Immudex

T-CELL ELISPOT PROFICIENCY PANEL 2021

September 2021

immudex
PRECISION IMMUNE MONITORING

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1. 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 with the opportunity 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 Cancer Immunotherapy Consortium of the Cancer Research Institute (CIC of CRI, USA) and the Association for Cancer Immunotherapy (CIMT, Europe). We are very honored to be responsible for conducting Proficiency Panels and continue the age-long efforts of CIC of CRI and CIMT to improve the immune monitoring assays' accuracy, robustness, and reliability. Read more about the Proficiency Panels here.

Immudex Proficiency Panels are conducted yearly, and the next will take place in 2022.

1.1. T-CELL ELISPOT PROFICIENCY PANEL 2021

In the T-cell ELISpot Proficiency Panel 2021, participants evaluated their proficiency in detecting 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 Assay. The participants included their own choice of antibodies, plates, enzyme, substrate, equipment, medium, etc. The PBMC samples and reagents were pre-tested at Mabtech according to instructions to ensure consistent results between vials and to check the viability of the cells. The viability of the tested PBMC samples was in the range of 94-98% after thawing and after one hour of rest. All pre-test results are shown in Appendix 3.

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 (PBS/DMSO).

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

In this Proficiency Panel:

- 29 laboratories from 11 countries participated.
- 22 participants were from Academia, and 7 participants were from industry.

2. ANALYSES

Each participant:

- Was assigned a confidential Laboratory Identification Number (Lab ID).
- Received instructions on how to perform the T-cell ELISpot proficiency test (Appendix 1).
- Received two pre-tested vials of PBMC samples (Lot 2010113745 and HHU20180918). The PBMC samples were pre-tested at Mabtech AB (Sweden) according to "Instructions for T-cell ELISpot Proficiency Panel 2021" (Appendix 1). The pre-test results were not shared with the participants before their proficiency test. The results from the pre-test are shown in Table 1 and Appendix 3.
- 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)
- Stimulated the two PBMC samples with Reagent 1, 2 and 3.
- 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 4 and Appendix 5).

The reported participant data was analyzed by Immudex and divided into four analyses as shown in Table 1. The calculated values from the data analysis are found in Appendix 6.

Table 1. Overview of PBMC samples and Reagents used for analysis and results obtained in the pre-test.

Data analysis no.	PBMC	Reagent	Pre-test result
1	2010113745	Reagent 1 (CMV) and Reagent 3 (Negative Control)	Negative
2	2010113745	Reagent 2 (CEFX) and Reagent 3 (Negative Control)	Medium response
3	HHU20180918	Reagent 1 (CMV) and Reagent 3 (Negative Control)	Low response
4	HHU20180918	Reagent 2 (CEFX) and Reagent 3 (Negative Control)	Medium/high response

3. RESULTS

In this year's T-cell ELISpot Proficiency Panel, 29 participants reported their data. The participants measured the number of IFN-γ secreting antigen-specific cells in two different PBMC samples (PBMC 2010113745 and HHU20180918) stimulated with CMV and CEFX peptide pools. In advance, the PBMCs were pre-tested by the external partner Mabtech AB (Sweden). PBMC 2010113745 was found to be negative for CMV and positive for CEFX, and PBMC HHU20180918 was positive for CMV and CEFX (Table 1).

The reported results from the participants are shown in Figures 1-2,5-6 on the following pages, and the raw data is presented in Appendix 4-5. All measurements were done in triplicates. Data analysis was combined for Reagent 1 or 2 and Reagent 3 for each PBMC sample with a total number of four data analyses (Table 1).

3.1. RESULTS FROM ANALYSIS OF PBMC 2010113745

The reported data for the measurements of IFN-γ secreting antigen-specific cells in PBMC 2010113745 stimulated with CMV and a negative control (analysis 1) is presented in Figure 1, whereas PBMC stimulated with CEFX and a negative control (analysis 2) is shown in Figure 2. In Figures 1A and 2A, the reported triplicate values are shown for stimulation with CMV/CEFX (reagent 1/2) and the negative control (reagent 3). In Figures 1B and 2B, the mean of the triplicates was calculated for CMV/CEFX-specific spots (reagent 1/2). The mean of the negative control triplicates (reagent 3) was subtracted from the mean of the CMV/CEFX-specific spots to background-correct, and the median value for all participants was determined. Negative background-corrected results were set to 0.

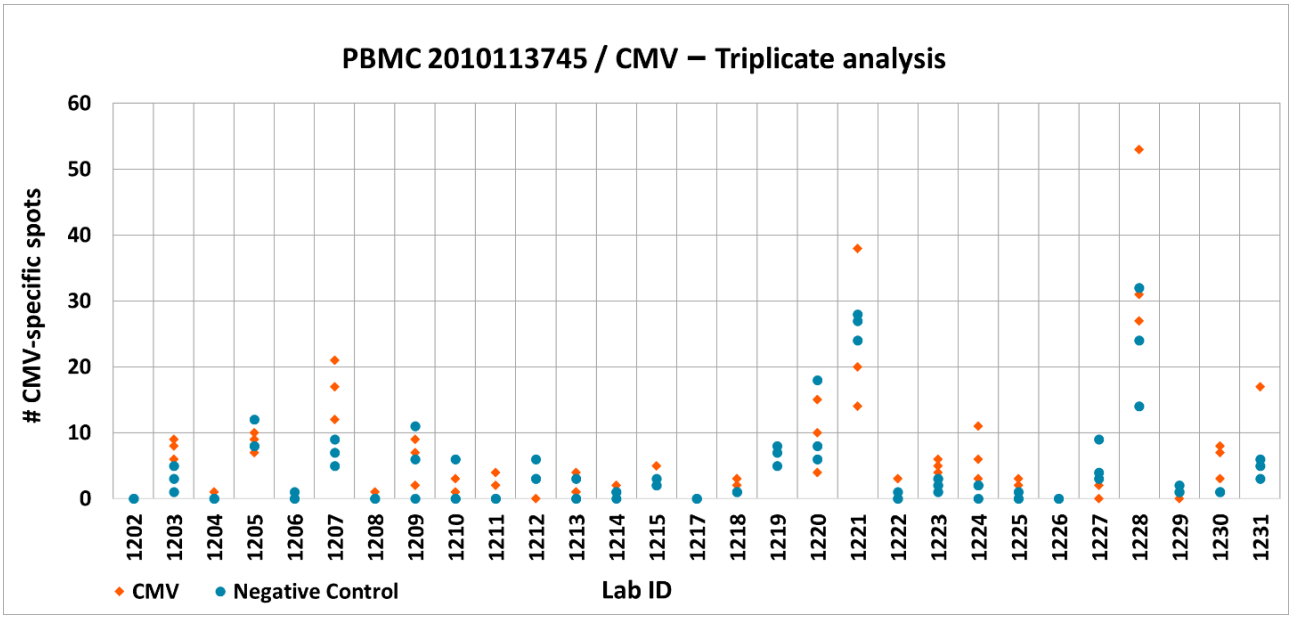

Figure 1A. Results from analysis of sample PBMC 2010113745 with Reagent 1 (CMV) and Reagent 3 (Negative control) (Analysis 1). Triplicate test values for CMV-specific spots (orange diamonds) and background spots (blue dots) per 200.000 PBMCs/well are shown.

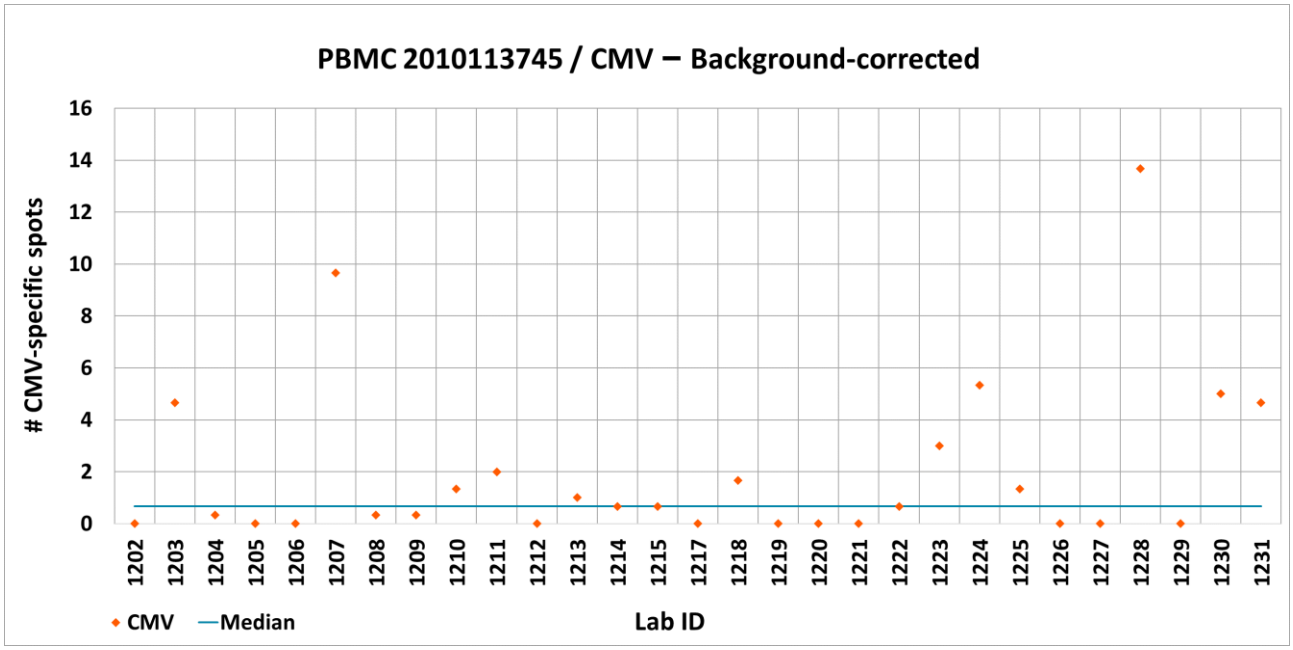

Figure 1B. Results from analysis of sample PBMC 2010113745 with Reagent 1 (CMV) and Reagent 3 (Negative control) (Analysis 1). The mean of CMV-specific spots subtracted the mean of background spots is shown (orange diamonds). Negative background-corrected results were set to 0. The median of all results is 0.67 spots/well and indicated by the blue line.

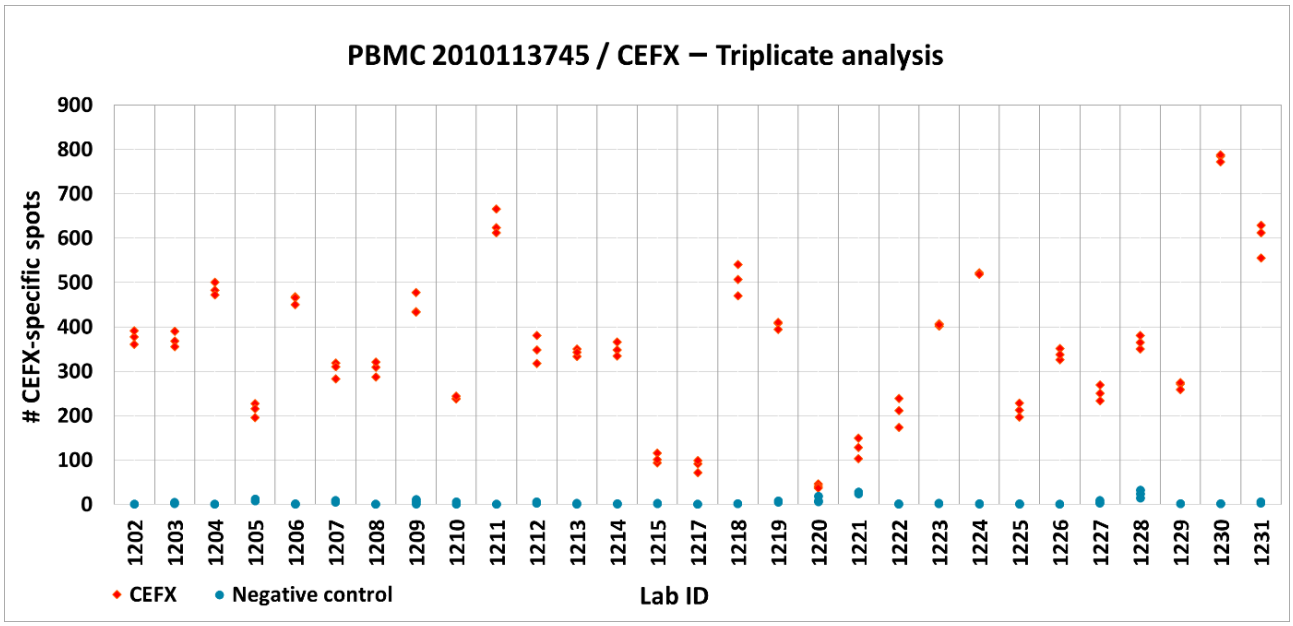

Figure 2A. Results from analysis of sample PBMC 2010113745 with Reagent 2 (CEFX) and Reagent 3 (Negative control) (Analysis 2). Triplicate test values for CEFX-specific spots (orange diamonds) and background spots (blue dots) per 200.000 PBMCs/well are shown.

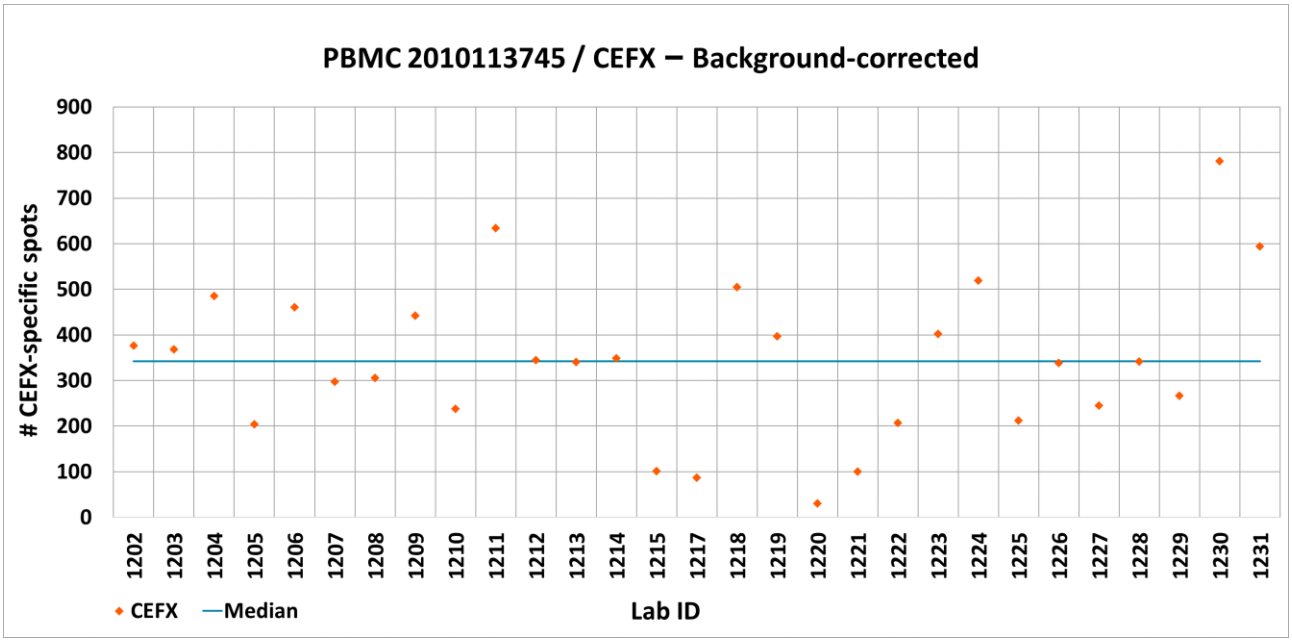

Figure 2B. Results from analysis of sample PBMC 2010113745 with Reagent 2 (CEFX) and Reagent 3 (Negative Control) (Analysis 2). The mean of CEFX-specific spots subtracted the mean of background spots is shown (orange diamonds). The median of all background-corrected test results is 342 spots/well and indicated by the blue line.

3.1.1. Evaluation of Test Results for PBMC 2010113745

3.1.1.1. PBMC 2010113745 stimulated with CMV

To compare the performance of each laboratory against all other participating laboratories for PBMC 2010113745 stimulated with CMV, the test results were background-corrected by calculating the mean of CMV-specific spots (reagent 1) for each laboratory and then subtracting the mean of background spots (reagent 3). The overall mean of the background-corrected results for all participants was calculated and overall mean ± 1-2 standard deviations (SD) was calculated and are shown in Figure 3.

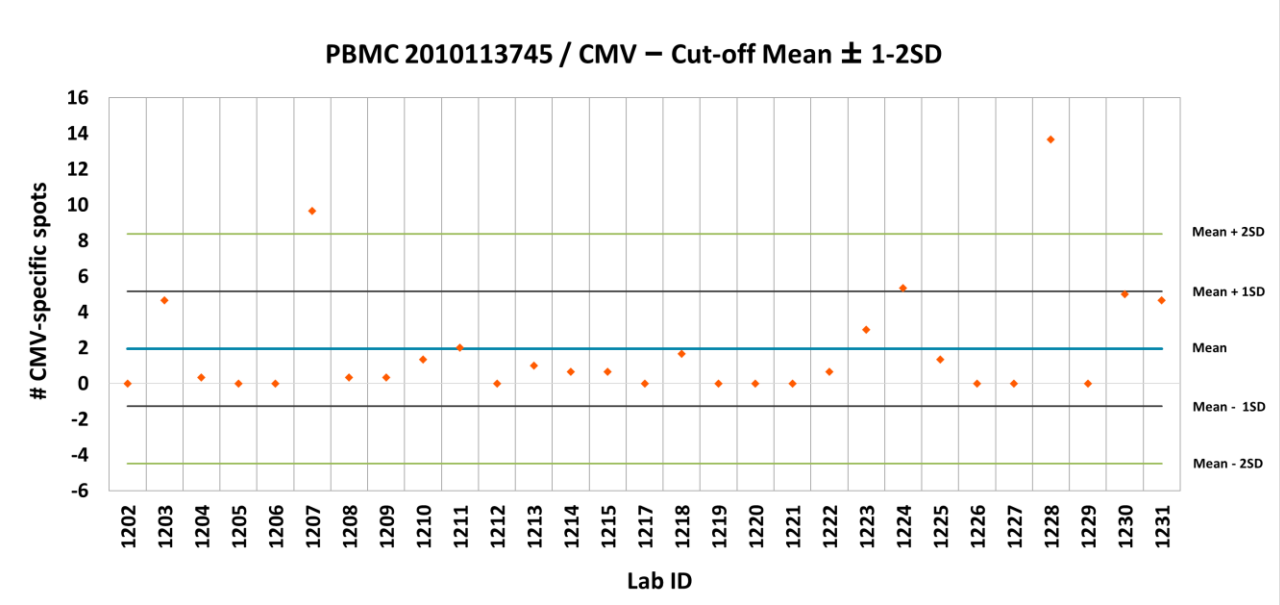

Figure 3. Analysis of PBMC 2010113745 with Reagent 1 (CMV) using mean ± 1-2SD as a cut-off. The orange diamonds show the mean of CMV-specific spots subtracted the mean of background spots. The blue line shows the mean of all results (1.94 spots). The grey lines are mean ± 1SD, and the green lines are mean ± 2SD. 26 of the 29 participants were considered "in the average range" as they were within the cut-off value of mean ± 1SD.

To evaluate the accuracy of each participants' measurements of the PBMC sample with no T-cells reactive to CMV (analysis 1), mean ± 1-2SD were as cut-off values. This measure shows how close each participant is to the average value reported by all participants. Table 2 shows which range a specific test result corresponds to. Mean ± 1SD were as a cut-off value to determine if participants were within the average range. Mean ± 1-2SD was used for data analysis because many of the participants' measurements were below or close to zero after subtracting the background values (reagent 3) from the test values (reagent 1).

Table 2. Definition of the test results.

Test Result	Corresponds to	Presented in the figures as
Mean ± 1SD	Within the average range	Grey lines
Mean ± 2SD	Near the average range	Green lines
>Mean + 2SD **<Mean - 2SD**	Far from the average range	Above/below green lines

3.1.1.2. PBMC 2010113745 stimulated with CEFX

The relative accuracy was used to evaluate the accuracy of each participants' measurements for PBMC 2010113745 stimulated with CEFX. The relative accuracy is defined as the background-corrected test result for each participant divided by the median value of the background-corrected test results for all participants. The relative accuracy tells how close each participant is to the average value reported by all participants. The median shown in Figure 2B was used as the average value to calculate the relative accuracy. See the example of calculation of the relative accuracy in Appendix 7. The individual laboratories' relative accuracies are presented in Figure 4, and the definition of what the values correspond to is listed in Table 3.

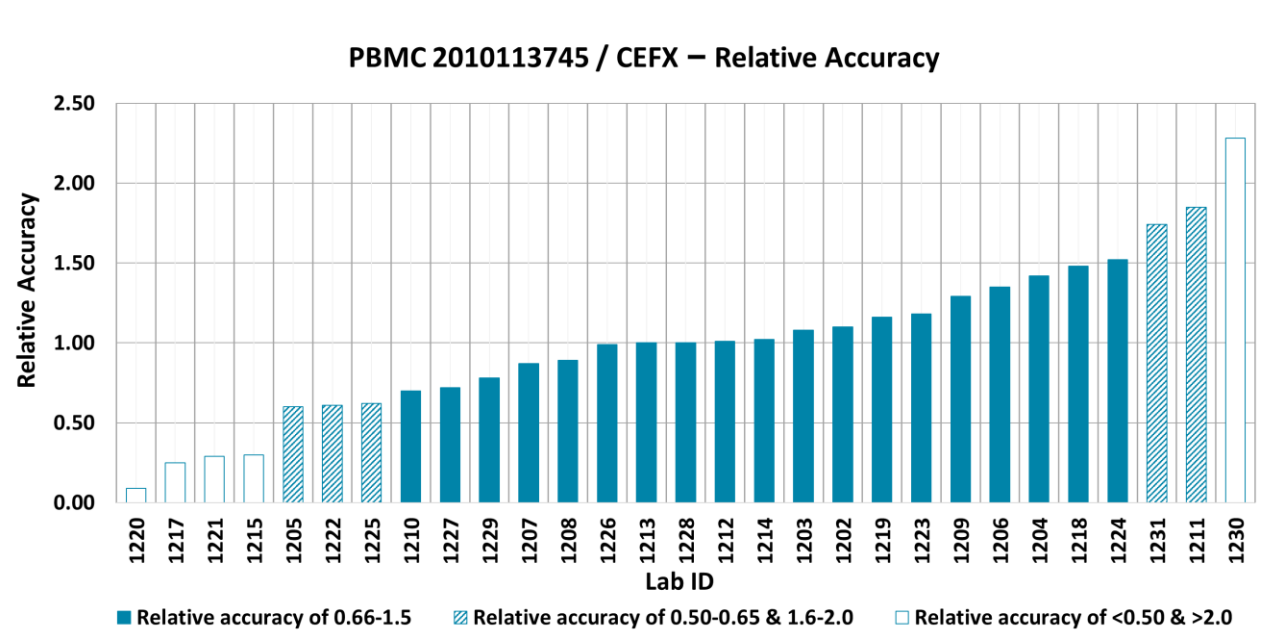

Figure 4. Relative accuracy for analysis of PBMC 2010113745 with Reagent 2 (CEFX). 19 of the 29 participants had a relative accuracy between 0.66-1.5 and are therefore considered "in the average range" (Blue filled columns).

Table 3. Definition of the relative accuracy.

Relative accuracy	Corresponds to	Presented in the figures as
0.66-1.50	Within the average range	Blue columns
0.50 – 0.65 **1.50-2.00**	Near the average range	Striped columns
<0.50 **>2.00**	Far from the average range	White columns

3.2. Results from Analysis of PBMC HHU20180918

The reported data for the measurements of IFN-γ secreting antigen-specific cells in PBMC HHU20180918 stimulated with CMV and a negative control (analysis 3) and CEFX and a negative control (analysis 4) is presented in Figure 5-6. In Figures 5A and 6A, the triplicate values are shown both for stimulation with CMV or CEFX and the negative control. In Figures 5B and 6B, the mean of the triplicates was calculated for CMV/CEFX-specific spots. The mean of the negative control triplicates was subtracted from the mean of the CMV/CEFX-specific spots to background-correct and the median value for all participants was determined. Negative background-corrected results were set to 0.

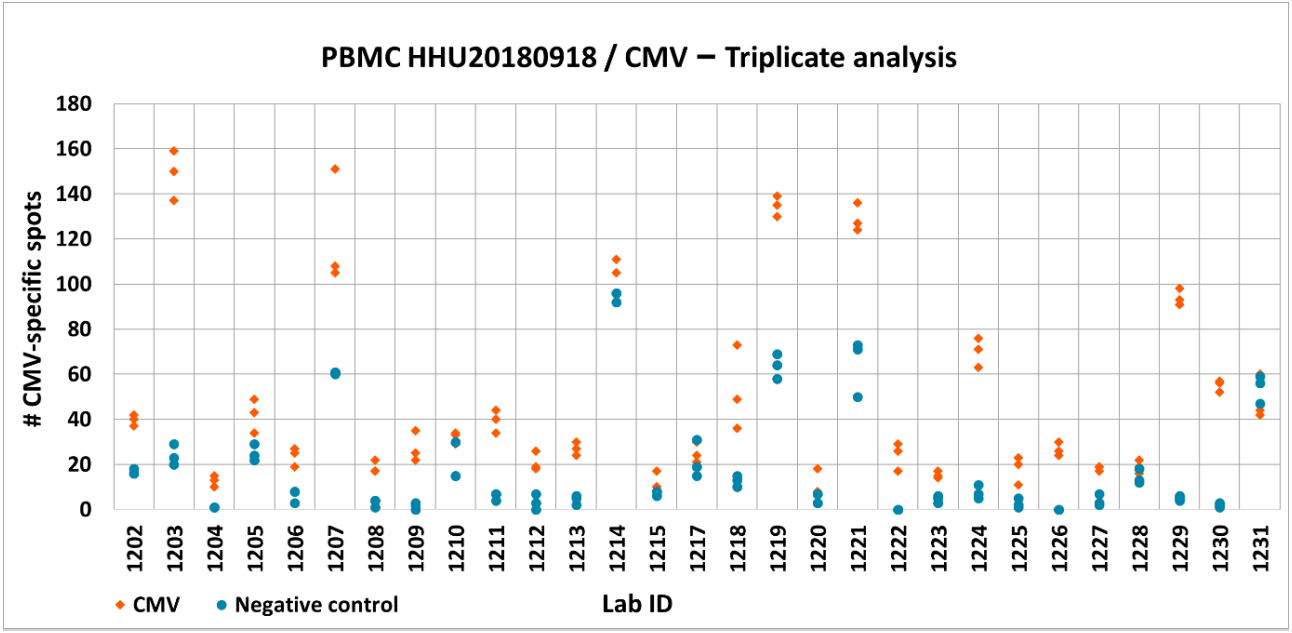

Figure 5A. Results from analysis of sample PBMC HHU20180918 with Reagent 1 (CMV) and Reagent 3 (Negative control) (Analysis 3). Triplicate test values for CMV-specific spots (orange diamonds) and background spots (blue dots) per 200.000 PBMCs/well are shown.

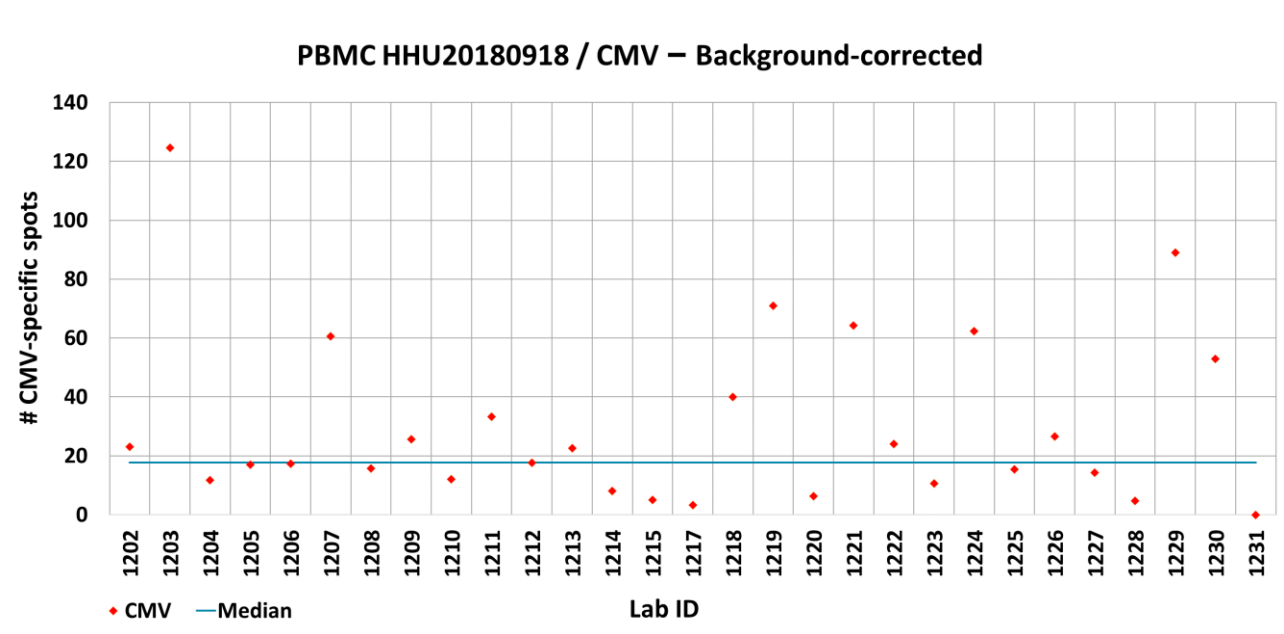

Figure 5B. Results from analysis of sample PBMC HHU20180918 with Reagent 1 (CMV) and Reagent 3 (Negative control) (Analysis 3). The mean of CMV-specific spots subtracted the mean of background spots is shown (orange diamonds). Negative background-corrected results were set to 0. The median of all results is 18 spots and indicated by the blue line.

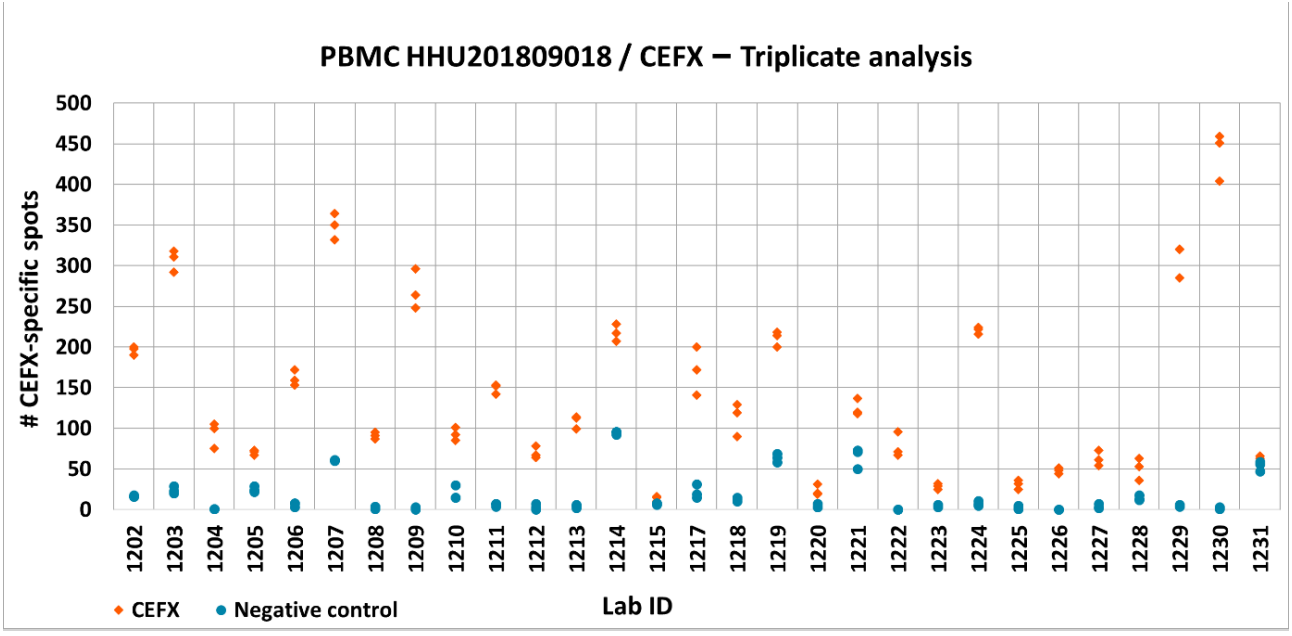

Figure 6A. Results from analysis of sample PBMC HHU20180918 with Reagent 2 (CEFX) and Reagent 3 (Negative control) (Analysis 4). Triplicate test values for CEFX-specific spots (orange diamonds) and background spots (blue dots) per 200.000 PBMCs/well are shown.

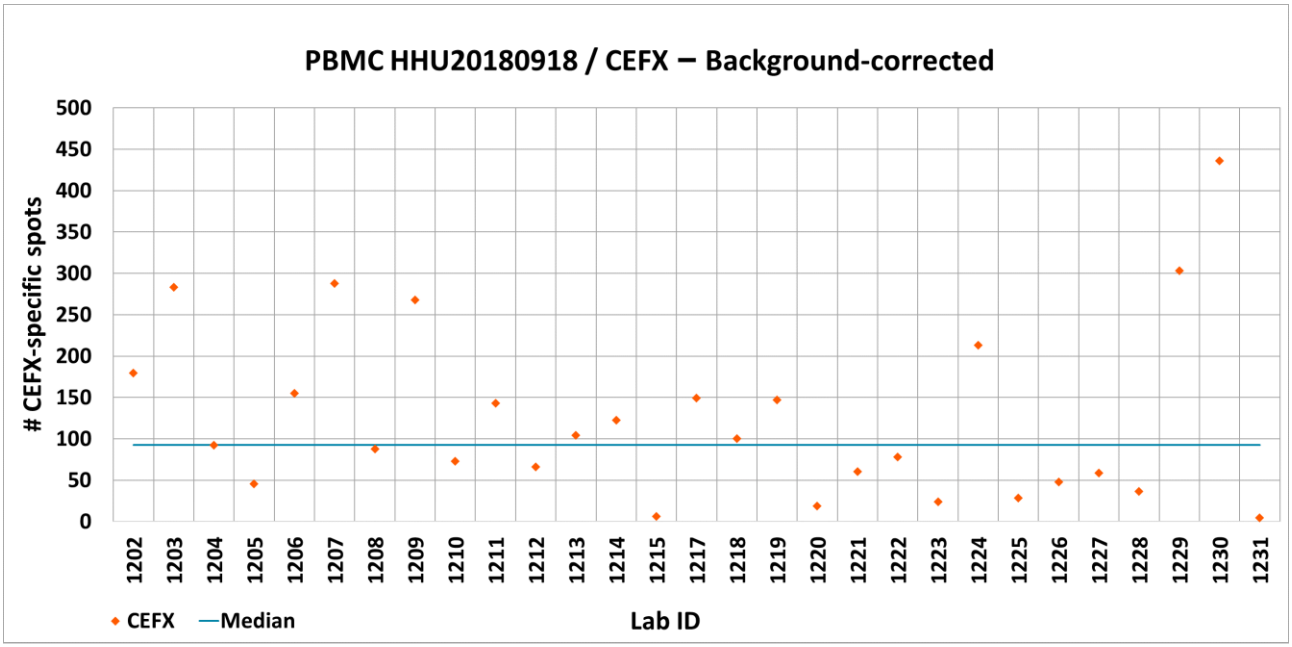

Figure 6B. Results from analysis of sample PBMC HHU20180918 with Reagent 2 (CEFX) and Reagent 3 (Negative Control) (Analysis 4). The mean of CEFX-specific spots subtracted the mean of background spots is shown (orange diamonds). The median of all results is 92 spots and indicated by the blue line.

3.2.1. Evaluation of Test Results for PBMC HHU20180918

The relative accuracy was used to compare and evaluate the accuracy of each participants' measurements for PBMC HHU20180918 stimulated with CMV and CEFX. The medians shown in Figures 5-6 were used as the average values to calculate the relative accuracy. See the example of calculation of the relative accuracy in Appendix 7. The individual laboratories' relative accuracies are presented in Figures 7-8 on the following pages, and the definition of what the values correspond to is listed in Table 3.

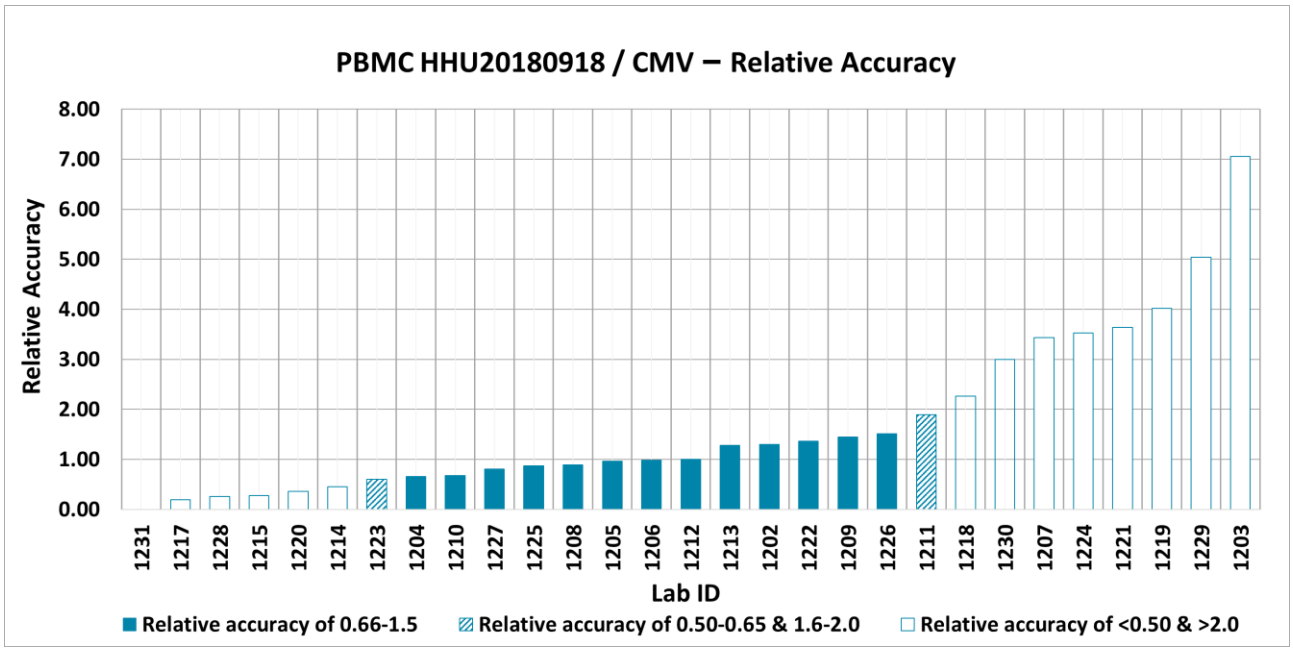

Figure 7. Relative accuracy for analysis of PBMC HHU20180918 with Reagent 1 (CMV). 13 of the 29 participants had a relative accuracy between 0.66-1.5 and are therefore considered "in the average range" (Blue filled columns).

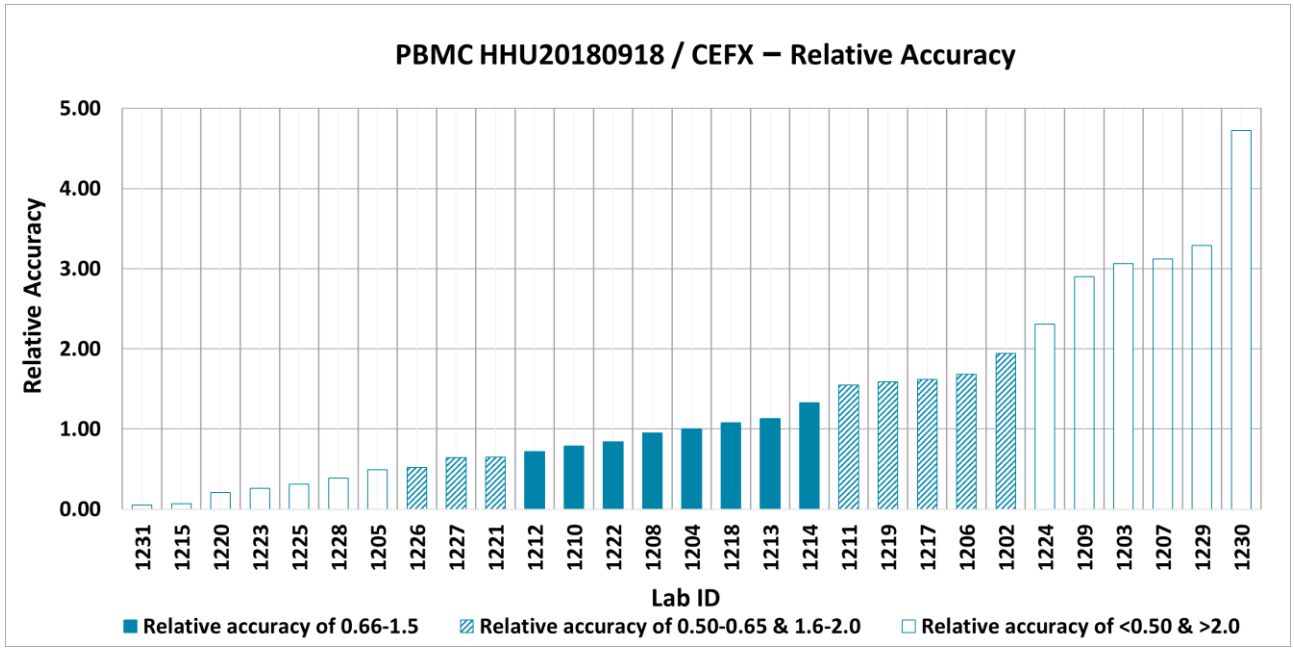

Figure 8. Relative accuracy for analysis of PBMC HHU20180918 with Reagent 2 (CEFX). 8 of the 29 participants had a relative accuracy between 0.66-1.5 and are therefore considered "in the average range" (Blue filled columns).

4. PROFICIENCY PERFORMANCE

The ability of each participant to identify IFN-γ secreting T-cells was described with an overall proficiency score. For each of the four analyses (Analysis 1-4), the laboratories were assigned a score between 1-3 (Table 4). The overall proficiency score was then defined by the average score obtained in the four analyses. Thus, a participant with an overall proficiency score 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.

Table 4. Definition of proficiency score.

Proficiency Score	Range	Mean ± 1-2SD (Analysis 1)	Relative Accuracy (Analysis 2-4)
3	Within the average range	mean ± 1SD	0.66-1.5
2	Near the average range	mean ± 2SD	0.50-0.65 or 1.6-2.0
1	Far from the average range	> mean + 2SD or < mean - 2SD	<0.50 or >2.0

Figure 9 shows the overall proficiency score for all the participating laboratories. 20 out of the 29 participating laboratories got an overall proficiency score of ≥ 2. This corresponds to 69%, in line with the T-cell ELISpot Proficiency Panel 2020, where 63% of the participants obtained an overall proficiency score of ≥ 2.

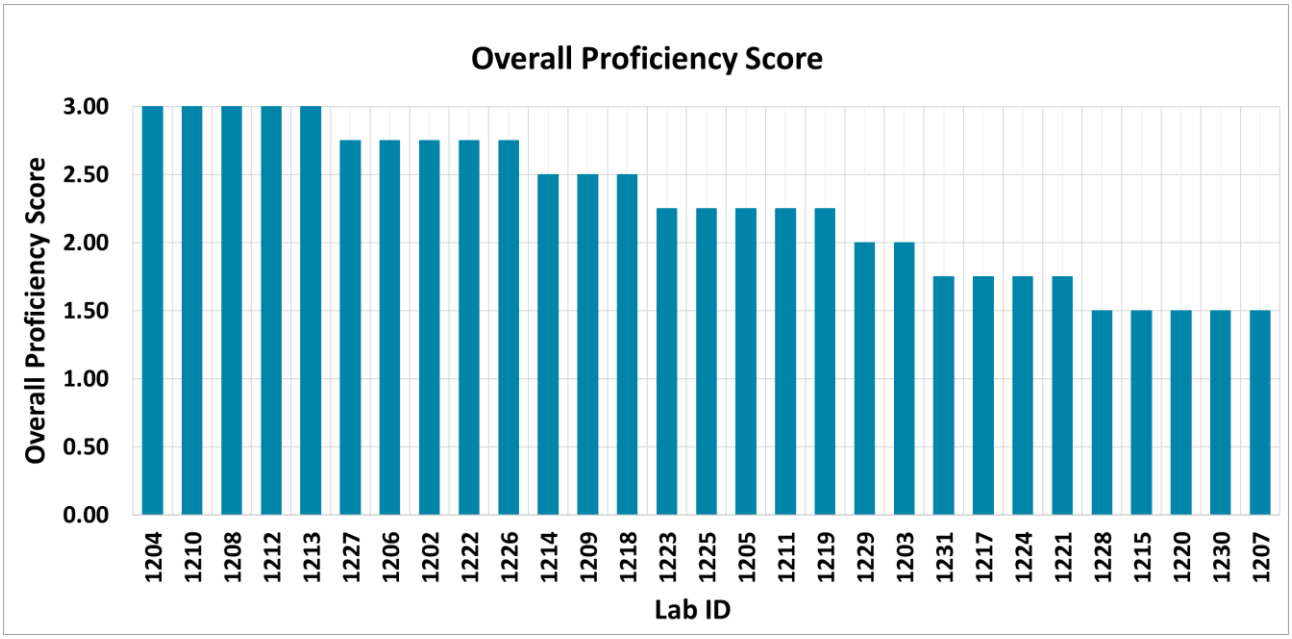

Figure 9. Overall Proficiency Score in the T-cell ELISpot Proficiency Panel 2021.

5. DISCUSSION

Immudex T-cell ELISpot Proficiency Panels provide a program for laboratories worldwide to assess their proficiency in identifying IFN-γ 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 (CMV and CEFX). 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 optimizations.

Variations in test results 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 general, the participating laboratories showed similar triplicate results. All participants had a protocol for ELISpot, and for most of the participants, the protocol covered all steps of the assay. Nearly all participants complied with the ELISpot harmonization guidelines.

In this proficiency panel, a negative sample (PBMC 2010113745) with no T-cells reactive to the tested CMV peptide pool (Analysis 1, Figure 1B) was included. 26 out of 29 participants were within the average range (based on mean ± 1SD as a cut-off value), demonstrating a general alignment and low risk of false positive measurements.

In the other three analyses (Analysis 2-4), samples with CMV/CEFX-specific T-cells were used. The sample with the highest panel median of 342 spots/well (Analysis 2, Figure 2B) was the one where most participants (19 out of 29) obtained results within the average range. For the other two samples with lower frequency of antigen-specific T cells (panel median of 18 (Analysis 3, Figure 5B) and 92 spots/well (Analysis 4, Figure 6B), respectively), results were less aligned. Here 13 and 8, respectively, of the 29 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, ii) there is a low risk of detecting false positives, and iii) Proficiency Panels are a valuable tool to evaluate proficiency in immune monitoring assays across different laboratories and ensure comparable results.

6. ACKNOWLEDGEMENTS

We thank Mabtech AB (Sweden) for quality control and ELISpot assay testing of PBMC samples and JPT Peptide Technologies (Germany) for providing peptide pools.

7. 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 to quantify 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.

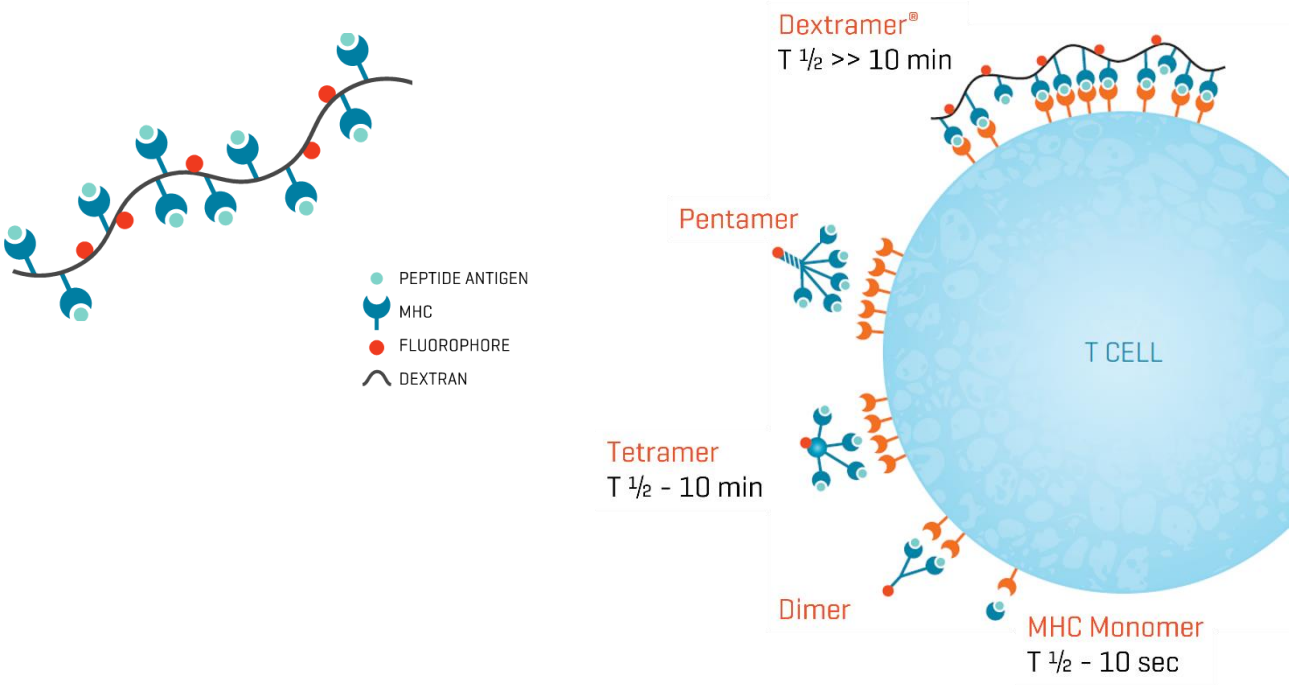

Figure 10 Schematic drawing of MHC Dextramer® and conventional MHC multimers binding to T-cell receptors (TCRs) on the surface of a T cell. MHC Dextramer® reagents are fluorescent-labeled MHC multimers that can bind simultaneously to multiple TCRs on a single T cell. This provides a solid and stable 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.

7.1. Resources from Immudex

We are committed to building a global community of proficiency in immune monitoring. Reach to us if you have questions or want to know more about the Immudex Proficiency Panels.

Proficiency Panels

Access the Immudex Proficiency Panels site, where you will find information about MHC Multimer and ELISpot Proficiency Panels.

Read more

Contact the Panel Coordinator

We are here to support you through all the process. From the proficiency testing to answering questions regarding deadlines, PBMC samples, data analysis. We want to ensure the process is easy for you.

proficiencypanel@immudex.com

Performance Reports

Curious about previous year's results? Find out more for MHC Multimer and ELISpot Proficiency Panels.

MHC Multimer Proficiency Panel reports

ELISpot Proficiency Panel Reports

Technical Support

Let us know if you experience difficulties or have questions. Immudex will help you get the most out of your Dextramer® products.

customer@immudex.com

8. APPENDIXES

8.1. APPENDIX 1: INSTRUCTIONS

Instructions for T-Cell ELISpot Proficiency Panel 2021

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 Cancer Immunotherapy Consortium of the Cancer Research Institute (CIC of CRI) and the Association for Cancer Immunotherapy (CIMT), Immudex conducts Proficiency Panels annually, allowing laboratories to assess their performance in monitoring antigen-specific T-cell responses.

In this T-cell 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 CIC of CRI and CIMT, see Appendix I.

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:	June 20, 2021
Final report from Immudex:	August, 2021

If you have questions, please contact Catharina Essendrup Dam, at proficiencypanel@immudex.com

Samples and Reagents Provided

- Two PBMC samples (Lot #2010113745 and Lot #HHU20180918)
- 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 total cell number and the percentage of viable cells. If a resting step is included, please count and record total cell number and the percentage of viable cells after the resting step, see Table 1 below.

Table 1 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				
HHU20180918				

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. 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 2 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 HHU20180918 *Reagent-1*	PBMC lot HHU20180918 *Reagent-1*	PBMC lot HHU20180918 *Reagent-1*	
F		PBMC lot HHU20180918 *Reagent-2*	PBMC lot HHU20180918 *Reagent-2*	PBMC lot HHU20180918 *Reagent-2*	
G		PBMC lot HHU20180918 *Reagent-3*	PBMC lot HHU20180918 *Reagent-3*	PBMC lot HHU20180918 *Reagent-3*	
H		**No cells – Medium**	**No cells – Medium**	**No cells – Medium**	

Report data

After completing the experiment, please report data and experimental details, using this link https://immudex.wufoo.com/forms/r1r3ep5z1hc1szi/

8.2. 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 pretested 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 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 reading process.
E2. Adequate adjustments for technical artefacts*.

F. Only allow trained personnel, which is trained per laboratory SOP, to 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).

8.3. Appendix 3: Pre-Test of PBMC Samples

Pre-test of PBMC 2010113745 and PBMC HHU20180918 was conducted using ELISpot assay performed by the external partner Mabtech AB (Sweden) according to "Instructions for T-cell ELISpot Proficiency Panel 2021" (Appendix 1). Three vials from each PBMC sample 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 6 PBMC samples was in the range of 94-98% after thawing and after one hour of rest.

Table. Results from the pre-test of 3 vials of PBMC 2010113745 and HHU20180918, where the test values represent the number of spots for each vial with Reagent 1-3. The mean for each of the two PBMCs was calculated for each reagent and then the background (test values from reagent 3) was subtracted and shown as a background-corrected value (BC).

PBMC batch	Reagent 1 (CMV)					Reagent 2 (CEFX)					Reagent 3 (Negative Control)			
	Test values			Mean	BC	Test values			Mean	BC	Test values			Mean
2010113745 (1)	1	0	0			93	115	107			0	1	0	
2010113745 (2)	2	0	0	1	0	180	143	151	145	144	1	1	0	1
2010113745 (3)	0	0	2			169	168	180			0	4	0	
HHU20180918 (1)	47	42	39			199	203	204			1	0	1	
HHU20180918 (2)	67	73	62	56	54	243	273	268	232	230	1	9	1	2
HHU20180918 (3)	61	45	72			244	219	237			0	3	0	

8.4. Appendix 4: 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.

Lab ID	Reagent 1 – CMV (Well B3-B5)			Reagent 2 – CEFX (Well C3-C5)			Reagent 3 – Negative Control (Well D3-D5)		
1202	0	0	0	361	377	391	0	0	0
1203	6	9	8	390	368	355	1	3	5
1204	0	0	1	500	483	472	0	0	0
1205	10	7	9	227	196	216	12	8	8
1206	1	0	0	468	466	450	0	0	1
1207	21	17	12	319	283	310	5	9	7
1208	0	0	1	309	321	287	0	0	0
1209	7	2	9	434	433	477	0	6	11
1210	6	1	3	239	238	244	6	0	0
1211	4	0	2	624	612	666	0	0	0
1212	6	0	3	381	348	318	3	6	3
1213	1	4	1	333	350	343	3	0	0
1214	2	1	1	348	366	334	1	0	1
1215	5	2	2	116	94	101	3	2	2
1217	0	0	0	71	91	99	0	0	0
1218	3	3	2	541	470	507	1	1	1
1219	8	5	7	408	410	394	7	8	5
1220	10	4	15	46	41	37	18	8	6
1221	20	14	38	103	149	128	28	24	27
1222	0	3	0	239	211	173	0	0	1
1223	4	5	6	402	407	405	2	1	3
1224	3	11	6	522	522	518	2	0	2
1225	2	3	0	212	228	197	1	0	0
1226	0	0	0	326	351	338	0	0	0
1227	0	3	2	269	250	233	3	9	4
1228	27	31	53	350	365	381	32	24	14
1229	2	2	0	271	274	259	2	1	1
1230	8	3	7	785	788	772	1	1	1
1231	5	6	17	612	555	629	3	5	6

8.5. Appendix 5: Results from Analysis of PBMC HHU20180918

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.

Lab ID	Reagent 1 – CMV (Well E3-E5)			Reagent 2 – CEFX (Well F3-F5)			Reagent 3 – Negative Control (Well G3-G5)		
1202	40	42	37	190	198	200	16	16	18
1203	159	150	137	292	318	311	20	29	23
1204	13	15	10	100	75	105	1	1	1
1205	43	49	34	73	67	71	22	24	29
1206	19	27	25	153	159	172	8	8	3
1207	108	151	105	350	364	332	61	61	60
1208	17	17	22	87	91	95	1	4	4
1209	22	25	35	296	264	248	3	0	2
1210	34	29	33	85	101	92	15	30	15
1211	40	44	34	153	152	142	7	4	7
1212	26	19	18	78	64	67	7	3	0
1213	27	24	30	99	113	114	6	5	2
1214	105	111	92	228	207	217	96	96	92
1215	10	10	17	15	16	10	8	8	6
1217	24	21	30	172	141	200	31	15	19
1218	49	36	73	129	119	90	15	13	10
1219	130	139	135	214	218	200	64	69	58
1220	18	6	8	20	31	19	7	3	3
1221	136	124	127	120	118	137	71	50	73
1222	29	26	17	96	67	71	0	0	0
1223	15	14	17	32	29	25	3	5	6
1224	71	76	63	224	222	216	11	5	7
1225	11	20	23	25	32	36	2	5	1
1226	24	30	26	51	48	44	0	0	0
1227	19	19	17	54	73	61	2	3	7
1228	16	22	19	53	63	36	13	18	12
1229	93	98	91	285	320	320	5	6	4
1230	57	56	52	451	459	404	1	2	3
1231	44	60	42	47	66	63	56	59	47

8.6. Appendix 6: Data Analysis Results for PBMC 2010113745 and HHU20180918

Analysis 1 – PBMC 2010113745 stimulated with CMV (reagent 1) and negative control (reagent 3)

Lab ID	Reagent 1 - CMV (Well B3-B5)			Mean of B3-B5	Reagent 3 - Negative Control (Well D3-D5)			Mean of D3-D5	Mean of B3-B5 – Mean of D3-D5 (Background-corrected test result)
1202	0	0	0	0	0	0	0	0	0
1203	6	9	8	8	1	3	5	3	5
1204	0	0	1	0	0	0	0	0	0
1205	10	7	9	9	12	8	8	9	0*
1206	1	0	0	0	0	0	1	0	0
1207	21	17	12	17	5	9	7	7	10
1208	0	0	1	0	0	0	0	0	0
1209	7	2	9	6	0	6	11	6	0
1210	6	1	3	3	6	0	0	2	1
1211	4	0	2	2	0	0	0	0	2
1212	6	0	3	3	3	6	3	4	0*
1213	1	4	1	2	3	0	0	1	1
1214	2	1	1	1	1	0	1	1	1
1215	5	2	2	3	3	2	2	2	1
1217	0	0	0	0	0	0	0	0	0
1218	3	3	2	3	1	1	1	1	2
1219	8	5	7	7	7	8	5	7	0
1220	10	4	15	10	18	8	6	11	0*
1221	20	14	38	24	28	24	27	26	0*
1222	0	3	0	1	0	0	1	0	1
1223	4	5	6	5	2	1	3	2	3
1224	3	11	6	7	2	0	2	1	5
1225	2	3	0	2	1	0	0	0	1
1226	0	0	0	0	0	0	0	0	0
1227	0	3	2	2	3	9	4	5	0*
1228	27	31	53	37	32	24	14	23	14
1229	2	2	0	1	2	1	1	1	0
1230	8	3	7	6	1	1	1	1	5
1231	5	6	17	9	3	5	6	5	5

*negative background-corrected values set to 0. Median: 0.67, Mean: 1.94, SD: 3.22.

Analysis 2 – PBMC 2010113745 stimulated with CEFX (reagent 2) and negative control (reagent 3)

Lab ID	Reagent 2 - CEFX (Well C3-C5)			Mean of C3-C5	Reagent 3 - Negative Control (Well D3-D5)			Mean of D3-D5	Mean of C3-C5 – Mean of D3-D5 (Background-corrected test result)	Relative accuracy (Background-corrected test result / median*)
1202	361	377	391	376	0	0	0	0	376	1.10
1203	390	368	355	371	1	3	5	3	368	1.08
1204	500	483	472	485	0	0	0	0	485	1.42
1205	227	196	216	213	12	8	8	9	204	0.60
1206	468	466	450	461	0	0	1	0	461	1.35
1207	319	283	310	304	5	9	7	7	297	0.87
1208	309	321	287	306	0	0	0	0	306	0.89
1209	434	433	477	448	0	6	11	6	442	1.29
1210	239	238	244	240	6	0	0	2	238	0.70
1211	624	612	666	634	0	0	0	0	634	1.85
1212	381	348	318	349	3	6	3	4	345	1.01
1213	333	350	343	342	3	0	0	1	341	1.00
1214	348	366	334	349	1	0	1	1	349	1.02
1215	116	94	101	104	3	2	2	2	101	0.30
1217	71	91	99	87	0	0	0	0	87	0.25
1218	541	470	507	506	1	1	1	1	505	1.48
1219	408	410	394	404	7	8	5	7	397	1.16
1220	46	41	37	41	18	8	6	11	31	0.09
1221	103	149	128	127	28	24	27	26	100	0.29
1222	239	211	173	208	0	0	1	0	207	0.61
1223	402	407	405	405	2	1	3	2	403	1.18
1224	522	522	518	521	2	0	2	1	519	1.52
1225	212	228	197	212	1	0	0	0	212	0.62
1226	326	351	338	338	0	0	0	0	338	0.99
1227	269	250	233	251	3	9	4	5	245	0.72
1228	350	365	381	365	32	24	14	23	342	1.00
1229	271	274	259	268	2	1	1	1	267	0.78
1230	785	788	772	782	1	1	1	1	781	2.28
1231	612	555	629	599	3	5	6	5	594	1.74

*Median: 342.

Analysis 3 – PBMC HHU20180918 stimulated with CMV (reagent 1) and negative control (reagent 3)

Lab ID	Reagent 1 - CMV (Well E3-E5)			Mean of E3-E5	Reagent 3 - Negative Control (Well G3-G5)			Mean of G3-G5	Mean of C3-C5 – Mean of G3-G5 (Background-corrected test result)	Relative accuracy (Background-corrected test result / median**)
1202	40	42	37	40	16	16	18	17	23	1.30
1203	159	150	137	149	20	29	23	24	125	7.06
1204	13	15	10	13	1	1	1	1	12	0.66
1205	43	49	34	42	22	24	29	25	17	0.96
1206	19	27	25	24	8	8	3	6	17	0.98
1207	108	151	105	121	61	61	60	61	61	3.43
1208	17	17	22	19	1	4	4	3	16	0.89
1209	22	25	35	27	3	0	2	2	26	1.45
1210	34	29	33	32	15	30	15	20	12	0.68
1211	40	44	34	39	7	4	7	6	33	1.89
1212	26	19	18	21	7	3	0	3	18	1.00
1213	27	24	30	27	6	5	2	4	23	1.28
1214	105	111	92	103	96	96	92	95	8	0.45
1215	10	10	17	12	8	8	6	7	5	0.28
1217	24	21	30	25	31	15	19	22	3	0.19
1218	49	36	73	53	15	13	10	13	40	2.26
1219	130	139	135	135	64	69	58	64	71	4.02
1220	18	6	8	11	7	3	3	4	6	0.36
1221	136	124	127	129	71	50	73	65	64	3.64
1222	29	26	17	24	0	0	0	0	24	1.36
1223	15	14	17	15	3	5	6	5	11	0.60
1224	71	76	63	70	11	5	7	8	62	3.53
1225	11	20	23	18	2	5	1	3	15	0.87
1226	24	30	26	27	0	0	0	0	27	1.51
1227	19	19	17	18	2	3	7	4	14	0.81
1228	16	22	19	19	13	18	12	14	5	0.26
1229	93	98	91	94	5	6	4	5	89	5.04
1230	57	56	52	55	1	2	3	2	53	3.00
1231	44	60	42	49	56	59	47	54	0*	0.00

*negative background-corrected values set to 0. **Median: 18.

Analysis 4 – PBMC HHU20180918 stimulated with CEFX (reagent 2) and negative control (reagent 3)

Lab ID	Reagent 1 - CEFX (Well F3-F5)			Mean of F3-F5	Reagent 3 - Negative Control (Well G3-G5)			Mean of G3-G5	Mean of F3-F5 – Mean of G3-G5 (Background-corrected test result)	Relative accuracy (Background-corrected test result / median*)
1202	190	198	200	196	16	16	18	17	179	1.94
1203	292	318	311	307	20	29	23	24	283	3.06
1204	100	75	105	93	1	1	1	1	92	1.00
1205	73	67	71	70	22	24	29	25	45	0.49
1206	153	159	172	161	8	8	3	6	155	1.68
1207	350	364	332	349	61	61	60	61	288	3.12
1208	87	91	95	91	1	4	4	3	88	0.95
1209	296	264	248	269	3	0	2	2	268	2.90
1210	85	101	92	93	15	30	15	20	73	0.79
1211	153	152	142	149	7	4	7	6	143	1.55
1212	78	64	67	70	7	3	0	3	66	0.72
1213	99	113	114	109	6	5	2	4	104	1.13
1214	228	207	217	217	96	96	92	95	123	1.33
1215	15	16	10	14	8	8	6	7	6	0.07
1217	172	141	200	171	31	15	19	22	149	1.62
1218	129	119	90	113	15	13	10	13	100	1.08
1219	214	218	200	211	64	69	58	64	147	1.59
1220	20	31	19	23	7	3	3	4	19	0.21
1221	120	118	137	125	71	50	73	65	60	0.65
1222	96	67	71	78	0	0	0	0	78	0.84
1223	32	29	25	29	3	5	6	5	24	0.26
1224	224	222	216	221	11	5	7	8	213	2.31
1225	25	32	36	31	2	5	1	3	28	0.31
1226	51	48	44	48	0	0	0	0	48	0.52
1227	54	73	61	63	2	3	7	4	59	0.64
1228	53	63	36	51	13	18	12	14	36	0.39
1229	285	320	320	308	5	6	4	5	303	3.29
1230	451	459	404	438	1	2	3	2	436	4.72
1231	47	66	63	59	56	59	47	54	5	0.05

*Median: 92.

8.7. Appendix 7: Calculation of the relative accuracy

Example of relative accuracy calculation of PBMC donor HHU20180918 stimulated with CEFX peptide pool

	# of spots								
Lab ID	**F3 CEFX**	**F4 CEFX**	**F5 CEFX**	**G3 Neg. control**	**G4 Neg. control**	**G5 Neg. control**	**Mean value subtracted background**	**Median for all participants**	**Relative Accuracy**
1206	153	159	172	8	8	3	155	92	$\frac{155}{92} = 1.68$