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“Fit-for-purpose” validation of a method involves experimental testing to establish that assay performance characteristics meet intended use of the data and the associated regulatory requirements.

Results and conclusions: Four CD4+ T cell response reportables (single or triple cytokine producers) were used to assess assay performance. Cytokine-producing antigen-specific CD4+ T cell frequencies exhibited acceptable inter-instrument, inter-operator, intra- and inter-assay precision (%CV<30%). The LLOQ, defined as the last consecutive dilution that can be measured with acceptable bias and CV was functional signature-specific and ranged between 0.01-0.05% in cytokine-producing CD4+ T cells. The LOD was determined using the isotype panel and determined to be lower than the LLOQ. Expression of cytokines of antigen-specific CD4+ T cells was detectable 24hr following staining procedure.

[illegible]

Two analytical runs (Run1, Run2) were performed by two operators (Op1, Op2) on two days and acquired on two cytometers (505, 555). Precision was assessed on all functional signatures of CMV and SEB stimulations. Dotted lines represent 100% reproducibility (A-C) or acceptable threshold of %CV (30%).

PBMC were stimulated with SEB and serially diluted (1:2) into non-stimulated controls. The %bias (non-weighted difference relative to the undiluted sample) and %CV of replicates was calculated. The LLOQ was established as the last consecutive dilution with acceptable bias and %CV (<30%). All functional signatures were assessed. 3_Cyt_SUM = all cytokine responses combined

Cytokine production was assessed over the course of 37 analytical runs. The cytokine-specific upper and lower limits are depicted and represent the average $\pm$ 3STD.

A STABILITY SUMMARY 1202

			IFN α	IL2 α	TNF α	3_CyL_Sum	IFN α IL2+TNF α	IFN α IL2+TNF α	IFN α IL2+TNF α	IFN α IL2+TNF α	IFN α IL2+TNF α
0h	CMV pp65	AVERAGE	0.066	0.027	0.079	0.084	0.025	0.001	0.044	0.006	0.002
10h	CMV pp65	AVERAGE	0.079	0.029	0.083	0.089	0.005	0.009	0.009	0.003	0.017
16h	CMV pp65	AVERAGE	0.066	0.027	0.079	0.084	0.025	0.001	0.044	0.006	0.002
24h	CMV pp65	AVERAGE	0.066	0.022	0.073	0.078	0.019	0.001	0.039	0.006	0.001
24h	CMV pp65	AVERAGE	0.077	0.027	0.079	0.085	0.024	0.001	0.039	0.005	0.003

B Non-weighted % difference

			IFN α	IL2 α	TNF α	3_CyL_Sum	IFN α IL2+TNF α	IFN α IL2+TNF α	IFN α IL2+TNF α	IFN α IL2+TNF α	IFN α IL2+TNF α
0h	CMV pp65	AVERAGE	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
10h	CMV pp65	AVERAGE	11.4%	0.0%	0.0%	0.0%	0.0%	0.0%	15.6%	2.1%	0.0%
16h	CMV pp65	AVERAGE	11.4%	0.0%	0.0%	0.0%	0.0%	0.0%	15.6%	2.1%	0.0%
24h	CMV pp65	AVERAGE	1.5%	20.4%	7.9%	0.4%	24.7%	0.0%	13.7%	0.0%	40.0%
24h	CMV pp65	AVERAGE	5.9%	0.0%	0.0%	1.1%	4.1%	0.0%	13.7%	18.1%	40.0%

The average of each functional cytokine signature at each acquisition time point depicted in A. %Non-weighted Difference relative to baseline (immediate acquisition) depicted in B.

- Signal specificity was demonstrated for all cytokines
- A positive response must be above assay noise (LOD)
- LLOQ was established for each cytokine signature, with a maximum of 0.42% for the triple-producing cytokine population
- Post-staining stability up to 24hrs
- For CD4+ T cell functional signatures with values >LLOQ
 - Frequencies >0.5% must exhibit %CV $\leq$ 30%