Cell Counting Using the BD Accuri™ C6 Flow Cytometer

Koen Verbrugghe, PhD

Marketing Applications Specialist
BD Biosciences

23-14763-00

The BD Accuri C6 Flow Cytometer System

- An affordable, full-featured, easy-to-use flow cytometer
- Two lasers and six detectors

Detection: Wide Dynamic Range

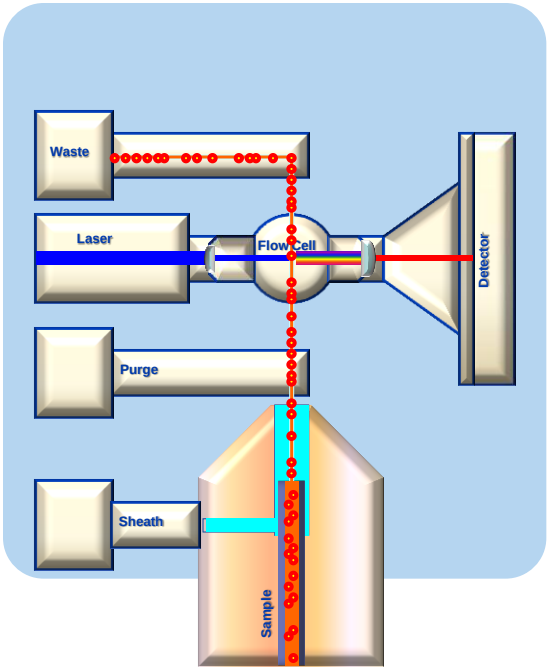
Advantages of Pre-optimized Voltage

- Greatly reduces the risk of lost data due to improper setup
  - Saves time and sample
- No specialist training or dedicated operator required
- Predictable, reproducible analysis relative to the sample type and application
- Attenuation filters (for bright signals) give controlled signal reduction
- Predictable fluorescence spillover
- Focus on the science of measuring fluorescence, not the art of setting voltages

BD

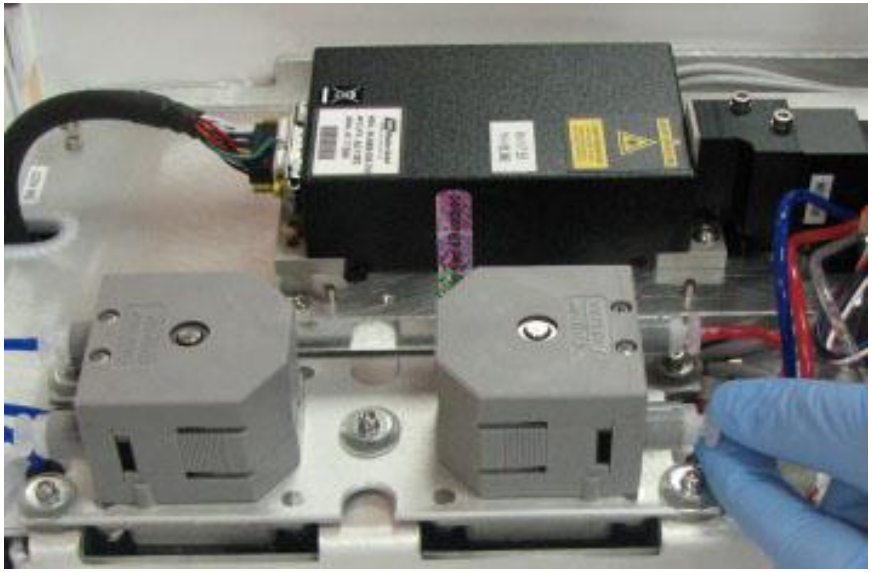
Fluidics

- Non-pressurized, peristaltic pump-driven system
- Patented pulse dampeners
- Volume is reported for measurement of cell concentration, eliminating the need for counting beads
- Minimum sample volume 50 µL
- Up to 10,000 events per second



Volume Determination

- During fluidic calibration, the motion of the pumps is related to pressure changes in the flow cell.
- During sample acquisition, the motion of the pumps is used to determine the sample volume.



Enhanced Sample Handling

- Many types of sample tubes may be used.
  - BD Falcon™ (“FACS”) tubes
  - Eppendorf®
  - Micro-capillary tubes
- Open system conducive to kinetic studies

- BD CSampler™ accessory for automated sample introduction

Intuitive Software

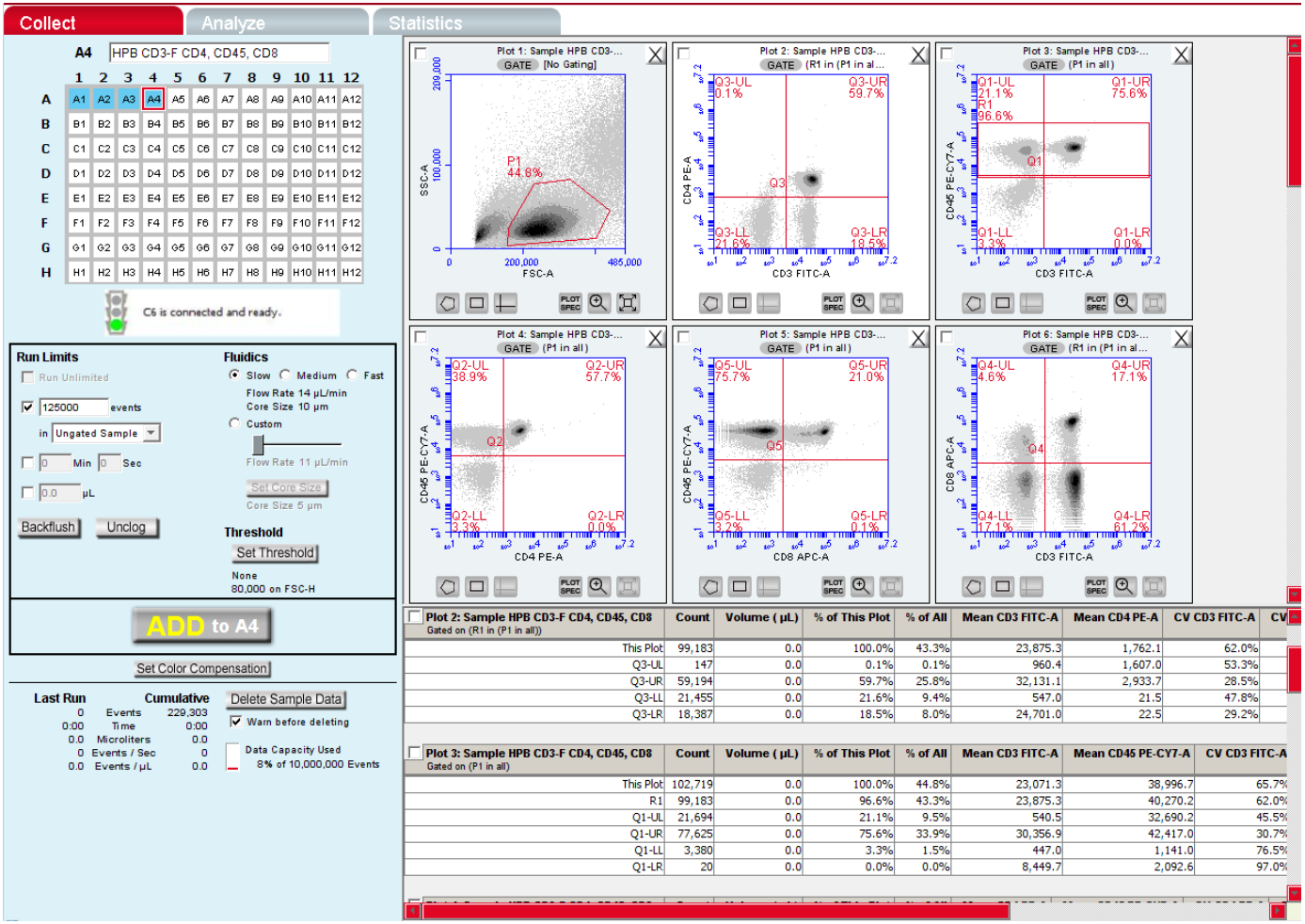
Sample Grid

Cytometer Status

Fluidics Controls

Run Criteria

Real-Time Updates



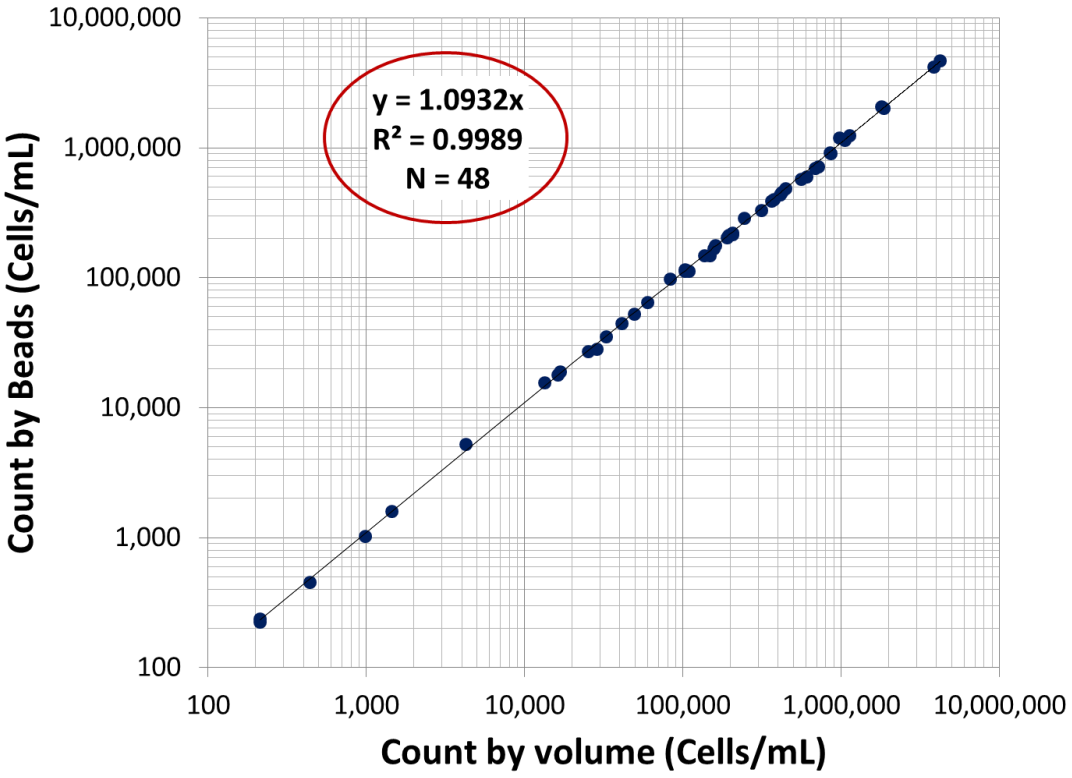
Histogram, Dot Plot, and Density Plot Display Area

Analysis and Gating Tools

Plot Statistics

BD

Verification of Counting on the BD Accuri C6



Comparison of absolute counts measured by direct volume vs counting beads.

Serial dilutions of Jurkat, 3T3, and U937 cells, and T cells, B cells, and platelet samples from four human peripheral blood donors were counted on the BD Accuri C6 by two methods.

X-axis values represent absolute cell counts measured against volume reported directly, while y-axis values are relative to the number of counting beads detected.

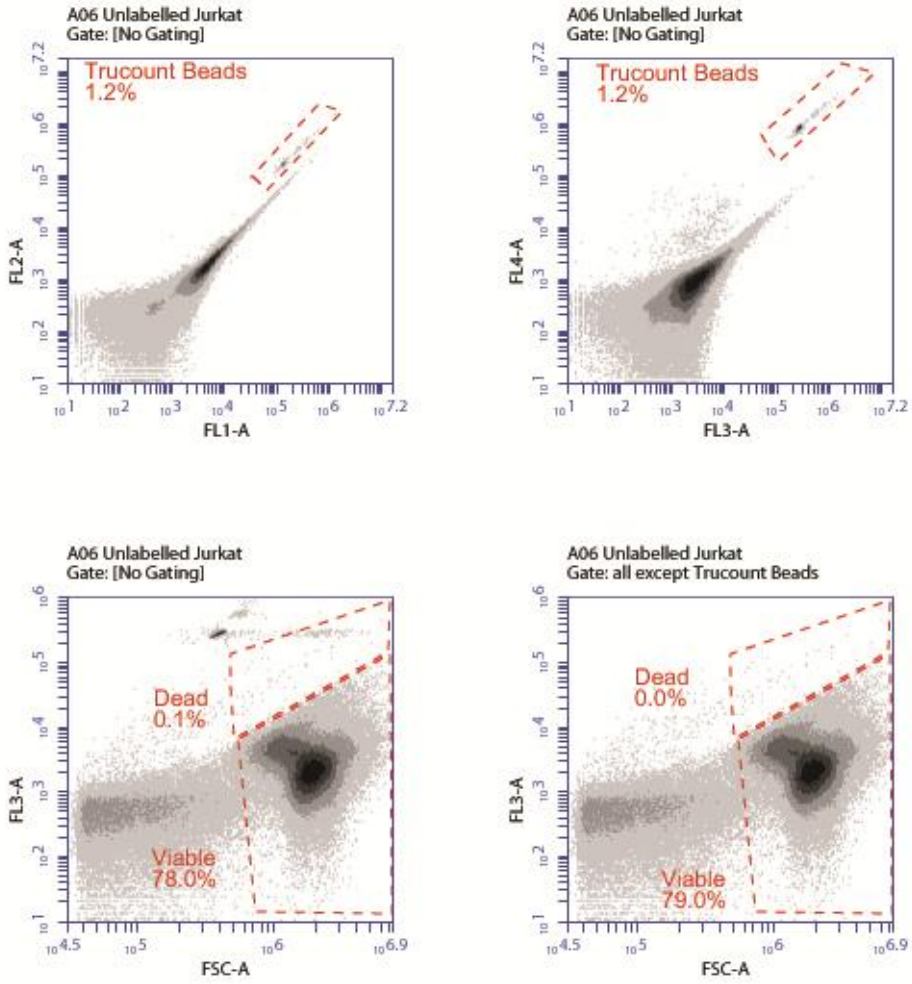
BD

Tips and Tricks

- Counting on any cytometer works best when determining relative counts comparing different samples.
- If an absolute count is needed, use controls of known concentrations to verify the accuracy of the method you want to use.
- Sample preparation is key
  - Optimize staining, minimize washes
  - Dilute cells properly (1,000–5 x 10^6 cells/mL),
  - Keep cells suspended, prevent clumping
- Follow instructions to calibrate the BD Accuri C6 for sample fluid type and sample volume and for proper instrument maintenance.
- If using beads, such as BD Trucount™ beads, take advantage of the beads' broad spectrum of fluorescence to separate them from the sample.
- Use the Zoom tool to more accurately set regions.

BD

Identifying and Excluding Beads for a More Accurate Count



Resources

- **A Guide to Absolute Counting**
  - How-to guide, instrument setup, etc
- **BD Accuri C6 Software User Guide**
  - Calibration instructions
- **Determining Cell Concentration by Direct Volume**
  - Example experiments with tips and tricks
- **Platelet Counting with the BD Accuri C6 Flow Cytometer**
  - Validation of using the BD Accuri C6 for counting platelets

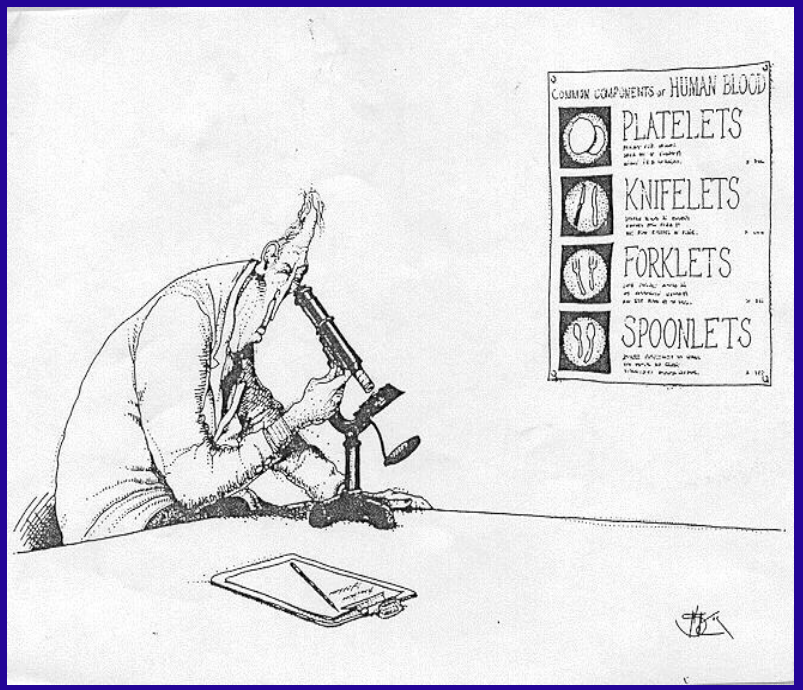
www.bdbiosciences.com/resources/accuri

BD

Acknowledgments

Stacey Roys

Platelet Counting on the BD Accuri™ C6 Flow Cytometer



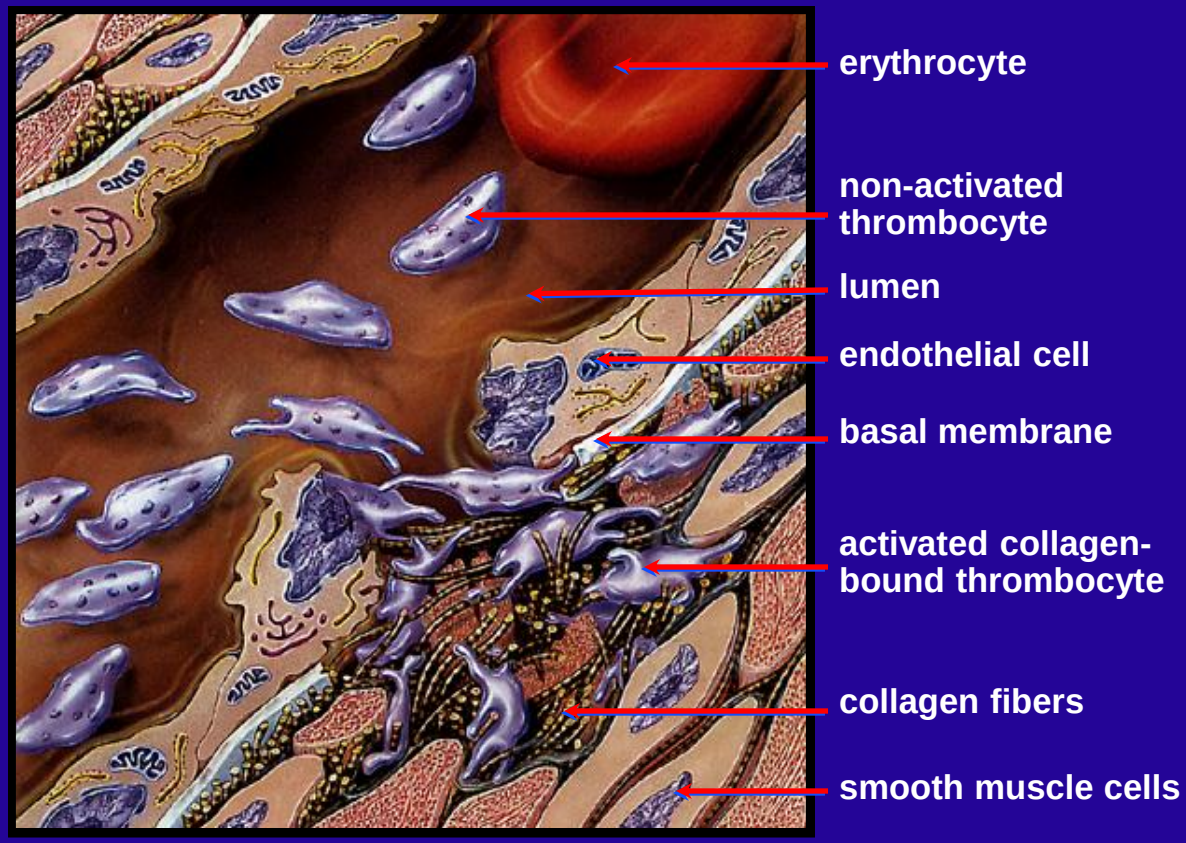
Paul Harrison
Oxford Haemophilia & Thrombosis Centre,
Churchill Hospital,
Oxford University Hospitals NHS Trust,
Oxford, UK

Haemostasis

The Normal In - Situ Process

FIG 2

- **Injured blood vessel**
- **Exposure of collagen**
- **Adhesion of platelets mediated by vWF**
- **Activation and de-granulation of platelets**
- **Aggregation of platelets**



Platelet Counting

In traditional haematological practice the platelet count is used to:

- **Assist in the diagnosis of various clinical disorders**

  Platelet counts may be increased, normal or decreased in different disease states, and its measurement can help in the diagnosis

- **Monitor patients receiving myelosuppressive treatments**

  Myelotoxic therapies (e.g. chemotherapy and radiotherapy) can lead to severe reductions in platelet counts which may then predispose patients to major bleeding problems.

- **Identify all thrombocytopenic patients at risk of significant bleeding.**

  The cause of thrombocytopenia is important, but irrespective of its origin, when the count falls below a certain level there are major clinical implications.

- **Platelet Counting in the Laboratory**

  Checking and standardizing Counts of purified Platelet Preparations.
  Platelet function – platelet counting after stimulation of samples by agonists

Platelet Count

Normal Platelet Count - 150 - 400 x 10^{9}/L

Above 40 :- Spontaneous bleeding uncommon
Bleeding only occurs after trauma/lesion
If spontaneous bleeding apparent then there may be an associated platelet function/coagulation defect

Below 40 :- Bleeding is common but not always present

Below 10 :- Severe Bleeding

Platelet transfusion threshold now set at 10 x 10^{9}/L

Be Aware of Counting Inaccuracies in severe thrombocytopenia

Methods of Platelet Counting/Analysis

- Manual Phase Contrast Microscopy - Oldest Method
- Impedance - Invented in 1953, Full automated counts became available in 1970's. Most widely used.
- Optical, Light Scatter - 1D analysis - 1970's
  2D analysis - 1990's
- Optical, Fluorescence - 1990's
- Immunological - Flow Cytometry – bead or RBC ratio
  - Cell Dyn Series – direct volume
- Immunological – Flow Cytometry – direct volume - Accuri

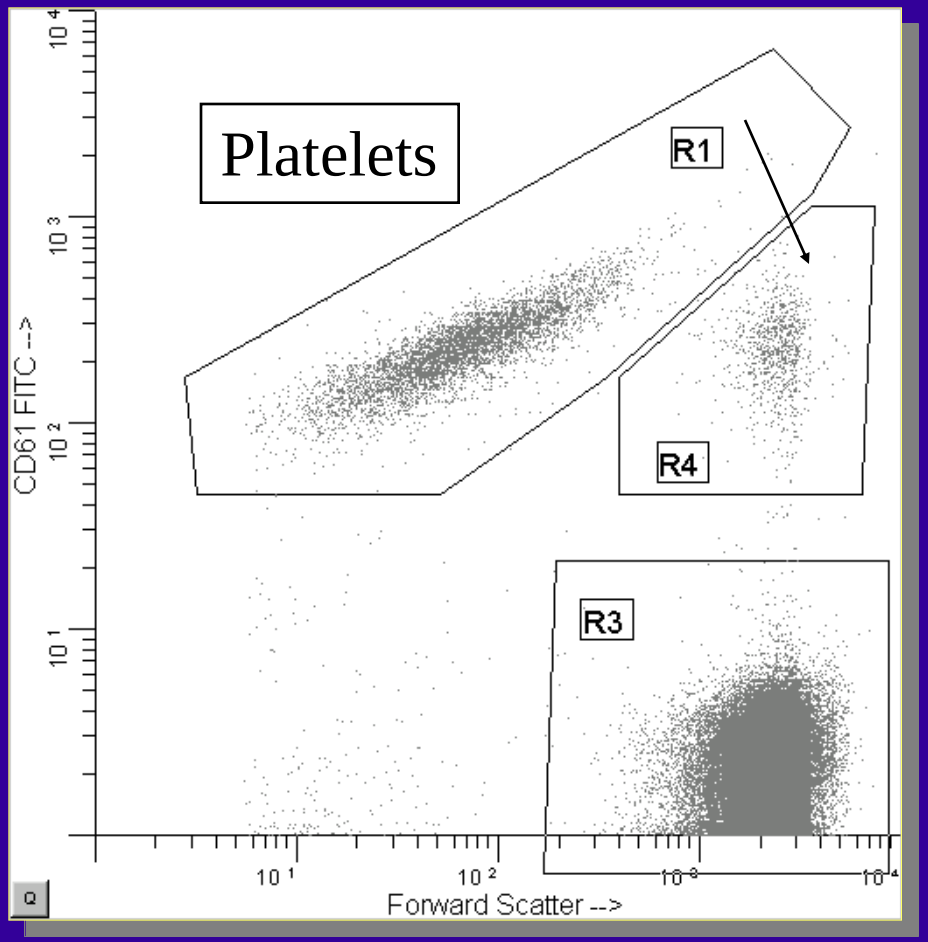
Consensus IRM Method

- Incubate 5 μl Blood with 5 μl of Anti-CD41 and CD61 for 15 minutes in PBS (0.1% BSA)
- Dilute 1:1000 in PBS (0.1% BSA)
- Flow Rate < 4000 events/second
- Collect at least 1000 platelet and 50,000 RBC events
- Bitmap analysis preferred - quad is OK though
- Correct for coincidence using equations
- Report corrected figure
- American Journal of Clinical Pathology, 2001, 115, 448-459 & 460-464.

PLATELET/RBC RATIO

For full method see Harrison et al, 2001, AJCP, 115, 448-59

& ICSH/ISLH, 2001, AJCP, 115, 460-4



Plt/RBC Coincidence

RBC

Correction of the Immuno-Platelet Count for Platelet / RBC and RBC / RBC Coincidence Events

Definitions:

P = Observed number of platelet events
R = Observed number of RBC events
C_{PR} = Observed number of Platelet / RBC coincidence events
RBC = Independent RBC count
P_{imm} = Immuno-platelet count
C_T = Total coincidence events
R_T = Total number of RBC
P_T = Total number of Platelets
C_{RR} = Number or RBC / RBC coincidence events

Calculation of C_{RR}:

		Explanation
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 platelet/RBC coincidence events.
2	$R_T = R + C_{PR} + C_{RR}$	The total number of RBC analyzed is equal to the number of RBC counted plus the number of platelet/RBC 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 RBC to platelets times the number of platelet/RBC coincidence events.
3a	$C_{RR} = C_{PR}(R + C_{PR} + C_{RR})/(P + C_{PR})$	Substituting 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 Immuno-Platelet Count and Total Coincidence %:

Now that we have C_{RR} we can use equations 1 and 2 to calculate P_T and R_T and then calculate P_{imm}

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

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

New Reference Method – What will we use it for?

- Method not designed to be utilised routinely –
  Exceptional cases?
- Assigning Values to Calibration Materials

  Recalibration of Instruments
- Checking the accuracy of different counting methods

  especially in thrombocytopenia

  Impedance

  Optical methods

  Immunoplt method

The Accuracy of Platelet Counting in Thrombocytopenic Blood Samples Distributed by the UK National External Quality Assessment Scheme for General Haematology

Barbara J. De la Salle, FIBMS,[1] Paul N. McTaggart, FIBMS,[1] Carol Briggs, FIBMS,[2] Paul Harrison, FRCPath,[3] Caroline J Doré,[4] Ian Longair,[2] Samuel J. Machin, FRCPath,[2] and Keith Hyde, FRCPath[1]

Key Words: Proficiency testing; Thrombocytopenia; Platelet count; Laboratory hematology; Transfusion

DOI: 10.1309/AJCP86JMBFUCFCXA

Abstract

A knowledge of the limitations of automated platelet counting is essential for the effective care of thrombocytopenic patients and management of platelet stocks for transfusion. For this study, 29 external quality assessment specimen pools with platelet counts between 5 and 64 × 10^9/L were distributed to more than 1,100 users of 23 different hematology analyzer models. The same specimen pools were analyzed by the international reference method (IRM) for platelet counting at 3 reference centers. The IRM values were on average lower than the all-methods median values returned by the automated analyzers. The majority (~67%) of the automated analyzer results overestimated the platelet count compared with the IRM, with significant differences in 16.5% of cases. Performance differed between analyzer models. The observed differences may depend in part on the nature of the survey material and analyzer technology, but the findings have implications for the interpretation of platelet counts at levels of clinical decision making.

Accurate and reproducible platelet counts are essential for the management of thrombocytopenic patients at risk of bleeding, such as patients undergoing cytotoxic therapy for hematologic malignancy. Current UK guidelines recommend a threshold of 10 × 10^9/L for prophylactic platelet transfusion and suggest that this threshold might be reduced further, to 5 × 10^9/L, for patients without risk factors.[1] This reduction would conserve valuable stocks of platelets for transfusion and reduce the exposure of patients to the risks associated with transfusion of blood components. The accuracy of platelet counts produced by routine automated hematology analyzers has been questioned,[2-4] and the limitations of platelet counting at these extremely low levels should be understood by people responsible for the care of patients.

The methods used for automated platelet counting are impedance, optical scatter, optical fluorescence, and immunologic flow cytometry. The traditional "gold standard" method was manual phase contrast microscopy,[5,6] although this method is time-consuming and imprecise at low counts.[7] The introduction of the international reference method (IRM) for platelet counting by flow cytometry[7-9] has improved the precision and accuracy of platelet counting at thrombocytopenic levels and offers a suitable comparator for routine platelet counting methods.

The UK National External Quality Assessment Scheme for General Haematology (UK NEQAS (H)) is uniquely able to undertake major "state-of-the-art" comparisons of equipment performance. Unlike many other external quality assessment (EQA) or proficiency testing providers, UK NEQAS (H) distributes the same in-house–prepared survey material to all instruments for the performance assessment of full blood count (FBC) parameters, including the platelet

Platelet counts using automated analyzers are overestimated (range 6 – 64 x 10^9/L) in 66.3% of specimens (405/611) and significantly overestimated in 16.5% of specimens (101/611) when compared with the IRM platelet count.

Overestimation of thrombocytopenic platelet counts may result in the substantial undertransfusion of platelets in high risk patients in need of platelet transfusion

Agrees with Segal HC, Briggs C, Kunka S, Casbard A, Harrison P, Machin SJ, Murphy MF Accuracy of platelet counting haematology analysers in severe thrombocytopenia and potential impact of platelet transfusion. Br J Haematol. 2005;128:520-525

Further studies in progress :-

UKNEQAS & BEST studies

Improvements in Calibration and Technology Required

Important that clinicians are aware of innaccuracy as decisions to give platelet transfusions are based upon inaccurate triggers

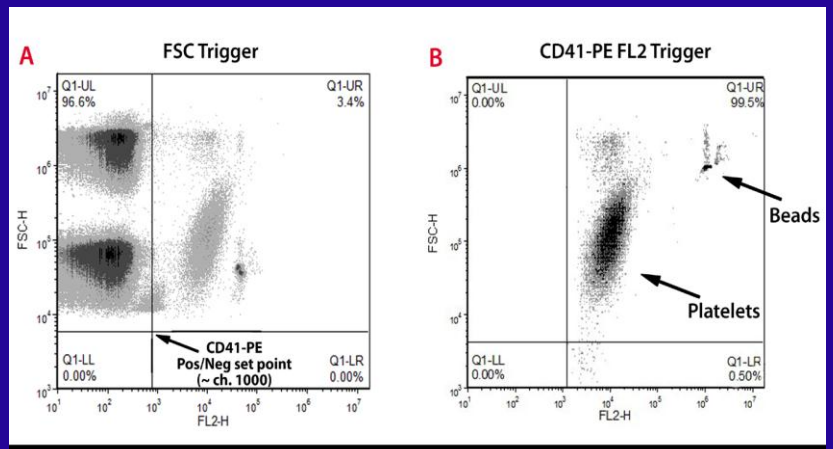
BD Accuri C6 Flow Cytometer

- Small, Affordable Flow Cytometer
- Peristaltic Pump, Laminar Flow Fluidics System
- Can Directly measure sample volume – absolute cell counts/concentrations
- Microprocessor controlled peristaltic pump allows flow rate through the sample introduction probe (SIP) to be measured
- Pressure drop in the SIP when fluid is pulled through is measured and a predetermined calibration constant is used to calculate the flow rate of sample
- The volume (in μl) appears as data in the statistics tales for any gated population
- Accuri Volume Validation beads of a known concentration used to validate the instrument by Accuri before delivery – used same calibration during this study

Accuri White Paper :- Improved Cell Counting (available online)

Platelet Method (based on Alugupalli et al, 2001) :-

- 1-2 μl of citrated whole blood diluted 1:10 with HBS/1% formaldehyde
- 20 μl aliquots incubated with 20 μl of CD-41PE for 20 minutes at RT
- Samples diluted with HBS/ 1% formaldehyde to 1 ml
- 5 μl of counting beads (spherotech, RFP-50-5 beads) added for comparison



C

Platelet Counts per μl whole blood (% CV)

Peripheral Blood	Accuri C6 Volume Method	Counting Bead w/ flow cytometer
Donor 1, Sample A	91,796 (1.82)	91,444 (1.27)
Donor 1, Sample B	114,172 (0.33)	127,908 (4.05)
Donor 2, Sample A	249,312 (1.45)	266,462 (4.70)
Donor 2, Sample B	251,176 (2.09)	288,893 (5.67)

Technical Bulletin :- A guide to Absolute Cell Counting Using the BD Accuri C6 Flow Cytometer (available online)

Summary of recommendations

Table 3. Summary of recommendations for absolute counting on the BD Accuri C6.

Area	Recommendations
Preventive maintenance	Follow recommended preventive maintenance routines.
Sample concentration	1,000–5 x 10^6 cells/mL
Cell suspension	Assess and minimize cell clumping.
Sample medium	Calibrate fluidics when necessary to account for liquid viscosity.
Sample type	Cell lines Primary cells Beads Bacteria*
Sample volume	12 x 75-mm tube: 300 µL–2 mL Users should verify other tube/plate types, calibrate fluidics when necessary.
Fluidics speed	Standard settings: Medium or Fast only Custom settings: Minimum settings are listed below. Appropriate flow rate and core size combinations are experiment specific and should be validated by the user. - Flow rate: ≥15 µL/min - Core size: ≥16 µm
Using the BD CSampler	Use the agitate function if necessary to maintain a homogeneous suspension. Avoid V-bottom, flat-bottom, and deep-well plates. Sample volume: - 96-well round- or U-bottom plates: 40–50% well capacity (150 µL–200 µL) - 12 x 75-mm tubes: 300 µL–2 mL
Troubleshooting	See the Troubleshooting section.

*For special considerations when counting bacteria and other small particles, see the BD Accuri Technical Bulletin Threshold and Analysis of Small Particles on the BD Accuri® C6 Flow Cytometer.

Platelet Count in blood
140-450 x 10^9/L
= 140-450 x 10^6/ml

1:1000 dilution as per IRM would give
~ 140-450 x 10^3/ml

Lowest Blood Count
1 x 10^9/L
1 x 10^6/ml
At 1:1000
~ 1000/ml

Optimization of Volume Counting Method on the Accuri C6

- Starting Point – Existing IRM method for platelet/RBC ratio (1:1000 final dilution factor)
- Comparison of Ratio and Volume Results in UKNEQAS reference samples
- Optimization of volumes and dilutions of blood used & flow rates
- Final Method (1:1000 final dilution factor)

    1) 20 μl of EDTA whole blood (mixed by inversion 6x) + 380 μl of Buffer (1:20 dil)

    2) 50 μl of diluted blood + 2.5 μl CD61-FITC in a total volume of 2500 μl (1:50 dil)

    3) Invert gently 6 x before analysis

    4) Analyse at Medium Flow Rate

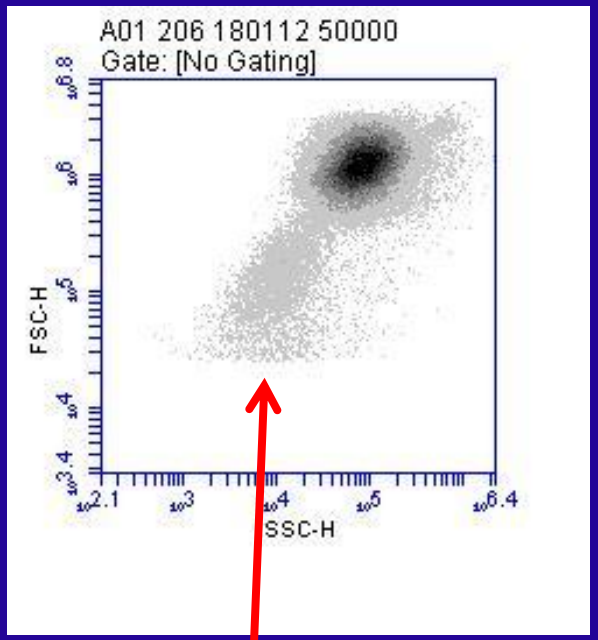
    5) Comparison of 100 μl or collection of at least 1000 platelet/50,000 RBC events

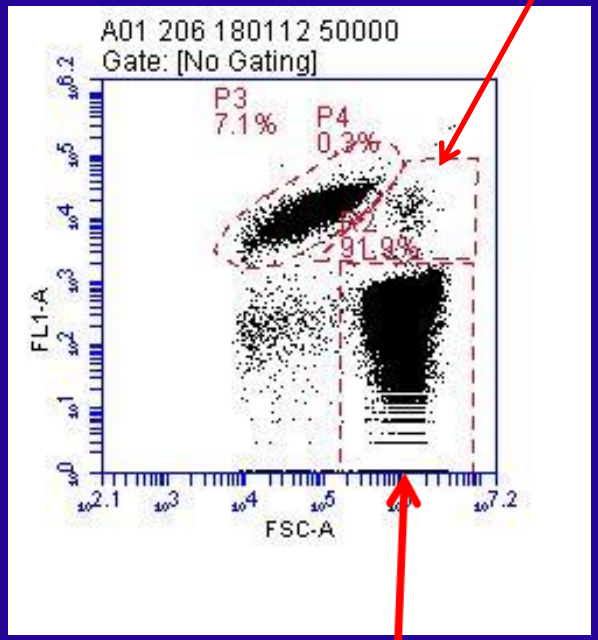
    6) Check coincidence events make no difference to final value

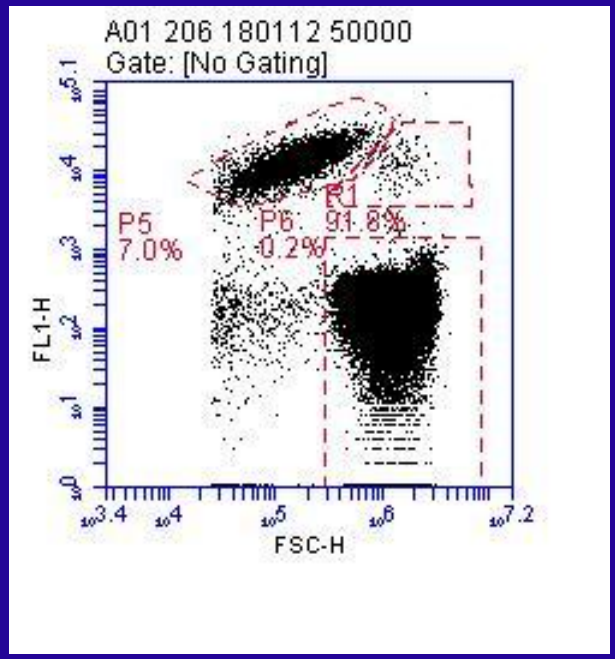
- Comparison of 144 EDTA blood samples from Haematology with a range of platelet counts (Accuri RBC ratio and Volume versus IRM (BD Facscalibur)
- Determination of Reproducibility at different counts and linearity

Optimised Settings for platelet counting

RBC/platelet coincidence





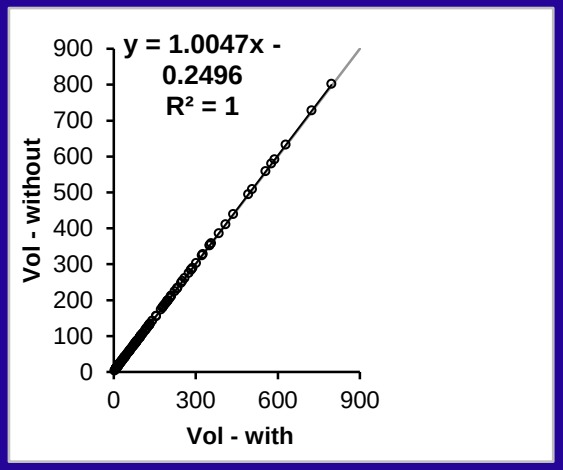


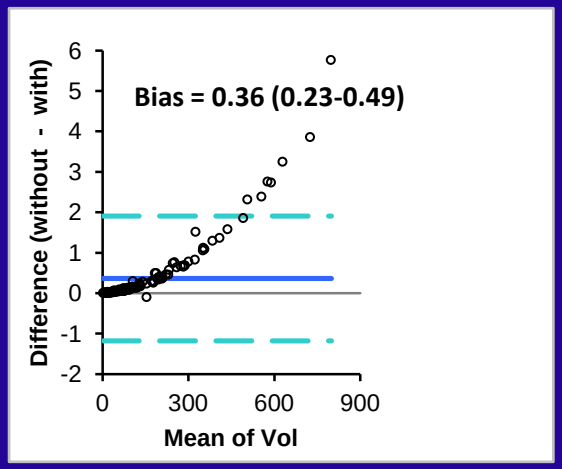
Use 25,000 FSC Threshold

Ensure that all RBC's are gated on if using ratio method

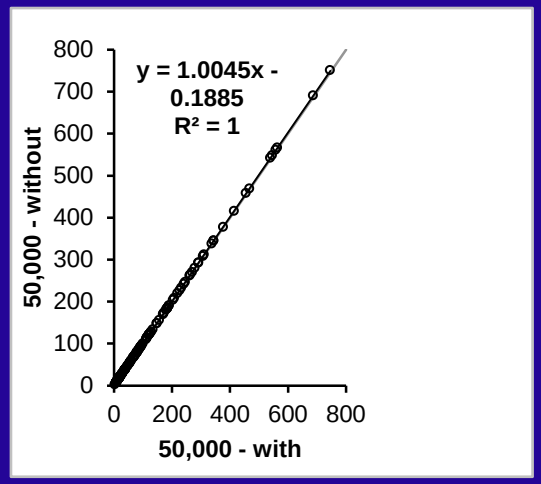
The Influence of Coincidence Correction on the platelet/RBC ratio at 1:1000

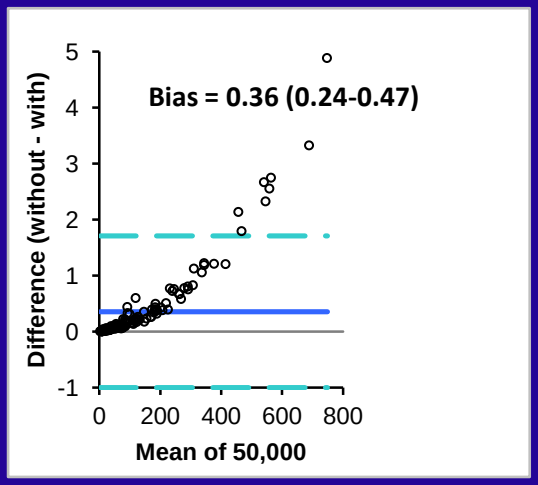
Accuri (100 µl)



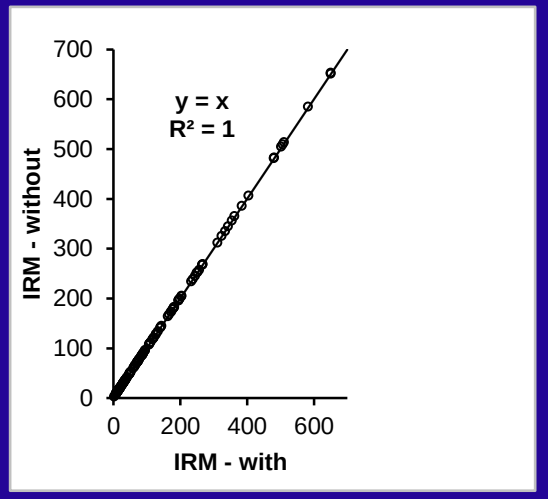


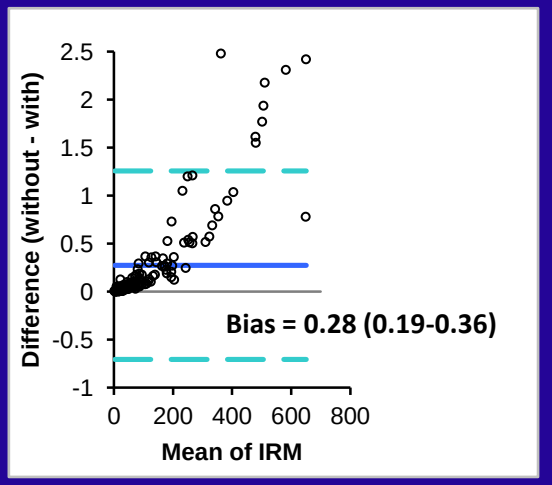
Accuri (1000/50000)



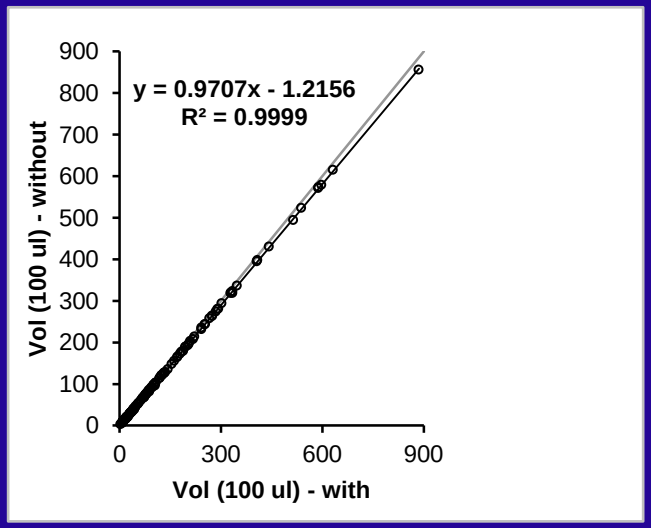


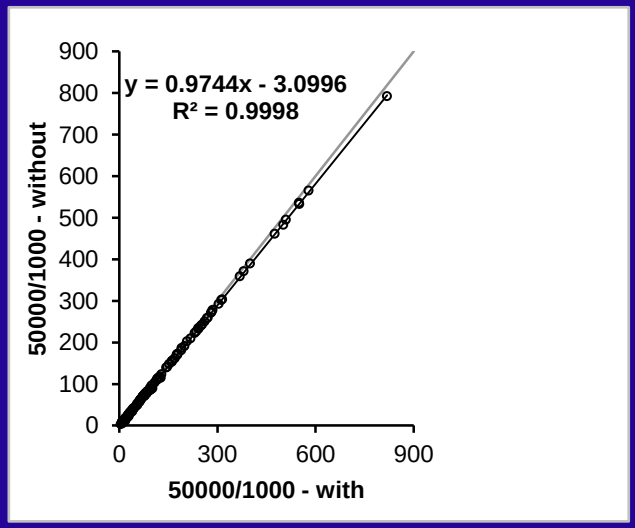
IRM (1000/50000)

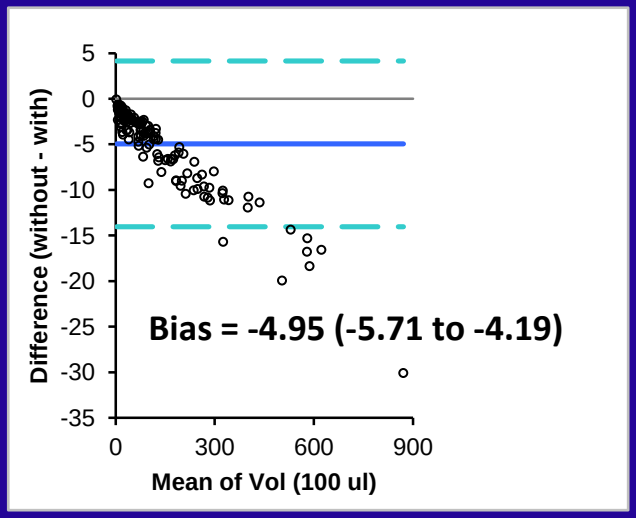


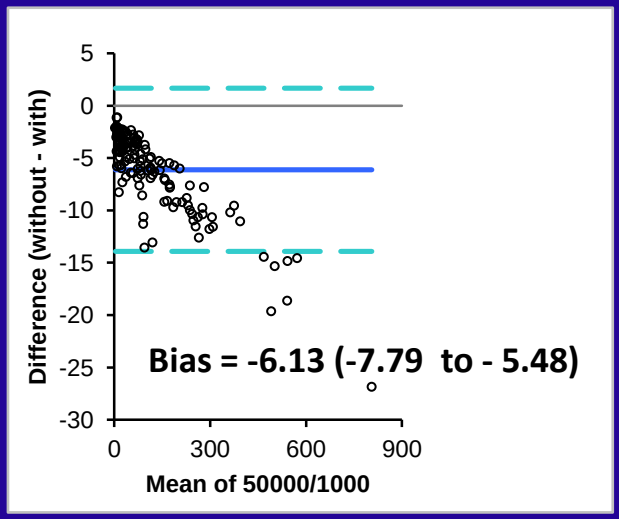


Accuri Volume Results (with and without platelet/RBC coincidence)

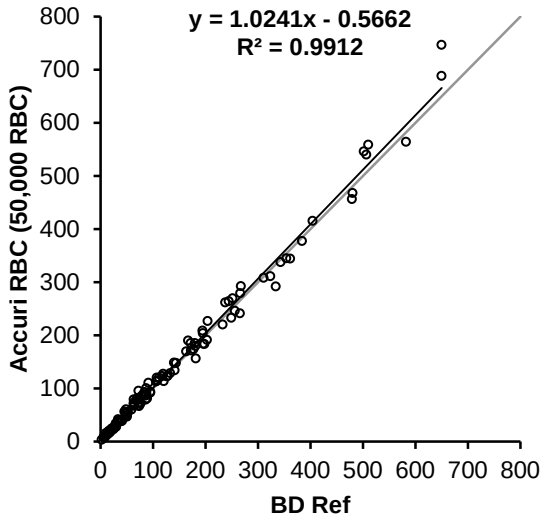


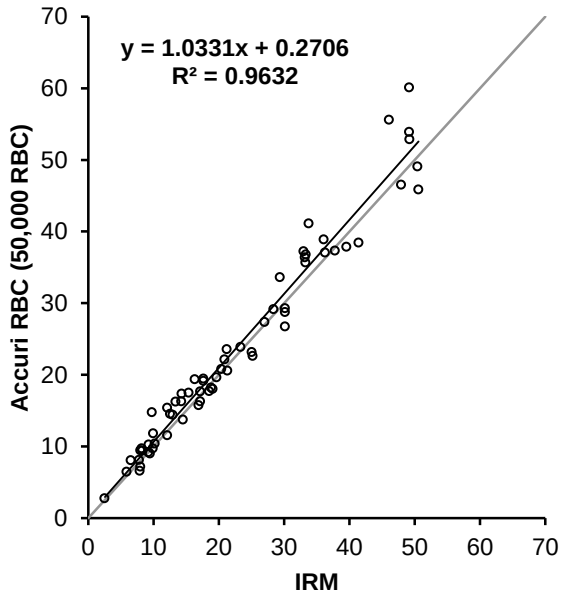


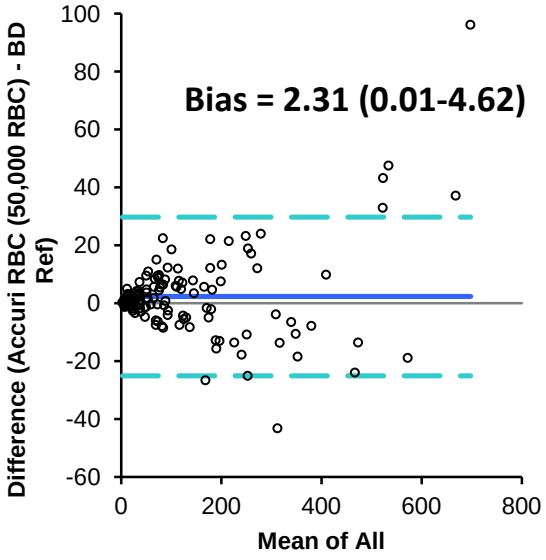


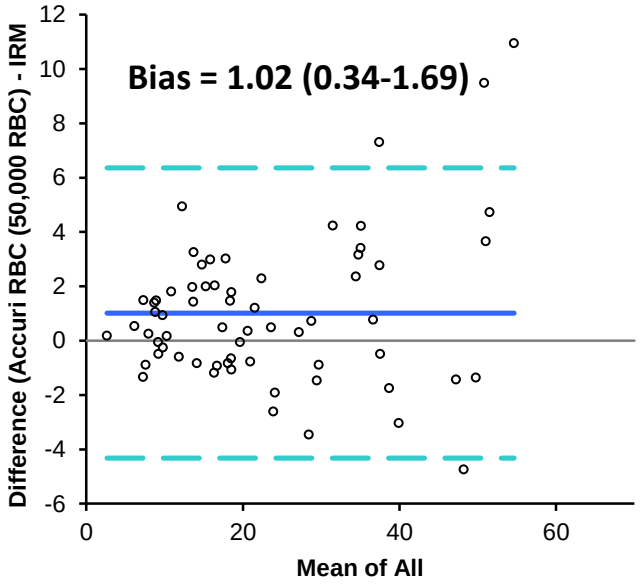


Accuri RBC Ratio (1000 platelet/50,000 RBC events) V IRM

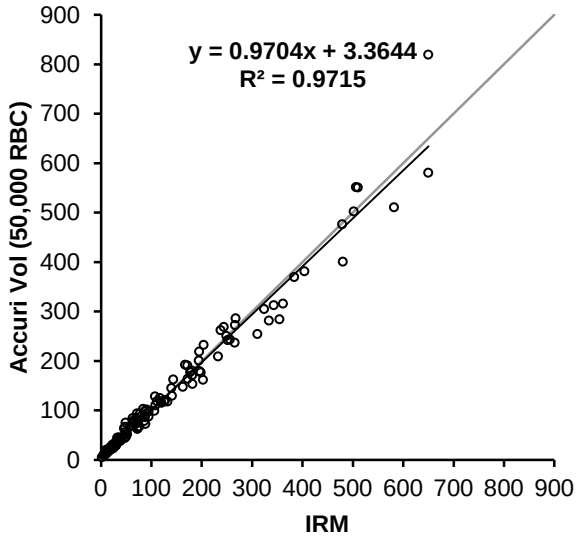


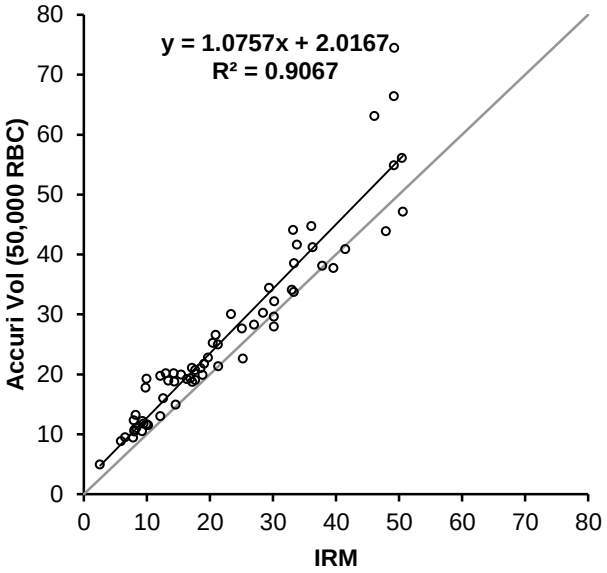


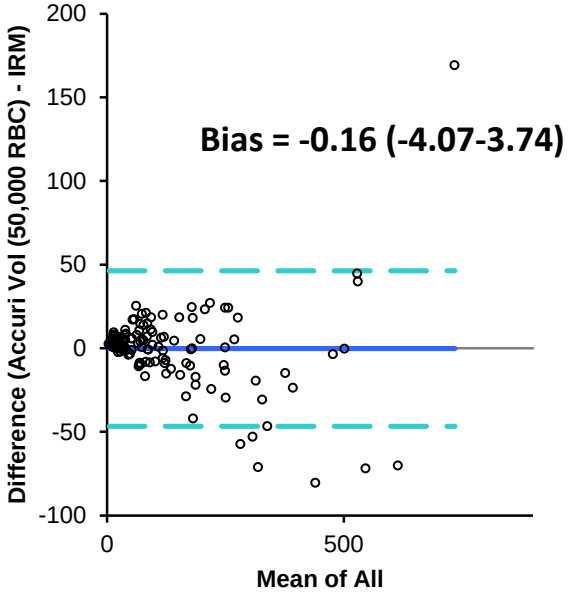


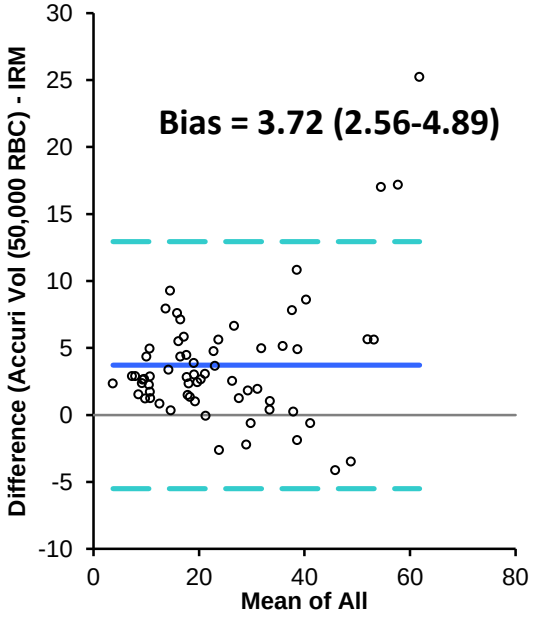


Accuri Volume (1000 platelet/50,000 RBC events) V IRM

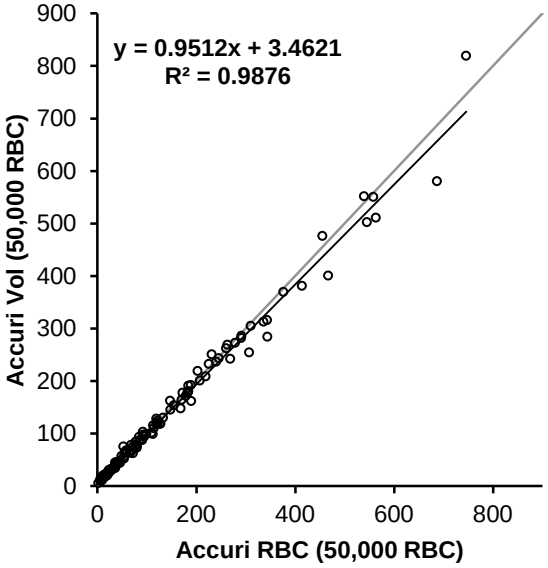


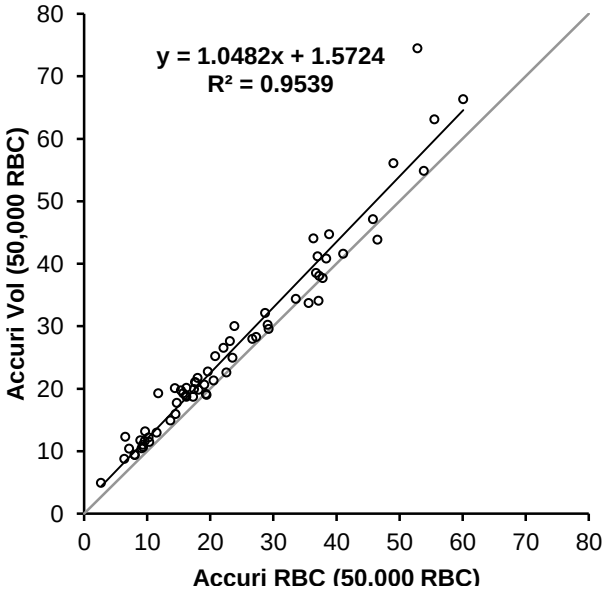


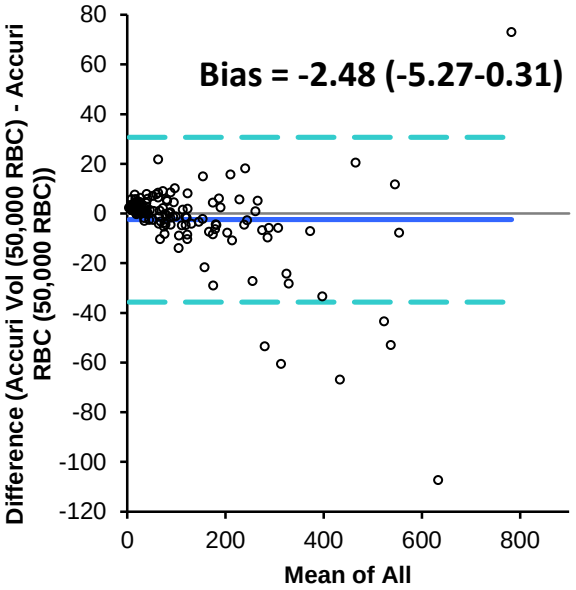


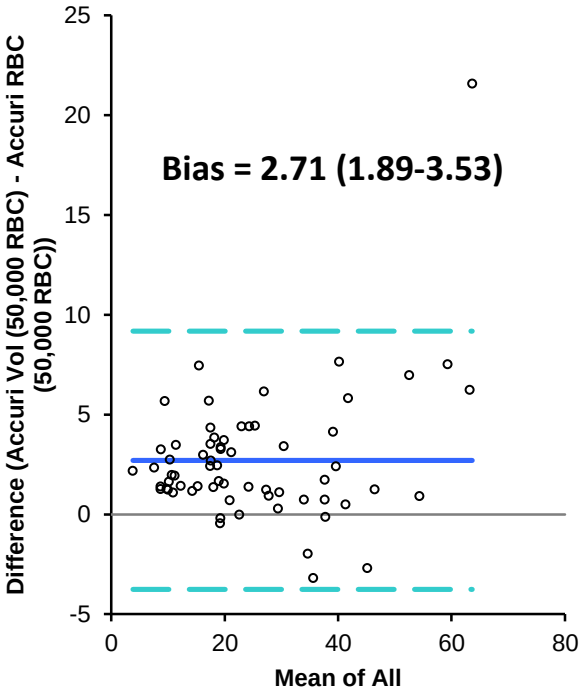


Accuri Volume (1000 platelet/50,000 RBC events) V Accuri RBC Ratio









Summary of Correlation and Bland Altman Data

	Comparison	R^2	Bias	R^2	Bias
		All counts		< 50 x 10^9/L	
1000 platelets/ 50,000 RBC	RBC ratio v IRM	0.99	2.31	0.96	1.02
	Vol v IRM	0.97	-0.16	0.91	3.7
	Vol v RBC ratio	0.99	-2.5	0.95	2.71
100 µl	RBC ratio v IRM	0.99	10.05	0.97	1.95
	Vol v IRM	0.98	9.03	0.91	3.3
	Vol v RBC ratio	0.99	-1.02	0.96	1.35

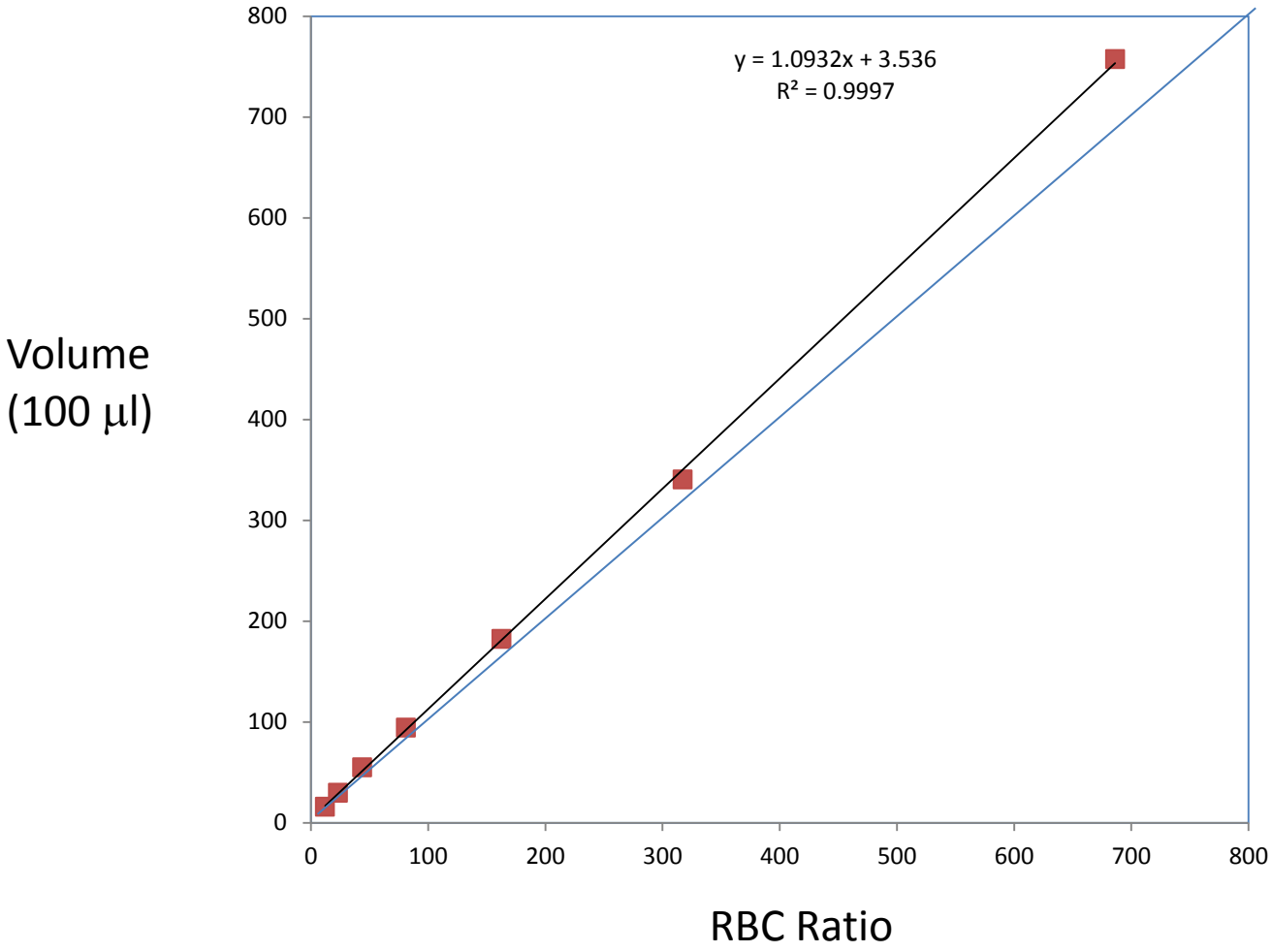
Reproducibility of Accuri RBC ratio and Volume at Beginning of Study

Sysmex Count	RBC ratio	Volume	Method/Volume
36	Mean = 38.7 CV = 4.5%	Mean = 40.9 CV = 12.8%	1000 platelets/ 50,000 RBC (Volume = 26.31 µl)
36	Mean = 36.8 CV = 3.2%	Mean = 37.1 CV = 8.9%	300 µl
11	Mean = 11.4 CV = 4.6%	Mean = 12.3 CV = 4.5%	100 µl
11	Mean = 11.3 CV = 4.84%	Mean = 12.5 CV = 8.05%	1000 platelets/ 50,000 RBC (Volume = 88.5 µl)
639	Mean = 698.5 CV = 2.94%	Mean = 783.5 CV = 8.0%	100 µl
639	Mean = 701.7 CV = 5.09%	Mean = 672.3 CV = 11.6%	1000 platelets/ 50,000 RBC (Volume = 1.6 µl)

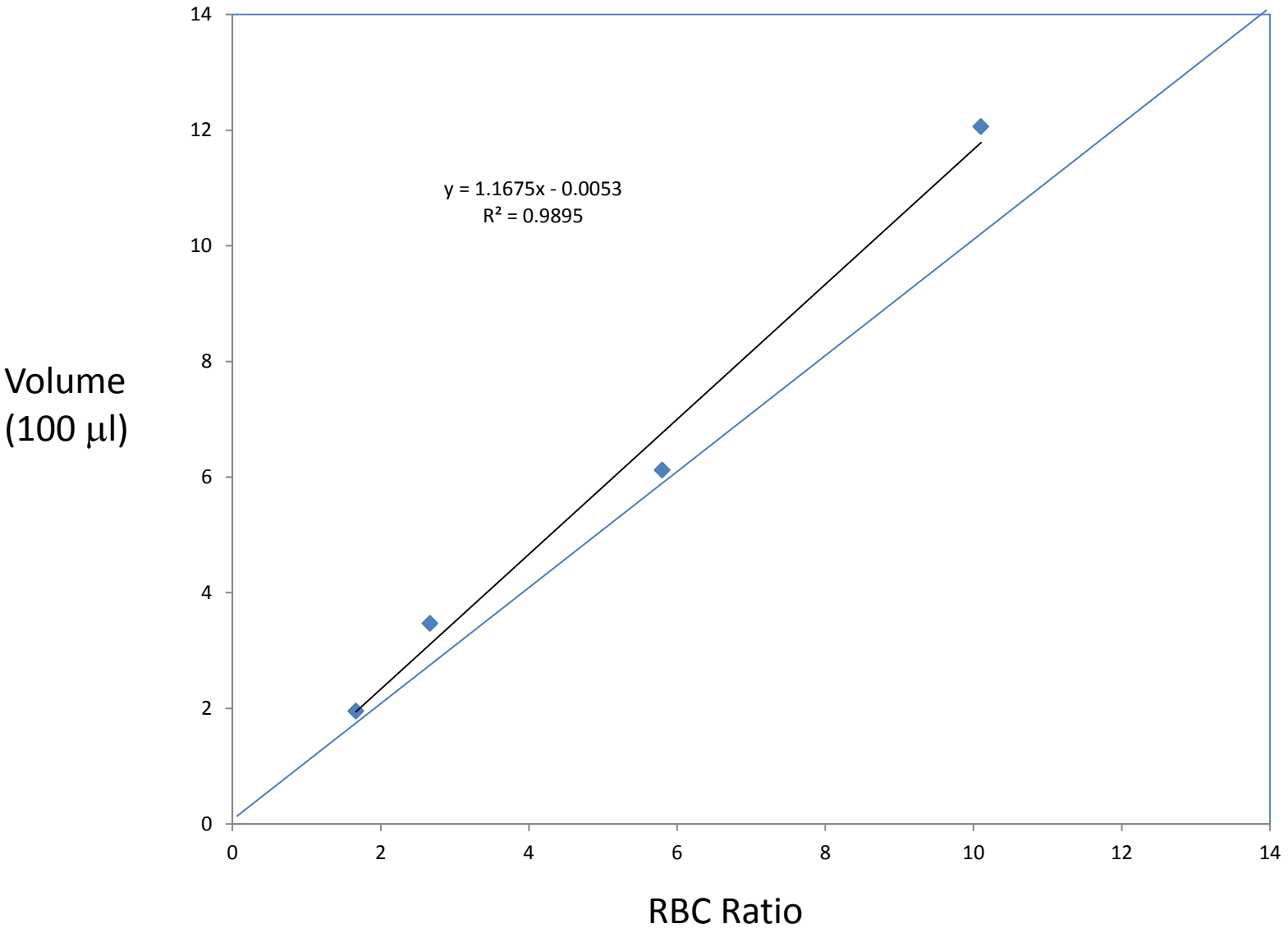
Reproducibility of Accuri RBC ratio and Volume at End of Study

Sysmex Count	RBC ratio	Volume	Method/Volume
36	Mean = 46.8 CV = 1.9%	Mean = 53.7 CV = 6.4%	100 µl
36	Mean = 44.3 CV = 4.9%	Mean = 53.0 CV = 4.1%	1000 platelets/50,000 RBC (Volume = 21.08 µl)
261	Mean = 258.7 CV = 5.8%	Mean = 253.8 CV = 9.3%	100 µl
261	Mean = 243.7 CV = 3.1%	Mean = 231.8 CV = 10.4%	1000 platelets/ 50,000 RBC (Volume =10.95 µl)
527	Mean = 510.9 CV = 3.0%	Mean = 561.7 CV = 3.6%	100 µl
527	Mean = 486.3 CV = 2.96%	Mean = 504.2 CV = 4.4%	1000 platelets/ 50,000 RBC (Volume =10.01 µl)

Linearity Check (Sample double diluted downwards)



Linearity Check (Sample double diluted downwards)



Summary

- Small, Affordable , Easy to Use and Reliable Flow Cytometer
- Can be used to measure platelet counts at all levels using either the platelet/RBC ratio IRM or direct volume method
- Both methods agree with each other and IRM performed on BD Facscalibur
- 1:1000 dilution is sufficient to eliminate need for coincidence correction with ratio
- Direct Volume method requires no additional measurement of RBC or reference bead counts (include platelet/RBC coincidence in results)
- If using RBC ratio method ensure that all RBC are gated on
- Reproducibility and Linearity of both methods are good
- CV's for volume method are higher (3-12%) than platelet/RBC ratio (2-6%)
- Volume method is good but will probably not replace the RBC ratio as reference method and counting in severe thrombocytopenia
- Provides an additional simple new method for counting platelets in blood samples and purified preparations.

Acknowledgements

Andrew Masters — Department of Haematology, Oxford University Hospitals NHS Trust

Kate Easten — BD Biosciences, Oxford, UK

ICSH/ISLH Reference platelet count task force