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Flow cytometric enumeration of CD34+ hematopoietic stem cells: A comparison between single- versus dual-platform methodology using the International Society of Hematotherapy and Graft Engineering protocol

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Abstract:

BACKGROUND: Flow cytometric enumeration of CD34+ hematopoietic stem cells (HSC) is the reference point for undertaking apheresis and evaluation of adequacy for peripheral blood stem cell (PBSC) engraftment.

AIMS: To determine whether single platform correlates with dual platform methods in CD34+ enumeration using ISHAGE protocol.

METHODS: Retrospective analysis of CD34 Enumeration assays on both peripheral blood and PBSC product samples using Beckman Coulter FC500 Flow Cytometer. The t test and correlation study was used to study the difference between single and dual platform methods in CD34+ enumeration.

RESULTS: We present our data on 152 samples comprising 41 peripheral blood samples collected before apheresis procedure and 111 samples collected from PBSC product. We observed strong positive correlation between single and dual platform methods for CD34+ counts in peripheral blood sample ($r = 0.92$; $P < 0.001$) and PBSC product sample ($r = 0.85$; $P < 0.001$).

CONCLUSION: In our study, both single versus dual platform had similar results in CD34+ cell counts. The single platform provides rapid results with ease of procedure. Errors with dual platforms are relatively common with respect to denominator. We recommend to use mean of total leukocyte count from two different hematology analyzer to minimize variation in dual platform.

Keywords:

CD34+ cells enumeration, flow cytometry, hematopoietic stem cells, International Society of Hematotherapy and Graft Engineering

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Introduction

Flow cytometric enumeration of CD34+ cells is commonly employed to assess the hematopoietic stem cell (HSC) numbers in peripheral blood, cord blood, and apheresis products used for peripheral blood stem cell (PBSC) transplantation. The CD34 antigen is stage-specific and identifies cells in the early stages of hematopoietic differentiation. Accurate enumeration of

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HSC's is important in estimating the adequate dose, the most useful indicator of the hematopoietic reconstitutive capacity of PBSC transplants. It will also help in planning for subsequent apheresis collection.

Early methods in CD34+ cells enumeration used simple forward scatter versus side scatter gating to select leukocytes as denominator.[1] The protocol was improved by subtracting nonspecific events from the CD34 events and the use of 7AAD dye to exclude dead cells.[2] Most centers follow CD34+ cells enumeration using the International Society of Hemototherapy and Graft Engineering (ISHAGE) guidelines using sequential Boolean gating strategy, dim CD45 expression by the CD34+, SS^{low} HPC.[3,4]

Two commonly followed methods for measuring CD34+ cells in the sample are single-platform and dual-platform methods. In dual platform, absolute CD34+ cell counts were derived from a flow cytometrically assessed percent CD34+ cells within leukocytes, combined with the assessment of the absolute leukocyte count from a hematology cell analyzer. Single-platform method uses fluorescent counting beads to this protocol and directly generating absolute CD34+ cell counts from a single flow cytometric assessment.[4]

The study compares single- versus dual-platform methods in CD34+ cell enumeration of HSCs in peripheral blood and PBSC product from a tertiary cancer center from South India.

Methods

Sample collection

It was a retrospective study from January 2014 to May 2018. The analysis was done on peripheral blood (before apheresis procedure) and samples aliquoted from PBSC product of autologous patients and allogeneic donors.

The complete blood count of samples was done using Beckman Coulter hematology analyzer LH750 in Central Laboratory. The daily three-level quality control steps were performed in the hematology analyzer before starting the assay. The CD34+ cells enumeration was performed on Beckman Coulter FC500 equipment using the Stem-Kit reagent. The method was based on the ISHAGE guidelines: four-parameter flow cytometry method (CD45FITC/CD34PE staining, side and forward angle light scatter). The assay was run using C×P protocol in blood bank.

Sample preparation

Freshly obtained samples with higher white blood cell (WBC) concentrations were diluted to 10, 000 WBC/μL with phosphate-buffered saline. The samples were stained in duplicate (Sample 1 and 2) with two color CD45-FITC/CD34-PE (20 μl) monoclonal reagents. Control reagent (20 μl CD45-FITC/IsoClonic Control-PE) to check the nonspecific binding of CD34 antibody was added. 7-aminoactinomycin D (7-AAD) viability dye (20 μl), a nucleic acid dye that binds to accessible base pairs (cellular DNA), distinguish between viable and nonviable cells was used. Red cell lysis was initiated with buffered ammonium chloride without washing. Just before flow cytometry acquisition, 100-μl fluorescent beads (polystyrene fluorescent microspheres in aqueous suspension medium) was added and thoroughly mixed with the sample for absolute CD34 enumeration by single-platform cell counting.[5]

Gating

The ISHAGE guidelines were followed for estimating the absolute number of CD34+ cells in single-platform method and percentage of CD34+ cells within leukocytes in dual-platform method.

Calculations

The assay was accepted if the number of CD34+ cells falls within 10% of the mean for the duplicate samples, if falls outside 10%, the assay was repeated. The value obtained with the control tube must represent less than 10% of the average value obtained from the tests tubes to validate the results.

Single-platform assay:[5] The single platform provides absolute CD34+ cell count without additional steps and calculated as

- CD34+ HSC Absolute Count (cells/μL) = MeanCD34+ Count (cells/μL) × Dilution

Dual-platform assay:[5] The percent of CD34+ cells was obtained from flow cytometer and the total leukocyte count (TLC) from the hematology analyzer. The absolute count was calculated by

- CD34+ HSC Absolute Count (cells/μL) = %CD34+ Count (cells/μL) × TLC from the hematology analyzer

Statistical analysis

The statistical difference between single and dual platform was compared through *t*-test. Pearson's correlation coefficient was used to evaluate the correlation between single- and dual-platform assays. $P < 0.05$ was considered statistically significant. All the analysis was done using SPSS version 18 (SPSS Inc., Chicago, IL, USA).

Results

During the study period, CD34+ cells enumeration was performed for 85 patients (70 autologous and 15 allogeneic). A total of 152 samples comprising 41 peripheral blood samples collected before the apheresis procedure and 111 samples collected from the PBSC product were analyzed for CD34+ cells enumeration.

Peripheral blood samples

The mean (SD) values for peripheral blood samples for single- and dual-platform assays were 59.8 ± 39.8 cells/μl and 55.9 ± 38 cells/μl, respectively. There was no statistically significant difference in the mean CD34+ counts between the two platforms in peripheral blood sample ($P = 0.112$) [Table 1].

Peripheral blood stem cell product

The mean CD34+ count in the single platform (1159.4 ± 922.3 cells/μl) was significantly higher than that in the dual platform (991.0 ± 951.63 cells/μl) with respect to the PBSC product ($P = 0.001$) [Table 1].

Pearson's correlation coefficient was used to evaluate significant correlations between single- and dual-platform assays of CD34+ in the peripheral blood sample and PBSC product sample. A strong positive correlation between single- and dual-platform assays of CD34+ counts in peripheral blood sample ($r = 0.92$; $P < 0.001$) and PBSC product sample ($r = 0.85$; $P < 0.001$) was noted [Table 2].

Discussion

CD34+ cells enumeration by flow cytometry has become a widely accepted technique to quantify HSCs for the management of stem cell transplantation. In the last two decades, several methods were made to simplify the enumeration procedure and adopt stringent gating strategies to minimize the errors in CD34+ cell counting. The ISHAGE guideline was a widely accepted and standardized protocol.[6] The ISHAGE guidelines recommend lyse no wash technique to minimize the cell loss during sample preparation.

The dual-platform assay was based on CD34+ cell percentage obtained from flow cytometer assay and TLC from hematology analyzer and multiplying the two values for absolute counts. This technique has its limitations due to its time-consuming exercise and calibration of equipment.[7] Adding known quantity of fluorescent beads in single-platform ISHAGE protocol allows the determination of absolute CD34+ cell counts without the need hematology analyzer. Single-platform assays have the potential to be more effective in reducing interlaboratory variation than dual-platform techniques.[8]

In the present study, we observed similar results in absolute CD34+ cell count in peripheral blood sample between the two methods ($P = 0.112$). However, in the PBSC product, we observed a difference in CD34+ cell counts between the two methods ($P < 0.001$).

The technical limitations of single platform are the accuracy and reproducibility of test result due to conventional pipetting of samples with high viscosity such as peripheral blood or PBSC product.[8] However, the pipette error can be overcome by using the reverse pipetting technique.

The guidelines-recommended reproducibility difference should be within 10% for CD34+ cell count from single-platform assay and the mean between the duplicates to be used for final calculations. The reproducibility of test results between Samples 1 and 2 was within limits in both peripheral blood and PBSC

Table 1: Enumeration of CD34 cells in peripheral blood and peripheral blood stem cell product using single- and dual-platform assay from a tertiary cancer center in South India

Sample	*n*	Mean±SD		*P*
		Single platform	Dual platform	
Peripheral blood	41	59.8±39.8	55.9±38.0	0.112
PBSC product	111	1159.4±922.3	991.0±951.6	<0.001

PBSC=Peripheral blood stem cell, SD=Standard deviation

Table 2: Correlation between single- and dual-platform assay of CD34+counts in peripheral blood sample and peripheral blood stem cell product sample from a tertiary cancer center in South India

Sample	*n*	*r*	*P*
Peripheral blood	41	0.92	<0.001
PBSC product	111	0.85	<0.001

PBSC=Peripheral blood stem cell

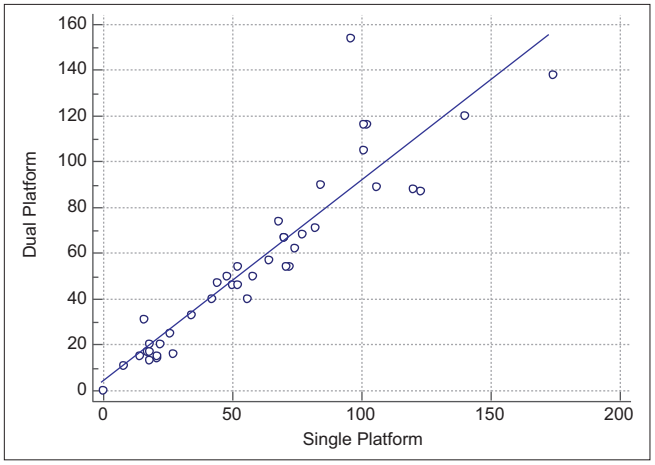

Figure 1: Correlation of single- and dual-platform assay for CD34 enumeration in peripheral blood sample from a tertiary cancer center in South India

product at our center. Those assays exceeding 10% in variation were repeated. Hence, we used single-platform assay for our final calculations as the method is rapid and straight forward offering high levels of standardization.

In dual platform, variation is relatively common with TLC than the CD34+ cell percentage. The inadequate mixing or pipetting of sample in hematology analyzer can be the possible reason for this difference. To overcome this, Naithani *et al*. used mean of TLC from two hematology analyzers.[9] However, in the present study, we used only one hematology analyzer to determine TLC, which was one of our limitations.

Single-platform assay has provisions for using the external quality control sample (Coulter: Stem-Trol Control Cells or BD: Trucount tubes). The control provides an antibody to antigen positive control for CD34 and CD45 staining in flow cytometry and their concentration is precisely calibrated for verifying each run.[8] Dual platform requires controls only for hematology analyzer and no additional controls for flow cytometry equipment.

Our present study had significant association between single- and dual-platform assays of CD34+ counts in the peripheral blood sample [Figure 1] and PBSC product sample [Figure 2]. Our results are consistent with those of Ngoma *et al*., Keeney *et al*., and Naithani *et al*.[4,9,10]

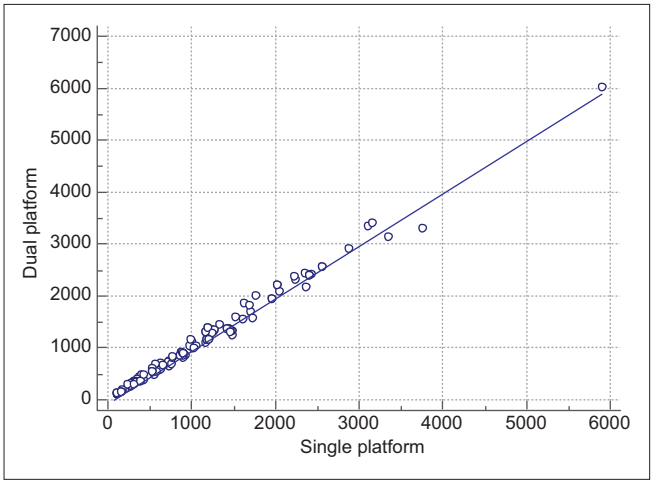

Figure 2: Correlation of single- and dual-platform assay for CD34 enumeration in PBSC product from a tertiary cancer center in South India

Conclusion

We conclude both single versus dual platform yield similar results in CD34+ cell counts. The single platform provides instant value with ease of procedure. However, when adopting dual-platform technique, we recommend using the mean of TLC obtained from two hematology analyzers for minimizing the variation.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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