Cytometry PART A
Journal of Quantitative Cell Science

Comparison of Flow Cytometric Methods for the Enumeration of Residual Leucocytes in Leucoreduced Blood Products: A Multicenter Study

Yang Zeng,[1]* Michelle Dabay,[2] Virginia George,[2] Shalini Seetharaman,[2] Monika de Arruda Indig,[3] Sharon Graminske,[3] Nicole Kimpel,[3] Anna Schmidt,[3] Amanda Boerner,[3] Sarai Paradiso,[4] Tatyana Delman,[4] Yunyao Li,[4] Viktoriya Litvak,[4] Farzad Oreizy,[1] Angela Chen,[1] Maryam Saleminik,[1] Fred Mosqueda,[1] Anna Lin,[1] Kevin Judge[1]

[1]BD Life Sciences, Medical Affairs and Corporate Clinical Development, San Jose, California 95131

[2]American Red Cross Holland Laboratory, Transfusion Innovation Department, Rockville, Maryland 20855

[3]Clinical Trial and Cellular Therapy Services, BloodCenter of Wisconsin, Milwaukee, Wisconsin 53233

[4]New York Blood Center, Clinical Laboratories, Long Island City, New York 11101

Received 22 September 2017; Revised 18 November 2017; Accepted 12 December 2017

Grant sponsor: BD Life Sciences

Additional Supporting Information may be found in the online version of this article

*Correspondence to: Yang Zeng, BD Life Sciences, Medical Affairs and Corporate Clinical Development, 2350 Qume Dr., San Jose, CA 95131 Email Yang_Zeng@BD.com

Published online 18 January 2018 in Wiley Online Library (wileyonlinelibrary.com) DOI: 10.1002/cyto.a.23318

Abstract

The BD FACSVia™ System features novel designs in hardware, software, and instrument QC. We compared the performance of the BD FACSVia System using the BD Leucocount™ kit with the BD FACSCalibur™ flow cytometer. Leucoreduced platelet (PLT, $n = 252$) and red blood cell (RBC, $n = 278$) specimens were enrolled at four sites. Each specimen was stained in four tubes using the BD Leucocount kit reagents and acquired on the two systems. BD Leucocount Control cells (high and low) were used to evaluate the inter-site reproducibility on the BD FACSVia System at three sites over 20 days. Deming regression and Bland–Altman analysis were performed to determine the WBC absolute counts on the BD FACSVia System vs. the BD FACSCalibur system. Assay accuracy for the range of 0–350 WBCs/μl was adequate. For samples with <25 WBCs/μl, the bias with 95% limits of agreement was 0.136 (–1.897 to 2.169) WBC/μl for PLTs ($n = 184$) and 0.170 (–2.025 to 2.365) WBC/μl for RBCs ($n = 193$). For inter-site reproducibility, the CV% was 6.46% (upper 95% CI 7.16%) for the PLT high control and 9.49% (10.52%) for the PLT low control. The CV% was 7.51% (8.32%) for the RBC high control and 10.76% (11.92%) for the RBC low control. The BD FACSVia System reported equivalent results of WBC absolute counts for leucoreduced PLT and RBC samples compared to the BD FACSCalibur system. The inter-laboratory reproducibility of the BD FACSVia System met study specifications. © 2018 The Authors. Cytometry Part A Published by Wiley Periodicals, Inc. on behalf of ISAC.

Key terms

leucodepletion; leucoreduced; residual Leucocytes; flow cytometry; performance evaluation

IN the past decades, performing leucoreduction for blood transfusion products has become a standardized practice in the US and Europe to improve clinical outcome, and also has been gradually adopted by nations in emerging markets worldwide (1–3). Depleting leucocytes below a specific level helps to prevent or reduce short and long term adverse effects in transfusion, such as febrile reaction and human leukocyte antigen (HLA) alloimmunization (4–7). Removal of >99.9% of leucocytes (or 3-log leucoreduction) in the red cell units and platelets can also significantly minimize potential transmission of viruses such as cytomegalovirus, and reduce transfusion-associated graft-versus-host disease (8–11). European countries, the USA, and Canada have published guidelines to enforce universal leucoreduction

aimed at improving the quality of blood components. This requires establishment of reliable quality control processes and standards for leucoreduced blood products, as well as rapid and robust cell counting methods to assess residual leucocytes.

The current US guidelines suggest that leucoreduced red cell units should contain <5,000,000 white blood cells (WBCs) per unit (12). To qualify apheresis platelet units, $<5 \times 10^6$ leucocytes should be in each apheresis bag. For whole blood-derived platelet pools, American Association of Blood Banks (AABB) standards require that leucocyte reduction ensures that 95% of the platelet units sampled contain $<8.3 \times 10^5$ leucocytes per unit. An integrated pack of six PLT units would have $<5 \times 10^6$ rWBCs (12). The Council of Europe requires the leucodepletion process to achieve $<1 \times 10^6$ per unit of red cells and $<0.2 \times 10^6$ residual leucocytes per unit of platelets from whole blood (13–15). Routine quality control of blood components demands fast, reliable, and sensitive methods of automated detection of low levels of leucocytes in final products for transfusion service.

Technologies to enumerate leucocytes in peripheral blood samples include automated leucocyte counters, flow cytometry, the manual Nageotte counting chamber, and volumetric capillary cytometry (16). Published studies from multiple institutions have compared performance of different technologies to evaluate sensitivity, precision, and robustness for leucocyte counting in leucodepleted blood components (16–24). Centralized blood banks and clinical laboratories have adopted flow cytometry as the method of choice because of its high sample throughput and outstanding intra- and inter-laboratory reproducibilities.

Traditional flow cytometers are based on old technologies, such as analog electronic systems, with less efficient workflow. The recently developed BD FACSVia™ System is a small flow cytometer with novel designs in optics, electronics and fluidic systems, as well as simplified instrument QC. To evaluate the performance of the BD FACSVia System in enumerating low levels of leucocytes in leucoreduced blood products, we conducted a multicenter method comparison and inter-laboratory reproducibility study. The method comparison was designed in accordance with the Clinical and Laboratory Standards Institute (CLSI) Guideline EP09-A3: *Measurement Procedure Comparison and Bias Estimation Using Patient Samples; Approved Guideline*—Third Edition (25). The inter-laboratory reproducibility study was based on the CLSI Guideline EP05-A3: *Evaluation of Precision of Quantitative Measurement Procedures; Approved Guideline*—Third Edition (26). We carried out this performance evaluation under Good Clinical Practices guidelines and the US Code of Federal Regulations. The study sites participating in the method comparison evaluation were the American Red Cross (ARC), Holland Lab in Rockville, Maryland; BloodCenter of Wisconsin (BCW) in Milwaukee, Wisconsin; New York Blood Center (NBC) in Long Island City, New York; and the BD MedLab (MED) in San Jose, California. Three of the four study centers, ARC, BCW, and MED, also participated in the inter-laboratory reproducibility study.

Table 1. Summary of specimen enrollment

STUDY SITE	SPECIMEN TYPE	ENROLLED EVALUABLE[a] SPECIMENS PER SITE	ANTICOAGULANT
American Red Cross (ARC)	RBC	87	CPD, CP2D
	PLT	81	CPD, CP2D
BloodCenter of Wisconsin (BCW)	RBC	94	ACDA, CPD, CP2D
	PLT	26	ACDA
New York Blood Center (NBC)	RBC	85	ACDA, CP2D
	PLT	77	ACDA
MedLab, BD (MED)	RBC	12	ACDA
	PLT	68	ACDA

CPD, citrate phosphate dextrose; CP2D, citrate phosphate double dextrose; ACDA, anticoagulant citrate dextrose A. BD, Becton, Dickinson and Company.

[a]Evaluable data was defined as meeting all applicable protocol requirements and containing complete traceable records.

Materials and Methods

Study Design

Method comparison. The leucoreduced PLT and RBC specimens were enrolled per the pre-defined inclusion and exclusion criteria and were prepared using the BD Leucocount™ kit. Samples were measured on the BD FACSVia and BD FACSCalibur instruments side-by-side at four study centers to determine the WBC absolute count on each system. Table 1 lists the number of specimens enrolled at each study site.

Inter-laboratory reproducibility. To evaluate the reproducibility of the WBC absolute count on the BD FACSVia System, the same BD Leucocount Control cells with high and low WBC concentrations were fluorescently labeled in BD Trucount™ tubes with the BD Leucocount kit. Controls were run once per day on the BD FACSVia System at three study centers for 20 operational days. At least two operators per site prepared samples and performed the inter-laboratory reproducibility study.

Specimen Enrollment for the Method Comparison

The institutional review board or ethics committee at each study center reviewed and approved the study protocol before proceeding to site initiation of investigational activity. On each study day, the phlebotomist at American Red Cross (Holland Lab, Rockville, MD) collected whole blood specimens from donors. The site's Informed Consent forms under the ARC Research Blood Donor Program were used. Samples from this site were apheresis PLTs and RBCs, or leucoreduced PLTs and RBCs derived from donor whole blood specimens by the ARC laboratory staff using their standard operational procedures. At other study sites, remnant leucoreduced PLT and RBC specimens together with autologous white blood cell sources such as same-donor leucocytes, non-leucoreduced PLTs or RBCs, or whole blood, were enrolled for the method comparison study. The remnant specimens were leftover PLTs

and RBCs from routine QC testing in blood centers, which otherwise would have been discarded as biological waste. Per enrollment needs, leucoreduced PLTs and RBCs with autologous white blood cell sources were also purchased from qualified vendors that produce apheresis PLTs and RBCs through standard procedures (AllCells, LLC, Alameda, CA; Stanford Blood Center, Palo Alto, CA). All PLT and RBC specimens used in the study were within 48 hours of leucoreduction as required by the BD Leucocount kit. Subjects' protected health information was de-identified from laboratory testing staff before enrollment. Anticoagulants in the enrolled leucoreduced PLTs and RBCs were citrate phosphate dextrose (CPD), citrate phosphate double dextrose (CP2D), or anticoagulant citrate dextrose A (ACDA), as shown in Table 1.

Distribution of PLTs and RBCs in the Method Comparison

To ensure that prepared PLT and RBC samples spanned the range of 0–350 WBCs/μl, especially within the critical range of $1-20$ WBCs/μl (equating to $\sim 1-5 \times 10^6$ per pack), four WBC bins were used to guide study enrollment. The first low bin contained <1 WBC/μl, the second low bin was defined as $1 \leq$ WBC $<$ 5 cells/μl, the third bin $5 \leq$ WBC $<$ 25 cells/μl, and the fourth bin had the highest WBC concentration range of $25 \leq$ WBC $<$ 350 cells/μl.

Preparation of PLT and RBC Samples

Method comparison. The specimens were initially assessed for WBC absolute count using a hematology cell counter to ensure that the WBC concentration in the leucoreduced PLT and RBC samples fell within the range of $0 <$ WBC $\leq$ 350 cells/μl. PLTs and RBCs did not have to be from the same donor. If both PLT and RBC products were obtained from the same donor, they were considered as independent samples. For each specimen, the WBC bins were determined based on the absolute count of WBC results from the BD FACSCalibur system.

Inter-laboratory reproducibility. Custom made PLT and RBC control cells (R&D Systems, Minneapolis, MN) were used as a stable cellular material to evaluate inter-laboratory reproducibility for the BD FACSVia System. Two concentration levels of PLT control cells, 7.6 ± 2.7 and 17 ± 4.3 cells/μl, and two concentration levels of RBC control cells, 7.2 ± 2.5 and 19 ± 4.8 cells/μl, were used. Controls were shipped from BD Life Sciences, San Jose to the study sites. Each of the three study sites stained each concentration level sample of PLTs and RBCs in duplicate in BD Trucount tubes, using the BD Leucocount kit, and the resulting stained samples were run on a BD FACSVia System.

Daily Instrument Setup and QC

The BD FACSVia System contains a blue laser (488 nm) and a red laser (640 nm) with four fixed voltage fluorescence detectors and two scatter detectors (FSC and SSC). The wavelengths of optical filters are 533/30 nm (FL1), 585/40 nm (FL2), >670 nm Long Pass (FL3), and 675/25 nm (FL4). At each study site, the BD FACSVia System was set up using BD™ Cytometer Setup and Tracking (CS&T) beads and BD FACSVia clinical software v1.0. CS&T beads are fluorochrome dyed polystyrene beads consisting of equal quantities of 3-μm bright, 3-μm mid, and 2-μm dim fluorescent microspheres. Three production lots of the CS&T beads were used at four study centers. The beads were prepared by adding two drops into 500 μl of deionized water, then run on the BD FACSVia instrument to generate an Instrument QC report. During instrument QC, BD FACSVia clinical software verified that CS&T bright beads were placed within the target fluorescence channels on the instrument. Fluorescence compensation was optimized based on the daily median fluorescence intensity (MFI) values of the bright beads. The bright bead robust coefficient of variation (rCV) was also measured during setup for each fluorescence channel and compared with preset QC criteria. Instrument sensitivity was calculated automatically during the CS&T setup process and subjected to predefined specifications. For quality control of optics, electronics, and fluidics of the system, a "Pass" on the Instrument QC report had to be obtained on the study day before sample acquisition. The BD FACSVia flow cytometer uses fixed detector voltages. Therefore, user adjustment and optimization of PMT settings are not part of the instrument setup.

On the BD FACSCalibur system, BD Calibrite™ beads were prepared per the reagent instructions for use and run on the BD FACSCalibur instrument using BD FACSComp™ software to set up the instrument. Optimized photomultiplier tube (PMT) voltages and compensation values were generated for the BD Leucocount assay by BD FACSComp software based on the measurement of the BD Calibrite beads. A Pass on the BD FACSComp QC report was obtained before sample test.

On each study day, the BD FACSVia and BD FACSCalibur systems also were qualified by running process controls. BD Leucocount Control cells were prepared using the BD Leucocount kit and run on both BD FACSVia and the BD FACSCalibur systems per the BD Leucocount Control instructions for use. The absolute count of WBCs in the BD Leucocount Control cells had to fall within the specification range provided by the manufacturer on both the BD FACSVia and BD FACSCalibur systems.

Enumeration of Residual WBCs

Samples were prepared according to the manufacturer's instructions included with the BD Leucocount kit. The kit includes BD Trucount tubes containing a lyophilized pellet with a predefined number of counting beads (~50,000 beads per tube). BD Leucocount™ reagent contains RNase for enzymatic digestion of RNA and a detergent that lyses red cells and that permeabilizes the WBC membrane. The tubes also contain propidium iodide to stain the nuclear DNA of white blood cells. Four hundred microliters of BD Leucocount reagent was mixed with 100 μl of leucoreduced PLTs or RBCs in a BD Trucount tube. Fluorescently labeled samples were vortexed and incubated in the dark for 5 min. BD Leucocount Control cells (R&D Systems), were treated the same as a specimen sample and were stained with the BD Leucocount kit in the same way. Three production lots of BD Leucocount kit were used in this study to prepare fluorescently labeled samples for flow cytometric analysis.

In the method comparison evaluation, for each PLT and RBC specimen, a total of four tubes of samples stained with BD Leucocount reagent were prepared for acquisition, with two tubes for the BD FACSVia System and two tubes for the BD FACSCalibur system. If prepared samples were not analyzed on cytometers immediately, they were kept at room temperature protected from light. Sample subsequently were analyzed within 24 h if PLTs and RBCs were prepared within 24 h of leucoreduction, or analyzed within 60 min if they were stained within 48 h of leucoreduction. In the inter-laboratory reproducibility evaluation, PLT and RBC control cells containing two concentration levels of WBCs were fluorescently labeled with BD Leucocount reagent in duplicated BD Trucount tubes per level. The stained and prepared BD Leucocount Control cell samples were analyzed on the BD FACSVia System within 60 min after preparation.

On the BD FACSVia System, optimized instrument settings were predetermined by the BD Leucocount test definition in BD FACSVia clinical software, at which labeled cells and beads can be readily identified and gated. Fluorescence compensation settings were preset to 0.0% for the Leucocount test definition. To best distinguish leucocyte signals from noise, the threshold of the BD Leucocount assay was automatically set at 2,500 for FL2-H (585/40 nm) in the BD FACSVia clinical software. For each sample, a fixed number of 10,000 bead events was counted as stopping criteria. The BD Leucocount test definition provides a locked Leucocount template with gates in place to view labeled cells and beads during acquisition and analysis. An FL1 (533/30 nm) vs. propidium iodide (FL2) dot plot is used in the template. The BD Trucount beads region is labeled for bead percentage and the WBCs region is labeled for the WBC% in total events in this dot plot. In our study, gating regions were adjusted manually by operators when necessary for optimal identification of WBCs and BD Trucount beads. Absolute counts of WBCs were determined automatically by BD FACSVia clinical software, and a report was generated after optimal gating was set for sample analysis. The BD FACSVia loader was used at each of the four study sites for high-throughput operation.

On the BD FACSCalibur instruments with BD CellQuest™ software, analysis of list-mode data was performed using the CellQuest template with an FL1 vs. propidium iodide (FL2) dot plot. A threshold of 300 was set on the FL2 fluorescence channel. A threshold of 332 was set on the FL3 fluorescence channel. Regions were set around the population of beads (R1) and the population of WBCs (R2), and they were adjusted manually as needed. For each sample, a fixed number of 10,000 bead events were counted as stopping criteria. The number of WBCs per microliter was calculated following the equation using the expression editor: $\frac{n \text{ events in R2} \times n \text{ beads in a Trucount tube}}{n \text{ events in R1} \times \text{volume of blood component added}}$.

Statistical Analysis

Data were analyzed with the SAS v9.3 program (SAS, Cary, NC), Analyse-it software (Analyse-it, Leeds, UK), and Microsoft® Excel software (Microsoft, Redmond, WA).

Method comparison. A total of 252 PLT and 278 RBC evaluable specimens were analyzed for the absolute count of WBCs measured on the BD FACSVia and BD FACSCalibur Systems. Statistical analysis was performed separately for each specimen type (PLT and RBC). No outliers were detected in the method comparison data (27). Data were also tested to fulfill a normal distribution in the entire WBC range, as well as in each WBC bin. Deming regression was performed in which WBC absolute counts from the test method (BD FACSVia System) were compared to the reference method (BD FACSCalibur) to determine analytical accuracy in the range of 0–350 WBCs/μl. Deming regression analysis demonstrates both constant and proportional bias using intercept and slope parameters.

To analyze and visually display the agreement between test and reference variables, especially for samples with low WBC counts, Bland–Altman plots were used to demonstrate constant bias between the BD FACSVia and BD FACSCalibur Systems. The BD FACSCalibur is considered the gold standard because it is an established and widely used method in clinical laboratories worldwide.

To further analyze samples in each WBC bin, difference between the two systems was determined by estimating the absolute and relative biases. The difference in WBC absolute count was calculated for each donor and pooled across donor samples in each WBC bin to generate an absolute bias with a 95% confidence interval (CI). The relative difference of the WBC absolute count of each donor sample was defined as the difference of the BD FACSVia output and BD FACSCalibur output divided by the BD FACSCalibur output times 100 (investigational – predicate/predicate × 100). The relative bias along with 95% CI was calculated by averaging the relative difference of donors based on the assignment for each bin and for all samples. Donor samples with 0 leucocytes per microliter on the BD FACSCalibur were excluded from calculation of relative bias.

Inter-laboratory reproducibility. A total of 40 acquisition runs (20 days × 2 replicates per run per day) was carried out on the BD FACSVia System per study site for each level of PLT and RBC control to evaluate precision. Analyses of variance were performed with the following random-effect factors: site, day, and within-run. Total reproducibility was calculated to include site, day, and within-run variability. The standard deviations (SDs) with 95% upper CIs and percent coefficient of variation (CV%) with 95% upper CIs were calculated for each factor, along with total reproducibility.

Results

Method Comparison Study

Regression analysis. Figure 1 shows Deming regression plots with regression slope, intercept, and R^2 for the whole WBC range (0–350 WBCs/μl) for PLT and RBC samples. Our four-center study demonstrated that BD Leucocount reagent with BD Trucount beads gave equivalent WBC absolute counts for the PLTs and RBCs on the two systems. The

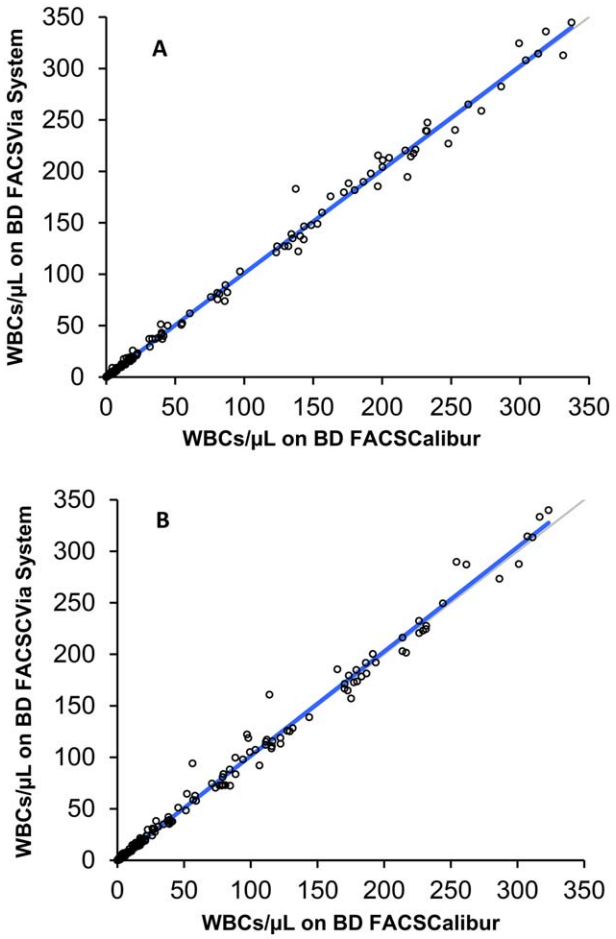

Figure 1. Method comparison of rWBCs in PLTs (**A**, $n = 252$) and RBCs (**B**, $n = 278$). (A) The intercept (95% CI) was 0.09 (–0.28, 0.45); the slope (95% CI) was 1.01 (0.99, 1.03); and R^2 was 1.00. (B) The intercept (95% CI) was 0.13 (–0.36, 0.61); the slope (95% CI) was 1.01 (0.99, 1.03); and R^2 was 0.99. The bold blue line indicates Deming regression fit. The thin line is the line of identity.

Deming regression results between the BD FACSVia and BD FACSCalibur Systems met study criteria, which were that the 95% CI of the regression slope shall be 0.9–1.1, R^2 shall be $\geq$0.90, and regression intercept shall be within $\pm$3 WBCs/µl.

Bland–Altman plots. In the areas of interest for blood banks, samples with WBCs <25 cells/µl were analyzed using the Bland–Altman method. Within 252 PLTs and 278 RBCs, there were 184 PLTs and 193 RBCs containing WBCs <25 cells/µl. The Bland–Altman test was applied to these samples to show the absolute bias between the BD FACSVia and BD FACSCalibur Systems with 95% limits of agreement, as presented in Figure 2. The absolute bias of 184 PLTs was 0.136 cells/µl with 95% limits of agreement (bias $\pm$1.96 SD) of $-$1.897 and 2.169 cells/µl, respectively. Similarly, Bland–Altman analysis of the 193 RBCs revealed an absolute bias of 0.170 cells/µl and 95% limits of agreement of $-$2.025 and 2.365 cells/µl, respectively.

The bias of the WBC absolute count between the two systems was further analyzed for samples in each WBC bin and for the overall method comparison data. Table 2 presents the analysis results. In the first low bin, WBC <1 cell/µl, the absolute bias was 0.07 cells/µl (0.02, 0.12) for PLT ($n = 56$) and $-$0.03 cells/µl (–0.08, 0.02) for RBC ($n = 59$). The high relative bias in the first low bin was due to very small denominators (WBC <1 cell/µl) in the calculation and was therefore not suitable to be used in bias estimation. In the next low leucocytes bin, in which residual leucocytes were >1 cell/µl but <5 cells/µl, the absolute bias was $-$0.05 cells/µl (–0.21, 0.11), relative bias 0.61% (–4.67%, 5.88%) for PLT ($n = 61$). In this bin, for RBC samples ($n = 72$), the absolute bias was 0.11 cells/µl (–0.08, 0.31), and the relative bias 5.73% (–1.75%, 13.20%). To meet standards proposed by the Council of Europe that the residual leucocyte number be $<1 \times 10^6$ per unit in leucoreduced blood components, equating to <3.3 cells/µl, a sensitive leucocyte enumeration method is critical

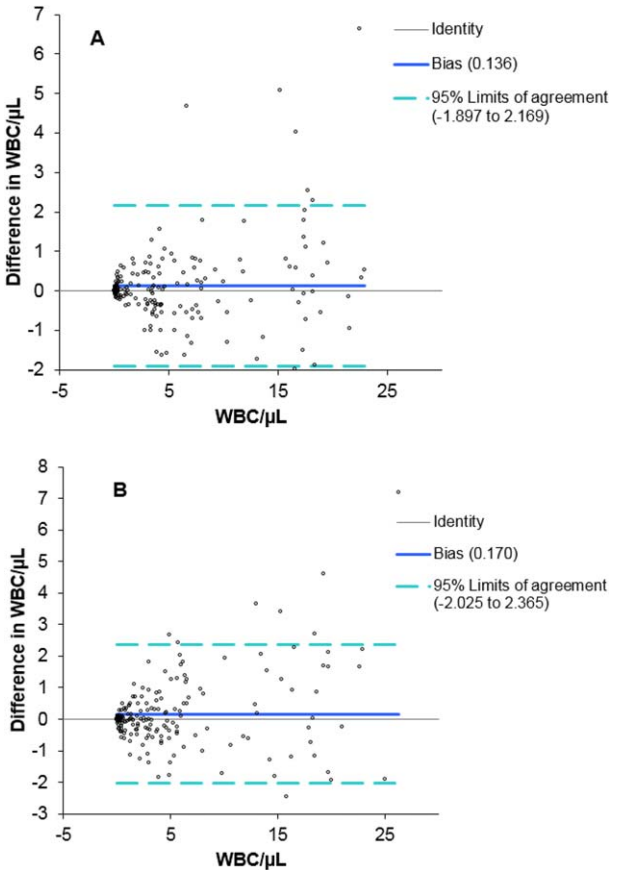

Figure 2. For samples containing WBCs <25 cells/µl, Bland–Altman plots showing the absolute difference of the WBC absolute count in PLTs (**A**, $n = 184$) and RBCs (**B**, $n = 193$). *Y*-axis: the difference in WBC absolute count between test (BD FACSVia) and predicate (BD FACSCalibur) was calculated as (WBCs/µl in BD FACSVia—WBCs/µl in BD FACSCalibur). *X*-axis: the mean absolute count of WBCs from donor samples measured on the BD FACSCalibur and BD FACSVia System. The absolute differences between BD FACSVia and BD FACSCalibur were calculated for each donor sample and pooled across all donor samples to estimate the bias for PLTs and RBCs, respectively. 95% Limits of agreement was defined as bias $\pm$1.96 standard deviation of the difference.

Table 2. Summary of bias for the PLT and RBC samples

SAMPLE TYPE	BIN (RANGE)	*N*	ABSOLUTE BIAS WITH 95% CI	RELATIVE %BIAS WITH 95% CI
PLT	$0 < \text{rWBC} < 1$	56 [49][a]	0.07 (0.02, 0.12)	46.47 (13.31, 79.61)
	$1 \leq \text{rWBC} < 5$	61	−0.05 (–0.21, 0.11)	0.61 (–4.67, 5.88)
	$5 \leq \text{rWBC} < 25$	67	0.36 (–0.03, 0.75)	3.29 (–1.07, 7.66)
	$25 \leq \text{rWBC} < 350$	68	1.33 (–1.18, 3.84)	1.43 (–0.49, 3.35)
	All PLT samples	252 [245][a]	0.46 (–0.22, 1.14)	10.74 (3.78, 17.71)
RBC	$0 < \text{rWBC} < 1$	59 [48][a]	−0.03 (–0.08, 0.02)	−0.16 (–20.62, 20.30)
	$1 \leq \text{rWBC} < 5$	72	0.11 (–0.08, 0.31)	5.73 (–1.75, 13.20)
	$5 \leq \text{rWBC} < 25$	62	0.42 (–0.02, 0.87)	3.33 (–0.38, 7.05)
	$25 \leq \text{rWBC} < 350$	85	1.84 (–0.56, 4.23)	2.56 (0.02, 5.10)
	All RBC samples	278 [267][a]	0.68 (–0.06, 1.41)	3.10 (–1.06, 7.27)

The absolute bias was calculated as the difference in WBC absolute count of each donor on the two instruments and pooled across donor samples in each WBC bin to generate an absolute bias with a 95% confidence interval (CI). The relative difference of the WBC absolute count of each donor sample was defined as: (investigational – predicate)/predicate × 100. The relative bias along with the 95% CI was calculated by averaging the relative difference of donors based on the assignment for each bin.

[a]In the calculation of relative bias, donor samples with 0 leucocytes per microliter on the BD FACSCalibur system were excluded from the calculation. The number in brackets was used to calculate relative bias.

to accurately count low levels of leucocytes. Our results in the first two low bins demonstrated that the BD FACSVia flow cytometer meets sensitivity requirements in residual leucocyte enumeration compared to the BD FACSCalibur instrument.

In the third leucocytes bin, in which residual leucocytes were >5 cells/μl but <25 cells/μl, the absolute bias for PLTs ($n = 67$) was 0.36 cells/μl (95% CI −0.03, 0.75), and the relative bias was 3.29% (–1.07%, 7.66%). For the leucoreduced RBC samples ($n = 62$), the absolute bias was 0.42 cells/μl (95% CI −0.02, 0.87) and the relative bias was 3.33% (–0.38%, 7.05%). This bin corresponds to the US standards for leucoreduced blood components, in which $<5 \times 10^6$ WBCs are required per unit. In the fourth bin ($25 \leq \text{WBC} < 350$ cells/μl), the BD FACSVia System had low relative bias for both PLTs and RBCs compared to the predicate, the BD FACSCalibur system.

Inter-Laboratory Reproducibility Study

Table 3 shows summary results of the SD and CV% with upper 95% CI of each variance component and total reproducibility. The inter-laboratory reproducibility results on four BD FACSVia Systems conducted at three study centers met our study expectation, which was that total CV% with 95% CI shall be ≤10% for the high controls and ≤30% for the low controls.

Discussion

In the method comparison study, we evaluated the BD Leucocount assay results on the new BD FACSVia System in comparison with the BD FACSCalibur system at four study centers using 252 PLT and 278 RBC samples for the range of 0–350 WBCs/μl. Within these leucoreduced blood products, 184 PLT and 193 RBC samples contained <25 WBCs/μl.

Table 3. Summary of inter-laboratory reproducibility on the BD FACSVia System

LEVEL	WBC MEAN (CELLS/μl)	SOURCE OF VARIANCE	SD WITH UPPER 95% CI	CV% WITH UPPER 95% CI
PLT high control	16.49	Site	0.30 (1.32)	1.82 (3.99)
		Day	0.63 (0.74)	3.81 (4.40)
		Within-run	0.81 (0.96)	4.89 (5.63)
		Total	1.07 (1.19)	6.46 (7.16)
PLT low control	7.30	Site	0.09 (0.40)	1.24 (2.73)
		Day	0.33 (0.39)	4.54 (5.25)
		Within-run	0.60 (0.71)	8.24 (9.50)
		Total	0.69 (0.78)	9.49 (10.52)
RBC high control	17.10	Site	0.48 (2.12)	2.80 (6.15)
		Day	0.79 (0.93)	4.59 (5.31)
		Within-run	0.90 (1.06)	5.24 (6.04)
		Total	1.28 (1.44)	7.51 (8.32)
RBC low control	6.76	Site	0.12 (0.53)	1.76 (3.87)
		Day	0.19 (0.22)	2.74 (3.16)
		Within-run	0.69 (0.82)	10.25 (11.82)
		Total	0.73 (0.81)	10.76 (11.92)

SD, standard deviation; CV, coefficient of variation.

Although leucocyte counting on samples with low WBC concentration is the major interest for blood banks and clinical test laboratories, the fourth WBC bin in Table 2 may resemble use cases in blood centers when leucodepletion filters malfunction and blood components fail quality control. Detection of PLT and RBC samples in the fourth bin on flow cytometers can be part of the laboratory workflow, although high WBC counts can also be obtained using a hematology analyzer.

We analyzed method comparison data using Deming regression and Bland–Altman methods for the whole WBC range, as well as for samples with <25 WBCs/µl. In addition, we further divided PLTs and RBCs into four WBC bins and analyzed agreement results (as measured by bias) between BD FACSVia System and BD FACSCalibur instrument. Our validation results demonstrated that for low to extremely low levels of leucocytes, the BD FACSVia System can provide results as accurate as the BD FACSCalibur system for leucoreduced PLT and RBC samples.

Counting low levels of WBCs in PLTs and RBCs with high precision has been a challenging task (28,29). A cross-comparison study demonstrated that BD Trucount bead tubes in combination with BD Leucocount reagent could improve precision, especially for counting low levels of WBCs (0–3 cells/µl) in leucoreduced PLTs and RBCs (18). This is because BD Trucount bead tubes contain a lyophilized bead pellet with a predetermined number of beads. The procedure requires only a single pipetting step during sample preparation and hence reduces overall assay variability. Our inter-laboratory reproducibility data in Table 3 demonstrated that on the new BD FACSVia System using BD Leucocount reagent with BD Trucount beads, we could achieve satisfactory reproducibility results for the absolute counts of WBCs in leucoreduced PLTs and RBCs across three sites.

Leucocyte removal from blood components before transfusion by leucodepletion filters prevents or at least delays leucocyte mediated adverse reactions. It also reduces risks of related infectious diseases caused by transfusion. Doing adequate process validation and providing routine monitoring of leucodepleted blood components using robust automatic cell counting methods are essential to ensure consistent production of high quality leucoreduced blood components. In these applications the BD FACSVia System provides a novel and effective flow cytometry solution.

Acknowledgments

The authors thank Jeannine Paliotta and Reika Nakari for data management support, Laurie Byrne for sample management support, and Charlene Bush-Donovan for reviewing the manuscript.

Conflict of Interest

Seven authors are employees of BD Life Sciences.

Literature Cited

1. Bassuni WY, Blajchman MA, Al-Moshary MA. Why implement universal leucoreduction. Hematol Oncol Stem Cell Ther 2008;1:106–123.
2. Pruss A, Kalus U, Radtke H, Koscielny J, Baumann-Baretti B, Balzer D, Dörner T, Salama A, Kiesewetter H. Universal leukodepletion of blood components results in a significant reduction of febrile non-hemolytic but not allergic transfusion reactions. Transfus Apher Sci 2004;30:41–46.
3. Blajchman MA. The clinical benefits of the leukoreduction of blood products. J Trauma 2006;60:S83–S90.
4. Wenz B, Gurtlinger KF, O'Toole AM, Dugan EP. Preparation of granulocyte-poor red blood cells by microaggregate filtration: A simplified method to minimize febrile transfusion reactions. Vox Sang 1980;39:282–287.
5. Sniecinski I, O'Donnell MR, Nowicki B, Hill LR. Prevention of refractoriness and HLA-alloimmunization using filtered blood products. Blood 1988;71:1402–1407.
6. Brand A. Alloimmune platelet refractoriness: Incidence declines, unsolved problems persist. Transfusion 2001;41:724–726.
7. Andreu G, Dewailly J, Leberre MC, Quarre MC, Bidet ML, Tardivel R. Prevention of HLA immunization with leukocyte-poor packed red cells and platelet concentrates obtained by filtration. Blood 1988;72:964–969.
8. de Graan-Hentzen YCE, Gratama JW, Mudde GC, Verdonck LF, Houbiers JG, Brand A, Sebens FW, van Loon AM, The TH, Willemze R, et al. Prevention of primary cytomegalovirus infection in patients with hematologic malignancies by intensive white cell depletion of blood products. Transfusion 1989;29:757–760.
9. Orlin JB, Ellis MH. Transfusion-associated graft-versus-host disease. Curr Opin Hematol 1997;4:442–448.
10. Raife TJ. Adverse effects of transfusions caused by leucocytes. J Intraven Nurs 1997; 20:238–244.
11. Dumont LJ, Luka J, VandenBroeke T, Whitley P, Ambruso DR, Elfath MD. The effect of leukocyte-reduction method on the amount of human cytomegalovirus in blood products: A comparison of apheresis and filtration methods. Blood 2001;97:3640–3647.
12. Roback JD, Grossman BJ, Harris T, Hillyer CD. Technical Manual of the American Association of Blood Banks, Karger Publishing, 17th ed.; 2011.
13. European Committee (Partial Agreement) on Blood Transfusion. Guide to the Preparation, Use and Quality Assurance of Blood Components, 18th ed. Strasbourg, France: Council of Europe Publishing; 2015.
14. Kelsey P. Guidelines for the use of platelet transfusion by British Committee for Standards in Haematology and Blood Transfusion Task Force. Br J Haematol 2003; 122:10–23.
15. Napier A. British Committee for Standards in Haematology, Blood Transfusion Task Force Guidelines on the clinical use of leucocyte-depleted blood components. Transfus Med 1998;8:59–71.
16. Conte R, Bontadini A, Cirillo D, Fruet F. Process control of filtered red blood cells: Which counting method? Transfus Med 1997;7:217–219.
17. Barnett D, Goodfellow K, Ginnever J, Granger V, Whitby L, Reilly JT. Low level leucocyte counting: A critical variable in the validation of leucodepleted blood transfusion components as highlighted by an external quality assessment study. Clin Lab Haematol 2001;23:43–51.
18. Dijkstra-Tiekstra MJ, Schrijver JG, van der Meer PF, Laport RF, Gratama JW, Levering WHBM, van Delden CJ, de Wildt-Eggen J. Crossover study of three methods for low-level white blood cell counting on two types of flow cytometer. Cytometry Part B 2003;54B:39–45.
19. van der Meer PF, Gratama JW, van Delden CJ, Laport RF, Levering WH, Schrijver JG, Tiekstra MJ, Keeney M, de Wildt-Eggen J. Comparison of five platforms for enumeration of residual leukocytes in leukoreduced blood components. Br J Haematol 2001;115:953–962.
20. Dzik S, Moroff G, Dumont L. A multicenter study evaluating three methods for counting residual WBCs in WBC-reduced blood components: Nageotte haemocytometry, flow cytometry, and microfluorimetry. Transfusion 2000;40:513–520.
21. Castegnaro S, Dragone P, Chieregato K, Alghisi A, Rodeghiero F, Astori G. Enumeration of residual white blood cells in leukoreduced blood products: Comparing flow cytometry with a portable microscopic cell counter. Transfus Apher Sci 2016;54:266–270.
22. Rebulla P, Dzik WH. Multicenter evaluation of methods for counting residual white cells in leukocyte-depleted red blood cells. The Biomedical Excellence for Safer Transfusion (BEST) Working Party of the International Society of Blood Transfusion. Vox Sang 1994;66:25–32.
23. Krailadsiri P, Seghatchian J, Rigsby P, Bukasa A, Bashir S. A national quality assessment scheme for counting residual leucocytes in unfixed leucodepleted products: The effect of standardization and 48 hour storage. Transfus Apher Sci 2002;26:73–81.
24. Rebulla P, Porretti L, Bertolini F, Marangoni F, Prati D, Smacchia C, Pappalettera M, Parravicini A, Sirchia G. White cell-reduced red cells prepared by filtration: A critical evaluation of current filters and methods for counting residual white cells. Transfusion 1993;33:128–133.
25. Budd JR, Durham AP, Gwise TE, Iriarte B, Kallner A, Linnet K. Clinical and Laboratory Standards Institute Guideline EP09-A3: Measurement Procedure Comparison and Bias Estimation Using Patient Samples. Approved Guideline, 3rd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2013.
26. McEnroe RJ, Durham AP, Goldford MD, et al. Clinical and Laboratory Standards Institute Guideline EP05-A3: Evaluation of Precision of Quantitative Measurement Procedures. Approved Guideline, 3rd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2014.
27. Krouwer JS, Tholen DW, Garber CC, Goldschmidt HMJ, Kroll MH, Linnet K, et al. Clinical and Laboratory Standards Institute Guideline EP09-A2: Method Comparison and Bias Estimation Using Patient Samples; Approved Guideline, 2nd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2002.
28. Dzik WH, Ragosta A, Cusack WF. Flow-cytometric method for counting very low numbers of leukocytes in platelet products. Vox Sang 1990;59:153–159.
29. Vachula M, Simpson SJ, Martinson JA, Aono FM, Hutchcraft AM, Balma DL, Van Epps DE. A flow cytometric method for counting very low levels of white cells in blood and blood components. Transfusion 1993;33:262–267.