Global Harmonization of a Validated 15-Color Pan-Leukocyte Spectral Flow Cytometry Panel for Human Whole Blood in Support of Translational and Clinical Applications

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INTRODUCTION

Flow cytometry assays are essential in clinical trials for evaluating drug efficacy, pharmacodynamics, potency, and toxicity. Their reliability depends on rigorous validation processes to ensure consistent data generation across multiple laboratories, a necessity for global trials. This study presents a multi-site validation of a 15-color pan-leukocyte flow cytometry assay for assessment of human whole blood using spectral cytometers.

NOVELTY

Harmonization of flow cytometry assays across multiple sites is essential for global clinical trials but poses unique challenges. These include harmonization of instrument hardware and settings, training of staff, and at each site. Typically, cross-site harmonization has been confined to clinical-grade instruments, which are limited to conventional cytometers. These instruments can typically assess fewer parameters than research-grade or spectral instruments, limiting the data that can be generated from a single sample. Herein, we describe the approaches used during successful cross-site validation of 15-color pan-leukocyte panel for the spectral cytometer at 3 test facilities on different continents. The procedures implemented for this study provide a framework for implementation of globalized flow cytometry assays for multi-site clinical trials.

Target	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

METHOD

Pre-installation testing was performed by the manufacturer to select six cytometers (two instruments per site) with the highest degree of similarity in terms of voltage gains and %CVs for each detector. This was followed by post-installation testing at each of the three laboratory sites. Hardware, software and assay-specific training were completed for each analyst involved in the study.

A 15-color, pan-leukocyte, lyse-no-wash assay for human whole was selected for cross-site validation, as it had previously been validated at the lead test site using whole blood from healthy donors. The assay (sold as a kit) identifies major leukocyte populations, including neutrophils, eosinophils, basophils, hematopoietic stem cells (HSCs), monocyte subsets, T cell subsets, B cells, NK cell subsets, and NKT cells (Gating scheme shown below). Absolute count tubes were utilized to allow precise enumeration of all subsets analyzed. Lot-matched controls were selected as the test samples for the validation to allow direct comparisons of samples processed at each site.

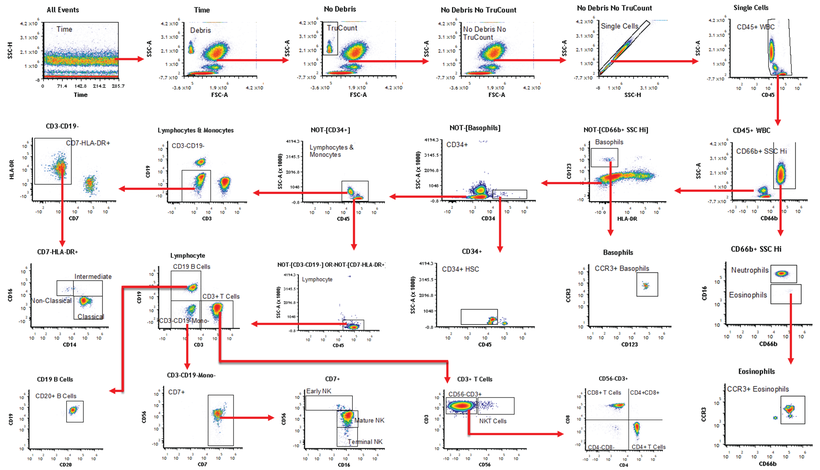
The following validation test scripts were performed:

- **Intra-assay precision:** At each site, 3 replicates were processed in a single run to evaluate reproducibility and consistency.
- **Inter-analyst precision:** At each site, 3 replicates were processed independently by 2-3 analysts to evaluate procedural robustness.
- **Inter-instrument precision:** 3 replicates per instrument were acquired using 2 instruments at the same site to evaluate instrument consistency.
- **Inter-site precision:** 3 replicates were prepared by different analysts at 2 different sites to assess similarity of results produced by each site.

Results were analyzed and the validation status of each reportable was evaluated by calculating the %CV (coefficient of variation). The following acceptance criteria were applied for each reportable:

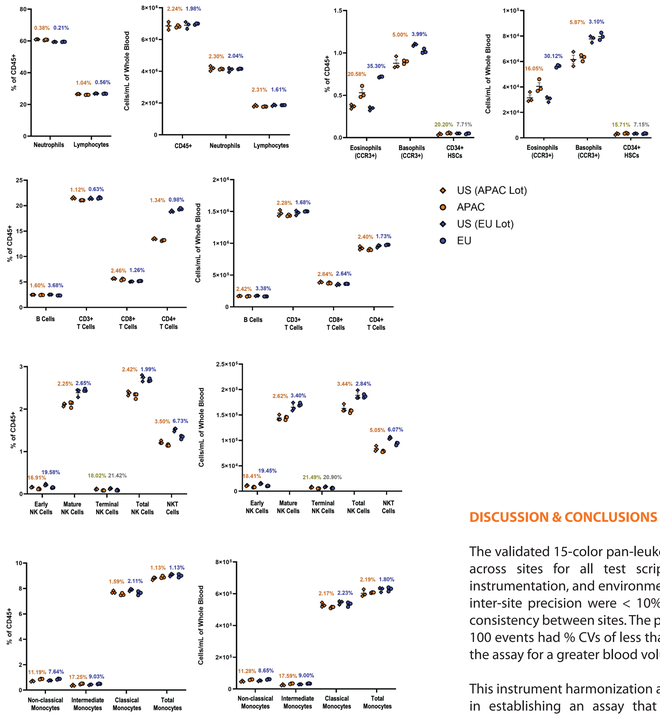
- The %CV was ≤ 30% for non-rare subsets, defined as >10% of CD45+ WBC and a minimum of 500 events
- The % CV was ≤ 40% for rare subsets. Rare subsets were defined as ≤ 10% of CD45+ WBC and a minimum of 100 gated events, or >10% of CD45+ and 100-500 events
- Subsets with < than 100 gated events were exempt from evaluation using the acceptance criteria.

GATING STRATEGY



RESULTS

A total of 48 parameters were evaluated, including both cell subset frequencies and absolute number of cells/mL. All reportables met the acceptance criteria for each of the validation test scripts, with the exception of 2 reportables that were *omitted from evaluation due to fewer than 100 events. Additionally, over 75% of the parameters assessed for all test scripts had % CVs of < 10%, indicating a high degree of consistency. The % CVs for intra-assay, shown in the table below, indicated minimal variability within assay runs and extremely high precision. Pearson correlation analysis between site averages further verified the assay's robustness, demonstrating strong agreement between test sites. Dot plots showing the inter-site precision results, with the % CV for each cross-site comparison are shown in **orange** (US-APAC) **blue** (US-EU) **gold** (US-APAC) or **grey** (US-EU).



Reportable	% CV: Intra-assay Precision US (EU)	EU	US (APAC)	APAC
Absolute CD45 Count	2.66	0.73	3.02	0.60
% of CD45+ WBC in (CD66b+ SSC Hi and Neutrophils)	0.24	0.11	0.19	0.10
Absolute Neutrophil count	2.73	0.80	3.10	0.51
% of CD45+ WBC in (Eosinophils and CCR3+ Eosinophils)	4.95	1.88	5.60	5.66
Absolute Eosinophil Count	5.70	2.06	10.20	5.69
% of CD45+ WBC in (Basophils and CCR3+ Basophils)	1.14	1.67	6.74	0.55
Absolute Basophil Count	2.78	1.43	7.55	1.41
% of CD45+ WBC in (CD7-HLA-DR+ and Non-Classical)	2.15	3.72	4.83	1.86
Absolute Non-Classical Monocyte Count	4.98	3.74	7.22	1.30
% of CD45+ WBC in (CD7-HLA-DR+ and Intermediate)	5.57	2.97	12.26	2.81
Absolute Intermediate Monocyte Count	3.80	3.43	14.75	2.35
% of CD45+ WBC in (CD7-HLA-DR+ and Classical)	1.01	0.82	1.10	0.76
Absolute Classical Monocyte Count	1.79	1.09	2.14	1.24
Total Monocytes % of CD45+ WBC	0.95	0.90	1.08	0.51
Absolute Total Monocyte Count	1.92	1.19	3.05	0.87
Absolute Lymphocyte Counts	2.23	0.75	2.65	0.89
% of CD45+ WBC in (CD19 B Cells and CD20+ B Cells)	0.65	1.40	0.87	1.93
Absolute B Cells Counts	2.01	2.23	2.13	2.47
% of CD45+ WBC in (CD7+ and Early NK)	7.84	10.21	5.10	7.42
Absolute Early NK Cell Count	7.61	9.21	7.83	5.70
% of CD45+ WBC in (CD7+ and Mature NK)	3.26	0.00	1.47	1.90
Absolute Mature NK Cell Count	4.02	0.80	3.14	2.42
% of CD45+ WBC in (CD7+ and Terminal NK)*	9.85	11.69	11.00	14.39
Absolute Terminal NK Cell Count*	11.38	10.27	13.67	15.42
Total NK Cells % of CD45+ WBC	2.28	1.08	1.76	1.90
Absolute Total NK Cell Count	3.69	0.25	3.78	2.50
% of CD45+ WBC in (Lymphocyte and CD3+ T Cells)	0.61	0.33	0.63	0.23
Absolute CD3+ T Cell Count	2.11	0.71	2.52	0.42
% of CD45+ WBC in (CD56-CD3+ and CD8+ T Cells)	0.83	0.84	1.08	1.31
Absolute CD8+ T Cell Count	2.92	1.49	2.19	1.03
% of CD45+ WBC in (CD56-CD3+ and CD4+ T Cells)	0.88	0.18	0.72	0.64
Absolute CD4+ T Cell Count	1.89	0.69	2.47	0.58
% of CD45+ WBC in (CD3+ T Cells and NKT Cells)	2.18	1.41	2.32	2.82
Absolute NK T Cell Count	1.70	0.79	5.09	3.09

DISCUSSION & CONCLUSIONS

The validated 15-color pan-leukocyte assay exhibited exceptional precision and reproducibility across sites for all test scripts. Its robustness under varying conditions of analysts, instrumentation, and environments underscores its suitability for global clinical trials. % CVs for inter-site precision were < 10% for over 75% of reportables, of indicating a high degree of consistency between sites. The parameters that were omitted from evaluation due to fewer than 100 events had % CVs of less than 30%, well within the established acceptance criteria. Scaling the assay for a greater blood volume per test could resolve this limitation.

This instrument harmonization and cross-site validation methods described herein are valuable in establishing an assay that performs consistently across multiple test sites. Pre- and post-installation harmonization by the manufacturer and the utilization of a single trainer for all test sites proved particularly valuable. a valuable tool for immune monitoring, supporting robust data generation and reliable conclusions in complex, multicenter clinical studies.