milliliter of DI water on the SIP and place the cytometer in standby mode by pressing the STANDBY button on the front of the cytometer.

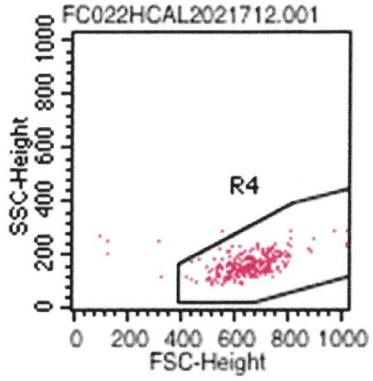
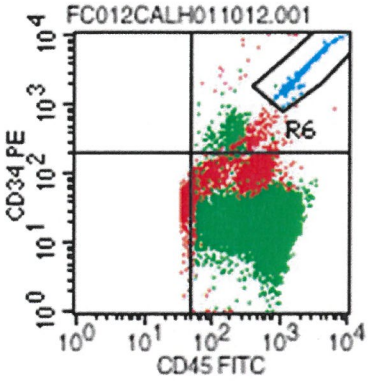
8.5.26 On page 2 of the Flow Cytometry Worksheet complete the entries for data collection through acquisition of testing samples.

8.6 Use the guidelines provided below for the BD SCE CQ Pro analysis of the data acquired from the SCE testing.

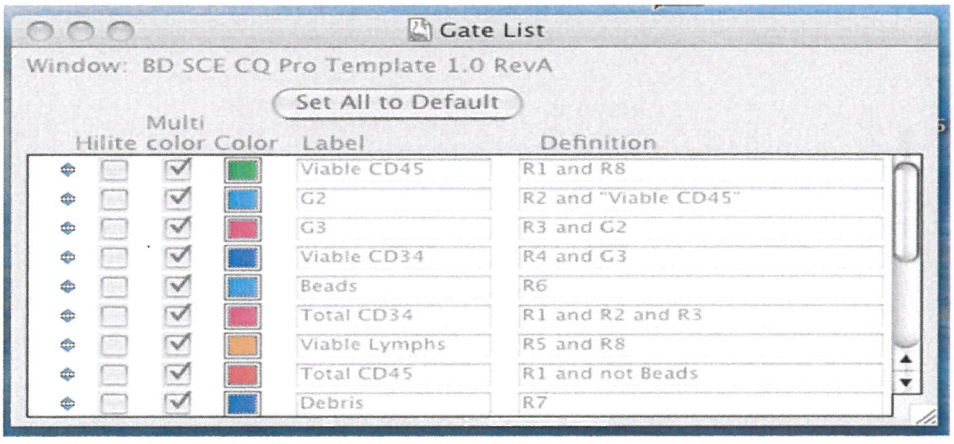
8.6.1 Analysis guidelines for BD SCE CQ Pro method:

Dot Plot	Explanation
 **Plot 8: 7-AAD vs SSC**	This plot contains the first gate to be adjusted and is used to identify viable cells (7-AAD$^{neg.}$) Adjust R8 to enclose the 7-AAD^{neg} events. The region extends beyond the top of the plot on the y-axis, beyond the far left of the plot on thex-zxis, and excludes positive-stained cells. **Caution: If there are a large number of red(dead) events outside R8, see page 34 of applicaton guide for troubleshooting information.**
 **Plot 7: 7-AAD vs SSC**	This plot is gated on the Total CD34 gate (R1 and R2 and R3) and is used during compensation optimization (FL3%-%Fl2). This plot displays total CD34 cells and confirms poper adjustment of region R8 on Plot 8. If the instrument is appropriately set up and compensated, the gated viable CD34 cells will exhibit the same level of fluouescence as the viable lymphocytes in Plot 8.
 **Plot 1: CD45 vs SSC**	This plot is ungated and is used to include all CD45 ^{dim} to CD45 ^{bright} events and excludes debris, platelets, and unlysed red blood cells (RBCs), which are all CD45 neg. Adjust the R1 leucocyte gate to extend above the top of the plot to include high SSC events that are CD45+. Adjust the left side of the gate to include all CD45+ cells, including dim CD45+ events that are CD34+. The right side can extend to the edge of the plot. Adjust polygon region R5 around the lymphocytes. Include only as many events as necessary to define the viable lymphocyte population displayed in Plot 6. **Tip** Display fewer events on this plot so that the limits of the lymphocytes are easier to define.

Dot Plot	Explanation
 **Plot 2: CD34 vs SSC**	This plot displays viable CD45 (G1) cells and is used to identify CD34+ cells. Adjust R2 to include all CD34+/low SSC (below 400)events. **NOTE** This region should exclude any platelets that form a streak between neutrphils and CD34+ events. See examples of this on page 3 of the application guide.
 **Plot 3: CD45 vs SSC**	This plot displays G2 (R2 and viable CD45+) cells and is use to further define the viable CD34+ CD45+ cell population Adjust R3 to include only those events that form a cluster with low to intermediate SSc and Cd45 dim expression. Adjust R3 to exclude any lymphocytes or monocytes seen to the right of the stem cell cluster.
 **Plot 6: FSC vs SSC**	This plot displays Viable Lymphs (R5 and R8) and is usde to establish the minimum FSC and SSC ranges for R4. Display region R4 on this plot and adjust the position os that only viable lymphs from R5 are included. R7 is an acquisition exclusion gate used to exclude debris. Region 7 should not exceed FSC channel 200 and SSC channel 200 and shold not encroach the lymphcytes population because events in R7 will be excluded from the data file.

Dot Plot	Explanation
 **Plot 4: FSC vs SSC**	This plot displays G3 (R3 and G2) cells and is used to identify viable stem cells (CD34+). Adjust R4 to include only those events that form a cluster with low to intermediate SSC and medium to high FSC. The gate serves to exclude platelets and debris that can show weak, nonspecific binding of CD34 and CD45. Its Lower FSC boundary is adjusted in Plot 6. Region R4 is the lymph/blast region and will be adjusted by scatter of lymphocytes. **Tip** This plot can be gated on R1 and R2 and R3 to display Total CD34+ cells instead of Viable CD34+ cells.
 **Plot 5: CD45 FITC vs CD34 PE**	This ungated multicolor plot is used to identify beads (R6). Optional: Adjust the quadrant marker to establish a visual lower limit of CD45 expression by the CD34+ events, as in Plot 1. The bead population appears in the top right corner (aqua). Allow R6 to extend beyond the plot boundary (x and y axes) to include all bead events. Verify that there are no cellular events in the bead gate.

8.6.2 BD Stem Cell Enumeration CQ Pro Gating definitions:



8.7 Print the results of testing to keep with the flow worksheet and save a PDF by performing the following steps:

8.7.1 Choose file>print.

8.7.2 In the bottom left corner of the print window choose PDF and scroll to Save PDF.

8.7.3 Enter the file name as it is for the data file and add the suffix PDF on the end. Example: JDOEPBMMDDYY.PDF.

8.8 If required for the specimen type, calculate the total viable CD34+ cells or Total viable CD34/kg values by multiplying the viable CD34/µl by the volume in microliters and divided by the recipient weight using the calculation fields provided on the flow cytometry worksheet.

8.9 Calculations must be reviewed by a second tech prior to reporting results.

8.10 Acquiring SCE assay samples on the FACSCanto II Flow Cytometer using FACSCanto software and BD Stem Cell application:

8.10.1 Power up the FACSCanto II flow cytometer and computer.

8.10.2 Log into windows: Administrator and enter the required password.

8.10.3 Launch BD FACSCanto Clinical software from the desktop icon.

8.10.4 From the drop down window find your name as user and enter your password.

8.10.5 Refer to sections 8.3.6-8.4 in procedure FLOW-GEN-039 for instructions on instrument setup, optimization, and process control testing.

8.10.6 Instrument performance checks must pass, control cell testing must be completed, and the results must fall within the assayed range of values for the control cell lot prior to staining test samples.

8.10.7 In the FACSCanto worklist "NAME" column enter SPECIMEN.

8.10.8 In the FACSCanto worklist "SAMPLE ID" column use the following guideline for entering sample identification information:

8.10.8.1 When relevant specimen type, enter pt. ID using the first initial of first name and first 3 letters of last name, abbreviation for sample type, date as mmddyy.

8.10.8.2 For fresh umbilical cord blood the file name structure is UCB (A, B, C etc.) mmddyy.

8.10.8.3 Specimen designations are as follows into the :

8.10.8.3.1 Peripheral Blood = PB

8.10.8.3.2 Leukapheresis (Peripheral blood stem cells) = L1, L2, L3 and so on. Add letters A, B, C when dealing with selected products or products for which multiple stages in

processing are monitored. Add MP for midpoint tests.

8.10.8.3.3 Peripheral blood stem cell reinfusion = LR. Add number designation if multiple reinfusions are performed.

8.10.8.3.4 Umbilical cord blood reinfusion = CBR

8.10.8.3.5 Bone Marrow=BMOR for samples taken from the OR bag or BMPO for post processed marrow.

8.10.9 In the "CASE NUMBER" column enter the product bar code or pt. history number (if no bar code assigned as with PB specimens).

8.10.10 Select "BD Stem Cell 7AAD" from the Panel drop down menu.

8.10.11 Click Run  to start the run.

8.10.11.1 A prompt appears asking to save the worklist; Choose No.

8.10.11.2 A prompt appears asking if manual run is OK; Choose OK.

8.10.11.3 At the next prompt choose Ignore.

8.10.12 Vortex the test tube on medium speed 2-4 second (vortex dial should be placed where arrow is drawn) before acquisition.

8.10.13 When prompted, manually install tube on the cytometer by doing the following:

8.10.13.1 Push the aspirator arm to the left.

8.10.13.2 Place the tube on the SIT, ensure that the tube is straight, and firmly push up until the tube comes to a complete stop and is fully seated.

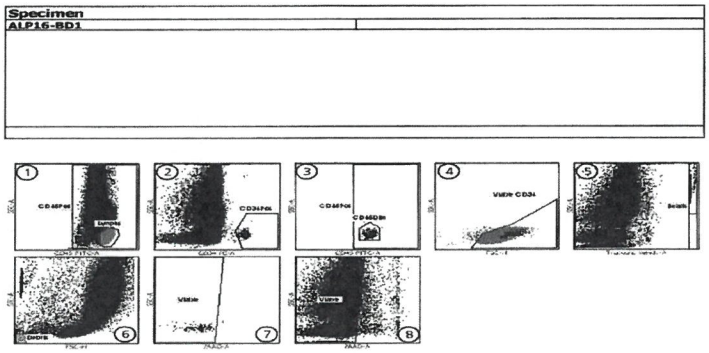
8.10.13.3 Center the aspirator arm under the tube so that the bottom of the tube sits centered above the three sensor pins on the aspirator arm.

8.10.14 After acquisition is complete the lab report appears on the screen.

8.10.15 Refer to the FACSCanto II *BD Stem Cell Enumeration Application Guide* for examples of various product types to help determine appropriate region placement. See report example 1 as a guide.

Example 1:

Example Lab Report The plots are displayed on the Lab Report in the following order.



Plot no.	Gate(s)	Plot no.	Gate(s)
①	CD45Pos, Lymphs	⑤	Beads
②	CD34Pos	⑥	Debris
③	CD45Pos, CD45Dim	⑦	Viable
④	Viable CD34	⑧	Viable

8.10.16 To make adjustments to regions, click on the plot to be changed (this action activates the plot and allows the user to modify gates) and click on the region to make adjustments as needed.

8.10.17 Once the analysis is complete, click on the Review button>Choose the appropriate User ID >click OK.

8.10.18 Print report to keep with the flow worksheet.

8.11 If required for the specimen type, calculate the total viable CD34+ cells or Total viable CD34/kg values by multiplying the viable CD34/µl by the volume in microliters and divided by the recipient weight using the calculation fields provided on the flow cytometry worksheet.

8.12 Calculations must be reviewed by a second tech prior to reporting results.

9 RELATED DOCUMENTS/FORMS

9.1 FLOW-GEN-012 FRM5 Stem Cell Laboratory Flow Cytometry Worksheet

9.2 FLOW-GEN-038 Enumeration of Viable CD34+ Stem Cells in Fresh Umbilical Cord Blood Using the BD Stem Cell Enumeration Kit: BD FACSCalibur and BD Cellquest Pro Version

9.3 FLOW-GEN-039 Enumeration of Viable CD34+ Stem Cells in Fresh Umbilical Cord Blood Using the BD Stem Cell Enumeration Kit: FACSCanto II Clinical Software Version

9.4 FLOW-GEN-023 Specimen Dilution Protocol (JA1)

10 REFERENCES

10.1 BD Stem Cell Enumeration Kit product insert: catalog # 344563.

10.2 BD Stem Cell Enumeration Application Guide for BD FACSCanto II Flow Cytometer and for BD FACSCalibur Flow Cytometer. Electronic version stored on the cytometer workstation instrument workstation desktop or on shared network drive.

11 REVISION HISTORY

Revision No.	Author	Description of Change(s)
03	M. Reese	1. Modified document title 2. Made corrections to document names in section 9

Signature Manifest

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FLOW-GEN-040 Using the BD Stem Cell Enumeration Kit for Hematopoietic Transplant Products and

Author

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Management

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Sandy Mulligan (MULLI026)		12 Oct 2020, 06:39:53 PM	Approved