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Validation and quality control of immunophenotyping in clinical flow cytometry

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Abstract

Clinical flow cytometry has evolved from two-parameter quantitative assessment of peripheral blood lymphocytes to six-parameter qualitative evaluation of bone marrow for hematopathology. Leukemia and lymphoma immunophenotyping represent an extremely important complement to morphology in the diagnosis and monitoring of hematopoietic malignancies. The complexity of five- and six-parameter analyses and the interpretation of the data rely on standardization and validation of the instrument, the reagents and the procedure. In addition, flow cytometry laboratories in the U.S. are required to document proficiency testing, sample preparation, method accuracy, specificity, sensitivity and precision. NCCLS and the U.S.–Canadian Consensus Conference have provided recommendations, but each laboratory is ultimately responsible for validating its own qualitative and quantitative procedures. This paper reviews procedures for validation and quality control of all aspects of the operation of a clinical flow cytometry service.

Keywords: Immunophenotyping; Flow cytometry; Phenotypic analysis; Leukemia diagnosis; Lymphoma diagnosis

1. Introduction

Flow cytometry is a dynamic technology which has allowed the multi-parameter analysis of heterogeneous cell populations to develop as a clinical service (Owens and Loken, 1995). Complex analyses are able to combine immunophenotyping of both surface and cytoplasmic antigens, DNA analysis and functional evaluations. Subsets of cells can be identified and characterized by patterns of maturation antigens and staining intensity which can assist in diagnostic and prognostic interpretations as well as the detection of minimal residual disease.

Three- and four-color immunophenotyping are not unusual in the clinical laboratory, allowing the simultaneous measurement of five or six different parameters, respectively (Nicholson et al., 1996). A recent consensus conference recommended five-parameter immunophenotyping to be the minimal standard for hematological malignancies, forward and right angle light scatter and three colors of fluorescence (Stelzer et al., 1997). With so many variables in these analyses, standardization and validation of instrumentation and methodology is essential to ensure the technical quality of the results (Brando and Sommaruga, 1993; Carter et al., 1992; Cavelli et al., 1993; National Committee for Clinical Laboratory Standards, 1992; Hurley, 1988, 1997a,c). Reagents must be well characterized for specificity and performance with different fluorochromes. All

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monoclonal-fluorochrome combinations must be critically evaluated for staining intensity, spectral overlap, and instrument compensation (Hurley, 1997b; McCoy et al., 1990; Muirhead, 1993). Furthermore, fluorescence patterns must be characterized for diagnostic combinations of antigens and diagnostic interpretation (McCoy and Overton, 1996).

In attempts to assist with standardization, the U.S.–Canadian Consensus Conference in 1997 and National Committee for Clinical Laboratory Standards (NCCLS) guidelines in 1998 provided recommendations for clinical flow cytometry in hematopathology (Borowitz et al., 1997; Davis et al., 1997; National Committee for Clinical Laboratory Standards, 1997a; Stelzer et al., 1997; Stewart et al., 1997; Braylan et al., 1997a,b). On the regulatory side, the Clinical Laboratory Improvement Act (CLIA 88) (Department of Health and Human Services, 1992) has influenced laboratory staffing, training, validation and documentation. As we enter the 21st century, the laboratory's responsibilities continue to increase. A recent publication by Hurley and Zito (1998b) offers approaches for CLIA compliance in the clinical flow cytometry laboratory and includes templates for appropriate forms to satisfy documentation.

The goal of this article is to discuss how regulatory oversight influences the laboratory's validation and quality control documentation, particularly in hematopathology. Although this article covers regulatory issues as they apply in the U.S., all laboratories world-wide are responsible for maintaining performance standards. Good Laboratory Practices include Standard Operating Procedures (SOPs), Quality Control (QC) and Quality Assurance (QA) as integral to providing good patient care (Hurley and Zito, 1998a). Regardless of requirements placed on manufacturers to provide analyte-specific reagents (ASR) or in vitro diagnostic (IVD) reagents, each laboratory should validate their own panels for sensitivity, specificity, and correlation with morphology and clinical findings. The following sections will consider laboratory responsibilities, reagent and instrument validations, QC, QA, and troubleshooting as a model for the integration of new technologies into diagnostic hematopathology.

2. Responsibilities of the clinical service

Flow cytometry falls under the Centers for Disease Control and Prevention (CDC) category of high complexity laboratory testing. Documentation of staff qualifications and training as well as analytical accuracy, sensitivity, precision and QC are required. In some states, such as California, personnel responsible for generating flow cytometric results must be licensed medical technologists who are required to have 12 h of continuing education yearly. Other states may not have the same personnel qualifications. At the very least, training and proficiency in the technology must be documented. Laboratories should develop SOPs for training, with supervisor verification of proficiencies in incremental responsibilities for each staff member. Instrument manufacturers and scientific societies also conduct training courses and provide certificates of training. Additionally, the American Society of Clinical Pathology (ASCP) offers a specialty exam for national certification in flow cytometry (QCYM).

2.1. Proficiency testing

All high-complexity laboratories must enroll in a proficiency testing (PT) program that meets CLIA regulations (Subpart I) (Department of Health and Human Services, 1992) and is approved by Health and Human Services (HHS). Regulations require that the laboratory inform HHS which programs it will use, list the tests performed for each program, participate in a program for at least a year before choosing a different program, and must notify HHS before changing. The laboratory must also authorize the PT program to release data to HHS.

Proficiency Testing samples must be treated the same as patient specimens, using the same personnel and work processes (Dorsey, 1975). Results must not be discussed with other laboratories and PT samples may not be outsourced under any circumstances. Documentation for handling, preparation, processing, analysis and interpretation of PT samples must be kept (Grannis et al., 1972). Final reports must be retained for a minimum of 2 years.

2.2. Sample handling

All laboratories must establish specimen requirements, recommended transport conditions and criteria for acceptability. Procedures for handling, packaging, labeling and transporting potentially infectious biological specimens have been published (Nicholson et al., 1994; National Committee for Clinical Laboratory Standards, 1990, 1997a). The U.S.–Canadian Consensus strongly recommended that every attempt be made to derive useful information from any specimen submitted for leukemia or lymphoma immunophenotyping analysis (Stelzer et al., 1997). Requesting physicians should always be informed about sample quality issues; any compromised specimen, whether evaluated or not, must be described in the test interpretation.

Clinical specimens must be appropriately identified — the minimum information is a unique patient identifier (for cytogenetic analyses, two identifiers may be required), test ordered and date of sampling. Other information helpful for interpretation include presumptive diagnosis, age, gender, date and time of specimen collection, source of specimen, name of physician, and recent treatment or medications. Confidentiality must be assured and documentation tracking the specimen's handling is essential. Tests may only be performed if requested by an authorized person. Verbal requests must be followed by written authorization. All requests must be retained at least 2 years.

2.3. Sample preparation

Sample preparation for flow cytometric analysis must consider the type of specimen submitted and the number of cells available for analysis (Stewart and Stewart, 1994). Peripheral blood, bone marrow, or tissue specimens should be processed to contain a suspension of the cells of interest, eliminating erythrocytes, at a concentration optimal for monoclonal staining ($0.5–1\times10^{7}$/ml; 0.1 ml per tube). All processing procedures must be written, approved and daily records maintained. Test records must be retained for at least 2 years.

Good laboratory practices and CLIA 88 require that all clinical testing be characterized for accuracy, specificity, sensitivity and precision (National Committee for Clinical Laboratory Standards, 1997b). However, for leukemia and lymphoma immunophenotyping by flow cytometry, there are no consensus standards or recommendations for these assessments; thus each laboratory must establish its own performance criteria (National Committee for Clinical Laboratory Standards, 1997a).

2.4. Accuracy

Analytical accuracy compares the test result to a reference, or 'gold' standard. In hematology, normal cell populations can be counted microscopically with a hemocytometer or with automated equipment and results compared for accuracy (Wooten and King, 1953). In abnormal populations, especially when the characterization is based on multi-parameter data, the comparison becomes complex. For hematopathology, the gold standard is morphology. The flow cytometric assessment of accuracy must therefore compare to morphology. With rare events, however, morphologic diagnosis is difficult. Flow cytometry allows characterization of cell populations with complex phenotypes and should also assess accuracy by comparison to previously characterized cells (Stewart and Stewart, 1997b). Sources of well-characterized cell populations are cryopreserved pedigreed specimens from another validated laboratory or relapse specimens from previously characterized cases. Another source of specimens for assessing accuracy can be cases diagnosed by cytogenetics and molecular biology. Documentation comparing at least 20 leukemia and lymphoma cases with complete histopathology and clinical findings should be on file to support analytical accuracy.

Another aspect of accuracy that should not be ignored is the qualitative staining pattern used to identify cell lineages. Descriptions of dim, moderate, and bright staining patterns can be diagnostic or prognostic and should be well characterized and documented. For example, when multiple antibodies conjugated to fluorochromes are used in combination, accuracy should be assessed for each antibody separately and the frequency and intensity results

compared to those obtained when using the combined reagents.

2.5. Specificity

Specificity of monoclonal reagents is defined by how well the antibody recognizes the correct antigenic target. Manufacturers are responsible for reagents having the correct specificity listed on their labels. However, specificity in a 'home-brew' diagnostic test has a broader meaning. For leukemia and lymphoma testing, the interpretation of the flow panel should be compared with morphology and clinical presentation to assess the 'specificity' of the testing (Zagursky et al., 1995). Each laboratory should establish its own expected rate of discrepancy between flow and morphology, most likely $<5\%$. The laboratory should then assess, on a case-by-case basis, the reason for a discrepancy, document the discrepancy as a QA assessment, and monitor trends quarterly.

Specificity of flow cytometric reagents can be assessed by consensus workshops, publications or by in-house validation. Most notable are the publications from the various Human Leukocyte Differentiation Antigen Workshops, the latest being Leucocyte Typing VI (Kishimoto et al., 1997), which aptly and succinctly summarize the vast body of testing results on most if not all of the monoclonal antibodies commonly used in leukemia and lymphoma immunophenotyping. Helpful data summaries, reviews, and citations pertaining to these reagents can be found on the HLDA Web page at http://www.mh-hannover.de/projekte/hlda7/hldabase/select.htm. Also refer to the PROW database (Protein Reviews on the Web). Other sources of specificity data are clinical texts and journal articles.

However, flow cytometry monoclonal reagents are considered 'home-brew' reagents and each laboratory must document staining performance, or 'diagnostic specificity' on both normal and abnormal cell populations, identifying both positive and negative staining (Stewart and Stewart, 1995). Clinical hematopoietic specimens will almost always contain normal along with any abnormal populations and thus will allow both evaluations in one specimen. Diagnostic immunophenotyping must be validated with pedigreed malignant specimens or well-characterized transfected cell lines used as positive controls.

2.6. Sensitivity

Reagent sensitivity describes the ability to detect the minimum staining intensity above non-specific or negative staining (Steen, 1991). The sensitivity of detection is dependent on the titration of monoclonal reagents (Stewart and Stewart, 1997a), the proper instrument setup and calibration, the number of cells counted and the flow rate of the instrument (Wittrup et al., 1994; Zucker et al., 1991). Documentation of reagent titrations and parallel testing of each new lot of antibody is required. Instrument calibration and documentation is also required.

2.7. Precision

Precision is a standard analytical parameter which measures the reproducibility of a single sample stained and analyzed in duplicate at least 10 times. NCCLS Guidelines recommend 20 replicates. Normal peripheral blood, cell lines, blood standards or CD Chex may be used. CD Chex are preserved white cell controls manufactured by Streck Laboratories. Quantitative mean and standard deviation for each monoclonal antibody should be documented and a 2 S.D. range developed. Determination of instrument precision is accomplished by running the same stained sample at least three times, with results within 2 S.D.

2.8. Analyte-specific reagents

In the United States, clinical laboratories, regulated under CLIA 88 and performing physician-ordered flow cytometric testing for leukemia and lymphoma immunophenotyping, are required to be qualified to perform high-complexity testing and must use reagents that are manufactured and labeled as Analyte Specific Reagents (ASRs).

Manufacturers of ASR must register their facility, list their products, follow current Good Manufacturing Practices (cGMP) under the new Quality System Regulations (QSRs), and comply with medical device reporting requirements. Manufacturers are restricted from providing any statement regarding

analytical or clinical performance, number of tests provided, or instructions for use. Advertising and promotional material for an ASR product must include the identity and purity (including source and method of acquisition) of the analyte-specific reagent and the identity of the analyte [Code of Federal Regulations, CFR 809.30(d), Federal Register, 1997].

The identity of the analyte for monoclonal antibody reagents is provided by the antigen distribution and supporting references. The HLDA references for when the antibody clone was first clustered are provided for the identity of the ASR. The labeling for analyte-specific reagents must include the reagent name, concentration, purity and quality, statement of warnings or precautions for users, date of manufacture, lot number, expiration date, storage instructions, net quantity of contents, name and place of business, and the following statement: ''Analyte Specific Reagent. Analytical and performance characteristics are not established'' [CFR 809.10(e)].

It is the responsibility of the testing laboratory using these reagents to validate their performance in 'home-brew' clinical assays. In addition, any laboratory reports using Class I ASRs must contain the following disclaimer: ''This test was developed and its performance characteristics determined by {laboratory name}. It has not been cleared or approved by the U.S. Food & Drug Administration'' [CFR 809.30(e)]. Further clarifying comments may also be included.

Research institutions and other non-clinical laboratories that use ASRs to make tests for purposes other than providing diagnostic information to patients and practitioner are not restricted under the ASR regulation [CFR 809.30(g)].

3. Validation of Immunophenotyping

How does a laboratory validate flow cytometric immunophenotyping? The basic components are the validation of the instrument, the individual reagents, and the staining protocols used to create a final interpretation (Whitehurst et al., 1975). Each step in the testing process must be specified and quality control measurements included to monitor performance (Belk and Sunderman, 1947; Sax et al., 1967; Shuey and Cebel, 1949; Paxton et al., 1989).

3.1. Instrument validation

Validation of instrument performance falls into two areas: (a) instrument setup and daily qualification of both light scatter and fluorescence measurements, and (b) cross-instrument performance using relevant clinical specimens.

3.2. Instrument setup and daily qualification

NCCLS recommends that the setup of a flow cytometer is comprehensive enough to assure proper optical alignment for adequate light scattering and fluorescence sensitivity and resolution as well as the proper degree of compensation to correct for spectral overlap when multiple fluorochromes are used. While hardware configuration may not allow the laboratory to align optics, a check of performance must be made and documented. In addition, since the hardware and optics are different for flow cytometers made by different manufacturers (the two major ones being Beckman-Coulter and Becton Dickinson, now known as BD Biosciences), the recommended procedures for instrument setup and performance qualification are instrument-specific.

3.3. Light scatter sensitivity and resolution

Optical alignment for optimal sensitivity and resolution of both forward (FSc) and side (SSc) scatter can be assessed by running uniformly sized beads that fall within the light scatter ranges observed with most clinical samples, at a constant PMT voltage on a daily basis. The mean FSc and SSc channel numbers and percent coefficient of variation (% C.V.) should be recorded. The acceptable ranges for each parameter can be established by first running the beads 20 times over a 5 day time period at the same PMT setting. Levy–Jennings graphs are then used to plot the values obtained daily and an action plan is established for what to do when any parameter falls outside of the expected range (Allen et al., 1969; Henry, 1959; Levy and Jennings, 1950). However, since beads and cells do not always behave similarly on a flow cytometer, it is also

recommended that the instrument be set up for running clinical samples using biological material and that the proper resolution of different cell types be determined in daily instrument qualification procedures (Henry and Segalove, 1952; Ladenson, 1975). Normal peripheral blood leukocytes processed in a similar manner as the clinical samples can be used for this purpose. For example, the lymphocytes can be set to fall within a specified range based on FSc. The PMT for SSc can then be adjusted so that the granulocytes fall within a specified range, with the final FSc vs. SSc cytogram required to demonstrate good separation of lymphocytes, monocytes and granulocytes.

3.4. Fluorescence sensitivity and resolution

Acceptance values for monitoring fluorescence sensitivity and resolution can be established by two methods: (a) recording the channel number and C.V. of calibration beads with a pre-determined, fixed laser power, filters, PMT voltages and gains, or (b) recording the high voltage and gain to position the beads in the same channel each time. For either approach, acceptance ranges can be established by using at least 20 replicate data sets collected over at least a 5 day period; new ranges must be developed each time a new lot of beads is phased into service. Regardless of the method used, procedures for what to do if any of the measured and plotted parameters do not fall within the acceptable range must also be established, followed and documented by the clinical laboratory. Values for the acceptable ranges displayed on the Levy–Jennings graphs for each parameter are typically updated based on each successive 20 day cycle of data collection. However, QC specifications for instrument performance must be established to allow for detection of significant trends or drifts over time which require corrective action.

3.5. Fluorescence compensation

Compensation should also be evaluated at the time of initial instrument setup and then monitored daily, preferably using a biological compensation control in addition to hard dyed beads provided by the instrument manufacturer. Depending on the number, type and combination of fluorochromes used in a particular staining protocol, the compensation between fluorescence signals must be properly set to prevent spill-over of one fluorescence signal into another (Bagwell and Adams, 1993). This is usually done automatically using hard dyed beads as seen in Fig. 1 for four-color compensation of FITC, PE, PerCP and APC on a FACSCalibur (BD Biosciences).

However, the use of a biological compensation control is also recommended. When performing four-color immunophenotyping using a single laser at 488 nm on a Coulter XL (Beckman-Coulter), and a combination of monoclonal antibodies directly conjugated to FITC, PE, PE-Cy5 and ECD, an appropriate biological compensation control would consist of normal donor peripheral blood lymphocytes stained with CD4–FITC, CD8–PE, CD2–PE/Cy5 and CD45–ECD, as seen in Table 1.

Hypothetically, in this example, there are 12 different combinations of fluorescence signals that need to be checked for compensation but, due to instrument design, the optical filters used, and the inherent spectral emissions of each fluorochrome, not all combinations can or need be considered. Using the Cytosetting Matrix Display on System II 3.0 Expo software in the Coulter XL, each of the four fluorescence PMTs are first adjusted to the proper settings based on the single color fluorescence histogram and compensated with the compensation matrix. For a complete discussion of four-color compensation, see Stewart and Stewart (1999).

4. Monitoring instrument performance

In the setup and qualification of a flow cytometer for daily use, NCCLS recommends a check of optical alignment, fluorescence resolution and intensity. Using the Coulter XL, a calibrator suspension of latex beads with a known concentration of fluorescence (DNA Check) can be used to verify an appropriate cell-wide laminar flow, a proper alignment between the laser beam and the cell at the interrogation point and a proper adjustment of laser power and/or PMT voltage. The ranges for the particles must be established, running 20 times during 5 days and keeping the PMT voltage constant. The placement of the beads must meet the established ranges and be recorded in the QC log. Any

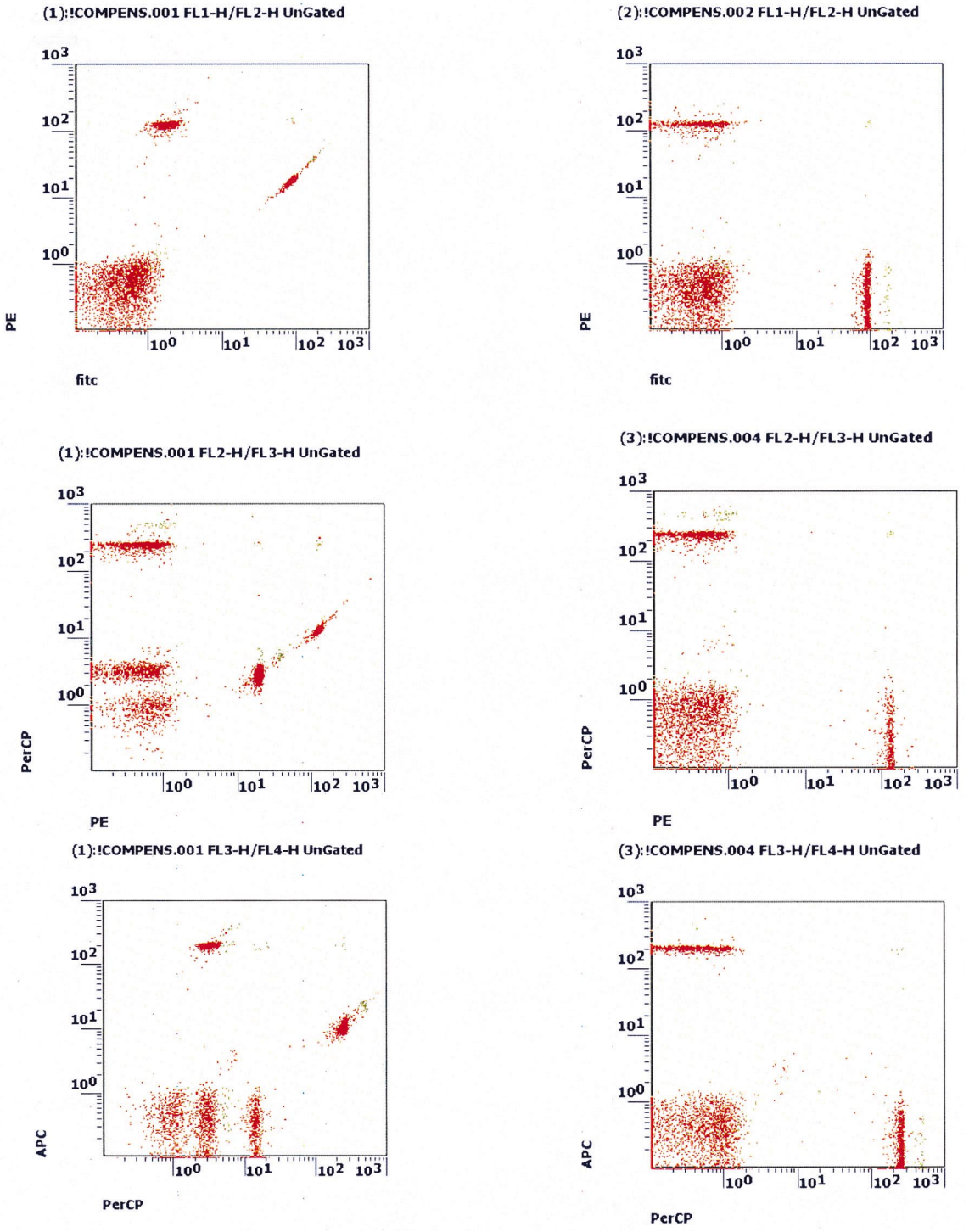

Fig. 1. Column A: fluorescent beads run without compensation on FACSCalibur (four color) FITC, PE, PerCP, APC. Column B: beads showing compensation for FL1−%FL2=1.2 and FL2−%FL1=23.3, FL2−%FL3=0, FL3−%FL4=2.5, FL4−%FL3=5.0.

Table 1
Signal volts and gain showing matrix compensation for four-color phenotyping on a single laser Coulter XL MCL instrument

Signal				Compensation					
Signal	Volts	Gain	Total gain	Signal out = $Y - \%X$					
				Y	$X \rightarrow$	FL1	FL2	FL3	FL4
FS	25	5.0	5.38	FL1			0.9	0.0	0.0
SS	402	20.0	44.12						
FL1	687	1.0		FL2		11.2		12.8	4.7
FL2	873	1.0							
FL3	718	1.0		FL3		0.0	31.1		2.1
FL4	909	1.0							
AUX	69	5.0	6.04	FL4		0.0	0.0	19.1	

values out of range must be investigated, corrective action taken and documented.

For the BD FacsCalibur, the fluidics and calibration check is performed by running CaliBrite Beads and allowing the instrument software to make adjustments in setup based on lot-specific performance expectations. Documentation of acceptable calibration is provided for record keeping. These records can be used for trend analysis of the laser power and voltage settings to indicate potential technical problems.

NCCLS recommends daily performance monitoring of fluorescence intensity, color compensation and verification of system performance using a QC (normal) specimen. Each laboratory must have QC procedures to monitor instrument performance. For hematopoietic immunophenotyping, color compensation and verification of performance can be evaluated with each specimen panel. Although every patient is different, the CD4/CD8/CD45/CD2 combination can be used to check compensation. Settings will rarely need to be changed.

Optics should not be changed by a clinical laboratory without specific validated procedures. It is more appropriate to have the manufacturer check and adjust optics during routine maintenance and to provide documentation for the laboratory.

4.1. Fluorescence linearity

Linearity of fluorescence detection should be checked on a monthly basis or as recommended by the instrument manufacturer (Vogt et al., 1989). Linearity should be constant unless the laser is unstable. Monitoring linearity is a check of PMT voltages. Using the same PMT settings used for clinical specimens, a set mixture of four to five multi-level fluorescence beads, each with a known relative fluorescence intensity level, should be run. Products are available from several manufacturers, for example Flow Cytometry Standards, or Spherotech. Acceptable mean fluorescence intensity (MFI) ranges for each bead in the mixture should be established by 20 replicate runs over a 5 day time period. These same bead mixtures should then be run once a month and the linearity, or the relative difference in MFI between each of the beads, should remain constant for each fluorescence PMT (Fig. 2). Graphs of linearity should be drawn following manufacturers' recommendations to accommodate Log 0.

4.2. Cross-instrument performance

Laboratories that are performing the same clinical immunophenotyping protocols on more than one instrument should include a semi-yearly cross-instrument comparison of at least five different and representative clinical samples stained with each of the standard protocols used in the laboratory. The results obtained for each sample should not differ between instruments by more than a pre-defined acceptable variance (Vogt et al., 1991, 1994). This cross-instrument sample testing process should be documented and corrective action plans must be established and followed when cross-comparison results fail to meet performance specifications.

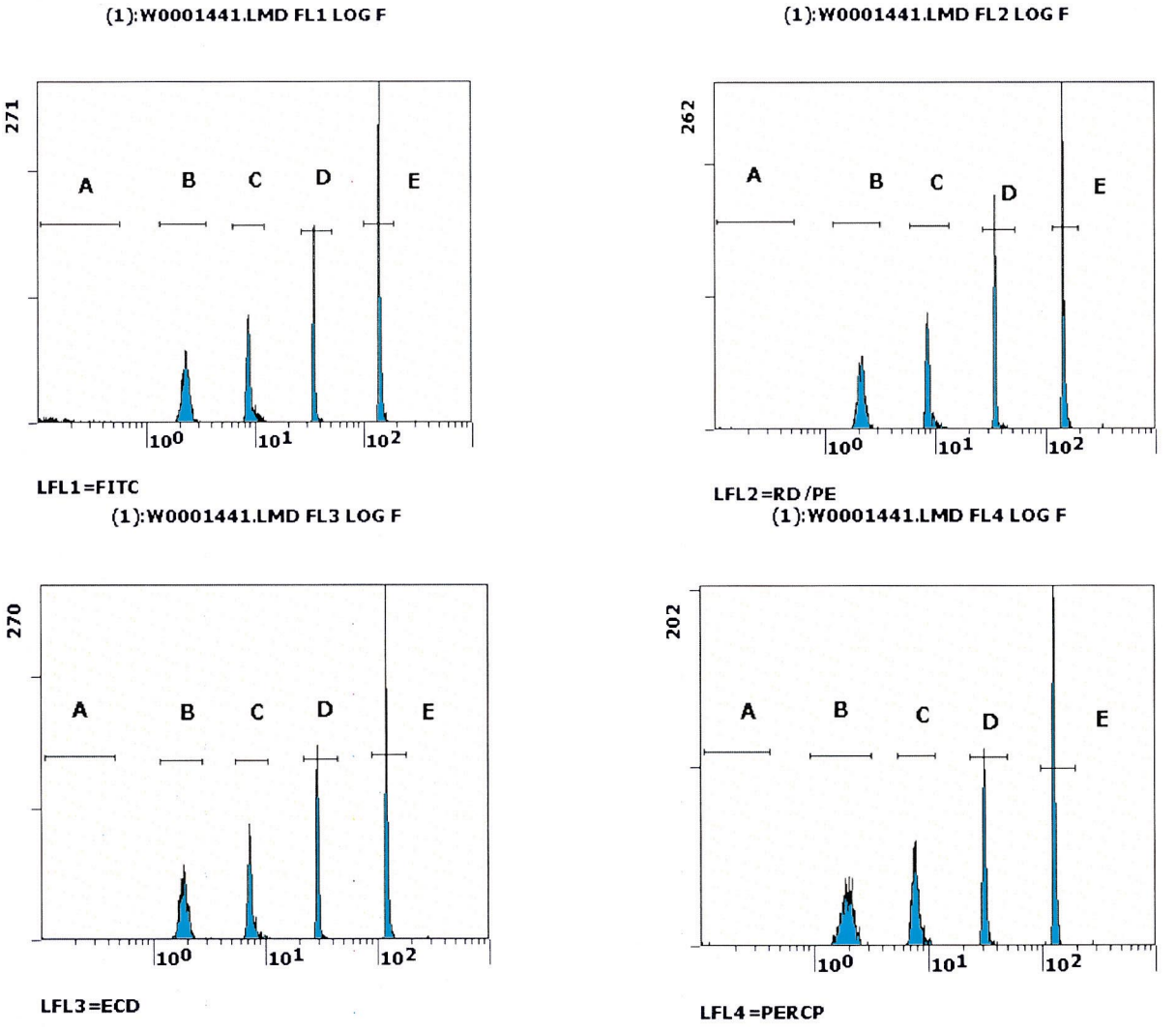

Fig. 2. Beads with five different fluorescence intensities for each fluorochrome. Setup regions A to E will provide the log intensity channel value. A plot of log intensity vs. bead number should provide a linear distribution.

5. Preanalytical QC

Laboratories must not only establish acceptance criteria for immunophenotyping specimens, but must have procedures to assess acceptability and documentation to assist in troubleshooting and the interpretation of results. NCCLS recommends a visual analysis on receipt to confirm specimen quality. Hemolysis, partial draw (especially in ACD tubes), temperature extremes and improper labeling should be documented before transfer to the testing laboratory.

6. Reagent and method validation

An immunophenotyping procedure first requires the selection of monoclonal reagents and fluorochromes which are to be used in the multi-parameter

analysis and interpretation of results. The hematopathology testing panel must be designed to clearly distinguish normal and abnormal immunoreactivity patterns based on differences in light scatter and/or fluorescence intensity. These pattern comparisons are integral to the interpretation of results. Therefore, the panel must include all relevant markers and reflect the visual expectations of the diagnostic interpreters.

The U.S.–Canadian Consensus Conference stopped short of recommending a diagnostic panel for leukemia and lymphoma immunophenotyping, however there was agreement on 42 determinants which can provide diagnostic information in these procedures (Stewart et al., 1997). Each specimen must be considered a unique case and fully evaluated to minimize missing any abnormality. The selection of panel reagents needs to balance the economic need for a minimal number of monoclonal antibodies to identify abnormal populations with the scientific need to detect abnormal antigen expression. The committee concluded that it is more important that each laboratory have adequate experience with the binding characteristics of their testing reagents than specifying what those reagents should be. How reagents are combined in testing panels is left up to the testing laboratory, but should be based on a target-oriented strategy (Table 2).

All the combinations have to be validated in each laboratory and the analysts must be familiar with the patterns associated with each combination. Different clones (under the same CD) may perform differently. Similarly, the same CD labeled with different fluorochromes will show a different level of intensity and possible color overlap. Fluorochrome intensity and antigen expression are both important in establishing the best antibody combination to evaluate antigen density (Schwartz et al., 1990, 1996a,b). Some tandem dye combinations can help to minimize these problems.

Different combinations of antibodies can be used to provide the same clinical interpretation and no single panel will accommodate all leukemia or lymphoma phenotypes. To compound the difficulty, new markers and reagents, when determined to be clinically valuable, must be validated by each laboratory. Any panel of monoclonal reagents used by a laboratory must be chosen not only for their technical performance, but also to satisfy the experience

Table 2
Some common tube combinations[a]

Population to identify	Three-color combination	Four-color combination
B cells	Kappa/Lambda/CD20 CD10/CD5/CD19 CD5/CD23/CD20	K/L/CD45/CD20 CD10/CD5/CD45/CD19 CD5/CD23/CD20/CD19
Plasma cells	CD38/cytoK/CD45 CD38/cytoL/CD45	
T cells	CD7/CD3/CD45 CD4/CD8/CD3	CD7/CD3/CD45/CD56 CD4/CD8/CD45/CD2
Myeloid	CD33/CD13/CD45 CD34/CD117/CD45 CD11b/CD16/HLA-DR	CD13/CD14/CD45/CD33 CD15/CD117/CD45/CD34 CD11b/CD16/CD45/HLA-DR
Myeloid w monocytic diff	CD64/CD13/CD45	CD64/CD14/CD45/CD33
Myeloid w megakariocytic diff	CD34/CD41/CD61 CD42b/CD61/CD45	CD41/CD61/CD45/CD34 CD42b/CD61/CD41/CD34
Red cell precursors	CD71/Glycophorin A/CD45	

[a] These combinations are for examples only and do not represent guidelines or consensus recommendations.

and expectations of the technical and medical personnel who must differentiate abnormal populations from normal cells.

The proper combination of monoclonal antibodies within a cocktail must consider antigen expression on normal and abnormal cells as well as the fluorochrome combinations to minimize interference between reagents. One example of potential interfering markers is bright CD15/CD16/CD45/CD13 on granulocytes. For multiple fluorochrome combinations, the laboratory must not only validate that each monoclonal performs as expected in the procedure, but must also prove that the performance is not affected by co-blocking of epitopes, fluorescence quenching, or energy transfer (Pollice et al., 1992).

Antibody cocktails may be purchased from the manufacturer or prepared by the laboratory. Whether reagents are mixed immediately before each use or stored as a premix solution, performance of each cocktail must be validated and expiration dates documented.

Each monoclonal antibody in each cocktail must be titrated individually for optimal signal-to-noise separation. However, potential interference between certain clones and/or fluorochromes necessitates also performing checkerboard titrations to select the final optimal working dilutions once the reagents are used in combination. For each new multi-color combination added to the panel, it is necessary to first validate that the performance (mean intensity fluorescence and percent positive) of each antibody when used in combination is comparable (within 2 S.D.) to the performance of each antibody used alone at the same concentration on the same target cell population. If reagents are stored in a pre-mixed cocktail format, it is also important to institute sufficient QC procedures, using appropriate control cells, to verify stability of performance of the cocktailed reagents over time. Stable cocktails will provide results wherein the mean channel and percent of gated cells will not differ more than 2 S.D. between cocktailed and single reagents. Documentation must be summarized and maintained in the laboratory and should include instrument photomultiplier tube (PMT) settings and compensation. No more than one combination of PMT and compensation may be used within a panel (Stewart and Stewart, 1999). The best approach is to set the PMT with the single reagent and then check compensation with the final cocktail.

7. Sample preparation

Integral to the analytical procedure, the laboratory must decide on and validate a sample preparation procedure. Erythrocyte lysis is the most commonly used method for preparing peripheral blood or bone marrow specimens for clinical immunophenotyping. Lysis can be performed either before staining with monoclonal antibodies or after. Whichever method for erythrocyte lysis is used must be validated prior to reporting clinical results. Lysis is optically monitored by observing a cloudy red suspension of cells change into a clear, translucent solution in 5 to 10 min. If red cells are still present after centrifugation and resuspension, the lysing process should be repeated.

Tissue specimens should be disaggregated and filtered to create a single cell suspension prior to staining. This laboratory procedure must also be written and validated. Cytological slides should be prepared and reviewed with the H&E slides of the tissue to assess the loss of fragile cells, such as Reed–Sternberg cells. Some large cells in fibrotic tissues may be difficult to remove and cell loss will occur during the processing. Other sampling errors are due to focal lymphoma in the lymph node, not seen in the isolated cells or in the fixed tissue. Cell fragility may be minimized by gentle tissue processing and teasing the tissue apart with a needle.

Reproducible monoclonal antibody staining relies on the proper proportion of antibodies to cells. For hematopoietic immunophenotyping, cell counts should be performed on all specimens to ensure correct proportions of cells to monoclonal antibodies and thus accurate staining intensity patterns. NCCLS recommends the following adjustments based on WBC count of peripheral blood. (There are no similar recommendations for bone marrow and each laboratory should establish and validate its own procedures.)

(a) WBC <1000 cells/μl: use 200 μl whole blood and adjust amount of lysing reagent appropriately;

(b) WBC 1000–10,000 cells/μl: use 100 μl whole blood and standard volume of lysing reagent;
(c) WBC 10,000–20,000 cells/μl: use 50 μl whole blood and adjust amount of lysing reagent appropriately;
(d) WBC >20,000: dilute whole blood with PBS to achieve WBC concentration 1000–10,000, then use 100 μl and standard volume of lysing reagent.

Light scatter patterns must be appropriate after lysing and staining.

7.1. Viability

Assessment of viability is crucial for evaluating leukemia and lymphoma specimens by flow cytometry because cell membrane integrity is required for antigen expression. Viability at the time of collection, the influence of transportation environment and the time before testing can all affect sample viability and compromise tumor cell detection.

By recent consensus recommendations, flow cytometry performed on leukemias and lymphomas should be held to less stringent viability restrictions than quantitative immunophenotyping (Stelzer et al., 1997). When viability is low, for example less than 30%, a gating discrimination strategy should be used to limit the analysis to live cells, minimizing nonspecific binding from dead cells which could lead to misdiagnoses. Several different methodologies have been used to report viability, trypan blue, 7-aminoactinomycin D (7-AAD), or propidium iodide (PI). The most common is trypan blue dye exclusion visualized microscopically with a hemocytometer. Dead cells stain blue because their membranes are broken, allowing the dye to enter the cell. Viability is reported as a percentage of the total cell population.

The exclusion of dead cells is now being commonly performed on the flow cytometer using dyes such as 7-AAD (Schmid et al., 1992) or PI (Sasaki et al., 1987) (see Fig. 3). Several others have been described and, for most common clinical instruments, a relatively simple dead cell discrimination can be performed simultaneously with immunophenotyping.

For example, using 7-AAD, the most common three-color application in single laser instruments is FITC in FL1, PE in FL2 and 7-AAD in FL3. In this setting, 7-AAD (E_{max} = 655 nm) is preferred over PI (E_{max} = 625 nm) because it has less spectral overlap with PE (E_{max} = 578 nm) In our experience with four-color immunophenotyping with a dual laser system (488 nm/635 nm on the FACSCalibur) the most powerful combination for excluding dead cells

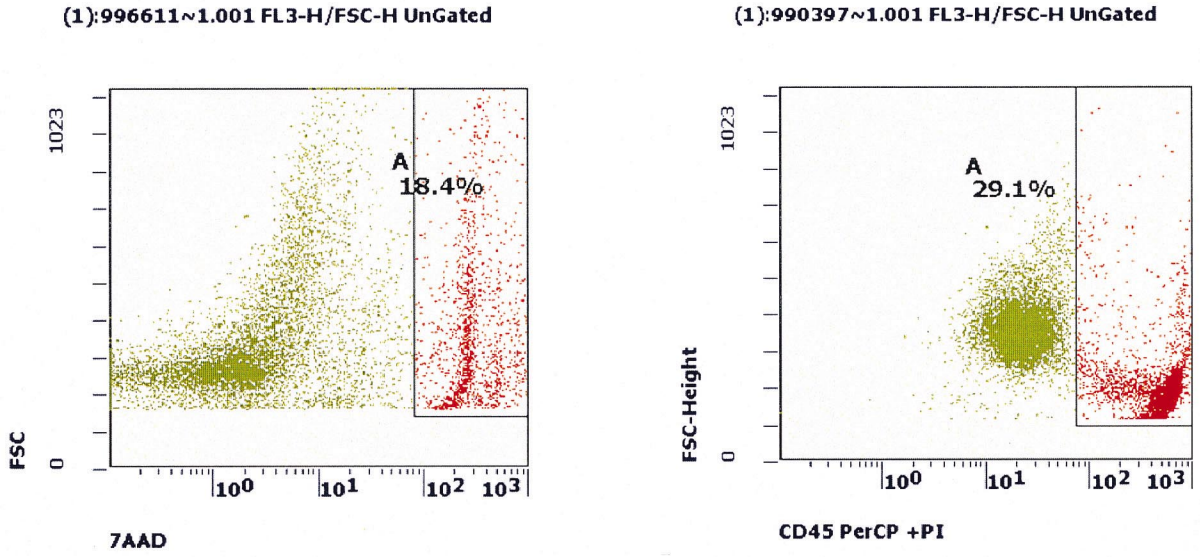

Fig. 3. Dead cell discrimination on two lymphoma tissues. (A) 7-AAD vs. Forward Scatter with 18.4% dead cells. (B) CD45+PI vs. FSC with 29.1% dead cells.

while evaluating four surface markers is FITC in FL1, PE in FL2, PerCP+PI in FL3, and APC in FL4.

7.2. Staining process QC

Once the instrument setup and performance validation is complete, a normal blood is run to check the staining process. For this purpose, there are some commercially available preserved cell preparations [e.g., CD Chex (Streck Labs), CD Chex Plus (Streck Labs), Immunotrol (Beckman-Coulter)] which contain most of the cellular markers evaluated in leukemia and lymphoma immunophenotyping. Staining each new lot of stabilized cells in triplicate will provide a range to be used for quality control. Most importantly, each leukemia or lymphoma specimen will contain a normal cell population which can be used to document appropriate staining patterns.

7.3. Isotype controls

The appropriate use of isotype controls is still controversial in the clinical flow cytometry community (Borowitz et al., 1997; National Committee for Clinical Laboratory Standards, 1997a). For quantitative immunophenotyping, the isotype control was included to provide a negative cell reference and to set up the axes for assessing the numbers of cells in each quadrant. However, immunophenotyping of leukemias and lymphomas is a qualitative assessment and does not usually require quadrant analysis. Furthermore, since all leukemias and lymphomas contain a mixture of cells, those cells that do not express the antigen will be the negative control for the ones that do.

Most of the commercially available antibodies have been selected by screening for the IgG class of immunoglobulin. For IgG class antibodies, however, it is important to recognize that some IgG subclasses will be more problematic due to their increased binding to Fc receptors present on various cell types. In general, the order of 'stickiness' is IgG2b> IgG2a>IgG1. As a result of their carbohydrate structure, some antigenic targets (e.g., CD15 and CD57) will invariably result in generation of only IgM class antibodies. High non-specific binding of IgM class antibodies is commonly observed and can be influenced by the donor, the target cell type, and the particular staining method used. For cytoplasmic staining, the use of isotype controls is recommended due to the significant influence of cell size on the degree of non-specific staining (Jacobberger and Bauer, 2000). It is important that each CD monoclonal antibody has the corresponding fluorochrome and isotype reagent to monitor non-specific binding performance.

The presence of non-specific binding of an isotype can affect the interpretation of the fluorescence intensity patterns and, as such, is critically important in the analysis of leukemia and lymphoma immunophenotyping.

7.4. Procedure control

Fluorescence staining intensity patterns are more easily interpreted if there is a common factor in each tube. For example, CD45 can be used as the 'anchor' in each tube of a multi-color cocktail, providing a reference population for comparisons to other antigenic expression patterns. The most important parameter, however, is experience-based. The analyst must understand the markers and the patterns used to identify an abnormal population. Running a normal control will provide a baseline for all the markers. Any deviation from the normal pattern should be reported and confirmed with other markers or technologies, such as cytogenetic or molecular techniques.

8. Data analysis and interpretation

Immunophenotyping by flow cytometry can be used to distinguish abnormal cell populations from normal cells and, in the process, identify the profile of antigens in the abnormal population (Almasri et al., 1992; Borowitz et al., 1993a; Braylan et al., 1993; Cheson et al., 1996; Hertler et al., 1988; Jamieson et al., 1993; Roth and Schmitz, 1996; Tomer and Harker, 1989). Various patterns of immunoreactivity with monoclonal antibodies are combined with light scatter to characterize populations of interest (Davis et al., 1994; Kilo and Dorfman, 1996; Loeffler et al., 1992; Segal et al., 1991; Wormsley et al., 1990; Davis and Bigelow, 1990; Shimenti et al.,

1992). The specific reagent combinations can be chosen to identify abnormal antigen expression and to characterize cells at various stages of maturation (Civin and Loken, 1992; Ford et al., 1995; Hurwitz et al., 1988; Macedo et al., 1995; Seivers et al., 1996).

The complement of monoclonal antibodies necessary to identify hematopoietic malignancies is not standardized. In fact, strategies for immunophenotyping must consider antibody specificity, fluorochrome, laboratory workload and, most importantly, appropriate multi-parameter combinations. Most laboratories use a comprehensive screening panel and add confirmatory monoclonal antibodies if needed (Hassett and Parker, 1995). Reimbursement for laboratory testing is a major factor, thus laboratories strive to make diagnoses with a minimal number of antibodies. The U.S.–Canadian Consensus Conference estimated that the average North American laboratory uses 19 antibodies to diagnose a leukemia and 16 for a lymphoma. European diagnoses often involve a larger number of monoclonal antibodies (Stewart et al., 1997).

Gating is one of the most important parameters in multi-parameter data analysis. In a diagnostic case, all cells should be evaluated. The U.S.–Canadian consensus recommended that the initial analysis include all viable cells. Further analyses on gated populations should only be performed if all cells of interest are contained within the analysis gate. Strategies for specific diseases may include gating on B cells to determine clonality or identification of leukemic blasts in a CD45 and right angle light scatter display.

In Fig. 4, gating by FSc vs. SSc is shown in the left panel and gating by CD45 vs. SSc for the same case is shown on the right. The better gating strategy uses CD45 to differentiate three populations (immature myeloid, immature monocytes and normal lymphocytes) (Borowitz et al., 1993b).

Data analysis of abnormal populations requires that fluorescence intensity be measured. The calculation of percent positive cells does not aid in the interpretation, while presence of inappropriate determinants or inappropriate intensity expression of antigens can be diagnostic (Bene et al., 1995; Press et al., 1989). Resolving dimly positive populations from negative populations is crucial in the accurate assessment of fluorescence intensity (Givan et al., 1991). There are no hard and fast rules for defining these populations; in fact, reagent combinations are generally optimized to provide discrimination between these populations based on the subjective review of the pathologist (Campana and Pui, 1993; Rameshwar et al., 1994). For critical markers, such as immunoglobulin light chains, in the diagnosis of

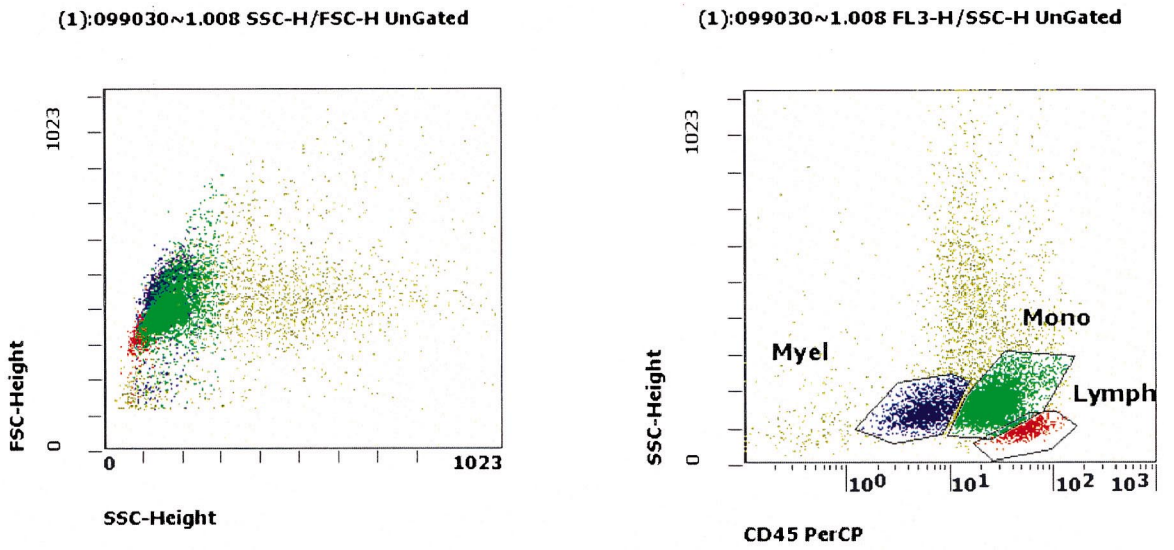

Fig. 4. Comparison of gating strategies: FSc vs. SSc on left; CD45 vs. SSc on right.

non-Hodgkin's lymphoma, the redundant use of antibodies to light chains using alternative fluorochromes, antibody pairs from different vendors, or monoclonal versus polyclonal reagents, can significantly enhance confidence in interpretation of monoclonality in the face of very low level expression. Diagnostic criteria for immunophenotyping were summarized in the U.S.–Canadian Consensus by Borowitz et al. (1997).

The interpretation of the flow cytometric data relies on an experienced diagnostic interpreter, usually a pathologist, reviewing all data, including morphology (Banks et al., 1992; Ryan et al., 1991). Drawing conclusions from either microscopy or flow cytometry is an experience-based skill and the final interpretation should reflect a synthesis of all available information about the case (Davis et al., 1997; Timm et al., 1995; Geisler et al., 1991). All reports must be sent to the requesting party and must also be retained for a minimum of 2 years.

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