Challenges associated with the development and validation of flow cytometry assays

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1 INTRODUCTION

Flow cytometry is a technology allowing multi-parametric analysis of thousands of particles per second and helps to adequately identify or functionally characterize complex cell populations of interest. It is often used in basic research, discovery, preclinical and clinical trials. With the increasing proportion of biologics in the pipeline, flow cytometry has proven itself to be an indispensable tool to assess safety, receptor occupancy (RO) or pharmacodynamics (PD).

Flow cytometry based methods can be challenging to develop and validate, given the cellular measurement involved and the lack of standardized cellular reference materials. An other important aspect in designing flow cytometry validations is the various purposes these assays can be used for. It is critical to know up front what the flow cytometer assay will be used for in order to conduct the appropriate validation to support GLP studies. There are currently no guidelines for the validation of flow cytometry methods to be used in the context of pre-clinical studies. Some initiatives have been taken by various working committees in the writing of guidance documents describing flow cytometry method validation. However, these recommendations have not yet been integrated in an official document released by the regulatory agencies as it has been done for other analytical methodologies.

The validation parameters commonly used for the validation of flow cytometry are presented in this poster as well as three case studies with validation designs adapted to address challenges such as inherent variability of functional endpoints, low frequency populations and sample stability limitations for shipment. For each case study, in addition to the validation parameters presented, all the validation parameters in Table 1 were also included (data not shown).

Table 1: Validation parameters commonly assessed in flow cytometry validations

Each laboratory has slightly different approaches for validation of flow cytometry methods. However, the following parameters are dealt with in a common manner.

Parameter	Assessment	Acceptance criteria
Antibody Titration	≥ 5 dilution/antibody	Optimal dilution: Clear and stable positive staining and minimal background level (negative population). The staining intensity of positive and negative cells are compared by calculating the signal over noise ratio using the MFI values.
Precision - Intra assay - Inter assay/analyst	≥ 5 samples Intra: ≥ 3 replicates/sample, 1 assay Inter: 1 replicate/sample, ≥ 3 assays (*done by different analysts*)	CV ≤ 20% (30% for low frequency populations)
Day-to-day variability	≥ 5 samples Collection over ≥ 3 occasions *Not applicable for terminal sample collections*	CV ≤ 20% (30% for low frequency populations)
Specificity	Isotype matched controls (IC) compared to specific antibodies	ICs should have a low signal and the antibodies should yield a positive signal
Antibody Interaction	Fluorescence minus one (FMO): the panel minus one of the antibodies vs. the full panel.	The FMO is expected to have a similar signal to the full panel when one antibody is removed, while showing low signal in the empty channel
Reference range	≥ 5 samples/sex	None (the mean, range and SD are reported)
Stability (pre/post staining)	≥ 3 samples/sex T=0 (reference): Samples are processed and acquired as soon as feasible T=X: Samples are processed X hours after collection TF= X: Samples are kept for X hours between staining completion and acquisition	Difference to the reference sample ≤ 25-30%

2 Case Study 1

Development of a PD marker by flow cytometry to assess the efficacy of a chemokine neutralizing antibody

Assay Design: The assay was required for the evaluation of pharmacodynamics (inhibition an agonist's granulocytes activation activity) in preclinical studies. Non-Human Primate (NHP) or rat blood from treated animals was incubated at 3 conditions and granulocyte activation was measured as CD11b expression by flow cytometry (MFI). The conditions tested included a negative control (PBS), a positive control (fMLP) and the test condition (agonist). The *results* were reported as normalized ratios (activated vs. non-activated).

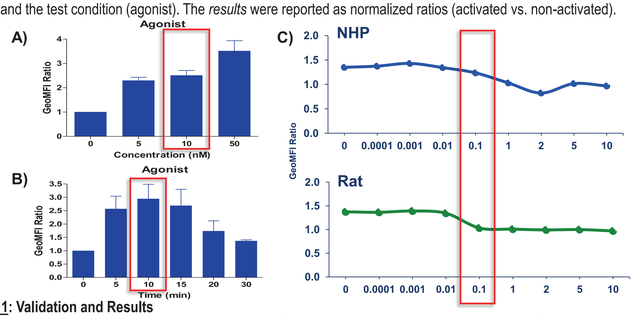

Figure 1: Validation and Results

*Commonly used validation parameters were tested (see Table 1) with the addition of parameters specifically related to the ex-vivo functional endpoint. **A)** The optimal agonist concentration was selected (10 nM). **B)** The stimulation time (10 minutes) was determined. The goal of these evaluations was to determine the optimal assay conditions to have good reproducibility while being able to detect any potential inhibition in study samples. **C)** The amount of drug required to inhibit the agonist's granulocyte activation (0.1 µg/µL) was assessed. The drug dose curve was included in the validation to determine at which concentration the compound was effective ex vivo in both species in order to determine if the doses selected for the toxicology studies were appropriate.*

Conclusion:

- Variability associated with the MFI values: Ratio between activated and non-activated was calculated for normalization.
- Concentration and timing of agonist: Should be taken into account when developing functional assays involving stimulation.
- In vitro drug titration: Specific to this validation, helped in the design of the tox studies (dose selection).

3 Case Study 2

Development of a flow cytometry assay for the measurement of basophil activation in the context of a Phase III clinical study

Assay Design: Human whole blood samples were spiked with the different controls (or compounds in the clinical study) and further stained with an anti-CCR3 and anti-CD63 antibody. The validations stimulation conditions tested included a negative control (PBS) and two positive controls (anti-FcεRI and fMLP). Basophils were identified as CCR3+ and upon activation, CD63 became externalized and present at the surface of the cells. Therefore, activated basophils were quoted as percentages of CD63+ cells from the CCR3+ population. A stimulation index (SI) was calculated for the positive controls (stimulated sample divided by negative control). Samples were considered positive if the % activated basophils was ≥ 5% and the SI was ≥ 2. For this study, commonly used validation parameters were tested (see Table 1), but extending the stability was critical since clinical samples were to be shipped to Canada from various countries around the world including Australia.

Storage condition	Stability Treatment	Acceptance criteria	Results Negative control (% of CD63+)	Results Anti-FcεRI (SI)	Results fMLP (SI)
Fresh blood (RT)	Blood from 10 donors were processed as soon a feasible (used as reference samples)	The percentage difference between the stability sample and the reference sample was to be within ±30% for at least 80% of donors.	24 hrs: ≤ 200%	24 hrs: ≤ 400%	24 hrs: ≤ 250%
Refrigerated blood (4°C)	Blood from the same donor samples were stained at least 24, 48 and 56 hours post collection		48hrs: ≤ 200% 56 hrs: ≤ 400%	48hrs: ≤120% 56 hrs: ≤ 90%	48hrs: ≤ 80% 56 hrs: ≤ 120%

Table 2: Stability Treatment and Results

The results following the stability treatments for most of the donors were not within 30% difference of the reference samples. However, all samples that tested positive with the reference samples (with the positive controls) remained positive after up to 56 hours post-blood collection (% activated basophils was ≥ 5% and the SI was ≥ 2) .

Conclusion:

- Stability limitations with whole blood: Not unusual for functional cell assays. However, given that the assay could be conducted as a qualitative screening assay to determine whether a compound tested positive or not, whole blood sample stability up to 56 hours post-collection was considered acceptable.

4 Case Study 3

Development of a flow cytometry assay for the measurement of regulatory T cells in rat whole blood and thymus

Assay Design: Rat blood and thymus samples were stained for regulatory T cells assessment using CD3, CD4, CD25 and FoxP3 as cell markers. Results were reported as percentage of lymphocytes and absolute counts (cells/µL of blood or cells/thymus).

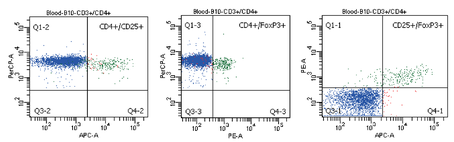

Figure 2: Example of flow cytometry analysis (Blood)

The frequency of regulatory T cells in rat blood was very low (< 1% of lymphocytes). Since the population is rare, the background in the assay could have an impact on the percentage of regulatory T cells reported. Consequently, a limit of detection parameter was included in the validation in addition to the commonly used validation parameters (see Table 1).

Limit of Detection Assessment (LOD): The LOD is used to determine the level of background noise in the overall relative percentage determined for each cell population of interest. Fluorescent minus one (FMO) controls were used to determine the LOD. The frequency of false positive events determines the lower limit of detection. LOD is calculated as the mean of all animals for each gated region of interest + 3SD. The LOD was assessed on 3 samples, each processed in 5 replicates for each FMO.

Parameters	Blood		Thymus
	CD4+/CD25+	CD4+/FoxP3+	CD4+/FoxP3+
LOD	0.29%	0.07%	0.02%
Reference range (% of Lymphocytes)	1.5 - 3.8%	1.7 - 3.8%	0.2 - 1.8%

Table 3: Blood and thymus LOD Results

LOD were calculated and compared to the reference range.

Conclusion:

- Limit of detection: For smaller populations, the LOD is important in the interpretation of the data especially, when considering the range of this population in blood and thymus. As an additional controls, FMOs were included as daily controls for future samples analysis.

5 CONCLUSION

- The parameters included in the validation of an assay should always depend on the purpose of the assay. Parameters, such as precision and stability, should always be included in validations. However, relevant additional parameters should be well thought of and included on a fit for purpose approach.
- In cases when limitations of the method are identified, then adaptations of the analysis or reporting strategies can be considered based on the purposes of the assay.