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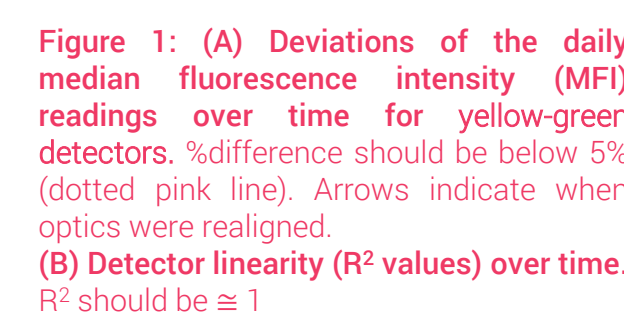
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Flow cytometry plays an important role for patient immune profiling in global clinical studies. Instrument standardization is critical to obtain transparent results across different instruments located in different labs. High parameter assay with deeper characterization of patients' immune subsets in clinical trials utilizing spectral flow cytometer requires developing new methods for instrument implementation and standardization. As there are no specific guidelines on performance qualification (PQ) and instrument standardization for spectral flow cytometers, an in-house workflow was developed for this purpose.

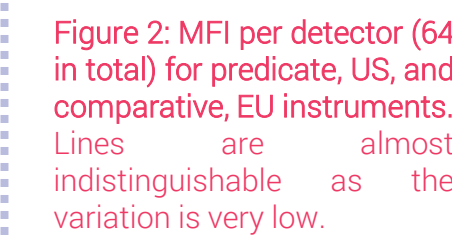
During Performance Qualification process, the instrument's optical alignment and system resolution were assessed by daily running of SpectroFlo® QC beads (Cytex® Biosciences) in the software's QC module. Stability of the lasers and detectors was assessed by daily acquisition of the SpectroFlo® QC beads in a user-defined acquisition module using default Cytex assay setting (CAS). Deviations of the daily median fluorescence intensity (MFI) readings were calculated against MFI target values established at installation and expressed as %difference. MFI deviation was calculated for all 64 detectors (as a representative data, only yellow-green laser is shown in Figure 1A). All the data show % difference within our acceptance criteria of <5%.

In addition, detector linearity was determined. SPHERO™ UltraRainbow calibration beads (Spherotech) were measured daily for ten subsequent 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 R² values were extrapolated and were within CLSI H62 acceptance criteria for each detector (R² $\geq$ 1) indicating the linearity of the detectors over time. Figure 1B shows R² values over time (up to 10 days) for Yellow-green detector, as a representative data. Data for all other detectors are similar.

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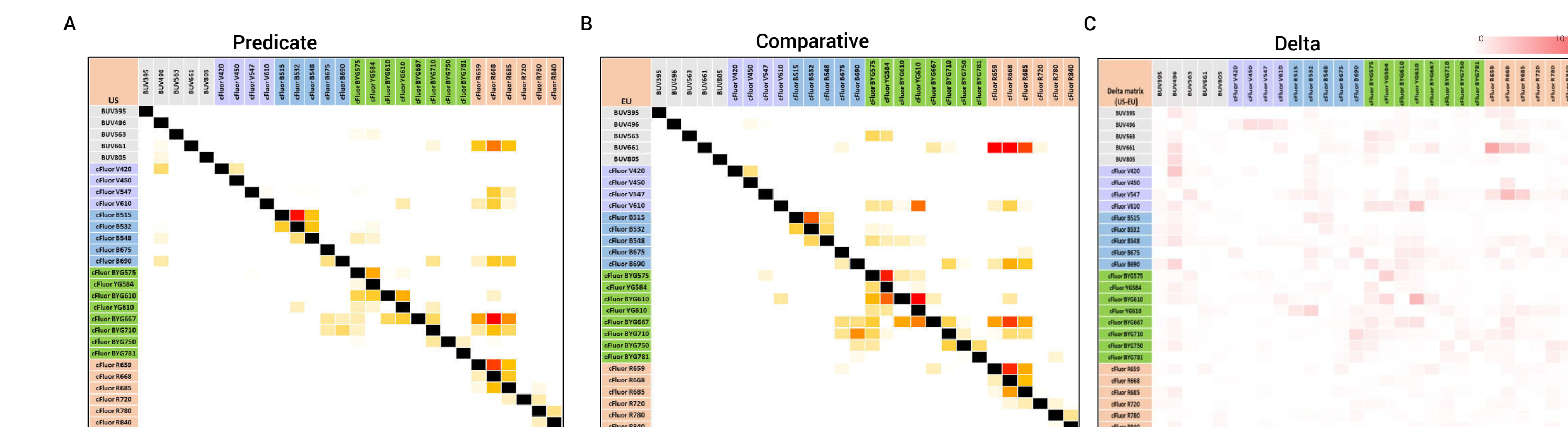
MFI target values, obtained by acquiring the same lot of SpectroFlo® QC beads with CAS in an acquisition module were compared to assess standardization between two Cytex® Aurora instruments in different locations (Figure 2). All detectors have %CV and % difference within the acceptance criteria of 5% (majority are even below 2-3%) which indicates that the two instruments are comparable.



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 a spillover spread matrix (SSM) was created for both predicate and comparative instrument (Figure 3, A and B). The resulting matrix is unique to each instrument and can be used as quality control and benchmarking tool to monitor instrument performance and support panel design.

SSM matrices for predicate and comparative instrument show a similar pattern, when compared side by side (Figure 3, A and B). To assess instrument comparison, the delta matrix between the two SSMs was calculated (Figure 3C). Values are within a delta of 10 which is in line with Cytek's observations for instrument comparison.

Figure 3: Heatmaps based on the SSM. (A) Predicate instrument, US. (B) Comparative instrument, EU. (C) Delta matrix of Predicate and Comparative instrument



The results shown here demonstrate that:

1. The instruments are comparable as variation of the MFI target values was between 2-5 %CV and the SSM delta matrix values, calculated between the SSM obtained from each instrument, are within Cytek's specifications for instrument comparison.
2. The performance between the two instruments was consistent as MFI output and populations frequencies obtained from immunophenotyping assay showed $\leq 20\%$ difference.

The standardization methods described above provide guidance on how to implement Cytex® Aurora instruments to generate transparent results for flow cytometry assays run in global clinical trials.

To assess comparability between instruments an 18-color pre-stained lyophilized PBMC kit (same lot number and reconstitution protocol) was used. MFI and population frequencies were used as a measure of assay performance between the instruments.

%difference between population frequency (Figure 4A and Table 1) and MFI (Figure 4B and Table 2) obtained from the two instruments is below our acceptance criteria of 20%, except for rare populations (with frequency below 5%), where a higher statistical variability is expected.

Figure 4: Results for assay performance comparison between predicate Instrument (US) and comparative instrument (EU) on 18-color pre-stained kit of lyophilized PBMC. (A) Population frequency comparison for different biological relevant populations. (B) Overlay of the MFI values for different markers.

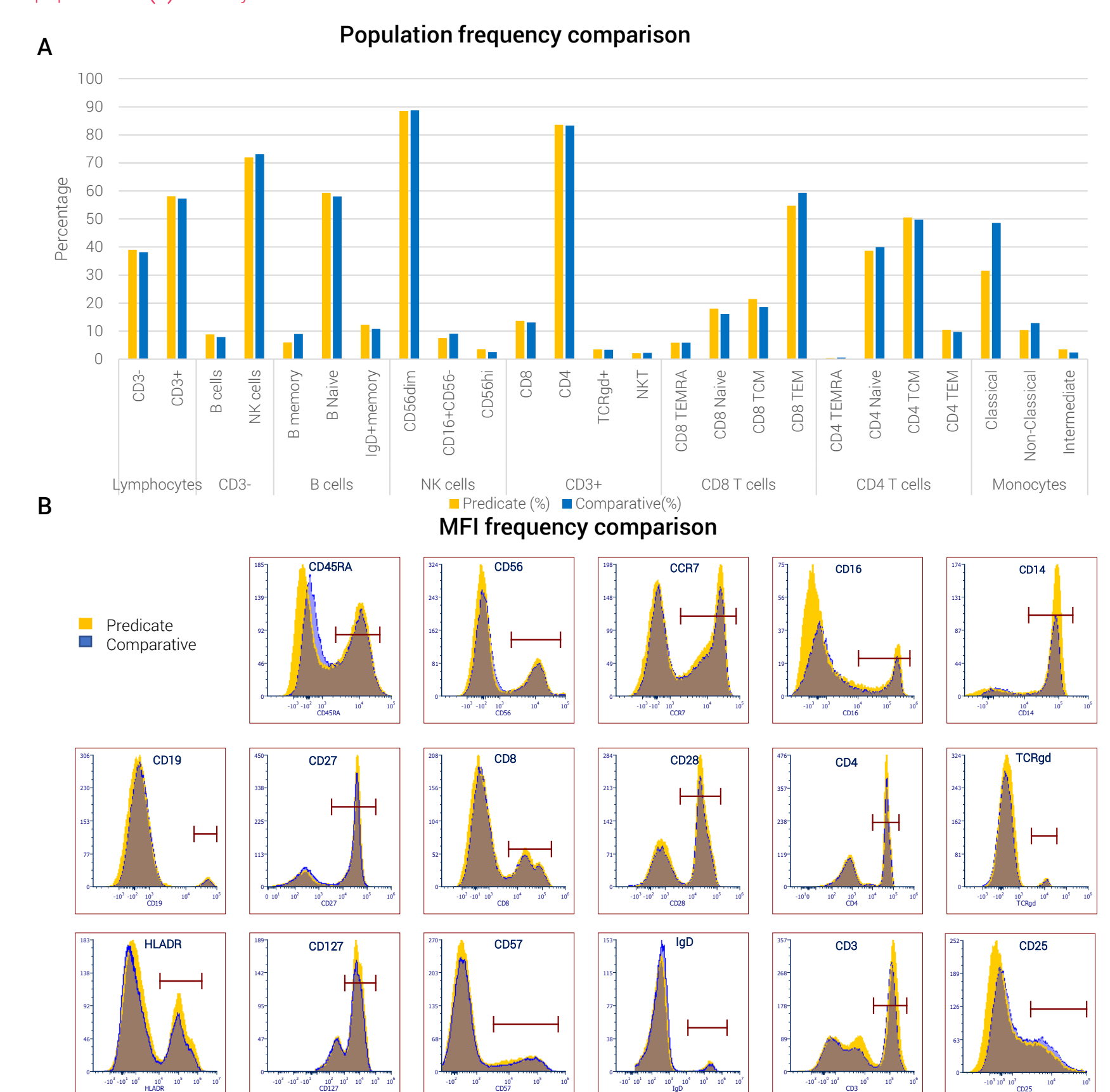


Table 1: %difference between predicate and comparative instrument for different population frequencies on 18-color pre-stained kit of lyophilized PBMC.

Parental population	Repetitors	%Inheritance
Lymphocytes	CD3	1.50
	CD4	1.01
	B cells	7.58
CD9	NK cells	1.09
	B memory	28.50*
B cells	B naive	1.51
	B memory	8.67
	CD56 ^{hi}	0.16
NK cells	CD14 ^{hi} CD56 ^{hi}	12.55
	CD56 ^{hi}	20.95**
	CD8	2.72
	CD4	0.22
CD8+	TCB β +	2.31
	TKT	4.95
	CD8 TEB β A	0.00
CD8 T cells	CD8 Naive	7.21
	CD8 TCM	9.10
	CD8 TSP	5.55
	CD4 TEB β A	36.76**
CD4 T cells	CD4 Naive	2.20
	CD4 TCM	1.00
	CD4 TSP	5.22
	Classical	30.43**
	Non-Classical	14.55
Monocytes	Intermediates	23.22

Values in bold exceed the criteria of 20% difference. (*) event count close to 100, (**) population frequency below 5%, (***) higher difference

Table 2: %difference between predicate and comparative instrument for different MFI frequencies on 18-color Pre-stained kit of lyophilized PBMC.

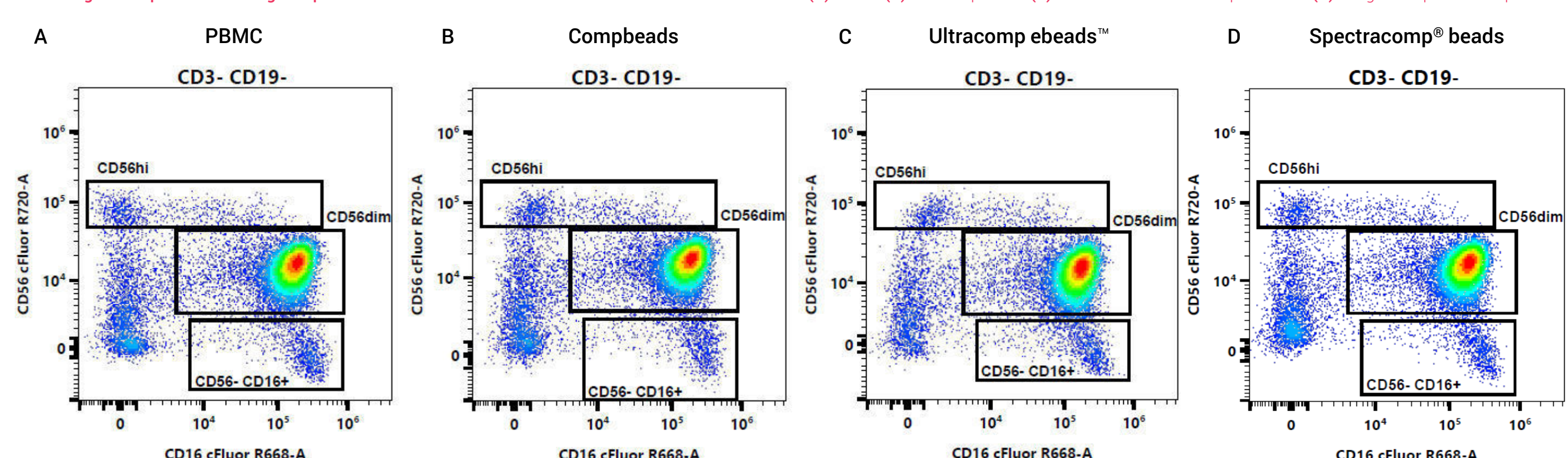
Marker	Fluorochrome	Wavelength
CD25	PE	3.84
CD45RA	BV295	0.28
CD56	BV237	1.15
CD57	BV421	0.20
CD16	eFluor 450	0.33
CD14	BV610	8.32
CD8	BV570	2.94
CD4	BV550	0.19
CD19	BV711	1.38
CD28	BV785	1.11
CD3	Alloca Fluor 488	11.18
CD57	PerCP-Cy5.5 710	2.94
HR	PE-Cy5.5-SP94	5.17
CD3	PE-Cy7	4.96
CD27	APC	1.52
CD127	APC-R700	1.46

$$\% \text{ difference} = \text{ABS} \left(\frac{\text{Mean Instrument 1} - \text{Mean Instrument 2}}{\frac{\text{Grand Mean} + (\text{C Mean Instrument 1} + \text{C Mean Instrument 2})}{2}} \right) \times 100$$

Analytical validation of the instrument was performed on PBMCs from 3 different donors using Cytex® cFluor™ IP Kit 14 Color (Cytex® Biosciences) for immunophenotyping of T, B, NK cells and monocytes.

During assay setup, different options for reference controls were evaluated with PBMCs (Figure 5). Error-free unmixing was achieved when using PBMCs or SpectraComp® beads. Testing of SpectraComp® beads as reference controls is suggested for spectral unmixing, to reduce errors due to PBMCs' batch-to-batch variability and optimize workflow.

Figure 5: 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



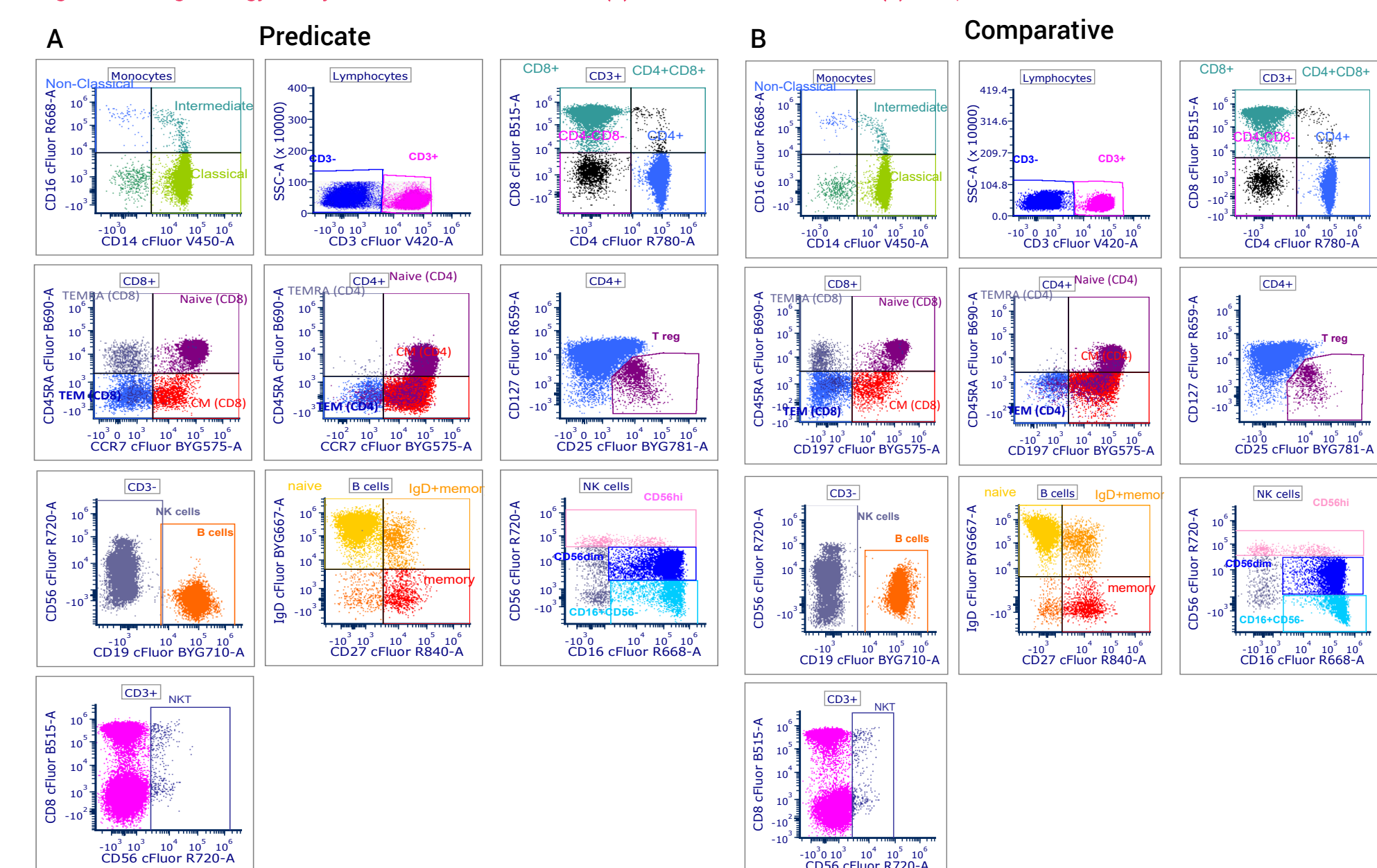
The same lot of immunophenotyping Cytex® Kit (Cytex® cFluor™ IP Kit 14 Color) was used on the predicate and comparative instruments with the same 3 PBMC donors to assess instrument standardization. Data show that both instruments provide visually similar profiles (Figure 6). In addition, it shows that the performance between the two instruments is consistent as the obtained population frequencies shown are below 20% difference, except for rare populations (with frequency below 5%) (Table 3).

Table 3: %difference between predicate and comparative instrument for different reportables over three donors stained with Cytek® cFluor™ IP Kit 14 Color.

Parental Population	Reportables	% difference		
		Donor 1	Donor 2	Donor 3
% Leuko	Lymphocytes	1.92	0.26	1.48
% Lymph	CD8	1.67	2.59	9.82
	T cells	0.10	0.65	1.25
	CD4+ T cells	2.29	2.41	5.89
	Naive	1.16	0.39	2.16
	CM	3.45	2.31	3.03
% CD4+ T	TEM	0.66	12.84	2.20
	TEMA	54.87	31.02	26.87
% T regulatory cells	Regulatory cells	5.42	9.25	5.68
	CD8+ T cells	1.21	1.98	8.04
% CD8+ T	Naive	0.13	2.98	5.68
	CM	12.95	12.82	6.64
	TEMA	5.57	11.66	8.92
% Lymph	TEM	4.20	5.66	1.45
	NKT cells	1.11	5.06	2.93
% Leuko	Monocytes	8.36	12.55	17.26
	CD56	2.56	2.05	2.69
% Mono	Intermediate	411.99	16.20	44.44
	Non-classical	46.24	71.33	34.93
% Lymph	B cells	1.61	9.52	13.98
	Naive	1.92	1.19	2.09
% B	Memory	15.23	3.18	6.85
	IgM+ memory	9.39	15.53	2.27
% Lymph	NK cells	9.34	3.02	2.81
	CD59b	0.16	16.84	10.36
% NK	CD56dim	10.10	3.97	3.73
	CD56+CD137+	0.20	19.07	20.47

Values in bold exceed the criteria of 20% difference; (*) nonstatic frequency below 5

Figure 6: Gating strategy for Cytex® cFluor™ IP Kit 14 Color (A) Predicate Instrument, US. (B) Comparative Instrument, EU



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flow cytometry panels for global clinical trials.
Connect with our scientific team to learn how we can enhance
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