STEM CELL LABORATORY (STCL)

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Flow Cytometry Staining and Analysis - 7-AAD Method

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FLOW-GEN-025
FLOW CYTOMETRY STAINING AND ANALYSIS- 7-AAD METHOD

1 PURPOSE

1.1 This procedure outlines the steps for measuring the viable CD34 content of manipulated blood and marrow products for which the BD SCE kit is not currently approved.

2 INTRODUCTION

2.1 The CD34 antigen is present on immature hematopoietic precursor cells in blood and bone marrow. It is also common practice to use mobilized peripheral blood as a source for transplant. The single-platform Stem Cell Enumeration (SCE) kit made by Becton Dickinson FDA approved for and has been validated by the STCL for use in normal and mobilized peripheral blood (PSC), fresh and thawed peripheral blood stem cells, fresh and thawed bone marrow, and fresh and thawed cord blood. However it has not been validated by for use in manipulated products (i.e. CD34 selected PSC and marrow). Alternately, this dual-platform lyse/no wash assay, validated for a previous study involving CD34 selection, may be used to determine the % of viable CD34+ cells in CD45+ population based on the ISHAGE gating strategy. This method involves using the % of CD34+ cells multiplied by the total white blood cell count obtained via hematology analyzer, the so-called 2 platform method.

3 SCOPE AND RESPONSIBILITIES

3.1 This procedure is to be used on products for which the BD Stem Cell Enumeration single platform method is not approved for use. (i.e. CD34 selection products or other manipulated products)

3.2 A minimum of 400µl of sample is needed.

3.3 The Stem Cell Laboratory Medical Directors, Manager, and Flow personnel are responsible for ensuring the requirements of this procedure are successfully met.

4 DEFINITIONS/ACRONYMS

4.1 SCE – Stem Cell Enumeration

4.2 BD – Becton Dickinson

4.3 7AAD- 7-amino actinomycin D for use in viability determination

4.4 TNC – Total nucleated cells

5 MATERIALS

5.1 PharM Lyse red cell lysing buffer 10x stock, BD Biosciences

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(Make 1x working solution by using 9 parts deionized, 0.2 micron filtered water to 1 part reagent. Store at 2-8°C for up to 2 weeks and warm to room temperature for use.)

5.2 7-amino actinomycin D (7-AAD), BD Biosciences

5.3 PBS with 1% BSA wash buffer, Gibco

5.4 Status Flow Process Control Cells, R&D Systems

5.5 Appropriate conjugated monoclonal antibodies (see panel below)

5.6 12x75 test tubes

5.7 Calibrated adjustable pipettes, 10,20,100,1000 µl volume

5.8 Pipette tips for 10-1000 µl volumes

6 EQUIPMENT

6.1 BD FACSCalibur flow cytometer or equivalent.

6.2 Vortex mixer

7 SAFETY

7.1 Wear all applicable personal protective equipment to include, but not limited to, gloves, lab coats, etc, when handling any/all potentially hazardous blood and body fluids.

8 PROCEDURE

8.1 Staining

8.1.1 Label a set of tubes for testing and a set of tubes for Status Flow positive control cells control (if not done earlier).

8.1.2 Warm the Pharm Lyse working solution to room temperature.

8.1.3 Add antibodies to the tubes according to the table below.

Tube #	FITC-FL-1	PE-FL-2	FL-3 (not needed in controls)
1	CD45 2µl	IgG1 2µl	7-AAD 15µl
2	CD45 2ul	CD34 4µl	7-AAD 15µl

8.1.4 If sample has a white blood cell of ≥ 40 million, dilute using wash buffer to a concentration below 40 million and note dilution factor on the worksheet

8.1.5 Add 50 µl of sample (or sample dilution) to the test tubes and 50 µl of control cells to the control tubes if not done with morning QC.

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8.1.6 Incubate in the dark for 15 minutes.

8.1.7 Add 450ul of Pharm Lyse working solution to the staining tubes.

8.1.8 Incubate 10 minutes in the dark at room temperature.

8.1.9 Tests sample may be stored in the dark at 2-8°C for up to 1 hour prior to acquisition, but should be acquired right away if possible.

8.1.10 Record all staining information in the appropriate places on the flow cytometry worksheet that was sent with the test sample.

8.2 Acquisition

8.2.1 Prior to acquiring test samples the flow cytometer must have passed daily quality control, compensation settings should be optimized, and the process controls must verify acceptability. Optimized lyse no-wash settings should be saved and used for this testing.

8.2.2 Find the template for CD34 Selection study acquisition in the routine test templates folder on the desktop of the flow cytometer workstation.

8.2.3 Open by double clicking on the icon.

8.2.3.1 Click on the acquire heading at the top of the page and scroll to connect to cytometer.

- The cytometer heading becomes active.
- The acquisition control box appears.
- The parameter description box opens.

8.2.3.2 Go to the parameter description box and enter the:

- Data file location -folder with date of acquisition
- Data file name-using first letter of first name, first 3 letters of last name, sample type/stage, date (i.e. MREEL1SELAMMDDYY)
- Name of operator
- Sample ID (bar code etc.)
- Patient ID (i.e. Last name, first name)

8.2.3.3 Go to the Acquire heading and scroll to counters to open counters window. (Click the green button in the counter window in order to view entire window).

8.2.3.4 Navigate to instrument settings window under cytometer heading and choose the correct lyse no wash setting.

8.2.3.5 Click **set** in instrument settings box. Quit box by clicking done.

8.2.3.6 Go to Threshold box (under Cytometer heading) and change Threshold to forward scatter (FS) if needed.

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8.2.3.7 Adjust the Threshold value as needed using the radio dial. This may be adjusted higher or lower if the debris population is more or less.

8.2.3.8 Go to the Acquisition control window and check setup mode.

8.2.3.9 Vortex Tube 1, place on sip, and click acquire in the acquisition control box.

8.2.3.10 Determine if the FS threshold should be adjusted and that the sample is correctly stained.

8.2.3.11 Once the sample is setup properly, remove the setup mode check and click acquire to begin saving data.

8.2.3.12 The template is set to acquire 300,000 events before stopping which is adequate to reach at least 100 CD34+ events in all the selection samples.

8.2.3.13 In the case of acquiring the sample from a final CD34 selection product, it is important to acquire enough events to determine the level of contamination from other cell types in the product (Leave tube on sip up to 15 minutes).

8.3 Analysis

8.3.1 Find the Viable CD34 analysis template for CD34 determinations in the Routine test template folder on the desktop.

8.3.2 Double-click to open.

8.3.3 Select all plots by pressing the cmd-A keys then press cmd-D to find data file to be analyzed.

8.3.4 Eliminate debris by adjusting Region 1 on plot 1 to eliminate events with very low forward and very low side scatter.

8.3.5 Plot 2 shows Debris-free (R1) events on a 7-AAD vs side scatter plot with most of the 7-AAD negative population below the 10^1 decade. Region 2 should be adjusted so that the dim to bright positive events are eliminated. These two gates establish the viable cell population.

8.3.6 Plot 3 displays viable cells and R5 is placed around viable CD34+ cells.

8.3.7 Plot 4 displays viable cells and R6 further characterizes CD34+ progenitor cells by their CD45/low SSC pattern while R7 is placed around lymphocytes to help identify a FSC/SSC lymphoblast gate in Plots 5 and 6.

8.3.8 In the post selection product analysis, some gate adjustments may be required due to the influence of the process on and fluorescence intensity (dim CD34+ events), and scatter properties.

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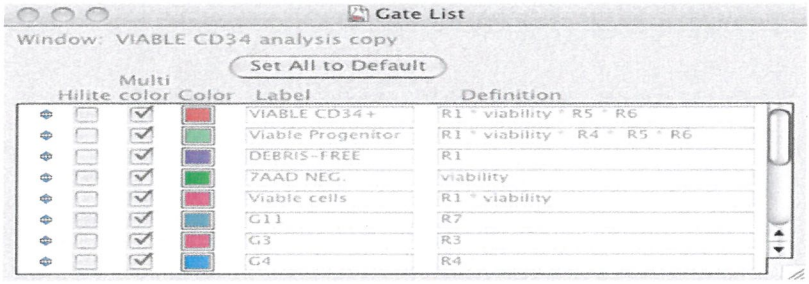
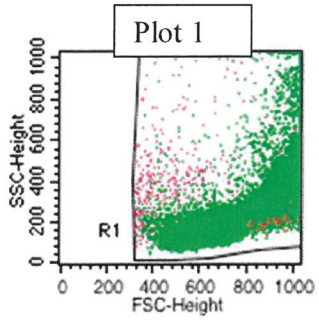
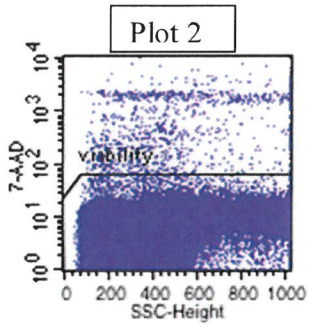
8.4 Analysis gate strategy and examples:



Pre-Selection analysis example

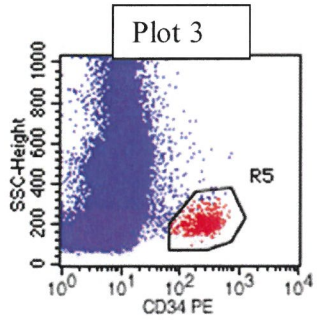


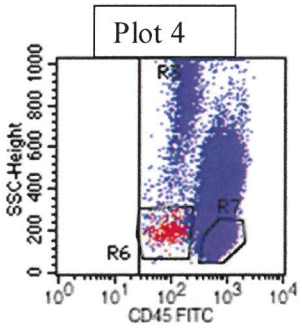


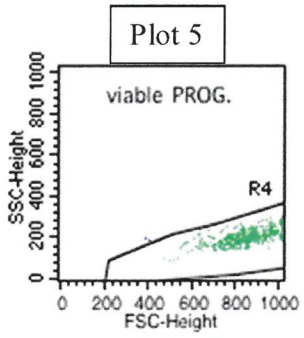
Gate: DEBRIS-FREE
Gated Events: 300973
Total Events: 306584

Region	Events	% Gated
R1	300973	100.00
viability	299784	99.60
R3	300973	100.00
R4	165103	54.86
R5	1401	0.47
R6	2622	0.87
R7	146476	48.67

R1 placement will vary based on FSC amplification and debris content





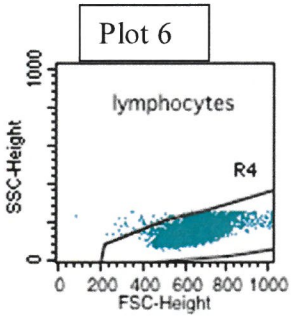


Gate: Viable cells
Gated Events: 299784
Total Events: 306584

Gate	Events	% Gated
VIABLE CD34+	1344	0.45
Viable Progenitor	1333	0.44
DEBRIS-FREE	299784	100.00
7AAD NEG.	299784	100.00
Viable cells	299784	100.00
G3	299784	100.00
G4	165000	55.04
G5	1381	0.46
G6	2553	0.85
G11	146328	48.81

Cell Viability: 99.60

% viable CD34+ of Viable CD45+: 0.44



R1= Debri free
R2= viable cells
R3 (G3)= CD45+
R4=lymph blast
R5= VIABLE CD34+
R6= CD45 DIM LOW SS
R7= LYMPHOCYTES

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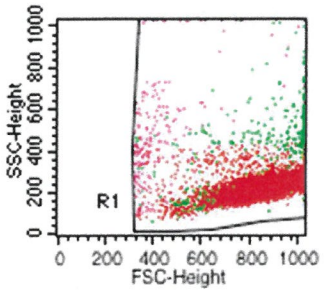
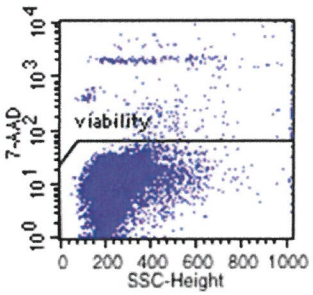
Post-Selection analysis example

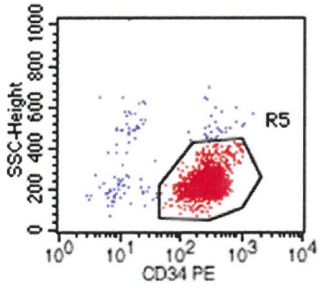


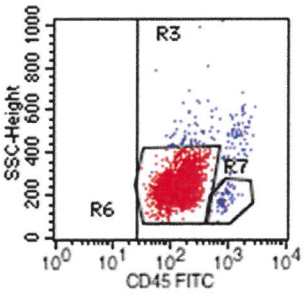
R1 placement will vary based on FSC amplification and debris content

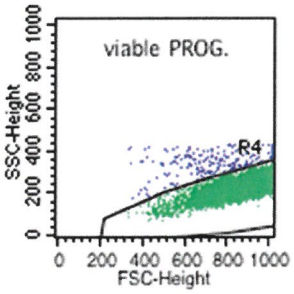


Gate: DEBRIS-FREE
Gated Events: 41583
Total Events: 53637

Region	Events	% Gated
R1	41583	100.00
viability	41171	99.01
R3	41583	100.00
R4	39461	94.90
R5	40168	96.60
R6	40198	96.67
R7	380	0.91







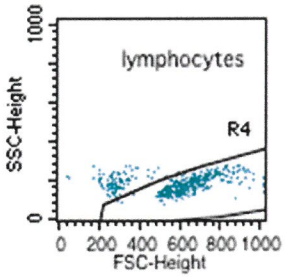
Gate: Viable cells
Gated Events: 41171
Total Events: 53637

Gate	Events	% Gated
VIABLE CD34+	39963	97.07
Viable Progenitor	38987	94.70
DEBRIS-FREE	41171	100.00
7AAD NEG.	41171	100.00
Viable cells	41171	100.00
G3	41171	100.00
G4	39420	95.75
G5	40019	97.20
G6	40048	97.27
G11	357	0.87

Cell Viability: 99.01

% viable CD34+ of Viable CD45+: 94.70

R1= Debri free
R2= viable cells
R3 (G3)= CD45+
R4=lymph blast
R5= VIABLE CD34+
R6= CD45 DIM LOW SS
R7= LYMPHOCYTES



In these examples: "Viable Progenitor" is defined by modified ISHAGE gating strategy. Viable CD34+ events have not yet been further characterized by the FSC/SSC gate.

8.5 Calculations:

8.5.1 % viable cells = 7-AAD neg. events/ (R1) events x 100

8.5.2 Total viable cells in product= TNC product x %viable

8.5.3 Absolute viable CD34 in product= % viable CD34+ progenitor cells x viable cells in the product.

8.6 If significant non-specific staining is observed in the Isotype control tube, then the background should be subtracted from the CD34%. This is not usually required.

9 RELATED DOCUMENTS/FORMS

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

9.2 FLOW-GEN-020 Stem Cell Laboratory Quality Management-Quality Control Policies For Flow Cytometry

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

9.4 FLOW-GEN-032 Fluorescence Overlap Compensation Optimization For The BD FACSCalibur Flow Cytometer

10 REFERENCES

10.1 D. Robert Sutherland, et. al. The ISHAGE Guidelines for CD34+ Cell Determination by Flow Cytometry. Journal of Hematotherapy 5:213-226(1996).

10.2 Jan W. Gratama, et. al. Flow Cytometric Enumeration of CD 34+ Hematopoietic Stem And Progenitor Cells. Semin Hematol 38:139-147 (2001)

10.3 CellQuest Pro Software Reference Manual

11 REVISION HISTORY

Revision No.	Author	Description of Change(s)
07	M. Reese	Corrected SOP titles in section 9

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Signature Manifest

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Quality

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Document Release

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Sandy Mulligan (MULLI026)		09 Oct 2020, 08:05:08 PM	Approved

Quick Approval

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Sandy Mulligan (MULLI026)		09 Oct 2020, 08:21:30 PM	Approved