Qualified Flow Cytometry Immunophenotyping Assays for Use in Clinical Trials

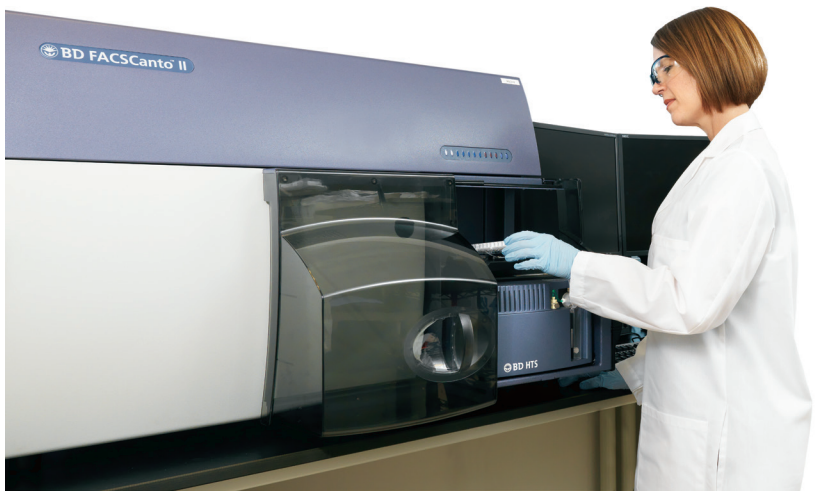
TBNK

T helper

DATASHEET

eurofins | Bioanalytical Services

Eurofins Bioanalytical Services is a global large molecule bioanalytical and biomarker service organization. With over twenty five years of experience supporting early discovery to large multi-center Phase III clinical trials for small to large sponsors, we offer industry-leading capacity while maintaining client-focused flexibility. Sites in St. Charles, USA, and Oxford, UK provide bioanalytical and biomarker services with regulatory expertise across multiple geographies, including GxP and CLIA capabilities.



eurofins | Bioanalytical Services

Qualified Flow Cytometry Immunophenotyping Assays for Use in Clinical Trials

BACKGROUND

Flow cytometry has been used in a clinical diagnostic setting for many years and is also widely utilized during pharmaceutical development for biomarker assessment. This continually developing technology allows complex multi-parameter measurement, which can be used to combine phenotypic identification of cellular populations with assessment of biological activity and even assess drug-target binding interactions. The many formats of this technology provide a robust and powerful technique for bioanalysis.

Flow Cytometry Panels for T-cell, B-cell and NK cell (TBNK) for the detection of T cells including the CD4 and CD8 subsets, B cells, NK cells, NKT cells lymphocytes and monocytes, and T-helper (Th) which identifies several key functional T lymphocyte subsets (Th1, Th2, Th17) 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. The 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.

METHOD

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

TABLE1: Panel design

RESULTS

ANTICOAGULANT COMPARISON

Whole blood from 3 donors was collected into citrate-theophylline-adenine-dipyridamol (CTAD), EDTA and sodium heparin tubes, processed and analyzed for both the TBNK and Th panels. The data showed that for these fresh samples the anticoagulant had no impact on the proportions of the various cell populations (see Figure 1 and Table 2). This provides confidence that these routine anticoagulant types will not adversely affect the assay, which allows flexibility at the clinical site for blood collection.

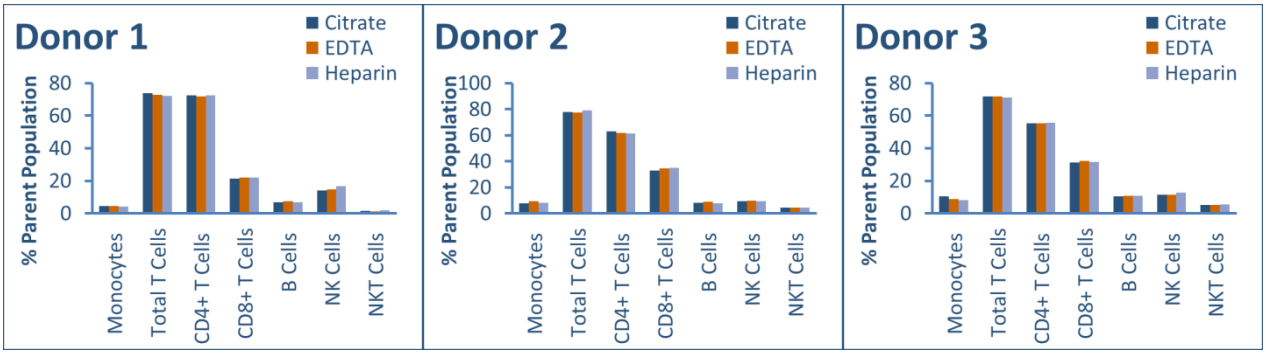


FIGURE 1. Effects of anticoagulant - TBNK panel

Donor	Anticoagulant	% 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 COMPARISON

LIVE/DEAD® Fixable Aqua stain was compared to 7-AAD as an alternative indicator of cell viability, using freshly lysed whole blood samples were spiked with heat-killed PBMCs and tested with and without subsequent fixation. 7-AAD produced good discrimination of live vs. dead cells for both freshly stained samples and stained samples held for 24 hours at +4°C (nominal), without fixation (shown in Figure 2). However, 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. Based on the results obtained, the LIVE/DEAD® Fixable Aqua dye was selected for use in the panels, due to stability when storing samples prior to analysis. This allows flexibility when analysing large numbers of samples on the flow cytometer.

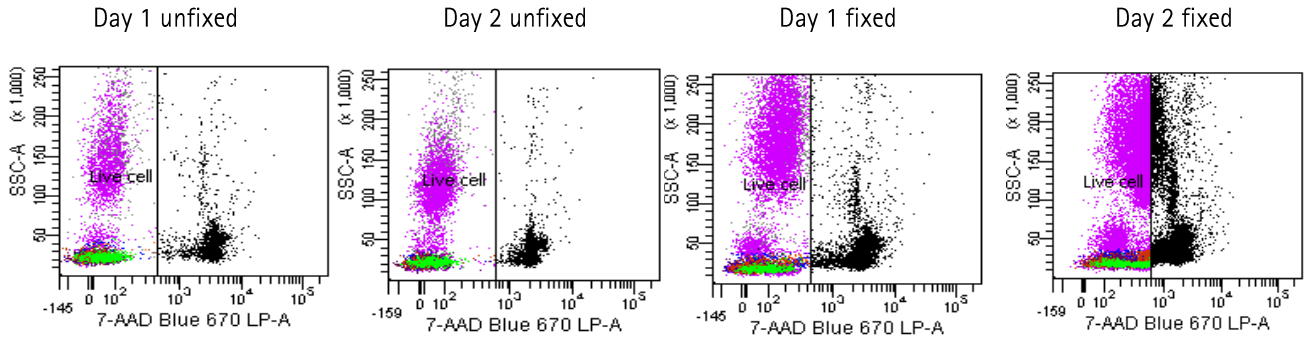


FIGURE 2. Comparison of viability stains

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 a baseline (20,000 lymphocyte events) for the various populations between -11.0% to 10.8%, for three donors (shown in Table 3). These populations included low frequency Th17 cell population (shown in Figure 3).

Donor Number	Lymphocyte Events Collected	CD4+	% Difference	Th1	% Difference	Th2	% Difference	Th17	% Difference
Donor 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
Donor 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
Donor 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.

The TBNK panel was assessed in a similar manner with the lowest acquisition threshold at 1,000 lymphocyte events. In line with the Th panel data the differences from the baseline acquisition (20,000 events) were between -12.1% and 5.0%.These results confirm that a minimum of 5,000 lymphocyte events collected for both TBNK and Th panels will allow application of reliable acceptance criteria. However, for the TBNK panel only 1,000 lymphocyte events would also fulfil this requirement. These criteria are useful where disease state affects biology, such as lymphopenia

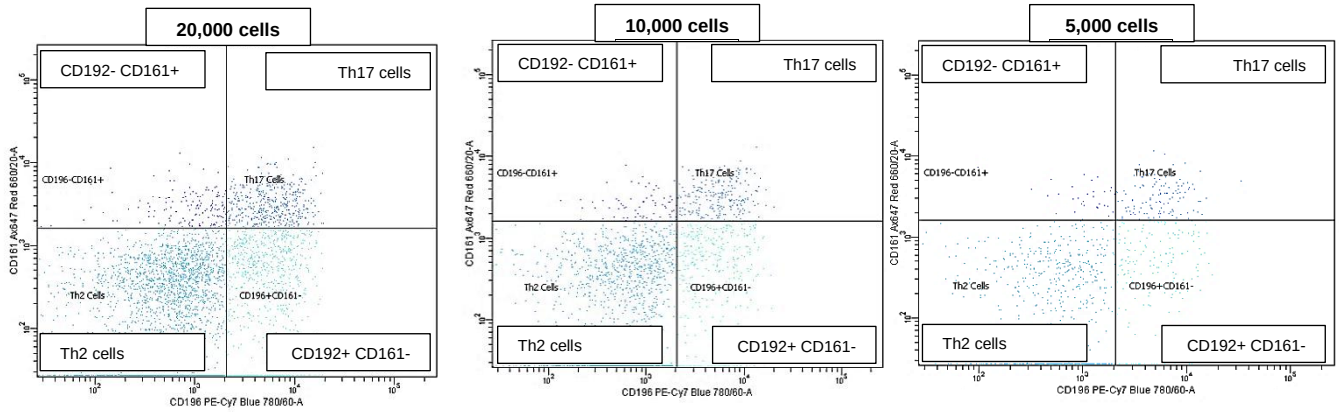


FIGURE 3. Decreasing acquisition cell number

INTRA-ASSAY (REPEATABILITY) PRECISION

Intra-assay precision was assessed using 6 replicates for both TBNK (Table 4) and Th panels (Table 5). 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%

Donor ID		Monocytes	Total T Cells	Th Cells	B Cells	NK Cells	Cytotoxic T Cells	NKT Cells
Donor 1	Mean	5.5	75.0	65.7	8.5	12.5	29.9	2.3
	%CV	10.5	0.9	0.4	11.8	4.2	1.0	6.7
Donor 2	Mean	13.0	68.5	49.2	11.9	15.9	40.0	5.5
	%CV	3.4	1.1	1.3	3.5	4.3	1.4	5.9
Donor 3	Mean	10.0	77.5	59.4	6.7	10.9	37.6	4.5
	%CV	7.2	0.8	1.2	6.3	5.8	1.9	4.3
IMMUNO-TROL™	Mean	20.3	64.9	60.7	13.6	16.9	33.4	3.0
	%CV	1.8	0.7	0.6	1.4	3.2	1.2	1.7

TABLE 4. Intra-assay precision - TBNK panel (lysed blood)

Donor ID		Helper T Cells %Parent	Th1 Cells %Parent	Th2 Cells %Parent	Th17 Cells %Parent
Donor 1	Mean	34.9	9.1	81.8	6.8
	%CV	6.9	13.3	1.3	6.9
Donor 2	Mean	24.3	9.0	77.4	8.1
	%CV	3.0	10.8	1.4	5.9
Donor 3	Mean	42.8	7.4	79.0	5.6
	%CV	3.5	11.0	0.9	4.7
IMMUNO-TROL™	Mean	59.9	17.8	88.9	0.3
	%CV	0.5	4.7	0.4	15.5

TABLE 5. Intra-assay precision - Th panel (lysed blood)

Intra assay precision was also assessed in isolated PBMC using 6 replicates. Acceptable precision was shown for the percentage population data of both the TBNK and Th panels, with maximum %CV for all parameters at 8.5% and 7.8% respectively.

INTER-ASSAY PRECISION

Inter-assay precision was assessed in whole blood and PBMC over 4 and 6 analytical runs respectively. 3 replicates of each fully stained TBNK and Th panel were analyzed each run.

Donor		Monocytes %Parent	Total T Cells %Parent	Th Cells %Parent	Cytotoxic T Cells %Parent	B Cells %Parent	NK Cells %Parent	NKT Cells %Parent
1	Mean	3.2	74.7	66.1	27.5	9.5	11.0	2.6
	%CV	49.3	3.2	1.5	16.1	14.4	9.9	16.9
2	Mean	7.3	68.3	49.5	39.6	13.0	15.0	5.8
	%CV	54.2	7.0	2.6	4.5	11.8	27.3	13.9
3	Mean	6.1	77.3	60.4	36.8	7.6	10.0	4.8
	%CV	42.3	3.1	2.5	3.8	16.6	19.7	12.6
IMMUNO-TROL™	Mean	20.5	65.7	59.9	33.8	13.9	15.2	3.8
	%CV	1.1	1.1	1.4	0.9	0.9	9.8	20.1

TABLE 6. Inter-assay precision - TBNK panel (lysed blood)

Donor		Helper T Cells %Parent	Th1 Cells %Parent	Th17 Cells %Parent	Th2 Cells %Parent
1	Mean	50.9	36.2	10.6	69.5
	%CV	4.7	7.8	6.3	3.1
2	Mean	44.5	22.8	5.4	84.2
	%CV	6.7	16.3	20.5	3.3
3	Mean	55.4	20.0	3.7	78.6
	%CV	3.3	19.9	10.1	1.3

TABLE 7. Inter-assay precision - Th panel (PBMC)

The TBNK results showed acceptable precision, with exception to the monocyte population, which was shown to be variable between donors and within runs. This variation was most prominent in the lysed blood assessments. The cause of this variability is uncertain.

Good precision between runs was found for the majority of Th panel populations, however variability was seen within the Th17 population data in the whole blood and PBMC assays. The low proportion of Th17 means low numbers of events were acquired and therefore the small values would contribute to the increased variability. This effect was most notable in Donor 2 PBMC samples with a %CV of 20.5%. In general, the PBMC samples were less affected.

INTER-ANALYST PRECISION

Inter-analyst precision was also assessed for both panels using PBMC and lysed whole blood. Acceptable precision was seen in all assessments, again the exception were the monocyte populations in the TBNK panel, which show larger %CV values as noted above for inter-assay precision.

The Th panel showed good precision between operators in both whole blood and PBMC. The %CV for the Th17 populations of some donors was >20%, most notably for Donor 2 PBMCs with Th17 %CV=29.1%. As noted previously this is likely to be due to the low frequency of the population.

In the whole blood analysis the Th17 population produced lower %CV values of 7.4% or less. However, the Th17 population in the stabilised blood preparation IMMUNO-TROL, assessed as a possible assay QC material, showed %CV=56.0%. In this case the IMMUNNO-TROL cells were not considered a suitable QC for Th17 analysis. All other cell populations showed acceptable precision and IMMUNO-TROL could be considered a suitable QC material. Figure 4 shows the inter analyst precision of the IMMUNO-TROL cells and the TBNK panel. Each analyst performed two independent runs.

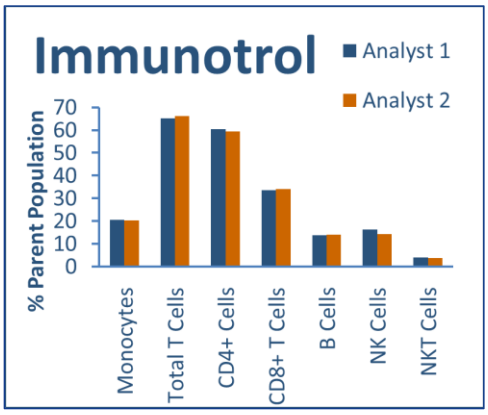


FIGURE 4. Inter-analyst precision - using IMMUNOTROL

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.

eurofins

Immunogenicity

- Assay development, method transfer, validation. Cut-point calculation
- Screen, Confirm, Titer. Advanced methodologies to address drug tolerance and soluble target interference
- Advanced cell based laboratory dedicated to GLP NAb assay development and sample analysis
- Experience and capacity for large volume sample analysis

Biosimilars

- Pre-developed assays for Trastuzumab, Bevacizumab, Cetuximab, Adalimumab, and more
- PK, Immunogenicity evaluation
- FcRN, FcγRI, II, III and C1q binding
- Cell based assays
  - Receptor binding, proliferation, ADCC

Biomarkers

- GLP/GCP/CLIA assay development, method transfer, validation
- Exploratory sample analysis using kits from any vendor across a wide variety of platforms
- Luminex, ELISA, RIA, MSD, GyroLab, Flow Cytometry, Singulex
- Full flow cytometry capabilities to support GxP studies
- Immunophenotyping, pharmacodynamics
- Cytokine release assays

Pharmacokinetics

- Large molecule specialists, capacity for large volume sample analysis
- Clinical and Pre-clinical PK studies
  - Exploratory / GxP
- Latest platforms including GyroLab and Singulex

Contact us today to discover how the Eurofins team can make the difference in your projects.

bioanalyticalservices@eurofins.com
US +1 844 522 7787
UK +44 (0)1235 444 100