

Guideline for Flow Cytometric Immunophenotyping: A Report From The National Institute of Allergy and Infectious Diseases, Division of AIDS

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BACKGROUND AND INTRODUCTION

Progressive and selective depletion of CD4-positive T lymphocytes is a hallmark of HIV infection and AIDS. Decreases in CD4-positive T cells are associated with increased risk for opportunistic infection and disease progression in HIV-infected individuals. In HIV infection, the CD4 T-cell count is the most commonly utilized laboratory measure for clinical prognosis, therapeutic monitoring, and entry criteria for clinical trials.

Currently, the enumeration of CD4 T cells involves three distinct measurements: the white blood count (WBC), the lymphocyte differential (percent lymphocytes), and the percentage of T lymphocytes that are CD4 positive (CD4 percent). The WBC and percent lymphocytes are routinely measured on whole blood samples using an automated hematology instrument. The CD4 percent is measured on whole blood by flow cytometric immunophenotyping. The sample is reacted with fluorochrome-labeled monoclonal antibodies that are specific for cell-surface antigens (e.g., CD4). When analyzed on a flow cytometer, cells that have bound the labeled antibody can be quantified on the basis of their fluorescence emission. Simultaneously, lymphocytes are resolved from other white blood cells (granulocytes, monocytes) based on size and granularity using light scatter properties. The resulting CD4% is used to calculate the CD4 cell number (i.e., absolute CD4, CD4 count) by the formula:

$$\text{WBC} \times \text{Percent Lymphocytes} \times \text{CD4\%} = \text{CD4 Cell number}$$

Studies have documented the intra- and interlaboratory variability in the measurement of CD4 T-lymphocyte counts and have attempted to evaluate the bias and imprecision attributable to a variety of technical and analytic factors, including: time from blood draw to analysis, staining and washing procedures, alignment and calibration of flow cytometers, specimen temperature, nonspecific binding of monoclonal antibodies, fixation techniques, electronic gating parameters, and corrections for nonlymphocytic contamination. The guidelines herein, as well as those developed by the Centers for Disease Control (CDC) (1) and National Committee for Clinical Laboratory Standards (NCCLS) (2), represent an effort to minimize the impact of these factors on interlaboratory variability in the measurement of CD4 cell numbers.

This guideline is not designed to comprise a detailed procedural manual for the clinical flow cytometry laboratory. Rather, the intent is to provide recommendations for the handling and processing of whole blood specimens for enumerating CD4 T lymphocytes and other immunophenotypes measured by flow cytometry. Where appropriate, the National Committee for Clinical Laboratory Standards (NCCLS) document "Clinical Applications of Flow Cytometry: Quality Assurance and Immunophenotyping of Peripheral Blood Lymphocytes" (2) is referenced to provide greater detail. Given the rapidity with which the technology of flow cytometry and our understanding of the pathogenesis of HIV disease are both evolving, it is expected that these and other such guidelines (NCCLS, CDC) will require periodic revision to reflect these changes.

SPECIMEN COLLECTION AND HANDLING

1.01. Biohazard Precautions

Proper biohazard precautions should be observed in the collection and handling of patient blood specimens (3–6).

1.02. Anticoagulant

Tripotassium ethylenediamine tetra-acetate (K_3EDTA) (lavender-top tube) is **required** as the anticoagulant for specimen collection for hematology (WBC and Differential) (7,8).

If a **separate specimen tube** is collected for flow cytometry, tripotassium ethylenediamine tetra-acetate (K_3EDTA), acid citrate dextrose A (ACD A) or sodium heparin can be used as the anticoagulant.

1.03. Specimen Identification

Specimens should be identified as to date and time of collection.

1.04. Specimen Holding

Specimens should be kept at room temperature (18–22°C) and transported to the Hematology and Flow Cytometry laboratories as soon as possible (7–13).

1.04.a. Hematologic analyses are optimally performed within 6 h of specimen draw, although this may vary for specific instrumentation and must be validated by individual laboratories.

1.04.b. Staining for flow cytometric analyses is optimally performed within 30 h of specimen draw.

1.04.c. Specimens not able to be processed (stained, lysed, and fixed) within 30 h of specimen draw are *not to be processed*, and a redraw should be requested.

1.05. Time of Collection/Patient Variables

Optimally, time of collection and patient variables (e.g., physical exertion, medication, etc.) should be kept consistent and specimen collection carried out under the same conditions for serial assays in the same patient (13).

1.06. Specimen Condition

Laboratories should be alert to suboptimal specimen conditions, such as hemolysis, lipemia, clotting, partial draw, abnormal specimen temperature (hot or cold to the touch).

1.06.a. Since reliability of the data may be suspect due to conditions such as these, suboptimal specimen conditions should be noted on the report form for use by the local physician.

1.06.b. Specimens exhibiting extremes of these conditions (including gross hemolysis, visible clots, or visibly frozen specimens) should be rejected and a redraw requested.

2. HEMATOLOGY: WBC AND DIFFERENTIAL

2.01. Intralaboratory Performance Documentation

The Hematology Laboratory **must** maintain documentation of *interlaboratory* and *intralaboratory* performance

conforming to accepted standards of practice (e.g., College of American Pathologists, National Committee for Clinical Laboratory Standards).

2.02. Timing of Hematology Analysis

The study site **must ensure** that the sample for WBC and differential is *drawn at the same time* as the specimen for flow cytometric analysis. Optimally the hematologic measurements are performed within 6 h of specimen draw, although this may vary for specific instrumentation and acceptability of longer times must be validated by individual laboratories.

2.03. WBC and Differential: Specific Recommendations

2.03.a. The white blood cell (WBC) and differential (including percent lymphocyte) counts **must** be performed on an automated instrument (14–15).

2.03.b. If the specimen is rejected or “flagged” by the machine, then a manual differential (optimally, a 400 cell count) may be performed. However, the *machine lymphocyte count is still the preferred number* for reporting purposes, *unless* the flag occurs in the *lymphocyte region*.

2.03.c. A cell designated as an “atypical lymph” or “LUC” (large unstained cell) should be included in the total lymphocyte number.

3. FLOW CYTOMETRY

3.01. Individual Laboratory Quality Assurance and Intralaboratory Standardization

It is the responsibility of the immunologist at each study site (site immunologist) to ensure that the Flow Cytometry Laboratory has established a normal reference range, carries out internal reproducibility studies, runs a normal fresh specimen on a daily basis, and maintains instrument performance standards (2,9,16–28). (see Appendix: Table 1 *and* see: NCCLS H42-T 10.0–10.2.4 for details of procedures for instrument standardization, calibration, and alignment.)

Prior to accepting specimens for flow cytometric analysis, each laboratory must have in place a comprehensive quality assurance protocol that includes intralaboratory standardization, quality control procedures, and proficiency testing.

3.01.a. Standardization of instrument optical alignment, spectral sensitivity, and fluorescence compensation must be performed daily, based on manufacturers’ specifications and settings established for individual laboratories.

3.01.b. Quality control must include monitoring and recording of instrument performance and cell preparation methods. Reagent stability should be assessed with lot changes or as otherwise needed. Parallel testing of lots represents a mandatory criterion of acceptable performance.

3.01.c. Participation in the NIAID DAIDS Quality Assessment Program is mandatory for laboratories performing immunophenotyping for NIAID DAIDS-supported studies.

Additional participation in a nationally recognized program on a thrice yearly basis is also desirable as an integral component of comprehensive quality assurance.

3.01.d. Normal Reference Range: Methodology for determination of a normal laboratory reference range is currently being established by the NCCLS and will be distributed as soon as it is available.

3.01.e. Reproducibility studies. The site immunologist should arrange for coded replicate peripheral blood specimens (e.g., 6 replicate samples from the same patient) to be sent to the Flow Cytometry Laboratory for routine analysis on a monthly basis. Results should be reported to the site immunologist, who is responsible for maintaining records of these studies. Percentages for replicate samples should vary by no more than $\pm 3\%$.

3.02. Specimen Collection and Handling

3.02.a. Optimally, time of collection and patient variables (e.g., medication; time of day; etc), which could affect flow cytometry results, should be kept consistent whenever serial testing is required (see Appendix: Table 2) (2,9,13).

3.02.b. Tripotassium ethylenediamine tetra-acetate (K₃EDTA) (lavender-top tube) is *required* as the anticoagulant for specimen collection for hematology (WBC and Differential) (7,8).

If a **separate specimen tube** is collected for **flow cytometry**, tripotassium ethylenediamine tetra-acetate (K₃EDTA), acid citrate dextrose A (ACD A), or sodium heparin can be used as the anticoagulant.

3.02.c. Specimens should be identified as to date and time of collection.

3.02.d. Specimens should be kept at room temperature (18–22°C) and transported to the Flow Cytometry Laboratory as soon as possible (7–13).

3.02.e. Staining for flow cytometric analyses is optimally performed within 30 h of specimen draw.

3.02.f. Laboratories should be alert to suboptimal specimen conditions, such as hemolysis, clotting, and temperature (see Section 1.06).

3.03. Minimum NIAID DAIDS Monoclonal Antibody Panel

| Monoclonal Antibody Combination (FITC/PE) | Cell Type Enumerated |
|--|--------------------------------------|
| 1. CD45/CD14 | % Lymphocytes in the “gating region” |
| 2. MsIgG ₁ /MsIgG ₂ ^a | Isotype Control ^a |
| 3. CD3/CD4 | CD4+ T Cells |
| 4. CD3/CD8 | CD8+ T Cells |
| 5. CD19 ^b | Total B-cell ^b |

^aSee 3.033.

^bSee 3.03b.

(See Refs. 29 and 30 for discussion)

3.03a. Monoclonal antibody combinations #1–4 are mandatory for all **adult** studies.

3.03b. Monoclonal antibody combinations #1–5 are mandatory for **pediatric** studies.

3.03c. Fluorochrome designations for monoclonal antibody combinations **must** be adhered to [fluorescein isothiocyanate (FITC), phycoerythrin (PE)].

3.03d. Lymphocytes are defined as CD45^{bright} CD14^{negative}.

3.03e. The cursor settings for the isotype control are not necessarily appropriate for all markers and should be used as the “base” reference for enumeration of lymphocyte subsets (3.09b).

A tube containing unstained patient cells may be used in addition to the isotype control tube and may be of value in evaluating autofluorescent vs. nonspecific binding properties of monoclonal antibody reagents in individual patient specimens.

3.03f. Values to be reported for **CD4 T** cells are the **CD3+ CD4+** percentage and for **CD8 T** cells are the **CD3+ CD8+** percentage.

Use of CD3 together with CD4 for determination of % CD4-positive T lymphocytes obviates the need for moving the cursor to exclude CD3⁻ CD4^{dim} cells.

The value to be reported for **Total CD3+ T cells** is the average percentage obtained for CD3-positive cells (i.e., single- and dual-positive) from all tubes that include CD3.

3.03g. Although inclusion of a marker for natural killer (NK) cells is not required for all NIAID DAIDS studies, an NK marker can be useful for accounting for additional cell populations in quality control (lymphosum: see 3.10c and Ref #28).

Staining with CD56 + CD16 using *the same* fluorochrome for both, paired with CD4 (or CD8) is recommended for NK cell analysis.

NK cells are CD3^{negative} and [CD56^{positive} and/or CD16^{positive}].

3.04. Immunofluorescent Staining

Proper staining for immunophenotyping includes control for cell number, specimen volume, and reagent concentration. Reagent concentration may have to be adjusted for high cell numbers. In particular, specimens from pediatric patients *less than four months* of age are likely to have high numbers of lymphocytes (31).

3.05. Lysing

Whole blood lysis methodology is **mandatory** for all laboratories processing NIAID DAIDS study specimens.

3.06. Fixation

Fixation of specimens for flow cytometric analysis is **recommended**. If specimens are not *analyzed within 2 h* after staining, specimens should be fixed using buffered 1–2% EM-grade paraformaldehyde in aqueous stock (Poly-

sciences, Warrington, PA) for a **minimum** of 30 min (9,32–36).

It should be noted that the fluorescence intensity of FITC is sensitive to even minor fluctuations of pH (37).

3.07. Flow Cytometer

Laser-based instrumentation is required, and the instrument *must have* 90° (side) light scatter (SSC, SS) capability (9).

3.07.a. Light scatter gating for the expected lymphocyte region must be carried out using linear *forward angle light scatter* (FSC, FS, FALS); *linear 90°* (side) scatter is preferable to log.

3.07.b. Fluorescence display/measurements should be log rather than linear fluorescence.

3.07.c. A standard subtraction/compensation protocol should be established for dual-color analysis using FITC/PE bead standards and/or appropriate mutually exclusive dual-color antibody-stained specimen(s). (See NCCLS H42-T, Sections 10.1.2.2 and 10.2.3 for detailed verification of color compensation.)

3.07.d. A *daily* Quality Control Maintenance Log must be maintained for the flow cytometer(s) being used for NIAID DAIDS samples. The maintenance log should contain all data relevant to instrument light scatter and fluorescence calibration, and fluorescence compensation.

Histograms and channel information for calibration bead runs, and fluorescence compensation (e.g., red minus green; green minus red) should be included for each analysis day.

3.08. Lymphocyte Gating

Lymphocyte gates set using linear forward angle light scatter and 90°/side scatter must be validated using CD45-FITC (pan-leukocyte) and CD14-PE (monocyte) monoclonal antibodies (Fig. 1) (9,38).

3.08.a. The lymphocyte population is defined as CD45^{bright} CD14^{negative} (Fig. 2).

3.08.b. Lymphocyte Gating. Two factors in *Lymphocyte Gating* greatly influence the validity of lymphocyte immunophenotyping data:

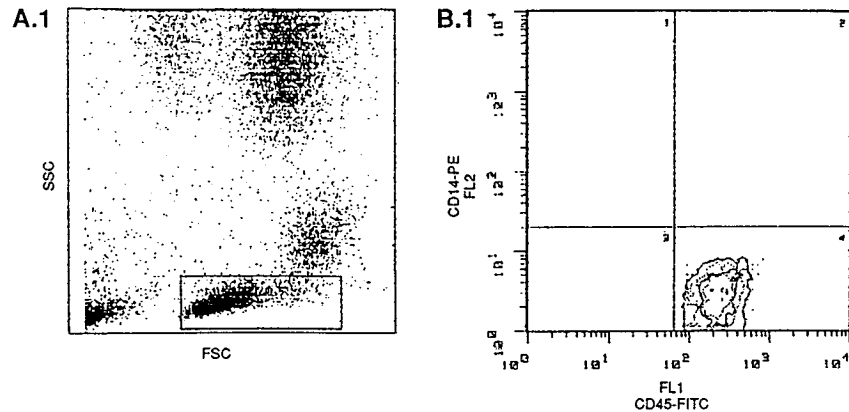
(1) The *proportion of all lymphocytes* present in the specimen that are contained within the boundaries of the lymphocyte light-scatter gate:

Optimally, at least 95% of all *lymphocytes* in each sample should be contained within the lymphocyte light-scatter gate.

Minimally, at least 90% of all *lymphocytes* in each sample must be contained within the lymphocyte light-scatter gate.

(2) The *presence of nonlymphocyte elements* present within the boundaries of the lymphocyte light-scatter gate: The *minimal acceptable percentage* of all cells that are within the light-scatter gate **that are lymphocytes as determined by phenotype**, is 85%. (See NCCLS H42-T,

FACScan[™] Flow Cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA)



EPICS[®] Profile[®] Analyzer (Coulter Corporation, Hialeah, FL)

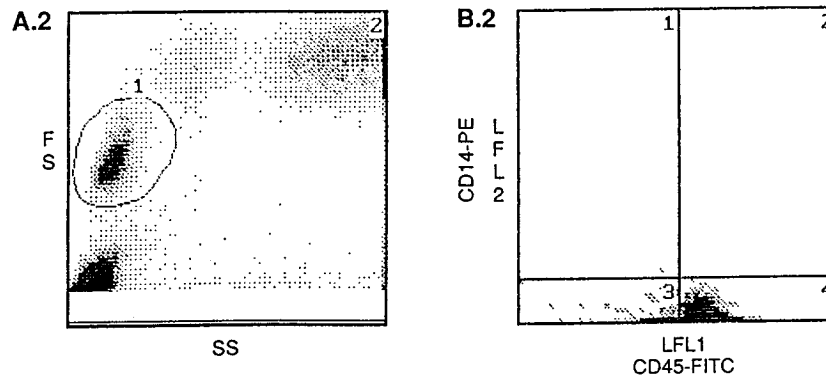


FIG. 1. **A.1 and 2.** Dual-parameter light scattergrams showing lymphocyte gate drawn to include at least 95% of all lymphocytes in the specimen and >85% CD45^{bright}CD14^{negative} lymphocytes within the gating

region. **B.1 and 2.** Dual-parameter fluorescence histograms showing the fluorescence distribution from the lymphocyte gates in A.1. and A.2. (B.1. = 97.9%; B.2. = 96.4%).

Sections 11.3–11.4 for more detailed presentation of lymphocyte gate optimization and verification of representative sampling.)

3.08.c. If the percentage of CD45^{bright}CD14^{negative} cells in the light scatter lymphocyte gate is <85%, the light scatter gate should be redrawn.

Care should be taken in redrawing the gate *not to exclude* additional lymphocytes.

If the CD45^{bright}CD14^{negative} population is still <85%, an aliquot of the same specimen could be restained (if the specimen is <30 h old).

If, after restraining, the CD45^{bright}CD14^{negative} population is still <85%, the Flow Cytometry Laboratory should notify appropriate study personnel to redraw the specimen.

If the CD45^{bright}CD14^{negative} population remains <85% in the redrawn specimen, the data should **not** be included in the protocol/study database.

3.08.d. If the CD14-positive population is >5%, the light-scatter lymphocyte gate should be redrawn to de-

crease the percentage of CD14-positive monocytes in the lymphocyte gate.

3.09. Enumeration of Lymphocyte Subsets: Fluorescence Analysis

3.09.a. Dual color fluorescence quadrant analysis. [NCCLS H42-T 12.4] Fluorescence data should be displayed as a histogram of log FITC immunofluorescence vs. log PE immunofluorescence.

This histogram is then divided into rectangular quadrants using horizontal and vertical cursors corresponding to the x and y axes.

Quadrant analysis provides the advantage of greater precision in identification of lymphocyte subsets based on co-expression of antigens defined by dual-color monoclonal antibody combinations.

Quadrants are selected to differentially contain cells/events that bind neither antibody, bind only FITC-conjugated antibody, bind only PE-conjugated antibody, or bind both antibodies (Fig. 3).

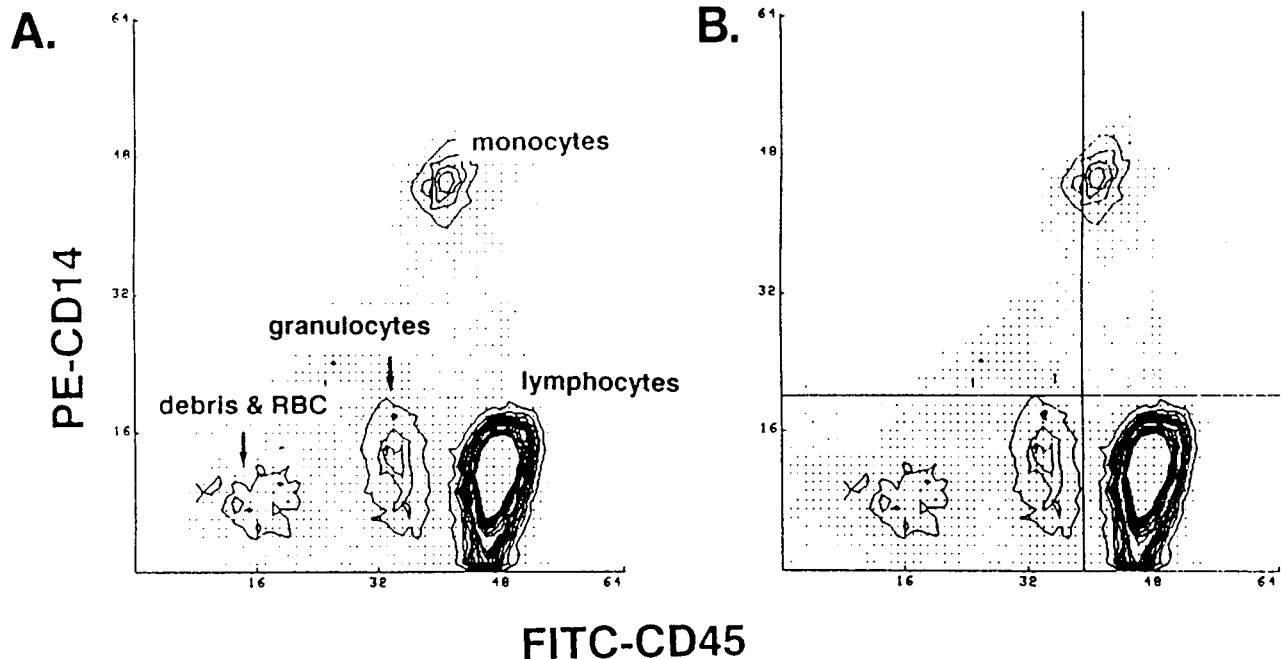


FIG. 2. A. Distribution of major leukocyte populations on a dual-parameter fluorescence histogram from an 'ungated' light scattergram, identified by staining with PE-conjugated CD14 and FITC-conjugated CD45. B. Fluorescence histogram with cursor correctly positioned for identification of CD45^{bright} cells (Reprinted from Reference 29).

3.09.b. Cursor settings for data analysis: Isotype control. Cursor settings obtained using the isotype control should be used as the "base" reference for enumeration of lymphocyte subsets.

The cursor settings obtained using the fluorescence signals of the isotype control *may not be appropriate* for all markers used.

The fluorochrome:protein ratio and/or the binding avidity of the specific monoclonal antibody used will necessarily differ from the isotype control and can result in aggregate formation via crosslinking. This can influence the appropriateness of the "base" cursor settings for fluorescence analysis for lymphocyte subsets.

3.09c. Correction of lymphocyte subset percentages. Percentages of lymphocyte subsets obtained from the flow cytometer **must be corrected** for CD45^{bright}CD14^{negative} values *between 85% and 95%* and may be carried out for values *above 95%*. Correction for CD45^{bright}CD14^{negative} values *above 95%* does not substantially affect the resulting value.

Correction is carried out by *dividing* the subset percentage *by* the percentage of CD45^{bright}CD14^{negative} lymphocytes in the lymphocyte gating region. Example:

$$\text{raw CD4 \%} = 41\%$$

$$\text{CD45}^{\text{bright}}\text{CD14}^{\text{negative}} \% = 92\%$$

$$\text{Corrected CD4 \%} = 41\% / 0.92 = 45\% (44.6\%)$$

3.10. Reference Ranges and Expected Results

3.10a. Each laboratory must establish age- and population-appropriate reference ranges, based on methodologies *currently in use*, in accordance with valid statistical criteria.

3.10b. It should be noted that pediatric reference ranges differ substantially, *particularly between birth and age 4*, from reference ranges for adult populations (31).

3.10c. Expected results. The CD3^{positive}CD4^{positive} + CD3^{positive}CD8^{positive} percentages should = Total CD3^{positive} ± 10%.

Lymphosum: For most specimens, the total of the corrected CD3^{positive} (Total T cells) + CD19^{positive} (Total B cells) + CD3^{negative}CD56^{positive} and/or CD16^{positive} (Total NK cells) percentages *ideally* should sum to between 95% and 105% (28).

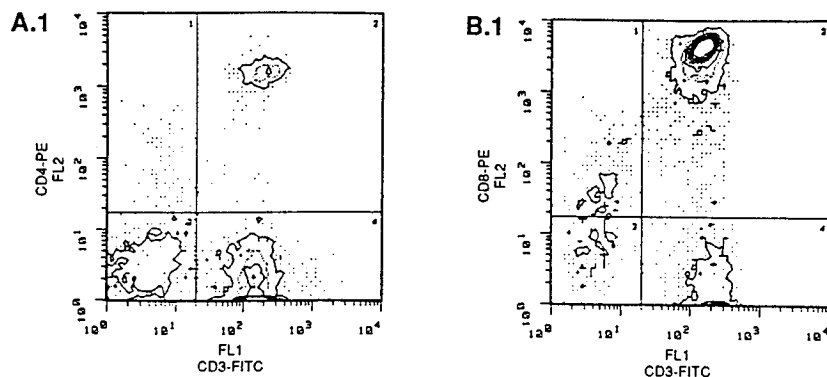
3.11. Laboratory Reports

Laboratory reports should optimally include lymphocyte subset percentages, absolute values, and laboratory reference ranges.

3.11a. Laboratory reports should specify the immunophenotype (CD designation) for all lymphocyte subsets enumerated (e.g., T-helper/inducer = CD3^{positive}CD4^{positive}, etc.).

3.11b. Values for lymphocyte subsets should be corrected for the lymphocyte representation in the gating region (3.09c.).

FACScan[™] Flow Cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA)



EPICS[®] Profile[®] Analyzer (Coulter Corporation, Hialeah, FL)

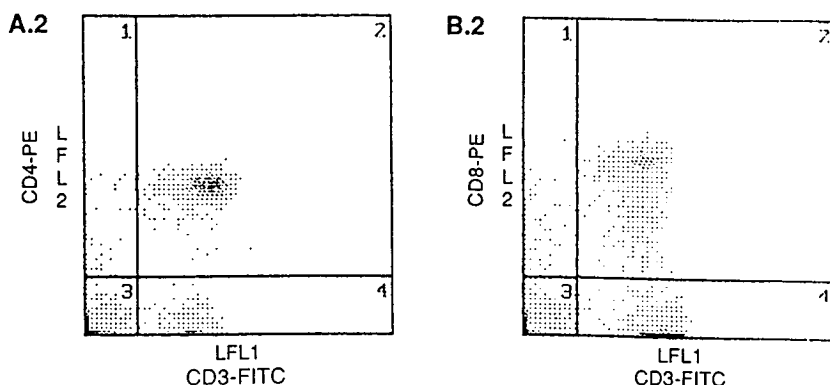


FIG. 3. Dual-parameter fluorescence histograms generated from the lymphocyte gating region, showing the fluorescence distributions for specimens stained with: A.1 & 2. CD3-FITC + CD4-PE and B.1 & 2. CD3-FITC + CD8-PE. The percentage of CD4-positive T cells is taken from Quadrant 2 of the A histograms (A.1. = 11.2; A.2. = 36.8); and

the percentage of CD8-positive T cells is taken from Quadrant 2 of the B histograms (B.1. = 73.6; B.2. = 30.1). The Total CD3-positive T cell percentage is obtained as the average of (Quadrant 2 + Quadrant 4) for the CD3/CD4 and the CD3/CD8 tubes.

3.11c. Absolute cell counts for lymphocyte subsets should be reported using *whole-number* hematologic cell counts obtained with automated instrumentation.

QUALITY ASSESSMENT PROGRAM

4.01 Participation

Laboratories performing flow cytometric enumeration of lymphocyte subsets for NIAID DAIDS protocols must participate in the Flow Cytometry Quality Assessment Program designated by the NIAID DAIDS Program Office.

For quality assessment specimens, laboratories are required to measure and report all phenotypes performed on NIAID DAIDS study specimens.

4.01a. NIAID DAIDS Flow Cytometry Laboratory Certification (for overview, see Appendix, Fig. 1).

4.01.a1. Laboratories will undergo quarterly evaluation for proficiency in determining lymphocyte phenotypes based upon shipments of three whole blood specimens per month for a total of nine specimens per quarter.

As of October 1, 1992, only CD3⁺CD4⁺ data will be used for this evaluation. In the future, laboratories will be

evaluated based upon results obtained with additional immunophenotypes (e.g., CD8).

The unsatisfactory **level of performance** is currently defined as 33% of a laboratory's CD4 analyses with residual values greater than or equal to $\pm 5\%$ and with deviates greater than or equal to 2. The residual value is a laboratory's CD4 value minus the median of all participating laboratories. The deviate is the residual divided by the interquartile range (IQR). The IQR is *three-quarters of the difference* between the 25th and the 75th percentiles of participating laboratories (16).

Example. For this example assume that only seven laboratories participate in the NIAID DAIDS flow cytometry quality assessment program for a particular month. Their median value for CD4% for one sample is 40%. The table below shows the values obtained by the individual laboratories, their residuals, their deviates, and whether or not their performance for that sample is acceptable. Note that $(7 + 1)/2 = 4$ so that the median is the fourth smallest value, or 40%. $(7 + 1)/4 = 2$ so the 25th percentile is the second smallest value, or 38%. The 75th percentile is the

second largest value, or 42%. Therefore, the interquartile range (IQR) is $0.75 * (42 - 38) = 3$.

| Reported lab CD4 value | Residual value = reported-40 | Deviate = residual/3 | Performance based on this sample |
|------------------------|------------------------------|----------------------|----------------------------------|
| A 29 | -11 | -3.67 | unacceptable |
| B 38 | -2 | -0.67 | acceptable |
| C 38 | -2 | -0.67 | acceptable |
| D 40 | 0 | 0 | acceptable |
| E 41 | 1 | 0.33 | acceptable |
| F 42 | 2 | 0.67 | acceptable |
| G 45 | 5 | 1.67 | acceptable |

4.01.a2. Laboratories will be rated Certified, Provisionally Certified, Probationary, or Suspended.

4.01.a3. Certified status. These laboratories may accept and analyze patient specimens from other NIAID DAIDS study sites.

Failure to perform at the satisfactory level for 1 quarter will result in a change of status from Certified to Provisionally Certified at which time the Site Immunologist/Flow Cytometry Laboratory Director will receive the following notification:

As the result of your unsatisfactory performance in the NIAID DAIDS Flow Cytometry Quality Assessment Program in the last quarter [DATE], your laboratory moved from Certified to Provisionally Certified. This does not restrict your analysis of specimens in current or future NIAID DAIDS protocols at your study site; however, until your laboratory attains the status of Certified, you may only accept specimens from NIAID DAIDS study sites for whom you currently perform analyses. Certified status may be regained after two consecutive quarters of satisfactory performance in the NIAID DAIDS Flow Cytometry Quality Assessment Program.

4.01.a4. Provisionally certified status. Laboratories are Provisionally Certified at the beginning of the certification process. Certified status is achieved by performing satisfactorily at the Provisionally Certified level for two consecutive quarters.

Provisionally certified laboratories may only accept specimens from other NIAID DAIDS study sites for whom they currently perform analyses.

Unsatisfactory performance for two consecutive quarters will result in a change of status from Provisional to Probationary and the site immunologist/flow cytometry laboratory director and study site principal investigator will receive the following notification:

As the result of your unsatisfactory performance in the NIAID DAIDS Flow Cytometry Quality Assessment Program in the last two quarters [DATES], your laboratory moved from Provisionally Certified to Probationary status. You are prohibited from analyzing specimens for any new single center or small (<6 study sites) multicenter NIAID DAIDS trials until your laboratory attains the

status of Provisionally Certified. You may continue current studies and accrue new patients to these studies and participate in any new large multicenter NIAID DAIDS Trials. Provisionally Certified status may be regained after two consecutive quarters of satisfactory performance in the NIAID DAIDS Flow Cytometry Quality Assessment Program.

4.01.a5. Probationary status. The site immunologist/Flow Cytometry Laboratory director and principal investigator will be notified of Probationary status by registered letter.

Progress of these laboratories will be closely monitored.

Provisionally Certified status may be regained after two consecutive quarters of satisfactory performance.

Laboratories that remain Probationary for three consecutive quarters will be recommended for Suspended status.

4.01.a6. Suspended status. Laboratories will be suspended by action of the Executive Committee(s) of the relevant NIAID DAIDS study group(s) (e.g., AIDS Clinical Trials Group (ACTG) Executive Committee; Multicenter AIDS Cohort Study (MACS) Executive Advisory Committee; Women Infant Transmission Study (WITS) Advisory Group, etc.).

The criteria for regaining Probationary status will be determined by a joint recommendation of the NIAID DAIDS Flow Cytometry Advisory Committee and the appropriate executive committee.

The NIAID DAIDS Site Immunologist/Flow Cytometry Laboratory director and principal investigator will be notified of Suspension by registered mail.

Until the laboratory achieves Probationary status, all NIAID DAIDS study specimens must be sent to a Certified laboratory for analysis.

4.01.a7. Reporting infractions. Laboratories that **do not report**, or are **late** in reporting their monthly proficiency testing data will be notified in writing within 10 working days following the due date.

For each late or nonreporting notification, laboratories will be penalized equivalent to one unsatisfactory sample.

4.01b. Other quality control data. Although certification status of study site flow cytometry laboratories is currently dependent only upon acceptable evaluation of CD4 percentage, performance is evaluated for all subsets analyzed in conjunction with NIAID DAIDS protocols (e.g., CD45, CD14, CD3, CD4, CD8, CD19) for the three monthly sendout specimens to be stained at individual study sites. Results from a fourth "unknown" specimen (prestained cells; one or two-color beads, etc.) designed to address specific aspects of flow cytometric analysis of lymphocyte subsets, are also evaluated for each study site's Flow Cytometry Laboratory to further assist maintenance of quality assurance of NIAID DAIDS flow cytometry data.

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Appendix Table 1
Quality Assurance Overview^a

| QA Function | Why | When | How (sample preparation) |
|--|--|---|--|
| Initial Instrument Setup | Optimize conditions for sample analysis | | |
| Align optical system | Maximize intensity, minimize variability | New instrument, major maintenance or repair | Homogeneous signals in all channels used for specimen analysis: long term stability (eg. uniform fluorescent plastic beads) |
| Establish values for alignment particles to be used in daily monitoring | Determine laboratory's expected range of values indicating acceptable alignment | Any time significant alignment changes are made | See Alignment |
| Establish Test Specific instrument settings | Optimize optically aligned instrument for analysis of test specimens | Introduction of test; after any significant change in specimen preparation or staining protocol | Specimen from healthy donor prepared using standard test protocol and antibody reagent giving well-resolved positives and negatives (eg. anti-CD3) |
| Establish values for sensitivity particles to be used in daily monitoring | Determine laboratory's expected range of values indicating acceptable fluorescence sensitivity | Any time significant changes are made in test specific instrument settings | See Test-Specific instrument conditions |
| Establish appropriate color compensation levels | Determine instrument settings for accurate analysis of dual fluorochrome labeled cells | Any time significant changes are made in test-specific fluorescence high voltage/gain settings | Lymphocytes stained with mutually exclusive antibodies bearing relevant fluorochromes (eg. FITC-anti-CD3, PE-anti-CD20; brightest possible reagents preferred) or plastic particles with intensities and spectral characteristics similar to bright antibody labeled cells whose values have previously been established under specified instrument conditions |
| Establish values for compensation particles to be used in daily monitoring | Determine laboratory's expected range of values indicating acceptable color compensation | When significant changes are made in color compensation settings | See Color Compensation settings |
| Daily Performance Monitoring | Minimize instrument variability; assure acceptable performance and reproducible results | Whenever samples are run or instrument malfunction is suspected | Long term stability; values established under conditions of optimal alignment (eg. uniform fluorescent beads) |
| Monitor optical alignment | Run reference material (alignment particles) at standard instrument settings to verify acceptable alignment | | See Initial Instrument setup (Alignment) |
| Monitor sensitivity | Run reference material (sensitivity particles) at test-specific settings to verify acceptable sensitivity | | See Initial Instrument Setup (Test-Specific settings) |
| Monitor color compensation | Verify that when measuring cells labeled with 2 different fluorochromes, double positives are being accurately counted | When lymphocytes labeled with two different fluorochromes are run | Freshly prepared positive control sample stained or plastic compensation particles whose values have previously been established after appropriate compensation levels were set (see Initial Instrument Setup-Color Compensation) |
| Verify system performance | Verify performance of samples preparation and staining method using samples for which expected results are known | Whenever test specimens are run; whenever sample prep or staining problems are suspected | Specimen from a healthy donor prepared according to standard test protocol using reagent for which laboratory reference limits are established |

^aAdapted with permission from National Committee for Clinical Laboratory Standards: Clinical applications of flow cytometry: Quality assurance and immunophenotyping of peripheral blood lymphocytes; Tentative Guideline. NCCLS Document H42-T (ISBN 1-56238-155-5). NCCLS, Villanova, PA, Vol. 12, No. 6, 1992.

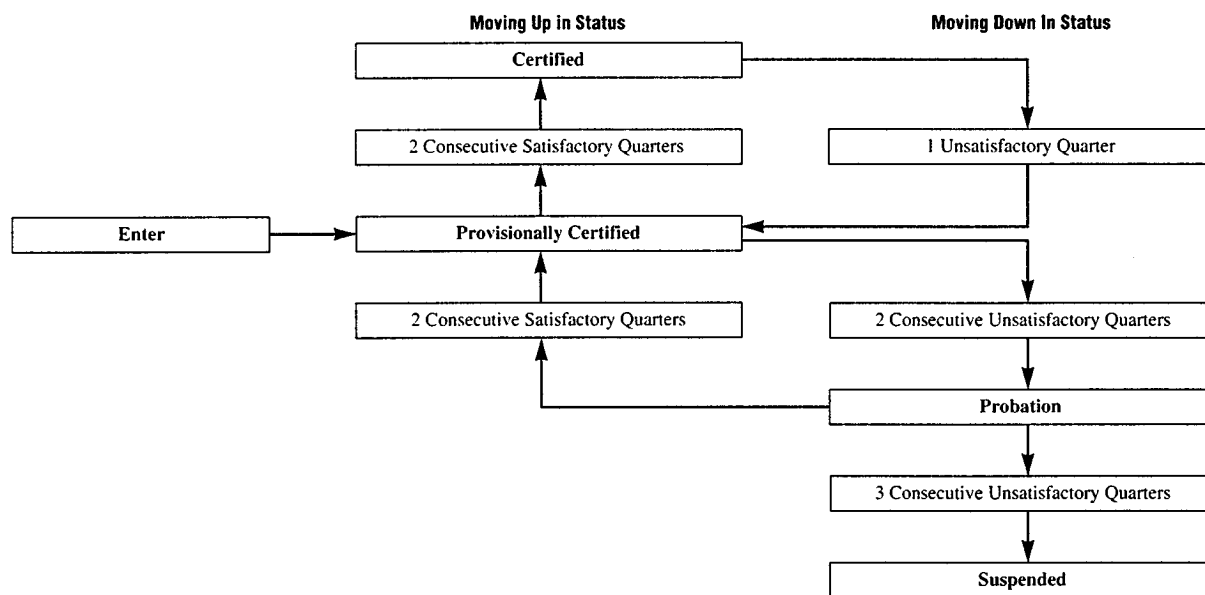
Table 2
Potential Sources of Artifacts in Immunophenotyping by Flow Cytometry^a

| Cause | Effect | Resulting Artifact |
|---|---|---|
| Medications/Drugs: | | |
| 1. Zidovudine (AZT) | Increased granulocyte fragility | Decreased light scatter resolution; Increased granulocyte contamination of mononuclear preparation |
| 2. Some Antibiotics (eg. cephalosporins) | Increased cellular autofluorescence | False positives if appropriate Negative control is not used |
| 3. Some chemotherapeutic agents (eg. daunorubicin) | Increased cellular autofluorescence | False positives if appropriate negative control is not used |
| 4. Nicotine | Increased lymphocyte counts | Elevated absolute values for lymphocyte subsets |
| 5. Corticosteroids | Increased lymphocyte margination; decreased CD4 levels | Overestimation of disease-related alterations |
| Biological Factors: | | |
| 1. Reticulocytosis | Incomplete red cell lysis, increased RBC contamination of mononuclear preps | Decreased light scatter resolution; RBC contamination of lymphocyte gate |
| 2. Strenuous exercise | Increased lymphocyte margination; decreased lymphocyte counts | Lowered absolute values for lymphocyte subsets |
| 3. Diurnal variation | Variable absolute lymphocyte count | Variable absolute values for lymphocyte subsets |
| 4. Specimen age and holding conditions | Variable granulocyte preservation and/or leukocyte viability | Increased granulocyte contamination of lymphocyte gates; false positive non-specific staining of dead cells |

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NIAID Division of AIDS Flow Cytometry Certification Program¹

Certification Program Schematic



Definitions for Schematic

| | |
|-----------------------------|---|
| Certified: | Satisfactory performance for CD4 percent determination |
| Provisionally Certified: | Initial level of certification or 1 unsatisfactory quarter as a certified lab |
| Probationary: | 2 consecutive quarters of unsatisfactory performance at provisionally certified level |
| Suspended: | 3 quarters of consecutive unsatisfactory performance at the probationary level |
| Quarter: | 3 month interval |
| Satisfactory Performance: | Performance meets established criteria |
| Unsatisfactory Performance: | Performance does not meet established criteria |

¹Beginning January 1994, the NIAID DAIDS switched from monthly to bimonthly shipment of specimens, with performance assessment every four months (trimester) instead of every three months (quarter).