

## **Cytometer harmonization, through BAT, as part of IVDR and key to conduct multicentre studies**

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Rather sooner than later, accreditation under the upcoming EU IVD-R 2017/746 Regulation (IVD-R) will be required by all laboratories in the European Union that uses or develops in-vitro medical tests with diagnostic value, including the so-called *laboratory-developed-tests* (LDT), now renamed *in-hospital in vitro devices* (IH-IVD) [1]. ISO 15189 is an important basis for compliance to the IVD-R for diagnostic laboratories. Health institutions that perform IH-IVD should comply with ISO 15189 standard, which is an important basis for compliance to the IVD-R [2]. To accurately use an IH-IVD assuring performance and correctness, a Quality Management System, including Quality Control System and Good Laboratory Practices must be implemented in agreement with ISO15189, which is rare in preclinical laboratories[2]. All work must be undertaken in a setting that promotes and ensures reproducibility, allowing results to be translated into useful applications for which standard operating protocols (SOPs) must be put in place. Even though all IVD techniques must adhere to ISO15189 criteria, flow cytometry and particularly flow cytometry LDTs, are undoubtedly one of the most difficult to accredit. Flow cytometers are highly customizable instruments, with difficult-to-standardize settings and variable fluorescence signal between cytometers [3]. In the case of multicolour panels, complexity increases due to assay development, sample preparation or complex data processing/analysis [4], which increase the difficulty of performing multicentric studies. To comply with section 5 of ISO15189 [5], referring to technical requirements for *in vitro* devices, and to evaluate the critical parameters, we performed a method transfer assay from a flow cytometer of our facility to a second instrument. The method was basophil activation test (BAT), a flow-cytometry-based multicolour *in vitro* assay that identifies activated basophils after challenge with the allergen or drug [6,7,8]. We performed correlation studies and determined the level of agreement between both instruments by comparing multiple parameters to identify any critical ones. From August to October 2022, BAT was performed following SOPs in anonymized clinical samples from patients with confirmed allergy to amoxicillin, clavulanic acid, cefuroxime, ceftriaxone, dipyrone or azithromycin. Two basic flow cytometers dedicated to routine testing in our in-hospital laboratory were compared (FACS Calibur model 2012:

Comparator Instrument (Eq#1) and FACS Calibur Model 2012: Test Instrument (Eq#2)), totaling 430 measures. Activated basophils were assessed either by %CD63, %CD203c<sup>high</sup>, CD203c mean fluorescence intensity (MFI), CD203c  $\Delta$ MFI CD203c MFI ratio and by stimulation index (SI: % marker of stimulated/% marker of non-stimulated cells) [6](Supplementary material). A cause-effect-based risk approach (as starting point of a Hazard Analysis and Critical Control Points) was applied following the International Council for Harmonisation (ICH) guideline Q9 on quality risk management, since it is considered a general tool to identify hazards and risks, and also to evaluate the impact of changing instruments in the performance of a test [9]. We searched putative critical factors that could influence cytometer performance with consequences on BAT results, compromising validation studies. Photomultiplier tubes (PMT) voltages, MFI, activation molecule (CD63 or CD203c), parameter to quantify activation (% or SI), basophil count, type of culprit drug, sample storage time and reagent lot were evaluated as potential hazards. Correlation studies were done on the continuous variables and on the binary results (positive or negative result). Pearson's was used to evaluate linear correlation between instruments, Bland-Altman to investigate the level of agreement and Cohens kappa coefficient (k) to assess the clinical significance of concordance [9]. Tolerance limits of agreement between instruments were set as  $k > 0.81$  (near perfect agreement). Testing of critical factors showed that PMT voltages could not be transferred from Eq#1 to Eq#2 without changing drastically the results, therefore they should be optimized individually on each instrument. Importantly correlation was not affected by sample storage, type of drug, or culprit therefore, they were not critical factors. The best way to compare both instrument's performance was by matching the fluorescence intensity (MFI) and background signal of the non-stimulated samples of both instruments. We analysed the different comparators in each cytometer, namely % and SI using CD63 or CD203c<sup>high</sup>, and CD203c MFI (total, ratio and  $\Delta$  MFI). Linear correlation was highest with %CD63;  $r = 0.9722$ ,  $p < 0.0001$ , followed by %CD203c<sup>high</sup>;  $r = 0.9643$ ,  $p < 0.0001$  and lowest with  $\Delta$ CD203cMFI ( $r = 0.9494$ ,  $p < 0.0001$ ). All comparators showed small bias between Eq#1 and Eq#2 with most of the values falling within the 95% limit of agreement, except for CD203c MFI and CD203c $\Delta$ MFI (14.33 and 15.61, respectively) meaning good agreement between instruments. k yielded near perfect agreement between instruments by using CD63 (threshold=2.5%) and %CD203c<sup>high</sup> (thresholds=2.5% and 5%). In terms of final tests results (n=36), k ranged from 0.82 to 0.89, giving near perfect agreement with SI for both markers, and percentage for both markers and thresholds assuring clinical consistency between cytometers, regardless of the sample storing conditions or the culprit used. Study design, gating strategy, concordance, level of agreement, mean bias  $\pm$ SD and LoA for each comparison, correlation values and risk-based approach can be found as supplementary material. By this risk-based approach, the best comparators were %CD63

(threshold=2.5%), line with others [10], and %CD203c<sup>high</sup> (both thresholds), since they gave good correlation and analytical and clinical k above acceptance limits, allowing to consider both instruments as equivalent. In turn, using only one comparator (i.e., CD203c MFI) can lead to non-representative results, highlighting the pertinence of more than one readout to obtain robust and reliable results. By this study, we intend to give a roadmap for clinical laboratories performing flow-cytometry based *in vitro* assays as part of their Quality System Management Plan to comply with the IVD-R accreditation process or engage in multicentre studies for which reproducible results between all participants is of utmost importance. Risk-based identification of critical points to compare between instruments will facilitate correlation studies, decrease bias, hand-time, and resources. Multicentre studies should perform risk analyses to confirm their critical points to be included on external quality programs for ongoing validation of tests and instrument's performance.

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### **Conflict of interests**

The authors declare that they have no conflicts of interest.

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