LeucoCOUNT™

Detection and Enumeration of Residual White Blood Cells in Leucoreduced Red Blood Cell and Platelet Products Using the LeucoCOUNT Kit*

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* For In Vitro Diagnostic Use.

Background and Rationale

Clinical studies have indicated that using leucocyte-reduced cellular blood components may prevent adverse transfusion reactions such as transfusion-associated graft-versus-host disease, transfusion-related bacterial sepsis, febrile reactions, transmission of cytomegalovirus, and alloimmunization to HLA antigens.[1–7] In areas of hematology, oncology, and transplantation, leucodepleted blood products have become the standard for support of patients in need of multiple transfusions.

Detection and enumeration of residual white blood cells (rWBCs) in filtered or depleted platelet and RBC packs is therefore an area of great and increasing interest in transfusion medicine and process control development. The American Association of Blood Banks (AABB) has published standards stipulating fewer than 5×10^8 leucocytes per transfused unit to prevent febrile transfusion reactions, and 5×10^6 for prevention of cytomegalovirus infection or alloimmunization.[8] Outside the United States, recognition of the value of leucodepletion has also led to an increase in the quality control of leucodepleted blood products.[9] Standards in Europe mandate leucoreduction to fewer than 1×10^6 leucocytes per unit.[10] Because the typical unit of erythrocytes or platelets is approximately 300 milliliters in volume, leucoreduction quality control assays must effectively detect 10 to 20 rWBCs per milliliter to confirm that the leucoreduction process is adequate.

The most common way to quantitate rWBCs is a manual count, performed using a light microscope and a large-volume hemacytometer, known as the Nageotte method. The Nageotte method is time and labor intensive, and is associated with a count underestimation bias and variation, even when technologists have had appropriate training.[11] Advancements in leucodepletion technology, which include high-efficiency filters, have led to a lower residual concentration of white cells in red cell and platelet products, and frequently result in leucocyte concentrations lower than the sensitivity range of the Nageotte method.[12]

Other enumeration methods have been validated and are becoming more widely accepted, including polymerase chain reaction, volumetric capillary cytometry, and flow cytometry.[12,13] Because of its many applications in the area of transfusion medicine, flow cytometry is already widely used in the blood bank setting. Flow cytometry provides a sensitive and rapid method of enumerating rWBCs in leucoreduced blood products. A distinct advantage that flow cytometry has over other methods of enumeration is its ability to process a large number of samples, and when necessary for better sensitivity, to increase the volume of sample collected.

Assay Design

There are important quality control factors to consider with assays in the blood bank and transfusion services environment. For detection and enumeration of rWBCs in leucodepleted blood products, assays have to be highly sensitive, accurate, and precise. The assay should show little variability between laboratories and/or users, should be useful for both RBC and platelet samples, and should demonstrate stability over a period of typical sample turn-around time.

Previously described flow cytometry methods have used a DNA dye to stain the rWBCs, usually propidium iodide (PI) or a combination of PI and thiazole orange. In this method, chicken red blood cells are used as an internal concentration standard.[15,16] The chicken cell concentration must be determined using a hematology analyzer before the cells are added to the test sample.[14] This method requires considerable sample handling time, introducing room for error.

The LeucoCOUNT Kit eliminates the need for a separate counting standard by combining the flow cytometric method with TruCOUNT™ absolute enumeration technology. The reagent is a combination of PI, RNase, and detergent in a unique buffer solution. TruCOUNT Tubes contain predispensed reference beads, which serve as an internal concentration standard. The result is a simple, precise assay for enumeration of rWBCs in leucoreduced blood products.

The following components are part of the LeucoCOUNT Kit:

rWBC Reagent:

- **Nucleic Acid Dye**—Propidium iodide (PI) is a DNA/RNA-specific dye that stains all nucleated cells such as leucocytes. The dye, excited at 488 nm, emits in the FL2 and the FL3 channel. The brightly stained leucocytes, detected in FL2, are easily distinguishable from any non-nucleated particles, such as erythrocytes and platelets, which are not stained with PI.

- **RNase**—RNase is included for the enzymatic digestion of any RNA present in the sample, which could otherwise be stained by the PI and provide falsely elevated counts.

- **Detergent**—The detergent incorporated into the staining reagent permeabilizes the cell membrane, which permits entry of the PI.

- **Buffers**—The reagent is appropriately buffered to provide a stable solution for the stained sample, and to optimize fluorescence and scatter properties.

TruCOUNT Tubes—TruCOUNT Absolute Count Tubes are 12 × 75-mm polystyrene tubes that contain a lyophilized pellet of fluorescent-dyed beads (4.2-µm). The pellet is restrained in the bottom of the tube by a stainless steel retainer. The number of beads in each pellet (beads per test) varies among lots and is printed on the foil pouch. The beads provide an internal reference for obtaining an absolute cell count. The reagent and sample are added directly to the TruCOUNT Tubes.

The LeucoCOUNT assay meets important laboratory requirements (Table 1), and provides features and benefits not associated with the Nageotte method of enumeration (Table 2).

Table 1. Laboratory assay requirements met by the LeucoCOUNT Kit

Requirement	LeucoCOUNT Feature	End Result
Precision in absolute counts	Internal reference particles (TruCOUNT) for determining absolute cell counts	Eliminates variability associated with using multiple instruments to determine absolute count
No cell loss because of sample processing	No-wash technique	Eliminates cell loss that can occur during cell-washing steps
Accurate identification of residual white cells	A nucleic acid dye to detect only nucleated cells. rWBC reagent and instrument settings optimized for separation of residual populations	Clear resolution of residual white cells from instrument noise and debris
Consistency in assay performance, and quality control in data acquisition and analysis	CD-Chex PLUS controls and a non-leucoreduced sample as a gating aid. Instrument setup instructions for consistency of settings	Residual white cells can be located consistently in the same gated region

Table 2. LeucoCOUNT features and benefits the Nageotte method lacks

Features	Benefits
Single reagent and protocol for both platelet and RBC samples	Simplified assay, reduced risk of errors in sample prep, efficient use of reagent
Commercially optimized reagent kit	Minimizes validation work for implementing a new study
High throughput	Increased lab productivity and cost reduction Nageotte: 1 sample: 30 min 10 samples: 3–4 hours LeucoCOUNT: 1 sample: 10 min 10 samples: 50 min
Loader capability	Automated sample acquisition increases productivity. Forty samples per rack when used with FACS Loader
Software for data processing	Enhanced ease-of-use with CellQuest software and LeucoCOUNT-specific experiment documents

Assay Optimization and Gating Strategy

When performing rare-event analysis in flow cytometry, it is critical to have the ability to discriminate the target from background noise or cellular debris. The LeucoCOUNT reagent has been optimized to stain only nucleated cells such as leucocytes, and to stabilize the stained cells in suspension to enhance detection by the cytometer. Instrument settings have also been optimized to maximize the separation between residual white cells and any background debris. These factors, combined with a consistent gating technique, make LeucoCOUNT an accurate method for detecting and enumerating rWBCs in leucoreduced blood products.

The LeucoCOUNT Kit provides a single reagent for enumerating rWBCs in both red blood cell and platelet products. The assay does not require additional anticoagulants* for sample processing, and can provide results for 10 samples in less than 1 hour. LeucoCOUNT is FACS Loader compatible for walk-away sample acquisition.

The typical staining pattern for the LeucoCOUNT assay is illustrated in Figure 1. Plot 1a shows a non-leucoreduced RBC sample. Plot 1b shows a leucoreduced RBC sample. Both samples were acquired and analyzed using CellQuest™ software. The TruCount reference beads emit fluorescence in FL1 and FL2. The PI-stained rWBCs appear on an FL1 vs FL2 dot plot as a discrete population. Event counts for the bead population (R1) and the rWBC population (R2) provide information necessary to calculate the absolute count of rWBCs. A whole blood sample rich in WBCs is prepared as a gating aid. A detailed description of gating and calculation strategies is given in the LeucoCOUNT package insert.

The assay detection limit is dictated by sample volume, which can be modified to increase sensitivity. Because there are 50,000 beads in a TruCount Tube, and the standard LeucoCOUNT protocol calls for 10,000 beads to be acquired, 20 µL of the recommended 100 µL sample volume is analyzed. In this case, a sample from a 300 mL pack with 1.5×10^4 rWBCs would on average yield 1 event in Region 2. Increasing the analysis to acquire 50,000 beads in the same sample would on average yield 5 rWBC events in Region 2, giving a more statistically robust result. If the 300 mL pack were to contain only 3000 rWBCs, the 50,000 bead collection volume would yield an average of 1 event in R2, while the standard 10,000 bead collection could yield no events.

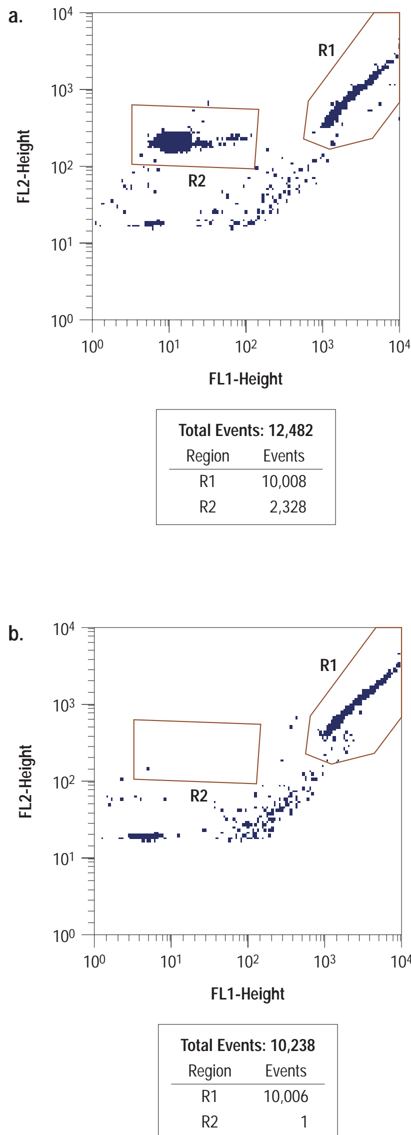


Figure 1. FL1 vs FL2 dot plots with data from a non-leucoreduced RBC unit (a) and a leucoreduced RBC unit (b) Region R1 contains the TruCOUNT bead population. Region R2 contains the residual white blood cell population.

* EDTA is not recommended when using LeucoCOUNT.

Performance of the LeucoCOUNT Assay

An understanding of the performance characteristics and boundaries associated with any assay is critical to appropriately interpreting the results. To validate the performance of the LeucoCOUNT assay, several studies were conducted. Determinations of accuracy, linearity, precision, interlaboratory reproducibility, and multi-platform comparison studies were performed at four sites. Not every site supplied data for every study. The following three clinical sites and one internal site were involved in the studies:

- New York Blood Center; New York, NY
- Broward Community Blood Center, Community Blood Centers of South Florida; Lauderhill, FL
- Civitan Regional Blood Center, (Lifesouth Community Blood Center); Gainesville, FL
- Applications Feasibility Laboratory, Becton Dickinson Immunocytometry Systems; San Jose, CA

The data presented here are pooled.

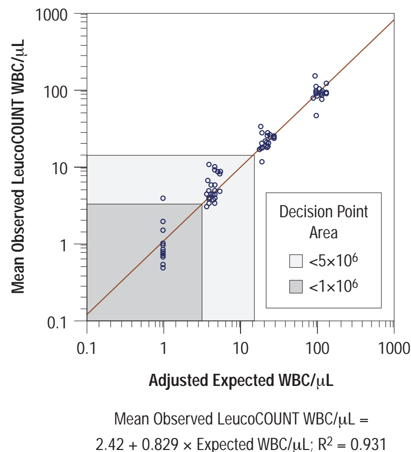


Figure 2. Regression of mean observed LeucoCOUNT WBC/μL versus expected WBC/μL for platelet packs (expected counts of 0 adjusted to 1 WBC/μL for presentation on logarithmic scale)

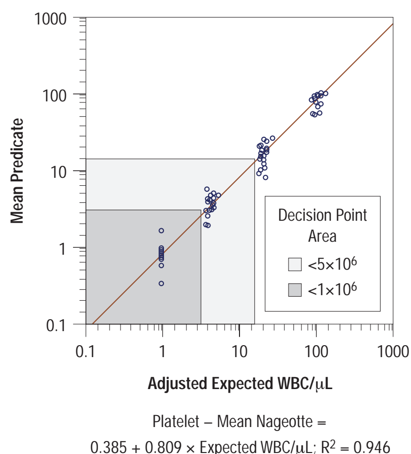


Figure 3. Regression of mean observed Nageotte WBC/μL versus expected WBC/μL for platelet packs (expected counts of 0 adjusted to 1 WBC/μL for presentation on logarithmic scale)

Accuracy

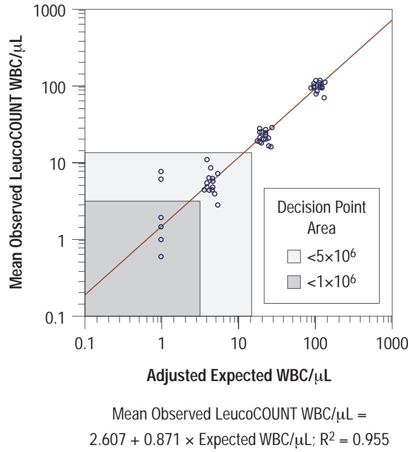
The current methods available for leucocyte depletion are very effective, but can leave detectable levels of rWBCs. To determine the accuracy of the LeucoCOUNT assay over a significant dynamic range, artificially produced samples were necessary, and a residual white cell dilution experiment was performed. Because performance could be affected by the assay matrix, samples were generated from both routine red blood cell and platelet packs. Five serial dilutions were made from non-leucoreduced samples using a double-filtered diluent to evaluate accuracy at four ranges of residual white cell concentration. The hematology analyzer count at the 500 WBC/μL level was used to estimate the expected count at each target concentration, and both the LeucoCOUNT and the Nageotte methods were compared to the expected value to obtain a percent bias.

The double-filtered diluent, containing ≤1 WBC/μL, represents the lowest clinically relevant decision point, or 1×10^5 residual white cells per 300 mL pack. The target dilution points of 4, 20, and 100 WBC/μL represent the decision points of 1×10^6, 5×10^6, and 1×10^7 rWBC/pack, respectively. Accuracy results determined using platelet samples are shown in Table 3 and Figures 2 and 3. Accuracy results determined using RBC samples are shown in Table 4 and Figures 4 and 5.

Table 3. Accuracy results for platelet samples

rWBC Target Value	Mean Bias	Method of Accuracy Determination*	Number of Samples	90% Lower Confidence Limit	90% Upper Confidence Limit
0 WBC/μL	0.7 rWBC/μL	L–E	21	0.47 rWBC/μL	0.9 rWBC/μL
4 WBC/μL	3.5%	[(L–E) ÷ E] × 100	21	–4.7%	11.8%
20 WBC/μL	–6.2%	[(L–E) ÷ E] × 100	21	–14.3%	1.8%
100 WBC/μL	–10.3%	[(L–E) ÷ E] × 100	21	–18.0%	–2.5%
0 WBC/μL	0.5 rWBC/μL	N–E	21	0.3 rWBC/μL	0.6 rWBC/μL
4 WBC/μL	–11.8%	[(N–E) ÷ E] × 100	21	–20.5%	–3.1%
20 WBC/μL	–19.5%	[(N–E) ÷ E] × 100	21	–27.8%	–11.1%
100 WBC/μL	–18.0%	[(N–E) ÷ E] × 100	21	–24.0%	–12.0%

* The method of accuracy determination is shown with mathematical symbols, where N = the mean absolute count determined using Nageotte, L = the mean absolute count determined using LeucoCOUNT, and E = the expected mean absolute count based on hematology.



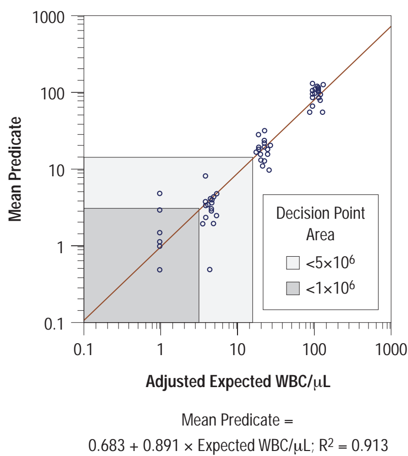
Mean Observed LeucoCOUNT WBC/μL = 2.607 + 0.871 × Expected WBC/μL; R^2 = 0.955

Figure 4. Regression of mean observed LeucoCOUNT WBC/μL versus expected WBC/μL for RBCs (expected counts of 0 adjusted to 1 WBC/μL for presentation on logarithmic scale)

Table 4. Accuracy results for RBC samples

rWBC Target Value	Mean Bias	Method of Accuracy Determination*	Number of Samples	90% Lower Confidence Limit	90% Upper Confidence Limit
0 WBC/μL	1.9 rWBC/μL	L–E	20	1.2 rWBC/μL	2.6 rWBC/μL
4 WBC/μL	25.1%	[(L–E) ÷ E] × 100	20	6.4%	43.7%
20 WBC/μL	1.1%	[(L–E) ÷ E] × 100	20	–6.6%	8.8%
100 WBC/μL	–8.2%	[(L–E) ÷ E] × 100	20	–14.0%	–2.5%
0 WBC/μL	0.8 rWBC/μL	N–E	19	0.3 rWBC/μL	1.3 rWBC/μL
4 WBC/μL	–22.4%	[(N–E) ÷ E] × 100	20	–37.2%	–7.6%
20 WBC/μL	–11.9%	[(N–E) ÷ E] × 100	20	–23.3%	–0.41%
100 WBC/μL	–10.0%	[(N–E) ÷ E] × 100	20	–18.0%	–2.1%

* The method of accuracy determination is shown with mathematical symbols, where N = the mean absolute count determined using Nageotte, L = the mean absolute count determined using LeucoCOUNT, and E = the expected mean absolute count based on hematology.



Mean Predicate = 0.683 + 0.891 × Expected WBC/μL; R^2 = 0.913

Figure 5. Regression of mean observed Nageotte WBC/μL versus expected WBC/μL for RBCs (expected counts of 0 adjusted to 1 WBC/μL for presentation on logarithmic scale)

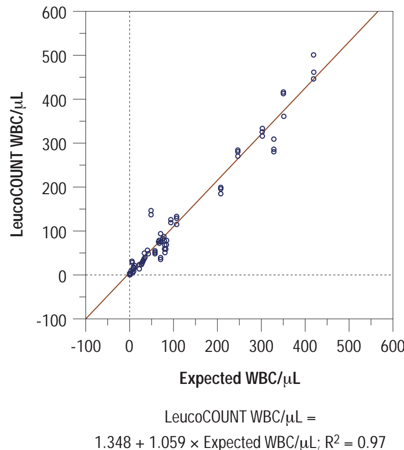
Linearity

To evaluate the linearity of the LeucoCOUNT assay in a model system, non-leucoreduced RBC and platelet samples were diluted using a double-filtered sample of the same type to reflect the following dilution percentages:

0%	=	Mean of absolute counts of double-filtered sample
0.01%	=	1:10 dilution of 0.1% sample
0.1%	=	1:10 dilution of 1.0% sample
1%	=	1:10 dilution of 10.0% sample
10%	=	1:10 dilution of non-leucoreduced sample
100%	=	Mean of absolute counts of non-leucoreduced sample

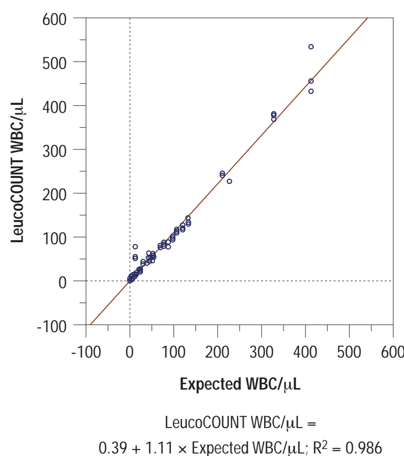
At the 0% dilution level, four replicates of 11 units were analyzed. At every other dilution point, four replicates of 16 units were analyzed. Data at or below the level of 450 rWBC/μL was used for this analysis.

The absolute count values recorded at the two ends of the dilution curve (0% and 100%) were used to obtain expected absolute counts for each of the other dilution points. These analyses were used to obtain estimates of slope, intercept, and the correlation coefficient. The linearity of the LeucoCOUNT system is demonstrated in Figures 6 and 7.



LeucoCOUNT WBC/μL = 1.348 + 1.059 × Expected WBC/μL; R^2 = 0.97

Figure 6. Linearity of the LeucoCOUNT Kit in the analysis of RBC samples



LeucoCOUNT WBC/μL = 0.39 + 1.11 × Expected WBC/μL; R^2 = 0.986

Figure 7. Linearity of the LeucoCOUNT Kit in the analysis of platelet samples

Precision and Interlaboratory Reproducibility

Reproducibility within and between laboratories is essential for valid comparison of data from multiple sites, and filter performance from site to site. Currently, the most common method of residual white cell enumeration is shown to have excessive variability across sites.[11,12] These two types of precision were evaluated for the LeucoCOUNT Kit in a site-to-site send-around study. For all studies reported, samples were transported and analyzed independently by each of the participating clinical sites.

Within-site precision was demonstrated with LeucoCOUNT at three sites. Leucoreduced RBC and platelet samples were spiked with CD-Chex PLUS cells and non-leucoreduced platelet samples, respectively. Four pairs of two identical samples were analyzed in duplicate at each of three sites. The standard deviations and coefficients of variation for LeucoCOUNT measurements were pooled according to sample type. Results for within-site precision in terms of mean SD and CV are 1.1 and 8.3% for pooled RBC values, and 2.3 and 10.0% for mean platelet values.

Site-to-site precision was demonstrated with LeucoCOUNT at three sites using routine RBC and platelet samples that had been leucoreduced. Seven RBC units, and 6 platelet units were evaluated in duplicate at three clinical sites. All samples in this study were found to have absolute counts ≤1 WBC/μL. Interlaboratory precision was determined using the mean percent difference across sites, pooled by sample type. The mean % difference for RBC samples was 11.2%, and for platelet samples was 8.6%.

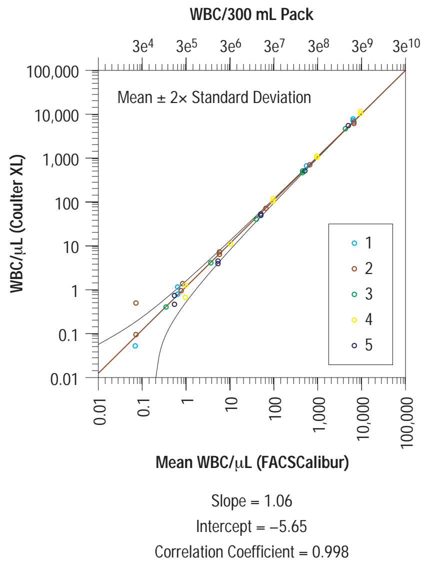


Figure 8. Regression plot of RBC samples on the Becton Dickinson FACSCalibur vs the Coulter XL

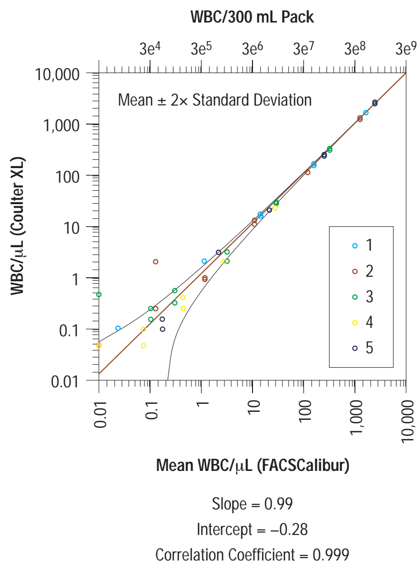


Figure 9. Regression plot of platelet samples on the Becton Dickinson FACSCalibur vs the Coulter XL

Flow Cytometry Platform Comparison

The LeucoCOUNT assay was designed for use on Becton Dickinson flow cytometers, but can be used on other systems as well. In order to validate the performance of the LeucoCOUNT assay on different instrument types, the following study was performed. Ten-fold serial dilutions were made for each of five non-leucoreduced platelet and RBC units. Dilutions were made from the original platelet or RBC unit using double-filtered units as the diluent. For each set of stained samples, two replicates were acquired and analyzed on the Becton Dickinson FACSCalibur™, and two were acquired on the Coulter XL. The samples evaluated in this study include an evenly distributed number of high, medium, and low levels of rWBCs stained with the LeucoCOUNT Kit. There was a strong linear relationship between those counts performed on the two instruments. Regression plots for RBC and platelet samples are shown in Figures 8 and 9, respectively.

Conclusion

The LeucoCOUNT assay effectively standardizes rWBC enumeration by improving upon previous flow cytometric methods of enumeration. LeucoCOUNT uses a lyse/no-wash procedure that incorporates a nucleic acid dye with TruCOUNT absolute counting technology. Our evaluations of LeucoCOUNT confirm that the assay design yields reliable, reproducible results. The LeucoCOUNT assay performed well in a series of studies to demonstrate accuracy, linearity, precision, and multi-platform performance. A standardized approach to rWBC enumeration in leucoreduced blood and platelet products significantly reduces the variability of the results, and cuts down significantly on sample processing time.

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