Flow Cytometric Devices

Draft Guidance for Industry and Food and Drug Administration Staff

DRAFT GUIDANCE

This guidance document is being distributed for comment purposes only.
Document issued on: October 14, 2014

You should submit comments and suggestions regarding this draft document within days of publication in the *Federal Register* of the notice announcing the availability of the draft guidance. Submit electronic comments to http://www.regulations.gov. Submit written comments to the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. Identify all comments with the docket number listed in the notice of availability that publishes in the *Federal Register*.

For questions about this document contact CDRH's Division of Immunology and Hematology Devices (DIHD) at 301-796-5480 and Kevin Maher at 301-796-6879 or by email at Kevin.Maher@fda.hhs.gov or CBER's Office of Communication, Outreach and Development (OCOD) by calling 1-800-835-4709 or240-402-7800.

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Devices and Radiological Health
Office of *In Vitro* Diagnostics and Radiological Health
Division of Immunology and Hematology
Hematology Branch

Center for Biologics Evaluation and Research

Preface

Additional Copies

Additional copies are available from the Internet. You may also send an e-mail request to CDRH-Guidance@fda.hhs.gov to receive a copy of the guidance. Please use the document number 1787 to identify the guidance you are requesting.

Additional copies of this guidance document are also available from the Center for Biologics Evaluation and Research (CBER) , Office of Communication, Outreach and Development, 10903 New Hampshire Ave., Silver Spring, MD 20993, Bldg. 71, Rm. 3128, 1-800-835-4709 or 240-402-7800, by email, ocod@fda.hhs.gov, or from the Internet at http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/default.htm.

Table of Contents

Flow Cytometric Devices

Draft Guidance for Industry and Food and Drug Administration Staff

This draft guidance, when finalized, will represent the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

I. Introduction

This guidance addresses certain issues that arise in premarket submissions for flow cytometric devices used as *in vitro* diagnostic devices for leukocyte immunophenotyping and provides suggestions on the content of submissions for these types of devices. It is intended to be used in conjunction with the other cited guidance documents referenced herein. In preparing your submission to the FDA, we recommend that you contact FDA's Office of *In Vitro* Diagnostics and Radiological Health (OIR) for additional information regarding your submission. This guidance focuses on issues relevant to flow cytometric device with its expanded scope of review topics that reflect the recognition of a flow cytometric device as an analytical system, which includes processing reagents, processing instrumentation, flow cytometers, and analytical software, in addition to the monoclonal antibody component. The information presented in this guidance is based on 1) current basic science, 2) clinical experience, and 3) previous submissions by manufacturers to the FDA. As advances are made in science and medicine, the content of this guidance will be re-evaluated and revised as necessary to accommodate new knowledge.

This guidance is directed toward immunophenotyping of leukocytes using monoclonal antibodies (mAb). However, the concepts may be applicable to related devices that utilize fluorochromes or fluorogenic substrates to measure ligand binding on solid particles in suspension, with or without monoclonal antibodies. This guidance does not cover microscopy devices utilizing fluorescent or chromogenic enzyme-substrate detection methods (e.g., immunohistochemical stains) nor does it cover the use of flow cytometry for cell enrichment and cell sorting/purification when used in cell therapy product manufacturing.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in Agency guidances means that something is suggested or recommended, but not required.

II. Background

Flow cytometry is a rapid, dynamic method of multi-parameter, multi-color (poly-chromatic) single-cell analysis. It is widely used in clinical laboratories for the enumeration of cell populations based on determination of cellular antigen expression using antibodies (monoclonal or polyclonal) or other appropriate fluorochrome labeled reagents.

Flow cytometry relies upon appropriate optimization of the reagent components, cytometer (configuration and settings), acquisition parameters, analysis protocols, and operator interventions in order to yield an accurate result. Because each of the above elements is a critical component in the generation of an accurate flow cytometric result, each element needs to be characterized and clearly defined in the pre-market submission before the device can be authorized for marketing. This guidance document is intended to outline these elements as they relate to validation of flow cytometric devices and to the preparation of a pre-market submission.

This guidance document addresses review issues associated with these devices. The suggestions, if followed, should lead to a timely premarket notification [510(k)] review and clearance. This document supplements other FDA documents regarding the specific content requirements of a premarket notification submission. You should also refer to 21 CFR 807.87 and CDRH's Device Advice http://www.fda.gov/medicaldevices/deviceregulationandguidance/default.htm .

Guidance on the content and format for abbreviated and traditional 510(k)s is available at http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm084365.htm.

Principle of the Test

Typically, a specific, fluorochrome-labeled mAb is added to a sample of whole blood or isolated cells in suspension. After an appropriate incubation period, the specimen may be treated with a reagent to lyse red blood cells and to fix the labeled cells for added stability. Following specimen processing, the labeled cells are aspirated by the cytometer, which distributes them as single cells in suspension and passes them one-at-a-time (single file) through a laser that excites the fluorochromes attached to the cell. The fluorescent and scattered light that emanates from each cell is sequentially collected by a series of filters and

mirrors, and the light is transduced, by the photomultiplier tubes (PMTs), to an electronic pulse that is counted by the cytometer's computer. The data for each collected parameter are interpreted with the use of acquisition and analysis software. This software typically utilizes 'gates' to permit the restriction of data analysis to specific subsets (e.g., lymphocytes) and integration cursors to permit the definition and enumeration of events that are considered 'positive' for that parameter (e.g., 75% of the lymphocyte population is CD3-FITC+).

III. Scope

The scope of this document is limited to flow cytometric devices identified and classified under 21 CFR 864.5220 with product code OYE. The recommendations found in this guidance do not supersede the requirements found in the guidance entitled "Class II Special Controls Guidance Document: Premarket Notifications for Automated Differential Cell Counters for Immature or Abnormal Blood Cells" (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm092780.htm) but, when this guidance addresses topics found in the special controls guideline, it provides recommendations about how those requirements should be addressed for flow cytometry devices.

IV. Policy

The following are certain review issues specific to flow cytometric devices that we would like to clarify:

A. Risks to Health

FDA has identified the risks of false negative test and false positive test results, both of which can lead to individual and/or public health consequences, as risks to health associated with this device. Failure of flow cytometric device to perform as indicated or an error in interpretation of the results may lead to inappropriate assessment and improper management of patients with significant implications. Specifically, a falsely low or falsely negative value in the setting of immunodeficiency could lead to premature treatment or in the setting of malignancy, to delayed or sub optimal treatment. A falsely high or falsely positive value in the setting of immunodeficiency could lead to delayed or sub optimal treatment or in the setting of malignancy to unnecessary testing and surgery.

We recommend that you also conduct a risk analysis, prior to submitting your premarket notification, to identify any other risks specific to your device. The premarket notification should describe the risk analysis method. If you elect to use an alternative approach to address a particular risk identified in this guidance document or have identified risks additional to those in this document, you should provide sufficient detail to support the approach you have used to address that risk.

B. Device Description

FDA considers a flow cytometric device to be the combination of reagents, instruments and software that are used to generate a clinical result. Although this document will focus on the validation of reagents for use in flow cytometric devices, consideration will also be given to instrumentation and analytical software, due to the interdependence of these components in the generation of an accurate result.

In your 510(k) submission, you should identify the regulation, the product code, and a legally marketed predicate device. You should include the following descriptive information to adequately describe your device.

1) Intended Use

For marketed systems, the intended use should describe what analytes and parameters (e.g., CD3, CD19, percent, absolute count, and cellular concentration) are measured with the device, and the clinical indications for which the device is used. For flow cytometry this information encompasses the patient population (e.g., gender, age, and condition, if specified), the clinical purpose (e.g., diagnostic, prognostic, and monitoring), the specimen type, and the specific instrumentation (e.g., manufacturer, model and software version) that the device is intended for.

An example of such an intended use for a flow cytometric device might read:

The ABC T-Type antibody reagent is intended for use with the ABC 2000 flow cytometer, ABC lysing reagent and ABC T-Type analysis software (v1.0). The system is intended for in vitro diagnostic use in the immunophenotypic identification and enumeration of dual CD3+CD4+, dual CD3+CD8+ and total CD3+ lymphocyte percentages and absolute counts, in peripheral whole blood by flow cytometry. This reagent is used to determine lymphocyte percentages and is to aid in the diagnosis of adults with immune deficiency.

Alternatively, instrument labeling may refer to reagents in a table or appendix which are intended for use with that instrument, and reagents may make reference to a separate list of compatible instruments that have demonstrated performance with those reagents. Compatible instruments in this instance refer to instruments having received FDA approval or premarket clearance and that have similar excitation and detection systems that are appropriate for the specified reagents.

Your intended use statement should be sufficiently similar to the predicate so that a decision of substantial equivalence can be made. If your device does not have a legally marketed predicate device with the same intended use, an alternative regulatory path [Premarket Approval (PMA) or “de novo” pathway] may need to be pursued, depending on the risk associated with the intended use of the new device.

2) Test Methodology

You should describe in detail the methodology and principles of your test. You should include a description of the reagents, instrumentation, analytical methods and reported parameters.

3) Reagent Characterization

You should provide data to show how your reagent combinations were selected (e.g., fluorochrome selection and concentration) such that they support the intended use. Fluorochrome should be selected for use with each antibody based on the antigen density expressed by the target cell population of interest. For example, you may choose a fluorochrome with the best quantum efficiency/yield as the antibody conjugate to identify the lowest antigen density, and vice versa. Reagents optimized for fluorescence intensity measures may be titrated to permit saturation binding while other intended uses may perform adequately with sub-saturating concentrations of reagent. The reagent concentration should be selected to account for the variance in cell concentrations and antigen density that is expected across the clinical spectrum for the intended use population. Once optimized, the device should function adequately for specimens having cell numbers within the limits for specimen cell counts that you provide in your labeling. Dilution curves for each component antibody, utilizing specimens with high antigen expression (i.e., median fluorescence intensity; (MFI) and elevated cells/µL, as appropriate), should be provided that describe the relationship between concentration of reagent and MFI and document binding from background through plateau (saturation). Graphical representation of curves should be provided (e.g., antibody concentration (x-axis) vs. MFI (y-axis)) along with the line data for your reagent optimization experiments. Include, when applicable, in a single graph, overlays of the curves indicating total binding, nonspecific binding, and net or specific binding. For more information see the discussion on Signal Detection Sensitivity (section 9.a). You should indicate on the curve the concentration selected for use in your device. If you intend to market a device with the intended use of discriminating different levels of antigen expression, you should provide data that indicate that your device incorporates a reagent concentration that will permit discrimination of dim, moderate, and high antigen expression as may be seen across the clinical spectrum of diseases for which your test is indicated.

4) Quality Control

You should describe the methods for quality control (QC) that will ensure accurate performance of your device over time. Describe each analytical parameter that is reported by your device, including fluorescence intensity measures, when relevant. The following information regarding your quality control materials should be provided:

a). Describe the use of control material and how it will assess the reliability of the reagents and the assay's procedural elements. Your device should be cleared with control material that has been previously cleared or that is being cleared in conjunction with your device. You should provide a plan for the development and validation of your control materials.

b). Describe the use of compensation QC and how it will correct for spectral overlap of one fluorochrome into the fluorescent spectrum of another if compensation is required for the assay. Degradation of some tandem dyes in multi-color cocktails may be different compared to when pipetted singly, particularly if the cocktails have been exposed to more or repetitive light or oxidizing conditions. If this happens, you may consider adding the affected tandem dye conjugates freshly to the cocktails of the other antibodies and to the compensation tube, in order to obtain the correct compensation. The use of fluorescence minus one (FMO) tubes may also be instructive.

c). Provide guidelines for visual inspection of all dot plots that is required to ensure correct function. You should provide representative dot plots and histograms of the analysis of control material to indicate the expected results along with limits for determining acceptable performance.

d). Describe what process controls may be added to control for steps that may differ between the processing of the control material and particular specimen types, if a matrix control is not available for all specimen types listed in the intended use.

e). The inclusion of internal quality checks is encouraged and should be described. Such measures may include the verification of internal precision of repeated measures using analytes repeated across tubes or the use of expected relationships to define a quality check (e.g., Lymphosum) [Ref. 1-4].

5) Instrumentation and Software

Due to differing configurations between instrument models and manufacturers, FDA recommends that sponsors identify the instrument(s) for which the reagent is intended, including manufacturer and model, in the application. Similarly, the acquisition and analysis software should be identified with software name and version. It is also recommended that the components (i.e., reagents, software, and instrumentation) be cleared together for the intended use.

If you are seeking clearance of an instrument that has been cleared for IVD use but contains un-cleared open channels, you should obtain prior or simultaneous clearance of those additional channels if the data to be collected in support of an application are generated on those channels. For instance, when a 4-color instrument is cleared for use with a 3-color reagent cocktail, the clearance applies only to the 3 channels that are established with the particular laser-filter-PMT configuration and the specified formulation of the cocktail. The clearance of the remaining channel could be achieved with a separate analytical and clinical validation of a unique reagent (e.g., new analyte). However, the stepwise migration of a previously cleared reagent may provide a more direct path toward clearance of the channel if it is used and marketed only for that use. For example, you may demonstrate equivalence of each of the new channels to a previously cleared channel by selecting reagents that have been previously been cleared (e.g., CD3-FITC, CD56-FITC) for comparison to a similar (e.g., isoclonal CD3 and CD56) antibody conjugated to the new fluorochrome that you will submit for clearance (e.g., CD3-PE-Cy5) in the new channel. In this process, the performance of CD3-FITC in channel 1 is directly compared to that of CD3-Pe-CY5 in channel 4. By testing

several antibodies that are directed against cell surface antigens known to have antigenic densities across the range of detection, equivalence can be demonstrated.

If the instrument contains both IVD and Research Use Only (RUO) or Investigational Use Only (IUO) functionalities, the RUO component should be isolated so as to not interfere with the IVD testing. For more information on RUO and IUO devices, see the guidance entitled "Distribution of In Vitro Diagnostic Products Labeled for Research Use Only or Investigational Use Only" (http://www.fda.gov/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm253307.htm). Neither the IVD software, the IVD labeling, nor your IVD marketing materials should make reference to the research parameters. Prior to beginning any studies, the instruments should be standardized according the manufacturer's specifications. If your device involves alternate instrument standardization, you should provide detailed methods in your instructions for use in your labeling that demonstrates how to standardize (e.g., establish optimal voltage and compensation settings) and verify proper functioning of the instrument.

If you are seeking clearance for use of your device on more than one instrument make and model, you should provide data to demonstrate equivalence across the instruments. Once an instrument has been fully validated, additional instruments may be directly compared to that instrument.

FDA recommends that manufacturers who wish to use 3rd party "Off-The-Shelf" (OTS) software with their devices refer to the guidance entitled "Guidance for Industry, FDA Reviewers and Compliance on Off-The-Shelf Software Use in Medical Devices" at http://www.fda.gov/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm073778.htm. Manufacturers who choose to use OTS software should ensure that their use of the software complies with the quality system regulations.

Please refer to the guidance entitled "Guidance for the Content of Premarket Submissions for Software contained in Medical Devices" at http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm089543.htm.

C. Analytical Performance Studies

We recommend that you participate in the pre-Submission program through the Office of *In Vitro* Diagnostics and Radiological Health (OIR) prior to performing your studies so that you can gain feedback from the agency regarding the appropriateness of your plan.

For each analyte (e.g., clinically valid reported phenotype), you should plan to select and validate each parameter for the stated intended use. Parameters reported from a flow cytometric device might include percentage of a population, an absolute count, or a measure of antigen density. Measures of antigen density can be expressed in the following ways: 1)

qualitative: positive/negative; 2) semiquantitative: dim/ bright or median fluorescence intensity; or 3) quantitative: antibodies bound per cell, molecules per cell, molecules of equivalent soluble fluorophore (MESF), or mean (or median) fluorescence intensity (MFI). Your application should be directed to those indications for which you provide supporting documentation. The application should present the device (a single reagent cocktail or panels including multiple cocktails) as it will be marketed.

Analytical performance may be conducted at one site. For comparison studies using clinical specimens and reproducibility studies, FDA recommends that you evaluate your assay performance at a minimum of three sites; one site may be in-house, and one should be in the US.

1) Specimen Types and Matrices

The analytical performance of your device should be demonstrated using specimen types that accurately reflect the intended use population. Depending on the target analyte, different approaches may be utilized in order to demonstrate performance. When the analyte is known to be expressed in both normal and pathologic states, specimens from peripheral blood of healthy individuals may be used to demonstrate some of the analytical performance characteristics. For example, CD19 binding curves might be initially performed on normal peripheral blood specimens but later confirmed with specimens from B cell lymphoma, if that is the intended use population and the binding characteristics (e.g., affinity and antigen density) are not expected to differ from and can be demonstrated to be representative of the intended use population. Other antigens may be expressed with different densities in the disease state or may only be expressed on pathologic specimens. In this case, the performance of the respective antibody cannot be completely demonstrated with specimens from healthy individuals. Depending on the prevalence of the condition and the specimen type, access to specimens may be limited. In this setting, alternative specimen types such as manipulated specimens (e.g., enriched, stimulated, etc.), cell lines, or lyophilized/stabilized control materials may be considered. However, great care should be taken when substituting specimen types for your validation studies, as differences in the density of antigen expression, post-translational processing of the target epitope, or lineage infidelity may yield results that do not accurately reflect the performance of your device in the intended use population.

Further, differences in specimen processing across different specimen types should be adequately assessed to demonstrate that those differences do not confound interpretation of the data. It should be noted that the use of specimen types other than those for which the device is intended (e.g., purified, cryopreserved or chemically stabilized) will require additional studies to demonstrate that the processing does not affect the scatter characteristics or antibody binding characteristics for each analyte under consideration, as some cell types or epitopes may be differentially affected by the processing.

For most analytes, your analytical studies should be performed with patient specimens from the intended use population, followed in decreasing preference, by specimens from healthy individuals, stabilized control cells, lyophilized control cells, cell cultures, and stimulated or

transformed cells. Any substitutions from the intended use population should be fully documented to describe the reason for substitution and the scientific validity for the substitution. Manipulated specimens or cell lines should not be used in your method comparison or analytical performance studies. Once chosen, one specimen matrix (specimen type, anticoagulant, or processing method) should be used for all studies. Additional matrices may be added for validation of different specimen or processing variables. In such case, a matrix comparison study should be performed to demonstrate substantial equivalence in assay performance.

You should describe how samples are selected with inclusion/exclusion criteria, and include a summary of the subject demographics. Include the number of samples tested, the number of measurements per sample and number of individuals represented. Provide an explanation if multiple measurements are tested per individual (e.g., duplicate draws, over time or multiple tests/readouts of the same draw). Specimens should be selected so that the sample distribution spans the measurement range of the assay and brackets important decision points, such as a cut-off.

Although the methods initially developed for processing blood may be demonstrated to be applicable to the study of bone marrow, cord blood, apheresis, or other types of samples, special care is often necessary to ensure that the cell concentration is appropriate for the amounts of antibody used, and methods for RBC lysis do not impact on the viability of cells of interest [Ref. 5]. You should describe the range of cellular concentrations with which your device will perform adequately and provide instructions regarding the appropriate steps to take when the cell concentrations are outside of this range.

2) Specimen Collection and Handling

Accurate flow cytometric analyses depend upon the proper preparation of specimens in order to preserve optimal ligand binding by the fluorochrome labeled antibodies. You should include in your validation protocol and product labeling all relevant process variables, including reagents, lysing protocol, fixation protocol, and wash steps that were used to demonstrate the operating characteristics of the device. If you intend to market these devices for use with alternative processing reagents, (e.g., different lysing solution), you should provide data to support their use with each reagent cocktail.

Anticoagulated whole blood is the recommended specimen type for immunophenotyping of peripheral blood lymphocytes as automated methods of cell preparation have been developed that permit reliable specimen staining and lysis with high precision. Alternative methods of cell preparation such as density centrifugation should be used with caution as cell subsets may be recovered to variable degrees and lead to erroneous phenotypic evaluation [Ref. 5, 6]. You should specify which anticoagulant(s) the device is to be used with, as this choice may affect device performance or specimen stability [Ref. 3, 6-8,15]. Anticoagulants used for specimen collection of whole blood for lymphocyte immunophenotyping include ethylenediaminetetraacetic acid (EDTA), sodium heparin, and acid citrate dextrose (ACD)

[Ref. 3, 5, 8]. Clinical performance characteristics should be determined with all specimen types or matrices for which your device is intended.

All validations submitted to the FDA in support of clearance or approval of a flow cytometric device should demonstrate the operating characteristics of the device with the chosen anticoagulant(s). Preanalytical variables, such as collection methods, matrix specific specimen stability, storage conditions, etc., should be described in detail for the device.

3) Analytical Specificity

Analytical specificity of a flow cytometric device is dependant upon the following variables:

- Reagent component (antibody and processing);
- Instrument component (cytometer configuration and standardization);
- Analytical component (gating software and cytometrist input); and
- Other specimen related characteristics (e.g., interfering therapeutic agents, auto-antibodies, or other endogenous factors).

a. Analytical Specificity: Reagents

Individual antibody specificity may be supported with literature citing the assignment of a particular antibody clone to a cluster of differentiation (CD) by the World Health Organizations' International Workshops on Human Leukocyte Differentiation Antigens. If the monoclonal antibody has not been characterized as such, you should provide details regarding how it has been characterized.

If the monoclonal antibody has not been characterized by the Workshop on Human Leukocyte Differentiation Antigens, the specificity of the individual components in the cocktail should be demonstrated by verifying that the binding of the antigen molecule with the antibody is as it is expected, based upon existing scientific evidence. The verification of non-CD classified antibodies may include demonstration of their binding to Western Blots of the target antigen, competitive inhibition of their binding to cells with recombinant target protein, and the lack of binding to other related proteins. Verification should also include a demonstration that the proper lineage or cell marker is identified using your flow cytometric method. In addition, specificity should be demonstrated by showing that the binding of each antibody in the multi-color reagent cocktail (single parameter analysis) is equal to that of the individual antibody-conjugates when used to stain the antigen expressing cells at the same concentration as in the final cocktail. If the patterns of expression or the numeric determinations of percent positive or fluorescence intensity differ significantly between the individual reagent and the final cocktail, interference between the components would be indicated. You should provide the protocol description, histograms, and dot plots of the analyses as well as the numeric data in tabular form to demonstrate that the combination of the reagent components in the cocktail does not alter the binding characteristics of the individual antibody.

b. Analytical Specificity: Instrumentation

Flow cytometry instrument components include, but are not limited to, fluidics, optics, laser source, signal amplification, and acquisition and analysis software. You should specify the instrumentation that was used for validation with your device. You should include in your description the instrument manufacturer, model, and software version. You should specify the instrument standardization process if it differs from the manufacturer's recommended procedure (e.g., voltages adjusted for washed specimens). If your device was validated on an instrument previously cleared for use, you should describe any modifications made to the instrument components or standardization process to accommodate your device.

c. Analytical Specificity: Specimen and Data Analysis

The analytical protocol and software components are considered part of the device. You should provide standardized protocols (Instructions for Use) that permit consistent performance across sites and across time for each analyte and intended use.

The Instructions for Use should describe the gating strategy used in the software for each reagent cocktail. Gating strategies may differ based on the reagents and intended use. For instance, you may intend to report that a population is positive/negative for a marker, that it falls within a defined fluorescence range with bins defining dim, moderate, and bright, or to quantify the antigen expression with fluorescence intensity standards, percent of population, numeric cellular concentration, etc. The labeling should provide detailed instructions for use that define how positive events are objectively distinguished from negative events. Optimally, the discrimination of positive events will be automated utilizing software algorithms that can identify populations based on their distribution or based on the use of paired isotype matched control antibodies. When such automated methods are not practical or possible, FDA recommends that the device be supplied with a software template for each antibody cocktail, which includes a standardized analysis strategy to manually identify the populations of interest and to objectively place cursors and perform compensation. The Instructions for Use will optimally include an atlas of representative analyses and common artifacts to permit the identification of relevant patterns of expression. Your Instructions for Use should provide a protocol for responding to such artifacts and instructions for interpretation and reporting of results.

d. Interfering Substances

You should evaluate the effects of potential interferents on your assay. Potential interferents include endogenous and exogenous sources, which may include autoantibodies and therapeutic reagents (e.g., rituxan, OKT3, etc). If interference is claimed to be minimized due to a wash step, then it should be demonstrated that potential interferents do not bind to cells with sufficient affinity that they resist elution with a wash step. Demonstrate that the potential interferents and remediation protocols do not alter the binding of your antibodies.

e. Carryover

An assessment should be made of the degree of carryover from one tube to the next during acquisition to demonstrate that neither stained cells nor unbound reagent is passed to the

subsequent tube. Testing for carryover is typically performed by running several specimens with high concentration of analyte followed by low concentration of analyte [Ref. 15]. While this method may be adequate for demonstrating cell carryover, additional studies may be warranted to demonstrate that no unbound reagent is carried over from stained specimens to tubes that have not been treated with fluorescent labels. These latter studies may be particularly relevant when the assay includes stained no-wash procedures, or utilizes nucleic acid binding or other novel dyes.

4) Analytical Sensitivity

Analytical sensitivity in flow cytometry encompasses two different concepts:

a. Signal Detection Sensitivity

The first definition of analytical sensitivity refers to the ability of the fluorochrome labeled antibody and appropriately standardized instrumentation, to permit the distinction between cells not expressing the antigen and those cells that express the antigen. This parameter is dependent upon the fluorescence intensity of the positively stained cells (specific staining plus nonspecific binding plus autofluorescence) relative to the cells that are negative (nonspecific binding plus autofluorescence). The specific fluorescence intensity is in turn dependant upon the antigen density of the analyte on the population of interest, the affinity of the monoclonal antibody, the concentration of antibody added to the cells, the spectral characteristics of the fluorochrome that is attached to the specific antibody (e.g., maximal absorption wavelength, stokes shift /excitation wavelength, emission wavelength, and energy transfer efficiency of tandem dyes), fluorochrome to protein (F:P) ratio, the flow cytometric device's detection sensitivity (lenses, mirrors, filters, PMT's, voltage, compensation, etc.), and the potential effect of other fluorochrome labeled antibodies in multi-color assays. This ability to discern a positive population from a negative one using your system should be demonstrated on antigen expressing specimens for each analyte in each reagent combination by utilizing specimens having antigen expression across the range of possibilities for the intended use population. The data can be drawn from the specimens performed in the comparison study. Data collected on each specimen should include the MFI of the negative population, MFI of the positive population, the ratio of positive MFI to negative MFI, and the effective delta (ED) for the populations. For most populations that appear to be normally distributed, the ED can be calculated as: ED = (Positive MFI – 2x SDp) – (Negative MFI + 2 x SDn) where SDp and SDn equal the standard deviation values for the positive and negative populations, respectively. Statistical summaries should be provided that describe each of the above parameters. Matched representative histograms should also be included for both the test reagents and the isotype controls (if used).

When the positive (or bright) population is not clearly separated from the negative (or moderate) population, a nonspecific binding curve may be useful to define the level of nonspecific binding of the fluorochrome labeled antibody at each tested concentration. This curve can be generated by pre-blocking the cells with saturating concentrations of unlabeled analyte-specific antibody, followed by incubation of pre-blocked cells with different concentrations of fluorochrome labeled antibody. Because all of the antigenic sites for the

antibody are pre-blocked with unlabeled antibody, the only fluorescence increase associated with the cell should be due to nonspecific adherence of the labeled antibody to the cell. Knowing the concentration of labeled antibody that was added, a curve of nonspecific binding can be generated and the data points used to subtract the non-specific fluorescence values from the Total Fluorescence Curve (generated from the non-blocked binding curve) to yield a Net or Specific Binding curve (see Figure 1, below).

An example of this approach would involve the following steps which are performed iteratively as needed:

1. Selection of the candidate fluorochrome labeled antibody
2. Acquisition of an un-conjugated isoclonal antibody or different monoclonal antibody that has high affinity for the same antigen of interest and which is capable of competitively blocking the candidate reagent (fluorochrome labeled antibody). This unconjugated antibody should be available at high enough concentrations to permit complete blocking of all available antigens on test samples.
3. Testing and selection of fresh specimens (within 6 hours of draw) that have been demonstrated to have cell numbers and antigen expression at the upper limits of the expected range. Note: failure to select the appropriate specimens may result in sub-optimal device performance and will limit the range over which you can demonstrate the performance of your device.
4. Select the appropriate blocking conditions:
   (a) Prepare a series of samples of the selected specimen(s) and pre-incubate them with serial dilutions of the unconjugated isoclonal antibody across a range of concentrations, including 0. Following incubation (optimal times and temperatures may vary for each antibody), the specimens are resuspended in the volume of the appropriate matrix intended for testing in your device (e.g., 100 uL; plasma if using whole blood).
   (b) To the resuspended specimens, a volume of labeled antibody is added and incubated for the time and temperature recommended for your device. Once completed, the specimen processing is completed according to the protocol for your device and the specimens are analyzed to demonstrate the concentration of unlabeled antibody required to maximally block the labeled antibody. Note: failure to completely block binding may indicate insufficient blocking by the unconjugated antibody (due to low concentrations or insufficient incubation time) or excessive nonspecific binding of the conjugated antibody and may indicate that additional testing is required. Repeat studies with higher concentrations of unconjugated antibody, lower concentrations of conjugated antibody, or other clones may be involved as appropriate.
5. Prepare Specimens for assessment of total and nonspecific binding:
   (a) Prepare two series of specimen tubes (e.g., one series labeled blocked and the other labeled stained; there should be 12 specimen tubes of each series) and aliquot samples of specimen known to express high antigen levels to each tube. To each tube of one series, add the unconjugated antibody at the optimal concentration

demonstrated in the preceding step and incubate to permit blocking of all available sites.

(b) Prepare a dilution series (n=12) of conjugated candidate antibody across a range of concentrations from undiluted (neet) to zero, with sufficient volume of each dilution to stain two samples. Add one volume of each dilution to the respective tube of the blocked series and add one volume of each dilution to the respective tube of the "stained" series. Incubate and complete processing according to your device protocol.

6. Analyze specimens.

(a) Create an analysis protocol that will permit you to set a gate on the population of interest (e.g., lymphocytes) and assess the percent and median fluorescence intensity of the negative and positive staining populations for each tube.

(b) Analyze each tube for each series

(c) Collect the median fluorescence intensity (MFI) of the positive peak for each tube of the stained series and plot on a graph the MFI on the y axis vs. the concentration of conjugated antibody added to each tube on the x-axis. This graph represents the "Total Binding" of the antibody.

(d) Collect the MFI for each tube of the blocked series and plot on a graph as an overlay to the preceding graph, the median fluorescence intensity on the y axis vs. the concentration of conjugated antibody added to each tube on the x-axis. This graph represents the "Non-specific Binding" of the antibody.

(e) For each concentration, calculate the net-MFI by subtracting the MFI of the blocked sample from the MFI of the stained sample for each concentration. Plot the net-MFI on the preceding graph. This graph represents the "Specific Binding Curve" for the antibody.

(f) Select a concentration of antibody that has an appropriate level of specific binding relative to non-specific binding for your Intended Use. Label the graph to indicate the selected concentration. Note: failure to demonstrate a concentration that has both a high level of specific staining and low non-specific binding may indicate that the monoclonal antibody is of low affinity and inadequate quality for your intended use.

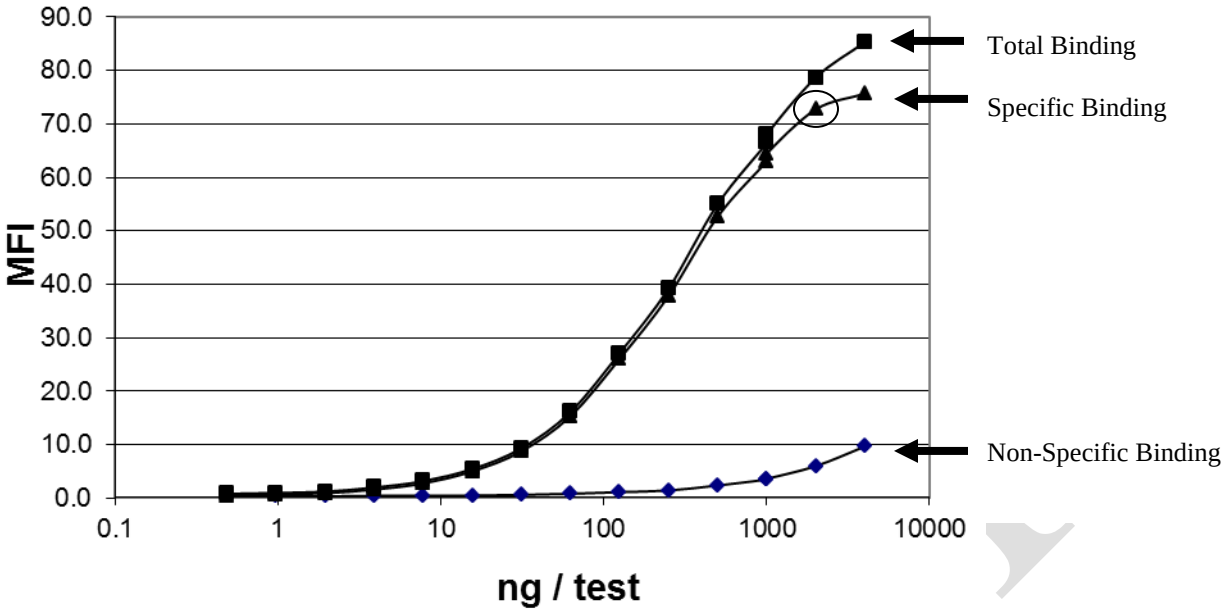

Figure 1. Representative binding curves. Circled triangle indicates concentrations selected for use in the device.

b. Enumeration of Rare Events

The second definition of analytical sensitivity refers to the lowest percentage of positively stained cells that can be quantified. The 2006 Bethesda International Consensus Recommendations on the Immunophenotypic Analysis of Hematolymphoid Neoplasia by Flow Cytometry [Ref. 9] suggests the following targeted sensitivities be permissible with the test system: B cell, 0.1%; T cell, 1%; Myelomonocytic, 0.5%; and Plasma cell, 0.1%. These parameters can be validated by spiking cell suspensions that are negative for the marker of interest with serially diluted aliquots of a specimen (or cell line) that expresses the analyte of interest. In many flow cytometric assays, the number of acquired events must be robust enough for clinically relevant precision. Verification that your system can detect the analyte of interest down to the claimed concentration can be demonstrated by determining the Limit of Blank (LOB), Limit of Detection (LOD), and Limit of Quantitation (LOQ) for your device. See CLSI EP17-A Protocols for Determination of Limits of Detection and Limits of Quantitation [Ref. 10]. CLSI EP17-A defines the LoB as "the highest value we expect to see in a series of results on a sample that contains no analyte." For a flow cytometric device, this would be the number of false positive events that may occur due to potential factors such as inaccuracies in cursor placement, distribution in fluorescence intensity of negative cells, non-specific binding, doublets, debris, etc.

Whether one or both of the above definitions of sensitivity are applied to the validation of a new device will depend upon the intended use of the device and the expected intensity of the analyte of interest. An intended use of detecting minimal residual disease using a marker

with dim expression would involve the demonstration of both signal detection sensitivity and enumeration of rare events.

5) Linear range

The linear range, or analytical measuring range, should be determined for all numerically reported parameters. Appropriate variables to consider include potential cell concentrations and potential antigen density. See CLSI EP06-A Evaluation of the Linearity of Quantitative Measurement [Ref. 11]. Note that the direct dilution (with buffer or plasma) of a specimen that contains a high concentration of cells with a particular phenotype may be adequate to demonstrate the linearity of the absolute count for a single platform method (e.g., cells/µL). However, this approach will not provide data on the linear range with respect to the percentage values since the relative proportions of cellular subsets should remain unchanged over the dilution scheme. In order to demonstrate linearity of the percentage of values, a sample with a high concentration of the phenotype of interest can be diluted with a specimen that lacks that phenotype.

6) Precision: Repeatability

Repeatability should be determined for each reported analyte and parameter for your device. As indicated above, the reported parameters, as determined by the intended use define the extent and type of studies that are required. For instance, if the medically significant value is a percentage, precision studies should be performed to demonstrate the precision for generating a percentage. If the measure is a cellular concentration (cells/µL), the precision should be determined relative to the number of events collected for the population of interest.

Repeatability should be demonstrated using clinical specimens that are prepared 20 times and analyzed in batch fashion. Specimen types may include whole blood, bone marrow, lymph nodes, or other specimen types as defined in the intended use. Effort should be made to utilize specimen types for which the device is intended. Because lyophilized mononuclear cells or cell lines may differ from the intended clinical specimens in matrices, antigen expression, or stability, the data generated using alternative specimen types may not reflect the actual level of precision that can be expected for your device using the intended clinical specimens.

You should define the repeatability of the device using specimens at the cutoff as defined for the intended use. If a clinical cutoff is not defined, provide measures of repeatability at 3 levels that span the reportable range from low, medium and high and across the expected range for the intended use population. These measures should be provided for each parameter of each analyte that is reported in the labeling. For instance, if CD38 is to be reported as percent positive, number of cells/µL, and fluorescence intensity, then precision should be determined for each of these parameters. If CD3 and CD3+CD4+ are reported, then precision for both analytes should be provided. Summary tables should be provided with references to files containing line data and representative histograms, etc. For statistical methods and additional considerations regarding protocol design, see CLSI EP5-A2

Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline-Second Edition [Ref. 12].

7) Precision: Reproducibility

Reproducibility is calculated similarly to repeatability, see section 11. "Precision: Repeatability" above, using test results assayed between users/instruments/sites, and over time (e.g., day to day) when those variables have the potential to contribute significantly to the total error in the system (site-to-site reliability should be calculated). FDA prefers that reproducibility be assessed in at least three testing sites, with at least one in the U.S.

We recommend that you design the reproducibility study to assess overall analytical performance (e.g., including sample preparation and analysis). You should characterize the reproducibility of the reagent/analytical systems for your clinical flow cytometric device using a set of well-characterized, positive samples. You should include specimens at the cutoff as defined for the intended use. If a clinical cutoff is not defined, provide measures of reproducibility that span the reportable range from low, medium and high and across the expected range for the intended use population. You should include two or more operators at each site and three or more instruments in the study, ensure that samples are masked and expected results are unknown to operators. Where specimen availability and stability permit, you should include at least 20 days and at least 2 replicates per day. See Section 6 for further discussion on specimen selection. Reagent lot-to-lot studies, using three different lots, may be conducted in-house. For monoclonal antibodies, these three lots should be unique in formulation in that the monoclonal source material should be distinct for each of the three lots, and conjugated with different fluorochrome lots if applicable.

You should demonstrate reproducible performance for each reported analyte in the assay, and provide the protocol (including statistical methods), results, and analysis for between-assay, between-instrument, between-operator, or other evaluations, as appropriate. You should perform separate calculations for each specimen tested for within-run and total precision for each instrument or method used. For qualitative assays, provide the percentage of results that are negative, borderline/equivocal (if appropriate), or positive for each test procedure in a frequency table. For quantitative/semi-quantitative or numeric results, you should present the coefficient of variation (CV) for each set of values for with-in run and total precision. If applicable, we recommend that you report the coefficients of variation (CV) with confidence intervals, for between- instruments, operators, device lots, and intra- and inter-assay, as appropriate; pair-wise correlation coefficients; scatter plots; and ANOVA analysis of data from all relevant elements of the reproducibility study. You should also report any additional metrics, as appropriate, and provide the data collected in tabular form. Identify any bias that you observe during your reproducibility studies, and provide an explanation to account for the bias.

Your protocol should stress the device to an appropriate degree, i.e., you should not use an assay system that is so robust as to obscure changes in sample integrity that may occur during preparation or assay steps. For statistical methods and additional considerations regarding

protocol design, see CLSI EP5-A2 Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline-Second Edition [Ref. 12].

8) Stability

Specimen stability: You should demonstrate that the anticoagulant, holding temperature, and preparation method maintain specimen integrity similar to freshly processed material. Your specimen stability study should include normal and abnormal specimens with analyte levels across the measuring range, and challenge the full range of the claimed specimen age. Until specimen stability studies are completed to demonstrate the maximum stability, you should maintain blood samples at 18-22°C and process within 6 hours of collection. You should state specimen stability and storage and shipping conditions (for unprocessed and processed specimens) in the Instructions for Use and provide data or appropriate literature references in the submission to substantiate any claims made. Please note that some antigens show relatively greater variability than others with specimen age. If your device uses cryopreserved cells, you should state the stability of the cryopreserved cells and provide data to demonstrate cell stability regarding the assayed parameters by your device.

Processed specimen stability: You should state the stability and storage condition of the processed/stained specimens and recommend how soon the processed samples need to be acquired on the instrument. You should provide data to support any claim made.

Reagent stability: In addition to the stability studies described for specimen stability, you should provide protocol descriptions and data to demonstrate the stability of your device and reagents (real-time, open-vial, freeze-thaw, shipping, etc). If you instruct the user to prepare antibody cocktails within the laboratory, you should state storage condition of the cocktails and how soon the cocktails should be used to stain the specimens.

D. Performance Studies Using Clinical Specimens

1) Study Population

For all studies using clinical specimens you should provide the following information:

- Inclusion/exclusion criteria (including a description of the sample collection protocol for stored samples);
- Clinical status (diagnosis, stage of illness, and signs/symptoms);
- Indicate how the diagnosis was made (criteria, laboratory tests, and physical examination);
- Indicate who made the diagnosis (i.e., specialist, generalist);
- Demographic information and the prevalence of disease, condition, signs/symptoms;
- A description of the samples matrix. The matrix should be consistent with intended population;
- Indicate if specimens will be collected fresh or if they were archived. If archived, indicate how they are stored and how their integrity is assessed; and
- A sound statistical basis for the determination of adequate sample size (N).

2) Expected Phenotypes

You should provide a clear description of the expected expression of each analyte for each intended specimen type, so as to permit the interpretation of results and appropriate lineage assignment. In addition to the expected expression in the targeted conditions, the expected expression should be provided for normal individuals (including all specimen types in the intended use) and for other conditions (common infections, disease states, or co-morbidities) that may be encountered clinically.

3) Normal and Abnormal Ranges

For each intended use, you should define a clinical decision point in order to permit the distinction between the normal and the abnormal state. You should demonstrate your assays ability to distinguish between healthy and diseased states by evaluating samples from each population.

a. Normal /Reference Range

FDA recommends that a normal reference range be established with analysis of anticoagulated whole blood samples from a minimum of 50 normal donors from each of three geographically diverse sites, which may include the manufacturer's own site. These donors should include all ethnic groups, genders, and ages encompassed by the intended use. You should test each cell isolation protocol claimed in the intended use with the 50 samples and establish a normal range of CDx positive lymphocytes using the new test with samples from a minimum of 50 normal persons (a healthy, asymptomatic population and/or in a population with similar signs or symptoms as the target population for the device). You should characterize the population study according to age, sex, geographic location, and symptoms of disease. You should state the population demographics and statistical method used in the package insert. All sample type(s)/matrices should be considered in the studies. A manufacturer may reference literature for a normal or expected value if the referenced studies used the same methods and materials and reported the same metrics. If using published reference values, the values should be independently validated with your device using specimens from the population(s) for which the device is intended. Line data and statistical summaries should be provided for relevant parameters, including percentage positive for individual markers and composite phenotypes and fluorescence intensity measures for each antibody.

b. Abnormal Ranges

Any claims or references to disease states should be supported with data utilizing a minimum of 50 specimens from the intended use population. The patient population/ patient conditions should span those that are expected to be tested with the device. A minimum of 10 samples should be tested for each disease or condition unless collection of such samples is impractical due to the rarity of the disease. The sampling for these disease states should be as diverse as possible and span the range of expected results. Determine the range of results for each reported analytical parameter for each specific claimed disease state. Line data and statistical

summaries should be provided for relevant parameters, including percentage positive for individual markers and composite phenotypes and fluorescence intensity measures for each antibody.

c. Selecting a Predicate or Reference Method

You should propose a predicate device or reference method for each analyte and Indications for Use against which you will seek clearance, and compare your device (or component analyte(s)) to the previously cleared (predicate) device. If your device measures both novel and established analytes, comparison to a predicate should be made where possible, with additional strategies employed for the remaining analytes.

Where no predicate exists, you should clearly define the methods that will be used to establish truth. If your device is the first of a kind or contains new analytes, you may support your application by demonstrating agreement with a clinically validated, standard of care, reference method, if one exists. For such studies, establishing truth may also require the demonstration of the performance of the assays relative to the expected phenotype of specimens with a documented diagnosis. As the expression patterns of different antigenic markers may vary within a diagnostic entity and between specimen types, a clearly defined statistical approach should be defined, bearing in mind that different approaches may be required for different analytes or conditions within a single device. When data are drawn from multiple sites, the analytical methods and diagnostic definitions should be consistent across the study sites (e.g., follow a single protocol) and have demonstrated reproducibility and accuracy. If a standard of care reference method for your device is not currently utilized as the standard of care for the condition of interest, clinical studies to support a premarket approval (PMA) may be involved.

When a diagnostic claim is intended for your device, comparison to a clinical diagnosis may be appropriate. When a target antigen is not consistently expressed in or is not pathognomonic for a particular condition, comparison of your device's result to a diagnosis may not be appropriate. Flow cytometric devices that have a monitoring or prediction claim should demonstrate the utility of the device over the course of disease, following treatment or remission. If a diagnosis is known to correlate with the expression of only a few markers in the panel, then a validation based on clinical diagnosis can only be applied to these markers. If additional analytes are present in the device for other purposes (e.g., staging), then additional validation criteria should be used for a full assessment of your device. You should include in your proposal a clear plan for validating each reported result.

For the method comparison study, the minimum number of specimens used should be adequate, statistically justified, and spread throughout the claimed analytical measurement range of the method or device. The comparison samples should be the same for all instruments. You should submit a detailed protocol describing how the samples were selected, specimens were processed, and data were collected. Also provide a statistical summary that includes graphical representation of the linear regression and a representative

number of histograms and dot plots, for all flow cytometric devices used. Deming regression and Bland-Altman plots should be utilized to evaluate the device's performance.

d. Statistical Analysis

For each study, you should present a plan for how data will be analyzed (e.g., identify independent and dependent variables). You should provide the reported reference range for each parameter for the candidate and reference methods. You should describe how the cut-off and reference range is determined and validated [Ref. 13, 14]. You should describe expected results and define or explain calculations. You should determine equivocal zones (if applicable) and describe if and how discrepant results will be resolved. You should provide the expected rate of clinical false positives and false negatives, if known. The data and statistical evaluation should be sufficient to determine if the device is substantially equivalent and/or safe and effective for all claimed specimen type(s)/matrices.

For all comparison data (e.g., versus the predicate device or instrument-to-instrument), you should provide the graphs and equations for the linear regression. You should provide representative histograms and dot plots to illustrate the expected appearance of labeled populations in normal and abnormal specimens as would be expected from the intended use population, for all flow cytometric devices used. You should provide analyses of median intensity measures and reported parameters for all relevant populations. You should include the statistical summaries from the normal and disease states and include the mean, SD, and the percent coefficient of variation (CV) for measures of precision. For each reported parameter, you should present all data, in tabular form, listed and sorted based on the submitted device. You should present the data from the disease states as a total and listed by age, ethnic group, and sex.

E. Labeling

Labeling must comply with the requirements set forth in 21 CFR 801 and 21 CFR 809.10, and must include adequate directions for use. These directions should include detailed instructions that define how gates and cursors are set for the intended use and how positive events are objectively distinguished from negative events. Optimally, the discrimination of positive events will be automated, utilizing software algorithms that can identify populations based on their distribution or based on the use of paired isotype matched control antibodies. When such methods are not practical or possible, FDA recommends that the device be supplied with a software template for each intended use, which includes a standardized analysis strategy to manually identify the populations of interest and to objectively place cursors or identify aberrant populations. If an electronic software template is not provided, detailed stepwise instructions should be provided to permit the recreation of the same analytical methods (population identification, gating, and cursor placement for definition of positive events) used in your submission for clearance or approval. Where stepwise instructions are provided in lieu of an analysis template, you should provide a user study to demonstrate the accuracy of results obtained with user generated analysis protocols.

Instructions will optimally include an atlas of representative analyses and common artifacts. Your instructions should provide a protocol for responding to such artifacts, and instructions for interpretation and reporting of results. When software algorithms for automated analysis are incorporated in your device, you should provide information regarding the identification of failures in the algorithm as well as a means to correct the analysis, if possible.

In addition to instructions for the identification of cells of interest, you should include, in your study design and instructions for use, the protocol for instrument calibration and instrument setup for analysis, if they are not provided or are different from those recommended in the manufacturer's instructions for the instrument.

Appendix

List of References

1. Giorgi, J.V., et al., *Quality control in the flow cytometric measurement of T-lymphocyte subsets: the multicenter AIDS cohort study experience. The Multicenter AIDS Cohort Study Group.* Clin Immunol Immunopathol, 1990. 55(2): p. 173-86.

2. Calvelli, T., et al., *Guideline for flow cytometric immunophenotyping: a report from the National Institute of Allergy and Infectious Diseases, Division of AIDS.* Cytometry, 1993. 14(7): p. 702-15.

3. Center for Disease Control, *The 1997 guidelines for performing CD4+ T-cell determinations in persons infected with human immunodeficiency virus (HIV).* Morbidity and Mortality Weekly Report, 1997. 46(RR-2).

4. Schnizlein-Bick, C.T., et al., *Use of CD45 gating in three and four-color flow cytometric immunophenotyping: guideline from the National Institute of Allergy and Infectious Diseases, Division of AIDS.* Cytometry, 2002. 50(2): p. 46-52.

5. CLSI, *Enumeration of Immunologically Defined Cell Populations by Flow Cytometry*, in *H42-A2*. 2007, Clinical and Laboratory Standards Institute: Wayne, Pennsylvania.

6. Paxton, H., et al., *Results of the flow cytometry ACTG quality control program: analysis and findings.* Clin Immunol Immunopathol, 1989. 52(1): p. 68-84.

7. Nicholson, J.K. and T.A. Green, *Selection of anticoagulants for lymphocyte immunophenotyping. Effect of specimen age on results.* J Immunol Methods, 1993. 165(1): p. 31-5.

8. Thornthwaite, J.T., et al., *Characteristics of monoclonal antibody measurements in human peripheral blood.* Ann N Y Acad Sci, 1986. 468: p. 144-59.

9. Wood, B.L., et al., *2006 Bethesda International Consensus recommendations on the immunophenotypic analysis of hematolymphoid neoplasia by flow cytometry: optimal reagents and reporting for the flow cytometric diagnosis of*

hematopoietic neoplasia. Cytometry B Clin Cytom, 2007. 72 Suppl 1: p. S14-22.

10. CLSI, *Protocols for Determination of Limits of Detection and Limits of Quantitation*, in *EP17-A* 2004, Clinical and Laboratory Standards Institute: Wayne, Pennsylvania.

11. CLSI, *Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach*, in *EP06-A*. 2003, Clinical and Laboratory Standards Institute: Wayne, Pennsylvania.

12. CLSI, *Evaluation of Precision Performance of Quantitative Measurement Methods*, in *EP05-A2* 2004, Clinical and Laboratory Standards Institute: Wayne, Pennsylvania.

13. CLSI, *Assessment of the Clinical Accuracy of Laboratory Tests Using Receiver Operating Characteristic (ROC) Plots*, in *GP10-A* 1995, Clinical and Laboratory Standards Institute: Wayne, Pennsylvania.

14. CLSI, *Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory* in *C28-A3c*. 2008, Clinical and Laboratory Standards Institute: Wayne, Pennsylvania.

15. Carter, P.H., et al., 1992, *Flow cytometric analysis of whole blood lysis, three anticoagulants, and five cell preparations.* Cytometry, 13(1): 1992: p. 68-74.