Global Validation of a Pan-Leukocyte Panel with Enumeration for Fresh Whole Blood

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Introduction:

Multiparameter flow cytometry assays provide insight into pharmacokinetics, pharmacodynamics, and mechanism of action of therapeutic candidates. As flow cytometry becomes increasingly important in clinical development, there is increased need for robust, standardized assays that can be reliably implemented across multiple global sites. However, implementation is complicated by short post-draw stability for fresh samples, limited availability of quantitative assay standards, and the requirement for rigorous harmonization and validation processes to ensure reproducibility across laboratories. To address these challenges, we performed a multi-site validation of a 15-color pan-leukocyte flow cytometry panel with enumeration for human whole blood. Validation was performed across 3 testing laboratories in the US, the EU, and the APAC region.

Method:

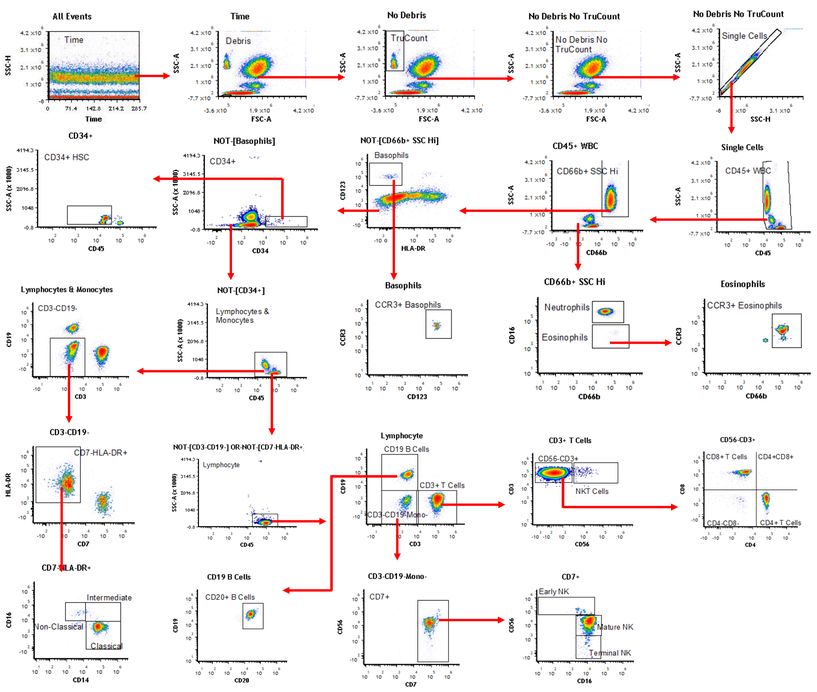
Sample Staining and Acquisition

Staining was performed using a 15-color human pan-leukocyte lyse-no-wash kit from Cytek® Biosciences. Antibodies for used for staining are listed in Table 1. Samples were stained in BD Trucount™ to determine absolute counts, reported in cells/mL of whole blood. Acquisition was performed using Cytek Aurora flow cytometers. Pre-installation testing was performed by the manufacturer prior to installation to identify instruments with a high degree of similarity. Data analysis was performed in FCS Express using the gating strategy shown below.

Table 1. Panel Composition

Marker	Clone	Fluorophore
CD8	SK1	cFluor® V450
HLA-DR	L243	cFluor® V505
CD45	HI30	cFluor® V547
CD4	SK3	cFluor® V610
CD16	3G8	cFluor® B515
CD34	4H11	cFluor® BYG575
CD123	6H6	cFluor® BYG610
CD193 (CCR3)	5E8	cFluor® BYG667
CD56	LT56	cFluor® BYG710
CD19	HIB19	cFluor® BYG750
CD14	MEM-15	cFluor® BYG781
CD7	CD7-6B7	cFluor® R659
CD20	2H7	cFluor® R685
CD66b	G10F5	cFluor® R720
CD3	SK7	cFluor® R780

Gating Strategy



Validation Test Scripts

Comprehensive testing at the US laboratory was performed using human whole blood in sodium heparin anticoagulant from 3 healthy donors. Testing at the EU and APAC laboratories was performed using one lot Streck CD-Chex controls per site. The following precision test scripts were performed at all test sites:

- **Intra-assay precision:** 3 replicates from each sample were processed in a single run to evaluate reproducibility and consistency.
- **Inter-analyst precision:** 3 replicates from each sample were prepared by 2 (US and EU sites) or 3 (APAC site) analysts to evaluate procedural robustness.
- **Inter-instrument precision:** At each site, 3 replicates per sample were acquired using 2 different instruments to evaluate consistency between instruments at a single testing laboratory.
- **Inter-site precision:** 3 replicates of lot-matched CD-Chex controls were prepared at 2 different sites to assess variability sites. Results from the US testing laboratory were compared to the results from the EU and APC testing laboratories.

Additionally, the following stability test scripts were performed at the US test site using whole blood in sodium heparin tubes from 3 healthy donors:

- **Processed sample stability:** To evaluate the stability of samples after staining and fixation, 12 replicates from each sample were prepared. 3 replicates per sample were acquired at each of the following time points from completion of staining: same day, 24±6 hours, 48±6 hours, and 72±6 hours
- **Unprocessed sample stability:** 3 replicates from each sample were assayed 24±6 hours, 48±6 hours, 72±6 hours, and 96±6 hours after sample collection. Samples were stored at ambient temperature on a nutating rocker prior to assay.

Statistical Analysis and Evaluation of Results:

Results were analyzed and the validation status of each reportable was evaluated by calculating the %CV (coefficient of variation) for all replicates from a single sample. When multiple samples were tested, the mean of the %CVs obtained from each donor was used to evaluate validation status. Stability test scripts were assessed by calculating the %CV of the of the results from the baseline time point and each subsequent time point tested.

- The %CV was ≤ 30% for non-rare subsets; defined as subsets with a frequency of > 10% of total CD45+ WBCs and a minimum of 500 events
- The %CV was ≤ 40% for rare subsets; defined as subsets with a frequency of ≤ 10% % of total CD45+ WBCs and a minimum of 100 gated events, or as subsets with a frequency of > 10% of total CD45+ WBCs and 100-500 events
- Subsets with fewer than 100 gated events were exempt from evaluation based on the acceptance criteria

Results:

Table 2. Intra-assay Precision Results (%CV)

Sample Type	CD-Chex Lot 1		CD-Chex Lot 2		Healthy Donors (US)				Mean - All Runs
Immune Cell Subset	EU	US	APAC	US	Donor 1	Donor 2	Donor 3	Mean	
CD45+	0.73	2.66	0.60	3.02	2.41	1.36	2.67	2.15	1.92
Neutrophils	0.80	2.73	0.51	3.10	5.75	1.57	3.15	3.49	2.51
Eosinophils	2.06	5.70	5.69	10.20	14.01	13.23	5.51	10.92	8.06
Basophils	1.43	2.78	1.41	7.55	4.40	5.85	0.95	3.73	3.48
CD34+ HSCs	1.82	5.07	10.83	18.26	19.66	13.69	12.82	15.39	11.73
Non-classical Monocytes	3.74	4.98	1.30	7.22	6.59	7.74	3.28	5.87	4.98
Intermediate Monocytes	3.43	3.80	2.35	14.75	7.50	10.43	3.41	7.11	6.52
Classical Monocytes	1.09	1.79	1.24	2.14	8.17	3.36	0.67	4.07	2.64
Total Monocytes	1.19	1.92	0.87	3.05	7.46	3.03	0.61	3.70	2.59
Lymphocytes	0.75	2.23	0.89	2.65	0.58	4.39	2.60	2.52	2.01
B Cells	2.23	2.01	2.47	2.13	3.48	5.05	5.20	4.58	3.22
Early NK Cells	9.21	7.61	5.70	7.83	3.18	9.38	7.55	6.70	7.21
Mature NK Cells	0.80	4.02	2.42	3.14	4.46	3.99	3.34	3.93	3.17
Terminal NK Cells	10.27	11.38	15.42	13.67	20.41	9.06	20.99	16.82	14.46
Total NK Cells	0.25	3.69	2.50	3.78	3.87	4.38	3.79	4.01	3.18
CD3+ T Cells	0.71	2.11	0.42	2.52	0.73	4.46	2.76	2.65	1.96
CD8+ T Cells	1.49	2.92	1.03	2.19	0.71	4.14	2.34	2.40	2.12
CD4+ T Cells	0.69	1.89	0.58	2.47	0.84	5.47	2.71	3.01	2.09
NKT Cells	0.79	1.70	3.09	5.09	3.50	2.83	2.89	3.07	2.84

Table 3. Inter-analyst Precision Results (%CV)

Immune Cell Subset	EU	APAC	US				Mean - All Comparisons
			Donor 1	Donor 2	Donor 3	Mean	
CD45+	3.91	3.34	2.08	7.46	3.64	4.39	4.09
Neutrophils	4.24	3.79	5.04	17.05	5.37	9.15	7.10
Eosinophils	5.03	8.48	13.48	12.33	9.59	11.80	9.78
Basophils	3.86	4.38	5.57	5.82	5.65	5.68	5.06
CD34+ HSCs	16.91	16.48	30.19	19.08	27.82	25.70	22.10
Non-classical Monocytes	4.77	7.14	6.62	9.90	4.85	7.12	6.66
Intermediate Monocytes	3.41	9.23	10.34	13.23	18.55	14.04	10.95
Classical Monocytes	4.13	3.57	11.12	6.89	3.44	7.15	5.83
Total Monocytes	3.91	3.91	10.27	6.32	3.49	6.69	5.58
Lymphocytes	3.46	3.08	0.63	3.77	2.15	2.18	2.62
B Cells	2.81	4.52	3.32	4.08	4.93	4.11	3.93
Early NK Cells	6.47	11.96	13.38	7.19	12.86	11.14	10.37
Mature NK Cells	3.66	4.18	4.39	6.60	2.69	4.56	4.30
Terminal NK Cells	10.97	7.11	18.93	9.74	21.20	16.62	13.59
Total NK Cells	3.75	4.19	5.19	6.17	2.98	4.78	4.46
CD3+ T Cells	3.51	3.14	0.73	4.25	3.09	2.69	2.94
CD8+ T Cells	2.63	3.51	1.50	4.58	2.05	2.71	2.85
CD4+ T Cells	3.75	3.57	1.23	4.77	3.04	3.01	3.27
NKT Cells	3.84	5.48	2.80	3.77	5.12	3.89	4.20

Table 2-5 Legend:
Grey shading indicates that the reportable had a mean of fewer than 100 gated events in the specified sample type and was not assessed for the sample type indicated. N/A indicates that the stability for the reportable was not assessed.
Blue text indicates a %CV between 10% and 20%.
Orange text without white or grey shading indicates a %CV between 20% and 30%.
Orange text with orange shading indicates a % CV between 30% and 40%.
Red text and with red shading indicates a %CV of greater than 40%.

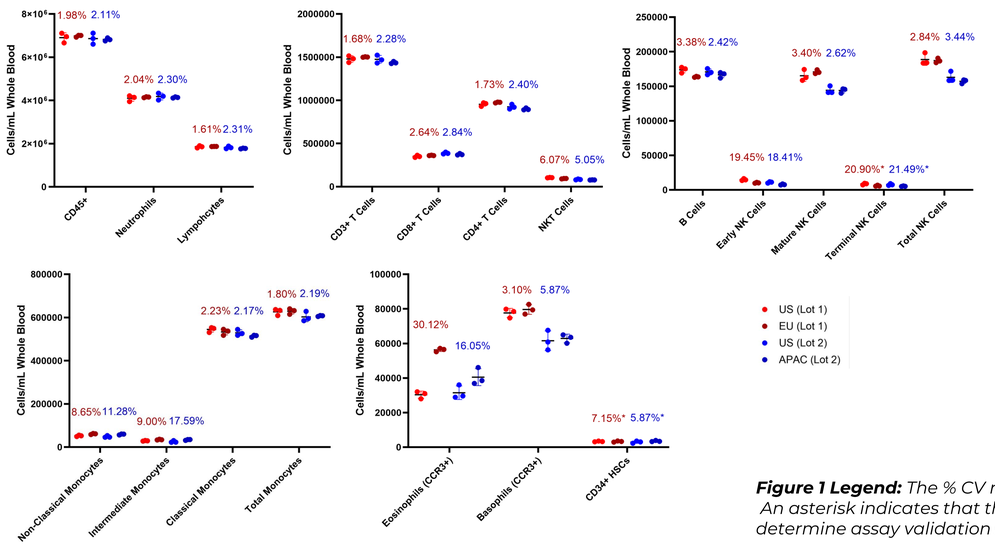
Table 4. Inter-instrument Precision Results (%CV)

Immune Cell Subset	EU	APAC	US			
			Donor 1	Donor 2	Donor 3	Mean
CD45+	3.83	1.13	9.74	3.65	6.95	6.78
Neutrophils	4.55	1.50	10.82	4.98	8.29	8.03
Eosinophils	21.43	4.37	21.61	22.85	17.47	20.64
Basophils	3.70	4.17	11.70	2.76	5.75	6.73
CD34+ HSCs	14.27	7.06	27.60	12.32	14.57	18.16
Non-classical Monocytes	4.27	5.02	13.37	5.08	6.25	8.23
Intermediate Monocytes	8.14	14.56	15.00	9.39	10.50	11.63
Classical Monocytes	4.36	2.92	12.46	2.28	8.64	7.79
Total Monocytes	4.08	2.81	12.21	2.67	7.64	7.50
Lymphocytes	2.37	1.08	10.14	1.66	4.26	5.36
B Cells	2.64	1.86	11.23	10.75	4.20	8.73
Early NK Cells	15.56	8.63	20.47	6.55	24.59	17.20
Mature NK Cells	3.65	2.34	10.80	2.32	6.42	6.51
Terminal NK Cells	10.47	6.10	16.36	35.13	24.86	25.45
Total NK Cells	3.48	2.12	11.03	2.86	6.99	6.96
CD3+ T Cells	2.46	1.40	9.92	1.62	6.16	5.90
CD8+ T Cells	2.38	4.14	10.25	2.60	5.74	6.20
CD4+ T Cells	2.47	3.37	9.73	1.86	6.44	6.01
NKT Cells	3.83	3.05	9.93	9.55	11.29	10.26

Table 5. Processed Sample Stability Results (%CV)

Immune Cell Subset	Baseline vs ~24 hours				Baseline vs ~48 hours				Baseline vs ~72 hours				Validated Stability
	Donor 1	Donor 2	Donor 3	Mean	Donor 1	Donor 2	Donor 3	Mean	Donor 1	Donor 2	Donor 3	Mean	
CD45+	5.80	3.53	4.31	**4.55**	6.86	2.26	3.57	**4.23**	6.67	1.75	5.24	**4.55**	72 hours
Neutrophils	13.86	5.74	6.03	**8.54**	15.75	3.79	4.68	**8.07**	17.49	2.61	5.51	**8.54**	72 hours
Eosinophils	19.94	14.34	18.00	**17.43**	17.31	17.22	30.47	**21.67**	29.77	43.01	45.18	**39.32**	N/A
Basophils	8.06	4.86	4.90	**5.94**	8.62	1.91	7.10	**5.88**	10.33	4.65	6.07	**7.02**	72 hours
CD34+ HSCs	21.93	14.41	13.47	**16.61**	13.54	8.61	9.30	**10.49**	16.18	13.21	14.47	**14.62**	72 hours
Non-classical Monocytes	10.44	7.52	4.14	**7.37**	9.62	11.13	7.89	**9.55**	10.39	6.80	9.46	**8.88**	72 hours
Intermediate Monocytes	18.71	11.66	8.40	**12.93**	7.15	13.96	7.52	**9.54**	3.98	13.54	7.51	**8.34**	72 hours
Classical Monocytes	7.04	12.16	6.31	**8.50**	5.36	11.13	3.79	**6.76**	7.06	9.76	4.98	**7.27**	72 hours
Total Monocytes	7.51	10.88	5.90	**8.10**	5.20	10.62	3.27	**6.36**	6.26	8.41	4.31	**6.33**	72 hours
Lymphocytes	2.60	3.08	2.73	**2.80**	1.09	1.57	3.20	**1.95**	2.32	1.88	4.76	**2.99**	72 hours
B Cells	5.12	4.29	3.96	**4.46**	4.02	3.92	3.08	**3.68**	4.81	3.90	5.11	**4.61**	72 hours
Early NK Cells	15.85	14.22	20.79	**16.95**	11.02	11.22	10.28	**10.84**	16.13	13.45	18.98	**16.19**	72 hours
Mature NK Cells	5.88	4.19	5.37	**5.14**	6.12	2.47	5.76	**4.78**	6.10	2.98	6.20	**5.09**	72 hours
Terminal NK Cells	18.34	10.11	8.11	**12.19**	12.00	12.73	9.32	**11.35**	13.53	21.42	10.48	**15.14**	N/A
Total NK Cells	7.33	3.45	5.28	**5.35**	6.51	2.45	5.60	**4.85**	7.36	2.70	6.06	**5.37**	72 hours
CD3+ T Cells	7.05	3.19	2.19	**4.14**	5.91	1.85	2.94	**3.57**	6.42	2.33	4.01	**4.25**	72 hours
CD8+ T Cells	6.95	3.82	2.41	**4.39**	5.65	2.40	3.93	**3.99**	6.03	2.63	3.76	**4.14**	72 hours
CD4+ T Cells	7.11	3.76	2.04	**4.30**	6.17	2.00	2.97	**3.72**	6.64	2.54	3.62	**4.27**	72 hours
NKT Cells	5.81	1.61	2.74	**3.38**	5.45	3.55	3.18	**4.06**	5.36	1.67	3.78	**3.60**	72 hours

Figure 1. Inter-site Precision Results



Absolute counts for all populations met the acceptance criteria for intra-assay, inter-assay, and inter-analyst precision at all 3 sites. Inter-site precision met the acceptance criteria for both the US-EU and US-APAC comparisons. The median % CVs for the intra-assay, inter-analyst, inter-instrument, and inter-site test scripts were 3.09%, 4.58%, 2.97%, and 6.25%, respectively. These results indicate extremely low variance for the assay. Processed sample stability was validated to 72 hours after sample preparation for all reportables tested. Unprocessed sample stability (or post-draw sample stability) was validated to 72 hours after sample collection for all reportables tested. Additionally, the majority of reportables met the acceptance criteria for unprocessed sample stability up to 96 hours after sample collection. Higher %CV results for all test scripts were typically correlated to smaller subset frequencies, as is expected based on the literature.

Figure 1 Legend: *The % CV result for each comparison is specified above each pair of scatter plots. An asterisk indicates that the reportable had fewer than 100 gated events and was not used to determine assay validation status.*

Table 6. Unprocessed Sample Stability Results (%CV)

Immune Cell Subset	Baseline vs ~48 hours				Baseline vs ~72 hours				Baseline vs ~96 hours				Validated Stability
	Donor 1	Donor 2	Donor 3	Mean	Donor 1	Donor 2	Donor 3	Mean	Donor 1	Donor 2	Donor 3	Mean	
CD45+	11.05	10.86	9.39	**10.43**	7.89	13.58	12.57	**11.35**	3.55	8.18	3.11	**4.95**	96 hours
Neutrophils	11.89	18.98	12.38	**14.42**	7.26	26.88	17.79	**17.31**	9.65	17.36	3.80	**10.27**	96 hours
Eosinophils	34.89	28.76	12.35	**25.33**	12.57	11.01	26.13	**16.57**	23.57	14.56	29.88	**22.67**	N/A
Basophils	10.12	9.96	7.78	**9.29**	4.94	6.99	6.57	**6.17**	7.80	6.08	3.07	**5.65**	96 hours
CD34+ HSCs	34.45	10.15	10.89	**18.49**	53.72	5.84	9.05	**22.87**	50.62	15.20	8.45	**24.76**	96 hours
Non-classical Monocytes	6.31	8.97	2.97	**6.08**	27.32	56.57	7.00	**30.30**	56.96	69.91	32.75	**53.21**	72 hours
Intermediate Monocytes	28.24	29.71	26.61	**28.19**	12.49	34.15	2.10	**16.25**	5.25	51.44	18.78	**25.16**	96 hours
Classical Monocytes	12.77	4.84	9.07	**8.89**	16.45	15.05	14.87	**15.46**	15.75	15.22	11.43	**14.13**	96 hours
Total Monocytes	11.93	4.98	9.08	**8.66**	13.07	9.23	12.84	**11.71**	10.98	9.30	7.50	**9.26**	96 hours
Lymphocytes	6.25	3.19	3.94	**4.46**	2.56	4.48	8.98	**5.34**	5.09	8.82	16.37	**10.09**	96 hours
B Cells	3.91	7.50	18.21	**9.88**	12.79	30.46	42.96	**28.74**	24.54	45.78	70.13	**46.82**	72 hours
Early NK Cells	19.06	5.24	9.79	**11.37**	11.83	8.81	13.95	**11.53**	8.98	20.69	11.97	**13.88**	96 hours
Mature NK Cells	4.89	4.48	7.95	**5.77**	8.61	9.78	16.99	**11.79**	17.15	17.98	26.01	**20.38**	96 hours
Terminal NK Cells	13.84	26.86	6.36	**15.68**	17.74	17.82	28.55	**21.37**	13.82	25.42	21.24	**20.16**	N/A
Total NK Cells	5.15	4.05	7.48	**5.56**	6.23	9.16	17.05	**10.81**	12.84	15.72	25.42	**17.99**	96 hours
CD3+ T Cells	6.06	3.43	5.75	**5.08**	2.81	4.83	12.84	**6.82**	4.99	8.89	19.92	**11.27**	96 hours
CD8+ T Cells	7.36	2.39	4.17	**4.64**	2.90	4.72	10.17	**5.93**	4.15	9.84	17.86	**10.62**	96 hours
CD4+ T Cells	5.67	3.41	6.41	**5.16**	2.97	6.95	13.67	**7.86**	5.59	12.17	21.31	**13.02**	96 hours
NKT Cells	5.18	4.16	4.45	**4.59**	8.23	14.93	14.51	**12.56**	14.34	21.88	21.39	**19.20**	96 hours

Discussion and Conclusions

The results of the precision test scripts indicate extremely robust performance for the 15-color pan-leukocyte panel under typical laboratory conditions. Additionally, the validated unprocessed and processed sample stabilities of at least 72 hours for all reportables permit additional logistical flexibility with regards to sample shipment and processing.

These data demonstrate high reproducibility and reliability across multiple sites globally. These results support the feasibility of harmonized flow cytometry assays in global clinical trials. Moreover, validation of absolute counts for most major leukocyte subsets substantiate the use of flow cytometry assays for generation of quantitative data.