

## Application Note 1

### Progenitor Cell Subsetting

#### with ProCOUNT™ \*

#### Introduction

The CD34<sup>+</sup> cell content of leukapheresis material used for stem cell transplantation is an important predictor of engraftment and time to neutrophil and platelet recovery.<sup>1</sup> ProCOUNT provides a quick, accurate method for determining the absolute number of CD34<sup>+</sup> cells in a variety of sample types, including leukapheresis and peripheral blood.

Many investigators are interested in studying the lineage commitment of the CD34<sup>+</sup> cells. The expression of cell-surface antigens associated with particular differentiation pathways can be used to estimate the percentage of CD34<sup>+</sup> cells that will become mono-myeloid cells (CD33, CD64),<sup>2,3</sup> B lymphocytes (CD19),<sup>4</sup> erythroid cells (CD71),<sup>5</sup> and megakaryocytes (CD41, CD61).<sup>6</sup> In addition, very immature CD34<sup>+</sup> stem cells that lack the CD38 antigen are thought to be responsible for long-term bone marrow reconstituting capability.<sup>7</sup>

The ProCOUNT™ reagents can be combined with APC<sup>‡</sup>-conjugated monoclonal antibodies against lineage-associated antigens for a more detailed analysis of the CD34<sup>+</sup> population. This application note describes using the FACSCalibur™ four-color option to excite APC-conjugated monoclonal antibodies added to the standard ProCOUNT reagents.

\* This product is not intended and has not been evaluated for use in diagnostic procedures. It is for research use only.

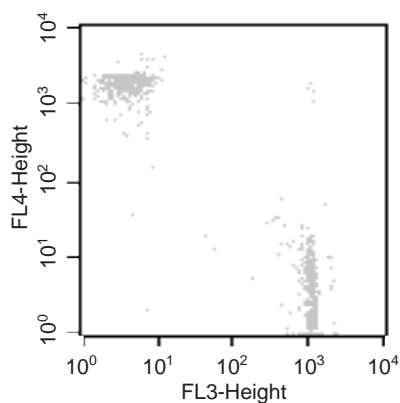
† US Patent Nos. 4,714,680; 4,965,204; and 5,035,994.

‡ US Patent No. 4,520,110; European Patent No. 76,695; Canadian Patent No. 1,179,942.

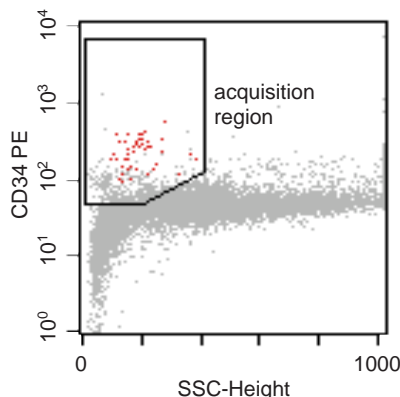
**Note:** Bottled concentrations of the APC-conjugated antibodies can be found on their vial labels. You may wish to confirm the appropriate titer for the sample type or cell concentration used in your experiment.

<b>Tube 1:</b> ProCOUNT CD34 Reagent + CD38 APC
<b>Tube 2:</b> ProCOUNT CD34 Reagent + CD33 APC
<b>Tube 3:</b> ProCOUNT CD34 Reagent + IgG <sub>1</sub> APC

**Table 1** Summary of stained samples.



**Figure 1** Correct FL4-%FL3 and FL3-%FL4 compensation.



**Figure 2** Acquisition gate around CD34<sup>+</sup>, low side scatter events (red).

## Materials and Methods

### Cells

Leukapheresis material was obtained from a clinical center (patient treated with chemotherapeutics and/or growth factors) and adjusted with 1X PBS/2% FBS/0.1% NaN<sub>3</sub> to a final cell concentration of approximately 1.5 x 10<sup>7</sup> cells/mL.

### Staining

CD38 and CD33 APC conjugates (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA) were previously titrated at 50 ng per test when used in a lyse/no-wash protocol. The antibodies were diluted to a concentration of 50 ng/5 μL in 1X PBS/0.2% gelatin/0.1% NaN<sub>3</sub>. For analysis of progenitor cell lineage-associated antigens, 50 μL of leukapheresis sample was stained with 20 μL of ProCOUNT CD34 reagent and 5 μL of CD38 APC or 5 μL of CD33 APC for 15 minutes at room temperature. An isotype control sample for the lineage marker antibodies was prepared by staining 50 μL of leukapheresis with 20 μL of ProCOUNT CD34 reagent and 5 μL of IgG<sub>1</sub> APC isotype control antibody (BDIS). Red blood cells were lysed by adding 450 μL of 1X FACS™ Lysing Solution (BDIS) to each tube and incubating for 30 minutes at room temperature. All samples were prepared in standard 12 x 75-mm polystyrene tubes.

For lyse/no-wash assays, optimum antibody concentration is often determined by maximum separation of positive and negative populations rather than by maximum staining level. At antibody concentrations required for maximum staining, there is frequently a considerable increase in the signal from negative cells that is caused by unbound antibody in the sample stream rather than by antibody bound to the cell. Because of this, it is extremely important that the isotype control matches the experimental antibody in concentration and fluorochrome-to-protein ratio. Different isotype tubes may be necessary if the experimental antibodies must be used at different concentrations.

### Flow Cytometry: Instrument Setup

A FACSCalibur flow cytometer equipped with the FL4 option was optimized manually using BDIS CaliBRITE™ beads (including PerCP\* and APC beads). The FL4 PMT voltage and FL3-%FL4 compensation were set using APC beads. Correct FL4-%FL3 and FL3-%FL4 compensation is shown in Figure 1.

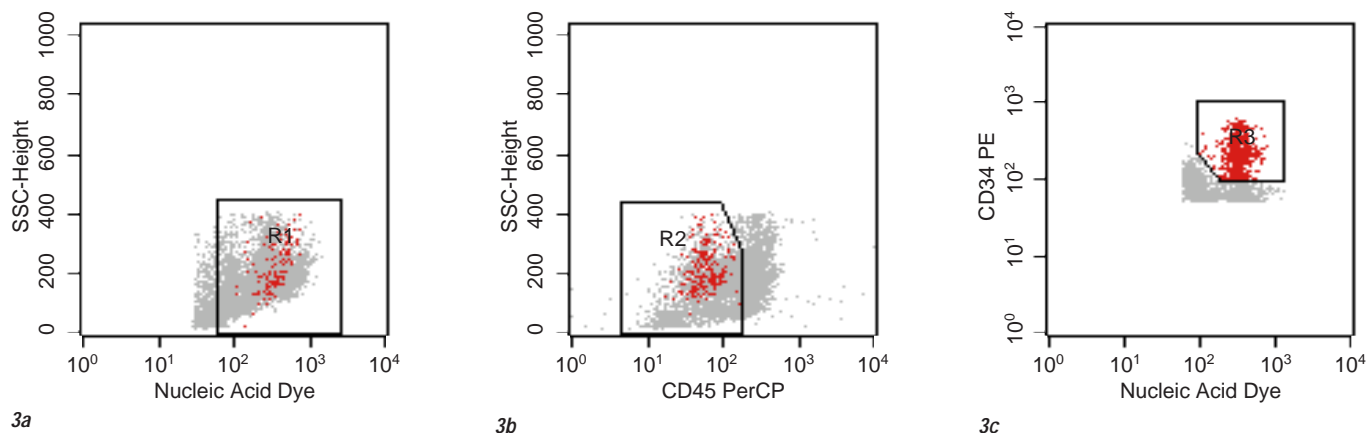
### Flow Cytometry: Sample Acquisition

Samples stained with the ProCOUNT CD34 reagent in combination with CD38 APC, CD33 APC, or IgG<sub>1</sub> APC were acquired using a live gate around the CD34<sup>+</sup>, low side scatter population (Figure 2). This ensures that all CD34<sup>+</sup> cells are collected in the data file, while minimizing the number of high side scatter events. At least 2,000 events were collected through the gate for each acquisition. A 20,000-event ungated file was also acquired from each tube. This allows the staining of the CD34<sup>+</sup> cells to be compared to the staining of known negative and positive cell populations within the four-color samples. In all cases, the acquisition threshold was set on the ProCOUNT green fluorescent nucleic acid dye.

\* US Patent No. 4,876,190.

## Results

The CD34<sup>+</sup> cells in the 2,000-event gated data files were identified using the ProCOUNT analysis method. Three plots were used: Nucleic Acid Dye vs SSC, CD45 PerCP vs SSC, and Nucleic Acid Dye vs CD34 PE<sup>+</sup> (Figure 3). Region R1 was drawn to include cells stained with the nucleic acid dye and exclude dye-dim debris (Figure 3a). Region R2 was drawn to select for CD45<sup>dim</sup> events and to exclude monocytes (Figure 3b). Regions R1 and R2 were then combined into an analysis gate (G1 = R1 and R2) which was applied to the Nucleic Acid Dye vs CD34 PE plot (Figure 3c). Region R3, around the bright CD34<sup>+</sup> cells, could then be easily drawn in this dot plot.



**Figure 3** Identification of CD34<sup>+</sup> cells. The file was gated during acquisition to exclude high side scatter events. For analysis, regions were drawn around the nucleic acid dye positive (R1), CD45<sup>dim</sup> (R2), and CD34<sup>bright</sup> (R3) cells. Only cells satisfying all three regions (R1 and R2 and R3) were considered to be true CD34<sup>+</sup> progenitor cells and are shown in red in each plot.

Once the CD34<sup>+</sup> progenitor cells had been definitively identified in the 2,000-event gated data files, lineage marker expression was analyzed using a multistep process.

First, the general level of nonspecific staining was determined using the ungated data file from the sample stained with ProCOUNT and IgG<sub>1</sub> APC (Figure 4, Step 1). The positive staining threshold was set so that no more than 2% of the events fell into the positive region.

This analysis threshold could then be applied to the ungated data files from the samples stained with ProCOUNT and CD38 APC or ProCOUNT and CD33 APC (Figure 4, Step 2). Finally, the same analysis threshold was applied to the CD34<sup>+</sup> cells identified in the corresponding gated data files (Figure 4, Step 3). This allows the level of staining of the CD34<sup>+</sup> cells with the APC-conjugated lineage markers (Step 3) to be directly compared with both isotype control staining (Step 1) and staining of the more mature cell populations in the leukapheresis sample (Step 2). A second leukapheresis sample is analyzed in Figure 5. The arrows in Figure 4 and Figure 5 indicate the progression of events in the analysis method. In all plots, positive events are shown in green and negative events in gray.

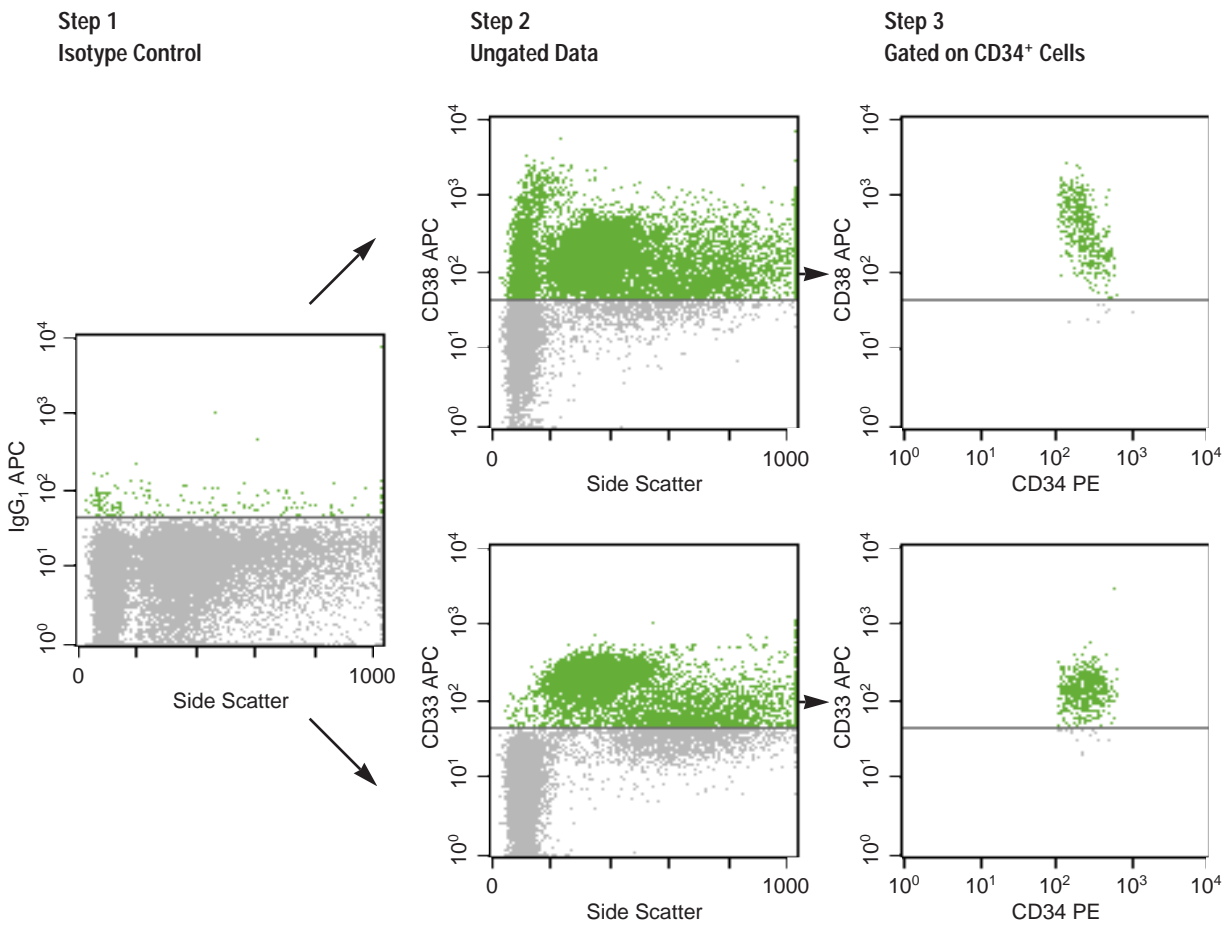
The majority of CD34<sup>+</sup> cells in both leukapheresis samples coexpress CD38 and are lineage committed. However, while over 95% of the CD34<sup>+</sup> cells in the first leukapheresis sample express the myeloid antigen CD33, only approximately 25% of the CD34<sup>+</sup> cells in the second sample are positive for this antigen. Results are shown in Table 2.

<b>Leukapheresis Sample #1:</b>
CD34 <sup>+</sup> CD38 <sup>+</sup> = 98.5%
CD34 <sup>+</sup> CD33 <sup>+</sup> = 97.2%
<b>Leukapheresis Sample #2:</b>
CD34 <sup>+</sup> CD38 <sup>+</sup> = 91.5%
CD34 <sup>+</sup> CD33 <sup>+</sup> = 22.9%

**Discussion and Summary**

The ProCOUNT reagents were combined with APC-conjugated monoclonal antibodies for a more detailed analysis of the CD34<sup>+</sup> progenitor cell population. The fourth fluorescence parameter allows for the measurement of progenitor cell lineage-associated antigens while maintaining the multiparameter classification of progenitor cells provided by the ProCOUNT reagents.

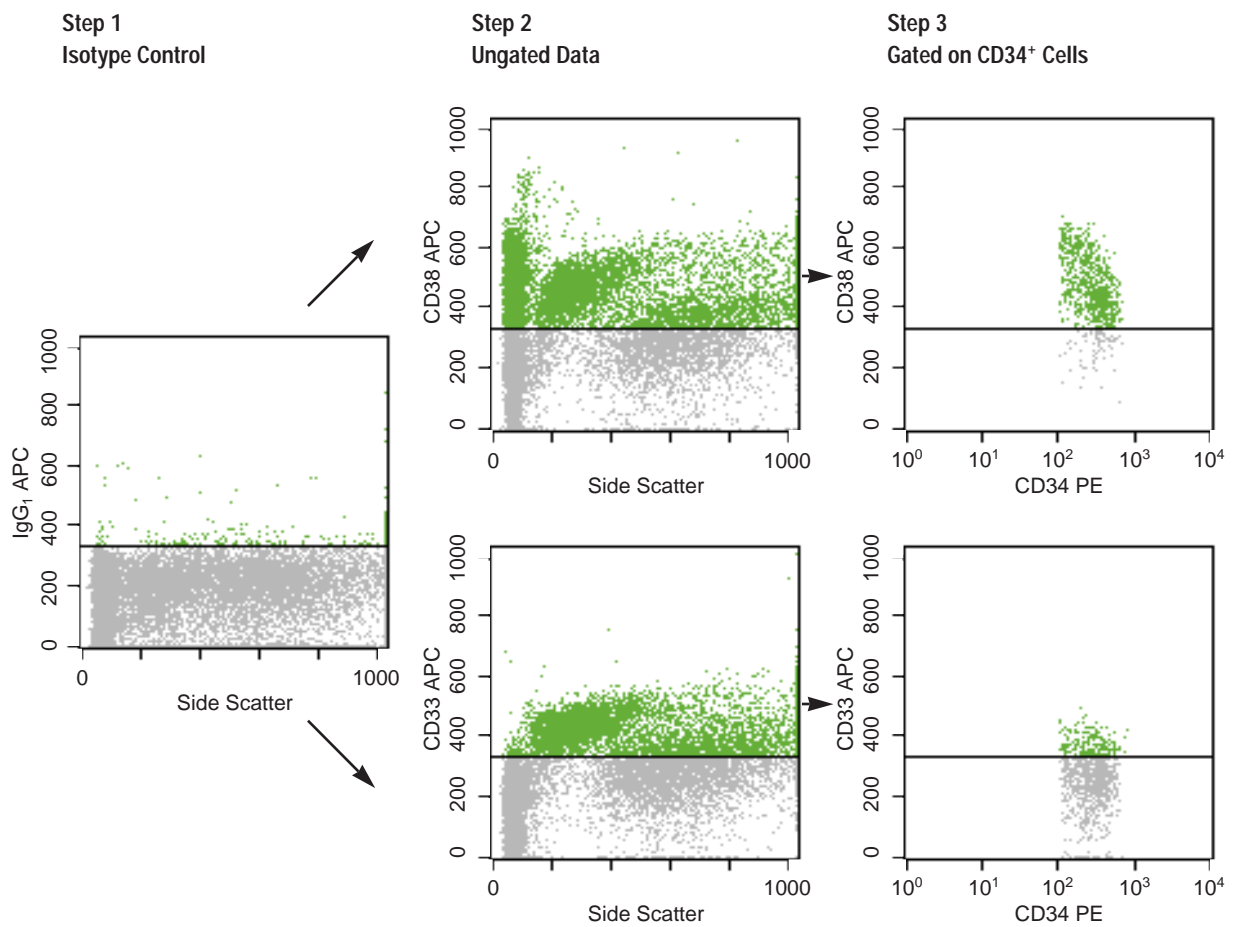
*Table 2 Percentages of CD34<sup>+</sup> cells coexpressing CD38 or CD33 in the two leukapheresis samples analyzed.*



**Figure 4** Analysis method for examining CD38 and CD33 expression on CD34<sup>+</sup> progenitor cells. The positive staining threshold was set on the isotype control sample such that no more than 2% of the events fell into the positive region. In all plots, positive events are shown in green and negative events in gray.

## Hints

- Fresh samples provide the best results with the subsetting monoclonal antibodies.
- If absolute counts of CD34<sup>+</sup> cells and/or their subsets are desired, stain samples in TRUCOUNT™ Absolute Count Tubes.
- Although ProCOUNT CD34 reagent was used in all subsetting sample tubes, the analysis method described also allows for the IgG<sub>1</sub> APC to be added to the ProCOUNT Control tube.



**Figure 5** Examining the expression of CD38 and CD33 on CD34<sup>+</sup> progenitor cells in a second leukapheresis sample.

*BDIS publishes this method*

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*as a service to investigators.*

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*Detailed support for non-flow*

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*cytometric aspects of this*

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*procedure may not be*

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*available from BDIS.*

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## References

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