Productivity and Efficiency of 6-Color BD Multitest and BD Trucount Technologies for Enumeration of Lymphocyte Subsets

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Application Note

This white paper discusses studies evaluating productivity when running patient samples using single-platform BD Multitest™ 6-color compared to a 4-color methodology. Comparisons were made on samples run with and without the BD FACS™ Sample Prep Assistant II (SPA II) and between BD FACSCanto™ II and BD FACSCalibur™ flow cytometers. The combination of the BD Multitest technology and the BD FACSCanto II flow cytometer resulted in the highest productivity and efficiency in terms of time savings and volume of reagents used.

Contents

Clinical rationale and application

Precise absolute $CD4^+$ T-lymphocyte values remain an accepted and important surrogate marker in management of patients with HIV infection. Physicians treating HIV-infected persons determine the appropriate staging and treatment in conjunction with absolute CD4 counts. Persons infected with HIV rely on CD4 counts for information on their immune status. Determining percentages or counts of $CD3^+CD4^+$ lymphocytes can be useful in monitoring human immunodeficiency virus (HIV)-infected individuals.[1] Individuals with HIV typically exhibit a steady decrease of $CD3^+CD4^+$ lymphocyte counts as the infection progresses.[2] $CD3^+CD4^+$ percentages or counts and the total number of T and B lymphocytes are used to characterize and monitor some forms of immunodeficiency[3-5] and autoimmune diseases.[6,7] NK lymphocytes identified as $CD3^-$ and $CD16^+$ and/or $CD56^+$ have been shown to mediate cytotoxicity against certain tumors and virus-infected cells.[8]

Most absolute T-cell counts and percentages are determined using two different instruments, a hematology analyzer and a flow cytometer (dual-platform technology [DPT]).[9] In 1997, the Centers for Disease Control (CDC) published guidelines addressing concerns related to DPT.[10] These guidelines still remain important for laboratories performing $CD4^+$ T-cell counts with this DPT technology.[10]

The introduction of Single-Platform Technology (SPT) has enabled the determination of both absolute CD4 counts and percentage of lymphocyte subsets from a single instrument (flow cytometer).[9] This technology uses both 6- and 4-color methodology.

Overview of the 4-color methodology

The 4-color BD Multitest system allows monitoring of CD4 and lymphocyte subsets. The full CDC-recommended lymphocyte subset profile for each specimen can be determined using a two-tube panel (CD3/CD8/CD45/CD4 and CD3/CD16$^+$CD56/CD45/CD19).[10]

This system uses 4-color BD Multitest reagents and a lyse/no-wash (LNW) sample preparation procedure that allows gating on CD45-stained lymphocytes. The system features automated sample analysis with BD Multiset™ software and is composed of BD Trucount™ absolute count tubes, BD FACSComp™ software, the BD FACSCalibur™ flow cytometer, and the BD FACS™ Loader for automated sample acquisition with BD Worklist Manager software. BD Trucount tubes contain a lyophilized pellet with a known quantity of fluorescent beads. Sample is added to the BD Trucount tube, into which the appropriate reagent has been added.

Once samples are stained using BD Multitest reagents and BD Trucount tubes, the system enumerates absolute counts (cells/µL) as well as lymphocyte percentages of mature T helper/inducer (CD3$^+$CD4$^+$), T suppressor/cytotoxic (CD3$^+$CD8$^+$), and total T (CD3$^+$) cells from the first tube. Using the second tube, the system enumerates mature T (CD3$^+$), B (CD3$^-$CD19$^+$), and NK (CD3$^-$CD16$^+$/CD56$^+$) subsets.

BD Multiset software performs flow cytometric data acquisition and automated analysis. Each sample is automatically analyzed using the CDC-recommended CD45 fluorescence and side scatter lymphocyte gating strategy for single-platform technology[10-13] and BD Trucount beads. A Laboratory Report is generated for each patient, illustrating the data plots and numeric results (percentage of lymphocytes and/or absolute counts). Patient results from each panel are also summarized in a Physician Report that includes normal range data and quality control features.

The BD FACSCalibur flow cytometer, a dual-laser, 4-color benchtop instrument, is equipped with an air-cooled 488-nm argon laser for excitation of FITC, PE, and PerCP in FL1, FL2, and FL3. The 4-color option adds a 635-nm red diode laser for excitation of APC in FL4. The instrument features automated setup with BD FACSComp software and BD Calibrite™ beads, software based instrument control, and pushbutton fluidic control. Combined with the BD FACS Loader, its automated sample loader, the BD FACSCalibur cytometer offers the high throughput and ease of use necessary to meet productivity requirements with improved performance.

The BD SPA II provides walk-away sample preparation. Its automated capabilities include sample tube cap piercing, blood and reagent aliquoting, incubating, and lysing and mixing steps. The software contains predefined protocols and can create customized protocols.

Overview of the 6-color methodology

The 6-color BD Multitest system also allows monitoring of CD4 and lymphocyte subsets. The full CDC-recommended lymphocyte subset profile for each specimen can be determined using a one-tube panel (CD3/CD16$^+$CD56/CD45/CD4/CD19/CD8).[10,14] The 6-color BD Multitest system consists of the BD FACSCanto II flow cytometer and BD Multitest 6-color TBNK reagent using BD Trucount tubes. The system features automated sample analysis and is composed of BD Trucount absolute count tubes, BD FACSCanto clinical software, the BD FACSCanto II flow cytometer, and the BD FACS Loader for automated sample acquisition. BD Trucount tubes each contain a lyophilized

pellet with a known quantity of fluorescent beads. Sample is added to the BD Trucount tube, into which the appropriate reagent has been added.

Once samples in the BD Trucount tubes are stained using BD Multitest 6-color TBNK reagents, the system enumerates absolute counts (cells/µL) as well as lymphocyte percentages of mature T helper/inducer ($CD3^+CD4^+$), T suppressor/cytotoxic ($CD3^+CD8^+$), total T ($CD3^+$) cells, B ($CD3^-CD19^+$), and NK ($CD3^-CD16^+/CD56^+$) subsets.

BD FACSCanto clinical software fully automates setup, acquisition, and analysis. BD FACS™ 7-color setup beads allow fully automated instrument setup. This system employs the CDC-recommended CD45 fluorescence and side scatter lymphocyte gating strategy for single-platform technology.[10-13] The BD FACSCanto II system is built with a blue laser (488 nm, air-cooled, 20 mW solid state) and a red laser (633 nm, 17 mW HeNe) excitation sources. The BD SPA II provides walk-away sample prep automation.

Comparison of 4-color BD Multitest and 6-color BD Multitest productivity

A productivity study for 6-color lymphocyte subsetting was conducted in collaboration with the University of Miami, Miller School of Medicine/Jackson Memorial Hospital. Using patient samples obtained from clinical settings, the study evaluated the 4- and 6-color methods run on the BD FACSCanto II and BD FACSCalibur flow cytometers, using either the BD SPA II or manual preparation. Time was recorded for completion of tasks in each step during the sample processing and running for both 4- and 6-color methods. Twenty samples were run on the two platforms twice a day for two consecutive days. The BD FACS Loader was used for running all tubes on both cytometers.

BD Multitest Reagent	Instrument	Sample Prep	Tubes per batch
4-color IMK	BD FACSCalibur	Manual Prep	40
4-color IMK	BD FACSCalibur	BD SPA II	40
4-color IMK	BD FACSCanto II	Manual Prep	40
4-color IMK	BD FACSCanto II	BD SPA II	40
6-color IMK	BD FACSCanto II	Manual Prep	60
6-color IMK	BD FACSCanto II	BD SPA II	60

Table 1. Study Design

Study methods

BD Multitest 4- and 6-color reagents use the same time-saving LNW method for direct immunofluorescent staining of human peripheral blood specimens. Whole blood is added directly to BD Trucount tubes and after staining, the data is acquired directly on the BD FACSCalibur or BD FACSCanto II flow cytometer to determine absolute count and percentages of cell populations of interest.

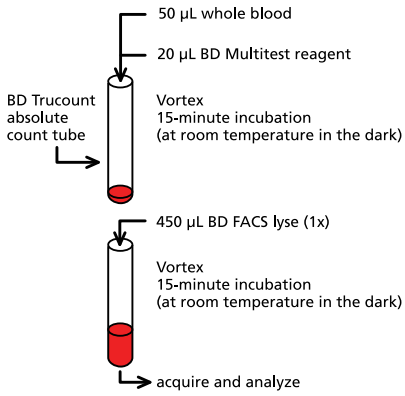


Figure 1. BD Multitest Sample Preparation

Staining procedure

For each specimen, 50 µL of EDTA anticoagulated whole blood is added directly to either one or two BD Trucount tubes containing the following reagents as shown in **Table 2.**

BD Multitest IMK Kit	1 Tube	20 µL	CD3 FITC/CD8 PE/CD45 Per CP/CD4 APC
	1 Tube	20 µL	CD3 FITC/CD16+CD56 PE/CD45 PerCP/CD19 APC
BD Multitest 6-color TBNK Reagent	1 Tube	20 µL	CD3 FITC/CD16+CD56 PE/CD45 PerCP-Cy™5.5/CD4 PE-Cy™7/CD19 APC/CD8 APC-Cy7*

*Cy is a trademark of Amersham Biosciences Corp.

Table 2. Sample Staining

The cells are incubated 15 minutes at room temperature in the dark. The erythrocytes are then lysed by adding 450 µL of 1x BD FACS™ lysing solution and incubating 15 minutes at room temperature in the dark. Samples are then ready to be analyzed on the cytometer as shown in **Figure 1.**

Acquisition was performed on either the BD FACSCalibur instrument using BD Multiset software with BD Worklist Manager software or the BD FACSCanto II instrument using BD FACSCanto clinical software. Automated gating was used for easy analysis of each subset population as shown in **Figures 2** and **3.**

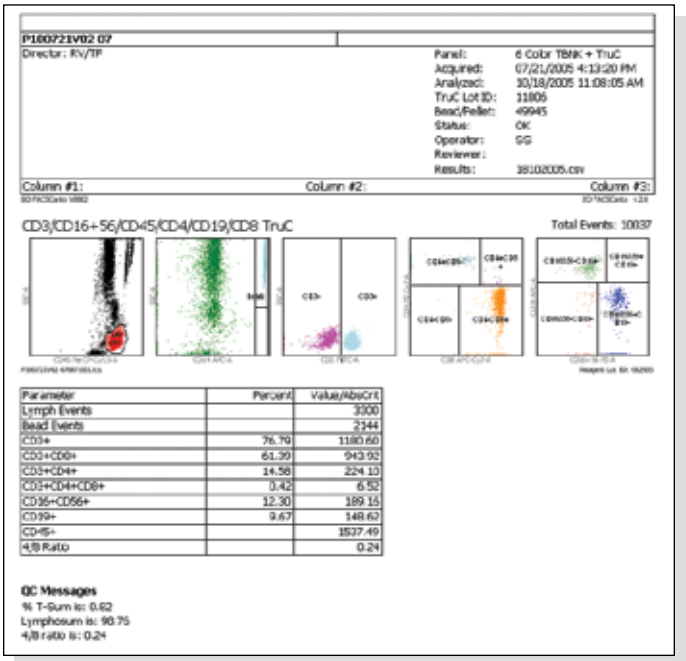


Figure 2. An example of 6-color result (analysis with BD Multitest 6-color TBNK reagents, BD FACSCanto clinical software, on the BD FACSCanto II flow cytometer)

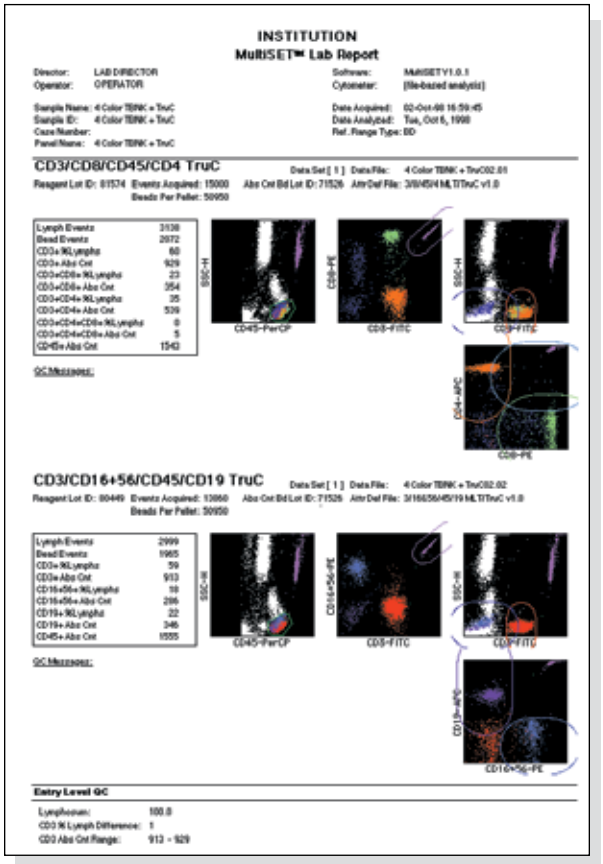


Figure 3. An example of 4-color result (analysis with BD Multiset software on the BD FACSCalibur flow cytometer)

Results

An overall productivity analysis compared the 6-color TBNK assay run on the BD FACSCanto II flow cytometer with the Loader to the 4-color IMK assay run on the BD FACSCalibur flow cytometer with the Loader. Samples were prepared both manually and using the BD SPA II. Instrument productivity was analyzed to understand the efficiency of running a BD FACSCanto II vs. BD FACSCalibur system. Average time was calculated for the tasks using results from 20 samples and two operators. The 20 samples were run as one batch. One sample equaled one tube for the 6-color assay and two tubes for the 4-color assay.

	6-Color Method	4-Color Method	% Avg. Time Savings
Number of tubes per sample	1	2	
Average total instrument time per sample batch (20 tubes)	45	74	39
Average total productivity time per sample batch (20 tubes) Manual sample preparation	100	158	37
Average total productivity time per sample batch (20 tubes) Automated sample preparation (BD SPA II)	134	186	28

Table 3. Productivity results summary (times listed are in minutes)

Time	BD FACSCanto II		BD FACSCalibur
	6-color TBNK	4-color IMK Kit	4-color IMK Kit
Set up instrument	11	9	8
Enter patient info into software	3	3	6
Optimize instrument	2	3	3
Acquire and analyze	29	58	57
Total instrument time	45	73	74

Table 4. Instrument productivity, 6-color vs. 4-color methodology (times listed are in minutes)

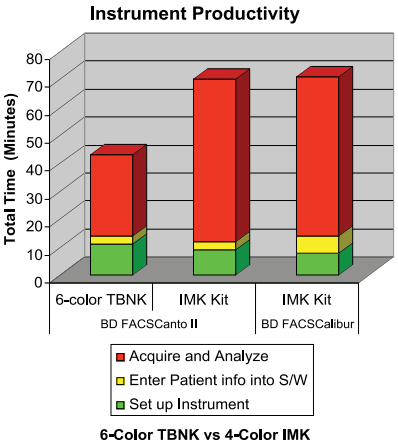


Figure 4. Instrument productivity, 6-color vs. 4-color methodology

An average savings of 39% on overall instrument time and 49% on acquisition and analysis time was achieved for the 6-color TBNK assay run on the BD FACSCanto II instrument compared to the 4-color IMK assay run on the BD FACSCalibur instrument. See **Table 4** and **Figure 4**.

Overall productivity (sample prep and instrument run)

When manual hands-on handling of sample preparation was added to the instrument time, the average total time savings was 37% for the 6-color TBNK assay run on the BD FACSCanto II instrument compared to the 4-color IMK assay run on the BD FACSCalibur instrument (both with the Loader). See **Table 5** and **Figure 5**. The average total time savings was 28% when the BD SPA II was used for sample preparation for both the 6-color TBNK assay run on the BD FACSCanto II instrument and the 4-color IMK assay run on the BD FACSCalibur instrument. See **Table 6** and **Figure 6**.

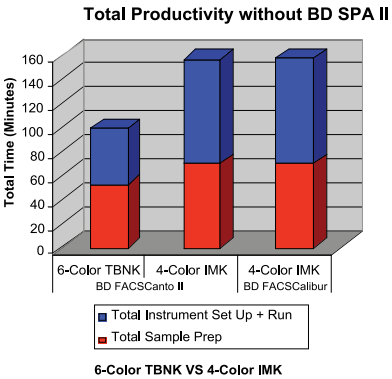


Figure 5. Overall (sample prep without BD SPA II, acquisition, and analysis) productivity graph, 6-color vs. 4-color methodology

Tasks	6-Color TBNK BD FACSCanto II	4-Color IMK BD FACSCanto II	4-Color IMK BD FACSCalibur
Total sample prep	53	70	71
Total instrument setup + run	47	87	87
Total time	100	157	158

Table 5. Overall (sample prep without BD SPA II, acquisition, and analysis) productivity table, 6-color vs. 4-color methodology (times listed are in minutes)

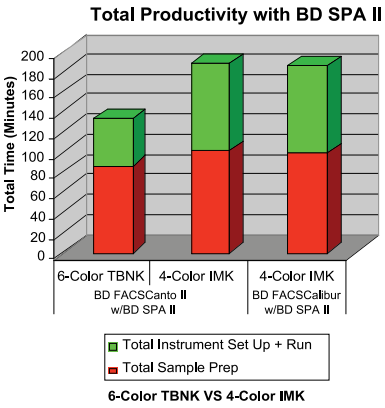


Figure 6. Overall (sample prep with BD SPA II, acquisition, and analysis) productivity graph, 6-color vs. 4-color methodology

Performance Evaluations

Tasks	6-Color TBNK BD FACSCanto II w/BD SPA II	4-Color IMK BD FACSCanto II w/BD SPA II	4-Color IMK BD FACSCalibur w/BD SPA II
Total sample prep	87	101	99
Total instrument setup + run	47	87	87
Total time	134	188	186

Table 6. Overall (sample prep with BD SPA II, acquisition, and analysis) productivity table, 6-color vs 4-color methodology (times listed are in minutes)

Accuracy of CD4 percentages and CD4 absolute counts: 4-color vs. 6-color

A clinical method comparison between the 6-color LNW assay and the 4-color LNW assay on the BD FACSCanto II systems was performed. Principal investigators were Phillip Ruiz, MD, PhD, at Jackson Memorial Hospital, and Michael J. Borowitz, MD, PhD at Johns Hopkins Medical Institutions.[14]

Figures 7 and **8** show the correlations for CD4 percentage and absolute counts from the study. Equivalent results were achieved using 4-color and 6-color BD Multitest (LNW) reagents. Similar correlation was observed with all other lymphocyte subsets.[14]

Conclusions

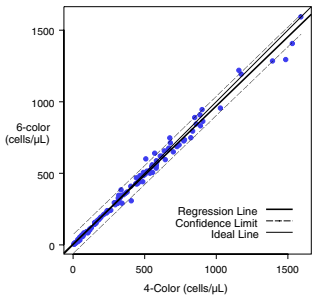
The 6-color BD Multitest system consists of the BD FACSCanto II flow cytometer and BD Multitest 6-color TBNK reagent with BD Trucount tubes. This system offers optimal time management and workflow in any laboratory setting. The BD SPA II adds automated capabilities, reduces hands-on time, and adds flexibility to the lab by increasing operator multitasking. The 6-color TBNK BD Multitest system is an accurate, fully functional system for clinical enumeration of CD4 T-cell counts and lymphocyte subsets. The use of automated CD45/SSC gating on lymphocytes provides consistency of gating for all samples.[10,13]

The 6-color method increases laboratory productivity, and the accuracy is equivalent to the 4-color method. The 6-color method using the BD FACSCanto II flow cytometer minimizes cost and labor by reducing the number of tubes, the volume of reagent required, and amount of patient sample needed. Sample acquisition and analysis are simple, and up to 39% savings on direct hands-on time is achieved when compared to the 4-color method using the BD FACSCalibur flow cytometer.

The 6-color TBNK BD Multitest system provides all the tools necessary for the clinical laboratory to meet today's and future CD4 enumeration needs.

Additional benefits to running 6-color method:

- Easier startup and shutdown
- Easier links to a Laboratory Information System (LIS)
- Single software program for setup, acquisition, and running
- Fewer tubes, reduced volumes of sample and reagent
- Fluidics cart for larger sheath fluid and waste tanks, allowing processing of more samples between refilling the sheath tank or emptying the waste tank



CD4_Abs
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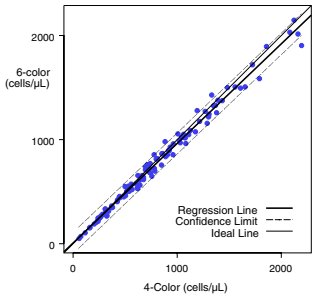
Intercept: 6.02
n: 117
r:0.995



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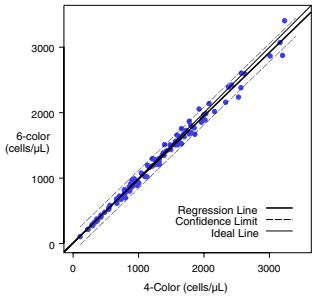
r:0.994



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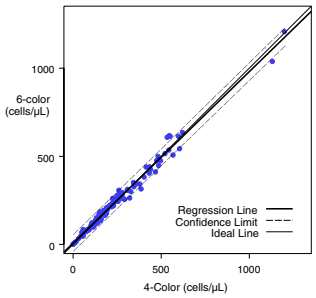
r:0.995



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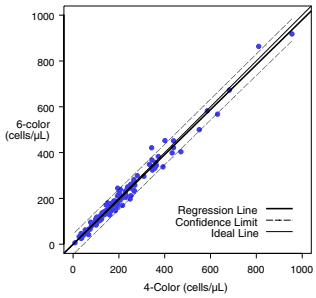
r:0.992



CD16_56_Abs
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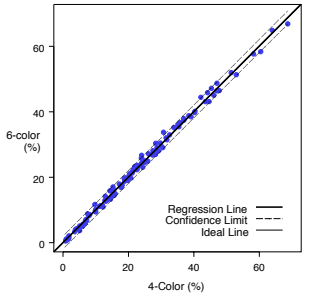
r:0.99

Figure 7. Absolute counts, 4-color vs. 6-color



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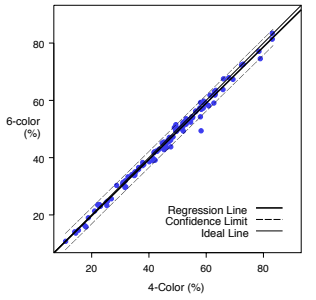

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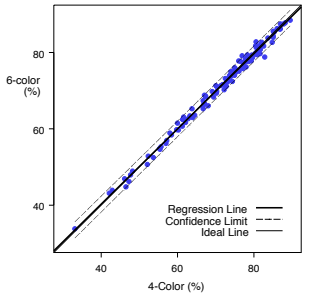

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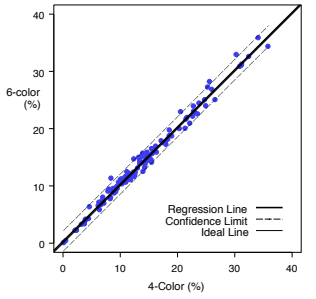

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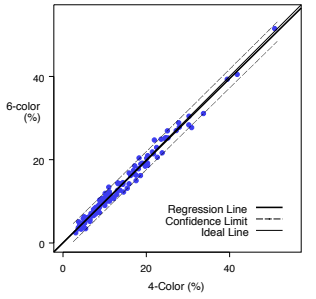

CD16_56_Pct
Statistics Summary
Slope: 0.985
Intercept: 0.0603
n:117
r: 0.992

Figure 8. Percentages, 4-color vs. 6-color

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