# Workflow and Troubleshooting for Performance Qualification of Cytek® Aurora Instrument in a Clinical Setting

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*"Performance qualification of Cytek® Aurora: Unleash the potential of high-parameter spectral flow cytometry for global clinical trials."*

## Background

The ability of developing high-parameter assays allows for a deeper characterization of patients' immune subsets with limited sample availability. Spectral flow cytometry is used to assess high-parameter immune profiling in global clinical trials. To date, there are no specific guidelines for performance qualification (PQ) of spectral flow cytometers. Here, we describe an in-house developed workflow for performing PQ of a Cytek® Aurora instrument in a clinical laboratory.

## Detector Stability

First, the instrument's optical alignment and system resolution were assessed by daily running of SpectroFlo® QC beads (Cytek® Biosciences) in the software's QC module (*i.e.*, daily QC). Then, stability of the lasers and detectors was assessed by daily acquisition of the SpectroFlo® QC beads in a user-defined acquisition module using default cytek assay setting (CAS). Deviations of the daily median fluorescence intensity (MFI) readings were calculated against MFI target values *i.e.*, MFI values at installation, and expressed as %difference. MFI deviation was calculated for all 64 detectors (shown in Figure 1, acceptance criteria are ±5% difference from baseline values). After installation, we noticed deviations from MFI target values that were near or exceeded acceptance criteria, especially for yellow-green and red channels (Figures 1D and 1E). After optics realignment – indicated by the arrows – the deviations were much smaller and more stable. These data indicate the importance of monitoring daily MFI deviation, as it allowed us to identify problems with the instrument's optics while daily QC was still passing acceptance criteria.

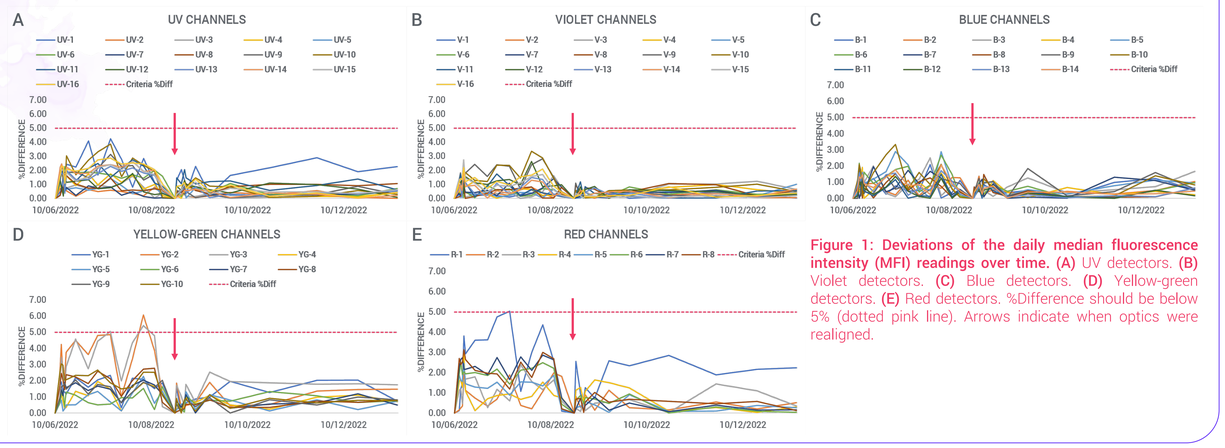


**Figure 1: Deviations of the daily median fluorescence intensity (MFI) readings over time. (A)** UV detectors. **(B)** Violet detectors. **(C)** Blue detectors. **(D)** Yellow-green detectors. **(E)** Red detectors. %Difference should be below 5% (dotted pink line). Arrows indicate when optics were realigned.

## Instrument Characterization

Cryopreserved peripheral blood mononuclear cells (PBMC) were single stained with anti-CD4 antibodies conjugated to 29 different fluorochromes whose emission span the full emission spectrum. CAS was used for acquisition. MFI output for each fluorochrome was recorded and cross stain index (CSI) matrix heat map (Figure 2A) and a spillover spread matrix (SSM) (Figure 2B) were created. The resulting matrices are unique to each instrument and can be used as quality control and benchmarking tool to monitor instrument performance and support panel design. Both matrices show that fluorochromes with similar emission patterns impact each other more than fluorochromes with more distinct emission patterns.

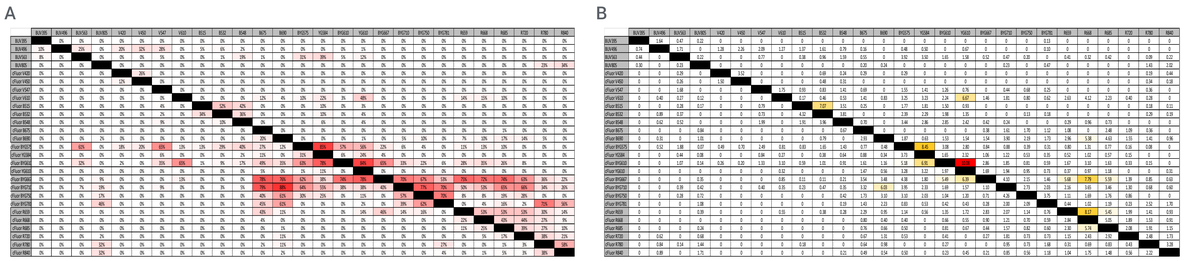


**Figure 2: Instrument characterization. (A)** Cross stain index matrix heatmap. **(B)** Spillover spread matrix heatmap.

## Detector Linearity

Detector linearity was determined using SPHERO™ UltraRainbow calibration beads (Spherotech), which were measured daily for ten subsequential days using default CAS. MFI values for all detectors were converted into molecules of equivalent fluorochrome (MEF) values and plotted into a regression line (MEF vs relative channel, values of beads provided by Spherotech). Slope, intercept and $R^2$ values were extrapolated and were within CLSI H62 acceptance criteria for each detector ($R^2 \cong 1$). Figure 3 shows $R^2$ values over time (up to 10 days) for each detector. Channels YG-6, YG-7, R-3, R4 and R-5 show lower $R^2$ values (< 0.98) on multiple days during the experiment (Figures 3D and 3E). Channels R-3 to R-5 have $R^2$ values below 0.95 on multiple days (Figure 3E). The lower $R^2$ values for Yellow-Green and Red detectors in this part of the emission spectrum (around 700 nm) is a general characteristic of the UltraRainbow calibration beads.* Therefore, we deem the spectral linearity of all detectors acceptable for this instrument.

*Personal communication with Spherotech Application Specialist.
*Ervaring met FACSLyric™ in het UZ Gent, Seminar by Malicorne Buysse.

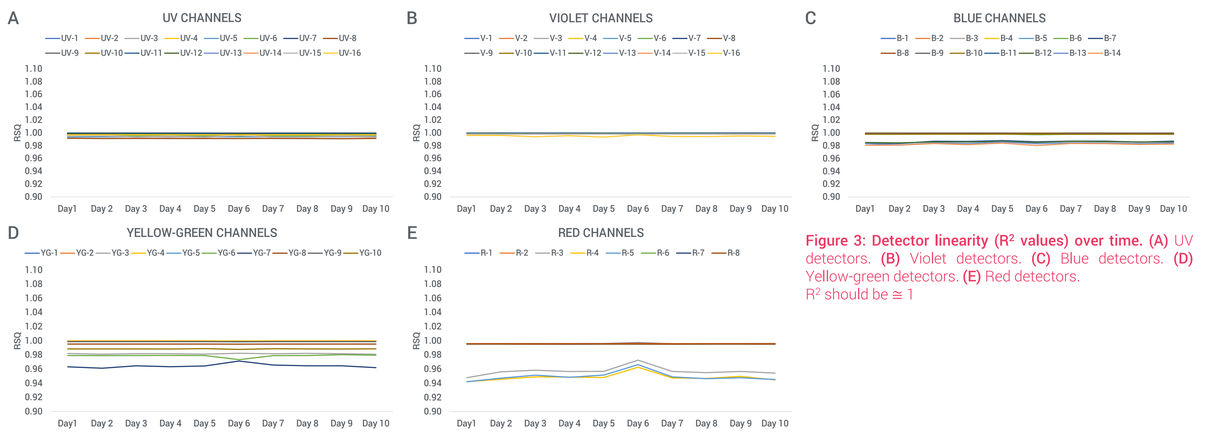


**Figure 3: Detector linearity ($R^2$ values) over time. (A)** UV detectors. **(B)** Violet detectors. **(C)** Blue detectors. **(D)** Yellow-green detectors. **(E)** Red detectors.
$R^2$ should be ≅ 1

## Analytical Validation – Unmixing With Cells Versus Beads

Analytical validation of the instrument was performed on PBMCs from different donors using Cytek® cFluor™ IP Kit 14 Color (Cytek® Biosciences) for immunophenotyping of T, B, NK cells and monocytes. During assay setup, different options for reference controls were evaluated. Unmixing with single stained controls were compared with PBMCs (Figure 4A) and compensation beads from different vendors: BD Biosciences (CompBeads; Figure 4B), Thermofisher (UltraComp ebeads™; Figure 4C) and Slingshot Biosciences (SpectraComp® beads; Figure 4D). Error-free unmixing was achieved when using PBMCs or SpectraComp® beads. Since quality of PBMCs can differ from batch-to-batch we strongly suggest to use SpectraComp® beads as reference controls for spectral unmixing.

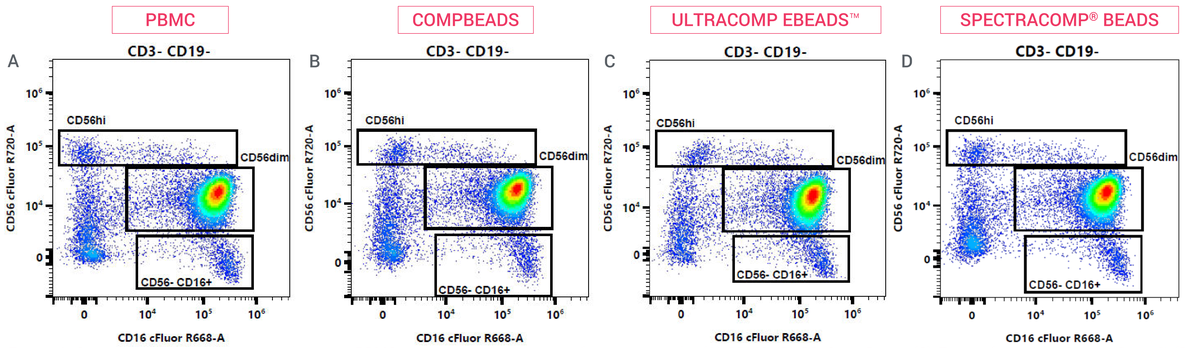


**Figure 4: Spectral unmixing comparison between PBMCs and beads for use as reference controls. (A)** PBMC **(B)** BD CompBeads **(C)** Thermofisher UltraComp eBeads™ **(D)** SlingShot SpectraComp® beads

## Conclusion

The process discussed here for performance qualification of a Cytek® Aurora instrument provides guidance to install multiple instruments and implement assays for global clinical trials. In addition, we show that monitoring different parameters over time can reveal certain deviations even when general quality controls still pass acceptance criteria.

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