BD Multitest™ 6-Color TBNK

50 Tests—Catalog No. 644611
50 Tests with BD Trucount™ Tubes—Catalog No. 337166

CE

IVD

12/2020

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1. INTENDED USE

BD Multitest™ 6-color TBNK reagent with optional BD Trucount™ tubes is a six-color direct immunofluorescence reagent for use with a suitably equipped BD flow cytometer to identify and determine the percentages and absolute counts of T, B, and natural killer (NK) cells, as well as the CD4 and CD8 subpopulations of T cells in peripheral blood.

BD Multitest™ 6-color TBNK reagent and BD Trucount™ tubes can be used with the BD FACS™ Loader and the BD FACS™ Universal Loader.

2. SUMMARY AND EXPLANATION

Human lymphocytes can be divided into three major subset populations based on their biologic function and cell-surface antigen expression: T lymphocytes ($CD3^+$), B lymphocytes ($CD19^+$), and NK lymphocytes ($CD16^+CD56^+$). BD Multitest™ 6-color TBNK reagent can be used with or without BD Trucount™ tubes to characterize these lymphocyte subset populations.

When the reagent is used with BD Trucount™ tubes, cytometer-specific BD software (see Table 2, Instruments section) calculates lymphocyte subset percentages and absolute counts using flow data from the BD Trucount™ beads and the sample.

When the reagent is used without BD Trucount™ tubes, cytometer-specific BD software calculates lymphocyte subset percentages directly from the sample flow data. If you enter lymphocyte data obtained from another instrument or method, the software can also calculate absolute counts.

Clinical Applications

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 total T and B lymphocytes are used to characterize and monitor some forms of immunodeficiency[3-5] and autoimmune diseases.[6,7]

Determining counts of $CD3^+CD4^+$ and/or $CD3^+CD8^+$ lymphocytes can be useful in the immunological assessment of SARS-CoV-2 infected individuals during the COVID-19 disease. Individuals with COVID-19 disease typically exhibit a decrease of $CD3^+CD4^+$ and/or $CD3^+CD8^+$ lymphocyte counts with increasing disease severity.[8,9,10]

In confirmed COVID-19 patients, $CD3^+CD4^+$ and/or $CD3^+CD8^+$ lymphocyte counts can also be used as an aid in determining the risk of intubation with mechanical ventilation, and the risk of mortality, in conjunction with clinical findings and the results of other laboratory testing.

NK lymphocytes identified as $CD3^-$ and $CD16^+$ and/or $CD56^+$ have been shown to mediate cytotoxicity against certain tumors and virus-infected cells.[11] NK-mediated cytotoxicity does not require class I or class II major histocompatibility complex (MHC) molecules to be present on the target cell.[12]

3. PRINCIPLES OF THE PROCEDURE

When whole blood is added to the reagent, the fluorochrome-labeled antibodies in the reagent bind specifically to leucocyte surface antigens. During acquisition, the cells travel past the laser beam and scatter the laser light. The stained cells fluoresce. These scatter and fluorescence signals, detected by the instrument, provide information about the cell's size, internal complexity, and relative fluorescence intensity. BD Multitest™ reagents employ fluorescence triggering, allowing direct fluorescence gating of the lymphocyte population[13–15] to reduce contamination of unlysed or nucleated red blood cells in the gate.

When BD Trucount™ tubes are used, a precise volume of sample is stained directly in a BD Trucount™ tube. The lyophilized pellet in the tube dissolves, releasing a known number of fluorescent beads. During analysis, the absolute number (cells/µL) of gated cells in the sample can be determined by comparing cellular events to bead events. If appropriate cytometer-specific BD software is used (see Table 2, Instruments section), absolute counts will be determined by the software. If manually performing data analysis using software such as BD CellQuest™ software, simply divide the number of positive cellular events by the number of bead events, and then multiply by the BD Trucount™ bead concentration.

4. REAGENT

Reagent Provided, Sufficient for 50 Tests

The BD Multitest™ 6-color TBNK reagent is provided in 1 mL of buffered saline with 0.1% sodium azide. It contains FITC-labeled CD3, clone SK7;[16–19] PE-labeled CD16, clone B73.1,[20–22] and PE-labeled CD56, clone NCAM16.2;[23] PerCP-Cy™5.5*–labeled CD45, clone 2D1 (HLe-1);[24] PE-Cy™7–labeled CD4, clone SK3;[25–27] APC-labeled CD19, clone SJ25C1;[28] and APC-Cy7–labeled CD8, clone SK1.[25,26]

CD3 reacts with the epsilon chain of the CD3 antigen/T cell antigen receptor (TCR) complex.[29] The CD3 antigen is present on 61% to 85% of normal peripheral blood lymphocytes.[30]

CD16 and CD56 together facilitate identification of the NK lymphocyte population.[11,14] CD16 recognizes a 50- to

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70-kilodalton (kDa) human NK lymphocyte antigen that is an Fc receptor for IgG.[20,21,31] CD16 reacts variably with granulocytes.[20] CD56 recognizes an extracellular immunoglobulin-like domain common to three molecular weight forms (120, 140, and 180 kDa) of the neural cell adhesion molecule (NCAM).[32–34]

CD45 recognizes human leucocyte antigens, 180 to 220 kDa, that are members of the T200 family.[35] The CD45 antigen is present on all human leucocytes, including lymphocytes, monocytes, granulocytes, eosinophils, and basophils in peripheral blood.[35] The CD45 antibody has been reported to react weakly with mature circulating erythrocytes and platelets.[35,36]

The CD4[26,37] antigen, 55 kDa,[17] is present on a T-lymphocyte subset[38,39] ($CD3^+CD4^+$) that comprises 28% to 58%[30] of normal peripheral blood lymphocytes.[17,26] The CD4 antigen is present in low density on the cell surface of monocytes and in the cytoplasm of monocytes.

CD19 recognizes a 90-kDa antigen that is present on human B lymphocytes.[28,40] The CD19 antigen is present on approximately 7% to 23% of human peripheral blood lymphocytes[30] and on splenocytes.[41] The CD19 antigen is present on human B lymphocytes at all stages of maturation.[42] CD19 does not react with resting or activated T lymphocytes, granulocytes, or monocytes.[43]

The CD8 antigen is expressed as a disulfide-linked bimolecular complex with a 32-kDa α subunit.[44,45] The CD8 antigen is present on a T-lymphocyte subset[17,26,38,39,46,47] as well as on a subset of NK lymphocytes.[48] The CD8 antigen is expressed on 19% to 48% of normal peripheral blood lymphocytes[30] and 60% to 85% of normal thymocytes.[17,26]

CD3, CD16, CD45, CD19, CD4, and CD8 antibodies are composed of mouse IgG_1 heavy chains and kappa light chains.

The CD56 antibody is composed of mouse IgG_{2b} heavy chains and kappa light chains.Concentration values of the conjugated antibodies are listed in Table 1:

Table 1 Concentration values

Reagent	Concentration (µg/mL)
CD3	2.3
CD16	1.65
CD56	1.1
CD45	6.0
CD4	1.5
CD19	2.3
CD8	6.3

BD Trucount™ tubes each contain a freeze-dried pellet of fluorescent beads in a single-use tube. Each BD Trucount™ pouch contains 25 tubes, sufficient for 25 tests.

Precautions

- Do not use the reagent if you observe any change in appearance. Precipitation or discoloration indicates instability or deterioration.
- The antibody reagent contains sodium azide as a preservative. However, take care to avoid microbial contamination, which can cause erroneous results.
- If using BD Trucount™ tubes, calibrate pipets to deliver exactly 50 µL of sample or perform the reverse pipetting technique (see Reverse Pipetting on page 16). See the pipet manufacturer's instructions for more information.
- Bead count varies by lot of BD Trucount™ tubes. It is critical to use the bead count shown on the current lot of BD Trucount™ tubes when entering this value in the software or when manually

calculating absolute counts. We recommend that you do not mix multiple lots of tubes in the same run.

- BD Trucount™ tubes are designed for use with a specific lyse/no-wash procedure. Do not attempt to threshold on forward scatter (FSC) for data collection.
- Do not use previously fixed and stored patient specimens. Whole blood samples refrigerated before staining can give aberrant results. Blast cells can interfere with test results. Hemolyzed samples should be rejected.
- Do not use BD Trucount™ controls with prepared BD Multitest™ 6-color TBNK samples. BD Trucount™ control beads can interfere with absolute count results.
- Use care to protect the tubes from direct light. Perform the procedure at room temperature (20°C–25°C).
- Visit regdocs.bd.com to download the Safety Data Sheet.

Storage and Handling

- Store the reagent at 2°C–8°C. Reagent in opened or unopened vials is stable until the expiration date shown on the vial label. Do not use after this expiration date.
- Do not freeze the reagent or expose it to direct light during storage or incubation with cells. Keep the reagent vial dry.
- Store BD Trucount™ tubes in their original foil pouch at 2°C–25°C. To avoid potential condensation, open the pouch only after it has reached room temperature and carefully reseal the pouch immediately after removing a tube. An unopened pouch is stable until the expiration date shown on the packaging. Do not open the pouch and use tubes after the expiration date. Use tubes within 1 hour after removal from the foil pouch. Use remaining tubes within 1 month after opening the pouch.

5. INSTRUMENTS

The BD Multitest™ 6-color TBNK reagent and BD Trucount™ tubes are designed for use on flow cytometers equipped with appropriate computer hardware and software. We recommend the BD systems listed in Table 2 for cytometer setup, acquisition, and analysis. See the corresponding reagent, cytometer, or software user documentation for details.

The BD FACS™ Loader and BD FACS™ Universal Loader can also be used with this product.

Ensure that the instrument is properly set up and passes daily quality control before use.

Table 2 Recommended BD systems

Flow cytometer	Setup beads	Setup software	Analysis software
BD FACSLyric™	BD® CS&T beads[a] BD® FC beads 7-color kit[b]	BD FACSuite™ Clinical software	BD FACSuite™ Clinical software
BD FACSCanto™ BD FACSCanto™ II	BD FACS™ 7-color setup beads[c]	BD FACSCanto™ clinical software v2.4 or later	BD FACSCanto™ clinical software v2.4 or later

a. To perform daily cytometer quality control.
b. To calculate compensation.
c. To set photomultiplier tube (PMT) voltages and fluorescence compensation, and check instrument sensitivity before use.

Use the specified software with:

- BD Trucount™ tubes to automatically calculate lymphocyte subset percentages and absolute counts

- 12 × 75-mm polystyrene tubes to automatically calculate lymphocyte subset percentages only

Alternatively, enter lymphocyte data obtained from another instrument or method to enable the software to calculate absolute counts in addition to lymphocyte subset percentages.

6. SPECIMEN COLLECTION AND PREPARATION

Collect blood aseptically by venipuncture into a BD Vacutainer® EDTA blood collection tube or equivalent.[51] The BD Multitest™ 6-color TBNK reagent and BD Trucount™ tubes have been validated with EDTA tubes.

A minimum of 100 µL of whole blood is required for this procedure. Follow the collection tube manufacturer's guidelines for the minimum volume of blood to be collected to ensure proper specimen dilution, especially when determining absolute counts using BD Trucount™ beads.

Anticoagulated blood stored at room temperature (20°C–25°C) must be stained within 24 hours of draw and must be analyzed within 6 hours of staining.

WARNING All biological specimens and materials coming in contact with them are considered biohazards. Handle as if capable of transmitting infection[49,50] and dispose of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Wear suitable protective clothing, eyewear, and gloves. Fixation has been reported to inactivate HIV.[57]

Interfering Conditions

The table lists the substances that were tested for interference with the BD Multitest™ 6-color TBNK reagent with optional BD Trucount™ tubes.

Testing for interference was performed in accordance with CLSI guidelines.[61] There was no detectable interference at the following concentrations.

Table 3 Non-interfering substances

Analyte	Concentration tested
Acetaminophen	156 µg/mL
Acetylsalicylic acid (Aspirin)	30 µg/mL
Albuterol	0.015 µg/mL
Atenolol	3 µg/mL
Atorvastatin	0.25 µg/mL
Azithromycin	3.7 µg/mL
Bilirubin, conjugated	2 mg/dL
Cobicistat	3.6 µg/mL
Efavirenz	12 µg/mL
Enoxaparin	2 µg/mL
Guaifenesin	1.5 µg/mL
Hydroxychloroquine	0.2 µg/mL
Ibuprofen	73 µg/mL
Insulin	37 µU/mL
Kaletra	15.5 µg/mL
Lisinopril	0.082 µg/mL

Table 3 Non-interfering substances

Analyte	Concentration tested
Maraviroc	0.888 µg/mL
Oseltamivir	0.133 µg/mL
Raltegravir	15 µg/mL
Remdesivir	16.32 µg/mL
Ritonavir	15 µg/mL
Tenofovir	0.978 µg/mL
Tocilizumab	149.4 µg/mL
Vancomycin	40 µg/mL

The following substances interfered with the assay at the indicated concentration:

Table 4 Interfering substances

Analyte	Concentration tested
Albumin[a,e]	6 g/dL
Bilirubin, unconjugated[b,e]	2 mg/dL
Erythrocytes[c,e]	$6x10^3$ cells/µL
Hemoglobin[c,e]	1000 mg/dL
Triglycerides[d,e]	1500 mg/dL

a. Albumin interferes as a result of its comparatively large concentration in the peripheral blood and its ability to bind as well as to release large quantities of ligands.[62]
b. Unconjugated Bilirubin may induce autofluorescence.[63]

c. The presence of red blood cells (RBCs) in the sample preparation can cause light interference and non-specific interactions leading to false test results.[64] Hemolyzed samples should be rejected. The hemoglobin concentration refers to free hemoglobin.
d. Immunomodulatory drugs used for treatment of HIV infection may cause lipemia. Lipemia is known to interfere in assays that use the transmission of light and impact the scattering of light.[65,66]
e. The listed endogenous substances interfere with the assay at higher than normal concentrations, i.e. hyperalbuminemia, unconjugated hyperbilirubinemia, erythrocytosis, hemoglobinemia, and hypertriglyceridemia. Interference caused by these endogenous substances is not uncommon and has been described in the literature.[62-66]

7. REAGENTS AND MATERIALS

Provided

- BD Multitest™ 6-color TBNK reagent (Catalog No. 644611), or
- BD Multitest™ 6-color TBNK reagent with BD Trucount™ tubes (Catalog No. 337166)

Required But Not Provided

- For BD FACSLyric™ flow cytometers:

  BD® CS&T beads (Catalog Nos. 656504, 656505)

  BD® FC Beads 7-color kit (Catalog No. 656867)

- For BD FACSCanto™ and BD FACSCanto™ II flow cytometers:

  BD FACS™ 7-color setup beads (Catalog No. 335775)

- BD FACS™ lysing solution (10X), 100 mL (Catalog No. 349202)

  BD FACS™ lysing solution contains diethylene glycol and formaldehyde. See the *BD FACS™ Lysing Solution* instructions for use (IFU) for precautions and warnings.

- Reagent-grade (distilled or deionized) water
- BD Vacutainer® EDTA blood collection tubes or equivalent

- Disposable 12 × 75-mm Falcon polystyrene tubes or equivalent (if not using BD Trucount™ tubes)
- Vortex mixer
- Micropipettor with tips
- Bulk dispenser or pipettor (450 µL) for dispensing 1X BD FACS™ lysing solution
- BD Multi-Check™ Control (Catalog Nos. 340911, 340912, 340913)
- BD Multi-Check™ CD4 Low Control (Catalog Nos. 340914, 340915, 340916)

8. PROCEDURE

Dilution Instructions for BD FACS™ Lysing Solution

Dilute the 10X concentrate 1:10 with room temperature (20°C–25°C) deionized water. The prepared solution is stable for 1 month when stored in a glass or high density polyethylene (HDPE) container at room temperature.

Reverse Pipetting

Accurate pipetting is critical when using a BD Trucount™ tube. Use the reverse pipetting technique to add the sample to a BD Trucount™ tube. For reverse pipetting, depress the button to the second stop. Release the button to draw excess sample into the tip. Press the button to the first stop to expel a precise volume of sample, leaving excess sample in the tip.

Staining the Cells

1. For each patient sample, label a 12 × 75-mm tube with the sample identification number.

   For absolute counts, label a BD Trucount™ tube in place of the 12 × 75-mm tube.

NOTE Before using a BD Trucount™ tube, verify that the BD Trucount™ bead pellet is intact and within the metal retainer at the bottom of the tube. If this is not the case, discard the BD Trucount™ tube and replace it with another. Do not transfer beads to another tube.

2. Pipette 20 µL of BD Multitest™ 6-color TBNK reagent into the bottom of the tube.

   If using a BD Trucount™ tube, pipette just above the stainless steel retainer. Do not touch the pellet.

3. Pipette 50 µL of well-mixed, anticoagulated whole blood into the bottom of the tube.

   NOTE Use the reverse pipetting technique to pipette sample onto the side of the tube just above the retainer. See Reverse Pipetting on page 16. Avoid smearing blood down the side of the tube. If whole blood remains on the side of the tube, it will not be stained with the reagent and can affect results.

4. Cap the tube and vortex gently to mix. Incubate for 15 minutes in the dark at room temperature (20°C–25°C).

5. Add 450 µL of 1X BD FACS™ lysing solution to the tube.

6. Cap the tube and vortex gently to mix. Incubate for 15 minutes in the dark at room temperature (20°C–25°C).

   The sample is now ready to be analyzed on the flow cytometer.

   CAUTION Some APC-Cy7 conjugates, and to a lesser extent PE-Cy7 conjugates, show changes in their emission spectra with prolonged exposure to paraformaldehyde. For overnight storage of stained cells, wash and resuspend in buffer without paraformaldehyde after 1 hour of fixation.

Flow Cytometry

- If samples are not to be analyzed immediately after preparation, store them in the dark at room temperature (20°C–25°C).
- Anticoagulated blood must be stained within 24 hours of draw and must be analyzed within 6 hours of staining.
- Vortex the cells thoroughly (at low speed) to reduce aggregation before running them on the flow cytometer.[52]
- If using the BD FACS™ Loader or BD FACS™ Universal Loader, vortex tubes immediately before placing them into the loader racks.
- Acquire and analyze data using BD FACSCanto™ clinical software or BD FACSuite™ Clinical software.
- Before acquiring samples, adjust the threshold to minimize debris and ensure populations of interest are included.
- See the appropriate instrument IFU for gating in the respective software. See BD FACSLyric™ flow cytometer for examples of lab reports.

Quality Control

In accordance with the College of American Pathologists (CAP) guidelines, we recommend running two levels of liquid control material (procedural control). These should be processed like patient samples to monitor the outgoing performance of the entire analytic process. This is done at least once each day patient testing is performed.[53] BD offers the BD Multi-Check™ Control and the BD Multi-Check™ CD4 Low Control.

Use commercial controls providing established values for percent positive and absolute counts with each run to assess system performance.

Representative Data

Visually inspect the CD45 vs SSC dot plot. The lymphocyte population should appear as a bright, compact cluster with low SSC. Monocytes

and granulocytes should also appear as distinct clusters. Do not proceed with analysis if populations are diffuse and there is little or no separation between clusters. Visually inspect all dot plots to ensure proper gating. See the following tables for a visual explanation of the gating hierarchy.

Table 5 BD Multitest™ 6-color TBNK reagent with BD Trucount™ tubes: CD3/CD16+CD56/CD45/CD4/CD19/CD8

No.	Plot	Populations of Interest
1	CD45 vs SSC	lymphocytes
2	CD19 vs SSC	BD Trucount™ beads
3	CD3 vs SSC	$CD3^-$ cells $CD3^+$ cells
4	CD8 vs CD4	$CD3^+$ cell subsets: $CD4^-CD8^+$ cells $CD4^+CD8^-$ cells $CD4^+CD8^+$ cells $CD4^-CD8^-$ cells
5	CD16+CD56 vs CD19	$CD3^-$ cell subsets: $(CD16+CD56)^-CD19^+$ cells $(CD16+CD56)^+CD19^-$ cells

Table 5 BD Multitest™ 6-color TBNK reagent with BD Trucount™ tubes: CD3/CD16+CD56/CD45/CD4/CD19/CD8

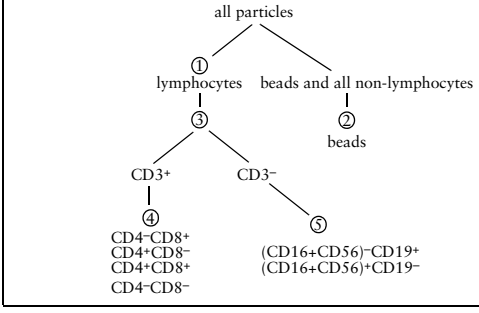
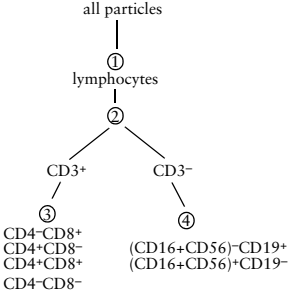
No.	Plot	Populations of Interest
Visual Representation of Hierarchy		
		

Table 6 BD Multitest™ 6-color TBNK reagent without BD Trucount™ tubes: CD3/CD16+CD56/CD45/CD4/CD19/CD8

No.	Plot	Populations of Interest
1	CD45 vs SSC	lymphocytes
2	CD3 vs SSC	CD3− cells CD3+ cells
3	CD8 vs CD4	CD3+ cell subsets: CD4−CD8+ cells CD4+CD8− cells CD4+CD8+ cells CD4−CD8− cells
4	CD16+CD56 vs CD19	CD3− cell subsets: (CD16+CD56)−CD19+ cells (CD16+CD56)+CD19− cells

Table 6 BD Multitest™ 6-color TBNK reagent without BD Trucount™ tubes: CD3/CD16+CD56/CD45/CD4/CD19/CD8

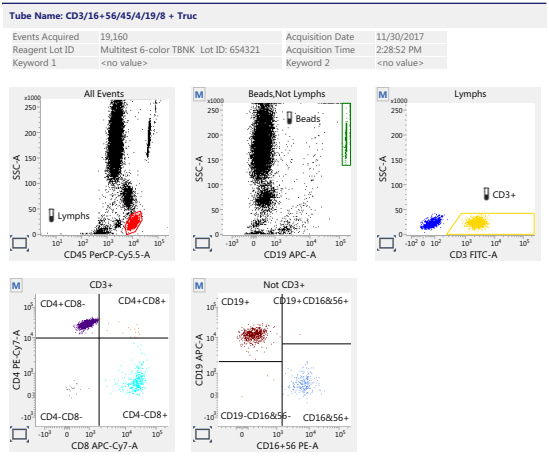
No.	Plot	Populations of Interest
Visual Representation of Hierarchy		



BD FACSLyric™ flow cytometer

A hematologically normal adult sample stained with BD Multitest™ 6-color TBNK reagent in a BD Trucount™ tube was acquired on a BD FACSLyric™ flow cytometer. See Figure 1 for representative data from a sample acquired using BD Trucount™ tubes. See the *BD FACSLyric™ Clinical Reference System*, which provides information on gating and troubleshooting.

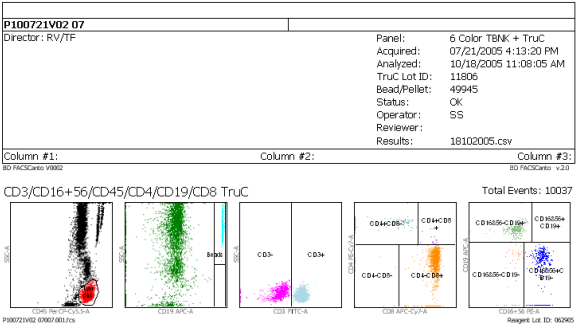
Figure 1 BD FACSLyric™ laboratory report showing data collected with BD Trucount™ tubes



BD FACSCanto™ flow cytometer

A hematologically normal adult sample stained with BD Multitest™ 6-color TBNK reagent in a BD Trucount™ tube was acquired on a BD FACSCanto™ flow cytometer. See Figure 2 for representative data from a sample acquired using BD Trucount™ tubes; see Figure 3 for representative data from a sample acquired without BD Trucount™ tubes. See the BD FACSCanto™ instrument IFU, which provides information on gating and troubleshooting.

Figure 2 BD FACSCanto™ laboratory report showing data collected with BD Trucount™ tubes



P100721V02 07

Director: RV/TF

Panel:	6 Color TBNK + TruC
Acquired:	07/21/2005 4:13:20 PM
Analyzed:	10/18/2005 11:08:05 AM
TruC Lot ID:	11806
Bead/Pellet:	49945
Status:	OK
Operator:	SS
Reviewer:	
Results:	18102005.csv

Column #1: Column #2: Column #3:

BD FACSCanto V0002 BD FACSCanto v.2.0

CD3/CD16+56/CD45/CD4/CD19/CD8 TruC

Total Events: 10037

Parameter	Percent	Value/AbsCnt
Lymph Events		3300
Bead Events		2144
CD3+	76.79	1180.60
CD3+CD8+	61.39	943.92
CD3+CD4+	14.58	224.10
CD3+CD4+CD8+	0.42	6.52
CD16+CD56+	12.30	189.16
CD19+	9.67	148.62
CD45+		1537.49
4/8 Ratio		0.24

QC Messages

% T-Sum is: 0.82
Lymphosum is: 98.76
4/8 ratio is: 0.24

Comments

Figure 3 BD FACSCanto™ laboratory report showing data collected without BD Trucount™ tubes

1010

003930

Director:

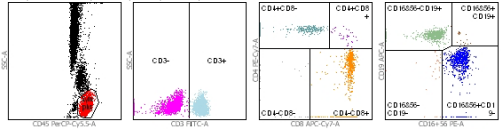
Panel:	6 Color TBNK
Acquired:	12/3/2008 3:20:06 PM
Analyzed:	12/3/2008 3:20:06 PM
Status:	OK
Operator:	PL
Reviewer:	
Results:	03122008.csv

WBC Count (x1000): Lymphs (%): Lymphs (x1000):

BD FACSCanto II V96300005 BD FACSCanto v.2.4

CD3/CD16+56/CD45/CD4/CD19/CD8

Total Events: 10045



003930003.001.fcs

Reagent Lot ID: 24771

Parameter	Percent	Value/AbsCnt
Lymph Events		4211
CD3+	62.48	0
CD3+CD8+	26.72	0
CD3+CD4+	35.55	0
CD3+CD4+CD8+	0.88	0
CD16+CD56+	18.93	0
CD19+	17.43	0
CD45+		0
4/8 Ratio		1.33

QC Messages

% T-Sum is: 0.21
Lymphosum is: 98.84
4/8 ratio is: 1.33

Comments

9. RESULTS

When using cytometer-specific BD software, results show positive cells as a percentage of lymphocytes. If BD Trucount™ tubes are used, or if hematology results are provided from another instrument or method,

the software also calculates the number of positive cells per microliter of blood (absolute count).

Calculating Absolute Counts

Single Platform Method. When BD Trucount™ tubes are used, the absolute number (cells/µL) of positive cells in the sample can be determined by comparing cellular events to bead events.

Absolute counts are calculated by cytometer-specific BD software using the following formula.

$$\frac{\#\text{events in cell population}}{\#\text{events in absolute count bead region}} \times \frac{\#\text{beads/test}^*}{\text{test volume}} = \text{cell population absolute count}$$

* This value is found on the BD Trucount tube foil pouch label and can vary from lot to lot.

Dual Platform Method. To have the software calculate absolute counts when using 12 × 75-mm polystyrene tubes (or equivalent) instead of BD Trucount™ tubes, enter either the absolute lymphocyte count, or the absolute white blood cell (WBC) count and the percentage of lymphocytes as determined by a hematology analyzer or other means. BD FACSCanto™ clinical software uses one of the following formulas to perform the calculation:

1. User provides absolute lymphocyte count per µL.

$$\frac{\#\text{events in cell population} \times \text{lymphocyte count per µL}}{\#\text{ lymphocytes acquired}} = \text{cell population absolute count}$$

2. User provides WBC count per µL and percentage of lymphocytes.

$$\frac{\#\text{events in cell population} \times \text{WBC count x (\% lymphocytes/100)}}{\#\text{ lymphocytes acquired}} = \text{cell population absolute count}$$

NOTE The accuracy of the absolute counts determined with the Dual Platform Method depends upon the accuracy of the values entered into the software.

10. LIMITATIONS

- Laboratories must establish their own normal reference intervals for the BD Multitest™ 6-color TBNK reagent parameters that can be affected by gender of patient, age of patient, and preparative technique. Race of patient[54] and individual variations of epitope expression[55] can also have an effect, although sufficient data is not available to establish this. Age, gender, clinical characteristics, and race of patients should be known when a reference interval is determined.[56] Reference intervals provided are for information only.
- The BD Multitest™ 6-color TBNK reagent is not intended for screening samples for the presence of leukemic cells or for use in phenotyping samples from leukemia patients.
- Absolute counts are not comparable between laboratories using different manufacturers' equipment.
- The BD Multitest™ 6-color TBNK reagent has not been validated by BD Biosciences for use with heparin or acid citrate dextrose (ACD) liquid anticoagulants in determining absolute counts with BD Trucount™ tubes.
- Corticosteroids may influence lymphocyte counts.

11. EXPECTED VALUES

Reference Intervals

Reference intervals for BD Multitest™ 6-color TBNK with and without BD Trucount™ Tubes were determined in a study using the BD FACSLyric™ flow cytometer.[56] Subjects were hematologically normal adults between the ages of 19 and 80 years. Similar studies carried out at different times using other BD flow cytometers used samples from different populations, which can contribute to differences in the reference intervals between studies.[57–60] See the first limitation in the preceding section for more information about reference intervals.

Table 7 Representative reference intervals for the BD Multitest™ 6-color TBNK reagent using BD Trucount™ tubes

Lymphocyte Subset	N[a]	Unit	Mean (%)	95% Range
$CD3^+$	134	%	71.95	57.52–83.11
		cells/µL	1,556.40	856–2,669
$CD3^+CD4^+$	134	%	46.63	31.45–62.38
		cells/µL	1,002.75	491–1,734
$CD3^+CD8^+$	134	%	23.05	9.55–38.32
		cells/µL	505.96	162–1,074
$CD3^-CD19^+$	134	%	13.84	5.89–24.21
		cells/µL	298.27	73–562
$CD3^-(CD16+CD56)^+$	134	%	13.56	5.17–30.36
		cells/µL	287.90	108–680

a. N = number of samples

12. PERFORMANCE CHARACTERISTICS

BD FACSLyric™ Flow Cytometer

Method comparison (BD FACSLyric™ flow cytometer)

Lymphocyte subset percentages and absolute counts were enumerated with the BD Multitest™ 6-color TBNK reagent in BD Trucount tubes and analyzed on the BD FACSLyric™ flow cytometer using BD FACSuite™ Clinical software version 1.0. The results were compared with results from the reagents analyzed on the BD FACSCanto™ II flow cytometer using BD FACSCanto™ clinical software version 2.4 or later.

Whole blood samples were collected at random at five clinical study sites. Method comparison statistics are reported for all cell subsets. See Table 8.

Table 8 Method comparison statistics for lymphocyte subsets (BD FACSLyric™ flow cytometer)

Lymphocyte Subset	N	Unit	R^2	Slope	Intercept	Range
$CD3^+$	297	%	0.99	1.00	1.23	0.75–94.18
		cells/µL	0.99	1.04	–1.26	4–6,422
$CD3^+CD4^+$	297	%	1.00	1.00	0.35	0.12–81.18
		cells/µL	0.99	1.04	–0.82	0–2,823
$CD3^+CD8^+$	297	%	1.00	1.01	0.12	0.20–82.46
		cells/µL	0.99	1.03	0.18	1–5,638
$CD3^-CD19^+$	297	%	0.99	1.02	–0.28	0.00–84.80
		cells/µL	0.99	1.01	–0.20	0–2,060
$CD3^-(CD16+CD56)^+$	297	%	0.99	1.00	–0.76	0.18–91.87
		cells/µL	0.98	0.94	–0.22	0–1,528

Within-site precision (BD FACSLyric™ flow cytometer)

A 21-day study was conducted at one site, BD Biosciences, to assess within-site precision. Estimates of precision for the enumeration of lymphocyte subset percentages and absolute counts were determined across four BD FACSLyric™ flow cytometers and four operators by acquiring two concentrations of analyte, CD-Chex Plus® CD4 Low control and CD-Chex Plus® control, stained in duplicate using four lots of the BD Multitest™ 6-color TBNK reagent. Two separate runs were analyzed during each of the 21 tested days for a total of 42 runs.

The following tables present standard deviations (SDs) and coefficients of variation (CVs) for within-site precision and repeatability of lymphocyte subset percentages and absolute counts, respectively.

Table 9 Within-site precision of lymphocyte subset percentages in low analyte concentration (CDL[a]) (BD FACSLyric™ flow cytometer)

Lymphocyte Subset	Mean (%)	SD (Repeatability)	SD (Within-site precision)
$CD3^+$	57.56	1.07	1.09
$CD3^+CD4^+$	10.91	0.69	0.73
$CD3^+CD8^+$	39.43	1.02	1.05
$CD3^-CD19^+$	21.92	0.86	0.87
$CD3^-(CD16+CD56)^+$	19.36	0.87	0.88

a. CDL = CD-Chex Plus CD4 Low control

Table 10 Within-site precision of lymphocyte subset percentages in normal analyte concentration (CDN[a]) (BD FACSLyric™ flow cytometer)

Lymphocyte Subset	Mean (%)	SD (Repeatability)	SD (Within-site precision)
$CD3^+$	77.01	0.88	0.90
$CD3^+CD4^+$	48.15	1.52	1.83
$CD3^+CD8^+$	21.78	0.78	0.80
$CD3^-CD19^+$	12.08	0.60	0.60
$CD3^-(CD16+CD56)^+$	10.35	0.62	0.63

a. CDN = CD-Chex Plus control

Table 11 Within-site precision of lymphocyte subset absolute counts in low analyte concentration (CDL) (BD FACSLyric™ flow cytometer)

Lymphocyte Subset	Mean (cells/µL)	%CV (Repeatability)	%CV (Within-site precision)
$CD3^+$	866.44	5.23	5.58
$CD3^+CD4^+$	164.18	8.03	8.44
$CD3^+CD8^+$	593.38	5.42	5.87
$CD3^-CD19^+$	329.94	6.32	6.59
$CD3^-(CD16+CD56)^+$	291.36	6.82	7.00

Table 12 Within-site precision of lymphocyte subset absolute counts in normal analyte concentration (CDN) (BD FACSLyric™ flow cytometer)

Lymphocyte Subset	Mean (cells/µL)	%CV (Repeatability)	%CV (Within-site precision)
$CD3^+$	1,728.97	4.40	4.47
$CD3^+CD4^+$	1,081.02	5.17	5.67
$CD3^+CD8^+$	488.98	5.91	6.00
$CD3^-CD19^+$	271.23	7.19	7.29
$CD3^-(CD16+CD56)^+$	232.54	7.86	7.95

Inter-site reproducibility (BD FACSLyric™ flow cytometer)

A study was conducted to assess inter-site reproducibility. A single lot of each process control, CD-Chex Plus® CD4 Low control and CD-Chex Plus® control, was provided to each of four clinical sites. The control samples were stained using the BD Multitest™ 6-color TBNK reagent. Two separate runs were analyzed during each of five non-consecutive tested days for a total of ten runs.

The following tables present standard deviations (SDs) and coefficients of variation (CVs) for reproducibility (total precision) of lymphocyte subset percentages and absolute counts, respectively.

Table 13 Inter-site reproducibility of lymphocyte subset percentages in low analyte concentration (CDL) (BD FACSLyric™ flow cytometer)

Lymphocyte subset	Mean (%)	SD
$CD3^+$	57.46	1.14
$CD3^+CD4^+$	12.19	0.73
$CD3^+CD8^+$	40.47	1.05
$CD3^-CD19^+$	21.97	0.84
$CD3^-(CD16+CD56)^+$	19.31	0.84

Table 14 Inter-site reproducibility of lymphocyte subset percentages in normal analyte concentration (CDN) (BD FACSLyric™ flow cytometer)

Lymphocyte subset	Mean (%)	SD
$CD3^+$	76.98	1.02
$CD3^+CD4^+$	51.91	1.09
$CD3^+CD8^+$	24.64	1.59
$CD3^-CD19^+$	12.21	0.65
$CD3^-(CD16+CD56)^+$	10.20	0.75

Table 15 Inter-site reproducibility of lymphocyte subset absolute counts in low analyte concentration (CDL) (BD FACSLyric™ flow cytometer)

Lymphocyte subset	Mean (cells/µL)	%CV
$CD3^+$	875.81	4.86

Table 15 Inter-site reproducibility of lymphocyte subset absolute counts in low analyte concentration (CDL) (BD FACSLyric™ flow cytometer)

Lymphocyte subset	Mean (cells/µL)	%CV
$CD3^+CD4^+$	185.79	7.28
$CD3^+CD8^+$	616.88	5.14
$CD3^-CD19^+$	335.03	6.37
$CD3^-(CD16+CD56)^+$	294.49	6.82

Table 16 Inter-site reproducibility of lymphocyte subset absolute counts in normal analyte concentration (CDN) (BD FACSLyric™ flow cytometer)

Lymphocyte subset	Mean (cells/µL)	%CV
$CD3^+$	1,742.38	5.48
$CD3^+CD4^+$	1,175.19	5.95
$CD3^+CD8^+$	557.86	8.32
$CD3^-CD19^+$	276.52	7.72
$CD3^-(CD16+CD56)^+$	231.03	9.48

Stability (BD FACSLyric™ flow cytometer)

A study was conducted to assess blood sample stability and stained sample stability using the BD Multitest™ 6-color TBNK reagent in BD Trucount™ tubes. The study measured:

- Changes associated with the storage of whole blood before staining
- Changes as a result of time between staining and data acquisition
- The combined effect of the two

Whole blood samples were tested up to 27 hours post draw and stained samples were tested up to 8 hours post stain. All samples were

maintained at room temperature (20°C–25°C) before staining or acquisition.

Based on the results of this study, we recommend staining samples within 24 hours of draw and analyzing samples within 6 hours of staining.

Linearity (BD FACSLyric™ flow cytometer)

Linearity was assessed for the BD FACSLyric™ flow cytometer using triplicate measurements of 11 equally spaced concentrations of WBCs. Lymphocyte subsets were observed to be linear across the following ranges. See Table 17.

Table 17 Linear ranges of lymphocyte subsets (BD FACSLyric™ flow cytometer)

Lymphocyte Subset	Range (cells/µL)
$CD3^+$	2–5,149
$CD3^+CD4^+$	5–2,964
$CD3^+CD8^+$	7–3,578
$CD3^-CD19^+$	0–968
$CD3^-(CD16+CD56)^+$	1–1,522

BD FACSCanto™ II Flow Cytometer

Method comparison with BD Trucount™ tubes (BD FACSCanto™ II flow cytometer)

Lymphocyte subset percentages and absolute counts were enumerated with BD Multitest™ 6-color TBNK reagent in BD Trucount™ tubes and analyzed on the BD FACSCanto™ II flow cytometer using BD FACSCanto™ clinical software v2.1. The results were compared with results from the reagent analyzed on the BD FACSCanto™ flow cytometer using BD FACSCanto™ clinical software v2.0.

Whole blood samples were collected at random at two clinical laboratories. Method comparison statistics are reported in Table 18.

Table 18 Method comparison statistics for subset percentages and absolute counts (BD FACSCanto™ II vs BD FACSCanto™ flow cytometer)

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
$CD3^+$	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

Method comparison with vs without BD Trucount™ tubes (BD FACSCanto™ II flow cytometer)

Lymphocyte subset percentages were determined using BD Multitest™ 6-color TBNK reagent without BD Trucount™ tubes and analyzed on the BD FACSCanto™ II flow cytometer using BD FACSCanto™ clinical software v2.4. The results were compared with results from the same reagent with BD Trucount™ tubes and analyzed on the BD FACSCanto™ II flow cytometer using BD FACSCanto™ clinical software v2.2.

Whole blood samples were collected internally at BD Biosciences. Method comparison statistics are reported in Table 19.

Table 19 Method comparison statistics for subset percentages (BD FACSCanto™ clinical software v2.4 vs v2.2 with BD FACSCanto™ II flow cytometer)

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
$CD3^+$	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

Precision with BD Trucount™ tubes (BD FACSCanto™ II flow cytometer)

Estimates of precision were determined at one site, BD Biosciences, using two specimens run in duplicate at two different levels of analyte concentration. Samples were run on three different instruments with three different operators (one operator and one instrument per day). Two separate runs were analyzed during each of the 21 days of testing for a total of 42 runs. Calibration with BD FACS™ 7-color setup beads was performed before each run for a total of 42 runs. One reagent lot and one calibrator lot were used for the duration of the study.

The following tables provide SDs and CVs for subset percentages and absolute counts for repeatability (same operator, method, equipment, time, and laboratory, as defined by Clinical and Laboratory Standards Institute [CLSI]) and within-device precision.

Table 20 Repeatability and within-device precision of subset percentages in low analyte concentration (CDL) (BD FACSCanto™ II flow cytometer)

Lymphocyte Subset (%)	Mean	SD Within Run	SD Within Device
$CD3^+CD4^+$	10.2	0.47	0.47

Table 20 Repeatability and within-device precision of subset percentages in low analyte concentration (CDL) (BD FACSCanto™ II flow cytometer)

Lymphocyte Subset (%)	Mean	SD Within Run	SD Within Device
$CD3^+CD8^+$	39.3	1.10	1.18
$CD3^+$	54.1	1.32	1.32
$CD3^-CD19^+$	26.0	1.07	1.09
$CD3^-(CD16+CD56)^+$	18.2	0.78	0.80

Table 21 Repeatability and within-device precision of subset percentages in normal analyte concentration (CDC) (BD FACSCanto™ II flow cytometer)

Lymphocyte Subset (%)	Mean	SD Within Run	SD Within Device
$CD3^+CD4^+$	46.3	0.76	0.80
$CD3^+CD8^+$	24.0	0.75	0.76
$CD3^+$	73.1	0.82	0.87
$CD3^-CD19^+$	15.3	0.57	0.60
$CD3^-(CD16+CD56)^+$	10.6	0.57	0.58

Table 22 Repeatability and within-device precision of absolute counts at low analyte concentration (CDL) (BD FACSCanto™ II flow cytometer)

Lymphocyte Subset (cells/µL)	Mean	%CV Within Run	%CV Within Device
$CD3^+CD4^+$	202.6	5.1	5.1
$CD3^+CD8^+$	780.1	4.9	5.1
$CD3^+$	1,074.5	4.2	4.3
$CD3^-CD19^+$	516.9	5.6	5.6
$CD3^-(CD16+CD56)^+$	361.1	5.4	5.6

Table 23 Repeatability and within-device precision of absolute counts at normal analyte concentration (CDC) (BD FACSCanto™ II flow cytometer)

Lymphocyte Subset (cells/µL)	Mean	%CV Within Run	%CV Within Device
$CD3^+CD4^+$	1,326.7	3.6	4.0
$CD3^+CD8^+$	686.8	4.5	4.5
$CD3^+$	2,091.5	3.3	3.6
$CD3^-CD19^+$	493.3	5.4	6.0
$CD3^-(CD16+CD56)^+$	303.7	5.9	6.3

Precision without BD Trucount™ tubes (BD FACSCanto™ II flow cytometer)

Estimates of precision were determined at one site, BD Biosciences, using two specimens run in duplicate at two different levels of analyte concentration. Samples were run on three different instruments with three different operators (one operator and one instrument per day). Two separate runs were analyzed during each of the 21 days of testing for a total of 42 runs. Calibration with BD FACS™ 7-color setup beads was performed before each run for a total of 42 runs. One reagent lot and one calibrator lot were used for the duration of the study.

The following tables provide SDs for repeatability and within-device precision of subset percentages.

Table 24 Repeatability and within-device precision of subset percentages in low analyte concentration (CDL) (BD FACSCanto™ II flow cytometer)

Lymphocyte Subset (%)	Mean	SD Within Run	SD Within Device
$CD3^+CD4^+$	11.0	0.56	0.77
$CD3^+CD8^+$	37.3	0.80	1.66
$CD3^+$	52.4	1.03	1.34

Table 24 Repeatability and within-device precision of subset percentages in low analyte concentration (CDL) (BD FACSCanto™ II flow cytometer)

Lymphocyte Subset (%)	Mean	SD Within Run	SD Within Device
$CD3^{-}CD19^{+}$	26.1	0.70	0.73
$CD3^{-}(CD16+CD56)^{+}$	19.5	0.72	0.84

Table 25 Repeatability and within-device precision of subset percentages in normal analyte concentration (CDC) (BD FACSCanto™ II flow cytometer)

Lymphocyte Subset (%)	Mean	SD Within Run	SD Within Device
$CD3^{+}CD4^{+}$	46.4	0.73	0.93
$CD3^{+}CD8^{+}$	23.9	0.58	1.12
$CD3^{+}$	72.8	0.81	0.90
$CD3^{-}CD19^{+}$	14.7	0.50	0.50
$CD3^{-}(CD16+CD56)^{+}$	11.4	0.64	0.66

Stability (BD FACSCanto™ II flow cytometer)

A stability study was conducted at two clinical laboratories to assess the stability of this BD Multitest™ reagent in a BD Trucount™ tube. The study measured:

- Changes associated with the storage of whole blood before staining
- Changes as a result of time between staining and data acquisition
- The combined effect of the two

Whole blood samples were tested up to 52 hours post draw and stained samples were tested up to 24 hours post stain. All samples were maintained at room temperature (20°C–25°C) before staining or acquisition.

Based on the results of this study, we recommend staining samples within 24 hours of draw and analyzing samples within 6 hours of staining.

Linearity (BD FACSCanto™ II flow cytometer)

Linearity of the 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.

Subset	Range (cells/µL)
CD4	1–4,494
CD8	2–2,922
CD3	4–7,382
CD19	0–863
CD16+CD56	0–435

BD FACSCanto™ Flow Cytometer

Method comparison with BD Trucount™ tubes (BD FACSCanto™ flow cytometer)

Lymphocyte subset percentages and absolute counts were enumerated with BD Multitest™ 6-color TBNK reagent in BD Trucount™ tubes and analyzed on the BD FACSCanto™ flow cytometer using BD FACSCanto™ clinical software v2.0. The results were compared with results from the BD Multitest™ IMK kit with BD Trucount™ tubes, which also were analyzed on the BD FACSCanto™ flow cytometer using BD FACSCanto™ clinical software v2.0.

Whole blood samples were collected at random at two clinical laboratories. Method comparison statistics are reported in Table 26.

Table 26 Regression analysis for subset percentages and absolute counts (BD Multitest™ 6-color TBNK reagent vs BD Multitest™ IMK kit reagent)

Lymphocyte Subset	N	Unit	R^2	Slope	Intercept	Range
$CD3^+CD4^+$	117	cells/µL	0.995	0.965	6.0	4–1,593
		%	0.998	1.0	0.0423	1–67
$CD3^+CD8^+$	117	cells/µL	0.994	0.956	7.01	51–2,146
		%	0.996	0.983	0.00592	11–83
Total $CD3^+$	117	cells/µL	0.995	0.968	13.5	107–3,403
		%	0.996	0.985	0.895	34–88
$CD3^-CD19^+$	117	cells/µL	0.992	0.973	6.97	1–1,207
		%	0.993	0.999	0.33	0–36
$CD3^-(CD16+CD56)^+$	117	cells/µL	0.99	0.98	–0.291	7–918
		%	0.992	0.985	0.0603	2–51

Method comparison with vs without BD Trucount™ tubes (BD FACSCanto™ flow cytometer)

Lymphocyte subset percentages were determined using BD Multitest™ 6-color TBNK reagent without BD Trucount™ tubes and analyzed on the BD FACSCanto™ II flow cytometer using BD FACSCanto™ clinical software v2.4. The results were compared with results from the same reagent with BD Trucount™ tubes and analyzed on the BD FACSCanto™ II flow cytometer using BD FACSCanto™ clinical software v2.2.

Whole blood samples were collected internally at BD Biosciences. Method comparison statistics are reported in Table 27.

Table 27 Method comparison statistics for subset percentages (BD FACSCanto™ clinical software v2.4 vs v2.2 with BD FACSCanto™ flow cytometer)

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
$CD3^+$	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

Precision with BD Trucount™ tubes (BD FACSCanto™ flow cytometer)

Estimates of precision were determined at one site, BD Biosciences, using two specimens run in duplicate at two different levels of analyte concentration. Samples were run on three different instruments with three different operators (one operator and one instrument per day). Two separate runs were analyzed during each of the 21 tested days for a total of 42 runs. Calibration with BD FACS™ 7-color setup beads was performed prior to each run for a total of 42 runs. One reagent lot and one calibrator lot were used for the duration of the study.

The following tables provide SDs and CVs for repeatability and within-device precision of subset percentages and absolute counts, respectively.

Table 28 Repeatability and within-device precision of subset percentages at low analyte concentration (CDL[a]) (BD FACSCanto™ flow cytometer)

Lymphocyte Subset (%)	Mean	SD Within Run	SD Within Device
$CD3^+CD4^+$	9.3	0.64	0.69
$CD3^+CD8^+$	41.9	1.07	1.29

Table 28 Repeatability and within-device precision of subset percentages at low analyte concentration (CDL[a]) (BD FACSCanto™ flow cytometer)

Lymphocyte Subset (%)	Mean	SD Within Run	SD Within Device
$CD3^+$	55.2	1.17	1.23
$CD3^-CD19^+$	25.0	0.89	0.89
$CD3^-(CD16+CD56)^+$	18.7	0.90	0.96

a. CDL = CD-Chex Plus CD4 Low control

Table 29 Repeatability and within-device precision of subset percentages at normal analyte concentration (CDC[a]) (BD FACSCanto™ flow cytometer)

Lymphocyte Subset (%)	Mean	SD Within Run	SD Within Device
$CD3^+CD4^+$	47.5	0.95	1.23
$CD3^+CD8^+$	24.5	0.65	0.81
$CD3^+$	74.3	0.86	0.90
$CD3^-CD19^+$	14.4	0.62	0.62
$CD3^-(CD16+CD56)^+$	10.6	0.61	0.62

a. CDL = CD-Chex Plus control

Table 30 Repeatability and within-device precision of absolute counts at low analyte concentration (CDL) (BD FACSCanto™ flow cytometer)

Lymphocyte Subset (cells/µL)	Mean	%CV Within Run	%CV Within Device
$CD3^+CD4^+$	175.9	7.6	8.0
$CD3^+CD8^+$	190.3	4.1	5.0
$CD3^+$	1,039.7	4.0	4.4
$CD3^-CD19^+$	471.4	5.7	6.0

Table 30 Repeatability and within-device precision of absolute counts at low analyte concentration (CDL) (BD FACSCanto™ flow cytometer)

Lymphocyte Subset (cells/µL)	Mean	%CV Within Run	%CV Within Device
$CD3^{-}(CD16+CD56)^{+}$	353.3	7.0	8.0

Table 31 Repeatability and within-device precision of absolute counts at normal analyte concentration (CDC) (BD FACSCanto™ flow cytometer)

Lymphocyte Subset (cells/µL)	Mean	%CV Within Run	%CV Within Device
$CD3^{+}CD4^{+}$	1,299.4	4.7	4.8
$CD3^{+}CD8^{+}$	671.0	4.7	5.4
$CD3^{+}$	2,030.5	4.2	4.2
$CD3^{-}CD19^{+}$	393.6	5.3	5.7
$CD3^{-}(CD16+CD56)^{+}$	289.9	7.9	7.9

Precision without BD Trucount™ tubes (BD FACSCanto™ flow cytometer)

Estimates of precision were determined at one site, BD Biosciences, using two specimens run in duplicate at two different levels of analyte concentration. Samples were run on three different instruments with three different operators (one operator and one instrument per day). Two separate runs were analyzed during each of the 21 tested days for a total of 42 runs. Calibration with BD FACS™ 7-color setup beads was performed prior to each run for a total of 42 runs. One reagent lot and one calibrator lot were used for the duration of the study.

The following tables provide SDs for repeatability and within-device precision of subset percentages.

Table 32 Repeatability and within-device precision of subset percentages at low analyte concentration (CDL) (BD FACSCanto™ flow cytometer)

Lymphocyte Subset (%)	Mean	SD Within Run	SD Within Device
$CD3^+CD4^+$	11.2	0.57	0.76
$CD3^+CD8^+$	37.2	0.96	1.56
$CD3^+$	52.4	1.28	1.42
$CD3^-CD19^+$	25.9	0.72	0.84
$CD3^-(CD16+CD56)^+$	19.4	0.74	0.90

Table 33 Repeatability and within-device precision of subset percentages at normal analyte concentration (CDC) (BD FACSCanto™ flow cytometer)

Lymphocyte Subset (%)	Mean	SD Within Run	SD Within Device
$CD3^+CD4^+$	46.5	0.86	1.05
$CD3^+CD8^+$	23.7	0.64	1.16
$CD3^+$	72.7	0.77	0.96
$CD3^-CD19^+$	14.8	0.51	0.60
$CD3^-(CD16+CD56)^+$	11.4	0.58	0.68

Stability (BD FACSCanto™ flow cytometer)

A stability study was conducted at two clinical laboratories to assess the stability of this BD Multitest™ reagent in a BD Trucount™ tube. The study measured:

- Changes associated with the storage of whole blood before staining
- Changes as a result of time between staining and data acquisition
- The combined effect of the two

Whole blood samples were tested up to 52 hours post draw and stained samples were tested up to 24 hours post stain. All samples were maintained at room temperature (20°C–25°C) before staining or acquisition.

Based on the results of this study, we recommend staining samples within 24 hours of draw and analyzing samples within 6 hours of staining.

Linearity (BD FACSCanto™ flow cytometer)

Linearity of the BD Multitest™ 6-color TBNK assay using BD Trucount™ tubes was assessed using triplicate measurements of nine concentrations of CD4+ T lymphocytes on the BD FACSCanto™ system. Results were observed to be linear across the following range.

Subset	Range (cells/µL)
CD4	4–2,234

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

All subset results were observed to be linear within these ranges.

13. EVALUATION IN COVID-19 PATIENTS

Clinical Performance

Clinical data was collected as part of an observational, retrospective study of PCR-confirmed COVID-19 patients admitted to hospital in Rome, Italy. Patients had CD4 and CD8 T cell absolute counts performed on hospital admission using the BD Multitest™ 6 color TBNK reagent with BD Trucount™ tubes run on the BD FACSLyric™ flow cytometer as part of standard care and were followed until death or discharge. The risk of intubation with mechanical ventilation (IMV), as well as mortality, were calculated for patients above and below the pre-established cutoffs of 250 CD4 T cells/µL and 100 CD8 T cells/µL. There were 141 patients included in the IMV analysis (patients who died prior to IMV were excluded) and 160 patients included in the mortality analysis. The absolute and relative risk of IMV and mortality are tabulated below with 95% confidence intervals, along with Kaplan-Meier analysis of time to event, reaching statistical significance (p-value<0.05) in all cases.

Data on risk of IMV

Table 34 CD4 T cell absolute count at hospital admission versus IMV: Contingency table with absolute and relative risk

	No IMV	IMV	Total	Abs Risk[a]	Abs Risk CI[b]	p-value	RR[c]	RR CI
CD4≥250	110	10	120	8.3%	4.1% 14.8%	<0.001	5.14	2.38 11.13
CD4<250	12	9	21	42.9%	21.8% 66.0%			
Total	122	19	141	13.5%				

a. Absolute risk
b. Confidence interval
c. Relative risk

Table 35 CD8 T cell absolute count at hospital admission versus IMV: Contingency table with absolute and relative risk

	No IMV	IMV	Total	Abs Risk	Abs Risk CI	p-value	RR	RR CI
CD8≥100	113	10	123	8.1%	4.0% 14.4%	<0.001	6.15	2.9 13.05
CD8<100	9	9	18	50.0%	26.0% 74.0%			
Total	122	19	141	13.5				

Table 36 CD4 and CD8 T cell absolute counts at hospital admission versus IMV: Contingency table with absolute and relative risk

	No IMV	IMV	Total	Abs Risk	Abs Risk CI	p-value	RR	RR CI
CD4≥250 or CD8≥100	118	13	131	9.9%	5.4% 16.4%	<0.001	6.05	2.94 12.45
CD4<250 and CD8<100	4	6	10	60.0%	26.2% 87.8%			
Total	122	19	141	13.5%				

Table 37 Combinations of CD4 and CD8 T cell absolute counts below and above cut-offs at hospital admission versus IMV

	No IMV	IMV	Total
CD4≥250 and CD8≥100	105 (93.8%)	7 (6.2%)	112
CD4<250 and CD8≥100	8 (72.7%)	3 (27.3%)	11
CD4≥250 and CD8<100	5 (62.5%)	3 (37.5%)	8
CD4<250 and CD8<100	4 (40.0%)	6 (60.0%)	10

Figure 4 Kaplan-Meier curves for time to IMV for patients above or below the CD4 and CD8 cutoffs.

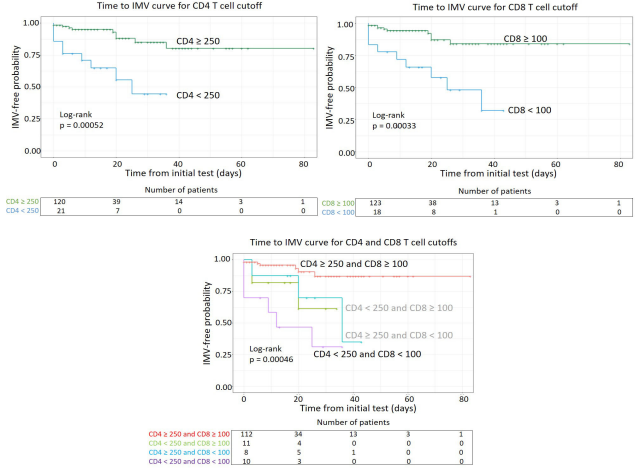


Table 38 Additional clinical evaluation statistics for IMV

	CD4 T cell cutoff		CD8 T cell cutoff		CD4 and CD8 T cell cutoffs[a]	
	Estimate	**CI**	**Estimate**	**CI**	**Estimate**	**CI**
Sensitivity	47.4%	24.4% 71.1%	47.4%	24.4% 71.1%	31.6%	12.6% 56.6%

Table 38 Additional clinical evaluation statistics for IMV

	CD4 T cell cutoff		CD8 T cell cutoff		CD4 and CD8 T cell cutoffs[a]	
Specificity	90.2%	83.4% 94.8%	92.6%	86.5% 96.6%	96.7%	91.8% 99.1%
PPV[b]	42.9%	21.8% 66.0%	50.0%	26.0% 74.0%	60.0%	26.2% 87.8%
NPV[c]	91.7%	85.2% 95.9%	91.9%	85.6% 96.0%	90.1%	83.6% 94.6%

a. Patients below both cutoffs are considered positive for risk of IMV
b. Positive predictive value
c. Negative predictive value

Data on risk of mortality

Table 39 CD4 T cell absolute count at hospital admission versus mortality: Contingency tables with absolute and relative risk

	Discharge	Death	Total	Abs Risk	Abs Risk CI	p-value	RR	RR CI
CD4≥250	114	17	131	13.0%	7.7% 20.0%	<0.001	4.52	2.64 7.74
CD4<250	12	17	29	58.6%	38.9% 76.5%			
Total	126	34	160	21.2%				

Table 40 CD8 T cell absolute count at hospital admission versus mortality: Contingency tables with absolute and relative risk

	Discharge	Death	Total	Abs Risk	Abs Risk CI	p-value	RR	RR CI
CD8≥100	116	18	134	13.4%	8.2% 20.4%	<0.001	4.58	2.71 7.76
CD8<100	10	16	26	61.5%	40.6% 79.8%			
Total	126	34	160	21.2%				

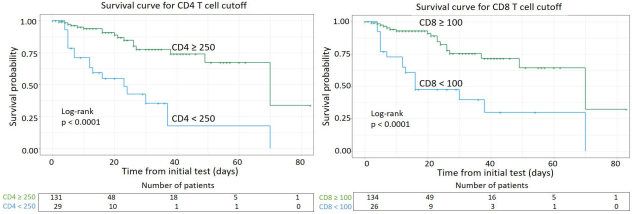
Table 41 CD4 and CD8 T cell absolute counts at hospital admission versus mortality: Contingency table with absolute and relative risk

	Discharge	Death	Total	Abs Risk	Abs Risk CI	p-value	RR	RR CI
CD4≥250 or CD8≥100	122	23	145	15.9%	10.3% 22.8%	<0.001	4.62	2.85 7.5
CD4<250 and CD8<100	4	11	15	73.3%	44.9% 92.2%			
Total	126	34	160	21.2%				

Table 42 Combinations of CD4 and CD8 T cell counts below and above cut-offs at hospital admission versus mortality

	Discharge	Death	Total
CD4≥250 and CD8≥100	108 (90.0%)	12 (10.0%)	120
CD4<250 and CD8≥100	8 (57.1%)	6 (42.9%)	14
CD4≥250 and CD8<100	6 (54.5%)	5 (45.5%)	11
CD4<250 and CD8<100	4 (26.7%)	11 (73.3%)	15

Figure 5 Kaplan-Meier curves for time to mortality for patients above or below the CD4 and CD8 cutoffs.



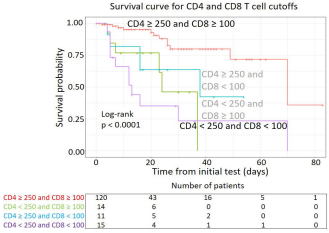


Table 43 Additional clinical evaluation statistics for mortality

	CD4 T cell cutoff		CD8 T cell cutoff		CD4 and CD8 T cell cutoffs[a]	
	Estimate	**CI**	**Estimate**	**CI**	**Estimate**	**CI**
Sensitivity	50.0%	32.4% 67.6%	47.1%	29.8% 64.9%	32.4%	17.4% 50.5%
Specificity	90.5%	84.0% 95.0%	92.1%	85.9% 96.1%	96.8%	92.1% 99.1%

Table 43 Additional clinical evaluation statistics for mortality

	CD4 T cell cutoff		CD8 T cell cutoff		CD4 and CD8 T cell cutoffs[a]	
PPV	58.6%	38.9% 76.5%	61.5%	40.6% 79.8%	73.3%	44.9% 92.2%
NPV	87.0%	80.0% 92.3%	86.6%	79.6% 91.8%	84.1%	77.2% 89.7%

a. Patients below both cutoffs are considered positive for risk of mortality.

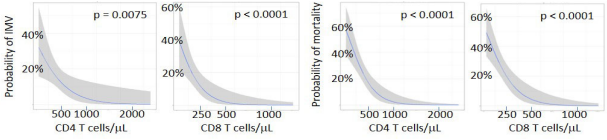
Additional Analysis

Univariate logistic regression identified a significant relationship between baseline CD4 or CD8 T cell counts and the probability of IMV or mortality for all patients in the study (Figure 6).

The odds ratios indicate odds of IMV and mortality increase by estimated 1.9-fold and 2.8-fold, respectively, for every 250 cell/μL decrease in CD4 T cell count; and by estimated 2.0-fold and 1.9 fold, respectively, for every 100 cell/μL decrease in CD8 T cell count.

Logistic regression and ANOVA (ANalysis Of VAriance) also showed that CD4 and CD8 T cell counts, when adjusted for the effect of each other, were each significant predictors of probability of IMV and mortality ($p = 0.026$ and 0.0041, respectively, for IMV, and $p = 0.001$ and 0.001, respectively, for mortality).

Figure 6 Fitted Probability of IMV and mortality based on CD4 or CD8 count across all patient data. P-values from ANOVA.



Instrument Platforms

The clinical performance data for BD Multitest™ 6 color TBNK reagent with BD Trucount™ tubes on BD FACSLyric™ is also applicable to BD FACSCanto™ and BD FACSCanto™ II. The data demonstrating performance equivalence between BD FACSLyric™ and BD FACSCanto™ II is provided in the Performance Characteristics section.

Cutoff Determination

Cutoffs for CD4 and CD8 T cell counts were established based on the analysis of published data[67-71] to identify confirmed COVID-19 patients with poor outcomes (ICU admission, mortality, and by extension IMV; 275 to 938 patients total) by maximizing Youden's index and corroborating the results with other reports not used in the analysis.

Results and Interpretation

Based on available clinical data, PCR-confirmed COVID-19 patients who have CD4 T cell count < 250 cells/µL or CD8 T cell count < 100 cells/µL, or both CD4 and CD8 T cell count below their respective cutoffs, are at increased risk for intubation with mechanical ventilation, and increased risk of mortality, during hospitalization. CD4 and CD8 T cell counts should be used in conjunction with clinical findings and the results of other laboratory testing. CD4 and/or CD8 T cell counts alone are not indicative of the need for intubation with mechanical ventilation or impending mortality.

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