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OPTIMIZATION AND QUALIFICATION OF AN IFN-γ ELISPOT ASSAY USING CRYOPRESERVED HUMAN PBMC SAMPLES

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PURPOSE

Monitoring cellular immune responses during drug development and clinical trials is critical for assessing the safety and efficacy of therapeutic interventions. ELISpot assays are widely used for this purpose due to their high sensitivity, quick turnaround, and high throughput. ELISpot assays typically use cryopreserved peripheral blood mononuclear cells (PBMCs) as starting material, followed by stimulation with peptide pools for a defined time, and subsequent detection of cytokine secretion using enzyme-linked antibody–mediated colorimetric detection. In our previous study, we evaluated the effects of resting cryopreserved PBMCs for different times on IFN-γ secretion by ELISpot assay and suggested that overnight resting (18–22 hours) improves functional assay quality, particularly for the IFN-γ ELISpot assay.

OBJECTIVE

In this poster, we aim to further optimize our ELISpot assay procedure, in order to perform IFN-γ ELISpot assay qualification and allow us to monitor cellular immune responses in the development and evaluation of CAR-T and TCR-T cell therapies and infectious disease vaccines.

METHODS

Mabtech IFN-γ ELISpot kits and procedures were used as the starting point for assay optimization. Cell culture media for cell resting and stimulation was optimized by comparing identical ELISpot assays run using either CTL-Test Media + 2mM L-glutamine or RPMI 1640 + 10% CultraPure HI-FBS. ELISpot kit type was optimized by comparing three kits with different antibody configurations and different enzymatic developments (3-step with ALP, 4-step with ALP, and 4-step with HRP). Mitogenic positive control concentration was optimized by comparing three concentrations of anti-CD3 antibody (5µg/mL, 1.25µg/mL, and 0.5µg/mL). Cell number was optimized by using serial dilutions of cells (200,000 to 6,250 cells per well) within the same ELISpot assay and determining which provided the highest number of spot-forming units (SFUs) that were not too numerous to count. Long-term stability of developed ELISpot plates was determined by quantifying SFUs initially as a baseline and re-quantifying SFUs at 18 time points over 140 days, measuring the difference in SFUs over time. Using these optimized parameters, three IFN-γ ELISpot assays were performed, encompassing both inter-operator and inter-assay comparisons, and repeatability and intermediate precision were calculated.

RESULTS

- Mabtech's ELISpot Plus: Human IFN-γ (HRP) kit, which uses a 4-step antibody configuration and HRP-based colorimetric development (4-Step HRP), was chosen for further use, as it produced an overall increase in signal (17% and 87%) and similar %CVs (<10%) compared to the 3-step ALP kit and 4-step ALP kit, respectively. An mitogenic positive control (anti-CD3 antibody) concentration of 1.25µg/mL was chosen as the optimal titer, as it produced mean SFUs similar to the 5µg/mL treatment and higher than the 0.5µg/mL treatment (**Figure 1**).
- RPMI 1640 + 10% CultraPure HI-FBS was chosen as the medium for cell resting and stimulation, as it produced a 3-fold lower background signal compared to CTL-Test serum-free medium (**Figure 2**).
- A cell concentration of 200,000 was optimal, as this treatment returned the highest SFU counts and lowest %CVs for both the mitogenic positive control (1272 SFU, 0.8%CV) and antigenic positive control (270 SFU, 4%CV), as well as a low background signal and lowest %CV for the negative control (4 SFU, 13.3%CV). Results were linear for treatments of 200,000 to 50,000 cells per well, with R^2 values for the mitogenic and antigenic positive controls of 0.9997 and 0.9643, respectively. (**Table 1, Figure 3**).
- Investigating long-term stability, the mean percent difference in SFU was <30% through Day 63, suggesting that the signal of developed ELISpot plates remains stable for at least two months (**Figure 4**).
- For three assays using these optimized parameters, repeatability was 11%CV, 13%CV, and 13%CV for Assays 1, 2, and 3, respectively. Inter-operator precision was 6%CV (Assay 1 vs Assay 2), and inter-assay precision was 24%CV (Assay 1 vs Assay 3) (**Table 2**).

Figure 1: *Mean spot-forming unit (SFU) counts compared across three different Mabtech IFN-γ ELISpot kits. Cells were stimulated by mock stimulation, antigenic positive control (CEF peptide pool), and three concentrations of mitogenic positive control (Anti-CD3 antibody). Error bars represent ±1SD of means.*

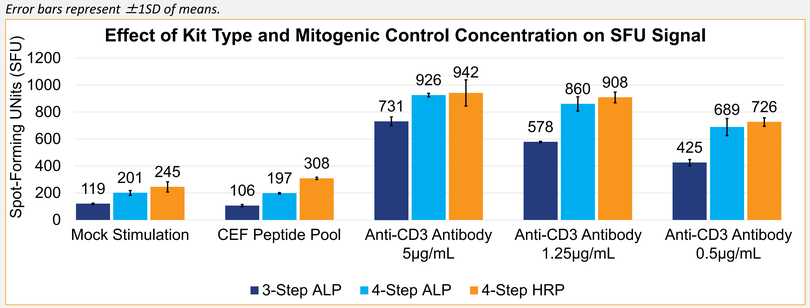


Figure 2: *Difference in mean spot-forming units (SFU) counted for assays using CTL-Test Media + 2mM L-glutamine or RPMI 1640 + 10% CultraPure HI-FBS for cell resting and stimulation. Error bars represent ±1SD of mean.*

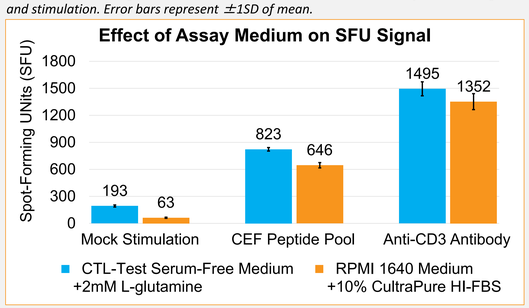


Table 1: *Spot-forming unit (SFU) counts and statistics for linearity experiment, testing variable cell number. Results correspond to cell stimulation by mitogenic positive control (Anti-CD3 antibody, **Top**) and antigenic positive control (CEF peptide pool, **Bottom**).*

Anti-CD3 Antibody (1.25µg/mL)						
Cell Number	Rep 1	Rep 2	Rep 3	Mean	SD	%CV
2.00E+05	1273	1282	1262	1272	10	1%
1.00E+05	842	759	790	797	42	5%
5.00E+04	379	338	337	351	24	7%
2.50E+04	138	92	131	120	25	21%
1.25E+04	35	45	55	45	10	22%
6.25E+03	21	20	20	20	1	3%
CEF Peptide Pool						
Cell Number	**Rep 1**	**Rep 2**	**Rep 3**	**Mean**	**SD**	**%CV**
2.00E+05	258	273	279	270	11	4%
1.00E+05	139	145	133	139	6	4%
5.00E+04	63	55	72	63	9	13%
2.50E+04	29	18	29	25	6	25%
1.25E+04	12	12	10	11	1	10%
6.25E+03	7	4	5	5	2	29%

Figure 3: *Linearity of relationship between Log10-transformed number of cells stimulated and number of spot-forming units (SFU) counted. Cell number was serially decreased twofold, from 2e5 cells to 6.25e3 cells. Results were linear from 2e5 to 5e4 cells. Results are separated by stimulation, with Anti-CD3 antibody (**Blue**) and CEF peptide pool (**Orange**) shown below. Results from mock stimulation are not included.*

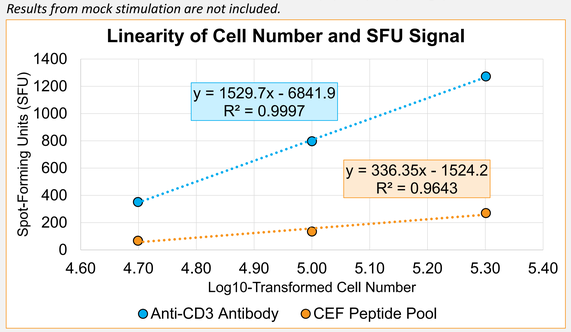


Figure 4: *Loss of spot-forming unit (SFU) signal over time, measured as mean percent difference between initial SFU count and SFU count on subsequent days. Stability measurement was limited to CEF peptide pool-stimulated replicates for which the initial measurement was >30 SFU. Stability was measured up to 140 days post-development. The cutoff for stability is represented by the blue line (30% difference).*

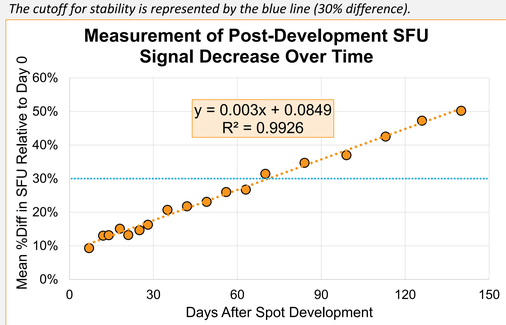


Table 2: *Spot-forming unit (SFU) counts and statistics for three IFN-γ ELISpot assays performed using optimized parameters. Intra-assay precision (repeatability) was calculated for each assay. Inter-operator and inter-assay precision were calculated by comparing Run 1 vs Run 2 and Run 1 vs Run 3, respectively.*

		SFU			Mean			%CV				
		Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Inter-Operator	Inter-Assay
CEF Peptide Pool Stimulation	Donor 1	32	29	66	29.3	28.7	45.0	9%	16%	40%	11%	39%
		29	24	34								
		27	33	35								
	Donor 2	436	390	383	443.0	396.3	374.0	5%	3%	2%	7%	10%
		425	411	370								
		468	388	369								
	Donor 3	446	473	651	469.7	457.7	634.7	6%	6%	5%	5%	17%
		500	474	652								
		463	426	601								
	Donor 4	32	22	41	35.0	30.7	35.7	15%	26%	13%	20%	12%
		32	32	33								
		41	38	33								
	Donor 5	92	119	58	94.0	109.7	50.0	6%	11%	14%	12%	34%
		100	114	46								
		90	96	46								
Anti-CD3 Antibody Stimulation	Donor 1	774	657	1055	621.0	644.3	850.3	21%	11%	24%	15%	27%
		545	567	842								
		544	709	654								
	Donor 2	1349	1251	1591	1370.3	1336.3	1590.0	1%	7%	2%	5%	8%
		1384	1327	1557								
		1378	1431	1622								
	Donor 3	74	68	104	67.3	86.3	86.0	15%	18%	22%	21%	22%
		56	95	67								
		72	96	87								
	Donor 4	656	642	640	617.0	674.0	599.7	8%	6%	6%	8%	7%
		637	717	587								
		558	663	572								
	Donor 5	571	389	138	474.3	459.7	127.7	20%	21%	8%	19%	66%
		473	418	127								
		379	572	118								
					Precision:			**11%**	**13%**	**13%**	**12%**	**24%**

CONCLUSION

By using these reagents and parameters, this IFN-γ ELISpot assay has been optimized to produce consistent, high-quality results within the acceptable ranges of linearity and precision to allow for assay qualification. This assay will be validated for use in GLP-regulated studies, to allow for the monitoring of cellular immune responses in the development and evaluation of CAR-T and TCR-T cell therapies and infectious disease vaccines.

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