
Validation Protocol for the BD Leucocount™ Kit

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Introduction

Before reporting patient test results, the flow cytometry laboratory must go through a process of validation to demonstrate that it can obtain performance specifications comparable to those established by BD Biosciences for the following performance characteristics:

- Accuracy (method comparison)
- Intra-assay precision (within-run precision)
- Inter-assay precision (between-run precision)
- Interference
- Carryover studies
- Linearity (analyte measurement range)
- Sensitivity

The process of validation includes the following steps:

- Review the instruction manual of the test to be implemented
- Review technical data sheets (TDSs), also known as Instructions for Use (IFU), of tests to be implemented
- Review standard operating procedures (SOPs)
- Review the validation protocol
- Collect appropriate specimens for the studies
- Perform testing
- Analyze data

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Specimens, Instruments, and Reagents

Specimens

Collect red blood cell (RBC) and platelet (PLT) samples according to the manufacturer's instructions.

Make sure that each sample is less than 24 hours old (since leucoreduction). Store RBC samples at 2°C–8°C until ready for staining. Store PLT samples at room temperature (20°C– 25°C) until ready for staining. Note: sample testing is allowed up to 48 hours post leucoreduction.

BD instruments and reagents

BD FACSVia™ cytometer

BD FACSVia™ Loader

BD™ CS&T beads (Catalog No. 656504 and 656505)

BD Leucocount™ controls (Catalog No. 341001, 341002, or 341003) or equivalent

BD Leucocount™ kit with BD Trucount™ tubes (Catalog No. 340523)

Falcon® test tubes (from Corning) or equivalent

BD FACS™ Clean solution (Catalog No. 340345)

BD™ Sheath additive (Catalog No. 660584)

BD™ Detergent Solution Concentrate (Catalog No. 660585)

Other reagents required

Reagent grade deionized or distilled water

Phosphate buffered saline (PBS) with 0.1% sodium azide

Statistical software

Data Innovations EP Evaluator® software or equivalent



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Accuracy Verification Protocol

Reference: CLSI documents EP-9 and EP-12

Accuracy is the closeness of the agreement between the measured value of an analyte and the true value of an analyte. Parallel studies are performed to determine the relative bias (accuracy) between the method under evaluation and the reference method. Bias is defined as the difference in mean values between each method or the average of the paired differences.

Specimens

- Include a minimum of 20 fresh samples (RBC and/or PLT) to be tested.
- Make sure that each sample is less than 24 hours old (since leucoreduction).
- Set up and run each sample on both the reference method and the BD method within the sample stability listed in the Technical Data Sheet (TDS).

Procedure

1. Set up the flow cytometer according to *BD Leucocount SOP 1: BD FACSVia Cytometer Startup Procedure*.
2. Prepare samples according to the Instructions for Use (IFU). Include controls in every run.
3. Acquire the samples on the cytometer.
4. Review all results and adjust gates as needed. Details are provided in the *BD Leucocount™ Application Guide For the BD FACSVia™ System*.
5. Enter the results in EP Evaluator or equivalent software for the BD platform and the reference method.

Analysis of results

When all the results have been entered into EP Evaluator or equivalent software, calculate the regression value (R^2). Intercept analysis may be performed if comparing dual-platform to single-platform results.

Check that the regression value agrees with the performance specifications shown in the following charts and in the TDS. If there are any highly discrepant values, check that there are no problems with specimen identification, data entry errors, or any method errors in the preparation or running of the samples.

Print the data chart, regression graph, and bias analysis graph if performed, and put the materials in the validation notebook.

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Performance specifications

Accuracy on the BD FACSVia vs the BD FACSCalibur™ flow cytometer, BD Leucocount kit, TDS 23-3434-08

Regression analysis

Specimen Type	R ²	Slope	Intercept
RBCs	0.99	1.01	0.13
PLTs	1.00	1.01	0.09

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Intra-assay (Within-run) Precision Verification Protocol

Reference: CLSI document EP-15

Precision is defined as the dispersion of replicate measurements. Precision is expressed quantitatively using standard deviation (SD) and coefficient of variation (CV).

In general, precision assessment requires a minimum of five to ten replicates of at least two levels of process controls or one specimen prepared and acquired five to ten times. Intra-assay precision is defined as replicate measurements that are tested in one run. The mean, SD, and CV are calculated.

Specimens

- Include a minimum of one sample or BD Leucocount (RBC or PLT) Control High or Low. Use a freshly opened tube of control.
- Each leucoreduced sample should be fresh, less than 24 hours old (since leucoreduction).

Procedure

1. Set up the flow cytometer according to *BD Leucocount SOP 1: BD FACSVia Cytometer Startup Procedure*.
2. Prepare samples according to the Instructions for Use (IFU).
3. Acquire the samples on the cytometer.
4. Review all results and adjust gates as needed. Details are provided in the *BD Leucocount™ Application Guide For the BD FACSVia™ System*.
5. Enter the results in EP Evaluator or equivalent software for the BD platform and the reference method.

Analysis of results

When all the results have been entered into EP Evaluator or the equivalent software, calculate the mean, SD, and CV.

Check that the CV and SD values agree with the performance specifications in the following charts and in the TDS. If there are any highly discrepant values, check that there are no problems with specimen identification, data entry errors, or any method errors in the preparation or running of the samples.

Print the data chart and associated graphs and put the materials in the validation notebook.



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Performance specifications

Precision of rWBC absolute counts, BD Leucocount kit, TDS 23-3434-08

SD and CV analysis

Control	Mean (absolute counts)	SD	%CV
BD Leucocount PLT Control Low	7.32	0.58	7.96
BD Leucocount PLT Control High	15.11	0.92	6.12

SD and CV analysis

Control	Mean (absolute counts)	SD	%CV
BD Leucocount RBC Control Low	7.58	0.68	9.03
BD Leucocount RBC Control High	16.50	1.12	6.81

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Inter-Assay (Between-run) Precision Protocol

Reference: CLSI document EP-15

Inter-assay precision is defined as replicate measurements that are tested on multiple runs. BD Leucocount RBC or PLT Control High and BD Leucocount RBC or PLT Control Low should be set up in triplicate and acquired at least three times in multiple runs, preferably by different technologists.

Specimens

- BD Leucocount RBC or PLT Control High
- BD Leucocount RBC or PLT Control Low

Procedure

1. Set up the flow cytometer according to *BD Leucocount SOP 1: BD FACSVia Cytometer Startup Procedure*.
2. Prepare samples according to the Instructions for Use (IFU).
3. Acquire the samples on the cytometer.
4. Review all results and adjust gates as needed. Details are provided in the *BD Leucocount™ Application Guide For the BD FACSVia™ System*
5. Enter the results in EP Evaluator or equivalent software for the BD platform and the reference method.

Analysis of results

When all the results for each analyte have been entered into EP Evaluator or equivalent software, calculate the mean, SD, and CV.

Check that the SD and CV values agree with the performance specifications in the following charts and the TDS. If there are any highly discrepant values, check that there are no problems with specimen identification, data entry errors, or any method errors in the preparation or running of the samples.

Print the data chart and associated graphs and put the materials in the validation notebook.

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Performance specifications

Total system precision of rWBC absolute counts, BD Leucocount kit, TDS 23-3434-08

SD and CV analysis

Control	Mean (absolute counts)	SD	%CV
BD Leucocount PLT Control Low	7.30	0.69	9.49
BD Leucocount PLT Control High	16.49	1.07	6.46

SD and CV analysis

Control	Mean (absolute counts)	SD	%CV
BD Leucocount RBC Control Low	6.76	0.73	10.76
BD Leucocount RBC Control High	17.10	1.28	7.51



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Interference Protocol

Reference: CLSI document EP-17

Procedure

1. Check the Technical Data Sheet (TDS) for a list of interfering substances.
2. List the interfering substances in the SOP with appropriate plans of action when present.
 - Do not use previously fixed specimens.
 - The presence of EDTA in the sample can interfere with results.
 - Samples without additive that are refrigerated before staining could give aberrant results.
 - Nucleated red cells contain nucleic acid and could be detected as residual WBCs in this assay.

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Carryover Protocol

Reference: CLSI document EP-17

The carryover specification for the BD FACSVia system is $\leq 0.50\%$. This value is published in the BD FACSVia Technical Specifications Sheet.

Specimens

BD Leucocount RBC or PLT Control High

BD Leucocount RBC or PLT Control Low

Procedure

1. Set up the flow cytometer according to *BD Leucocount SOP 1: BD FACSVia Cytometer Startup Procedure*.
2. Prepare at least five replicates of the BD Leucocount RBC or PLT Control High and BD Leucocount RBC or PLT Control Low according to the Instructions for Use (IFU).
3. Acquire the BD Leucocount RBC or PLT Control High followed by BD Leucocount RBC or PLT Control Low, alternating five times on the cytometer.
4. Review all results and adjust gates as needed. Details are provided in the *BD Leucocount™ Application Guide For the BD FACSVia™ System*.
5. Enter the results in EP Evaluator or equivalent software for the BD platform and the reference method.

Analysis of results

When all the results have been entered into EP Evaluator or equivalent software, compare the acquired results between the high and low controls. The results for the BD Leucocount RBC or PLT Low control should be within the stated intra-assay specifications. Verify that there are no entry errors or mistakes in the analysis to account for the differences shown.

Print the data chart and carryover graph and put the materials in the validation notebook.



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Linearity Verification Protocol

Reference: CLSI document EP-6

The purpose of this protocol is to determine at what point the absolute count for the assay is not linear, and the deviation from the expected value.

Specimens

A non-leucoreduced sample containing a high number of WBCs is serially diluted to cover the analyte measured range (AMR) as listed in the following table, and all the dilutions are prepared and run once.

The sample can be run on a hematology instrument to determine the starting concentration of WBCs, at least 1,400/μL.

Perform the dilution of the sample in the following manner:

1. Make two-fold serial dilutions with PBS such that the WBCs are diluted down to approximately zero cells.

Tube	Dilution Factor	Expected WBC	Volume of PBS	Volume of Previous Dilution
1	neat/undiluted	1,400	0 μL	800 μL of whole blood
2	1:2	700	800 μL	800 μL of neat/undiluted
3	1:4	350	800 μL	800 μL of 1:2
4	1:8	175	800 μL	800 μL of 1:4
5	1:16	88	800 μL	800 μL of 1:8
6	1:32	44	800 μL	800 μL of 1:16
7	1:64	22	800 μL	800 μL of 1:32
8	1:128	11	800 μL	800 μL of 1:64
9	1:256	6	800 μL	800 μL of 1:128
10	1:512	3	800 μL	800 μL of 1:256
11	1:1,024	2	800 μL	800 μL of 1:512
12	1:2,048	0	800 μL	800 μL of 1:1,024



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Procedure

1. Set up the flow cytometer according to *BD Leucocount SOP 1: BD FACSVia Cytometer Startup Procedure*.
2. Create serial dilutions of the sample to be tested.
3. Prepare samples according to the Instructions for Use (IFU).
4. Acquire samples on the cytometer.
5. Review all results and adjust gates as needed. Details are provided in the *BD Leucocount™ Application Guide For the BD FACSVia™ System*.
6. Enter the results in EP Evaluator or equivalent software for the actual value of each dilution and the predicted value of each dilution. If there are any highly discrepant values, check that there are no problems with specimen identification or any other method errors.

Analysis of results

Check that the regression values for the all analytes meet the statistical significance requirements of your laboratory.

Print the data chart and put the materials in the validation notebook.

Performance specifications

Linearity for BD Leucocount Kit, TDS 23-3434-08.

Linearity for the BD Leucocount Kit was assessed for the BD FACSVia system and was determined to be 0–350 WBCs/ μ L.



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Sensitivity (Lower Detection Limit) Protocol

Reference: CLSI document EP-17

The minimum detection limit (lowest concentration that can be distinguished from zero) is determined by assaying replicates of the lowest detectable result as determined by dilution. A specimen near the limit of detection (LOD) is acquired to ensure that the results obtained are not lower than what is reported by the analyte measurement range (AMR).

Specimens

Three to five specimens with known WBCs are serially diluted with PBS until the expected WBC result is zero. See the Linearity procedure previously described.

Each specimen, at the lowest detectable result dilution as defined by the linearity protocol, should be stained three times according to the BD protocol and acquired five times.

Procedure

1. Set up the flow cytometer according to *BD Leucocount SOP 1: BD FACSVia Cytometer Startup Procedure*.
2. Prepare diluted samples with expected WBC results of 10 or lower according to the Instructions for Use (IFU).
3. Acquire the samples on the cytometer.
4. Review all results and adjust gates and histograms as needed.
5. Enter the results in EP Evaluator or equivalent software for the BD platform and the reference method.
6. Calculate the mean, SD, and %CV. A %CV of 10% to 20% is acceptable for each subset. If there are any highly discrepant values, check that there are no problems with specimen identification or any other method errors.

Analysis of results

When all the results have been entered into EP Evaluator or equivalent software, compare to the lowest linearity value for each subset to ensure that the values obtained are not lower than the lowest results reported in the analyte measurement range.

Print the data chart and put the materials in the validation notebook.



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Stability Study Information

All stability testing was done by BD Biosciences and clinical trial sites and RBC sample stability was determined to be 48 hours when held at 2°C–8°C. Stability of PLT samples was determined to be 48 hours when held at room temperature 20°C–25°C.

In some cases samples may be received beyond the manufacturer's stated stability testing specifications. Extended stability can be performed and samples used beyond the manufacturer's timing with proper documentation. If the CV exceeds the inter-assay precision when compared to time 0, then the stability of the sample is in question.

If extended stability is to be tested, the following method is suggested.

Specimens

- Collect a minimum of two samples, RBC and/or PLT, according to manufacturer's guidelines.
- Samples should be fresh (less than two hours old since leucoreduction), to be considered for time 0 testing.
- Store PLT samples at room temperature (20°C–25°C) and RBC samples at 2°C–8°C during the time period for testing.
- Prepare and stain the samples according to the BD Leucocount kit IFU every day for five consecutive days.

Procedure

1. Set up the flow cytometer according to *BD Leucocount SOP 1: BD FACSVia Cytometer Startup Procedure*.
2. Prepare samples according to the Instructions for Use (IFU). Include controls in every run.
3. Acquire the samples on the cytometer.
4. Review all results and adjust gates as needed. Details are provided in the *BD Leucocount™ Application Guide For the BD FACSVia™ System*.
5. Enter the results in EP Evaluator or equivalent software for the BD method and the reference method.

Analysis of results

When all the results have been entered into EP Evaluator or equivalent software, calculate the CV.

Check that the CV value agrees with the inter-assay precision compared to time 0. If there are any highly discrepant values, check that there are no problems with specimen identification or any other method



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errors. If there is a significant difference in the CV of the results compared to time 0, then the data point at which there is no significant difference is the stability time allowable.

Print the data chart and CV graph and put the materials in the validation notebook.

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