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Validating Flow Cytometry Assays For Use During Clinical Trial Biomarker Assessments

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Purpose

Here we present preliminary validation data from two flow cytometry assays; a 'TBNK panel' to identify several of the major leukocyte populations (T cells including the CD4 and CD8 subsets, B cells, NK cells, NKT cells lymphocytes and monocytes) and a 'T helper panel' which identifies several key functional T lymphocyte subsets (Th1, Th2, Th17), in both whole blood and cryopreserved PBMCs. These populations are often assessed during clinical trials with biopharmaceuticals as PD endpoints. These preliminary data demonstrate the utility of both whole blood and cryopreserved PBMCs for flow cytometry.

Methods

Peripheral blood samples were collected into a range of standard anticoagulant blood tubes, or sodium heparin CPT™ tubes (BD Biosciences) for PBMC preparation. PBMCs were either used fresh or cryopreserved in vapor phase nitrogen. PBMCs or whole blood samples, with erythrocytes removed using PharmLyse™ (BD Biosciences), were initially stained using LIVE/DEAD® Fixable Aqua stain (Invitrogen), followed by staining with lineage-specific, fluorochrome-conjugated antibodies (various vendors), as indicated Table 1. Samples were fixed in a 1% formaldehyde solution before analysis using a 3-laser FACSCanto II™ flow cytometer (BD Biosciences), run under FACSDiva™ (v6.1.2) software. Prior to any sample analysis the manufacturer's QC checks and calibration using CS&T™ beads (BD Biosciences) were performed to confirm system performance and adjust settings against the baseline values.

Panel *	BV421	BV510	Ax488	PE	PerCP Cy5.5	PECy7	Ax647	APC-Cy7
TBNK	CD3	Fix L/D Aqua	CD45	CD14	CD4	CD19	CD16 / CD56	CD8
Th	CD3	Fix L/D Aqua	-	CD183	CD4	CD196	CD161	CD8

Table 1. Panel descriptions. *Gating control panels are not shown.

Results

Anticoagulant Comparison: Whole blood from 3 Donors was collected into CTAD, EDTA and sodium heparin tubes, analyzed for both the TBNK and Th panels. The data showed that for these fresh samples the choice of anticoagulant had no impact on the proportions of the various cell populations (Figure 1, Table 2).

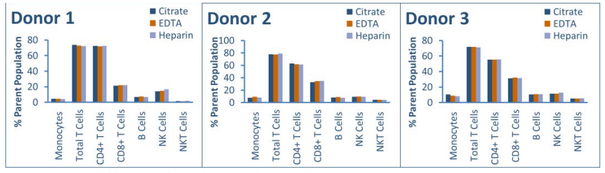

Figure 1. Effects of anticoagulant - TBNK panel.

Donor	Anti-coagulant	% Parent Population Monocytes	Total T Cells	CD4+ T Cells	CD8+ T Cells	B Cells	NK Cells	NKT Cells
1	CTAD	4.5	73.8	72.3	21.3	6.9	14.2	1.5
	EDTA	4.5	72.8	72.0	22.0	7.6	14.9	1.3
	Heparin	4.2	72.1	72.3	21.9	6.8	16.7	1.9
2	CTAD	7.9	78.0	62.8	33.0	8.3	9.4	4.3
	EDTA	9.2	77.2	61.9	34.4	8.9	9.7	4.3
	Heparin	8.0	79.0	61.3	35.0	7.9	9.5	4.5
3	CTAD	10.5	71.7	55.4	31.3	10.6	11.3	5.3
	EDTA	8.9	71.9	55.2	32.4	10.9	11.6	5.2
	Heparin	8.2	71.1	55.6	31.5	10.9	12.7	5.7

Table 2. Effect of anticoagulant type on analysis of cell populations (data from n=3 donors).

Viability Stain: LIVE/DEAD Fixable Aqua stain was compared to 7-AAD as indicators of cell viability. Freshly lysed whole blood samples were spiked with heat-killed PBMCs and tested with and without subsequent fixation. In the presence of formaldehyde there appeared to be a marked increase in background staining for 7-AAD when the stained samples were held for 24 hours, which reduced the discrimination between the live and dead populations (Figure 2).

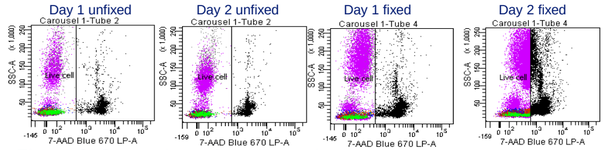

Figure 2. Comparison of viability stains.

Impact of Number of Events Collected: Acquisition of 5,000 or more lymphocyte events for Th panel showed consistently reproducible results, with percentage differences from the baseline (20,000 lymphocyte events) for the various populations between -11.0% to 10.8%, for three donors. For the TBNK panel the lowest acquisition threshold at 1,000 lymphocyte events and the differences from the baseline acquisition (20,000 events) were between -12.1% and 5.0%.

Donor	Lymphocyte Events Collected	CD4+	% Difference	Th1	% Difference	Th2	% Difference	Th17	% Difference
1	20000	48.8	-	28.4	-	71.2	-	9.0	-
	10000	48.6	-0.4	28.4	0.0	72.0	1.1	8.6	-4.4
	5000	49.2	0.8	28.3	-0.4	72.6	2.0	9.4	4.4
2	20000	44.5	-	17.3	-	85.9	-	4.2	-
	10000	44.8	0.7	18.5	6.9	86.0	0.1	3.9	-7.2
	5000	45.2	1.6	15.4	-11.0	86.7	0.9	4.2	1.2
3	20000	54.0	-	15.8	-	79.0	-	3.7	-
	10000	54.1	0.2	16.8	6.3	80.6	2.0	3.5	-5.4
	5000	53.8	-0.4	16.6	5.1	79.5	0.6	4.1	10.8

Table 3. Impact of varying acquisition number on proportions of collected T helper cells. Assessment of difference for from a baseline collection of 20,000 lymphocytes from 3 donors using isolated PBMCs.

Precision: Intra-assay precision was assessed using n=6 replicates for both TBNK and Th panels using lysed whole blood. %CV values between replicates for the TBNK panel did not exceed 10.5%, and for the Th panel did not exceed 15.5% (data not shown). The Th panel showed good precision inter-analyst precision in both whole blood and PBMCs. %CV for the Th17 populations of some donors was >20% likely due to low frequency of the population. In the whole blood analysis the Th17 population produced %CV values of 7.4% or less (IMMUNNO-TROL™ cells were not considered a suitable QC for Th17 analysis as the %CV was 56%).

Assay Transfer: Cross-laboratory Assay reproducibility tests using cryopreserved PBMCs from 3 donors was assessed. PBMCs were prepared and analyzed at the originating laboratory (Lab 1, UK). PBMC samples, aliquots of original reagents, electronic copies of the cytometer protocols, analytical procedure and data reporting templates, were dispatched to a second laboratory (Lab 2, USA), where analyses were run on a FACSCanto II instrument with an identical configuration to Lab 1. The mean results from two independent assays at Lab 2 were compared to the mean data from 6 assays run at Lab 1.

The data presented in Table 4 (TBNK) show good inter-assay precision within each lab for most of the lymphocyte populations; T cells, B cells, NK cells, CD4 and CD8 subsets, and 2/3 donors for the low frequency NKT cell population (CVs <20%). Inter-lab reproducibility for T cells, NK cells, CD4 and CD8 subsets was also acceptable with between lab differences of <20%. Both laboratories found the monocyte population gave within-lab inter-assay precision >20%. On examination this was considered to be due to the gating strategy used.

Donor	Lab		Mono-cytes	Total T Cells	CD4+ T cells	CD8+ T cells	B Cells	NK Cells	NKT Cells
1	Lab 1	Mean	9.3	69.9	48.5	39.9	7.5	18.9	7.8
		%CV	20.9	2.3	3.3	3.0	11.0	4.5	13.5
	Lab 2	Mean	4.9	66.4	48.6	39.7	9.1	19.6	6.3
		%CV	17.9	4.0	10.4	15.9	7.4	14.1	12.9
		% diff	-46.9	-5.1	0.2	-0.3	22.3	3.9	-19.3
2	Lab 1	Mean	8.6	72.8	43.9	41.2	12.5	12.5	5.3
		%CV	27.6	1.9	4.1	3.1	5.4	4.2	10.1
	Lab 2	Mean	4.3	69.9	43.7	41.1	13.8	12.3	3.3
		%CV	20.7	2.0	4.3	4.2	3.8	0.3	42.9
		% diff	-50.1	-4.1	-0.3	-0.2	10.3	-1.7	-37.3
3	Lab 1	Mean	13.5	68.2	53.5	38.2	9.7	18.0	2.9
		%CV	17.1	2.4	1.7	1.5	9.3	5.5	14.7
	Lab 2	Mean	8.8	64.1	51.7	39.0	12.4	18.0	1.3
		%CV	49.0	4.1	3.7	3.5	12.8	3.3	8.0
		% diff	-34.6	-6.0	-3.4	2.1	28.2	-0.1	-54.8

Table 4. Inter-laboratory reproducibility for TBNK panel using frozen PBMCs.

The inter-laboratory data for the B cells showed differences >20%, but within 30%, whilst the low frequency NKT cell population showed between lab variations >35% for 2/3 donors. These latter differences may have been due to the 3 month difference between the assessments and changes within the PBMC samples. This will be further investigated by conducting parallel assays at both laboratories to remove sample age as a covariate. In addition the monocyte gating strategy will be reviewed and reassessed to reduce the inter-assay variations observed.

Conclusion

Panels for TBNK and Th detection have been developed and partially validated, showing suitable precision between donors and runs, including for lower frequency cell populations such as Th17 cells. Analysis of anticoagulants showed no impact and LIVE/DEAD was chosen as the viability stain based on performance with formaldehyde-fixed cells. Both assays are robust to as low as 5,000 cells (Th panel) and 1,000 cells (TBNK panel). Using a core set of assays, the method was transferred successfully between two analytical sites. Some inter-assay and inter-operator variability was observed in monocytes and Th17 populations and these may require additional assessment to determine the source of these variations. Inter-laboratory reproducibility was demonstrated for the main lymphocyte populations, with some evidence that difference in sample age may contribute to true inter-lab comparisons for B cells and lower frequency populations.