BD Tritest™ CD3/CD19/ CD45

For determining percentages and absolute counts of human T and B lymphocytes in erythrocyte-lysed whole blood

Tests	Catalog No.
50 Tests	340381
50 Tests with BD Trucount Tubes	340405

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IVD **Rx Only**

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1. INTENDED USE

BD Tritest™ CD3 fluorescein isothiocyanate (FITC)/CD19 phycoerythrin (PE)/CD45 peridinin chlorophyll protein (PerCP) is a three-color direct immunofluorescence reagent for use with a suitably equipped flow cytometer to identify and determine the percentages and absolute counts of mature human T ($CD3^+$) and B ($CD19^+$) lymphocytes in erythrocyte-lysed whole blood. When used with BD Trucount™ tubes, absolute counts of these populations can be enumerated from a single tube.

BD Tritest reagent and BD Trucount tubes can be used with the BD FACS™ Loader. The reagent can be used with or without an isotype control.

2. SUMMARY AND EXPLANATION

Human lymphocytes can be divided into three major populations based on their biologic function and cell-surface antigen expression: T lymphocytes, B lymphocytes, and NK lymphocytes.

Clinical Applications

Total T and B lymphocytes are used to characterize and monitor some forms of immunodeficiency[1–3] and autoimmune diseases.[4,5]

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 Tritest reagents employ fluorescence triggering, allowing direct fluorescence gating of the lymphocyte population[6–8] to reduce contamination of unlysed or nucleated red blood cells in the gate.

When BD Trucount tubes are used, a known 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 positive cells in the sample can be determined by comparing cellular events to bead events. If appropriate software such as BD Multiset™ software is used, absolute counts will be determined by the software. If manually performing data analysis using software such as BD CellQuest™ Pro software, simply divide the number of positive cellular events by the number of bead events, then multiply by the BD Trucount bead concentration.

4. REAGENT

Reagent Provided, Sufficient for 50 Tests

BD Tritest CD3/CD19/CD45 reagent is provided in 1 mL of buffered saline with bovine serum albumin and 0.1% sodium azide. It contains FITC-labeled CD3, clone SK7;[9–11] PE-labeled CD19, clone SJ25C1;[12] and PerCP-labeled CD45, clone 2D1 (HLe-1).[13]

CD3 identifies T lymphocytes and recognizes the epsilon chain of the CD3 antigen/T-cell antigen receptor (TCR) complex.[14] This complex is composed of at least six proteins witha range in molecular weight from 20–30 kilodaltons (kDa).[15] The antigen recognized by CD3 antibodies is noncovalently associated with either α/β or γ/δ TCR (70–90 kDa).[16]

CD19 identifies B lymphocytes and recognizes a 90-kDa antigen that is present on human B lymphocytes at all stages of maturation but is lost on plasma cells.[17] The CD19 antigen might be involved in activation and proliferation of B lymphocytes.[17]

CD45 identifies leucocytes and recognizes a 180- to 220-kDa human leucocyte antigen that is a member of the leucocyte common antigen (LCA) family.[18]

The CD3, CD19, and CD45 antibodies are composed of mouse γ_1 heavy chains and kappa light chains.

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

Concentration values are listed in the following table:

Reagent	Concentration (µg/mL)
CD3 FITC	2.0
CD19 PE	2.0
CD45 PerCP	6.25

Precautions

- For In Vitro Diagnostic Use.
- 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.

  WARNING All biological specimens and materials coming in contact with them are considered biohazards.

Handle as if capable of transmitting infection[19,20] 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.[21]

- BD FACS™ lysing solution is required and contains diethylene glycol and formaldehyde. Refer to the *BD FACS Lysing Solution* instructions for use (IFU) for warnings.
- If BD Trucount tubes are used, the addition of a precise volume of blood is critical to achieving the result. Pipettes must be calibrated to deliver exactly 50 µL of sample. An electronic pipette that operates in the reverse pipetting mode is available through BD. If this or a similar pipette is not used, perform the reverse pipetting technique (see Reverse Pipetting in Section 7 for a brief description). Refer to the pipette 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. Do not mix multiple lots of tubes in the same assay.
- 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.

Storage and Handling

- Store the reagent at 2°C–8°C. Do not use after the expiration date shown on the label.
- 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. Examine the desiccant each time you open the pouch. If the desiccant has turned from blue to lavender, discard the remaining tubes. Use tubes within 1 hour after removal from the foil pouch and do not use beyond the expiration date indicated on the packaging.

5. INSTRUMENT

The BD Tritest CD3/CD19/CD45 reagent and BD Trucount tubes are designed for use on flow cytometers equipped with appropriate computer hardware and software. We recommend the BD FACSCalibur™, BD FACSort™, or BD FACScan™ flow cytometer; however, results can be achieved using other platforms. The flow cytometer must be equipped with a 488-nm laser capable of detecting light scatter (forward and side) and three-color fluorescence with emission detectable in three ranges: 515–545 nm, 562–607 nm, and >650 nm. The instrument must be able to threshold or discriminate using the >650-nm channel. The BD FACS Loader can also be used with this product.

We recommend using BD Calibrite™ beads and BD FACSComp™ software, version 2.0 or later, for setting the photomultiplier tube (PMT) voltages, setting the fluorescence compensation, and checking instrument sensitivity before

use. For users of flow cytometers manufactured by companies other than BD, refer to the manufacturer's instructions for setting up three-color immunophenotyping.

BD has developed software applications such as BD Multiset that automatically calculate absolute counts when BD Trucount tubes are used. However, other software packages can be used for data acquisition and analysis and the absolute counts can be calculated manually.

6. SPECIMEN AND COLLECTION PREPARATION

Collect blood aseptically by venipuncture[22,23] into a sterile EDTA BD Vacutainer® blood collection tube (lavender top). BD Tritest CD3/CD19/CD45 reagent and BD Trucount tubes have been validated with both liquid and dry formulations of EDTA. 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 with BD Trucount beads.

Obtain a white blood cell (WBC) count and a differential white cell count from the same whole blood sample before staining to ensure that the WBC count is within the linear range (see Section 11, Performance Characteristics: Linearity) or to calculate absolute counts from percentages.

When determining absolute counts, anticoagulated blood stored at room temperature (20°C–25°C) must be stained within 6 hours of draw and then analyzed within 6 hours of staining. For percentages, blood samples held at room temperature can be stained within 24 hours of draw and then analyzed within 24 hours of staining.

Interfering Conditions

Do not use previously fixed and stored patient specimens. Whole blood samples refrigerated before staining can give aberrant results. Samples obtained from patients taking immunosuppressive drugs can yield poor resolution.[24] Blast cells can interfere with test results. Hemolyzed samples should be rejected.

7. PROCEDURE

Reagent Provided

- BD Tritest CD3/CD19/CD45 (Catalog No. 340381), or
- BD Tritest CD3/CD19/CD45 with BD Trucount tubes (Catalog No. 340405)

Reagents and Materials Required But Not Provided

- BD Calibrite 3 beads (Catalog No. 340486)
- BD FACS lysing solution (10X), 100 mL (Catalog No. 349202). Refer to the BD FACS *Lysing Solution* IFU for dilution instructions and warnings.
- Reagent-grade (distilled or deionized) water
- BD Vacutainer EDTA blood collection tubes or equivalent
- Falcon®* disposable 12 x 75-mm polystyrene test tubes or equivalent (if not using BD Trucount tubes)
- Vortex mixer

* Falcon is a registered trademark of Corning Incorporated.

- Micropipettor with tips
- Bulk dispenser or pipettor (450 µL) for dispensing BD FACS lysing solution
- BD FACSFlow™ sheath fluid (Catalog No. 342003) or equivalent

  CAUTION Use only BD FACSFlow sheath fluid diluent to dilute BD Calibrite beads.

- BD Trucount™ Controls (Catalog No. 340335), necessary if using BD Trucount tubes
- Lysable whole blood control (available commercially)

Staining the Cells

BD Tritest reagents can be used with or without an isotype control to assess the amount of nonspecific antibody binding. If you want to use a control, BD Tritest™ IgG_1/IgG_1/CD45 isotype control reagent (Catalog No. 340385) is available.

Lyse red blood cells following staining using diluted (1X) BD FACS lysing solution. Use care to protect the tubes from direct light. Perform the procedure at room temperature (20°C–25°C). See Precautions in Section 4 and Interfering Conditions in Section 6.

Reverse Pipetting

If BD Trucount tubes are used, the addition of a precise volume of blood is critical to achieving the result. If a BD electronic pipette or a similar pipette that delivers a precise volume of blood is not used, perform reverse pipetting. This technique takes advantage of two stops in a pipette.

- For normal pipetting, the button is depressed to the first stop. Sample is drawn up by releasing the button, then expelled by pressing to the first stop again.
- For reverse pipetting, the button is depressed to the second stop. When the button is released, excess sample is drawn up into the tip. A precise volume of sample is expelled by pressing the button to the first stop, leaving excess sample in the tip.

Staining

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

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

   NOTE Before use, 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.

2. Pipette 20 µL of BD Tritest CD3/CD19/CD45 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 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.

   If using a BD Trucount tube, accuracy is critical. Use the reverse pipetting technique to pipette sample onto the side of the tube just above the retainer.

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.

Flow Cytometry

If samples are not to be analyzed immediately after preparation, store them in the dark at room temperature (20°C–25°C).

Vortex the cells thoroughly (at low speed) to reduce aggregation before running them on the flow cytometer.[25] If using the BD FACS Loader, vortex tubes immediately before placing them into the loader racks. Acquire and analyze list-mode data using the appropriate software such as BD CellQuest Pro or BD Multiset. Before acquiring samples, adjust the threshold to minimize debris and ensure populations of interest are included.

Quality Control

Run a control sample daily from a normal adult subject or a commercially available whole blood control to optimize instrument settings and as a quality control check of the system.[23] The BD Tritest isotype control reagent is optional to set fluorescence markers for detecting the presence of nonspecific staining.

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

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.

See Figure 1, Figure 2, and Figure 3 for representative data from a hematologically normal adult sample stained with CD3/CD19/CD45 in a BD Trucount tube.

Figure 1 Ungated CD45 vs SSC dot plot (1 = CD45$^+$ lymphocytes)

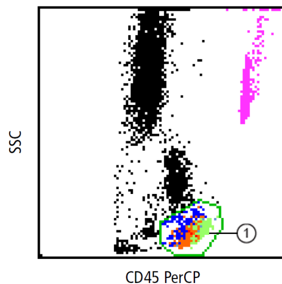


Figure 2 Ungated CD3 vs CD19 dot plot with BD Trucount bead gate (1 = Absolute count beads)

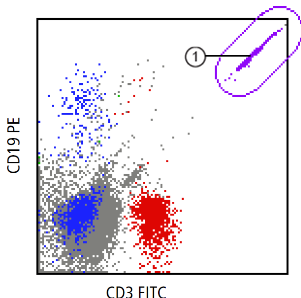
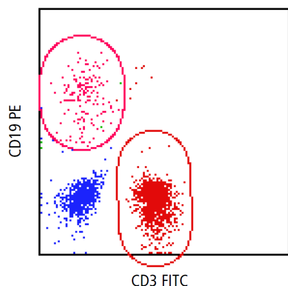


Figure 3 Lymphocyte-gated CD3 vs CD19 dot plot



8. RESULTS

Results are reported as the percentage of positive cells per lymphocyte population or as the number of positive cells per microliter of blood (absolute count).

Calculating Absolute Counts

During analysis, the absolute number (cells/µL) of positive cells in the sample can be determined by comparing cellular events to bead events. If BD Multiset software is used, absolute counts will be determined automatically. For manual data analysis using BD CellQuest Pro or other software, simply divide the number of positive cellular events by the number of bead events, then multiply by the BD Trucount bead concentration.

The absolute count of the cell population (A) can be obtained using the following equation:

$A = X/Y \times N/V$, where:

- X is number of positive cell events
- Y is the number of bead events
- N is the number of beads per test, which is found on the BD Trucount foil pouch and can vary from lot to lot
- V is the test volume

9. LIMITATIONS

- Laboratories must establish their own normal reference ranges for the BD Tritest CD3/CD19/CD45 reagent parameters that can be affected by sex of patient, age of patient, and preparative technique. Race of patient can also have an effect,[26] although sufficient data is not available to establish this. Age, sex, clinical characteristics, and race of patients should be known when a reference range is determined.[27] Reference ranges provided are for information only.
- BD Tritest CD3/CD19/CD45 reagent has not been validated for use with heparin or acid citrate dextrose (ACD) liquid anticoagulants in determining absolute counts with BD Trucount tubes.
- BD Tritest CD3/CD19/CD45 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 manufacturer's equipment.

10. EXPECTED VALUES

Reference Ranges

The reference ranges for CD3/CD19/CD45 shown in Table 1 were determined at BD Biosciences in San Jose, CA, and at four clinical centers: Cleveland Clinic Foundation, Cleveland, OH; Johns Hopkins Hospital, Baltimore, MD; Institute of Tropical Medicine, Antwerp, Belgium; and University of North Carolina Hospital, Chapel Hill, NC.

Subjects were hematologically normal adults between the ages of 18 and 65 years.

Table 1 Representative reference ranges for CD3/CD19/CD45 reagent parameters in hematologically normal adults

Subset	n	Mean	Lower 2.5 Percentile	Upper 97.5 Percentile
B lymphocytes (%)	516	14	6	25
Total T lymphocytes (%)	516	72	55	84
B lymphocytes (cells/µL)[a]	516	280	90	660
Total T lymphocytes (cells/µL)[a]	516	1,410	690	2,540

a. Absolute counts rounded to the nearest 10 cells/µL.

These reference ranges are pooled ranges. Refer to the first limitation for more information about reference ranges.

11. PERFORMANCE CHARACTERISTICS

Performance of the reagents was established by testing at BD Biosciences laboratories in San Jose, CA, at an external clinical center in the US or Europe, or at a combination of sites.

Accuracy

Lymphocyte subset percentage enumerations with BD Tritest CD3/CD19/CD45 were compared with results from BD Simultest™ CD3/CD19. Absolute counts were compared to BD Simultest results and lymphocyte counts obtained on a hematology analyzer.

Aliquots of the same blood sample from normal and abnormal donors were analyzed. Regression statistics reported in Table 2 indicate that the results are substantially equivalent.

Table 2 Regression analysis

Subset	n	Slope	Intercept	r	Range
B lymphocytes (%)	167	0.94	1.6%-positive	0.94	0–44
Total T lymphocytes (%)	167	0.91	5.7%-positive	0.96	24–94
B lymphocytes (cells/µL)	166	0.97	24 cells/µL	0.95	0–1,370[a]
Total T lymphocytes (cells/µL)	166	0.93	118 cells/µL	0.95	130–3,710[a]

a. Absolute counts rounded to the nearest 10 cells/µL.

Within-Specimen Reproducibility

Ten aliquots of specimens from three samples representing high, medium, and low CD4 counts were assessed. The %-positive results were as follows (SD = standard deviation):

- % CD19: mean = 14, pooled SD = 0.8
- % CD3: mean = 66, pooled SD = 1.1

The results for absolute counts are shown in Table 3.

Table 3 Within-specimen reproducibility for BD Tritest CD3/CD19/CD45 reagent

Subset	Level	Mean	SD	CV[a] (%)
B lymphocytes (cells/µL)	High	1,197	160	13
	Med	253	23	9
	Low	104	24	23

Table 3 Within-specimen reproducibility for BD Tritest CD3/CD19/CD45 reagent

Subset	Level	Mean	SD	CV[a] (%)
Total T lymphocytes (cells/µL)	High	3,202	308	10
	Med	1,922	157	8
	Low	672	122	18

a. CV = coefficient of variation

Stability

A stability study was conducted to assess the effect of time with respect to BD Tritest reagent performance specifications. The study measured: 1) changes associated with the storage of whole blood before staining, 2) changes as a result of time between staining and data acquisition, and 3) the combined effect of the two.

Based on the results of this study, we recommend staining samples within 6 hours of draw and analyzing within 6 hours of staining for absolute counts; or staining samples within 24 hours of draw and analyzing within 24 hours of staining for percentages.

Cross-Reactivity

CD3 and CD19 have no known cross-reactivity to nonlymphocyte-formed elements in blood; however, this CD19 clone has been observed to react with follicular dendritic cells in germinal centers of lymphoid tissue by histochemical staining.[28]

Linearity

The linearity was assessed by testing within a WBC concentration of 2.5 x 10^3 to 31 x 10^3 WBC/µL and a lymphocyte concentration of 2.0 x 10^2 to 16.7 x 10^3 lymphocytes/µL. Results were observed to be linear within the $CD19^+$ range (52 to 2.4 x 10^3 cells/µL) and the $CD3^+$ range (125 to 11.3 x 10^3 cells/µL).

WARRANTY

Unless otherwise indicated in any applicable BD general conditions of sale for non-US customers, the following warranty applies to the purchase of these products.

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