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# Validation of a 25-color spectral flow cytometry assay for immunoprofiling of human whole blood samples from clinical trials

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## Introduction

Advances in high parameter flow cytometry have enabled increasingly comprehensive characterisation of immune cell populations in peripheral blood and other relevant matrices. Spectral cytometry, in particular, allows simultaneous detection of large numbers of fluorochromes with minimised spillover challenges and improved resolution of in-depth cellular subsets. To support the growing demand for standardised, multiparametric immunophenotyping solutions, ICON implemented and validated a 25-colour spectral immunoprofiling panel (Cytex SKU R7 40002) designed for broad immune monitoring applications.

The panel is intended for use on the Cytex Aurora 5 laser (5L) platform, leveraging the system's full spectrum capabilities. Importantly, the panel was intentionally designed using only four of the five available lasers, thereby reserving one laser's spectral space for sponsor requested drop in markers. This provides additional flexibility for incorporating study specific targets, for example in support of target occupancy studies.

The 25-colour backbone enables high resolution immunophenotyping across major innate and adaptive immune lineages. Table 1 provides a high level summary of the subsets that can be reported from the panel.

**Table 1: High level immune subsets and associated marker coverage**

Immune subset	Key markers	Notes
T-cells	CD3, CD4, CD8, CD45RA, CD25, CD127, CCR7, TCR $\gamma\delta$ , CD197, CD27, CD28	Total T-cells and CD4 <sup>+</sup> /CD8 <sup>+</sup> profiling, naïve–memory (central, effector), regulatory T cells, gamma-delta T-cells, and more granular T-subset identification such as early/late effector memory cells
B-cells	CD19, CD20, CD27, CD38, IgD, IgM	Differentiation of naïve, memory (switched, unswitched), transitional, and marginal-zone-like B-cells, and plasmablasts
NK-cells	CD56, CD16, CD3	Resolves early, mature, and terminal NK-cell populations, and NKT-cells
Monocytes	CD14, CD16, HLA-DR	Identification of classical, intermediate, and non-classical Monocytes
Dendritic cells	CD123, HLA-DR, CD1c, CD11c, CD141	Plasmacytoid and conventional DCs
Other	CD16, CD123, CD38	Granulocytes, Basophils

Together, these populations provide a broad yet structured overview of systemic immune composition suitable for immunomonitoring in clinical and translational studies.

To ensure robustness across commonly used sample types, and to obtain broad sample stability information, we evaluated assay performance in multiple matrices, including Cytochex preserved whole blood, K<sub>2</sub>EDTA whole blood, sodium heparin (NaHep) whole blood, and isolated PBMCs. These matrices reflect the typical clinical trial sample collection tubes for flow cytometry assessments and this information enables optimal matrix selection based on subsets of interest and clinical design.

The validation design aligned with the fit for purpose, type 2 approach, outlined in CLSI chapter H62 (Validation of Assays Performed by Flow Cytometry) <sup>1</sup>. Key performance characteristics evaluated included precision within and between runs, instruments and operators, stability of samples, as well as post-staining stability, and cocktail stability.

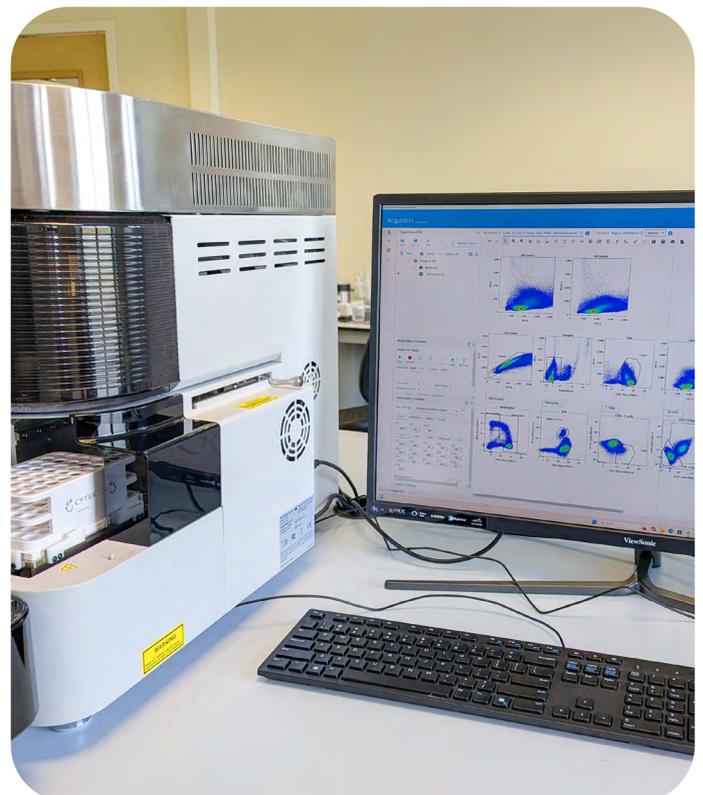
The overall objective was to provide an off the shelf, validated 25-colour immunophenotyping assay that ICONs flow laboratories can readily deploy for immune profiling in clinical trials. By establishing baseline performance across instruments and sample types - and by preserving space for customisable study specific markers – this panel provides in-depth immune cell monitoring in longitudinal multi-center studies, supported by ICON’s global flow cytometry laboratories.

## Materials and methods

Peripheral blood samples were collected in-house from healthy adult volunteers. All volunteers provided written informed consent before participation. PBMC samples for validation of antibody cocktail stability were purchased from BioIVT.

Samples were processed according to the protocol as provided by Cytex Biosciences, with minor modifications.<sup>2</sup> See Table 2 and Table 3 for used materials, and the list of used antibodies. In summary, 4.0 mL whole blood of the relevant matrix was lysed, washed, and 2·10<sup>6</sup> cells were stained with an antibody cocktail containing 23 antibodies. TCR $\gamma\delta$  and CCR7 antibodies were added separate from and prior to adding the cocktail. After incubation, cells were washed and fixed using 1% paraformaldehyde solution.

Single-stain spectral reference controls were either generated using cells or beads, ensuring that the fluorescence level was higher than observed in a typical study sample. Autofluorescence was recorded from unstained samples in the relevant matrix and included as an additional spectral parameter. Both raw spectral and unmixed FCS files were retained to enable retrospective re-analysis/unmixing, if deemed necessary.



Samples were analysed on Cytex Aurora 5L Spectral Flow Cytometers. This cytometer is equipped with 5 lasers (355nm, 405nm, 488nm, 561nm, 640nm) and full-spectrum detection across all lasers. Cytex SpectroFlo software 3.3.0 was used for acquisition and unmixing, and FCS Express 7.26 (clinical) was used for data analysis. Data was reported in MS Excel.

Identification of the subsets is described in the appendix.

**Table 2: List of assay materials**

Material	Manufacturer	Catalog number
Pharm Lyse lysing buffer (10x)	BD Biosciences	555899
Cell staining buffer	BioLegend	420201
Brilliant Stain Buffer Plus	BD Biosciences	566385
4% Paraformaldehyde solution in PBS	Thermo Fisher Scientific	J19943.K2
DPBS	Gibco	14190
UltraComp eBeads Plus Compensation Beads	Thermo Fisher Scientific	01-3333-42

**Table 3: List of used antibodies**

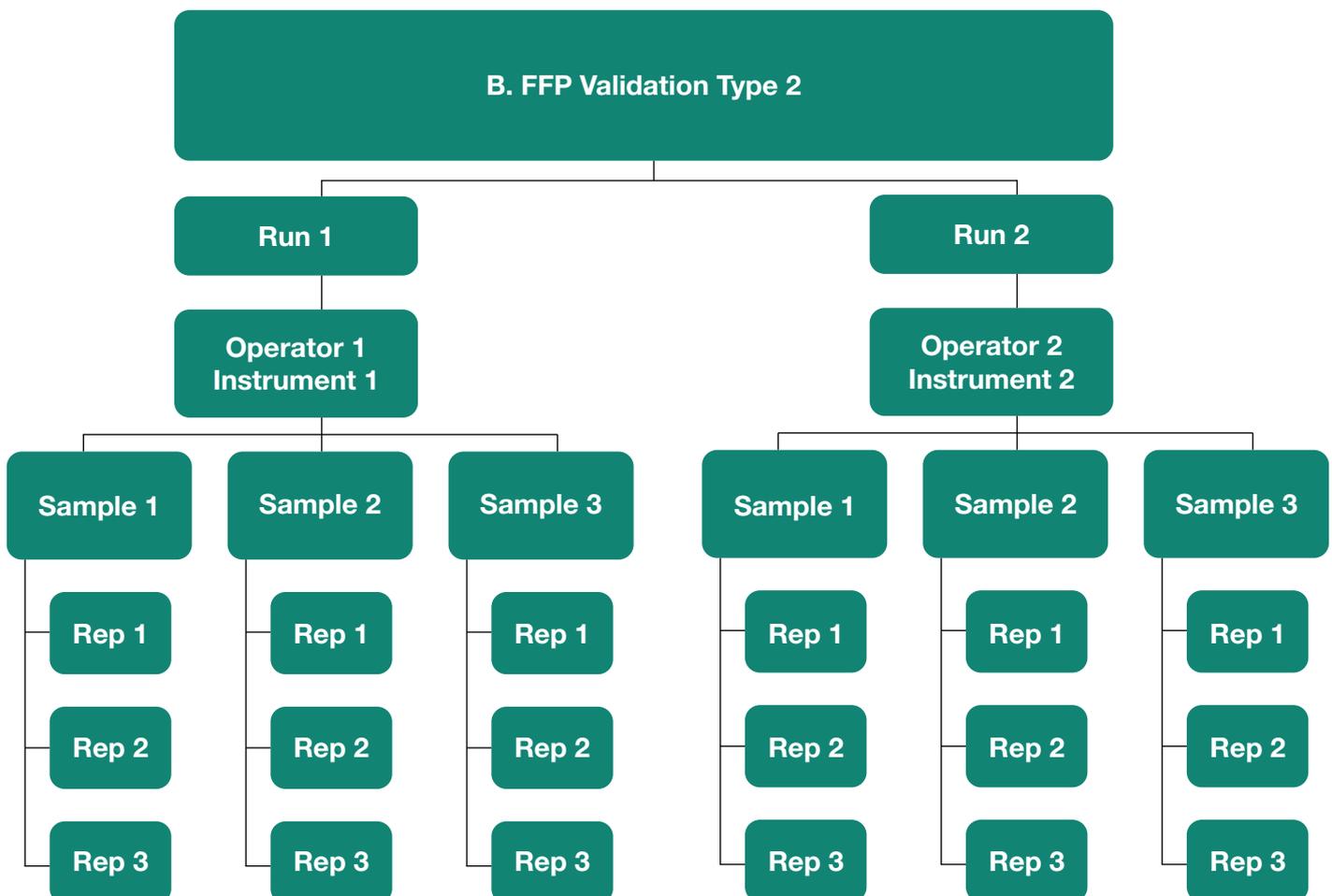
Target	Manufacturer	Catalog number	Clone	Fluorochrome
CD197 (CCR7)		353208	G043H7	BV421
IgM		314522	MHM-88	BV510
CD3		300436	UCHT1	BV570
CD28	BioLegend	302946	CD28.2	BV650
CD38		303528	HIT2	BV711
CD56 (NCAM)		362556	5.1H11	BV750
CD279 (PD-1)		329930	EH12.2H7	BV785
CD45RA			HI100	cFluor V450
CD20			2H7	cFluor V547
CD141			M80	cFluor B515
CD8			SK1	cFluor B532
CD14			63D3	cFluor B548
HLA-DR			L243	cFluor B690
CD25			BV96	cFluor BYG575
CD4			SK3	cFluor YG584
CD16	Cytex 25-color Immunoprofiling Assay	R7-40002	3G8	cFluor BYG610
IgD			IA6-2	cFluor BYG667
TCR $\gamma\delta$			B1	cFluor BYG710
CD11c			CD11c	cFluor BYG781
CD127			A019D5	cFluor R659
CD1c			L161	cFluor R668
CD19			HIB19	cFluor R685
CD123			6H6	cFluor R720
CD45			2D1	cFluor R780
CD27			QA17A18	cFluor R840

## Validation parameters and experimental design

The design of the validation was based on the guidance provided in CLSI chapter H62 (Validation of assays performed by flow cytometry)<sup>1</sup>. The following assessments were performed, in line with the recommendation for a Fit-For-Purpose Validation, Type 2. See Table 4 below and Figure 1 for details.

**Table 4: Overview of validation parameters**

Validation parameter	Donors	Replicates	Number of runs	Purpose, Remarks
Reproducibility/ repeatability	Intra-assay	6	3	4 Within run precision Between run precision Between Operator variability Between Instrument variability
	Inter-assay	6	3	
	Between Operator	6	3	
	Between Instrument	6	3	
		<b>Donors</b>	<b>Timepoints</b>	
Stability	Whole blood stability	6	6	1 per timepoint Sample stability
	Stained cell stability	3	5	Processed sample stability
	Cocktail stability	QC (PBMC)	4	Stability of antibody cocktail



**Figure 1. Schematic representation of the reproducibility/repeatability experimental design. Image copied from CLSI chapter H62. This experimental setup was performed twice, to generate data from 6 individual donors.**

Reproducibility and repeatability parameters were assessed using blood collected in K3EDTA tubes. Assessment of sample stability was done in multiple blood collection tubes, including K3EDTA (stored at 4°C and room temperature (RT)), NaHep (stored at 4°C), and CytoChex BCT® (stored at RT). Stained cell stability was assessed using K3EDTA whole blood, and cocktail stability in commercial PBMC samples.

## Data evaluation and criteria

A minimum of 400 gated cells was set as main requirement for main subsets to be evaluated, in order to avoid increased variability introduced by a limited numbers of gated cells. General acceptance criteria were a CV of 20.0% for evaluation of reproducibility and repeatability of the assay and change from baseline of 20.0% in case of stability assessments.

For populations known to be rare, 100 gated events were required as a minimum, and a CV% and %Change of 30.0% were deemed acceptable. Calculations were performed according to the formulas below:

$$CV\% = \frac{\text{st.dev.}}{\text{average}} * 100$$

$$\% \text{ Change from baseline} = \frac{\text{freq. at timepoint} - \text{freq. at baseline}}{\text{average (baseline and timepoint)}}$$

## Results (Reproducibility/repeatability)

In this section we present the summarised data for within- and between-run variability. In summary, the 25-color assay demonstrated excellent reproducibility, with CV's typically <10.0% for the larger cell subsets, and for many of the smaller subsets <20.0%. See the sections and tables below for detailed results. Individual donor results will become available as well through the ICON website.

### T-cells and T-cell subsets

Major T Cell Subsets: For the larger T-cell compartments – including total T-cells (as % of mononuclear cells (MNC), TCR $\gamma\delta$ <sup>+</sup> T-cells, CD4<sup>+</sup> T helper cells, CD8<sup>+</sup> cytotoxic T-cells, the panel demonstrated excellent reproducibility: within run precision was well within acceptable CV, with maximum observed CV's mostly <10.0%. The between-run precision showed higher variability but max CV's still below 20.0%.

More granular T-cell subsets include early and late effector memory helper and cytotoxic T-cells, intermediate and terminal effector memory cytotoxic T-cells, CD45RA re expressing effector memory cells, as well as CD4/CD8 double positive and double negative T-cell populations. For these smaller subsets, the within run precision was still excellent, however the between run variability increased significantly, with many CV's >30.0%. See Table 5 for a detailed results summary.

**Table 5: Reproducibility/repeatability T-cells and T-cell subsets, showing min and max % CV across 4 runs**

	Freq of parent gate (avg at baseline)	Intra-assay		Inter-assay		Remarks
		Min CV	Max CV	Min CV	Max CV	
T-cells of MNC	71.9%	0.4%	1.0%	0.4%	2.0%	
gd T-cells	3.0%	1.6%	5.5%	3.5%	5.1%	
NK T-cells	2.3%	1.3%	6.6%	4.8%	12.5%	
CD4+ Th of abTCR	67.7%	0.1%	1.2%	0.5%	1.3%	
Treg of CD4+ Th	6.1%	1.8%	6.2%	1.3%	5.6%	
non Treg of CD4+ Th	93.9%	0.1%	0.4%	0.1%	0.3%	
Th Naïve of CD4+ Th	43.6%	0.5%	2.6%	9.2%	18.2%	

**Table 5: Reproducibility/repeatability T-cells and T-cell subsets, showing min and max % CV across 4 runs, continued**

	Freq of parent gate (avg at baseline)	Intra-assay		Inter-assay		Remarks
		Min CV	Max CV	Min CV	Max CV	
CD27+CD28+ Th Naïve of Th Naïve	99.9%	0.0%	0.1%	0.0%	0.2%	
CD8+ Tc of abTCR	27.7%	0.2%	1.1%	1.1%	1.6%	
Tc Naïve of CD8+ Tc	52.3%	0.5%	2.7%	2.4%	5.5%	
CD27+CD28+ Naïve of Tc Naïve	95.5%	0.1%	2.0%	0.1%	3.0%	
TcEM of CD8+ Tc	21.4%	1.7%	5.4%	2.6%	9.3%	
TcEEM of TcEM	60.3%	0.4%	2.3%	3.3%	12.2%	
TcELEM of TcEM	5.6%	2.2%	6.1%	0.7%	10.9%	
TcIEM of TcEM	22.0%	1.6%	5.6%	2.1%	16.5%	
CD27-CD28- TcEMRA of TcEMRA	29.6%	0.6%	5.6%	2.4%	11.5%	
DNT of abTCR	1.1%	1.1%	6.7%	5.6%	19.1%	
ThCM of CD4+ Th	41.0%	0.2%	8.1%	29.3%	46.7%	
CD27+CD28+ ThCM of ThCM	96.7%	0.1%	0.7%	2.2%	2.5%	
ThEM of CD4+ Th	14.6%	1.0%	13.8%	30.8%	54.7%	
ThEEM of ThEM	75.8%	0.5%	1.8%	6.2%	9.4%	
ThELEM of ThEM	23.4%	1.5%	4.4%	20.9%	26.6%	
ThTEM of ThEM	0.2%	5.1%	20.6%	55.1%	69.1%	<100 events in multiple donors
ThEMRA of CD4+ Th	0.9%	2.3%	12.5%	101.2%	132.0%	
CD27-CD28-ThEMRA of ThEMRA	0.9%	4.2%	67.6%	47.2%	130.7%	<100 events in multiple donors
TcCM of CD8+ Tc	17.6%	0.9%	9.3%	12.4%	29.2%	
CD27+CD28+ TcCM of TcCM	93.7%	0.1%	1.2%	0.8%	1.4%	
TcTEM of TcEM	12.1%	1.2%	7.6%	6.2%	25.9%	
TcEMRA of CD8+ Tc	8.7%	0.6%	6.0%	9.9%	24.7%	
DPT of abTCR	1.1%	1.2%	6.2%	5.4%	26.2%	

**B-cells and B-cell subsets:**

For B cell subsets including total B-cells, naïve and memory (switched/unswitched) B cells, and IgD/CD27 double negative B-cells, the panel delivered consistently high precision. Event threshold was set at 400 events for major populations and at 100 for plasmablasts. Within run precision was <20.0% for these subsets. Marginal zone B-cells and plasmablasts demonstrated higher variability. These subsets also showed the highest variability in the between run variability evaluation. See Table 6 below for detailed results.

**Table 6: Reproducibility/repeatability T-cells and T-cell subsets, showing min and max % CV across 4 runs**

	Freq of parent gate (avg at baseline)	Intra-assay		Inter-assay		Remarks
		Min CV	Max CV	Min CV	Max CV	
B-cells of MNC	10.4%	0.8%	3.7%	2.9%	7.1%	
B Memory of B-cells	14.3%	0.7%	4.3%	0.9%	7.7%	
Switched of B Memory	96.4%	0.2%	1.6%	0.4%	2.6%	
Unswitched of B Memory	6.8%	8.2%	15.9%	11.4%	11.4%	<400 events in multiple donors
Marginal zone B cells of B-cells	3.9%	1.9%	6.7%	10.6%	21.5%	
Naïve B cells of B-cells	73.8%	0.1%	1.8%	0.1%	1.8%	
Plasmablasts of B-cells	0.6%	6.8%	35.0%	19.1%	65.8%	<100 events in multiple donors
DN B-cells [IgD-CD27-] of B-cells	7.3%	1.4%	3.8%	4.0%	13.7%	

**NK-cells, monocytes, dendritic cells and basophils**

For the major subsets in this panel – monocytes (including classical, intermediate and non-classical), NK-cells (including early, mature and terminal), TCRγδ<sup>+</sup> T-cells – the panel showed excellent reproducibility, with all subsets having CV's <20.0% for within-run precision. Between-run variability was higher, but most subsets still well within the acceptable variability. For Dendritic cells and basophils the observed maximum CV's were >20.0% but <30.0%. See Table 7 below for detailed results.

**Table 7: Reproducibility/repeatability on NK-cells, monocytes, dendritic cells and basophils, showing highest observed % CV across 4 runs**

	Freq of parent gate (avg at baseline)	Intra-assay		Inter-assay		Remarks
		Min CV	Max CV	Min CV	Max CV	
Monocytes of MNC	4.4%	3.8%	9.4%	10.6%	19.2%	
cMono of Monocytes	71.4%	0.9%	3.8%	1.2%	6.4%	
inMono of Monocytes	6.3%	2.1%	8.8%	6.4%	12.8%	
ncMono of Monocytes	20.0%	2.7%	6.4%	3.4%	12.3%	
NK of MNC	11.0%	1.0%	4.7%	1.7%	4.9%	
Early NK of NK	3.1%	2.7%	10.6%	2.8%	7.4%	
Mature NK of NK	89.9%	0.3%	1.0%	0.3%	0.6%	
Terminal NK of NK	0.9%	4.3%	15.0%	11.7%	16.1%	
T cells of MNC	67.7%	0.3%	1.0%	0.4%	2.2%	
pDC of MNC	0.1%	3.2%	16.1%	19.0%	25.0%	
cDC of MNC	0.2%	5.6%	10.5%	7.2%	14.0%	
cDC CD16- of cDC	86.7%	0.9%	6.4%	1.1%	10.1%	
cDC CD16+ of cDC	15.6%	7.9%	8.4%	8.9%	21.6%	<100 events in multiple donors
Basophils of MNC	0.3%	6.0%	18.5%	6.4%	23.2%	

## Results (sample stability)

In this section we present a high-level overview of sample stability outcomes. Data is shown for the most stable sample collection/storage condition per subsets. In summary, T-cells and T-cell subsets provided the longest stability in K3EDTA blood stored at 2-8°C. For B-cells and it's subsets, as well as monocytes, NK-cells, Dendritic cells and basophils, the longest stability was provided by the Cytochex BCT. A selection of the data is shown below.

### T-cells and T-cell subsets

For the larger T-cell subsets – including total T-cells, CD4<sup>+</sup> T helper cells (including naïve, central-memory and effector-memory subsets), NK T-cells and CD8<sup>+</sup> cytotoxic T-cells - samples remained stable for up to 240 hours in K3EDTA tubes, stored at 2-8°C. Regulatory T-cells, and naïve, central memory subset phenotyping of cytotoxic T-cells was stable for 72h.

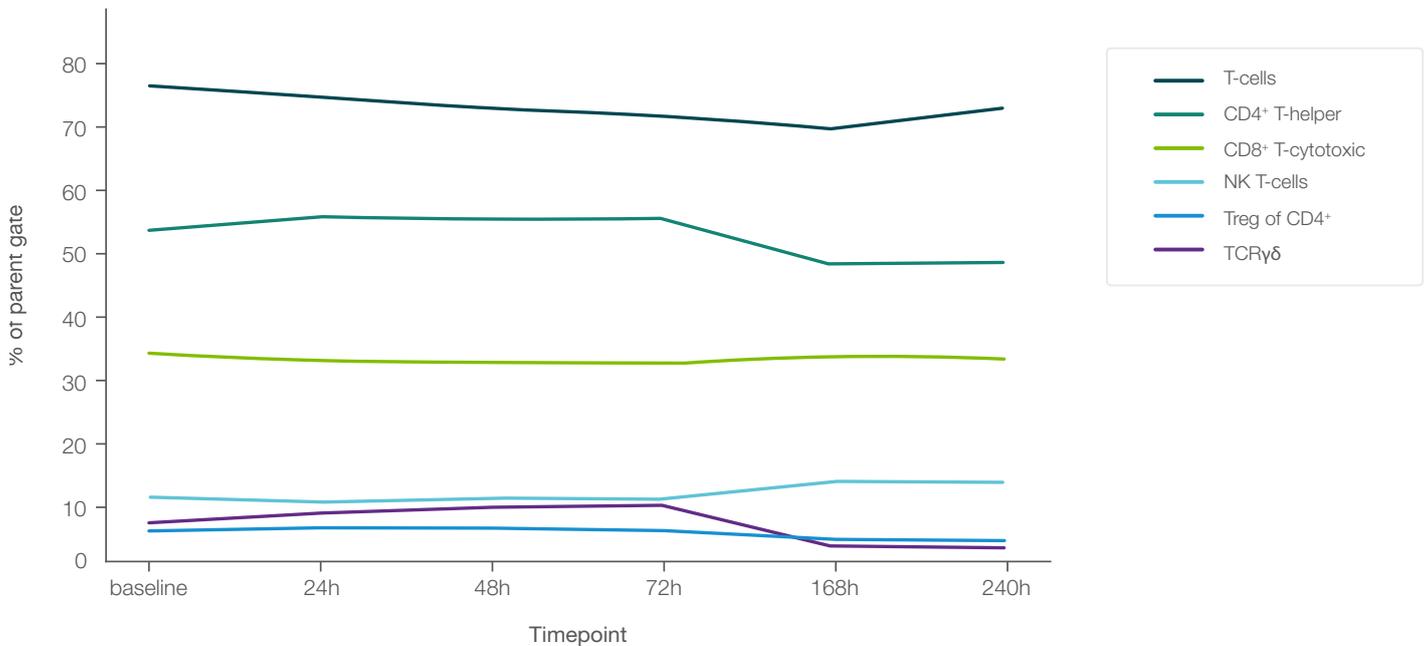
For  $\gamma\delta$  T-cells, no adequate stability was observed, as the average change from baseline already reached 25.9% after 24h. Detailed results are provided in Table 8 below.

For extended T-cell phenotyping – including early and late effector memory helper and cytotoxic T-cells, intermediate and terminal effector memory cytotoxic T-cells, CD45RA<sup>+</sup> re expressing effector memory T-cells, and CD4/CD8 double positive and double negative T-cells – most subsets remained within <20% change from baseline after 72 hours (data not shown).

**Table 8: Stability of T-cells and T-cell subsets in K3EDTA whole blood stored at 2-8°C**

	Freq of parent gate (avg of baseline)	% Change (24h)	% Change (48h)	% Change (72h)	% Change (168h)	% Change (240h)
T-cells	76.1%	2.4%	4.9%	6.5%	11.2%	8.9%
TCR $\gamma\delta$	6.6%	25.9%	42.9%	39.8%	71.6%	86.9%
NK T-cells	10.8%	8.1%	6.9%	7.1%	16.3%	19.0%
CD4 <sup>+</sup> Th of abTCR	53.3%	5.2%	4.1%	4.8%	15.9%	17.6%
Treg of CD4 <sup>+</sup> Th	5.4%	9.5%	10.6%	8.1%	29.9%	36.4%
CD8 <sup>+</sup> Tc of abTCR	33.7%	4.2%	4.9%	5.3%	7.7%	9.9%

**Stability of major T-cell subsets in K3EDTA whole blood (stored at 2-8° C)**



**Figure 2. Stability of T-cells and selected larger subsets in K3EDTA whole blood (stored at 2-8°C). The average values (percentage of parent gate) across 6 donors is shown.**

Data from the other conditions (K3EDTA (RT), NaHep (2-8 °C), and CytoChex BCT (RT)) is not shown here. In general, stability was shorter in the other non-fixed samples, but comparable in the Cytochex BCT.

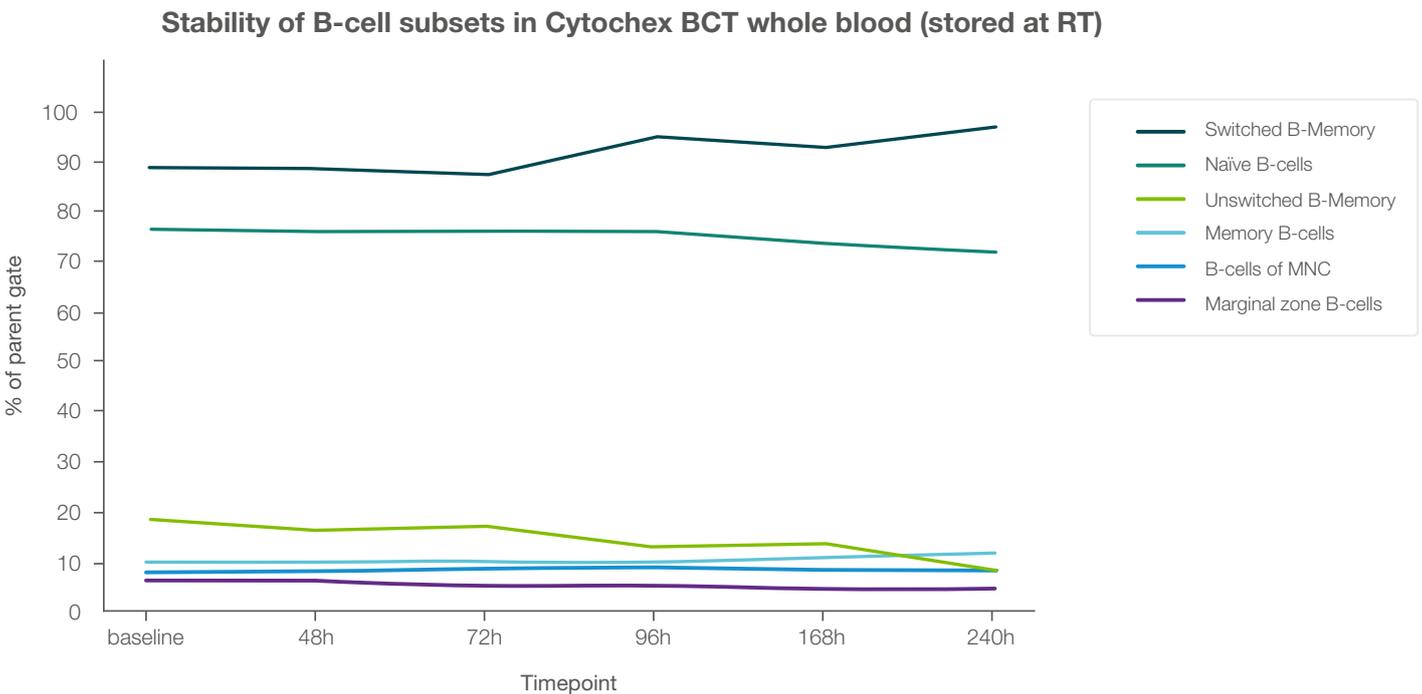
## B-cells and B-cell subsets

Large B-cell subsets- including total B-cells, memory, switched memory, and naïve B-cells can be reliably detected after 10 days of storage in Cytochex BCT, stored at RT. Smaller B-cell subsets such as unswitched memory B-cells were found to be less stable, with plasmablasts (from total B-cells) already failing at the 48h timepoint. Plasmablasts from CD20-CD27<sup>+</sup>, Double Negative B-cells, and marginal zone B-cells failed at 72h. Unswitched remain stable for 72h. Detailed results are provided in Table 9 below.

**Table 9: Stability of B-cells and B-cell subsets in Cytochex BCT whole blood stored at RT**

	Freq of parent gate (avg of baseline)	% Change (48h)	% Change (72h)	% Change (96h)	% Change (168h)	% Change (240h)
B-cells of MNC	8.2%	2.2%	3.5%	5.0%	5.5%	7.6%
Memory B-cells	9.7%	5.8%	8.0%	4.9%	15.7%	18.4%
Switched of B-Memory	88.4%	0.9%	3.0%	6.8%	4.7%	9.0%
Unswitched of B-Memory <sup>1</sup>	18.6%	3.4%	18.3%	54.4%	31.1%	92.8%
Marginal zone B-cells of B-cells	6.3%	12.3%	23.0%	22.5%	38.5%	38.1%
Naïve B cells of B-cells	76.1%	1.4%	1.9%	1.4%	4.1%	6.7%
Plasmablasts of B-cells	0.4%	49.5%	52.8%	92.2%	111.6%	NC
DN B-cells	7.2%	19.6%	28.4%	26.3%	46.6%	53.3%

1) Subset present in 3 out of 6 donors, with 1 donor fluctuating around the threshold (set at > 400 events)



**Figure 3. Stability of B-cells and selected subsets in Cytochex BCT whole blood (stored at RT). The average values (percentage of parent gate) across 6 donors is shown.**

Data from the other conditions (K3EDTA (RT and 2-8 °C), NaHep (2-8 °C) is not shown here. In general, stability was shorter in these tubes, although results in K3EDTA stored at 2-8°C showed better stability for plasmablast detection.

## NK-cells, monocytes, dendritic cells and basophils

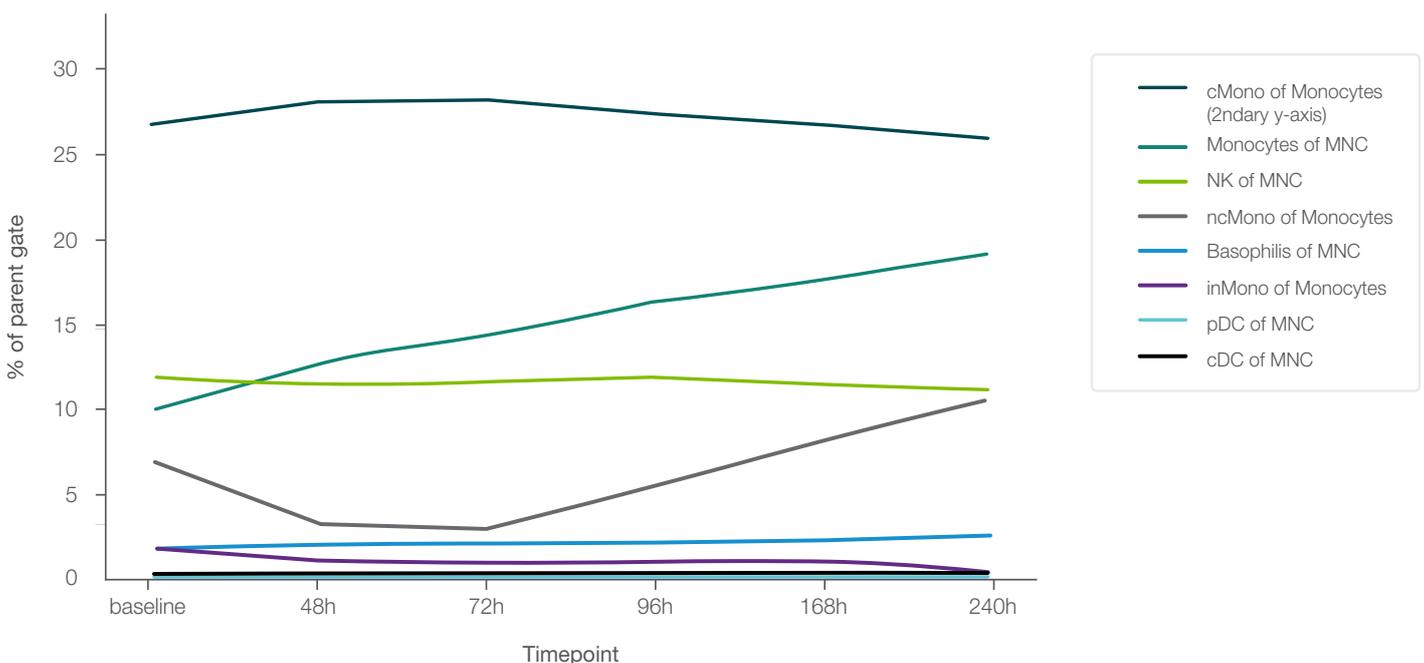
Major subsets in this panel – non-classical monocytes, NK-cells (including early and mature), NKT cells, cDC (including total and CD16<sup>-</sup>) and Basophils – can be reliably detected up to 240h of storage in Cytochex BCT, stored at RT. Basophils show >20.0% change, but since this is considered a small subset broader criteria are applied.

Out of the smaller subsets, intermediate and non-classical monocytes are not stable in this matrix. When stored in K3EDTA at 2-8°C, extended stability is observed for intermediate monocytes of up to 48h. Non-classical monocytes are stable up to 48 when stored in NaHep tubes, stored at 2-8°C (data not shown). Terminal NK cells are stable up to 48h, this stability is not improved in other matrices.

**Table 10: Stability of NK-cells, monocytes, dendritic cells and basophils in CytoChex BCT whole blood stored at RT**

	Freq of parent gate (avg of baseline)	% Change (48h)	% Change (72h)	% Change (96h)	% Change (168h)	% Change (240h)
Monocytes of MNC	10.0%	24.7%	36.6%	48.5%	55.4%	63.0%
cMono of Monocytes	88.9%	5.1%	5.5%	3.0%	2.3%	4.5%
inMono of Monocytes	1.7%	54.0%	83.3%	96.2%	82.4%	95.9%
ncMono of Monocytes	6.8%	68.1%	75.9%	27.8%	31.0%	48.0%
NK of MNC	11.8%	3.6%	3.1%	4.0%	3.5%	7.9%
Early NK of NK	2.2%	8.1%	9.2%	9.5%	11.6%	15.8%
Mature NK of NK	90.7%	1.6%	2.9%	4.7%	7.5%	9.7%
Terminal NK of NK	0.2%	18.2%	43.9%	47.4%	77.9%	96.2%
T cells of MNC	59.8%	6.4%	10.3%	12.1%	12.0%	13.1%
pDC of MNC	0.2%	21.3%	39.3%	56.2%	79.6%	130.3%
cDC of MNC	0.3%	5.9%	8.2%	5.3%	12.6%	6.4%
cDC CD16- of cDC	99.4%	0.3%	0.3%	0.2%	0.2%	0.3%
Basophils of MNC	1.8%	17.3%	16.6%	21.0%	22.5%	31.3%

**Stability of B-cell subsets in Cytochex BCT whole blood (stored at RT)**



**Figure 4. Stability of monocytes, NK-cells, dendritic cells, and basophils in CytoChex BCT whole blood (stored at RT). The average values (percentage of parent gate) across 6 donors is shown.**

## Stained cell stability

Overall, stained cell stability observed on major T-cell subsets was excellent, up to 96h after completion of sample processing. NK T-cells and Regulatory T-cells were an exception, with especially the T-regs lacking stability post 24h storage. See table below for detailed results.

For extended T-cell phenotyping - including early and late effector memory helper and cytotoxic T-cells, intermediate and terminal effector memory cytotoxic T-cells, CD45RA<sup>+</sup> re expressing effector memory T-cells, and CD4/CD8 double positive and double negative T cells - most subsets remained within <20% as well up to 96h, except for ThEMRA of CD4<sup>+</sup> Th which is <0.5% in most donors (data not shown).

**Table 11: Stained cell stability of T-cells and T-cell subsets**

	Freq of parent gate (avg of baseline)	% Change (24h)	% Change (48h)	% Change (72h)	% Change (96h)
T-cells	68.8%	0.4%	1.0%	2.4%	5.2%
TCRγδ	3.1%	1.9%	4.5%	3.2%	4.0%
NK T-cells	3.0%	17.0%	20.2%	22.6%	17.9%
CD4 <sup>+</sup> Th of abTCR	66.7%	0.3%	0.9%	0.7%	0.7%
Treg of CD4 <sup>+</sup> Th	5.8%	29.4%	50.0%	74.1%	85.6%
Th Naïve of CD4 <sup>+</sup> Th	40.0%	9.2%	8.0%	8.0%	4.8%
ThCM of CD4 <sup>+</sup> Th	48.1%	7.6%	7.2%	6.4%	4.5%
ThEM of CD4 <sup>+</sup> Th	11.7%	7.2%	4.2%	8.2%	6.6%
CD8 <sup>+</sup> Tc of abTCR	28.2%	1.3%	0.6%	0.4%	0.5%
Tc Naïve of CD8 <sup>+</sup> Tc	50.0%	0.8%	0.8%	1.3%	2.9%
TcCM of CD8 <sup>+</sup> Tc	17.4%	7.3%	7.1%	6.1%	2.7%
TcEM of CD8 <sup>+</sup> Tc	24.1%	3.7%	3.7%	6.2%	6.7%

For B-cells and B-cell subsets shorter stained cell stability was observed, with most of the populations having stability for 24h. Beyond 24h for some of the subsets the %changes are >20.0%. See table below for detailed results.

**Table 12: Stained cell stability of B-cell and B-cell subsets**

	Freq of parent gate (avg of baseline)	% Change (24h)	% Change (48h)	% Change (72h)	% Change (96h)
B-cells of MNC	7.4%	8.4%	28.2%	44.1%	50.1%
B Memory of B-cells	10.3%	9.8%	11.1%	9.0%	6.9%
Switched of B Memory	90.3%	2.4%	4.4%	4.9%	4.5%
Unswitched of B Memory	14.0%	10.6%	NC	40.9%	NC
Marginal zone B cells of B-cells	4.8%	11.6%	14.4%	13.9%	20.5%
Naïve B cells of B-cells	79.1%	2.2%	1.6%	1.1%	1.6%
Plasmablasts of B-cells <sup>1</sup>	0.7%	8.2%	25.4%	31.2%	18.6%
DN B-cells	5.1%	10.4%	19.3%	17.3%	23.8%

1) Plasmablasts only found in 2 out of 3 donors (threshold set at >100 events)

NK-cells (early and mature), Monocytes (total, classical and non-classical), NK-Tcells, gd T-cells, cDC (total and CD16-) all demonstrated 48h stained cell stability. pDC and Basophils are stable up to 24h.

**Table 13: Stained cell stability of NK-cells, monocytes, dendritic cells and basophils**

	Freq of parent gate (avg of baseline)	% Change (24h)	% Change (48h)	% Change (72h)	% Change (96h)
Monocytes of MNC	7.1%	8.4%	10.8%	24.4%	41.4%
cMono of Monocytes	63.4%	7.9%	13.6%	17.0%	21.2%
inMono of Monocytes	8.9%	NC	NC	NC	NC
ncMono of Monocytes	25.1%	8.3%	4.1%	14.1%	29.4%
NK of MNC	13.8%	6.0%	6.2%	3.8%	3.4%
Early NK of NK	3.7%	16.2%	15.4%	20.7%	25.7%
Mature NK of NK	89.0%	3.4%	6.2%	4.6%	3.8%
Terminal NK of NK	0.8%	106.5%	137.0%	97.1%	91.7%
T cells of MNC	65.6%	0.3%	2.1%	3.0%	5.3%
pDC of MNC	0.2%	29.5%	55.9%	64.1%	69.3%
cDC of MNC	0.3%	9.5%	29.5%	47.4%	58.5%
cDC CD16- of cDC	90.9%	5.5%	7.1%	8.1%	7.2%
Basophils of MNC	0.5%	22.8%	38.8%	43.2%	60.1%

### Antibody cocktail stability

Stability of stored antibody cocktail was compared to a freshly prepared cocktail, and demonstrated that cocktail can be stored for up to 4 weeks for all major cell populations described in this paper. See table below for detailed results. Majority of the in-depth subsets were also properly resolved using stored antibody cocktail, however for a few subsets we found higher %change values. These were all considered rare populations, i.e. plasmablasts, dendritic cells, and ThEMRA of T-helper cells.

**Table 14: Antibody cocktail stability, tested on PBMC samples**

	% Change 1 week	% Change 2 weeks	% Change 4 weeks
T-cells	1.7%	1.6%	5.5%
TCR $\gamma\delta$	1.5%	18.4%	0.3%
NK T-cells	2.1%	2.8%	3.4%
CD4+ Th of abTCR	0.2%	0.2%	0.1%
Treg of CD4+ Th	2.9%	1.1%	5.5%
Th Naïve of CD4+ Th	4.3%	0.0%	1.7%
ThCM of CD4+ Th	4.2%	0.2%	3.1%
ThEM of CD4+ Th	2.7%	1.5%	8.8%
CD8+ Tc of abTCR	0.4%	0.3%	1.1%
Tc Naïve of CD8+ Tc	0.7%	0.7%	2.5%
TcCM of CD8+ Tc	5.3%	8.9%	2.0%
TcEM of CD8+ Tc	4.8%	0.6%	6.1%
B-cells of MNC	0.9%	20.4%	2.9%
B Memory of B-cells	0.5%	4.1%	6.6%

**Table 14 continued: Antibody cocktail stability, tested on PBMC samples**

	% Change 1 week	% Change 2 weeks	% Change 4 weeks
Switched of B Memory	0.2%	0.4%	1.0%
Unswitched of B Memory	2.3%	3.1%	NC
Marginal zone B cells of B-cells	11.3%	9.4%	NC
Naïve B cells of B-cells	0.6%	0.5%	0.5%
Plasmablasts of B-cells	13.0%	8.1%	27.5%
DN B-cells	14.3%	0.0%	1.7%
Monocytes of MNC	9.2%	5.8%	7.5%
cMono of Monocytes	0.9%	0.1%	0.0%
inMono of Monocytes	4.6%	0.1%	2.2%
ncMono of Monocytes	4.4%	4.4%	3.8%
NK of MNC	3.6%	3.9%	0.5%
Early NK of NK	6.1%	3.3%	2.9%
Mature NK of NK	0.7%	0.1%	0.5%
Terminal NK of NK	7.0%	13.6%	NC
T cells of MNC	1.1%	1.6%	2.5%
pDC of MNC	1.3%	0.8%	0.5%
cDC of MNC	0.7%	5.4%	8.6%
cDC CD16 - of cDC	1.0%	1.1%	1.3%
Basophils of MNC	6.9%	3.3%	9.9%

## Discussion/conclusions

We successfully implemented and validated the Cytex 25-color spectral immunoprofiling assay. The assay demonstrated excellent reproducibility and repeatability. Reproducibility between analysts and instruments was well within acceptance criteria for most larger cell subsets. As expected, smaller subsets showed higher variability due to lower event counts, lower reported frequencies, and more challenging gating requirements.

A key strength of this panel – and of high-parameter flow cytometry in general – is the ability to apply extensive negative-gating strategies. This enables more accurate identification of subsets that would otherwise overlap with phenotypically similar populations. For example, monocytes were more precisely defined when dendritic cells and other confounding populations were excluded using the panel's negative-gating possibilities.

We evaluated multiple sample collection tubes to gain a comprehensive understanding of subset stability under conditions commonly used in clinical studies. For many larger subsets, samples collected in K3EDTA (stored at 4°C) and Cytochex BCT (stored at room temperature) showed stability for up to 10 days. While this level of stability is expected for Cytochex, the largely comparable stability observed in K3EDTA was unexpected. This finding is particularly valuable given that fixation in Cytochex tubes can interfere with detection of certain antigens – depending on both the antigen and antibody clone – making K3EDTA stored at 2 - 8°C a viable alternative in those cases.

The demonstrated stability of stained cells (24–96 hours, depending on subset) and the stability of the antibody cocktail support efficient sample processing and acquisition scheduling.

Overall, our results show that this assay is robust and ready for off-the-shelf use in global clinical trials.

## Considerations and future perspective

The validation results presented here represent part of an even larger dataset. Additional data - including frozen PBMC stability and pre-isolation whole blood stability (K3EDTA and Cytochex BCT) - will be incorporated into updated versions of this paper on the ICON website in the coming months.

In addition, we have expanded the panel with additional B-cell, and T-cell marker modules, bringing the total panel to 41 markers, enabling further in depth phenotyping. Validation of this panel is scheduled in 2026. The panel and modules will be transferred and cross-validated into additional ICON flow cytometry laboratories.

While implementing the 25-colour panel, we have developed an unsupervised analysis pipeline specifically tailored to this panel. For more details, refer to our companion paper on the future of flow cytometry data analysis.<sup>3</sup>



## Contact

Many sponsors choose to outsource customised assays and need a partner with the expertise to address their trial requirements. ICON has the scientific expertise to implement a broad range of flow cytometric assays in clinical trials. We cultivate a partnership with sponsors to provide scientific expertise, full-service assay development, and validation, followed by high- quality sample analysis to drive successful clinical trials forward.

To learn more about ICON's globally harmonised spectral cytometry network or to discuss your project needs visit [ICONplc.com/labs](https://iconplc.com/labs) or email [globalflowcytometryrequests@iconplc.com](mailto:globalflowcytometryrequests@iconplc.com).

## Contributions

Contributions to this work were made by members of ICON's global flow cytometry team: Frank Beltman, Fabienne Stoffers, Mariska van der Veen, Patrick Veerman, Ron Suk, Long Nguyen and Henko Tadema.

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## Appendix: Cell subset and marker-definition

The table below provides the gating strategies that were used for the reported cell subsets.

Cell type	Marker expression	Parent population
Singlets	SSC-A / SSC-H	Time
Total leukocytes	CD45+	Singlets
SSC low	SSC-A - low / CD16 -/+	Total leukocytes
Non-granulocytes	CD1c- / CD123-	SSC low
B-cell panel		
B-cells	CD3- TCRγδ- CD14- CD16- NOT(CD19- CD20-)	Non-granulocytes (MNC)
Naïve	CD3- TCRγδ- CD14- CD16- NOT(CD19- CD20-) IgD+ CD27-	B-cells
Marginal zone-like	CD3- TCRγδ- CD14- CD16- NOT(CD19- CD20-) IgD+ CD27+	B-cells
CD27+ IgD-	CD3- TCRγδ- CD14- CD16- NOT(CD19- CD20-) IgD- CD27+	B-cells
B memory	CD3- TCRγδ- CD14- CD16- NOT(CD19- CD20-) IgD- CD27+ CD20+	CD27+ IgD-
Switched B memory	CD3- TCRγδ- CD14- CD16- NOT(CD19- CD20-) IgD- CD27+ CD20+ IgM-	B memory
Unswitched B memory	CD3- TCRγδ- CD14- CD16- NOT(CD19- CD20-) IgD- CD27+ CD20+ IgM+	B memory
CD20- CD27+	CD3- TCRγδ- CD14- CD16- NOT(CD19- CD20-) IgD- CD27+ CD20-	CD27+ IgD-
Plasmablasts	CD3- TCRγδ- CD14- CD16- NOT(CD19- CD20-) IgD- CD27+ CD20-CD38+	CD20- CD27+
DN B-cells	CD3- TCRγδ- CD14- CD16- NOT(CD19- CD20-) IgD- CD27-	B-cells
T-cells		
abTCR	CD19- CD20- CD14- CD11c- CD3+ TCRγδ-	Non-granulocytes (MNC)
TCRγδ	CD19- CD20- CD14- CD11c- CD3+ TCRγδ+	Non-granulocytes (MNC)
T-cells	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- AND +	abTCR and TCRγδ
NKT	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56+	abTCR
CD4+ T Cells {Th}	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8- CD4+	Non-NKT
Th Naïve	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8- CD4+ CD45RA+ CD197+	CD4+ T Cells {Th}
Th Naïve (CD27+ CD28+)	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8- CD4+ CD45RA+ CD197+ CD27+ CD28+	Th Naïve
ThCM	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8- CD4+ CD45RA- CD197+	CD4+ T Cells {Th}
ThCM (CD27+ CD28+)	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8- CD4+ CD45RA- CD197+ CD27+ CD28+	ThCM
ThEM	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8- CD4+ CD45RA- CD197-	CD4+ T Cells {Th}
ThEEM	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8- CD4+ CD45RA- CD197- CD27+ CD28+	ThEM
ThELEM	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8- CD4+ CD45RA- CD197- CD27- CD28+	ThEM
ThTEM	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8- CD4+ CD45RA- CD197- CD27- CD28-	ThEM
ThEMRA	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8- CD4+ CD45RA+ CD197-	CD4+ T Cells {Th}

T-cells		
ThEMRA (CD27-CD28-)	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8- CD4+ CD45RA+ CD197- CD27- CD28-	ThEMRA
Treg	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8- CD4+ CD127- CD25+	CD4+ T Cells {Th}
CD8+ T Cells {Tc}	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8+ CD4-	Non-NKT
Tc Naïve	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8+ CD4- CD45RA+ CD197+	CD8+ T Cells {Tc}
Tc Naïve (CD27+ CD28+)	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8+ CD4- CD45RA+ CD197+ CD27+ CD28+	Tc Naïve
TcCM	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8+ CD4- CD45RA- CD197+	CD8+ T Cells {Tc}
TcCM (CD27+ CD28+)	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8+ CD4- CD45RA- CD197+ CD27+ CD28+	TcCM
TcEM	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8+ CD4- CD45RA- CD197-	CD8+ T Cells {Tc}
TcEEM	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8+ CD4- CD45RA- CD197+ CD27+ CD28+	TcEM
TcELEM	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8+ CD4- CD45RA- CD197+ CD27- CD28+	TcEM
TcTEM	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8+ CD4- CD45RA- CD197+ CD27- CD28-	TcEM
TcIEM	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8+ CD4- CD45RA- CD197+ CD27+ CD28-	TcEM
TcEMRA	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8+ CD4- CD45RA+ CD197-	CD8+ T Cells {Tc}
TcEMRA (CD27- CD28-)	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8+ CD4- CD45RA+ CD197- CD27- CD28-	TcEMRA
DP T-cells	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8+ CD4+	Non-NKT
DN T-cells	CD19- CD20- CD14- CD11c- CD3+ TCRγδ- CD56- CD8- CD4-	Non-NKT

Monocytes, NK-cells, gd T-cells, Dendritic Cells and Basophils		
Non B-cells	CD19- CD20-	SSC low (MNC)
Non TBNK	CD19- CD20- CD3- CD56-	SSC low (MNC)
CD4- CD123+	CD19- CD20- CD3- CD56- CD4- CD123+	Non TBNK
CD4dim CD123-	CD19- CD20- CD3- CD56- CD4dim CD123-	Non TBNK
CD4+ CD123+	CD19- CD20- CD3- CD56- CD4+ CD123+	Non TBNK
Pre-monocytes	CD19- CD20- CD3- CD56- CD4dim CD123- HLA-DR+ CD1c-	CD4dim CD123-
HLA-DR+ CD1c+	CD19- CD20- CD3- CD56- CD4dim CD123- HLA-DR+ CD1c+	CD4dim CD123-
Classical Monocytes	CD19- CD20- CD3- CD56- CD4dim CD123- HLA-DR+ CD1c- CD14++ CD16-/ dim	Pre-monocytes
Intermediate Monocytes	CD19- CD20- CD3- CD56- CD4dim CD123- HLA-DR+ CD1c- CD14+ CD16+	Pre-monocytes
Non-Classical Monocytes	CD19- CD20- CD3- CD56- CD4dim CD123- HLA-DR+ CD1c- CD14- CD16+	Pre-monocytes
Non T-cells	CD19- CD20- HLA-DR- CD3-	Non B-cells
Pre NK	CD19- CD20- HLA-DR- CD3- CD14- CD123-	Non T-cells
Early NK	CD19- CD20- HLA-DR- CD3- CD14- CD123- CD56- CD16-	Pre NK

## Monocytes, NK-cells, gd T-cells, Dendritic Cells and Basophils

Mature NK	CD19- CD20- HLA-DR- CD3- CD14- CD123- CD56dim CD16+	Pre NK
Terminal NK	CD19- CD20- HLA-DR- CD3- CD14- CD123- CD56- CD16++	Pre NK
T-cells	CD19- CD20- CD3+	Non B-cells
CD4- TCRγδ+	CD19- CD20- CD3+ CD4- TCRγδ+	T-cells
CD4- TCRγδ-	CD19- CD20- CD3+ CD4- TCRγδ-	T-cells
TCRγδ	CD19- CD20- CD3+ CD4- TCRγδ+ CD56- CD11c-	CD4- TCRγδ+
NK T-cells	CD19- CD20- CD3+ CD4- TCRγδ- CD56+ CD8+	CD4- TCRγδ-
pDC	CD19- CD20- CD3- CD56- CD4+ CD123+ HLA-DR+ CD11c-	CD4+ CD123+
cDC	CD19- CD20- CD3- CD56- CD4dim CD123- HLA-DR+ CD1c+ HLA-DR+ CD11c+	HLA-DR+ CD1c+
cDC CD16-	CD19- CD20- CD3- CD56- CD4dim CD123- HLA-DR+ CD1c+ HLA-DR+ CD11c+ CD16-	cDC
cDC CD16+	CD19- CD20- CD3- CD56- CD4dim CD123- HLA-DR+ CD1c+ HLA-DR+ CD11c+ CD16+	cDC
Basophils	CD19- CD20- CD3- CD56- CD4- CD123+ HLA-DR- CD38+	CD4- CD123+