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Guidelines for Performing Single-Platform Absolute CD4⁺ T-Cell Determinations with CD45 Gating for Persons Infected with Human Immunodeficiency Virus

Guidelines for Using the QuantiFERON[®]-TB Test for Diagnosing Latent *Mycobacterium tuberculosis* Infection

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Centers for Disease Control and Prevention

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Guidelines for Performing Single-Platform Absolute CD4⁺ T-Cell Determinations with CD45 Gating for Persons Infected with Human Immunodeficiency Virus

Prepared by

Francis F. Mandy, Ph.D.¹

Janet K.A. Nicholson, Ph.D.²

J. Steven McDougal, M.D.³

¹Bureau of HIV/AIDS, STD and TB

Population and Public Health Branch

Health Canada

Ottawa, Ontario, Canada

²Office of the Director

and ³Division of AIDS, STD, and TB Laboratory Research

National Center for Infectious Diseases, CDC

Summary

These guidelines were developed by CDC for laboratorians who perform immunophenotyping for detection and enumeration of CD4⁺ T-cells and other lymphocyte subsets in persons infected with human immunodeficiency virus (HIV). The guidelines describe single-platform technology (SPT), a process in which absolute counts of lymphocyte subsets are measured from a single tube by a single instrument. SPT incorporates internal calibrator beads of known quantity in the analysis of specimens by three- or four-color flow cytometry. With CD45 gating, the relative numbers of beads and lymphocyte subsets are enumerated, and their absolute numbers and percentage values are calculated. This report supplements previous recommendations published in 1997 (CDC. 1997 revised guidelines for performing CD4⁺ T-cell determinations in persons infected with human immunodeficiency virus [HIV]. MMWR 1997;46[No. RR-2]) that describe dual-platform technology, a method in which absolute counts are derived from measurements obtained from two instruments—a flow cytometer and hematology analyzer. The new recommendations address concerns specific to the implementation of SPT as well as other general topics such as laboratory safety and specimen handling.

Introduction

Obtaining accurate and reliable measures of CD4⁺ T lymphocytes (CD4⁺ T cells) is essential to assessing the immune system and managing the health care of persons infected with human immunodeficiency virus (HIV) (1–4). The pathogenesis of acquired immunodeficiency syndrome (AIDS) is largely attributable to the decrease in the number of T cells that bear the CD4 receptor (5–9). Progressive depletion of CD4⁺ T cells is associated with an increased likelihood of severe HIV disease and an unfavorable prognosis (10–13). Accordingly, the U.S. Public Health Service (PHS) has recommended that CD4⁺ T-cell levels be monitored every 3–6 months in all HIV-infected persons (14). Measurement of CD4⁺ T-cell levels has been used to establish decision points for initiating prophylaxis for *Pneumocystis carinii* pneumonia and other opportunistic infections and for initiating and monitoring antiretroviral therapy (15–20). CD4⁺ T-cell levels are also a criterion for categorizing HIV-related clinical conditions according to

CDC's classification system for HIV infection and surveillance case definition of AIDS among adults and adolescents (21).

Single-platform technology (SPT) is designed to enable determinations of both absolute and percentage lymphocyte subset values using a single tube. Until recently, most absolute T-cell numbers were derived from three measurements determined with two different instruments, a hematology analyzer and a flow cytometer (dual-platform technology [DPT]). Hence, the CD4⁺ T-cell number is the product of three laboratory measurements: the white blood cell count, the percentage of white blood cells that are lymphocytes (differential), and the percentage of lymphocytes that are CD4⁺ T cells (determined by flow cytometry). In 1997, CDC published guidelines addressing concerns related to DPT (22); those guidelines remain appropriate for laboratories performing CD4⁺ T-cell counts with this technology.

On November 14–15, 2001, a third national conference on CD4⁺ immunophenotyping was held in Orlando, Florida, to discuss scientific and technologic advances in the development and production of reagents, instrumentation, and software that have occurred since publication of the 1997

The material in this report originated in the National Center for Infectious Diseases, James M. Hughes, M.D., Director; Division of AIDS, STD, and TB Laboratory Research, Jonathan E. Kaplan, M.D., Acting Director.

guidelines. The conference was attended by representatives from public health, private, and academic laboratories as well as product manufacturers. These guidelines reflect a consensus of that conference, reviewed by attendees, and specifically related to the performance of SPT.

Development of new guidelines was driven by advances in knowledge and experience with new approaches to enumerate CD4⁺ T cells. First, a gating strategy for identifying lymphocytes using CD45 fluorescence and side-scattering characteristics is now the preferred method for identifying lymphocytes accurately and reproducibly. Second, three- or four-color flow cytometry has been demonstrated to be superior to two-color methods for measuring CD4⁺ and CD8⁺ T-cell counts (23). Finally, the availability of Food and Drug Administration (FDA)-approved commercial microfluorosphere counting reagents for SPT has resulted in decreased interlaboratory variability (24,25). Consequently, SPT is the preferred method in an increasing number of laboratories (4).

Recommendations

I. Laboratory Safety

- A. Use universal precautions with all specimens (26).
- B. Adhere to the following safety practices (27–29):
 1. Wear laboratory coats and gloves when processing and analyzing specimens, including reading specimens on the flow cytometer.
 2. Never pipette by mouth. Use safety pipetting devices.
 3. Never recap needles. Dispose of needles and syringes in puncture-proof containers designed for this purpose.
 4. Handle and manipulate specimens (e.g., aliquot, add reagents, vortex, and aspirate) in a class I or II biological safety cabinet.
 5. Centrifuge specimens in safety carriers.
 6. After working with specimens, remove gloves and wash hands with soap and water.
 7. For stream-in-air flow cytometers, follow the manufacturer's recommended procedures to eliminate the operator's exposure to any aerosols or droplets of sample material.
 8. Disinfect flow cytometer wastes. Before adding waste materials to the waste container, add a sufficient volume of undiluted household bleach (5% sodium hypochlorite) so that the final concentration of bleach will be 10% (0.5% sodium hypochlorite) when the container is full (e.g., add 100 mL of undiluted bleach to an empty 1,000-mL container).

9. Disinfect the flow cytometer as recommended by the manufacturer. One method is to flush the flow cytometer fluidic chambers with a 10% bleach solution for 5–10 minutes at the end of the day and then flush with water or saline for at least 10 minutes to remove excess bleach, which is corrosive.
10. Disinfect spills with household bleach or an appropriate dilution of mycobactericidal disinfectant. **Note:** Organic matter will reduce the ability of bleach to disinfect infectious agents. NCCLS recommendations regarding how to disinfect specific areas should be followed (30). For use on smooth, hard surfaces, a 1% solution of bleach is usually adequate for disinfection; for porous surfaces, a 10% solution is needed (30).
11. Ensure that all samples have been properly fixed after staining and lysing but before analysis. **Note:** Some commercial reagents employ a single-step, lyse and fix method that reduces the infectious activity of cell-associated HIV by 3–5 logs (31,32); however, these reagents have not been evaluated for their effectiveness against other agents (e.g., hepatitis virus). Cell-free HIV can be inactivated with 1% paraformaldehyde within 30 minutes (33–35).

II. Specimen Collection for Single-Platform Technology

- A. Anticoagulant
 1. Use tripotassium ethylenediamine tetraacetate (K₃EDTA, 1.5 ± 0.15 mg/mL blood) or heparin (36–39), and perform the test within the time frame allowed by the SPT manufacturer. Because acid citrate dextrose is added as a liquid to blood collection tubes, its use would make calculating accurate final sample volume difficult and is not recommended. With this absolute counting technology, use of an accurate sample volume is critical.
 2. Reject specimens that cannot be processed within 72 hours.
- B. Collect blood specimens by venipuncture (40) into evacuated tubes containing K₃EDTA anticoagulant, completely expending the vacuum in the tubes.
 1. Use pediatric tubes to obtain specimens from children, and ensure that the tube is full.
 2. Mix the blood well with the anticoagulant to prevent clotting.

- C. Label all specimens with the date, time of collection, and a unique patient identifier. Ensure that patient information and test results are accorded confidentiality.

III. Specimen Transport

- A. Maintain and transport specimens at room temperature (64°–72°F [18°–22°C]) (39,41–43). Specimens should not be exposed to extreme temperatures that could allow them to freeze or become too hot. Temperatures >99°F (37°C) might cause cellular destruction and affect flow cytometry measurements (39). In hot weather, pack the specimen in an insulated container. If necessary, place this container inside another containing an ice pack and absorbent material. This method helps retain the specimen at ambient temperature. The effect of cool temperatures (i.e., <39°F [4°C]) on CD45 gate-based immunophenotyping results is not clear (39,43).
- B. Transport specimens to the immunophenotyping laboratory as soon as possible.
- C. For transport to locations outside the collection facility, follow state or local guidelines. One method for packaging such specimens is to place the tube containing the specimen in a leakproof container (e.g., a sealed plastic bag) and to pack this container inside a cardboard canister containing sufficient material to absorb all the contents should the tube break or leak. Cap the canister tightly. Fasten the request slip securely to the outside of this canister with a rubber band. For mailing, this canister should be placed inside another canister containing the mailing label.
- D. For interstate shipment, follow federal guidelines for transporting diagnostic specimens (available at <http://www.cdc.gov/od/ohs/biosfty/shipregs.htm>). **Note:** Use overnight carriers with an established record of consistent overnight delivery to ensure arrival the following day. Check with these carriers for their specific packaging requirements.
- E. Obtain specific protocols and arrange appropriate times of collection and transport from the facility collecting the specimen.

IV. Specimen Integrity

- A. Inspect the tube and its contents immediately upon arrival.
- B. Take corrective actions if any of the following occur:
 1. If the specimen is hot or cold to the touch but not obviously hemolyzed or frozen, process it

but note the temperature condition on the worksheet and report form. Do not rapidly warm or chill specimens to bring them to room temperature because this may adversely affect the immunophenotyping results (39). Abnormalities in light-scattering patterns may reveal a compromised specimen.

2. If blood is hemolyzed or frozen, reject the specimen and request another.
3. If clots are visible, reject the specimen and request another.
4. If the specimen is received >72 hours after collection, reject it and request another.

V. Specimen Processing

- A. Perform the test within 48 hours (preferred), but no later than 72 hours after drawing the blood specimen (44).
- B. Place the samples on a gentle blood rocker for 5 minutes to ensure that the samples are uniformly distributed.
- C. Pipette blood volumes accurately and in a reproducible manner. A reverse pipetting technique is recommended (Box).
- D. Vortex sample tubes to mix the blood and reagents and break up cell aggregates. In addition, vortex samples immediately after the lyse/fixation step and before analysis to disperse cells optimally.
- E. Incubate all tubes in the dark during the staining procedure.
- F. A lyse/no-wash method is required for SPT. Follow directions provided by the manufacturer.
- G. Immediately after processing the specimens, cap the tubes and store all stained samples in the dark and under refrigeration (39°–50°F [4°–10°C]) until flow cytometric analysis. These specimens should not be stored for longer than 24 hours unless the laboratory can demonstrate that scatter and fluorescence patterns do not change for specimens for stored longer periods.

VI. Monoclonal Antibody Panels

- A. CD45 is required to aid in the identification of lymphocytes. Lymphocytes are brightly positive for CD45 and have low light-scattering characteristics.
- B. Monoclonal antibody panels must contain appropriate antibody combinations to enumerate CD4⁺ and CD8⁺ T-cells and to ensure the quality of the results (Table 1).

BOX. Accurate pipetting technique

Use of SPT to obtain absolute CD4 counts requires accurate and precise measurement of blood and beads. Reverse pipetting technique is recommended for dispensing these products.

Testing Pipetting Precision

The precision of pipetting should be evaluated periodically (e.g., monthly) to ensure the accuracy of results. Retain all records of this evaluation procedure for quality assurance purposes.

- Using the reverse pipetting technique, pipette 10 replicates of blood and record the weights. Select a volume normally used in the performance of the assay.
- Using the reverse pipetting technique, pipette 10 replicates of bead suspension and record the weights (this applies to methods in which the beads must be pipetted into the tubes).
- Calculate the mean, standard deviation, and coefficient of variation (CV). The CV for replicates should be <2% (Table 2).

Testing Pipetting Accuracy

The following procedure can be used to test the pipette and how accurately it measures volume. Water is used because the weight of 1 μL of water is 1 μg .

- Using the reverse pipetting technique, pipette 10 replicates of distilled water and record the weight. (100 μL of water should weigh 0.1000 grams.) (Table 2)
- Calculate the mean, standard deviation, and CV. The CV must be <2% (range: 0.098–0.102).

Procedures

The following information is consolidated from operational instruction manuals from several pipette manufacturers. Complete information and more detailed instructions are contained in specific pipette instruction manuals; some of these are available online. Read the manufacturer's manual carefully before beginning the pipetting procedure.

- Select the desired volume (with manual pipettes, higher volumes should be set first; if adjusting from a lower to a higher volume, first surpass the desired volume and then slowly decrease the volume until the required setting is reached).
- If applicable, select the desired mode (e.g., reverse pipette). This is recommended for optimal precision and reproducibility.
- Reverse pipetting can be done with a manual pipette by pressing the control button slightly past the first stop when aspirating, taking up more liquid than will be dispensed, then pressing the control button only to the first stop when dispensing. A small volume will remain in the tip after dispensing.
- Select an appropriate tip (usually color matches the color of the control button).

Prerinsing

The following procedures will help ensure optimal precision and accuracy.

- Volumes >10 μL : Prerinsing pipette 2–3 times for each new tip (this involves aspirating and dispensing liquid several times). Reasons for prerinsing include the following:
 - to compensate for system pressure, for slight differences in temperature between pipette and liquid, and for properties of the liquid;
 - to clear the thin film formed by the liquid on the inside of the pipette. Without prerinsing, retention of a thin film on the inside wall of the tip would cause the first volume to be too small. The thickness and nature of this film, and therefore the potential source of error, will vary depending on the nature of the liquid being pipetted.
- Volumes <10 μL : Do not prerinsing pipette, but rinse tip after dispensing to ensure that the whole volume was dispensed. For smaller volumes, prerinsing is not recommended because the dispensed volume would be too great.

Filling

- Make sure tip is securely attached.
- Hold pipette upright.
- When aspirating, try to keep the tip at a constant depth below the surface of the liquid.
- Glide control button slowly and smoothly (electronic pipettes perform this step automatically).
- When pipetting viscous liquids (e.g., whole blood), leave the tip in the liquid for 1–2 seconds after aspirating before withdrawing it.
- After liquid is in the tip, never lay the pipette on its side.

Dispensing

- Hold the tip at an angle, against the inside wall of the vessel/tube if possible.
- Glide control button slowly and smoothly (electronic pipettes perform this step automatically).

Other Recommendations

- To ensure optimal performance, the temperature of the pipetted solution and the pipette and tips should be the same (volume errors may occur because of changes in air displacement and viscosity of the liquid). Do not pipette liquids with temperatures >70°C.
- Volume errors may also occur with liquids that have a high vapor pressure or a density/viscosity that differs greatly from water. Water is most commonly used to calibrate pipettes and to check inaccuracy and imprecision. A pipette could possibly be recalibrated for liquids with densities that vary greatly from that of water.
- Pipettes should be checked regularly for precision and accuracy.
- Regular maintenance (e.g., cleaning) should be performed either by the user or a service technician according to manufacturer's instructions.

TABLE 1. Three- and four-color monoclonal antibody panels

Panel	Monoclonal antibodies	Notes
Three-color panel	CD3/CD4/CD45*	Gate using CD45 and side scatter; measure CD3 ⁺ /CD4 ⁺ T cells and CD3 ⁺ T cells
	CD3/CD8/CD45*	Gate using CD45 and side scatter; measure CD3 ⁺ /CD8 ⁺ T cells and CD3 ⁺ T cells
	CD3/CD19/CD45 [†]	Gate using CD45 and side scatter; measure CD3 ⁺ T cells and CD19 ⁺ B cells
Four-color panel	CD45/CD3/CD4/CD8 [§]	Gate using CD45 and side scatter; measure CD3 ⁺ /CD4 ⁺ T cells, CD3 ⁺ /CD8 T cells, and CD3 ⁺ T cells
	CD45/CD3/CD19/CD16 and/or CD56 [†]	Gate using CD45 and side scatter; measure CD19 ⁺ B cells, CD3 ⁺ T cells, and CD3 ⁺ /CD16 and/or CD56 ⁺ NK [¶] cells

* Recommended three-color panels.

[†] Recommended for specimens obtained from children.[§] Recommended four-color panel.[¶] Natural killer.**TABLE 2. Evaluation of pipetting precision and accuracy**

Replicate no.	Accuracy	Precision	Precision
	100 μ L of water (grams)	100 μ L of blood (grams)	100 μ L of microbeads (grams)
1	0.1036	0.1072	0.1056
2	0.1018	0.1071	0.1056
3	0.1020	0.1067	0.1055
4	0.1026	0.1069	0.1056
5	0.1008	0.1067	0.1052
6	0.1002	0.1060	0.1055
7	0.0989	0.1072	0.1056
8	0.1019	0.1090	0.1047
9	0.1009	0.1070	0.1050
10	0.1027	0.1066	0.1050
Mean	0.1015	0.1070	0.1053
Standard deviation	0.0014	0.0008	0.0003
% coefficient of variation	1.35	0.73	0.31

1. CD4 T-cells are identified as being positive for CD3 and CD4.
2. CD8 T-cells are identified as being positive for CD3 and CD8.

C. Three-color monoclonal antibody panels

1. Three-color monoclonal antibody panels should fulfill the following basic requirements: enumerate CD4⁺ and CD8⁺ T-cells, validate the CD45 gate used, and provide some assessment of tube-to-tube variability.
2. Three-color monoclonal antibody panels must consist of at least two tubes, each with the same lineage marker. For the examples described previously, CD3 is the common lineage marker in each tube. Differences between replicate CD3 results should be $\leq 2\%$.
3. CD19⁺ B-cell values may be important in assessing immune status of pediatric patients.

D. Four-color monoclonal antibody panels

1. Addition of CD45 to a single tube containing CD3, CD4, and CD8 allows the identification of lymphocytes based on CD45 and side scatter and the enumeration of CD4⁺ and CD8⁺ T-lymphocytes.
2. CD19⁺ B-cell values may be essential for assessing the immune status of pediatric patients.
3. Use of a second tube containing a natural killer (NK) cell marker together with CD3 and CD19 can help to assess the recovery and purity of the lymphocytes within the CD45/side-scatter gate.

VII. Negative and Positive Controls for Immunophenotyping

Note: An isotype control is not needed.

A. Positive methodologic control

1. Use the methodologic control to determine whether procedures for preparing and processing the specimens are optimal. Prepare this control each time specimens from patients are prepared.
2. Use either a whole-blood specimen from a control donor or commercial materials validated for this purpose.
3. If the methodologic control falls outside established normal ranges, determine the reason. **Note:** The purpose of the methodologic control is to detect problems in preparing and processing the specimens. Biologic factors that cause only the whole-blood methodologic control to fall outside normal ranges do not invalidate the results from other specimens processed at the same time. Poor lysis or poor labeling in all specimens, including the methodologic control, invalidates results.

B. Positive control for evaluating reagents

1. Use the positive control to test the labeling efficiency of new lots of reagents or when the labeling efficiency of the current lot is questioned. Prepare this control only when needed (i.e., when reagents are in question) in parallel with reagent lots of known acceptable performance. **Note:** New reagents must demonstrate similar results to those of known acceptable performance.
2. Use a whole-blood specimen or other human lymphocyte preparation (e.g., cryopreserved or commercially obtained lyophilized lymphocytes or stabilized whole blood).

VIII. Flow Cytometer Quality Control

- A. Verify optical alignment daily. Usually, clinical flow cytometers that are capable of three- and four-color immunophenotyping have fixed optical systems, i.e., the relative position of the flow cell with respect to the optical elements is fixed. In such systems, the instrument operator cannot optimize alignment but must verify that the instrument meets the manufacturer's specifications for optical alignment. Regardless of whether the alignment is user adjustable, it should be checked with alignment standards, such as wide-spectrum fluorescent microfluorospheres with measurable light-scatter characteristics. Daily monitoring of optical alignment ensures that the cytometer gives acceptably bright fluorescence measurements and that homogeneous

peaks are produced for all parameters to be used in sample analysis (45).

1. Use a stable calibration material (e.g., microfluorospheres labeled with fluorochromes) that has measurable and known forward-scatter, side-scatter, and fluorescence properties in each channel to be used for sample analysis.
 2. Verify acceptable optical alignment by establishing that calibration particles meet manufacturer- or laboratory-defined criteria for brightness and homogeneity.
 3. Align stream-in-air flow cytometers daily (at a minimum) and stream-in-cuvette flow cytometers (most clinical flow cytometers are this type) as recommended by the manufacturer.
- B. Standardize fluorescence and light-scatter signals daily. This ensures that the flow cytometer is operating within manufacturer- or laboratory-defined acceptance ranges under test-specific conditions each day and that its performance is consistent from day to day.
1. Select machine settings that are appropriate for antibody/fluorochrome-labeled, whole-blood specimens.
 2. Use microfluorospheres or other stable standardization material to place the scatter and fluorescence peaks in the same narrow range of scatter and fluorescence channels each day. Adjust the flow cytometer as needed.
 3. Retain machine standardization settings for the remaining quality control procedures (sensitivity and color compensation) and for reading the specimens.
- C. Determine fluorescence resolution daily. The flow cytometer must differentiate between the dim peak and autofluorescence in each fluorescence channel (45).
1. Unstained and lysed fresh whole blood is suitable for adjusting the photomultiplier tube (PMT) voltages. The autofluorescence from the unstained lymphocytes should be completely on scale (i.e., <5% of cells within the lymphocyte light-scatter gate fall in channel 0 in each fluorescence scale) and should fall within the lower left quadrant of the dot plot for every PMT/detector in use.
 2. Evaluate standardization/calibration material or cells to verify that cells with low-level fluorescence can be resolved from autofluorescence

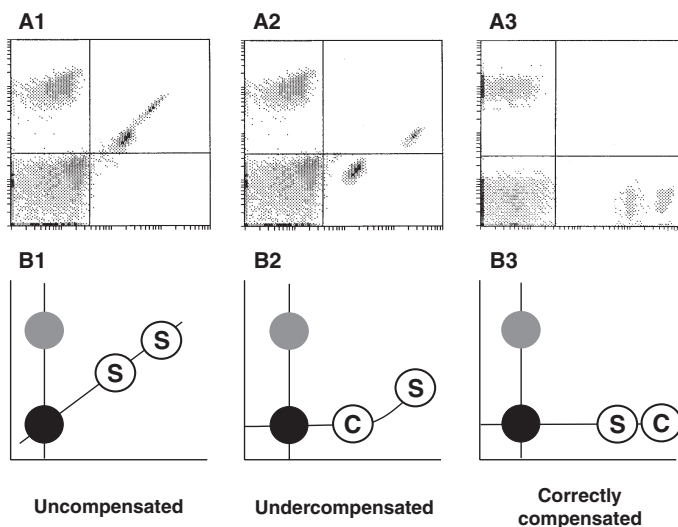
(e.g., microbeads with low-level and negative fluorescence, CD56-labeled lymphocytes, or dim cells in CD8-labeled lymphocytes).

3. Establish a minimal acceptable distance between peaks; monitor this difference, and correct any daily deviations.

D. Compensate for spectral overlap daily (Figure 1). Compensation is the process of correcting for spectral overlap of one fluorochrome into the filter window being used to monitor another fluorochrome. In most instruments used clinically, this correction is done by adjusting the electronic compensation circuits on the flow cytometer to place populations not expected to be dual positive for two fluorochromes into orthogonal fluorescence quadrants with no overlap into the double-positive quadrant. At the same time, avoiding overcompensation is essential because this may cause dual-positive cells to be incorrectly classified as single positive. The following procedures may be performed manually, or the software on the flow cytometer may perform the spectral compensation automatically.

1. Select the compensation control so it will match the brightest specimen signal. Use either

FIGURE 1. The multicolor compensation concept



Histograms A1–A3 and their respective schematic depictions, B1–B3, illustrate uncompensated, undercompensated, and correctly compensated two-color displays, respectively. The solid circles represent the cell populations for which accurate color compensation is desired. The open circles labeled C represent the compensation controls being used to adjust color compensation settings. If the intensity of the color compensation control does not exceed the fluorescence intensity of the sample to be analyzed, that sample will be undercompensated (A2, B2). If the intensity of the color compensation control exceeds the fluorescence intensity of the sample to be analyzed, the sample is less likely to be undercompensated (A3, B3).

microbead or cellular compensation material containing four populations for three-color immunofluorescence (no fluorescence, phycoerythrin [PE] fluorescence only, fluorescein isothiocyanate [FITC] fluorescence only, and a population that is positive for only the third color) or five populations for four-color (the four described previously and a population that is positive for only the fourth color).

2. Analyze this material, and adjust the electronic compensation circuits on the flow cytometer to place the fluorescent populations in their respective fluorescence quadrants with no overlap into the double-positive quadrant (Figure 1). With three fluorochromes, compensation must be carried out in an appropriate sequence: FITC, PE, and the third color, respectively (46). For four-color monoclonal antibody panels, follow the flow cytometer manufacturer's instructions for four fluorochromes. Avoid overcompensation.

3. If standardization or calibration particles (microbeads) have been used to set compensation, confirm proper calibration by using lymphocytes labeled with FITC- and PE-labeled monoclonal antibodies and a third-color- or fourth-color-labeled monoclonal antibody for three-color or four-color panels, respectively. So that separate cell populations can be recognized without overlap, cells in individual tubes may be separately stained with each different fluorochrome-labeled antibody and then combined in a single tube for analysis. These populations should have the brightest expected signals. **Note:** Using a dimmer-than-expected signal to set compensation can result in suboptimal compensation for the brightest signal.

4. Reset compensation when photomultiplier tube voltages or optical filters are changed.
5. Commercially available software can analyze data without compensation and perform the compensation automatically. When using this software, follow manufacturer's instructions for this procedure.

E. Repeat all four instrument quality control procedures (section VIII A–C) whenever instrument problems occur or if the instrument has been serviced.

F. Maintain instrument quality control logbooks and monitor them continually for changes in any of the

parameters. In the logbook, record instrument settings, peak channels, and coefficient of variation (CV) values for materials used to monitor or verify optical alignment, standardization, fluorescence resolution, and spectral compensation. Reestablish target fluorescence levels for each quality control procedure when lot numbers of beads are changed or the instrument has been serviced.

IX. Sample Analyses

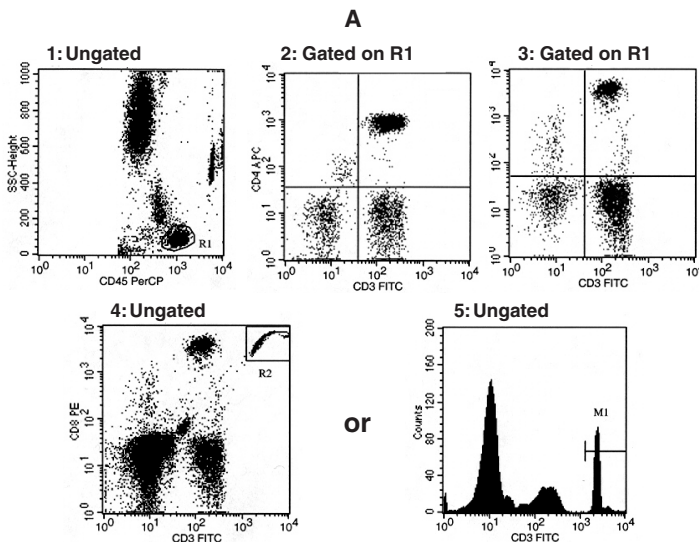
- With single-platform absolute count determination, use of the lyse/no-wash sample processing is mandatory. The lymphocyte population is identified as having bright CD45 fluorescence and low side-scattering properties (Figure 2). Set the threshold or discriminator as recommended by the manufacturer. Adjust side scatter so that all leukocyte populations are visible. Draw a gate on the bright CD45⁺ cell population and analyze the cells in that population (47).
- Count at least 2,500 gated lymphocytes in each sample to ensure that enough cells and beads have been counted to provide an accurate absolute lymphocyte value.

X. Data Analysis

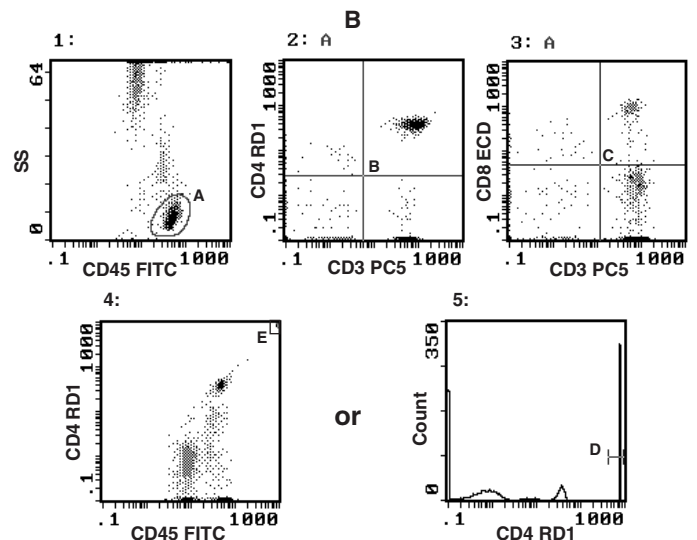
A. CD45 gating

- Lymphocytes are identified by being brightly labeled with CD45 monoclonal antibody and having low side-scattering properties. Two typical examples of a four-color SPT analysis based on CD45 gating are illustrated (Figure 2).
- Establish criteria for cluster identification based on a clear definition of lymphocytes that does not include basophils (less bright CD45, low side scatter) or monocytes (less bright CD45, moderate side scatter). **Note:** Care must be taken to include all lymphocytes. CD45 fluorescence may be slightly less with B cells than with T cells (the major cluster of lymphocytes). NK cells have bright CD45 fluorescence but have slightly more side-scattering properties than the majority of the lymphocytes.
- CD45/side-scatter gates for lymphocytes are assumed to contain >95% lymphocytes. Lymphocyte purity is assumed to be high with the CD45/side-scatter gating strategy; therefore, correction of lymphocyte subset values is not needed (47).
- If an estimate of lymphocyte recovery is needed (i.e., percentage of total lymphocytes within the CD45/side scatter gate), all the B and NK cells must be immunophenotyped as well.

FIGURE 2. Results of single-platform technology (SPT) performed by using CD45 gating with four colors



A. A typical illustration of how the four-color SPT will appear on a Becton Dickinson Biosciences (San Jose, CA) FACSCalibur™ instrument monitor. Dot plots 1–3 illustrate the CD45 versus side-scattering, the CD4 versus CD3, and the CD8 versus CD3 dot plots, respectively. Graphs 4 and 5 illustrate the two options available to monitor the presence of the microfluorospheres used for absolute cell counting calculations. The microfluorospheres in graphs 4 and 5 are located in regions R2 and M1, respectively.



B. A typical example of the four-color SPT on a Beckman Coulter (Miami, FL) Epics XL™ instrument monitor. Dot plots 1–3 illustrate the CD45 versus side scatter, the CD4 versus CD3, and the CD8 versus CD3 dot plots, respectively. Graphs 4 and 5 illustrate the two options available to monitor the presence of the microfluorospheres. The microfluorospheres in graphs 4 and 5 are located in regions E and D, respectively.

Note: Validation of a CD45/side-scatter gate is recommended during its initial use to help determine the CD45 and side-scatter characteristics of T, B, and NK cells and to ensure their inclusion in the gate.

- B. Set cursors based on the tube containing CD3/CD4 and CD3/CD8 so that the negative and positive cells in the histogram are clearly separated.
- C. Analyze each patient or control specimen with lymphocyte gates and cursors for positivity set for that particular patient or control.
- D. Include the following analytic reliability checks, when available:
 1. With SPT, an additional analytical tool can be used to check the accuracy of the absolute count; time can be used as a parameter to determine how long it takes to obtain a microfluorosphere count that represents a unit volume of blood analyzed. Optimally, if blood pipetting was performed without noticeable error and the beads were accurately added to the tubes, the time required to analyze a microliter of whole blood should be constant. Follow manufacturer's instructions to set time as an active parameter. If more or less time is required for a sample to accumulate the usual number of microspheres, this may indicate a serious counting problem and specimen processing should be repeated.
 2. Optimally, the sum of the percentages of CD3⁺CD4⁺ and CD3⁺CD8⁺ cells should equal the total percentage of CD3⁺ cells \pm 5%, with a maximum variability of <10%. **Note:** For specimens containing a considerable number of T $\gamma\delta$ T-cells (48,49), this reliability check may exceed the maximum variability.

XI. Data Storage

- A. Store list-mode data for all specimens analyzed. This allows for reanalysis of the raw data, including re-drawing of gates. At a minimum, retain hard copies of the CD45/side-scatter gate and correlated dual-histogram data of each sample's fluorescence.
- B. Retain all primary files, worksheets, and report forms for 2 years or as required by state or local regulation, whichever is longer. Data can be stored electronically. Disposal after the retention period is at the discretion of the laboratory director.

XII. Data Reporting

- A. Report all data in terms of CD designation, with a short description of what that designation means. **Note:** CD4⁺ T cells are T-helper cells. The correct cells to report for this value are those that are positive for both CD3 and CD4. Similarly, CD8⁺ T-cells are T-suppressor/cytotoxic cells and are positive for both CD3 and CD8. Do not include other cell types (non-T cells) in CD4 and CD8 T-cell determinations.
- B. Report lymphocyte subset values as follows:
 1. Report both percentages and absolute counts.
 2. With SPT, determine the absolute counts directly from the flow cytometers. These calculations are usually handled by software that reports calculated results. The following formula should be used:

$$\frac{\text{No. of events in the bright CD45 region}}{\text{No. of events in the microfluorosphere region}} \times \frac{\text{Total no. of microfluorospheres added}}{\text{Volume of blood added}}$$

- C. Report data from all relevant monoclonal antibody combinations with corresponding reference limits of expected normal values (e.g., CD4⁺ T-cell absolute number and percentage). Reference limits for immunophenotyping test results must be determined for each laboratory (45). Separate reference ranges must be established for adults and children, and the appropriate ranges must be reported for patient specimens.

XIII. Quality Assurance

- A. Ensure the overall quality of the laboratory's CD4⁺ T-cell testing by monitoring and evaluating the effectiveness of the laboratory policies and procedures for the preanalytic, analytic, and postanalytic testing phases. The practices and processes to be monitored and evaluated include the following:
 - methods for collecting, handling, transporting, identifying, processing, and storing specimens;
 - information provided on report forms for test requests and results;
 - instrument performance, quality control protocols, and maintenance;
 - reagent quality control protocols;
 - process for reviewing and reporting results.

- employee training and education, which should consist of the following:
 - basic training by flow cytometer manufacturers and additional training involving hands-on workshops for flow cytometer operators and supervisors;
 - education of laboratory directors regarding flow cytometric immunophenotyping through workshops and other programs;
 - continuing education regarding new developments for all flow cytometric immunophenotyping personnel through meetings and workshops;
 - adherence to federal and state regulations for training and education;
- assurance of satisfactory performance. Laboratories must fully participate in a performance evaluation program and demonstrate acceptable level of performance. When proficiency testing programs have been approved by the Centers for Medicare & Medicaid Services (formerly, the Health Care Financing Administration) as meeting the requirements of the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88) (none are currently approved for CD4⁺ T-cell testing), laboratories must satisfactorily participate.
- review and revision (as necessary or at established intervals) of the laboratory's policies and procedures to ensure adherence to the quality assurance program. All staff involved in the testing should be informed of any problems identified during the quality assurance review, and corrective actions should be taken to prevent recurrences.

B. Document all quality assurance activities.

Evaluation and Validation of a Newly Adopted SPT in the Laboratory

When a laboratory adopts the new SPT, specimens should be tested in parallel by using both the current and the new method to characterize any systematic differences in the methods. Laboratorians should use statistical tools that provide useful information for the comparison studies. Linear least squares regression analyses are helpful in establishing good correlations between the new and established methods. If no error is detected with the new method, the r^2 value will ap-

proach 1.0. However, regression-type scatter plots provide inadequate resolution when the errors are small in comparison with the analytical range and do not characterize the relationship between the two methods (50–52).

A bias scatter plot provides laboratorians with a more useful tool for determining bias. These simple, high-resolution graphs plot the differences in the individual measurements of each method (result of old method—result of new method) against measurements obtained with one of the methods (result of old method) (50). Such graphs provide an easy means of determining if bias is present and distinguishing whether bias is systematic, proportional, or random/nonconstant. The laboratorian can visually determine the magnitude of these differences over the entire range of values. When sufficient values are plotted, outliers or samples containing interfering substances can be identified. The laboratorian can then divide the data into ranges relevant to medical decisions and calculate the systematic error (mean of the bias) and the random error (standard deviation of the bias) to gain insight into analytical performance at the specified decision points (50–52).

Several detailed guidelines and texts provide additional information regarding quality goals, method evaluation, estimation of bias, and bias scatter plots (50–54). Once a new method is accepted and implemented, the laboratory will need to confirm or redefine its normal range and should continue to monitor the correlation between the results and the patient's clinical disease data to ensure that no problems have gone undetected by the relatively few samples typically tested during method evaluations.

Discussion

More than 1.6 million CD4⁺ T-cell measurements are performed yearly by the approximately 600 testing laboratories in the United States (55). This figure is based on the reported number of tests performed annually by laboratories participating in CDC's Model Performance Evaluation Program (MPEP) for T-lymphocyte immunophenotyping in 1996. These measurements are performed with flow cytometers using either multiplatform technology or SPT. SPT was introduced for clinical application in 1996, and its wide-scale implementation is relatively new. In 2000, results of two independent multicenter studies of SPT were reported (24,25). Those and subsequent reports on SPT and CD45 gating (56–60) have increasingly encouraged adoption of these improved testing practices (61,62). The resulting outcomes associated with SPT and CD45 gating include a) increased confidence in results, b) more reproducible results, c) increased ability to resolve discrepant problems, d) decreased propor-

tion of unacceptable specimens received for testing, e) decreased proportion of specimens requiring reanalysis, and f) fewer incidents that could pose biohazard risks (61).

Although these guidelines for SPT use might foster improved laboratory practices, developing comprehensive guidelines for every aspect of CD4⁺ T-cell testing (including some laboratory-specific practices) is not possible. Moreover, measuring the outcomes associated with the adoption of these guidelines is inherently difficult. First, the guidelines lack evaluation protocols that can adequately account for the interactions among the recommendations. No weight of importance has been assigned for the individual recommendations that address unique steps in the testing process; hence, the consequences of incompletely following the entire set of recommendations are uncertain. Second, because published data are not available for every aspect of the guidelines, certain recommendations are based on the experience and opinion of knowledgeable persons. Recommendations made on this basis, in the absence of data, may be biased and inaccurate. Finally, variations in testing practices and interactions among the practices (e.g., how specimens are obtained and processed, skill of laboratory personnel [such as with pipetting], testing methods used, test-result reporting practices, and compliance with other voluntary standards and laboratory regulations) complicate both the development of guidelines that will fit every laboratory's unique circumstances and the assessment of the value of implementing the guidelines.

The first CDC recommendations for laboratory performance of CD4⁺ T-cell testing (63) were written so as not to impede development of new technology or investigations into better ways to assess the status of the immune system in HIV-infected persons. Developments in the technology have resulted in an assay that is technically less complicated and more accurate. These single-platform methods are now being implemented in as many as one fourth of the laboratories in the United States (MPEP data). In addition, other T-cell phenotypic markers are being investigated as prognostic indicators or markers of treatment efficacy, alone and in combination with other cellular markers (64,65).

These guidelines for SPT are intended for domestic implementation. Several alternative methods are available that require fewer reagents and involve more cost-effective gating algorithms. Some of these alternative methods may be compatible with current U.S. clinical laboratory methods; however, to date they have not been validated for domestic applications. As published validation data accumulate from multisite studies for methods such as PanLeucogating (66) and primary CD4 gating (67, 68), these potentially more cost-effective options will be considered as alternative or substitute

methods. In the future, guidelines should be harmonized to include all methods that meet domestic performance standards to ensure consistent high quality.

References

1. Turner BJ, Hecht FM, Ismail RB. CD4⁺ T-lymphocyte measures in the treatment of individuals infected with human immunodeficiency virus type 1. *Arch Intern Med* 1994;154:1561-73.
2. Fei DT, Paxton H, Chen AB. Difficulties in precise quantitation of CD4⁺ T lymphocytes for clinical trials: a review. *Biologicals* 1993;21:221-31.
3. Hoover DR, Graham NM, Chen B, et al. Effect of CD4⁺ cell count measurement variability on staging HIV-1 infection. *J Acquir Immune Defic Syndr* 1992;5:794-802.
4. Mandy F, Nicholson J, Autran B, Janossy G. T-cell subset counting and the fight against AIDS: reflections over a 20-year struggle. *Cytometry (Clinical Cytometry)* 2002;50:39-45.
5. DeWolf F, Roos M, Lange JM, et al. Decline in CD4⁺ cell numbers reflects increase in HIV-1 replication. *AIDS Res Hum Retroviruses* 1988;4:433-40.
6. Giorgi JV, Nishanian PG, Schmid I, Hultin LE, Cheng H, Detels R. Selective alterations in immunoregulatory lymphocyte subsets in early HIV (human T-lymphotropic virus type III/lymphadenopathy-associated virus) infection. *J Clin Immunol* 1987;7:140-50.
7. Lang W, Perkins H, Anderson RE, Royce R, Jewell N, Winkelstein W, Jr. Patterns of T-lymphocyte changes with human immunodeficiency virus infection: from seroconversion to the development of AIDS. *J Acquir Immune Defic Syndr* 1989;2:63-9.
8. Masur H, Ognibene FP, Yarchoan R, et al. CD4 counts as predictors of opportunistic pneumonias in human immunodeficiency virus (HIV) infection. *Ann Intern Med* 1989;111:223-31.
9. Smith RD. The pathobiology of HIV infection. A review. *Arch Pathol Lab Med* 1990;114:235-9.
10. Hanson DL, Chu SY, Farizo KM, Ward JW. Distribution of CD4⁺ T lymphocytes at diagnosis of acquired immunodeficiency syndrome-defining and other human immunodeficiency virus-related illnesses. The Adult and Adolescent Spectrum of HIV Disease Project Group. *Arch Intern Med* 1995;155:1537-42.
11. Stein DS, Korvick JA, Vermund SH. CD4⁺ lymphocyte cell enumeration for prediction of clinical course of human immunodeficiency virus disease: a review. *J Infect Dis* 1992;165:352-63.
12. Fahey JL, Taylor JM, Detels R, et al. The prognostic value of cellular and serologic markers in infection with human immunodeficiency virus type 1. *N Engl J Med* 1990;322:166-72.
13. CDC. 1994 revised classification system for human immunodeficiency virus infection in children less than 13 years of age. Official authorized addenda: human immunodeficiency virus infection codes and official guidelines for coding and reporting ICD-9-CM. *MMWR* 1994;43(No. RR-12).
14. CDC. Recommendations for prophylaxis against *Pneumocystis carinii* pneumonia for adults and adolescents infected with human immunodeficiency virus. *MMWR* 1992;41(No. RR-4).
15. CDC. 1995 revised guidelines for prophylaxis against *Pneumocystis carinii* pneumonia for children infected with or perinatally exposed to human immunodeficiency virus. *MMWR* 1995;44(No. RR-4).
16. Friedland GH. Early treatment for HIV: the time has come. *N Engl J Med* 1990;322:1000-2.

17. Goldman AI, Carlin BP, Crane LR et al. Response of CD4 lymphocytes and clinical consequences of treatment using ddI or ddC in patients with advanced HIV infection. *J Acquir Immune Defic Syndr* 1996;11:161–9.
18. De Gruttola V, Gelman R, Lagakos S. Uses of CD4-lymphocyte count in AIDS treatment decisions. *Infect Agents Dis* 1994;2:304–13.
19. CDC. USPHS/IDSA guidelines for the prevention of opportunistic infections in persons infected with human immunodeficiency virus: a summary. *MMWR* 1995;44(No. RR-8).
20. CDC. Recommendations for counseling persons infected with human T-lymphotropic virus types I and II. Recommendations on prophylaxis and therapy for disseminated *Mycobacterium avium* complex for adults and adolescents infected with human immunodeficiency virus. *MMWR* 1993;42(No. RR-9):1–20.
21. CDC. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *MMWR* 1992;41(No. RR-17).
22. CDC. 1997 revised guidelines for performing CD4+ T-cell determinations in persons infected with human immunodeficiency virus (HIV). *MMWR* 1997;46(No. RR-2).
23. Gelman R, Wilkening C. Analyses of quality assessment studies using CD45 for gating lymphocytes for CD3+4*%. *Cytometry (Communications in Clinical Cytometry)* 2000;42:1–4.
24. Schnizlein-Bick CT, Spritzler J, Wilkening CL, et al. Evaluation of TruCount absolute-count tubes for determining CD4 and CD8 cell numbers in human immunodeficiency virus-positive adults. *Clin Diagn Lab Immunol* 2000;7:336–43.
25. Reimann KA, O’Gorman MRG, Spritzler J, et al. Multisite comparison of CD4 and CD8 T-lymphocyte counting by single- versus multiple-platform methodologies: evaluation of Beckman Coulter Flow-Count fluorospheres and the tetraONE system. *Clin Diagn Lab Immunol* 2000;7:344–51.
26. CDC. Perspectives in disease prevention and health promotion update: universal precautions for prevention of transmission of human immunodeficiency virus, hepatitis B virus, and other bloodborne pathogens in health-care settings. *MMWR* 1988;37:377–88.
27. CDC. Agent summary statement for human immunodeficiency viruses (HIVs) including HTLV-III, LAV, HIV-1, and HIV-2. *MMWR* 1988;37(S-4):1–17.
28. CDC. Updated U.S. Public Health Service guidelines for the management of occupational exposures to HBV, HCV, and HIV and recommendations for postexposure prophylaxis. *MMWR* 2001;50(No. RR-11).
29. CDC, National Institutes of Health. Biosafety in microbiological and biomedical laboratories. Fourth edition. Washington, DC: US Government Printing Office, 1999.
30. National Committee for Clinical Laboratory Standards. Protection of laboratory workers from instrument biohazards and infectious disease transmitted by blood, body fluids, and tissue; approved guideline. Wayne, PA: National Committee for Clinical and Laboratory Standards 1991; NCCLS document M29-A (vol. 17, no. 20).
31. Nicholson JK, Browning SW, Orloff SL, McDougal JS. Inactivation of HIV-infected H9 cells in whole blood preparations by lysing/fixing reagents used in flow cytometry. *J Immunol Methods* 1993;160:215–8.
32. Aloisio CH, Nicholson JK. Recovery of infectious human immunodeficiency virus from cells treated with 1% paraformaldehyde. *J Immunol Methods* 1990;128:281–5.
33. Cory JM, Rapp F, Ohlsson-Wilhelm BM. Effects of cellular fixatives on human immunodeficiency virus production. *Cytometry* 1990;11:647–51.
34. Lifson JD, Sasaki DT, Engleman EG. Utility of formaldehyde fixation for flow cytometry and inactivation of the AIDS associated retrovirus. *J Immunol Methods* 1986;86:143–9.
35. Martin LS, Loskoski SL, McDougal JS. Inactivation of human T-lymphotropic virus type III/lymphadenopathy-associated virus by formaldehyde-based reagents. *Appl Environ Microbiol* 1987;53:708–9.
36. National Committee for Clinical Laboratory Standards. Additives to blood collection devices: EDTA; proposed standard. Wayne, PA: National Committee for Clinical Laboratory Standards, 1989; NCCLS document H35-P (vol 9, no. 14).
37. National Committee for Clinical Laboratory Standards. Reference leukocyte differential count (proportional) and evaluation of instrumental methods; approved standard. Wayne, PA: National Committee for Clinical Laboratory Standards, 1992; NCCLS document H20-A (vol. 12, no. 1).
38. Nicholson JK, Green TA. Selection of anticoagulants for lymphocyte immunophenotyping. Effect of specimen age on results. *J Immunol Methods* 1993;165:31–5.
39. Paxton H, Bendele T. Effect of time, temperature, and anticoagulant on flow cytometry and hematological values. *Ann N Y Acad Sci* 1993;677:440–3.
40. National Committee for Clinical Laboratory Standards. Procedures for the collection of diagnostic blood specimens by venipuncture; approved standard—fourth edition. Wayne, PA: National Committee for Clinical Laboratory Standards, 1998; NCCLS document H3A4. (vol 18, no. 7).
41. Shield CF, III, Manlett P, Smith A, Gunter L, Goldstein G. Stability of human lymphocyte differentiation antigens when stored at room temperature. *J Immunol Methods* 1983;62:347–52.
42. McCoy JR, Jr., Carey JL, Krause JR. Quality control in flow cytometry for diagnostic pathology. I. Cell surface phenotyping and general laboratory procedures. *Am J Clin Pathol* 1990;93:S27–S37.
43. Ekong T, Kupek E, Hill H, Clark C, Davies A, Pinching A. Technical influences on immunophenotyping by flow cytometry. The effect of time and temperature of storage on the viability of lymphocyte subsets. *J Immunol Methods* 1993;164:263–73.
44. Bergeron M, Nicholson JKA, Phaneuf S, et al. Selection of lymphocyte gating protocol has an impact on the level of reliability of T-cell subsets in aging specimens. *Cytometry (Clinical Cytometry)* 2002;50:53–61.
45. National Committee for Clinical Laboratory Standards. Clinical applications of flow cytometry: quality assurance and immunophenotyping of lymphocytes; approved guideline. Wayne, PA: National Committee for Clinical Laboratory Standards, 1998; NCCLS document H42-A. (vol. 18, no. 21).
46. Mandy FF, Bergeron M, Recktenwald D, Izaguirre CA. A simultaneous three-color T cell subsets analysis with single laser flow cytometers using T cell gating protocol. Comparison with conventional two-color immunophenotyping method. *J Immunol Methods* 1992;156:151–62.
47. Nicholson JKA, Hubbard M, Jones B. Use of CD45 fluorescence and side-scatter characteristics for gating lymphocytes when using the whole blood lysis procedure and flow cytometry. *Cytometry (Communications in Clinical Cytometry)* 1996;26:16–21.
48. Margolick JB, Scott ER, Odaka N, Saah AJ. Flow cytometric analysis of gamma delta T cells and natural killer cells in HIV-1 infection. *Clin Immunol Immunopathol* 1991;58:126–38.

49. De Paoli P, Gennari D, Martelli P, et al. A subset of gamma delta lymphocytes is increased during HIV-1 infection. *Clin Exp Immunol* 1991;83:187–91.
50. Kringle RO, Bogovich M. Statistical procedures. In: Burtis CA, Ashwood ER, eds. *Textbook of clinical chemistry*. Philadelphia: WB Saunders, 1999:265–309.
51. National Committee for Clinical Laboratory Standards. Method comparison and bias estimation using patient samples; approved guideline—Second edition. Wayne, PA: National Committee for Clinical Laboratory Standards, 2002; NCCLS document. EP9-A.2. (vol. 15, no. 17).
52. National Committee for Clinical Laboratory Standards. Preliminary evaluation of quantitative clinical laboratory methods; approved guideline. Wayne, PA: National Committee for Clinical Laboratory Standards, 1998; NCCLS document EP10-10-A (vol. 18, no.6).
53. Shultz EK. Selection and interpretation of laboratory procedures. In: Burtis CA, Ashwood ER, eds. *Tietz textbook of clinical chemistry*. Philadelphia: WB Saunders, 1999:310–9.
54. Koch DD, Peters T. Selection and evaluation of methods. In: Burtis CA, Ashwood ER, eds. *Tietz textbook of clinical chemistry*. Philadelphia: WB Saunders, 1999:320–35.
55. CDC. Results of the 1996 T-lymphocyte immunophenotyping questionnaire survey mailed to laboratories participating in the Model Performance Evaluation Program. Atlanta, GA: US Department of Health and Human Services, Public Health Service, CDC, 1996:16.
56. Schnizlein-Bick CT, Mandy FF, O’Gorman MRG, et al. Use of CD45 gating in three- and four-color flow cytometric immunophenotyping: guideline from the National Institute of Allergy and Infectious Diseases, Division of AIDS. *Cytometry (Clinical Cytometry)* 2002;50:46–52.
57. Bergeron M, Faucher S, Ding T, Phaneuf S, Mandy F. Evaluation of a universal template for single-platform absolute T-lymphocyte subset enumeration. *Cytometry (Clinical Cytometry)* 2002;50:62–8.
58. Gratama JW, Kraan J, Keeney M, Granger V, Barnett D. Reduction of variation in T-cell subset enumeration among 55 laboratories using single-platform, three or four-color flow cytometry based on CD45 and SSC-based gating of lymphocytes. *Cytometry (Clinical Cytometry)* 2002;50:92–101.
59. Whitby L, Granger V, Storie I, et al. Quality control of CD4⁺ T-lymphocyte enumeration: results from the last 9 years of the United Kingdom National External Quality Assessment Scheme for immune monitoring (1993–2001). *Cytometry (Clinical Cytometry)* 2002;50:102–10.
60. Mandy F, Bergeron M, Houle G, Bradley J, Fahey J. Impact of the International Program for Quality Assessment and Standardization for Immunological Measures Relevant to HIV/AIDS: QASI. *Cytometry (Clinical Cytometry)* 2002;50:111–6.
61. Rosner ER, Siragusa MT. Laboratory standardization in CD4 testing: results of a nationwide impact evaluation study [Abstract A1]. Presented at the Clinical Applications of Cytometry 10th Annual Meeting, August 1995. *Cytometry (Communications in Clinical Cytometry)* 1996;26:78–97.
62. Harwell TS. Are there differences between laboratories that use or fail to use CDC’s guidelines to measure CD4⁺ and CD8⁺ T cells? *Cytometry (Communications in Clinical Cytometry)* 1995;21:256–7.
63. CDC. Guidelines for the performance of CD4⁺ T-cell determinations in persons with human immunodeficiency virus infection. *MMWR* 1992;41(No. RR-8).
64. Giorgi JV. Phenotype and function of T cells in HIV disease. In: Gupta S, ed. *Immunology of HIV infection*. New York: Plenum Medical Book Company, 1996:181–99.
65. Giorgi JV, Hultin LE, McKeating JA, et al. Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *J Infect Dis* 1999;179:859–70.
66. Glencross D, Scott LE, Jani IV, Barnett D, Janossy G. CD45-assisted PanLeucogating for accurate, cost-effective dual-platform CD4⁺ T-cell enumeration. *Cytometry (Clinical Cytometry)* 2002;50:69–77.
67. Janossy G, Jani I, Gohde W. Affordable CD4⁺ T-cell counts on ‘single platform’ flow cytometers I. Primary CD4 gating. *Br J Haematol* 2000;111:1198–208.
68. Janossy G, Jani IV, Kahan M, Barnett D, Mandy F, Shapiro H. Precise CD4 T-cell counting using red diode laser excitation: for richer, for poorer. *Cytometry (Clinical Cytometry)* 2002;50:78–85.

Guidelines for Using the QuantiFERON®-TB Test for Diagnosing Latent *Mycobacterium tuberculosis* Infection

Prepared by
Gerald H. Mazurek, M.D.
Margarita E. Villarino, M.D.
Division of Tuberculosis Elimination
National Center for HIV, STD, and TB Prevention

Summary

Until 2001, the only test used to diagnose latent tuberculosis infection (LTBI) was the tuberculin skin test (TST). However, in 2001, a new test (QuantiFERON®-TB or QFT; manufactured by Cellestis Limited, Carnegie, Victoria, Australia) that measures the release of interferon-gamma in whole blood in response to stimulation by purified protein derivative was approved by the Food and Drug Administration. This statement provides interim recommendations for using and interpreting QFT. As with TST, interpretation and indicated applications of QFT differ for persons according to their risk for LTBI and for developing tuberculosis (TB). This report provides guidance for public health officials, health-care providers, and laboratorians with responsibility for TB control activities in the United States in their efforts to incorporate QFT testing for detecting and treating LTBI. Regardless of the test used to identify LTBI, testing should be primarily targeted at diagnosing infected patients who will benefit from treatment.

Introduction

In 2001, the QuantiFERON®-TB test (QFT) (manufactured by Cellestis Limited, Carnegie, Victoria, Australia) was approved by the Food and Drug Administration (FDA) as an aid for detecting latent *Mycobacterium tuberculosis* infection (1). This test is an in vitro diagnostic aid that measures a component of cell-mediated immune reactivity to *M. tuberculosis*. The test is based on the quantification of interferon-gamma (IFN- γ) released from sensitized lymphocytes in whole blood incubated overnight with purified protein derivative (PPD) from *M. tuberculosis* and control antigens.

Tuberculin skin testing (TST) has been used for years as an aid in diagnosing latent tuberculosis infection (LTBI) and includes measurement of the delayed type hypersensitivity response 48–72 hours after intradermal injection of PPD. TST and QFT do not measure the same components of the immunologic response and are not interchangeable. Assessment of the accuracy of these tests is limited by lack of a standard for confirming LTBI.

As a diagnostic test, QFT 1) requires phlebotomy, 2) can be accomplished after a single patient visit, 3) assesses responses to multiple antigens simultaneously, and 4) does not boost anamnestic immune responses. Compared with TST, QFT results are less subject to reader bias and error. In a CDC-sponsored multicenter trial, QFT and TST results were mod-

erately concordant (overall kappa value = 0.60). The level of concordance was adversely affected by prior bacille Calmette-Guérin (BCG) vaccination, immune reactivity to nontuberculous mycobacteria (NTM), and a prior positive TST (2). In addition to the multicenter study, two other published studies have demonstrated moderate concordance between TST and QFT (3,4). However, one of the five sites involved in the CDC study reported less agreement (5).

Limitations of QFT include the need to draw blood and process it within 12 hours after collection and limited laboratory and clinical experience with the assay. The utility of QFT in predicting the progression to active tuberculosis has not been evaluated.

This report provides interim recommendations for using and interpreting QFT results based on available data. As with TST, interpretation and indicated applications of QFT differ between those persons at low risk and those at increased risk for LTBI. This report should assist public health officials, health-care providers, and laboratorians who are responsible for TB control activities in the United States in their efforts to incorporate QFT testing for detecting and treating LTBI.

QFT Performance, Interpretation, and Use

Tuberculin testing is performed for persons who are 1) suspected as having active TB; 2) at increased risk for progression to active TB; 3) at increased risk for LTBI; or 4) at low risk for LTBI, but are tested for other reasons (Table 1).

The material in this report originated in the National Center for HIV, STD, and TB Prevention, Harold W. Jaffe, M.D., Director, and the Division of Tuberculosis Elimination, Kenneth G. Castro, M.D., Director.

QFT Performance

Aliquots of heparinized whole blood are incubated with the test antigens for 16–24 hours.* The antigens included in the test kits are PPD from *M. tuberculosis* (tuberculin)[†] and PPD from *Mycobacterium avium* (avian sensitin). The kits also include phytohemagglutinin (a mitogen used as a positive assay control), and saline (negative control or nil). After incubation, the concentration of IFN- γ in the separated plasma is determined by enzyme-linked immunosorbent assay (ELISA).

QFT results are based on the proportion of IFN- γ released in response to tuberculin as compared with mitogen, or (tu-

berculin – nil) / (mitogen – nil) \times 100 = percentage tuberculin response.[§] The difference in the amount of IFN- γ released in response to tuberculin as compared with avian sensitin is expressed as (avian – nil) – (tuberculin – nil) / (tuberculin – nil) \times 100 = percentage avian difference. A computer program is available from the test manufacturer that performs these calculations and interprets the test results.[¶]

QFT Interpretation

Interpretation of QFT results (Table 2) is stratified by estimated risk for infection with *M. tuberculosis*, in a manner simi-

* Additional technical information is available from the manufacturer at <http://www.cellestis.com>.

[†] PPD from *M. tuberculosis* is referred to by the manufacturer and in FDA documents as human PPD.

[§] Percentage tuberculin response is referred to by the manufacturer and in FDA documents as percentage human response.

[¶] Available at <http://www.cellestis.com>.

TABLE 1. Interim recommendations for applying and interpreting QuantiFERON[®]-TB (QFT) (Cellestis Limited, Carnegie, Victoria, Australia)

Reason for testing	Population	Initial screening	Positive results	Evaluation
Tuberculosis (TB) suspect	Persons with symptoms of active TB	Tuberculin skin testing (TST) might be useful; QFT not recommended	Induration \geq 5 mm	Chest radiograph, smears, and cultures, regardless of test results
Increased risk for progression to active TB, if infected	Persons with recent contact with TB, changes on chest radiograph consistent with prior TB, organ transplants, or human immunodeficiency virus infection, and those receiving immunosuppressing drugs equivalent of \geq 15 mg/day of prednisone for \geq 1 month*	TST; QFT not recommended	Induration \geq 5 mm	Chest radiograph if TST is positive; treat for latent TB infection (LTBI) after active TB disease is ruled out
	Persons with diabetes, silicosis, chronic renal failure, leukemia, lymphoma, carcinoma of the head, neck, or lung, and persons with weight loss of \geq 10% of ideal body weight, gastrectomy, or jejunoileal bypass*	TST; QFT not recommended	Induration \geq 10 mm	
Increased risk for LTBI	Recent immigrants, injection-drug users, and residents and employees of high-risk congregate settings (e.g., prisons, jails, homeless shelters, and certain health-care facilities) [†]	TST or QFT	Induration \geq 10 mm; percentage tuberculin response \geq 15 [§]	Chest radiograph if either test is positive; confirmatory TST is optional if QFT is positive; treat for LTBI after active TB disease is ruled out; LTBI treatment when only QFT is positive should be based on clinical judgment and estimated risk
Other reasons for testing among persons at low risk for LTBI	Military personnel, hospital staff, and health-care workers whose risk of prior exposure to TB patients is low, and U.S.-born students at certain colleges and universities [†]	TST or QFT	Induration \geq 15 mm; percentage tuberculin response \geq 30 [§]	Chest radiograph if either test is positive; confirmatory TST if QFT is positive; treatment for LTBI (if QFT and TST are positive and after active TB disease is ruled out) on the basis of assessment of risk for drug toxicity, TB transmission, and patient preference

* QFT has not been adequately evaluated among persons with these conditions; it is not recommended for such populations.

[†] QFT has not been adequately evaluated among persons aged $<$ 17 years, or among pregnant women; it is not recommended for such populations.

[§] The following additional conditions are required for QFT to indicate *Mycobacterium tuberculosis* infection: 1) mitogen – nil and tuberculin – nil are both $>$ 1.5 IU, and 2) percentage avian difference is \leq 10.

lar to that used for interpreting TST with different cut-off values. QFT results indicative of *M. tuberculosis* infection include the following three criteria:

1. (mitogen – nil) and (tuberculin – nil) are both ≥ 1.5 IU; and
2. percentage avian difference ≤ 10 ; and
3. percentage tuberculin response ≥ 15 (increased risk for LTBI) or ≥ 30 (low risk for LTBI).

Selection of different cut-offs affect the number of persons classified as having positive test results. Using 15 as the percentage tuberculin response cut-off for interpreting a QFT test as positive identifies approximately the same number of persons compared with using a TST induration cut-off of 10 mm. Using 30 as the percentage tuberculin response cut-off for interpreting a QFT test as positive identifies approximately the same number of persons compared with using a TST induration cut-off of 15 mm. The test is considered negative if (mitogen – nil) ≥ 1.5 IU but (tuberculin – nil) $< 15\%$ (mitogen – nil). Results are considered indeterminate if (mitogen – nil) < 1.5 IU, which might be observed among anergic persons.

Using QFT for Persons at Increased Risk for LTBI

QFT can aid in detecting *M. tuberculosis* infections among certain populations who are at increased risk for LTBI (6). These populations include recent immigrants (i.e., immigrated within the previous 5 years) from high-prevalence countries where tuberculosis case rates are $\geq 30/100,000$, injection-drug users, residents and employees of prisons and jails, and health-care workers who, after their preemployment assessment, are

considered at increased risk for exposure to tuberculosis. For these populations, a percentage tuberculin response of ≥ 15 should be considered a positive QFT result.

Using QFT for Persons at Low Risk for LTBI

CDC discourages use of diagnostic tests for LTBI among populations at low risk for infection with *M. tuberculosis* (6). However, initial testing is occasionally performed among certain population groups for surveillance purposes or where cases of active, infectious tuberculosis might result in extensive transmission to highly susceptible populations. These populations include military personnel, hospital staff and health-care workers whose risk of prior exposure to TB was low, and U.S.-born students at higher education institutions (e.g., as a requirement for admission to U.S. colleges and universities). For these populations, a percentage tuberculin response of ≥ 30 should be considered a positive QFT result.

Recommendations

The highest priority of targeted tuberculin testing programs remains one that identifies persons at increased risk for TB who will benefit from treatment for LTBI. Following that principle, targeted tuberculin testing should be conducted among groups at risk for recent infection with *M. tuberculosis* and those who, regardless of duration of infection, are at increased risk for progression to active TB.

TABLE 2. QuantiFERON®-TB (Cellestis Limited, Carnegie, Victoria, Australia) results and interpretation

M – N* (IU/mL)	T – N† (IU/mL)	Avian difference (%)	Tuberculin response (%)§	Report and interpretation	Interpretation
≤ 1.5		All other response profiles		Interferon-gamma (IFN- γ) response to mitogen is inadequate	Indeterminate
≥ 1.5		All other response profiles		Percentage tuberculin response is < 15 or not significant	Negative: <i>Mycobacterium tuberculosis</i> infection unlikely
≥ 1.5	≥ 1.5	≤ 10	≥ 15 but < 30	Percentage tuberculin response is 15–30	Conditionally positive: <i>M. tuberculosis</i> infection likely if risk is identified, but unlikely for persons who are at low risk
≥ 1.5	≥ 1.5	≤ 10	≥ 30	Percentage tuberculin response is ≥ 30	Positive: <i>M. tuberculosis</i> infection likely

* M – N is the IFN- γ response to mitogen minus the IFN- γ response to nil antigen.

† T – N is the IFN- γ response to purified protein derivative from *M. tuberculosis* minus the IFN- γ response to nil antigen; this must be ≥ 1.5 IU/mL for a patient to be considered QuantiFERON-TB–positive for *M. tuberculosis* infection. If T – N < 1.5 IU/mL, the persons are deemed negative for *M. tuberculosis* infection, regardless of their percentage tuberculin response and percentage avian difference results.

§ A percentage tuberculin response cut-off of 15% is used for persons with identified risk for tuberculosis infection, whereas a cut-off of 30% is used for persons with no identified risk factors.

The role of QFT in targeted testing has not yet been defined, but QFT can be considered for LTBI screening as follows:

- initial and serial testing of persons with an increased risk for LTBI (e.g., recent immigrants, injection-drug users, and residents and employees of prisons and jails);
- initial and serial testing of persons who are, by history, at low risk for LTBI but whose future activity might place them at increased risk for exposure, and others eligible for LTBI surveillance programs (e.g., health-care workers and military personnel); or
- testing of persons for whom LTBI screening is performed but who are not considered to have an increased probability of infection (e.g., entrance requirements for certain schools and workplaces).

Before QFT testing is contemplated, arrangements should be made with a qualified laboratory. Those arrangements should include quality assurance and collection and transport of blood within the required 12 hours.

Confirmation of QFT results with TST is possible because performance of QFT does not affect subsequent QFT or TST results. The probability of LTBI is greatest when both the QFT and TST are positive. Considerations for confirmation are as follows:

- When the probability of LTBI is low, confirmation of a positive QFT result with TST is recommended before initiation of LTBI treatment. LTBI therapy is not recommended for persons at low risk who are QFT-negative or who are QFT-positive but TST-negative.
- TST can also be used to confirm a positive QFT for persons at increased risk for LTBI. However, the need for LTBI treatment when QFT is positive and the subsequent TST is negative should be based on clinical judgment and perceived risk.
- Negative QFT results do not require confirmation, but results can be confirmed with either a repeat QFT or TST if the accuracy of the initial test is in question.

Contraindications

Because of insufficient data on which to base recommendations, QFT is not recommended for

- evaluation of persons with suspected tuberculosis. Active tuberculosis is associated with suppressed IFN- γ responses, and in prior studies, fewer persons with active TB had positive QFT results than TST results. The degree of suppression appears to be related to the severity of disease and the duration of therapy. Studies are under way that compare the sensitivity of QFT and TST among persons with untreated active TB.
- assessment of contacts of persons with infectious tuberculosis, because rates of conversion of QFT and TST af-

ter a known exposure to *M. tuberculosis* have not been compared, and concordance of QFT and TST after exposure and with serial LTBI screening have not been studied.

- screening of children aged <17 years, pregnant women, or for persons with clinical conditions that increase the risk for progression of LTBI to active TB (e.g., human immunodeficiency virus infection). Further studies are needed to define the appropriate use of QFT among these persons.
- detection of LTBI after suspected exposure (i.e., contact investigation after a resident or employee is diagnosed with active TB or a laboratory spill of *M. tuberculosis*) of persons participating in longitudinal LTBI surveillance programs. The approach of using QFT for initial screening, followed by QFT and TST 3 months after the end of the suspected exposure, has not been evaluated.
- confirmation of TST results because injection of PPD for TST might affect subsequent QFT results. Although QFT is not recommended for confirmation of TST results, QFT can be used for surveillance <12 months after a negative TST, if the initial QFT is negative.
- diagnosis of *M. avium* complex disease.

Conclusions

These interim recommendations are intended to achieve a high rate of acceptance and completion of testing for LTBI among groups who have been identified for targeted testing. Testing programs using TST or QFT should only be implemented if plans are also in place for the necessary follow-up medical evaluation and treatment (e.g., chest radiograph or LTBI treatment) of persons who are diagnosed with LTBI and quality laboratory services are ensured.

References

1. Food and Drug Administration, Center for Devices and Radiological Health. QuantiFERON[®]-TB - P010033 [Letter]. Rockville, MD: Food and Drug Administration, 2002. Available at <http://www.fda.gov/cdrh/pdf/P010033b.pdf>.
2. Mazurek GH, LoBue PA, Daley CL, et al. Comparison of a whole-blood interferon gamma assay with tuberculin skin testing for detecting latent *Mycobacterium tuberculosis* infection. *JAMA* 2001;286:1740–7.
3. Pottumarthy S, Morris AJ, Harrison AC, Wells VC. Evaluation of the tuberculin gamma interferon assay: potential to replace the Mantoux skin test. *J Clin Microbiol* 1999;37:3229–32.
4. Streeton JA, Desem N, Jones SL. Sensitivity and specificity of a gamma interferon blood test for tuberculosis infection. *Int J Tuberc Lung Dis* 1998;2:443–50.
5. Bellete B, Coberly J, Barnes GL, et al. Evaluation of a whole-blood interferon-gamma release assay for the detection of *Mycobacterium tuberculosis* infection in 2 study populations. *Clin Infect Dis* 2002;34:1449–56.
6. CDC. Targeted tuberculin testing and treatment of latent tuberculosis infection. *MMWR* 2000;49(No. RR-6):1–51.

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