BECTON DICKINSON

MultiTEST CD3 FITC/ CD8 PE/CD45 PerCP/ CD4 APC Reagent

50 Tests per Vial–**Catalog No. 340499**
50 Tests per Vial with TruCOUNT Tubes–
Catalog No. 340491

For determining percentages and absolute counts of human helper/inducer and suppressor/cytotoxic T lymphocytes in erythrocyte-lysed whole blood.

For In Vitro Diagnostic Use.
Pour diagnostic in vitro. Enr. n°:S64202
Zur in vitro Diagnostik.
Per uso diagnostico in vitro.
Para uso diagnóstico in vitro.

4/01 23-3600-02

1. INTENDED USE

BD MultiTEST™ CD3 fluorescein isothiocyanate (FITC)/CD8 phycoerythrin* (PE)/ CD45 peridinin chlorophyll protein† (PerCP)/ CD4 allophycocyanin* (APC) is a four-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 lymphocytes ($CD3^+$), suppressor/cytotoxic ($CD3^+CD8^+$) T-lymphocyte subsets, and helper/inducer ($CD3^+CD4^+$) T-lymphocyte subsets in erythrocyte-lysed whole blood. When used with TruCOUNT™ Tubes, absolute counts of these populations can be enumerated from a single tube.

BD MultiTEST reagent and TruCOUNT Tubes can be used with the FACS Loader.

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 natural killer (NK) lymphocytes.

Clinical Applications

Suppressor/cytotoxic lymphocytes are a subset of T lymphocytes ($CD3^+$) that are $CD8^+$. Helper/inducer lymphocytes are a subset of T lymphocytes ($CD3^+$) that are $CD4^+$. $CD3^+CD8^+$ and $CD3^+CD4^+$ percentages or counts are used to characterize and monitor some forms of immunodeficiency[1–3] and autoimmune diseases.[4,5]

Determining percentages or counts of helper/ inducer T lymphocytes can be useful in monitoring human immunodeficiency virus (HIV)-infected individuals.[6] Individuals with HIV typically exhibit a steady decrease of helper/inducer T lymphocyte counts as the infection progresses.[7]

The percentage of suppressor/cytotoxic lymphocytes lies outside the normal reference range in some autoimmune diseases.[8] The relative percentage of the $CD8^+$ subset is elevated in many patients with congenital or acquired immune deficiencies such as severe combined immunodeficiency (SCID)[1] or acquired immune deficiency syndrome (AIDS).[6]

The Centers for Disease Control (CDC) recommends using reagent combinations containing CD3 antibodies for determining the percentage of T-lymphocyte subsets in HIV-infected subjects.[9] The MultiTEST CD3/CD8/CD45/CD4 reagent allows helper/ inducer T lymphocytes to be identified and enumerated separately from contaminating $CD3^-CD4^+$ monocytes.[10–12]

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* US Patent No. 4,520,110; European Patent No. 76,695; and Canadian Patent No. 1,179,942
† US Patent No. 4,876,190

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. MultiTEST reagents employ fluorescence triggering, allowing direct fluorescence gating of the lymphocyte population[10–12] to reduce contamination of unlysed or nucleated red blood cells in the gate.

When TruCOUNT Tubes are used, a known volume of sample is stained directly in a 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 MultiSET™ is used, absolute counts are determined by the software. If manually performing data analysis using software such as CellQuest™, simply divide the number of positive cellular events by the number of bead events, then multiply by the TruCOUNT bead concentration.

4. REAGENT

Reagent Provided, Sufficient for 50 Tests

BD MultiTEST CD3/CD8/CD45/CD4 reagent is provided in 1 mL of buffered saline with 0.1% sodium azide. It contains FITC-labeled CD3, clone SK7;[13–15] PE-labeled CD8, clone SK1;[16,17] PerCP-labeled CD45, clone 2D1 (HLe-1);[18] and APC-labeled CD4, clone SK3.[16,17,19]

CD3 identifies T lymphocytes and recognizes the epsilon chain of the CD3 antigen/T-cell antigen receptor (TCR) complex.[20] This complex is composed of at least six proteins that range in molecular weight from 20 to 30 kilodaltons (kDa).[21] The antigen recognized by CD3 antibodies is noncovalently associated with either α/β or γ/δ TCR (70 to 90 kDa).[22]

CD8 identifies suppressor/cytotoxic T lymphocytes and recognizes an antigen expressed on the 32-kDa α subunit of a disulfide-linked bimolecular complex.[23] The cytoplasmic domain of the α subunit of the CD8 antigen is associated with the protein tyrosine kinase $p56^{lck}$.[24] The CD8 molecule interacts with class I major histocompatibility complex (MHC) molecules resulting in increased adhesion between the $CD8^+$ T lymphocytes and the target cells.[25–27] Binding of the CD8 molecule to class I MHC molecules enhances the activation of resting T lymphocytes.[25–27]

CD45 identifies leucocytes and recognizes a 180- to 220-kDa human leucocyte antigen that is a member of the leucocyte common antigen (LCA) family.[28]

CD4 identifies helper/inducer T lymphocytes and recognizes the CD4 antigen, Mr 59 kDa,[29] which interacts with class II molecules of the major histocompatibility complex (MHC) and is the primary receptor for the human immunodeficiency virus (HIV).[30,31] The cytoplasmic portion of the antigen is associated with the protein tyrosine kinase $p56^{lck}$.[24]

CD3, CD8, CD45, and CD4 antibodies are composed of mouse γ_1 heavy chains and kappa light chains.

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

Precautions

1. For In Vitro Diagnostic Use.
2. Do not use the reagent if you observe any change in appearance. Precipitation or

discoloration indicates instability or deterioration.

3. The antibody reagent contains sodium azide as a preservative; however, take care to avoid microbial contamination, which can cause erroneous results.

   WARNING: Sodium azide is harmful if swallowed. Keep out of reach of children. Keep away from food, drink, and animal feedingstuff. Wear suitable protective clothing. If swallowed, seek medical advice immediately and show this container or label. Contact with acids liberates very toxic gas. Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in lead or copper plumbing where explosive conditions can develop.

4. **WARNING**: All biological specimens and materials coming in contact with them are considered biohazards. Handle as if capable of transmitting infection[32,33] and dispose of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Wear suitable protective clothing and gloves. Fixation has been reported to inactivate HIV.[34]

5. FACS™ Lysing Solution* is required and contains diethylene glycol and formaldehyde. Refer to the *FACS Lysing Solution* package insert for warnings.

6. If 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 (see #7 in Reagents and Materials Required but Not Provided). 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.

7. Bead count varies by lot of TruCOUNT Tubes. It is critical to use the bead count shown on the current lot of 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.

8. 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

1. Store the reagent at 2–8°C. Do not use after the expiration date shown on the label.

2. Do not freeze the reagent or expose it to direct light during storage or incubation with cells. Keep the reagent vial dry.

3. Store TruCOUNT Tubes in their original foil pouch at 2–25°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 MultiTEST CD3/CD8/CD45/CD4 reagent and TruCOUNT Tubes are designed for use on a flow cytometer equipped with appropriate computer hardware and software. BD recommends the FACSCalibur™ or FACSort™ flow cytometer; however, results can be achieved using other platforms. The flow cytometer must be equipped with 635 nm

* US Patent Nos. 4,654,312; 4,902,613; and 5,098,849

and 488 nm lasers and must be capable of detecting light scatter (forward and side) and four-color fluorescence with emission detectable in four ranges: 515–545 nm, 562–607 nm, >650 nm, and 652–668 nm. It must be able to threshold or discriminate using the >650 nm channel. The BD FACS Loader can also be used with this product.

BD recommends using CaliBRITE™ beads and FACSComp™ software, version 4.0 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 four-color immunophenotyping.

BD has developed software applications such as MultiSET that automatically calculate absolute counts when 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 COLLECTION AND PREPARATION

Collect blood aseptically by venipuncture[35,36] into a sterile K_3 EDTA (ethylenediamine-tetraacetic acid) VACUTAINER® blood collection tube (lavender top). MultiTEST CD3/CD8/CD45/CD4 reagent and TruCOUNT Tubes have been validated with both liquid and dry formulations of K_3 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 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.

Anticoagulated blood stored at room temperature (20–25°C) must be stained within 48 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.[37] Blast cells can interfere with test results. Hemolyzed samples should be rejected.

7. PROCEDURE

Reagent Provided

- MultiTEST CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC (BD Catalog No. 340499), or
- MultiTEST CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC with TruCOUNT Tubes (BD Catalog No. 340491)

Reagents and Materials Required but Not Provided

1. CaliBRITE 3 and APC beads (BD Catalog Nos. 340486 and 340487, respectively)
2. FACS Lysing Solution (10X), 100 mL (BD Catalog No. 349202). Refer to the *FACS Lysing Solution* package insert for dilution instructions and warnings.
3. Reagent-grade (distilled or deionized) water
4. K_3 EDTA VACUTAINER blood collection tubes (BD Catalog No. 366457), or equivalent
5. Disposable 12 x 75-mm Falcon™ capped polystyrene test tubes (BD Catalog No. 352058), or equivalent (if not using TruCOUNT Tubes)

6. Vortex mixer
7. Micropipettor with tips (BD Electronic Pipette, Catalog No. 343246 [US], or 343208 [Europe]; Pipetman®, Rainin Instrument Co Inc; or equivalent)
8. Bulk dispenser or pipettor (450 µL) for dispensing FACS Lysing Solution
9. Sheath fluid (FACSFlow™, BD Catalog No. 340398 [US and Latin America] or 342003, or equivalent)
10. TruCOUNT Controls (BD Catalog No. 340335), necessary if using TruCOUNT Tubes
11. Lysable whole blood control (available commercially)

Staining the Cells

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

Reverse Pipetting

If 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 TruCOUNT Tube in place of the 12 x 75-mm tube.

   NOTE: Before use, verify that the TruCOUNT bead pellet is intact and within the metal retainer at the bottom of the tube. If this is not the case, discard the TruCOUNT Tube and replace it with another.
2. Pipette 20 µL of MultiTEST CD3/CD8/CD45/CD4 reagent into the bottom of the tube.

   If using a 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 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 (20–25°C).
5. Add 450 µL 1X 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 (20–25°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 (20–25°C).

Vortex the cells thoroughly (at low speed) to reduce aggregation before running them on the flow cytometer.[38] If using the FACS Loader, vortex tubes immediately before placing them into the loader racks. Acquire and analyze list-mode data using MultiSET or CellQuest software. 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.[36]

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 Figures 1, 2, and 3 for representative data from a hematologically normal adult sample stained with CD3/CD8/CD45/CD4 in a TruCOUNT Tube.

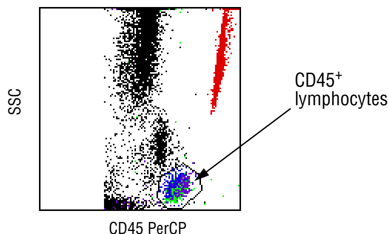

Figure 1 Identification of lymphocytes in CD45 vs SSC dot plot

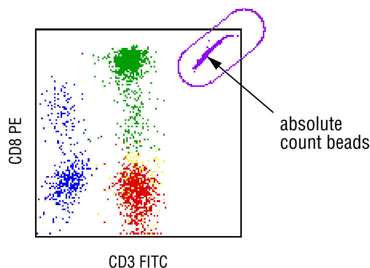

Figure 2 TruCOUNT absolute count bead events in CD3 vs CD8 dot plot

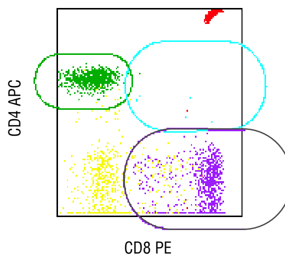

Figure 3 Identification of $CD3^+CD8^+$ events and $CD3^+CD4^+$ events in CD8 vs CD4 dot plot

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 MultiSET software is used, absolute counts will be determined by the software.

For manual data analysis using CellQuest or other software, simply divide the number of positive cellular events by the number of bead events, then multiply by the TruCOUNT bead concentration:

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

* This value is found on the TruCOUNT Tube foil pouch label and might vary from lot to lot.

9. LIMITATIONS

1. Laboratories must establish their own normal reference ranges for the MultiTEST CD3/CD8/CD45/CD4 reagent parameters that can be affected by sex of patient, age of patient, and preparative technique. Race of patient[39] and individual variations of epitope expression[40] can also have an effect, 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.[41] Reference ranges provided are for information only.
2. MultiTEST CD3/CD8/CD45/CD4 reagent has not been validated for use with heparin or acid citrate dextrose (ACD) liquid anticoagulants in determining absolute counts with TruCOUNT Tubes.
3. MultiTEST CD3/CD8/CD45/CD4 reagent is not intended for screening samples for the presence of leukemic cells or for use in phenotyping samples from leukemia patients.
4. Absolute counts are not comparable between laboratories using different manufacturers' equipment.

10. EXPECTED VALUES

Reference Ranges

The reference ranges for CD3/CD8/CD45/CD4 shown in Table 1 were determined at three clinical investigation centers in the United States. Subjects were hematologically normal adults between the ages of 18 and 65 years. Refer to the first limitation for more information about reference ranges.

Table 1. Representative Reference Ranges for CD3/CD8/CD45/CD4

Subset	n	Mean	95% Range
Helper/inducer T lymphocytes (%)	164	45	33–58
Suppressor/cytotoxic T lymphocytes (%)	164	24	13–39
Total T lymphocytes (%)	164	72	56–86
Helper/inducer T lymphocytes (cells/µL)	164	941	404–1612
Suppressor/cytotoxic T lymphocytes (cells/µL)	164	511	220–1129
Total T lymphocytes (cells/µL)	164	1513	723–2737

11. PERFORMANCE CHARACTERISTICS

Performance of the reagents was established by testing at BD Biosciences laboratories in San Jose, CA and at three clinical laboratories in the US.

Accuracy

Lymphocyte subset percentage and absolute counts enumerated with MultiTEST CD3/CD8/CD45/CD4 in TruCOUNT Tubes were compared with results from TriTEST™ CD3/CD4/CD45 or CD3/CD8/CD45 in TruCOUNT Tubes.

Whole blood samples from normal and abnormal donors were collected at random at two clinical laboratories and evaluated in both systems. Regression statistics reported in Table 2 indicate that the results are substantially equivalent.

Table 2. Regression Analysis

Subset	n	R	Slope	Intercept	Range
Helper/inducer T lymphocytes (%)	124	0.998	0.996	–0.434	1–62
Suppressor/cytotoxic T lymphocytes (%)	124	0.997	1.018	–0.383	13–78
Total T lymphocytes (%)	124	0.995	1.002	0.254	22–90
Helper/inducer T lymphocytes (cells/μL)	124	0.982	1.015	–7.692	93–1904
Suppressor/cytotoxic T lymphocytes (cells/μL)	124	0.988	1.001	2.494	132–2229
Total T lymphocytes (cells/μL)	124	0.981	1.028	–20.451	189–2987

Within-Specimen Reproducibility

Estimates of within-sample reproducibility were determined at three clinical laboratories from five replicates of each sample collected from normal and abnormal donors. Means, standard deviations (SD) and/or coefficients of variation (CV) are provided for subset percentages and absolute counts greater than 100 cells/μL in Tables 3 and 4.

Table 3. Within-Specimen Reproducibility of Subset Percentages

Subset	n	Mean (%)	SD
Helper/inducer T lymphocytes (%)	46	26.1	0.85
Suppressor/cytotoxic T lymphocytes (%)	46	42.0	0.93
Total T lymphocytes (%)	46	72.0	1.06

Table 4. Within-Specimen Reproducibility of Absolute Counts

Subset	n	Mean (cells/μL)	SD	% CV
Helper/inducer T lymphocytes (cells/μL)	38	565.2	62.92	7.02
Suppressor/cytotoxic T lymphocytes (cells/μL)	46	687.8	54.02	6.79
Total T lymphocytes (cells/μL)	46	1219.9	101.57	6.34

Stability

A stability study was conducted to assess the stability of MultiTEST reagent in TruCOUNT Tubes. 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. Whole blood samples were tested up to 48 hours post draw and stained samples were tested up to 24 hours post stain. All samples were maintained at room temperature (20° to 25°C) before staining or acquisition.

Based on the results of this study,* we recommend staining samples within 48 hours of draw and analyzing samples within 24 hours of staining.

*Data available at BD Biosciences

Cross-Reactivity

The CD8 antibody reacts with NK lymphocytes[42] as well as with suppressor/cytotoxic T lymphocytes. The CD4 antibody reacts with monocytes as well as with helper/inducer T lymphocytes.[19]

Linearity

Linearity was assessed within a WBC concentration of 0.2 x 10^3 to 29.7 x 10^3 WBC/µL and a lymphocyte concentration of 0.1 x 10^3 to 9.0 x 10^3 lymphocytes/µL. Results were observed to be linear within the $CD3^+CD4^+$ range, the $CD3^+CD8^+$ range, and the $CD3^+$ range.

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