Validation Protocol for the BD™ Stem Cell Enumeration (SCE) 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) of tests to be implemented
- Review the standard operating procedure (SOPs)
- Review the validation protocol
- Collect appropriate specimens for the studies
- Perform testing
- Analyze data

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Specimens, Instruments, and Reagents

Specimens

EDTA, ACD-A, heparin, and CPD anticoagulants were validated with this assay. For leucopheresis, a mixture of ACD-A and heparin, and EDTA, can also be used with this assay.

The following specimens can be analyzed with this kit: normal and mobilized peripheral blood, fresh and thawed leucopheresis products, fresh and thawed bone marrows, and fresh and thawed cord blood.

BD instruments and reagents

BD FACSCanto™ II or BD FACSCalibur™ cytometer

BD FACS™ 7-color setup beads (Catalog No. 335775) for the BD FACSCanto II instrument or BD Calibrite™ beads (Catalog No. 340486 and 340487) for the BD FACSCalibur instrument

BD™ Stem Cell Control kit (Catalog No. 340991)

BD™ Stem Cell Enumeration kit with BD Trucount™ tubes (Catalog No. 344563)

Falcon® test tubes (from Corning) or equivalent

BD FACS™ Shutdown solution (Catalog No. 334224)

BD FACS™ Clean solution (Catalog No. 340345)

BD FACS™ Rinse solution (Catalog No. 340346)

BD FACSFlow™ sheath fluid (Catalog No. 342003)

Other reagents required

Phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA)

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 in EDTA and any other anticoagulants to be tested.
- Make sure that each sample is less than 24 hours old (since draw or thaw).
- Include both normal and mobilized specimens.
- Set up and run each sample on both the reference method and the BD method within the sample stability as listed in the Technical Data Sheet (TDS).

Procedure

1. Set up the flow cytometer according to *Stem Cell Enumeration SOP 1: BD FACSCanto™ II 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 Stem Cell Enumeration Application Guide for BD FACSCanto II Flow Cytometers*.
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 FACSCanto II flow cytometer, BD Stem Cell Enumeration Kit, TDS 23-7867-04

Regression analysis for subset percentages and absolute counts by specimen type

Specimen Type	Variable	N	R^2	Slope	Intercept
Peripheral blood	Viable CD34	188 (normal: 57, mobilized: 131)	0.94	0.96 (0.93, 0.99)	–0.07 (–0.21, 0.07)
	%CD34 in CD45	188 (normal:57, mobilized: 131)	0.98	0.99 (0.93, 1.06)	0.00 (–0.01, 0.00)
Leucopheresis	Viable CD34	341 (fresh: 232, frozen: 109)	0.97	0.96 (0.94, 0.98)	–0.02 (–0.2, 0.17)
	%CD34 in CD45	341 (fresh: 232, frozen: 109)	0.95	0.96 (0.94, 0.99)	–0.02 (–0.03, 0.00)
Cord blood	Viable CD34	241 (fresh: 124, frozen: 117)	0.88	0.97 (0.93, 1.02)	–0.52 (–0.92, –0.11)
	%CD34 in CD45	241 (fresh: 124, frozen: 117)	0.87	1.02 (0.94, 1.09)	–0.02 (–0.04, 0.01)
Bone marrow	Viable CD34	148 (fresh: 75, frozen: 73)	0.95	1.00 (0.96, 1.03)	–0.02 (–0.13, 0.10)
	%CD34 in CD45	148 (fresh: 75, frozen: 73)	0.89	1.21 (1.13, 1.28)	–0.05 (–0.10, 0.00)

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Accuracy on the BD FACSCalibur flow cytometer, BD Stem Cell Enumeration Kit, TDS 23-7867-04

Regression analysis for subset percentages and absolute counts by specimen type

Specimen Type	Variable	N	R^2	Slope	Intercept
Peripheral blood	Viable CD34	167 (normal: 52, mobilized; 115)	0.94	1.00 (0.96, 1.03)	–0.13 (–0.32, 0.05)
	%CD34 in CD45	167 (normal: 52, mobilized; 115)	0.97	0.97 (0.9, 1.05)	0.00 (–0.01, 0.01)
Leucopheresis	Viable CD34	342 (fresh: 232, frozen: 110)	0.97	0.98 (0.96, 0.99)	0.04 (–0.06, 0.15)
	%CD34 in CD45	342 (fresh: 232, frozen: 110)	0.96	0.98 (0.96, 1.00)	–0.02 (–0.03, 0.00)
Cord blood	Viable CD34	245 (fresh: 122, frozen: 123)	0.94	0.96 (0.93, 0.99)	–0.28 (–0.52, –0.04)
	%CD34 in CD45	245 (fresh: 122, frozen: 123)	0.87	1.02 (0.92, 1.11)	–0.02 (–0.05, 0.02)
Bone marrow	Viable CD34	151 (fresh: 73, frozen: 78)	0.94	0.96 (0.92, 1.00)	–0.03 (–0.09, 0.03)
	%CD34 in CD45	151 (fresh: 73, frozen: 78)	0.88	1.23 (1.15, 1.31)	–0.08 (–0.12, –0.03)

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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 will require 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 Stem Cell Control High or Low.
- Each peripheral blood sample should be fresh, less than two hours old (since draw).
- Use a freshly opened tube of BD Stem Cell Controls.

Procedure

1. Set up the flow cytometer according to *Stem Cell Enumeration SOP 1: BD FACSCanto™ II 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 Stem Cell Enumeration Application Guide for BD FACSCanto II Flow Cytometers.*
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.

Validation Protocol for the BD™ Stem Cell Enumeration (SCE) Kit

Performance specifications

Precision of $CD34^+$ absolute counts and percentages, BD Stem Cell Enumeration Kit, TDS 23-7867-04

SD and CV analysis (BD FACSCanto II flow cytometer)

Control	Mean (%CD34)	SD	%CV	Mean (CD34 cells/µL)	SD	%CV
BD Stem Cell Control High	0.58	0.03	4.7	35.3	1.83	5.2
BD Stem Cell Control Custom Low	0.15	0.01	9.6	8.8	0.9	10.3

SD and CV analysis (BD FACSCalibur flow cytometer)

Control	Mean (%CD34)	SD	%CV	Mean (CD34 cells/µL)	SD	%CV
BD Stem Cell Control High	0.58	0.03	4.7	35.5	1.69	4.8
BD Stem Cell Control Custom Low	0.14	0.01	8.2	8.8	0.75	8.6

Validation Protocol for the BD™ Stem Cell Enumeration (SCE) Kit

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 Stem Cell High and BD Stem Cell Low Controls should be set up in triplicate and acquired at least three times in multiple runs, preferably by different technologists.

Specimens

- BD Stem Cell High Control
- BD Stem Cell Low Control

Procedure

1. Set up the flow cytometer according to *Stem Cell Enumeration SOP 1: BD FACSCanto™ II 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 Stem Cell Enumeration Application Guide for BD FACSCanto II Flow Cytometers*.
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.

Validation Protocol for the BD™ Stem Cell Enumeration (SCE) Kit

Performance specifications

Total system precision of $CD34^+$ absolute counts and percentages, BD Stem Cell Enumeration Kit, TDS 23-7867-04

SD and CV analysis (BD FACSCanto II flow cytometer)

Control	Mean (%CD34)	SD	%CV	Mean (CD34 cells/µL)	SD	%CV
BD Stem Cell Control High	0.58	0.03	4.7	35.3	1.83	5.2
BD Stem Cell Control Custom Low	0.15	0.01	10.2	8.8	0.95	10.8

SD and CV analysis (BD FACSCalibur flow cytometer)

Control	Mean (%CD34)	SD	%CV	Mean (CD34 cells/µL)	SD	%CV
BD Stem Cell Control High	0.58	0.03	5.1	35.5	2.02	5.7
BD Stem Cell Control Custom Low	0.14	0.01	9.4	8.8	0.82	9.3

Validation Protocol for the BD™ Stem Cell Enumeration (SCE) Kit

Interference Protocol

Reference: CLSI document EP-17

Procedure

1. Check the Instructions for Use (IFU) 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 and stored specimens.
    - Any samples which are clotted or hemolyzed should be rejected for testing.

Validation Protocol for the BD™ Stem Cell Enumeration (SCE) Kit

Carryover Protocol

Reference: CLSI document EP-17

The carryover specification for the BD FACSCanto II cytometer is ≤0.1%. The carryover specification for the BD FACSCalibur cytometer is ≤1%. These values are published in the respective Technical Specifications Sheets.

Specimens

BD Stem Cell Control High

Procedure

1. Set up the flow cytometer according to *Stem Cell Enumeration SOP 1: BD FACSCanto™ II Cytometer Startup Procedure*.
2. Prepare at least five replicates of the BD Stem Cell Control High according to the Instructions for Use (IFU).
3. Aliquot 1 mL of PBS into five additional tubes.
4. Acquire the BD Stem Cell Control High followed by a tube of PBS, alternating five times on the cytometer. Acquire each PBS tube for the 15-minute maximum time in BD FACSCanto™ clinical software.
5. Review all results and adjust gates as needed. Details are provided in the *BD Stem Cell Enumeration Application Guide for BD FACSCanto II Flow Cytometers*.
6. 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 lymphocyte and PBS (blank) specimens. The results for the PBS tubes should be within the stated carryover specifications of the instrument. Verify that there are no entry errors or mistakes in the analysis to account for the differences shown.

Note: there may be very few events recorded due to the very low carryover in the BD FACSCanto II system. The FITC threshold may need to be decreased to have at least one event collected and recorded.

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

Validation Protocol for the BD™ Stem Cell Enumeration (SCE) Kit

Linearity Verification Protocol

Reference: CLSI document EP-6

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

Specimens

A sample containing a high number of CD34 cells (at least 2,000 CD34 cells/µL) is serially diluted to cover the analyte measured range (AMR) as listed in the following tables, and all the dilutions are prepared and run once.

Alternatively, a sample with a CD34 count of lower than 500 CD34 cells/µL, such as the BD Stem Cell Enumeration Kit or other CD34 enumeration assay, can be concentrated by centrifuging the sample at 350*g* for five minutes and aspirating the plasma. The sample can then be tested to determine the starting concentration of CD34 cells.

Perform the dilution of the sample in the following manner:

1. Make two-fold serial dilutions with PBS with 0.5% BSA such that the CD34 count is diluted down to approximately zero CD34 cells.

Tube	Dilution Factor	Expected CD34 counts	Volume of PBS with 0.5% BSA	Volume of Previous Dilution
1	neat/undiluted	2,000	0 µL	800 µL of whole blood
2	1:2	1,000	800 µL	800 µL of neat/undiluted
3	1:4	500	800 µL	800 µL of 1:2
4	1:8	250	800 µL	800 µL of 1:4
5	1:16	125	800 µL	800 µL of 1:8
6	1:32	63	800 µL	800 µL of 1:16
7	1:64	32	800 µL	800 µL of 1:32
8	1:128	16	800 µL	800 µL of 1:64
9	1:256	8	800 µL	800 µL of 1:128
10	1:512	4	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

Validation Protocol for the BD™ Stem Cell Enumeration (SCE) Kit

Procedure

1. Set up the flow cytometer according to *Stem Cell Enumeration SOP 1: BD FACSCanto™ II 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 Stem Cell Enumeration Application Guide for BD FACSCanto II Flow Cytometers*.
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 Stem Cell Enumeration Kit, TDS 23-7867-04

Linearity for BD Stem Cell Enumeration Kit was assessed for the BD FACSCalibur and BD FACSCanto II system within a WBC concentration up to 4.0 x 10^4 WBCs/µL. Linearity for BD Stem Cell Enumeration Kit was determined to be 0–1,000 $CD34^+$ cells/µL.

Validation Protocol for the BD™ Stem Cell Enumeration (SCE) Kit

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 CD34 counts are serially diluted with PBS with 0.5% BSA until the expected lymphocyte 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 *Stem Cell Enumeration SOP 1: BD FACSCanto™ II Cytometer Startup Procedure*.
2. Prepare diluted samples with expected CD34 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.

Validation Protocol for the BD™ Stem Cell Enumeration (SCE) Kit

Reference Range Verification Protocol

Reference: CLSI document EP-28

The reference range as published by the manufacturer is acceptable if the verification studies determine that the patient population undergoing tests at the laboratory falls within +/-2 SD of the published reference range. In this case, samples from at least 20 healthy individuals need to be acquired to verify the published reference range. If patient demographics are outside the ages of 18 to 65, samples from at least 120 healthy individuals need to be acquired to determine the appropriate reference range. Some state licensures require that the reference range be validated in healthy donors according to age, gender, and a mix of races.

Specimens

- Include a minimum of samples from 20 healthy individuals to be tested.
- Each sample should be fresh, less than two hours old (since draw).
- Set up and run each sample on both the reference method and the BD method within a close time frame.

Procedure

1. Set up the flow cytometer according to *Stem Cell Enumeration SOP 1: BD FACSCanto™ II 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 Stem Cell Enumeration Application Guide for BD FACSCanto II Flow Cytometers*.
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, calculate the mean, SD, and +/-2 SD of the data generated.

Check that the SD value agrees with the performance specifications in the following chart. If there are any highly discrepant values, check that there are no problems with specimen identification or any other method errors.

The study may be expanded to include more specimens if the results of the validation study are inconclusive, or the parallel (accuracy) studies have demonstrated a significant positive or negative bias.

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Print the data chart and put in the validation notebook.

Performance specifications

Reference ranges for BD Stem Cell Enumeration Kit, TDS 23-7867-04

The following reference intervals were determined using healthy adults.

Representative adult reference intervals

Measures Reported	n	Median	SD	95% Reference Interval	
				Lower (90% conf. bounds)	Upper (90% conf. bounds)
CD34 absolute counts (cells/µL)	169	2.2	1.7	0.7 (0.6, 0.8)	6.9 (6.1,7.9)
%CD34 cells	169	0.037	0.025	0.013 (0.011, 0.014)	0.11 (0.096, 0.12)

We recommend that laboratories and other users establish their own reference intervals for their patient populations using the BD Stem Cell Enumeration Kit to reflect potential sources of variability such as patient gender, race, and age, and sample preparation techniques.

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Stability Study Information

All stability testing was done by BD Biosciences and clinical trial sites and sample stability was determined to be 24 hours when held at 2° to 8°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 in the appropriate anticoagulant.
- Samples should be fresh (less than two hours old since draw), to be considered for time 0 testing.
- Store samples at room temperature and upright during the time period for testing.
- Prepare and stain the samples according to the Stem Cell Enumeration protocol every day for five consecutive days.

Procedure

1. Set up the flow cytometer according to *Stem Cell Enumeration SOP 1: BD FACSCanto™ II 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 Stem Cell Enumeration Application Guide for BD FACSCanto II Flow Cytometers*.
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 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.

Validation Protocol for the BD™ Stem Cell Enumeration (SCE) Kit

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

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