Pharmaceutical Research, Vol. 26, No. 12, December 2009 (© 2009)
DOI: 10.1007/s11095-009-9972-5

Opinion

An Approach to the Validation of Flow Cytometry Methods

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Received June 2, 2009; accepted September 14, 2009; published online October 14, 2009

Abstract. This publication outlines an approach for the validation of flow cytometry methods used in the analysis of a wide range of biomarkers. It is written as a guidance document for method validation in a GLP environment, and from the viewpoint of the pharmaceutical industry, but its relevance is wide-ranging. The approach to method validation described is intended as a starting point for further discussion, as well as providing reference material to colleagues developing fit-for-purpose flow cytometry methods. Pre-validation steps are discussed as prerequisite assessments to determine method and reagent suitability, and to minimise variables during the full validation process. The guide to method validation takes account of the many flow cytometry assay types in use, and provides guidance on the types of assessments necessary to produce a fit-for-purpose method suitable for use in a regulatory environment.

KEY WORDS: biomarker; flow cytometry; GLP; method validation; regulatory.

INTRODUCTION

This publication outlines an approach to the validation of flow cytometry methods used in the analysis of a wide range of biomarkers. The content is based on the existing regulatory guidelines for bioanalysis of small molecules, with additional material that is specific to flow cytometry. It is written as a guidance document for method validation in a Good Laboratory Practice (GLP) environment and from the viewpoint of the pharmaceutical industry, but its relevance is wide-ranging and can equally be applied to Good Clinical Practice (GCP) or non-regulatory environments, including forensic science, health service and environmental science laboratories, veterinary medicine and the agrochemical industry.

Analytical biomarkers, and associated technologies, used in support of the development of potential drug candidates are critical in many stages of the pharmaceutical industry, including drug syntheses, formulation study support bulk pharmaceutical stability monitoring, determination of efficacy, assessment of physiochemical and functional characteristics, bioavailability, toxicity, and assessment of clinical endpoints. Ensuring these biomarker measurements are relevant, robust, objective, accurate and, therefore, fit for purpose is an essential factor in the drug discovery process.

Method validation, as opposed to qualification of the biology, is the process of defining the properties of an analytical method and demonstrating that it is acceptable for its intended purpose. Guidelines, from a variety of regulatory bodies, already exist for use in the pharmaceutical industry to provide a framework for designing and performing bioanalysis validation studies for small molecules (1–3). Equally, clinical diagnostic guidelines published in the US through the Clinical and Laboratory Standards Institute (CLSI) address disease identification, prevention and treatment, whereas in Europe, the Clinical Trials Regulations set the standard for maintaining clinical trial quality through the implementation of GCP. Although these publications set a benchmark for the process of laboratory analysis during clinical trials, detailed guidelines for the validation of methods for the quantification of biomarkers are still not clearly defined and are not currently addressed by any regulatory documentation (4–6). Biomarkers that are analysed using liquid chromatography–mass spectrometry techniques can be validated using a similar approach to that described for small molecules, with the added complication of endogenous analyte levels that mean a zero-background authentic matrix is not always available. Working groups have also generated recommendations for validating chromatographic and ligand-binding assays (5,7), but nothing is currently available for flow cytometry methods performed in a GLP environment. Primarily, methods for regulatory submission must (at the minimum) include studies demonstrating accuracy, precision, selectivity, sensitivity, stability and robustness.

GLP is concerned with the organisational process and conditions under which non-clinical, health and environmental safety studies are planned, performed, monitored, recorded, reported and archived (8). GLP does not assess the quality of the science performed, but ensures processes are in place to achieve a regulatory quality standard of work. Setting standards for assay methods gives confidence in the data produced, and ensures consistency between and within laboratories.

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0724-8741/09/1200-2551/0 © 2009 Springer Science + Business Media, LLC

This report provides practical guidance to applying validation criteria to flow cytometric analyses, with the aim to harmonise the practices employed and provide guidelines/ recommendations of acceptance standards. This approach should be viewed with the understanding that validation requirements are continually altering and vary extensively, and as such will need to be adjusted and modified to tailor to the type of analytical method employed in individual environments. Each laboratory is accountable for providing sufficient data to show that methods provide acceptable performance to meet the objectives of their use, by carrying fit-for-purpose method validation, following the principles of GCP (where applicable) and GLP. Local definitions may vary, but for the purposes of this paper, *fit for purpose* is based on the definition of Lee *et al*. (2006), which describes an approach to method validation that is tailored to match that of the intended use of the data produced (5). As a result, the fit-for-purpose approach is in fact a continuous process that should be reviewed as the application of the biomarker method changes.

The discussion is divided into two areas: (i) pre-validation steps, describing initial investigational work that needs to be addressed prior to the start of the method validation itself, and (ii) validation parameters, which describes aspects of the method that need to be fully assessed before a method can be deemed fit for purpose.

PRE-VALIDATION STEPS

This aim of this stage is to identify any aspects that may render the assay unusable and to minimise variables during the validation process. The pre-validation steps are critical to establish suitability of a method prior to starting validation studies. The range of sample types analysed by flow cytometry are wide and various. Cell preparations are rarely purified and often heterogeneous, with analytes being targeted by light scatter and multiple antibody staining. Such an approach requires careful assay optimisation.

Questions to ask prior to embarking on validating a method:

1. What are the objectives of the assay?
2. What performance criteria are acceptable for the assay in this application?
3. What confidence level is required in the results obtained from the assay; what are the criteria for detecting change?
4. How will consistency in quality within and between every run performed be ensured?
5. Will the assay quality withstand increased throughput if high sample numbers are required?

Literature Review

A literature search should be performed to provide basic knowledge of feasibility of the assay and guidance for the rest of the pre-validation steps, including the investigation of the following:

- Sex differences: for example, Natural Killer cell numbers are lower in human females than males (9).
- Genetic variation: for example, $CD3^+$ cells are significantly lower in the Chinese population (9).
- Species differences: for example, CD14 Expression on rabbit granulocytes is significantly higher than any other species (10).
- Diurnal variation: for example, CD11b shows significant diurnal variability in basal levels (11).

This process should be subject to continual review.

Reagent Choice

Choice of suitable reagents is critical in assay development.

Antibodies, Labels and Dyes

Antibodies, although targeted at the same receptor and conjugated to the same fluorochrome, will vary by manufacturer due to clonal differences, staining intensities, etc. When identifying appropriate reagents, the implications of choosing an antibody classified as for research use only (RUO), *in vitro* diagnostic (IVD) or analyte specific reagent (ASR) should be considered. For example, an antibody classified as RUO (which may not be subject to good manufacturing regulations and hence subject to greater between-batch variability) may be inappropriate in a biomarker assay for clinical trials.

Choice of fluorochrome or dye will be limited by instrument optics and may be conjugated or used indirectly. Knowledge of antigen density will also drive both the selection of fluorochrome, and choice of direct or indirect staining, as these can aid in amplifying a weak signal. The fluorochrome cocktail may be influenced by compensation issues, e.g. combinations of phycoerythrin (PE) and propidium iodide (PI) stain are very difficult to compensate due to the extended spectral overlap of PI. Stability of fluorochromes and dyes also vary e.g. PerCP is particularly sensitive to photobleaching, and certain laser intensities will adversely affect signal.

Tandem dyes may be considered for multicolour experiments when using a 488 nm laser. Due to the nature of these dyes variability between lots may result in inadequate energy transfer, low fluorescence intensity, dye degradation and non-specific binding. However between-batch variability should be evaluated in the use of any antibody and where appropriate the same lot used for the duration of an experiment/study.

An isotype control, where applicable and/or available, should be chosen to match your antibody of choice in terms of both concentration and fluorochrome. In some circumstances, an antibody blocking agent may be a more appropriate choice of non-specific control.

Fixatives, Permeabilising and Lysing Solutions

There are a multitude of cell treatment reagents available for use during sample preparation, all of which have slightly different properties. These include reagents described as stabilising agents, e.g., CytoChex (Streck Laboratories); fixatives, e.g., formaldehyde solution; lysing solutions, e.g., Facs-Lyse (BD Biosciences); hypotonic saline; fix and lyse solutions, e.g., Optilyse B and C (Beckman Coulter); permeabilising solutions, e.g., saponin, methanol; FacsPerm (BD Biosciences); Intrastain A&B (Dako); and blocking

solutions, e.g., brefeldin A, monensin. These primary reagent actions do not limit the range of their use; e.g., saponin can be used to lyse red blood cells, whilst also permeabilising white cells. Reagent choice may be initially driven by the literature and then by the individual requirements of the sample type and assay objectives; e.g., certain lysing reagents can adversely affect cell morphology, making them unsuitable for assays monitoring shape change. This stage of choosing reagents may include a certain amount of trial and error, particularly in the absence of literature guidance.

Antibody Titre

It is recommended that an antibody titration be carried out to ensure that antibodies are used efficiently yet still remain in excess. Consideration needs to be given to disease states where a large range of responses may be expected. Titration should be performed relative to the initial Ig concentration of the chosen antibody, the number of washing steps involved, and manufacturer recommendations. Common dilutions are neat, 1/10, 1/20, 1/50, 1/1000, dependent on the concentration of cells in the sample; e.g., BD Biosciences recommend 20 microlitres per 1 x 10^6 cells. Identifying the 'leveling' off of stained cell number indicates the maximum dilution to use, and the antibody should be used slightly more concentrated than this. Manufacturers will generally recommend an antibody concentration greater than that identified by assay-specific titration experiments, so further investigation can lead to more economic use of the reagent.

Selectivity / Specificity

Due to the nature of the sample types measured by flow cytometry, confidence is needed that the assay methodology will identify the intended target or analyte in the sample material in the presence of other components. A demonstration is necessary that the substance being quantified is the intended one. This need only be a single assessment, capturing the key component of the assay. There are various possible options, including the use of commercial control material, although this has very limited applicability, being largely aimed at clinical users:

- Spiking or competitive binding experiments, for example, using LPS as a competitive agent to compete for CD14 binding sites.
- Cell sorting and image analysis, for example, sorting cell sub-types and staining to demonstrate the stimulated effect by micropscopy, e.g., shape change
- Use of positive control, for example, the use of ionomycin to increase calcium flux, demonstrated by an increase in Indo-1 staining
- Commercial QC, for example, CD Chex CD4 low stabilised blood cells to monitor CD4 staining.

Sample Collection Criteria

Many matrices are subject to flow cytometry analysis, and each will have its own impact on the validation process. Any change in sample matrix type will necessitate the re-evaluation of the method performance. It is essential that strict criteria regarding sample collection be defined at this early stage. Factors to be considered include the following:

Choice of Anticoagulant. The anticoagulants commonly used in flow cytometry are ethylenediaminetetraacetic acid (EDTA), lithium heparin (LH), and citrate. The optimal anticoagulant will be dependent on the nature of the assay; e.g., EDTA is not suitable for functional assays requiring free calcium; certain anticoagulants will affect basal analyte values and therefore assay range. Identified requirements need to be communicated clearly, e.g., coated tubes, rather than solid EDTA.

Sample Handling. Treatment of the sample during and after collection needs to be investigated. Factors to be considered include the following:

- Site of the bleed: Due to variability in analyte levels at different sites of the body, a consistent approach to the site of bleed should be taken; for example, differences in results may be seen between samples taken at rat vena cava *versus* the tail vein.
- Sequence of blood collection: for example, platelet analysis requires samples to be taken neither from the initial blood drawn nor from the end of a large bleed.
- Sampling trauma, as a sample that is difficult to draw may contain activated cells.
- Treatment of sample post bleed: for example, a sample may need to be taken into a chilled tube or require rolling immediately.
- Chance of contamination.
- Centrifugation effects on plasma/serum/cells.
- Sample storage and shipping requirements.

At this point there is no quantifiable quality control measure to monitor these errors; therefore, it is important to emphasise to the responsible individual the rationale for following this procedure. Monitoring the compliance to specific details is an important factor in generating valid data.

Assay Feasibility

Consideration should be given to the demands on the assay for a given study. For example, is there sample stability to cope with the throughput required? Assay feasibility is an initial look at stability; a full evaluation would be necessary as part of the main validation (see "Validation Parameters"). Points to consider include the following:

- Analytical laboratories must clearly define their minimal requirements; e.g., 2-hour sample stability prior to analysis limits location and transport options.
- What cell separation/purification steps will be used? How will, for example, lysing, magnetic bead separation or density gradient columns affect the cells?
- Is a Peripheral Blood Mononuclear Cell (PMBC) preparation more suitable than whole blood? If a separation method for white cells is used, percentage and proportional recovery should be determined; i.e., is there selective loss of cells of interest? Precision of this recovery should also be determined for comparison across runs.
- Is there a functional change of a surface marker expression over time?

- Determination of agonist time course and optimum assay temperature, etc: Are the optimum conditions feasible in the study situation?
- Assay robustness: If the assay needs to run in triplicate, is this feasible performed in a high-throughput study?
- Stabilising agents *versus* fixatives: Is antibody binding required? Are there epitope stability issues? Cells may need to remain viable if they will be further stimulated as part of the assay.
- Suitability of chosen antibody with fixed or lysed tissue.
- Conditions for storing samples, e.g., ice *vs*. 37°C, with or without mixing/rolling. Does the sample need time to stabilise post-collection?

Gold Standard Comparisons

A gold standard is an assay methodology or benchmark that is widely accepted as providing the definitive read-out. If available, a brief comparison to gold standard is recommended prior to the full validation stage. For example, a comparison of bone marrow cellularity by cytological staining would be used to compare to a flow cytometry cell differential. This will provide an estimate of bias (difference between the test result and the accepted reference value), and a comparison of specificity and sensitivity. Results should identify an advantage over the gold standard. This needs not be performance related but may cover local aspects, such as throughput, availability of instrumentation, etc. In the example above, flow cytometry offers more flexibility and faster readout of a larger number of cells.

Defining an Appropriate Readout

Identification of a robust readout is imperative in order to obtain valid data. This needs to take into account at the endpoint of the analysis, given that it may have a history of other use; for example, a research assay that will subsequently be used for clinical Proof of Mechanism. Care needs to be taken that data can be consistently measured between and within assays, and that the readout is appropriately sensitive and robust across the predicted sample types. The measure to be used may be 'unconventional' and is often not simply a change in EC50. Some examples are dose ratio following therapy, or variation in basal scatter to show morphology change.

Robustness

Robustness is the measure of susceptibility of a method to any small changes that may occur during the running of the assay. This could be temperature, pH, operator-related changes, or the logistics of transporting the samples. It is important to assess this initially at the pre-validation stage, ideally under the same conditions as those to be used in studies requiring this support.

VALIDATION PARAMETERS

Having successfully completed pre-validation steps, the following aspects should be incorporated into the formal validation of the method. Due to the specific nature of each assay, it may not be possible to address all of these aspects. Flow cytometry methods are mainly quasi-quantitative or, at best, relative quantitative assays, and should be validated using a fit-for-purpose approach such as described by Lee *et al.* for ligand binding assays. (5).

The steps below are summarised in Table I, which is designed to act as a quick reference guide.

Accuracy

The accuracy of a method is the closeness of the measured result to the true value for the sample. Accuracy is determined by comparing test results from the new method with results from an existing alternate method (gold standard) that is known to be accurate. The mean value should be within 15% of the actual value (12). The deviation of the mean from the true value serves as the measure of accuracy. Data should be generated 5 times per concentration and at a minimum of 3 concentrations in the relevant range.

In many flow cytometry assays it is not always possible to determine accuracy, e.g., when comparing changes in specific cell surface markers in an individual. There is no easy answer, and often no option; however, it may be possible to include internal or commercial QC / calibration material where available, such as receptor density beads. Acceptability of QC data should be based on Westgard or similar rules (13), and the generation of QC data over time will act as a useful guide for monitoring accuracy in longitudinal studies. Use of a positive control material in certain assays may be appropriate to confirm assay performance.

Precision

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly. The precision measurements should be compiled using the appropriate final assay readout. Precision should be determined for both intra- and inter-assay data. Although not a measure of method reproducibility, intra- and inter-subject variability should also be determined. For example, patient data should be determined over repeated time points in order to ascertain natural variability and hence give confidence to a meaningful change in data during therapy. The following precision measurements are interrelated and should all be taken into consideration in order to determine assay reproducibility. During these measurements other variables should be minimised, e.g., multiple analysts, etc. For percentage positive cells it is critical that precision is determined at the lower limit of detection to give sufficient confidence at this level.

Intra-assay

Intra-assay precision is determined using replicate samples (minimum of 5, 10 is recommended) prepared and analysed in a single batch. It is recommended that the precision determined should not exceed 15% (ideally, less than 10% and lower than the inter-assay precision) of the coefficient of variation (CV) (12). However, acceptable limits should be determined locally, dependent on the nature and intended use of the assay.

Table I. Applicability of Validation Parameters for Flow Cytometry Methods

Validation parameters	DNA assay	*Ex vivo* stimulation	Cell surface expression	Receptor density	Intracellular staining	Activation markers	Functional assays	Comments
Accuracy	Y	N	(Y)	Y	N	Y	N	Where specific QC material or internal standards are avaliable
Precision								
Intra-assay	Y	Y	Y	Y	Y	Y	Y	
Inter-assay	Y	N	Y	Y	Y	N	N	Samples may need to be stabilised or fixed prior to storage
Intra-subject	Y	Y	Y	Y	Y	Y	Y	
Inter-subject	Y	Y	Y	Y	Y	Y	Y	
Incurred Sample Reproducibility	Y	N	Y	Y	Y	N	N	Possible only with stable or stabilised study samples, see interassay precision
Stability								
Raw Material	Y	Y	Y	Y	Y	Y	Y	
Prepared Sample	Y	Y	Y	Y	Y	Y	Y	
Assay Range								
ULOQ (linearity)	Y	(Y)	N	Y	N	N	N	*Ex-vivo* stimulation assays: ULOQ is the maximum test compound concntartion for which the assay gives a readout
LLOQ (sensitivity)	Y	(Y)	Y	Y	Y	Y	Y	*Ex-vivo* stimulation assays: LLOQ may be the minimum curve depth or curve parameter readout
Reference Ranges	Y	(Y)	Y	Y	Y	Y	Y	*Ex-vivo* stimulation assays: Assay range is the test compound concentration range within which assay gives a readout
Instrument Monitoring								
Inter-instrument Validation	Y	Y	Y	Y	Y	Y	Y	
Instrument Monitoring	Y	Y	Y	Y	Y	Y	Y	

Inter-assay

Inter-assay precision is determined using replicate samples prepared and analysed in batches over several occasions (5 replicates per run on at least three occasions). It is recommended that the precision determined should not exceed 15% of the CV (12). Due to the unstable nature of many flow cytometry samples, it is not possible to use the same sample repeatedly, and, therefore, a fixed or reference material should be used where available, for example, Streck CD Chex QC reference for CD3 analysis, which uses a stabilised whole blood preparation.

Intra-subject

An individual may show natural variability in a given parameter over time. The level of this variation for an individual should be measured over several occasions in order to determine the lower limit of sensitivity of the assay with respect to defining a meaningful change. Care should be taken to minimise variables such as diurnal variation, although these experiments could be used to assess such variation where evidence may be absent from the literature. Minimum recommended data should be generated from a minimum of 5 subjects sampled over three timepoints.

Inter-subject

There may be natural variation in some parameters between individuals, and this may extend over a much larger range than within a subject, for example, genetic differences in CCR5 expression. Initially, analysis should be carried out on a minimum of ten different subjects (sampled at the same time of day if indicated), but this data set should be reviewed over time as additional information becomes available. These data can be used to generate a provisional reference range for the parameter, which is reflective of the intended sample population. These data can also be used to set limits for further study design, e.g., for powering a clinical study and determining placebo-to-dosed ratios. In many cases, wide inter-subject

variability means that results can only be compared within a subject, with an individual acting as their own control, or the data should be normalised before comparison.

Incurred Sample Reproducibility

Following a publication on the reanalysis of bioanalytical samples (14), a discussion was held at an AAPS (American Association of Pharmaceutical Scientists) Workshop in February 2008 (15) regarding incurred sample reproducibility. The conclusion was for laboratories to begin to compile incurred sample reproducibility data during sample analysis in pharmacokinetic (PK) studies, or during validation of newly developed methods. Comparison of replicate data from approximately 20 different individual samples, re-assayed once each, was suggested, using the 4:6:20 or 4:6:30 criteria for bioanalysis of small and large molecules, respectively [3, 7]. It was also suggested that if repeat analysis of a sample is not possible, justification for the exclusion of this aspect of the validation should be included in the study file. Equally, if incurred sample reproducibility is unsatisfactory, an investigation needs to carried out and a conclusion reached regarding the suitability of the assay. This approach should also be considered for flow cytometry methods, where possible, in order to show alignment to the wider regulatory environment in which these assays take place. Although there are currently no official regulatory recommendations regarding incurred sample reproducibility, a formal publication is likely at some stage, and it is necessary to have some process in place to address this issue. Incurred sample reproducibility can be considered a measure of precision, but only once stored sample stability has been established.

Stability

Stability testing defines the length of time the sample is suitable for analysis, both before and after preparation. The stability of an analyte in a given matrix under specific conditions should be determined to identify method stability limits, including time, temperature and freeze-thaw cycles. These experiments must include stability at the various stages of analysis, including storage prior to acquisition. The stability determined in a given set of conditions is relevant only to those conditions and should not be extrapolated to other conditions, such as changes in anticoagulant, matrices, etc. Consideration should be given to what is appropriate for your sample or methodology and any relevant potential variables/ effects on stability evaluated. This may also include the effects of test compound up to and during data acquisition.

Samples should be tested over an appropriate period of time, both pre- and post-processing, and compared to their fresh result. For routine testing in which many samples are prepared and analysed each day, it is essential that processed sample stability be determined to allow for delays such as instrument breakdowns or overnight analyses. Additional stability experiments may include the following:

- Freeze-thaw cycles where applicable, 3 cycles in triplicate samples
- Short term on the bench whilst processing, in process stability
- Effects of storage on autosampler, including effects within a plate

Assay Range

Assay range describes the concentrations over which the analyte can be measured. Due to the absence of available standards, the measuring ranges for flow cytometry assays are commonly user-defined and often with no upper limit. It is crucial to accurately determine the negative/positive boundary of the detection method, and this equates to the lower limit of quantification.

Points for consideration also include the following:

- For rare event analysis, it is important to establish a minimum number of relevant events to provide a statistically meaningful result.
- Assay range may also be directly related to the assay readout parameter, for example, in *ex-vivo* stimulation curves where test compound causes a change in EC50. An assay ranges exists relating to the concentrations of test compound within which the assay gives a meaningful readout.
- Establishment of normal reference range for your population of interest

INSTRUMENT MONITORING

Instrument monitoring is an important part of the method validation process. Once pre-analytical variables have been established and controlled for, it is necessary to ensure that the data produced are accurate, reproducible, and, where applicable, comparable between instruments and laboratories. When developing procedures for monitoring instrument performance, it is important that operators are aware of the manufacturer's guidelines when considering the following factors:

Calibration

Fluorescent microbeads of a pre-defined fluorescent intensity can be used to measure the instruments capacity to resolve negative and positive populations. Instrument manufacturers use standardised calibration packages to allow a number of performance indicators (e.g., linearity, dynamic range and detection threshold) to be measured, and subsequently detect if the parameters are within acceptable ranges. Monitoring of these performance indicators can give an indication of the instruments response to fluorescent signals over time. Gradual changes in calibration performance may indicate deterioration or misalignment of the lasers/ optical system.

Instrument Quality Control

As well as monitoring pre-analytical processes, quality control material can be used to ensure that the performance of the instrument is within acceptable limits. Ideally, both internal and external quality control measures should be utilised: commercially available internal quality control allows day-to-day reproducibility and the acceptance ranges of the assay to be monitored; external quality control allows for inter-laboratory assay/instrument comparisons.

Instrument quality control is especially significant when multiple instruments are used to generate data. Similarly,

whilst all initial validation may take place using the flow cytometer of choice, it may also be necessary to determine the suitability of transferring an assay to a backup instrument. It is important to demonstrate cross-validation of instruments, especially when differences in optical bench arrangements mean that instrument optimisation procedures differ, or when sampling methods differ (e.g., carryover from manual sampling *versus* auto-sampling on a carousel or plate). Therefore, the analysis of quality control material can be used to demonstrate an acceptable level of correlation in inter-instrument performance for a given assay, ensuring the integrity of the data.

In order to successfully monitor instrument performance, it is essential that calibration and quality control data be logged over time. Consideration should also be given to the validation of the instrument software, measures to ensure the reproducibility of the data (e.g., locking of protocols and gates, when possible, to meet GLP requirements), and training of staff. Therefore, proper documentation of maintenance and operating procedures, as well as the competency of instrument operators, allows for consistency in the generation of data between multiple users.

CONCLUSIONS

The approach to method validation described here is intended as a starting point for further discussion, and it provides reference material to colleagues needing guidance in the use of flow cytometry methods within the regulatory environment. The authors will welcome feedback and suggestions. It is hoped that, with wider discussion, common guidelines can be agreed upon and published for use across industry, thus providing global consistency and greater compliance during the use of this increasingly widespread methodology.

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