# Validation of a Flow Cytometry Assay on Cytek® Aurora to Monitor Immune Cells in Peripheral Whole Blood for Clinical Trials

Jan Spitaels[1], Feyzâ Matisli[1], Amber Baele[2], Leen Catrysse[2], Silke De Waele[1], Miet De Baere[1], Veronica Nash[2] and Nithianandan Selliah[2]

[1]Cerba Healthcare Belgium, Division CRI; [2]Cerba Research, Ghent, Belgium and New York, USA

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## Introduction

Immune monitoring of patients enrolled in clinical trials for drug development is of pivotal importance to support evaluation of drug safety and efficacy. The ability to develop high parameter panels with spectral flow cytometry, allows for a deeper characterization of patient samples and for an exhaustive picture of immune system dynamic in response to a specific pipeline asset. Here, we describe the validation workflow of a 14-color assay designed to characterize T cells, B cells, NK cells, monocytes, and subsets thereof for use in immune monitoring of patients with hematological malignancies for global clinical trials.

## Flow Cytometry Assay Description

A 14-color Flow cytometry assay, BL_TBNKM_CR14C, was developed at Cerba Research from Cytek cFluor™ immunoprofiling 14 colors kit, initially created by Cytek® Biosciences. The assay was validated on a Cytek® Aurora (UV/V/B/YG/R, 64 detectors) for the purpose of monitoring T cells, B cells, NK cells, monocytes, and their subsets in peripheral whole blood (WB), collected in Cyto-Chex® BCT (Streck). Panel configuration, gating strategy and panel endpoints are shown in Figure 1A, 1B, and Table 1, respectively.

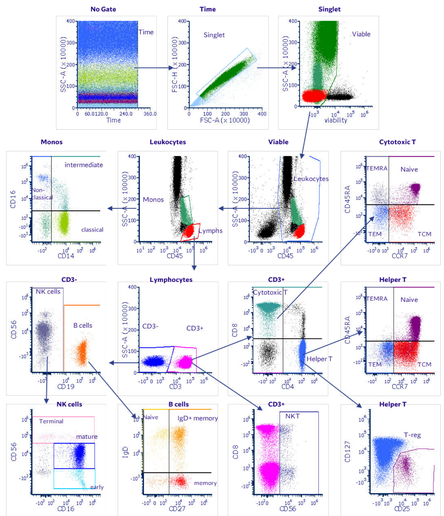
Figure 1A: Panel Configuration

| Violet | | Blue | | Red | |
|---|---|---|---|---|---|
| Marker | Fluorochrome | Marker | Fluorochrome | Marker | Fluorochrome |
| CD3 | V420 | CD8 | B515 | CD127 | R659 |
| CD14 | V450 | CCR7 | BYG575 | CD16 | R668 |
| CD45 | V547 | IgD | BYG667 | CD56 | R720 |
| | | CD45RA | B690 | CD4 | R780 |
| | | CD19 | BYG710 | viability | ViaDye Red |
| | | CD25 | BYG781 | CD27 | R840 |

Table 1: Reportable populations and respective immunophenotype

| Population | Immunophenotype |
|---|---|
| Monocytes | SSC high, FSC high, CD45+ |
| Monocytes, classical | SSC high, FSC high, CD45+, CD14++, CD16- |
| Monocytes, intermediate | SSC high, FSC high, CD45+, CD14++, CD16+ |
| Monocytes, non-classical | SSC high, FSC high, CD45+, CD14+, CD16++ |
| Lymphocytes | SSC low, FSC high, CD45++ |
| T Cells | SSC low, FSC high, CD45++, CD3+ |
| CD4+ T Cells | SSC low, FSC high, CD45++, CD3+, CD4+, CD8- |
| CD4+ T Cells, Naïve | SSC low, FSC high, CD45++, CD3+, CD4+, CD8-, CCR7+, CD45RA+ |
| CD4+ T Cells, Central memory (CM) | SSC low, FSC high, CD45++, CD3+, CD4+, CD8-, CCR7+, CD45RA- |
| CD4+ T Cells, Effector memory (TEM) | SSC low, FSC high, CD45++, CD3+, CD4+, CD8-, CCR7-, CD45RA- |
| CD4+ T Cells, RA+Effector memory (TEMRA) | SSC low, FSC high, CD45++, CD3+, CD4+, CD8-, CCR7-, CD45RA+ |
| T regulatory cells | SSC low, FSC high, CD45++, CD3+, CD4+, CD8-, CD127low/-, CD25+ |
| CD8+ T Cells | SSC low, FSC high, CD45++, CD3+, CD8+, CD4- |
| CD8+ T Cells, naive | SSC low, FSC high, CD45++, CD3+, CD8+, CD4-, CCR7+, CD45RA+ |
| CD8+ T Cells, Central memory (CM) | SSC low, FSC high, CD45++, CD3+, CD8+, CD4-, CCR7+, CD45RA- |
| CD8+ T Cells, Effector memory (TEM) | SSC low, FSC high, CD45++, CD3+, CD8+, CD4-, CCR7-, CD45RA- |
| CD8+ T Cells, RA+Effector memory (TEMRA) | SSC low, FSC high, CD45++, CD3+, CD8+, CD4-, CCR7-, CD45RA+ |
| Double positive T cells | SSC low, FSC high, CD45++, CD3+, CD4+, CD8+ |
| Double negative T cells | SSC low, FSC high, CD45++, CD3+, CD4-, CD8- |
| B cells | SSC low, FSC high, CD45++, CD3-, CD19+ |
| B cells, naive | SSC low, FSC high, CD45++, CD3-, CD19+, CD27-, IgD+ |
| B cells, memory | SSC low, FSC high, CD45++, CD3-, CD19+, CD27+, IgD- |
| NK cells | SSC low, FSC high, CD45++, CD3-, CD16+, CD56-/+ |
| NK cells, CD56hi | SSC low, FSC high, CD45++, CD3-, CD16+, CD56++ |
| NK cells, CD56dim | SSC low, FSC high, CD45++, CD3-, CD16+, CD56dim |
| NK cells, CD56-CD16+ | SSC low, FSC high, CD45++, CD3-, CD16+, CD56- |
| NKT cells | SSC low, FSC high, CD45++, CD3+, CD56+ |

Figure 1B: Gating strategy (FCS Express™ (De Novo Software))



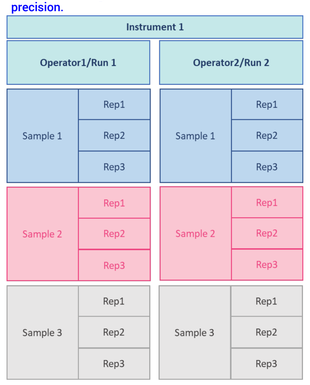

## Analytical Validation

Assay validation is critical to ensure that the data obtained from an assay are interpretable and transparent between multiple time points for a patient, within large patient cohorts, tested at different locations around the world.

https://cerbaresearch.com/posters/evidence-for-the-utility-of-artificial-intelligence-ai-and-image-analysis-in-ki-67-quantification-in-solid-tumors/

Validation parameters to evaluate depend on the context of use (COU) of the assay. Precision (repeatability, reproducibility, inter-operator variability), and sample stability were evaluated to validate the assay for exploratory endpoints.

Figure 2: Method validation experimental design. The optimized design allows for calculation of all precision.




## Instrument Set-up and Assay Optimization

### Assay settings and reference controls

Assay-specific settings were created by adjusting forward scatter (FSC) gain and threshold from the default Cytek Assay Settings (CAS), generated during installation and operational qualification (IQ/OQ). These settings have optimized median fluorescence intensity (MFI) target values, used by the software during daily quality control (QC) run to set voltages for acquisition. The gains are automatically updated each day following daily QC. Reference controls created with SpectraComp® beads (Slingshot Biosciences) were used for error-free unmixing of samples.

### Viability dye titration

Titration of the viability dye, ViaDye™ Red, was performed to establish the optimal concentration to discriminate dead cells in Cyto-Chex® BCT. A 3-point titration was performed in WB from an apparently healthy donor, starting from the manufacturer's recommendation which is 1:100. For the second and third concentration, 5-fold and 10-fold sequential dilutions were performed, respectively. An aliquot of heat-treated WB (incubated 1 minute at 60°C) was mixed in a 1:1 ratio with non-heat-treated WB of the same donor to generate a portion of dead cells that could be detected by the dye. The 1:500 dilution of ViaDye™ Red (final dilution of 1:50,000) was defined as the optimal dilution that shows a reduction in the non-specific staining of monocytes and granulocytes, with a discrete separation of dead-live cells (Fig 3A). NxN plots generated from data obtained during feasibility run show no unmixing errors (Fig 3B).

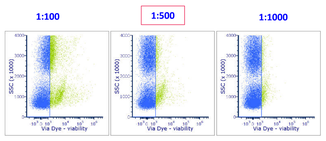



Figure 3A: Viability Dye titration

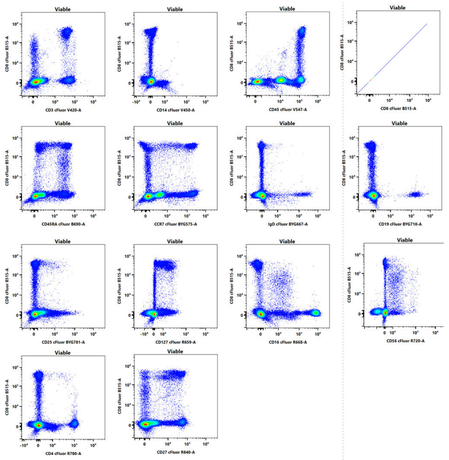


Figure 3B: NxN plot (SpectroFlo® (Cytek® Biosciences)) for unmixing evaluation of feasibility run that uses 1:500 dilution of Viadye™

## Precision

Precision evaluation describes the closeness of agreement between individually measured values. It is assessed by means of repeatability (intra-assay or within-run) and reproducibility (inter-assay or between-run) experiments and evaluated by the percentage of variation among measurements, expressed by percentage of coefficient of variation (%CV).

Acceptance criterion of ≤25%CV was applied for all the reportables. Higher imprecision (30-35%CV) was accepted for rare populations *(≤5% of parent population or ≤100 events)* or populations with dimly expressed antigens. For reproducibility assessment, overall %CV of 25 was applied per sample.

For reproducibility (Table 2) and repeatability (Table 3), %CV for primary reportables are within the acceptance criteria. Highlighted in **bold** is the value above its acceptance criterion, relative to population with a frequency of <5% of parent population, where a higher variability is expected.

$$\%CV = \frac{SD}{MEAN} \times 100$$

Figure 4A: Repeatability calculation

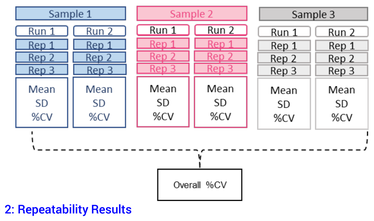



Table 2: Repeatability Results

| Reportables | Parental population | Sample 1 Op 1 Run1 | Sample 1 Op 2 Run 2 | Sample 2 Op 1 Run1 | Sample 2 Op 2 Run 2 | Sample 3 Op 1 Run1 | Sample 3 Op 2 Run 2 | Overall %CV |
|---|---|---|---|---|---|---|---|---|
| | | %CV | | | | | | |
| Lymphocytes | % Leuko | 2,63 | 2,95 | 0,11 | 4,44 | 2,52 | 4,76 | 2,90 |
| T Cells | % Ly | 0,27 | 0,64 | 0,22 | 0,24 | 1,28 | 0,66 | 0,55 |
| CD4+ T Cells | % T | 0,31 | 0,15 | 0,60 | 0,34 | 0,55 | 0,35 | 0,39 |
| Naïve | % CD4+ | 0,09 | 0,49 | 0,16 | 1,00 | 1,36 | 1,39 | 0,75 |
| Central memory (CM) | | 1,44 | 0,93 | 3,12 | 1,27 | 1,00 | 0,97 | 1,46 |
| Effector memory (TEM) | | 4,01 | 0,59 | 7,80 | 2,52 | 1,55 | 1,54 | 3,00 |
| RA+Effector memory (TEMRA) | | 15,06 | 15,23 | 13,29 | 8,11 | 32,73 | 13,32 | 16,29 |
| T regulatory cells | | 2,98 | 2,39 | 4,33 | 4,15 | 3,59 | 5,23 | 3,78 |
| CD8+ T Cells | % T | 0,72 | 0,62 | 1,47 | 1,16 | 0,08 | 0,28 | 0,72 |
| Naïve | % CD8+ T | 0,91 | 0,25 | 1,01 | 0,95 | 1,40 | 1,77 | 1,05 |
| Central memory (CM) | | 2,19 | 2,04 | 16,59 | 2,32 | 2,20 | 2,41 | 4,63 |
| Effector memory (TEM) | | 1,14 | 1,16 | 1,27 | 1,32 | 2,76 | 5,31 | 2,16 |
| RA+Effector memory (TEMRA) | | 2,82 | 6,98 | 2,99 | 0,47 | 1,19 | 5,75 | 3,37 |
| NKT cells | % Ly | 4,74 | 5,80 | 2,67 | 3,05 | 8,76 | 8,51 | 5,59 |
| Monocytes | % Leuko | 5,37 | 5,34 | 1,80 | 6,70 | 2,81 | 3,53 | 4,26 |
| Classical | % Mono | 1,35 | 1,87 | 1,38 | 0,51 | 0,74 | 0,68 | 1,09 |
| Intermediate | | 13,28 | 8,14 | 15,73 | 11,84 | 6,29 | 10,64 | 10,99 |
| Non-classical | | 6,03 | **56,73*** | 4,49 | 7,59 | 18,94 | 13,93 | 17,95 |
| B cells | % Ly | 3,63 | 6,10 | 4,88 | 7,72 | 4,76 | 3,63 | 5,12 |
| Naïve | % B | 2,59 | 3,36 | 12,87 | 9,84 | 5,01 | 2,54 | 6,03 |
| Memory | | 7,90 | 1,12 | 2,32 | 8,47 | 4,13 | 1,07 | 4,17 |
| IgD+ memory | | 4,62 | 0,97 | 12,63 | 4,09 | 19,01 | 7,54 | 8,14 |
| NK cells | % Ly | 1,30 | 1,56 | 2,00 | 0,44 | 2,38 | 1,32 | 1,50 |
| CD56hi | % NK | 3,36 | 13,11 | 14,77 | 15,34 | 14,50 | 13,74 | 12,47 |
| CD56dim | | 1,02 | 0,42 | 0,35 | 0,69 | 0,94 | 0,53 | 0,66 |
| CD56-CD16+ | | 8,06 | 6,65 | 4,13 | 12,11 | 6,21 | 1,58 | 6,46 |

Figure 4B: Reproducibility calculation

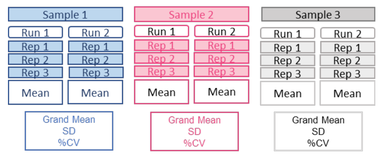



Table 3: Reproducibility Results

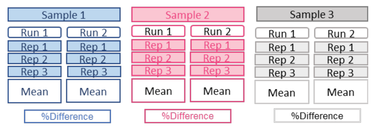
| Reportables | Parental population | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|---|
| | | %CV | | |
| Lymphocytes | % Leuko | 3.71 | 1.9 | 5.05 |
| T Cells | % Ly | 0.28 | 0.14 | 0.83 |
| CD4+ T Cells | % T | 1.01 | 0.38 | 1.53 |
| Naïve | % CD4+ | 0.59 | 0.37 | 0.32 |
| Central memory (CM) | | 1.77 | 0.42 | 9.64 |
| Effector memory (TEM) | | 8.13 | 14.06 | 5.24 |
| RA+Effector memory (TEMRA) | | 2.53 | 0.91 | 2.29 |
| T regulatory cells | | 0.32 | 0.19 | 0.79 |
| CD8+ T Cells | % T | 0 | 0.09 | 0.98 |
| Naïve | % CD8+ T | 5.37 | 2.96 | 0.49 |
| Central memory (CM) | | 4.74 | 0.33 | 0.26 |
| Effector memory (TEM) | | 3.49 | 1.5 | 5.46 |
| RA+Effector memory (TEMRA) | | 8.38 | 11.34 | 5.87 |
| NKT cells | % Ly | 1.97 | 1.94 | 6.25 |
| Monocytes | % Leuko | 2.26 | 0.44 | 0.79 |
| Classical | % Mono | 17.68 | 0.4 | 7.7 |
| Intermediate | | **37.07*** | 0.56 | 14.14 |
| Non-classical | | 8.61 | 3.96 | 2.89 |
| B cells | % Ly | 4.87 | 10.42 | 1.34 |
| Naïve | % B | 14.04 | 6.41 | 5.29 |
| Memory | | 8.22 | 1.66 | 6.6 |
| IgD+ memory | | 0.49 | 0.94 | 1.12 |
| NK cells | % Ly | 18.88 | 16.2 | 13.02 |
| CD56hi | % NK | 0.16 | 0.18 | 0.17 |
| CD56dim | | 6.25 | 3.91 | 0.26 |
| CD56-CD16+ | | 0.59 | 0.37 | 0.32 |

## Inter-operator variability

Inter-operator variability is assessed to document the compatibility of an assay setup by more than one operator. Acceptance criterion of ≤20% difference between operators was applied.

For inter-operator variability, %difference for primary reportables are within acceptance criteria. Highlighted in **bold** are the values above acceptance criteria relative to the populations with a frequency of <5% of parent population, where a higher variability is expected.

Figure 4C: Inter-Operator variability calculation




$$\%difference = ABS\left(\frac{Mean\ Operator\ 1 - Mean\ Operator\ 2}{Mean\ of\ (Mean\ Operator\ 1\ and\ Mean\ Operator\ 2)} \times 100\right)$$

*ABS: absolute value

Table 4: Inter-operator variability

| Reportables | Parental population | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|---|
| | | %CV | | |
| Lymphocytes | % Leuko | 5.25 | 2.69 | 7.15 |
| T Cells | % Ly | 1.05 | 0.35 | 1 |
| CD4+ T Cells | % T | 0.4 | 0.19 | 1.17 |
| Naïve | % CD4+ | 0.02 | 0.05 | 2.08 |
| Central memory (CM) | | 0.83 | 0.53 | 0.45 |
| Effector memory (TEM) | | 2.5 | 0.59 | 13.63 |
| RA+Effector memory (TEMRA) | | 11.49 | 19.88 | 7.41 |
| T regulatory cells | | 3.58 | 1.29 | 3.24 |
| CD8+ T Cells | % T | 0.46 | 0.27 | 1.11 |
| Naïve | % CD8+ T | 0.01 | 0.13 | 1.39 |
| Central memory (CM) | | 7.59 | 4.19 | 0.7 |
| Effector memory (TEM) | | 6.7 | 0.46 | 0.36 |
| RA+Effector memory (TEMRA) | | 4.94 | 2.13 | 7.72 |
| NKT cells | % Ly | 11.85 | 16.04 | 8.29 |
| Monocytes | % Leuko | 2.78 | 2.75 | 8.84 |
| Classical | % Mono | 3.19 | 0.63 | 1.11 |
| Intermediate | | **25.00*** | 0.57 | 10.89 |
| Non-classical | | **52.43*** | 0.79 | 20 |
| B cells | % Ly | 12.18 | 5.6 | 4.09 |
| Naïve | % B | 6.89 | 14.74 | 1.89 |
| Memory | | 19.85 | 9.07 | 7.48 |
| IgD+ memory | | 11.62 | 2.35 | 9.33 |
| NK cells | % Ly | 0.69 | 1.32 | 1.58 |
| CD56hi | % NK | **26.69*** | **22.91*** | 18.41 |
| CD56dim | | 0.23 | 0.26 | 0.23 |
| CD56-CD16+ | | 8.84 | 5.53 | 0.36 |

## Sample stability

Antigen expression, cellular composition and cellular viability can change over time in an anticoagulant blood collection tube. Evaluation of sample stability determines the time window when a sample can be tested, and data can be considered reliable. Samples from 6 different apparently healthy donors were processed at baseline (within 2 hours after collection or upon receipt), and at 24h, 48h, 72h, 96h and 168h from baseline time. Samples were kept refrigerated (2°C – 6°C) until processed.

Stability was established at 96h, which is the latest time point where a maximal change of 20% from the baseline was achieved for at least 80% of the samples, for most of the reportables.

Exceptions: T-regulatory cells, and B cells reportables (primary reportables are shown in Table 5)

$$\%change = ABS\left(\frac{Time\ point\ x - Baseline}{Baseline} \times 100\right)$$

*ABS: absolute value

Table 5: Stability Results

| Reportables | Parental population | Stability |
|---|---|---|
| T Cells | % Ly | 96h |
| CD4+ T Cells | % T | 96h |
| Naïve | % CD4+ | 96h |
| Central memory (CM) | | 96h |
| RA+Effector memory (TEMRA) | | 96h |
| T regulatory cells | | 48h |
| CD8+ T Cells | % T | 96h |
| Naïve | % CD8+ T | 96h |
| Effector memory (TEM) | | 96h |
| RA+Effector memory (TEMRA) | | 96h |
| NKT cells | % Ly | 96h |
| Classical | % Mono | 96h |
| Intermediate | | 96h |
| Non-classical | | 96h |
| B cells | % Ly | fail |
| Memory | % B | 72h |
| IgD+ memory | | fail |
| NK cells | % Ly | 96h |
| CD56hi | % NK | 96h |
| CD56-CD16+ | | 96h |

## Conclusions

The workflow discussed here provides guidance on how to validate a high parameter panel to be implemented for patient immune profiling in global clinical trials. We showed that assay endpoints for BL_TBNKM_CR14C assay meets acceptance criteria for repeatability, reproducibility and inter-operator variability, in accordance with CLSI H62 guidelines. Sample stability was defined at 96 hours for most of the reportables. This panel is validated to be used in global clinical trials for exploratory purposes only. It will be most useful for hematological malignancies and cell and gene therapy trials.

Cerba Research
www.cerbaresearch.com
Flow Cytometry Science Team
flowcytometry@cerbaresearch.com

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