

Division of Laboratory Sciences Laboratory Protocol

Analyte:	Laboratory 3- CD4+ T-lymphocytes (helper T cells) and CD8+ T cells (suppressor/inducer T cells)
Matrix:	Whole Blood
Method:	TruCount Method
Branch:	HIV Immunology and Diagnostics Branch
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Laboratory Procedure Manual

Analyte: **CD4+ T-lymphocytes (helper T cells) and CD8 T cells (suppressor/inducer T cells)**

Matrix: **Whole Blood**

Method: **TriCount Method**

as performed by: Lee Lam, Marjorie Hubbard
HIV Immunology and Diagnostics Branch
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Important Information for Users

CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

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Public Release Data Set Information

This document details the Lab Protocol for NHANES 2001-2002 data.

A tabular list of the released analytes follows:

Dataset name	Variable name	Description
I03_b	LBXCD4	CD4 cell counts (cells/mm ³)
	LBXCD8	CD8 cell counts (cells/mm ³)

Immunophenotyping Fresh Frozen Whole-Blood Specimens Using the BD TruCOUNT Method

1. Principle

Human immunodeficiency virus (HIV) infection is characterized by a decrease, and eventually, a depletion of CD4+ T-lymphocytes (helper T cells). By using immunophenotyping, patient specimens are tested for the proportion of lymphocytes that are T cells, B cells, natural killer (NK) cells, CD4+ T cells (helper T cells), and CD8+ T cells (suppressor/inducer T cells). This is done by incubating anti-coagulated whole blood with monoclonal antibodies to the various cellular antigens that identify specific cell populations (phenotypes), and then lysing the blood to remove red blood cells. The antibodies are conjugated to fluorescent tags that emit light of a certain frequency when excited by a laser beam. The specimens are analyzed on a flow cytometer to determine the proportion of cells of a particular phenotype (that emit light at the right wavelength).

The TruCount method (TriTEST 3-color and MultiTEST 4-color) uses TruCount tubes which contain a lyophilized pellet containing a known quantity of fluorescent beads. A precise quantity of whole blood is added to the tubes, and the lymphocytes are stained with TriTEST or MultiTEST monoclonal antibodies (mAb). The absolute count of a full lymphocyte subset profile (CD3+, CD3+CD4+, CD3+CD8+, CD3-CD19+, CD3-CD16/56+) can be determined in four tubes with TriTEST and two tubes with MultiTEST by calculating the ratio of region events for each subset to bead events using the BD Biosciences-developed software, MutlitiSET.

2. Safety Precautions

- A. FOLLOW UNIVERSAL PRECAUTIONS. Assume that blood and body fluids from all patients are infectious. Handle all specimens used as controls or reagents at Biosafety Level 2.
- B. Wear gloves for handling blood or body fluids. Change gloves when they become contaminated and wash hands after handling specimens. Do not handle door knobs, telephones, or other community objects while wearing gloves.
- C. Wear protective clothing (lab coat, plastic apron, gown, face shield, etc.) while working with potentially infectious materials. Leave protective clothing in lab area. Wear closed-toe shoes (no sandals).
- D. Use a biological safety cabinet, mechanical pipetting devices when possible, and centrifuge specimens in safety carriers.
- E. Lab work surfaces should be decontaminated with a 10% bleach solution following any spill and at the completion of work activities. If a vacutainer is broken or blood is spilled, cover with 10% bleach and plastic-backed absorbent paper or "diaper" and let sit for 10 minutes. Double-glove, wipe up, and dispose of paper and gloves in an autoclave pan. Use 70% isopropanol for surfaces that might be damaged by bleach, such as the inside of centrifuges.
- F. Dispose of contaminated materials in a plastic bag inside an autoclave pan with at least 2 inches of water. All liquids should be discarded into bleach equivalent to 10% of the total

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volume. 12×75 mm tubes with phenotyping leftover solutions are discarded in a special tube canister or jug with 10% bleach.

- G. Personnel should wash their hands after removal of protective clothing and before leaving the laboratory.
- H. Do not remove the covers to the flow cytometers when the lasers are on. If instructed to remove the cover by Becton Dickinson for troubleshooting purposes, turn the power off and unplug the power cord.

3. Specimen Collection, Storage, and Handling Procedures; Criteria for Specimen Rejection

- A. Enumeration of CD4+ lymphocytes from NHANES 1999-2002 HIV-positive participants and age-matched controls was performed on cryopreserved whole blood using the method reported by Fiebig et. al. (11).
- B. An appropriate specimen consists of anticoagulated whole blood. Specimens must be collected in EDTA and processed within 30 hours of draw for Becton Dickinson's TruCOUNT procedures. The specimens must meet the following criteria. They must:
 - (1) be labeled with the patient's name or local ID, date of collection, and CDC number (specimen number or DASH number).
 - (2) be transported to the lab as soon as possible. They should be shipped by Federal Express or AirBorne from locations outside Atlanta so that they are received the day after they are drawn and are temperature controlled to 6°C–39°C.
 - (3) be processed within 48 hours of draw and preferably within 30 hours of draw for EDTA-anticoagulated specimens.
- C. Several 0.5-ml aliquots of the well-mixed whole blood are made in 2-ml cryovials and stored at –70°C until the day of testing.

4. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

Not applicable for this procedure

5. Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials, Equipment and Instrumentation

A. Reagents

- (1) Monoclonal antibodies (mAb) - BD Biosciences
- (2) Four Color: BD MultiTEST Reagents
- (3) MultiTEST IMK Kit (#340503) with TruCOUNT tubes (#340504)
- (4) MultiTEST CD3/CD8/CD45/CD4(#340499) with TruCOUNT tubes(#340491)
- (5) MultiTEST CD3/CD16+56/CD45/CD19 (#340500) with TruCOUNT tubes (#340492)

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- (6) Monoclonals are stored at 4°C–8°C and are stable for the dating period on the label. Freezing and exposure to light should be avoided. Use undiluted.
- (7) Fluorochrome: CD45 PerCP, CD3 FITC, CD4 APC, CD8 PE, CD19 APC, CD16+56 PE.
- (8) Fluorescence-activated cell sorter (FACS) lysing solution, 10× (#349202). Dilute the concentrate 1:10 with distilled water to make working lysing solution. Make fresh monthly.
- (9) Phosphate-buffered saline (PBS), 0.01 M, pH 7.2 (Formula 4550C)
- (10) FACS wash solution: sterile PBS, pH 7.2 with 0.1% sodium azide and 2.0% fetal calf serum. FACS should be heat-inactivated for 30 min at 56°C and Millipore-filtered. Store and use FACS wash solution at 4°C for 3 months.

B. Supplies

- (1) 12×75 mm test tubes with caps (Falcon #352054).
- (2) Dispensing bottles for 2 ml, 300 µl, and 450 µl volumes.
- (3) Repeating micropipettors to deliver 10 µl and 100 µl.
- (4) Micropipettor tips.
- (5) BD precision pipettor, 50 µl fixed volume.
- (6) Bleach (hypochlorite, 10%).
- (7) Surgical gloves.

C. Equipment

- (1) Vortex mixer.
- (2) Biological safety cabinet (class 1 or 2).
- (3) Tube rotator.
- (4) Becton Dickinson FACSort or FACSCalibur flow cytometer.
- (5) Centrifuge.

6. Calibration and Calibration Verification Procedures

A. FACSComp Procedure

The FACSComp software uses CaliBRITE Beads (plastic micro-spheres) to adjust the FACScan, FACSort, or FACSCalibur settings for immunophenotyping analysis. The FACSort or FACSCalibur contains sensitive photoelectric components with variable gain

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settings. The CaliBRITE Beads are used to ensure that the instrument provides consistent readings from day to day by adjusting the gain settings and fluorescence compensation values to standard samples of known light scatter and fluorescent intensity.

FACSComp performs the following adjustments:

- (1) Gating singlet events;
- (2) Adjusting PMT voltage; and
- (3) Adjusting fluorescence compensation.

B. Preparation of the CaliBRITE Beads.

BD CATALOG #310486-CaliBRITE 3 and #340487-APC beads. Pipette FACSCFlow sheath reagent (#340398) into two 12×75 mm plastic tubes (Falcon #352054), 0.5 ml for the unlabeled + activated protein C (APC) beads and 1 ml for the mixture of unlabeled fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll- α protein (PerCP), and APC beads. Gently mix the unlabeled + APC beads by inversion and add one drop to the 0.5 ml tube and the 1 ml tube. Gently mix the FITC, PE, PerCP and APC beads and add one drop of each to the 1 ml tube. Cap the tubes and mix by gently inverting several times. Harsh mixing can cause high background noise. Make fresh daily.

C. Select the FACSComp software.

On the first screen, enter your initials under Operator ID. Make sure the lot identification for the current batch of CaliBRITE Beads is correct in the software (matches the bead lot numbers on the insert from the CaliBRITE box). The suffix letter is important because it tells the software what the minimum signal-to-noise separation is for that lot. Acquire the unlabeled + APC bead mixture; if "PMT's" set successfully, acquire the mixed beads after gently inverting the tube several times to mix. If all tests passed successfully, print the results. See BD's FACSComp manual for detailed instructions for performing FACSComp on the three different flow cytometers.

D. Recording of Results.

The results are recorded in the FACScan, FACSort, or FACSCalibur FACSComp Results logbook and transferred onto Levy-Jennings plots to monitor instrument performance.

E. Acceptance Criterion

- (1) FSC noise should be less than 50. If after eliminating air bubbles and repeating the sensitivity test with a fresh bead dilution the noise is still over 50, request a service call from BD.
- (2) If any of the results when plotted on the Levy-Jennings charts are out of range, make new dilutions of the beads and run again. One point slightly out of range on one day does not have to be rejected; but if a trend seems to be developing, a service call may be required.

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F. Chicken Red Blood Cell (CRBC) Procedure

- (1) After FACSComp has been performed successfully, set up the CRBC tube for CRBC calibration. In a 12×75 mm Falcon #352054 tube, add 1.5 ml of isoton and one drop of well mixed CRBC.
- (2) Select the “CRBC Acq.alias” document and “Connect to Cytometer”. Under “Counter” the flow rate should be 400–1000 events/second. Under Cytometer, select “Instrument Settings” and select the “CRBC Settings” from the CRBC folder. Under Cytometer, select “Detectors/Amps”. Vortex the CRBC mixture and acquire. The “mean” bars should be centered over the peaks
- (3) Set GeoMean of M1 to predetermined target values found in the Instrument Calibration Log Sheet in the CRBC Values & Settings Book by raising and lowering voltages FL1, FL2, FL3, and FL4 of the detectors/amps. Clicking on their respective arrows will adjust the average (target) values [i.e. M1, M2, M3, M4] accordingly. Mode for FL1, FL2, and FL3 should be set to “Log”. Examples of target values are: FL1 = 725, FL2 = 681, FL3 = 644.
- (4) Record the adjusted FL1, FL2, FL3, and FL4 voltages and GeoMean values in the Instrument Calibration Log. Rinse the cytometer with dH₂O.
- (5) CRBC calibration is useful for monitoring the consistency of settings for research studies.

7. Procedure Operating Instructions; Calculations; Interpretation of Results

A. Lyse/No Wash Procedure (LNW)

Used for single-platform lymphocyte subset determination panel

- (1) Remove the cryovials from the –70°C freezer in batches of no more than 10. Make sure to compare the specimen number or patient ID on the specimen vial to the paperwork (transmittal form, etc.) Thaw quickly in a 37°C water bath.
- (2) Log in specimen under appropriate project spreadsheet.
- (3) CD-Chex will be used as a QC control and frozen whole-blood pool as normal control.
- (4) Label TruCOUNT tubes as follows:
 - (a) Write the panel and antibody added on the top part of the tube (i.e. 4C-A, 4C-B).
 - (b) Write the specimen’s assigned lab ID on the lower part of the tube (i.e. A-Z, etc).
 - (c) 4 Color (4C)-4C-1 label 1 tube or 4C-2 label 2 tubes.
 - (d) The antibodies used in the panel are located in the refrigerator in a box labeled “MultiSET antibodies in use”.
- (5) Pipette 20 µl of the appropriate antibody into the corresponding labeled tube.

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- (6) Make sure specimens are well mixed before adding 50 μ l (using BD pipette) of frozen whole blood to the corresponding tube.
- (7) Vortex briefly at vortex setting 4 to 6.
- (8) Incubate at room temperature in the dark for 15 minutes.
- (9) Add 450 μ l (0.45 ml) of FACS lysing solution found in the cabinet and vortex well.
- (10) Incubate at room temperature in the dark for 10 minutes.
- (11) Add 50 μ l of TruCOUNT control beads (from refrigerator) to the CD-Chex tube.
- (12) Incubate at room temperature in the dark for at least 15 minutes before acquiring the sample. Acquire within 2 hours of set-up.
- (13) MultiSET Acquisition and Analysis
 - (a) TruCOUNT 3Color-4 tube and 4Color-2 tube LNW panels can be performed using BD MultiSet software.
 - (b) Open MultiSET software from the Apple menu and then open appropriate schedule (XXX.sch) from the CLIA Lab folder on the Desktop. Follow the prompting messages from the program to proceed.
 - (c) Sign in with your initials.
 - (d) Make sure to select the appropriate locations for data files, reports, and export document storage.
 - (e) MultiSET data files are saved in CLIA LAB PROJECTS FOLDER on the DeskTop, then save into the ICL folder in which a new patient folder with the patient ID and date (e.g. MI9901_120303) has been designated, and finally save into into a folder named MultiSET. Both the patient and normal go into this folder.
 - (f) Make sure the appropriate absolute bead count, reagents, and control bead lot ID's are filled in and saved.
 - (g) Fill out the schedule as follows:

SAMPLE NAME:	Local patient ID
SAMPLE ID:	SpecNo
CASE NUMBER:	Project name and number (i.e., ICL P137)
PANEL NAME:	Appropriate panel ID (i.e., 4C-1)
 - (h) Open Cytometer from the Apple menu and make sure the LNW instrument settings file from the current day's FACScmp results is selected before clicking the Run Test Command.
- (14) Append statistics to the same file created for the project.

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(15) Run tests and use Manual Gate for analysis; print report before going to the next sample.

(16) Save schedule before quitting the program when finished.

B. Calculations

Calculations for the proportion of lymphocytes that are of particular cell type are determined in the MultiSET software. This software calculates the proportion of cells that are positive with a particular monoclonal antibody and reports them in whole numbers. Additionally, the values are automatically corrected for the purity of lymphocytes inside the gate (% of bright CD45+, CD14- cells of all cells in the gate). All results reported are corrected for lymphocyte purity. Additional calculations are meant for data quality control. These include adding the CD3+CD4+ and CD3+CD8+ results to equal the CD3 results (+10%) and accounting for all of the lymphocytes in the specimen by adding the T, B, and NK cells together to total 90%–110%.

The absolute counts of each subset are calculated automatically by the following formula:
absolute CD4 or CD8 or CD3 Count = (# of target population events / # of bead events collected) × (# of beads per test from the package insert / test volume of 50 µl).

8. REPORTING RESULTS

T cell (CD3+) results are determined from the average of the 4 or 2 CD3 values. T helper/inducer cell values are from the cells that are positive for both CD3 and CD4 (CD3+CD4+). T cytotoxic/suppressor cell values are from cells that are positive for both CD3 and CD8 (CD3+CD8+). B cell values are those cells that are CD19+. NK cells are determined to be negative for CD3 but positive for CD16 and/or CD56 (CD3-[CD16/56]+).

Results are reported as cells/µl, with a reference range obtained from 129 control donors that were obtained through the CDC Serum Bank. These donors are composed of adult men and women who were healthy at the time of blood drawing. Racial composition of this group was mixed. The data set from these donors was analyzed using SAS and the results for CD3+, CD3+CD4+, and CD3+CD8+ are from a univariate analysis, with the 5th and 95th percentiles being the low and high values. The values for CD19 and CD3-(CD16/56)+ were log-transformed before univariate analysis was performed.

One hundred and seven samples of this set of control donors were also frozen in aliquots and tested and analyzed on the same flow cytometer using the BD MultiTest TruCount method. The correlation coefficient (R) of the fresh whole-blood absolute CD4 counts compared to the frozen whole-blood absolute CD4 counts was 0.9655, with a slope of 0.92489, intercept of 4.6, and mean bias of -67.2.

If a WBC with differential is available from a hematology complete blood count (CBC), a cross check can be done on the CD4 count using the CD4 percent from the flow cytometry analysis on frozen whole blood and the calculated absolute lymphocyte count from the CBC on fresh whole blood using the following formula:

$$(\%CD4 \times \text{absolute lymphocyte count} / 100) = \text{absolute CD4 count.}$$

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9. Quality Control and Quality Assurance

- A. A freshly drawn peripheral whole-blood specimen from a healthy donor must be run as a daily quality control with each run of patients for red blood cell (RBC) lysing control.
- B. CD-Chex Plus Normal control (Streck Labs #213326) is run using each antibody for precision of absolute counts.

(1) Reagent login:

When a new batch of reagents (whole-blood control, TruCOUNT controls, TruCOUNT tubes and antibodies, CaliBRITE beads, etc.) is received, log it in on the appropriate Reagent Record sheet recording the batch number, lot number, quantity, expiration date, date received, and initials. Mark the batch number in red on the reagent vial. When a new batch is opened, record the date of first use, and record the \pm expected range of values on the appropriate QC sheet in the TruCOUNT QA/QC notebook. Place a copy of the package insert in the TruCOUNT 3-Color and 4-Color QA/QC book. Prior to putting new lots of reagents or kits into use, a lot-to-lot reagent check is performed in parallel with the current lot in use. These results are recorded on the reagent login sheet. If results of the new lot are not within 5% of the previous lot, repeat with different specimens; if it is still out of this range, do not use the new lot.

(2) Setup

(a) Set up the whole-blood control* with the patient samples using the same procedure, reagents, etc. as the patient samples. Add TruCOUNT bead controls to the whole-blood control according to the TruCOUNT control procedure. Only one level (high, medium, or low) needs to be used.

(b) Acquire on the flow cytometer in the same manner as the participant samples.

(3) Analysis

(a) Analyze the patient samples and whole-blood control in the appropriate software (MultiSET).

(b) Record the whole-blood control percent and absolute values and the TruCOUNT bead counts on the appropriate pages and columns for cytometer in the TruCOUNT 3-Color and 4-Color QA/QC book.

(c) Check to see if the values are within the expected range (\pm) for that lot number.

(4) Procedure for failed run:

(a) If the values of either the whole-blood control or the TruCOUNT control beads are out of the expected range, vortex and acquire again on the same cytometer. If they are in the expected range, continue with analysis and reporting of the patient samples.

(b) If the values of either the whole-blood control or the TruCOUNT control beads are still out of the expected range, set them up again with samples from a fresh vial,

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acquire on the same cytometer, and analyze. If they are in the expected range, continue with analysis and reporting of the patient samples.

- (c) If the whole-blood control values are still out of the expected range, acquire the whole-blood control and the patient samples on the backup cytometer.** If they are in the expected range, continue with analysis and reporting of the patient samples.
- (d) If the whole-blood control values are still out of the expected range, try a new lot number of control or place a service call to BD Biosciences.
- (e) If the patient samples are acquired on more than one cytometer and the percent and absolute values are essentially the same, the patient results can be reported if there is a problem with the whole-blood control. Otherwise, the whole-blood control and the TruCOUNT control should be within the expected range before reporting results.

* Currently CD-Chex Plus normal.

** Currently FACSCalibur for 4-Color and FACSCan for 3-Color

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11. REFERENCES

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