Overcoming Challenges for Flow Cytometry in Clinical Regulated Biomarker Analysis

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Introduction

Multi-colour flow cytometry is a powerful laser-based technology, which is widely used for cellular characterisation and functional analysis. It is a powerful analytical tool with several major advantages; providing multi-parameter analysis of different cellular components, on large numbers of events, in a quantifiable manner. Modern cytometry can combine measurements on surface expressed targets with analysis of intra-cellular signalling and functional endpoints, including determination of drug-receptor occupancy - all in a single sample. Collecting >100,000 target population events in less than a minute also allows delivery of robust analytical datasets for rare and infrequent populations of interest.

The benefits of flow cytometric analysis are exploited at all stages of a drug development program, from early discovery and proof of mechanism to post-marketing clinical testing, placing it in multiple regulatory environments (R&D, GLP, GCP and even GMP). Each stage of drug development provides its own set of challenges, both in method development and assay validation. Validating flow cytometry methods in support of regulated clinical trial analysis can be particularly challenging, as they are mostly cell-based analyses. However, not all of the challenges are laboratory-based and often the most significant factors relate sample preparation and logistical issues at clinical sites.

Here we present data on sample stability obtained during development of a flow analysis to support clinical trials and show it is possible to resolve some common issues to ensure clinical trial samples provide high quality data for regulatory assessment.

Methods

Peripheral blood samples were collected into sodium citrate CPT™ tubes (BD Biosciences) and either tested as whole blood, or processed to peripheral blood mononuclear cells (PBMC) . PBMCs were cryopreserved at -70ºC and thawed for analysis. Samples were stained with an antibody cocktail containing CD3 and CD19 and analysed on a FACSCanto II™ flow cytometer (BD Biosciences) using FACSDiva™ (v6.1.2) software. Prior to analysis the cytometer was calibrated using CS&T™ beads (BD Biosciences) to determine the difference in output compared to baseline measurements.

Assay reproducibility
a) when cell events are limited
The mean values of cell populations from three donor PBMC samples were used to set acceptance criteria limits for analysis. Repeat acquisition using a decreasing number of lymphocyte events was used with the following maximum event acquisition targets : 50000, 10000, 5000, 1000 and 100 events. Changes in the percentages of the cell populations were monitored as an indication of variability.

b) Long term stability for batching samples for analysis
Multiple vials of donor samples were stored at -70ºC (nominal) to mimic sample storage at clinical sites. The samples were stored for up to 18 weeks to allow samples to be batched before performing analysis. A change in the percentage of the population present in the sample of >20% was used to define the point of unacceptable sample instability during storage.

Abbreviations
CD – Cluster of Differentiation
CPT™ - Cryopreservation Tube
DMSO - Dimethyl sulfoxide
FBS – Foetal Bovine Serum
Fc - Fragment crystallizable region
FMO – Fluorescence Minus One
MESF – Molecules of Equivalent Soluble Fluorochrome
PBMC – Peripheral Blood Mononuclear Cell

Validation of sample collection and processing effects on marker expression and proportions of cell populations
The effect of cryopreservation on $CD3^+$ cells was assessed in PBMC samples. Whole blood samples were processed to PBMCs using CPT™ vacutainers (BD Biosciences) and then cryopreserved in 90% FBS/10% DMSO. The effect of the preservation process on the level of surface marker expression and the relative percentages of $CD3^+$ populations was assessed.

Results

Assay reproducibility when cell events are limited
Collection of 5000 events or more in acquisition (as seen in Table 1 and Figure 1) consistently produced results with percentage differences from the baseline measurement (50,000 events) were 5.3% or less. Based on this result, it was concluded that collection of a minimum of 5,000 lymphocyte events should be assigned as the acceptance criteria.

This result shows that even samples with lower lymphocyte counts were able to produce results that were comparable the 'baseline' using higher lymphocyte counts, but showed more variability. This can be especially useful in studies where low cell numbers are expected (e.g. lymphopenia) or when samples are not processed effectively at the clinical site.

Donor	Lymphocyte events acquired	B Cells (Percentage of CD19+ CD3- Lymphocytes)	% Difference from 50000 events	T Cells (Percentage of CD3+ Lymphocytes)	% Difference from 50000 events
Donor 1	50000	6.0	-	81.0	-
	10000	7.1	17.5	77.3	-4.6
	5000	6.2	2.5	83.1	2.5
	1000	6.4	6.7	83.5	3.1
	100	8.8	45.8	82.6	1.9
Donor 2	50000	10.5	-	60.6	-
	10000	9.8	-6.7	61.6	1.7
	5000	10.3	-2.4	60.9	0.5
	1000	10.8	2.4	60.2	-0.7
	100	12.8	21.4	64.9	7.1
Donor 3	50000	8.6	-	56.1	-
	10000	7.6	-11.1	58.2	3.8
	5000	8.1	-5.3	57.0	1.6
	1000	6.7	-22.2	59.7	6.5
	100	7.6	-11.1	54.2	-3.4

Table 1. Determination of assay reproducibility for the use of the method if cell events are limited.

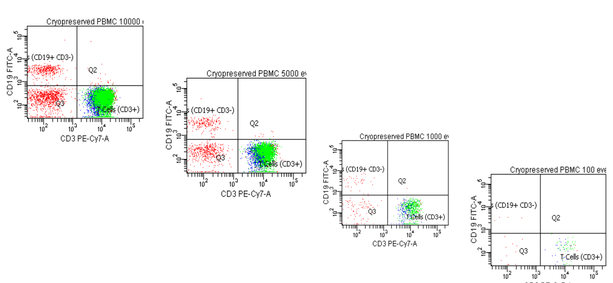

Figure 1. The effect of decreasing cells events on cryopreserved PBMCs from the three healthy donors.

Long term stability for batching samples for analysis Sample storage is often a problem with extended trials. Therefore assessing the stability of the samples in storage that mimic storage at the trial site is important. Three donor sample sets were tested with blood drawn and processed to PBMCs before being cryopreserved. The samples were stored at -70ºC, to mimic storage at the clinical site. Various time-points up to 18 weeks were tested (Table 2 and Figure 2). The cells of interest ($CD3^+$) were found to be stable throughout this period, without detrimental effect to the proportion of the cell population. This result was reflected in all three donors.

This data provides a good indicator that samples can be collated and analysed in groups, rather than splitting up patient profiles, if the sample time-points fall within the assessed stability window.

	T Cells (CD3+ of Lymphocytes)					
Stability Time point	Donor 1	% Difference from Baseline	Donor 2	% Difference from Baseline	Donor 3	% Difference from Baseline
Baseline	82.1	-	60.6	-	73.1	-
4 Weeks	81.7	-0.5	68.8	13.4	77.3	5.7
8 Weeks	82.7	0.7	70.6	16.4	76.5	4.6
12 Weeks	75.8	-7.7	65.2	7.6	65.2	-10.8
18 Weeks	78.8	-4.1	66.5	9.7	70.8	-3.1
Mean	80.2	-	66.3	-	72.6	-
Stdev	2.92	-	3.8	-	4.88	-
%CV	3.6	-	5.7	-	6.7	-

Table 2. Stability of Donor PBMC stored at -80ºC for up to 18 weeks.

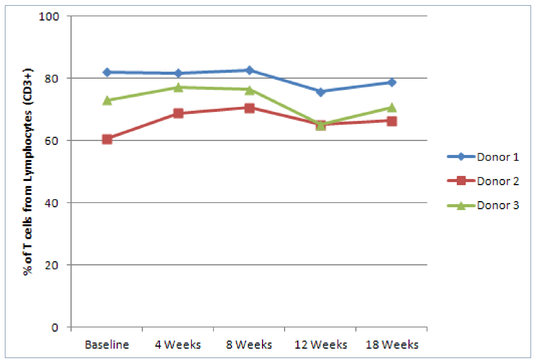

Figure 2. Stability of Donor PBMC stored at -80ºC for up to 18 weeks.

Validation of sample collection and processing effects on marker expression and proportions of cell populations
Storage, or cryopreservation, reduced CD3 expression by approximately the same proportions in all three of the donors tested. This allowed comparison of the CD3 expression levels between the donors, as the rank order for expression of CD3 was not altered (Table 3 and Figure 3).

In addition the short-term storage data shows <20% change in MESF for samples stored for 48 hours before processing. Therefore for shipments where processing can be performed within 48 hours, it is feasible to ship samples to test facilities without any additional processing or cryopreservation.

The effect of cryopreservation on the PBMC cell populations of interest was assessed. There was no evidence of an alteration by the freezing process. This comparison was performed for fresh and frozen PBMC from the same donor and time-point. The differences observed between the cell populations were <10% (data not shown).

	MESF CD3+ T Cells					
Sample Type	Donor 1	% Difference from Fresh Blood	Donor 2	% Difference from Fresh Blood	Donor 3	% Difference from Fresh Blood
Fresh Blood	187937	-	192607	-	135022	-
48 Hour Stored Blood	164268	-12.6	162629	-15.6	115336	-14.6
Frozen PBMC	130312	-30.7	125623	-34.8	91840	-32.0

Table 3. Effect of sample processing and storage on cell surface marker expression.

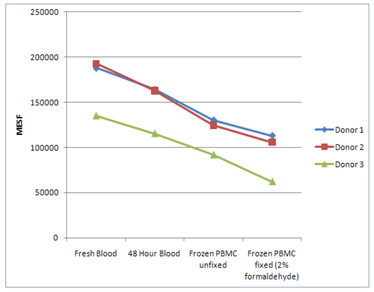

Figure 3. Effect of sample processing and storage on cell surface marker expression.

Conclusions

For the analysis of B cells and T cells, the proportion of each population was shown to be unaffected by lower event acquisition, where > 5,000 events were collected. Therefore for reproducible and reliable results a lower limit of 5,000 lymphocyte events should be acquired.

Sample stability of CD3+ T cells showed the relative proportion of the T cell population was not affected by storage at -70ºC (nominal) for up to 18 weeks. This enables collation of trial samples into groups for analysis; reducing costs, increasing throughput and allowing whole donor profile testing which removes inter-assay variations.

Validation of sample collection methods and processing showed that any effects on CD3 marker expression were consistent and allowed comparison of samples that have been handled in a similar manner, since cryopreservation, or blood storage, did not affect the rank order of the marker between donors.

This approach to sample validation can be applied to flow cytometry analyses on other sample types, enabling the use of this powerful multiparameter technology during clinical trial bioanalysis.