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Qualification of an IFN-γ Elispot Assay in the Bioanalytical Laboratory

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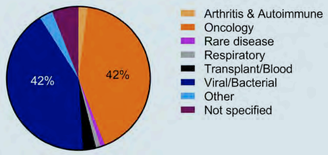
PURPOSE

Introduction

Immune Monitoring assays, such as flow cytometry and Elispot (Enzyme-linked immunosorbent spot) have been utilized in the research arena for decades. Adapting such complex assays into the clinical realm has a host of challenges, with an increasing emphasis on compliance to industry standards in a regulated environment being at the forefront (FDA BMV guidelines, May 2018). An intensified focus on biomarkers in the drug development process combined with technological advances has led to the growth in popularity of multifaceted assays such as Intracellular Cytokine Staining (ICS) and Elispot.

The Elispot assay provides a powerful tool in the development of new vaccines and novel immunotherapy agents. The emergence of global disease outbreaks has led to an expansion of studies focused on vaccine development for infectious agents. Breakthroughs in understanding the immune system in recent years have brought a new wave of treatments using immune system modulators, such as checkpoint inhibitors, as well as other immuno-oncology treatment advancements. (Figure 1.)

Figure 1. A graphical representation highlighting the relevant therapeutic areas that utilized ELISPOT in clinical trials in 2017



Regulatory Guidance

Elispot and other immune monitoring assays such as intracellular cytokine staining (ICS) provide unique challenges as no reference material or gold standard can be utilized. It is important to note that FDA Bioanalytical Method Validation guidance is not always applicable (Table 1), or may need to be adapted to the unique properties of the assay (Table 2). Numerous global harmonization studies have been carried out for Elispot, creating optimized protocols and guidelines (Janetzki et al., 2008, 2015), as well as targets for precision and linearity (Maecker et al., 2008). IFN-γ is the most common analyte measured with the Elispot assay in clinical studies. Utilizing optimized protocols and guidelines in established literature, a qualification plan was developed for an IFN-γ Elispot including target criteria. In this study, we address essential components in qualifying an Elispot assay; precision, accuracy, specificity, limit of detection (LOD), and linearity of the assay.

Table 1. Recommended Components of Bioanalytical Method Validation (FDA, May 2018)

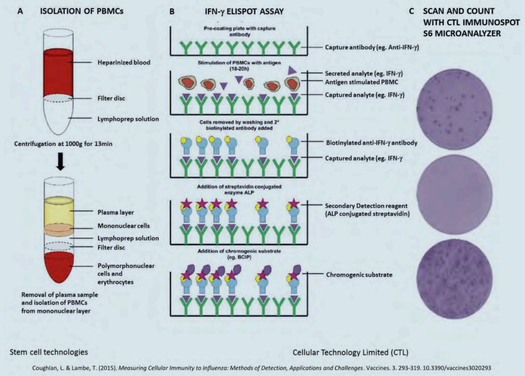
BMV	Application to Elispot
Reference Standard	Not Applicable
Critical Reagents	Identified, monitored
Calibration curve	Not Applicable
Quality Control Samples	Control treatments/trending sample
Accuracy	Addressed by proficiency testing
Precision	Repeated testing of same donor sample/treatment
Sensitivity	Statistical testing at lower limit
Selectivity and specificity	Irrelevant peptide treatment
Reproducibility	Inter-lab testing
Stability	LTS of key reagents /same donor

Table 2. Feasibility of Bioanalytical Method Validation Parameters in Elispot

Achievable	Adapted	Not Applicable
•Precision	•Accuracy	•Reference Standard
•Reproducibility	•Quality Control Samples	•Calibration Curve
•Critical Reagents	•Sensitivity	
•Stability	•Selectivity and Specificity	

METHODS

Figure 2. Elispot Workflow



Coughlan, L. & Lambe, T. (2015). *Measuring Cellular Immunity to Influenza: Methods of Detection, Applications and Challenges.* Vaccines. 3. 293-319. 10.3390/vaccines3020293

Figure 2 outlines the workflow of the Elispot assay. Cryopreserved PBMCs (CTL CRYO ABC media kit) were thawed, rested overnight in CTL test media, and then added to a coated plate containing treatments. Peptide pools that correspond to Cytomegalovirus, Epstein-Bar, Influenza (CEF and CMVpp65), as well as human skeletal muscle alpha actin, were all purchased from JPT Peptide Technologies. PHA-L was purchased from Sigma. After incubation for 20-22 hours, cells were washed off the membrane and the plate was developed according to the CTL INF-γ kit protocol. Plates were scanned and counted using an Immunospot S6 microanalyzer. Exported files were analyzed with Excel and Graphpad Prism.

RESULTS

Criteria:

- Precision: Both standard deviation (SD) and %CV will be reported for wells > 30 spots. For wells with fewer than 30 spots only SD will be reported. Precision (%CV) for samples with a mean spot count of greater than 100 will be ≤ 25%. For samples with a mean spot count of ≥30 spots/well up to 100 spots/well the % CV will be ≤ 50%.
- Specificity: Expected outcome of negative control peptide and media (background) wells is low or no reactivity (<10 spots/well)
- LOD: 3x median background of the assay. Statistical testing will not occur below the LOD.
- Range: The range of the assay is defined as cell number per well where the results are linear and proportionality is maintained.

Table 3. Inter-Batch Precision Data

		Donor 1			Donor 2			Donor 3		
	Treatment	Mean Spot Count/well			Mean Spot Count/well			Mean Spot Count/well		
Batch 001	CEF	271.3			207.0			10.3		
	pp65	240.5			0.3			2.7		
Batch 002	CEF	319.0			250.3			19.3		
	pp65	283.3			2.3			4.3		
Batch 003	CEF	264.0			180.7			15.0		
	pp65	217.0			0.7			1.3		
Batch 004	CEF	322.0			202.3			16.7		
	pp65	216.0			0.7			1.3		
		Mean	SD	% CV	Mean	SD	% CV	Mean	SD	% CV
	CEF-002	294.1	30.7	10.4%	210.1	29.2	13.9%	15.3	3.8	24.7%
	pp65	239.2	31.5	13.2%	1.0	0.9		2.4	1.4	

Figure 3. Specificity Experiment Utilizing Skeletal Actin Peptide Pool

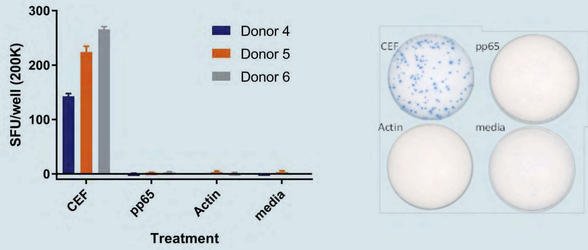
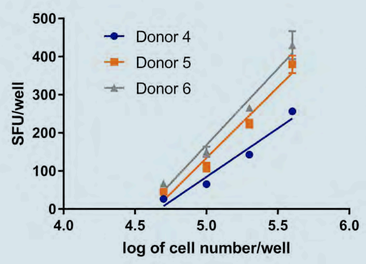


Table 4. Proportionality expressed as a percentage of 200,000 cells/well

	Donor 4		Donor 5		Donor 6	
cells plated per well	spots/well	% of 200K	spots/well	% of 200K	spots/well	% of 200K
400,000	128.2	90%	189.8	85%	215.2	81%
200,000	142.7	100%	224.0	100%	265.7	100%
100,000	130.7	92%	220.7	99%	300.7	113%
50,000	105.3	74%	181.3	81%	266.7	100%
25,000	69.3	49%	90.7	40%	208.0	78%

Figure 4. Linearity of the INF-γ Response from 50,000 - 400,000 Cells Per Well



	R square	P value
Donor 4	0.95	0.02
Donor 5	0.97	0.02
Donor 6	0.98	0.01

Results Summary:

- A qualification plan with target criteria was developed based as closely as possible on BMV, Elispot harmonization guidance, and peer review articles.
- Precision of this INF-γ Elispot assay meets the criteria specified (<25% CV) for donors with a mean spot count of >100 spots/well, with an inter-batch range from 10.4 to 13.9% CV.
- Specificity was demonstrated with a mean spot count < 10 spots/well for PBMCs treated with media control, or skeletal actin peptide pool.
- The assay was linear from 50,000 - 400,000 cells/well.
- The LOD of the assay was determine to be 3 spots (data not shown), below which statistical testing will not occur.

CONCLUSION

- Validation of complex cell based assays can be accomplished by adapting components of traditional BMV using published best practices in the field.
- We have qualified an INF-γ Elispot assay that will provide precise, specific, reproducible data on the antigen specific T-cell response of patients.
- Elispot assays can be utilized throughout the drug development process in diverse areas such as vaccine development, immuno-oncology, evaluation of immunogenicity of biologics, and auto-immune diseases.

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