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1997 Revised Guidelines for Performing CD4+ T-Cell Determinations in Persons Infected with Human Immunodeficiency Virus (HIV)

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1997 Revised Guidelines for Performing CD4+ T-Cell Determinations in Persons Infected with Human Immunodeficiency Virus (HIV)

Summary

*These revised guidelines were developed by CDC for laboratories performing lymphocyte immunophenotyping assays in human immunodeficiency virus-infected persons. This report updates previous recommendations (*MMWR *43[No. RR-3]) and reflects current technology in a field that is rapidly changing. The recommendations address laboratory safety, specimen collection, specimen transport, maintenance of specimen integrity, specimen processing, flow cytometer quality control, sample analyses, data analysis, data storage, data reporting, and quality assurance.*

INTRODUCTION

Accurate and reliable measures of CD4+ T-lymphocytes (CD4+ T-cells) are essential to the assessment of the immune system of human immunodeficiency virus (HIV)-infected persons (*1–3*). The pathogenesis of acquired immunodeficiency syndrome (AIDS) is largely attributable to the decrease in T-lymphocytes that bear the CD4 receptor (*4–8*). Progressive depletion of CD4+ T-lymphocytes is associated with an increased likelihood of clinical complications (*9,10*). Consequently, the Public Health Service (PHS) has recommended that CD4+ T-lymphocyte levels be monitored every 3–6 months in all HIV-infected persons (*11*). The measurement of CD4+ T-cell levels has been used to establish decision points for initiating *Pneumocystis carinii* pneumonia prophylaxis (*12*) and antiviral therapy (*13*) and to monitor the efficacy of treatment (*14–16*). CD4+ T-lymphocyte levels also are used as prognostic indicators in patients who have HIV disease (*17,18*) and recently have been included as one of the criteria for initiating prophylaxis for several opportunistic infections that are sequelae of HIV infection (*19,20*). Moreover, CD4+ T-lymphocyte levels are a criterion for categorizing HIV-related clinical conditions by CDC's classification system for HIV infection and surveillance case definition for AIDS among adults and adolescents (*21*).

Most laboratories measure absolute CD4+ T-cell levels in whole blood by a multiplatform, three-stage process. The CD4+ T-cell number is the product of three laboratory techniques: the white blood cell (WBC) count; the percentage of WBCs that are lymphocytes (differential); and the percentage of lymphocytes that are CD4+ T-cells. The last stage in the process of measuring the percentage of CD4+ T-lymphocytes in the whole-blood sample is referred to as "immunophenotyping by flow cytometry" (*22–28*). Immunophenotyping refers to the detection of antigenic determinants (which are unique to particular cell types) on the surface of WBCs using antigen-specific monoclonal antibodies that have been labeled with a fluorescent dye or fluorochrome (e.g., phycoerythrin [PE] or fluorescein isothiocyanate [FITC]). The fluorochrome-labeled cells are analyzed by using a flow cytometer, which categorizes individual cells according to size, granularity, fluorochrome, and intensity of fluorescence. Size and granularity, detected by light scattering, characterize the types of

WBCs (i.e., granulocytes, monocytes, and lymphocytes). Fluorochrome-labeled antibodies distinguish populations and subpopulations of WBCs. Although flow cytometric immunophenotyping is a highly complex technology, methodology for performing CD4+ T-cell determinations has become more standardized between laboratories. The publication of several sets of guidelines addressing aspects of the CD4+ T-lymphocyte testing process (e.g., quality control, quality assurance, and reagents for flow cytometric immunophenotyping of lymphocytes) has contributed to this standardization (*29–32*).

The CDC guidelines concerning CD4+ T-cell determinations (*33*) were first published in the *MMWR* in 1992 to provide laboratorians with the most complete information about how to measure CD4+ T-lymphocytes in blood from HIV-infected persons by using flow cytometry. These guidelines were based on data from scientific literature, information from discussions with technical experts, and experience with related voluntary standards for flow cytometric analyses (*29*). The 1992 guidelines concluded that more data were needed and that revisions would be published as additional information became available and as important innovations in technology were made. In 1993, a national conference was convened by CDC with sponsorship from the Food and Drug Administration (FDA), National Institutes of Health, and Association of State and Territorial Public Health Laboratory Directors. The objectives of the conference were to review data collected after 1992 and to obtain input about the efficacy of the 1992 guidelines. As a result of the 1993 conference, the revised guidelines for performing CD4+ T-cell determinations in HIV-infected persons were published in 1994 (34).

Since 1994, the field of CD4+ T-cell testing has rapidly expanded. Flow cytometric analyses of T-cell subsets using three- and four-color approaches (in contrast to the two-color approach addressed in previous reports [*35,36*]), flow cytometric analyses for measuring both the proportion and the absolute numbers of CD4+ T-lymphocytes, and other methods for deriving an absolute CD4+ T-cell count in a blood sample are now commercially available. (Some of these other methods do not depend on the multi-stage process and are collectively referred to in this report as single-platform methods.) Moreover, data evaluating some of the parameters of two-color flow cytometric testing and the routine testing practices of laboratories that provide these testing services have been collected. A second national conference on CD4+ T-lymphocyte immunophenotyping was held in Atlanta, Georgia, on December 12–13, 1995, to discuss these changes. Information shared at the conference and new data collected about laboratory testing practices serve as the basis for the revisions and additions that have been made to the 1994 guidelines. These changes include a) quality assurance (namely, revision of the recommended monoclonal panel to provide a cost-effective solution for laboratories using three-color and four-color approaches), b) the importance of following manufacturers' instructions when using tests and testing devices approved by the FDA, c) recommendations for laboratories performing three- and four-color T-lymphocyte immunophenotyping (TLI), and d) recommendations about the validation and verification procedures that laboratories should conduct before implementing new tests.

RECOMMENDATIONS

I. Laboratory Safety

A. Use universal precautions with all specimens (*37*).

B. Establish the following safety practices (*38–44*):

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., aliquoting, adding reagents, vortexing, and aspirating) 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. Add a volume of undiluted household bleach (5% sodium hypochlorite) to the waste container before adding waste materials 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 fluidics with a 10% bleach solution for 5–10 minutes at the end of the day, 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. For specific procedures about how areas should be disinfected, see reference 44. For use on smooth, hard surfaces, a 1% solution of bleach is usually adequate for disinfection; for porous surfaces, a 10% solution is needed (*44*).

11. Assure that all samples have been properly fixed after staining and lysing, but before analysis. **Note:** Some commercial lysing/fixing reagents will reduce the infectious activity of cell-associated HIV by 3–5 logs (*45*); however, these reagents have not been evaluated for their effectiveness against other agents (e.g., hepatitis virus). Buffered (pH 7.0–7.4) 1%–2% paraformaldehyde or formaldehyde can inactivate cell-associated HIV to approximately the same extent (*45–48*). Cell-free HIV can be inactivated with 1% paraformaldehyde within 30 minutes (*49*). Because the commercial lysing/fixing reagents do not completely inactivate cell-associated HIV and the time frame for complete inactivation is not firmly established, stained and lysed samples should be resuspended and retained in fresh 1%–2% paraformaldehyde or formaldehyde through flow cytometric analysis.

II. Specimen Collection

A. Select the appropriate anticoagulant for hematologic testing and flow cytometric immunophenotyping.

1. Anticoagulant for hematologic testing:

   a. Use tripotassium ethylenediamine tetra-acetate (K_3EDTA, 1.5 ± 0.15 mg/mL blood) (*50,51*), and perform the test within the time frame allowed by the manufacturer of the hematology analyzer, not to exceed 30 hours.

   b. Reject a specimen that cannot be processed within this time frame unless the hematology instrumentation is suitable for analyzing such specimens. **Note**: Some hematology instruments are capable of generating accurate results 12–30 hours after specimen collection (*52*). To ensure accurate results for specimens from HIV-infected persons, laboratories must validate their hematology instrument's ability to give the same result at time 0 and at the maximum time claimed by the manufacturer when using specimens from both persons infected with HIV and those not infected.

2. Anticoagulant for flow cytometric immunophenotyping, depending on the delay anticipated before sample processing:

   a. Use K_3EDTA, acid citrate dextrose (ACD), or heparin if specimens will be processed within 30 hours after collection. **Note**: K_3EDTA should not be used for specimens held for >30 hours before testing because the proportion of some lymphocyte populations changes after this period (*53*).

b. Use either ACD or heparin, not K_3EDTA, if specimens will be processed within 48 hours after specimen collection.

c. Reject a specimen that cannot be processed within 48 hours after specimen collection and request another.

B. Collect blood specimens by venipuncture (*54*) into evacuated tubes containing an appropriate anticoagulant, completely expending the vacuum in the tubes.

1. Draw specimens from children in pediatric tubes to avoid underdrawing.

2. Mix the blood well with the anticoagulant to prevent clotting.

C. Draw the appropriate number of tubes:

1. Use one tube containing K_3EDTA when a) hematology and flow cytometric immunophenotyping will be performed in the same laboratory on the same specimen or b) a single measurement is performed on the flow cytometer that results in an absolute number. **Note**: For single-platform methods that do not use determinations from a hematology analyzer or from conventional flow cytometers to derive absolute CD4+ T-cell numbers, follow the manufacturer's recommendations for anticoagulant and maximum times between specimen collection and testing.

2. In all other circumstances, draw two separate tubes (K_3EDTA for hematologic determinations and K_3EDTA, ACD, or heparin for flow cytometric immunophenotyping).

D. Label all specimens with the date, time of collection, and a unique patient identifier.

1. Assure that patient information and test results are accorded confidentiality.

2. Provide on the submission form pertinent medications and disease conditions that may affect the immunophenotyping test (Appendix).

III. Specimen Transport

A. Maintain and transport specimens at room temperature (64–72 F [18–22 C]) (*52,55–57*). Avoid extremes in temperature so that specimens do not freeze or become too hot. Temperatures >99 F (37 C) may cause cellular destruction and affect both hematology and flow cytometry measurements (*52*). In hot weather, packing the specimen in an insulated container and placing this container inside another containing an ice pack and absorbent material may be necessary. This method helps retain the specimen at ambient

temperature. The effect of cool temperatures (i.e., 39 F [4 C]) on immunophenotyping results is not clear (*52,57*).

B. Transport specimens to the immunophenotyping laboratory as soon as possible.

C. For transport to locations outside the collection facility but within the state, follow state or local guidelines. One method for packaging such specimens is to place the tube containing the specimen in a leak-proof container (e.g., a sealed plastic bag) and to pack this container inside a cardboard canister containing sufficient material to absorb all the blood 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. **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 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 (*52*). Abnormalities in light-scattering patterns will 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 >48 hours old (from the time of draw), reject it and request another.

V. Specimen Processing

A. Hematologic testing

*49 CFR parts 100–171 (56 FR 47158).

1. Perform the hematologic tests within the time frame specified by the manufacturer of the specific hematology instrument used (time from blood specimen draw to hematologic test). (See Note under II.A.1.b.)

2. Perform an automated WBC count and differential, counting 10,000–30,000 cells (*58*). If the specimen is rejected or "flagged" by the instrument, a manual differential of at least 400 cells can be performed. If the flag is not on the lymphocyte population and the lymphocyte differential is reported by the instrument, the automated lymphocyte differential should be used.

3. If absolute counts are determined by using a single-platform method, hematology results are not needed for this determination.

B. Immunophenotyping

1. For optimal results, perform the test within 30 hours, but no later than 48 hours, after drawing the blood specimen (*59,60*).

2. When centrifuging, maintain centrifugation forces of no greater than 400 g for 3–5 minutes for wash steps.

3. Vortex sample tubes to mix the blood and reagents and break up cell aggregates. Vortex samples immediately before analysis to optimally disperse cells.

4. Include a source of protein (e.g., fetal bovine serum or bovine serum albumin) in the wash buffer to reduce cell clumps and non-specific fluorescence.

5. Incubate all tubes in the dark during the immunophenotyping procedure.

6. Before analysis on the flow cytometer, be sure all samples have been adequately fixed. Although some of the commercial lysing/fixing reagents can inactivate cell-associated HIV, all tubes should be fixed after staining and lysing with 1%–2% buffered paraformaldehyde or formaldehyde. **Note**: The characteristics of paraformaldehyde and formaldehyde may vary between lots. They may also lose their effectiveness over time. Therefore, these fixatives should be made fresh weekly from electron-microscopy–grade aqueous stock.

7. Immediately after processing the specimens, store all stained samples in the dark and at refrigerator temperatures (39–50 F [4–10 C]) until flow cytometric analysis. These specimens should be stored for no longer than 24 hours unless the laboratory can demonstrate that scatter and fluorescence patterns do not change for specimens stored longer.

8. If absolute counts are determined on the flow cytometer, follow the manufacturer's recommended protocols.

VI. Monoclonal Antibody Panels

A. Monoclonal antibody panels must contain appropriate monoclonal antibody combinations to enumerate CD4+ and CD8+ T-cells and to ensure the quality of the results (*61*).

1. CD4 T-cells must be identified as being positive for both CD3 and CD4.

2. CD8 T-cells must be identified as being positive for both CD3 and CD8.

B. Two-color monoclonal antibody panels

1. The recommended two-color immunophenotyping antibody panel (Table 1), delineated by CD nomenclature (*62*) and fluorochrome, provides data useful for defining the T-cell population and subpopulations; determining the recovery and purity of the lymphocytes in the gate; setting cursors for positivity; accounting for all lymphocytes in the sample; monitoring tube-to-tube variability; and monitoring T-cell, B-cell, and natural killer (NK)-cell levels. The following internal controls are included in the panel:

   a. CD3 Monoclonal antibody in tubes 3–6 serves as a control for tube-to-tube variability and is also used to determine T-cell populations. **Note**: All CD3 values in this six-tube panel should be within 3% of each other. If the CD3 value of a tube is >3% of any of the others, that tube should be repeated (i.e., new aliquot of blood labeled, lysed, and fixed).

   b. Monoclonal antibodies that label T-cells, B-cells, and NK-cells are used to account for all lymphocytes in the specimen (*61*).

TABLE 1. Recommended two-color monoclonal antibody panel

Tube no.	Fluorescein isothiocyanate (FITC)	Phycoerythrin (PE)	Cell populations identified
1	CD45	CD14	Lymphocytes, monocytes, and granulocytes
2	Isotype	Isotype	—
3	CD3	CD4	CD4+ T-cells
4	CD3	CD8	CD8+ T-cells
5	CD3	CD19	T-cells, B-cells
6	CD3	CD16 and/or CD56	T-cells, NK-cells

2. An abbreviated two-color panel should only be used for testing specimens from patients for whom CD4+ T-cell levels are being requested as part of sequential follow-up, and then only after consulting with the requesting clinician. Because some of the internal controls are no longer included, when using an abbreviated panel, the immunophenotyping results should be reviewed carefully to ensure that CD3+ T-cell levels are similar to those determined previously with the full recommended panel. When discrepancies occur, the specimens must be reprocessed by using the full recommended two-color monoclonal antibody panel.

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 lymphocyte gate used, and provide some assessment of tube-to-tube variability.

2. For determining T-cell subset percentages, the third color should be used to identify lymphocytes by following one of two procedures (Table 2):

   a. Use CD45 as the third color to identify lymphocytes as those cells that are bright CD45+ but have low side scattering properties. In this case, the panel would consist of the following monoclonal antibodies: CD3/CD4/CD45; CD3/CD8/CD45; and CD3/CD19/CD45 (Table 2, Panel A).

   b. Use lineage markers (T-cell, B-cell, and NK-cell) to identify lymphocytes (*63*). The panel would consist of the following monoclonal

TABLE 2. Three-color monoclonal antibody panels*

Panel	Monoclonal antibodies	Notes
A	CD3/CD4/CD45	Gate on CD45 and side scatter; measure CD3+CD4+ T-cells
	CD3/CD8/CD45	Gate on CD45 and side scatter; measure CD3+CD8+ T-cells
	CD3/CD19/CD45†	Gate on CD45 and side scatter; measure CD3+ and CD19+ T-cells
B§	CD3/CD19/CD16 and/or CD56	For absolute counts of T-, B-, and NK-cells
	CD3/CD4/CD8	To determine CD3+, CD3+CD4+, and CD3+CD8+ cells
	Isotype control	For nonspecific fluorescence

*Suggested three-color panels. (See Section VI.C.)
†Recommended for specimens obtained from children (32).
§This panel may be used for systems determining absolute cell numbers directly from the flow cytometer. Percentage determinations are calculated from the absolute numbers. (See Section VI.C.2.b.)

antibodies: CD3/CD19/CD16 and/or CD56; CD3/CD4/CD8; and an isotype control (Table 2, Panel B). **Note**: Software on the flow cytometer must be capable of using the information obtained from these monoclonal antibody combinations to correctly identify lymphocytes and to extrapolate that information to determine the percentage of lymphocytes that are CD4+ and CD8+ T-cells (*63*). **Note**: A single tube containing CD3, CD4, and CD8 monoclonal antibodies is not appropriate for determining the percentage of lymphocytes that are CD4+ or CD8+ T-cells because there is no method to validate the lymphocyte gate in this tube without the addition of another tube for that purpose. Lymphocyte gate purity and recovery cannot be determined. Internal quality control measures may be obtained by adding another tube containing CD3 (e.g., CD3, CD19, and CD16 and/or CD56).

3. A three-color monoclonal antibody panel must consist of at least two tubes, each with the same lineage marker. For the examples above, CD3 is the common lineage marker in each tube. Differences between replicate CD3 results should be ≤2%. **Note:** The variability of a CD3 result between two tubes is approximately half that of four tubes.

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. A four-color monoclonal antibody panel must consist of at least two tubes, each with the same lineage marker. A second tube containing CD45, CD3, CD19, and CD16 and/or CD56 is recommended.

VII. Negative and Positive Controls for Immunophenotyping

A. Negative (isotype) reagent control

1. Use this control to determine nonspecific binding of the mouse monoclonal antibody to the cells and to set markers for distinguishing fluorescence-negative and fluorescence-positive cell populations.

2. Use a monoclonal antibody with no specificity for human blood cells but of the same isotype(s) as the test reagents. **Note**: In many cases, the isotype control may not be optimal for controlling nonspecific fluorescence because of differences in F/P ratio, antibody concentration between the isotype control and the test reagents, and other characteristics of the immunoglobulin in the isotype control. Additionally, isotype control reagents from one manufacturer are not appropriate for use with test reagents from another manufacturer.

3. The isotype control is not needed for use with CD45 because CD45 is used to identify leukocyte populations based on fluorescence intensity.

4. For monoclonal antibody panels containing antibodies to CD3, CD4, and CD8, the isotype control may not be needed because labeling with these antibodies results in fluorescence patterns in which the unlabeled cells are clearly separated from the labeled cells. In these instances, the negative cells in the histogram are the appropriate isotype control.

5. The isotype control must be used when a monoclonal antibody panel contains monoclonal antibodies that label populations that do not have a distinct negative population (e.g., some CD16 or CD56 monoclonal antibodies).

B. Positive methodologic control

1. The methodologic control is used to determine whether procedures for preparing and processing the specimens are optimal. This control is prepared 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. Ideally, this control will match the population of patients tested in the laboratory. (See Section XII.D.)

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.

C. Positive control for testing reagents

1. Use this 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 lots of reagents 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).

VIII. Flow Cytometer Quality Control (*29*)

A. Align optics daily. This ensures that the brightest and tightest peaks are produced in all parameters. **Note**: Some clinical flow cytometers can be aligned by laboratory personnel whereas others can be aligned only by qualified service personnel.

1. Align the flow cytometer by using stable calibration material (e.g., microbeads labeled with fluorochromes) that has measurable forward scatter, side scatter, and fluorescence peaks.

2. Align the calibration particles optimally in the path of the laser beam and in relation to the collection lens so the brightest and tightest peaks are obtained.

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 daily. This ensures that the flow cytometer is performing optimally each day and that its performance is the same from day to day.

1. Select machine settings that are optimal for fluorochrome-labeled, whole-blood specimens.

2. Use microbeads or other stable standardization material to place the scatter and fluorescence peaks in the same scatter and fluorescence channels each day. Adjust the flow cytometer as needed.

3. Maintain records of all daily standardizations. Monitor these to identify any changes in flow cytometer performance.

4. 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.

1. Evaluate standardization/calibration material or cells that have low-level fluorescence that can be separated from autofluorescence (e.g., microbeads with low-level and negative fluorescence or CD56-labeled lymphocyte preparation).

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

D. Compensate for spectral overlap daily. This step corrects the spectral overlap of one fluorochrome into the fluorescence spectrum of another.

1. Use either microbead or cellular compensation material containing three populations for two-color immunofluorescence (no fluorescence, PE fluorescence only, and FITC fluorescence only), four populations for three-color immunofluorescence (the three above plus a population that is positive for only the third color), or five populations for four-color (the four above plus 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). If three fluorochromes are used, compensation must be carried out in an appropriate sequence: FITC, PE, and the third color, respectively (*64*). For four-color monoclonal antibody panels, follow the flow cytometer manufacturer's instructions for four fluorochromes. Avoid over-compensation.

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) that recognize separate cell populations but do not overlap. These populations should have the brightest expected signals. **Note**: If a dimmer-than-expected signal is used to set compensation, suboptimal compensation for the brightest signal can result.

4. Reset compensation when photomultiplier tube voltages or optical filters are changed.

E. Repeat all four instrument quality control procedures whenever instrument problems occur or if the instrument is serviced during the day.

F. Maintain instrument quality-control logs, and monitor them continually for changes in any of the parameters. In the logs, record instrument settings, peak channels, and coefficient of variation (CV) values for optical alignment, standardization, fluorescence resolution, and spectral compensation. Re-establish fluorescence levels for each quality-control procedure when lot numbers of beads are changed.

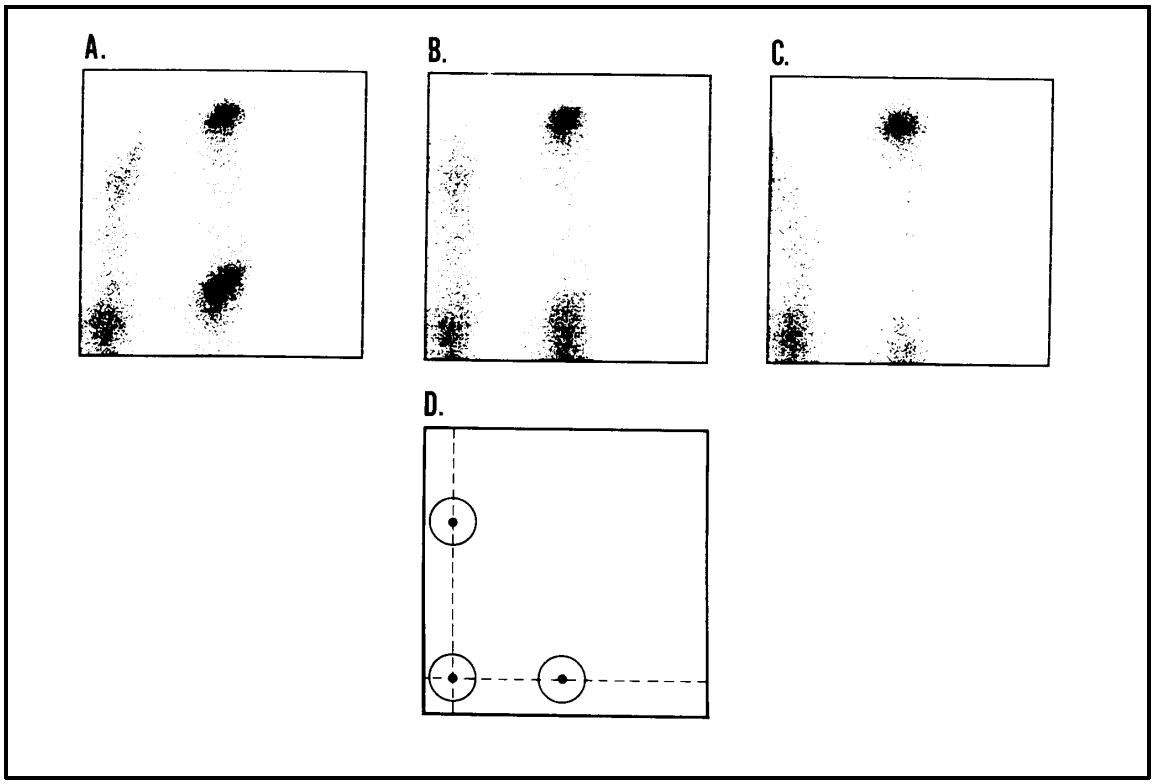
IX. Sample Analyses

A. For the two-color immunophenotyping panel using a light-scatter gate, analyze the sample tubes of each patient's specimen in the following order: 1) The tube containing CD45 and CD14 (gating reagent): read this tube first so that gates can be set around the lymphocyte cluster; 2) Isotype control: set cursors for differentiating positive and negative populations so that ≤2% of the cells are positive; and 3) Remaining tubes in the panel.

1. Count at least 2,500 gated lymphocytes in each sample. This number ensures with 95% confidence that the result is ≤2% standard deviation (SD) of the "true" value (binomial sampling). **Note**: This model assumes that variability determined from preparing and analyzing replicates is ≤2% SD. Each laboratory must determine the level of variability by preparing and analyzing at least eight replicates of the last four tubes in the recommended panel. Measure variability when first validating the methodology used and again when methodologic changes are made.

2. Examine light-scattering patterns on each sample tube. Determine whether lysis or sample preparation, which can affect light scattering, is the same in each sample tube of a patient's specimen. Deviation in a particular tube usually indicates sample preparation error, and the tube should be repeated (i.e., a new aliquot of blood should be stained and lysed).

FIGURE 1. Determination of appropriate compensation for spectral overlap*



*A dot plot analysis of a specimen labeled with fluorescein isothiocyanate (FITC)-CD3 (horizontal axis, green fluorescence) and phycoerythrin (PE)-CD8 (vertical axis, orange fluorescence) demonstrates undercompensation (Panel A), proper compensation (Panel B), and overcompensation (Panel C). Imaginary lines intersecting the peaks of green-only or orange-only fluorescence and the negative control should be parallel to each axis (example, Panel D). Note that this combination of antibodies (from the suggested monoclonal antibody panel) gives the brightest signals for each fluorochrome and may be used for verifying spectral compensation.

B. For three- or four-color monoclonal antibody panels using a CD45/side scatter gate, determine the lymphocyte population based on bright CD45 fluorescence and low side scattering properties. Draw a gate on this population and analyze the cell populations using this gate (*65*).

X. Data Analysis

A. Light-scatter gate (for two-color panels).

1. Reading from the sample tube containing CD45 and CD14, draw lymphocyte gates using forward and side light-scattering patterns and fluorescence staining.

   a. When using CD45 and CD14 and light-scattering patterns for drawing lymphocyte gates, define populations on the following basis:

      - Lymphocytes stain brightly with CD45 and are negative for CD14.
      - Monocytes and granulocytes have greater forward and side light-scattering properties than lymphocytes.
      - Monocytes are positive for CD14 and have intermediate to high intensity for CD45.
      - Granulocytes are dimly positive for CD14 and show less intense staining with CD45.
      - Debris, red cells, and platelets show lower forward scattering than lymphocytes and do not stain specifically with CD45 or CD14.

   b. Using the above characteristics, draw a light-scattering gate around the lymphocyte population (*66*). **Note**: Other methods for drawing a lymphocyte gate must accurately identify lymphocytes and account for non-lymphocyte contamination of the gate.

2. Verify the lymphocyte gate by determining the recovery of lymphocytes within the gate and the lymphocyte purity of the gate.

   a. Definitions

      - The lymphocyte recovery (previously referred to as the proportion of lymphocytes within the gate) is the percentage of lymphocytes in the sample that are within the gate.
      - The lymphocyte purity of the gate is the percentage of cells within the gate that are lymphocytes. The remainder may be monocytes, granulocytes, red cells, platelets, and debris.

   b. Optimally, the lymphocyte recovery should be ≥95%.

   c. Optimally, the lymphocyte purity of the gate should be ≥90%.

d. Optimal gates include as many lymphocytes and as few contaminants as possible.

e. Lymphocyte recovery within the gate using CD45 and CD14 can be determined by two different methods: light-scatter gating and fluorescence gating (Figures 2 and 3). **Note**: The number of lymphocytes identified will be the same whether determined by light-scatter gating or by fluorescence gating.

- Lymphocyte recovery determined by light-scatter gating is done as follows: first, identify the lymphocytes by setting a relatively large light-scatter gate (Figure 2, Panel A), then set an analysis region around CD45 and CD14 lymphocyte reactivity (bright CD45-positive, negative for CD14) (Figure 2, Panel B). Determine the number of cells that meet both criteria (total number of lymphocytes). Set a smaller lymphocyte light-scatter gate that will be used for analyzing the remaining tubes (Figure 2, Panel C). Determine the number of cells that fall within this gate and the CD45/ CD14 analysis region (bright CD45-positive, negative for CD14) (Figure 2, Panel D). This number divided by the total number of lymphocytes times 100 is the lymphocyte recovery. The advantage of this method is that it can easily be done on most software programs.
- Lymphocyte recovery determined by fluorescence gating is done as follows. First, identify lymphocytes by setting a fluorescence gate around the bright CD45-positive, CD14-negative cells (Figure 3, Panel A), then set an analysis region around a large light-scatter region that includes lymphocytes (Figure 3, Panel B). The number of cells that meet both criteria is the total number of lymphocytes. Set a smaller lymphocyte light-scatter gate that will be used for analyzing the remaining tubes (Figure 3, Panel C). Determine the number of cells that fall within this gate and the CD45/CD14 analysis region (bright CD45+, negative for CD14) (Figure 3, Panel D). This number divided by the total number of lymphocytes times 100 is the lymphocyte recovery. The advantage of this method is that the light-scatter pattern of lymphocytes can be easily determined. **Note**: Some instrument software packages automatically determine lymphocyte recovery by fluorescence gating; others do not.

f. The lymphocyte purity of the gate is determined from the CD45 and CD14 tube by calculating the percentage of cells in the light-scattering gate that are bright CD45-positive and negative for CD14.

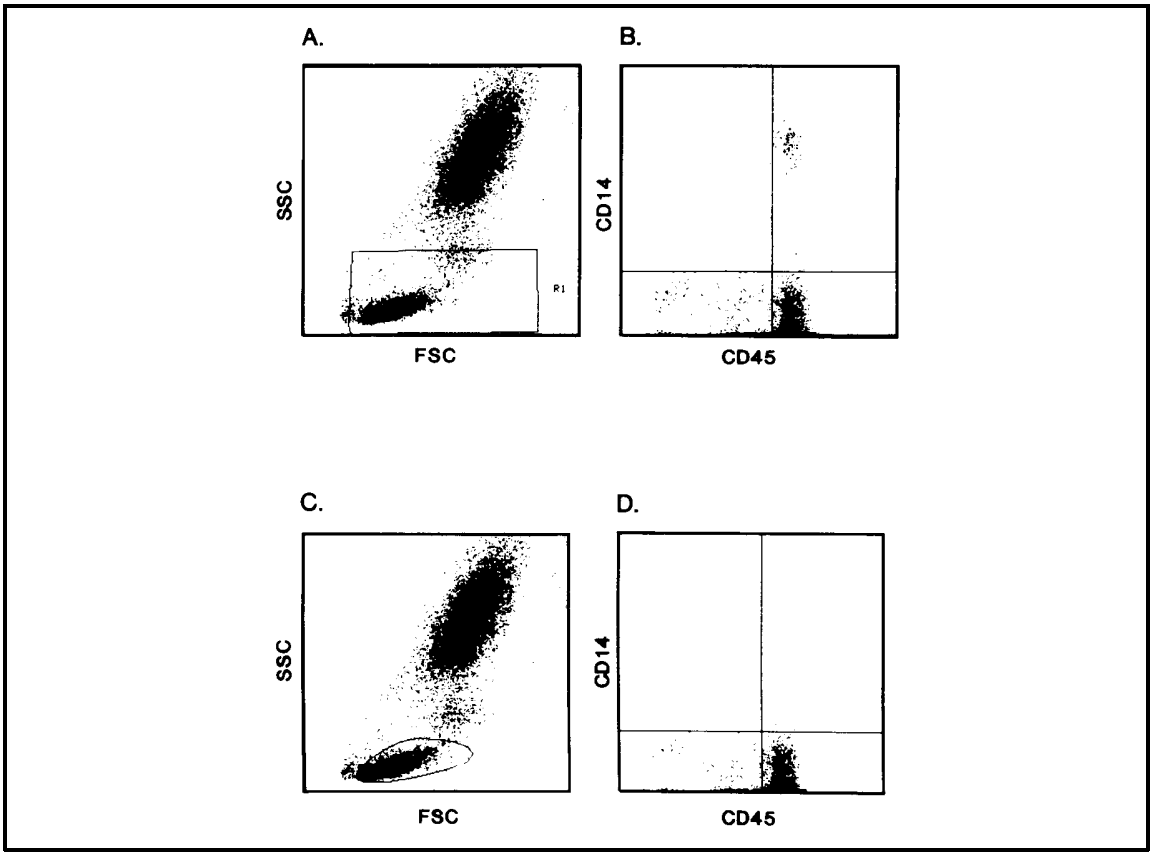
g. If the recommended recovery and purity of lymphocytes within the gate cannot be achieved, redraw the gate. If minimum levels still cannot be obtained, reprocess the specimen. If this fails, request another specimen.

B. CD45 gating (for three- and four-color monoclonal panels)

1. Identify lymphocytes as cells brightly labeled with CD45 and having low side scattering properties.

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. B-cells may have slightly less CD45 fluorescence than the 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.

FIGURE 2. Light-scatter gating technique for determining lymphocyte recovery and purity*



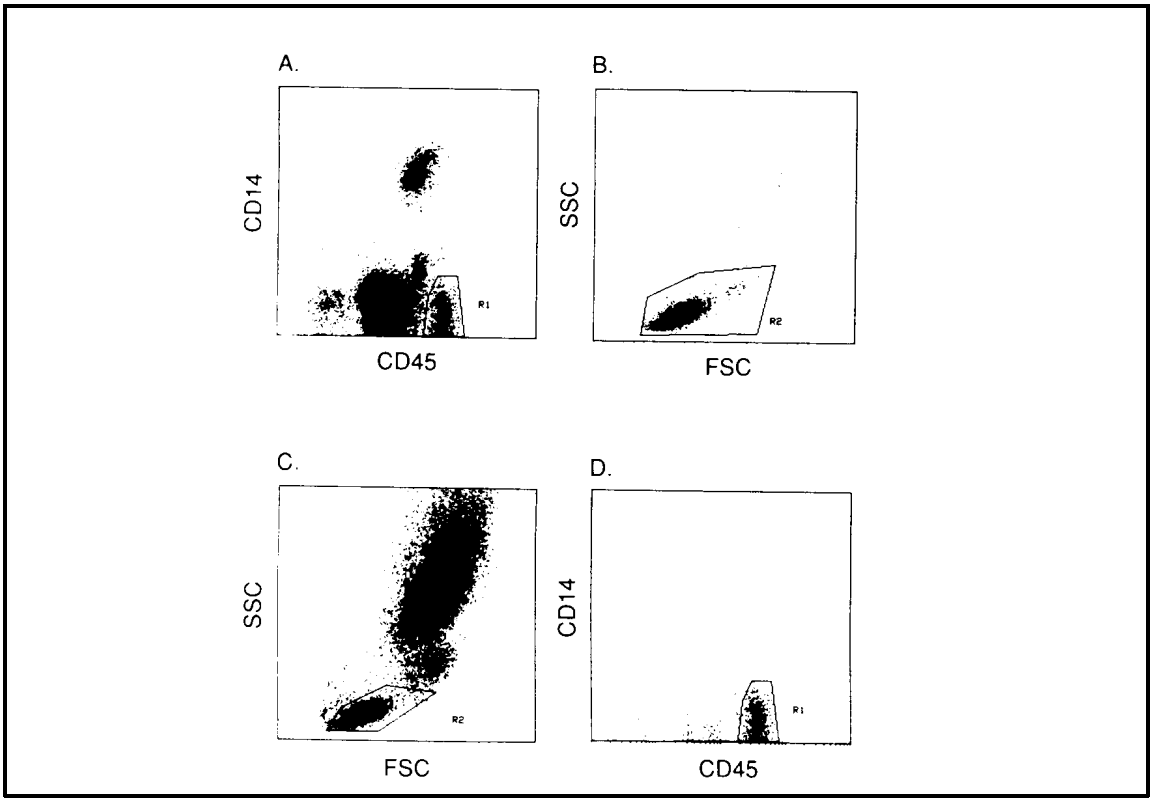
*Panel A shows a large light-scatter region drawn around the lymphocytes (R1). Panel B is the two-parameter histogram of CD45/CD14 fluorescence gated on R1. The number of cells in quadrant 4 (bright CD45-positive, negative for CD14) is the total number of lymphocytes (3,951 cells). Panel C is the same light-scatter histogram as Panel A except that a smaller light-scatter gate has been drawn, which will be used for analyzing the remaining tubes from that specimen. Panel D is the CD45/CD14 histogram from gated R2. The number of gated lymphocytes is in quadrant 4 (3,889 cells). The total number of gated cells in R2 is 4,298. From these numbers, the lymphocyte recovery is (3,889/3,951) x 100, or 98.4%. The lymphocyte purity is (3,889/4,298) x 100, or 90.5%.

3. CD45/side scatter gates for lymphocytes are assumed to contain >95% lymphocytes, and no further corrections need be made to the percentage subset results (*65*).

4. Lymphocyte recovery cannot be determined without using a panel of monoclonal antibodies that identify T-, B-, and NK-cells. **Note**: Validation of a CD45/side scatter gate is recommended when beginning to use CD45/ side scatter gates to help determine the CD45 and side scatter characteristics of T-, B-, and NK-cells and to ensure their inclusion in the gate.

C. Set cursors using the isotype control so that <2% of cells are positive. **Note**: If an isotype control is not used, set cursors based on the tube containing CD3 and CD4 so that the negative and positive cells in the histogram are clearly separated. These cursors may be used for the remaining tubes. If

FIGURE 3. Fluorescence gating technique for determining lymphocyte recovery and purity*



*Panel A shows a region (R1) drawn around the lymphocytes identified by fluorescence (bright CD45-positive, negative for CD14). Panel B is the two-parameter light-scatter histogram gated on R1. A relatively large light-scatter region (R2) is set around the lymphocytes. The number of cells in this region is the total number of lymphocytes (3,846). Panel C shows the light-scatter histogram with a smaller light-scatter gate drawn (R2), which will be used for analyzing the remaining tubes from that specimen. Panel D is the CD45/CD14 histogram from gated R2, Panel C. The number of gated lymphocytes is in R1 (3,771 cells). The total number of gated cells in R2, Panel C is 4,139. From these numbers, the lymphocyte recovery is (3,771/3,846) x 100, or 98.0%. The lymphocyte purity is (3,771/4,139) x 100, or 91.1%.

CD16 and/or CD56 are included in a monoclonal antibody panel, an isotype control may be needed to help identify negative cells.

D. Analyze the remaining samples with the cursors set. **Note**: In some instances, the isotype-set cursors will not accurately separate positive and negative staining for another sample tube from the same specimen. In such cases, the cursors can be moved on that sample to more accurately separate these populations. The cursors should not be moved when fluorescence distributions are continuous with no clear demarcation between positively and negatively labeled cells.

E. Analyze each patient or control specimen with lymphocyte gates and cursors for positivity set for that particular patient or control.

F. When spectral compensation of a particular specimen appears to be inappropriate because FITC-labeled cells have been dragged into the PE-positive quadrant or vice-versa (when compensation on all other specimens is appropriate) (*67*), repeat the sample preparation, prewashing the specimen with phosphate-buffered saline (PBS) (pH 7.2) to remove plasma before monoclonal antibodies are added.

G. Include the following analytic reliability checks, when available:

1. Optimally, at least 95% lymphocyte recovery (proportion of lymphocytes within the lymphocyte gate) should be achieved. Minimally, at least 90% lymphocyte recovery should be achieved. **Note**: These determinations can only be made when using either CD14 and CD45 to validate the gate or when using T, B, and NK reagents to validate a gate.

2. Optimally, ≥90% lymphocyte purity should be observed within the lymphocyte gate. Minimally, ≥85% purity should be observed within the gate.

3. Optimally, the sum of the percentage of CD3+CD4+ and CD3+CD8+ cells should equal the total percentage of CD3+ cells within ±5%, with a maximum variability of ≤10%. **Note**: In specimens containing a considerable number of T γδ cells (*68,69*), this reliability check may exceed the maximum variability.

4. Optimally, the sum of the percentage of CD3+ (T-cells), CD19+ (B-cells), and CD3-(CD16 and/or CD56)+ (NK-cells) should equal the purity of lymphocytes in the gate ±5% (*61*), with a maximum variability of ≤10%. If the data are corrected for lymphocyte purity (see XII.B.), the sum should ideally equal 95%–105% (or at a minimum 90%–110%).

XI. Data Storage

A. If possible, store list-mode data on all specimens analyzed. This allows for reanalysis of the raw data, including redrawing of gates. At a minimum, retain hard copies of the lymphocyte gate and correlated dual histogram data of the fluorescence of each sample.

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. If using light-scatter gates, report data as a percentage of the total lymphocytes and correct for the lymphocyte purity of the gate. For example, if the lymphocyte purity is 94% and the CD3 value is 70%, correct the CD3 value by dividing 0.70 by 0.94 and then multiply the result by 100 to result in a T-lymphocyte value of 74%.

C. Report absolute lymphocyte subset values when an automated complete blood cell (CBC) count (WBC and differential) has been performed from blood drawn at the same time as that for immunophenotyping.

1. Calculate the absolute values by multiplying the lymphocyte subset percentage (from flow cytometry data) by the absolute number of lymphocytes (from WBC and differential). **Note**: The hematology laboratory providing the CBC (WBC and differential) must perform satisfactorily in a hematology proficiency testing program approved by the Health Care Finance Administration (HCFA) as meeting the requirements of the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88).*

2. Report both percentages and absolute counts when these are available. **Note:** If absolute counts are determined directly on the flow cytometer, report these results.

*42 CFR part 493 § 493.801–493.865.

D. Report data from all relevant monoclonal antibody combinations with corresponding reference limits of expected normal values (e.g., CD4+ T-cell percentage and absolute number of CD4+ T-cells). Reference limits for immunophenotyping test results must be determined for each laboratory (*29*). Separate reference ranges must be established for adults and children, and the appropriate ranges must be used for patient specimens.

XIII. Quality Assurance

A. Assure 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:

1. Methods for collecting, handling, transporting, identifying, processing, and storing specimens.
2. Information provided on test request and results report forms.
3. Instrument performance, quality-control protocols, and maintenance.
4. Reagent quality-control protocols.
5. Process for reviewing and reporting results.
6. Employee training and education, which should consist of:

    a. Basic training by flow cytometer manufacturers and additional training in hands-on workshops for flow cytometer operators and supervisors.

    b. Education of laboratory directors in flow cytometric immunophenotyping through workshops and other programs.

    c. Continuing education in new developments for all flow cytometric immunophenotyping personnel through attendance at meetings and workshops.

    d. Adherence to federal and state regulations for training and education.

7. Assurance of satisfactory performance. Laboratories must successfully participate in a performance evaluation program. When proficiency testing programs are approved by HCFA as meeting the requirements of CLIA '88 (none are currently approved for CD4+ T-cell testing), laboratories must satisfactorily participate.

8. Review and revision (as necessary, or at established intervals) of the laboratory's policies and procedures to assure 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 the corrective actions should be taken to prevent recurrences.

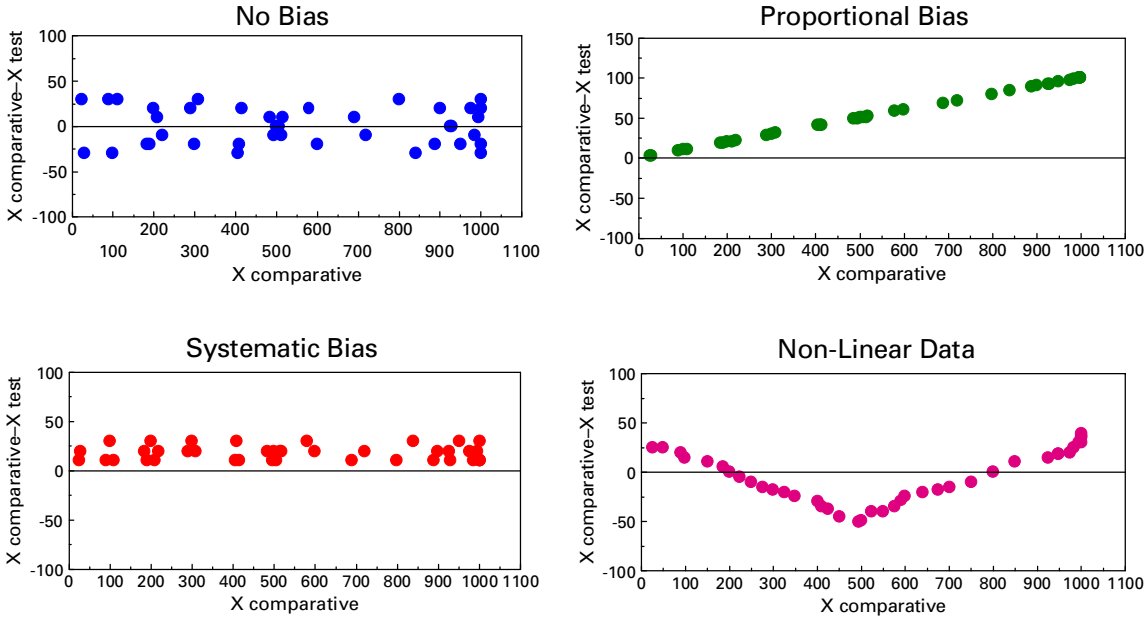
B. Document all quality assurance activities.

LABORATORY VALIDATION OF SINGLE-PLATFORM CD4+ T-CELL METHODS

When performing method-validation studies on the new single-platform methods for enumerating CD4+ T-cell populations, laboratorians must consider that these assays may determine the absolute CD4+ count using methodologies that are very different from multi-platform techniques. In most clinical settings, multi-platform methods do not perform at the level of a gold standard. Still, the single-platform methods must be compared with accepted methods or testing procedures. When no optimal standard exists and bias is present, the amount of error contributed by each method cannot be determined. Therefore, if results yielded from a single-platform method are significantly different from those obtained using a multi-platform method, the new method is not necessarily in error. Conducting a large-scale study correlating results from single-platform methods with clinical disease data to establish new medical decision points may be the only surrogate for comparison with a gold standard. Laboratories should not adopt methods that yield results significantly different from multi-platform methods until these studies can be performed, published, and accepted by the scientific and medical communities.

Traditional method comparison tools may be used for validation of single-platform methods that compare favorably with multi-platform methods. Single-platform methods, as the name implies, derive the absolute CD4+ T-cell counts from a single measurement and therefore have the potential to yield a less variable (although not necessarily more accurate) analysis than multi-platform methods, which utilize a combination of hematology and flow cytometry measurements. Laboratorians should utilize statistical tools that provide useful information about these new methodologies but that do not presume that either the comparative or test method is definitive. Linear least squares regression analysis must be conducted based on the assumption that no error exists in the comparative method, and regression-type scatter plots provide inadequate resolution when the errors are small in comparison to the analytical range (*70,71*). The bias scatterplot may provide laboratorians with a more useful tool for determining bias (Figure 4). These simple, high resolution graphs plot the difference in the individual measurements of each method ($X_{\text{test method}} - X_{\text{comparative method}}$) against those by one of the methods ($X_{\text{comparative method}}$) (*70*). Such graphs provide an easy means of determining if bias is present and distinguishing if bias is systematic, proportional, or random/non-constant. The laboratorian may visually determine the significance of these differences over the entire range of values, and when sufficient values are plotted, outliers and/or samples containing interfering substances can be identified. The laboratorian may then divide the data into ranges relevant to

FIGURE 4. Simplified examples of bias scatter plots



medical decisions and calculate the systematic error (mean of the bias), the random error (standard deviation of the bias), and total error (the greatest absolute 95% error limit of the systematic error twice the random error) to gain insight into analytical performance at the specified decision points (*70,71*). Several detailed guidelines and texts can provide laboratorians with additional information regarding quality goals, method evaluation, estimation of bias, and bias scatter plots (*70–76*). Once a new method is accepted and implemented, the laboratory 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

On the basis of the reported number of tests performed annually by laboratories participating in CDC's Model Performance Evaluation Program for T-lymphocyte immunophenotyping in 1995, more than 1.6 million CD4+ T-cell measurements are performed yearly by the approximately 600 testing laboratories in the United States (*77*). Most of these measurements are made by using multi-platform flow cytometric methods, although new single-platform methods (both flow cytometric and others) are available (*78–85*). Recommendations concerning CD4+ T-lymphocyte immunophenotyping have focused on the more complex multi-platform process of measuring CD4+ T-cells. The recommendations for testing have increasingly been adopted (*86*), and as a result, laboratorians have reported improved testing practices (*86,87*). Testing outcomes associated with following the recommendations include a) increased confidence in results, b) more reproducible results, c) increased ability to resolve discrepant problems, d) decreased proportion of unacceptable specimens received for

testing, e) decreased proportion of specimens requiring reanalysis, and f) decreased incidents that could pose biohazard risks (*86*).

Although data suggest that guidelines for CD4+ T-cell lymphocyte immunophenotyping have improved many laboratory practices, developing guidelines that address 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 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 were not available as the basis for every guideline, some recommendations are based on experience and expert opinion. 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, laboratory personnel credentials and experience, testing methods used, test-result reporting practices, and compliance with other voluntary standards and laboratory regulations) complicate both development of guidelines that will fit every laboratory's unique circumstances and measurement of the value of guideline implementation.

When the first CDC recommendations for laboratory performance of CD4+ T-cell testing were developed, the guidelines 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. Presentations at the second national conference in Atlanta indicated that although CD4+ T-cell testing by multi-platform flow cytometry is still being performed by most laboratories, other single-platform methods are being implemented. In addition, alternative T-cell phenotypic markers are being investigated as prognostic indicators or markers of treatment efficacy, alone and in combination with other markers (*88*).

Participants at the second national conference emphasized the need for monitoring the intralaboratory and interlaboratory accuracy, precision, and reliability of current and new procedures. Decisions about implementing and modifying procedures should be based on performance data collected to assess the extent to which the quality goals established by providers and users of laboratory testing services are achieved (*76*). In testing areas where no absolute gold standards exist (e.g., CD4+ T-cell enumeration), method validation and verification processes are even more critical. Laboratorians should continue to rely on as many sources of information and data as possible to help in their decision processes. Factors that have contributed to improved testing practices and that are important resources for laboratorians include regulatory* and voluntary laboratory standards (*29,31,32,34,89*); manufacturer's recommendations; proficiency testing and performance evaluation program data; information shared at scientific conferences, meetings, and training sessions; and publications in scientific literature.

*42 CFR part 493 § 493.801–493.865.

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APPENDIX. Effects of medications and other biologic factors on immunophenotyping results

Causative agent	Effect	Result
Zidovudine (AZT)	Increased granuloctye fragility; red blood cells (RBCs) less susceptible to lysing reagents	Poor light-scattering (increased granulocyte and RBC contamination of lymphocyte gates)
Some antibiotics (e.g., cephalosporins)	Increased cellular auto-fluorescence	Increased autofluorescence
Corticosteroids	Decreased CD4+ T-cell levels	Decreased CD4+ T-cell percentage and absolute number
Strenuous exercise	Decreased lymphocyte counts	Lower absolute lymphocyte subset values
Diurnal variation	Changes in absolute lympho-cyte counts	Variable absolute lymphocyte subset values (not as prevalent in human immunodeficiency virus infection)

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