An Interlaboratory Study of a Candidate Reference Method for Platelet Counting

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Abstract

A multinational interlaboratory task force explored the important variables of platelet reference counting and developed a candidate flow cytometric reference method based on the RBC/platelet ratio. A multicenter comparison was performed to determine whether the method met the necessary criteria and was precise enough to be recommended as a new reference method. Each laboratory analyzed serial dilutions of normal specimens, stabilized material, and at least 60 patient specimens with a range of platelet counts from 1 to 400 · 10^3/μL (1-400 · 10^9/L). Pooled analysis of the serial dilutions showed that RBC-platelet and RBC-RBC coincidence events became negligible at sufficiently high dilutions (ie, >1:1,000). All laboratories demonstrated excellent intra-assay and acceptable interlaboratory precision. Two antibodies (CD61 and CD41) were used for identifying platelets and individually gave acceptable results, but in a minority of samples, staining differences were observed. The optimum method thus uses a double-labeling procedure with a final dilution factor of 1:1,000. The study demonstrated that this method meets the criteria for a reference platelet count.

The current reference method for platelet counting is a manual phase contrast microscope chamber count.[1,2] However, this method exhibits a number of important limitations with high interoperator imprecision in the order of 10% to 25%.[3,4] Although the method has been used for many years, not only is it laborious for calibration of hematology analyzers, but it also is unsuited for the evaluation of low and ultralow platelet counts because of its imprecision. Single-channel impedance counters with or without focused flow are not suitable either because of inherent coincidence effects between RBCs and platelets. Impedance counters are also prone to interference from nonplatelet particulate matter and cannot adequately resolve small RBCs or RBC fragments from normal platelets and normal-sized RBCs from large platelets.[5-8] This is particularly relevant because of the recent trend in clinical decision making to lower the platelet count levels for platelet transfusions to less than 20 · 10^3/μL (20 · 10^9/L).[9] Therefore, to calibrate and determine which instruments can perform reliable platelet counts, there is a demand for a reliable reference counting method that is accurate for the entire range of platelet counts that are encountered clinically. This not only should result in improved calibration of hematology analyzers but also may ultimately increase the accuracy of platelet counting at very low levels, facilitating reexamination and, perhaps, redefinition of existing platelet transfusion thresholds.[10-15]

A flow cytometric platelet counting procedure has been advocated as a potential reference method and has been the subject of review by the International Council for Standardization in Haematology (ICSH) Expert Panel on Cytometry.[3,4,16-19] The ICSH panel originally identified the important variables associated with this method. This, in turn, enabled the International Society of Laboratory Hematology

(ISLH) Task Force to develop a working protocol for a candidate reference count method and organize a multilaboratory evaluation of this method. The principle of the method involves labeling EDTA-anticoagulated whole blood platelets with a suitable antiplatelet monoclonal antibody (fluorescein isothiocyanate [FITC] or otherwise conjugated). After optimal dilution, the platelets are enumerated from the ratio of fluorescent platelet events to the number of unlabeled RBCs. This gives the so-called RBC/platelet (PLT) ratio, and the platelet count is calculated from an accurate RBC count of the sample.[3,4] Providing that the blood sample is well mixed and that coincidence events (RBC-RBC and RBC-platelet) are accounted or controlled for, the derived platelet count then is independent of pipetting and dilution artifacts that are known to potentially influence other procedures (eg, fluorescent bead–based counts).[3,18] Previous studies have shown that although the new RBC/PLT ratio agrees well with the phase count, the method is very much more precise and, therefore, potentially should supersede manual counting as the new international reference method.[3,4]

The ISLH set up a task force for platelet counting in September 1999, designed a consensus protocol, and undertook an interlaboratory comparison with 10 participants with the aim of demonstrating that the RBC/PLT ratio has the potential to be recommended as the new reference method. In the present study, fresh blood specimens from healthy adult volunteers and from patients were analyzed. In addition, stabilized material from 3 sources was analyzed by all participants. Data were collated by April 2000 and presented at the ISLH 12th International Symposium on Technical Innovation in Laboratory Haematology (Banff, Alberta). All participants successfully completed the initial study, and after presentation and thorough round table discussion, a modified final consensus protocol was generated. An additional interlaboratory study was undertaken to demonstrate that this method is reliable. It was concluded that the new candidate reference method for platelet counting should become the platelet count derived from the RBC/PLT ratio. The final method is published in another article in this issue of the *Journal*.[20]

Materials and Methods

Participating Laboratories

Laboratories participating in the study were as follows: Abbott Laboratories, Santa Clara, CA; ABX, Montpelier, France; Bayer Diagnostics, Tarrytown, NY; Beckman Coulter, Miami, FL; Sysmex, Kobe, Japan; Loma Linda University, Loma Linda, CA; Maine Medical Center Research Institute, Portland; William Beaumont Hospital, Royal Oak, MI; and University College London, London, England (UCL).

Blood Specimens

All blood specimens were anticoagulated with dipotassium EDTA or tripotassium EDTA and were obtained after informed consent from healthy adult volunteers or as the residual material from patient specimens collected for clinical testing purposes. All specimens were tested within 6 hours after collection.

Consensus Materials

The following were provided to all participating laboratories to minimize interlaboratory variation: (1) 2 FITC-labeled antiplatelet antibodies: anti-CD61 (clone RUU-PL 7F12,[21] lot number 13222, Becton Dickinson, San Jose, CA) and anti-CD41 (clone P2,[22] lot number 26, Beckman Coulter, Hialeah, FL); (2) bovine serum albumin, fraction V (BSA; Sigma, St Louis, MO); (3) Millex-GV filters, mean pore size 0.22 µm (Millipore Intertech, Bradford, MA); and (4) preserved blood samples with different RBC/PLT ratios provided by R&D Systems (Minneapolis, MN), Beckman Coulter (Miami, FL), and UK National External Quality Assessment Scheme (NEQAS; Sheffield, England).

Initial Consensus Platelet Counting Method

After a first meeting of the ISLH Task Force in September 1999, a consensus protocol was written. All test samples were obtained from EDTA-anticoagulated (dipotassium EDTA or tripotassium EDTA) blood samples and analyzed within 6 hours of venipuncture. Specimens were maintained in the upright position at 18 C to 22 C and were *not* placed on any type of rocking or mixing device before assay. Samples were rejected if there was any evidence of hemolysis or clotting.

All quantities of blood, antibodies, and buffer were divided into aliquots using calibrated positive displacement pipettes, and the outside of the plastic tip was wiped carefully with tissue paper in an upward manner to remove excess fluid without disturbing the fluid inside the pipette tip. Five microliters of well-mixed blood (slowly inverted at least 8 times) was pipetted into the bottom of a Falcon polystyrene tube (Becton Dickinson) as a single bead of blood. Polypropylene or polystyrene tubes preferably should be used throughout specimen processing, and plain glass surfaces should be avoided. Five microliters of anti-CD61-FITC (12.5 µg/mL), an anti–glycoprotein (Gp) IIIa monoclonal antibody (clone RUU-PL 7F12),[21] or 5 µL of anti-CD41-FITC (100 µg/mL), an anti-GpIIbalpha monoclonal antibody (clone P2),[22] then was immediately pipetted into the same tube as a separate bead adjacent to, but not

touching or mixed with, the bead of blood. Then 100 µL of filtered (via 0.22 µm filters, Millex-GV) phosphate-buffered saline containing 0.1% BSA (PBSA; fraction V, Sigma; warmed to room temperature) was immediately pipetted into the bottom of the tube, and the blood was mixed with antibody and buffer by gently rocking the tube rack. After incubation for exactly 15 minutes at ambient room temperature in the dark, 890 µL of PBSA was added to give a final volume of exactly 1 mL (equivalent to 1:200 dilution of blood). Further final dilutions from 1:500 to 1:8,000 were made in separate Falcon tubes by adding appropriate volumes of the diluted blood solution to PBSA. The dilution series was prepared by diluting the more concentrated specimen into the diluent, and mixing was performed by at least 8 gentle inversions before aspiration of diluted blood. Specimens were kept in the dark at ambient room temperature until analyzed. Flow cytometric analysis then was performed on the mixed diluted blood using each laboratory's calibrated flow cytometer.

Flow Cytometers Used in the Study

A fluorescent flow cytometer with hydrodynamic focusing and the capability of measuring forward scatter, side scatter, and fluorescence is recommended. Each participating laboratory used different flow cytometers **Table 1**. Discriminators typically were set on a low value of forward scatter. Samples were aspirated at optimum flow rate (according to each laboratory), and a minimum of 2,000 platelet events or 50,000 RBC events were collected. Instruments used, individual settings, and calibration and control procedures are summarized in Table 1.

Reference Hematology Analyzers Used in the Study

Each participating laboratory performed a standard whole blood count using a variety of commercial analyzers (using impedance, optical, or immunologic methods), including the Cell-Dyn 4000 (Abbott Laboratories), the Sysmex 9500 or XE-2100 (Sysmex), the Beckman Coulter GEN-S and STKS (Beckman Coulter), and the ADVIA 120 System (Bayer). Individual calibration and control methods were the standard procedures used by each laboratory.

Flow Cytometric Data Analysis

RBC/PLT ratios were calculated from plotting cytometry scattergrams of cell size (log forward scatter) vs granularity (log side scatter) **Figure 1** and fluorescence (log FL1) vs cell size (log forward scatter) **Figure 2**. Figure 1 clearly shows the platelet (A) and RBC clouds (B). The use of both scattergrams is recommended, as they each provide visual clues to the labeling status of the platelets within the sample being analyzed. Quadrant and bitmap analyses are shown in Figure 2. These clearly show the resolution of platelets from

Table 1
Flow Cytometers, Discriminator Settings, and PMT Settings for Each Participating Laboratory*

Laboratory	Flow Cytometer	Flow Rate	Discriminator	PMT Settings	Daily Quality Control Procedure
Abbott Laboratories, Santa Clara, CA	FACScan	Low	Threshold = 52; FS-H = 200	FS = E00 log, gain = 2.00; SS = 309 log, gain = 1.00; FL1 = 582 log, gain = 1.00; FL2 = 630 log, gain = 1.00	CaliBRITE beads: FS, SS, FL1, and FL2
Bayer Diagnostics, Tarrytown, NY	FACScan	Low; 12 µL/min	FS = 52	FS = E00 log, gain 2.0; SS = 340 log, gain 1.0; FL1 = 725 log, gain 1.0	CaliBRITE beads: FS, SS, FL1, FL2 FACStation FACS-Comp 4.1 CVs <2.00
Beckman Coulter, Miami, FL	XL-MCL	Low	FS = 1.0	FS = 48V, gain = 1.0; SS = 7V, gain = 10.0; FL1 = 838V, gain = 1.0	Flow-Check beads (FS, FL1, FL2, and FL3 HPCV <1.5; FL4 <1.7); MFI, Flow-Set
Loma Linda University, Loma Linda, CA	FACScan	High	SS = 140	FS = E00 log; SS = 270 log; FL1 = 674 log	Becton Dickinson 3-color beads
Maine Medical Center Research Institute, Portland	FACScan	Low	FS = 199	FS = E00 log; SS = 304 log; FL1 = 606 log	CaliBRITE beads
Sysmex, Kobe, Japan	FACScalibur	Low	SS = 200	FS = E00 log; SS = 392 log; FL1 = 640 log	CaliBRITE beads
William Beaumont Hospital, Royal Oak, MI	FACScan	High	FS-H = 196	FS = E00 log; SS = 314 log; FL1 = 591 log	Flow-Check beads; CaliBRITE beads
University College London Hospital, London, England	XL-MCL	Medium	FS = 1.0	FS = 200V, gain = 1.0; SS = 706V, gain = 2.0; FL1 = 700V, gain = 1.0	Flow-Check beads for FS, FL1, FL2, FL3, and FL4 HPCVs <1.6 and MFI check

CV, coefficient of variation; FL1, log fluorescence at 528 nm; FL2, log fluorescence at 575 nm; FL3, log fluorescence at 620 nm; FL4, log fluorescence at 675 nm; FS, forward scatter; FS-H, forward scatter height; HPCV, half-peak coefficient of variation; MFI, mean fluorescence intensity; PMT, photomultiplier tube; SS, side scatter; V, voltage.
* FACScan, CaliBRITE beads, FACStation, FACS-Comp 4.1, and FACScalibur, Becton Dickinson, San Jose, CA; Coulter XL-MCL, Flow-Check beads, and Flow-Set, Beckman Coulter, Miami, FL.

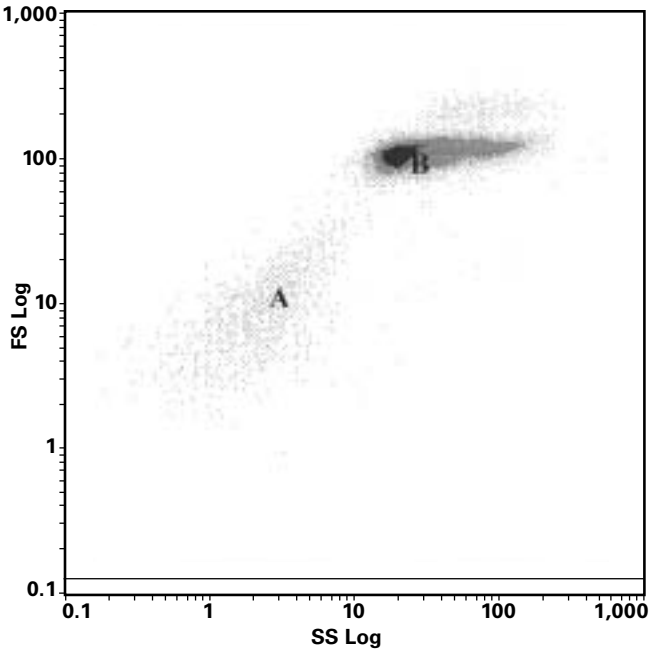

Figure 1 Flow cytometry scattergram (log side scatter [SS] vs log forward scatter [FS]) of all detected events (Coulter XL-MCL) within a blood sample diluted 1:1,000. The platelet cloud is shown on the lower left side (**A**) and is clearly resolved from the RBC cloud on the upper right side (**B**). For proprietary information, see Table 1.

noise and debris, RBCs, and PLT-RBC coincidence events. The final platelet counts were calculated by dividing the known RBC count of the same sample (determined by using the individual laboratory's choice of a calibrated automated hematology analyzer) by the RBC/PLT ratio. This derived count does not take into account the coincidence events. For calculation of a derived platelet count taking into account RBC-RBC and platelet-RBC coincidence, see **Appendix 1**. Calculation of the number of RBC-RBC coincidence events requires some explanation. Briefly, a platelet-RBC coincidence event means that a platelet and an RBC are within the observation volume at the same time. An RBC-RBC coincidence event means that 2 RBCs are within the observation volume at the same time. Thus, for either kind of event, there must be at least 1 RBC in the volume and 1 other cell, either a platelet or an RBC. The chance that the other cell will be an RBC is greater than the chance it will be a platelet by an amount equal to the true ratio of RBCs to platelets. Thus, if one multiplies the number of platelet-RBC events by the ratio of RBCs to platelets, the number of RBC-RBC events that must have occurred is derived.

Interlaboratory Comparisons

Each participating laboratory performed serial dilutions of 5 normal samples using both monoclonal antibodies to determine the optimum assay conditions on the instrument

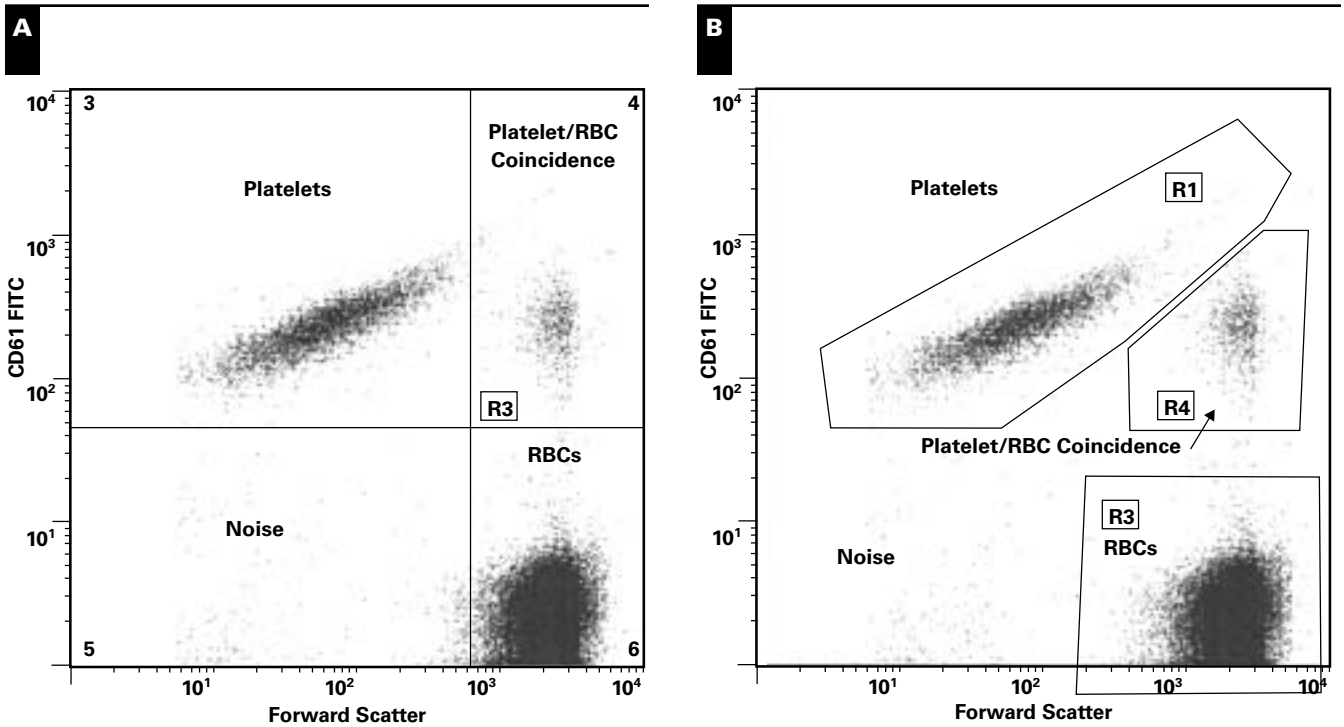

Figure 2 Flow cytometry scattergram (log fluorescence at 528 nm [FL1] vs log forward scatter [FS]) of all events collected in Figure 1. The fluorescent platelets are clearly resolved from noise and debris, RBCs, and RBC/platelet coincidence events. **A**, Quadrant analysis. **B**, Bitmap analysis. FITC, fluorescein isothiocyanate.

used in that laboratory. Pathologic samples were avoided at this stage to ensure adequate optimization. After determining the optimum dilution to minimize coincidence events, each laboratory analyzed the stabilized blood samples in duplicate using both monoclonal antibodies, CD41 and CD61. To minimize interlaboratory variables, the samples were analyzed within 10 days of receipt, and the date of analysis was recorded. For each sample run, the data that were pooled included the final dilution, the total number of events collected, and the number of platelet, RBC, and platelet-RBC coincidence events using either quadrant or bitmap analysis. All data were sent to the Maine Medical Center Research Institute for data processing and calculation of corrected platelet counts.

Within-Laboratory Studies

Each laboratory conducted its own study of patient samples. Data were collected as described, but each laboratory processed its own data. All centers performed a within-assay precision study on 5 samples with a range of platelet counts. Each sample was assayed 11 times with separate dilution tubes to calculate the within-laboratory coefficient of variation (CV) at different levels of platelet counts. Each center also assayed at least 60 patient samples (20 with platelet counts <20 $\cdot 10^3/\mu L$ [<20 $\cdot 10^9/L$], 20 between 20 and 120 $\cdot 10^3/\mu L$ [between 20 and 120 $\cdot 10^9/L$], and 20 between 120 and 400 $\cdot 10^3/\mu L$ [between 120 and 400 $\cdot 10^9/L$]).

Comparison of Final Double Labeling Consensus Method With the Single Labeling Procedures

During the ISLH Task Force consensus meeting (April 2000) a slightly modified final platelet counting procedure was recommended (for details see ICSH/ISLH[20]). The method is identical to the initial consensus method described with a number of small differences. These included a recommended final dilution factor of 1:1,000 (consensus finding, see "Results" section) and use of a double-labeling method with both anti-CD41 and anti-CD61. The double-labeling procedure was chosen because of substantial differences between CD41 and CD61 labeling results observed in a small number of samples by some of the laboratories. It also was decided that if at least 1,000 platelet events and 50,000 RBC events were collected, the assay would be statistically valid as shown previously.[3] These conditions provide a good balance between coincidence elimination and analysis time, particularly in severely thrombocytopenic samples. A small interlaboratory study was undertaken by 4 centers measuring at least 25 samples with a range of platelet counts. All samples were measured using anti-CD41, anti-CD61, and the double-labeling method. Data were sent to UCL and collated, and the 3 labeling methods were compared.

Comparison With the Manual Platelet Count

The anti-CD61, anti-CD41, and anti-CD61/anti-CD41 labeling methods were compared with the existing reference method. Manual platelet counts were performed via a standard phase microscopy method.[1,2] A 1:20 dilution of EDTA-anticoagulated whole blood was prepared in a diluent of 1% ammonium oxalate. Lower dilutions were made for specimens with a platelet count of less than 100 $\cdot 10^3/\mu L$ (<100 $\cdot 10^9/L$). The suspension then was mixed on a mechanical mixer for 10 to 15 minutes to allow for the complete lysis of all RBCs. A clean, dust-free Neubauer chamber was filled with the suspension and left in a moist chamber for 10 to 30 minutes to allow the platelets to settle. The preparation then was examined using the 40$\cdot$ objective; the platelets appeared as small but refractive particles. The total number of platelets appearing in 50 small squares on the chamber was counted and was equivalent to the platelet count $\cdot 10^9/L$. Both sides of the chamber were counted to check for reproducibility. The maximum observed difference allowed between the slides was 10%; a new preparation was counted if the difference exceeded 10%. The final count then was taken as the mean value of the 2 counts.

Results

Dilution Studies

The pooled data from the dilution curves for all laboratories is shown in **Figure 3** and **Figure 4**. The range of platelet counts was 140 to 403 $\cdot 10^3/\mu L$ (140-403 $\cdot 10^9/L$). Figure 3 shows data for all individual laboratories plotted as a function of the percentage of error vs dilution. At low dilutions (<1:500), the coincidence error was extremely variable between the different laboratories. This may be caused by variations in flow cytometer flow rate and geometry in terms of observation volume. When all the dilution data are combined (Figure 4), the total error due to coincidence consistently dropped to less than 5% at a dilution of more than 1:1,000, and further dilution did not seem to decrease the error below 2%. The recommended acquisition rate (events per second), which takes into account flow rate and dilution, should be less than 3,000 events per second. A potential problem with high dilutions is that the analysis time increases, especially in thrombocytopenic samples in which the number of platelet events is relatively low. Coincidence correction using the formulas given in Appendix 1 seems to be useful for correcting counts obtained at low dilution; at sufficiently high dilutions, however, the error due to coincidence becomes almost negligible. In addition, correcting for coincidence may introduce additional sources of error and is not recommended for dilutions greater than 1:1,000 (see "Discussion" section).

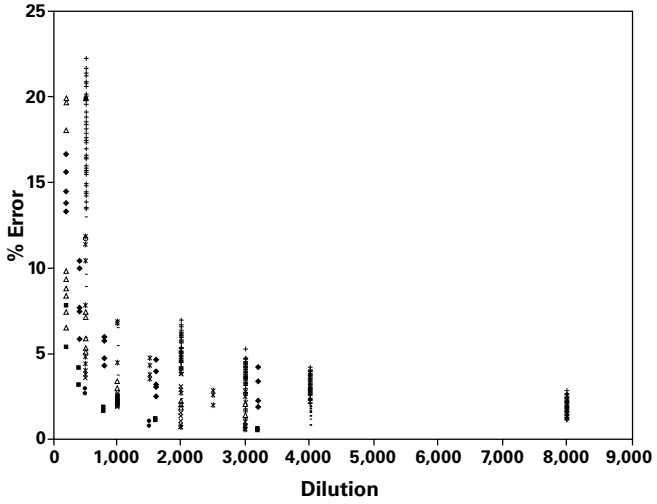

Figure 3 Percentage coincidence error vs dilution. All laboratory data are shown and were collected by bitmap analysis using both monoclonal antibodies (anti-CD41 and anti-CD61).

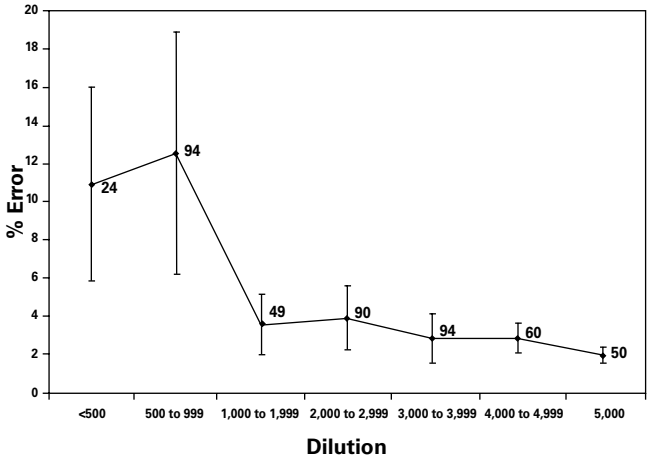

Figure 4 Combined coincidence error (mean ± SD) (number of data points shown on each point) vs dilution. The level of coincidence drops to less than 5% below a dilution of 1:1,000 but does not go below 2%.

Stabilized Blood Preparations

Eleven different stabilized blood preparations with a range of platelet counts were analyzed by 9 laboratories by either bitmap or quadrant analysis using both monoclonal antibodies. **Figure 5** and **Figure 6** summarize the data. Lysis problems were reported with the UK NEQAS low (UCL-L) material; this is reflected in the range of results obtained (4 laboratories reported lysis and did not attempt to analyze the sample). The R&D systems standards were analyzed by only 8 of the 9 laboratories. Several laboratories reported differences in the labeling intensity between CD41 and CD61 for the Beckman Coulter (BC) preparations. This probably caused the inconsistent results obtained, especially for the BC-1 material. **Figure 7** and **Figure 8** show correlation graphs between quadrant and bitmap analysis and between CD41 and CD61 labeling. The overall comparison

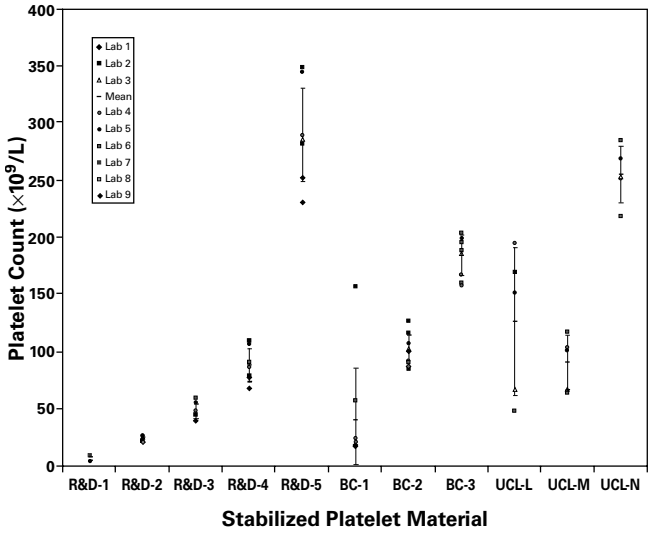

Figure 5 Summary of laboratory platelet counts (mean ± SD, indicated by central and outlying dashes, respectively) obtained using anti-CD61 with 11 different stabilized blood preparations. R&D Systems standards (R&D; 1-5), Beckman Coulter standards (BC; 1-3), and University College London UK NEQAS standards (UCL-L-N). Values are given in Système International units; conventional units are $\cdot 10^3/\mu L$, and the multiplier is 1.0. For proprietary information, see Table 2.

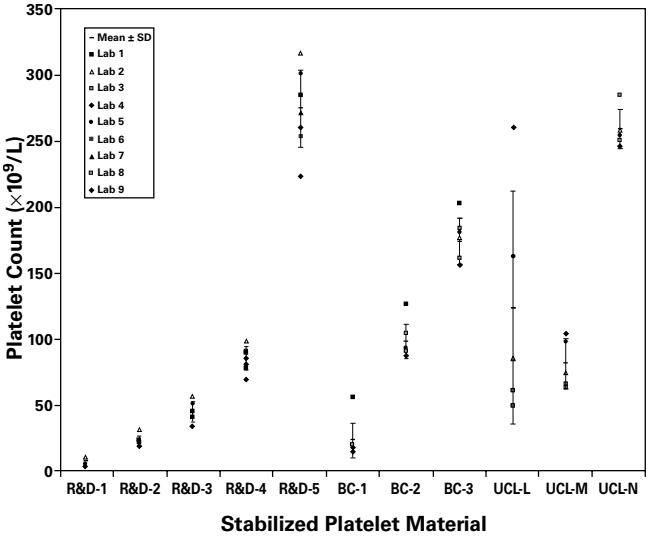

Figure 6 Summary of laboratory platelet counts (mean ± SD, indicated by central and outlying dashes, respectively) obtained using anti-CD41 with 11 stabilized blood preparations. R&D systems standards (R&D; 1-5), Beckman Coulter standards (BC; 1-3), and University College London UK NEQAS standards (UCL-L-N). Values are given in Système International units; conventional units are $\cdot 10^3/\mu L$, and the multiplier is 1.0. For proprietary information, see Table 2.

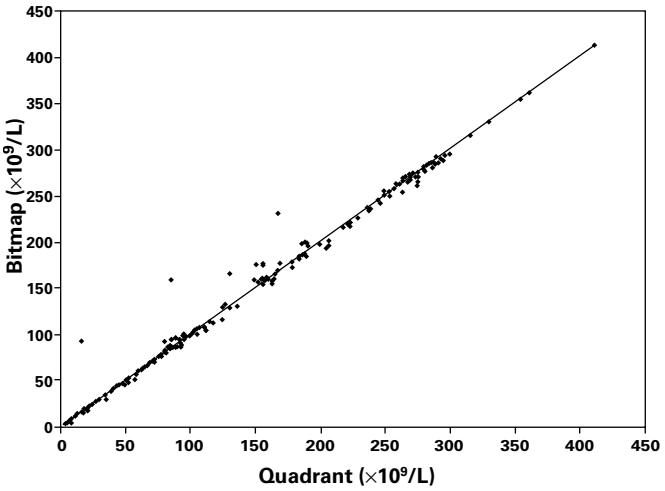

Figure 7 Comparison of all bitmap analysis– and quadrant analysis–derived platelet counts on the stabilized blood samples obtained from all participating laboratories (n = 303). y = 0.9983x + 1.228; R^2 = 0.9922. Values are given in Système International units; conventional units are $\cdot 10^3/\mu L$, and the multiplier is 1.0.

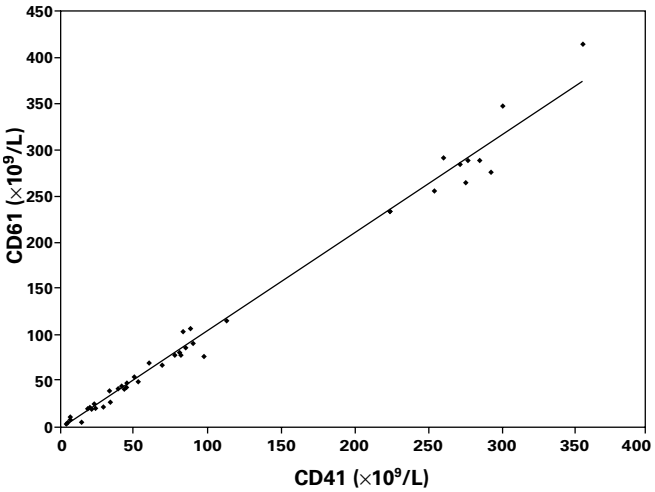

Figure 8 Comparison of CD41 and CD61 antibody labeling on the platelet counts from all participating laboratories; R&D Systems standards only. y = 1.0537x – 2.1027; R^2 = 0.9891. Values are given in Système International units; conventional units are $\cdot 10^3/\mu L$, and the multiplier is 1.0. For proprietary information, see Table 2.

of quadrant vs bitmap analysis of the samples (n = 303) was excellent, with only 1 laboratory reporting a systematic difference that was limited to 1 SD. The 3 samples with higher bitmap than quadrant values are the result of better platelet mapping. Several laboratories expressed a preference for bitmap analysis of the data in this particular study, as it was sometimes difficult to place the quadrant gate without overlap of events into other quadrants. For the monoclonal antibody comparison, only the data from the 5 R&D Systems preparations (n = 55) were used because of the problems reported with the other 2 materials. In this subset of data, the overall agreement between the 2 monoclonal antibodies was excellent. A summary of the interlaboratory variation using CD61, CD41, and bitmap analysis is shown in **Table 2**. Similar interlaboratory CVs using either antibody were obtained for the R&D, BC-2, BC-3, UCL-L, UCL-M, and UCL-N preparations.

Within-Laboratory Variation

An example of typical within-laboratory data from 1 participant (UCL) is shown in **Table 3**. Overall, the CV percentage was excellent, less than 5% for most samples, including severely thrombocytopenic samples. This is representative of the findings of other participants.

Comparison Using Patient Specimens

Comparison of duplicate, bitmap, and quadrant analyses gave essentially identical results (data not shown). Most laboratories, though, expressed a preference for bitmap analysis because, in a minority of samples, it is sometimes difficult to draw the quadrant gate to resolve large platelets and RBCs into their correct areas.

Comparison of Single and Double Labeling

Results of the double-labeling procedure agreed with those for both CD41 and CD61 alone in 4 participating laboratories. Pooled data are shown in **Figure 9**; all platelet counts are summarized in Figures 9A through 9C and platelet counts less than 100 $\cdot 10^3/\mu L$ (<100 $\cdot 10^9/L$) in Figures 9D through Figure 9F. The overall correlation between CD41 and CD61 and with CD41 and CD61 dual labeling was excellent with no apparent assay bias.

Comparison With Manual Phase Count

Figure 10 shows the comparison of CD41, CD61, and CD41 and CD61 labeling with the manual phase method in 1 laboratory. Although the overall correlations were excellent, note the bias toward the manual method, particularly at higher counts. This highlights the problem with the manual phase method in this laboratory. Other studies previously have shown that there was no apparent assay bias between the two methods.[3,4]

Discussion

The goals of the present study were to evaluate a candidate reference method for platelet counting and to determine its transferability. So-called indirect platelet counts can be derived from the RBC/PLT ratio obtained by flow cytometry

Table 2
Interlaboratory Comparison of Stabilized Blood Samples Using CD61 and CD41 Analyzed by Bitmaps*

	CD61			CD41		
Standard	**Mean**	**SD**	**CV (%)**	**Mean**	**SD**	**CV (%)**
R&D 1	6.19	1.44	23.2	6.11	1.87	30.6
R&D 2	22.73	1.82	8.0	22.99	3.89	16.9
R&D 3	47.10	6.44	13.7	44.45	7.28	16.4
R&D 4	87.34	14.20	16.3	84.79	8.94	10.5
R&D 5	289.96	40.45	13.9	273.75	28.84	10.5
BC-1	38.82	45.66	117.6	23.39	13.16	56.3
BC-2	99.69	14.18	14.2	97.63	13.56	13.9
BC-3	183.74	17.91	9.7	173.82	17.30	10.0
UCL-L	125.29	65.37	52.2	123.43	88.05	71.3
UCL-M	89.21	23.85	36.7	80.87	18.68	23.1
UCL-N	255.49	24.83	9.7	258.62	14.98	5.8

BC, Beckman Coulter, Miami, FL: R&D, R&D Systems, Minneapolis, MN; UCL-L-N, University College London UK NEQAS standards.
* Mean and SD are given as $\cdot 10^3/\mu L$ ($\cdot 10^9/L$).

Table 3
Coefficients of Variation Obtained With Five Samples With a Range of Platelet Counts*

	Platelet Count ($\times 10^9/L$)			
	Impedance	**CD61 (Mean)**	**SD**	**CV (%)**
Bitmap	259	283	4.3	1.5
Quadrant	259	283	4.3	1.5
Bitmap	97	134	2.7	2.0
Quadrant	97	128	2.9	2.3
Bitmap	45	50	3.5	7.0
Quadrant	45	50	3.6	7.2
Bitmap	20	22	0.8	3.5
Quadrant	20	22	0.9	4.0
Bitmap	3	3.4	0.1	4.1
Quadrant	3	3.7	0.2	4.3

* Samples were analyzed 11 times each. Values are given in Système International units; conventional units are $\cdot 10^3/\mu L$, and the multiplier is 1.0.

and require only an accurate RBC count performed on a calibrated hematology analyzer to calculate the result. Platelets are simply resolved from RBC or debris by specific labeling with suitable monoclonal antibodies.[3,4] After labeling and optimal dilution of the sample, the number of platelet-RBC and RBC-RBC coincidence events is reduced to a level that should not influence the final derived count. The data from the present study clearly demonstrate that a dilution factor of at least 1:1,000 is required to reduce the coincidence level error to acceptable limits (Figure 4). Previous studies have suggested dilution factors ranging from 1:400 to 1:2,000.[3,4,9] Higher dilutions tend to give identical results but require longer analysis times, particularly in thrombocytopenic samples. This does not present a problem, but it is possible that very prolonged analysis times may result in some degree of cell settling, which obviously will influence the ratio and alter the derived count. At a dilution of 1:1,000, typical analysis times are well within reasonable time limits without substantial RBC settling, even in severely thrombocytopenic samples. Platelet doublets or triplets at this dilution also will be insignificant. The recommended acquisition rate (events per second) that takes into account flow rate and dilution ideally should be less than 3,000 events per second. Interestingly, the coincidence error observed in this interlaboratory study does not seem to fall below 2% at higher dilutions. This phenomenon will be studied further and possibly is related to WBC-platelet complexes. Therefore, caution should be exercised if samples with high WBC counts are analyzed. Provided samples are analyzed optimally, ie, at 1:1,000 and less than 3,000 events per second, coincidence correction is not necessary. However, the inclusion of PLT-RBC and RBC-RBC coincidence events is necessary if a laboratory is setting up and optimizing its method and also for monitoring potential problems in certain pathologic samples (eg, those with high platelet counts).

Comparison of the interlaboratory data obtained with the stabilized blood samples was encouraging (Figures 5 and 6). One of the stabilized blood samples performed well, with

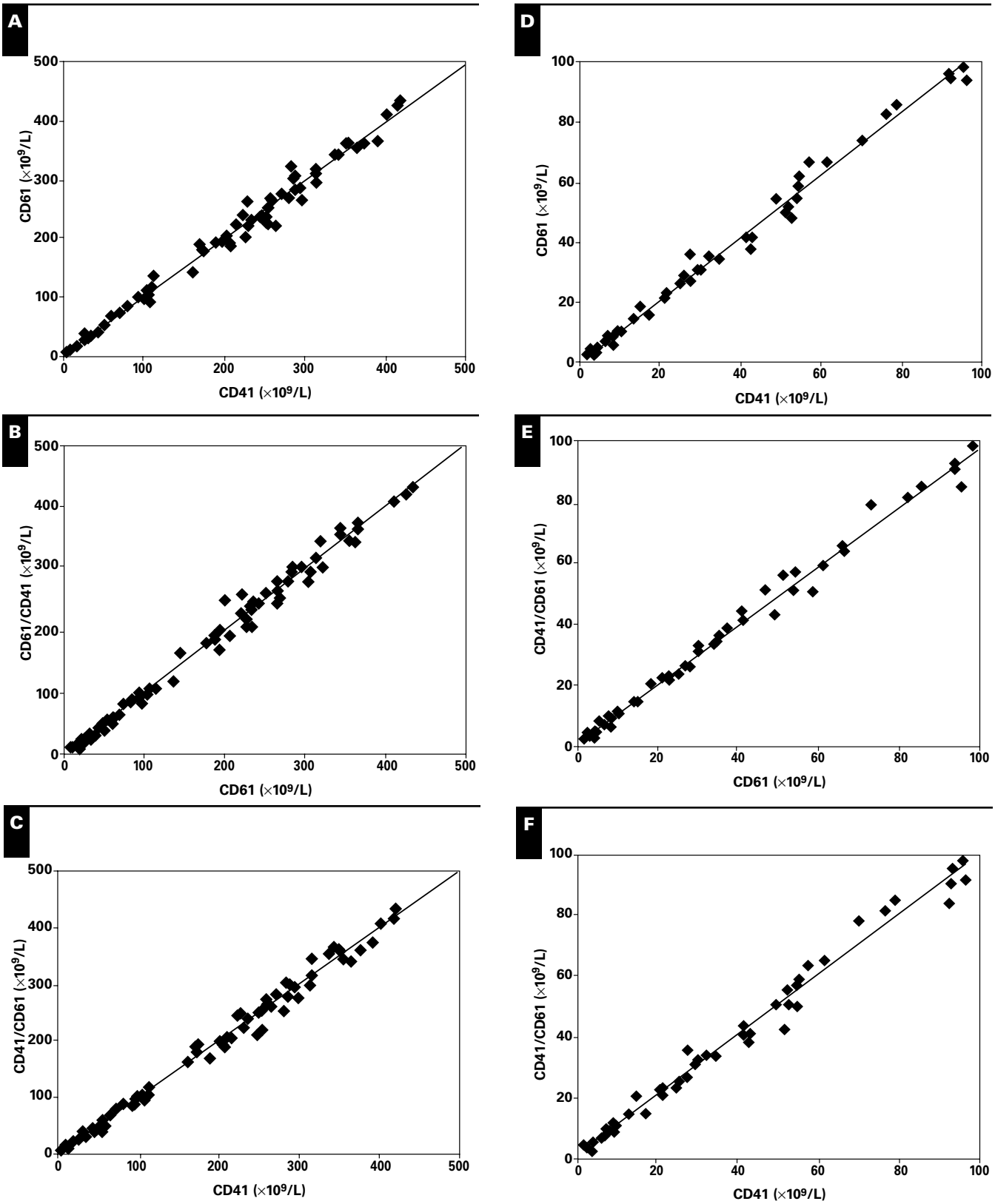

Figure 9 Comparison of CD41, CD61, and CD41/CD61 (all bitmap analyzed) platelet counts. Data were obtained from 4 participating laboratories. **A-C**, Comparisons at all platelet counts. **D-F**, Platelet counts <100 · 10^9/L. Values are given in Système International units; conventional units are · 10^3/μL, and the multiplier is 1.0. **A**, y = 0.9911x + 1.3507; R^2 = 0.9912. **B**, y = 1.0054x – 0.5803; R^2 = 0.9923. **C**, y = 1.0009x + 0.1252; R^2 = 0.9923. **D**, y = 1.0310x – 0.0034; R^2 = 0.9907. **E**, y = 0.9655x + 0.6369; R^2 = 0.9912. **F**, y = 0.9947x + 0.5546; R^2 = 0.9862.

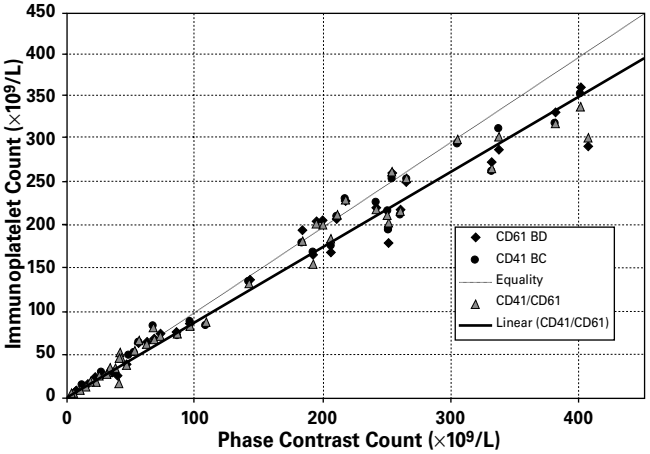

Figure 10 Comparison of CD41, CD61, and CD41/CD61 labeling with the manual phase contrast microscopy method in 1 laboratory. y = 0.8704x + 4.8822; R^2 = 0.9778. BD, Becton Dickinson, San Jose, CA; BC, Beckman Coulter, Miami, FL. Values are given in Système International units; conventional units are $\cdot 10^3$/μL, and the multiplier is 1.0.

CVs between 8.0% and 30%, even for counts less than 10 $\cdot$ 10^3/μL (<10 $\cdot$ 10^9/L; Table 2). For the other 2 preparations, there were problems with differing degrees of sample stability; some laboratories encountered RBC lysis during analysis. Despite these problems, the overall correlation between bitmap and quadrant analysis of the preparations was excellent in all laboratories (Figure 7). Comparison of CD61 and CD41 labeling gave similar results with one of the preparations (Figure 8). CD41 labeling tends to count slightly lower than CD61, particularly at the higher levels (Figure 8). The others were omitted from this comparison because many laboratories reported differences in the degree of labeling obtained. Because transport of the materials to the various laboratories was an uncontrollable variable (transport time, temperature, and handling), some degree of error between laboratories is not surprising. Comparison of typical within-laboratory CVs obtained with fresh blood samples demonstrates that the method is robust and superior to manual phase contrast microscopy (Table 3).[3,4] Analysis of stabilized samples with a range of counts may provide a means of ensuring assay performance within and between laboratories, provided the long-term stability of materials can be ensured.

Comparison of within-laboratory results from patient samples obtained using anti-CD61 or anti-CD41 revealed essentially identical data except in a minority of samples (data not shown). Some participants reported a small number of significant discrepancies between the derived platelet counts using the 2 antibodies. Although the antibodies label the same receptor on the platelet surface, they recognize unique epitopes. This may explain the discrepant results in a minority of samples. Therefore, to account for the rare possibility that use of a single antibody may not give an accurate count, it was agreed that a double-labeling procedure should be a "failsafe" way of ensuring accuracy of the counting method; the proposed reference method thus uses a combination of both antibodies for labeling.[20] A separate interlaboratory study was undertaken to ensure that the procedure was optimized. The data essentially demonstrated that this method gives identical results to a single-labeling method using either antibody alone (Figure 9). Previous studies have demonstrated that a variety of antibodies can be used to perform platelet labeling, including anti-GpIb.[4] One could argue that 2 antibodies raised against 2 different receptors should be used (eg, anti-GpIIb/IIIa and anti-GpIb). Although anti-GpIb antibodies work extremely well,[4,17] problems with internalization of this receptor into the open canalicular system during platelet activation have been reported with a substantial loss in mean fluorescence intensity of the entire platelet population.[23] The ISLH Task Force concluded that antibodies raised against 2 distinct epitopes on the GpIIb/IIIa complex should be adequate for the following reasons: (1) The receptor is up-regulated on the plasma membrane during platelet activation. (2) The receptor is rarely absent or decreased (eg, rare cases of Glanzmann thrombasthenia that should be easily screened out). (3) In the rare case of potential autoantibodies blocking labeling, only 1 of the 2 epitopes theoretically will be blocked.

Furthermore, although different monoclonal antibodies can be used, any given antibody must be proven to exhibit a sufficiently high fluorescent signal of the entire platelet population (ie, greater than the first log decade compared with an isotype control) to enable optimal resolution of all platelet events from noise and debris and RBCs (Figure 2).

Thus, the method satisfies the criteria for a reference method, particularly as it agrees well with the manual phase method as shown in previous studies.[3,4] The method is rapid, simple, and reproducible and can be set up easily by any laboratory with a fluorescent flow cytometer. The method should be a valuable tool for determining the accuracy of platelet counting in thrombocytopenia but is useful for assigning accurate platelet counts to samples (controls and calibrators) used in the calibration of hematology analyzers. This should result in greater harmonization of laboratory hematology practice and improve the accuracy of platelet counting, particularly in thrombocytopenic samples. Unlike impedance analyzers, the method clearly not only resolves large platelets from RBCs but also discriminates platelets from nonfluorescent platelet-like particles.[3,4] Certain samples with particles such as microcytes and fragmented RBCs also should be analyzed with caution, as the gates may require adjustment to perform optimal analysis, and the impedance RBC count may be inaccurate.

In the present study, all participants demonstrated that they could set up and run a reliable method that performed quite adequately within their laboratories and well in the interlaboratory comparison. This method, therefore, should replace manual phase contrast microscopy and may help to improve the calibration of hematology analyzers and the accuracy of platelet counting in thrombocytopenic samples.

Appendix 1
Coincidence Correction Calculations

Formula to Derive the Platelet (PLT)/RBC Ratio

PLT/RBC Ratio = $P/(R + C_{PR})$

Where P is the observed number of platelet events; R, the observed number of RBC events; C_{PR} the observed number of RBC/PLT coincidence events; RBC, the independent RBC count; P_{PLT}, the immunoplatelet count; C_T, the total coincidence events; R_T, the total number of RBCs; P_T, the total number of platelets; and C_{RR}, the number of RBC/RBC coincidence events.

$$\text{Ratio} = \frac{P_T}{R_T} = \frac{P + C_{PR}}{R + C_{PR} + C_{RR}} \quad \text{By definition}$$

$$= \frac{P + C_{PR}}{R + C_{PR} + (RC_{PR} + C^2_{PR})/P} \quad \text{Substitute value for } C_{RR}$$

$$= \frac{P(P + C_{PR})}{PR + PC_{PR} + RC_{PR} + C^2_{PR}} \quad \text{Distribute P}$$

$$= \frac{P(P + C_{PR})}{(R + C_{PR})(P + C_{PR})} \quad \text{Factor term in denominator}$$

$$= \frac{P}{(R + C_{PR})} \quad \text{Cancel common term}$$

Calculation of C_{RR}

1 $P_T = P + C_{PR}$
The total number of platelets that were analyzed is equal to the number of platelets counted plus the number of RBC/PLT coincidence events.

2 $R_T = R + C_{PR} + C_{RR}$
The total number of RBCs analyzed is equal to the number of RBCs counted plus the number of RBC/PLT coincidence events, plus the number of RBC/RBC coincidence events.

3 $C_{RR} = (R_T/P_T)C_{PR}$
The number of RBC/RBC coincidence events is equal to the true ratio of RBCs to platelets times the number of RBC/PLT coincidence events.

3A $C_{RR} = C_{PR}(R + C_{PR} + C_{RR})/(P + C_{PR})$
Substitute equations 1 and 2 into equation 3.

3B $PC_{RR} + C_{PR}C_{RR} = RC_{PR} + C_{PR}^2 + C_{PR}C_{RR}$
Multiply both sides by $P + C_{PR}$.

4 $C_{RR} = (RC_{PR} + C_{PR}^2)/P$
Solve for C_{RR}.

Calculation of Platelet Count and Total Coincidence Percentage

With the calculation for C_{RR}, equations 1 and 2 can be used to calculate P_T and R_T and then to calculate P_{PLT}:

$P_{PLT} = (P_T/R_T)RBC$

$\%C_T = 100(C_{PR} + C_{RR})/(P_T + R_T)$

From [1]University College London, London, England; [2]Maine Medical Center Research Institute, South Portland, ME; [3]Bayer Diagnostics, Tarrytown, NY; [4]Beckman Coulter, Miami, FL; [5]Sysmex, Kobe, Japan; [6]Loma Linda University, Loma Linda, CA; [7]ABX Diagnostics, Pessac, France; [8]Abbott Laboratories, Santa Clara, CA; and [9]Centers for Disease Control and Prevention, Atlanta, GA.

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References

1. England JM, Rowan RM, Bins M, et al. Recommended methods for the visual determination of white cell and platelet counts. *WHO LAB*. 1988;88:1.
2. Brecher G, Schneiderman M, Cronkite EP. The reproducibility of the platelet count. *Am J Clin Pathol*. 1953;23:15-21.
3. Harrison P, Horton A, Grant D, et al Immunoplatelet counting: a proposed new reference procedure. *Br J Haematol*. 2000;108:228-235.
4. Davis B, Bigelow NC. Indirect immunoplatelet counting by flow cytometry as a reference method for platelet count calibration. *Lab Haematol*. 1999;5:15-21.
5. Rowan RM. Platelet counting and assessment of platelet function. In: Koepke JA, ed. *Practical Laboratory Hematology*. New York, NY: Churchill Livingstone; 1991:157.
6. Hammerstrom H. Spurious platelet counts in acute leukemia with DIC due to cell fragmentation. *Clin Lab Haematol*. 1992;14:239-243.
7. Dickerhoff R, Von Ruecker A. Enumeration of platelets by multiparameter flow cytometry using platelet specific antibodies and fluorescent platelet particles. *Clin Lab Hematol*. 1995;17:163-172.
8. Springer W, von Ruecker A, Dickerhoff R. Difficulties in determining prophylactic transfusion thresholds of platelets in leukemia patients. *Blood*. 1998;92:2183-2184.
9. Ault KA. Platelet counting: is there room for improvement? *Lab Haematol*. 1996;2:265-269.
10. Rebulla P, Finazzi G, Marangoni F, et al. The threshold for prophylactic platelet transfusion in adults with acute myeloid leukemia. *N Engl J Med*. 1997;337:1870-1875.
11. Ancliff PJ, Machin SJ. Trigger factors for prophylactic platelet transfusion. *Blood Rev*. 1998;12:234-238.

12. Norfolk DR, Ancliffe PJ, Contreras M, et al. Consensus Conference on Platelet Transfusion, Royal College of Physicians of Edinburgh, 27-28 November 1997. Synopsis of background papers. *Br J Haematol.* 1998;101:609-617.
13. Gmur J, Burger J, Schanz U, et al. Safety of stringent prophylactic platelet transfusion policy for patients with acute leukemia. *Lancet.* 1991;338:1223-1226.
14. Murphy WG. Prophylactic platelet transfusion in acute leukaemia [letter]. *Lancet.* 1992;339:120-121.
15. Kickler TS, Rothe M, Blosser L, et al. Improving platelet transfusion therapy using the ImmunoPLT method on the CELL-DYN 4000. *Lab Haematol.* 1998;4:80-87.
16. Ault KA, Mitchell J, Knowles C, et al. Implementation of the immunological platelet count on a haematology analyser, the Abbot CELL-DYN 4000. *Lab Haematol.* 1997;3:125-128.
17. Tanaka C, Isii T, Fujimoto K. Flow cytometric platelet enumeration utilising monoclonal antibody CD42a. *Clin Lab Haematol.* 1996;118:265-269.
18. Matzdorff AC, Kuhnel G, Kemkes-Matthes B, et al. Quantitative assessment of platelets, platelet microparticles and platelet aggregates. *J Lab Clin Med.* 1998;131:507-517.
19. Groner W, Mayer K, Chapman E. An indirect platelet count using a platelet specific monoclonal antibody and flow cytometry can produce reliable platelet counts for assessing thrombocytopenia [abstract]. *Blood.* 1994;84(suppl 1):687a.
20. International Council for Standardization in Haematology Expert Panel on Cytometry and International Society of Laboratory Hematology Task Force on Platelet Counting. Platelet counting by the RBC/platelet ratio method: a reference method. *Am J Clin Pathol.* 2001;115:460-464.
21. Wong DA, Springer TA. CD61 (B3) cluster report. In: Schlossman SF, Boumsell L, Gilks W, et al, eds. *Leukocyte Typing V: White Cell Differentiation Antigens.* Oxford, England: Oxford University Press; 1995:1664-1665.
22. Koksch M, Rothe G, Kiefel V, et al. Fluorescence resonance energy transfer as a new method for the epitope-specific characterization of anti-platelet antibodies. *J Immunol Methods.* 1995;187:53-67.
23. Michelson AD, Ellis PA, Barnard MR, et al. Downregulation of the platelet surface glycoprotein Ib-IX complex in whole blood stimulated by thrombin, adenosine diphosphate, or an in vivo wound. *Blood.* 1991;77:770-779.