⟨127⟩ FLOW CYTOMETRIC ENUMERATION OF CD34+ CELLS

INTRODUCTION

The CD34 antigen is expressed on the surface of almost all human hematopoietic stem and progenitor cells. The absolute number of CD34+ hematopoietic stem cells (HSCs) has been shown to correlate with in vitro colony-forming unit (CFU) assay activity and with clinical engraftment in hematopoietic grafts prepared from peripheral blood, bone marrow, and cord blood sources. The single-platform, flow cytometric CD34+ cell enumeration method described here is based on a clinical laboratory protocol,[1] established by the International Society of Hematotherapy and Graft Engineering (ISHAGE), now known as the International Society for Cellular Therapy (ISCT). This protocol can be used to enumerate CD34+ cells in samples of peripheral blood, leukapheresis products, bone marrow, and cord blood. CD34+ cell enumeration by flow cytometry is a rare event analysis, which requires specific gating instructions that are provided in this chapter. Furthermore, the USP CD34+ Cell Enumeration System Suitability RS has been developed to assess the reagents and ensure the correct gating during data acquisition and analysis.

IDENTIFICATION OF CD34+ HEMATOPOIETIC STEM CELLS

CD34+ cell enumeration by flow cytometry is a rare event analysis. For the analysis of CD34+ HSCs, cell samples are stained with fluorescently labeled antibodies against both the HSC antigen CD34 and the pan-leukocyte antigen CD45. Five parameters —forward light scatter (FSC), side light scatter (SSC), CD34 staining, CD45 staining, and viability dye staining are combined in a sequential, or Boolean, gating strategy to identify viable CD34+ cells.

CD34+ HSCs have FSC and SSC characteristics similar to lymphocytes; expressing both CD45 and CD34, and exhibiting dim CD45 expression and low SSC characteristics. Viability dye does not stain live cells, allowing the exclusion of dead cells from the analysis of viable cell preparations. For analysis of nonviable, fixed (preserved) cell preparations such as the USP CD34+ Cell Enumeration System Suitability Reference Standard (RS), either the flow cytometer viable cell analysis gate is fully opened to include all cells, or the *Viability dye* is omitted from the analysis.

ENUMERATION CONSIDERATIONS

The CD34+ cell enumeration method described here relies on the use of synthetic fluorescent microspheres (counting beads) as internal enumeration controls. Homogeneous counting beads are added to the cell sample at a known concentration and volume, or the counting beads may be procured, pre-aliquoted, and lyophilized in special sample tubes, in which all subsequent cell staining and processing steps are conducted. To avoid the loss of counting beads, the wash steps are omitted, and protein-containing sample buffers are used. An ammonium chloride-based, red blood cell (RBC)-lysing protocol is used for fresh cell preparations; no lysing is necessary for frozen, thawed, or fixed cell preparations.

After the cells are stained and processed, the counting beads and cells are simultaneously analyzed on a flow cytometer. The number of CD34+ cells/µL in the cell sample can be directly calculated by comparing the absolute number of target CD34+ cells and the number of counting beads detected in the same data file. The USP CD34+ Cell Enumeration System Suitability RS is used to verify that the correct reagents and flow cytometer gating parameters were used.

EQUIPMENT SPECIFICATIONS

The following equipment is needed:

- A pipettor capable of accurately reverse-pipetting microliter volumes
- A flow cytometer with the following minimum specifications (see *Flow Cytometry* ⟨1027⟩):
    - Detection capabilities for FSC; SSC; "green" fluorescence emission (range, 510–550 nm); "yellow" fluorescence emission (range, 560–590 nm); and "red" fluorescence emission (>600 nm)
    - Light-scatter and fluorescence measurements that are correlated to time
    - Light-scatter resolution allowing for the identification of lymphocytes, monocytes, granulocytes, and fluorescent counting beads
    - Detection and data acquisition rates of at least 5000 cells/s
    - A 488-nm laser excitation source
    - Fluorescence intensity sensitivity allowing for the detection of cellular autofluorescence
    - Analysis software, including a data file structure in the Flow Cytometry Standard (FCS) or equivalent, with spectral overlap correction capability

FLOW CYTOMETER INSTRUMENT SETUP AND CONSIDERATIONS

[NOTE—For general information, see ⟨1027⟩ and the flow cytometer manufacture's recommendations.]

[1] Sutherland DR, Keeney M, Gratama JW. Enumeration of CD34+ hematopoietic stem and progenitor cells. *Curr Protoc Cytom.* 2003;*6*:6.4.1–6.4.24.

Ensure that the sheath fluid receptacle is filled, the waste fluid receptacle is empty, and all caps are tightly closed. Verify that the laser power, system vacuum, and pressure are properly set.

Using fluorochrome-conjugated calibration microspheres, verify proper system alignment by measuring the average detection rate, mean fluorescence, mean FSC, mean SSC, and the respective calculated coefficient of variance (CV) values as measured by each detector. All values should fall within the ranges recommended by the manufacturer of the flow cytometer instrument. Adjust the detector threshold to ensure that all counting bead events are included.

Use either antibody-binding microspheres or preserved cells, such as the USP CD34+ Cell Enumeration System Suitability RS, to establish a matrix of detector values while adjusting for spectral overlap (compensation). Note that although either microspheres or cells can be stained with anti-CD34 phycoerythrin (PE) and anti-CD45 fluorescein isothiocyanate (FITC) antibodies, only fresh cells can be stained with *Viability dye*.

REAGENTS

Phosphate-buffered saline (PBS): 138 mM sodium chloride, 2.7 mM potassium chloride, 8 mM dibasic sodium phosphate, and 1.47 mM monobasic potassium phosphate. [NOTE—pH 7.0–7.4. If needed, adjust pH with hydrochloric acid.]

Dilution buffer: 1% (w/v) bovine serum albumin or human serum albumin in *PBS*

10X Stock ammonium chloride RBC lysis buffer: 1.5 M ammonium chloride, 0.01 M sodium bicarbonate, and 0.01 M ethylenediaminetetraacetic acid (EDTA)[2]

Diluted (1X) RBC lysis buffer: *10X Stock ammonium chloride RBC lysis buffer* diluted 1:10 with water

Anti-CD34 PE: PE-conjugated mouse IgG1 anti-human CD34 antibody (clones QBEnd10, 8G12, 581, Birma K3, or equivalent class II or class III PE-conjugated anti-CD34 antibodies), appropriately titrated

Anti-CD45 FITC: FITC-conjugated mouse IgG1 anti-human CD45 antibody (clones J33, T29/33, or HLE-1 or equivalent FITC-conjugated anti-CD45 antibodies), appropriately titrated

Viability dye: 1 µg/mL of 7-aminoactinomycin D (7-AAD) or equivalent, freshly diluted from a stock solution of 100 µg/mL[3]

Instrument calibration beads: Fluorochrome-conjugated instrument calibration microspheres[4]

Suspended counting beads: Fluorochrome-conjugated counting beads, formulated in liquid suspension[5]

Lyophilized counting bead tube: Fluorochrome-conjugated counting beads, formulated and lyophilized in a counting tube[6]

System suitability standard USP CD34+ Cell Enumeration System Suitability RS: Reconstitute the entire vial of USP CD34+ Cell Enumeration System Suitability RS with 0.5 mL of distilled water.

Cell samples: Fresh, fixed, or cryopreserved and thawed cell suspensions containing CD34+ cells, with a minimum of 100,000 nucleated cells/sample. Cell samples may include peripheral blood, leukapheresis product, cord blood, or bone marrow.

SAMPLE PREPARATION

Use reverse-pipetting techniques for all sample dilutions and transfers. Dilute *Cell samples* with *Dilution buffer* to obtain a total nucleated cell concentration of 10–30 × 10^6 cells/mL. Add *Anti-CD45 FITC* and *Anti-CD34 PE* to a 12-mm × 75-mm polystyrene tube (or a *Lyophilized counting bead tube*), followed by *Viability dye* to a final concentration of 1 µg/mL. Add 100 µL of well-mixed *Cell samples* to the bottom of the tube, and mix. Incubate for 20 min protected from light. Add 2 mL of *Diluted (1X) RBC lysis buffer* to fresh samples or add 2 mL of *Dilution buffer* to a fixed or frozen and thawed sample; vortex to mix. For fresh *Cell samples*, incubate for 10 min protected from light; for fixed or frozen then thawed *Cell samples*, no incubation time is needed. Place samples on ice. Samples should be analyzed within 1 h of lysis.

Immediately before acquiring data on the flow cytometer, pipet 100 µL of well-mixed *Suspended counting beads* (if not using *Lyophilized counting bead tubes*) to the prepared *Cell samples* described above. Cap the tube, and gently mix by inversion. Do not add counting beads to *Cell samples* intended to be used for adjustment of the instrumentation compensation matrix.

Immediately proceed to flow acquisition to collect a minimum of 75,000 CD45+ events and a minimum of 100 CD34+ cells. Acquire and analyze data by creating regions and logical gates (manually, or by using software appropriate for the flow cytometer), as described in *Table 1*.

System Suitability Requirements

Use the USP CD34+ Cell Enumeration System Suitability RS to verify that the gating strategy described below allows the detection and quantitation of CD34+ HSCs. Note that for fixed *Cell samples*, the viability gate (*R1* in *Table 1*) must be opened to include all events; alternatively, the *Viability dye* can be omitted. Calculate the number of CD34+ HSCs/µL in USP CD34+ Cell Enumeration System Suitability RS.

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[2] A suitable fixative-free RBC lysis reagent can be obtained from BD Biosciences: BD Pharm Lyse™ Lysing Buffer Catalog No. 555899, BD Ammonium Chloride Lysing Solution (10X) Catalog No 344563, or equivalent.

[3] Suitable reagents can be obtained from BD Biosciences as part of a kit, Catalog No. 344563, or BD Biosciences Catalog No. 555899, or equivalent.

[4] A suitable reagent can be obtained from BD Biosciences: Catalog No. 641319, or equivalent.

[5] Suitable reagents can be obtained from Dako: Catalog No. K2370 (in a kit) or Dako Catalog No. S2366, or equivalent.

[6] A suitable reagent can be obtained from BD Biosciences: Catalog No. 340334.

Acceptance Criteria

The results should fall within the range provided in the USP CD34+ Cell Enumeration System Suitability RS certificate.

DATA ACQUISITION AND ANALYSIS

For flow acquisition, collect a minimum of 75,000 CD45+ events and a minimum of 100 CD34+ cells. Acquire and analyze data by creating regions and logical gates (manually, or using software appropriate for the flow cytometer) as described in *Table 1*. The best results are obtained when cell and bead data (events) are displayed as bivariate dot plots. *Table 1* contains representative dot plots and gating strategies for fresh cell samples. Dot plot displays may vary depending on the cell sample, flow cytometer, and software used. Troubleshooting guidelines can be found in *Table 2*.

Table 1. Dot Plot Descriptions and Gating Instructions

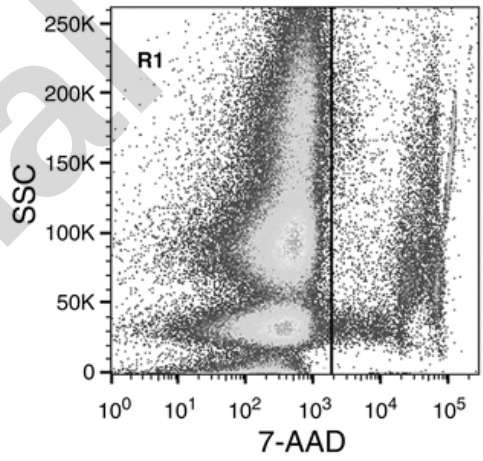
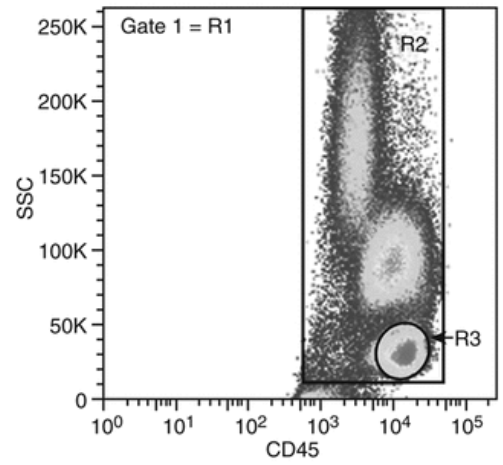
Step	Format	Purpose	Gating Instructions	Representative Dot Plots and Gating Strategies
1	SSC (linear) vs. *Viability dye* fluorescence (log)	Define viable cells	• Display all unselected events. Create region *R1* around events with little-to-no fluorescence. • Included in the region *R1*: viable cells, debris. • Excluded from the region *R1*: dead and dying cells, counting beads.	
2	SSC (linear) vs. CD45 fluorescence (log)	Define viable CD45+ leukocytes and lymphocytes	• Display *R1*-selected events. Create region *R2* around events with CD45+ fluorescence, excluding debris (i.e., events with very low SSC). Within *R2*, create region *R3* around events with high CD45+ fluorescence and low SSC. • Included in the region *R2*: viable CD45+ leukocytes. • Included in the region *R3*: viable lymphocytes. • Excluded from the region *R2*: cell debris, platelets, and unlysed RBCs. • Excluded from the region *R3*: granulocytes, monocytes, and other non-lymphocytes.	

Table 1. Dot Plot Descriptions and Gating Instructions *(continued)*

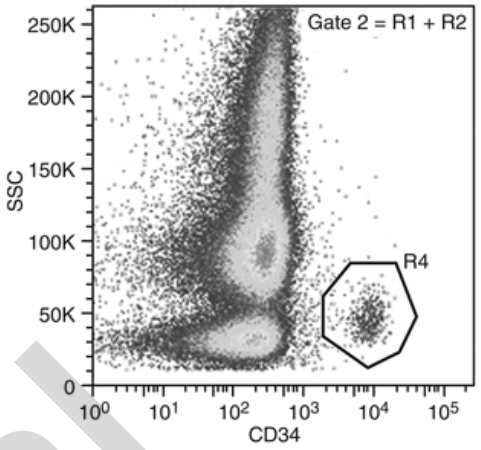
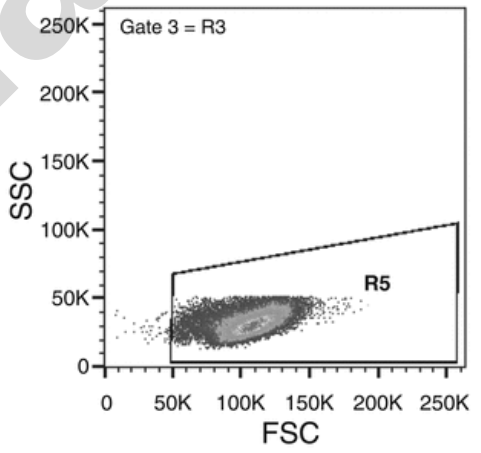
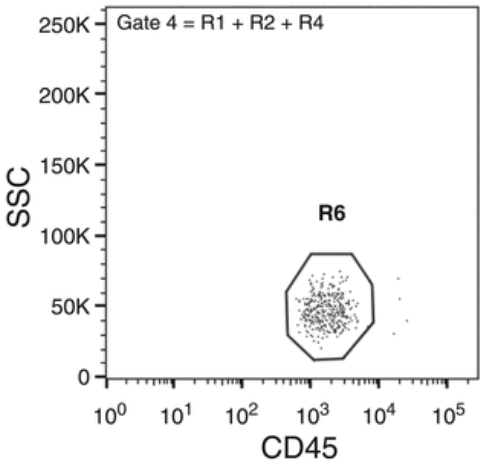
Step	Format	Purpose	Gating Instructions	Representative Dot Plots and Gating Strategies
3	SSC (linear) vs. CD34 fluorescence (log)	Define viable CD34+ cells	• Display *R2*-selected events. Create region *R4* around events with CD34+ fluorescence and low SSC. • Included in the region *R4*: viable CD34+ cells. • Excluded from the region *R4*: CD34- cells.	
4	SSC (linear) vs. FSC (linear)	Define lymphocytes and blasts	• Display *R3*-selected events. Create region *R5* to exclude debris (low SSC and low FSC). • Included in the region *R5*: viable lymphocytes and blasts. • Excluded from the region *R5*: small debris and events with high SSC values.	
5	SSC (linear) vs. CD45 fluorescence (log)	Define CD45 dim CD34+ cells	• Display *R4*-selected events. Create region *R6* around events with low-to-intermediate SSC and intermediate CD45 expression. • Included in the region *R6*: viable CD34+ HSCs. • Excluded from the region *R6*: cells that express high levels of CD45.	

Table 1. Dot Plot Descriptions and Gating Instructions *(continued)*

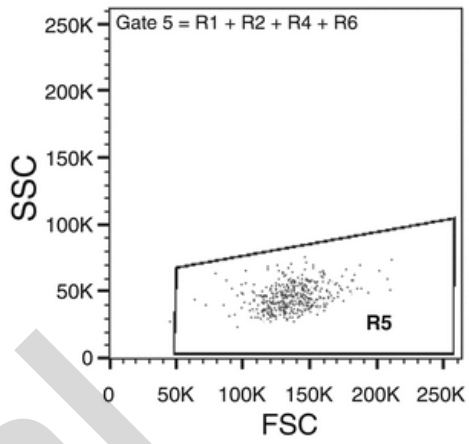
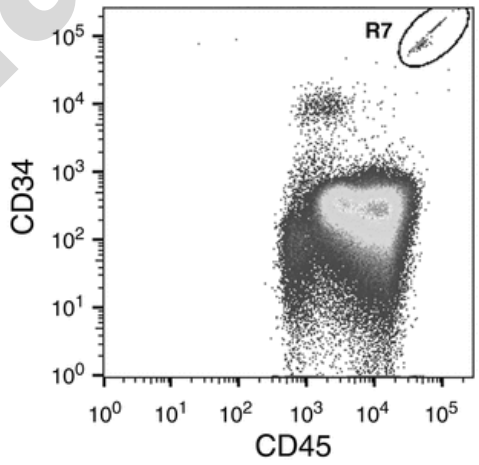
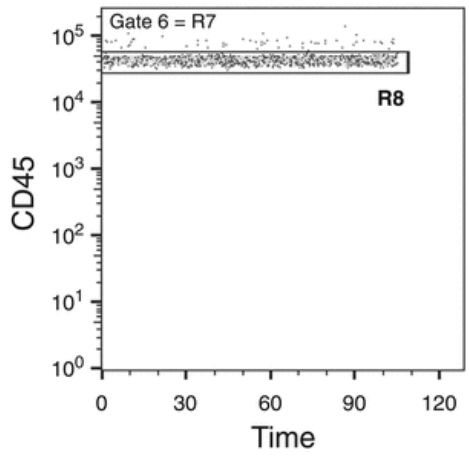
Step	Format	Purpose	Gating Instructions	Representative Dot Plots and Gating Strategies
6	SSC (linear) vs. FSC (linear)	Confirm CD34+ HSCs	• Display *R6*-selected events, and paste a copy of the *R5* gate parameters into the plot. Events in *R5* have light-scatter characteristics similar to lymphocytes and blasts, confirming the identity of the events as CD34+ HSCs. • Included in the region *R5*: confirmed viable CD34+ HSCs. • Excluded from the region *R5*: debris and other irrelevant events. • For counting bead enumeration, proceed to either Step 7 or Step 8 according to the recommendations of the bead manufacturer.	
7	CD34 fluorescence (log) vs. CD45 fluorescence (log)	Quantify total counting beads	• Display all ungated events. Create region *R7* around events with both high CD34+ and high CD45+ fluorescence. • Included in the region *R7*: total counting beads. • Excluded from the region *R7*: cells and debris.	
8	CD45 fluorescence (log) vs. time (linear)	Quantify "singlet" (e.g., unaggregated) counting beads	• When recommended by the counting bead manufacturer, display *R7*-selected events. Create *R8* around events with lower fluorescence intensity. • Included in the region *R8*: singlet counting beads. • Excluded from the region *R8*: counting bead aggregates.	

Table 2. Troubleshooting Guidelines (see also ⟨1027⟩)

Problems	Possible Reasons	Solutions	Comments
Unable to clearly define the bead gate (*R7*) in *Table 1*, graph 7 due to fluorescent debris	Degraded fresh cell sample; fixed sample; too many platelets; incomplete RBC lysis; nucleated RBCs (e.g., in cord blood)	Use a far-red fluorescence channel (>600 nm), instead of FITC, to gate counting beads.	Counting beads are highly fluorescent in all channels. Autofluorescence interference decreases dramatically at longer wavelengths (>600 nm).
Unable to find CD34+ population in *Table 1*, graph 3	Incorrect compensation settings for PE and *Viability dye*	Repeat the spectral compensation setup procedure, and adjust settings accordingly.	Make sure that counting beads are not in the compensation matrix sample, because they can interfere with compensation settings.
Unsure of gating for singlet or total beads	High levels of counting bead aggregates	Follow the counting bead manufacturer's recommendation for including or excluding aggregates.	Counting bead concentration calculations will vary by manufacturer.
Want to use a different CD34 antibody clone	Multi-parametric flow cytometry considerations	Alternative antibody clones must be carefully validated against the specified CD34 antibodies. Choose a class II or class III antibody that detects all CD34 isoforms and glycoforms.	Class I antibodies do not detect all CD34 glycoforms.
Want to use a different CD45 antibody clone	Multi-parametric flow cytometry considerations	Alternative antibody clones must be carefully validated against the specified CD45 antibodies. Choose an antibody that detects all isoforms and all glycoforms of CD45.	—

CALCULATION OF ABSOLUTE CD34 NUMBER

The absolute number of viable CD34+ cells/µL is calculated as follows:

$$\text{Viable CD34+ cells/µL} = (\text{number of CD34+ cells})/(\text{number of counting beads}) \times (\text{bead concentration} \times DF)$$

DF = sample dilution factor

The number of CD34+ cells is determined from the viable CD34+ cells (region *R5* from *Table 1*, Step 6). Depending on the recommendations of the counting bead manufacturer, the number of counting beads is determined from either the total counting beads (region *R7* from *Table 1*, graph 7) or the singlet counting beads (region *R8* from *Table 1*, graph 8). The bead concentration is specified by the counting bead manufacturer.

ADDITIONAL REQUIREMENTS

USP Reference Standards ⟨11⟩

USP CD34+ Cell Enumeration System Suitability RS