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Clinical Applications of Flow Cytometry: Immunophenotyping of Leukemic Cells; Approved Guideline

This documents provides performance guidelines for the immunophenotypic analysis of leukemic and lymphoma cells using immunofluorescence-based flow cytometry; for sample and instrument quality control; and precautions for acquisition of data from leukemic cells.

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Clinical Applications of Flow Cytometry: Immunophenotyping of Leukemic Cells; Approved Guideline

Abstract

The increasing acceptance of immunophenotyping for the proper management of patients with leukemia and lymphoma necessitates the development of guidelines for the appropriate performance of these techniques in the clinical laboratory. NCCLS document H43-A—*Clinical Applications of Flow Cytometry: Immunophenotyping of Leukemic Cells; Approved Guideline*, addresses issues of safety, specimen collection and transportation, sample preparation, immunofluorescent staining, instrument quality control, data acquisition, and data storage for the application of flow cytometry to the immunophenotypic analysis of these disorders. This document builds on NCCLS document H42-A— *Clinical Applications of Flow Cytometry: Quality Assurance and Immunophenotyping of Peripheral Blood Lymphocytes*.

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Foreword

H43-A establishes performance guidelines for the immunophenotyping of specimens of leukemia and lymphoma by flow cytometry. It is designed to help clinical laboratories using different commercially available instruments and reagents obtain comparable results and to aid these laboratories in the development of quality assurance procedures that are specifically applicable to leukemia and lymphoma.

This document follows a related document, H42-A— *Clinical Applications of Flow Cytometry: Quality Assurance and Immunophenotyping of Lymphocytes; Approved Guideline.* In some respects, some sections of the current document—particularly those that cover specimen collection and transportation, safety, instrument quality control, and data storage—are similar to those in H42. However, issues specific to the study of samples of leukemia and lymphoma are covered in sections on sample preparation, sample staining and quality control procedures, data acquisition, data analysis, and result reporting and interpretation.

There has been a substantial expansion of the application of flow cytometry in leukemia since the initial publication of this proposed guideline as H43-P in 1993. Instrumentation has improved, routine use of three or even four color flow cytometry has expanded, and the number of publications devoted to clinical applications of this technology has greatly increased. The principles outlined in the proposed guideline served as a starting point for other publications on immunophenotyping of leukemic cells from Europe and Japan, and for a North American group whose work is still unpublished at this writing. The subcommittee felt that incorporating all of these efforts, and updating all the changes in this rapidly moving field would have required an extensive rewriting of this document, and unnecessarily duplicate the efforts of these and other groups. At the same time, the principles outlined in this document are still valid and useful for laboratories providing the clinical service of leukemia phenotyping. This document is therefore presented as a final guideline with relatively few changes from those in the provisional document, except those raised by the community with regard to underlying principles. The reader is encouraged to consult the publications and documents under development on specific issues regarding consensus practice in different geographic regions.

Standard Precautions

Because it is often impossible to know which might be infectious, all patient blood specimens are to be treated with standard precautions. Guidelines for specimen handling are available from the U.S. Centers for Disease Control and Prevention [*MMWR* 1987;36(supple 2S): 2S-18S]. NCCLS document M29—*Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue*, deals specifically with this issue.

Key Words

Autofluorescence, cluster differentiation system, color compensation, density gradient centrifugation, dual parameter display, fluorescein isothiocyanate, fluorescence intensity, forward angle light scatter, gate, histogram, immunophenotyping, list mode, 90° light scatter, phycoerythrin, procedural control, tandem conjugate, viability.

Acronyms

(ACD)	acid citrate dextrose
(ALL)	acute lymphocytic leukemia
(AML)	acute myeloid leukemia
(amp)	amplifier
(BSC)	biological safety cabinet
(BSL-2)	Biosafety Level 2
(CV)	coefficient of variation
(EDTA)	ethylene-diaminetetraacetic acid
(FALS)	forward angle light scatter
(IC)	isotope control
(kd)	kilodalton
(LS)	light scatter
(MCF)	mean channel fluorescence
(mV)	millivolt
(PE)	phycoerythrin
(PI)	propidium iodide
(RBC)	red blood cell
(TdT)	terminal deoxynucleotidyl transferase
(V)	volt
(WBC)	white blood cell

Clinical Applications of Flow Cytometry: Immunophenotyping of Leukemic Cells; Approved Guideline

1 Introduction

This document represents the efforts of the NCCLS Subcommittee on Flow Cytometry to extend the guidelines for immunophenotyping by flow cytometry to studies of leukemia and lymphoma. To this end, it builds upon these guidelines established in document H42—*Clinical Applications of Flow Cytometry: Quality Assurance and Immunophenotyping of Lympho-cytes*, and indeed, several sections of this document are similar to those in H42. However, the subcommittee recognizes that the use of flow cytometry to characterize cases of leukemia presents several specific problems that are not covered in H42. Issues specific to the study of leukemia and lymphoma that are covered in this document include:

- patient groups to be included;
- sample preparation techniques particular to neoplastic specimens;
- reagent panels to be employed;
- types of methodologic controls required and the necessary frequency of their use;
- rules and precautions to be followed in acquisition of data from leukemic specimens;
- goals and methods of analysis unique to leukemic samples, with emphasis on dual or multiparameter analysis; and
- guidelines for interpretation and reporting of data.

An overview of the differences between pheno-typing neoplastic samples and nonneoplastic lymphocytes is provided in Section 3.

2 Scope

This document establishes performance guidelines for the immunophenotypic analysis of samples from patients with leukemia and lymphoma using immunofluorescence-based flow cytometry. It is clear that immunophenotypic analysis of hematologic neoplasms is crucial for the accurate diagnosis and classification of these complex malignancies. However, the subcommittee believes strongly that, in current practice, flow cytometric data must be interpreted in the context of additional information obtained using more conventional morphologic and cytochemical methods. Because this document does not address this other information, it will not propose a modification of existing classification systems of leukemia, or provide recommendations for criteria used to diagnose leukemias. Similarly, because this document is intended primarily for laboratory workers in flow cytometry, it cannot describe all the possible clinical situations in which flow cytometric analysis of leukemia is appropriate. Other organizations and committees, including the French–American–British group, the National Cancer Institute, the College of American Pathologists, and the International Council for Standardization in Haematology, among others, are engaged in formulating broader recommendations.

This document includes guidelines for phenotyping cases of acute leukemias, chronic lymphoid leukemias, and non-Hodgkin's lymphomas. In current practice, there is little acceptance for the phenotyping of chronic myeloid leukemias before they enter the blast phase. For this reason, the term "chronic leukemia" is sometimes used to imply chronic lymphoid leukemia. The subcommittee also recognizes that most of the principles used to approach chronic lymphoid leukemias can also be applied to the study of lymphomas so that these problems are considered together. However, certain special problems unique to the study of lymphomas are treated separately. In most places in the text, the term "leukemia" is used generically to imply either acute or chronic leukemia or lymphoma. However, the subcommittee recognizes the fact that problems laboratories have in studying acute and chronic leukemias often differ with respect to reagent selection, gating strategies, data analysis, and interpretation of results.

Finally, the subcommittee recognizes that there is growing interest in the use of flow cytometry to investigate minimal involvement of blood or bone marrow (so called minimal residual disease [MRD]) by acute leukemia detection; however, the subcommittee believes that approaches are not sufficiently standardized to investigate at this time and are therefore outside the scope of this document.

At present, there are no generally agreed-upon standards for precision, accuracy, and interlaboratory comparability of leukemia analysis by flow cytometry. Therefore, it is each laboratory's responsibility to establish instrument performance criteria and staining characteristics for its own specific reagents.

3 Overview

In a previous document, this subcommittee outlined general principles that are useful for performing flow cytometry on blood samples. Some of these principles are directly applicable to the study of leukemic samples. As currently practiced, immunophenotyping by flow cytometry is used in conjunction with morphologic and other information to help in the diagnosis and classification of leukemia. Although evolution of technology may someday change how this information is used, the current document is written from this perspective. Because neoplastic cells differ in many ways from normal cells, and because the overall goals of leukemia phenotyping are different from those of lymphocyte subset enumeration, in several important respects special procedures are necessary for the study of leukemias. This section outlines the most important differences; detailed information is then presented in appropriate sections to follow.

3.1 Goals

The principal goal of leukemia phenotyping is to determine the expression of lineage- and differentiation-associated antigens on leukemic cells. This differs from that of blood lymphocyte phenotyping, whose aim is to enumerate antigenically defined lymphocyte subsets. In the case of acute leukemia, phenotypic characterization aids in the classification of the leukemia. In the case of chronic lymphoid leukemias, one of two situations may pertain. First, where blood, bone marrow, or other tissue is completely replaced by neoplastic lymphoid cells, flow cytometry is again used to determine immunophenotype and thus aid in classifying the leukemia. Second, in some circumstances the goal of flow cytometry is to determine if a population of lymphocytes is leukemic or not. In this situation, gating strategies and data interpretation are often different.

3.2 Quality Control Procedures

Instrument setup for leukemia phenotyping is generally similar to that used for lymphocyte enumeration. However, in the case of leukemia, it is often extremely important to be able to distinguish dim reactivity from either back-ground autofluorescence or nonspecific staining. Section 10.2 addresses the importance of appropriate negative control reagents and also points out how to construct a panel of test reagents to include reagents that can also potentially serve as negative controls. Section 11 describes procedures for ensuring that the instrument is capable of resolving low-intensity fluorescence.

Several reagents used for leukemia phenotyping may not react with normal blood lymphocytes, and special procedures for verifying their reactivity may have to be instituted.

3.3 Sample Preparation

Although the methods described for processing blood may be applicable to the study of leukemic blood samples, additional types of samples are often received for leukemia or lymphoma analysis. While bone marrow can be processed by erythrocyte lysis techniques, special care is often necessary to ensure that the cell concentration is appropriate for the amounts of antibody being used. Alternatively, density gradient separation techniques may be used, although these are discouraged in lymphocyte subset enumeration because of the selective loss of certain subsets; this is not a consideration in the analysis of leukemia. Finally, lymph nodes or other tissues may be received and special procedures followed in order to produce a representative cell suspension.

3.4 Reagents

The reagent panels used for leukemia phenotyping are potentially much more extensive than those used for enumerating lymphocyte subsets (see Section 9). No specific required panel is described in this document, but several principles are stated. First, the best reagents for characterizing cases of acute and chronic leukemia are different. Second, many different types of multicolor reagent panels can be constructed. Because visual examination of dual parameter displays plays such an important role in data analysis and interpretation (see Section 13 and Appendix C), the laboratory should devise a few panels and become familiar with the patterns of reactivity seen with these reagents on either scatter versus fluorescence or dual fluorescence plots.

3.5 Sample Analysis

When a stained leukemic sample is analyzed, it is vital to ensure that the signals acquired represent those of the leukemic cells. Obviously, the safest course is not to set any restrictive acquisition gates. However, in some cases, leukemic cells may only represent a portion of the cells to be analyzed, and it would be desirable to eliminate normal cells, provided that doing so did not also eliminate leukemic cells. Setting a gate to exclude some high 90° light scatter (LS) cells (i.e., granulocytes) is the most common way of doing this. In many cases, leukemic cells will not occupy a unique region on the forward angle light scatter (FALS) versus 90° LS display, and it will not be possible to draw a gate to selectively acquire leukemic cells. Under these circumstances, alternate gating strategies that use immuno-fluorescence may help to identify the leukemic population (see Section 12.5.4).

It is recommended that all signals be acquired unless it can be conclusively shown that those to be excluded do not belong to the leukemic population. Review of a morphologically stained preparation from the sample to be analyzed may help determine which signals are of greatest interest. Because the data that are thus collected may be complex and contain several different populations, many different gates may have to be set later when the data are analyzed. For this reason, it is recommended that data be acquired in list mode.

3.6 Data Analysis and Interpretation

3.6.1 Data Analysis

The goal of data analysis in leukemia is to determine which antibodies in a panel react with leukemic cells or, in some cases of chronic leukemia, to determine if a population of cells in a specimen expresses an abnormal combination of antigens. To this end, descriptions of "percent reactivity" of several monoclonal antibodies may not be informative, particularly in specimens that contain a mixture of leukemic and nonleukemic cells. Moreover, the conventional method of analyzing histograms—in which a cursor is placed at the boundary of a negative control and events in the test histogram to the right of this boundary are considered positive—is often not appropriate for analyzing distributions in leukemias because control histograms often show considerable overlap with test histograms containing low-intensity positives. Fluorescence intensity becomes an important variable.

For these reasons, data analysis in leukemia is different from that in enumeration of lymphocyte subpopulations. Comparison of histograms of single parameter test data to controls may be appropriate for specimens in which the great majority of the cells are leukemic. However, in many cases, substantially more information is gained when dual parameter displays of data — either of correlated fluorescence or of scatter versus fluorescence—are analyzed (see Section 13.4).

3.6.2 Data Interpretation

Once each of the antibodies in the panel has been analyzed and it has been determined which ones react with leukemic cells in a specimen, some synthesis of the data should be done. However, note that the diagnosis of acute leukemia requires morphologic assessment of blood or bone marrow to determine that an appropriate percentage of blasts is present; the main role of

immunophenotyping is to assign lineage to the leukemia. Even in chronic leukemia, where demonstration of an abnormal phenotype may help to establish the diagnosis of leukemia, it may be dangerous to make a specific diagnosis in the absence of morphologic correlation. Thus, it is recommended that information from immunophenotyping be reported primarily in descriptive form; data can be used in a diagnostic report provided that additional information is also used to establish the most accurate diagnosis. Obviously, close communication between the flow cytometry laboratory and the physician responsible for making the diagnosis is essential.

Results from flow cytometry studies should be reported to give the composite immunophenotype of the leukemic cells, with some estimate of the number of leukemic cells present in the sample if this is possible. The interpretation should include the cell lineage of the tumor, and it might also include descriptions of unexpected results such as aberrant antigen expression. Although some general rules can be laid out for deducing lineage based on patterns of antibody reactivity, it is not possible to provide a simple, unambiguous algorithm that can cover all possible outcomes.

4 Definitions of Terms

Analysis boundary, *n* - dividing line (cursor) placed on a histogram or dual parameter display that discriminates events that are considered positive or reactive with a particular antibody from those that are negative or nonreactive. An analysis boundary is most commonly set on a histogram obtained using a negative control antibody so that a fixed small proportion of events is considered positive. Traditional analysis boundaries are often inappropriate for analyzing leukemic samples.

Autofluorescence, *n* - The intrinsic fluorescence of unstained cells is generally caused by pyrimidines and flavin nucleotides. The level of autofluorescence is a function of the excitation wavelength and varies with the cell type being analyzed and/or the state of cellular activation. Cultured cell lines and macrophages usually demonstrate higher levels of autofluorescence.

Cluster differentiation (CD) system, *n* - The identification of monoclonal antibodies with similar patterns of reactivity with human cells has been the focus of five international workshops. Each group of antibodies was assigned a CD number. Not all antibodies in a CD group react with identical portions (epitopes) of their target antigen. An antigen recognized by a given cluster of antibodies (e.g., CD4) is referred to as a "CD antigen" (e.g., CD4 antigen). An antibody that belongs to a given cluster is referred to as CD "x," with the manufacturer's nomenclature given in parenthesis (e.g., CD4 [Leu3a]).

Color compensation, *n* - Electronic or mathematical subtraction of a fraction of one signal from a second, typically used in correcting for overlapping fluorescence from one fluorochrome in the wavelength region where the second is to be measured so that populations stained exclusively with each fluorochrome appear at right angles to each other.

Density gradient centrifugation, *n* - A physical separation procedure whereby a heterogeneous population of cells of varying densities is layered onto a medium of known density and sedimented by centrifugation resulting in the separation of cells according to density. Practically speaking, it is used to separate lighter mononuclear cells from denser granulocytes and erythrocytes.

Dual parameter display (dot plot, contour plot, cytogram, two-parameter histogram), *n* - A graphic representation of data in which correlated values for two different parameters measured on the same cell are plotted on an *x*,*y* grid.

Epitope (antigenic determinant), *n* - That portion of an antigen against which the specific binding region of a monoclonal antibody reagent is directed. Epitopes may be linear sequences of as few as six amino acids or conformationally determined sections of the antigen; each antigen typically contains multiple epitopes.

FITC (fluorescein isothiocyanate), *n* - The most common fluorochrome for cell

phenotyping. Fluorescein conjugates absorb maximally at approximately 490 nm, close to the 488-nm emission of argon lasers, and emit near 525 nm. Each conjugated fluorescein molecule adds a net negative charge to the antibody and therefore may change its potential binding characteristics.

Fluorescence intensity, *n* - A measurement of the amount of fluorochrome bound to a particle or cell. Increasing intensity is reflected in a fluorescence signal appearing in a higher channel number. Under appropriate conditions, fluorescence intensity can be related to the number of binding sites a cell has for a particular fluorochrome-conjugated reagent.

Forward angle light scatter (Low angle; FALS; FSC; FS), *n* - Measurement of light at a low radial angle relative to the incident light source. Measured values are a function of the cross-sectional area and refractive index of a cell or particle and the wavelength used for measurement. It is commonly used as an estimate of the relative size of a cell or particle.

Gate, *n* - A set of parameters used to electronically select particular cells to be evaluated. Typically, a region of interest is defined based on one set of parameters (such as FALS versus 90° LS) and other parameters (such as fluorescence) are evaluated only for cells within that defined region.

Immunophenotyping, *n* - Identification of cell surface antigens (markers) characteristic of subsets of leukocytes utilizing fluorescence-labeled antibody reagents that recognize cell-associated molecules.

Linear amplification, *n* - A linear amplifier (amp) produces a signal output proportional to the input signal amplitude. For example, a linear amp could have output varying from 1 to 5 V as the input signal varies from 0.01 to 0.05 V.

List mode, *n* - A data storage format in which individual values of all parameters for each cell in a sample are retained, allowing maximum flexibility for subsequent analysis.

Live gate, *n* - Also called an acquisition gate, this represents a set of parameters used to collect data on a population of cells for further analysis. A live gate is typically set when the population of interest represents only a minor fraction of the total population but when one or more parameters clearly distinguishes this population from the larger population of less important cells.

Logarithmic amplification, *n* - A logarithmic amplifier produces an output signal value proportional to the logarithm of the input signal value. A four-decade log amp might, for example, have an output of 0 V for a 1-mV input; 2.5 V for a 10-mV input; 5 V for a 100-mV input; 7.5 V for a 1-V input; and 10 V for a 10-V input. Log amps are useful when analyzing samples containing cells whose measured parameters differ by orders of magnitude.

90° light scatter, *n* - (right angle [90° LS], wide-angle, side scatter [ss], or orthogonal light scatter): Measurement of light at right angles to the incident light source. This measurement is related to cytoplasmic granularity, membrane irregularity and/or nuclear shape of a cell or particle.

Phycoerythrin (PE), *n* - One of several phycobiliprotein-based fluorochromes, derived from algae or bacteria, which can be conjugated to antibodies for use in immunophenotyping. PE has a molecular weight of 240,000 kD. Although it absorbs light maximally at 545 nm, there is sufficient absorption at the 488-nm excitation by argon lasers to provide a usable signal in the red/orange (575 nm) emission range. (**NOTE**: Denaturation of PE does not cause a spectral shift but instead quenches the fluorescence.) Aggregation of PE/immunoglobulin conjugates causes nonspecific binding.

Procedure control, *n* - Specimen that is drawn, prepared, and stained with the same protocol used for patient specimens. It is used for the following: (1) in instrument setup and monitoring for setting test-specific instrument settings and establishing color compensation settings; and (2) as the control specimen for sample and data analysis.

Quality assessment, external, *n* - External quality assessment refers to a system of retrospectively and objectively comparing results from different laboratories by means of

surveys organized by an external agency. The main object is to establish between-laboratory and between-instrument comparability that is, if possible, in agreement with a reference standard (where one exists). External quality assessment schemes may be regional, national, or international. They may also be limited to the users of a particular instrument. It is sometimes also referred to as "proficiency testing," especially when the external agency is a regulatory agency.

Quality assurance, *n* - The practice that encompasses all procedures and activities directed toward ensuring that a specified product is achieved and maintained.

Quality control, *internal,* *n* - Internal quality control is the set of procedures undertaken in a laboratory for the continual assessment of work carried out within the laboratory and evaluation of the results of tests to decide whether the latter are reliable enough to be released to the requesting clinician. The procedures should include tests on control material and statistical analysis of patients' data. The main object is to ensure day-to-day consistency of measurement or observation that is, if possible, in agreement with an agreed indicator of truth such as control material with assigned values.

Resolution, *n* - The ability to discriminate between cells or particles that have different signal intensities. The ability to resolve discrete populations is a function of biological factors (heterogeneity of signal within each population, difference in signal intensities between populations), as well as instrumental factors (sensitivity, measurement CV).

Sample (patient), *n* - An aliquot prepared from the patient specimen and used to obtain information by means of a specific laboratory test.

Sensitivity, *n* - In this document, the ability to distinguish signals of interest from background optical or electronic 'noise' or biological signals such as those due to autofluorescence or nonspecific reagent binding. **NOTE:** ISO's *International Vocabulary of Basic and General Terms in Metrology* ***(VIM 93-5.10)*** defines 'sensitivity' as the change in response of a measuring {system or} instrument divided by the corresponding change in the stimulus. A significant scientific dispute exists regarding this term, its underlying concept and its definition, with the opposing view defining 'sensitivity' in a manner similar to VIM 93's definition for 'limit of detection.'

Single parameter display (histogram), *n* - The frequency distribution of measured signal intensities (i.e., channel numbers) observed for cells within a population.

Specimen (patient), *n* - A volume of whole blood, bone marrow, body fluid, lymph node or other tissue, appropriately collected, transported, and processed to provide a sample for performing one or more laboratory tests.

Subclass control, *n* - A subclass control is an immunoglobulin of the same isotype (class or subclass) as the monoclonal antibody of interest but without specificity for any known human antigens. Most monoclonal antibodies used in flow cytometry are either IgM or various subclasses of IgG (IgG1, IgG2a, IgG2b, or IgG3). This type of control is typically used to estimate Fc-mediated, nonspecific binding for antibody reagents of a given subclass.

Tandem conjugate, *n* - A chemical complex of two fluorochromes arranged so that some of the energy emitted from the first is used to excite the second. In practice, the most common tandem conjugates link phycoerythrin or other fluorochromes, which can be excited at 488 nm, to a fluorochrome that normally is excited at a longer wavelength than 488 nm, and that emits in the far red region. This makes three-color analysis practical with a single-laser instrument.

Terminal deoxynucleotidyl transferase (TdT), *n* - A nuclear enzyme found in immature cells, principally of the lymphoid lineage, but under some circumstances, in blast cells of myeloid lineage.

Threshold, *n* - The level of signal above which a measured value is believed to be significantly different than background "noise." Threshold for instrument fluorescence sensitivity is the level of signal

found for what is believed to be a nonfluorescent object. Threshold for determination of positive antibody staining is the level of signal found for cells not believed to react specifically with a given antibody reagent.

Viability dye, *n* - A dye that may serve to identify nonviable cells by virtue of its ability to penetrate cells with damaged membranes and subsequently bind to double-stranded nucleic acid. Propidium iodide (PI), is the most commonly used viability dye. It absorbs 488-nm argon laser light and emits in the red spectrum near 620 nm. This can be detected independently of fluorescein-labeled cells but only with appropriate filter/photomultiplier tube conditions when other fluorescent dyes are present. Propidium iodide (PI) cannot be used as an indication of viability after fixation of cells by most methods because all cells become permeable and take up PI.

5 Safety

There are many biologic agents that may be present in blood and body fluids. Because any sample might contain an active agent of infection, which might result in morbidity or mortality if improperly handled, all specimens should be treated as though they are potentially infectious. "Standard precautions" should be observed.

5.1 Specimen Collection

All human body fluids and tissues should be treated as potentially infectious. Blood should be collected as outlined in NCCLS document *M29—Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue*. Take care to prevent needle stick injuries. Needles should be discarded in a puncture- resistant container and they should not be resheathed, purposefully bent, cut, or removed from the syringe.

5.2 Safety Attire

Gloves and laboratory coats should be worn by all personnel who process specimens. If splashing or splattering of the specimen is anticipated, masks and protective eyewear should be worn to prevent exposure of eyes, nose, and mouth. Gloves should be changed and hands washed after completion of the work and before leaving the laboratory.

5.3 Biological Safety Cabinets

If possible, all work should be done in a biological safety cabinet (BSC, Class I or II). If this is not possible, at the very least all procedures that have the potential for creating droplets or aerosols (e.g., vortexing, opening evacuated tubes) should be performed in a BSC (Class I or II).

5.4 Specimen Containers

All specimens should be divided into aliquots and put into tubes that have caps, or microtiter plates that have covers (preferably sealed covers). The caps and covers should be in place except when adding to or removing from the containers.

5.5 Centrifugation

If specimens are centrifuged, care should be taken to avoid aerosol formation (i.e., in sealed vessels or inside safety carriers).

5.6 Pipetting

Pipetting by mouth is *not* allowed. Manual pipetting devices must be used.

5.7 Sharp Devices

Use of "sharps" (e.g., needles, glass Pasteur pipets, and glass containers) should be avoided as much as possible.

5.8 Blood Spills

If a blood spill occurs, it should be decontaminated at once with an appropriate agent such as 1:10 volume dilution of 0.71 mol/L sodium hypochlorite (i.e., undiluted household bleach) or appropriately diluted mycobacteriocidal hospital disinfectants.

5.9 Waste Disposal and Specimen Inactivation

Specimens and all disposable laboratory supplies should be collected in puncture-resistant containers designed specifically for such materials and disposed of by appropriate

means (e.g., incineration or autoclaving).

An alternative technique for specimen inactivation is to mix samples with bleach, retain for 24 hours, and dispose of properly.

For more information on the handling and disposal of medical waste, refer to the most current edition of NCCLS document GP5—*Clinical Laboratory Waste Management*.

5.10 Specimen Storage

It is preferable to analyze only samples in which infectious agents have been inactivated. For flow cytometric analysis, after the last centrifugation step in the labeling procedure, samples should be resuspended in a solution of buffered (pH 7.0 to 7.4) paraformaldehyde or formaldehyde (commonly 0.1 to 2.0%) and stored at 4 °C until analysis. The minimum storage time in paraformaldehyde/formaldehyde required to inactivate potentially infectious agents is not clearly established. Fixation is also used for stabilization of the sample so that it may be analyzed at a later date. Because specimen preparation and fixation techniques may vary depending on lysing agents and type/concentration of fixatives used, the minimum and maximum time the specimens should be stored in fixative varies. For validity of the analysis, the concentration of paraformaldehyde/formaldehyde and maximum storage time should be verified by the laboratory.

5.11 Unfixed Specimens

When unfixed specimens are to be run on the flow cytometer, the laboratory must be able to demonstrate that appropriate biosafety procedures are in place to minimize exposure to infectious material. Because aerosol generation in flow cytometers with a stream-in-air is a potential hazard, special attention must be exercised to operate the instrument in accordance with the manufacturer's biosafety recommendations.

5.12 Equipment Disinfection

The waste container on the flow cytometer should contain 1:10 of its volume of 0.71 mol/L sodium hypochlorite (undiluted household bleach.)

Laboratory equipment should be properly disinfected with a solution known to inactivate infectious agents or a freshly made 10% solution of household bleach. It is particularly important that instruments be properly disinfected before repair or maintenance (see the most current edition of NCCLS document I17—*Protection of Laboratory Workers from Instrument Biohazards*).

6 Specimen Collection

6.1 Patient Information

A test requisition should accompany all specimens. This should include a unique patient identifier, presumptive diagnosis, age, gender, pertinent medication or recent treatment (including dates of chemotherapy or radiation), date and time of specimen collection, name of physician, and the source of the specimen (such as bone marrow aspirate or cerebrospinal fluid). Pertinent laboratory information (such as white blood cell (WBC) count, differential, morphological, cytochemical and cytogenetic findings) should also be available.

6.2 Sample Collection Techniques

6.2.1 General

All body fluids (e.g., blood; bone marrow aspirates; and pleural, ascitic or cerebrospinal fluids) should be considered potentially infectious. Biosafety Level 2 (BSL-2) practices should be followed during the collection and handling of body fluids. Every attempt should be made to provide the laboratory with fresh specimens for analysis. When specimen analysis is delayed by shipment to a remote laboratory, a freshly prepared and stained smear or tissue imprint and/or tissue section should accompany specimens for reference.

6.2.2 Venipuncture

NCCLS document H3—*Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture* should be followed for the collection of blood specimens.

6.2.3 Bone Marrow Aspirates

Approved procedures are not available for the collection of bone marrow specimens. Specimens should be collected under sterile conditions and procedures should follow those outlined according to standard medical practice for venipuncture as they apply.

6.2.4 Other Body Fluids or Tissue Samples

Numerous additional sources of specimens are useful in the laboratory evaluation of the patient with neoplastic disease. Examples may include lymph node biopsy, organ aspirates or washings, and various other surgical and/or biopsy material. Specific guidelines for specimen collection are not available and these types of specimens are rarely, if ever, collected by the laboratory. These specimens should be collected under sterile conditions according to standard medical practice. Once received in the laboratory, they should be handled in accord with standard precautions (see Section 5).

6.3 Anticoagulants

The choice of anticoagulant depends upon specimen type, transportation, and storage requirements (see Section 7), and in some cases, method of sample preparation (see Section 8).

6.3.1 Blood Specimens

Ethylene-diaminetetraacetic acid (EDTA), acid citrate dextrose (ACD, solution A), or heparin may be used. If a white cell count and differential are to be made from the same specimen used for flow cytometry, EDTA is the anticoagulant of choice. **NOTE**: White cell count must be performed within six hours of collection to ensure an accurate count.

6.3.2 Bone Marrow Aspirates

There is no consensus on the anticoagulant of choice. Heparin or ACD may be used.

6.3.3 Other Body Fluids

Heparin, EDTA, and ACD are appropriate anticoagulants, although samples preserved with ACD and held overnight may be vulnerable to low viability. No anticoagulant is used with tissue suspensions.

6.4 Labeling of Specimen

6.4.1 Requirements

Specimen(s) should be labeled with a unique patient identifier. When multiple specimens from the same patient are collected for analysis, the source of the specimens should be clearly indicated on the tube or vessel (i.e., "blood and bone marrow from the same patient").

6.4.2 Specimen Age

Because specimen age is a critical variable in flow cytometric analysis, specimens must be labeled with the date and time of collection. This is particularly important in cases where chemotherapy and/or radiation treatments were given.

6.4.3 Biohazard Label

Specific labeling of specimens as "infectious" is not required because all specimens should be regarded as potentially infectious and standard precautions must be followed.

7 Specimen Transport

7.1 Specimen Handling and Packaging

All specimens must be considered infectious and must be handled in such a way as to minimize risks for the laboratory worker and third-party carriers who transport specimens. Procedures for handling, packaging, labeling and transporting specimens have been developed that list federal regulations which should be referred to for specific details. (See the most current edition of NCCLS document H5— *Procedures for the Handling and Transport of Diagnostic Specimens and Etiologic Agents*.) Briefly, specimen test tubes or vials (preferably unbreakable) should be placed in a secondary container with sufficient absorbent material to absorb the entire contents of the vessel if breakage should occur.

Blood smears on glass slides should be carefully wrapped or packaged to eliminate

sharp edges and corners. The secondary container should have sufficient insulating capacity to maintain transportation temperatures at room temperature (18 to 22 °C). Styrofoam containers of one-inch thickness are usually suitable for this purpose.

7.2 Specimen Integrity

Handling and transportation procedures must maintain the viability of the specimen. Specimen integrity is the composite product of anticoagulant, storage conditions (time and temperature), and sample preparation procedure. Specimens can be maintained at room temperature (18 to 22 °C) and, ideally, they should be processed and fixed for flow cytometric analysis immediately after collection. Cold (4 °C) storage may be useful for prolonged storage of some specimens.

Many laboratories perform leukemia phenotyping on transcontinentally shipped specimens. Although the anticoagulant used and the length of storage may affect the relative recovery of neoplastic cells, in some circumstances it may not affect the overall goals of phenotyping. However, in cases in which leukemic cells are only a minor proportion of those present, differential recovery may greatly influence the ability to detect them. Chemotherapy and/or radiation treatment may also drastically affect cell recovery. Laboratories should demonstrate that, in their specific application, stored specimens give equivalent results to freshly processed ones.

8 Sample Preparation

The goal of sample preparation is to process a specimen from a patient with suspected leukemia or lymphoma into a representative sample suitable for introduction into the flow cytometer for sample analysis. All pertinent cellular and antigenic parameters for lymphoid, monocytic, and myeloid cells should be maintained. Irrelevant cells (e.g., erythrocytes) that may slow or interfere with analysis, should or may be removed. When neoplastic cells are present at low frequency, avoidance of their differential loss during cell preparation may be critical.

8.1 Visual Specimen Evaluation

Observable specimen problems are of two types:

- Those that indicate that the sample is altered or damaged; these call for immediate specimen rejection.

- Those that suggest that specimen mishandling may have occurred: These call for further evaluation during sample preparation. Mishandling problems should be recorded because they may prove to be informative when preparing, analyzing, and interpreting data from the specimen.

8.1.1 Hemolysis

Hemolysis indicates that the blood was exposed to conditions that can cause erythrocyte lysis, which suggests that leukocytes may also be damaged. Severely hemolyzed specimens should be rejected.

8.1.2 Clotted Blood

Even a partial clot may cause selective loss or alteration of certain subpopulations. Significantly clotted specimens should be rejected.

8.1.3 Partial Draw

For some anticoagulants (i.e., ACD), a partially filled tube may produce hypertonic conditions that are deleterious to cells. Depending on the magnitude of the underfill, one may accept or reject the specimen. If the specimen is accepted, the laboratory should have previously demonstrated that the observed underfill does not affect final results, and the underfill should be recorded for consideration during preparation, analysis, and interpretation.

8.1.4 Temperature Extremes

If the specimen tube was mailed to the laboratory, it may have been exposed to temperature extremes and it should be examined upon receipt to determine if it is unusually warm or cold to the touch. Even if all other evaluation criteria are satisfied, this observation should be noted for further consideration during preparation, analysis, and

interpretation.

8.1.5 Improper Specimen Labeling

All specimens should be labeled with a unique patient identifier. If one is not present, then the laboratory should follow its established policy for dealing with unlabeled specimens and, if adequate identification cannot be obtained, the specimen should be rejected.

8.2 Selection of a Sample Preparation Procedure

8.2.1 Erythrocyte Lysis versus Density Gradient Methods

In general, the more a cell suspension is manipulated or processed, the greater the opportunity for cell loss. The advantage of an erythrocyte lysis method is that it involves a minimum of manipulation. Moreover, this procedure is generally faster and it is more likely to maintain the distribution of leukocytes found in the original specimen. However, a lysis procedure assumes that all leukocyte subsets are equally tolerant to the lysis method used. This may not be the case with leukemic blood samples or marrow samples.

The advantage of density gradient methods is that leukemic cells are almost always recoverable in the lighter fractions of density gradients and may in fact be enriched, although under some circumstances one might enrich for cells of no interest. This procedure also takes longer and data on the relative frequency of leukemic cells in the processed specimen become difficult to interpret. Under some circumstances, it may be necessary to subject the material that is removed from a density gradient to an additional erythrocyte lysis step.

Alternative methods for cell preparation are discussed in Appendix B.

8.2.2 Selection of Lysing Agent

Several lysing techniques are available. These include water, tris-buffered ammonium chloride, and hypotonic buffer. Several proprietary lysing reagents are also available from instrument or monoclonal antibody manufacturers. When using commercial reagents, the manufacturer's recommended protocol should always be followed unless data are available that confirm that any modifications do not adversely affect results. See Appendix B.

8.2.3 Lymph Node or Extranodal Tissue—Sample Preparation for Lymphoproliferative Disorders

Any tissue biopsy specimen or fine needle aspiration biopsy with a suspected diagnosis of a lymphoproliferative disorder can be used to prepare a cell suspension suitable for flow cytometric immunophenotyping. The goal of tissue transportation, handling, and preparation is to maximize the cellular yield while maintaining cell viability; therefore, rapid and gentle processing is imperative.

Tissues that undergo a biopsy for a suspected lymphoproliferative disorder should be received by the laboratory immediately after removal in the fresh state, at room temperature, and on saline-moistened gauze in a sterile container. The specimen should be examined and divided according to individual laboratory protocol. Processing may include dividing the tissue for permanent fixed sections, touch imprints, frozen tissue for immunohistochemistry and molecular biology, cytogenetics, or for culturing. Portions submitted for flow cytometry should be selected to minimize the amount of normal tissue (i.e., fat, connective tissue, and residual normal tissue) and to maximize the lymphoid tissue.

Tissue for flow cytometric immunophenotyping should be placed in a sterile plastic container, such as a Petri dish or capped test tube which contains several milliliters of an isotonic nutrient media (such as RPMI 1640, at 4 °C). Other isotonic media, such as phosphate-buffered saline, McCoy's modified media, or Hank's buffered salt solution may be used. Nonisotonic solutions are unacceptable.

Single-cell suspensions are usually easily achieved in lymphoid tissue by mechanical disaggregation of lymphoid cells. Enzymatic disaggregation may be used provided it can be shown not to alter surface antigen expression or viability.

Mechanical disaggregation can be accomplished by a number of techniques. Disaggregation is easily performed in a sterile Petri dish partially filled with nutrient media. Scalpel blade and forceps may be used to finely mince the tissue. Alternatively, hypodermic needles may be used to gently tease apart the tissues, which should then result in a cloudy appearance in the surrounding nutrient media. Gentle disaggregation using metal mesh screens can be used as well, or a Pasteur pipet, or a syringe fitted with a large bore needle may be used to gently aspirate and expel minced tissue fragments suspended in the media.

Cellular material obtained by fine needle aspiration biopsy should be directly placed into nutrient media and immediately transported to the laboratory. In most cases, the material will already be a single-cell suspension and only subsequent filtering will be required.

All cell suspensions should be filtered to remove large clumps of cells and residual connective tissue. The suspension can be filtered through nylon mesh, generally 50 μm or greater in size. Alternatively, cells may be drawn up into or expelled out of a syringe with the hub fitted over with fine nylon mesh.

Density gradient separation may be used if there is the possibility of nonlymphoid contaminating cells, but in most cases this is not required. If the cell suspension contains numerous red blood cells, these may be removed by either density gradient separation or by lysis techniques as described for blood and bone marrow.

Once the cell suspension is made, cells can be held in nutrient media at room temperature or preferably, at 4 °C for short periods of time. The viability of cell suspensions over time is unpredictable; therefore, it is recommended that after checking viability and adjusting cell count/sample concentration, samples should be stained, fixed, and analyzed as soon as possible.

8.3 Adjustment of Cell Counts

Manufacturers' recommended amounts of antibody to be used generally assume normal ranges for the number of target cells to be stained. This assumption may not be valid in leukemic specimens unless cell concentrations are adjusted.

For samples that have been separated on density gradients, cell concentration should be adjusted to 1 to 5 x 10^6/mL, and from 50 to 200 μL used for staining, depending on the antibody concentration to be used.

For samples processed by direct lysis techniques, it is necessary to obtain some estimate of the white count before staining because leukemic patients often have abnormal white counts. Moreover, bone marrow samples are variably diluted with blood and their cellularity can vary greatly.

Thus, some specimens may not have sufficient lymphocytes in the whole blood or marrow aliquot for flow cytometric analysis. Conversely, normal concentrations of antibody reagents may be insufficient to saturate all binding sites in densely cellular specimens which may lead to false negative results. Each laboratory must have a procedure to identify specimens with abnormal lymphocyte counts and correct for them. The following guidelines may be used for sample adjustment:

(1) WBC count less than 1,000/μL (1 x 10^9/L): Use 200 μL of whole blood and adjust the volume of lysing reagent appropriately.

(2) WBC count of 1,000 to 10,000/μL (1-10 x 10^9/L) (some laboratories would say up to 20,000): Use 100 μL of whole blood and a standard volume of lysing agent.

(3) WBC count of 10,000 to 20,000/μL (1-20 x 10^9/L): Use 50 μL of whole blood and lyse accordingly.

(4) WBC count greater than 20,000/μL (greater than 20 x 10^9/L): Dilute with phosphate buffered saline so that the concentration is in the range of (2) or (3) and use 100 μL of whole blood dilution.

When processing bone marrow by direct lysis techniques, it is often necessary to dilute the sample 1:10 in PBS before performing either a

manual count or an automated count. The WBC count should then be adjusted to the range specified above. Some lysing reagents may perform differently on bone marrow than on blood, especially with respect to the scatter characteristics of the WBCs. It is the responsibility of the laboratory to determine how bone marrow specimens differ from blood specimens when processed by erythrocyte lysis techniques.

8.4 Evaluation of Sample Viability

Because nonviable cells may nonspecifically bind to many antibodies, it is generally necessary to determine the viability of the leukemic cells in a sample before analyzing them. This is particularly important when a specimen has been a long time in transport, or when there is some doubt about how a specimen was handled. If separated cells are used, visual methods using Trypan blue or other vital dyes may be satisfactory. It is also possible to use propidium iodide (PI) or 7-aminoactinomycin D (7-AAD) as a fluorescent viability dye and to monitor sample viability on the basis of dye exclusion. If this is done, it is essential to use unfixed samples (see Section 5.11), since PI or 7-AAD may redistribute over all cells after fixation and storage. If alternative viability probes are used for post-fixation viability discrimination, the laboratory must verify, using representative patient specimens, that such probes give results comparable to those obtained using a standard viability dye (i.e., PI, trypan blue) in unfixed samples. Whatever method is used, the laboratory is responsible for determining the concentration of viability dye to use to give optimal resolution between live and dead cells.

8.5 Sample Preparation for Immunoglobulin Staining

Anti-immunoglobulin antibodies are unique among reagents commonly used in clinical flow cytometry in that they react with a substance that is present in high concentration in patient plasma. Accordingly, special procedures have to be followed to ensure that the technique will detect immunoglobulin on the surface of cells. Moreover, many cell types will bind immunoglobulin nonspecifically via Fc receptors, and steps may have to be taken to make sure that cell-associated immunoglobulin is intrinsic and not extrinsic (or cytophilic).

8.5.1 Removal of Immunoglobulin

Samples processed by density gradient centrifugation will have serum immunoglobulin removed adequately, although immunoglobin may remain bound to the surface of some cells. Of course, the cells must not be resuspended in medium containing human serum. For whole blood or bone marrow specimens, simple washing with buffer before staining with anti-immunoglobulin reagents may be adequate to remove Ig.

8.5.2 Cytophilic Immunoglobulin

Although the washing procedure described above probably removes some cytophilic immunoglobulin, in some cases further steps are needed. One method requires incubation of the sample for one hour at 37 °C in plasma-free medium, followed by washing.

The effect of cytophilic immunoglobulin can, in some circumstances, be mitigated by the appropriate construction of antibody panels. For example, B cells generally have lower levels of Fc receptors than do monocytes, granulo-cytes, and some T cells, so that combining anti-immunoglobulin antibodies with a pan-B antibody (called B cell gating) can help insure that immunoglobulin expression is only considered on the B cell population. In this case the non-B cells will often demonstrate the presence of cytophilic Ig though this will not interfere with analysis of the B cells. In addition, for light chain analysis, simultaneous detection of kappa and lambda expression can also be useful because cytophilic Ig binding generally involves both kappa- and lambda-bearing immunoglobulin molecules, and such cells appear to be doubly labeled (provided there is not a monoclonal immunoglobulin component!). In contrast, B cells express only one light chain and appear singly labeled.

8.6 Sample Preparation to Block Non-specific Fc Binding

Some leukemic cells have Fc receptors that result in nonantigen mediated binding of some

or all antibodies in a panel unless steps are taken to prevent this. Since most monoclonal antibodies used for immunophenotyping are of the IgG isotype, murine IgG fractions at a concentration of 2 mg/mL final volume may be used. Fc binding may not be as much of a problem if cells are stained by a whole blood or marrow erythrocyte lysis technique, provided unwashed cells are used, because immuno-globulin present in the plasma often saturates possible Fc binding sites. This obviously cannot be employed for analysis of surface immunoglobulin, in which IgG must be removed. (See Section 8.5.)

It should be noted that in some unusual cases, particularly in monocytic leukemias, it may not be possible to overcome Fc binding completely. Because antibodies of different subclasses have different affinities for Fc receptors, the pattern of antibody reaction in such a case may be unusual and difficult to interpret.

9 Immunofluorescence Staining of Surface Antigens in Leukemic Specimens

9.1 Reagents

The central concept that underlies leukemia immunophenotyping is that leukemic cells express cell surface antigens that are related to normal cells of a particular lineage and that these antigens can be detected with appropriate antibodies. It is important to note, however, that leukemia cells may exhibit aberrant or unusual patterns of cell surface antigen expression that are different from those of normal circulating blood or bone marrow cells. In addition to unique combinations of antigen expression, leukemic blasts may exhibit aberrant antigen density manifested as abnormal fluorescence intensity (often dim) when compared with normal hematopoietic cells. Thus, information on presence, absence, or level (fluorescence intensity) of cell surface antigens provides useful information for characterizing leukemic cell populations.

A wide variety of monoclonal antibodies are used to phenotype cases of leukemia. These can be divided into those that react primarily with B cells, T cells, NK cells, or cells of myeloid or monocytic lineages. In addition, several reagents are relatively nonspecific as to lineage, but nonetheless they may play an important role in phenotyping some cases of leukemia. Unfortunately, many reagents, while primarily reacting with cells of one lineage, are not absolutely specific in that they react with a subset of normal or neoplastic cells of other lineages. Thus, many leukemias react with reagents whose primary reactivity is against cells of more than one lineage. Such cases are frequently considered to be examples of "mixed lineage" leukemias. However, considerable controversy exists in the literature concerning these leukemias; no precise definition of "mixed lineage" exists, and this document does not provide one. Lack of an acceptable definition of mixed lineage leukemias may, in part, reflect the increasing sensitivity of flow cytometers and the corresponding ability to detect antigens of low frequency or with few epitopes on the cell surface.

Because antibodies vary in their degree of lineage specificity and because many leukemias lack one or more antigens expected to be present on normal cells of a particular lineage, it is recommended that a certain degree of redundancy be built into a panel used for leukemia phenotyping. Where possible, it is thus recommended that lineage assignment be confirmed with an antibody from a second CD grouping that is known to react principally with the suspected cell lineage. Note, however, that failure to detect the confirmatory marker does not exclude the suspected lineage because leukemias commonly exhibit loss of various antigens. It is not possible to specify a minimum (or maximum) panel that should be used for phenotyping all cases of leukemia.

In general, the best reagents for phenotyping cases of acute leukemia are different from those that are most useful for cases of chronic leukemia and lymphoma.

9.1.1 Reagents for Phenotyping Acute Leukemia

Cell surface antigen expression is generally used to assign a given case of acute leukemia into one of three broad general categories: B-lymphoid, T-lymphoid or myeloid, and

reagents can be divided into those that react primarily (though not exclusively) with these cell types.

9.1.1.1 Reagents That React Primarily With B Cells

- CD19 is expressed on nearly all cells from B-lineage acute leukemias, although it is occasionally expressed in myeloid leukemia as well. Recently, CD79a has been found to react with most B-lineage acute leukemias. It has been reported to react in cases of acute myeloid leukemias (by immunohistochemical methods).

- CD20 is less often expressed than CD19 on cells from acute B-lineage leukemias.

- CD22 is usually expressed on cells from B-lineage acute lymphocytic leukemia (ALL), but it is often dim, and it is more strongly expressed in the cytoplasm than on the surface.

- The common ALL antigen CD10 is expressed in the great majority of cases of B-lineage acute leukemia, although it is not specific for B-cell lineage because it reacts with a significant number of cases of T-ALL.

- Surface IgM (±IgD), with monoclonal immunoglobulin light chain, is expressed on a small subset of B-lineage acute leukemias of a more mature stage of B-cell differentiation.

9.1.1.2 Reagents That React Primarily With T Cells

- CD7 is the T-associated marker that is most frequently expressed in T-lineage ALL, but it is not specific because it is found in a distinct subset of acute myeloid leukemia.

- CD2, the sheep erythrocyte (E) rosette receptor, is present in most cases of T-lineage ALL, but it may again be present in myeloid leukemia.

- CD5 is present in most cases of T-lineage ALL, but it is often dimly expressed.

- CD3 is uncommonly expressed on the surface of cases of T-lineage ALL, but when present it is highly specific for T lineage. However, it is almost always present in the cytoplasm of cases of T-lineage ALL, and it is probably the most sensitive and specific marker for T-cell derivation.

- Many cases of T-lineage ALL express CD1, or coexpress CD4 and CD8 indicating their relationship to immature (thymic) T cells. The immature nature of T-ALL is further indicated by expression of TdT. Among these, CD4 is probably the least specific because it is found in many cases of myeloid leukemia.

- Most cases of T-ALL express the nonlineage-specific markers CD71 (transferrin receptor) and CD38, while many lack HLA-DR.

9.1.1.3 Reagents That React Primarily With Myeloid Cells and Other Miscellaneous Reagents

- CD13 and/or CD33 are expressed in nearly all cases of acute myeloid leukemia. When these are expressed in the absence of the lymphoid-associated antigens noted above, this finding is strongly suggestive of myeloid leukemia.

- CD13 and CD33 may occasionally be expressed on B- or T-lineage ALL, but the significance of this is controversial.

- CD14, an antigen characteristic of normal monocytes, is frequently though not invariably expressed in myeloid leukemias that display monocytic differentiation. It is also expressed in some lymphoid leukemias.

- More mature myeloid antigens, such as CD15 and CD11b, are generally expressed in myeloid leukemias that show more morphologic evidence of differentiation.

- CD41 and CD61 react with platelet-associated glycoproteins and may be useful to detect megakaryoblastic leukemias.

- Glycophorin may be useful to detect erythroleukemias.

- While not lineage-specific, the stem cell marker CD34 is present in many cases of acute leukemia and may help distinguish abnormal from normal myeloid cells in heterogeneous populations.

- HLA-DR is found in most cases of AML and B-lineage ALL, but it is frequently absent from cases of T-ALL and acute promyelocytic leukemia.

- TdT is expressed in the great majority of cases of B- and T-lineage ALL, indicating their derivation from an immature precursor cell. It is occasionally expressed in acute myeloid leukemia.

- Antimyeloperoxidase (MPO), Cdw65 and CD117 have been shown to react preferentially with acute myeloid leukemias. Some investigators have proposed to define acute myeloid leukemias immunopheno-typically by the coexpression of two or more of CD13, CD33 anti-MPO, Cdw65 and CD117.

9.1.2 Reagents Used in Phenotyping Chronic Lymphoid Leukemia and Lymphoma

The task of phenotyping studies in chronic leukemia and lymphoma is first to demonstrate the presence of a phenotypically abnormal population of lymphocytes and second to classify it as being of T- or B-cell lineage. The appropriate choice of reagents often allows both these tasks to be accomplished at the same time. B-cell clonality can be determined based on restricted expression of immuno-globulin light chains, but phenotypic identification of clonal T-cell processes is not generally possible with current reagents.
However, unusual patterns of antigen expression (such as CD5 positivity on B cells or lack of CD7 on T cells) may allow identification of abnormal cells. Once cells are identified, they may be further subclassified using additional B-cell or T-cell reagents.

9.1.2.1 Reagents That React Primarily with B Cells

- Because chronic leukemias are generally derived from mature B cells and are clonal proliferations, they therefore express surface membrane immunoglobulin and show immunoglobulin light chain restriction; intensity of expression of immunoglobulin may help distinguish among different types of B-cell tumors.

- Pan- or mature B markers, such as CD19, CD20, CD22 and CD37 are generally positive on chronic B-cell leukemias and are rarely if ever, positive on T-cell tumors.

- CD10 is often expressed on B-cell lymphomas and leukemias derived from follicular center B cells and is characteristically absent from B-cell CLL.

- The distinction between chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SL) and mantle cell lymphoma (MCL) has prognostic significance and has attracted the attention of numerous investigators. CLL/SL is usually CD23+, CD5+ and may exhibit dim CD20 fluorescence. FMC7 is negative. MCL is usually CD23-, CD5+, bright CD20 and FMC7+. CD103 has been strongly associated with hairy cell leukemia. The clinical setting and the coexpression of CD25 and CD11c usually are sufficient to define this entity.

9.1.2.2 Reagents That React Primarily with T Cells

- Most chronic T cell leukemias express pan- or mature T markers such as CD3, CD2 and CD5.

- Because they are derived from mature T cells, most chronic T leukemias express either CD4 or CD8, but not both. $CD4^+$ lymphomas are more common than $CD8^+$ lymphomas. Expression of CD4 is associated with certain specific variants of T cell lymphoma such as Mycosis fungoides/ Sezary leukemia, while CD8 is expressed in other diseases, such as large granular lymphocyte syndrome.

- CD5 is present not only on most T cell leukemias but is also expressed on a subset of B cell leukemias and lymphomas, particularly B cell chronic lymphocytic leukemia, though its presence

is not specific for that diagnosis.

- T cell proliferations can frequently be recognized as abnormal because of loss of a normal pan-T antigen; CD7 is the marker most commonly lost. In some cases, abnormal T cell proliferations can be recognized by abnormal intensity of expression of a normal T antigen.

- The majority of T cell lymphomas express the αß T cell receptor while a minority are derived from γδ T cells. The latter frequently lack both CD4 and CD8.

- With the exception of CD5, aberrant expression of T-associated antigens is uncommon in chronic B cell leukemias.

9.1.2.3 Predominantly Myeloid and Other Miscellaneous Reagents

- Certain myeloid-associated markers can react with subsets of lymphoid leukemias. For example, CD11c is often expressed in a subset of B-cell chronic lymphocytic leukemia and it is highly expressed in hairy cell leukemia.

- The stem cell-associated marker CD34 is essentially always absent from chronic B- and T-cell leukemias.

- Natural killer-associated markers, such as CD57 and CD56, are often expressed in large granular, lymphoproliferative diseases.

- Activation markers, such as CD25, CD30, and, (in the case of T cells) HLA-DR are often expressed in lymphomas derived from transformed lymphocytes.

- CD38 is often highly expressed in plasma cell neoplasms.

9.1.3 Assembly of Reagent Panels

As noted above, no specific panel can be recommended for leukemia phenotyping. Although under some circumstances, single-color immunofluorescence may be adequate, in most cases dual or even multicolor immuno-fluorescence analyses are preferred because these are generally most economical in cells and in preparation time. It is strongly recommended that a laboratory choose a panel and gain experience with how leukemias react when stained with that panel. Provided data are collected in list mode; there may be multiple possible ways of analyzing the same data (see Section 13). Appendix C illustrates some examples of how acute leukemias can be analyzed with a panel of reagents using both a dual- and single-color approach.

9.2 Optimization of Staining Protocol

When commercially available reagents have a detailed protocol for their use, the protocol should be followed exactly. This is true for procedures for antibody staining, red cell lysis, and fixation.

The following variables have typically been examined by the manufacturers in arriving at their recommended procedure.

9.2.1 Volume

Where sample plus reagent volume exceeds 0.2 mL, mixing during incubation and/or an increased time of incubation may be required. A staining buffer (pH 7.2) containing at least 1% protein is recommended for density-purified cell preparations.

9.2.2 Temperature

Room temperature (18 to 22 °C) is recommended for staining samples for immunopheno-typing.

9.2.3 Time of Incubation With Reagent

The length of time needed to reach saturation is affected by temperature, sample volume, and fluorochrome. PE-conjugated reagents may require longer incubation times compared to FITC conjugates. Typical staining times recommended by the manufacturer vary from 10 to 30 minutes.

Any deviation from the standard laboratory or manufacturer's protocol should be documented in a laboratory protocol journal to compare results with those obtained using the recommended procedure.

9.2.4 Cell Count

The amount of antibody recommended for staining with most commercial reagents is sufficient to stain 100 μL of blood, assuming approximately normal leukocyte counts and frequencies or 0.5 to 2 x 10^6 separated mononuclear cells. Methods for adjusting sample concentration are described in Section 8.3.

9.2.5 Reagent Concentration

Most commercial reagents have recommended amounts for staining a fixed volume of blood (or number of separated cells) in a fixed time. These recommended amounts are typically derived by determining the amount of reagent required to give the following:

- the maximum separation between the positive/negative populations, and
- the maximum fluorescence intensity of the positive population.

For each lot of a given reagent, the laboratory should verify that the manufacturer's antibody gives comparable positive/negative resolution and comparable positive-staining intensity with previous lots using the laboratory's method of sample preparation. Antibodies may be of sufficient concentration to provide adequate staining of cells when diluted past the manufacturer's recommended concentration, but this should be verified in titration experiments.

For any deviation from the manufacturer's recommended staining conditions (e.g., time, volume, temperature, or cell number), the laboratory must determine the minimal amount (volume or weight) of reagent required to give optimal positive/negative resolution and optimal positive-staining intensity.

NOTE: Excess reagent may cause increased nonspecific staining of negatives, resulting in decreased resolution of positives and negatives, or it may cause quenching, resulting in decreased staining intensity and decreased resolution of positives and negatives.

10 Sample Quality Control

10.1 Verification of a Leukemic Population

Bone marrow or blood samples submitted for immunophenotyping should include information about the number and nature of leukemic cells present in the specimen. Where possible, morphologic examination of the cell population to be analyzed is useful to confirm that leukemic cells are present. In the case of lymph nodes, it may be desirable to review a touch imprint or cytocentrifuge preparation, or a frozen or permanent tissue section to correlate the nature of the material present in the cell suspension with that of the initial sample. In acute leukemia and some lymphomas, populations of blast cells can usually be resolved by flow cytometry based on FALS and orthogonal light scatter characteristics or in some cases, by correlation of scatter and expression of certain antigens. Agreement between the flow cytometric assessment of abnormal cells and the morphologic impression is an important first step in assuring sample quality. In the chronic leukemias and indolent lymphomas, a novel population may not be resolved using light scatter characteristics, but morphologic correlation is again important.

10.2 Negative Controls

Negative reagent controls are analyzed to establish the level of background fluorescence resulting from autofluorescence and nonspecific binding of primary and secondary antibodies.

10.2.1 Immunoglobulin Subclass Controls

Panels of monoclonal antibodies to human leukocytes that are typically used to characterize leukemic cells are composed of antibodies of several subclasses of immunoglobulin. Because the different subclasses show varying degrees of nonspecific binding, it is advisable to run negative controls for each subclass to estimate Fc-receptor-mediated nonspecific binding, even if steps were taken to block this (see Section 8.6). Incubations with these reagents are set up to parallel those of the target cells with test antibodies. It is desirable that the negative controls run

with the subclass should be employed at the same immunoglobulin concentration as the test primary monoclonal antibodies if indirect immunofluorescence assays are employed. If cells are to be tested with direct antibody conjugates, it is desirable that the controls run with the subclass are of a similar specific activity (fluorochrome to protein ratio). Some laboratories elect to pool control reagents of different immunoglobulin sub-classes. This practice is not recommended. *If pooled subclass controls are used, it is critical to reanalyze low-level positive immunofluorescence profiles in the context of a subclass-matched control.*

It is evident that different control reagents, albeit of the same subclass, may give variable staining intensities. This can be evaluated by staining a defined target cell with the subclass negative control and with monoclonal antibodies not reactive with that target. In this way, it is possible to confirm that the test and the control antibodies are matched reagents that give similar levels of nonspecific binding. As an evolution of this rationale, it is possible to coordinate panels of monoclonal antibodies to compare the binding of monoclonal antibodies of the same subclass that typically have mutually exclusive patterns of reactivity with subsets of hematopoietic cells (i.e., CD19 and CD33). In this way, test antibodies may also double as control reagents.

NOTE: *It is emphasized that dim reactivity of test antibodies cannot be interpreted with certainty in the absence of appropriate negative controls.*

Polyvalent antibodies are frequently used to detect the expression of cell-surface immunoglobulin molecules on neoplastic hematopoietic cells. Standardization of these reagents may pose difficulties because, unlike monoclonal antibodies, they are heterogeneous. It is necessary to analyze appropriate control reagents in parallel to determine the level of nonspecific binding of anti-immunoglobulin reagents. Manufacturers may prepare matched control reagents that have been optimally titered and absorbed. However, even when appropriate negative controls show no staining, a high level of false-positive staining with anti-immunoglobulin reagents may occur because these reagents may bind to cytophilic immunoglobulin adsorbed onto Fc receptors. Procedures for preparing the specimen to minimize this are described in Section 8.5.

10.2.2 Autofluorescence Control

Different subpopulations of leukocytes show varying levels of autofluorescence. In general, higher levels of autofluorescence are noted in cells that contain granules. For this reason, myeloid leukemias typically show higher levels of autofluorescence than lymphoid leukemias. To determine the level of background autofluorescence of the target cells, a control tube, in which the staining reagents have been omitted, is analyzed in parallel. Although this control is not required, it may be of considerable value in assessing the relative contributions of granularity and nonspecific antibody binding to background fluorescence.

10.3 Positive Controls

Positive controls are required to confirm that the methods used to prepare target cells, the immunologic reagents, and the staining procedures are performing appropriately. Two types of positive control procedures can be described. In the first, cells of known reactivity are incubated with the test reagents to verify that the reagents react under the test conditions. In the second, reagents expected to react with the target cells are included in the panel to show that the preparative, staining, and analytical procedures are functioning appropriately.

10.3.1 Positive Reagent Control

The reactivity of immunologic reagents with defined target cells should be periodically characterized. Most of the monoclonal antibodies included in leukemia typing panels react with subpopulations of normal leukocytes. Reactivity of these immunologic reagents can be verified by analyzing blood leukocytes or other sources of positive targets such as cryopreserved cells from lymphoid tissues. Various target cell types may be discriminated by resolving leukocyte subpopulations by electronic bit-mapped gating of FALS versus 90° LS properties. It may be necessary to identify an alternative

source of positive target cells to test the antibodies that cannot be assayed in this fashion. Such alternatives include normal bone marrow, appropriate human leukemic cells, human leukemic cell lines, or commercially available preparations of preserved human leukocytes. If human leukemic cells are to be employed as target cells in positive reagent controls, they should be viably cryopreserved, maintained in liquid nitrogen, and properly thawed for each control analysis. The cell viability and recovery must be established and recorded each time the cells are processed, stained, and analyzed. Alternatively, human leukemic cell lines maintained in culture may constitute the panel of target cells of known reactivity. However, this is not practical for most laboratories.

Reactivity of immunologic reagents can normal-l. be verified during the course of sample analysis based on their reactivity with leukemic cells or residual normal cells in leukemic samples. The reactivity of immunologic reagents with control target cells of known reactivity should be verified within laboratory-defined intervals if a positive and negative target cell population is not encountered during this interval. Antibodies should also be analyzed to document their reactivities when reagent lots are changed. In this way, the reactivity of a new lot can be compared directly to that of the lot of reagent in ongoing use. Any difference in the fluorescence intensity of a known target population stained with a new lot of antibody should be recorded.

10.3.2 Procedural Control

The most appropriate procedural control demonstrates that target cells in the sample are capable of reacting under the test conditions. To this end, an antibody that has broad reactivity with hematopoietic target cells should be included in every typing panel. Examples of such monoclonal antibodies include those that recognize the leukocyte common antigen (CD45) and framework determinants of HLA class I antigens. Because the vast majority of samples contain T lymphocytes, it is possible to consider their reactivity with pan-T lymphocyte antibodies as evidence that the immunoassay is functioning. This approach has the disadvantage that it does not directly determine the ability of the target cells of interest to bind to a test antibody. This is a relevant concern in instances when hematopoietic malignancies are more fragile, and thus of lower viability, than normal lymphocytes. *Results in which the leukemic cells in a sample fail to react with any of the reagents in a test panel should be interpreted with caution*.

Under some circumstances, a specimen from a normal donor that is drawn, prepared, and stained in parallel with the patient specimen may serve as a positive procedural control. It is not practical, or necessary, to analyze this type of procedural control sample on a daily or even a weekly basis if the laboratory is active and if within-run positive and negative control results on a leukemic specimen demonstrate appropriate reactivity. However, for laboratories that do not process a high volume of leukemic specimens, such a sample should be prepared and analyzed when leukemic samples are tested. It should be noted that an immuno-fluorescence-stained blood sample from a normal donor is recommended as the optimum material for determining instrument settings (see Section 11.1). It is acceptable to use this same sample as the procedural control sample and to analyze the reactivity of monoclonal antibodies that identify the subpopulations of leukocytes that are represented.

11 Instrument Quality Control

Currently, there are no standards that can be used to check the accuracy of flow cytometric test results. Hence, verifying reproducibility of instrument performance is an essential element of daily quality assurance within the flow cytometry laboratory. Instrument performance must be monitored under the same conditions used to run test samples. Instrument quality control, therefore, involves two procedures:

- ***Instrument setup:*** Identification of appropriate conditions for analysis of test samples and establishment of acceptance values for materials to be used in daily instrument monitoring (described in Section 11.1).

- ***Instrument monitoring***: Use of reference samples or materials with stable properties to monitor instrument performance and to reproduce test-specific analysis conditions (described in Section 11.2).

Many of the issues involved in instrument quality control for immunophenotypic analysis of hematologic malignancies are similar to those encountered in analysis of nonneoplastic leukocytes. However, the issues of sensitivity and color compensation take on particular importance. Neoplastic leukocytes frequently exhibit aberrant intensities of antigen expression and/or aberrant patterns of antigen coex-pression (Section 9). If instrument sensitivity is inadequate, dim antigen expression may not be detected. If color compensation is excessive, a population expressing one antigen may be incorrectly considered to lack another. Therefore, it becomes particularly important to validate instrument performance by using samples with characteristics as similar as possible to those of test samples.

Table 1 summarizes the various tasks involved in instrument quality assurance and the general properties of samples that are useful in performing each task.

Table 1: Quality Assurance (QA) Overview

QA Function	Why	When	What (Sample Properties)
Initial Instrument Setup	**Optimize Analysis Conditions**	**New Test or Instrument Conditions**	See below
Align optical system (11.1.1.1)	Maximize intensity, minimize variability	New instrument, major maintenance or repair	Homogeneous signals in all channels used for specimen analysis; long-term stability (e.g., uniform fluorescent plas-tic beads)
Establish acceptance values for paricles to be used in alignment monitoring (11.1.1.2)	Determine laboratory's expected range of values indicating acceptable alignment	Whenever significant alignment changes are made	See alignment
Establish test-specific instrument set-tings giving: (a) optimal light scatter resolution (11.1.2.1) and (b) optimal fluorescence resolution (11.1.2.2)	Optimize optically aligned instrument for analysis of test specimens	Introduction of test; after any significant change in specimen preparation or staining protocol	"Normal" specimens prepared using laboratory's standard method, labeled with 1) reagent giving 'bright' staining and good resolution of positive from negative cells (e.g., CD3); 2) reagent giv-ing "dim" positive staining (e.g., CD2 or CD56); and 3) reagents bearing relevant fluorochromes that give 'bright' mutually exclusive labeling (e.g., FITCCD4 and PE-CD8)
Establish acceptance values for mate-rials to be used in intensity monitoring (11.1.2.2(2))	Determine laboratory's expected range of values indicating acceptable ability to resolve dim positive cells from negatives	Introduction of test; after any significant instrument maintenance or change in test-specific instrument settings	Material with intensities lower than relevant unlabeled leukocyte; 'normal' specimen labeled with 'dim' positive reagent (e.g. CD2 or CD56)
Establish acceptance values for mate-rials to be used in compensation monitoring (11.1.2.2(4))	Determine laboratory's expected range of values indicating acceptable ability, when carrying out two-color analysis, to resolve dual positive cells from cells labeled only with one reagent or the other	Whenever filters are replaced; whenever instrument parameters that affect fluorescence amplification are altered (e.g., detector voltage/gain settings)	Cells labeled with mutually exclusive reagents bearing relevant fluorochromes (e.g., FITC-CD4 and PE-CD8) or plastic particles with intensities and spectral characteristics similar to bright antibody-positive cells or a mixture of samples separately labeled, with reagent giving a range of dim-to-bright intensities conju-gated to relevant fluorochromes (e.g., FITC-CD8 and PE-CD8)
Daily Performance Monitoring	**Assure Acceptable Performance**		
Monitor optical alignment (11.2.1)	Verify acceptable alignment	**Whenever Samples Are Run**	**Stable; Established Acceptance Values**
		Whenever samples are run or instrument malfunction is suspected	Reference material with acceptance va-lues established under conditions of optimal alignment (e.g., uniform fluores-cent beads); see Initial Instrument Setup
Monitor intensity performance and fluorescence resolution (11.2.2)	Verify reproducible intensity measure-ments and acceptable sensitivity	Whenever samples are run or instrument malfunction is suspected	Reference material with acceptance values established under test-specific settings and conditions of optimal alignment
Monitor color compensation (11.2.3)	Verify that after labeling with two different fluorochromes, dual-labeled cells can be accurately distinguished from single-labeled cells	When specimens labeled with two different fluorochromes are run	Freshly prepared specimen stained with relevant fluorochromes or plastic particles with acceptance values established under appropriate compensation settings (see Initial Instrument Setup)
Verify system performance (11.2.4)	Verify performance of preparation and staining methods using sample for which expected results are known	Whenever test specimens are run; whenever sample preparation or staining prob-lems are suspected	'Normal' specimen prepared using laboratory's standard test protocol and reagents with established laboratory reference values

11.1 Instrument Setup

Instrument setup procedures first establish that instrument performance is acceptable under some initial (frequently manufacturer-defined) set of conditions and then identify optimal conditions for analysis of representative test samples. Once optimal, test-specific conditions are identified, acceptance ranges for materials to be used in daily performance monitoring and reference ranges for specific markers are established under those same conditions.

The following instrument setup procedures should be carried out when the flow cytometer is first received, when major maintenance or repair is performed, and when any significant change in specimen preparation or staining protocols is made.

11.1.1 Optical Alignment

11.1.1.1 Optimization of Optical Alignment

Proper alignment of the optical components of the flow cytometer (e.g., laser, flow cell, focusing lenses, collecting lenses, photodetectors) should be established using the manufacturer's recommended alignment materials and procedures. Alignment particles are typically uniform plastic particles incorporating a fluorescent dye; other materials may also be recommended by the manufacturer.

The goal in optimizing alignment is to simultaneously achieve maximum signal intensity (maximum mean channel number) and minimum signal variability (minimum coefficient of variation [CV]) for all parameters that will be used to analyze test specimens, including forward and side scatter.

For instruments with fixed optical systems, where alignment is not carried out by the user on a daily basis, alignment should be verified using manufacturer-recommended particles to determine whether instrument performance meets specifications.

11.1.1.2 Establishment of Acceptance Values for Alignment Particles to be Used in Daily Monitoring

In some cases, manufacturers provide expected values for their particles when used, for example, with specific instruments, instrument settings, and filters. In other cases, the laboratory must determine optimum settings for its own instrument-alignment particle combination and establish its own expected values.

Two different methods for monitoring reproducibility of instrument alignment are commonly used. Method 1 monitors the reproducibility of mean intensities and CVs for alignment particles under fixed instrument conditions (i.e., specified laser power, filters, and PMT high voltages and gains) as shown in Figure 1, Panel A. Differences in measured particle intensity thus give a direct indication of how much instrument performance varies from day to day. Method 2 monitors the reproducibility of instrument conditions (typically PMT high voltage and/or gain) required to achieve specified mean intensities (channel numbers) for the alignment particles, as shown in Figure 1, Panel B. Differences in instrument setting(s) required to achieve a specified intensity do not indicate as directly how much instrument performance varies on a daily basis, but they can nonetheless be used to establish acceptance ranges for performance monitoring.

Whichever method is used, alignment particles should be run under conditions of optimal alignment a total of 20 or more times over a minimum of at least 5 separate days, while collecting data for all parameters that will be used to analyze test specimens. The ranges of expected values for mean channel number and CV (Method 1), or for PMT settings required to achieve specified mean channel values (Method 2), along with relevant instrument settings should be recorded in an instrument log book for subsequent use as acceptance ranges in daily monitoring. Any new lot of alignment particles should be run in parallel with the old lot to establish acceptance ranges for the new lot.

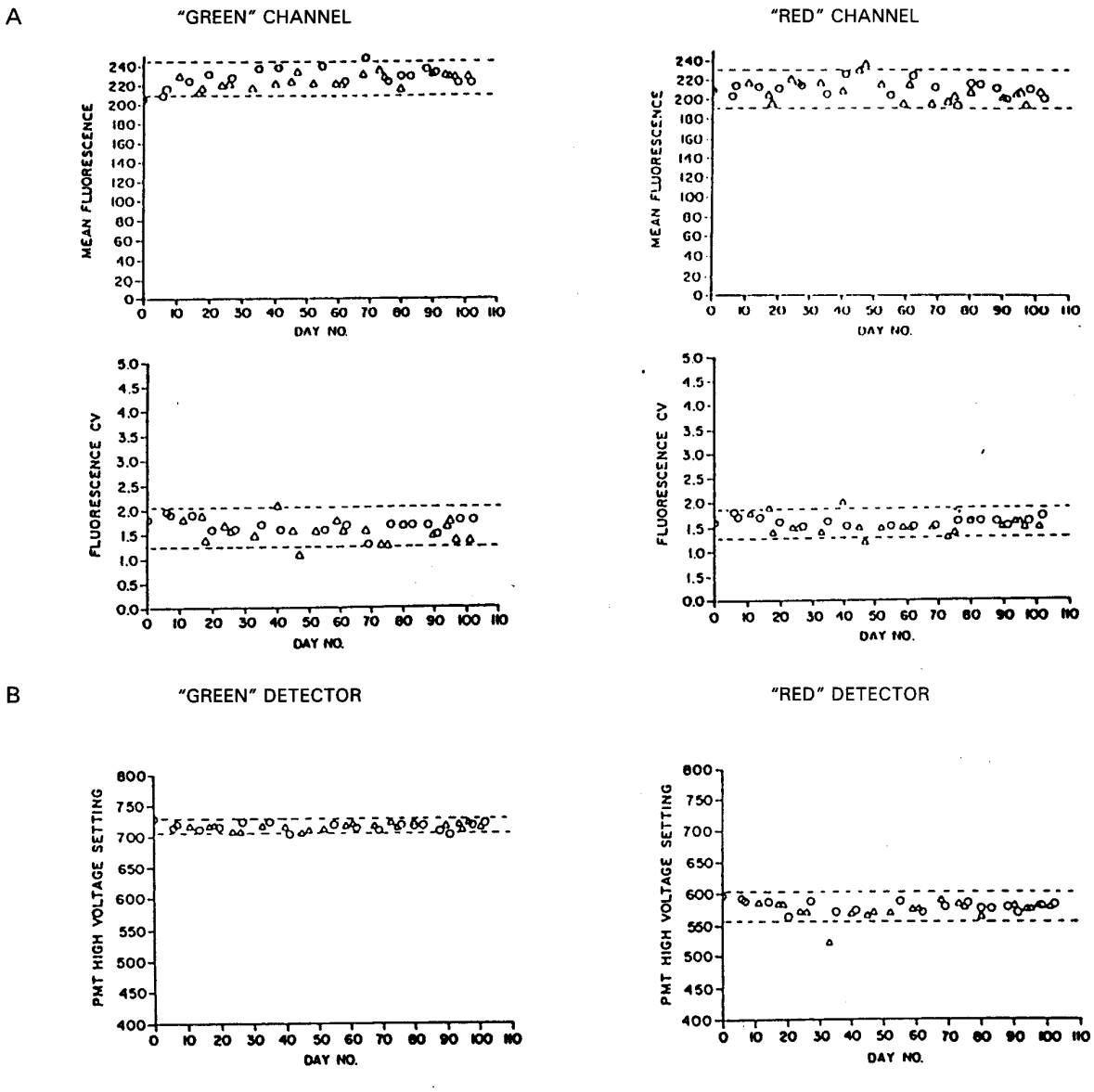

Figure 1. Methods for Instrument Performance Monitoring. Circles and triangles represent values obtained by two different operators; broken lines represent acceptance ranges for parameters shown (mean ± 2 SD).

A. Mean intensity and coefficient of variation (CV) of fluorescent polystyrene particles were evaluated under constant instrument conditions (filters, laser power, detector high voltage/gain settings, and linear amplification).

B. Detector high voltage required to reproduce mean channel number ± 1 channel for FITC-conjugated calf thymocyte nuclei was evaluated using fixed laser power, filter sets, and logarithmic amplification. **NOTE:** Out-of-range value in "red" channel on day 32 was due to propidium iodide carryover.

11.1.2 Test-Specific Instrument Settings

Alignment particles often have significantly different LS and fluorescence intensities than lymphocytes stained with fluorochrome-labeled antibodies. Therefore, it may be necessary to "fine tune" instrument settings for analysis of test specimens. This is done using blood prepared using the laboratory's standard method and cells that are labeled with:

- reagent that gives relatively bright staining intensity and good resolution of stained and unstained cells (e.g., normal lymphocytes stained with CD3), and

- reagent that gives dim but consistently greater staining intensity than unstained cells (e.g., normal lymphocytes stained with CD2 or CD56, normal monocytes stained with CD13, or a leukemic sample with known low density expression of a particular antigen).

Selection of appropriate test-specific settings involves two steps:

(1) optimization of LS resolution to allow clear visualization of the lymphocyte population by selection of appropriate amplification settings for both FALS and 90°LS, and

(2) optimization of fluorescence resolution to allow clear discrimination of negative, dimly stained, and brightly stained populations:

    - for single-color analysis, appropriate fluorescence amplification settings must be identified,

    - for dual-color analysis, appropriate fluorescence amplification and color compensation settings must be identified.

11.1.2.1 Optimization of Light Scatter Resolution

For a normal blood sample, the lymphocyte population should be well resolved from other leukocyte populations, and the shapes of all leukocyte clusters should be visible. For most abnormal samples, the predominant LS cluster(s) should fall on scale and within the general region defined by normal leukocytes. However, this may not be the case in all specimens and, where available, morphological information about the size and frequency of abnormal cells should be utilized in selecting appropriate LS gains.

(1) Establishment of Appropriate Fluorescence Intensity Settings

Fluorescence amplification/gain settings should be chosen to place the positive peak of the "bright" control at or above the midpoint of the fluorescence intensity scale; however, no positive cells should accumulate in the highest channel of the histogram. It is possible that significant numbers of unstained patient cells will be as bright as "dim" controls because they are totally unpredictable with respect to their cytoplasmic content, which is the primary source of autofluorescence in unstained cells. Amplification should be selected to place as many of the unstained cells as possible above the lowest channel and still not allow accumulation of positive cells in the highest channel. When dual-color analysis is to be carried out, appropriate fluorescence amplification/gain settings should be verified with samples that represent "dim" and "bright" staining with each fluorochrome of interest.

Once a test-specific amplification is selected, it is important to determine whether instrument sensitivity is adequate to discriminate between unlabeled and "dim" cells at these settings. On some instruments, this may be done by comparing the "blank" signal obtained when no cell is present (using triggering electronics to activate the detectors for an appropriate period of time). Alternatively, a "blank" particle (one with autofluorescence lower than that of normal lymphocytes) may be run. If the instrument can discriminate between a "blank" signal and that of unlabeled cells, its sensitivity should be sufficient to discriminate between unlabeled and dimly labeled cells. If unlabeled cells give the same signal as a "blank," it may not be possible to discriminate between unlabeled and dim cells, and instrument performance should be further optimized.

(2) Establishment of Acceptance Values for Materials to be Used in Daily Intensity Monitoring

It has been suggested that level of expression of specific cell surface antigens varies as a function of maturation, differentiation, and/or disease state. While consensus on the clinical utility of such variations has not yet been reached, use of a consistent intensity scale from day to day is recommended as one way to speed the process of gathering the necessary information.

Two different approaches to daily intensity monitoring are possible. One approach is to use freshly prepared blood specimens following establishment of instrument operating parameters with a standard material. If the intensity value determined for the "normal" sample falls within the laboratory's established intensity range for the measured antibody (using appropriate statistical analysis), unknowns can then be analyzed. The values obtained for the normal sample that day, as well as corresponding instrument settings, must be recorded. Modification of instrument settings based on the "normal" must be justified based on the statistics of the population norm for the laboratory. The problem of variability can be reduced if daily verification is done using a reagent that gives relatively stable intensities from individual to individual (e.g., CD3).

A second method, which leads to more reproducible intensity settings, is to use a reference material with stable staining and/or fluorescence intensity characteristics similar to those of antibody-stained lymphocytes. Ideally, it should contain populations with fluorescence intensities similar to unstained and dimly antibody-stained lymphocytes (i.e., when the instrument is able to resolve these populations, it should also be able to resolve dimly stained lymphocytes). Acceptance values for the intensity reference material are then established by running the material under test-specific conditions a total of 20 or more times over a period of at least five days. These values and all relevant instrument settings are then recorded in the instrument log book for use in daily monitoring. Any new lot of reference material should be run in parallel with the old lot to establish acceptance ranges for the new lot.

(3) Establishment of Appropriate Color Compensation Settings

The goal of color compensation is to assure that when both fluorescein-(FITC) and phycoerythrin (PE)-conjugated antibodies are used to label a cell sample, cells colabeled with both reagents can be accurately distinguished from cells labeled only with one reagent or the other. This corresponds to selecting instrument conditions under which cells labeled only with FITC give no greater signal than unstained cells on the PE histogram and vice versa. Electronic subtraction is typically used to correct (compensate) for the fact that a small proportion of FITC fluorescence is detected using "PE" filters and, conversely, a small proportion of PE fluorescence is detected using "FITC" filters. Alternatively, compensation may be done mathematically using appropriate software with uncompensated list mode files.

The degree of compensation required depends on the spectral characteristics of the fluorochromes and filters used, and on the level of signal amplification. Therefore, compensation settings must be re-evaluated whenever filters or photomultiplier tubes are replaced or when instrument parameters (which affect fluorescence amplification) are altered (e.g., photomultiplier high voltage and/or gain settings). This is true whether compensation levels are established using cells or plastic particles. Note that if the intensity scale is reset daily to a constant position using a stable intensity reference material, this corresponds to maintaining a constant system amplification from day to day. Therefore, if this method is used, compensation settings need only be re-evaluated when filters or detectors are replaced.

(a) Use of cells to establish compensation settings

Lymphocytes stained with mutually exclusive antibodies bearing the relevant fluorochromes are the proper reference materials for establishing appropriate compensation settings or for verifying that compensation settings chosen using plastic particles are appropriate.

Ideally, the antibody combination used to establish compensation settings should be chosen from antibodies in the test panel giving the highest fluorescence intensities (e.g., CD4/ CD8) because this will make undercompensation more visually evident (see Figures 2 and 3, panel A). For example, if normal lymphocytes are used as the reference material for establishing compensation settings, the following samples might be evaluated:

Tube 1: FITC-CD4 labeled
Tube 2: PE-CD8 labeled
Tube 3: admixture of Tubes 1 and 2

If higher values are found for percent dual positive in dual-color (tube 3) than in single-color samples (tubes 1 and 2), undercompensation is occurring and compensation settings must be increased to avoid overestimation of dual-labeled cells (see Figures 2 and 3, panel A).

Note that it is also possible to overcompensate (electronically overcorrect; see Figures 2 and 3, panel C). Overcompensation will not affect detection of singly labeled cells, but may cause underestimation of dual-labeled cells, especially when they are dimly positive (compare Figures 2 and 3, panels B and C). Comparison of single-color values for normal lymphocytes labeled with FITC-CD8 or PE-CD8 with dual-color values obtained after admixture of these two specimens is particularly useful in detecting overcompensation because dim and bright cells are present for each color.

The lowest compensation settings that give satisfactory agreement between single-color and dual-color samples should be selected to avoid overcompensation. When in doubt, undercompensate. Satisfactory agreement means that the difference in percent positive determined from single- versus dual-color samples should be no greater than the difference between replicate determinations on single-color samples. Equivalently, cells labeled only with FITC should give no greater signal than the negative control at the PE detector. Conversely, cells labeled only with PE should give no greater signal than the negative control at the FITC detector.

(b) Use of particles to establish compensation settings

Plastic particles labeled with FITC and PE, which have stable spectral characteristics and intensities similar to those of antibody-labeled cells, may also be useful in the initial choice of approximate compensation levels. For some particles, the manufacturer provides expected values for particle intensity that have been established under test-specific conditions on a particular type of instrument. In other cases, the laboratory assigns its own values for particle intensities under test-specific conditions.

If the plastic particles do not have precisely the spectral characteristics as stained leukocytes (something that is difficult to accomplish), particle-based compensation settings may be somewhat different than those that would be chosen using stained leukocytes. Therefore, compensation settings initially chosen using any plastic particles should always be verified using stained lymphocytes as described in Section 11.1.2.2 (3)(a).

If the laboratory uses plastic compensation particles with manufacturer-defined values to establish compensation settings, manufacturer-recommended procedures should be followed. However, the compensation levels chosen should still be verified using lymphocyte preparations such as those described in Section 11.1.2.2 (3)(a).

If the laboratory uses plastic compensation particles without manufacturer-defined values, the particles should have fluorescence intensities at least as bright as the brightest antibody-labeled samples under test conditions to avoid possible undercompensation. Approximate compensation settings should be chosen by running the particles under test-specific instrument settings and then verified using lymphocyte preparations such as those described in Section 11.1.2.2.(3)(a).

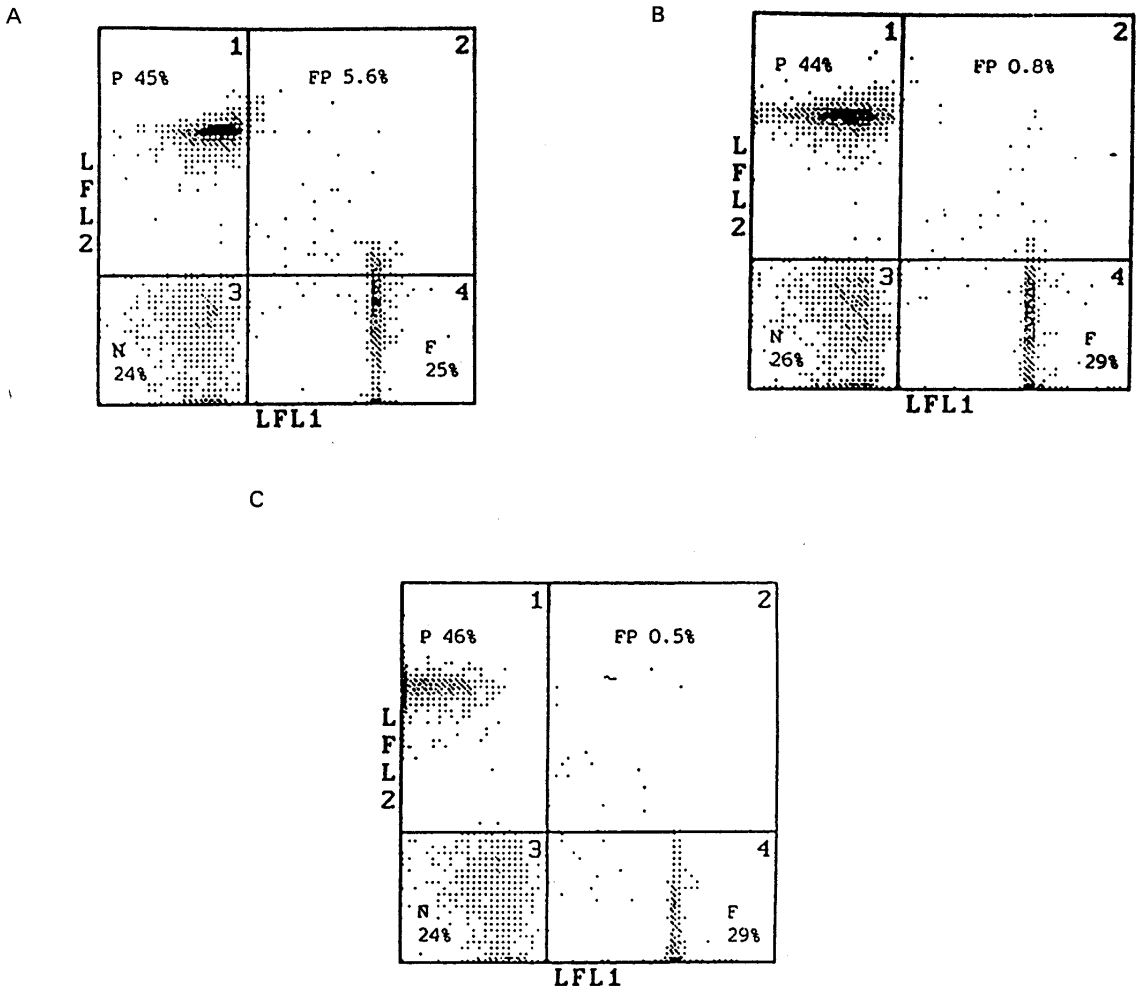

Figure 2. Effect of Undercompensation and Overcompensation on Two-color Analysis of Mutually Exclusive Markers. Microspheres labeled with no fluorochrome (N), FITC only (F), PE only (P), or FITC and PE (FP) were analyzed at several different compensation settings. LFL1 = all fluorescence collected using a 525 ± 20 nm bandpass filter; LFL2 = all fluorescence collected using a 575 ± 15 nm bandpass filter.
Panel A: Slight undercompensation—average LFL2 signal of FITC beads greater than average LFL2 signal of unlabeled beads; average LFL1 signal of PE beads slightly greater than average LFL1 signal of unlabeled beads.
Panel B: Correct compensation—average LFL2 signal of FITC beads same as average LFL2 signal of unlabeled beads; average LFL1 signal of PE beads same as average LFL1 signal of unlabeled beads.
Panel C: Slight overcompensation—average LFL2 signal of FITC beads less than average LFL2 signal of unlabeled beads; average LFL1 signal of PE beads less than average LFL1 signal of unlabeled beads.

A

B

C

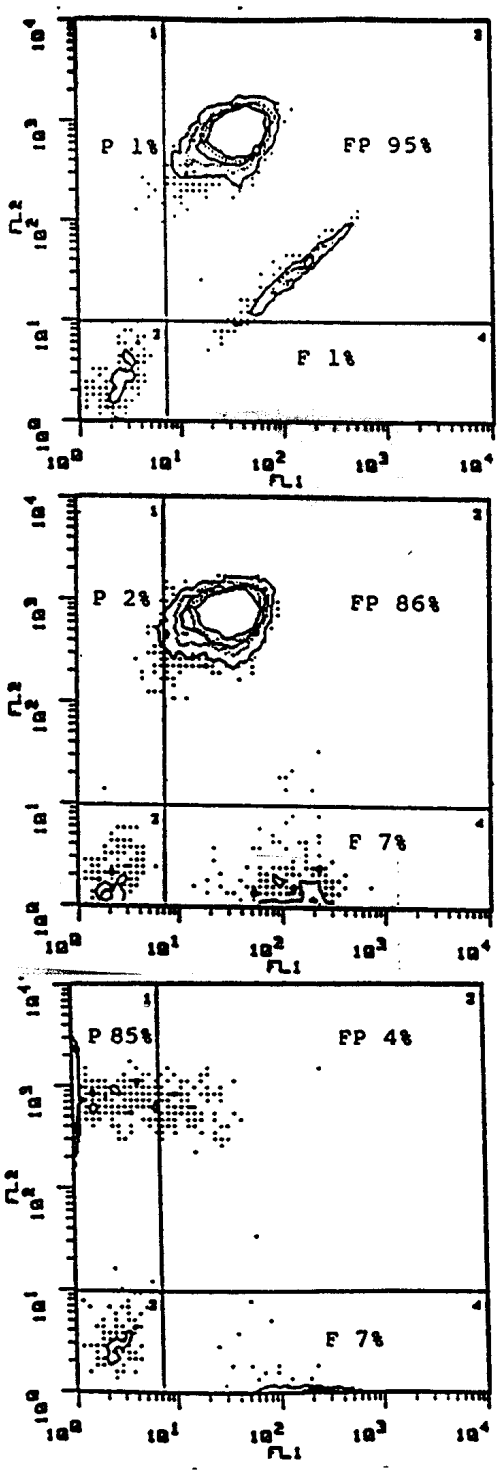

Figure 3. Effect of Under- and Overcompensation on Two-color Analysis of Coexpressed Markers. Specimen from patient with B-CLL was labeled with FITC-CD5 (FL1) and PE-CD20 (FL2) and analyzed at several different compensation settings for frequency of normal T cells (F; $CD5^{bright}$); normal B cells (P; $CD20^{bright}$); neoplastic B cells (FP; $CD5^{dim}$, $CD20^{bright}$); and antibody-negative cells (N). Percent CD20-positive (quadrant 1), CD5/CD20 dual-positive (quadrant 2), negative (quadrant 3), and CD5-positive (quadrant 4) events are indicated by numbers in each region.

Figure 3 (Continued).

Panel A: Undercompensation—use of perpendicular analysis thresholds as shown results in inclusion of normal T cells as false positives in region 2 (dual-positive region).
Note the strong diagonal nature of the normal T cell cluster, which is characteristically seen when two parameters are strongly correlated (here because both FL1 and FL2 signals reflect FITC intensity). Unless a sample known to contain no FITC-positive cells has been previously analyzed and found to give no significant events in quadrant 2, it is not possible to say whether the CLL population is truly double positive.
Panel B: Correct compensation—normal T cells have average FL2 similar to that of antibody negatives and are correctly counted as single positives in quadrant 4; CLL cells are dual positive. Note, however, that this can only be said with confidence if a sample known to contain no FITC-positive cells has been previously analyzed and found to give no significant events in quadrant 2.
Panel C: Overcompensation—use of perpendicular analysis thresholds show results in analysis of most CLL cells as CD5 negative. Normal T cells are barely visible along the *X* axis and have apparent FL2 intensity significantly less than that of antibody-negative cells; CLL cells have apparent FL1 intensity significantly less than that of antibody-negative cells.

4) Establishment of Acceptance Values for Materials to be Used in Daily Compensation Monitoring

Expected values for compensation particles may be provided by the manufacturer for some instruments with fixed optical systems. For other particles and/or instruments that are user adjustable, expected values are established by running the material under test-specific conditions and at established intensity and compensation settings a total of 20 or more times over a period of at least 5 days. Mean fluorescence intensities for each population of interest (FITC only, PE only, and negative for both) along with all relevant instrument settings and compensation settings are recorded in the instrument log book for use in daily monitoring. Any new lot of compensation reference particles should be run in parallel with the old lot to establish acceptance ranges for the new lot.

Expected values for compensation reference particles must be re-established whenever compensation settings are re-evaluated (i.e., whenever filters are replaced or when instrument parameters that affect fluorescence amplification are altered significantly [e.g., photomultiplier high voltage and/or gain settings]).

11.2 Monitoring Instrument Performance and Reproducibility

For daily performance monitoring, control or reference materials are reassayed under test-specific instrument settings and the values obtained compared to the acceptance values previously established under conditions of optimal performance. Consistent quality assurance requires that each of the relevant functions listed in Table 1 be performed regularly. Note, however, that a separate sample is not necessarily required for each function. One quality control material may serve several functions, or a mixture of several materials may be formulated to minimize the time required to verify proper instrument performance. For example, an intensity control containing both cellular and plastic particles of varying intensities was described that can be used to assess instrument reproducibility with respect to LS, fluorescence sensitivity, and amplifier response characteristics. Recording values for certain instrument settings (e.g., laser current required to achieve a given power output and laser power required to achieve a given forward scatter intensity for a standard particle) may also assist in identifying trends that require preventive maintenance (i.e., cleaning optics and laser tube replacement) and in avoiding down-time due to catastrophic failure.

In addition to improving confidence in test data, information provided by the various reference materials can be of significant value in troubleshooting. If ambiguous or inconsistent test results are obtained, reassaying the various reference materials can immediately establish whether faulty instrument performance is responsible. If this is not the case, possible errors in sample handling, preparation, or staing should be investigated.

11.2.1 Verification of Optical Alignment

To verify optical alignment:

(1) Run alignment particles under instrument settings determined at the time of initial instrument setup.

(2) Record the mean channel number and CV, or instrument parameters (PMT settings) required to reproduce a specified mean channel number, for all parameters to be analyzed for test specimens in the daily log book and on Levy-Jennings plots.

If values are not within the range of acceptance, alignment should be optimized before proceeding.

11.2.2 Verification of Fluorescence Resolution

To verify reproducibility of intensity scale and instrument sensitivity, use the following procedures:

- If freshly stained samples are used to verify instrument sensitivity, establish that the dimly stained positive control can be distinguished from the unstained control.

- If intensity reference particles (e.g., fluorochrome-labeled beads or nuclei) are used to verify instrument sensitivity, run them under test-specific settings established at the time of initial setup and establish that "dim" particles can be distinguished from "blank" ones.

- If a constant intensity scale is being used, run intensity reference particles under test-specific instrument settings and establish that their mean channel values fall within the ranges established at the time of initial instrument setup.

- Record mean fluorescence channel and CV for all populations of interest (e.g., unstained and dimly stained) in the daily log book and on Levy-Jennings plots.

  If mean fluorescence values are not within the range of acceptance, reasons for altered instrument sensitivity should be determined before the analysis of test specimens and they should be corrected as necessary.

11.2.3 Verification of Appropriate Color Compensation

If dual-color analysis is being run, verify appropriate color compensation settings using the following procedures:

- If freshly stained samples are used to verify compensation settings, establish that single-color and dual-color samples give equivalent results [see Section 11.1.2.2 (3)(a)]

- If compensation particles (e.g., FITC- and PE-labeled beads) are used to verify compensation settings, run them under test-specific settings and establish that their values fall within the expected ranges determined at the time of initial instrument setup.

- Record mean fluorescence intensity for each population of interest (FITC only, PE only, and negative for both) in the daily log book and on Levy-Jennings plots.

If particle values are not within the range of acceptance, compensation settings should be re-evaluated using antibody-stained lymphocytes.

11.2.4 Verification of Overall System Performance

To verify overall system performance use the following procedures:

- Run the positive procedure control.

- Verify that the LS resolution of all leukocyte populations is acceptable.

- Verify that the percent antibody-positive lymphocytes falls within laboratory-established ranges for the markers selected.

If this positive control does not meet laboratory criteria, remedial action should be taken. Instrument performance and/or staining procedure should be checked to determine the source of the problem. Any problems identi

fied using this sample must be rectified before the analysis of test specimens.

12 Data Acquisition

12.1 Instrument Configuration

Acquisition of data from a sample involves a number of steps that ensure appropriate, high-quality data. The first steps for sample analysis involve proper configuration of the instrument. Appropriate color compensation should be determined and standard scatter and immunofluorescence gains established (Section 11). The instrument configuration should minimize biohazard to the operator and maximize decontamination of fluid waste. Samples should be appropriately fixed before analysis, and waste should be collected into vessels containing decontaminating agents such as chlorine bleach.

12.2 Order of Analysis

The order of analysis is as follows:

(1) controls for light scatter gating validation

(2) negative controls for definition of intensity threshold above which true positives are calculated

(3) samples stained with reagents for immuno-phenotyping of leukemia

12.3 Verification of Acceptable Specimen Viability

Because of the biohazard concerns, it is recommended that all samples be appropriately fixed before they are analyzed on the flow cytometer. However, because there is no consensus on a method applicable to fixed leukemic cell preparations which can be used to distinguish those cells that were nonviable prior to fixation (see Section 8.4), the determination of viability is most often done on stained cell preparations just prior to fixation (see Section 5.11). Whatever method is used, the laboratory is responsible for determining the concentration of viability dye required to give optimal resolution between live and dead cells.

Each laboratory should have an established method for assessing specimen viability and sample viability (see Section 8.4). Each laboratory should establish a minimum proportion of nonviable cells that can be included in the analysis and not significantly influence the measurement of interest. Nonviable cells are a significant source of false-positive staining.

If specimen viability is below the established laboratory minimum, test results are suspect and this should be noted in the report.

12.4 Representative Sampling

Samples should be well mixed immediately before introduction into the instrument and, if possible, during data accumulation to avoid the possibility of artifacts. Representative sampling is especially important when specimen LS characteristics are abnormal, because this may indicate size and/or density differences that could affect relative sedimentation rates.

12.5 Optimization of Gating to Include and Maximally Enrich the Leukemic Population

12.5.1 Preliminary Differential Cell Count

The presence and percent of leukemic cells in the patient's bone marrow (or blood or other fluid) should be estimated from a manual differential morphologic cell count on a Wright–Giemsa-stained preparation. For many cases it is also necessary to examine a Wright-stained preparation made from the actual cell preparation to be immunophenotyped immediately before immunostaining. The frequency of leukemic cells may either be lower in this specimen than in the diagnostic specimen prepared for histology due to the dilution of the marrow sample with blood, or higher than in the diagnostic specimen due to the enrichment of leukemic cells by the gradient separation.

Similarly, in the case of lymphoma, it is often invaluable to review frozen sections or Wright–Giemsa-stained touch imprints of a portion of the submitted lymph node to determine proportions of different types of cells in the specimen. Morphologic review of either leukemia or lymphoma specimens gives the

cytometer operator and those persons analyzing and interpreting the flow cytometric data important information, which may be used to base gating or analysis criteria and is especially important when the specimen does not contain a majority of leukemic cells. For example, the presence of large numbers of morphologically normal granulocytes in the preparation would suggest that signals with high 90° LS may be ignored (gated out) during analysis.

12.5.2 Distinguishing Between Normal and Abnormal Scatter Regions

To optimize the resolution of blood leukocytes, FALS and 90° LS signal gains should be reproduced daily. This information is important in decisions to ignore certain signals (e.g., high 90° LS scatter cells likely to be normal granulocytes) in the later analysis of patient leukemia samples. In some cases, leukemic cells may be readily recognized within a distinct region not occupied by normal leukocytes. Unfortunately, leukemic cells may not always occupy such a region. Under these circumstances, both nonleukemic and leukemic cells are included in any LS gate used for acquisition. Morphologic correlation may help to determine where abnormal cells are likely to be found. Occasionally, cells are so large as to be off scale. In this case, LS gains may have to be adjusted appropriately.

12.5.3 Setting Gates on the Patient Sample to Include the Leukemic Cells

Acquisition gates should be set on a bivariate distribution, most commonly FALS versus 90° LS, but occasionally acquisition gates can be set on fluorescence versus scatter, as discussed above. *Do not exclude acquisition of any population of cells — particularly those with low-moderate 90E LS — without justification, i.e., by "back-gating" on immuno-fluorescence to verify that they are not the leukemic population. Failure to acquire a subset of signals takes the risk that the leukemic population, or an important leukemic subpopulation, may be missed. This may lead to a complete misdiagnosis*.

The general rule must be that exclusion of any significant subpopulation is more safely done later during analysis, rather than during acquisition, because the latter would result in permanent loss of immunophenotypic information on the excluded cells. It is not possible to prescribe a uniform methodology to reliably exclude potentially contaminating mature cell types. This difficulty is magnified because the immature normal marrow precursors of these lineages are even harder to distinguish from leukemic blast cells. In specimens with a low percentage of leukemic cells, it is often impossible to reliably exclude a proportion of nonleukemic cells from the gate or to precisely estimate the numbers of these "contaminating" cells, even with the use of sophisticated multicolor backgating. For this reason, in order to avoid missing the leukemic cells, it is vital to avoid too small an acquisition gate.

12.5.4 Alternate Gating Strategies

Under some circumstances, it may be desirable to set an acquisition gate to enrich for the leukemic population. Although leukemic cells can often be specifically identified and gated during analysis, if the leukemic cell population constitutes only a minority of the cells in a sample, most of the acquired data may relate to normal elements, and the leukemic events may be so few in number as to make accurate phenotypic analysis impossible. A live (acquisition) gate can be set on a scatter display, to enrich, for example, for large cells or on a scatter versus fluorescence display to collect only cells of a particular phenotype. An example of the latter application might include acquisition of cells labeled with a pan B antibody to evaluate kappa and lambda light chain staining on a population enriched for B cells. If a viability dye is used, a fluorescence gate can be set to collect data on only viable cells. *If an acquisition gate is set, it is crucial that the caution noted in Section 12.5.3 above be followed. Moreover, an ungated record from the same tube should always be acquired before the gated record.*

12.6 Analysis of Negative Control Sample(s)

Each patient sample must have its own negative reagent control (see Section 10.2). In analyzing negative control sample (s), it is necessary to do the following:

- Acquire single-color or correlated dual-parameter immunofluorescence data using defined gates.

- Compare the mean signal intensities for the negative control to the established laboratory value for the negative reagent control on a normal sample.

Greater than expected intensity of the negative reagent control is common when analyzing leukemic samples, particularly in myeloid leukemias, because of increased auto-fluorescence. It also may indicate increased nonspecific binding due to reagent problems in binding to Fc receptors; poor specimen viability; or artifactual uptake of free, nonprotein-bound fluorochromes remaining in the instrument, particularly when analyzing fixed specimens. To evaluate whether intensity is greater than expected, it is necessary to reproduce the immunofluorescence intensity scale(s) on a daily basis using an appropriate calibration material.

Problems due to autofluorescence can often be distinguished from problems due to artifactual uptake of fluorochrome by analyzing an unstained sample.

If the channel number at the upper boundary of the negative control sample is higher than the correct value for the normal negative control, it should be so noted on the test record.

12.7 Analysis of Samples Stained for Phenotype Determination

In analyzing samples stained for phenotype determination, it is again necessary to acquire single-color or correlated two-parameter immu-nofluorescence data with the same gates and settings used for acquiring the negative control sample.

Ideally, a sufficient number of positive cells should be counted to assure that variability due to this type of error is no greater than the variability among replicate preparations of a single specimen. Counting errors for estimation of positive cells are expected to be proportional to the square root of the number of positive cells counted (i.e., if the population of interest comprised 5% of the total number of cells and 10,000 cells are evaluated, the expected count is 500 ± 22; if 1,000 cells are evaluated, the expected count is 50 ± 7). In practice, at least 200 to 1,000 cells of interest should be evaluated, but this ideal may not be achievable in patients with severely hypoplastic marrow. The ideal number of events to collect also depends on the relative purity of the sample for leukemic cells.

In some cases, the population of interest may represent only a small subset of the total number of cells in the sample. For example, a small number of lymphoma cells may be present in a background of a large number of normal bone marrow cells. Under these circumstances, it may be appropriate to redefine acquisition gates for a particular stained tube to enhance the number of cells of interest. For example, when looking for B-cell lymphomas, samples stained with both a pan B marker and kappa or lambda immunoglobulin light chain can be analyzed by setting a fluorescence gate and acquiring an enriched population of B cells. If such a procedure is used, an initial acquisition should be performed as described above (Section 12.5) and examined to identify alternate gating parameters. A second acquisition run using these new parameters can then be carried out to collect a data file enriched for the population of interest.

13 Data Analysis

13.1 Goals

The goal of data analysis in leukemia is to accomplish the following objectives:

- Determine if at least some of the leukemic cells in a given sample are positively labeled with a given antibody.

- Determine, where possible, what proportion of leukemic cells are so labeled.

- Provide some descriptive information about the relative intensity of expression of a given label.

Unlike the case with analysis of lymphocyte subpopulations, many reagents, when applied to leukemias, give distributions in which low-intensity positives show considerable overlap with negatives. Analysis of such data is not

straightforward. Moreover, this problem is compounded further in specimens in which there are variable numbers of leukemic cells along with normal cells that may or may not label with the antibodies of interest. Ease (and to some extent accuracy) of analysis depends on several things:

- the number of leukemic cells in the sample;
- the intensity of expression of a given label, which in turn depends upon fluorochrome, instrument performance, and staining conditions (as well as the underlying pathobiology);
- the ability of leukemic cells to be separated from normal cells on the basis of LS characteristics; and
- the judicious design of reagent combinations, which, when analyzed using multiparameter analysis, can help distinguish normal and abnormal cells.

13.2 Initial Procedures

13.2.1 Identification of Populations to be Analyzed

During the course of acquisition, a gate may have been set to include only the leukemic cells (see Section 12.5). More frequently, however, both nonleukemic and leukemic cells will have been acquired, and attempts should be made to distinguish them at the time of analysis. Because the best method of analyzing a particular sample may not always be known in advance, data should be collected in list mode, whenever possible.

13.2.2 Examination of Light Scatter

Usually the first correlated display examined is FALS versus 90° LS. On this display, there may be only a single population of cells visible, or in some cases, more than one population may be identified. In some cases, there may be a major population of low 90° LS and an additional, well-resolved population with scatter properties of normal neutrophils. In the latter case, an analysis gate may be set on the nonneutrophil population. Any ambiguity in scatter pattern should always be resolved on the side of including questionable events in an analysis gate or analyzing using multiple gates. When multiple gates are set, they can be analyzed individually using the principles outlined below. Where possible, separate descriptive statements about "large" versus "small," or "agranular" versus "granular" populations can then be made. Intraspecimen variation of the position of populations defined by LS should be minimal and, if experienced, indicates a problem in either sample preparation or instrument function.

13.2.3 Examination of Scatter vs Fluorescence

In cases in which there are mixtures of cell types present, correlated displays of either FALS or 90° LS versus fluorescence may demonstrate distinct clusters of events that were not apparent from examination of scatter plots alone. Such modes of analysis may be particularly helpful to distinguish between leukemic and nonleukemic cell populations, particularly when displays are correlated with morphologic examination of the material. Examples of more markers that may help to distinguish leukemic and nonleukemic cells include the following markers:

- CD45–absent from erythroid cells and cell debris;
- CD3 and/or CD20—strongly expressed on mature T or B lymphocytes, respectively;
- CD56–present on some large granular lymphocytes;
- CD14—present on monocytes; and
- CD15—strongly expressed on mature granulocytes.

It is important to note that the above markers can also be expressed in a wide variety of leukemias so that one cannot assume that cells with the properties described above are nonleukemic in any particular sample. Nonetheless, because antigen density often differs between normal and neoplastic cells, examination of FALS versus fluorescence displays with markers such as these may help to distinguish leukemic and nonleukemic populations. Thus, for example, CD45 expression is usually of lower intensity on blasts in acute leukemia than it is on normal lymphocytes or

monocytes. Similarly, CD20 is often of lower density on B-CLL or B-lineage acute leukemia than it is on normal B cells. In addition, under some circumstances scatter versus fluorescence displays may be useful for gating if one is interested only in expression on a phenotypically defined subset, such as expression of immunoglobulin light chain on CD19- or CD20-positive B cells. In such a case, a display of FALS versus PE fluorescence, for example, can be used to identify a population and the FITC fluorescence histograms of that gated population analyzed using the principles outlined below. As another example, if a viability dye is used, display of scatter versus fluorescence can be used to identify viable cells for analysis.

13.3 Single-Color Analysis

In single-color analysis, data are usually collected as a single parameter histogram of cell count versus fluorescence intensity.

Analysis of one-parameter histograms of single-color data in samples with heterogeneous populations is prone to error and should be avoided wherever possible. If single color data are to be analyzed, the preferred method is to use dual-parameter displays of FALS (or, less commonly, 90° LS) versus fluorescence intensity. The principles to be used are those discussed in Section 13.4 except that LS is substituted for one of the fluorescence parameters. The ability of a dual parameter display to provide information not available from examination of a simple single-parameter histogram is illustrated in Figure 4.

13.4 Dual Parameter Analysis

In dual parameter analysis, the display to be analyzed is either a dot plot or contour map correlating the expression of two parameters. Dual parameter analysis is frequently considered synonymous with dual-color analysis, though this is not so. Nevertheless, because dual-color analysis is most commonly used, most of the discussion focuses on it; it should be recognized, however, that the same principles can be applied to displays of any two parameters.

13.4.1 Dual-Color Analysis

In the most commonly used method of analysis, data are displayed as a plot of log FITC immunofluorescence on the *x*-axis and log PE immunofluorescence on the *y*-axis. Such a plot is commonly divided into rectangular quadrants by the use of two perpendicular boundaries. The quadrants are selected to differentially contain those events that bind neither antibody (“double negatives”), bind only FITC-conjugated antibody, bind only PE-conjugated antibody, or bind both antibodies ("double positives"). If color compensation was not accurately set, use of rectangular analysis boundaries may result in either false positives or false negatives, particularly false double positives. Although rectangular quadrants are the most common method of setting analysis boundaries, it should be realized that this method is not appropriate unless the positive distributions are clearly separated from those obtained with the negative control antibody, as outlined above in consideration of one-color analysis. Frequently, examination of such a two-color display reveals two or more distinct clusters of events, recognizable by the distribution of dots on a dot plot or by the contour levels on a contour map. In general, it is best to compare each recognizable cluster in the test sample individually with the cluster(s) produced with the appropriate negative control antibody. Several situations may be obtained, as illustrated in Figures 9-13.

Some combinations of these are possible, but the principles described can be extended to consideration of these unusual cases.

13.4.2 Other Dual Parameter Analysis

As mentioned above, under some circumstances it may be appropriate to display dual-parameter data in the form of scatter versus fluorescence rather than FITC versus PE. The principles for analyzing such displays are similar to those noted above in Section 13.4.1. In general, the goal is to use the displays to identify distinct clusters of events that are separate from each other; only in such circumstances are analysis boundaries appropriate. When distributions containing low-intensity positives overlap with negative control distributions, boundaries can at best, provide an estimate of percent-positive cells. It should be noted that when heterogeneous cell populations are present, the background autofluorescence of different populations is frequently different. Because of this,

nonorthogonal boundaries may have to be set.

A special case of a dual parameter display that some have found particularly useful for analyzing cases of acute leukemia has been a correlated display of CD45 fluorescence and right angle light scatter. Because blasts generally have lower CD45 intensity than normal lymphocytes and monocytes, they are often analysis gate. Obviously, to perform dual color analysis of antigen expression on leukemic cells it would be necessary to perform

better separated from these normal elements on this display than on a display of FALS and RALS (See Figure 14). Thus, if CD45 is used as a common reagent in all tubes in the reagent panel, an analysis gate may be set on this display, and the antigenic phenotype of the leukemic population can, in many cases, be more accurately assessed than by using a display of FALS and RALS to set an three color analysis, as one fluorochrome is devoted to CD45.

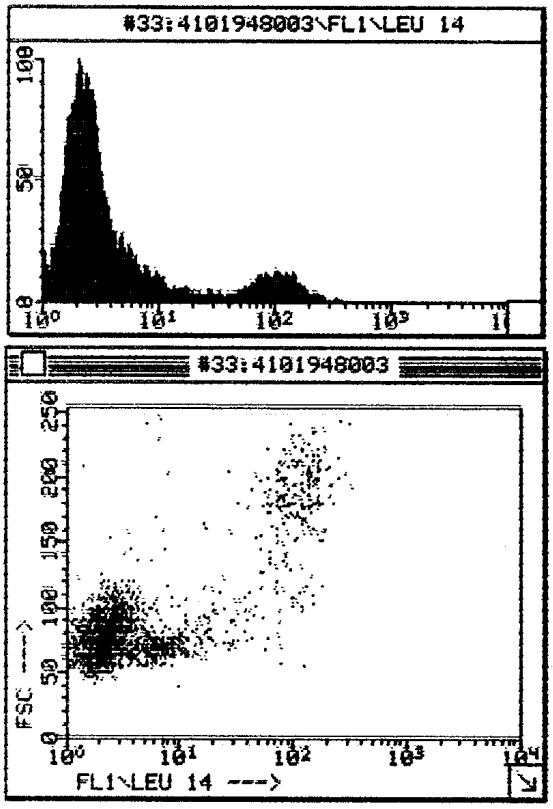

Figure 4. Comparison of a One-parameter and Two-parameter Display in a Sample with Heteroge

neous Populations. From the correlated dot-plot, it is easy to see that there are two distinct CD22 $(Leu14)^+$ populations: a dim one composed of cells that are on average smaller than CD22-negative cells, and a bright one composed of large cells. This information is much harder to glean from examination of the one-parameter histogram.

When the sample contains a uniform population of cells or when several populations can be distinguished from each other on the LS display and gated separately, analysis of single-parameter histograms of fluorescence intensity may be appropriate. In such cases the distribution must be analyzed to meet the principal goal of leukemic cell analysis, namely, to determine if an antigen is present on a leukemic cell. To do this, the shape of the histogram must be compared to that of a histogram given by cells stained with an antibody determined not to be reacting with the leukemic cell ("negative control antibody," [see Section 10.2]).

Several basic situations may be obtained when the histogram to be analyzed (hereafter the "test histogram") is compared with that from a nonreactive antibody. Analysis of each case is slightly different (see Figures 5-8).

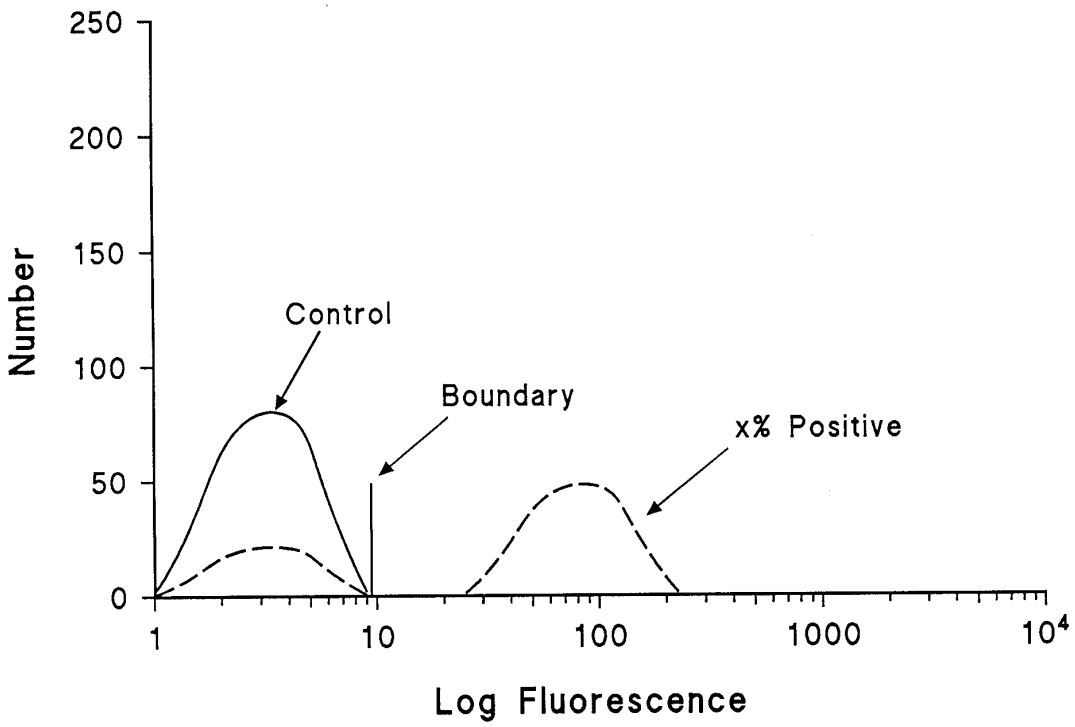

Figure 5. The test histogram is clearly resolved from that of the nonreactive antibody histogram (i.e., a positive result). There may not be a small peak that coincides with the negatively stained sample, but if there is, this returns completely to baseline before the rise of the second peak.

To analyze this case, an analysis boundary is set using the negative control. All events of a higher channel number than (to the right of) this boundary are considered positive. On the negative control sample, these are false positives, and they are typically chosen as less than 2%. Whatever method is chosen for selection, the analysis boundary should be well defined and reproducible. With this pattern of reactivity, it is appropriate to record the percentage of cells reacting with the antibody in question. Some measure of the relative intensity value obtained should be recorded. This could be a qualitative statement, such as "bright," or the mean or modal channel of the positive population could be recorded. Many methods for estimating quantitative fluorescence exist, and each laboratory should validate its own chosen method.

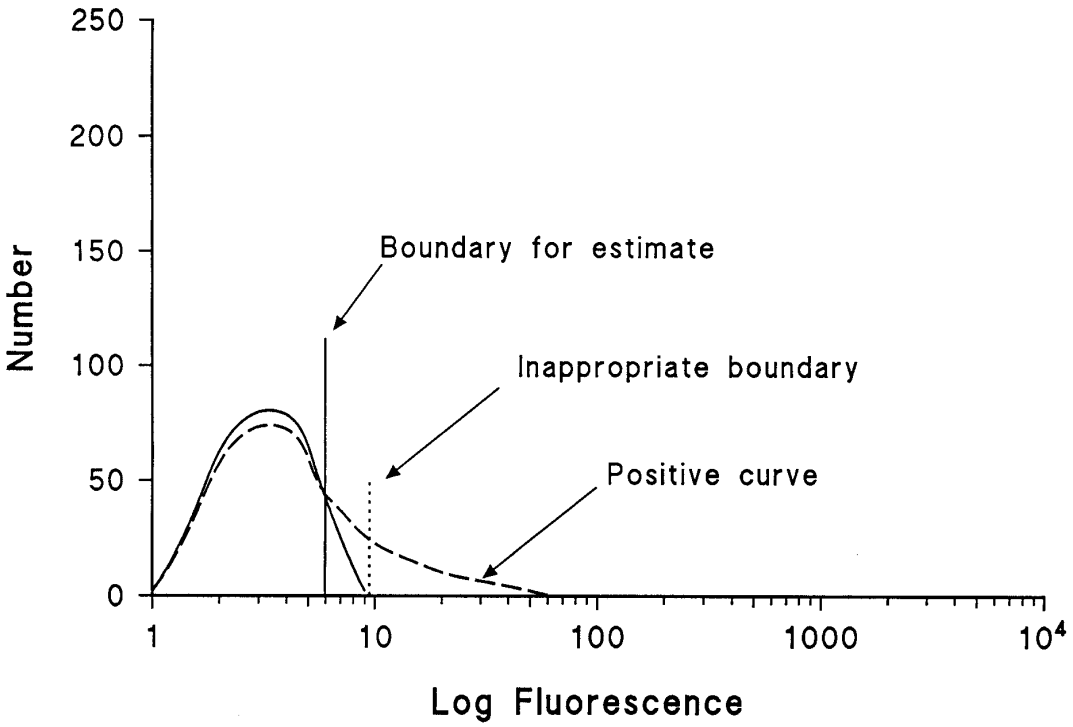

Figure 6. The left-hand portion of the test histogram overlaps with the negative control histogram, and the modal peak is the same, but the test histogram has a distinct "shoulder" not apparent on the negative one.

To analyze this case, the two curves should first be scaled in proportion to the total number of events collected. They then may be analytically subtracted on a channel-by-channel basis. Alternatively, an analysis boundary can be set where the shapes of two histograms diverge, and the false-positive events from the negative histogram (now typically much greater than 2%) subtracted from the positive one. It is recognized that the resultant value is inaccurate and only represents an approximation of the actual number of positive events. The relative intensity of such events are generally reported as "dim." If more quantitative methods are used, the mean or median channel, and not the mode (which is not appropriate to these distributions) is then recorded. It is inappropriate to set the analysis boundary as in Figure 5, and then report the percent positive as only those events at a higher channel number than this boundary.

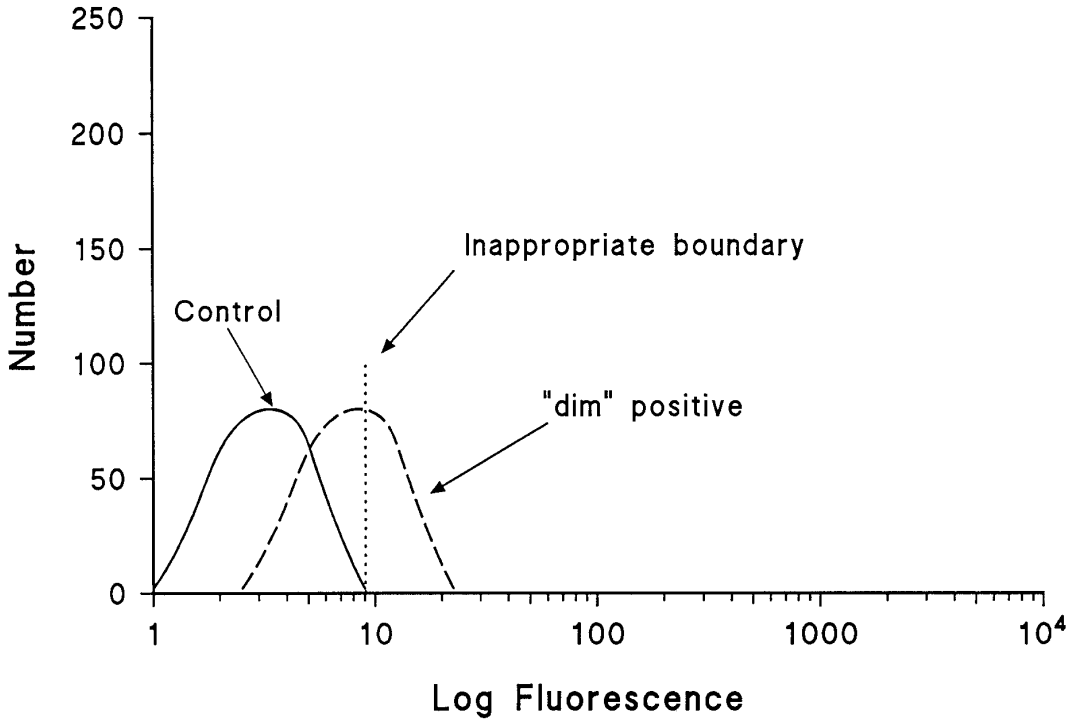

Figure 7. The shape of the test and negative control histograms are the same, but the test histogram is shifted several channels to the right of the negative control.To analyze this case, no boundary is appropriate, and no quantitative estimate of the percentage of positive cells can be obtained. Results should be recorded as "dim," or if quantitative methods are being used, the mean, median, or modal channel recorded. The most difficult part of analyzing this type of histogram is to be certain that a shift of a few channels in fact represents low levels of antigen expression and not nonspecific staining. For this to be true, it is necessary that (1) the instrument is analytically capable of resolving the presumed difference, (2) the mean channel of the test histogram is judged to be significantly and reproducibly higher than that obtained from cells stained with other test antibodies in the panel that are considered nonreactive, and (3) the antibody used to obtain the positive result has been appropriately titered, not only on a positive but also a negative target, and is known to give a negative histogram that overlaps with those seen in the sample.

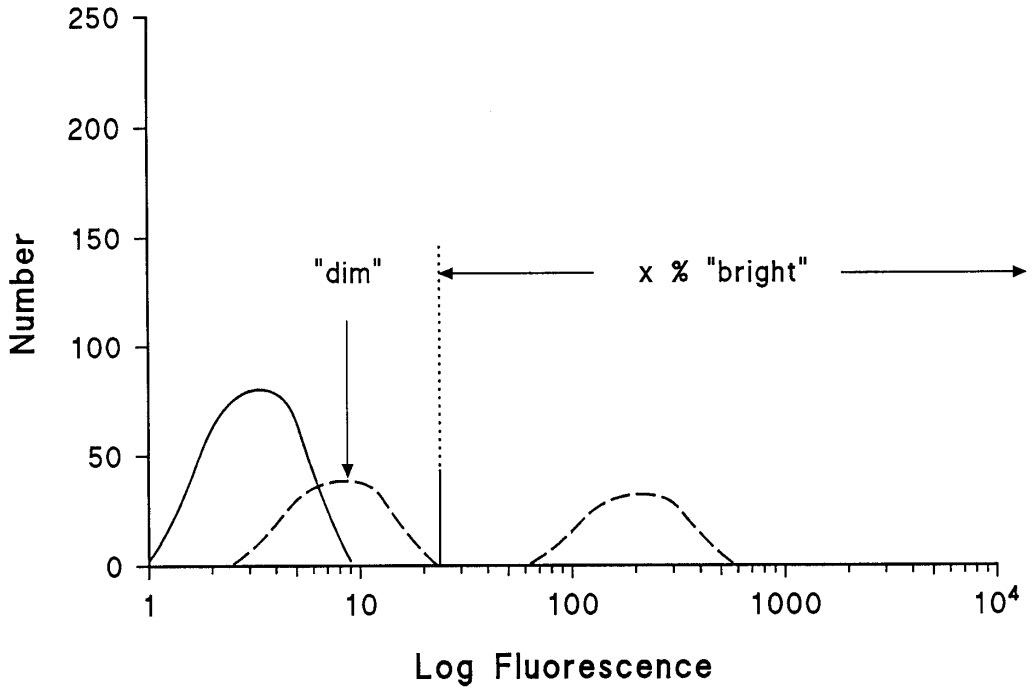

Figure 8. Some combination of the cases presented previously (in the example shown, Figures 5 and 7) may be found.

To analyze this case, an analysis boundary is set, not based on the negative control histogram but, rather, based on the point that the more dimly stained population in the test sample returns to baseline. It is appropriate to enumerate events to the right of this boundary as "bright," as in the case in Figure 5. For analysis of the more dimly stained population, its distribution should be compared to that of the negative control population and the principles previously outlined under Figure 6 or (in the case illustrated) Figure 7 should be applied.

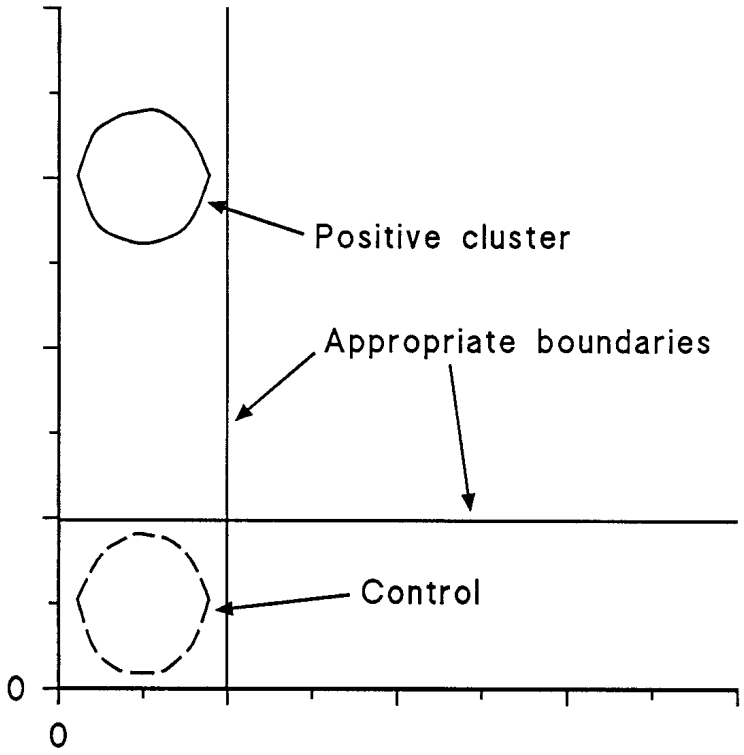

Figure 9. The cluster is distinctly separate from the negative control cluster and falls entirely within one of the quadrants defined by setting perpendicular markers around the control cluster.

Analysis of this situation is straightforward. Perpendicular markers are set on the negative control cluster. These are typically set so that there are less than 2% false positives in the FITC-only, PE-only, and FITC + PE quadrants. For the test sample, the percent of events falling in each quadrant should be recorded. These may also be noted as "bright" or, if quantitative methods are being used, the mean channel should be recorded.

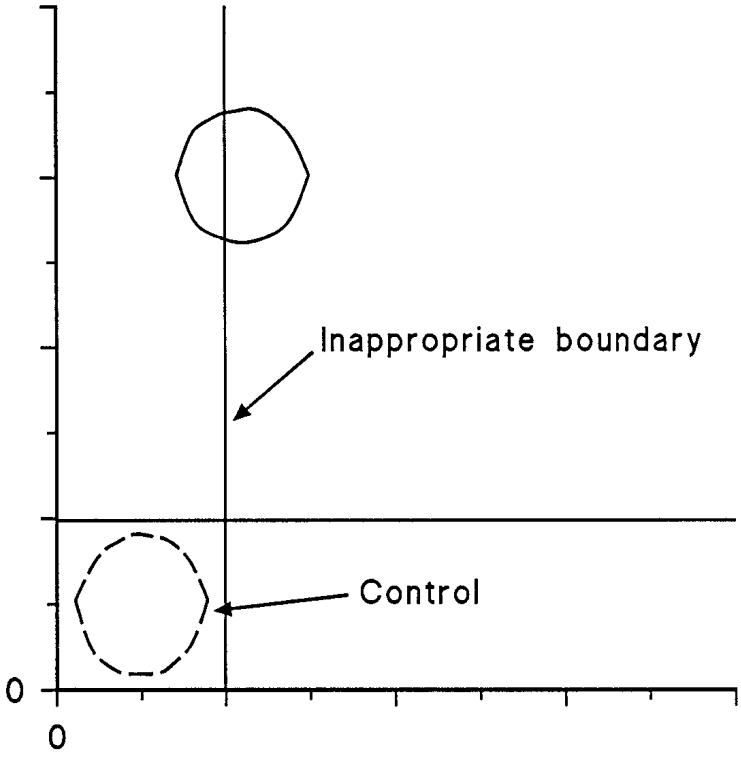

Figure 10. The cluster is distinctly separate from the negative control cluster but overlaps one of the perpendicular boundaries so that some events fall in the FITC + PE quadrant.

Analysis of this situation is slightly more complicated. For the dimension in which there is clear separation from the control cluster, the method outlined in Figure 9 is appropriate. To determine the double-positive events, one should compare the projection of the cluster from the test sample along either the *x*- or *y*-axis to that of the negative control cluster and use the principles outlined in either Figure 11 or Figure 12 that follow, depending upon the shape of the display.

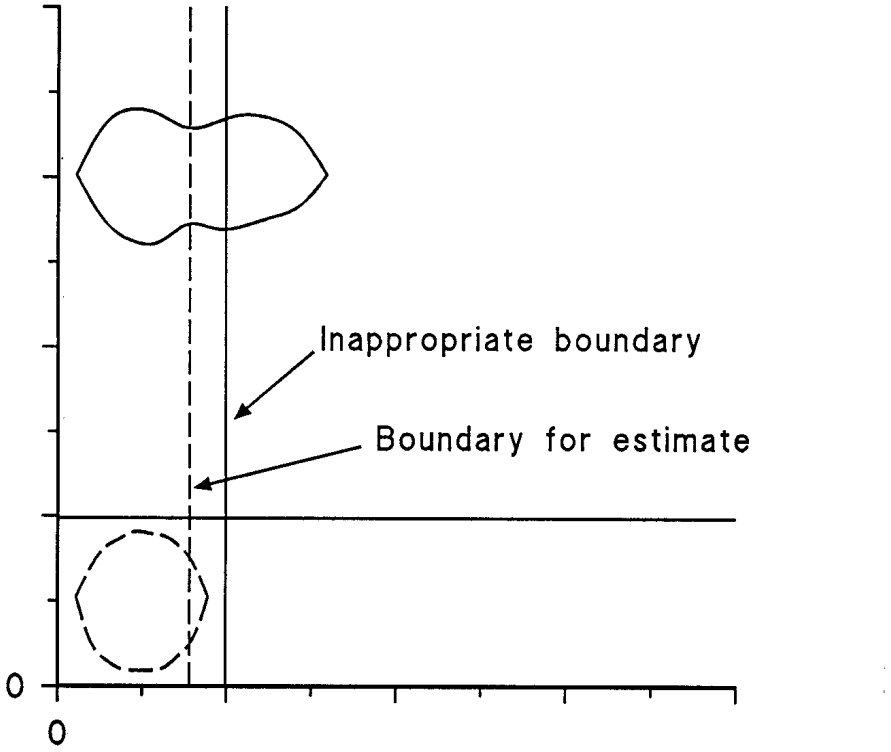

Figure 11. The left-hand portion of the cluster is parallel to, or overlaps the negative control cluster, but there is a distinct "tail" of events parallel to either the *x*- or *y*- axes.

This situation should be analyzed along the same principles mentioned previously for consideration of single-color analysis (Figure 6) and indeed data within this cluster may be redisplayed in single-histogram format and that method applied directly. A marker may be set on the negative control cluster where the shapes of the two clusters diverge (this is again best seen with a contour plot or histogram rather than a dot plot) and the false-positive events (typically much greater than 2%) subtracted from the events in the test cluster. The resultant events should be considered an approximation of the actual number of positive events and they should be recorded as "dim," or the mean channel of the positive region should be recorded if quantitative methods are used.

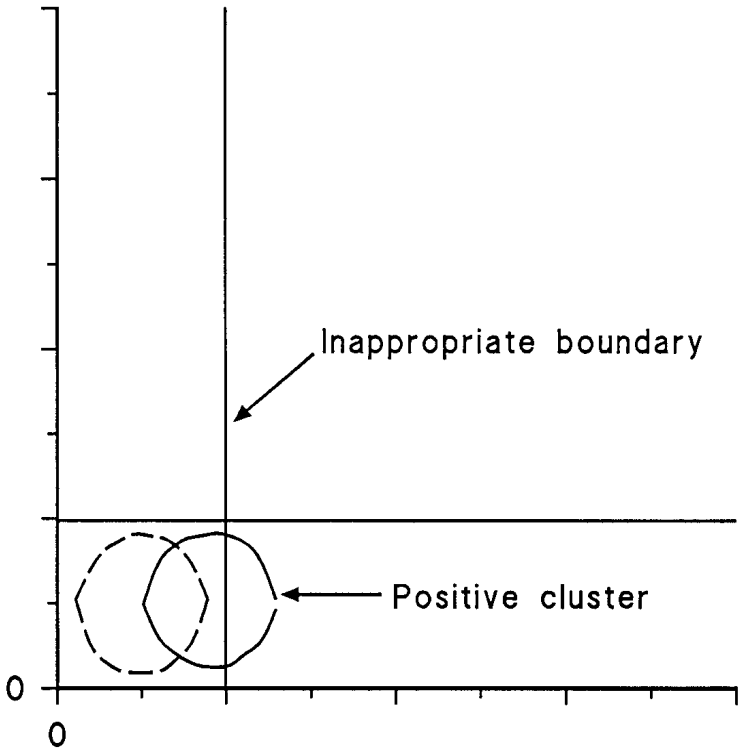

Figure 12. The shape of the cluster is identical to that of the negative control cluster (this is best appreciated on contour displays) and overlaps the control cluster, but the cluster from the test sample is shifted several channels parallel to either the *x*- or *y*- axes.

This situation should be analyzed along the same principles as were used previously under single-color analysis (Figure 7). Recording of percent-positive cells is inappropriate for these distributions, and results should be reported as "dim" or the mean channel should be recorded if quantitative methods are used. In addition to the cautions noted above, note that several commercial phycoerythrin conjugates, even more so than fluorescein conjugates, are prone to show dim, nonspecific staining of this form. For this reason, special care should be taken before interpreting dim phycoerythrin staining as positive.

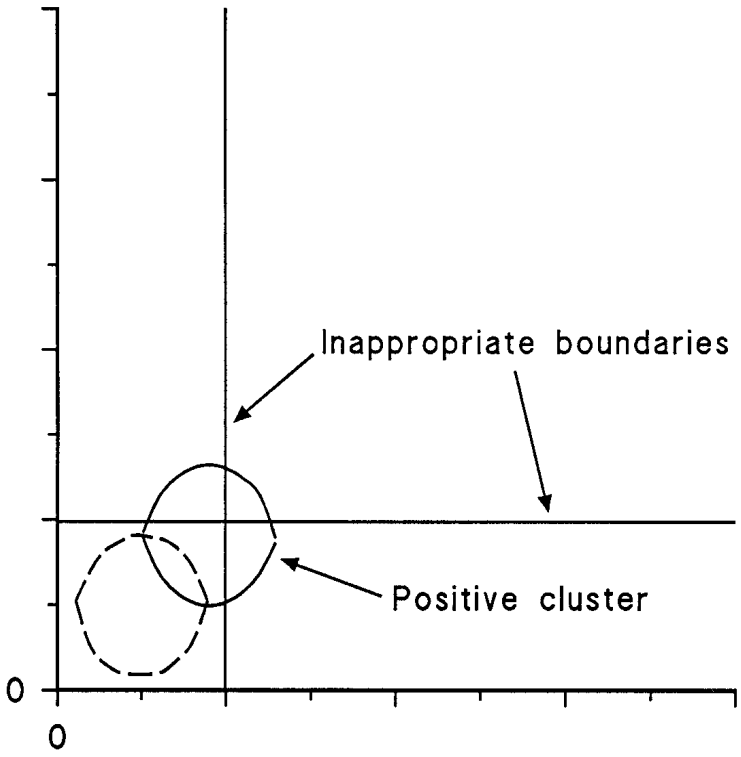

Figure 13. The cluster overlaps the negative control cluster, and there is either a shift in mean channel or a tail of events at a more or less 45° angle to the axes.

Analysis of this situation in principle is no different from Figure 11 and Figure 12. One could describe the reactivity in terms of "dim," doubly positive events and use the *x*- and *y*- projections of the clusters to determine if setting a marker is appropriate. However, interpretation of data with this distribution is hazardous because it is frequently seen with nonspecific staining, while simultaneous, dimly expressed markers in leukemia are a rather infrequent occurrence. The reagent panel should be chosen to minimize the likelihood of achieving this result.

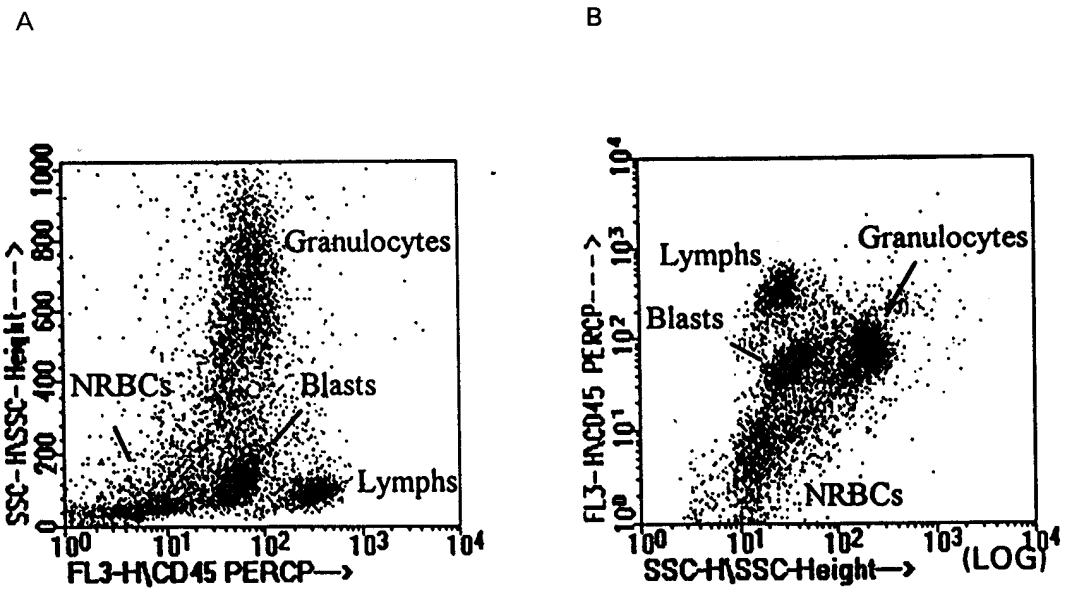

Figure 14. Dual parameter display of CD45 and RALS from a leukemic bone marrow using a linear scale for RALS with CD45 displayed on the abscissa (A) or a logarithmic scale for RALS with CD45 displayed on the ordinate (B). In both displays, major normal populations of bone marrow are resolved, and blasts occupy a region of low CD45 and low RALS relatively free of other normal elements. A gate set on this region for analysis of the leukemic phenotype would be relatively more pure than one set using conventional light scatter gating in which blasts, lymphocytes and nucleated red cells would overlap.

13.5 Other Analytic Methods

Many current commercial software products permit multiparameter data of mixed populations to be analyzed using color to identify particular subpopulations. In these applications, one can identify a discrete cluster of events on the one dual parameter display in which they are most clearly separated from others, and then "see" where these events appear on other dual-parameter displays. The principal advantage of such methods is that it allows analysis of complex data without having to commit oneself to a specific analysis gate. It also provides a powerful visual picture of the expression of antigens in combination. However, it can sometimes be cumbersome to analyze large panels of antibodies this way, and comparing results with a particular antibody to a control may not be straightforward.

After all antibodies in a panel have been analyzed using one of the methods outlined above, it should be possible to give a qualitative description of the antigens expressed in a particular case of leukemia. Note that using arbitrary criteria such as "20% positive" for determining if an antigen is or is not present on a leukemic cell was avoided, because such a term rarely has any meaning for addressing this question. The next step is to incorporate this information into a report and to synthesize and interpret the information to provide the maximum information possible as to the nature of the leukemia.

14 Data Reporting and Interpretation

14.1 Introduction

In the analysis of leukemias there are two initial and fundamental considerations:

(a) When presented with an appropriate clinical picture and a proliferation of blasts in bone marrow or blood that exceeds the limits established by consensus classifications, such as the French-American-British (FAB) classification, the diagnosis of acute leukemia is established. Consequently, immuno-phenotypic analysis contributes to assigning lineage (and therefore treatment regimen) and not to establishing the diagnosis.

(b) In contrast, when presented with a proliferation of mature (nonblastic) and variably atypical-appearing lymphoid elements that are increased in number (absolute lymphocytosis), immunopheno-typic analysis contributes to establishing a diagnosis by defining characteristic phenotypic abnormalities.

It is advisable to determine whether one is dealing with situation (a) or (b) before attempting to interpret or report any data. It should be recognized, however, that some cases may be clinically and morphologically ambiguous and, in some of these cases, immunophenotyping may clarify the diagnosis.

14.2 Acute Leukemia

14.2.1 Types of Samples to be Analyzed

The diagnosis of acute leukemia must be established before sending the specimen for immunophenotypic analysis. If not, all data need to be interpreted with caution and in the light of relevant clinical and laboratory findings. A diagnosis of "acute leukemia" should not be rendered in a report that otherwise lacks appropriate fundamental information. It is neither useful nor relevant to perform immuno-phenotypic analysis on samples from patients with chronic myelogenous leukemia or myelodys-plastic syndrome in the chronic phase. These diagnoses are complex and depend on clinical, morphologic, and cytogenetic criteria. They cannot be made from immunophenotypic composites. Having established that the patient whose sample is being examined has acute leukemia (or blastic transformation of either chronic myelogenous leukemia or a myelodys-plastic syndrome), morphologic verification of the presence of blasts should be done by an experienced person. Established morphologic and cytochemical classifications, such as the French-American-British (FAB) classifications, are useful guidelines. The approximate percentage of blasts should be determined and correlated with LS and immunophenotype.

If a sample does not contain blasts, there is no justification for proceeding with monoclonal antibody staining. This would result in an unnecessary expense and may

produce confusing results.

14.2.2 Reporting of Immunophenotypic Data

The composite immunophenotype should be described. For example, "Blasts coexpressing HLA-DR and CD19 present in this sample. These cells constitute approximately 56% of all mononuclear cells examined."

The alternate method of reporting positivity using percentage may be confusing, for example:

HLA-DR 62%
CD19 .....75%

It is not clear whether the HLA-DR value of 62% means that 62% of the blasts are HLA-DR positive or whether the sample contains only 62% blasts, all of which are HLA-DR positive.

If percentages are used, they should meet the following criteria:

- They should only apply to distributions in which the population on which an antigen is expressed is clearly separated from the negative population (see Section 13.3), and

- They should only be used to imply that a subset of the gated (i.e. the leukemic) population expresses a particular antigen.

Thus, for example, it may be appropriate to say in a case of chronic granulocytic leukemia in mixed lymphoid and myeloid blast crisis that 50% of the blasts in the sample express CD19 and CD10 antigens, and a different 50% express CD33. Although many leukemias can demonstrate heterogeneous expression of particular antigens on leukemic cells, the significance of this is not yet known.

14.2.3 Guidelines for Interpretation of Immunophenotypic Data

(1) **Acute Myeloid Leukemia**

Virtually all acute myeloid leukemias (with the exception of acute progranulocytic leukemia [FAB M3]) are HLA-DR positive. Therefore, the coexpression of HLA-DR and one more myeloid-associated antigen (i.e., CD13 and/or CD33) is consistent with acute myeloid leukemia (AML), although such a diagnosis requires more information. Further confirmation may be obtained by expression of other antigens, LS characteristics, and ultimately by review of Wright-Giemsa and cytochemical stains. In the absence of morphologic or cytochemical evidence of myeloid differentiation, myeloid leukemia can still be diagnosed on the basis of myeloid antigen expression (so-called "FAB M0" leukemia) provided lymphoid antigens are not expressed. Acute myeloid leukemias that have monocytic features will also often coexpress CD14 or CD4 and they may have cytochemical evidence (such as nonspecific esterase) or ultrastructural evidence of monocytic differentiation. Coexpression of lymphoid antigens, such as CD19, TdT, or CD2, may be interpreted by some persons as indicative of a mixed-lineage acute leukemia or an unclassifiable leukemia. This is a controversial issue. There does not appear to be sufficient consensus at this time for the subcommittee to adopt a position on how such cases should be interpreted and reported. It may be prudent to make statements such as "acute myeloid leukemia with coexpression of CD2." This informs the treating physician of the predominant lineage of the leukemic process as assessed by traditional and immuno-phenotypic criteria and it also informs the physician of the coexpression of unanticipated antigens. The subcommittee recognizes that there is not even consensus as to what constitutes an "unanticipated" or "aberrant" antigen. For example, while many would consider CD19 or CD2 expression in myeloid leukemia to be "aberrant," most would not consider expression of CD7 to be so.

Acute megakaryocytic leukemias (FAB M7) may be recognized by their expression of platelet antigens such as CD61 and CD41. They may be HLA-DR negative or positive, and variably coexpress myeloid-associated antigens.

Acute erythroleukemia (FAB M6) may be difficult to distinguish from acute myeloid leukemias with significant dyserythropoiesis. In fact, the terminal phase of an otherwise classical erythroleukemia is usually an M1 or M2 variant of acute myeloid leukemia.

(2) **B-cell Precursor Acute Leukemia**

The coexpression of HLA-DR- and B cell-associated antigens, such as CD19, CD22, or CD20, with or without CD10, is consistent with B-cell precursor acute leukemia. Again, other information is required for an absolute diagnosis, although bright coexpression of CD19 and CD10 is essentially only seen in B-lineage leukemias. This group of leukemias is almost always TdT positive. The coexpression of the above-mentioned antigens with myeloid antigens CD13 and/or CD33 is a subject of controversy. The subcommittee again recommends a conservative approach to this issue when reporting data. Therefore, reports might contain a statement such as "acute leukemia, precursor B-cell type with coexpression of myeloid antigen(s)." As in the previous example (13.2.3.1), a statement is made with regard to the predominant lineage by traditional and immunophenotypic criteria and it also informs of the presence of unanticipated antigens.

Acute B-cell leukemia of the L3 variant, the leukemic phase of Burkitt's lymphoma, is a well-defined, biologic and clinical entity. It is a highly aggressive lymphoproliferative process that expresses B-cell associated antigens and monotypic surface membrane immunoglobulin (SmIg). L3 leukemia has a characteristic morphology. Rare, non-L3 ALL cases express SmIg, but the clinical significance of this is uncertain. Distinguishing B-cell acute leukemia from the leukemic phase of certain malignant lymphomas may be difficult.

(3) **T-cell Acute Leukemia**

The usual T-cell acute leukemia is HLA-DR negative and T-cell antigen-positive, most commonly expressing CD7 but also often CD2 and CD5. Most are TdT-positive. Further immunophenotypic characterization may be accomplished with additional T-cell-associated antigens such as CD1, CD4 and CD8. Like B-precursor ALL, T-ALL can also express the myeloid-associated antigens CD13 and CD33, and even HLA-DR. Because of the lack of absolute specificity of these reagents, interpretation of results—even to determine major lineage—is often impossible. Cytoplasmic CD3 expression is reported to be the most specific marker of T cell differentiation.

14.3 Leukemic Phase of Chronic Lympho-proliferative Disorders

14.3.1 B-Cell Lymphoproliferative Disorders

Having established that there is an abnormal proliferation of variably atypical lymphoid elements in blood, bone marrow, or body fluids, it is necessary to identify immunophenotypic abnormalities that are characteristic of neoplastic as opposed to reactive states. The most frequent occurrence is the isotopic restriction of cell surface immunoglobulin light chain expression (kappa or lambda), which generally serves to demonstrate monoclonality. If a single light chain is expressed and the morphologic and clinical picture is consistent with a nonreactive lymphoproliferative process, this should be reported as such. Although immunophenotypic information can sometimes be useful in helping to subclassify chronic B-cell lymphoproliferative disorders (e.g., CD5 expression in chronic lymphocytic leukemia), caution should be exercised in making a diagnosis such as "chronic lymphocytic leukemia" or another clinical diagnosis in a report without coordinating the phenotypic information with other clinical and laboratory measurements. Unambiguous terms such as "monoclonal, kappa-positive, $CD5^+$ B-cell lymphoproliferative process" may be more appropriate.

14.3.2 T-Cell Lymphoproliferative Disorders

T-cell lymphoproliferative disorders that have a circulating (leukemia) component are relatively rare. Although clonal T-cell lymphoproliferative disorders can in theory be detected by their restricted use of T-cell receptor variable region genes, in practice few T-cell leukemias can be detected in this way. The diagnosis of a T-cell lymphoproliferative disorder is best established by demonstrating overwhelming T-cell predominance with T-cell antigen restriction in *morphologically neoplastic* cells. Failing this, T-cell neoplasia can often be suggested based on either of the following findings:

- "Anomalous" expression of T-cell-associated antigens, i.e., coexpression of CD4 and CD8, or

- Deletion of one or more pan-T-cell antigens, i.e., $CD3^+$, $CD7^-$.

Care must be exercised in interpreting minor populations of cells expressing these aberrant phenotypes because small numbers of aberrant cells can be seen in nonneoplastic conditions.

Cases that demonstrate an anomalous immunophenotype need to be confirmed as clonal by other techniques. Southern blots, or other DNA-based technology such as PCR, are most useful to establish the presence of a clonal process.

When reporting T-cell lymphoproliferative disorders, a conservative use of words is recommended. For example, a statement such as "atypical T-cell immunophenotype suggestive of Sezary cell leukemia" may be preferable to an outright diagnosis of "Sezary cell leukemia."

Because any lymphohematopoietic malignancy may coexist with normal lymphohematopoietic elements that have overlapping LS and immunophenotypic characteristics, discretion should be used in their identification. To do so requires sophisticated analysis using multiparameter analysis combining LS and, often, multiple fluorochromes. Complex cases may have to be referred to specialized laboratories with experienced personnel.

15 Data Storage

The possibility of patients or their legal representatives contesting diagnostic implications derived in part from flow cytometric phenotypic analysis makes it incumbent on the laboratory to be able to demonstrate and verify the process used in arriving at the reported test results. Both the data stored and the methods used to obtain the data must be thoroughly documented.

15.1 Information to be Stored

Patient data files should include all parameters used to obtain test results. These data include:

- Low angle light scatter
- 90° light scatter
- One, two, three or more fluorescence parameters (for single or multicolor analysis)

The variability observed in LS patterns in patient samples requires that data be stored in list mode (cell-by-cell) form because gating may not be assignable *a priori*. List mode data storage will not offset the effect of improper instrument settings or improper sample preparation. Instrument setup and sample preparation are information data sets that must be stored with the list mode data collected by the instrument.

Quality assurance/quality control data records (files) should include all parameters, analysis regions, and analytical results used to verify system (instrument and method) performance.

15.2 Types of Data Storage

15.2.1 Paper Hard Copy

Because data storage is recommended as list mode, paper hard copy is reserved for data presentation. All histograms and analysis regions used in arriving at the reported test results (e.g., gating regions and analysis boundaries), whether obtained from the list mode or from real-time data, must be recorded on the hard copy of the relevant histogram. A unique specimen identifier should also be recorded with each histogram and a cross reference to the originating list mode data file.

15.2.2 Archival Files

A variety of data storage media may be used, depending on the particular systems available in the laboratory, including floppy disks, removable hard disks, optical disks, and magnetic tape.

If data storage software does not provide for storage of relevant gate information and analysis regions, these data must be recorded as paper hard copy (e.g., amorphous gating regions) or with the test record (e.g., orthogonal gates and analysis boundaries). If the data presentation software does not permit the display of all cell regions in the parameters on which gates are used, separate, ungated data must be made available to permit the clinician

to ascertain the validity of the gated portion with respect to the entire data set.

15.2.3 Duration of Storage

Patient data should be kept for a minimum of two years, or as required by state and federal law, whichever is longer. After that, it is the responsibility of the laboratory director to determine whether to save or discard the data. Quality assurance/quality control data should be kept for a minimum of 2 years.

Data may be stored for as long thereafter as the laboratory director considers necessary, provided storage space is available.

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Appendix A. Fixation and Permeabilization for Detection of Intracellular Antigens

Several differentiation antigens are expressed in the cytoplasm of cells before being incorporated into the surface membrane. Because of this, it is sometimes important to be able to detect antigens in cytoplasm. In addition, it is sometimes important to determine expression of the nuclear antigen TdT in cases of acute leukemia. To detect intracellular antigens, the cell membrane must be rendered permeable. However, special procedures are required to ensure not only that permeabilization occurs but also that the epitope of interest is preserved. The methods outlined below allow not only detection of intracellular epitopes but also simultaneous detection of surface and cytoplasmic constituents. Similar procedures can be used for the simultaneous analysis of a membrane epitope and DNA.

A.1 Fixation and Permeabilization

When cells are fixed, changes occur in the tertiary structure of proteins that can destroy the epitope sought. Because different fixatives affect different proteins in different ways, there is no single fixation method for all proteins. Accordingly, each antibody–antigen system may require a unique fixation system and this should be tested before a final protocol is established. In addition, the best reagents for preserving epitopes are generally not the best for permeabilizing cells.

In some circumstances, one is interested only in cytoplasmic epitopes, while in others it might be more appropriate to perform dual-color analysis, staining surface epitopes with one fluorochrome and cytoplasmic epitopes with another. In the former case, if surface membrane staining is not required, cells may be directly fixed with a noncrosslinking fixative. Alcohols are most commonly used for this purpose. They fix cells by denaturing protein and extracting lipid. While denaturing may destroy the epitope sought, permeabilization readily occurs. Thus, alcohols are most often used for staining intracellular epitopes. If epitope degradation occurs, other fixatives should be tested. PE- and PE-tandem fluorochromes are particularly susceptible to degradation by alcohol fixation. Acetone may preserve some epitopes better than alcohols do. It should be noted that alcohol or acetone-fixed cells generally give scatter signals that are substantially different from those of aldehyde-fixed cells.

Paraformaldehyde and ultrapure formaldehyde are excellent fixatives for postantibody-stained surface membranes on viable cells. Aldehydes fix cells by crosslinking proteins and crosslinking continues to take place as long as cells are in the aldehyde solution, albeit at a continuously decreasing rate. Aldehyde-fixed cells are not very permeable, which causes poor intracellular antigen detection. Aldehydes may also be used for prefixation of cells before surface membrane staining, though some membrane epitopes may be destroyed by prestaining fixation. If postfixation staining is to be used, comparison between fixed and viable cells should be performed.

To stain both a surface and an internal epitope, a combination of fixation procedures is recommended. Whenever possible, surface membrane staining should be performed using viable cells followed by 1% (para) formaldehyde fixation. Cells treated in this way must be permeabilized. A 30-to-60 min treatment with a suitable, noncrosslinking fixative, such as one of the alcohols, can be used to permeabilize the cells without destroying the surface marker fluorochrome. In some circumstances, and with some fixatives such as formalin-acetone, the fixation and permeabilization steps can be combined.

A.2 Verification of Specific Antibody Binding

Immunoglobulins bind with low affinity nonspecifically to dead cells in a nonsaturable way. In contrast, antibodies bind to their epitopes in a saturable way with high affinity. This provides the basis for detection of specific antibody binding to desired epitopes in fixed (dead) cells. It also requires that the antibody to the desired epitope have a high specific activity. Antibodies obtained from commercial suppliers may not always meet this criterion.

To determine the efficacy of any given antibody, both the specific antibody and an isotype control (IC) immunoglobulin are adjusted to the same IgG concentration. Serial dilutions of each are made and used to stain the cells in suspension. The mean channel fluorescence (MCF) is then determined for each sample and the antibody (signal) MCF is divided by the IC (noise) MCF. The concentration of antibody that provides the highest signal to noise ratio should be selected. Acceptable antibodies always have ratios greater than 3.0. If the antibody fails this test, it is not a good antibody for use in flow cytometry.

A.3 Controls

Suitable controls should always be analyzed along with the test samples. These controls should include an instrument verification control, as well as staining verification controls — both positive and negative. Cell lines that express the epitopes for the specific antibodies are most convenient for the former purpose. Isotype controls should be run on cells subjected to the same fixation and permeabilization procedures as the test antibodies. In addition, for antigens that can be expressed on either the surface or within cells, nonpermeabilized cells should be reacted with the test antibodies to ensure that any positive reactivity noted with permeabilized cells in fact represents intracellular and not surface antigen expression.

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Appendix B. Alternative Methods for Cell Preparation

B.1 Buffy Coat And Lysis

In cases where the lymphocyte count is very low, the sample is centrifuged and the buffy coat removed for staining with the Whole Blood Procedure. This step serves to concentrate the leukocytes for subsequent analysis.

Erythrocyte concentration is a factor that is associated with adverse effects. If an excessive number of red cells are collected with the buffy coat, the stained aliquot will contain an unusually large number of red cells. The excessive erythrocytes may overload the lysing reagent and result in incomplete lysis and a poorly delineated population for electronic gating.

B.2 Discontinuous Density Gradient Techniques

The general concept involves creating a discontinuous gradient by layering blood over a separation medium of high density (~ 1.077 g/mL) followed by centrifugation. Erythrocytes and granulocytes, of higher density, sediment at the bottom of the tube, while lymphocytes and monocytes, of lower density, are retained at the plasma-separation medium interface. The most common preparations consist of the synthetic polymer ficoll, which also causes erythrocyte agglutination, mixed with an iodinated x-ray contrast medium. The ultimate quality of such a preparation is a function of cell size and, to a greater extent, cell density. When performed under optimal conditions, approximately 70% of the mononuclear cells can be recovered with less than 5% granulocyte contamination. However, monocytes may constitute up to 20% of the cell suspension. Because all of these cell populations are defined by a limited range of sizes and/or densities, there is some overlap of adjacent populations. It is this overlap that creates a potential for inadequate separation, reduced yield, or biased cell loss if a sample is suboptimal due to either extrinsic or intrinsic factors.

B.3 Factors With Adverse Effects

B.3.1 Age of Specimen

Storage of a blood specimen results in a progressive increase in the proportion and type of cells contaminating the mononuclear isolate. Within a few hours, the percentage of monocyte contamination begins to increase and at about 24 hours granulocyte contamination becomes apparent. For these reasons, density gradient separations should generally not be used on specimens over 24 hours old.

B.3.2 Storage Conditions

Storage of anticoagulated blood under some conditions, but not others, results in significant loss of certain lymphocyte subpopulations during the subsequent separation and up to a 70% decrease in the proportion of T cells. Therefore, specimen storage at room temperature is preferred.

B.3.3 Storage of Separated Cells

Once isolated, the mononuclear cell suspension may be stored in an enriched (tissue culture grade) medium at 4 °C for staining the next day.

B.3.4 Examination of Stained Smear

If the quality of a specimen is in doubt, and a density gradient separation is done, the final preparation should be examined in a stained smear to assess contamination by nonlymphoid cells and a decision made concerning the relative suitability for analysis and the gating appropriate for that specimen.

B.4 Erythrocyte Agglutination Techniques

Dextran, Plasmagel (gelatin), methyl cellulose, and ficoll are high-molecular weight polymers that cause the sedimentation of erythrocytes as a result of rouleaux formation when mixed with whole blood. The technique is simple but it leaves a leukocyte suspension consisting predominantly of granulocytes and it is not generally recommended as a method for lymphocyte preparation. Overall leukocyte recovery is in the range of 50 to 70%.

B.5 Monocyte Depletion

These procedures physically remove monocytes from either whole blood or enriched lymphocyte suspension based on functional properties of the monocytes. Incubation with iron particles permits the phagocytosis of the particles by the monocytes and subsequent removal with a magnet. Similarly, incubation of the cell suspension in a glass vessel at 37 °C allows the monocytes to adhere to glass. The nonadherent cells (lymphocytes) can then be washed off.

These procedures have variable effects on the lymphocytes, commonly resulting in the loss of up to 45% of the cells. Because of this loss, and the fact that some lymphocytes have limited ability for adherence, these techniques are generally not recommended for lymphocyte preparation.

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Appendix C. Examples of Phenotyping Cases of Acute Leukemia

Two cases of acute leukemia stained with the same two-color panel of antibodies are illustrated. Data are presented in two different ways, but they lead to the same interpretation.

C1 Case I: B-Precursor Acute Lymphocytic Leukemia

In method 1, a gate or bitmap is set on a FALS versus 90° LS display (A) to include all mononuclear cell events. Dual-color data are then displayed based on this gate. Quadrant markers are set based on the position of the cluster of events from the isotype control tube (B), but these are used throughout as estimates of the boundary between positive and negative regions, rather than as a means of calculating events in each of the four quadrants. From © it is clear that the main cluster of gated events is doubly positive for CD19 and CD10. There are a small number of $CD19^{+}CD10^{-}$ events, which likely represent residual normal B cells. The double negative events can be inferred to consist mainly of T cells based on the minor cluster of events in (D), (E), and (F) staining with CD5, CD3, and CD7, respectively. (Calculation of quadrant percentages may be useful here to demonstrate that each of these markers is expressed on a similar percentage of cells, in this case 10%.) Consideration of the position of the major cluster of events further demonstrates that the leukemic cells are positive for CD22, HLA-DR, CD13, CD38, and CD34, but lack CD20, CD33, and the T antigens mentioned above.

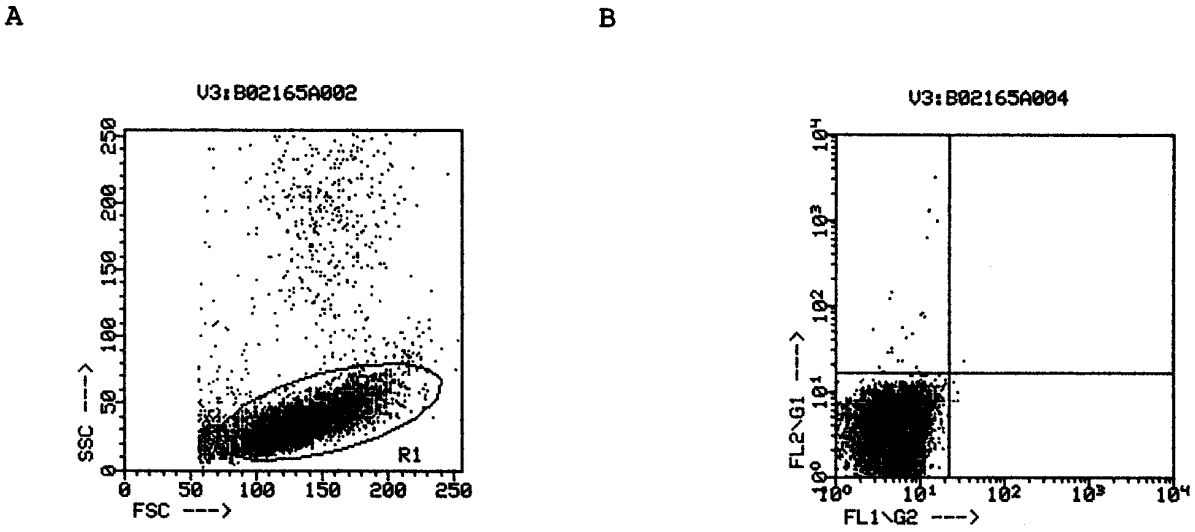

Figure C1. CASE I, Method 1

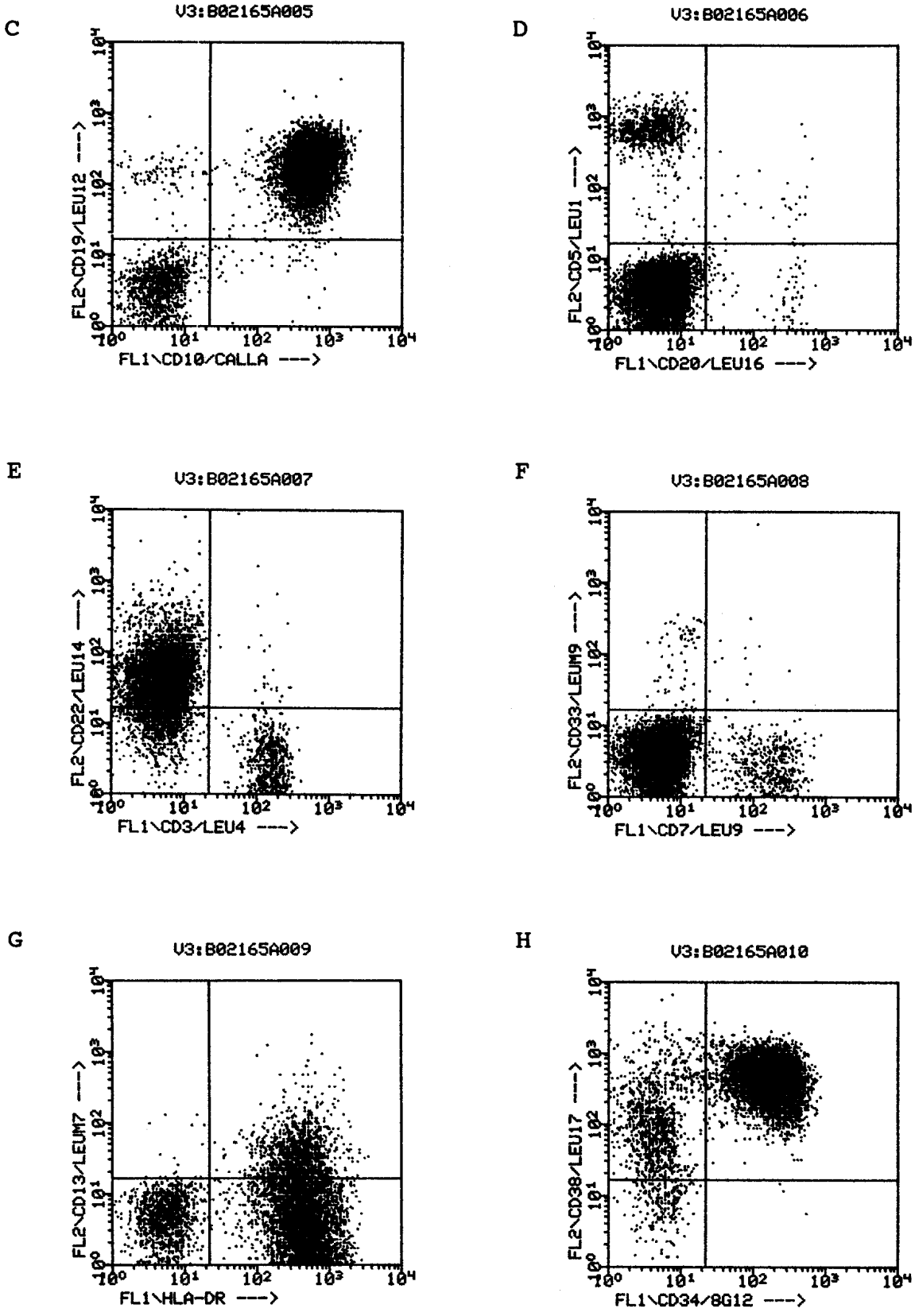

Figure C1. CASE I, Method 1 (Continued)

In method 2, a gate is set again to exclude high 90° LS events, but this time dual-parameter displays of FALS and fluorescence are displayed. From these displays it can be seen that there is considerable size heterogeneity among the leukemic cells, but the T markers only react with a subpopulation of the smaller cells. Again, the leukemic population can be inferred to have the same phenotype as in the previous case. Note that the contour map display gives a better understanding of the shape of the fluorescence distributions than does the dot plot. Thus, for example, CD22 can be seen to be homogeneously expressed on the leukemic cells at a relatively low antigen density, while the distinct minor subpopulation of $CD22^{bright+}$ small lymphocytes represents normal B cells.

From either display, interpretation of this case is straightforward. Coexpression of CD19 and CD10 is characteristic of B-precursor ALL, and B lineage is further confirmed by expression of CD22. CD13 should be noted to be aberrantly expressed, but in the presence of an otherwise clear B-precursor phenotype, it should not be interpreted to imply that this represents myeloid leukemia.

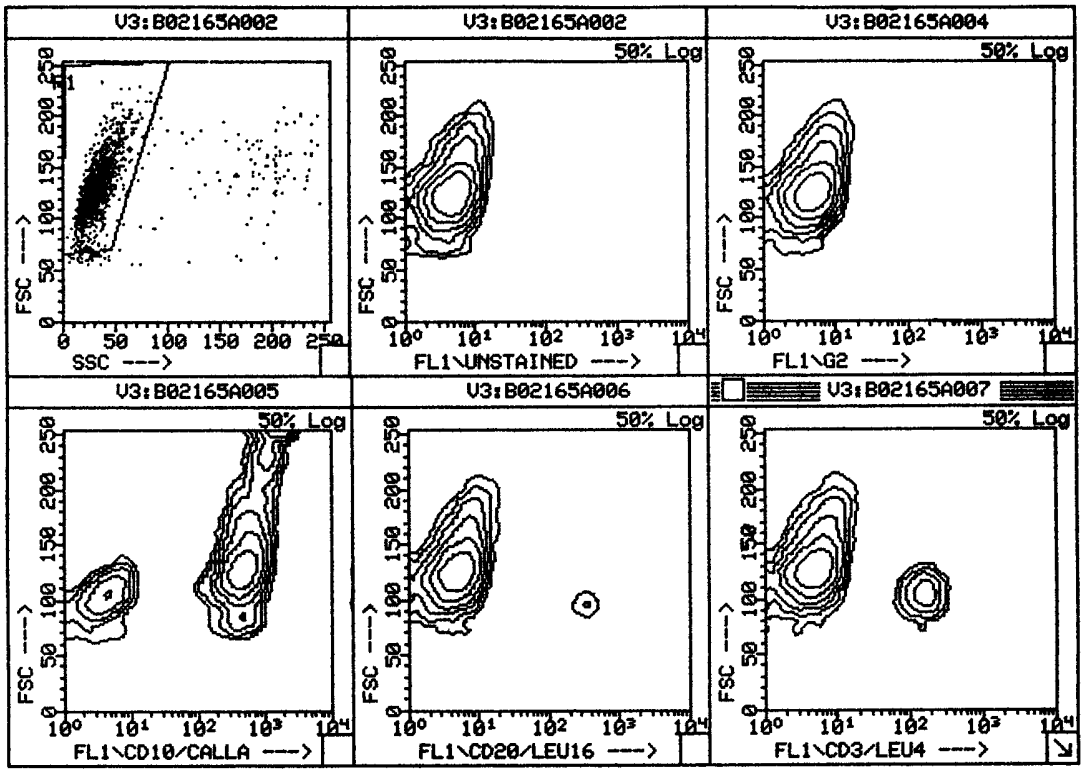

Figure C2. CASE I, Method 2

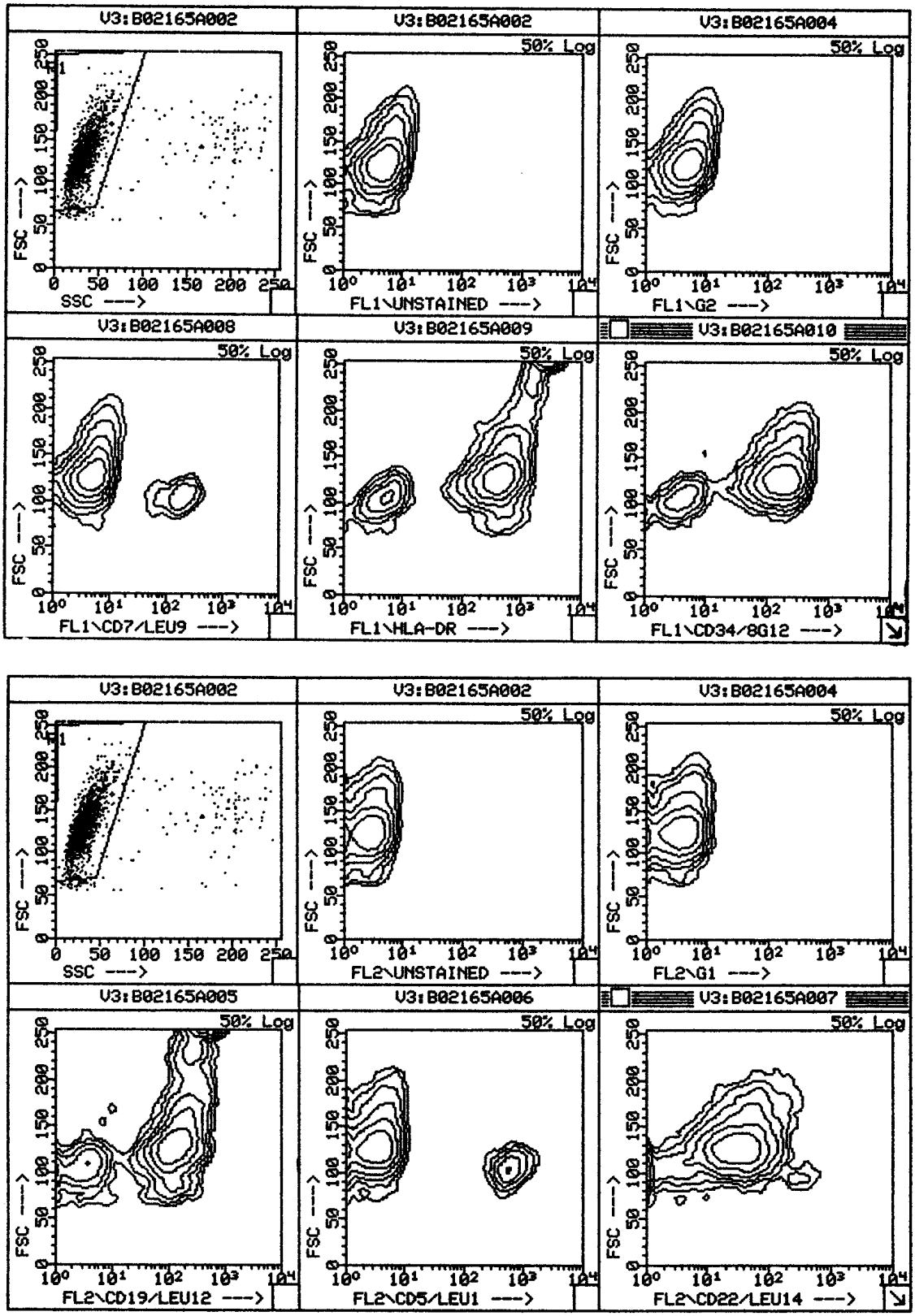

Figure C2. CASE I, Method 2 (Continued)

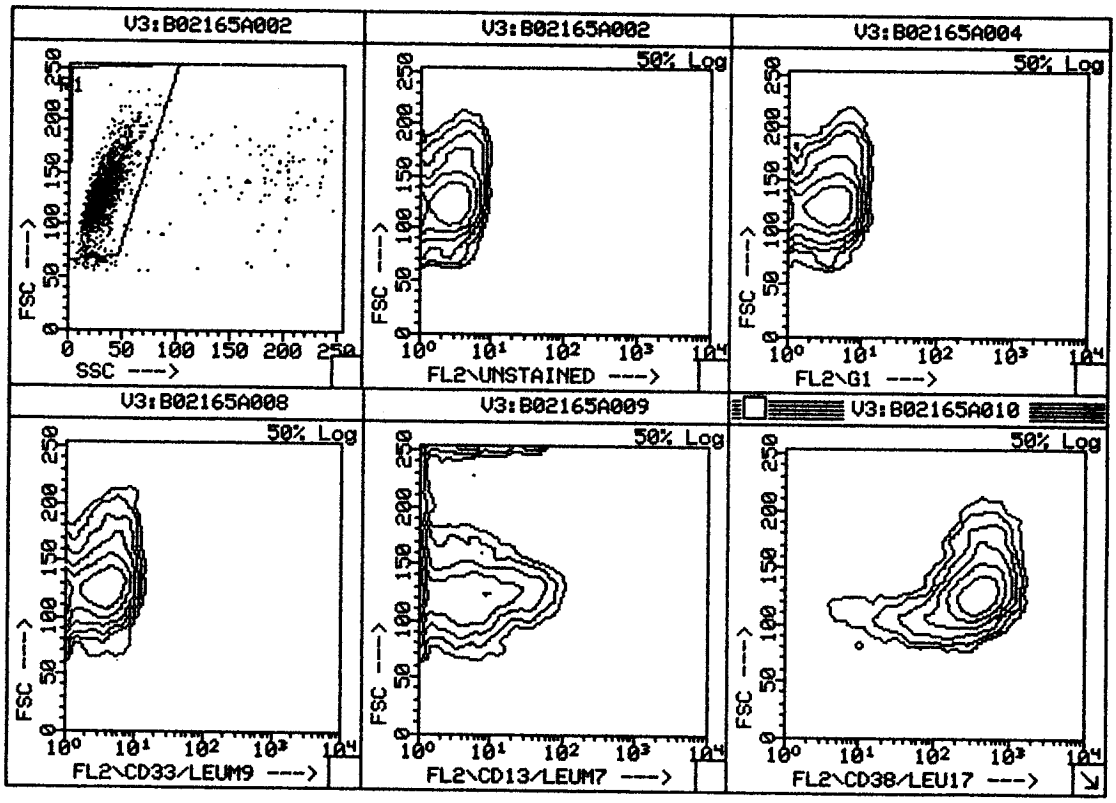

Figure C2. CASE I, Method 2 (Continued)

CASE II: Acute myeloid leukemia

In method 1 a gate is set as before on a FALS versus 90° LS display, but this time a population of high FALS and moderate 90° LS cells are identified and are distinct from smaller cells. A separate gate could be set on the latter population; these cells have the phenotype of normal lymphocytes (not shown). Dual fluorescence display shows that the high FALS-moderate, 90° LS population has higher autofluorescence based on an unstained control (B), and positioning quadrant markers on the isotype control © makes it clear that there is no staining for CD19, CD10, CD20, CD5, CD3, or CD22 (D through F). There is, however, homogeneous expression of CD33, as well as expression of CD13, HLA-DR, and CD38, although CD34 is negative.

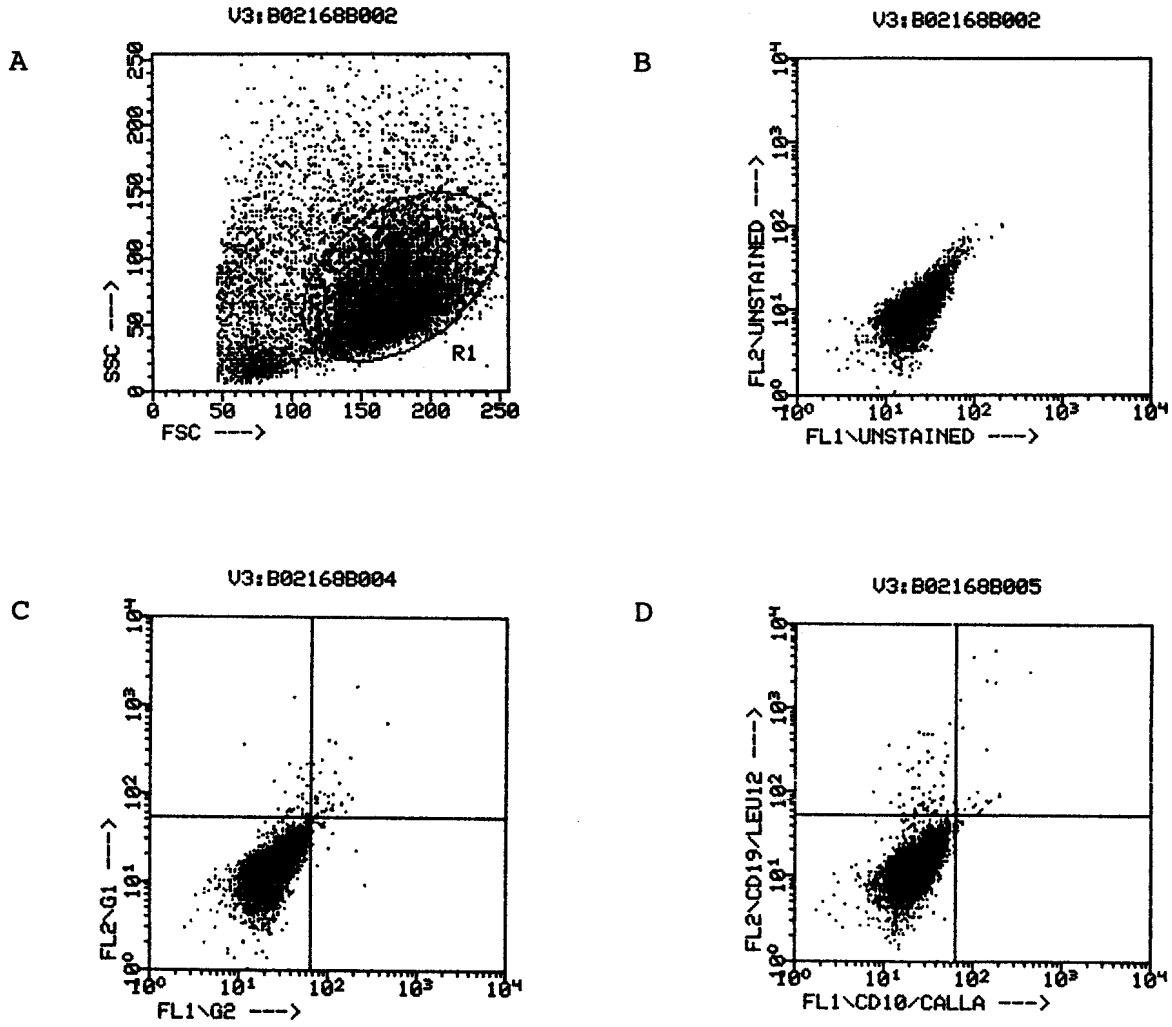

Figure C3. CASE II, Method 1

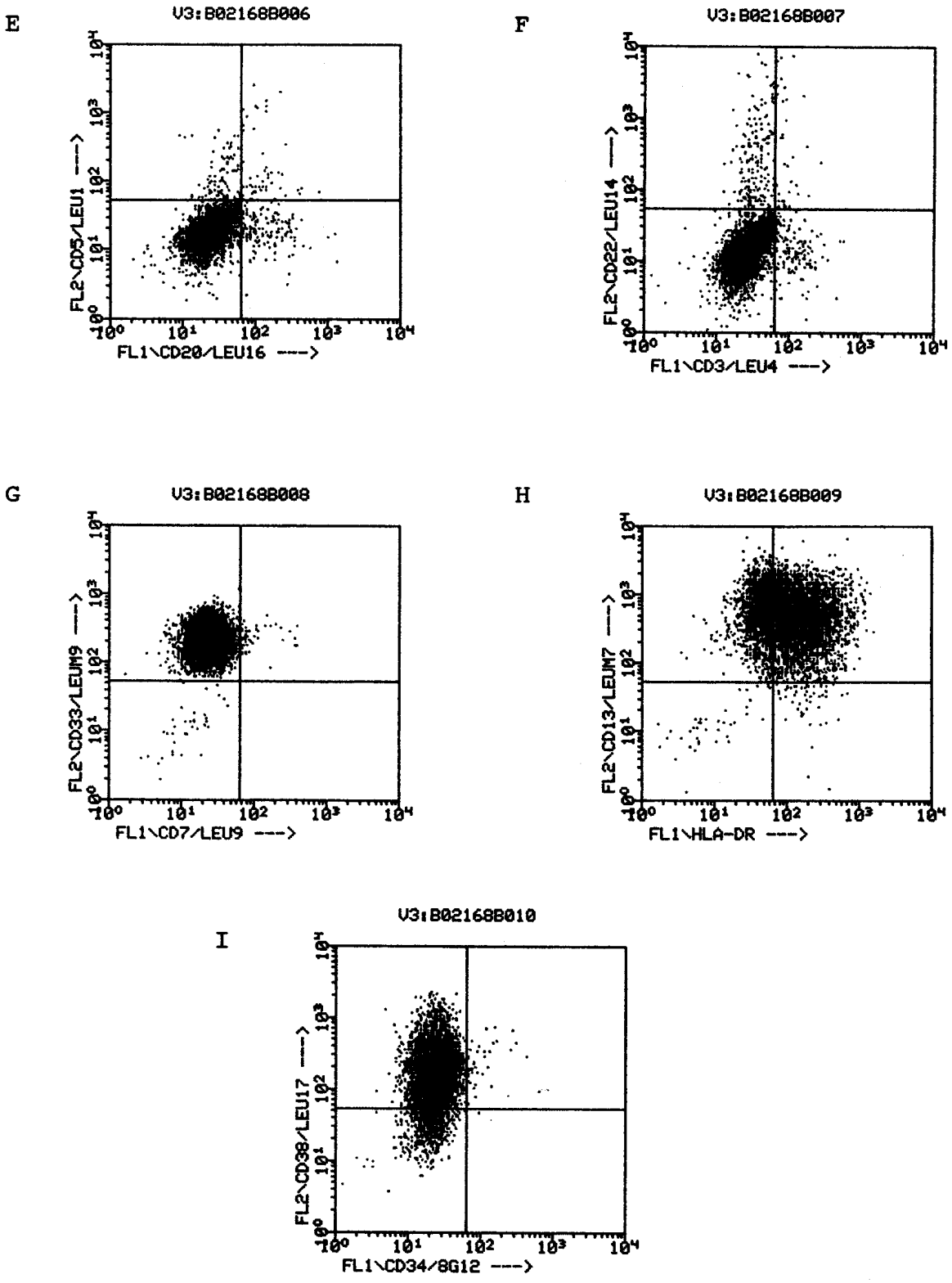

Figure C3. CASE II, Method 1 (Continued)

In method 2, a gate is set to exclude only high 90° LS events consistent with mature granulocytes. From the dual-parameter display, it can be seen that the larger cells display the same phenotype noted in method 1 above, while the smaller cells are heterogeneous, with partial expression of both B and T markers and HLA-DR. By inference, these are normal lymphocytes.

In this case, expression of both CD13 and CD33 in the absence of any evidence of B or T antigens, coupled with the unusual LS pattern with relatively high 90° LS is a clear indication of myeloid leukemia.

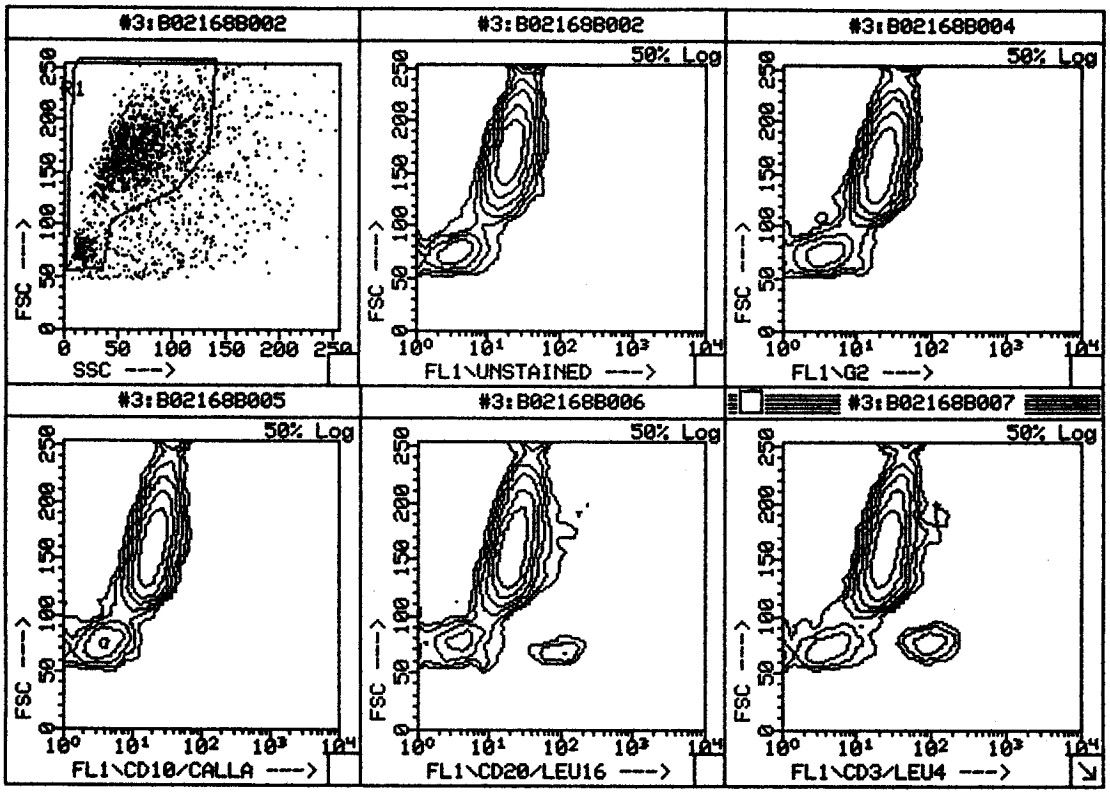

Figure C4. CASE II, Method 2

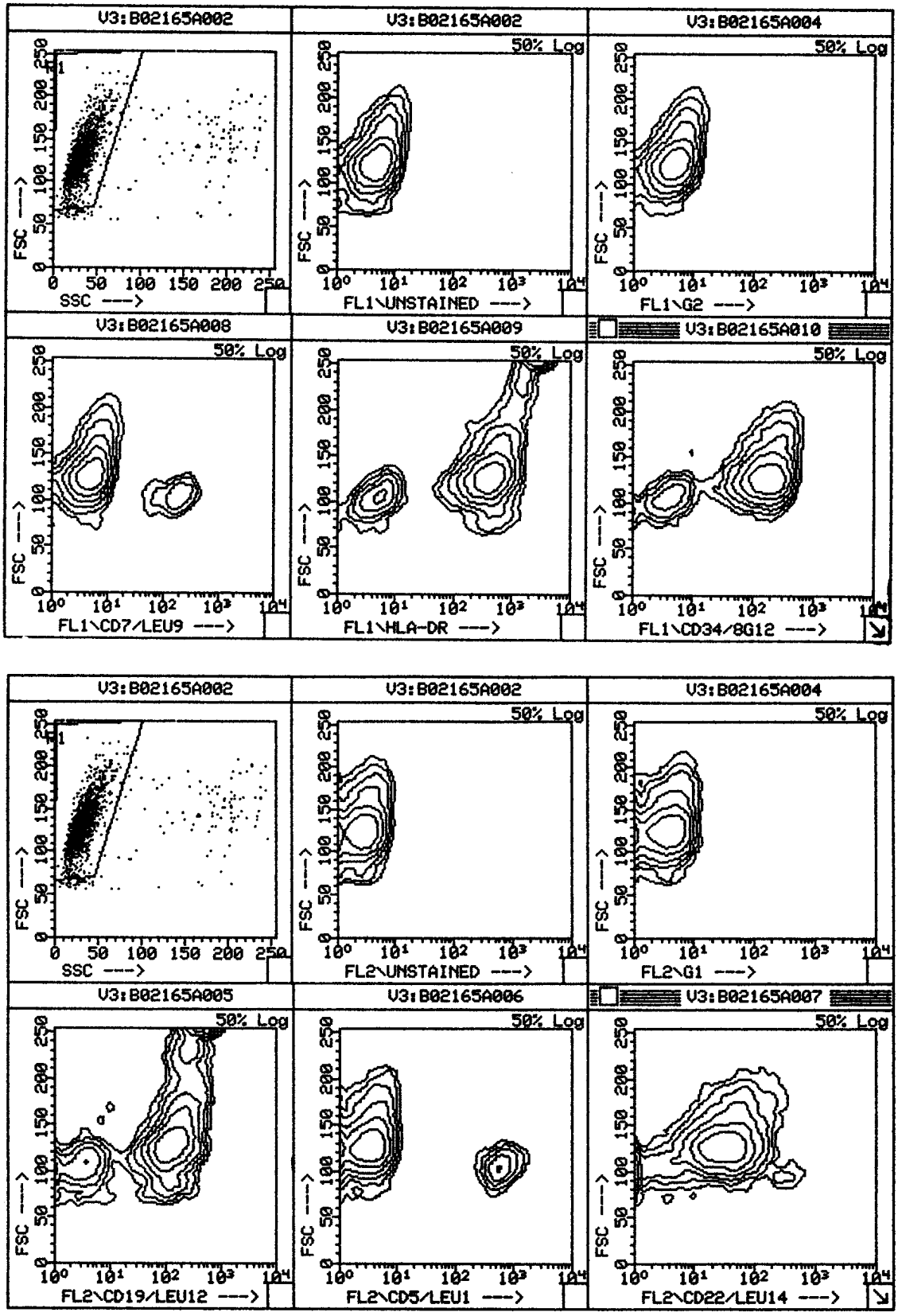

Figure C4. CASE II, Method 2

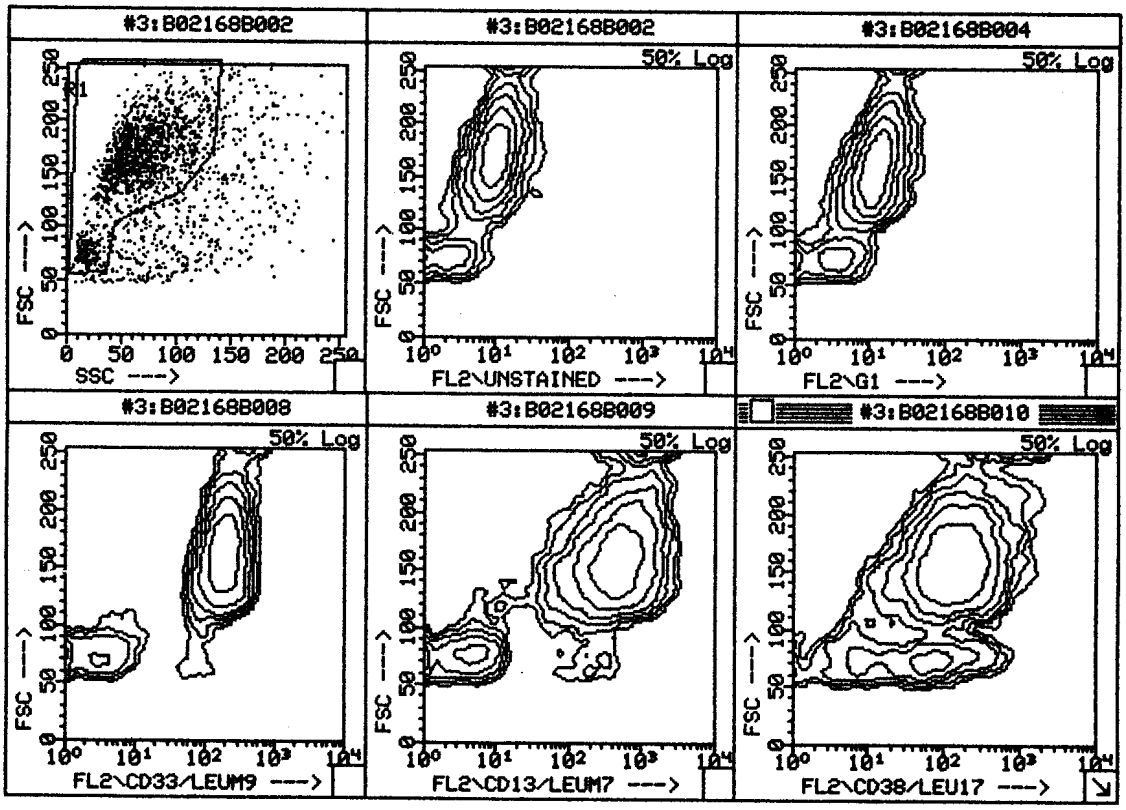

Figure C4. CASE II, Method 2 (Continued)

Summary of Comments and Subcommittee Responses

H43-P: Clinical Applications of Flow Cytometry: Immunophenotyping of Leukemic Cells; Proposed Guideline

General

1. I would encourage some discussion regarding the utility of CD 45/side scatter gating strategies in the next consensus level version of the document. The advantages of this approach to the analysis of acute leukemias, especially in assessing early relapse or minimal residual disease, which have come to light subsequent to the original drafting of the proposed guideline are notable.

- **The subcommittee agrees. A discussion of this method as a possible approach to leukemia phenotyping has been added to Section 13.4.2.**

2. Anticoagulated blood contains "plasma" not serum. Thus all sections that refer to "serum" in the context of anticoagulated blood should be changed to "plasma."

- **The subcommittee agrees; the text has been revised.**

Section 8.4

3. The paper on performing viability measurements on fixed samples has been referenced but not indicated as a possible approach in Section 8.4. Because of the potential health hazard associated with sample analysis using viable cells, you may wish to include this procedure. We have used this procedure for evaluating viability from over 5,000 patients without a problem.

- **As stated in Section 12.3 on sample analysis, the published methods for postfixation viability discrimination have not yet been sufficiently validated in leukemic samples across multiple laboratories to warrant their recommendation as standard practice. For example, the method of Riedy et al. has been documented to provide data on fixed whole blood preparations containing primarily mature leukocytes which are comparable to that obtained with propidium iodide in unfixed whole blood preparations. However, there is also a published abstract which indicates that the ethidium monoazide viability probe is less well excluded by viable immature leukocytes and/or hematopoietic cells than by viable mature leukocytes. The subcommittee nonetheless agrees that it would be desirable to identify a postfixation viability discrimination method suitable for use with leukemic samples, and has therefore modified the language in Sections 8.4 and 12.3 to reflect this need.**

Section 8.6

4. It is scientifically incorrect to use 5% normal serum to block with. Anyone who takes the time to compute the normal serum immunoglobulin concentration would never make this incorrect recommendation. Section 8.6 needs to be rewritten to clarify this. See Reference: C.C. Stewart, Cell preparation for the identification of leukocytes, methods in cell biology, chapter 39,411-426, 1990.

- **The subcommittee agrees. Section 8.6 has been rewritten taking the point raised into account and the reference provided is included in the bibliography.**

5. In Section 8.5 it is recommended that human serum immunoglobulin should be removed. In Section 8.6 it is recommended that one adds the IgG back, this will be most confusing to the reader.

- **The subcommittee agrees that this language is potentially confusing. Section 8.6 has been modified to clarify that it applies only to assays other than immunoglobulin.**

Section 14.2 1

6. I would not agree with the statement that "it is neither useful nor relevant to perform immunophenotypic analysis on samples from patients with chronic myelogenous leukemia or myelodysplasia syndrome in the chronic phase." This statement is not correct because three-color but not two- or one-color is extremely useful and diagnostic. I would suggest instead that the statement read: It has not yet been established whether immunophenotypic analysis is useful on samples..."

- **The subcommittee recognizes that multicolor analysis may provide information not obtainable by single or dual color analysis. In principle, the subcommittee agrees with the fact that it has not yet been established whether one can define myelodysplastic/myelo-proliferative processes (in chronic phase) immunophenotypically. However, in the absence of a blastic transformation, the need for (and expense) of immunophenotypic analysis has not been defined.**

Section 14.2.3

7. I would urge some further clarification in Section 14.2.3(1) regarding the use of platelet associated antigens in the diagnosis of AML M7. In particular, CD 61 and CD 42 are preferred markers of Megakaryocytic differentiation relative to CD 41, which is a more mature platelet antigen. I suggest the inclusion of a statement indicating that the discordance between CD 41 and CD 42 expression can be useful in the recognition of aberrant M7 blasts cells and provide a distinction from the not uncommon problem of cytophilic platelets.

- **The subcommittee agrees that the problem of cytophilic platelets may create confusion. A Wright-Giemsa stained cytospin is usually sufficient to evaluate this.**

Related NCCLS Publications*

GP5-A **Clinical Laboratory Waste Management; Approved Guideline (1993)**. Offers guidance on safe handling and disposal of chemical, infectious, radioactive, and physical waste generated in the clinical laboratory.

H1-A4 **Evacuated Tubes And Additives For Blood Specimen Collection—fourth Edition; Approved Standard (1996)**. Discusses requirements for blood collection tubes and additives including heparin, EDTA, and sodium citrate.

H3-A3 **Procedures For The Collection of Diagnostic Blood Specimens by Venipuncture—third Edition; Approved Standard (1991)**. Discusses methods for the collection of blood specimens by venipuncture and appropriate training program aimed at increasing analyte integrity and minimizing laboratory error. Includes a 24-step protocol for specimen collection, recommendations for "order of draw" and considerations for performing venipuncture on children.

H5-A3 **Procedures For The Handling And Transport of Diagnostic Specimens And Etiological Agents—third Edition; Approved Standard (1994)**. Gives proper packaging, handling, and transport requirements for medical specimens including federal regulations.

H18-A **Procedures For The Handling And Processing of Blood Specimens; Approved Guideline (1990).** Addresses the multiple factors associated with the handling and processing of specimens, factors that can introduce imprecision or systematic bias into test results.

H20-A **Reference Leukocyte Differential Count (Proportional) And Evaluation of Instrumental Methods; Approved Standard (1992).** Discusses automated differential counters and establishes a reference method based on the visual (or manual) differential count for leukocyte differential counting to which an automated or manual test method can be compared, and an experiment to carry out the comparison. Describes procedures for collecting specimens; preparing blood films and requirements for acceptable wedge and spun films; Romanowsky staining; the formed elements; variant leukocyte forms; and a protocol for examining blood films. Details procedures for determining inaccuracy, and within-run and between-run imprecision; procedures for determining clinical sensitivity; and statistical methods for determining inaccuracy and imprecision.

H26-A **Performance Goals For The Internal Quality Control of Multichannel Hematology Analyzers; Approved Standard (1996)**. Gives recommended performance goals for analytical accuracy and precision based on mathematical models for the following measurements: hemoglobin concentration, erythrocyte count, leukocyte count, platelet count, and mean corpuscular volume.

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* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

Related NCCLS Publications (Continued)

H42-A **Clinical Applications of Flow Cytometry: Quality Assurance And Immunophenotyping of Peripheral Blood Lymphocytes; Approved Guideline (1997)**. Offers guidelines on establishing quality assurance procedures for immunophenotyping lymphocytes by flow cytometry.

M29-A **Protection of Laboratory Workers From Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue; Approved Guideline (1997)**. This document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of bloodborne infection from laboratory instruments and materials; and recommendations for the management of bloodborne exposure.

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