FLOW CYTOMETRY: FROM THE SCIENTISTS PERSPECTIVE

A Question & Answer with Priscillia Bresler, PhD Scientist, Medpace Labs, Belgium.

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ANSWERED QUESTIONS:

- In what regulatory environment does Medpace validate flow cytometry testing?
- What are the main steps involved in flow cytometry validations?
- How do you establish the performance and robustness of an assay?
- Can accuracy and linearity assay be performed in flow cytometry?
- How does Medpace stay objective and unbiased when doing data analysis?
- How do you ensure analysis is accurate and reliable?
- What is the advantage of having IVD tests in flow cytometry?
- How do you verify the specimen integrity?
- How do you detect immediate errors or monitor test performances?
- What is next for flow cytometry in clinical development?

IN WHICH REGULATORY ENVIRONMENT DOES MEDPACE VALIDATE FLOW CYTOMETRY TESTING?

Medpace's flow cytometry validations follow regulations such as Good Clinical Practice (GCP), Clinical Laboratory Improvement and Amendments (CLIA), and College of American Pathologists (CAP). These regulations are mandatory to implement good clinical practices and principles to analyze patient samples from a clinical trial.

Additionally, the flow cytometry area follows the Clinical and Laboratory Standards Institute (CLSI) guidelines which are committed to achieving global harmonization and standardization between facilities and to reduce interlaboratory and inter-operator variabilities. CLSI guidelines are our main reference to provide guidance for flow cytometry cell-based assays in order to develop high quality, validated methods following quality assurance procedures.

WHAT ARE THE MAIN STEPS INVOLVED IN FLOW CYTOMETRY VALIDATIONS?

Most flow cytometry assays are cell-based assays and are considered to be Laboratory Developed Tests (LDTs). They must be optimized and developed to meet Quality Assurances requirements. Medpace mostly uses flow cytometry in exploratory endpoints using Research Use Only (RUO) products, but the flow cytometry technique can also be used as a patient-inclusion or exclusion criteria.

Before starting the validation of a cell-based flow cytometry assay, the scientist needs to understand the objective of the assay, how the assay is to be implemented, and what the best method is to provide trustworthy results to the sponsor for their clinical trial.

A flow cytometry validation includes assay optimization and assay characterization:

- Assay optimization includes the titration of the antibody and the creation of the assay on the instrument (including compensation and the spill over values).
- Assay characterization is performed to determine if the assay is fit for the intended use. The characterization includes precision, stability, instrument carry-over and analytical measuring range of the assay.

HOW DO YOU ESTABLISH THE PERFORMANCE AND ROBUSTNESS OF AN ASSAY?

Performance: Methods used to establish the performance of an assay include precision, stability, analytical measuring range, and reference ranges.

Precision: The precision establishes the degree of agreement among test results when the procedure is applied in a repeated manner to one or multiple stained specimens. The precision confirms if the assay yields reproducible results with precise measurement.

Precision can be measure in two ways:

1. Variation between separate runs (inter-run precision or reproducibility)
2. Variation within a run (repeatability)

Three parameters can induce variability in assay results:

1. The biological variation between samples
2. Instrument fluctuation
3. Sample processing by different operators

Therefore, multiple samples and operators are used during reproducibility.

Stability: The stability is defined by the time point whereby the sample does not have a significant loss of the property or the integrity of a measurand compared to the baseline. The effect of shipping conditions, shipping delays, short- or long-term cryopreservation of a specimen, and the effect of processing delay (time between the staining and acquisition) must be evaluated in the stability section of the initial validation plan.

Analytical measuring range (AMR): The sensitivity of an assay is determined by the lowest reportable results of a specific measurement. The sensitivity includes the Limit of Detection (LOD), the Limit of Blank (LOB) and the Lower Limit of Quantitation (LLOQ).

For quasi-quantitative assays, the analytical sensitivity includes LOD, LOB and LLOQ. The LOB is the highest signal that can be measured by the instrument in the absence of the measurand and can be assessed by using Florescence Minus One (FMO) control tube.

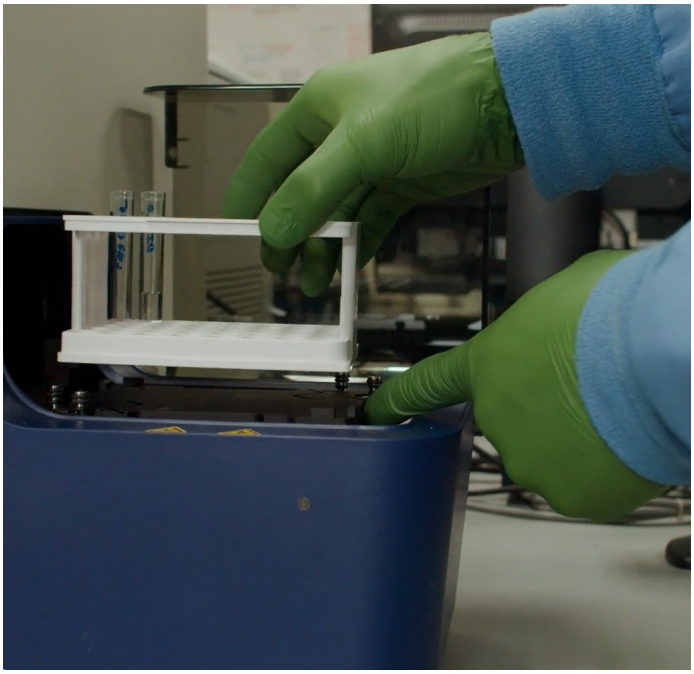
The LOD is related to the LOB and is defined as the lowest concentration of measured that can be detected by the instrument but not quantified.

The LLOQ is the lowest level of measurand that can be detected by the instrument with an acceptable criteria of 30% to 35% Coefficient of Variation (CV) For cell-

based assay like flow cytometry, the approach is to create samples near the LLOQ through serial dilutions of stained specimens into unstained specimens. The LLOQ maybe equal but never lower than the LOD.

Reference Ranges: Reference ranges are the limits (minimum and maximum) of the distribution for a measurands. Reference ranges are usually used to diagnostic abnormal populations and to flag critical values on laboratory reports. In flow cytometry, references ranges can be used in IVD tests such as TBNK assay, except if the population in normally absent in healthy donors. In LDTs, reference intervals are not always available or appropriate if we consider the complexity of the qualitative multiparametric analysis.

Robustness: The robustness of an assay is the capacity of a defined method to remain unaffected by deliberated changes. We address the robustness of an assay by analyzing the same sample using a different antibody lot to determine the variability between both lots. During the reproducibility we start up and shut down the instrument and run the performance check of the instrument before doing a second run and analyzing the variability between two runs.



CAN ACCURACY AND LINEARITY ASSAY BE PERFORMED IN FLOW CYTOMETRY?

A flow cytometry cell-based assay includes several components including the fluorochromes (type and number), the lasers, the type/configuration of instruments, reagents, cellular targets and type, and the stability of the specimen. These parameters can vary and expend depending on the assay. Therefore, using flow cytometry to design and validate an assay including all these components is challenging. For these reasons, we cannot apply all the same methods used to establish the performance of an assay as in chemistry or hematology because of the lack of reference materials with known values. Among the methods that cannot always be evaluated there is testing for accuracy and the linearity.

Accuracy: The accuracy is the degree of agreement between a measured quantitative value and the true known quantitative value. The accuracy is assessed by calculating the bias between the standard reference material with defined target values and the measured value obtained by the laboratory.

Example: It is extremely challenging to determine the accurate expression level of a specific cell-surface (CD45) or intracellular marker (FoxP3) because the true known standard reference material for this specific marker in the appropriate specimen type is not available. For specific IVD assays, we can find commercial QC samples and run accuracy, however the known reference ranges are broad, so each laboratory needs to establish its own range for assay acceptance criteria.

Linearity: The linearity of an analytical method is the capability to show results proportional to the concentration of analyte in a sample, within a given range. Linearity cannot be assessed without a calibration curve and is only applicable for quantitative methods. In quantitative analytical methods such as hematology or chemistry, we can establish the linearity of the analyte where a mathematically verified relationship between the observed values and the true concentrations of the analyte can be determined.

In the case of flow cytometry, most of the results generated are quasi-quantitative or qualitative in nature. The reportable results obtained from a flow cytometry assay are usually expressed as a percentage of a specific population of cells, the number of cells per µ or the Mean Fluorescence Intensity (MFI). Therefore, linearity cannot always be assessed.

However, the Receptor Occupancy (RO) assay is the only category of flow cytometric assay in which the linearity must be evaluated. RO assay describes the relationship between the drug and its target, then, we can use the drug as a reference standard with known concentrations. Usually we assess the linearity of the RO assay using healthy donor samples pre-incubated ex vitro with known drug concentrations from a full to partial saturation of the targeted receptor.

HOW DOES MEDPACE STAY OBJECTIVE AND UNBIASED WHEN DOING DATA ANALYSIS?

Being biased in flow cytometry is something to avoid at all cost. Therefore, we have to implement several controls in flow cytometry assays to stay objective when performing an analysis.

First we can define a good gating strategy to exclude debris, dead cells, doublets and fluidic instability as they promote the occurrence of nonspecific events. Additionally, during the initial validation it is crucial to make sure that no carryover is detected.

When performing deep analysis for an official data transfer, it is important for the Scientist to stay transparent and communicate with the sponsor to reach a consensus about the gating strategy. It is highly recommended to share the baseline timepoint for sponsor approval. This baseline gating can be applied to the future timepoints and any changes can be mentioned in the final report for sponsor review.

Secondly, we include FMO (fluorescence minus one), isotypes or internal controls. For instance, by using an FMO condition we are staining a defined sample without the antibody of interest. The negative population as defined based on the FMO conditions used as a negative control. Applying this FMO gating strategy on a fully stained sample allows us to better define, without any subjectivity, positive cells for this marker.

The use of isotype control can be implemented as well. Isotype controls are conjugated immunoglobulin antibodies from the same species but with an irrelevant specificity. Isotype controls allow us to detect and exclude the presence of nonspecific binding during assay optimization and data analysis. However, the disadvantage of using an isotype control is that they do not contain the same fluorescence-to-protein ratio as the primary antibody.

Finally, another method is to include an internal negative control, such as a population that is known to not express the antigen of interest. However, it is better to use a population that shows the same autofluorescence as the population of interest.

HOW DO YOU ENSURE THE ANALYSIS IS ACCURATE AND RELIABLE?

Before performing flow cytometry analysis, we need to assess the performance of the cytometer before any acquisition using Cytometer Setup and Tracking beads (CS&T). Moreover, the maintenance of the instrument (cleaning, preventive maintenance) needs to be done regularly and documented. After each compensation (baseline, preventive maintenance) adjustment, the PhD scientist validates the new compensations by following a set of defined acceptance criteria.

The reliability of an analysis is based on the proper usage of antibodies as well. Each time a new antibody is used, the PhD scientist needs to make sure that the bias between the old and new lot is acceptable. Importantly, every operator processing samples must be properly trained. The PhD scientist ensures that the bias between the trainer and the trained operator is acceptable to process clinical patient samples. As flow cytometry samples are extremely precious, after each sample acquisition, the PhD scientist analyzes and reviews flow cytometry data to provide high quality results to the sponsor. If necessary, the PhD scientist can ask for selected samples to be rerun or analyzed, should there be potential discrepancies.

WHAT IS THE ADVANTAGE OF HAVING IVD TESTS IN FLOW CYTOMETRY?

In Vitro Diagnostics (IVD) tests are extremely useful in flow cytometry as we are using reagents such as antibody cocktails approved by the Food Drug Administration (FDA). FDA approvals ensure that IVD tests provide consistent results and can be used for primary endpoint clinical trials. IVD tests are less time consuming, less prone to errors compared to LDTs. Moreover, IVD tests provide accuracy and reliable results because of the utilization of QC materials.

HOW DO YOU VERIFY THE SPECIMEN INTEGRITY?

In flow cytometry we are frequently using precious and sensitive samples with short stability; therefore, during the initial validation of the assay, the stability before and after sample processing (staining) has to be evaluated to have an idea of the acceptable timeframe in which a sample can be either stored or analyzed. Each time a patient sample is received, the specimen integrity will be verified by visually inspecting for the presence of a clot or hemolysis. Moreover, we make sure to process the sample as quickly as possible after reception in the laboratory to start the processing within the stability window defined during the initial validation. We make sure to include a live/dead dye in the flow cytometry panels to exclude debris and dead cells in the analysis because these components can lead to non-specific binding.

HOW DO YOU DETECT IMMEDIATE ERRORS OR MONITOR TEST PERFORMANCES?

Each time an assay is validated, the precision (within run and between run imprecision) is performed to address the performance and the robustness of the assay.

For flow cytometry IVD assays (non-LDTs), we include Quality Control materials to ensure the reliability and performance of the assay. The QC material is similar to the specimen matrix but with a longer shelf life.

For LDTs, QC material does not exist. One option is to use in-house QC materials such as positive controls (cell lines, activated PBMCs) or commercialized lyophilized cells. Each time a sample is acquired, an in-house QC material can be run before or after to validate the assay performance.

An alternative procedure is to test duplicated specimen. Each replicate will be run by two different operators with a different mixture of antibody and then reviewed by the scientist. The consistency between both analyses will highly validate the test performance.

Finally, the PhD scientist check immediate errors by reviewing data immediately after processing and acquisition so that it can be repeated or corrected if necessary.

WHAT IS NEXT FOR FLOW CYTOMETRY IN CLINICAL DEVELOPMENT?

Flow cytometry is a well-known technique that can be used to complement other techniques and areas such as ELISA or molecular biology. In 10 years, we can imagine the clinical pipeline embarked by a single fresh whole blood patient sample starting from two assays performed by flow cytometry.

The first assay will be an immunophenotyping assay and the second will be to use Fluorescent Activated Cell Sorter to sort for specific cell subsets for genomic testing by the molecular biology area. The ELISA team will then take the plasma of this sample and perform a multiplex cytokine analysis using the Mesoscale Discovery technique. The collaboration of scientists from three different platforms increases the data output from one single patient sample.

FULL-SERVICE CLINICAL DEVELOPMENT

Medpace is a scientifically-driven, global, full-service clinical contract research organization (CRO) providing Phase I-IV clinical development services to the biotechnology, pharmaceutical and medical device industries. Medpace's mission is to accelerate the global development of safe and effective medical therapeutics through its high-science and disciplined operating approach that leverages local regulatory and deep therapeutic expertise across all major areas including oncology, cardiology, metabolic disease, endocrinology, central nervous system and anti-viral and anti-infective.