



## STEM CELL LABORATORY (STCL)



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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

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# 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

### 1 PURPOSE

- 1.1 This procedure provides instructions for flow cytometric evaluation of fresh umbilical cord blood specimen for viable CD34+ stem cell enumeration using the BD Stem Cell Enumeration (SCE) staining kit, the BD FACSCalibur flow cytometer, and BD Cell Quest Pro software SCE template.

### 2 INTRODUCTION

- 2.1 The BD™ SCE kit provides a single tube viable CD34 assay for fresh umbilical cord blood. The reagent is combined with test samples in individual BD Trucount Tubes to obtain absolute cell counts. When a sample is added to the reagent, the fluorochrome-labeled antibodies in the reagent bind specifically to the cell surface. Additionally, the lyophilized pellet in the BD Trucount tube dissolves, releasing a known number of fluorescent beads. The dye 7-AAD is added to assess viability of the cells. Cells that are 7-AAD+ are not viable. Ammonium chloride is added to lyse erythrocytes before the sample is acquired on a flow cytometer. During analysis of the sample, the concentration of viable CD34+ cells, viable CD45+ cells, and percentage of viable CD34+ cells in the viable CD45+ cell population, are calculated.

### 3 SCOPE AND RESPONSIBILITIES

- 3.1 This procedure should be used when performing flow cytometric assays to enumerate viable CD34+ stem cells in fresh UCB specimens. It is the responsibility of the laboratory director, manager, and flow cytometry staff, to ensure the requirements of this procedure are successfully met.

### 4 DEFINITIONS/ACRONYMS

- 4.1 SCE - Stem Cell Enumeration
- 4.2 CCB - Carolinas Cord Blood Bank
- 4.3 BD - Becton Dickinson, manufacturer of flow cytometry instruments, software, and reagents.
- 4.4 Single-platform method – This is a flow cytometry method for directly enumerating cells of interest (CD34) by using a known number of beads/volume of sample. Results are reported as number of CD34+ progenitor cells per µl of specimen tested.
- 4.5 Test specimen - The test tube that holds the UCB or dilution of UCB to be added to the testing tubes.
- 4.6 Testing Tubes - The tubes used in the staining step in which antibodies and test specimen are mixed.

- 4.7 Reverse pipetting – A method of pipetting using an adjustable air displacement pipette that allows for a more accurate volume measure upon dispense, particularly with viscous fluids. The plunger is pressed all the way down prior to inserting the tip into the fluid, then, released all the way up. The plunger is then pressed only to the first stop to dispense the volume dialed into the pipette.
- 4.8 UCB - Fresh (not thawed) umbilical cord blood that has been red cell depleted and volume reduced.
- 4.9 SIP – Sample injection probe where the testing tube is installed on the flow cytometer.
- 4.10 CQ – CellQuest Pro BD flow cytometry acquisition and analysis software.
- 4.11 Menu – Refers to the CellQuest pro menu bar in the software experiment document.
- 4.12 Dot Plot - A two parameter box used to display events acquired by a flow cytometer.
- 4.13 Acquisition region (or gate) – The region drawn around the designated population of events/cells that determine when the acquisition will stop, signaling that the data file with the information needed to perform an analysis has been stored and that the testing tube can be uninstalled from the SIT.
- 4.14 Region - A line or box drawn on a dot plot to define a specific population of events/cells during flow cytometry acquisition or analysis.
- 4.15 Gate - used to define events/cells of interest on a flow cytometry plot. Gates may be defined by one or more regions and may be used to sequentially define events/cells from enumeration of a specific population such as CD34+ progenitor cells. The logical operators AND, OR, and NOT are used to define logical gates.
- 4.16 Sequential gating - The process of using regions and gates around populations of events based on various parameters including light scatter and fluorescent antibody staining intensity. This can also be referred to as hierarchical gating in which gates (parent population) can contain subgates (subpopulations).
- 4.17 CCBB Worksheet Packet – The packet of forms delivered with the UCB specimen includes, Graft Characterization Form, the Progenitor Assay Form, Flow Cytometry Worksheet, and additional bar code labels with lab letter ID.

## 5 MATERIALS

- 5.1 BD Stem Cell Enumeration Kit
  - 5.1.1 CD34 PE/CD45 FITC reagent
  - 5.1.2 7-aminoactinomycin-D (7AAD) reagent
  - 5.1.3 10X ammonium chloride lysing solution
  - 5.1.4 50 BD Trucount tubes (2 foil pouches of 25)
- 5.2 Specimen Dilution reagent-PBS/ 1% BSA, Gibco BRL

- 5.3 12 mm x 75 mm, 5 ml non sterile polystyrene test tubes and caps, Fisher brand or equivalent
- 5.4 Test tube rack
- 5.5 Traceable timers, VWR or equivalent
- 5.6 Bleach – Clorox household or equivalent for waste tank.
- 5.7 FACSFlow Sheath fluid – BD
- 5.8 Colored Markers-Sharpie

## 6 EQUIPMENT

- 6.1 FACSCalibur™ Flow Cytometer, Becton Dickinson
- 6.2 Vortex mixer, VWR or equivalent
- 6.3 Adjustable pipettes and tips 20, 200, 1000 microliter (μl), Rainin
- 6.4 Automated pipette and tips 2000 microliter, Rainin

## 7 SAFETY

- 7.1 Use universal precautions and wear appropriate personal protective equipment (PPE) when working with the biological materials described in this procedure.
- 7.2 Use particular caution with the following reagents used in this procedure. Refer to Material Safety Data Sheet for these products or Duke Occupational and Environmental Safety Office website for more information on these warnings.
  - 7.2.1 Monoclonal antibodies used in testing.
  - 7.2.2 Sodium azide warning
  - 7.2.3 7-AAD
  - 7.2.4 BD Trucount tubes
  - 7.2.5 Cobalt chloride warning
  - 7.2.6 Silica warning
  - 7.2.7 Ammonium Chloride


## 8 PROCEDURE

- 8.1 UCB Specimen Receipt:
  - 8.1.1 Upon receipt of the specimen and worksheet packet at the designated drop off location in the STCL flow cytometry section, open the worksheet packet to the *FLOW-GEN-012 (FRM5) Stem Cell Laboratory Flow Cytometry Worksheet*. The example below shows how the specimen information section of the worksheet should be completed. The dilution factor (Dil Fac.) is entered by the STCL staff at the staining step.

InfoCard #: FLOW-GEN-012 FRM5 Rev. 04 Effective Date: 12 Sep 2012  
Accession # \_\_\_\_\_  
(Not Required for banked UCB Units)

**FLOW-GEN-012 FRM5 STEM CELL LABORATORY FLOW CYTOMETRY WORKSHEET**

Circle or write NA when request is not applicable

<b>Sample ID (Attach labels with donor, recipient or patient sample info)</b> <u>NA</u>	<b>Bar Code label</b> <u>NA</u>  W1582 14 491061 S Y <b>UCB LAB ID:</b> <u>A</u>
<b>If NMDP Product:</b> <input type="checkbox"/> OUTGOING <input type="checkbox"/> INCOMING <u>NA</u>	<b>Recipient wt.</b> <u>NA</u> <b>kg</b>

**Sample classification (✓):** ☐ Adult ☐ Pediatric ☐ Allogeneic ☐ Auto ☒ UCB Bank  
☐ To be concentrated ☐ Other \_\_\_\_\_

**Sample source (circle the appropriate test condition):**  
☒ UCB <sup>Fresh</sup> ☐ Thawed infusion ☐ Other \_\_\_\_\_  
☐ PBSC ☐ Midpoint ☐ Preliminary ☐ Final ☐ Thawed infusion ☐ Other \_\_\_\_\_  
☐ Bone Marrow ☐ OR Bag ☐ Post process ☐ Other \_\_\_\_\_  
☐ PB mobilized ☐ Immune Reconstitution (IR) \_\_\_\_\_ (Provide IR time point if known)  
☐ Donor lymphocyte infusion (DLI)  
☐ Control Cell Lot# \_\_\_\_\_ Exp. Date \_\_\_\_\_

**Proficiency Test**  
Date/Time PB or Product Collected 05/05/2014 @ 1420 NA  
Date/Time Product Sampled for tests 05/06/2014 @ 1129 By MW NA  
Cell Concentration 33.1 x10<sup>6</sup>/ml Product volume 21.50 mls  
**To be completed by STCL flow cytometry staff for stem cell product collections:**  
Date/Time stained 5-6-14 @ 1242 BY JS Dil. Factor X 1  
**For Banked UCB: Sample stained within 4hr of sampling time** ✓ tech/date: JS 15-6-14 NA

- 8.1.2 If any information is missing, contact the CCBB laboratory manager or designee and request the required information.
- 8.1.3 Observe the recorded sampling date/ time (05/06/2014@1120 in the example provided) of each specimen to determine if staining will be within 4 hours of the sample draw, then initial and date that this was checked in the space provided "For Banked UCB" under Date/Time Stained.
- 8.1.4 For quality assurance purposes, if the specimen is >4 hours past sampling at the time of staining, note this by writing "Specimen >4 hours post sampling at time of staining" on the comment line below the reagent lot information section of the Flow Cytometry Worksheet.
- 8.1.5 Arrange the worksheet packets in order by lab letter ID (i.e. alphabetical order) and remove a bar code label from each packet to place in the specimen receipt log book. Complete the log book entry after staining the test samples.
- 8.1.6 Verify that the specimen identification matches that of the corresponding worksheet packet for both the barcode label and for the lab letter ID.
- 8.1.7 Verify that the reagents to be used for testing have met quality control requirements. (This may be determined in advance of specimen receipt).
- 8.1.8 For the reagents used in this testing, enter the lot and expiration date in the designated fields on the Flow Cytometry Worksheet prior to specimen staining.

## 8.2 Specimen Staining:

8.2.1 Prior to running test samples on any day of testing, low and high process controls with known assayed CD34+ absolute count values are run to confirm staining and system integrity. The results of this testing must be acceptable prior to running test samples.

8.2.2 Gather staining supplies:

8.2.2.1 BD SCE kit (component lots marked in current use).

8.2.2.2 12 x 75 mm non-sterile polystyrene test tubes with caps

8.2.2.3 1x working solution of red cell lysing reagent:

- Determine volume of 1X lysing agent that will be required for the day and make a 1:10 working solution from the 10X Ammonium Chloride reagent using 1 part 10X reagent and 9 parts reagent grade DI water. Make only enough for each day of use.

8.2.2.4 PBS with 1% BSA used for sample dilution.

8.2.2.5 1000, 200 and 20 µl Adjustable pipettes and tips

8.2.2.6 2000 µl Automated pipette and tips

8.2.2.7 Test tube staining rack

8.2.2.8 Traceable timers.(2)

8.2.3 The following tube setup is shown as a guideline only. Other designs may be used as long as specimen bar code is matched with staining tubes:

8.2.3.1 Arrange (up to 3 specimens /rack, one specimen per row horizontally) the testing tubes and specimen in order as illustrated below and in alphabetical order from the forward facing side of the rack by lab letter ID (i.e. row one = lab ID “A” specimen, row three = lab ID “B” specimen, row 5 = lab ID “C” specimen, or row one = lab ID “X” specimen, row three = lab ID “Y” specimen, row 5 = lab ID “Z” specimen).:

	Col 1	Col 2 open	other	other	Col 5 open	Col 6 open	Col 7	Col 8					
Row 5	SCE						Dil	Sp.					
Open row 4													
Row 3	SCE						Dil	Sp.					
Open Row 2													
Row 1	SCE						Dil	Sp.					

### Forward facing side of rack

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

- SCE= Trucount tube (vertical column 1st column position from left to right)
- Open position (i.e. open column 2nd position)
- Dil=Dilution tube 12x75mm polystyrene tube (If needed)
- Sp=The UCB Specimen tube
- Once the specimen is placed next to the row of tubes, match the bar code labels to the specimen and apply one barcode label to each tube in the row.

8.2.4 Dispose of unused labels in the labeled “Shred-it” drawer at each cytometer table. The contents must be shredded at the end of each work day.

8.2.5 UCB specimens with cell concentrations of 40.0 x10<sup>6</sup> cells/ milliliter or greater must be diluted using the specimen dilution reagent.

**NOTE: Bring the concentration below this mark using whole value dilutions (i.e. x 2, x 3 etc.). The final diluted sample volume should be no less than 300µl in order to provide enough volume to complete testing. The original specimen volume used in the dilution should not be less than 50µl. Table 1 provides guidelines for making dilutions.**

8.2.6 The dilution factor must be entered in the designated field (Dil Fac.) on the Flow Cytometry Worksheet.

**Table 1 Use the following guidelines when preparing dilutions:**

DILUTION TYPE		SPECIMEN VOLUME (Reverse Pipette method)	DILUTION REAGENT (Reverse Pipette method)
2-FOLD	1:2	150 µl	150 µl
3-FOLD	1:3	100 µl	200 µl
4-FOLD	1:4	100 µl	300 µl
5-FOLD	1:5	100 µl	400 µl
10-FOLD	1:10	50 µl	450 µl
15 FOLD	1:15	50 µl	700 µl
20 FOLD	1:20	50 µl	950

8.2.7 Refer to Table 2 reagent volume requirements when adding the antibodies for testing. Use the antibodies in the designated “IN USE” box or if a replacement vial is required, replace from the vial compartment labeled with green “Ready For Use” labels.

**NOTE: If a new lot of reagent must be opened it must first pass laboratory established quality control testing before being put into use for test specimens.**

**Table 2: Use the following guidelines when preparing process controls and test samples as recommended by the manufacturer:**

1. Using the pipetting guidelines in 8.2.8 below 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)		Cells (μl)	
		Normal pipetting		Reverse pipetting	
		CD34	7-AAD	High	Low
7-AAD control	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 2-3, and incubate for 20 minutes in the dark at room temperature.					
3. Add 2 ml of 1X ammonium chloride lysing solution to each tube.					
4. Cap the tubes, vortex gently 2-3 seconds, 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.

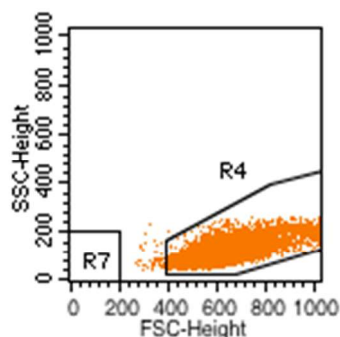
- 8.2.8 Use the following pipetting guidelines when adding antibodies and specimen:
- 8.2.8.1 Adjust the pipette volume setting to the correct volume.
  - 8.2.8.2 Keep the pipette upright and hold the Trucount tube in the hand opposite the pipetting hand while dispensing the antibody being sure to avoid touching the bead pellet with the pipette tip.
  - 8.2.8.3 Use the same tip when adding the same antibody reagent to multiple testing tubes if the addition is to a clean tube.
  - 8.2.8.4 Use a clean tip between different antibody vials or if the tube receiving antibody contains another agent.
  - 8.2.8.5 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.



- 8.2.8.6 Use the reverse pipetting method when making specimen dilution or adding 100µl of specimen (or specimen dilution if required) to Trucount tubes.
- 8.2.8.7 Hold the Trucount tube in the opposite hand to the pipetting hand.
- 8.2.8.8 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).
- 8.2.8.9 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.
- 8.2.8.10 Use a clean tip each time specimen is added to a Trucount tube.
- 8.2.9 Cap all testing tubes, color code them by row using colored markers.
- 8.2.10 Place the original UCB specimen tube on the sample storage rack corresponding to the day of testing. Test specimens for the week are disposed of the following Monday prior to start of testing.
- 8.2.11 Dispose of the dilution tube, if applicable.
- 8.2.12 Record the staining time information on the Flow Cytometry Worksheet based on the timer setting compared to the cytometer computer clock time.
- 8.3 Preparing for UCB Specimen Acquisition:
  - 8.3.1 The BD FACSCalibur flow cytometers used for this testing must have passed laboratory established quality control testing using BD Calibrite beads and 4-color lyse/no-wash (LNW) assay with BD FACSComp™ on the day of use and prior to the initiation of test sample acquisition. This process is outlined in procedure FLOW-GEN-014.
- 8.4 Follow the steps below using the High process control to optimize the FACSComp Lyse/No Wash settings:
  - 8.4.1 Open the **Routine Test Templates** folder on the workstation desktop.
  - 8.4.2 Open the **UCB testing** folder and double click on the BD SCE CQ Pro Template icon.
  - 8.4.3 Navigate to the “Acquire” menu and scroll to “Connect to cytometer”. A browser window and acquisition control window will open.
  - 8.4.4 Navigate to the “Cytometer” menu and click and scroll to instrument settings. A window will open displaying the current settings.
  - 8.4.5 Click on the “Open” button and navigate to the designated file location: FACStation (hard drive)/BD files/instrument settings.
  - 8.4.6 Double click on CALIB FILE.LNW. Click on “SET”, then, “DONE” at the bottom of the instrument settings window.

- 8.4.7 Navigate to the “Cytometer” menu and scroll down to open the Detector Threshold window, and Compensation window.
- 8.4.8 Choose the FL-1 threshold in the threshold window and use the radio dial to set to the threshold value in current use for the instrument (posted at each cytometer).
- 8.4.9 Check the sheath and waste tanks prior to acquisition of samples.
  - 8.4.9.1 See pages 55-59 in the BD FACSCalibur instructions for Use (Part No. 643271 Rev. A, November 2007) for detailed instructions for removing the sheath and waste tanks.
  - 8.4.9.2 If the level is near or at the refill line drawn on the tank, the sheath tank should be removed, taken to the FACSFlow cube filling station and filled with BD FACSFlow. **Do not fill past the designated fill line.**
  - 8.4.9.3 The waste tank should be emptied in the designated waste sink.
  - 8.4.9.4 Add 400 milliliters of undiluted bleach to the empty tank using the designated “Bleach” beaker at the waste sink area.
  - 8.4.9.5 Replace the tanks following the guidelines in the BD FACSCalibur instructions for Use (Part No. 643271 Rev. A, November 2007) pages 55-59.
  - 8.4.9.6 Once the tanks are in place perform a prime of the instrument by pressing the PRIME button on the front of the cytometer.
  - 8.4.9.7 When the STANDBY button lights up, the instrument is finished with the Prime.
- 8.4.10 Press the RUN and Hi button on the front of the cytometer, put the High control tube on the SIP.
- 8.4.11 In the acquisition control window, select the Setup checkbox, and then click Acquire to begin instrument optimization.
- 8.4.12 Select Cytometer>Detectors/Amps. Observe Plot 6 (FSC vs SSC dot plot, Figure 1) and if necessary, adjust the FSC gain in the Detectors/Amps window so that the viable lymphocytes fall between channels 400 and 600.

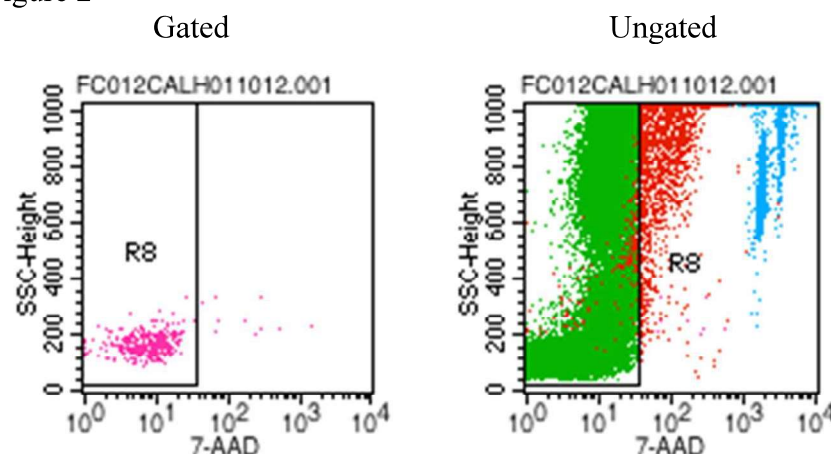
Figure 1



R7 is an acquisition exclusion gate use to exclude debris and should not exceed FSC channel 200 and SSC channel 200. It should not encroach on the lymphocyte population as cells in R7 are excluded from the data file.

- 8.4.13 **In the Compensation window, increase the FL3-%FL2 compensation value by 4 from the initial LNW settings value.**
- 8.4.14 Observe the two 7-AAD vs SSC plots (Figure 2). One is un-gated and one is gated on the CD34 Total gate. Both plots display R8. Because this sample does not have 7-AAD in the tube, the majority CD34 cells fall in the negative gate (R8).

Figure 2



On the ungated plot, adjust R8 to include the majority of the 7-AAD<sup>neg</sup> cells. On the gated plot, verify that the majority of the CD34 cells are within R8. Adjust FL3%-%FL2 compensation if the main population is falling outside of R8.

**CAUTION: Do not overcompensate. The ungated CD34 population should have the appearance in Figure 2 gated with the events well off the y-axis.**

- 8.4.15 In the acquisition control window, select Pause, then Abort, and remove the tube from the sip.
- 8.4.16 Go to the cytometer heading>Instrument settings>Save and name the optimized settings BD SCE.

**NOTE: If samples are run intermittently throughout the day, these settings must be retrieved when needed for this testing via the Cytometer>Instrument settings route.**

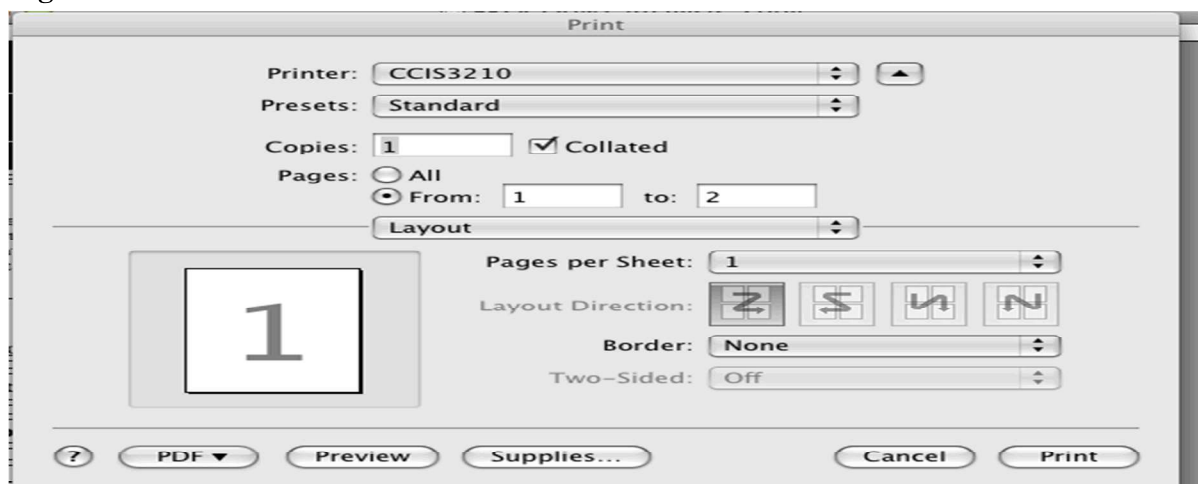
#### 8.5 Acquiring samples:

- 8.5.1 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.
- 8.5.2 In the parameter description window, specify the file name and storage location. File naming convention for control cells is as follows: Lot(H/L)mmddyy (eg.FC012H012212). File naming convention for processed cord blood is as follows: UCB -lab letter ID-mmddyy (e.g. UCBA012212)
- 8.5.3 Create a data storage folder on the specified drive location corresponding to the current date.
- 8.5.4 Go to the Acquire menu heading and scroll to open the Acquisition and Storage window.
- 8.5.5 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 viable 45+ events or after 900 seconds.
- 8.5.6 For test samples change the Event count to 300,000 viable 45+ events or after 900 seconds and leave all other parameters the same as the control setup.
- 8.5.7 Open the custom keywords window from the Acquire menu.
- 8.5.8 In the Value fields, enter the Trucount bead count, Dilution Factor (1 if not diluted), and Sample staining volume (100 µl)
- 8.5.9 Click to check the SETUP box in the Acquisition Control window (also found in the browser window).
- 8.5.10 Vortex the test tube on medium speed for 2-4 seconds; remove the cap, and load the tube on the SIP.

**NOTE: 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.**

- 8.5.10.1 If using the loader, place the tube in carousel position 1 and replace the loader cover.

- 8.5.10.2 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.**
- 8.5.10.3 If performing work without a loader, install the testing tube on the SIP making sure the tube is pushed securely onto the SIP.
- 8.5.11 Click Acquire in the acquisition window (optionally use the browser acquire window).
- 8.5.12 Observe the acquisition plots to make sure the events are showing on the screen display as they should be.
  - 8.5.12.1 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.
- 8.5.13 Return to the acquisition window and proceed as follows:
  - 8.5.13.1 Click on STOP, then ABORT.
  - 8.5.13.2 Remove the check from the SETUP box.
  - 8.5.13.3 Click ACQUIRE to begin saving the data file.
  - 8.5.13.4 When a testing 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.
  - 8.5.13.5 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.
- 8.5.14 On page 2 of the Flow Cytometry Worksheet complete the entries for data collection through acquisition of testing samples.
- 8.6 Analysis
  - 8.6.1 Follow the guidelines in the table 3 below for placing regions and gates.
  - 8.6.2 Once the analysis is complete, print a paper copy and save a PDF by going to the Print window (See in Figure 3 below) and clicking on PDF in the lower left corner. Save the PDF using the same naming convention as the data file name and store it in the same location as the original FCS file.

**Figure 3**

Refer to the following gate definitions for the BD Stem Cell Enumeration analysis plots using the BD CellQuest Pro template.

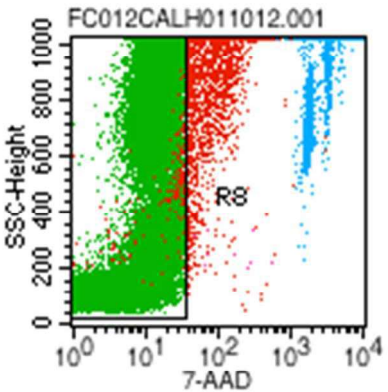
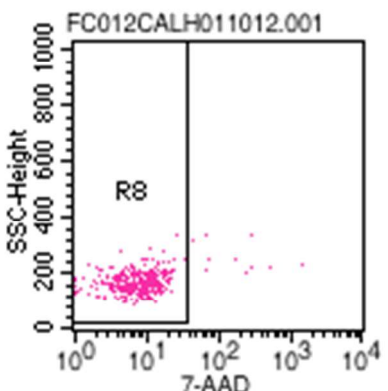
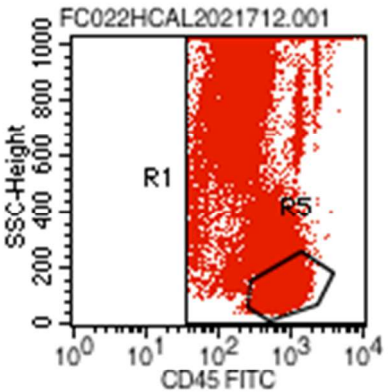
Gate List

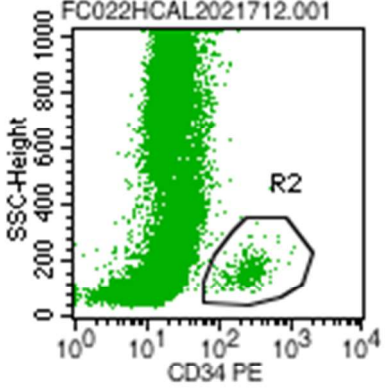
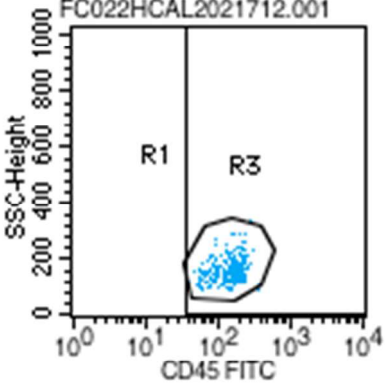
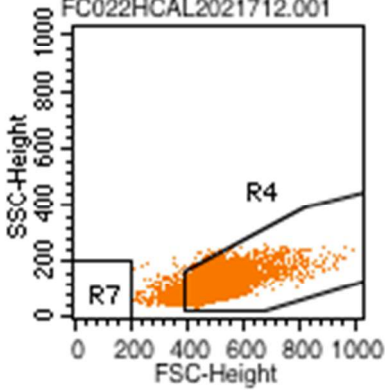
Window: BD SCE CQ Pro Template 1.0 RevA

Set All to Default

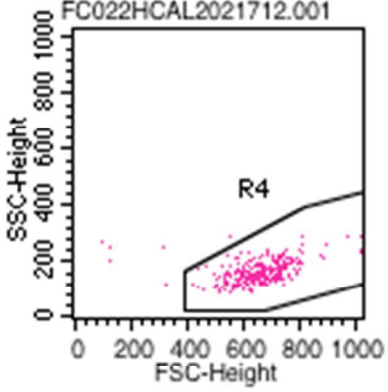
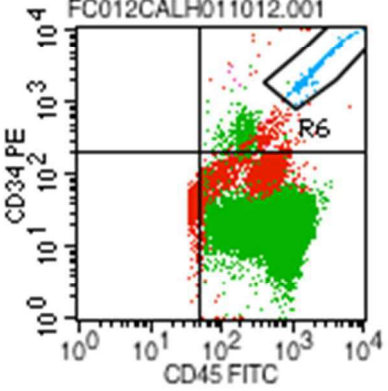
	Multi	Hilite	color	Color	Label	Definition
	<input type="checkbox"/>	<input checked="" type="checkbox"/>		Viable CD45	R1 and R8	
	<input type="checkbox"/>	<input checked="" type="checkbox"/>		G2	R2 and "Viable CD45"	
	<input type="checkbox"/>	<input checked="" type="checkbox"/>		G3	R3 and G2	
	<input type="checkbox"/>	<input checked="" type="checkbox"/>		Viable CD34	R4 and G3	
	<input type="checkbox"/>	<input checked="" type="checkbox"/>		Beads	R6	
	<input type="checkbox"/>	<input checked="" type="checkbox"/>		Total CD34	R1 and R2 and R3	
	<input type="checkbox"/>	<input checked="" type="checkbox"/>		Viable Lymphs	R5 and R8	
	<input type="checkbox"/>	<input checked="" type="checkbox"/>		Total CD45	R1 and not Beads	
	<input type="checkbox"/>	<input checked="" type="checkbox"/>		Debris	R7	

**Table 3: Gating Strategy:**

Dot Plot	Explanation
 <p><b>Plot 8: 7-AAD vs SSC</b></p>	<p>This plot contains the first gate to be adjusted and is used to identify viable cells (7-AAD<sup>neg</sup>.) Adjust R8 to enclose the 7-AAD<sup>neg</sup> events. The region extends beyond the top of the plot on the y-axis, beyond the far left of the plot on the x-axis, and excludes positive-stained cells.</p> <p><b>Caution: If there are a large number of red(dead) events outside R8, see page 34 of application guide for troubleshooting information.</b></p>
 <p><b>Plot 7: 7-AAD vs SSC</b></p>	<p>This plot is gated on the Total CD34 gate (R1 and R2 and R3) and is used during compensation optimization (FL3%-%F12).</p> <p>This plot displays total CD34 cells and confirms proper adjustment of region R8 on Plot 8.</p> <p>If the instrument is appropriately set up and compensated, the gated viable CD34 cells will exhibit the same level of fluorescence as the viable lymphocytes in Plot 8.</p>
 <p><b>Plot 1: CD45 vs SSC</b></p>	<p>This plot is ungated and is used to include all CD45<sup>dim</sup> to CD45<sup>bright</sup> events and excludes debris, platelets, and unlysed red blood cells (RBCs), which are all CD45<sup>neg</sup>.</p> <p>Adjust the R1 leucocyte gate to extend above the top of the plot to include high SSC events that are CD45<sup>+</sup>. Adjust the left side of the gate to include all CD45<sup>+</sup> cells, including dim CD45<sup>+</sup> events that are CD34<sup>+</sup>. The right side can extend to the edge of the plot.</p> <p>Adjust polygon region R5 around the lymphocytes. Include only as many events as necessary to define the viable lymphocyte population displayed in Plot 6.</p> <p><b>Tip</b> Display fewer events on this plot so that the limits of the lymphocytes are easier to define.</p>

Dot Plot	Explanation
<p data-bbox="248 615 487 646"><b>Plot 2: CD34 vs SSC</b></p> 	<p data-bbox="833 237 1391 331">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.</p> <p data-bbox="833 363 1391 489"><b>NOTE</b> This region should exclude any platelets that form a streak between neutrophils and CD34+ events. See examples of this on page 3 of the application guide.</p>
<p data-bbox="248 1087 487 1119"><b>Plot 3: CD45 vs SSC</b></p> 	<p data-bbox="833 709 1391 804">This plot displays G2 (R2 and viable CD45+) cells and is used to further define the viable CD34+ CD45+ cell population.</p> <p data-bbox="833 804 1391 951">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.</p>
<p data-bbox="248 1560 487 1587"><b>Plot 6: FSC vs SSC</b></p> 	<p data-bbox="833 1150 1391 1266">This plot displays Viable Lymphs (R5 and R8) and is used to establish the minimum FSC and SSC ranges for R4.</p> <p data-bbox="833 1266 1391 1360">Display region R4 on this plot and adjust the position so that only viable lymphs from R5 are included.</p> <p data-bbox="833 1360 1391 1518">R7 is an acquisition exclusion gate used to exclude debris. Region 7 should not exceed FSC channel 200 and SSC channel 200 and should not encroach the lymphocytes population because events in R7 will be excluded from the data file.</p>



Dot Plot	Explanation
<p data-bbox="248 615 472 646"><b>Plot 4: FSC vs SSC</b></p> 	<p data-bbox="833 174 1399 331">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.</p> <p data-bbox="833 363 1399 520">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.</p> <p data-bbox="833 552 1399 636"><b>Tip</b> This plot can be gated on R1 and R2 and R3 to display Total CD34+ cells instead of Viable CD34+ cells.</p>
<p data-bbox="248 1094 613 1125"><b>Plot 5: CD45 FITC vs CD34 PE</b></p> 	<p data-bbox="833 678 1399 741">This ungated multicolor plot is used to identify beads (R6).</p> <p data-bbox="833 741 1399 835">Optional: Adjust the quadrant marker to establish a visual lower limit of CD45 expression by the CD34+ events, as in Plot 1.</p> <p data-bbox="833 867 1399 1014">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.</p>

- 8.7 Enter the CD34 results of testing into the designated fields on the Graft Characterization Form.
- 8.8 Input the required information relating the test acquisition on the Flow Cytometry Worksheet.
- 8.9 Attach the printed results to the appropriate worksheet(s) and file in the designated location.

## 9 RELATED DOCUMENTS/FORMS

- 9.1 FLOW-GEN-012 (FRM 5) Stem Cell laboratory Flow Cytometry Worksheet
- 9.2 FLOW-FORM-012 Graft Characterization

## 10 REFERENCES

- 10.1 BD CellQuest Pro Reference Manual, Part No.643274 Rev. A, November 2007
- 10.2 BD FACSCalibur Instructions for Use , Part No. 643271, Rev. A, November 2007
- 10.3 BD Stem Cell Enumeration Kit Product insert 04/2010, Catalogue no.344563.
- 10.4 BD Stem Cell Enumeration Application Guide For BD FACSCalibur Flow Cytometer. Electronic version shortcut stored on the cytometer workstation desktop and on shared network drive.

## 11 REVISION HISTORY

Revision No.	Author	Description of Change(s)
03	Melissa Reese	Corrected title of FLOW FORM-012 Section 9.

## Signature Manifest

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### FLOW-GEN-038 Enumeration of Viable CD34+ Stem Cells in Fresh... BD FACSCalibur and BD Cellquest Pro

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