1994 Revised Guidelines for the Performance of CD4+ T-Cell Determinations in Persons with Human Immunodeficiency Virus (HIV) Infection

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Summary

This document contains revised guidelines developed by CDC for laboratories performing lymphocyte immunophenotyping assays in human immunodeficiency virus-infected persons. The recommendations in this document reflect 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

Human immunodeficiency virus (HIV) is a retrovirus that infects cells that possess the CD4 receptor (*1–3*). This infection causes the depletion of CD4+ T-cells, which is a major clinical finding in progressive infection (*2–5*). Depletion in these cells is associated with increased clinical complications and is a measure of immunodeficiency. Among persons with HIV infection, CD4+ T-lymphocyte determinations are used in clinical decisions for prognosis and therapy (*5–8*) because they have been found to be useful for predicting the onset of opportunistic diseases (*4*). These determinations are also used as a surrogate for therapy outcome (*7,8*). In addition, persons with CD4+ T-cell levels <200 cells/µl, or 14%, are now classified as having acquired immunodeficiency syndrome (AIDS) using CDC's revised classification system (*9*).

Recently, CDC published guidelines for laboratories performing assays to enumerate CD4+ T-cell levels (*10*). These guidelines addressed issues about hematology measures as well as flow cytometric measures, which are combined for enumerating CD4+ T-cells. As technology evolves, revisions in the guidelines may be necessary. A number of laboratories have raised questions regarding the 1992 guidelines and have helped resolve some of the controversial issues in that document. In addition, new technologies for enumerating CD4+ T-cells have been explored and are being validated. As a result, revisions reflecting current technology have been made to the 1992 guidelines to help guide laboratories in proper quality assurance (QA) and quality control (QC).

RECOMMENDATIONS

A. Laboratory safety
    1. Use universal precautions with all specimens (*11*).
    2. Establish the following safety practices (*12–18*):
        a. Wear laboratory coats and gloves when processing and analyzing specimens, including reading specimens on the flow cytometer.
        b. Never pipette by mouth. Use safety pipetting devices.

    c. Never recap needles. Dispose of needles and syringes in puncture-proof containers designed for this purpose.

    d. Handle and manipulate specimens (aliquoting, adding reagents, vortexing, and aspirating) in a class I or II biological safety cabinet.

    e. Centrifuge specimens in safety carriers.

    f. After working with specimens, remove gloves and wash hands with soap and water.

    g. 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.

    h. 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 undiluted bleach to an empty 1,000-mL container).

    i. 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.

    j. 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 18. In general, for use on smooth, hard surfaces, a 1% solution of bleach is adequate for disinfection; for porous surfaces, a 10% solution is needed (*18*).

    k. 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 (*19*), however, these reagents have not been evaluated for their effectiveness against other agents such as hepatitis virus. Buffered (pH 7.0–7.4) 1%–2% paraformaldehyde or formaldehyde can inactivate cell-associated HIV to approximately the same extent (*19–22*). Cell-free HIV can be inactivated with 1% paraformaldehyde within 30 minutes (*23*). Because the commercial lysing/fixing reagents do not completely inactivate cell-associated HIV, and the time frame for complete inactivation is not firmly established, it is good practice to resuspend and retain stained and lysed samples in fresh 1%–2% paraformaldehyde or formaldehyde through flow cytometric analysis.

B. Specimen collection

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

    a. Anticoagulant for hematologic testing:

      i. Use tripotassium ethylenediamine tetra-acetate (K_3EDTA, 1.5 ± 0.15 mg/mL blood)(*24,25*) and perform the test within the time frame allowed by the manufacturer of the hematology analyzer, not to exceed 30 hours.

      ii. 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 (*26*). 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 HIV-infected as well as HIV-uninfected persons.

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

      i. Use K_3EDTA, acid citrate dextrose (ACD), or heparin if specimens will be processed within 30 hours after collection.

      ii. Use either ACD or heparin, NOT K_3EDTA, if specimens will be processed within 48 hours after specimen 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 (*27*).

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

2. Collect blood specimens by venipuncture (*28*) into evacuated tubes containing an appropriate anticoagulant, completely expending the vacuum in the tubes.

    a. Draw pediatric specimens in pediatric tubes to avoid underdrawing.

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

3. Draw the appropriate number of tubes:

    a. When hematology and flow cytometric immunophenotyping will be performed in the same laboratory on the same specimen, use one tube containing K_3EDTA.

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

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

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

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

C. Specimen transport

1. Maintain and transport specimens at room temperature (18–22 C) (*26,29–31*). Avoid extremes in temperature so that specimens do not freeze or become too hot. Temperatures above 37 C may cause cellular destruction and affect both the hematology as well as flow cytometry measurements (*26*). In hot weather, it may be necessary to pack the specimen in an insulated container and 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 (4 C) on immunophenotyping results is not clear (*26,31*).

2. Transport specimens to the immunophenotyping laboratory as soon as possible.
3. 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, such as a sealed plastic bag, and 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.
4. For interstate shipment, follow federal guidelines (*32*) 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 as well.
5. Obtain specific protocols and arrange appropriate times of collection and transport from the facility collecting the specimen.

D. Specimen integrity
   1. Inspect the tube and its contents immediately upon arrival.
   2. Take corrective actions if the following occur:
      a. 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 (*26*). Abnormalities in light-scattering patterns will reveal a compromised specimen.
      b. If blood is hemolyzed or frozen, reject the specimen and request another.
      c. If clots are visible, reject the specimen and request another.
      d. If the specimen is >48 hours old (from the time of draw), reject it and request another.

E. Specimen processing
   1. Hematologic testing
      a. 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 B.1.a.ii.)
      b. Perform an automated white blood cell (WBC) count and differential, counting 10,000 to 30,000 cells (*33*). 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.
   2. Immunophenotyping
      a. For optimal results, perform the test within 30 hours, but no later than 48 hours, after drawing the blood specimen (*34,35*).
      b. Use a direct two- or three-color immunofluorescence whole-blood lysis method. Use the "stain, then lyse" procedure.
      c. Use a monoclonal antibody panel that contains appropriate monoclonal antibody combinations to enumerate CD4+ and CD8+ T-cells and to ensure

the quality of the results (*36*). A recommended two-color immunopheno-typing antibody panel is in Table 1, listed by CD nomenclature (*37*) and fluorochrome. The results from this panel provide 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 in sequential patient specimens. The following internal controls are included in the panel:

  i. 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 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 (new aliquot of blood labeled, lysed, and fixed).

  ii. Monoclonal antibodies that label T-cells, B-cells, and NK-cells are used to account for all lymphocytes in the specimen (*36*). **Note**: 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. The greatest danger in using an abbreviated panel is that the internal controls (noted above) are no longer included. For this reason, the immunophenotyping results should be reviewed carefully to ensure that T-cell levels are similar to those determined previously with the full recommended panel. When discrepancies occur, the specimens must be reprocessed using the full recommended two-color monoclonal antibody panel.

d. Three-color monoclonal antibody panels can be used if the quality of immunophenotyping results from the three-color combinations can be

TABLE 1. Recommended 2-color monoclonal antibody panel for lymphocyte immuno-phenotyping

FITC*	PE†	Reason for using
CD45	CD14	To draw gates; lymphocytes are brightly positive for CD45 and negative for CD14
Isotype	Isotype	To set cursors
CD3	CD4	To measure CD4+ T-cells; only cells positive for both CD4 and CD3 should be considered CD4+ T-cells
CD3	CD8	To measure CD8+ T-cells; only cells that are positive for both CD8 and CD3 should be considered CD8+ T-cells. The remainder of the CD8 cells (CD3-) are natural killer (NK)-cells
CD3	CD19§	To measure B-cells for quality assurance and to help account for all lymphocytes
CD3	CD16 and/or CD56§	To measure NK-cells (negative for CD3, positive for CD16 and/or CD56) for quality assurance and to help account for all lymphocytes.

*Fluorescein isothiocyanate.
†Phycoerythrin or RD-1 (Coulter™).
§A minimal acceptable 2-color panel omits these two monoclonal antibody combinations. See Note under specimen processing (E.2.c.ii) before deciding to use an abbreviated panel.

assured and the panel has been validated using specimens from both HIV-infected and HIV-uninfected persons. Assurance of the results includes a) validating the gating strategies used so that the quality of the gate is known (i.e., lymphocyte recovery and purity) (see Section I.2.) and b) a method for evaluating nonspecific fluorescence in the unlabeled population. Validation of a three-color panel includes labeling specimens with both the two-color panel and the proposed three-color panel then determining whether the differences in results for a particular population (e.g., CD4+ T-cells) by both methods are within the variability expected from replicates in the laboratory. (See Section H.2.)

e. Use premixed two- or three-color monoclonal antibodies at concentrations recommended by the manufacturer. **Note**: If, instead, two or three single-color reagents are combined, each must be titered with the other(s) to determine optimal concentrations for use (10 µL antibody A with 5 µL antibody B, 5 µL antibody A with 10 µL antibody B, etc., for two-color; 10 µL antibody A with 5 µL antibody B and 5 µL antibody C, 5 µL antibody A with 10 µL antibody B and 5 µL antibody C, etc., for three-color). **Note**: Reagents from different manufacturers are likely to be different in their epitope specificity, fluorochrome/protein (F/P) ratio, and protein concentrations. Because of these differences, combining reagents from different manufacturers is not generally recommended. Optimal antibody concentrations are those in which the brightest signal is achieved with the least amount of noise (nonspecific binding of antibody to the negative population) (i.e., the best signal-to-noise ratio). The nonspecific binding should be no greater than that of an isotype control. The way to evaluate the appropriate concentration of antibodies when combined is to evaluate the fluorescence histogram in a tube in which only one antibody is added and compare it with the histogram from a tube in which more than one antibody is added. The single-parameter histograms from both tubes should be similar. In addition, the percent positive cells for the cell population by both methods should be within the expected variability established in the laboratory. (See Section H.2.)

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

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

h. Include a source of protein (e.g., fetal bovine serum or bovine serum albumin) in the wash buffer to reduce cell clumps and autofluorescence.

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

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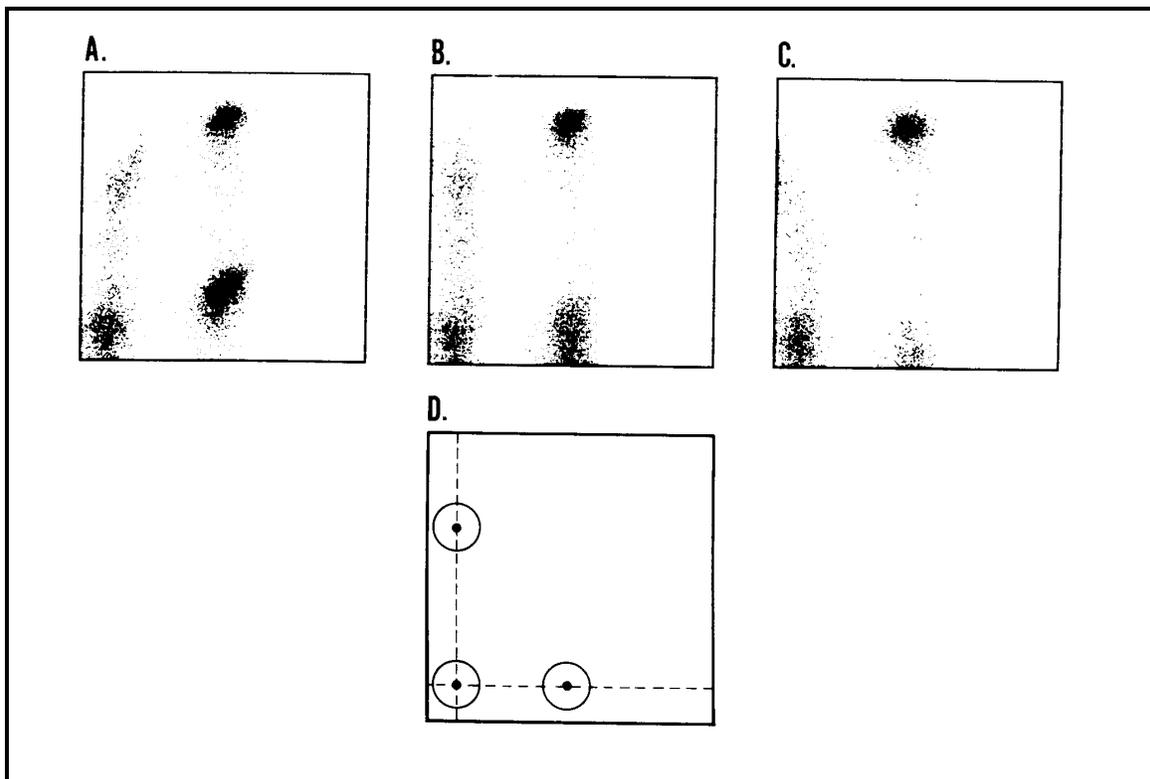
over time. Therefore, these fixatives should be made fresh weekly from electron microscopy-grade aqueous stock.

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

F. Negative and positive controls for immunophenotyping
   1. Negative (isotype) reagent control
      a. Use this control with each specimen 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.
      b. 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. At this time there is no solution to these problems.
   2. Positive methodologic control
      a. Use this control to determine whether procedures for preparing and processing the specimens are optimal. This control is prepared each time patient specimens are prepared.
      b. Use a whole blood specimen from a control donor. Ideally, this control will match the population of patients tested in the laboratory (see Section K.4.).
      c. If this 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 reasons that cause only this 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, as well as the methodologic control, invalidates the results.
   3. Positive control for testing reagents
      a. 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 (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.
      b. Use a whole blood specimen or other human lymphocyte preparation (cryopreserved or lyophilized lymphocytes).

G. Flow cytometer quality control (*38*)
   1. Align optics daily. This assures that the brightest and tightest peaks are produced in all parameters. **Note**: Some clinical flow cytometers can be aligned

by laboratory personnel and others can be aligned only by qualified service personnel.

   a. Align the flow cytometer using stable calibration material (such as microbeads labeled with fluorochromes) that has measurable forward scatter, side scatter, FITC, and PE peaks.

   b. 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.

   c. 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.

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

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

   b. 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.

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

   d. Retain machine standardization settings for the remaining quality control procedures (sensitivity and color compensation) as well as for reading the specimens.

3. Determine fluorescence resolution daily. The flow cytometer must differentiate between the dim peak and autofluorescence in each fluorescence channel.

   a. Evaluate standardization or 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).

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

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

   a. Use either microbead or cellular compensation material containing three populations for two-color immunofluorescence (no fluorescence, PE fluorescence only, and FITC fluorescence only) or four populations for three-color immunofluorescence (the three above plus a population that is positive for only the third color). **Note**: microbeads are generally only available for FITC and PE and not for a third fluorochrome.

   b. 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, it is important that compensation be carried out in an appropriate sequence: FITC, PE, and the third color, respectively (*39*). Take care to avoid overcompensation.

    c. If standardization or calibration particles (microbeads) have been used to set compensation, confirm this by using lymphocytes labeled with FITC- and PE-labeled monoclonal antibodies (and a third-color-labeled monoclonal antibody for three-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.

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

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

6. Maintain instrument quality control logs, and monitor them continually for changes in any of the parameters. In the logs, record instrument settings as well as peak channels and coefficient of variation (CV) values for optical alignment, standardization, fluorescence resolution, and spectral compensation. Reestablish fluorescence levels for each quality control procedure when lots of beads are changed.

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.
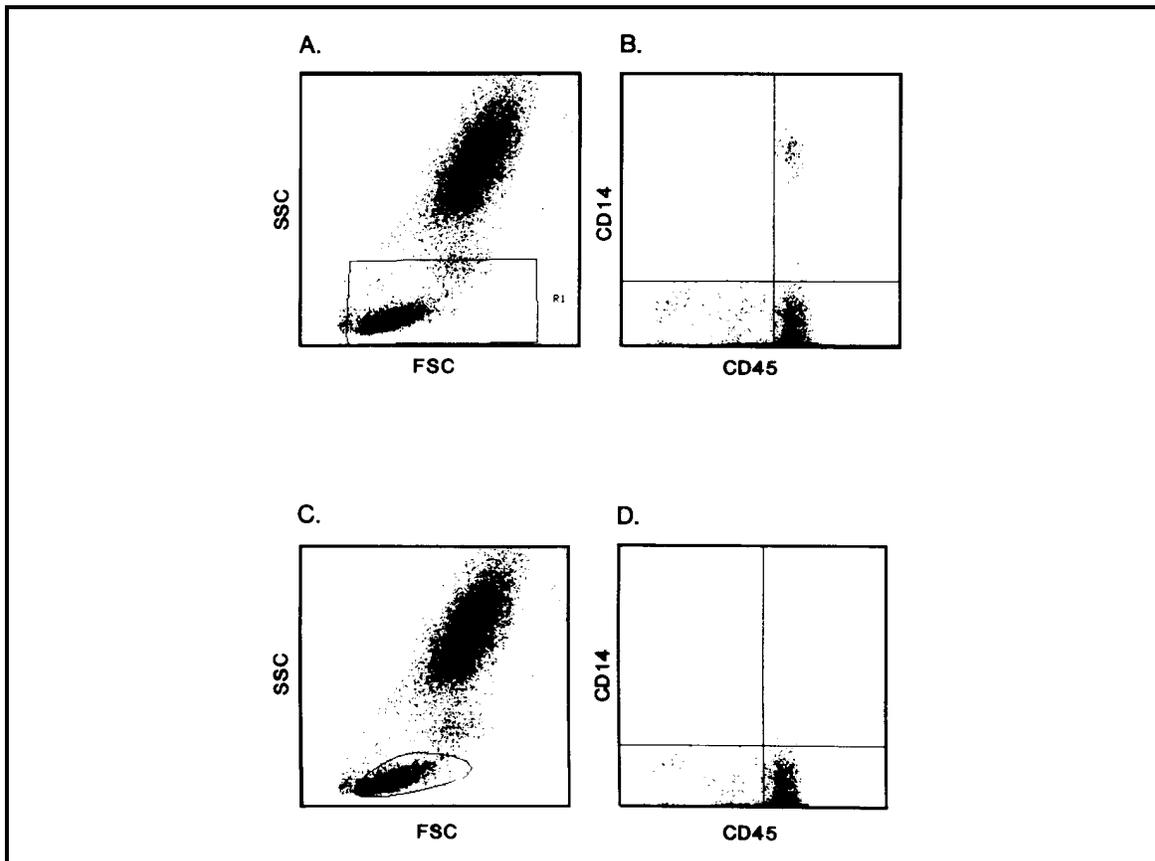
H. Sample analyses
   1. For the two-color immunophenotyping panel, analyze the sample tubes of each patient's specimen in the following order:
      a. The tube containing CD45 and CD14 (gating reagent): read this tube first so that gates can be set around the lymphocyte cluster.
      b. Isotype control: set cursors for differentiating positive and negative populations so that ≤2% of the cells are positive.
      c. Remaining tubes in the panel.
   2. Count at least 2,500 gated lymphocytes in each sample. This number assures 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.
   3. 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 (a new aliquot of blood is stained and lysed).
I. Data analysis
   1. Reading from the sample tube containing CD45 and CD14, draw lymphocyte gates using forward and side light-scattering patterns and fluorescence staining. **Note**: Other methods of drawing lymphocyte gates may be used with three-color monoclonal antibody panels. These may include using CD45-bright positivity and low side scattering patterns or reading from a tube containing labeled T, B, and NK cell populations to identify lymphocytes. If verified, these methods may be used instead of light scatter gating using the CD45 and CD14 tube.
      a. When using CD45 and CD14 and light-scattering patterns for drawing lymphocyte gates, define populations on the following basis:
         i. Lymphocytes stain brightly with CD45 and are negative for CD14.
         ii. Monocytes and granulocytes have greater forward and side light-scattering properties than lymphocytes.
         iii. Monocytes are positive for CD14 and have intermediate intensity for CD45.
         iv. Granulocytes are dimly positive for CD14 and show less intense staining with CD45.
         v. 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 (*40*). **Note**: Other methods for drawing a lymphocyte gate must accurately identify lymphocytes and account for nonlymphocyte 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
   i. 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.
   ii. 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. The lymphocyte recovery should optimally be at least 95%.
c. The lymphocyte purity of the gate should optimally be at least 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

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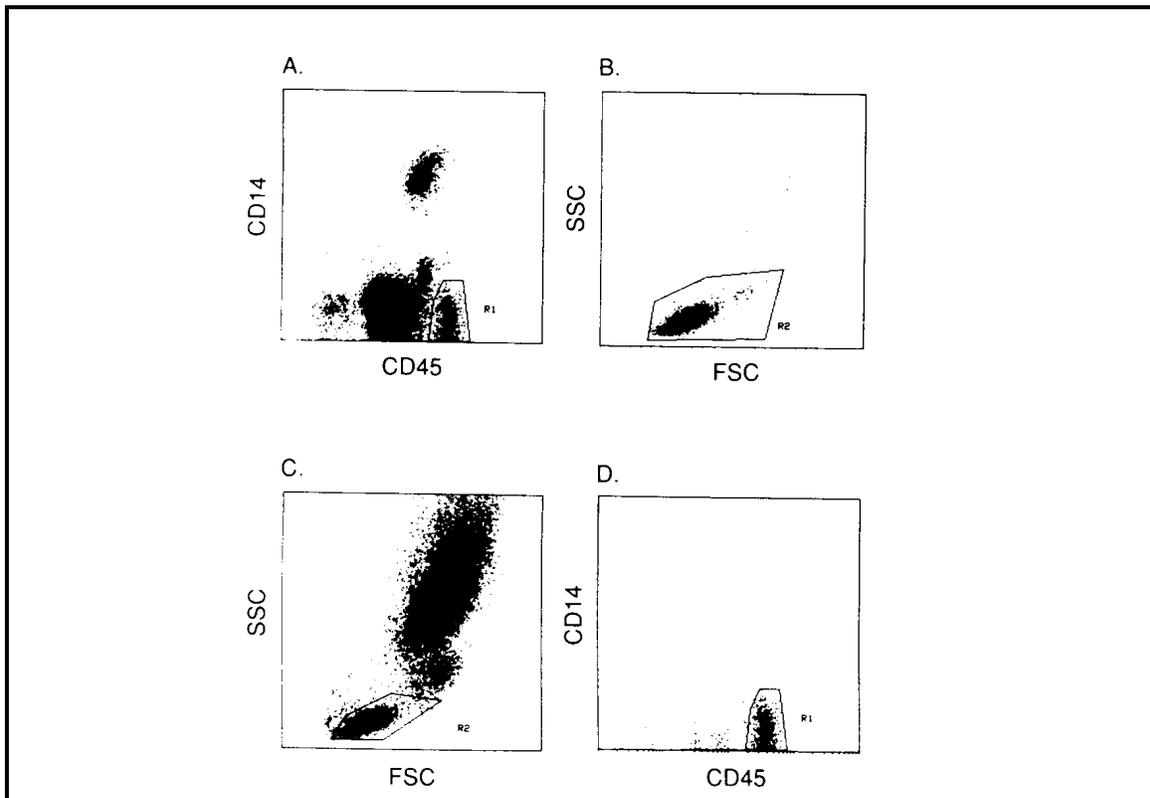

*Panel A shows a rather large light scatter region drawn around the lymphocytes (R1). Panel B is the two-parameter histogram of CD45/CD14 fluorescence gated on R1. The number of cells in quadrant 4 (bright CD45-positive, negative for CD14) is the total number of lymphocytes (3,951 cells). Panel C is 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%.

be the same whether determined by light scatter gating or by fluorescence gating.

i. 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 as well as 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.

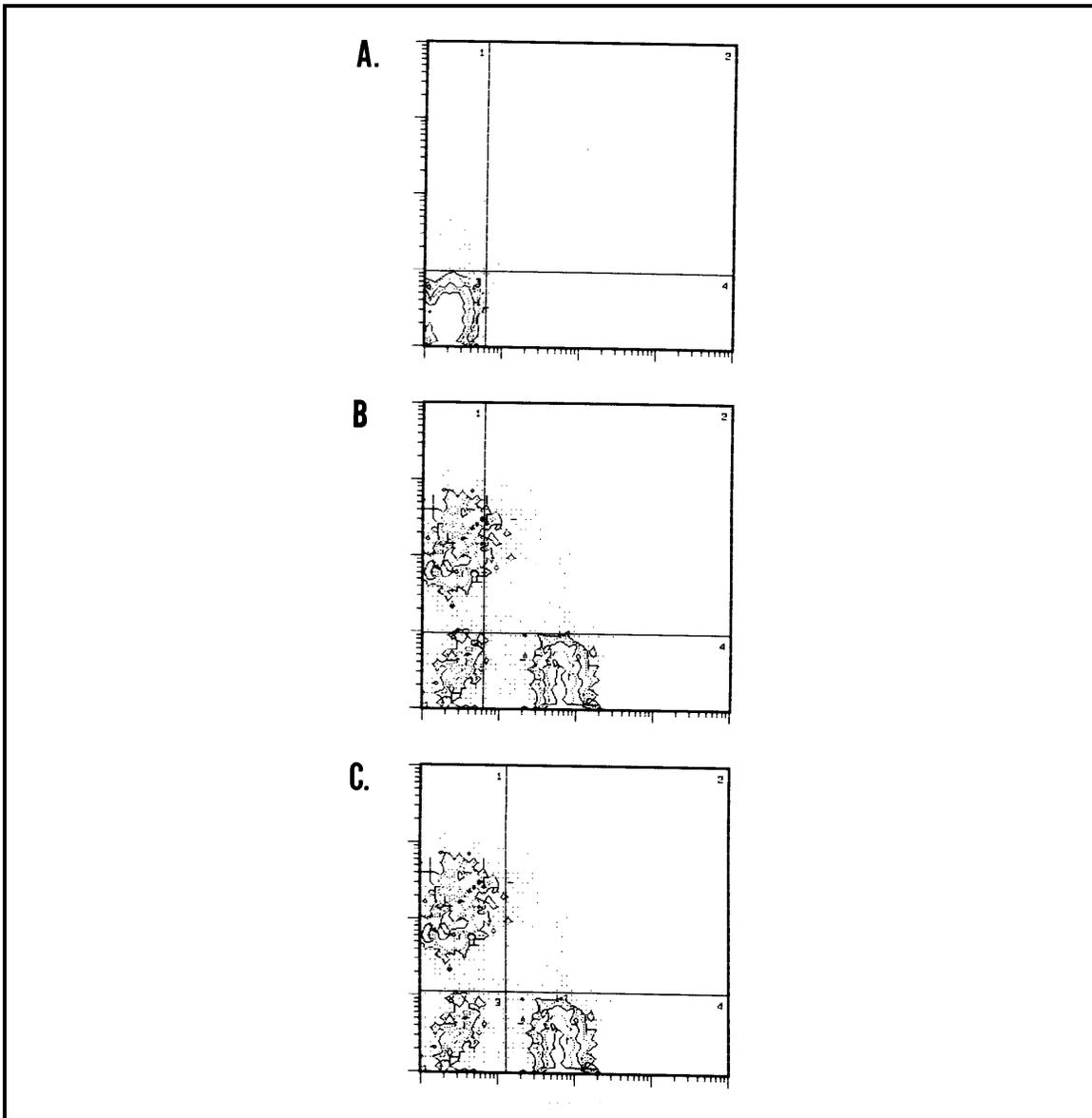**FIGURE 3. Fluorescence gating technique for determining lymphocyte recovery and purity***

*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%.

ii. 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 as well as 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.

3. Set cursors using the isotype control so that <2% of cells are positive.

4. Analyze the remaining samples with the cursors set based on the isotype control. **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 (Figure 4). This should not be done when fluorescence distributions are continuous with no clear demarcation between positively and negatively labeled cells.

5. Analyze each patient specimen or normal control specimen with light-scattering gates and cursors for positivity set for that particular patient or control.

6. Where 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)(*41*), repeat the sample preparation, prewashing the specimen with phosphate-buffered saline (PBS), pH 7.2, to remove plasma before monoclonal antibodies are added.

7. Include the following analytic reliability checks:

a. 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.

b. Optimally, at least 90% lymphocyte purity should be observed within the lymphocyte gate. Minimally, at least 85% purity should be observed within the gate.

c. Optimally, the sum of the %CD3+CD4+ and %CD3+CD8+ cells should equal the total %CD3+ cells within ±5%, with a maximum variability of ≤10%.

Note: In specimens containing a considerable number of Tγ/δ cells (*42,43*), this reliability check may excede the maximum variability.

   d. Optimally, the sum of the %CD3+ (T-cells), %CD19+ (B-cells), and the %CD3-(CD16 and/or CD56)+ (NK-cells) should equal the purity of lympho-cytes in the gate ±5% (*36*), with a maximum variability of ≤10%. If the data

FIGURE 4. Justification for moving cursors that were set with the isotype control*

*A specimen was labeled with the isotype control (Panel A), and cursors were placed to include ≤2% of the cells in quadrants 1, 2, or 4. A sample from the same patient was labeled with CD3 (T-cells, horizontal axis) and CD19 (B-cells, vertical axis) monoclonal antibodies (Panels B and C). the placement of the cursor determined from the isotype control (Panel B) includes some of the CD19+ B-cells in the double-positive quadrant, which is not appropriate. In Panel C, the marker was moved to include all the CD19+ B-cells in quadrant 1, which more accurately estimates the proportion of B-cells.

are corrected for lymphocyte purity (see K.2.), the sum should equal 95%–105% (or a minimum of 90%–110%).

J. Data storage

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

2. 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.

K. Data reporting

1. 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 (determined from tube 3 in the suggested two-color panel). Similarly, CD8+ T-cells are T-suppressor/cytotoxic cells, and these are positive for both CD3 and CD8 (tube 4 in the two-color panel). It is important not to include other cell types (non-T-cells) in CD4 and CD8 determinations.

2. 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 give a T-lymphocyte value of 74%.

3. 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.

   a. 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).

   b. Report both percentages and absolute counts when these are available.

4. 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. Separate reference ranges must be established for adults and children, and the appropriate ranges must be used for patient specimens. See reference 38 for methods for determining these limits.

L. Quality assurance

1. 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:

   a. Methods for collecting, handling, transporting, identifying, processing, and storing specimens.
   b. Information provided on test request and results report forms.
   c. Instrument performance, quality control protocols, and maintenance.
   d. Reagent quality control protocols.
   e. Process for reviewing and reporting of results.
   f. Employee training and education, which should consist of:
      i. Basic training by flow cytometer manufacturers and additional training in hands-on workshops for flow cytometer operators and supervisors.
      ii. Education of laboratory directors in flow cytometric immunophenotyping through workshops and other programs.
      iii. Continuing education in new developments for all flow cytometric immunophenotyping personnel through attendance at meetings and workshops.
      iv. Adherence to federal and state regulations for training and education.
   g. 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.
   h. 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 taken to prevent recurrences.
2. Document all quality assurance activities.

DISCUSSION

Though there is no standard for immunophenotyping using flow cytometry, laboratories now have several detailed guidelines to follow (*10,38,44,45*). Proficiency testing programs have shown that laboratory performance for CD4+ T-cell percentages has improved over the last several years (*46–48*). In addition, CLIA '88 requires that certain levels of laboratory quality control and performance be attained to qualify the laboratory for clinical testing. This QC and performance requirement pertains to immunophenotyping using flow cytometry.

Absolute lymphocyte subset values are obtained from three separate determinations: a) the WBC, b) the leukocyte differential, and c) the percent positive cells from flow cytometry. Even though the flow cytometry results have improved in interlaboratory performance programs, the hematology results have been less carefully studied. This is primarily because most recommendations for hematology measurements state that differentials must be done within 6 hours of blood drawing (*24,25*). With these time constraints, it is not possible to evaluate performance in proficiency testing programs because these specimens do not usually arrive in the laboratory until the following day. Further improvements in absolute lymphocyte subset values, including absolute CD4+ T-cells, can be achieved through improving the hematology determinations. Newer hematology technology may produce accurate WBC and differential

determinations on blood drawn 24 hours earlier, but time limitations for the blood must be carefully tested to validate these instruments.

The intralaboratory analytic variability (CV) in determining the WBC count using an automated leukocyte counter is 2.2%–7.7%, and 9.3%–17.6% using a hemocytometer. The lymphocyte differential varies from 1.9% to 5.3% for automated counts and from 12.5% to 27% for manual counts (*33*). Therefore, the variability in the absolute number of lymphocytes in the blood reflects the combined variability of the WBC count and the lymphocyte differential. Biologic variability is even greater: about 10% diurnally and 13% week to week (*49*).

Estimates of interlaboratory variability (SD) in flow cytometric immunophenotyping results have been derived from proficiency testing and performance evaluation data (*46,47*; CDC, Model Performance Evaluation Program, unpublished data). An analysis of data from the College of American Pathologists surveys between 1989 and 1991 of more than 200 laboratories showed that the SD of the percentage of CD4+ T-cells was 4.7% to 8.4%, with the lower number associated with CD4 T-cell percentages near 25% and the higher with percentages near 50% (*46*). For duplicate measurements, the SD of the percentage of CD4+ T-cells was about 3% when the specimen contained 45% CD4+ T-cells. The results furnished to CDC by 280 laboratories participating in the MPEP for T- lymphocyte immunophenotyping in March 1991 indicated the same trends. For samples of CD4+ values in the range of 1% to 16%, the SD of the percentage of CD4+ T-cells was about 2.5%; for samples with CD4+ values between 16% and 24%, the SD was about 3.4%. In the National Institutes of Allergy and Infectious Diseases, Division of AIDS quality assurance program, the SDs ranged from 2.7% for HIV-negative specimens to 2.6% for HIV-positive specimens with >10% CD4+ T-cells and 1.9% for HIV-positive specimens with ≤10% CD4+ T-cells (*47*).

Limited information is available on the degree of interlaboratory variability in CD4+ T-cell counts. In a multicenter proficiency testing study (*48*) of seven laboratories for the year 1987, interlaboratory CVs for the percentage and absolute number of CD4+ T-cells on normal specimens were 6% and 29.4%, respectively. This study has been ongoing and, through rigorous quality assurance and training, CV values have been reduced each year. Subsequently, in 13 laboratories in 1991, CVs for the percentage and absolute number of CD4+ T-cells on normal specimens were 5.1% and 7.0%, respectively (*48*).

To bypass the variability of absolute CD4+ T-cell numbers, alternative technologies to enumerate CD4 cells are being or have been developed by several manufacturers. These technologies will require less technical expertise and be less expensive and time-consuming than flow cytometry. Additionally, since these procedures derive the absolute CD4 cell numbers from one measurement rather than three measurements (WBC, differential, and flow cytometry), the variability of the CD4 cell number by these technologies should be less than that of flow cytometry and hematology combined. All these new methodologies vary greatly in the procedures by which the CD4 cell numbers are obtained. They measure CD4 in different ways: on T-cells, on lymphocytes, or in whole blood lysates. Because of these differences, quality control for each of these procedures will differ. Careful validation of these methodologies under a variety of conditions is needed. It is likely that these technologies will be found in clinical laboratories in the near future, and it is imperative that manufacturers and clinical

laboratorians work together to establish QC guidelines and help ensure the quality of the CD4 cell results.

This document reflects current information on QA/QC procedures for immunophe- notyping to determine CD4+ T-cell levels in HIV-infected persons. Revisions made to the 1992 guidelines (*10*) are the result of additional data, new methodology, and bet- ter understanding of variables that contribute to how specimens are processed and analyzed. This technology continues to evolve. These guidelines will be revised again as newer techniques and reagents are developed and more data become available.

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APPENDIX

Effects of medications and other biologic factors on immunophenotyping results

Causative agent	Effect	Result
Zidovudine (AZT)	Increased granulocyte fragility; red blood cells (RBCs) less susceptible to lysing reagents	Poor light-scattering (increased granylocyte and RBC contamination of lymphocyte gates)
Some antibiotics (e.g., cephalosporins)	Increased cellular auto-fluorescence	Increased autofluorescence
Nicotine	Decreased lymphocyte counts	Lowered absolute lymphocyte subset values
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 lymphocyte count	Variable absolute lymphocyte subset values (not as prevalent in Human Immunodeficiency Virus infection)

MMWR