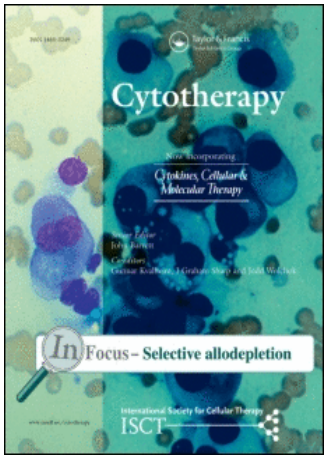

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An internal positive control for the enumeration of CD45$^+$ and CD34$^+$ cells by flow cytometry allows monitoring of reagent and operator performance

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Background

Three-color flow cytometry assays are used to determine CD34$^+$ cell doses prior to stem cell transplantation. These assays require high-quality reagents that are dispensed accurately to ensure reproducible results. We have developed a flow cytometry assay for CD34$^+$ cells with an integral positive control (KG1a cells) for monitoring reagent and operator performance.

Methods

The method was validated using samples from 127 allogeneic donations (42 BM, 85 PBSC) from healthy donors and 195 autologous donations (46 BM, 149 PBSC) from patients in remission from hematologic malignancies. The mean, SD and range of CD45$^+$ and CD34$^+$ cell counts were determined for each donation type. An internal control was used to assess performance of reagents and operators by comparison with a predetermined target value and an experienced operator.

Results

Replicate studies showed the method to be accurate and precise, with KG1a cells at 97.7 ± 3.9% of the target value and a CV of 4.0%. In routine use over 322 samples, the accuracy was 91.7 ± 17.7% of the target value, with a CV of 19.3%. Investigations into the cause of the reduced precision showed that reagents performed consistently well but operator performance was variable, with two of six operators significantly under-dispensing KG1a cells.

Discussion

This study validates our three-color flow cytometry assay and demonstrates that KG1a cells may be used to monitor test performance in the routine working environment. In addition to monitoring performance within a single laboratory, its wider use in multicenter studies may be helpful regarding standardization of methods.

Keywords

CD34+, flow cytometry.

Introduction

The use of flow cytometry to determine CD34$^+$ cell doses prior to HPC transplantation is well established [1–5]. The accuracy of the methods has been improved with the introduction of standardized guidelines based on the ISHAGE gating strategy. This uses the low CD45 expression of CD34$^+$ HPC and their forward (FSC) and side (SSC) scatter characteristics to improve discrimination from other cells and cell debris [6,7]. The use of the vital dye, 7-aminoactinomycin-D (7-AAD), has allowed non-viable cells to be identified and excluded from dose calculations [8,9]. This modification has proved particularly important where storage, transit and manipulation of HPC collections may affect cell viability. The complexity of modern flow cytometry assays that use three or more fluorescent markers to identify cell populations makes day-to-day assessment of reagents used in the assay important, particularly where a single-platform, lyse no-wash method [8,10] is used, as recommended in the European reference protocol for quality assessment of autologous collections [11].

Preserved KG1a cells (Stem-Trol™ control cells; Beckman-Coulter, High Wycombe, UK) express the

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CD34 class III epitope and the CD45 leukocyte common Ag at densities that approximate normal immature human hematopoietic cells [12], and are used as a control to validate CD34 flow cytometry assays. However, this does not assess performance in subsequent assays where variation in operator dispensing of samples and reagents can affect results. We have developed a modified single-platform lyse no-wash protocol based on the ISHAGE gating strategy with 7-AAD, using KG1a cells as an integral control. The method allows enumeration of $CD45^+$ and $CD34^+$ cells and provides an internal quality control in each individual assay, thereby confirming accurate dispensing and reagent performance.

Methods

All BM and PBSC donations were collected and tested with written, fully informed consent for processing, testing and subsequent storage of the stem cells. Anti-coagulated (Na_2EDTA) samples from 127 healthy allogeneic hematopoietic stem cell transplant (HSCT) collections (42 BM and 85 mobilized PBSC) and 195 autologous HSCT collections (46 BM and 149 PBSC) from patients with hematopoietic malignancies in remission were tested to determine the WBC count using the LH750 hematology analyzer (Beckman-Coulter) and by flow cytometry to determine $CD45^+$ and $CD34^+$ cell counts, as outlined below.

Flow cytometry

Three tubes containing FITC-conjugated CD45-reactive Ab (10 μL, clone J33; Beckman-Coulter) and the vital dye 7-AAD (10 μL, 0.4 mg/mL (w/v); Molecular Probes, Paisley, UK) were prepared as described in Table 1, using air-displacement pipettes with a 'wet tip' dispensing technique [13]. PE-conjugated CD34 Ab (10 μL, clone 8G12; Becton-Dickinson, Plymouth, UK) was added to two of the tubes. One of the two $CD34^+$ test tubes was spiked with 10 μL non-viable KG1a cells (Stem-Trol™; Beckman-Coulter). The remaining tube contained mouse γ1 Ab (5 μL, clone X40; Becton-Dickinson) conjugated with PE as an isotype control. Following the addition of 50 μL diluted cells (maximum 1×10^6 cells/tube), the tests were incubated in the dark at room temperature for 20 min before contaminating red cells were lysed with ammonium chloride solution (IOTEST III; Beckman-Coulter) for 10 min. Flowcount™ microbeads (50 μL; Beckman-Coulter) were added to each tube immediately prior to reading on a Cytomics FC500 flow cytometer (Beckman-Coulter) using RXP/CXP software and the gating strategy outlined in Figure 1. The mean $CD45^+$ and $CD34^+$ cell counts in the replicate tubes were calculated by direct counting of events in the A and P gates (Figure 1), respectively. Background events were identified using the isotype control and subtracted from the CD34 cell counts.

Quality control

The flow cytometer was subjected to daily quality control checks using Flow-Check™ Fluorospheres (Beckman-Coulter), as recommended by the manufacturer. KG1a cell counts were determined by direct counting of non-viable cells falling into the Stem-Trol™ gate in test 2 and subtracting other non-viable CD34 cells present in test 1 (Table 1). To compensate for the different volumes of KG1a cells and Flowcount™ microbeads used (10 μL vs. 50 μL), the KG1a cell count was multiplied by five. The suppliers of the KG1a cells specify a target value of 1250 cells/μL with an allowable variation of ± 15% (1062.5–1437.5 cells/μL). These were adopted as target values to measure the reliability of the assay. For the purpose of this study, all results were accepted, even where KG1a cell counts fell outside the target range, so that factors that might affect the accuracy and precision of the method could be investigated. The factors investigated included

Table 1. Reagents used in the modified three-color flow cytometry assays

	Flow cytometry channel			
Tube	FL1	FL2	FL4	KG1a cells
Isotype	CD45–FITC(J33)	Mouseγ1–PE (X40)	7-AAD	–
Test 1	CD45–FITC (J33)	CD34–PE (8G12)	7-AAD	–
Test 2	CD45–FITC (J33)	CD34–PE (8G12)	7-AAD	+

Tests 1 and 2 are replicates, with non-viable KG1a cells added as an integral control in test 2 only.

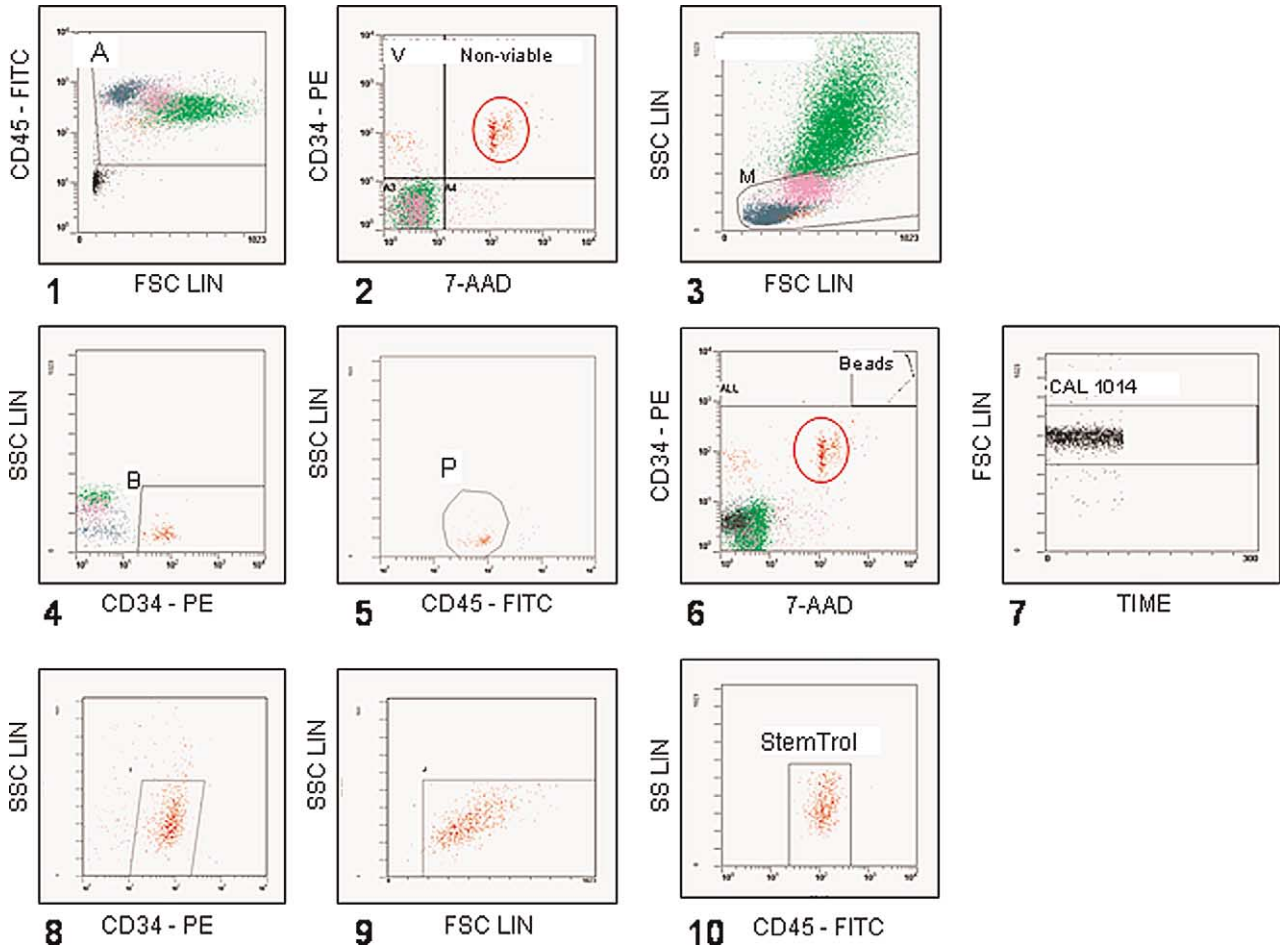

***Figure 1**. Gating strategy for enumeration of viable CD45$^+$ and CD34$^+$ cells. Viable CD34$^+$ progenitor cells (P gate) were defined through sequential gating of CD45-positive (A gate, plot 1), viable (V gate, plot 2), low SSC and low to medium FSC characteristics (M gate, plot 3), CD34-positive (B gate, plot 4) and low SSC and low CD45 expression (P gate, plot 5). Non-viable KG1a control cells are circled in red. Gates are included within the protocol for color tracking (data not shown). KG1a control cells were sequentially gated through CD45 expression (gate A, plot 1), non-viable (7-AAD positive; plot 2), CD34-positive (plot 8), SSC and FSC characteristics (plot 9) and low CD45 expression (plot 10). Flowcount™ microbeads were gated in plots 6 and 7 and used to allow direct counting as described by the manufacturers.*

(1) collection type, (2) dispensing accuracy of the operator, (3) KG1a cell batch and (4) Flowcount™ microbead batch.

Accuracy and precision studies

The accuracy and precision of the method were first assessed using 10 µL aliquots of standardized KG1a cells in eight replicate tests of a PBSC collection by an experienced operator (operator 1). Secondly, six different operators each tested 10 µL KG1a cells with two PBSC collections (2a and 2b). In addition to the KG1a cells, the accuracy and precision were also assessed by direct comparison of CD45$^+$ cell counts with WBC counts determined using the LH750 hematology analyzer, and through analysis of the CD45/WBC ratio.

Statistics

Results are expressed as mean $\pm$ SD. Statistical analyzes used an unpaired two-tailed *t*-test to compare two means. A *P*-value of < 0.05 was considered significant, < 0.01 very significant and < 0.001 extremely significant. The coefficient of variation (CV) was determined by dividing the SD by the mean, and was expressed as a percentage.

Results

The first study of eight replicate tests performed by a single operator showed excellent accuracy and precision,

Table 2. Results of replicate and routine sample tests showing the accuracy and precision of the method

	WBC $\times 10^6$/mL	CD45 $\times 10^6$/mL	CD34 $\times 10^6$/mL	CD45/WBC	KG1a cells/μL	% target KG1a cells
Precision study 1 ($n=8$)						
Min.	176.0	168.0	1.7	0.93	1135.0	90.8
Max.	183.0	191.0	2.0	1.08	1280.0	102.4
Mean	180.3	183.9	1.8	1.02	1220.6	97.7
SD	2.1	7.6	0.1	0.04	49.1	3.9
CV (%)	1.2	4.2	6.1	4.37	4.0	4.0
Precision study 2a ($n=6$)						
Min.	244.0	229.0	5.6	0.90	1120	89.6
Max.	254.0	273.1	6.4	1.12	1455	116.4
Mean	250.7	253.9	6.0	1.01	1238.5	99.1
SD	5.2	14.8	0.3	0.07	116.5	9.3
CV (%)	2.1	5.8	4.5	7.17	9.4	9.4
Precision study 2b ($n=6$)						
Min.	187.0	186.5	2.5	0.91	1180	94.4
Max.	205.0	204.8	2.8	1.03	1280	102.4
Mean	199.3	194.8	2.6	0.98	1234	98.7
SD	7.2	6.6	0.1	0.05	38	3.0
CV (%)	3.6	3.4	4.1	4.85	3.1	3.1
BM collection ($n=88$)						
Min.	4.2	2.7	0.0	0.53	455	36.4
Max.	53.0	46.0	1.1	1.14	2370	189.6
Mean	17.1	15.1	0.2	0.88	1152.5	92.2
SD	8.5	7.6	0.2	0.12	277.4	22.2
CV (%)	NA	NA	NA	13.64	24.1	24.1
PBSC collection ($n=234$)						
Min.	25.1	28.3	0.1	0.20	520	41.6
Max.	461.0	487.2	63.3	1.26	1800	144
Mean	280.9	297.9	3.4	1.06	1144.6	91.6
SD	82.0	89.0	5.4	0.09	196.4	15.7
CV (%)	NA	NA	NA	8.49	17.1	17.1

Study 1 shows the results of eight replicate tests on a single PBSC sample by a single experienced operator (operator 1). Study 2 shows the results of two PBSC samples (2a and 2b) each tested once by six different operators. Aliquots, 10 μL, of KG1a control cells were used in studies 1 and 2, and demonstrated excellent accuracy and precision. As a consequence this volume was used for the remaining BM ($n=88$) and PBSC ($n=234$) collections tested.

with KG1a cells at $97.7 \pm 3.9\%$ of the target value at 1220.6 ± 49.1 cells/μL (Table 2). All replicates were within $\pm 15\%$ of the target (ranging between 1135 and 1280 cells/μL), with a CV of 4.0%. The counts were $183.9 \pm 7.6 \times 10^6$/mL for $CD45^+$ cells and $1.8 \pm 0.1 \times 10^6$/mL for $CD34^+$ cells and showed good reproducibility, with a CV of 4.2% and 6.1%, respectively. The $CD45^+$ cell counts were slightly higher than the WBC counts (183.9 ± 7.6 vs. $180.3 \pm 2.1 \times 10^6$/mL), although this difference did not reach statistical significance ($P = 0.2343$).

The second study, using 10 μL aliquots of KG1a cells with two PBSC samples (2a and 2b) tested once each by six different operators, also showed high accuracy and precision (Table 2). The mean KG1a cells were close to the target value, at 1238.5 ± 116.5 cells/μL ($99.1 \pm 9.3\%$ of target) and 1234.0 ± 38.0 cells/μL ($98.7 \pm 3.0\%$ of target) for samples 2a and 2b, respectively. Only one of the 12 tests showed a KG1a cell count outside the target range, at 1455 cells/μL ($+16.4\%$ of the target value). The CV for CD45, CD34 and KG1a cell counts ranged between 3.1% and 9.4%.

The $CD45^+$ and $CD34^+$ cell counts obtained for the 88 BM and 234 PBSC collections are also summarized in Table 2. The BM collections had $CD45^+$ and $CD34^+$ cell counts of $15.1 \pm 7.6 \times 10^6$/mL and $0.2 \pm 0.2 \times 10^6$/mL, respectively, whereas the PBSC collections had $CD45^+$ cell counts of $297.9 \pm 89.0 \times 10^6$/mL and $CD34^+$ cell counts of $3.4 \pm 5.4 \times 10^6$/mL. The cell counts were within expected ranges for both BM and PBSC collections [14,15] and showed that the total $CD34^+$ cell content of PBSC donations was approximately twice that obtained from BM for both allogeneic (410.5 ± 296.0 vs. $199.1 \pm 107.0 \times 10^6$) and autologous ($246.1 \pm 312.6$ vs. $136.3 \pm 97.2 \times 10^6$) donations.

The accuracy and precision were assessed further through a direct comparison of WBC and $CD45^+$ cells counts (Table 2). This showed BM collections had higher WBC counts ($17.1 \pm 8.5 \times 10^6$/mL) than $CD45^+$ cell counts ($15.1 \pm 7.6 \times 10^6$/mL), which probably reflected the presence of nucleated red blood cells that do not express CD45 in the BM collections that are identified as WBC by the hematology analyzer [7]. A review of 10 films from BM collections indicated that nucleated RBC represented $15.1 \pm 5.7\%$ (range 8.3–23.7%) of the total nucleated cells present. For PBSC collections, the WBC counts were on average 6.1% lower (Table 2) than the respective $CD45^+$ cell counts ($280.9 \pm 82.0 \times 10^6$/mL vs. $297.9 \pm 89.0 \times 10^6$/mL). This difference reached statistical significance ($P = 0.0322$) and suggested that our flow cytometry assay may have marginally overestimated $CD45^+$ cell counts compared with the hematology analyzer. Despite this, the difference was within the acceptable limits defined by the manufacturers and probably reflected variation in calibration of the instruments and deterioration or loss of Flowcount™ microbeads either in storage or during acquisition [16]. A plot of WBC against $CD45^+$ cell counts (Figure 2) confirmed a strong linear relationship between WBC and $CD45^+$ cells in PBSC donations, with slope $y = 1.061x$, and excellent data correlation (R^2), 0.9629. The CD45/WBC ratio (Table 2) was 1.06 ± 0.09, further indicating that CD45 cell counts were on average approximately 6% higher than WBC in PBSC collections. Interestingly, the CV was better for the CD45/WBC ratio than for the KG1a cells (8.49% vs. 17.1%), suggesting that CD45 cell counts were determined with greater precision than the KG1a cells used as an integral control.

A comparison of duplicate tubes in which one contained non-viable KG1a cells showed no significant difference in viable $CD34^+$ cells/μL ($y = 1.0298x$, $R^2 = 0.9928$). The incidence of cells positive with the isotype control was low (range 0 to 12 cells/μL) but occurred primarily with BM collections (2.0 ± 2.2 cells/μL) rather than PBSC (0.4 ± 0.8 cells/μL), representing $1.7 \pm 3.3\%$ of $CD34^+$ cells identified in BM and $0.7 \pm 1.9\%$ in PBSC collections.

Factors affecting accuracy and precision of KG1a cells

The mean KG1a control cell count for all 322 determinations of routine samples was 1147 ± 221 cells/μL. This was within 8.2% (103 cells/μL) of the target value of 1250 cells/μL specified by the manufacturer. Unfortunately, the CV was high, at 19.3%, suggesting the precision of KG1a

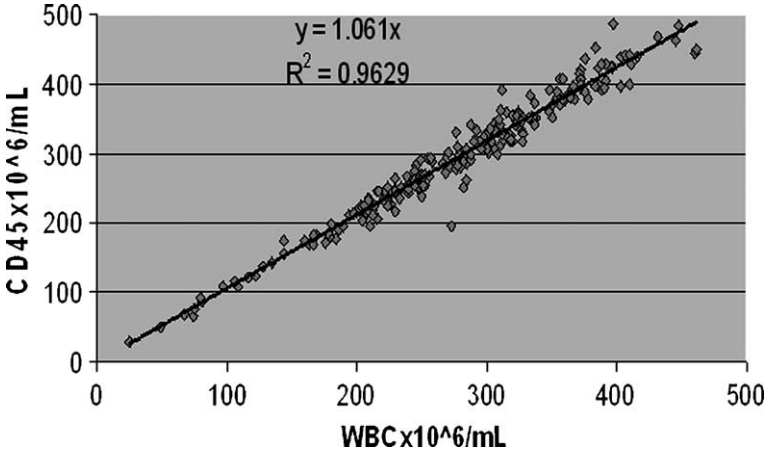

Figure 2. Scatter graph comparing the WBC count determined using the hematology analyzer, with $CD45^+$ cell count determined by our flow cytometry method for 234 PBSC collections.

cell enumeration was lower in routine use than in the initial accuracy and precision studies. In order to investigate the possible causes for the high CV, we reviewed our results for the different batches of critical reagents used (KG1a cells and Flowcount™ microbeads) and the operators performing the tests. Five batches of KG1a cells were used in the study, and their cell counts ranged between 1169 ± 204 and 1112 ± 244 cells/μL, suggesting acceptable consistency between control cell batches. Similarly, three different batches of Flowcount™ microbeads were used in the study and with these the KG1a cell counts ranged from 1110 ± 233 to 1202 ± 226 cells/μL, suggesting that the microbeads were also acceptable and provided consistent results between batches.

Six operators participated in the study; three (operators 1–3) were experienced at flow cytometry and three (operators 4–6) were relatively inexperienced. A comparison of KG1a control cell counts for experienced and inexperienced operators identified an extremely significant difference (1181 ± 198, $n = 210$, vs. 1082 ± 246 cells/μL, $n = 112$, $P = 0.0003$) between the two groups. Despite this, there was no difference in the CD45/WBC ratios for PBSC collections between the two groups (1.06 ± 0.06, CV = 5.7%, $n = 151$, vs. 1.06 ± 0.07, CV = 6.6%, $n = 83$, $P = 1.000$).

The KG1a control counts for the individual operators (Figure 3) showed that operator 1, who was the most experienced, obtained results closest to the target KG1a value, at 1219 ± 214 cells/μL (97.5% of the target value), and this was used to benchmark other results. Results from operators 2 and 3 were similar to operator 1, whereas results from the less experienced operators showed significant differences. Operator 4 performed 37 tests and averaged below the target range, at 1031.6 ± 204.6 cells/μL (82.5% of the target value), and was significantly different from operator 1 ($P = 0.0003$). Results for operator 5 were also significantly different ($P = 0.0067$) from operator 1, with a KG1a cell count of only 1071.6 ± 227.0 cells/μL (85.7% of the target value). Those for operator 6 were closer to the target values and were not significantly different from operator 1 ($P = 0.0986$), at 1123 ± 288 cells/μL (89.8% of the target value).

Discussion

We have developed a flow cytometry assay using non-viable KG1a cells as an integral control. The assay described allows $CD45^+$ and $CD34^+$ cell enumeration using an ISHAGE-based protocol that defines $CD34^+$ cells by FSC/SSC characteristics and expression of both

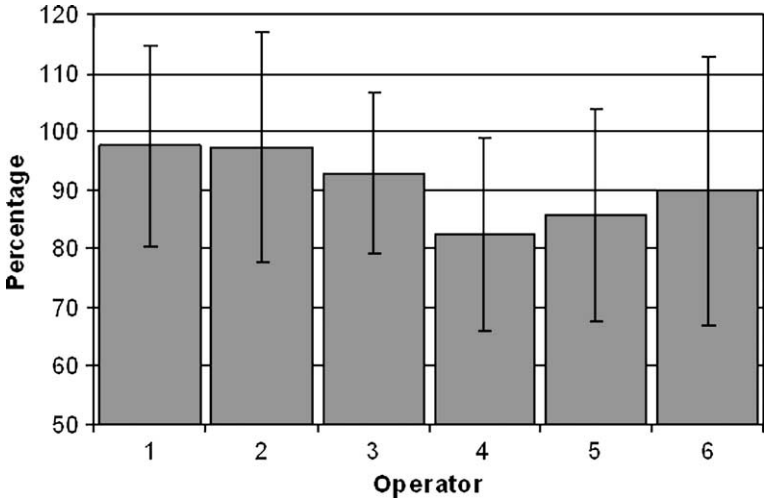

Figure 3. *A bar chart comparing operator performance. The mean KG1a cell counts for each operator are shown as a percentage of the target value (1250 cells/μL). The error bars show SD. Statistical analyses were performed to compare each operator with operator 1, who was the most experienced. Operator 1:* n = *36, 1219.0* ± *213.7, CV = 17.3% (97.5* ± *17.1% of target). Operator 2:* n = *45, 1217.4* ± *246.8, CV = 20.3% (97.4* ± *19.7%,* P = *0.9752). Operator 3:* n = *129, 1162.3* ± *170.7, CV = 14.7% (93.0* ± *13.7%,* P = *0.1501). Operator 4:* n = *37, 1031.6* ± *204.6, CV = 19.8% (82.5* ± *16.4%,* P = *0.0003). Operator 5:* n = *34, 1071.6* ± *227.5, CV = 21.2% (85.7* ± *18.2%,* P = *0.0067). Operator 6:* n = *41, 1123.2* ± *287.8, CV = 25.6% (89.9* ± *23.0%,* P = *0.0986). KG1a cell counts for operators 4 and 5 were statistically different from operator 1.*

CD45 (low/+) and CD34 markers. In addition, the method incorporates a positive integral control (KG1a cells) that can be used to confirm that CD45, CD34 and 7-AAD reagents have been included in the assay tubes and are working accordingly. The dispensing accuracy of the laboratory staff can also be assessed and monitored for quality control purposes.

The accuracy and precision of the method was initially determined by eight replicate tests, including KG1a cells at $97.7 \pm 3.9\%$ of the target value with a CV of 4.0%. In routine use over 322 tests, the accuracy remained acceptable, with KG1a cells achieving $91.7 \pm 17.7\%$ of the target value, although the precision was disappointing, with a CV of 19.3%. In order to investigate possible causes for the reduced precision of the method in routine use, we compared KG1a cell counts achieved using (1) different batches of critical reagents, (2) the experience of the operators and (3) the collection type. There was no significant difference in mean KG1a cells for the critical reagents, including the KG1a cell batch and the Flowcount™ microbeads, nor between BM and PBSC collection types (Table 2). However, a comparison of KG1a control cell counts for experienced and inexperienced operators identified an extremely significant difference (1181 ± 198, $n = 210$ vs. 1082 ± 246 cells/μL, $n = 112$, $P = 0.0003$) between the two groups.

The significant differences in KG1a cell enumeration between operators are most probably attributable to the accuracy and precision of operators in dispensing cells and reagents. As there was no difference ($P = 1.000$) in the CD45/WBC ratios for PBSC collections between experienced (1.06 ± 0.06, $n = 151$, CV = 5.7%) and inexperienced (1.06 ± 0.07, $n = 83$, CV = 6.6%) operators, this would suggest that the samples and Flowcount Fluorospheres were dispensed accurately and consistently, and the operator variation was primarily attributable to dispensing the KG1a cells. Two of the inexperienced operators in particular (4 and 5) could be identified because their results differed significantly from those of the benchmark operator (operator 1). Operator 4 was the least experienced and this was reflected in the KG1a cell results, which were significantly below the target range of 1062.5–1437.5 cells/μL. Operator 5 was more experienced than operator 4, but the results suggest that that operator also under-dispensed reagents compared with operator 1. In addition to the variation in KG1a cell counts between operators, the CV values were higher in routine use for all operators, with operator 3 showing the lowest CV, at 14.7%, and operator 6 the highest, at 25.6%. This was in contrast with the eight replicate tests by operator 1 shown in Table 2, where a CV of only 4.0% was achieved. This may reflect differences between routine and non-routine tests. In our laboratory in Bristol the routine tests are often performed under time pressure related to the clinical urgency of the results, and at variable times of the day and night.

This study supports previous reports that accurate dispensing is an important factor in single-platform, no-wash flow cytometry assays [7,13]. The use of positive-displacement pipettes, which are more accurate and precise than standard air-displacement pipettes, may be beneficial at improving test reproducibility between operators. In a separate study of eight replicate tests comparing standard air-displacement with positive-displacement pipettes, we showed that the latter gave a lower CV using KG1a cells for both experienced (3.2% vs. 5.1%) and inexperienced (4.8% vs. 12.3%) operators (data not shown).

In conclusion, this study validates our three-color flow cytometry assay, and demonstrates that non-viable KG1a cells may be used as part of an integrated quality assurance program to monitor test performance in the routine working environment. The method has an advantage over other quality-monitoring schemes as the control is integral to each test. As $CD34^+$ cells often occur at low frequency, the presence of KG1a control cells is valuable because it provides direct evidence that an active anti-CD34 reagent was used in each test. In addition to monitoring performance within a single laboratory, its wider use in multicenter studies may be helpful regarding standardization of methods by allowing direct comparison of techniques between laboratories.

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