Global Validation of a Flow Cytometry Panel for Clinical Trials: Detection of Ki67, Granzyme B and FoxP3 in T and NK cell Populations

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Abstract

Flow cytometry is critical in immunotherapy and cell and gene therapy trials by evaluating the impact of innovative medicines on the immune system. To support global clinical trials, assays must be standardized and validated across international sites. A flow cytometry panel using peripheral blood mononuclear cells (PBMCs) was developed to characterize T cell subsets – including memory T cells, Tregs, and γδ T cells – and NK cells, and to assess intracellular markers such as Ki67 (proliferation), Granzyme B (activation), and FoxP3 (Tregs). Due to inconsistent Granzyme B staining in PBMCs, the panel was validated in whole blood (WB) and transferred from the US lab to labs in Europe, Australia, and Taiwan. A Trucount tube was added to the WB panel to allow for absolute count calculation. Detecting low-expression intracellular markers required protocol optimization. Two fixation/permeabilization methods were compared: BD FACS™ Permeabilizing Solution 2 (BD Biosciences) and eBioscience™ FoxP3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific). FoxP3 was only detectable using the eBioscience™ FoxP3 buffer, which also yielded higher Ki67+ cell percentages. Due to low endogenous Ki67 expression in healthy donors, custom Ki67+ Trucytes – developed in collaboration with Slingshot Biosciences – were spiked into WB to support standardized Ki67 detection across all sites. Validation included precision analysis, sample stability testing and inter-lab performance comparison. Repeatability, reproducibility, and inter-operator and inter-instrument variability met the acceptance criteria of ≤25% coefficient of variance (CV) and ≤20% difference, respectively, for all primary reportables, except for a few rare populations such as endogenous Ki67+ cells. In contrast, spiked-in Ki67+ Trucytes met all precision requirements. Sample stability assessment showed that most reportables remained stable for up to 96 hours post-collection, while a few were stable between 48 and 96 hours. Assay performance across all sites was evaluated using BD™ Multicheck Control material (BD Biosciences), which showed consistent results among labs. Overall, this multi-site validation demonstrates that a protocol developed in one lab can be successfully transferred and implemented across global sites. This panel was developed and validated for exploratory assessment in clinical trials.

Panel Configuration

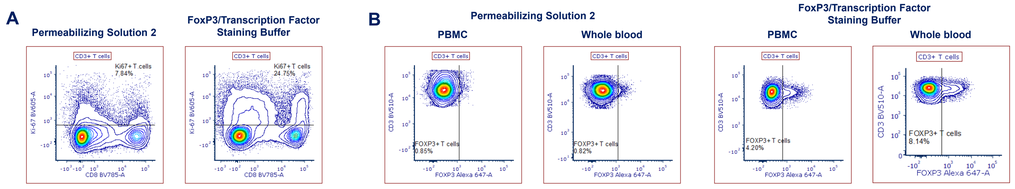
Detector (laser)	FL1 (405)	FL2 (405)	FL3 (405)	FL4 (405)	FL5 (405)	FL6 (488)	FL7 (488)	FL8 (488)	FL9 (488)	FL10 (640)	FL11 (640)	FL12 (640)
Fluorochrome (Filter)	**BV421 (448/45)**	**BV510 (528/45)**	**BV605 (606/36)**	**BV711 (715/50)**	**BV785 (755LP)**	**FITC (527/32)**	**PE (586/42)**	**PerCP-Cy5.5 (700/54)**	**PE-Cy7 (783/56)**	**APC/AF647 (660/10)**	**AF700 (720/30)**	**APC-H7/eFluor780 (783/56)**
Tube 1	TCRγδ	CD3	Ki67	ICOS	CD8	CD4	PD-1 + IgG4	CD16	Granzyme B	CD56	CD45	CD14 + Viability
Tube 2	CCR7	CD3	Ki67	CD25	CD8	CD4	PD-1 + IgG4	CD45RA	Granzyme B	FoxP3	CD45	CD14 + Viability
Tube 3*											CD45	

**Trucount tube; Lyse/No Wash*

Panel Optimization

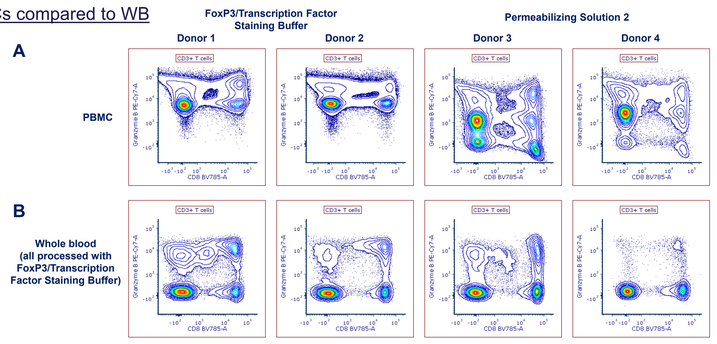
1. Comparison of fixation/permeabilization methods

BD FACS™ Permeabilizing Solution 2 (BD Biosciences; 340973) and eBioscience™ FoxP3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific; 00-5523-00) were evaluated for their effectiveness in detecting the intracellular markers Ki67 and FoxP3. Both buffers allowed Ki67 staining in PBMCs spiked with activated TCRγδ cells; however, the eBioscience™ FoxP3 buffer resulted in a 3-fold higher percentage of Ki67+ T cells (Figure A). Notably, FoxP3 detection was only successful with the eBioscience™ FoxP3 buffer, showing a 5-fold increase in FoxP3+ T cells in PBMCs and a 10-fold increase in WB compared to the Permeabilizing Solution 2 buffer (Figure B). The flow cytometry plots are representative of multiple donors. PBMCs and WB samples were derived from different donors.



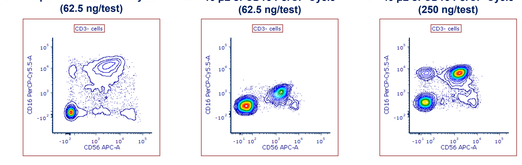
2. Granzyme B staining is suboptimal in PBMCs compared to WB

Granzyme B (GZMB) staining in PBMCs was suboptimal. The eBioscience™ FoxP3 buffer produced high background in the GZMB-negative population, while the Permeabilizing Solution 2 buffer yielded three distinct GZMB populations: negative, intermediate, and positive (Figure A). In contrast, GZMB staining in WB using the eBioscience™ FoxP3 buffer showed a typical staining pattern, with the GZMB-negative population centered near zero, and a single GZMB+ population clearly defined (Figure B). The flow cytometry plots are representative of multiple donors. PBMCs and WB samples were derived from different donors.



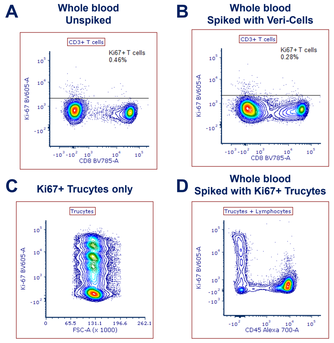
3. Increased CD16 antibody volume needed for staining NK cells in WB

Switching from the PBMC panel to the WB panel impacted CD16 staining of NK cells. While 10 µL (62.5 ng/test) of CD16 PerCP-Cy5.5 was sufficient for clear NK cell detection in PBMCs (Figure A), it was not adequate in WB due to reduced resolution (Figure B). This reduction is attributed to the presence of neutrophils in WB, which also express CD16 and interfere with CD16 staining of NK cells. Therefore, the antibody was retitrated for use in WB, and a final volume of 40 µL (250 ng/test) was selected, which provided a resolution comparable to that observed in PBMC (Figure C). The flow cytometry plots are representative of multiple donors. PBMCs and WB samples were derived from different donors.



4. Ki67+ Trucytes for standardized detection of Ki67+ cells

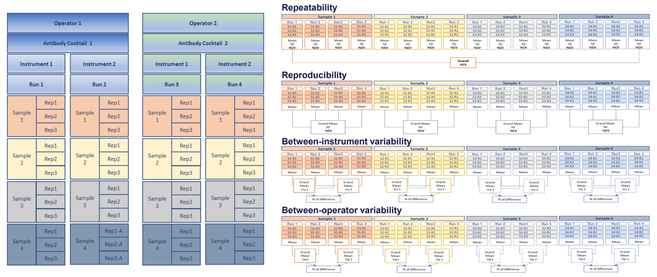
Ki67 expression levels are typically low or absent in healthy donors (Figure A). Spiking WB with Veri-Cells™ Activated (Surface) PBMC (BioLegend; 426703) did not result in an increased frequency of Ki67+ cells (Figure B). To overcome this limitation, Ki67+ Trucytes were developed in collaboration with Slingshot Biosciences. These Trucytes were specifically designed to reflect the gradient-like Ki67 expression observed in clinical samples (Figure C). When spiked into WB, the Ki67+ Trucytes enabled reliable detection of Ki67+ cells in this panel. Note that these Trucytes are CD45-negative (Figure D). The flow cytometry plots are representative of multiple donors.



Panel Validation

1. Precision

Precision was determined on four WB samples from apparently healthy donors, spiked with Ki67+ Trucytes and Veri-Cells™ Activated (Surface) PBMC, processed in triplicates by two operators and acquired on two instruments for a total of four different runs. For repeatability (or intra-assay precision) and reproducibility (or inter-assay precision), acceptance criteria of ≤25%CV between replicates/runs were applied. Higher imprecision (35%CV) was accepted for rare populations (≤5% of parent or ≤100 events) or populations with dimly expressed antigens. For between-instrument and between-operator variability, acceptance criteria of ≤20% difference between instruments/operators were applied.



All primary reportables met the acceptance criteria for repeatability, reproducibility, between-instrument variability, and between-operator variability, except for certain Ki67+ reportables. The increased variability in these cases is expected due to the typically low endogenous Ki67 expression levels in healthy donors. In contrast, Ki67+ reportables derived from Ki67+ Trucytes are within the acceptance criteria.

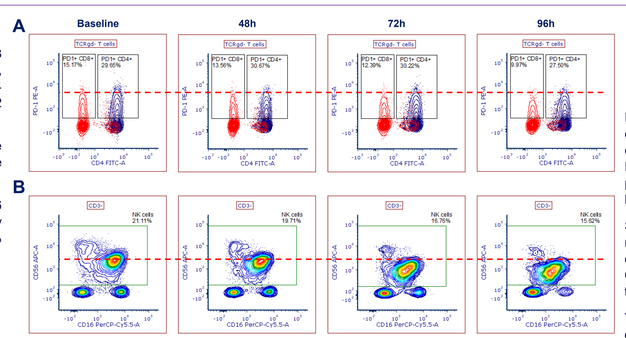
Reportables	Repeatability Overall %CV	Reproducibility %CV	Inter-instrument % difference	Inter-operator % difference
Lymphocytes (% singlets)	2.98	3.15	4.26	2.64
T cells (% Ly)	0.64	0.66	0.44	1.05
CD4+ T cells (% T)	0.88	0.97	0.36	1.64
CD4+ T cells (% Ly)	1.23	1.62	0.81	2.69
PD-1+ CD4 T cells (% CD4 T)	1.61	2.86	1.83	3.52
ICOS+ CD4 T cells (% CD4 T)	2.57	1.99	0.32	1.89
Ki67+ CD4 T cells (% CD4 T)	14.10	25.62	39.25	19.63
CD8+ T cells (% T)	0.90	2.48	1.91	3.56
CD8+ T cells (% Ly)	0.69	1.89	1.48	2.52
PD-1+ CD8 T cells (% CD8 T)	1.29	0.99	0.21	1.16
ICOS+ CD8 T cells (% CD8 T)	3.86	1.77	1.70	0.85
Ki67+ CD8 T cells (% CD8 T)	19.85	29.23	42.76	26.21
GranzymeB+ CD8 T cells (% CD8 T)	2.56	3.27	0.25	5.65
TCRγδ+ T cells (% T)	3.62	6.37	10.04	4.58
TCRγδ+ T cells (% Ly)	3.20	6.35	10.42	3.51
PD-1+ TCRγδ+ T cells (% TCRγδ T)	4.55	4.64	6.23	5.08
ICOS+ TCRγδ+ T cells (% TCRγδ T)	5.00	5.47	6.46	4.59
Ki67+ TCRγδ+ T cells (% TCRγδ T)	27.41	33.58	39.89	40.98
GranzymeB+ TCRγδ+ T cells (% TCRγδ T)	1.96	2.66	3.01	2.58
NK cells (% Ly)	1.19	2.57	3.37	0.62
PD-1+ NK cells (% NK)	16.52	15.80	17.92	14.15
ICOS+ NK cells (% NK)	10.65	19.18	13.73	5.56
Ki67+ NK cells (% NK)	9.89	15.14	5.56	17.63
GranzymeB+ NK cells (% NK)	2.88	3.99	4.04	3.06
CD56bright CD16- NK cells (% NK)	6.34	14.95	16.19	17.45
CD56dim CD16+ NK cells (% NK)	5.74	9.10	5.89	13.66
CD25+ CD4 T cells (% CD4 T)	1.29	2.11	1.50	3.15
CD4+ Naive T cells (% CD4 T)	0.52	1.19	1.41	1.47
CD4+ Tcm cells (% CD4 T)	1.24	2.60	3.17	3.20
CD4+ Tem cells (% CD4 T)	1.19	1.60	0.21	2.31
CD4+ Temra cells (% CD4 T)	2.44	4.46	1.00	7.12
Treg cells (% CD4 T)	8.99	3.62	5.23	2.67
Treg cells (% Ly)	9.85	4.31	4.46	5.29
Ki67+ Treg cells (% Treg)	7.67	6.89	7.58	2.81
CD25+ CD8 T cells (% CD8 T)	4.08	3.18	3.20	4.49
CD8+ Naive T cells (% CD8 T)	2.76	6.58	6.14	7.03
CD8+ Tcm cells (% CD8 T)	3.65	7.28	6.84	8.82
CD8+ Tem cells (% CD8 T)	3.66	6.00	3.30	5.05
GranzymeB+ CD8+ Tem cells (% CD8 Tem)	4.79	9.39	9.47	13.18
CD8+ Temra cells (% CD8 T)	1.79	3.36	4.74	2.99
Ki67+ Trucytes T1 (% of Total cells)	5.46	2.77	4.36	0.26
Ki67+ Trucytes T1 (MFI Ki67)	5.60	5.03	8.71	0.30
Ki67+ Trucytes T2 (% of Total cells)	5.95	4.02	4.37	2.23
Ki67+ Trucytes T2 (MFI Ki67)	8.84	4.14	5.97	2.41

2. Sample Stability

Sample stability was determined on six WB samples from apparently healthy donors, processed at baseline (approximately 24 hours post-collection), and at 48 hours, 72 hours and 96 hours from collection time.

Stability was established at the latest time point where ≤20% change from the baseline was achieved for at least 80% of the samples.

Sample stability was established up to 96 hours post-collection for most of the primary reportables, except for "PD-1+ CD8 T cells (% CD8 T)" and "NK cells (% Ly)".

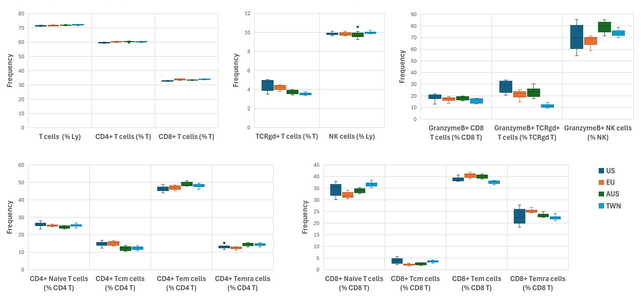


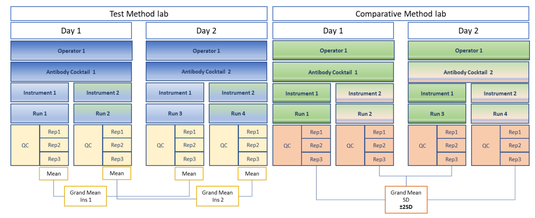
Reportables	% change from baseline 48h	72h	96h
PD-1+ CD4 T cells (% CD4 T)	2.67	1.24	8.53
PD-1+ CD8 T cells (% CD8 T)	10.59	25.53	35.45
NK cells (% Ly)	6.86	33.36	46.92

3. Sample Correlation

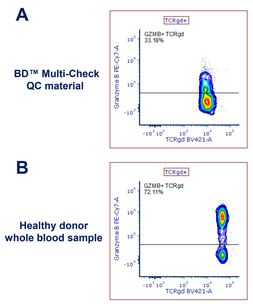
Sample correlation was determined using BD™ Multi-Check QC material (BD Biosciences; 349700), processed in triplicates by one operator and acquired on two instruments for a total of four different runs at each lab in two separate days. Acceptance criteria of ≤20% difference between sites and/or the mean value of the test method lab is within the 2SD of the mean value of the comparative method lab was applied. Rare populations or populations with dimly expressed antigens were not considered for performance correlation assessment.

Frequency of each population indicated in the box and whisker plots below are from each lab.





Reportables	% difference US-EU	US-AUS	US-TWN
Lymphocytes (% singlets)	7.07	13.41	1.06
T cells (% Ly)	0.33	0.62	1.05
CD4+ T cells (% T)	1.01	1.27	0.94
CD4+ T cells (% Ly)	1.35	1.90	2.00
CD8+ T cells (% T)	3.04	1.81	3.37
CD8+ T cells (% Ly)	3.39	2.45	4.46
GranzymeB+ CD8 T cells (% CD8 T)	9.21	4.06	16.71
TCRγδ+ T cells (% T)	7.45	18.51	22.12
TCRγδ+ T cells (% Ly)	7.08	17.92	21.23
GranzymeB+ TCRγδ+ T cells (% TCRγδ T)	23.25	17.87	59.48
NK cells (% Ly)	0.09	2.18	1.44
GranzymeB+ NK cells (% NK)	3.54	12.81	6.16
CD56dim CD16+ NK cells (% NK)	0.91	1.08	1.04
CD4+ Naive T cells (% CD4 T)	1.63	5.14	1.75
CD4+ Tcm cells (% CD4 T)	4.28	17.70	15.96
CD4+ Tem cells (% CD4 T)	1.40	5.64	3.68
CD4+ Temra cells (% CD4 T)	6.23	9.88	8.23
CD8+ Naive T cells (% CD8 T)	7.10	1.86	6.12
CD8+ Tcm cells (% CD8 T)	43.16	34.71	9.41
CD8+ Tem cells (% CD8 T)	4.38	3.28	3.35
CD8+ Temra cells (% CD8 T)	10.70	3.06	2.60



Conclusion

The data presented in this poster highlight the importance of a comprehensive assay optimization prior to initiating validation experiments. Furthermore, the results demonstrate that a protocol developed and validated in one lab can be successfully transferred and implemented across global sites.

Sample correlation between labs globally is challenging. However, data shown here indicate that commercially available QC material (BD™ Multi-Check) can be used to assess panel performance across labs located in different countries.

This panel was developed and validated for exploratory use in clinical trials.

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