

Flow Cytometry Assay Validation as a Biomarker versus in a Drug Development Setting

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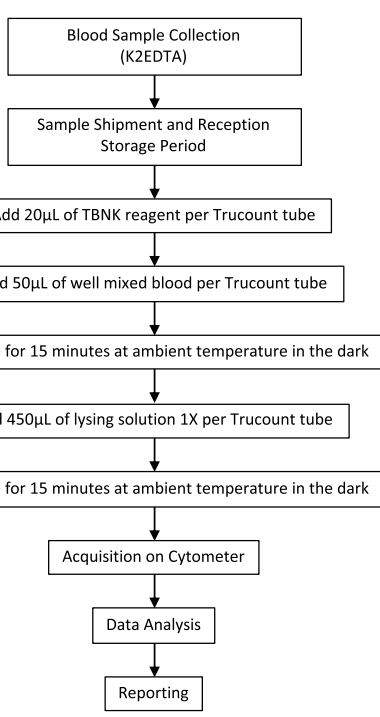
CAPRION

Introduction

This poster will describe the validation of 2 methods: the first is the TBNK assay validated for a CAP/CLIA setting; the second is T cell Functional assay for a secondary endpoint in drug development GLP setting. Commonalities and difference in both validation strategies will be highlighted.

TBNK Assay

Methods



Reportable Results

Population	Phenotype	Units
T cells	SSC ^{low} , CD45 ^{high} , CD3 ⁺	Relative % of Lymphs Abs cnt (cells/µL)
CD4 T cells	SSC ^{low} , CD45 ^{high} , CD3 ⁺ , CD4 ⁺	Relative % of Lymphs Abs cnt (cells/µL)
CD8 T cells	SSC ^{low} , CD45 ^{high} , CD3 ⁺ , CD8 ⁺	Relative % of Lymphs Abs cnt (cells/µL)
B cells	SSC ^{low} , CD45 ^{high} , CD3 ⁺ , CD19 ⁺ , CD16/CD56 ⁻	Relative % of Lymphs Abs cnt (cells/µL)
NK cells	SSC ^{low} , CD45 ^{high} , CD3 ⁺ , CD19 ⁻ , CD16/CD56 ⁺	Relative % of Lymphs Abs cnt (cells/µL)

Laser	Blue, 488 nm		Violet, 405 nm						Yellow Green, 561 nm			Red, 640 nm		UV, 355 nm				
Filter	695/40 BP	530/30 BP	780/60 BP	710/50 BP	660/20 BP	610/20 BP	525/50 BP	450/50 BP	780/80 BP	678/30 BP	610/20 BP	585/15 BP	780/80 BP	730/45 BP	670/14 BP	740/35 BP	515/30 BP	379/28 BP
Fluor	PerCP-Cy5.5	FITC							PE-Cy7			PE	APC-Cy7		APC			
Ag	CD45	CD3							CD4			CD16 CD56	CD8		CD19			
Clone	2D1	SK7							SK3			B73.1 NCAM 16.2	SK1		SJ25C1			

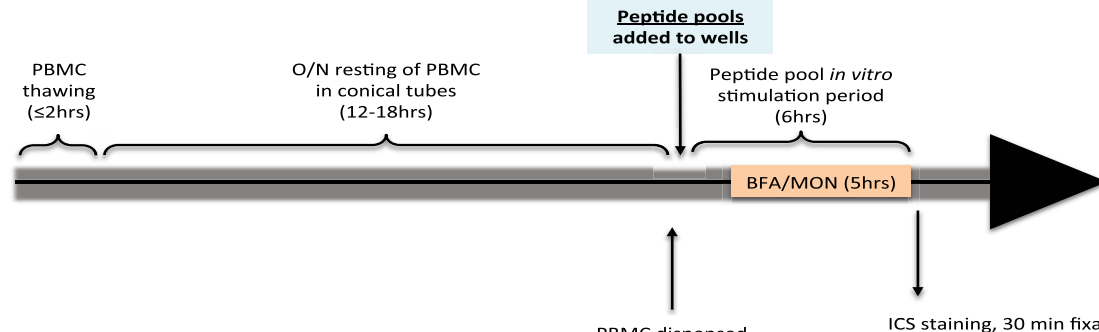
Validation Samples

Whole blood collected in Cyto-Chex® BCT (Streck) stored at 2-4°C/ CD-CHEX CD34® (Streck)

Validation Plan

	TBNK (modified)
Regulatory Setting	CAP/CLIA
Intended Use of Data	- This test is labeled as an <i>in vitro</i> diagnostic - Patient care and treatment - Patient enrollment in a clinical trial - Provided to investigators associated with patient ID
Accuracy	- Compare to QC manufacturer's target values - Proficiency Testing survey - Sample exchange with another laboratory
Sensitivity Functional (LLOQ)	Accept manufacturer's claims
Precision	
Intra-Assay	Matrix samples (K ₂ EDTA)—6 samples 2 operators
Inter-Operator	QC material—4 runs total, 2 operators, 2 instruments
Inter-Equipment	
Inter-Run	
Linearity	Accept manufacturer's claims
Sample Stability	Confirm manufacturer's claims
Processed Sample Stability	Confirm manufacturer's claims

TBNK Assay T cell Functional Assay



CD4 T cells

IFNγ
IL2
TNFα
IFNγ, IL2 and TNFα, in combination

CD8 T cells

IFNγ
Granzyme B
Perforin
IFNγ, Granzyme B and Perforin, in combination

Reportable Results

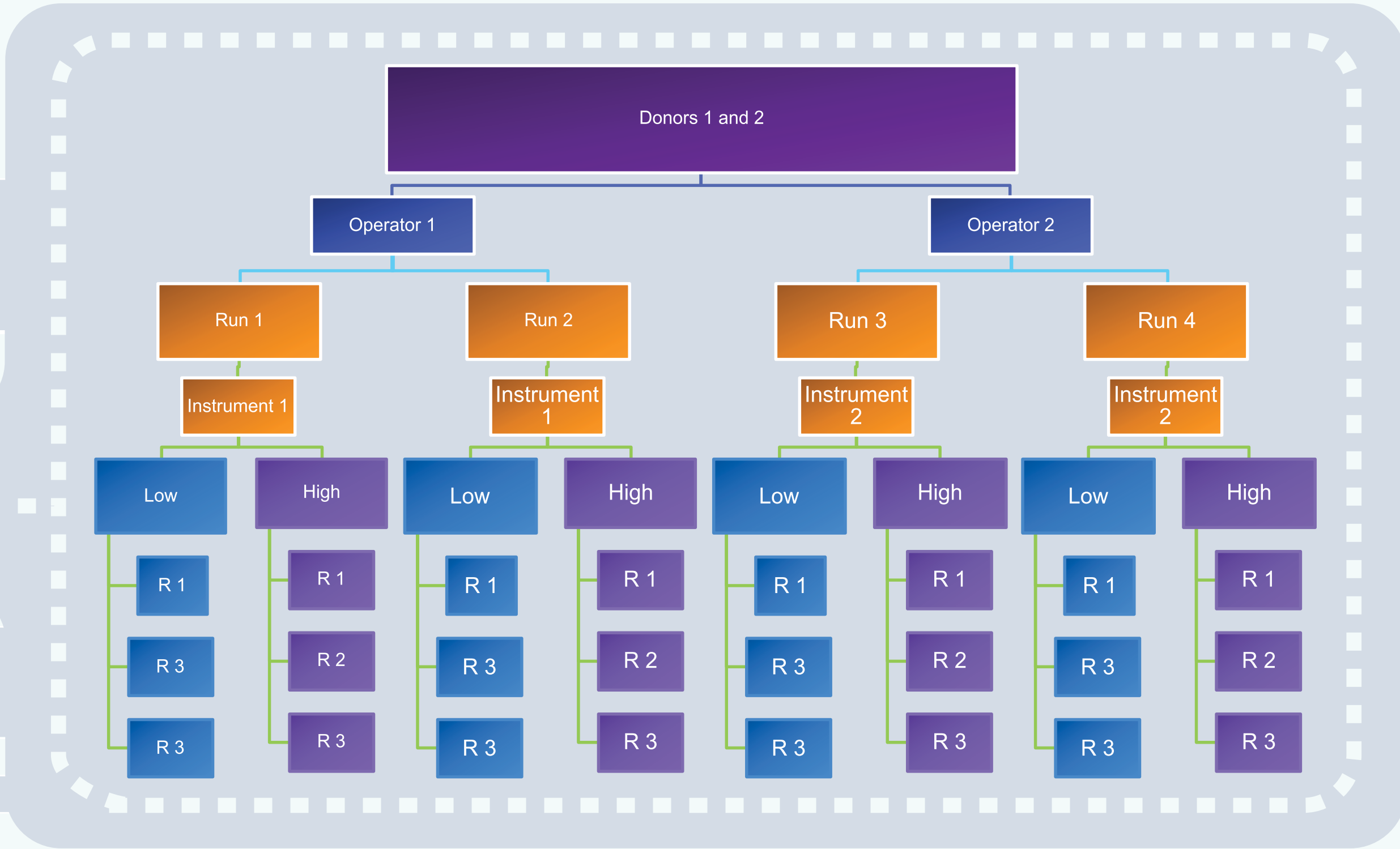
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Filter	695/40 BP	530/30 BP	780/80 BP	710/90 BP	660/20 BP	610/20 BP	525/50 BP	450/50 BP	780/80 BP	670/30 BP	610/20 BP	585/15 BP	780/80 BP	730/45 BP	740/35 BP
Fluor	FITC	PE-Cy5.5						PE-Cy7		PE	APC-Cy7		APC	Alexa 700	APC
Isotype	IFN- γ	CD4	CD8	CD3	CD16 (vAbR)	CD3	CCR7		IFN- γ	IFN- γ	CD4 (R4)	CD8	IFN- γ		
Clone	MOFIC 11	SK3	RPA-18	0323	MS12	SP34-2	G0H11.1		SK3	MOFIC 11	MOFIC 11	R2729a	MOFIC 11		
Fail	IFN- γ	CD4	CD8	CD3	CD16	CD3	CCR7		Graysen B	Perforin			R-2	TNFr1	
Clone	B27	SK3	RPA-18	0323	MS12	SP34-2	CCR7		GB1	B-D38			GB1		

CD4 and CD8 naïve and memory subsets

Leukapheresis packs from 2 healthy donors

- Cryopreserved PBMC/ Pre-screened for responsiveness CMV pp65 peptide pool/Will serve as QC material
- Ex vivo stimulation
 - High sample--SEB (Provides a strong positive response)
 - Low sample-- CMV pp65 peptide pool (antigen specific, physiologically relevant response)

	T cell Functional Assay
Regulatory Setting	GLP-like
Intended Use of Data	Efficacy endpoint in clinical trials for novel vaccines Secondary endpoints Relative percentage of CD4 T cells producing •IFNγ •IL2 •TNFα Exploratory endpoints • All other reportable results
Accuracy	Not possible
Sensitivity Functional (LLOQ)	Admix stimulated and unstimulated samples. 12 two-fold dilutions
Precision	
Intra-Assay	
Inter-Operator	
Inter-Equipment	
Inter-Run	
Linearity	Plot LLOQ data (dilution vs observed) Establish (n=6) Establish (n=3)



Results

Intra-assay precision
Mean, SD, %CV of replicates
Mean of all %CV
Acceptability criteria
CV ≤ 25%, CV ≤ 30% near LLOQ

Inter -Assay Precision Summary (absolute counts)					
Population	T cells	CD4 T cells	CD8 T cells	B cells	NK cells
Low QC n=4 runs	0.62	1.87	0.67	1.51	0.93
High QC n=4 runs	1.10	1.64	0.85	0.34	2.71

Intra -Assay Precision Summary (absolute counts)					
Population	T cells	CD4 T cells	CD8 T cells	B cells	NK cells
Gand Mean %CV	3.6%	3.5%	3.5%	4.1%	3.8%
Range	1.3 to 6.5%	1.3 to 6.7%	1.1 to 5.6%	1.3 to 7.5 %	0.6 to 7.0%
Intra -Assay Precision Summary (relative percentage)					
Population	T cells	CD4 T cells	CD8 T cells	B cells	NK cells
Gand Mean %CV	0.5%	0.8%	1.1%	2.5%	1.5%
Range	0.1 to 0.8%	0.3 to 1.2%	0.9 to 1.6%	0.9 to 4.6%	0.4 to 2.6%

Inter -Assay Precision Summary (relative percentage)					
Population	T cells	CD4 T cells	CD8 T cells	B cells	NK cells
Low QC n=4 runs	0.2	1.46	0.343	0.843	0.243
High QC n=4 runs	0.2	1.46	0.343	0.843	0.243

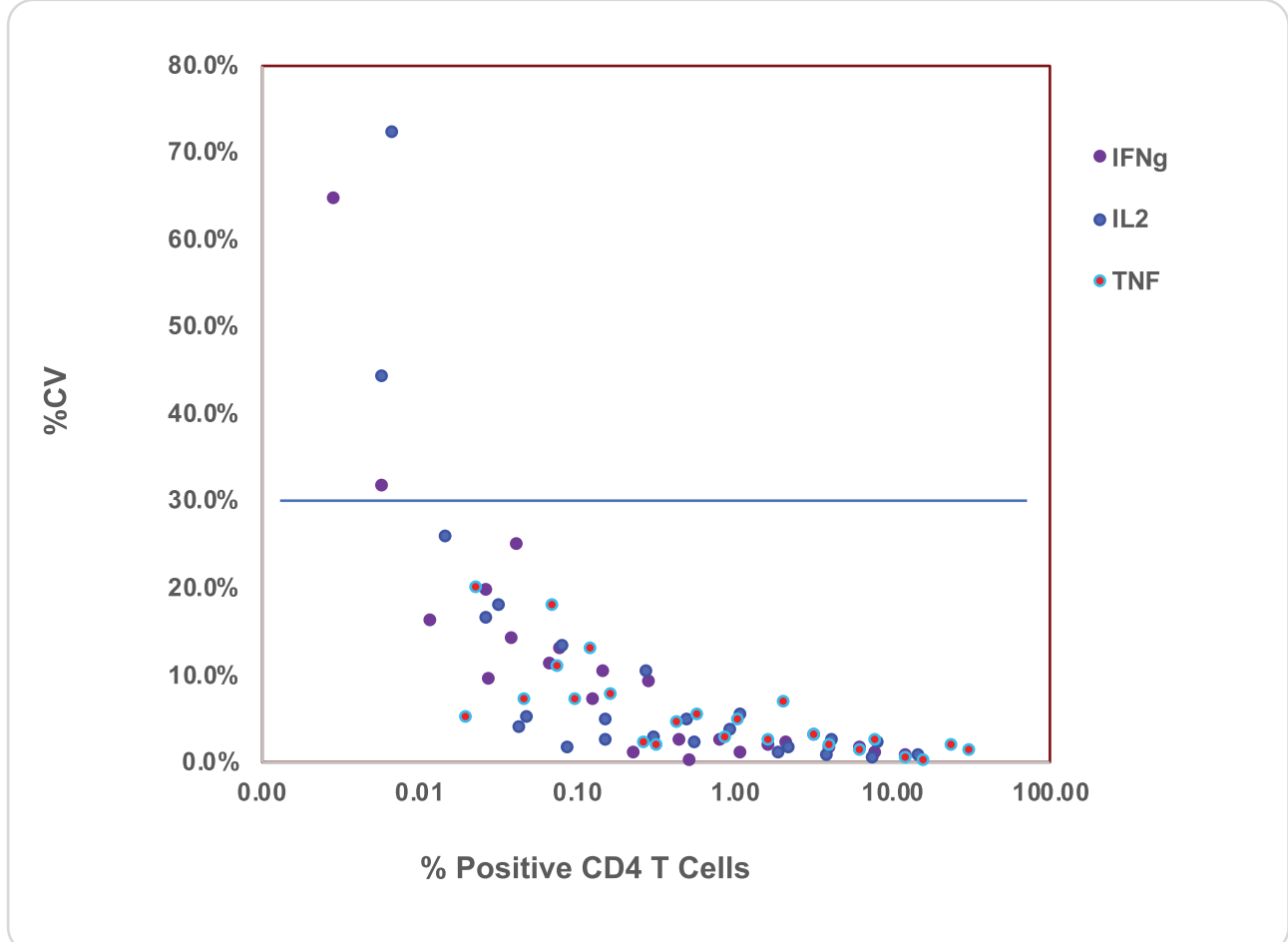
Accuracy	
Low QC Lot 1	92.9% – 101.3%
High QC Lot 1	91.1% - 109.2%
Low QC Lot 2	90.1% – 113.2%
High QC Lot	92.0% - 116.7%

Conclusions

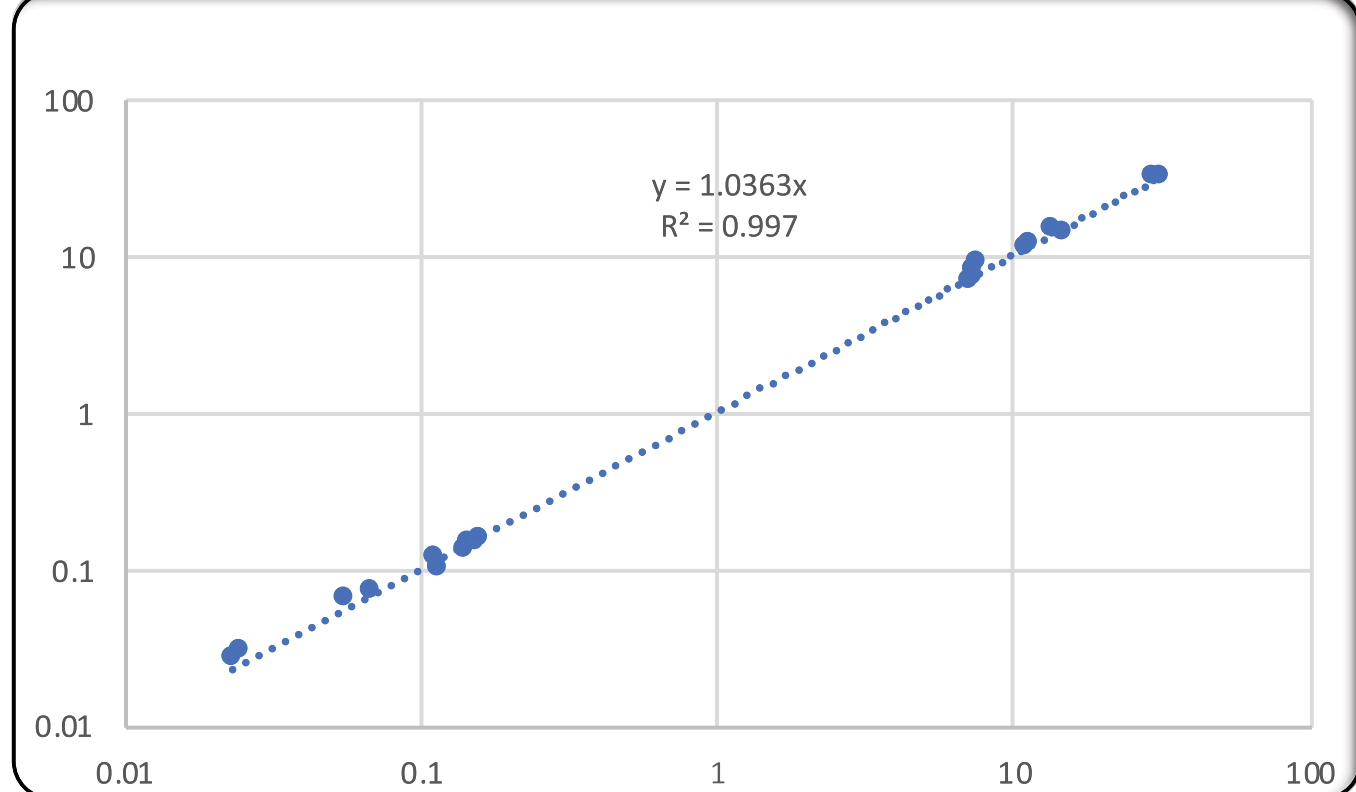
All methods must be validated appropriately to the intended use of the data.
Flow cytometric methods can deliver high quality data in a CAP/CLIA and in a drug development setting.

Intra-Assay Precision Summary (4 runs per sample)			
	IFN	IL2	TNF
Grand Mean %CV	5.89%	7.58%	4.62%
Range	0 to 15.81%CV	0 to 27.5 %CV	0 to 12.3 %CV
Inter-Assay Precision Summary			
Donor 1 Low (n=4 runs)	7.51	6.30	4.10
Donor 1 High (n=4 runs)	4.24	4.44	3.27
Donor 2 Low (n=4 runs)	7.82	11.38	7.71
Donor 2 High (n=4 runs)	6.44	9.97	7.01
Grand Mean %CV	6.50	8.02	5.52

Functional Sensitivity (LLOQ)



Inter-Operator and Inter-Instrument

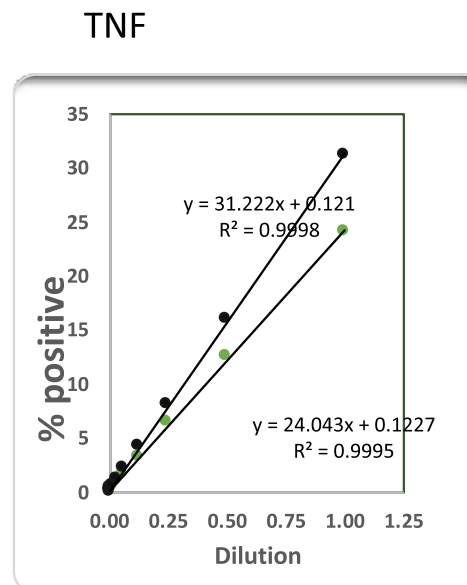
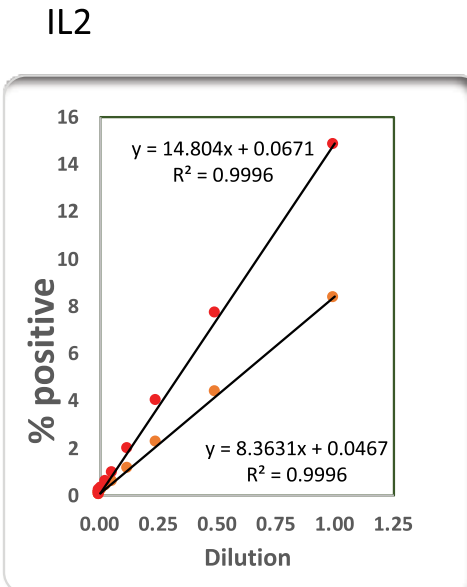
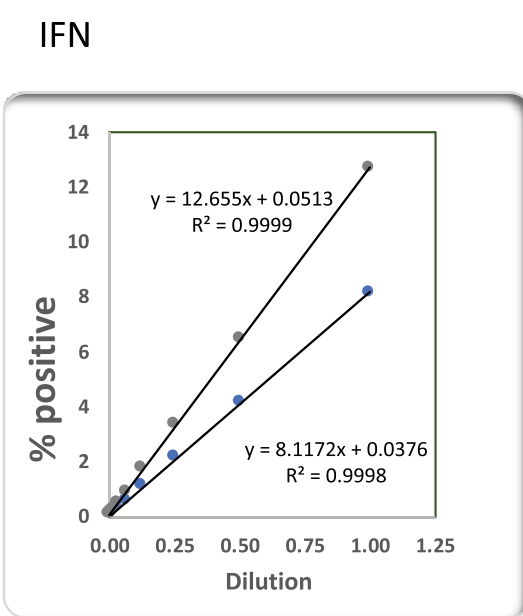


References

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Linearity



Donor 1 top line/ Donor 2 bottom line