Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

Contents

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
- Reference range validation
- Stability studies

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

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

Specimens, Instruments, and Reagents

Specimens

EDTA anticoagulated peripheral blood

All testing done by BD Biosciences was performed using EDTA anticoagulated samples. Any claims for stability are based on the use of EDTA as an anticoagulant. If other anticoagulants are to be used for your testing, then all validation testing should be done on each anticoagulant. Other anticoagulants that could be tested include sodium heparin and ACD. ACD anticoagulated samples cannot be used in single-platform testing using BD Trucount™ tubes, since the volume of the anticoagulant dilutes the absolute counts generated by the flow assay.

BD instruments and reagents

BD FACSCanto™, BD FACSCanto™ II, or BD FACSCalibur™ cytometer

BD FACS™ Sample Preparation Assistant III (SPA III) for automated sample preparation (optional)

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

BD™ Multi-Check Whole Blood Control (Catalog No. 340912) and BD™ Multi-Check CD4 Low Control (Catalog No. 340915) or equivalent

BD Multitest™ reagents with BD Trucount™ tubes: Choose the product being tested from the following list.

- BD Multitest™ IMK kit with BD Trucount™ tubes (Catalog No. 340504)
- BD Multitest™ CD3/CD8/CD45/CD4 with BD Trucount™ tubes (Catalog No. 340491)
- BD Multitest™ CD3/CD16 + CD56/CD45/CD19 with BD Trucount™ tubes (Catalog No. 340492)
- BD Multitest™ 6-color TBNK reagent with with BD Trucount™ tubes (Catalog No. 337166)

Or BD Multitest™ reagents without BD Trucount™ tubes: Choose the product being tested from the following list.

- BD Multitest™ IMK kit (Catalog No. 340503)
- BD Multitest™ CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC reagent (Catalog No. 340499)
- BD Multitest™ CD3 FITC/CD16 + CD56 PE/CD45 PerCP/CD19 APC reagent (Catalog No. 340500)
- BD Multitest™ 6-color TBNK reagent (Catalog No 644611)

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Falcon® test tubes (from Corning) or equivalent

BD FACS™ lysing solution (Catalog No. 349202)

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)

BD Pharmingen™ stain buffer (BSA) (Catalog No. 554657)

Other reagents required

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

Procedure

1. Set up the flow cytometer according to *Lymphocyte Enumeration SOP 1: BD FACSCanto™ Cytometer Startup Procedure*.
2. Set up the SPA III (if being used) according to *Lymphocyte Enumeration SOP 4: Immunofluorescent Labeling of Whole Blood with BD Multitest™ 6-color TBNK Reagents or 4-color IMK Reagents, BD FACS™ SPA III Preparation*.
3. Prepare samples according to the TDS, either manually or with the SPA III. Include controls in every run.
4. Acquire the samples on the cytometer.
5. Review all results and adjust gates and histograms as needed.
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, 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 is no problem with specimen identification, data entry errors, or any method errors in the preparation or running of the sample.

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

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

Performance specifications

Accuracy with BD Trucount tubes, BD Multitest 6-color TBNK, TDS 23-10834-03

BD FACSCanto II flow cytometer vs BD FACSCanto flow cytometer

Regression analysis for subset percentages and absolute counts

Lymphocyte Subset	N	Unit	r^2	Slope	Intercept	Range
$CD3^+CD4^+$	104	cells/µL	0.997	0.94	20.96	6–2,079
		%	0.994	1.0	0.47	1–57
$CD3^+CD8^+$	104	cells/µL	0.987	0.93	35.43	62–3,462
		%	0.989	1.0	0.44	11–82
Total $CD3^+$	104	cells/µL	0.976	0.93	60.19	217–3,952
		%	0.971	0.99	1.77	50–92
$CD3^-CD19^+$	104	cells/µL	0.980	0.96	4.25	0–820
		%	0.985	1.0	–0.04	0–38
$CD3^-(CD16 + CD56)^+$	104	cells/µL	0.953	0.91	2.30	15–633
		%	0.964	1.0	–0.5	2–33

Accuracy with vs without BD Trucount tubes, BD Multitest 6-color TBNK, TDS 23-10834-03

BD FACSCanto™ clinical software v2.4 without BD Trucount tubes vs v2.2 with BD Trucount tubes with BD FACSCanto flow cytometer

Regression analysis for subset percentages

Lymphocyte Subset	N	Unit	r^2	Slope	Intercept	Range
$CD3^+CD4^+$	52	%	0.995	0.979	0.567	1–62
$CD3^+CD8^+$	52	%	0.992	0.989	0.542	10–68
Total $CD3^+$	52	%	0.988	1.000	–0.173	36–88
$CD3^-CD19^+$	52	%	0.989	1.037	–0.553	0–37
$CD3^-(CD16 + CD56)^+$	52	%	0.988	0.997	0.178	4–40

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

Accuracy with vs without BD Trucount tubes, BD Multitest 6-color TBNK, TDS 23-10834-03

BD FACSCanto clinical software v2.4 without BD Trucount tubes vs v2.2 with BD Trucount tubes with BD FACSCanto II flow cytometer

Regression analysis for subset percentages

Lymphocyte Subset	N	Unit	r^2	Slope	Intercept	Range
$CD3^+CD4^+$	52	%	0.994	0.996	–0.001	1–61
$CD3^+CD8^+$	52	%	0.993	1.006	0.310	11–68
Total $CD3^+$	52	%	0.982	1.012	–0.919	36–87
$CD3^-CD19^+$	52	%	0.985	0.985	0.039	0–35
$CD3^-(CD16 + CD56)^+$	52	%	0.986	1.034	–0.485	5–40

Accuracy with BD FACSCanto flow cytometer, BD Multitest IMK kit (4-color) with BD Trucount tubes, TDS 23-3602-07

BD FACSCanto clinical software v2.1 with the BD FACSCanto II flow cytometer vs BD FACSCanto clinical software v2.0 with the BD FACSCanto flow cytometer

Regression analysis for subset absolute counts and percentages

Lymphocyte Subset	N	Unit	r^2	Slope	Intercept	Range
$CD3^+CD4^+$	104	cells/µL	0.986	0.95	18.25	11–1,905
		%	0.994	1.01	0.20	2–57
$CD3^+CD8^+$	104	cells/µL	0.988	0.95	28.36	68–3,577
		%	0.993	1.00	0.34	11–81
Total $CD3^+$	104	cells/µL	0.991	0.97	27.59	221–3,873
		%	0.984	0.97	2.72	52–92
$CD3^-CD19^+$	104	cells/µL	0.979	0.97	2.37	0–834
		%	0.986	0.97	0.32	0–38
$CD3^-(CD16 + CD56)^+$	104	cells/µL	0.961	0.88	10.56	20–606
		%	0.957	0.93	0.19	2–32

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

Accuracy with BD FACSCanto flow cytometer, BD Multitest IMK kit (4-color) with BD Trucount tubes, TDS 23-3602-07

BD FACSCanto clinical software v2.0 with the BD FACSCanto flow cytometer vs BD Multiset™ software with the BD FACSCalibur flow cytometer

Regression analysis for subset absolute counts and percentages

Lymphocyte Subset	N	Unit	r^2	Slope	Intercept	Range
$CD3^+CD4^+$	108	cells/µL	0.991	0.97	10.80	3–3,211
		%	0.998	0.99	0.26	1–70
$CD3^+CD8^+$	108	cells/µL	0.983	0.96	24.60	68–2,754
		%	0.996	1.00	0.27	10–81
Total $CD3^+$	108	cells/µL	0.987	0.99	–6.27	75–5,257
		%	0.993	1.00	–0.17	40–93
$CD3^-CD19^+$	108	cells/µL	0.990	1.02	–7.93	0–2,527
		%	0.994	0.99	–0.05	0–42
$CD3^-(CD16 + CD56)^+$	108	cells/µL	0.981	0.95	10.80	1–1,374
		%	0.989	1.00	0.39	2–45

Accuracy with BD FACSCalibur flow cytometer, BD Multitest IMK kit (4-color) with BD Trucount tubes, TDS 23-3602-07

BD Multitest CD3/CD8/CD45/CD4 with BD Trucount tubes vs BD Tritest™ CD3/CD4/CD45 and CD3/CD8/CD45 with BD Trucount tubes

Regression analysis for subset absolute counts and percentages

Subset	n	R	Slope	Intercept	Range
Helper/inducer T lymphs (%)	124	1.0	0.996	–0.434	1–62
Suppressor/cytotoxic T lymphs (%)	124	1.0	1.018	–0.383	13–78
Total T lymphs (%)	124	1.0	1.002	0.254	22–90
Helper/inducer T lymphs (cells/µL)	124	0.98	1.015	–7.692	93–1,904
Suppressor/cytotoxic T lymphs (cells/µL)	124	0.99	1.001	2.494	132–2,229
Total T lymphs (cells/µL)	124	0.98	1.028	–20.451	189–2,987

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

Accuracy with BD FACSCalibur flow cytometer, BD Multitest IMK kit (4-color) with BD Trucount tubes, TDS 23-3602-07

BD Multitest CD3/CD16 + CD56/CD45/CD19 with BD Trucount tubes vs BD Tritest™ CD3/CD16 + CD56/CD45 and CD3/CD19/CD45 with BD Trucount tubes

Regression analysis for subset absolute counts and percentages

Subset	n	R	Slope	Intercept	Range
NK lymphs (%)	126	0.97	0.97	–0.85	3–40.0
B lymphs (%)	126	0.99	0.98	–0.25	0–59
T lymphs (%)	126	0.99	0.99	1.74	21.5–90.0
NK lymphs (cells/µL)	126	0.96	0.92	–5.44	37–901
B lymphs (cells/µL)	126	0.98	0.95	1.77	3–877.6
T lymphs (cells/µL)	126	0.98	1.01	–10.18	100–2,883

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

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 Multi-Check Whole Blood Control, both normal and CD4 low.
- Each peripheral blood sample should be fresh, less than two hours old (since draw).
- Use a freshly opened tube of BD Multi-Check Controls.

Procedure

1. Set up the flow cytometer according to *Lymphocyte Enumeration SOP 1: BD FACSCanto™ Cytometer Startup Procedure*.
2. Set up the SPA III (if being used) according to *Lymphocyte Enumeration SOP 4: Immunofluorescent Labeling of Whole Blood with BD Multitest™ 6-color TBNK Reagents or 4-color IMK Reagents, BD FACS™ SPA III Preparation*.
3. Prepare samples according to the Technical Data Sheet (TDS), either manually or with the SPA III. Include controls in every run.
4. Acquire the samples on the cytometer.
5. Review all results and adjust gates and histograms as needed.
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 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 is no problem with specimen identification, data entry errors, or any method errors in the preparation or running of the sample.

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

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

Performance specifications

Precision with BD Trucount tubes, BD Multitest 6-color TBNK, TDS 23-10834-03

SD analysis for subset percentages (BD FACSCanto II flow cytometer)

Lymphocyte Subset (%)	CDL[a] SD		CDC[b] SD	
	Within Run	Within Device	Within Run	Within Device
$CD3^+CD4^+$	0.47	0.47	0.76	0.80
$CD3^+CD8^+$	1.10	1.18	0.75	0.76
Total $CD3^+$	1.32	1.32	0.82	0.87
$CD3^-CD19^+$	1.07	1.09	0.57	0.60
$CD3^-(CD16 + CD56)^+$	0.78	0.80	0.57	0.58

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

CV analysis for absolute counts (BD FACSCanto II flow cytometer)

Lymphocyte Subset (%)	CDL[a] %CV		CDC[b] %CV	
	Within Run	Within Device	Within Run	Within Device
$CD3^+CD4^+$	5.1	5.1	3.6	4.0
$CD3^+CD8^+$	4.9	5.1	4.5	4.5
Total $CD3^+$	4.2	4.3	3.3	3.6
$CD3^-CD19^+$	5.6	5.6	5.4	6.0
$CD3^-(CD16 + CD56)^+$	5.4	5.6	5.9	6.3

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

Precision with BD Trucount tubes, BD Multitest 6-color TBNK, TDS 23-10834-03

SD analysis for subset percentages (BD FACSCanto flow cytometer)

Lymphocyte Subset (%)	CDL[a] SD		CDC[b] SD	
	Within Run	Within Device	Within Run	Within Device
$CD3^+CD4^+$	0.64	0.69	0.95	1.23
$CD3^+CD8^+$	1.07	1.29	0.65	0.81
Total $CD3^+$	1.17	1.23	0.86	0.90
$CD3^-CD19^+$	0.89	0.89	0.62	0.62
$CD3^-(CD16 + CD56)^+$	0.90	0.96	0.61	0.62

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

CV analysis for absolute counts (BD FACSCanto flow cytometer)

Lymphocyte Subset (%)	CDL[a] %CV		CDC[b] %CV	
	Within Run	Within Device	Within Run	Within Device
$CD3^+CD4^+$	7.6	8.0	4.7	4.8
$CD3^+CD8^+$	4.1	5.0	4.7	5.4
Total $CD3^+$	4.0	4.4	4.2	4.2
$CD3^-CD19^+$	5.7	6.0	5.3	5.7
$CD3^-(CD16 + CD56)^+$	7.0	8.0	7.9	7.9

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

Precision without BD Trucount tubes, BD Multitest 6-color TBNK, TDS 23-10834-03

SD analysis for subset percentages (BD FACSCanto II flow cytometer)

Lymphocyte Subset (%)	CDL[a] SD		CDC[b] SD	
	Within Run	Within Device	Within Run	Within Device
$CD3^+CD4^+$	0.56	0.77	0.73	0.93
$CD3^+CD8^+$	0.80	1.66	0.58	1.12
Total $CD3^+$	1.03	1.34	0.81	0.90
$CD3^-CD19^+$	0.70	0.73	0.50	0.50
$CD3^-(CD16 + CD56)^+$	0.72	0.84	0.64	0.66

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

SD analysis for subset percentages (BD FACSCanto flow cytometer)

Lymphocyte Subset (%)	CDL[a] SD		CDC[b] SD	
	Within Run	Within Device	Within Run	Within Device
$CD3^+CD4^+$	0.57	0.76	0.86	1.05
$CD3^+CD8^+$	0.96	1.56	0.64	1.16
Total $CD3^+$	1.28	1.42	0.77	0.96
$CD3^-CD19^+$	0.72	0.84	0.51	0.60
$CD3^-(CD16 + CD56)^+$	0.74	0.90	0.58	0.68

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

Precision with BD FACSCanto II flow cytometer, BD Multitest IMK kit (4-color), TDS 23-3602-07

Repeatability of subset percentages

Lymphocyte Subset (%)	CDL[a] SD	CDC[b] SD
$CD3^+CD4^+$	0.53	0.81
$CD3^+CD8^+$	1.33	0.78
Total $CD3^+$	0.96	0.63
$CD3^-CD19^+$	0.86	0.54
$CD3^-(CD16 + CD56)^+$	0.87	0.51

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

Repeatability of absolute counts

Lymphocyte Subset (cells/µL)	CDL[a] %CV	CDC[b] %CV
$CD3^+CD4^+$	5.9	3.6
$CD3^+CD8^+$	3.8	4.7
Total $CD3^+$	3.5	2.7
$CD3^-CD19^+$	6.2	5.5
$CD3^-(CD16 + CD56)^+$	5.9	6.0

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

Precision with BD FACSCanto flow cytometer, BD Multitest IMK kit (4-color), TDS 23-3602-07

Repeatability of subset percentages

Lymphocyte Subset (%)	MCL[a] SD	MCN[b] SD
$CD3^+CD4^+$	0.73	1.04
$CD3^+CD8^+$	0.97	1.03
Total $CD3^+$	1.16	1.15
$CD3^-CD19^+$	0.70	0.68
$CD3^-(CD16 + CD56)^+$	1.03	0.89

a. MCL = BD Multi-Check CD4 Low control
b. MCN = BD Multi-Check control

Repeatability of absolute counts

Lymphocyte Subset (cells/µL)	MCL[a] %CV	MCN[b] %CV
$CD3^+CD4^+$	5.8	5.7
$CD3^+CD8^+$	5.1	6.4
Total $CD3^+$	3.2	3.9
$CD3^-CD19^+$	4.9	7.0
$CD3^-(CD16 + CD56)^+$	6.6	8.0

a. MCL = BD Multi-Check CD4 Low control
b. MCN = BD Multi-Check control

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

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 Multi-Check Whole Blood Control and BD Multi-Check CD4 Low Control should be set up in triplicate and assayed for three to five consecutive days.

Specimens

- BD Multi-Check Whole Blood Control
- BD Multi-Check CD4 Low Control

Procedure

1. Set up the flow cytometer according to *Lymphocyte Enumeration SOP 1: BD FACSCanto™ Cytometer Startup Procedure*.
2. Set up the SPA III (if being used) according to *Lymphocyte Enumeration SOP 4: Immunofluorescent Labeling of Whole Blood with BD Multitest™ 6-color TBNK Reagents or 4-color IMK Reagents, BD FACS™ SPA III Preparation*.
3. Prepare samples according to the Technical Data Sheet (TDS), either manually or with the SPA III. Include controls in every run.
4. Acquire the samples on the cytometer.
5. Review all results and adjust gates and histograms as needed.
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 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 is no problem with specimen identification, data entry errors, or any method errors in the preparation or running of the sample.

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

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

Performance specifications

In the following tables, “Within Device” precision is equivalent to inter-assay or between-run precision.

Precision with BD Trucount tubes, BD Multitest 6-color TBNK, TDS 23-10834-03

SD analysis for subset percentages (BD FACSCanto II flow cytometer)

Lymphocyte Subset (%)	CDL[a] SD		CDC[b] SD	
	Within Run	Within Device	Within Run	Within Device
$CD3^+CD4^+$	0.47	0.47	0.76	0.80
$CD3^+CD8^+$	1.10	1.18	0.75	0.76
Total $CD3^+$	1.32	1.32	0.82	0.87
$CD3^-CD19^+$	1.07	1.09	0.57	0.60
$CD3^-(CD16 + CD56)^+$	0.78	0.80	0.57	0.58

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

CV analysis for absolute counts (BD FACSCanto II flow cytometer)

Lymphocyte Subset (cells/µL)	CDL[a] %CV		CDC[b] %CV	
	Within Run	Within Device	Within Run	Within Device
$CD3^+CD4^+$	5.1	5.1	3.6	4.0
$CD3^+CD8^+$	4.9	5.1	4.5	4.5
Total $CD3^+$	4.2	4.3	3.3	3.6
$CD3^-CD19^+$	5.6	5.6	5.4	6.0
$CD3^-(CD16 + CD56)^+$	5.4	5.6	5.9	6.3

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

Precision with BD Trucount tubes, BD Multitest 6-color TBNK, TDS 23-10834-03

SD analysis for subset percentages (BD FACSCanto flow cytometer)

Lymphocyte Subset (%)	CDL[a] SD		CDC[b] SD	
	Within Run	Within Device	Within Run	Within Device
$CD3^+CD4^+$	0.64	0.69	0.95	1.23
$CD3^+CD8^+$	1.07	1.29	0.65	0.81
Total $CD3^+$	1.17	1.23	0.86	0.90
$CD3^-CD19^+$	0.89	0.89	0.62	0.62
$CD3^-(CD16 + CD56)^+$	0.90	0.96	0.61	0.62

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

CV analysis for absolute counts (BD FACSCanto flow cytometer)

Lymphocyte Subset (cells/µL)	CDL[a] %CV		CDC[b] %CV	
	Within Run	Within Device	Within Run	Within Device
$CD3^+CD4^+$	7.6	8.0	4.7	4.8
$CD3^+CD8^+$	4.1	5.0	4.7	5.4
Total $CD3^+$	4.0	4.4	4.2	4.2
$CD3^-CD19^+$	5.7	6.0	5.3	5.7
$CD3^-(CD16 + CD56)^+$	7.0	8.0	7.9	7.9

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

Precision without BD Trucount tubes, BD Multitest 6-color TBNK, TDS 23-10834-03

SD analysis for subset percentages (BD FACSCanto II flow cytometer)

Lymphocyte Subset (%)	CDL[a] SD		CDC[b] SD	
	Within Run	Within Device	Within Run	Within Device
$CD3^+CD4^+$	0.56	0.77	0.73	0.93
$CD3^+CD8^+$	0.80	1.66	0.58	1.12
Total $CD3^+$	1.03	1.34	0.81	0.90
$CD3^-CD19^+$	0.70	0.73	0.50	0.50
$CD3^-(CD16 + CD56)^+$	0.72	0.84	0.64	0.66

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

SD analysis for subset percentages (BD FACSCanto flow cytometer)

Lymphocyte Subset (%)	CDL[a] SD		CDC[b] SD	
	Within Run	Within Device	Within Run	Within Device
$CD3^+CD4^+$	0.57	0.76	0.86	1.05
$CD3^+CD8^+$	0.96	1.56	0.64	1.16
Total $CD3^+$	1.28	1.42	0.77	0.96
$CD3^-CD19^+$	0.72	0.84	0.51	0.60
$CD3^-(CD16 + CD56)^+$	0.74	0.90	0.58	0.68

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

Precision with BD FACSCanto II flow cytometer, BD Multitest IMK kit (4-color), TDS 23-3602-07

SD analysis for subset percentages (BD FACSCanto II flow cytometer)

Lymphocyte Subset (%)	CDL[a] SD	CDC[b] SD
$CD3^+CD4^+$	0.53	0.82
$CD3^+CD8^+$	1.34	0.80
Total $CD3^+$	0.98	0.67
$CD3^-CD19^+$	0.86	0.56
$CD3^-(CD16 + CD56)^+$	0.87	0.52

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

CV analysis for subset absolute counts (BD FACSCanto II flow cytometer)

Lymphocyte Subset (cells/µL)	CDL[a] %CV	CDC[b] %CV
$CD3^+CD4^+$	5.9	3.8
$CD3^+CD8^+$	3.9	4.7
Total $CD3^+$	3.6	2.9
$CD3^-CD19^+$	6.4	5.6
$CD3^-(CD16 + CD56)^+$	6.1	6.0

a. CDL = CD-Chex Plus CD4 Low control
b. CDC = CD-Chex Plus control

Precision with BD FACSCanto flow cytometer, BD Multitest IMK kit (4-color), TDS 23-3602-07

SD analysis for subset percentages (BD FACSCanto flow cytometer)

Lymphocyte Subset (%)	MCL[a] SD	MCN[b] SD
$CD3^+CD4^+$	0.77	1.18
$CD3^+CD8^+$	1.17	1.15
Total $CD3^+$	1.22	1.21
$CD3^-CD19^+$	1.05	0.77
$CD3^-(CD16 + CD56)^+$	1.14	1.03

a. MCL = BD Multi-Check CD4 Low control
b. MCN = BD Multi-Check control

CV analysis for subset absolute counts (BD FACSCanto flow cytometer)

Lymphocyte Subset (cells/µL)	MCL[a] %CV	MCN[b] %CV
$CD3^+CD4^+$	6.5	5.9
$CD3^+CD8^+$	5.8	7.1
Total $CD3^+$	4.1	4.8
$CD3^-CD19^+$	7.1	7.8
$CD3^-(CD16 + CD56)^+$	8.2	9.9

a. MCL = BD Multi-Check CD4 Low control
b. MCN = BD Multi-Check control

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

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.
    - The presence of lipemia typically requires that the sample be washed with PBS with 0.1% sodium azide to remove the lipids.
    - Subset percentages can be reported after washing. However, absolute counts cannot.
    - Any samples which are clotted or hemolyzed should be rejected for testing.

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

Carryover Protocol

Reference: CLSI document EP-17

The carryover specification for the BD FACSCanto cytometer and 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

One sample in EDTA anticoagulant, with a high lymphocyte count (7,000 to 9,000 lymphocytes) or BD Multi-Check Whole Blood Control.

Procedure

1. Set up the flow cytometer according to *Lymphocyte Enumeration SOP 1: BD FACSCanto™ Cytometer Startup Procedure*.
2. Prepare at least five replicates of the high lymphocyte sample according to the Technical Data Sheet (TDS), either manually or with the SPA III.
3. Aliquot 1 mL of PBS into five additional tubes.
4. Acquire a high lymphocyte sample followed by a tube of PBS, alternating five times on the cytometer. Acquire each PBS tube for the five-minute maximum time in BD FACSCanto clinical software.
5. Review all results and adjust gates and histograms as needed.
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.

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

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

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 lymphocytes (at least 9,000 lymphocytes/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 lymphocyte count of lower than 9,000/µL, as determined by a CBC or other method, 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 lymphocytes.

Perform the dilution of the sample in the following manner:

1. Make two-fold serial dilutions with BD Pharmingen stain buffer (BSA) such that the CD45 count is diluted down to approximately three CD45 cells.

Tube	Dilution Factor	Expected CD45 counts	Volume of Stain Buffer	Volume of previous dilution
1	Neat	9,000	0 µL	800 µL of whole blood
2	1:2	4,500	800 µL	800 µL of Neat
3	1:4	2,250	800 µL	800 µL of 1:2
4	1:8	1,125	800 µL	800 µL of 1:4
5	1:16	563	800 µL	800 µL of 1:8
6	1:32	281	800 µL	800 µL of 1:16
7	1:64	141	800 µL	800 µL of 1:32
8	1:128	71	800 µL	800 µL of 1:64
9	1:256	36	800 µL	800 µL of 1:128
10	1:512	18	800 µL	800 µL of 1:256
11	1:1,024	9	800 µL	800 µL of 1:512
12	1:2,048	5	800 µL	800 µL of 1:1,024
13	1:4,096	2	800 µL	800 µL of 1:2,048
14	1:8,192	1	800 µL	800 µL of 1:4,096
15	1:16,385	<1	800 µL	800 µL of 1:8,192

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

Procedure

1. Set up the flow cytometer according to *Lymphocyte Enumeration SOP 1: BD FACSCanto™ Cytometer Startup Procedure*.
2. Create serial dilutions of the sample to be tested.
3. Prepare samples according to the Technical Data Sheet (TDS).
4. Acquire samples on the cytometer.
5. Review all results and adjust gates and histograms as needed.
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 is no problem with specimen identification or any other method errors.

Analysis of results

Check that the regression values for 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 Multitest 6-color TBNK, TDS 23-10834-03

Linearity for BD Multitest 6-color TBNK assay using BD Trucount tubes was assessed for the BD FACSCanto II system within a WBC concentration of 0 to 3.3 x 10^4 WBCs/µL. Results were observed to be linear across the following range.

Linearity for BD Multitest 6-color TBNK assay for BD FACSCanto II flow cytometer

Subset	Range
$CD3^+CD4^+$	1–4,494
$CD3^+CD8^+$	2–2,922
Total $CD3^+$	4–7,382
$CD3^-CD19^+$	0–863
$CD3^-(CD16 + CD56)^+$	0–435

Linearity for BD Multitest 6-color TBNK assay for BD FACSCanto flow cytometer

Subset	Range (cells/µL)
CD4	4–2,234

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

The absolute counts were measured for other lymphocyte subsets across the previously indicated $CD4^+$ T-lymphocyte range.

The subset ranges were:

- 158 to 1,125 cells/µL for $CD8^+$ T lymphocytes
- 498 to 3,356 cells/µL for $CD3^+$ T lymphocytes
- 71 to 447 cells/µL for $CD19^+$ B lymphocytes
- 0 to 1,559 cells/µL for $CD16^+$ and $CD56^+$ NK lymphocytes

Linearity for BD Multitest IMK (4-color), TDS 23-3602-07

Linearity for BD FACSCanto II flow cytometer

Subset	Range (cells/µL)
CD4	1–3,669
CD8	2–2,324
CD3	6–5,998
CD19	1–857
CD16+CD56	1–447

Linearity for BD FACSCanto flow cytometer

Subset	Range (cells/µL)
CD4	29–5,827
CD8	22–4,076
CD3	48–9,627
CD19	5–1,131
CD16+CD56	4–671

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

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 lymphocyte counts are serially diluted with BD Pharmingen stain buffer with 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 *Lymphocyte Enumeration SOP 1: BD FACSCanto™ Cytometer Startup Procedure*.
2. Prepare diluted samples with expected CD45 results of 20 or lower according to the Technical Data Sheet (TDS).
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 is no problem 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 BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

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 *Lymphocyte Enumeration SOP 1: BD FACSCanto™ Cytometer Startup Procedure*.
2. Prepare samples according to the Technical Data Sheet (TDS). Include controls in every run.
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 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 is no problem 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.

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

Print the data chart and put in the validation notebook.

Performance Specifications

The following reference intervals were determined using healthy adults between the ages of 18 and 65 years.

Reference intervals using BD Trucount tubes, BD Multitest 6-color TBNK, TDS 23-10834-03

Representative reference intervals for BD Multitest 6-color TBNK reagent

Lymphocyte Subset	N	Mean (%)	95% Range
$CD3^+CD4^+$	123	46.4	28.2–62.8
$CD3^+CD8^+$	123	24.0	10.2–40.1
Total $CD3^+$	123	71.1	49.1–83.6
$CD3^-CD19^+$	123	14.9	6.5–27.0
$CD3^-(CD16 + CD56)^+$	123	11.7	4.1–25.2

Lymphocyte Subset	N	Mean (cells/µL)	95% Range
$CD3^+CD4^+$	123	1,106	441–2,156
$CD3^+CD8^+$	123	583	125–1,312
Total $CD3^+$	123	1,705	603–2,990
$CD3^-CD19^+$	123	354	107–698
$CD3^-(CD16 + CD56)^+$	123	266	95–640

Reference intervals using BD Trucount tubes, BD Multitest IMK kit (4-color), TDS 23-3602-07

Representative reference intervals for BD Multitest CD3/CD8/CD45/CD4

Lymphocyte Subset	N	Mean	95% Range
Helper/inducer T lymphs (%)	164	45	33–58
Suppressor/cytotoxic T lymphs (%)	164	24	13–39
Total T lymphs (%)	164	72	56–86
Helper/inducer T lymphs (cells/µL)	164	941	404–1,612
Suppressor/cytotoxic T lymphs (cells/µL)	164	511	220–1,129
Total T lymphs (cells/µL)	164	1,513	723–2,737

Representative reference intervals for BD Multitest CD3/CD16 + CD56/CD45/CD19

Lymphocyte Subset	N	Mean	95% Range
NK lymphs (%)	164	13	5–26
B lymphs (%)	164	14	5–22
T lymphs (%)	164	72	56–86
NK lymphs (cells/µL)	164	267	84–724

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

Stability Study Protocol

All stability testing done by BD Biosciences was performed using EDTA anticoagulated samples. Any claims for stability are based on the use of EDTA as an anticoagulant. If other anticoagulants are to be used for your testing, then stability testing should be done. Other anticoagulants that could be tested include sodium heparin and ACD. ACD anticoagulated samples cannot be used in single-platform testing, since the volume of the anticoagulant affects the absolute counts generated by the flow assay.

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

Specimens

- Collect a minimum of two samples collected 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 samples every day for five consecutive days.

Procedure

1. Set up the flow cytometer according to *Lymphocyte Enumeration SOP 1: BD FACSCanto™ Cytometer Startup Procedure*.
2. Prepare samples according to the Technical Data Sheet (TDS). Include controls in every run.
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 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 is no problem with specimen identification or any other method errors. If there is 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.

Validation Protocol for BD Multitest™ IMK and BD Multitest™ 6-color TBNK Reagents for Lymphocyte Subset Enumeration

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