Optimization, Validation and Implementation of a 25-color Flow Cytometry Assay on Cytek® Aurora Instrument to Monitor Immune Cells in Whole Blood for Global Clinical Trials

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Abstract

Immune monitoring of patients enrolled in clinical trials for drug development is of pivotal importance to support evaluation of drug safety and efficacy, especially for hematological malignancies and/or cell and gene therapy trials. The ability to develop high parameter panels with spectral flow cytometry allows for deeper characterization of patient samples in response to a specific drug. Here, we describe the implementation workflow of a 25-color assay designed to characterize major human immune subpopulations for T, B, NK cells, monocytes, dendritic cells, basophils, and subsets.

25-color immunoprofiling cFluor® kit (Cytek®) was optimized at Cerba Research in blood collected in Cyto-Chex® tube. A comprehensive titration of the ViaDye™ red fixable viability dye showed that live-dead cells can be discriminated without nonspecific staining in Cyto-Chex® tubes. Optimal reference controls were determined by comparing spectral signatures of single stained controls between cells and beads. For beads, SpectraComp® from Slingshot Biosciences was an acceptable alternative to cells for most fluorochromes with some exceptions.

After panel optimization, analytical validation was performed allowing for assessment of repeatability, reproducibility, and inter-operator variability. Data shows that all primary populations are within acceptance criteria according to CLSI H62 guidelines.. The assay was transferred to a second testing site with assessment of repeatability, reproducibility, and assay performance correlation with same donor blood samples (data pending).

Instruments in two locations, US & Belgium, were standardized with Median Fluorescence Intensity (MFI) alignment. Data were acquired to assess MFI, spillover spreading matrix (SSM) and performance comparison with PBMC sample. All parameters assessed show both instruments are standardized and ready for multi-site global clinical trials.

Panel Configuration

Violet		Blue		Yellow green		Red	
Specificity	Fluorochrome	Specificity	Fluorochrome	Specificity	Fluorochrome	Specificity	Fluorochrome
CCR7	BV421	CD141	cFluor B515	CD25	cFluor BYG575	CD127	cFluor R659
CD45RA	cFluor V450	CD8	cFluor B532	CD4	cFluor YG584	CD1c	cFluor R668
IgM	BV510	CD14	cFluor B548	CD16	cFluor BYG610	CD19	cFluor R685
CD20	cFluor V547	HLADR	cFluor B690	IgD	cFluor BYG667	CD123	cFluor R720
CD3	BV570			TCRgd	cFluor BYG710	CD45	cFluor R780
CD28	BV650			CD11c	cFluor BYG781	ViaDye Red	
CD38	BV711					CD27	cFluor R840
CD56	BV750						
PD-1	BV785						

Figure 1: Panel configuration of Cytek® 25-color immunoprofiling assay, cFluor® reagent kit. 18-antibodies are within the kit and 7 Brilliant Violet antibodies are from BioLegend.

Method

Whole blood collected in Cyto-Chex® BCT were obtained from 3 apparently healthy donors. Cyto-Chex® tubes were refrigerated (2-8°C) until processed.

Antibody titration was not performed as the reagent kit is optimized by Cytek for all the antibodies.

ViaDye Red viability dye was prepared as manufacturer's recommendation. Titration of the ViaDye was performed with healthy donor blood collected in Cyto-Chex® tubes, and half the volume was killed at 65ºC for one minute and mixed with fresh blood.

200µL of WB was stained as described in the Cytek biosciences validation report (DOC-00327 Rev. B,).

Stained samples were acquired on 5-laser Cytek Aurora instrument with default Cytek assay settings (CAS) with optimized MFI target values established during the instrument installation qualification.

Data were analyzed using FCS Express software (clinical edition version 7.0).

Results

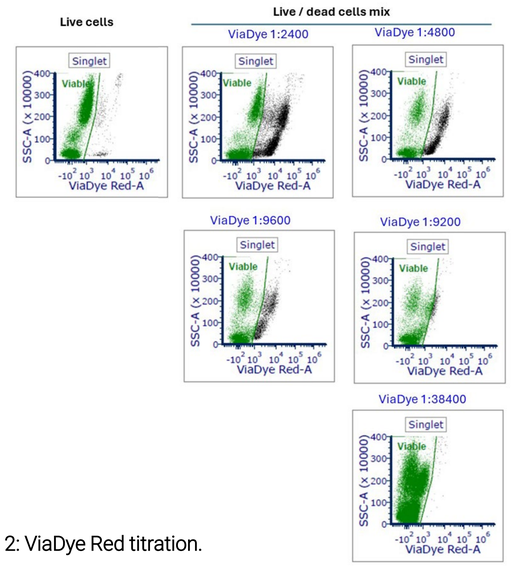

Figure 2: ViaDye Red titration.

ViaDye Red viability dye was titrated with a 5-point titration to optimize the signal to noise ratio and nonspecific staining of populations in Cyto-Chex® tubes. Titration of the ViaDye Red was performed with healthy donor blood collected in Cyto-Chex® tubes, and half the volume was killed at 65ºC for one minute and mixed with fresh blood (1:1). A 5-point titration was done starting from the dilution selected to be optimal in another assay, and 2-fold dilutions were performed. The concentration of the dye resulting in error-free unmixing, and lack of nonspecific binding was chosen. For all the precision experiments, 1:9600 dilution was made and 5µL was added per 200µL whole blood.

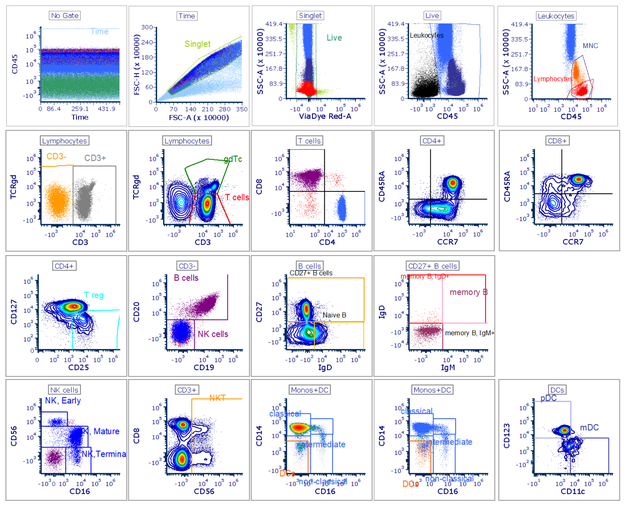

Figure 3a: Representative plots of one sample to highlight primary populations.

Figure 3b: Precision (repeatability and reproducibility) and inter-operator variability assessment.

Whole blood samples from 3 apparently healthy donors were processed in triplicate by two operators and acquired on one instrument.

For precision, data show all the primary reportables are within the acceptance criteria of <25%CV. Inter-operator variability assessment show that all the primary reportables are within the acceptance criteria of <20% difference between operators.

$$\%CV = \frac{SD}{Mean} \times 100 \qquad \% \text{ difference} = \frac{MFI_{\text{reference instrument}} - MFI_{\text{value instrument of choice}}}{MFI_{\text{reference instrument}}} \times 100$$

	%CV Repeatability						%CV Reproducibility			%difference inter-operator			Grand Mean of Operator 1 and Operator 2		
	Donor 1		Donor 2		Donor 3										
	Operator 1	Operator 2	Operator 1	Operator 2	Operator 1	Operator 2	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
Repotables	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2									
Monocytes (% of Leukocytes)	3.03	3.78	2.02	0.50	0.96	1.25	0.24	0.84	2.39	0.34	1.19	3.38	5.82	5.03	3.55
Monocytes, classical (% of Monocytes)	0.37	0.54	0.56	0.46	1.03	0.79	0.04	1.25	1.45	0.06	1.77	2.06	5.41	4.34	3.24
Monocytes, intermediate (% of Monocytes)	1.84	2.22	1.06	7.32	14.13	8.09	6.63	1.99	11.90	9.37	2.82	16.82	3.69	4.71	5.05
Monocytes, non-classical (% of Monocytes)	10.37	12.16	5.03	0.54	4.61	12.51	1.19	3.63	11.16	1.68	5.13	15.79	3.44	9.00	3.56
Dendritic cells (DC) (% of Monos+DC)	7.97	2.00	2.03	3.21	1.08	2.79	4.84	0.83	1.39	6.84	1.17	1.97	5.70	9.41	6.78
Plasmacytoid DC (pDC) (% of DC)	1.38	3.11	1.74	2.68	1.19	4.93	2.39	2.48	3.25	3.38	3.51	4.60	47.20	60.23	36.95
Myeloid DC (mDC) (% of DC)	5.34	2.77	2.43	5.57	5.37	8.57	2.82	0.61	4.12	3.99	0.87	5.83	25.82	21.51	36.58
Lymphocytes (% of Leukocytes)	1.96	3.69	0.78	0.41	1.25	3.09	6.20	3.67	4.95	8.76	5.19	6.99	22.14	30.58	21.83
gdT Cells (% of lymphocytes)	3.88	0.58	1.67	3.13	6.32	5.38	4.22	1.35	1.25	5.96	1.90	1.76	10.28	10.15	7.76
gdT Cells (% of CD3+)	4.50	1.01	1.72	3.06	6.17	5.37	4.18	1.36	1.07	5.91	1.93	1.51	15.47	14.54	10.13
T Cells (CD3+TCRgd-) (% of lymphocytes)	1.56	0.70	0.31	0.49	0.58	0.59	0.67	0.23	0.18	0.95	0.33	0.25	56.36	59.84	68.92
T Cells (CD3+TCRgd-) (% of CD3+)	0.89	0.17	0.28	0.54	0.67	0.60	0.77	0.23	0.02	1.09	0.32	0.02	84.77	85.67	90.03
CD4+ T Cells (% of T Cells)	0.29	0.63	0.08	0.21	0.34	0.16	0.89	0.19	0.12	1.25	0.26	0.16	58.49	55.79	73.64
CD4+ T Cells, Naive (% of T Cells)	0.47	1.15	0.36	0.46	0.25	0.77	2.74	1.18	1.06	3.88	1.67	1.50	23.12	32.93	45.20
CD4+ T Cells, Naive (% of CD4+ T Cells)	0.75	1.40	0.27	0.28	0.09	0.62	1.84	1.36	0.94	2.61	1.93	1.33	39.53	59.03	61.38
CD4+ T Cells, Central memory (TCM) (% of T Cells)	4.12	2.56	1.92	3.64	3.10	0.52	1.21	3.65	1.06	1.71	5.17	1.50	25.70	11.94	17.95
CD4+ T Cells, Central memory (TCM) (% of CD4+ T Cells)	4.12	2.24	1.89	3.63	3.45	0.37	0.33	3.46	0.96	0.47	4.89	1.35	43.94	21.40	24.38
CD4+ T Cells, Effector memory (TEM) (% of T Cells)	11.32	2.60	3.00	4.71	6.97	3.27	4.41	0.52	5.56	6.24	0.74	7.86	9.56	9.91	10.22
CD4+ T Cells, Effector memory (TEM) (% of CD4+ T Cells)	11.23	2.65	3.05	4.79	6.67	3.40	5.30	0.38	5.69	7.50	0.54	8.05	16.35	17.77	13.88
CD4+ T Cells, RA+Effector memory (TEMRA) (% of T Cells)	5.59	0.00	2.05	4.32	10.18	9.45	4.42	0.23	5.24	6.25	0.33	7.41	0.11	1.01	0.27
CD4+ T Cells, RA+Effector memory (TEMRA) (% of CD4+ T Cells)	5.88	5.26	1.68	4.18	9.10	9.49	7.86	0.00	5.14	11.11	0.00	7.27	0.18	1.81	0.37
T regulatory cells (% of T Cells)	2.63	4.48	2.18	2.10	2.65	0.61	5.68	1.67	1.18	8.03	2.36	1.67	3.70	2.83	3.80
T regulatory cells (% of CD4+ T Cells)	2.49	3.88	2.03	2.08	2.62	0.63	4.77	1.81	1.05	6.75	2.56	1.49	6.32	5.07	5.16
CD8+ T Cells (% of lymphocytes)	1.69	1.41	0.28	0.45	1.29	0.22	1.24	0.76	0.21	1.75	1.08	0.30	21.51	21.37	16.64
CD8+ T Cells (% of T Cells)	0.49	0.76	0.07	0.65	0.70	0.43	0.57	0.99	0.38	0.80	1.40	0.54	38.15	35.70	24.14
CD8+ T Cells, naive (% of T Cells)	1.82	3.05	0.33	0.49	1.41	0.61	0.17	2.37	0.97	0.24	3.35	1.37	9.57	12.37	11.40
CD8+ T Cells, naive (% of CD8+ T Cells)	1.36	2.36	0.25	1.10	0.70	0.20	0.42	1.38	0.57	0.59	1.95	0.81	44.50	57.91	68.52
CD8+ T Cells, Central memory (CM) (% of T Cells)	14.89	5.61	4.77	5.56	5.47	1.59	5.76	0.97	6.60	8.14	1.37	9.33	3.07	1.46	1.32
CD8+ T Cells, Central memory (CM) (% of CD8+ T Cells)	14.50	6.04	4.78	5.48	4.78	1.91	5.27	0.29	5.98	7.45	0.41	8.46	8.06	4.10	5.48
CD8+ T Cells, Effector memory (TEM) (% of T Cells)	2.83	1.43	0.52	3.07	1.90	0.46	0.07	4.01	2.50	0.09	5.68	3.53	14.06	8.99	4.25
CD8+ T Cells, Effector memory (TEM) (% of CD8+ T Cells)	3.23	1.49	0.59	2.42	2.54	0.71	0.68	4.96	2.76	0.96	7.02	3.90	36.87	25.17	17.59
CD8+ T Cells, RA+Effector memory (TEMRA) (% of T Cells)	3.21	3.96	1.76	0.78	3.02	1.22	0.70	4.58	2.44	0.99	6.48	3.45	4.03	4.58	2.03
CD8+ T Cells, RA+Effector memory (TEMRA) (% of CD8+ T Cells)	3.66	4.69	1.84	0.09	3.64	0.99	0.09	3.59	2.75	0.13	5.07	3.88	10.58	12.82	8.41
B cells (% of lymphocytes)	0.37	0.68	0.46	0.25	0.97	0.84	1.09	1.22	1.89	1.54	1.73	2.67	17.13	19.31	12.21
B cells, naive (% of B cells)	1.68	2.29	4.68	7.43	0.41	0.32	2.03	0.42	0.39	2.88	0.59	0.55	18.32	27.64	72.78
B cells, memory (% of B cells)	0.52	1.74	0.45	0.89	2.28	1.58	0.50	0.16	0.14	0.70	0.23	0.20	35.03	18.69	11.68
B cells, memory, IgD+ (% of B cells)	7.68	12.39	9.30	12.84	3.71	5.06	8.69	2.78	1.68	12.30	3.93	2.38	0.81	1.27	7.56
NK cells (% of lymphocytes)	2.70	2.39	0.59	0.23	0.69	1.48	1.59	2.27	0.93	2.24	3.21	1.31	16.34	10.78	11.18
NKT cells (% of lymphocytes)	3.60	2.62	0.31	0.78	1.87	1.63	1.80	0.16	0.33	2.54	0.23	0.46	4.06	7.40	2.16

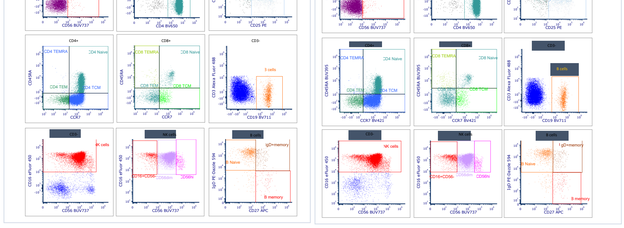

Figure 4: Instrument performance correlation.

18-color pre-stained kit of lyophilized PBMC was used to assess instrument performance in two instruments located in different labs (one in US lab and the other in EU lab). Every single population falls in the same gate placement, and the data from both instruments look comparable. % difference between instruments is less than 20% for all primary reportables (data not shown).

Summary/Conclusion

Detection of dead cells in Cyto-Chex® tube with viability dye is a challenge, as the fixation in this blood collection tube allows for most viability dyes to enter all the cells. Careful titration of ViaDye Red viability dye shows that the dead cells could be excluded from the further analysis of immune cell subsets. This is critical as clinical samples are shipped overnight and there is potential for cell death during the shipment, and removal during data analysis is required to obtain high quality data.

All the validation parameters assessed for the 25-color assay, are within the acceptance criteria and as such this assay is qualified for testing in clinical trials.

Inter-lab (inter-instrument) sample correlation for the 25-color assay was not completed, but the instrument performance comparison performed on PBMC samples, show that both instruments are comparable and will provide comparable data for any global clinical trials.

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