T-CELL ELISPOT PROFICIENCY PANEL 2020

January 2021

CONTACT IMMUDEX
ProficiencyPanel@immudex.com

FOR MORE INFORMATION
www.proficiencypanel.com

IMMUDEX.COM

Content

INTRODUCTION TO PROFICIENCY PANELS

The ability to compare data generated by different laboratories is a powerful tool to ensure alignment and drive improvements in research and development.

Immudex Proficiency Panels are programs that provide laboratories worldwide to assess their proficiency in monitoring antigen-specific T-cell responses. It is a non-profit service offered to increase the proficiency among researchers and clinicians who perform the immune monitoring assays, MHC multimer, and T-cell ELISpot. The Proficiency Panels are open to any laboratory, independent of geographic location or field of interest.

In 2013, Immudex took over the Proficiency Panels from the CIC of CRI (Cancer Immunotherapy Consortium of the Cancer Research Institute, USA) and the CIMT (Association for Cancer Immunotherapy, Europe). We are very honored to have the Proficiency Panels' responsibility and continue the age-long efforts of CIC and CIMT to improve the immune monitoring assays level of accuracy, robustness, and reliability. Please check out the science behind the Proficiency Panels here.

Immudex Proficiency Panel runs yearly, and the next will run in March-June 2021.

T-CELL ELISPOT PROFICIENCY PANEL 2020

In the T-cell ELISpot Proficiency Panel 2020, participants evaluated the accuracy to detect the number of IFN-γ secreting antigen-specific cells in two different PBMC samples using ELISpot assay and standardized peptide pools.

Each participant received two pre-tested PBMC samples and tested them according to the instructions but with their own protocol for direct human IFN-γ ELISpot Assays. The participants included their own choice of antibodies, plates, enzyme, substrate, equipment, medium, etc.

Each participant determined the spot count per well after stimulation with two standardized peptide pools (JPT's PepMixTM HCMVA (pp65), and JPT's CEFX Ultra SuperStim Pool) or a negative control reagent.

This report shows the participants' test results and overall performance without revealing their name and affiliation.

In this Proficiency Panel:

- 30 laboratories from 12 countries participated.
- 19 participants were from Academia, and 11 participants were from industry.

ANALYSES

Each participant:

- Was assigned a confidential Identification Number (Lab ID).
- Received instructions on how to perform the T-cell ELISpot proficiency test (Appendix 1).
- Received two pre-tested vials of PBMCs (LOT 2010113745 and 2010113367). Pretesting was conducted at an external laboratory according to the instructions and results are shown in Appendix 5.
- Received three vials of reagents:
    - Reagent 1 (JPT's PepMixTM HCMVA (pp65) >90%; PM-PP65-2)
    - Reagent 2 (JPT's CEFX Ultra SuperStim Pool 90%; PM-CEFX-2)
    - Reagent 3 (Negative control: PBS/DMSO)
- Was encouraged to analyze samples with their own standard protocol to reflect routine sample analysis conducted in their laboratory.
- Was recommended to look at the "Assay Harmonization Guidelines" (Appendix 2).
- Reported their results back to Immudex after their analysis (Appendix 3 and Appendix 4)

RESULTS

27 participants in this year's T-Cell ELISpot Proficiency Panel reported their data. This is shown in Figures 1-4 on the following pages, and the raw data is presented in Appendixes 3-4. All measurements were done in triplicates.

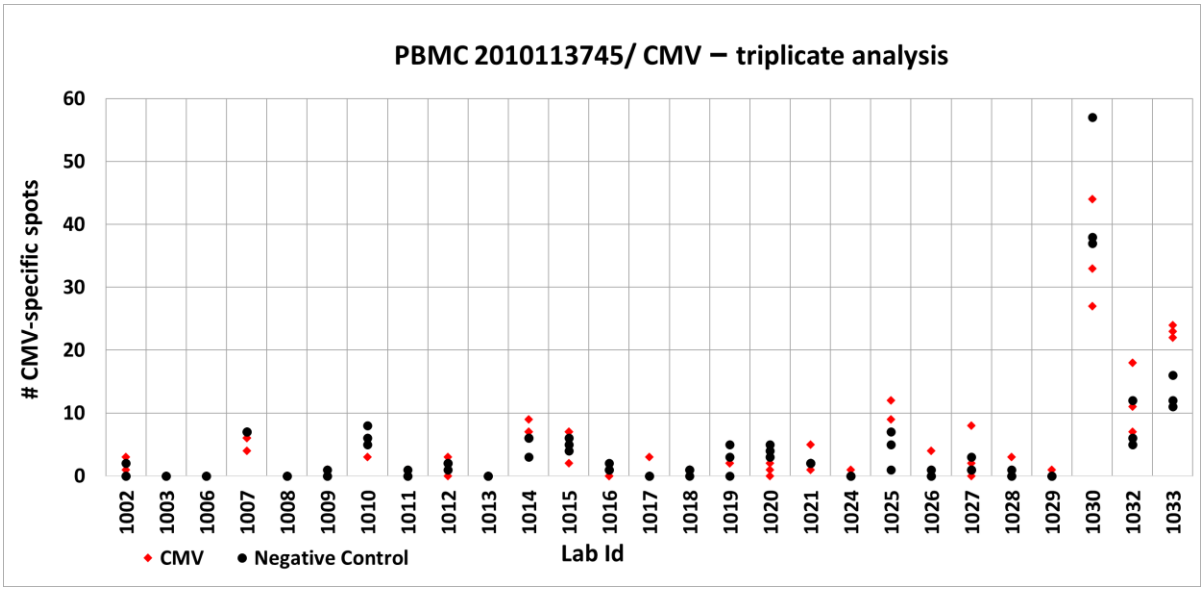

Figure 1A. Results from analysis of sample PBMC 2010113745 with Reagent 1 and Reagent 3. CMV-specific spots (red diamonds) and background spots (black dots) per 200.000 PBMCs (n=3).

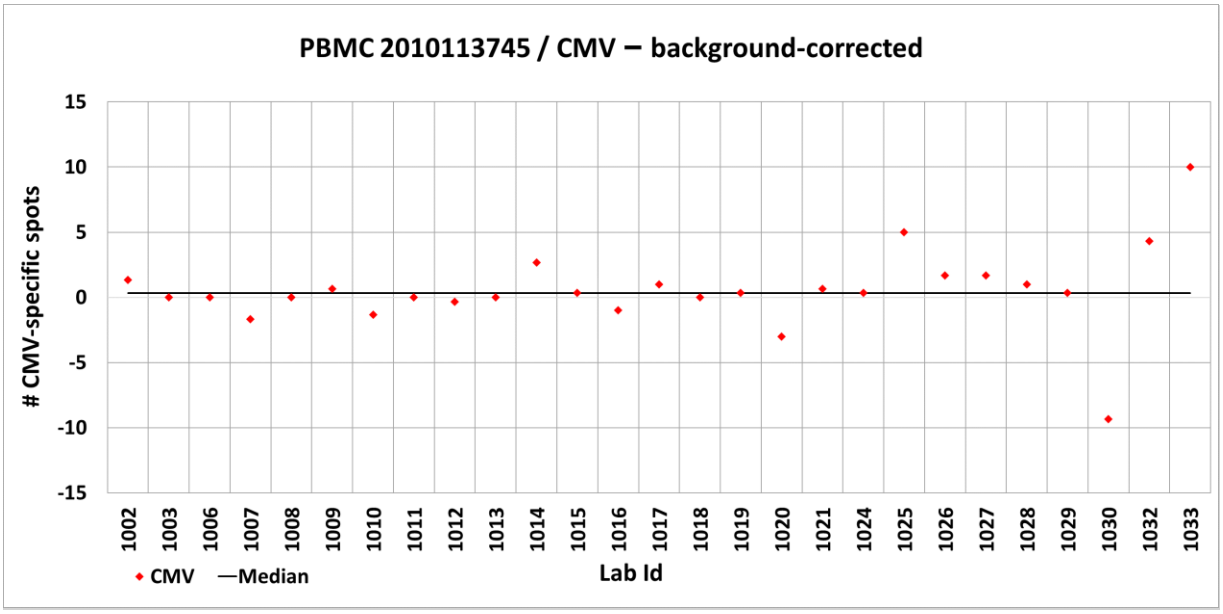

Figure 1B. Results from analysis of sample PBMC 2010113745 with Reagent 1 and Reagent 3. The mean of CMV-specific spots subtracted the mean of background spots is shown (red diamonds). The median of all results was 0.33 spots and indicated by the black line.

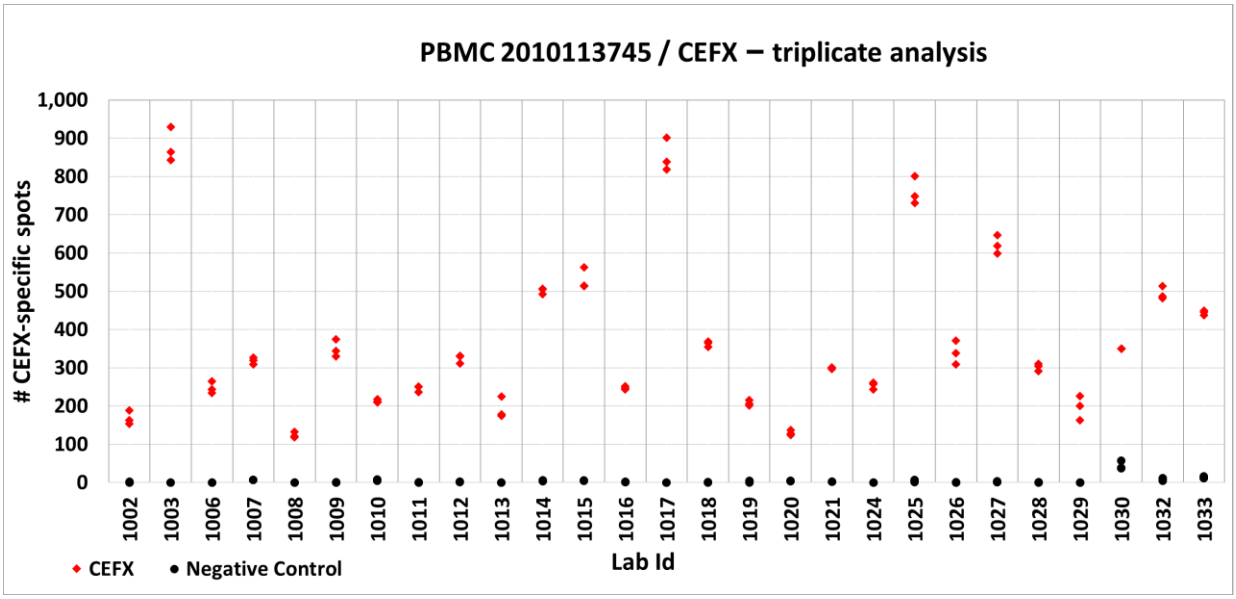

Figure 2A. Results from analysis of sample PBMC 2010113745 with Reagent 2 and Reagent 3. CEFX-specific spots (red diamonds) and background spots (black dots) per 200.000 PBMCs (n=3).

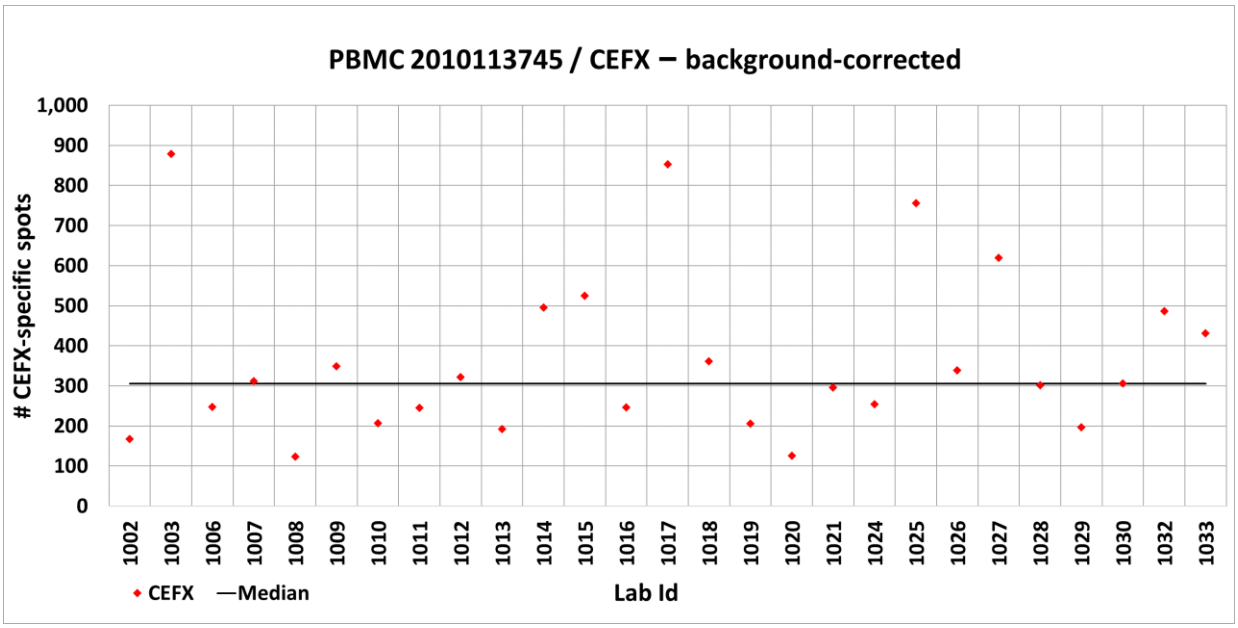

Figure 2B. Results from analysis of sample PBMC 2010113745 with Reagent 2 and Reagent 3. The mean of CEFX-specific spots subtracted the mean of background spots is shown (red diamonds). The median of all results was 308 spots and indicated by the black line.

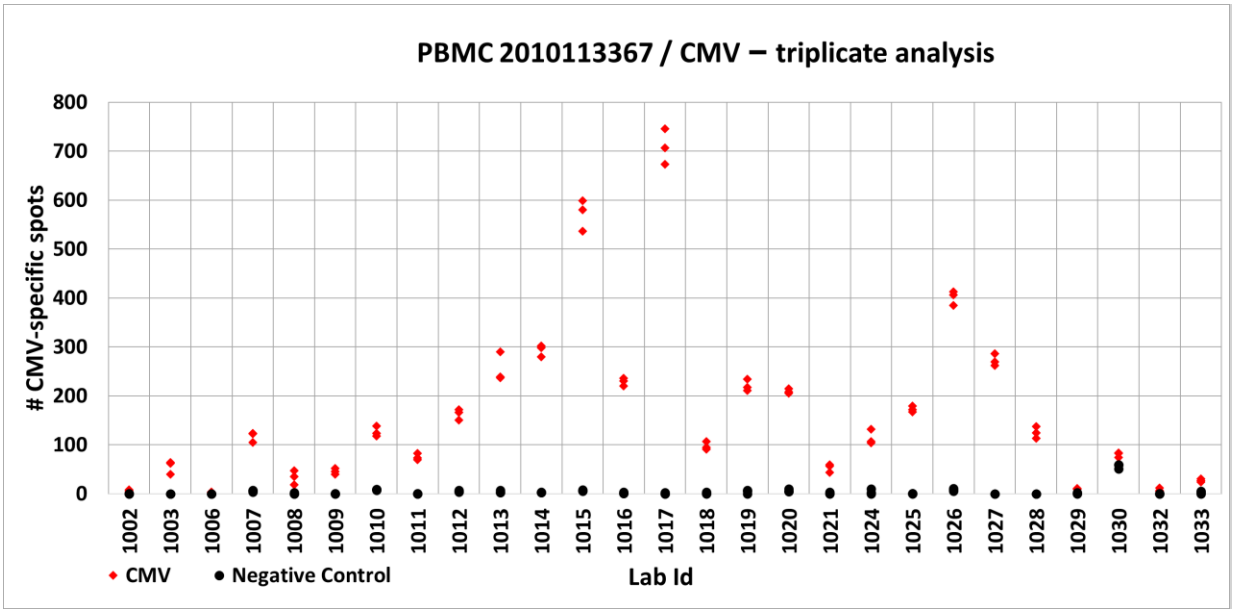

Figure 3A. **Results from analysis of sample PBMC 2010113367 with Reagent 1 and Reagent 3.** CMV-specific spots (red diamonds) and background spots (black dots) per 200.000 PBMCs (n=3).

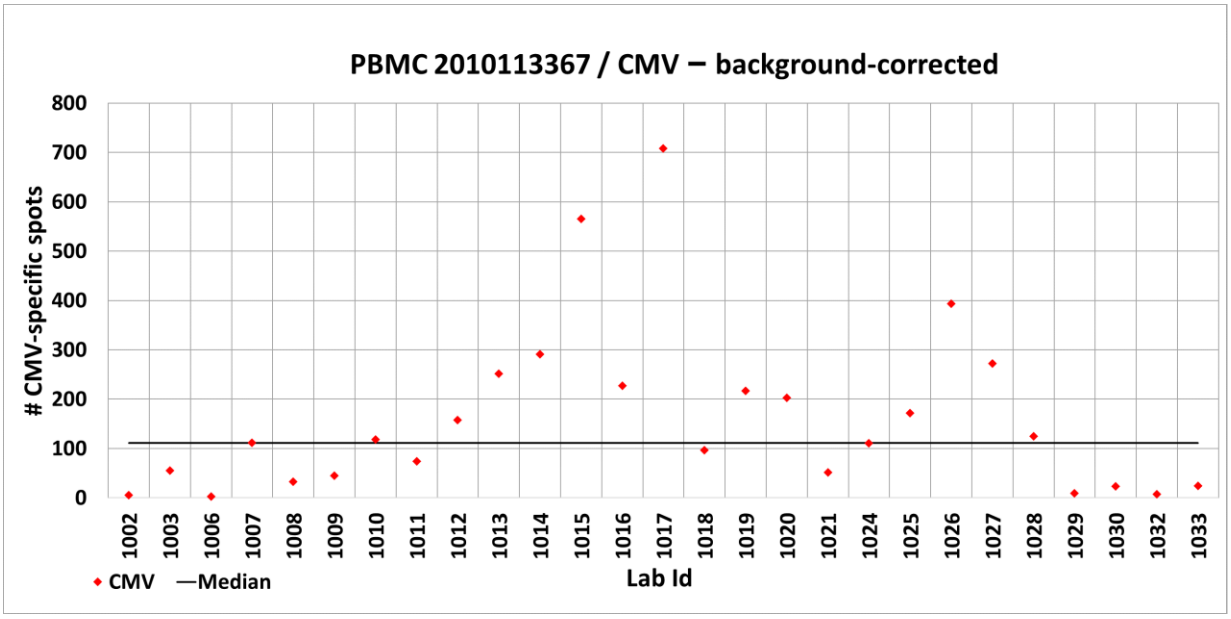

Figure 3B. **Results from analysis of sample PBMC 2010113367 with Reagent 1 and Reagent 3.** The mean of CMV-specific spots subtracted the mean of background spots is shown (red diamonds). The median of all results was 111 spots and indicated by the black line.

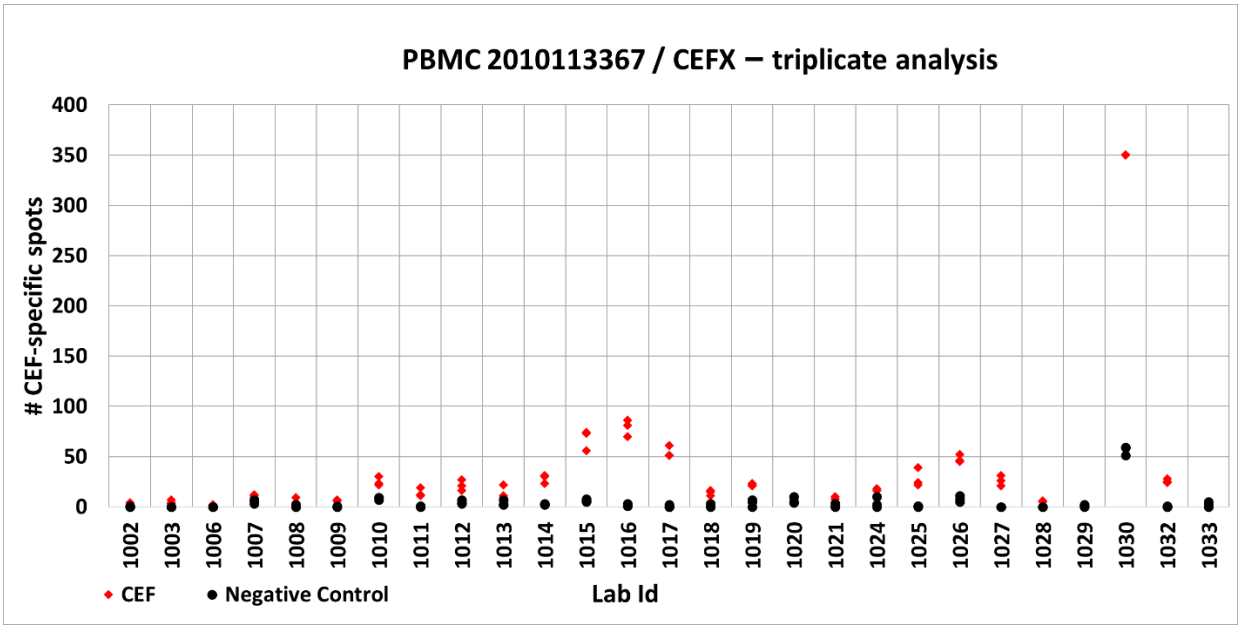

Figure 4A. Results from analysis of sample PBMC 2010113367 with Reagent 2 and Reagent 3. CEFX-specific spots (red diamonds) and background spots (black dots) per 200.000 PBMCs (n=3).

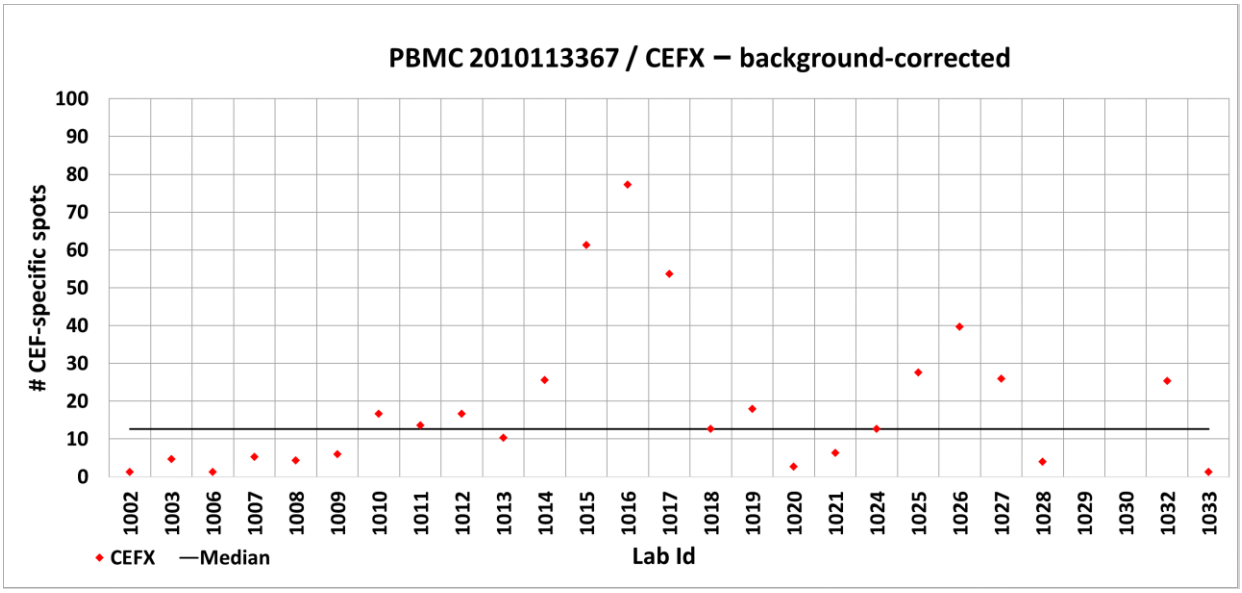

Figure 4B. Results from analysis of sample PBMC 2010113367 with Reagent 2 and Reagent 3. The mean of CEFX-specific spots subtracted the mean of background spots is shown (red diamonds). The median of all results was 13 spots and indicated by the black line.

PROFICIENCY PANEL TESTING RESULTS

To evaluate the accuracy of each participants' measurements, we used the relative accuracy. The relative accuracy tells you how close each participant is to the average value reported by all participants. The medians shown in Figures 2-4 were used as the average values to calculate the relative accuracy, see example of calculation in Appendix 6. The individual laboratories' relative accuracies are presented in Figures 5-7 on the following pages, and the definition of what the values correspond to is listed in Table 1.

Table 1 Definition of the relative accuracy

RELATIVE ACCURACY	CORRESPONDS TO	PRESENTED IN THE FIGURES AS
<0.50 **> 2.00**	Far from the average range	White columns
0.50 – 0.65 **1.50-2.00**	Near the average range	Striped columns
0.66-1.50	Within the average range	Black columns

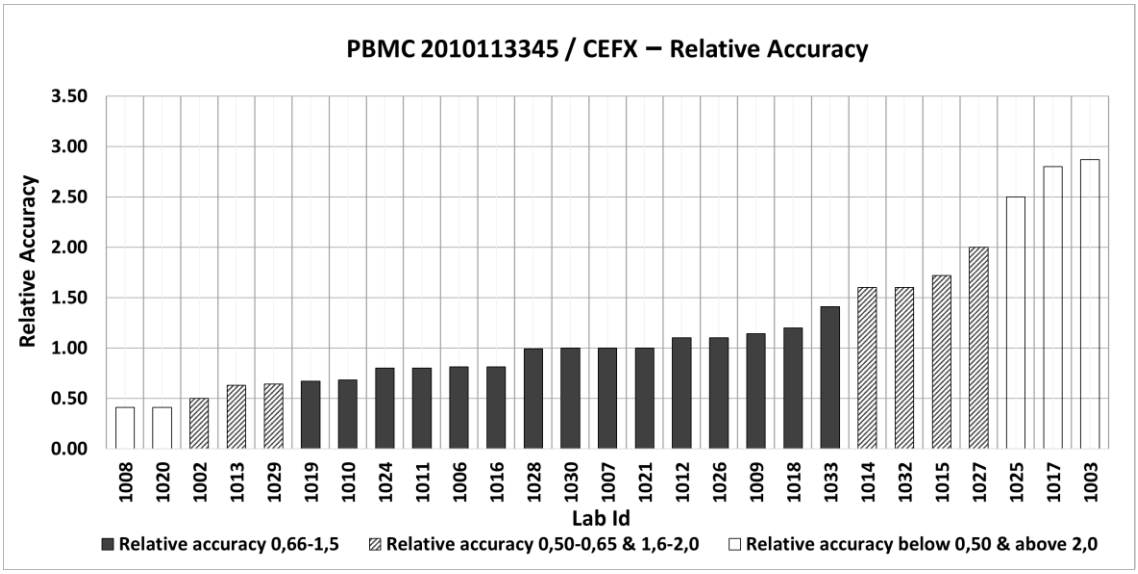

Figure 5. Relative accuracy for analysis of PBMC 2010113745 with Reagent 2 (CEFX). 15 of the 27 participants had a Relative Accuracy between 0,66-1,5 and are therefore considered "in the average range" (Black filled columns).

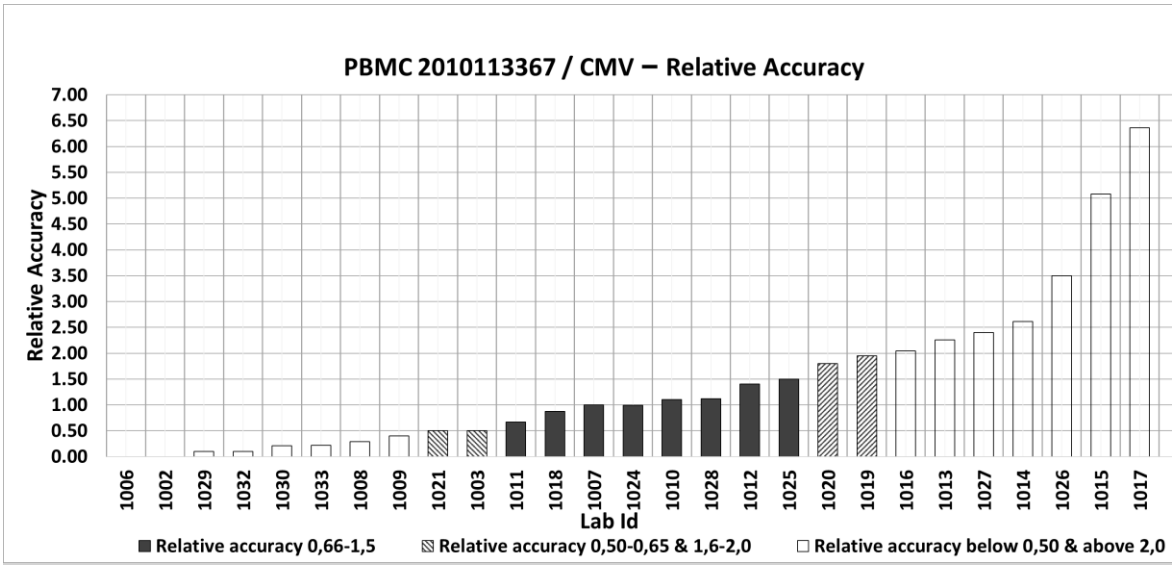

Figure 6. Relative accuracy for analysis of PBMC 2010113367 with Reagent 1 (CMV).
8 of the 27 participants had a Relative Accuracy between 0,66-1,5 and are therefore considered "in the average range" (Black filled columns).

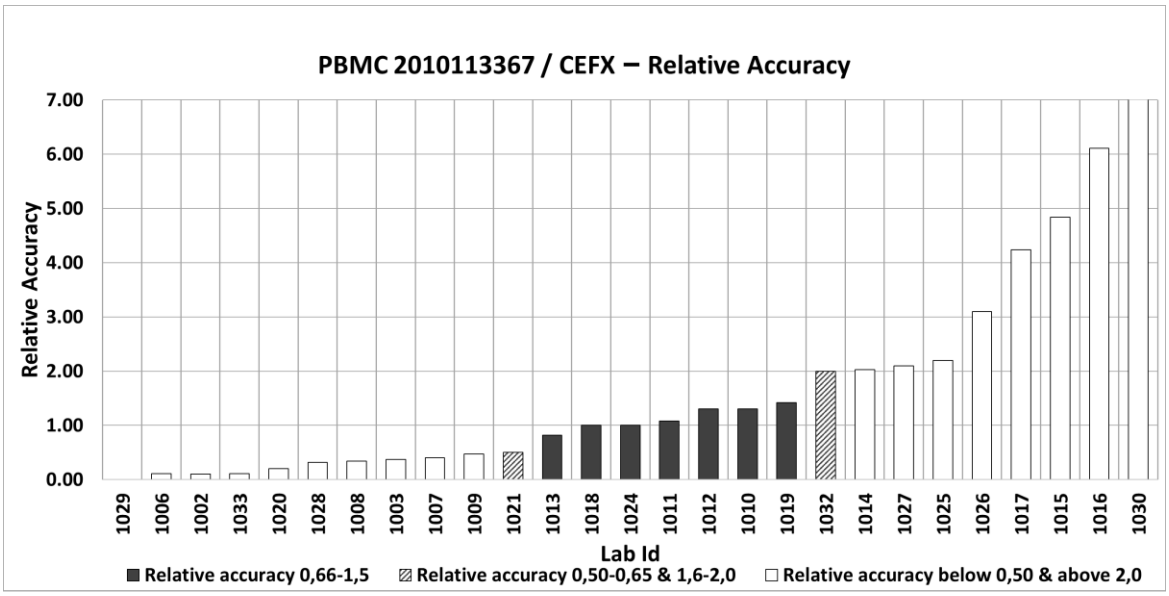

Figure 7. Relative accuracy for analysis of PBMC 2010113367 with Reagent 2 (CEFX).
7 of the 27 participants had a Relative Accuracy between 0,66-1,5 and are therefore considered "in the average range" (Black filled columns).

For the analysis of sample PBMC 2010113745 with Reagent 1 (CMV), the panel median was 0.33 pots (Figure 1B). Because many of the participants' measurements were below or close to zero after subtracting the background values, we decided to leave out the relative accuracy analysis and instead use the mean +/- 2xSD cut-off to determine if participants were within the average range (Figure 8).

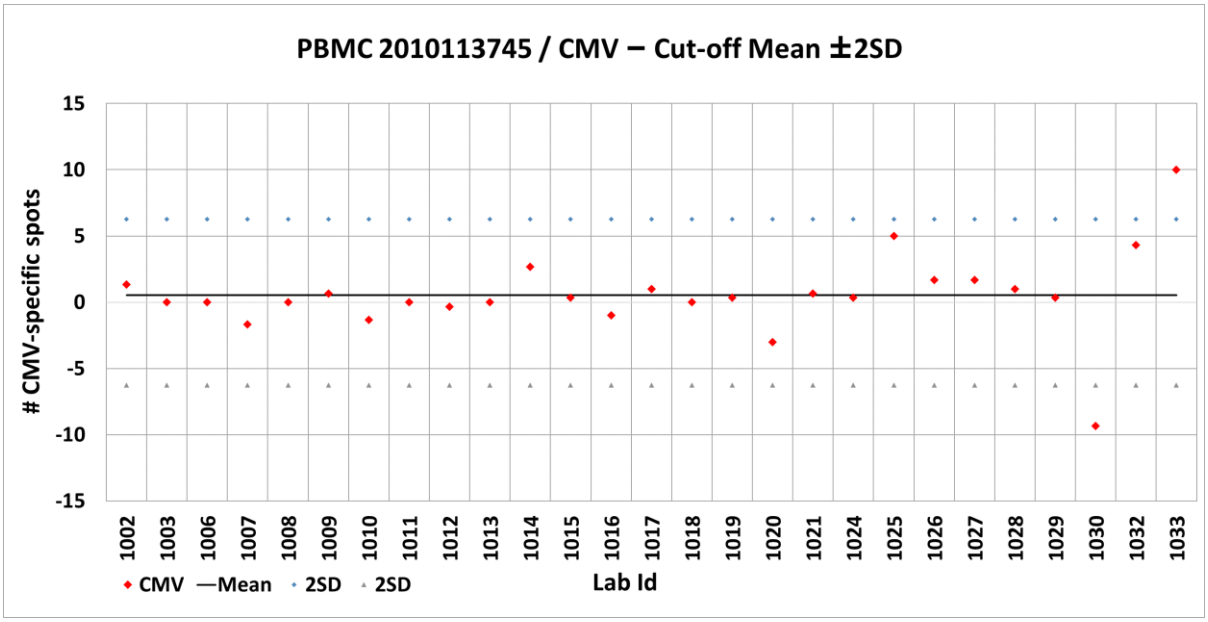

Figure 8. Analysis of PBMC 2010113367 with Reagent 1 (CMV) using mean +/- 2x SD as cut-off. The red diamonds show the mean of CMV-specific spots subtracted the mean of background spots. The black line shows the median of all results (0.33 spots). The blue and green dots are +/- 2x Standard Deviation. 25 of the 27 participants were considered "in the average range" as they were within the cut-off value of Mean ±2SD.

PROFICIENCY PERFORMANCE

Each participants' ability to identify the specific cytokine-secreting T-cells were described with an overall proficiency score. For each of the analyses, the laboratories were assigned a score between 1-3. The overall proficiency was then defined by the average score obtained over the four analyses. Thus, a participant with an overall proficiency of "3" is in the average range on all four measurements and has the highest possible score. A participant with an average score of "1" is far from average on all four measurements and has the lowest possible score.

Proficiency Score	
Score 3	Assigned to results within the average range. (Relative Accuracy between 0,66 and 1,5, and within the mean 2x SD cut-off value for the first analysis).
Score 2	Assigned to results near the average range. (Relative Accuracy = 0,50-0,65 or 1,6-2,0, and outside the mean 2x SD cut-off value for the first analysis).
Score 1	Assigned to results far from the average range. (Relative Accuracy below 0,50 or above 2,0).

Figure 9 shows the overall proficiency score. 17 out of the 27 participating laboratories got an overall proficiency score of ≥ 2. This corresponds to 63%, which is in line with the T-cell ELISpot Proficiency Panel 2019, where 62% of the participant obtained an overall proficiency score of ≥ 2.

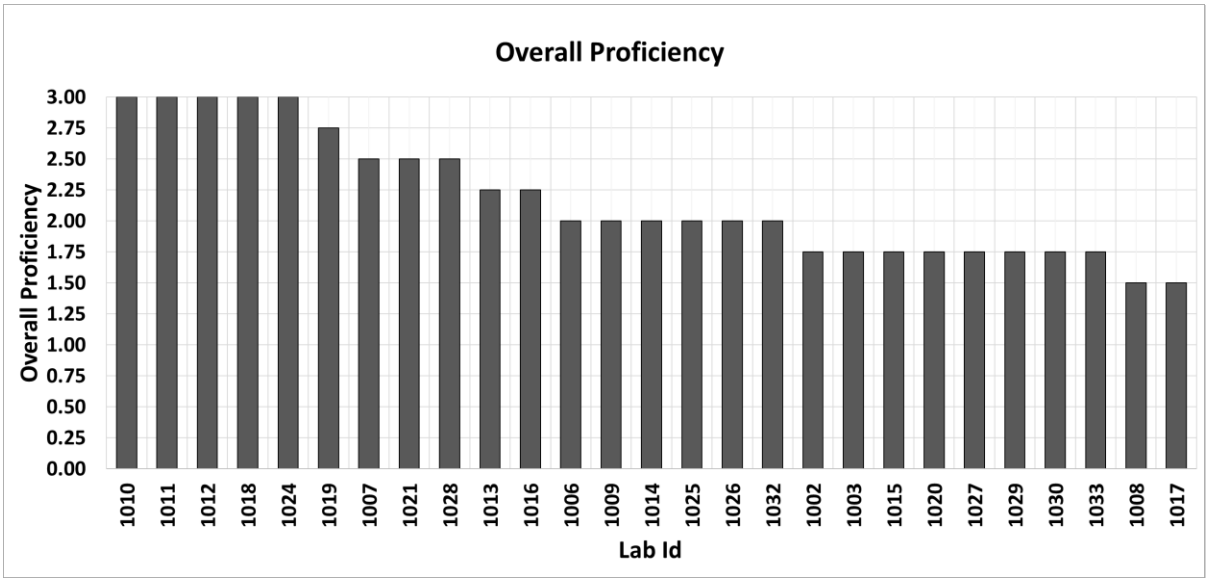

Figure 9. Overall Proficiency in the T-cell ELISpot Proficiency Panel 2020.

DISCUSSION

Immudex T-cell ELISpot Proficiency Panels provide a program for laboratories worldwide to assess their proficiency in identifying specific cytokine-secreting T-cells using ELISpot. Evaluation of laboratory performance is essential to ensure alignment and drive research and development improvements. Harmonized laboratory performance is of high importance in multicenter trials, where clinical results from different sites are compared to evaluate treatment response in immunotherapeutic research and development.

In this T-cell ELISpot Proficiency Panel, participants used their own laboratory-specific procedure to determine the number of IFN-γ secreting cells after stimulation with two different standardized peptide pools. In this report, each participant can see how aligned their obtained results are with the rest of the participants. This critical knowledge provides each participant with the opportunity to evaluate their assay protocol. To ensure and sustain their ability to identify IFN-γ secreting T-cells accurately, reproducibly, and in alignment with other researchers across sites, or to identify necessary protocol optimization.

Variations are seen between sites. Factors like high performing serum/medium, overnight resting, assessment of apoptotic cells may explain some of the differences observed. Look at the ELISpot harmonization guidelines to learn more (Appendix 2).

In this proficiency panel, we included a negative sample (PBMC 2010113367) with no T-cells reactive with the tested CMV peptide pool (Figure 1B). 25 out of 27 participants were within the average range of the panel median (based on the mean 2x SD cut-off value), demonstrating a general alignment and low risk of false positive measurements.

In the other three analyses samples with specific T cells was used. The sample with the highest panel median, 308 spots (Figure 2B), was the one where most participants (15 out of 27) obtained results within the average range. For the other two samples with lower frequency of antigen-specific T cells (panel median of 111 and 13 spots, respectively) results were less aligned (Figure 3B and Figure 4B). Here 8 and 7, respectively of the 27 participants were within the average range.

Conclusively, this Proficiency Panel shows that i) T-cell ELISpot assays are more harmonized across different laboratories when looking at high T-cell responses, and ii) Proficiency Panels are a useful tool to evaluate immune monitoring assays' proficiency across different laboratories and ensure comparable results.

ACKNOWLEDGEMENTS

We thank Mabtech AB for quality control and ELISpot assay testing of PBMC samples, JPT Peptide Technologies for providing peptide pools, and Sylvia Janetzki for providing helpful guidance and advice.

ABOUT IMMUDEX

Based in Copenhagen, Denmark, with North American operations based in Fairfax, Virginia, Immudex manufactures MHC Dextramer® for the detection of antigen-specific T cells. Under an agreement with the US Cancer Immunotherapy Consortium (CIC) and the European Cancer Immunotherapy Consortium (CIMT), Immudex also provides MHC Multimer and Elispot proficiency panel services worldwide.

Immudex' MHC Dextramer® products are utilized for the quantification or sorting of antigen-specific T cells in life science research, in-vitro diagnostics, as well as the development of immunotherapeutics and vaccines. The primary focus is research-use-only products for the immune monitoring of immunotherapy development and monitoring of CMV cellular immunity in transplant and other immune-deficient patients. In Europe, the CE marked Dextramer® CMV Kit is approved for in vitro diagnostic use for the quantification of CMV-specific T cells. USA FDA 510(k) clearance for the CMV kit was granted in March 2017. GMP Grade reagents are available.

Our state-of-the-art dCODE Dextramer® reagents enable massive multiplexing of antigen-specific T-cell detection. Detection of over 1000 CD8+ T-cell specificities from a single blood sample has been achieved.

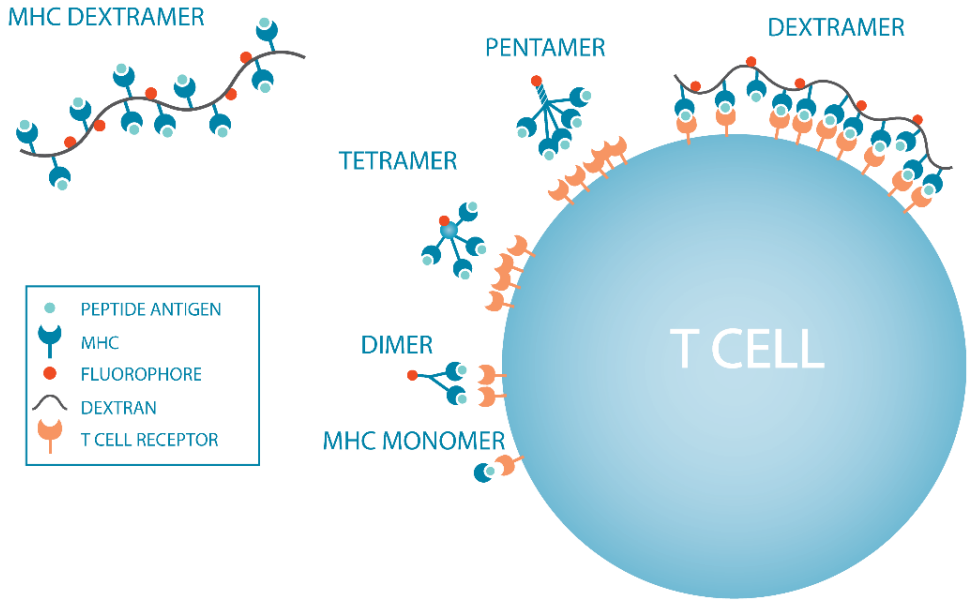

Figure 10 Schematic drawing of MHC Dextramer® and conventional MHC multimers binding to T-cell receptors (TCRs) on the surface of a T cell.
MHC Dextramer® reagents are fluorescent-labeled MHC multimers that can bind simultaneously to multiple TCRs on a single T cell. This provides a strong and stable interaction between the MHC Dextramer® reagents and the T cell, enabling detection of antigen-specific T cells with even low affinity for the MHC-peptide complex.

APPENDIX1: INSTRUCTIONS

Introduction

The ability to compare data generated by different laboratories is a powerful tool to ensure alignment and drive improvements in research and development. Supported by CIC and CIMT, Immudex conducts Proficiency Panels annually, allowing laboratories to assess their performance in monitoring antigen-specific T-cell responses.

In this ELISpot Proficiency Panel, participants evaluate their accuracy to detect the number of IFN-γ secreting antigen-specific cells in two different PBMC samples. The participants must determine the spot count per well as a result of stimulation with three different reagents: JPT's PepMixTM HCMVA (pp65), CEFX Ultra SuperStim Pool, and a negative control reagent.

Each participant is asked to test the PBMC samples according to these instructions, but following their own protocol for direct human IFN-γ ELISpot Assays, including own choice of antibodies, plates, enzyme, substrate, equipment, medium, and other miscellaneous chemicals and tools to perform the assay. We encourage participants to analyze samples with their own protocol to reflect routine sample analysis. We also recommend participants to have a look at the "Assay harmonization guidelines" provided by the Cancer Immunotherapy Consortium of the Cancer Research Institute (CIC of CRI) and the Association for Cancer Immunotherapy (CIMT), see Appendix 1.

After analysis, participants report their results to Immudex. Results and performance from all participants are presented in a final report where participants' name and affiliation are kept anonymous.

Deadlines and Immudex contact

Data submission:	November 15, 2020
Final report from Immudex:	End of December 2020

If you have questions, please contact Rikke Yding Tingleff, PhD, at proficiencypanel@immudex.com

Samples and Reagents provided

- Two PBMC samples (LOT #2010113745 and LOT #2010113367)
- Reagent-1 (PepMixTM HCMVA (pp65); JPT Product Code: PM-PP65-2)
- Reagent-2 (CEFX Ultra SuperStim Pool; JPT Product Code: PM-CEFX-2)
- Reagent-3 (Negative control PBS/DMSO).

PBMC samples and reagents are shipped in a liquid nitrogen shipper. Instructions for how to unload the samples and return the shipper is included. Please store samples at ≤ -150°C until you run the ELISpot assay and return the liquid nitrogen shipper promptly.

Experimental setup

ELISpot Step-by-Step

A. Antibody coating
B. Cell incubation
C. Cytokine capture
D. Detection antibodies
E. Streptavidin-enzyme conjugate
F. Addition of substrate
G. Analysis

Please use your own currently established protocol for the IFN-γ ELISpot assay, but follow the general instructions listed here.

General instructions

1. One 96-well plate is required for the assay. Coat columns 3-5 of the plate according to your own IFN-γ ELISpot protocol. Coat 3x8 = 24 wells in total. See plate setup in Table 2 next page.

2. Thaw the two PBMC vials and count the cells using your laboratory's preferred procedure.

For each PBMC vial, record the total cell number and the percentage of viable cells. If a resting step is included, please count, and record the total cell number and the percentage of viable cells after the resting step. See Table 1 below.

Table 2 PBMC status

	Right after thawing		After resting (if you include a resting step)	
PBMC lot	Total cell number	% Viable cells	Total cell number	% Viable cells
2010113745				
2010113367				

3. Dilute Reagents:
Reagent 1, Reagent 2, and Reagent 3 contain approximately 100µl and must be diluted 1:10 with the medium used for the assay.

4. Plate PBMC samples and add Reagents exactly as outlined in Table 2 (data are reported in this format).

- Row B3-5, C3-5, D3-5, E3-5, F3-5, G3-5:
  Plate 200,000 viable cells/well in 50 µl medium/well. Add Reagents at 50 µl/well. The final volume of cells and Reagent should be 100 µl.

- Row A3-5 and H3-5:
  Add 100 µl medium/well (no cells or Reagent), to enable assessment of false-positive spots.

5. Perform the assay, following your own established protocol.

Table 3 Plate overview

	1-2	3	4	5	6-12
A		**No cells – Medium**	**No cells – Medium**	**No cells – Medium**	
B		PBMC lot 2010113745 *Reagent-1*	PBMC lot 2010113745 *Reagent-1*	PBMC lot 2010113745 *Reagent-1*	
C		PBMC lot 2010113745 Reagent-2	PBMC lot 2010113745 *Reagent-2*	PBMC lot 2010113745 *Reagent-2*	
D		PBMC lot 2010113745 *Reagent-3*	PBMC lot 2010113745 *Reagent-3*	PBMC lot 2010113745 *Reagent-3*	
E		PBMC lot 2010113367 *Reagent-1*	PBMC lot 2010113367 *Reagent-1*	PBMC lot 2010113367 *Reagent-1*	
F		PBMC lot 2010113367 *Reagent-2*	PBMC lot 2010113367 *Reagent-2*	PBMC lot 2010113367 *Reagent-2*	
G		PBMC lot 2010113367 *Reagent-3*	PBMC lot 2010113367 *Reagent-3*	PBMC lot 2010113367 *Reagent-3*	
H		**No cells – Medium**	**No cells – Medium**	**No cells – Medium**	

Report data

After the end experiment, please report data and experimental details, using this link https://immudex.wufoo.com/forms/qob8keo0byq6hc/

APPENDIX 2: ASSAY HARMONIZATION GUIDELINES

Initial ELISpot Harmonization Guidelines to Optimize Assay Performance (based on previously published recommendations from the CIC/CRI and CIMT ELISpot panel programs).

A. Use only pre-tested and optimized serum or serum-free media, allowing for low background: high signal ratio.

B. Establish laboratory SOP for ELISPOT testing procedures, including:
B1. Counting method for apoptotic cells for determining adequate cell dilution for plating.
B2. Duration of the resting period (i.e. overnight) of cells before plating and incubation.

C. Test each condition at least in triplicates.

D. Add optimal cell number per well for sufficient antigen presentation and highest signal to noise ratio.

E. Establish SOP for plate reading, including:
E1. Human auditing during the reading process.
E2. Adequate adjustments for technical artifacts. *

F. Let only trained personnel (per laboratory SOP) conduct assays.

*For details see Nature Protocols 2015 (Guidelines for the automated evaluation of Elispot assays by Janetzki, Sylvia et al.; 2015. Nat Protoc. 2015).

APPENDIX 3: RESULTS FROM ANALYSIS OF PBMC 2010113745

This table shows the triplicate values that the participants reported for analysis with the three reagents. The values represent the number of spots read for each sample.

Reagent 1 (CMV) / Reagent 2 (CEFX)/ Reagent 3 (Negative Control)

Lab Id	Well B3-5 CMV			Well C3-5 CEFX			Well D3-5 Negative Control		
1002	3	1	2	163	189	153	0	0	2
1003	0	0	0	843	864	929	0	0	0
1006	0	0	0	265	244	234	0	0	0
1007	6	4	6	309	327	320	7	7	7
1008	0	0	0	121	118	133	0	0	0
1009	1	1	1	344	330	374	1	0	0
1010	3	6	6	212	218	210	6	8	5
1011	1	0	0	251	250	236	0	0	1
1012	1	3	0	331	330	311	2	2	1
1013	0	0	0	225	178	174	0	0	0
1014	7	7	9	505	492	506	6	3	6
1015	7	7	2	515	562	513	4	5	6
1016	0	0	1	244	252	247	1	2	1
1017	0	3	0	901	818	838	0	0	0
1018	0	1	1	368	365	354	0	1	1
1019	5	2	2	216	202	206	5	0	3
1020	2	0	1	137	124	128	5	4	3
1021	2	1	5	297	301	297	2	2	2
1024	0	1	0	244	261	258	0	0	0
1025	6	4	4	620	636	628	0	0	2
1025	7	12	9	801	731	748	5	7	1
1026	4	1	1	371	309	338	0	1	0
1027	2	0	8	619	647	599	1	3	1
1028	0	1	3	310	292	304	0	0	1
1029	0	1	0	200	163	226	0	0	0
1030	27	33	44	350	350	350	38	57	37
1032	11	18	7	482	513	486	6	5	12
1033	22	23	24	438	446	449	11	16	12

APPENDIX 4: RESULTS FROM ANALYSIS OF PBMC 2010113367

This table shows the triplicate values that the participants reported for analysis with the three reagents. The values represent the number of spots read for each sample.

Reagent 1 (CMV) / Reagent 2 (CEFX)/ Reagent 3 (Negative Control)

Lab Id	Well E3-5 CMV			Well F3-5 CEFX			Well G3-5 Negative control		
1002	3	8	6	4	1	0	0	1	0
1003	62	40	64	7	4	3	0	0	0
1006	3	2	3	1	1	2	0	0	0
1007	122	105	123	9	12	11	6	3	7
1008	35	47	18	4	2	9	0	2	0
1009	52	45	40	7	6	6	0	0	1
1010	123	118	138	30	23	22	9	9	7
1011	82	69	73	11	12	19	0	1	0
1012	172	150	166	21	16	27	4	3	7
1013	290	239	237	11	9	22	2	2	7
1014	298	302	280	23	31	30	2	2	3
1015	599	536	580	73	56	74	6	5	8
1016	220	236	230	86	70	81	1	1	3
1017	707	746	673	51	51	61	0	0	2
1018	95	107	91	11	16	15	0	3	1
1019	234	217	211	22	21	23	0	7	5
1020	205	208	215	8	10	9	10	5	4
1021	56	59	43	8	10	5	1	3	0
1024	132	107	104	16	16	18	0	2	10
1025	113	98	105	24	22	22	0	2	2
1025	179	167	172	22	39	24	1	1	0
1026	413	406	385	52	45	46	11	8	5
1027	262	286	269	31	21	26	0	0	0
1028	137	113	124	1	6	5	0	0	0
1029	9	11	10	0	0	2	1	2	0
1030	82	83	74	350	350	350	59	59	51
1032	6	5	12	28	25	24	0	0	1
1033	30	27	24	3	4	4	0	2	5

APPENDIX 5: PRE-TEST OF PBMC BATCHES

Elispot assay performed according to "Instruction for the Elipspot assay" using PBMC 2010113345 and PBMC 201011367. 2 vials from each PBMC batch were pre-tested with all three reagents:

- Reagent-1 (PepMixTM HCMVA (pp65); JPT Product Code: PM-PP65-2)
- Reagent-2 (CEFX Ultra SuperStim Pool; JPT Product Code: PM-CEFX-2)
- Reagent-3 (Negative control PBS/DMSO).

The viability of all 4 PBMC samples was in the range of 94-96% after thawing, and after one hour rest.

Table 4 shows pre-testing results, where the values represent the number of read spots for each sample.

Table 5 shows the mean value subtracted from the background (Reagent 3).

Table 6 shows the mean of the values listed in **Table 5.**

Table 4 Results from the pre-test.

PBMC batch	Reagent 1			Reagent 2			Reagent 3		
2010113745 (1)	3	2	2	213	236	223	3	6	1
2010113745 (2)	3	1	2	237	259	240	5	3	1
2010113367 (1)	118	113	133	9	16	16	5	6	3
2010113367 (2)	165	185	174	20	19	21	4	3	4

Table 5 Mean values of the results from Table 4 subtracted the background (reagent 3).

PBMC Batch	Reagent 1	Reagent 2
2010113745 (1)	0	221
2010113745 (2)	0	242
2010113367 (1)	116	9
2010113367 (2)	171	16

Table 6 Men values of the results from Table 4.

PBMC Batch	Reagent 1	Reagent 2
2010113745	0	232
2010113367	144	12

APPENDIX 6 CALCULATION OF THE RELATIVE ACCURACY

Table 7 Example of relative accuracy calculation of PBMC donor 2010113345 stimulated with CEFX peptide pool

	# of spots							
Lab ID	D3 DMSO/ PBS	D4 DMSO/ PBS	D5 DMSO/ PBS	C3 CEFX	C4 CEFX	C5 CEFX	Mean value subtracted back-ground	Median for all participants	Relative Accuracy
1002	0	0	2	163	189	153	167.67	306	$\frac{167}{306} = 0.5$