Published in final edited form as:

Adv Cell Gene Ther. 2018 September ; 1(2): . doi:10.1002/acg2.15.

Method comparison study of peripheral blood CD34+ count performed on an Abbott CELL-DYN Sapphire hematology analyzer versus flow cytometry reference procedure (modified ISHAGE)

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Abstract

Introduction: CD34+ cell enumeration is a critical parameter used to determine the timing of apheresis collections of hematopoietic progenitor cell products (HPC(A)). Automated hematology analyzers equipped with flow cytometry capabilities may be a solution to the problem of limited access to standard flow cytometry testing.

Methods: We compared CD34+ cell enumeration using a reference flow cytometry procedure employing modified International Society of Hematotherapy and Graft Engineering (ISHAGE) analysis with a hematology analyzer /flow cytometer hybrid (CELL DYN (CD)Sapphire) using a sequential gating analysis designed to emulate the ISHAGE gating strategy.

Results: CD34+ cell values obtained from the ISHAGE and CD Sapphire analysis were plotted and compared in a linear regression analysis which showed a high degree of correlation ($R^2=0.96$). No statistically significant ($p=0.53$) differences in CD34+ cell enumeration values were observed between the flow cytometer and automated hematology analyzer using manual analysis schema.

Conclusions: We have demonstrated that an automated hematology analyzer equipped with a flow module can provide CD34+ cell enumeration results in the peripheral blood for clinical decision algorithms without the need for a dedicated flow cytometry laboratory.

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Conflict of Interest Statement: Authors STA and SMM have received honoraria from Abbott Laboratories in association with presenting the data from this study submitted to ACGT. All other authors have no conflicts of interest relevant to the manuscript submitted to ACGT.

Ethics Statement: The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The study conformed to the US Federal Policy for the Protection of Human Subjects.

Clinical Implications:

Extended access of clinically important peripheral blood CD34+ cell enumeration to assess mobilization status and guide collection procedures. Access to these results on off-hours and weekends will allow optimized care for stem cell collection patients and donors.

Keywords

CD34+ count; ISHAGE analysis; CELL DYN Sapphire; stem cell harvest; HPC(A)

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is a potentially curative treatment for various hematologic malignancies such as acute myelogenous leukemia, lymphoma, and plasma cell dyscrasias¹. Clinically useful sources of hematopoietic progenitor cells (HPCs) used for HSCT include bone marrow, mobilized peripheral blood, and umbilical cord blood. Of the three HPC sources, currently the most commonly used is mobilized peripheral blood collected by apheresis (HPC(A)). The frequent use of HPC(A) is due to the relative ease of collection (non-surgical procedure), donor convenience, and the ability to safely collect large numbers of HPCs. Since the number of circulating HPCs in peripheral blood is normally very low at baseline, donors undergo pharmacologic stimulation to increase the number of HPCs in the peripheral blood in a process called mobilization. Granulocyte colony stimulating factor (G-CSF) administered subcutaneously daily for five days prior to apheresis collection is the mobilization agent of choice at most centers^{2, 3}. G-CSF stimulation results in an increase of white blood cell (WBC) counts including a variable increase in circulating HPCs. Enumeration of circulating CD34+ cells in mobilized donors and patients is considered to be the best predictor of a successful HPC(A) collection⁴.

In order to optimize resource utilization, some centers obtain a preharvest peripheral blood sample to determine the circulating CD34+ cell count prior to initiating HPC(A) collections on donors and patients. The pre-harvest peripheral blood CD34+ cell count can be used to predict the final CD34+ cell yield that can be expected from the collection procedure. By using estimation algorithms for HPC(A) collection outcomes, a clinician can decide whether the donor should be collected on the anticipated collection day, usually beginning on the fifth day of G-CSF stimulation. In patients undergoing autologous HPC(A) collection, this ability to predict mobilization adequacy can be used to prevent low CD34+ cell yield collections and to adjust collection procedure duration to maximize the probability of collecting sufficient HPCs.

The current recommended method for CD34+ cell enumeration by the International Society of Hematotherapy and Graft Engineering (ISHAGE) utilizes clinical flow cytometry instrumentation and sophisticated data analysis protocols⁴. The availability of this type of specialized testing is usually limited to routine clinical laboratory day shifts (Monday to Friday, 9AM – 5PM) whereas other clinical laboratories such as Hematology are available to perform testing 7 days a week, 24 hours a day. Contemporary automated hematology analyzers can be equipped with advanced testing methods which can include flow cytometry. Such multifunctional analyzers may thus offer a solution for providing afterhours flow cytometry testing in real time, so results can be used in patient management. We set out to compare whether a flow cytometry-capable hematology analyzer could be used to accurately measure peripheral blood CD34+ cell counts as compared to the reference method using clinical flow cytometry and the ISHAGE protocol^{5, 6, 7}.

MATERIALS AND METHODS

Patient Samples

A total of 98 EDTA-anticoagulated residual samples were collected from patients/ donor who were referred for standard CD34+ testing as part of the pre-transplantation collection process. All patients included in the study had previously provided informed consent for specimen analysis and the study was approved by the institutional review board at MSKCC. Samples were divided, stained and tested within 2–4 hours of collection.

Reference Flow Cytometric CD34 Determination (modified ISHAGE):

Flow cytometric evaluation of the collected peripheral blood was performed using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA) and FACS Diva software (BD Biosciences). Samples were stained and analyzed within 2–4 hours of collection. Duplicate aliquots containing 0.5×10^6 mononuclear cells were incubated with anti-CD45 FITC (fluorescein isothiocyanate- BD#340664), anti-CD34 PE (phycoerythrin- Beckman Coulter #IM 1871) monoclonal antibodies at room temperature in the dark for 20 minutes. Red blood cells were lysed with fixative-free ammonium chloride (10x NH₄CL Lysing Solution, Beckman Coulter #IM 3514, diluted with reagent grade water to 1x). 7-amino-actinomycin D (7AAD)(Beckman Coulter 3IM3422) was then added. Samples were mixed, kept at room temperature in the dark for 15 minutes and then stored for <1 hr on wet ice in the dark until acquisition/ analysis.

Analysis was performed using a modification of the International Society of Hematotherapy and Graft Engineering (ISHAGE) method previously described⁷. Briefly, compensations were set to avoid spectral overlap for CD34 PE and 7AAD ; 7AAD versus FSC plots were used to define non-debris regions examined in subsequent dot plots. Similarly, the FL1 secondary threshold was adjusted on the CD45 FITC versus SSC dot plot to exclude red cells, platelets, and other debris but not any CD45 FITC cells. The sequential Boolean gating strategy defined by ISHAGE was then applied to identify CD34+ cells. Viability was then evaluated on the FSC versus 7AAD dot plot instead of the traditional SSC vs 7AAD plots as this technique avoids overestimation of percent viability which can sometimes be seen with traditional gating strategies. Absolute CD34+ cell numbers were calculated using the dual platform method with total white blood cell counts measured on an Advia 2120 hematology analyzer (Siemens Healthcare, Germany.)

Modified Hematology Analyzer (Abbott CELL-DYN Sapphire)

The CD Sapphire hematology analyzer (Abbott, Santa Clara CA) is equipped with a 488 blue diode laser and 3 fluorescent detectors capable of providing limited phenotypic analysis similar to a basic flow cytometer. White blood cell and reticulocyte discrimination is accomplished by integrating data from 4 angles of light scatter (0°-ALL, 7°-IAS, 90°-DSS and 90°-PSS) with a single fluorescence channel (FL3) measuring propidium iodine staining to formulate a Multi-Angle- Polarized- Scatter-Separation (MAPSS) process. There is additional capability for automated determinations of CD3/4/8 subsets as well as CD61 based immunoplatelet counts through the use of the appropriate antibodies and fluorescent measurements of FITC/PE staining. The automated CD3/4/8 process has been further

modified to include a number of leukocyte antigens used in characterizing a variety of lymphoid and myeloid markers including CD34+ progenitors^{8,9}.

For CD Sapphire analysis, patient specimens were run in duplicate. Briefly, 200µl of the anticoagulated blood collected as described above were allocated to two 13×75mm non-anticoagulated tubes and each stained with 10µl of anti-CD45 FITC (BD) and 10 µl of anti-CD34-PE (Coulter). The 2 tubes were incubated for 20 minutes at room temperature and then each placed in a Sapphire rack immediately adjacent to a blank 13×75 mm tube. Processing the CD34 sample on the Sapphire requires placing a blank tube in position 1–7 of a 10 tube rack immediately followed by the two prepared sample tubes. The mixture was processed using the automated CD3/4/8 (automated monoclonal antibody) mode. In contrast to the standard full blood count mode where four angles of light scatter are gathered to generate a five- part differential, in the CD3/4/8 mode information is obtained only for the 0°-ALL and 7°-IAS optical scatter which for this instrument are approximations of forward and side scatter. This mode is selected from the main menu and the sample processor is activated. No sample washing was required and red cell lysis was accomplished by the automated procedure in the analyzer. Analysis time was approximately 8 minutes. The data from the analyzed samples is stored in list mode using a file structure that is compatible with fcs (flow cytometry standard) files. Once the sample processing is completed, retrieval of the fcs file is accomplished by a menu driven utility driven via analyzer software and data is downloaded to external storage media. Each file contains results from both analyzed tubes and the fcs files are then analyzed on windows based computer using FCS Express Version 4.0 (De Novo Software, Thornhill, ON, CA) software.

As the CD Sapphire functions as a hematology analyzer, it employs a time-gated versus event gate acquisition typically found on standard diagnostic flow cytometers. This difference limits the number of events that can be recorded per analysis on the Sapphire to 20,000 events. Optimal event numbers for a rare event analysis like CD34 determination typically require between 60,000 –100,000 events. The number of events available for analysis using Sapphire acquisition is further enhanced to an average of 80,000–100,000 events by merging FCS files, a feature routinely available in the FCS software used for analysis. All sample data is applied to a pre-defined scatterplot template specifically designed for CD34 stem cell identification (fixed SGS). Batch analysis was available so multiple samples could be analyzed during one session further reducing the time required by the technologist to process multiple samples.

An alternative method was employed to analyze data since the populations of interest were noted to vary from plot to plot so an effort was made to control for this drift or variability in the staining pattern. This required manual adjustment/application of gates on sequential dot plots and was designed to emulate the ISHAGE strategy (manual SGS). Briefly, the initial gate used the IAS vs CD45 (FITC-FL1) scattergram to select for leukocytes gating out any debris in the specimen. A subsequent plot of IAS vs CD34 (PE-FL2) was used to set a positive region for CD34+ and this population was then back-gated to the original IAS vs CD45 plot to define the “true” positive CD34+ population. Absolute CD34+ numbers were calculated using the white blood cell count measured via the Sapphire’s full blood count

mode. A comparison of the modified ISHAGE method and the manual SGS gating strategy is demonstrated in Figure 1.

Data Analysis

The CD34+ cell enumeration data obtained by ISHAGE protocol, the manual SGS, and the fixed SGS were analyzed using Excel 2007 (Microsoft, Washington) and with Analyse-it (Leeds, United Kingdom).

Truth tables were also constructed at various clinically relevant thresholds (0, 5, 10, 15, 20, and 40 CD34+ cells/uL) to determine the sensitivity and specificity of the manual and fixed SGS as compared to ISHAGE protocol.

A paired, two tailed t-test was also used to ascertain whether or not there was any significant discrepancy between the FACSCalibur and the CD Sapphire.

RESULTS

CD34+ cell values obtained from both the BD FACSCalibur (ISHAGE protocol) and CD Sapphire (manual SGS) were plotted and a linear relationship was observed (Figure 2). Linear regression analysis demonstrated a high correlation ($R^2 = 0.96$), slope of 0.89, and an intercept of 8.18. The data points ranged from 0 to 400 CD34+ cells/uL. A paired, two-tailed t-test showed that there was no significant difference ($p = 0.53$) between the data enumerated by each of the methods (ISHAGE protocol vs manual SGS) of CD34+ cell enumeration.

A residual plot (Figure 3) was used to display the difference in values of CD34+ cells obtained through the CD Sapphire where the BD FACSCalibur is used as the reference standard. At lower levels of CD34+ cells/uL (< 100) counts, there was less difference in results between the CD Sapphire and the reference method. The difference results appeared to distribute normally with no overt positive or negative bias.

The reference platform was also compared to data generated from a fixed SGS analysis algorithm on the CD Sapphire (Figure 4). A statistically significant difference ($p = 0.02$) was observed between the two methods of CD34+ cell enumeration using this method. A low correlation ($R^2 = 0.55$) was observed between the two platforms when using linear regression analysis.

The sensitivity and specificity performance characteristics of the CD Sapphire with manual SGS were determined at six clinically relevant thresholds (0, 5, 10, 15, 20, and 40 CD34+ cells/uL). At each CD34+ cell level, the sensitivity of the CD Sapphire exhibited markedly high sensitivity (98%) when using the manual SGS (Table 1). The specificity was found to be also high (87%) at CD34+ cell levels at or above 10 CD34/uL. When a fixed SGS algorithm was applied, the sensitivity of the assay remained high (96%), however the specificity of the test was observed to decrease, ranging from 50–71% (Table 1).

DISCUSSION

Timely access to peripheral blood CD34+ cell counts in the setting of determining the adequacy of mobilization of HPC(A) donors and patients is critical to the development and implementation of evidence based decisions. Due to the complexity of flow cytometric enumeration of specific cell populations such as CD34+ HPCs, availability of this critical testing is often limited in many centers to weekdays and routine work hours. Unfortunately, donors and patients are not always optimally mobilized precisely on the fifth day of G-CSF stimulation which may not be compatible with these operational hours. Suboptimal mobilization more commonly occurs in heavily pre-treated (chemotherapy) autologous donors¹⁰ which can lead to delayed collection cycles that begin later into the work week and frequently extend into the weekend and/or afterhours. The lack of CD34+ cell count testing during non-business time periods is a barrier to optimal utilization of evidence based medicine to best manage donor/patient collections. A single instrument method for CD34+ cell enumeration which is available 24/7 and provides real time results would benefit patients by allowing care providers to use prediction models to estimate collection harvest yields and to customize mobilization and collection strategies to best accomplish the goals of care.

In an attempt to accomplish this goal, we adapted a modern hematology analyzer with flow capability to provide CD34+ cell enumeration on peripheral blood. Two analysis algorithms were developed for the CD Sapphire derived data. The first, a sequential gating strategy (manual SGS) which emulates the key features of the ISHAGE protocol used analysis gating templates which relied upon minor adjustment of analytic gates to best fit the sample cell populations. The second algorithm attempted to analyze the data with fixed gates (fixed SGS) to as to eliminate the need for user intervention at the analysis step. While the development of a “turnkey” solution for CD34+ cell counting is the ultimate goal, we observed that the lack of gate adjustments specific for each sample resulted in unacceptable reductions in test specificity at the various clinically relevant CD34+ cell levels. We conclude that manual gate adjustments are still an integral and required component of successful CD34+ cell enumeration at this time until more advanced automatic gating computer algorithms can be developed and applied.

One important difference between the ISHAGE protocol and the CD Sapphire platform as used in this study is the viability marker. The ISHAGE protocol recommends the use of 7-AAD (peak emission ~650 nm) as the viability marker due to its fluorescence spectral characteristics which make proper analysis of the data less dependent upon compensation. Due to the incorporation of propidium iodide (PI) into the buffer system of the CD Sapphire, there was no way to implement a substitution of viability marker without re-engineering the reagent system. PI as a viability marker (peak emission ~620 nm) has the additional drawback of a wide spectral overlap with the PE fluorochrome (peak emission ~575 nm) which can result in difficulties separating the PI signal from PE signal often requiring compensation manipulations during data acquisition and analysis. Of note, the manual SGS results were determined without compensation adjustments when compared to the ISHAGE protocol. Because the samples that were run were all fresh (less than 8 hours old) peripheral blood samples from mobilized donors and patients, viability measurements play a minimal

role. We hypothesize that using the CD Sapphire platform to analyze previously cryopreserved products, where viability often plays a substantial role, will need some level of compensation to achieve a similarly high correlation. Alternatively, viability measurements could be accomplished separately using standard methods and incorporated into the final reported results. The reference method used in this study was dual platform flow cytometry, which has been gradually supplanted by single platform methodologies as the state of the art, in order to increase accuracy and precision of the results. Our Center has recently adopted a single platform assay as well (BD-Truecount Tubes) where we observed a correlation coefficient of 0.89, an intercept of 0 and a slope of 0.72 when performing a Demming regression analysis for method comparison (data not shown). Due to the adequate level of agreement between our single and dual platform methods we believe that the results of this study are still relevant for the purpose of peripheral blood CD34 enumeration. Of note, CD34 enumeration using this method would likely be considered a laboratory developed test (LDT) and prior to implementation, it would require validation against the currently approved flow cytometer based method in use clinically in order to comply with relevant accrediting and regulatory requirements.

The availability of alternative platforms such as automated hematology analyzers with flow capabilities presents a new paradigm for extended access of clinically important, albeit low volume, tests such as peripheral blood CD34+ cell enumeration. The CD Sapphire offers the ability to perform CD34+ cell counts on an instrument that is expected to be available for testing 24/7 as is the case with all hematology analyzers. We have demonstrated that the CD34+ cell counts obtained from the CD Sapphire and analyzed with a manual sequential gating algorithm is able to produce robust, clinically actionable data since there is a high level of correlation and, that at multiple CD34+ cell level decision points, the test has both high sensitivity and specificity. This hybrid platform may be especially useful in medical facilities with size, staffing, and other resource limitations since no additional laboratory space or specialty training is required to perform the assay.

Acknowledgements:

Sources of Support: This work was funded in part through the NIH/NCI Cancer Center Support Grant P30 CA008748

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Figure 1.
Modifies ISHAGE vs Manual Sequential Gating Strategy

Figure 2.
Linear correlation of modified ISHAGE vs manual SGS

Figure 3.
Difference plot of manual SGS vs modified ISHAGE reference

Figure 4.
Linear correlation of modified ISHAGE vs fixed SGS

Performance characteristics of manual SGS and fixed SGS on CD Sapphire for CD34+ cell enumeration

Table 1.

		5 CD34/uL	10 CD34/uL	15 CD34/uL	20 CD34/uL	40 CD34/uL
Manual SGS	Sensitivity	99%	99%	100%	100%	98%
	Specificity	67%	94%	96%	89%	87%
	Positive Predictive Value	96%	99%	99%	96%	89%
	Negative Predictive Value	89%	94%	100%	100%	97%
Fixed SGS	Sensitivity	99%	99%	99%	97%	96%
	Specificity	50%	71%	58%	61%	70%
	Positive Predictive Value	93%	94%	88%	86%	78%
	Negative Predictive Value	86%	92%	93%	89%	94%