Platelet Counting by the RBC/Platelet Ratio Method

A Reference Method

International Council for Standardization in Haematology Expert Panel on Cytometry[*] *and International Society of Laboratory Hematology Task Force on Platelet Counting*[†]

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Abstract

The International Council for Standardization in Haematology (ICSH) and the International Society of Laboratory Hematology (ISLH) recommend the counting of specifically labeled platelets relative to the RBCs with a fluorescence flow cytometer, together with an accurate RBC count determined with a semiautomated, single-channel aperture-impedance counter as a reference method for the enumeration of platelets. Fresh EDTA-anticoagulated venous blood specimens are measured within 4 hours of the draw. The specimen is prediluted (1:20) and the platelets labeled with two monoclonal antibodies specific to a cluster of differentiation common to all platelets. A final 1:1,000 dilution is made and at least 50,000 events with a minimum of 1,000 platelet events are counted with a flow cytometer to determine the RBC/platelet ratio. The platelet count is then calculated from this ratio and the RBC concentration of the original blood specimen.

The International Council for Standardization in Haematology (ICSH) has recommended reference methods for the determination of hemoglobin,[1] packed RBC volume,[2] and the enumeration of erythrocytes and leukocytes.[3] Thus, there remains the need for a reference method for the enumeration of platelets to complete the set of methods required for (whole blood) calibration of the traditional CBC count and for assignment of values to hematology analyzer calibration materials.

The ICSH has defined a *reference method* as "a clearly and exactly described technique for a particular determination which, in the opinion of a defined authority, provides sufficiently accurate and precise laboratory data for it to be used to assess the validity of other laboratory methods for this determination. The accuracy of the reference method must be established by comparison with a definitive method where one exists, and the degree of inaccuracy and imprecision must be stated."

Lacking a true reference method for the platelet count, the ICSH Expert Panel on Cytometry previously has recommended selected methods for assigning platelet count values to fresh blood used for whole blood calibration of hematology analyzers.[4] These selected methods include hemocytometer phase contrast microscopy with an ammonium oxalate diluent[5] and the determination of the RBC/platelet (PLT) ratio measured with an aperture-impedance counter with a hydrodynamic focused flow stream (indirect platelet counting).

Obtaining accurate, precise, and reliable platelet counts as a reference for the calibration of hematology analyzers has been a continuing problem for 2 reasons: (1) difficulties discriminating the small platelet signals from those of debris and spurious noise, and (2) the elimination of interference with the platelet count by substantially more numerous erythrocytes.

With the increasing use of fluorescent flow cytometry, there has been renewed interest in indirect platelet counting, ie, the determination of the RBC/PLT ratio. These indirect counting methods are performed on a flow cytometer after the platelets have been labeled fluorescently with a monoclonal antibody specific to a cluster of differentiation common to all (resting and activated) platelets. A number of such antibodies have been described, including anti-CD42a[6-8]; anti-CD41b[8]; anti-CD61[9]; and anti-CD41, anti-CD42, and anti-CD61.[10,11] Studies have shown that fluorescent labeling with any one of a number of commercially available monoclonal antibodies seems to be specific and is claimed to be essentially universal, ie, all platelets are labeled in a large variety of blood specimens.[9,11] The preparation and counting with modern flow cytometers is practical, with good separation between the cell types for discrimination purposes. However, RBC-RBC, RBC-platelet, and WBC-platelet coincidences remain a problem for cell counting by flow cytometry, since the sensing zone generally is defined only by the Gaussian distribution of the laser light and is typically greater than 1,000 µm. Because the sensing volume is defined poorly, coincidence correction generally is precluded, and serial dilutions must be used to verify the absence of errors due to coincidence.

Recommendation

The ICSH now recommends that an indirect platelet count, ie, the counting of specifically labeled platelets relative to the RBCs with a fluorescence flow cytometer, together with an accurate RBC count determined with a semiautomated, single-channel, aperture-impedance particle counter, provides a method sufficiently accurate and precise to be used for whole blood calibration of cell counters and for assigning values to calibration materials.

Principle of the Method

An EDTA-anticoagulated blood specimen is prediluted in a sterile buffered solution. The platelets then are stained with specific fluorescent antibodies. The stained platelets in solution are diluted to the counting concentration, and the platelets and RBCs are counted on a flow cytometer with thresholds set to discriminate platelets from RBCs on the basis of fluorescence amplitude and scatter amplitude. The RBC/PLT ratio is determined, and the platelet count is calculated from an accurate RBC count of the sample, obtained using a cell counter that meets previous ICSH specifications.[3]

Blood Specimens

Fresh venous blood specimens are collected from apparently healthy donors by syringe or evacuated container and anticoagulated with EDTA, dipotassium salt, or tripotassium salt, 3.7 to 5.4 µmol/mL of blood (K_2EDTA, anhydrous, Chemical Abstracts Service [CAS] 25102-12-9, formula weight of 368.4, 1.4-2.0 mg/mL; K_3EDTA, dihydrate, CAS 6550-24-8, formula weight of 442.5, 1.6-2.4 mg/mL). Only K_2EDTA should be used when the same specimens also are required for hematocrit and mean corpuscular volume measurements.[12] The containers must have sufficient air remaining to allow for proper mixing. The specimen should be rejected if there is visual hemolysis or if microclots are present. Maintain the specimens at room temperature (18 C-22 C), and process within 4 hours of obtaining the specimen. Do not place specimens on a rocking or other "mixing" device. To ensure a homogeneous distribution of RBCs and platelets, thoroughly mix the specimen by at least 8 gentle, complete inversions before predilution and antibody labeling.[13]

Glassware

Care must be taken to avoid the adherence of platelets to any surface of any container used in storing the original specimen or any diluted sample. Therefore, plain glass surfaces must be avoided, and polypropylene or polystyrene must be used throughout specimen processing.

Instrument Specifications

For platelet and RBC enumeration, a fluorescent flow cytometer with hydrodynamic focusing and the capability of measuring forward light scatter and fluorescence is used. The instrument should have sufficient sensitivity to scattered and fluorescein fluorescent light to reliably count fluorescein isothiocyanate–labeled spherical particles of 2 µm in diameter.

For the whole blood RBC count, a semiautomated, single-channel, aperture-impedance particle counter is used. The instrument should have an orifice diameter of 80 to 100 µm and a length 70% to 100% of the diameter, and the volume displaced during the counting period must be known to within an accuracy of 1%, traceable to a national or international metrologic standard.[3] (NOTE: At present, no optical particle counters are available that meet the requirement of an accurately known and traceable volume displacement.)

Reagents

Diluent

The diluent is phosphate-buffered saline (PBS), 0.01 mol/L, pH 7.2 to 7.4 with 0.1% bovine serum albumin (BSA). Steps are as follows:

1. Dissolve 1.15 g dibasic, anhydrous sodium phosphate

(Na_2HPO_4; CAS, 7558-79-4; formula weight, 142.0) in approximately 750 mL of deionized or distilled water.

2. Add 210 mg monobasic, anhydrous potassium phosphate (KH_2PO_4; CAS, 7778-77-0; formula weight, 136.1), 8.0 g sodium chloride (NaCl; CAS, 7647-14-5; formula weight, 58.44), 200 mg potassium chloride (KCl; CAS, 7447-40-7; formula weight, 74.55), and 1.0 mL BSA, fraction V. Dilute to 1,000 mL with deionized or distilled water. Store at 4 C to 8 C.

3. Filter the diluent through a low-binding, low-release membrane filter, with a mean pore diameter of 0.20 to 0.25 µm before use. (Good results have been obtained with, eg, Millex-GV filters, Millipore Intertech, Bradford, MA.[13])

Staining Solution

For the staining solution, directly conjugated, fluorescein isothiocyanate–labeled, antibodies against 2 distinct epitopes on the glycoprotein IIb/IIIa complex of platelets are used: antibodies to CD41 and CD61.

NOTE: In a series of 65 blood specimens with platelet counts ranging from 15 to 900 $\cdot 10^3$/µL (15-900 $\cdot 10^9$/L), no difference in labeling was found in 17 specimens; in 25 specimens, more platelets were stained by anti-CD41 than by anti-CD61; and in 23 specimens, more were stained by anti-CD61 than by anti-CD41 **Table 1**. The reference method thus requires a double-staining technique.

Laboratories must verify that a specific clone or batch of antibodies results in adequate fluorescent staining of the platelets. The antibody (antibodies) must give a high enough fluorescent signal of the entire platelet population so that it is completely resolved from noise and debris and the RBCs on a plot of log FL1 (fluorescence at 528 nm) vs log FS (forward scatter). (This usually was greater than the first log decade when the background fluorescence was set with a matched isotype control.)

Procedure

Enumeration of RBCs and Platelets

Steps are as follows:

1. Pipette 5 µL of the well-mixed (at least 8 complete, gentle inversions of the specimen tube) blood specimen into 100 µL of the filtered PBS-BSA diluent.

2. Add 5 µL of the anti-CD41 and 5 µL of the anti-CD61 staining solution and incubate for 15 minutes, in the dark, at ambient temperature (18 C-22 C).

NOTE: Consistent results have been obtained when the blood and the anti-CD41 and anti-CD61 staining solutions are pipetted as separate beads in the bottom of the reaction tube and the PBS-BSA diluent is added.[13]

3. After 15 minutes, prepare a final 1:1,000 dilution for counting by adding 4.85 mL of the PBS-BSA diluent. Mix well by gentle inversions to ensure proper and equal distribution of RBCs and platelets.

4. With the flow cytometer, count a minimum of 50,000 events with a minimum of 1,000 platelet events. The flow cytometer settings must ensure an acquisition rate of less than 3,000 events per second. Events that are positive for RBC scatter signal, as well as for platelet fluorescence, are considered RBC-platelet coincidence events and are added to both the RBC and the platelet events.

Either quadrant or bitmap analysis is acceptable, but bitmap analysis is recommended. The use of positive displacement pipettes is recommended.

Determination of RBC Concentration

Following the ICSH reference method for enumeration of erythrocytes (and leukocytes),[3] determine the RBC concentration of the original blood specimen using a semiautomated, single-channel, aperture-impedance particle counter.

Platelet Count Determination

From the flow cytometry data, determine the RBC/PLT ratio, R, to at least 3 decimal places by using the following formula:

R = RBC Events/Platelet Events

Divide the RBC count determined in the original specimen by this ratio, R, to arrive at the platelet count.

For example:

RBC Count, 5.44 $\cdot 10^6$/µL (5.44 $\cdot 10^{12}$/L); R = 20.4896

Table 1
Staining Results

Staining by Anti-CD41 vs Anti-CD61	No. of Samples	Difference (%)		
		Mean	SD	Total Range
No difference (± 1%)	17	0	—	—
Anti-CD41 greater than anti-CD61 (> 1%)	25	4.8	3.04	1.1-12.2
Anti-CD41 less than anti-CD61 (< –1%)	23	4.8	4.62	1.02-19.9

Platelet Count = 5.44 · 10^6/μL (5.44 · 10^{12}/L) Divided by 20.4896 = 265.5 · 10^3/μL (265.5 · 10^9/L)

NOTE: In a series of 357 apparently healthy, racially mixed, male and female volunteers, median age 26 years, a mean ± SD ratio of 21.572 ± 5.134 was found.

Coincidence

Once the assay has been optimally set up, application of a coincidence correction is not required. However, application of the coincidence correction equations given in Appendix 1[13] is recommended during setup of the assay to ensure optimal conditions (eg, dilution, flow, or acquisition rate) are achieved and to monitor potential problems with some pathologic specimens (eg, very high platelet counts).

Imprecision

As in all cell counting methods, a fundamental limitation on the reproducibility of the result is provided by the statistical variation in the number of cells sampled: the coefficient of variation (CV) is the square root of the reciprocal of the number of cells counted. In the reference method described, a proportion, R, is derived from the ratio of the two counts (number of platelets, N_{Plt}, and number of RBCs, N_{RBC}) after a fixed number, N, of total cells has been analyzed.

The theory of propagation of errors indicates that the CV of a ratio is given by the variation of the individual measurements added in quadrature:

$$CV^2 = (1/N_{Plt}) + (1/N_{RBC})$$

Owing to the relatively large number of RBCs (N_{RBC} = approximately N_{total}) and substituting (N_{RBC}/R) for N_{Plt}, the equation can be simplified and expressed as a function of the RBC/PLT ratio:

$$CV^2 = (R + 1)/N_{total}$$

and

$$CV\ (\%) = [\sqrt{(R + 1)/N_{total}}] \cdot 100$$

The mean of normal values for the RBC/PLT ratio is around 20. At this value and with an N_{total} of 50,000, the statistical imprecision is 2%. Practical experience has demonstrated the replicate precision for the method to be about 1.7% and 3% at platelet counts of 280 and 40 · 10^3/μL (280 and 40 · 10^9/L), respectively.

Sources of Interference

Although labeling with the monoclonal antibodies used in this method has been shown to be effective with a wide variety of specimens,[12] there exists the potential for specimen-specific interference. Interference could result from an absence of the binding sites or blocking of the staining reaction in certain specimens. A summary of potential interferences is listed in **Table 2**. Thus, it is important to ensure that whole blood specimens used for the calibration of hematology analyzers do not exhibit any of the conditions listed.

Evaluation and Transferability

The method, staining with anti-CD41 and anti-CD61 in parallel, has been tested in 11 laboratories (in France, Japan, the United Kingdom, and the United States); staining with a combination of anti-CD41 and anti-CD61 has been tested in 4 laboratories (in the United Kingdom and the United States). Each laboratory analyzed stabilized material and at least 60 patient specimens with a range of platelet counts.

Table 2
Potential Sources of Interference With Platelet Counts in Selected Conditions*

Error Source	Effect	Condition	Frequency
High platelet count (platelet activation)	Unstable count	Thrombocythemia; thrombocytosis	Occasional
Autoantibodies	Blocking of binding site	Idiopathic thrombocytopenic purpura	Rare
Congenital platelet disorders	Lack of binding site glycoprotein IIb/IIIa	Glanzmann thrombasthenia	Very rare
Platelet aggregation	Platelet clumps	Aged blood; EDTA effect	Occasional
Platelet-WBC adherence	Platelets outside gate	Following cardiac bypass; EDTA	Occasional
Cold agglutinins	RBC clumps	Agglutinin-related disorder	Occasional
Fragmented RBCs	Incorrect RBC count	Hemolytic anemia; disseminated intravascular coagulation; HUS; artificial heart valve	Rare
Fragmented WBCs (including apoptotic fragments)	False platelet count	Leukemia, especially chronic lymphocytic; chemotherapy	Occasional
Abnormal platelet size	Platelets outside gate	Thrombocytopenia; May-Hegglin anomaly	Occasional

EDTA, ethylenediaminetetraacetic acid; HUS, hemolytic uremic syndrome.
* Adapted from Groner W. Draft ICSH reference method for obtaining the ratio of the RBC count to platelet count. ICSH Expert Panel on Cytometry, internal document.

All laboratories demonstrated excellent intra-assay and acceptable interlaboratory precision. The overall correlations between anti-CD41 and anti-CD61 and each with anti-CD41 and anti-CD61 dual labeling was excellent with no apparent bias. The method, using a calibrated automated hematology analyzer instead of a reference particle counter to determine the number of RBCs, also has been shown to be a valuable tool for determining the accuracy of platelet counting in thrombocytopenia.[13]

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The use of trade names is for identification only and does not constitute endorsement by the Public Health Service of the US Department of Health and Human Services.

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