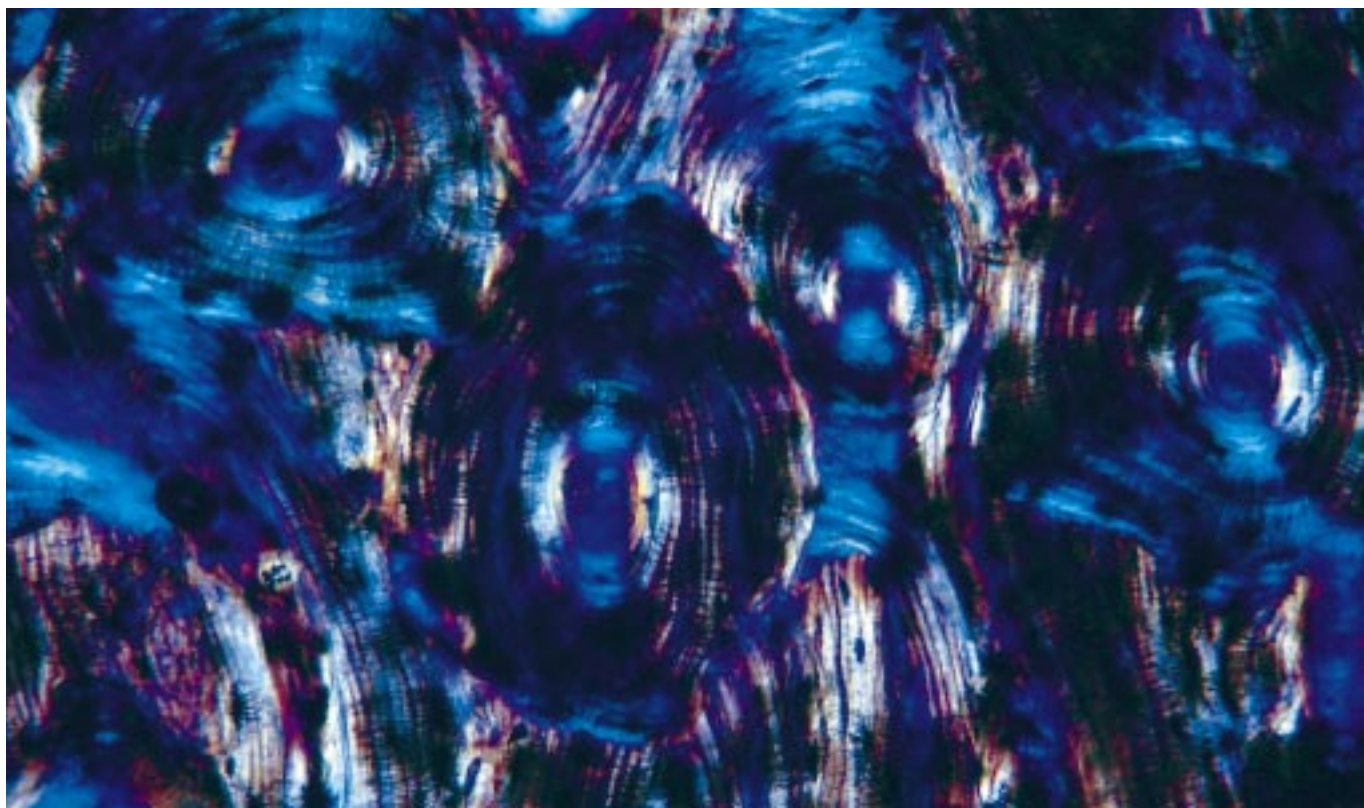


# ProCOUNT™

Setting the Standard for  
Progenitor Cell Enumeration

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Cross section of human bone (50x magnification)



## Acknowledgments

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# Progenitor Cell Enumeration in Transplant Studies

## Introduction

Transplantation of hematopoietic progenitor cells obtained from the bone marrow of a patient or HLA-matched donor is used increasingly in the treatment of blood disorders, malignancies, and genetic abnormalities. When used in treating hematologic malignancies, stem cell transplants enable physicians to destroy diseased bone marrow using high-dose chemo/radiotherapy and replace it with healthy progenitor cells that repopulate the marrow and produce normal blood cells. In addition, patients with solid tumors can be rescued from the lethal hematologic toxicity caused by high-dose chemotherapy with stem cell infusions. Recent studies indicate that progenitor cell therapy, used in conjunction with high-dose chemotherapy, results in clinically relevant improvements in the long-term survival rates among patients with certain types of cancer.<sup>1,2,3,4</sup> Stem cell transplantation is under study as a treatment for other non-malignant hematopoietic diseases such as hemoglobinopathies, immunodeficiencies, and aplastic anemia.<sup>5,6</sup>

## Historical Approaches

Bone marrow was initially the primary source of stem cells for transplantation. A bone marrow transplant procedure requires anesthesia, an operating room, and usually a short hospital stay. With the arrival of mobilization regimes (G-CSF, GM-CSF, and chemotherapy), peripheral blood has become the preferred source of stem cells. Several publications show that peripheral blood stem cell (PBSC) transplants are less traumatic to the patient, and they shorten the time to engraftment and provide adequate numbers of stem cells for either autologous or allogeneic transplants.<sup>3,4,7,8,9</sup>

In early studies, physicians relied on nucleated cell counts to quantitate the number of hematopoietic progenitor cells infused into patients. Colony forming unit (CFU) assays allowed for crude estimations of specific primitive cell populations. The variability and length of time to obtain results for the CFU assay (14 days) precludes its clinical usefulness as an assay for quantitation and identification of progenitor cells.

## CD34

In the mid-1980s, a cell surface molecule was discovered and later cluster designated as CD34. This molecule is present on immature hematopoietic cells and all hematopoietic colony-forming cells in bone marrow and peripheral blood, including the unipotent and pluripotent progenitor cells.<sup>1</sup> The relative frequency of the CD34<sup>+</sup> cell population is 1% to 4% of the mononuclear cells in normal bone marrow, and less than 0.1% in normal peripheral blood.<sup>1,10,11</sup>

An accurate measure of the CD34 cell count, also referred to as the absolute count, is necessary for dose requirement protocols in stem cell transplantation. Quantitating the CD34<sup>+</sup> cell population can also be useful during mobilization and during or after leukapheresis procedures. One can determine

whether mobilization has been successful and when is the best time to begin leukapheresis by measuring blood levels. The total number of cells collected during a leukapheresis session can also be estimated, indicating whether or not more stem cells will be required for a transplantation dose.

Several studies have investigated the relationship between the number of CD34<sup>+</sup> cells infused and the time to engraftment.<sup>2,3,4,7</sup> The relative dose of CD34<sup>+</sup> cells transplanted into a patient is determined by the total number of CD34<sup>+</sup> cells infused per kilogram body weight. The threshold dose value is estimated to be 2 to 5 x 10<sup>6</sup> CD34<sup>+</sup> cells/kg.<sup>7,8,9</sup>

### Flow Cytometry

Fluorochrome-conjugated monoclonal antibodies directed against the CD34\* molecule can be used to identify the CD34<sup>+</sup> cells using flow cytometry. Flow cytometric applications used for CD34<sup>+</sup> cell identification and enumeration provide a rapid, quantitative and reproducible method to evaluate the progenitor cell population.

Significant site-to-site variation has been observed with the current flow cytometric methods used to determine the percent and absolute numbers of CD34<sup>+</sup> cells.<sup>12</sup> This variability can be attributed to different sample preparation techniques, reagents, and analysis methods. Some groups have attempted to standardize the methodologies, in particular the Milan protocol,<sup>13,15</sup> and ISHAGE protocol.<sup>11</sup> Results from studies using current methods show a high degree of site-to-site variability in the assessment of the CD34<sup>+</sup> percentages<sup>13,14,15,16</sup> and absolute counts.<sup>16</sup> CD34 analysis methods clearly remain a limiting factor in determining the true number of progenitor cells required for successful engraftment. Therefore, a robust standardized approach to CD34<sup>+</sup> or progenitor cell enumeration will further the research in this rapidly developing field.

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## Assay Design Criteria

Certain critical parameters must be considered in the design of an accurate and robust flow cytometric assay for identifying and enumerating the infrequent CD34<sup>+</sup> cell population. These parameters include: variability associated with the hematology analyzers, sample pipetting, sample processing, and cell identification. The ProCOUNT™ system addresses these parameters in order to obtain an accurate assessment of the CD34<sup>+</sup> or progenitor cell population as summarized in Table 1. A comparison of ProCOUNT's advantages to other published CD34 enumeration methods are summarized in Table 2.

Table 1. Critical Factors for Progenitor Cell Enumeration

Critical Factor	ProCOUNT Solution	Result
<i>Absolute Count Variability</i>	Use of an internal reference particle (TRUCOUNT™) for determining absolute cell counts	Eliminates site-to-site variability due to hematology counter. Eliminates variability associated with using multiple instruments to determine a single answer.
<i>Sample Pipetting</i>	Accurate and precise sample addition using a reverse pipetting technique	Eliminates intra-site variability resulting from pipetting errors of the sample.
<i>Sample Processing Cell Loss</i>	Use of a no-wash technique	Eliminates cell loss that may occur during cell washing steps.
<i>Accurate Identification of the CD34<sup>+</sup> Cells</i>	Use of a nucleic acid dye as the threshold reagent Standardized gating strategy	Clear resolution of the CD34 <sup>+</sup> cells from instrument noise and debris. The CD45 <sup>dim</sup> progenitor cells are not eliminated. Consistency in data analysis.

Table 2. Comparison of ProCOUNT to Other Assay Methods

Feature	ProCOUNT	ISHAGE	MILAN
<i>CD34, class III (protease-resistant) antibody, phycoerytherin (PE) conjugated</i>	•	•	• *
<i>Isotype (IgG<sub>1</sub>) control reagent</i>	•	•	•
<i>Whole blood lyse technique</i>	•	•	•
<i>Threshold reagent, ie nucleic acid dye</i>	•		
<i>No-wash technique</i>	•		
<i>Preformulated, titered reagents</i>	•		
<i>Direct absolute count determinations (elimination of the hematology counter)</i>	•		

\*A FITC-conjugated CD34 may also be used.

## PIPETTING VARIATION

When pipetting material such as blood or a leukapheresis sample, the precision and accuracy of usual pipetting techniques is less than that estimated at the time of pipette calibration. This phenomenon occurs because the viscosity of the sample (blood) is greater than the material used to calibrate the pipette (water). This translates to variable and artificially low amounts of sample being added.

Use of reverse pipetting reduces the variability associated with the amount of sample being added since the technique reduces the viscosity dependency associated with conventional pipetting techniques.

To understand the relative magnitude of this problem, a pipetting study was performed. Five operators used a single water-calibrated pipette. Multiple replicates (50  $\mu$ L, n = 20) of a whole blood sample were measured for each pipetting technique and are summarized in the following table.

Comparison of Pipetting Techniques (5 Persons)

	Average Dispensed Weight	Pooled CV	Volume*
<i>Conventional Pipetting</i>	47.3 mg (45.0 - 48.2)	2.91% (1.75 - 4.41%)	43.8 $\mu$ L (41.7 - 44.6)
<i>Reverse Pipetting</i>	54.0 mg (52.6 - 57.0)	1.49% (1.37 - 1.67%)	50.0 $\mu$ L (48.7 - 52.8)

\*Assumes the density of whole blood is 1.08. Ranges shown in parentheses.

Use of the reverse pipetting technique significantly reduces the variability in the amount of sample delivered. It is for this reason that Becton Dickinson highly recommends the reverse pipetting technique and preferably an automated pipettor for absolute counting applications.

## Absolute Count Variability

Currently, many laboratories determine absolute counts of a cell subset by multiplying the percentage of cells obtained using flow cytometry by the WBC count obtained from a hematology analyzer. Studies have reported that this methodology produces consistent results within an institution, but variability between sites remains high and can be attributed to the variability introduced by the hematology analyzers.<sup>17,18</sup> Determination of accurate and reproducible absolute counts in any flow cytometric assay requires incorporating a volumetric measurement into the assay. The ProCOUNT assay incorporates TRUCOUNT Absolute Count Tubes to volumetrically determine the absolute cell count, thereby eliminating any variability associated with the hematology-derived absolute counts. This approach uses a known number of reference particles per microliter of sample. The TRUCOUNT method allows for the determination of absolute cell counts independent of sample volume acquired on the flow cytometer.

## Sample Pipetting

Accurately and precisely dispensing the sample is critical to the assay accuracy and precision when a lyse-no-wash method is used. Reverse pipetting technique, preferably with an automated pipettor, results in a reproducible addition of sample volume (see side bar).

## Sample Processing Cell Loss

Maintaining sample integrity with respect to sample content, ie, avoiding any loss of cells, mandates that minimal sample manipulation is employed. Any potential cell loss caused by cell washing contributes to assay variability and should be avoided. A lyse-no-wash sample staining technique eliminates the potential for cell loss and simplifies the sample staining procedure.

## Accurate Identification of CD34<sup>+</sup> Cells

### Thresholding

It is critical in rare-event analysis to be able to discriminate the target from background noise or cellular debris.<sup>19</sup> Progenitor cells stain dimly with CD45, therefore use of this antibody as the threshold discriminator would exclude a portion of the cells for analysis. Use of a nucleic acid dye, as in the ProCOUNT kit, reliably defines the threshold that is both well resolved and independent from the classification parameter, ie, scatter- and antibody-related fluorescence. This ensures that all cells (CD45<sup>dim</sup>/CD34<sup>+</sup> cells) are included in the analysis. Figure 1 shows the ambiguity of using CD45 as the threshold parameter versus using a nucleic acid dye on various sample types in a lyse-no-wash format.

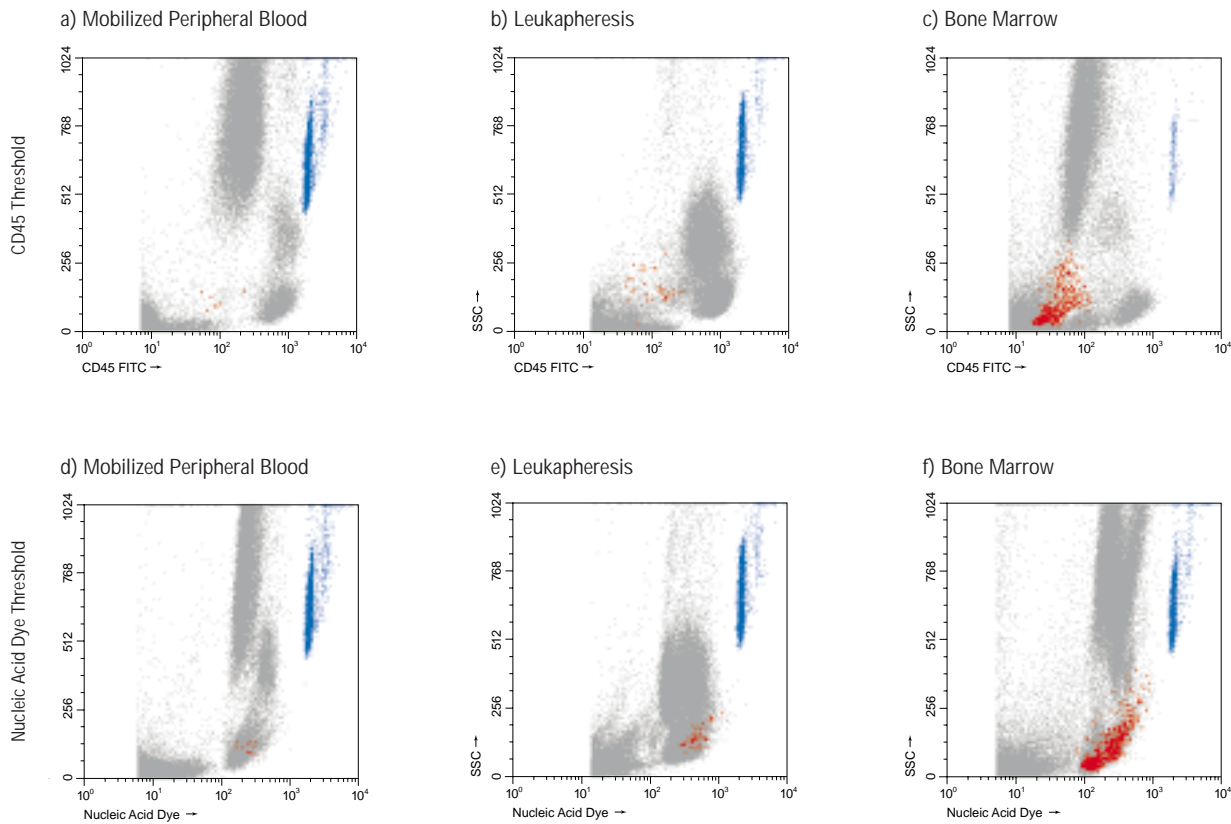


Figure 1. Comparison of a CD45 Threshold vs Nucleic Acid Dye Threshold on Various Sample Types

### Resolution of Progenitor Cells

Selecting the appropriate monoclonal antibody and fluorophore combination is a key component of accurate stem cell identification. The ProCOUNT kit uses a class III CD34 antibody, Anti-HPCA-2 conjugated to phycoerythrin\* (PE), the highest signal-to-noise fluorophore. The combination of Anti-HPCA-2 conjugated to PE enables reliable identification of the progenitor cells in samples where the enrichment process required a protease treatment. Progenitor cells have other distinct features, in addition to CD34 expression, that are used to facilitate their identification and enumeration by flow cytometry. Table 3 describes some of these salient features.

When analyzing the data collected by the flow cytometer, it is important to use a consistent and standardized gating strategy that incorporates both inclusion and exclusion criteria. Currently, ProCOUNT utilizes a manual gating strategy based on the flow cytometric characteristics of progenitor cells as shown in Table 3. Development of an automated software algorithm similar to the manual gating strategy is in progress. This automated analysis software will further reduce data analysis variability.

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

Table 3. Flow Cytometric Characteristics of Progenitor Cells Used by ProCOUNT

Cell Features	Flow Cytometric Characteristics of Progenitor Cells
<i>CD34 expression</i>	Dim to strong reactivity with CD34 (Anti-HPCA-2).
<i>CD45 expression</i>	Dim to negative reactivity with CD45 (Anti-HLe-1).
<i>DNA/RNA content, nucleic acid dye uptake</i>	Relatively bright staining because of the large amount of DNA/RNA present in the progenitor cells.
<i>Size (forward scatter)</i>	Progenitor cells are 8 to 10 $\mu\text{m}$ in diameter, and are co-located in the same forward scatter space as large granular lymphocytes. They may extend into the monocyte region.
<i>Granularity (side scatter)</i>	Progenitor cells have low side scatter, similar to lymphocytes and large granular lymphocytes.

## Overview of ProCOUNT Progenitor Cell Enumeration Kit

The ProCOUNT Progenitor Cell Enumeration kit is designed as a two-step, lyse-no-wash assay. The 50-test kit consists of a CD34 reagent, an isotype control reagent and TRUCOUNT Absolute Count Tubes to determine the absolute cell counts volumetrically. The ProCOUNT analysis strategy fulfills the need for a consistent and standardized data analysis method. The ProCOUNT kit is intended for identifying and enumerating CD34<sup>+</sup> cells. CD34<sup>+</sup> cells may be present in peripheral blood, leukapheresis, bone marrow, cord blood, and enriched samples.\* As illustrated in Figure 2, ProCOUNT is easy to use and requires minimal sample handling or processing.

### ProCOUNT Assay Components

CD34 Reagent	Control Reagent
<i>Nucleic acid dye</i>	Nucleic acid dye
<i>CD34 (HPCA-2) PE</i>	Concentration matched IgG <sub>1</sub> PE
<i>CD45 PerCP</i>	CD45 PerCP
<i>TruCount tube</i>	TRUCOUNT tube

\*ProCOUNT is under US regulatory review for use with peripheral blood, mobilized peripheral blood, and leukapheresis products.

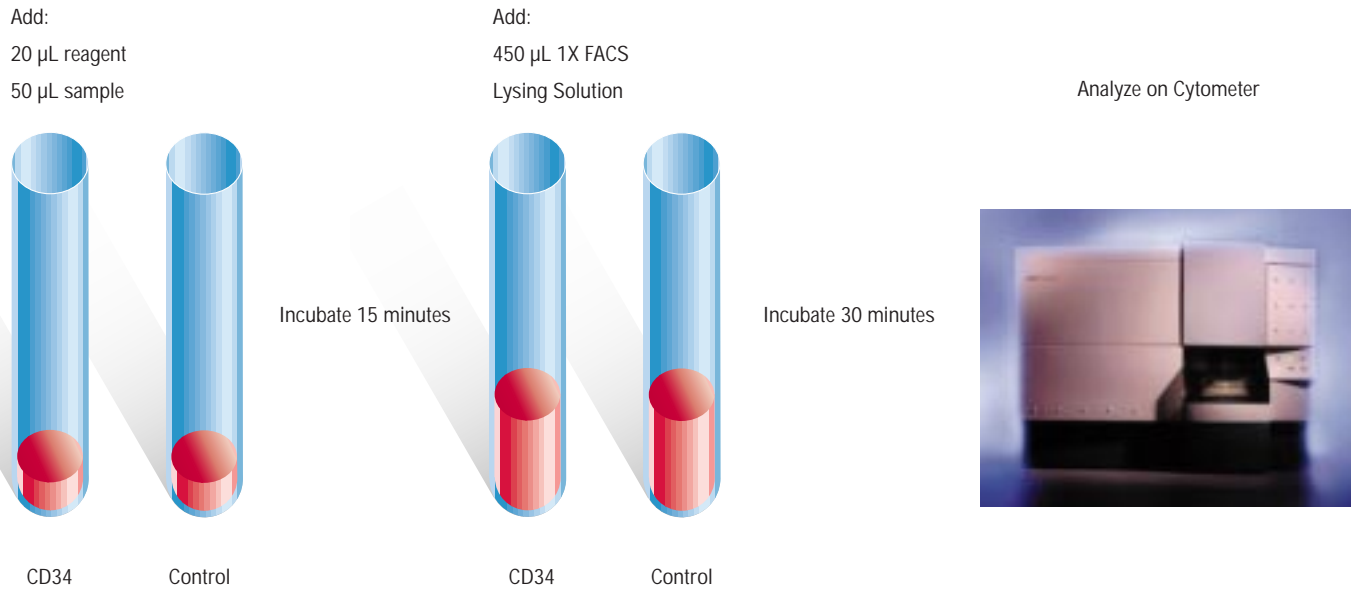


Figure 2. ProCOUNT Assay Methodology

**Nucleic Acid Dye** — The DNA/RNA dye allows for inclusion of all intact nucleated cells such as leucocytes and nucleated red blood cells in subsequent analytical steps. Non-nucleated events (cellular debris, mature erythrocytes, and platelets) are excluded by this reagent component.

**CD34 PE** — The CD34 monoclonal antibody, clone 8G12, recognizes a class III (protease-resistant) epitope of the human progenitor cell antigen (HPCA). The CD34 antigen is present on immature hematopoietic precursor cells and all hematopoietic colony-forming cells in bone marrow and blood, including unipotent and pluripotent progenitors.<sup>1</sup>

**IgG<sub>1</sub> PE** — The antibody, used as the isotype control, is specific for keyhole limpet hemocyanin, an antigen not expressed on human cells or human cell lines. IgG<sub>1</sub> conjugated to PE is used as a concentration-matched isotype-specific negative control to evaluate nonspecific binding and to alert the user to any sample-associated debris that might occur in the CD34 enumeration region.

**CD45 PerCP\*** — CD45 (Anti-HLe-1) is an antibody specific for the human leucocyte antigen. It is present on all leucocytes but is expressed at distinctly lower levels on hematopoietic progenitor cells. This parameter, in conjunction with the side scatter parameter, assists in discriminating the CD34<sup>+</sup> cells from lymphocytes, monocytes, granulocytes, dying cells, cell debris, platelet clumps, and nucleated erythrocytes.

\* US Patent No. 4,876,19.

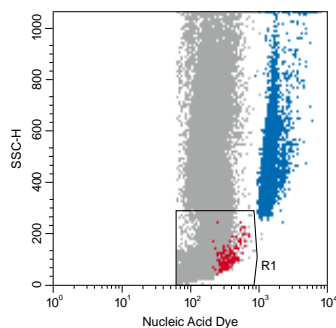
TRUCOUNT Tubes — The TRUCOUNT Absolute Count Tubes contain a lyophilized pellet of a known number of fluorescent beads. The beads provide an internal reference through which an absolute cell count is obtained. The pellet is contained in the bottom of the tube by a stainless steel retainer.

The reagents provided in the ProCOUNT kit combined with a consistent and standardized gating strategy provide an accurate method for identifying and enumerating CD34<sup>+</sup> progenitor cells in a variety of different sample types.

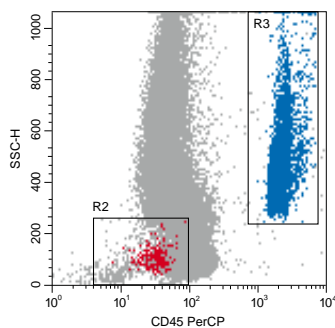
#### Data Analysis

Due to the relative infrequency of the CD34 cells, and their flow cytometric similarity with other cell populations, a multiparameter gating strategy using established progenitor cell characteristics is required. Combination or logical gates are used to effectively eliminate unwanted clusters of cells such as granulocytes, monocytes, and debris. The analysis strategy utilizes the granularity (SSC), nucleic acid dye expression (FL1), CD45 expression (FL3) and antigen expression of the CD34 (FL2) cells to aid in distinguishing them from other cells. Figure 3 shows the multiparameter gating strategy that is used with the ProCOUNT kit. The first two steps (3a and 3b) identify the location of the progenitor cells by removing any cellular debris (FL1, nucleic acid dye) and eliminates any events that stain brightly with CD45 (FL3). The use of side scatter (SSC) in Figures 3a and 3b removes any events that have high SSC which is not a characteristic of progenitor cells. Figure 3c shows only those events that are contained in regions R1 and R2 (and the bead region R3). This results in a clear separation of the progenitor cells from other events in the FL1 (nucleic acid dye) vs. FL2 (CD34) dot plot. Drawing regions around the progenitor cells and the beads completes the steps necessary to calculate the absolute count of progenitor cells. A detailed description of the gating strategy is in the ProCOUNT Progenitor Cell Enumeration Kit package insert.

3a



3b



3c

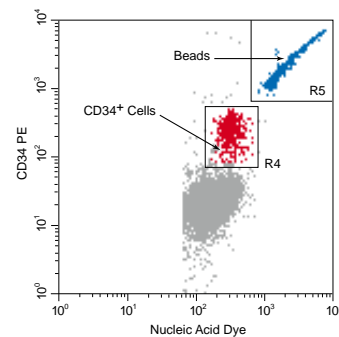


Figure 3. ProCOUNT Multiparameter Data Analysis Gating Strategy

## Performance

Understanding performance characteristics and boundaries associated with any assay are critical to providing meaningful results. To assess the performance of the ProCOUNT assay, several studies were conducted to determine its accuracy, linearity, precision, and interlaboratory reproducibility.

### Accuracy

A CD34<sup>+</sup> cell-spiking experiment was performed to assess the accuracy of the ProCOUNT kit. A known number of CD34<sup>+</sup> selected progenitor cells were added to normal peripheral blood to obtain samples that contained a wide range of CD34<sup>+</sup> cells/ $\mu$ L. ProCOUNT was then used to determine the number of absolute CD34<sup>+</sup> cells in each sample. The results are shown in Figure 4.

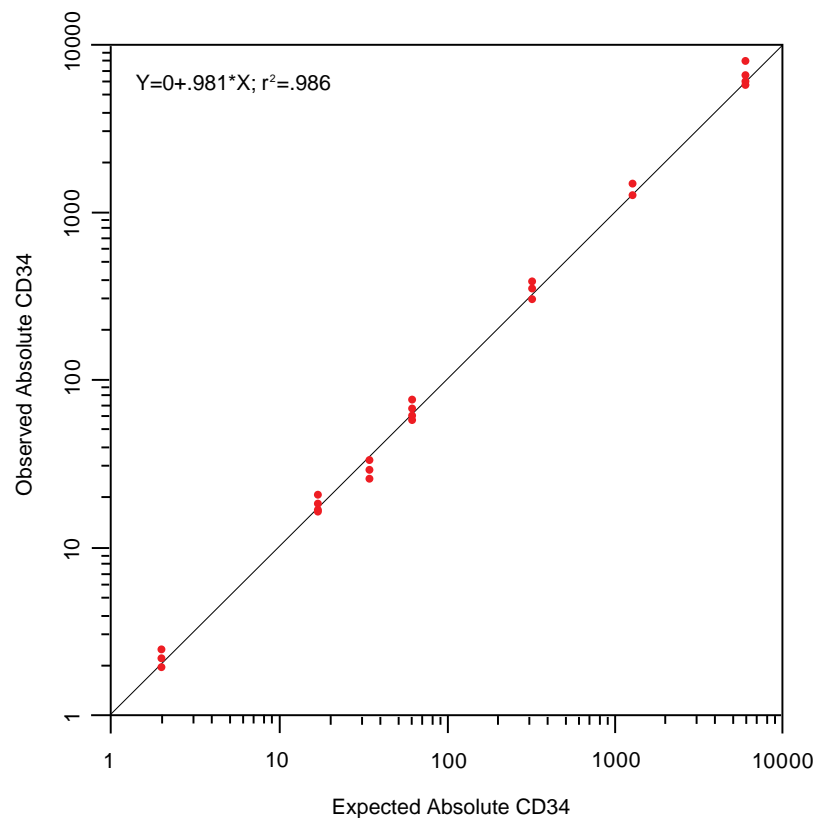


Figure 4. CD34<sup>+</sup> Cell Spiking: Observed versus Expected Absolute CD34<sup>+</sup> Cells/ $\mu$ L.

#### Regression performed using a no-intercept model.

NOTE: In this experiment the lowest point represents a normal peripheral blood. The highest point is a calculated point based upon a four-fold dilution of the stock prior to analysis. It is important to note that the slope obtained is directly linked to the absolute count of the original spiked-in material. Use of an automated hematology instrument to count the spiked material may yield slopes in the range of 0.8 to 1.2, depending upon how pure the original material was and the calibration of the hematology counter.

To compare the accuracy of ProCOUNT with other methodologies, the spiking model system described above was performed using KG-1a and CD34<sup>+</sup> selected cells and processed using ProCOUNT and the ISHAGE method.<sup>11</sup> The observed CD34<sup>+</sup> cell counts for each model and method are presented in Figure 5 and Figure 6.

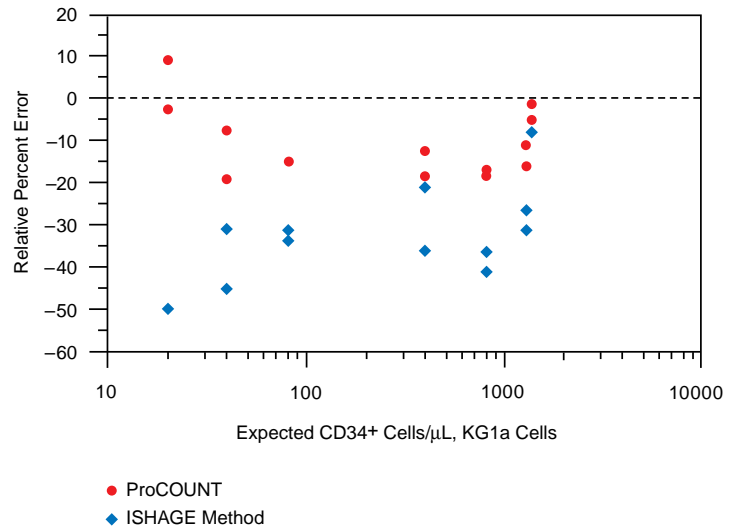


Figure 5. Accuracy Evaluation—Recovery of KG-1a Cells. Expressed as a Relative Percent Error vs. the Expected Absolute CD34<sup>+</sup> Cell Count.

In the KG-1a model system, using the ISHAGE method, a lower count was observed compared to both ProCOUNT and the expected value. To verify that this was not due to differences intrinsic to the cell line, a CD34<sup>+</sup> selected cell preparation was substituted for the KG-1a cell line and the experiment repeated.

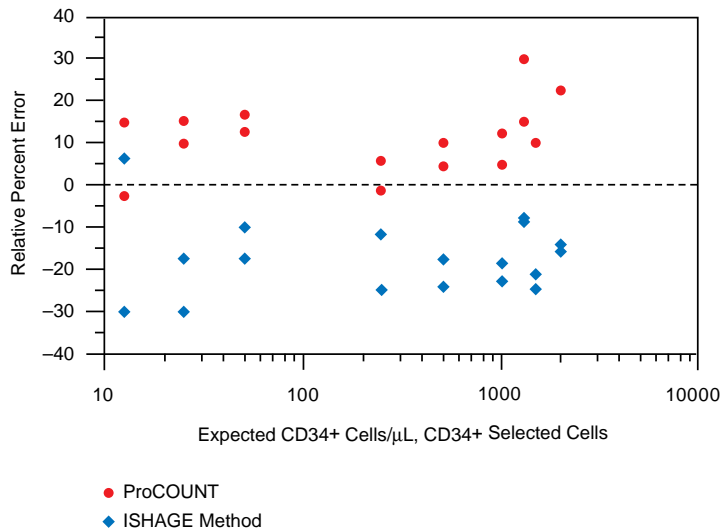


Figure 6. Accuracy Evaluation—Recovery of CD34<sup>+</sup> Selected Cells. Expressed as a Relative Percent Error vs. the Expected Absolute CD34<sup>+</sup> Cell Count.

Figure 5 confirms that the lower absolute CD34 counts observed with the ISHAGE method is independent of cell type. Several factors may account for the systematic bias with the ISHAGE method compared to ProCOUNT or the expected values. These include cell loss during the washing or lysis step, systematic exclusion or inclusion of cells during analysis, and variability introduced from the hematology analyzer used to calculate the absolute counts.

### Linearity

To evaluate assay linearity in a model system, enriched CD34<sup>+</sup> selected progenitor cells were diluted in normal whole blood to obtain an evenly distributed range (0%, 25%, 50%, 75% and 100% of original stock, using four replicates at each dilution level). The linearity of the ProCOUNT system is demonstrated in Figure 7.

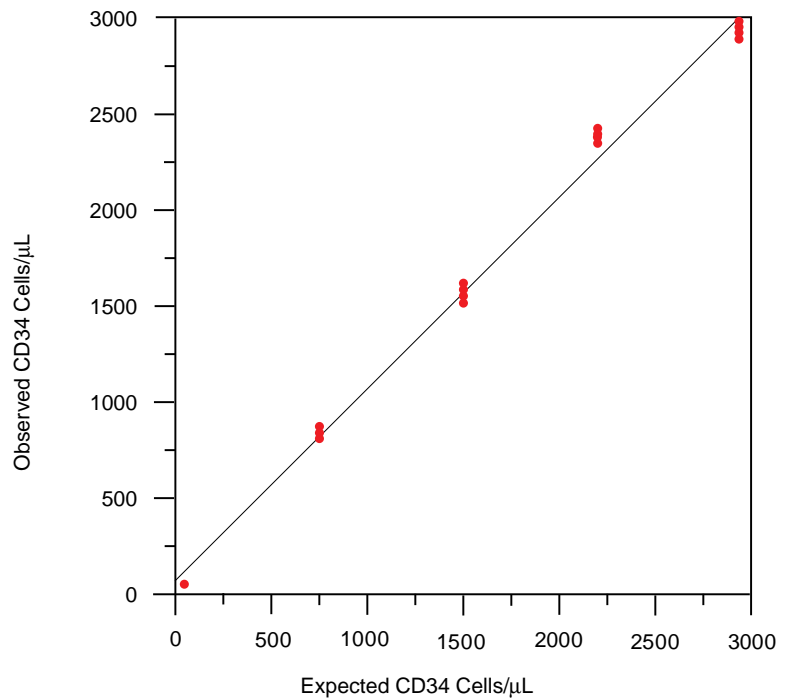


Figure 7. CD34<sup>+</sup> Linearity Study Using ProCOUNT

The ProCOUNT assay exhibits a linear response over a wide dynamic range (0 to 2,000 cells/μL).

### Precision and Inter-laboratory Reproducibility

Good intra- and inter-laboratory reproducibility is essential for valid comparison of data from multiple sites. Most methodologies currently used for progenitor cell enumeration have been observed to have a high amount of variability across sites.<sup>12,13,14,16</sup>

## RELATIVE FREQUENCY AND COUNT STATISTICS IN RARE EVENT ASSAYS

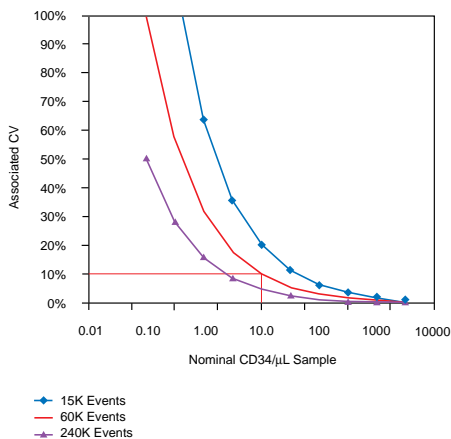
The lower bound on the precision (expressed as coefficient of variation) of any counting system is determined by the number of events counted. The fewer events counted, the higher the coefficient of variation (CV). This relationship can be expressed by the formula:

$$CV = \frac{SD}{Mean} = \frac{\sqrt{\#CD34^+ \text{ events}}}{\#CD34^+ \text{ events}} = \frac{1}{\sqrt{\#CD34^+ \text{ events}}}$$

In most counting assays the errors introduced by these counting statistics are negligible compared to other errors. When counting 1,000 events, the imprecision introduced by counting is only  $1/\sqrt{1000}$  or 3.2%.

The figure below shows the CV as a function of the absolute CD34 count for total events collected; 15,000, 60,000 and 240,000 and assuming a nucleated cell count of 5,000 cells/ $\mu$ L and 1,000 beads/ $\mu$ L.

The CV is lower when the concentration of CD34 cells is higher, and when more total events are collected. The decrease in CV going from 15,000 total events to 60,000 total events is substantial. The decrease in CV going from 60,000 total events to 240,000 total events is less dramatic. Therefore, collecting 60,000 total events is a compromise, which provides adequate precision in the range of the assay.



### Theoretical Counting Statistics Limitations

Minimum expected coefficients of variation associated with varying concentrations of CD34 cells as a function of list-mode data file size.

## Intra-laboratory Precision

Intra-laboratory precision was demonstrated with ProCOUNT at three sites, with three to four samples at each site, and nine to twelve replicates for each sample. Samples include normal peripheral blood, mobilized peripheral blood, and leukapheresis. The results were pooled into three subgroups based upon their CD34 counts, and the results are summarized in Table 4.

Table 4. Intra-Laboratory (Stain to Stain) Precision of ProCOUNT at Three Sites

### Absolute CD34 Counts

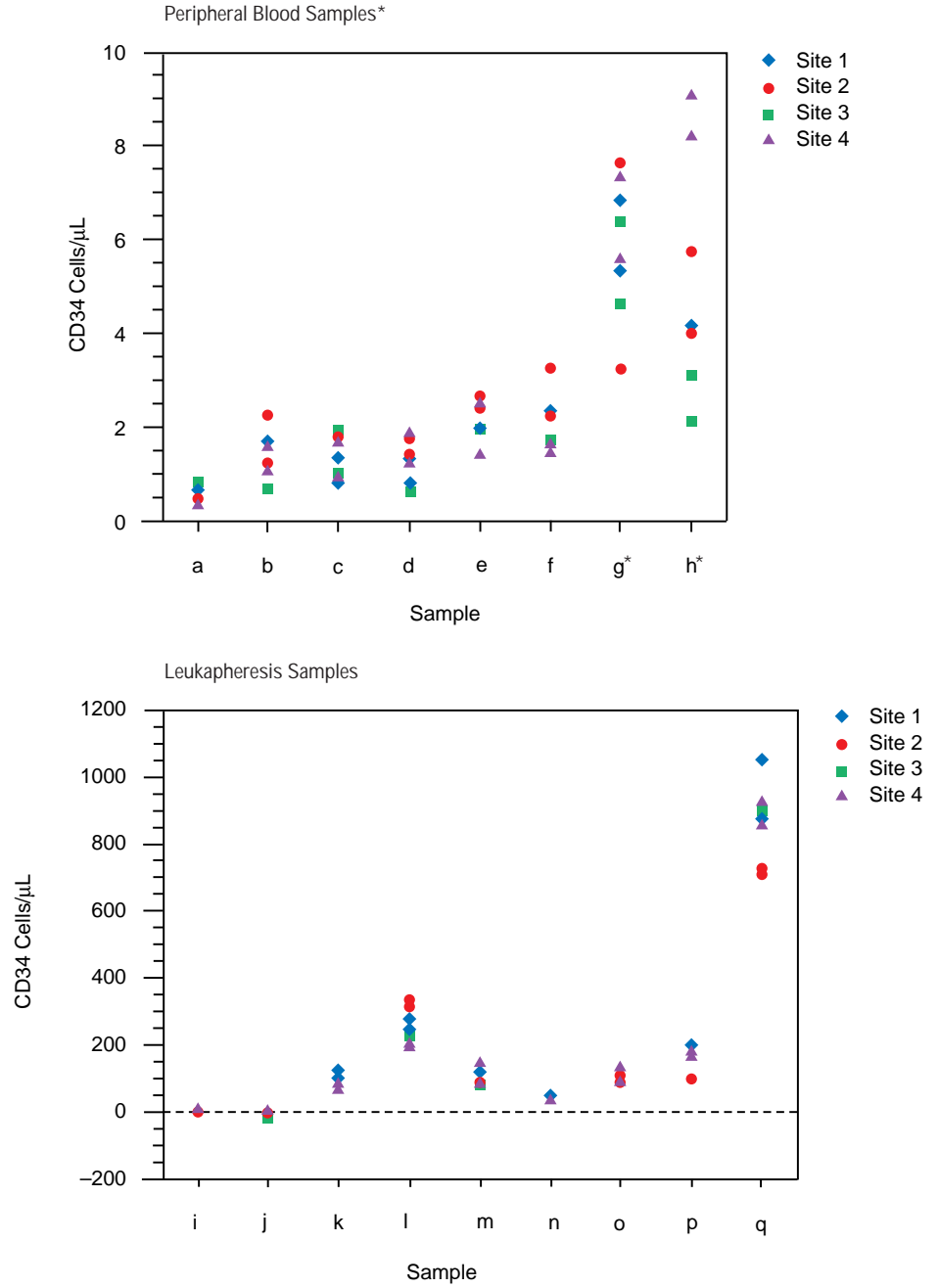
CD34 Cells/ $\mu$ L	Mean	CV% (observed)	CV% (90% confidence Limits)
$\leq 5$	3.8/ $\mu$ L	13%	16%
$>5$ and $\leq 200$	117/ $\mu$ L	8%	10%
$>200$	332/ $\mu$ L	6%	8%

### Percent CD34

% CD34 Cells/CD45	Mean	CV% (observed)	CV% (90% confidence Limits)
$\leq 5$ CD34 cells/ $\mu$ L	0.07%	14%	16%
$>5$ and $\leq 200$ CD34 cells/ $\mu$ L	0.38%	7%	9%
$>200$ CD34 cells/ $\mu$ L	1.4%	5%	5%

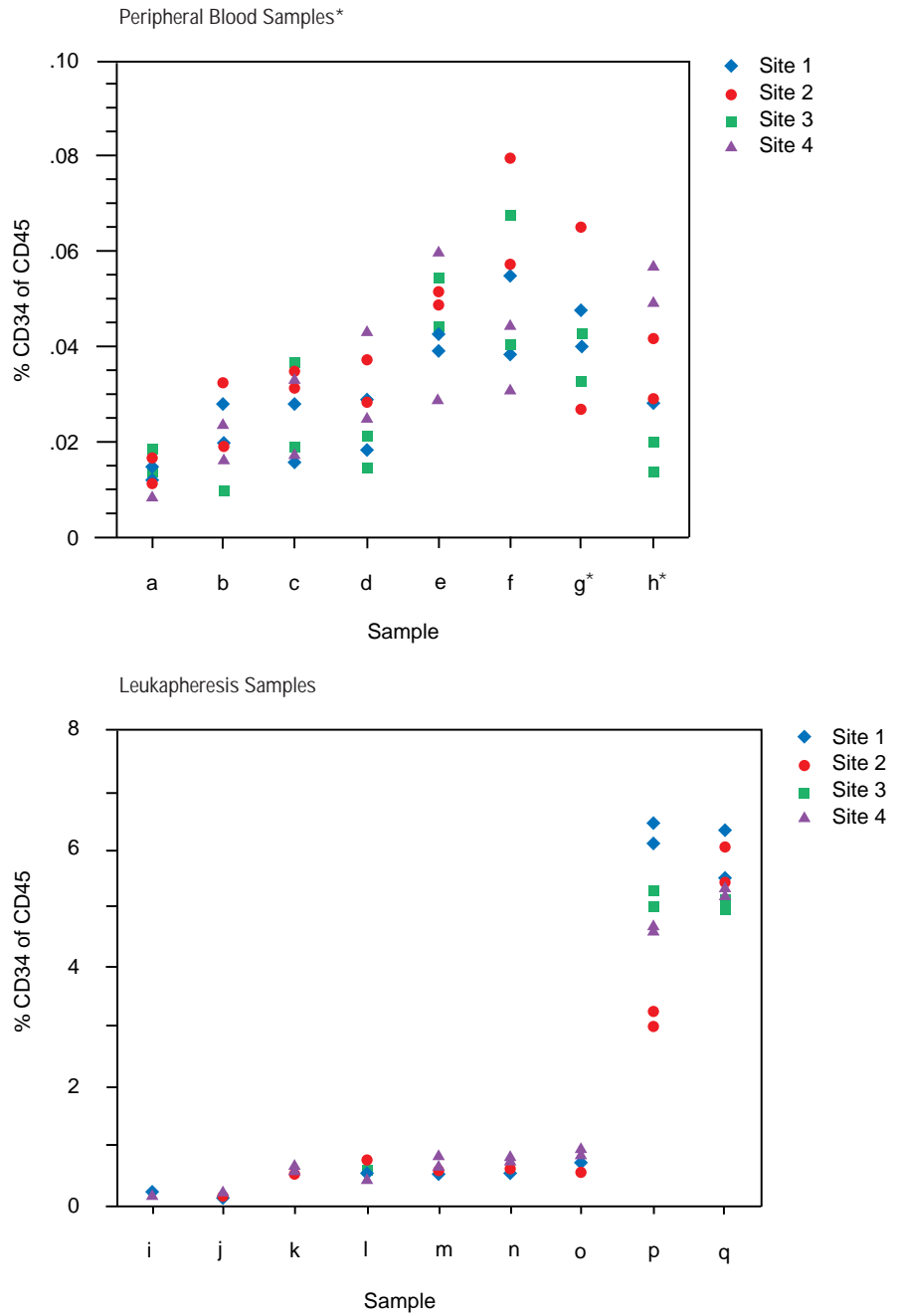
## Inter-laboratory Precision

To estimate the site-to-site reproducibility of ProCOUNT, 17 peripheral blood and leukapheresis samples were evaluated at four sites. Each site prepared and analyzed the samples, which were up to 48 hours old, using ProCOUNT.<sup>20</sup> The variability for each sample for CD34 cells/ $\mu$ L and percent CD34 are shown in Figures 8 and 9.



\* Samples g and h are mobilized peripheral blood samples.

Figure 8. Inter-laboratory Reproducibility of ProCOUNT, CD34 Cells/μL, in Peripheral Blood Samples and Leukapheresis Samples



\* Samples g and h are mobilized peripheral blood samples.

Figure 9. Inter-laboratory Reproducibility of ProCOUNT, Percent CD34, in Peripheral Blood Samples and Leukapheresis Samples

Several studies have been published on multisite reproducibility of CD34 percents<sup>12,13,14,15,16</sup> and absolute counts.<sup>16</sup> The inter-laboratory results from this multicenter study using ProCOUNT can be compared to results from other studies. For all studies reported, samples were transported and analyzed independently by each of the participating clinical sites. The various multicenter studies are summarized in Table 5.

Table 5. Multicenter Studies on Inter-laboratory Reproducibility of CD34 Percents and Absolute Counts

Multicenter Study	# Sites	# Samples	Method	Reported Results
<i>North American</i> <sup>4</sup>	10*	21 <sup>†</sup>	various	CD34%
<i>Nordic Group WSIF</i> <sup>5</sup>	24	12	Milan	CD34%
<i>United Kingdom</i> <sup>6</sup>	15 <sup>†</sup>	28	various	CD34% and cells/ $\mu$ L
<i>ProCOUNT Study</i>	4	17 <sup>§</sup>	ProCOUNT	CD34% and cells/ $\mu$ L

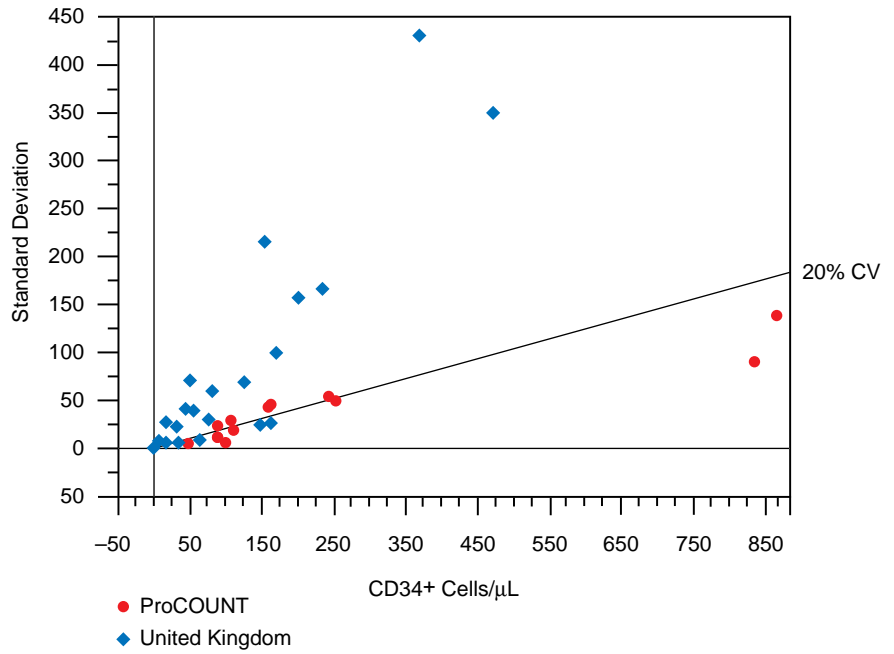
\* One site reported four results using four different methods.

<sup>†</sup> Sites were clustered in groups of five, so each sample was analyzed by five sites, not all 15.

<sup>‡</sup> Five samples were duplicates.

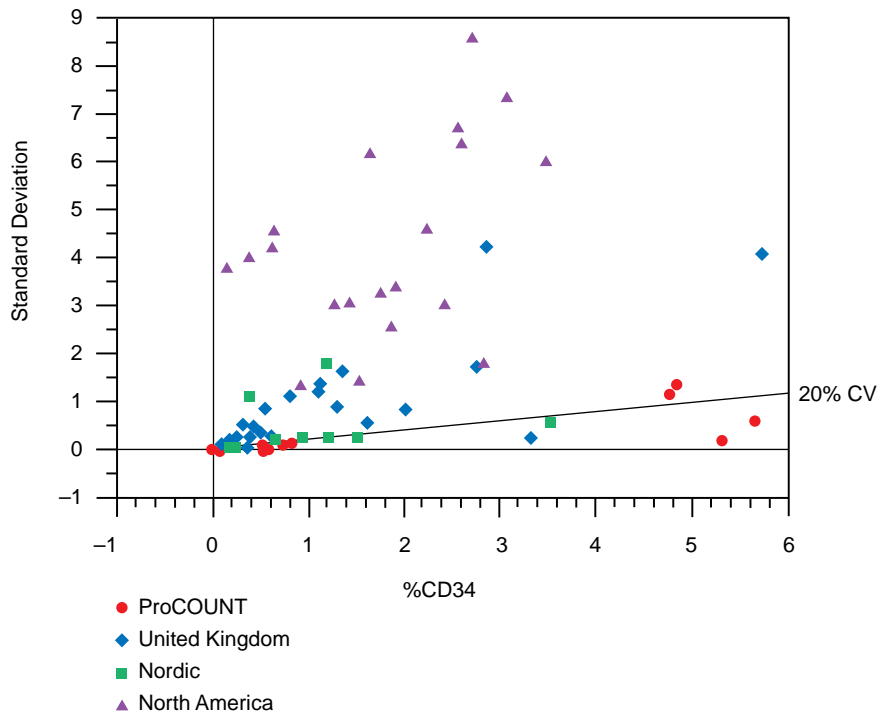
<sup>§</sup> Each sample was run in duplicate.

Figures 10 and 11 graphically show the published data from each of the studies. Figure 10 shows the standard deviation across sites vs mean absolute CD34 count for two of the studies and Figure 11 shows the standard deviation across sites vs the percent CD34 for the four studies. All sites in the Nordic and ProCOUNT studies standardized on one method (Milan and ProCOUNT, respectively), while the sites used their own methods in the United Kingdom and North America studies. Since the Nordic and North America studies reported results for CD34 percent only, the only study we can compare the ProCOUNT study to for absolute counts is the United Kingdom study. For absolute CD34 cells/ $\mu$ L, the ProCOUNT trial showed much lower variability across sites than observed in the United Kingdom study. For CD34 percent, the ProCOUNT trial showed lower variability across sites than the Nordic trial (Milan method), and much lower variability across sites than observed in the North American and United Kingdom studies.



Observations below the line represent a site-to-site CV of <20%.

Figure 10. Comparison of Standard Deviation vs. Mean for CD34 Absolute Counts in Two Multisite Trials



Note: United Kingdom, one sample with a CD34 percent of 19.31 and standard deviation of 6.82 is not shown. Observations below the line represent a site-to-site CV of <20%.

Figure 11. Comparison of Standard Deviation vs. Mean for CD34 Percents in Four Multisite Trials

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## Conclusion

A standardized approach to CD34<sup>+</sup> cell enumeration significantly reduces the variability of the assay. The ProCOUNT assay effectively standardizes CD34<sup>+</sup> progenitor cell enumeration by improving upon current methodologies. ProCOUNT uses a lyse-no-wash process that incorporates a nucleic acid dye for reliable thresholding and TRUCOUNT absolute counting technology. Our evaluations of ProCOUNT confirm that the assay design yields an accurate, linear, and precise assay. The performance of the assay was excellent, within any given laboratory, for both intra- and inter-laboratory testing, a prerequisite for effective standardization.

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