Journal of Immunological Methods 524 (2024) 113588

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Validation of a double-color ELISpot assay of IFN-γ and IL-4 production in human peripheral blood mononuclear cells

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ARTICLE INFO

Keywords:
Validation
ELISpot
Cell-mediated immunity
hPBMCs
IFN-γ
IL-4

ABSTRACT

The Enzyme-Linked ImmunoSpot (ELISpot) assay detects cytokines secreted during T cell-specific immune responses against pathogens. As this assay has acquired importance in the clinical setting, standard bioanalytical evaluation of this method is required. Here, we describe a formal bioanalytical validation of a double-color ELISpot assay for the evaluation of IFN-γ and IL-4 released by T helper 1 and T helper 2 cells, respectively. As recommended by international guidelines, the parameters assessed were: range and detection limits (limit of detection, LOD; upper and lower limit of quantification, ULOQ and LLOQ), Linearity, Relative Accuracy, Repeatability, Intermediate Precision, Specificity and Robustness. The results obtained in this validation study demonstrate that this assay meets the established acceptability criteria. ELISpot is therefore a reliable technique for measuring T cell-specific immune responses against various antigens of interest.

1. Introduction

The host's ability to fight viral or bacterial infection and prevent the development of serious diseases requires the coordinated activation of several components of the immune system, which ultimately leads to the production of neutralizing and antigen-binding antibodies as well as antiviral T cells (Tan et al., 2021). The adaptive immune system confers protective immunity, counteracting pathogens in an antigen-specific manner. This adaptive system consists of three main types of lymphocytes: B cells (antibody-producing cells), $CD4^+$ T cells (helper T cells) and $CD8^+$ T cells (cytotoxic or killer T cells) (Murphy and Weaver, 2016). Most licensed human vaccines elicit the activation of B cells and consequently induce protective antibody responses, with neutralizing antibodies being the most common mechanism of action (Piot et al., 2019; Plotkin et al., 2018). T-cell responses, however, are also an important source of protection. In response to most acute viral infections, both B and T cells can bind viral proteins through their antigen receptors; they therefore become activated, expand, differentiate, and begin secreting effector molecules to help control the infection. When reinfection occurs, pathogen-specific Memory B Cells (MBCs) rapidly proliferate and differentiate into protective plasma cells that secrete antigen-specific IgG immunoglobulin (Kim et al., 2019; Knox et al., 2019). Meanwhile, the two T-cell populations are also activated: the reactivated memory $CD4^+$ T cells 'help' activate the MBCs and secrete cytokines to activate the innate cells (Ruterbusch et al., 2020), while the memory $CD8^+$ T cells directly kill the virus-infected cells by means of cell-cell contact and the consequent release of cytolytic molecules (Schmidt and Varga, 2018). These memory populations are pathogen-specific and coordinate in order to rapidly eliminate the infective agent, thereby preventing the onset of disease and reducing the possibility of transmission. It is therefore crucial to assess the entire set of immune memory responses specific to a certain pathogen in order to determine whether vaccination can induce a long-lasting, multilayer defense.

During the recent COVID-19 pandemic, much evidence demonstrated that a key role in recovery from disease was played not only by neutralizing antibodies, but also by memory $CD8^+$ and/or $CD4^+$ T helper (Th) 1 T cells. This implies that T cells play a role in providing

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https://doi.org/10.1016/j.jim.2023.113588
Received 11 September 2023; Received in revised form 21 November 2023; Accepted 27 November 2023
Available online 29 November 2023

long-term protection against SARS-CoV-2, prompting researchers to evaluate T cell responses following vaccination, too (Iqbal, 2020). Zhang et al. (2022) analyzed $CD4^+$ and $CD8^+$ memory T cells in depth following vaccination with four different COVID-19 vaccines based on three different vaccine technologies. Basically, these authors found that, while antibody levels tended to decline, memory T cells remained comparatively stable over time, further suggesting that T cell responses might provide more durable protection than antibody-mediated immunity.

Given the increasing importance of assessing cell-mediated immunity against pathogens in the clinical setting, the validation of assays such as Enzyme-Linked ImmunoSpot (ELISpot) is required. Since the ELISpot technique was introduced, it has rapidly become a widely applied method for the detection of cellular immune responses (Janetzki, 2018; Kiecker et al., 2004), not least in vaccine development. This method has a relatively wide quantitative range and offers unique sensitivity, revealing cytokine secretion at the single cell level. To date, however, no standard bioanalytical evaluation of this method of testing clinical samples is widely available in the literature (Barabas et al., 2017; Maecker et al., 2008). For example, how to establish ELISpot detection cut-offs (limit of detection, LOD; upper and lower limit of quantification, ULOQ and LLOQ), whether to implement confirmatory cut-off points, and how to set assay acceptance criteria of precision, linearity and accuracy during validation and sample analysis are matters of choice.

Here, we describe the development and formal bioanalytical validation of a double-color ELISpot assay to evaluate the release of IFN-γ and IL-4 in human Peripheral Blood Mononuclear Cells (hPBMCs). To demonstrate that the assay is suitable for its intended purpose, the parameters commonly recommended by international guidelines were assessed: range and detection limits (LOD, LLOQ and ULOQ), Linearity, Relative Accuracy, Repeatability, Intermediate Precision, Specificity and Robustness.

2. Materials and methods

2.1. hPBMCs thawing

Uncharacterized hPBMCs samples from three donors (lot. HHU20181108, lot. HHU20181220, lot. HHU20181218), purchased from CTL (Cellular Technology Limited, Cleveland, OH), were used. hPBMCs were quickly thawed in a water bath at 37 °C (±2 °C) and Phosphate Buffered Saline (PBS) 1×, supplemented with 2.5 mM Ethylenediaminetetraacetic acid (EDTA) and 20 μg/ml Deoxyribonuclease I (DNase I), was then used as a thawing medium. The cells were then centrifuged at 350*g* for 5 min at RT (22 ± 2 °C). After washing with the thawing medium, the hPBMCs were counted by means of a CTL-LDC™ Live/Dead Cell Counting Kit (CTL-LDC-100-2, ImmunoSpot® Cleveland, OH) in accordance with the manufacturer's instructions. Live cells were counted by means of the CTL Cell Counting™ Software (ImmunoSpot®, Cleveland, OH).

2.2. ELISpot assay

A commercial ELISpot kit (HIFNIL4-2 M/5, ImmunoSpot® Cleveland, OH) was used in accordance with the manufacturer's protocol. Briefly, 96-well plates were activated with 70% Ethanol, coated with capture solution and incubated at 4 °C for at least 16 h. After incubation, the capture solution was washed with Dulbecco's Phosphate Buffered Saline (D-PBS) 1×, and 100 μl of stimulation media was plated in each well. Specifically, CTL medium supplemented with 0.75% of Dimethyl sulfoxide (DMSO) (Sigma Aldrich) was used as a negative control, while Staphylococcal enterotoxin B (SEB; 1 μg/ml) (Merck KGaA, Darmstadt, Germany) was added to the CTL medium as a positive control. To evaluate Relative Accuracy, Repeatability, Intermediate Precision, Specificity and Robustness, 3×10^5 hPBMCs were plated into ELISpot plates, while to evaluate Linearity, hPBMCs were plated at different concentrations (from 300,000 to 62,915 cells per well) in 1.25-fold dilution steps.

Plates were incubated for 44 h at 37 °C in 5% CO_2 in a humidified incubator. Regarding robustness, the incubation time was 44 ± 2 h.

After incubation, signal detection was performed in accordance with the manufacturer's step-by-step protocol. Stimulated hPBMCs were discarded from the plate, and the wells were washed before the addition of anti-human interferon-γ (IFN-γ) (FITC) and interleukin-4 (IL-4) (Biotin) to the detection solution. The tertiary solution (a mix of secondary antibodies, anti-FITC-HRP and anti-Strep-AP), was added to the washed plates, and finally the Developer Solutions were used to develop the reaction. All solutions are supplied with commercial ELISpot kit (HIFNIL4-2 M/5, ImmunoSpot® Cleveland, OH). After signal development, plates were left upside down to dry overnight and subsequently scanned with the automated spot counter (ImmunoSpot® CTL S6 Analyzer, ImmunoSpot® Cleveland, OH) to enumerate spots. Each spot count in each well was quality checked by operators, who manually removed debris or fibers improperly counted as spots. Spot counts greater than the ULOQ (1500 spots) were reported as "Too Numerous To Count" (TNTC).

2.3. Validation protocol design

The validation design enabled us to distinguish the different components contributing to the overall variability of the assay, thereby guaranteeing the performance of the ELISpot assay in terms of its range and detection limits (LOD, ULOQ and LLOQ), Linearity, Relative Accuracy, Repeatability, Intermediate Precision, Specificity and Robustness. Each condition was run in triplicate by two different operators per day and repeated on two different days. Moreover, to assess the parameters of linearity, accuracy, precision, and robustness, three different lots of hPBMCs were used. This experimental design yielded a minimum of 9 determinations for each parameter, thus covering the reportable range for the procedure.

2.4. Statistical methods

To assess linearity and relative accuracy, the Geometric Mean (GM) was calculated from all replicates of IFN-γ and IL-4 spot counts. The arithmetic mean was calculated for the other validation parameters.

Values greater than the Upper Limit Of Quantitation (ULOQ) were classified as TNTC. TNTC values and values below the Lower Limit Of Quantitation (LLOQ) were replaced using estimation approach suggested by Persson and Rootzen (1977). The Persson-Rootzen method is applicable when at least two numerical values between LLOQ and ULOQ are available to directly calculate the statistic of interest. If all values are TNTC, the desired statistic resulted as TNTC, while it was Not Determined (N/D) in all other cases.

Regarding linearity and relative accuracy, no acceptance criteria are available in the literature. To validate the parameter of linearity, we therefore determined the acceptance criterion from the slope of the regression line. A bootstrap-type simulation consisting of 1000 replicates per dilution was carried out by means of the statistical software R (version 4.2.2, 2022-10-31 ucrt). For each dilution, the simulations consisted of repeated samplings with replacement of the observed 36 values (Table 1S and 2S, supplementary materials). The GM was calculated for each sample extracted and a linear regression model was estimated for each replicate and dilution; this yielded the distribution of the slopes. Finally, the 99% confidence interval of the distribution of the slopes provided the range of acceptance for linearity.

Relative Accuracy (RA) is the ratio of the observed GM to the predicted GM. In general, the acceptance range of RA can be expressed as

$$b^{-th} \leq RA \leq b^{th}$$

Table 1
Relationships among RA tolerances, thresholds, and base logarithms. RA.low and RA.up represent the lower and upper bounds of the relative accuracy acceptability criterion, respectively. Calculation was performed on a total of 36 biological replicates, obtained from four independent tests performed by two operators in two different days with three different lots of hPBMCs. In each test a triplicate of each dilution point was performed.

Base logarithm	Threshold	RA.low	RA.up
1.25	0.5	89.4%	111.8%
1.50	0.5	81.6%	122.5%
2.00	0.5	70.7%	141.4%
1.25	1.0	80.0%	125.0%
1.50	1.0	66.7%	150.0%
2.00	1.0	50.0%	200.0%
1.25	2.0	64.0%	156.2%
1.50	2.0	44.4%	225.0%
2.00	2.0	25.0%	400.0%

Table 2
SEB-stimulated IFN-γ and IL-4 dilutional linearity results: geometric mean (GM) per dilution was calculated from a total of 36 biological replicates, obtained from four independent tests performed by two operators in two different days with three different lots of hPBMCs. In each test a triplicate of each dilution point was performed. Log of the GM of SFU/well was calculated for SEB-stimulated IFN-γ and IL-4 dilutional linearity samples.

Cytokine	Sample (cell dilution)	LogDil	GM	LogGM
IFN-γ	1/1	0	1491.752	32.749
IFN-γ	1/1.25	1	1379.096	32.397
IFN-γ	1/1.56	2	1188.698	31.731
IFN-γ	1/1.95	3	908.837	30.528
IFN-γ	1/2.44	4	665.457	29.131
IFN-γ	1/3.05	5	487.843	27.740
IFN-γ	1/3.81	6	342.187	26.151
IL-4	1/1	0	365.960	26.452
IL-4	1/1.25	1	319.828	25.848
IL-4	1/1.56	2	232.941	24.427
IL-4	1/1.95	3	168.173	22.967
IL-4	1/2.44	4	121.441	21.508

where *b* is the base of logarithm and *th* is the threshold (Table 1).

To obtain a standard reference, we set a threshold of 1 on the absolute value of the logarithm of the RA. This choice is suitable for any logarithm base and represents a compromise between RA tolerance and the extension of the dilution range. The higher the threshold, the greater the difference between the observed and expected GMs, while low values place too great a restriction on the extension of the dilution range (Table 1).

3. Results

3.1. Protocol definition and establishment of range and detection limits

In this validation study, the following parameters were analyzed: range and detection limits (LOD, ULOQ and LLOQ), Linearity, Relative Accuracy, Repeatability, Intermediate Precision, Specificity, and Robustness.

A theoretical LOD of 15 spots/well was applied during the validation experiments, with LOQ = LOD*3.3, corresponding to 50 spots/well (Waerlop et al., 2022).

We defined the ULOQ on the basis of morphology and the ability to count clearly separated single spots, while considering that our automated reader system is able to count up to approximately 1500 spots per well.

The Coefficient of Variation (CV) % calculated among values was set as: Not Determined (N/D) when the number of spots per well was <50; ≤ 50% CV when the number of spots per well was between 50 and 150; ≤ 25% CV for a number of spots >150.

3.2. Linearity

To assess dilutional linearity, samples were tested in triplicates by two operators on two different days (two operators each day). The slope of the regression line between the Log of the GM of the 36 values obtained for IFN-γ and IL-4 was evaluated in relation to the Log of sample dilution. The assay was considered to have acceptable linearity if the slope of the regression line was between −1.41 and − 1.04. To establish linearity, a minimum of five concentrations (GM values different from 0) appropriately distributed across the range are required.

The results (Table 2) obtained on analyzing IFN-γ demonstrated the linearity of the assay for the first seven dilution points (1:1–1:3.81), which showed a slope of −1.132; regarding IL-4, the linearity of the assay was ascertained for the first five dilution points (1:1–1:2.44), which showed a slope of −1.277 (linearity parameters in Table 3 and regression lines in Fig. 1.)

3.3. Relative accuracy

The accuracy of an analytical procedure is typically tested by comparing measured results with expected values. To evaluate Relative Accuracy (RA), we used the GM calculated on replicates from Linearity data (Table 2) as measured results, while expected values were calculated by sequentially dividing the GM observed at 1/1 dilution by the 1.25-fold serial dilutions.

The formula used to evaluate accuracy was 100*(GM Observed / GM Calculated). The accuracy range was chosen according to the dilution factor used (1:1.25 dilution factor with a range of 80–125%). The analytical method was considered accurate, as the GM of the values obtained for IFN-γ and IL-4 fell within 80–125% CV in relation to the expected GM value calculated on the neat sample results.

As shown in detail in Table 4, SEB-stimulated hPBMCs met the RA criteria in the dilution range from 1/1 to 1/3.81 for IFN-γ and from 1/1 to 1/2.44 for IL-4.

3.4. Repeatability

Repeatability (also termed intra-assay precision) refers to the precision of the assay under the same operating conditions. To test for repeatability, we used the data from Linearity assay of SEB-stimulated and unstimulated samples at 1/1 dilution (CTL Medium supplemented with DMSO). The arithmetic mean was calculated per operator and replicate; this yielded 6 reportable values (O1-R1, O1-R2, O1-R3, O2-R1, O2-R2, O2-R3), which were used to calculate the CV% (Table 3S and 4S, supplementary materials). Acceptability criteria were set as follows: Not Determined (N/D) when the number of spots per well was <50; ≤ 50% CV when the number of spots per well was between 50 and 150; ≤ 25%

Table 3
SEB-stimulated IFN-γ and IL-4 dilutional linearity results: statistics of regression. The statistics of interest were the slope of regression line (Slope), the coefficient determination R-squared (R^2), the standard error (St.Err), the degrees of freedom (DF), statistics t (t stat), the lower bound of 95% confidence interval (CI95% lower) and the upper bound of 95% confidence interval (CI95% upper). Analysis was performed on a total of 36 biological replicates, obtained from four independent tests performed by two operators in two different days with three different lots of hPBMCs. In each test a triplicate of each dilution point was performed.

Parameter	IFN-γ	IL-4
Slope	−1.132	−1.277
R^2	0.960	0.980
St.Err	0.097	0.099
DF	5.000	3.000
t stat	2.571	3.182
CI95% lower	−1.382	−1.591
CI95% upper	−0.883	−0.962

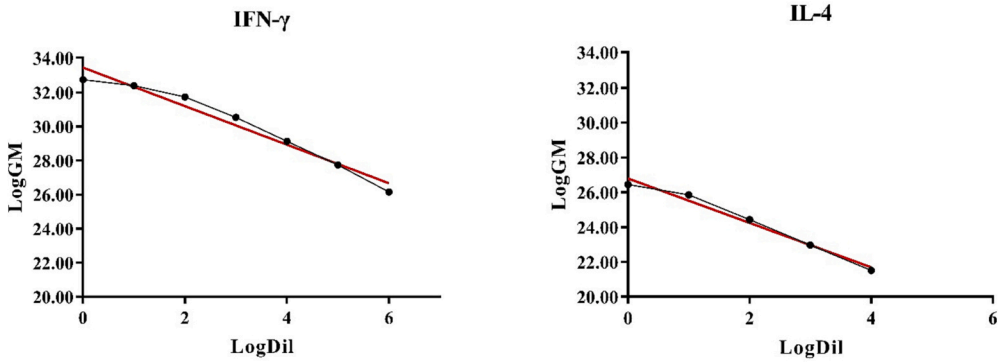

Fig. 1. SEB-stimulated IFN- γ and IL-4 dilutional linearity: regression lines between the Log of the Geometric Mean (LogGM) of SFU/well for IFN-γ and IL-4 was evaluated in relation to the Log of sample dilution (LogDil). Analysis was performed on a total of 36 biological replicates, obtained from four independent tests performed by two operators in two different days with three different lots of hPBMCs. In each test a triplicate of each dilution point was performed.

Table 4
SEB-stimulated IFN-γ and IL-4 relative accuracy results. Observed Geometric mean (Obs GM) per dilution was calculated from a total of 36 biological replicates, obtained from four independent tests performed by two operators in two different days with three different lots of hPBMCs. In each test a triplicate of each dilution point was performed. Expected Geometric Mean (Exp GM) was calculated from the Obs GM of neat sample, which was serially divided for the dilution factor (1,25). Relative accuracy was calculated as the ratio of the Obs GM to Exp GM and expressed as percentage.

Cytokine	Sample (cell dilution)	Fold Dilution	Obs GM	Exp GM	Relative accuracy
IFN-γ	1/1	1.00	1491.752	1491.752	100%
IFN-γ	1/1.25	1.25	1379.096	1193.402	116%
IFN-γ	1/1.56	1.56	1188.698	954.721	125%
IFN-γ	1/1.95	1.95	908.837	763.777	119%
IFN-γ	1/2.44	2.44	665.457	611.022	109%
IFN-γ	1/3.05	3.05	487.843	488.817	100%
IFN-γ	1/3.81	3.81	342.187	391.054	88%
IFN-γ	1/4.77	4.77	242.571	312.843	78%
IL-4	1/1	1.00	365.960	365.960	100%
IL-4	1/1.25	1.25	319.828	292.768	109%
IL-4	1/1.56	1.56	232.941	234.214	99%
IL-4	1/1.95	1.95	168.173	187.371	90%
IL-4	1/2.44	2.44	121.441	149.897	81%
IL-4	1/3.05	3.05	80.899	119.918	67%
IL-4	1/3.81	3.81	46.640	95.934	49%
IL-4	1/4.77	4.77	42.730	76.747	56%

CV for a number of spots >150/well.

As shown in Table 5, SEB-stimulated and unstimulated hPBMCs met the assay acceptance criteria for Repeatability for both IFN-γ and IL-4.

3.5. Intermediate precision

Intermediate Precision was evaluated in order to assess the variability of the analytical procedure over two different days. To this end, the data from Linearity assay of SEB-stimulated and unstimulated samples at 1/1 dilution were used. The %CV was calculated on the same 36 results obtained at 1/1 dilution in the test of Linearity (Table 3S and 4S, supplementary materials). Acceptability criteria were set as follows: Not Determined (N/D) when the number of spots per well was <50; ≤ 50% CV when the number of spots per well was between 50 and 150; ≤ 25% CV for a number of spots >150/well.

As shown in Table 6, SEB-stimulated and unstimulated hPBMCs met the assay acceptance criteria for Intermediate Precision for both IFN-γ and IL-4.

3.6. Specificity

The specificity of a given analytical procedure refers to the ability of the assay to measure the analyte of interest without any interference by other components present during operating steps of the procedure. We evaluated specificity by averaging the triplicates run by two operators on two different days (two operators each day) and testing 10 conditions overall (Table 5S and 6S, supplementary materials):

- CONDITION 1/2: wells in which hPBMCs were not plated were stimulated with medium or positive control but were processed with all the reagents necessary for the IFN-γ and IL-4 ELISpot assay; acceptability criteria were set as follows: IFN-γ and IL-4 spot counts ≤ LOD on stimulation with medium or positive control.
- CONDITION 3/4: 300,000 hPBMCs stimulated with medium or positive control were processed with all the reagents necessary for the IFN-γ and IL-4 ELISpot assay, with the exception of all detection reagents; acceptability criteria were: IFN-γ and IL-4 spot counts ≤ LOD on stimulation with medium or positive control.
- CONDITION 5/6: 300,000 hPBMCs stimulated with medium or positive control were processed with all the reagents necessary for the IFN-γ and IL-4 ELISpot assay, with the exception of the detection reagent for IFN-γ; acceptability criteria were: IFN-γ spot counts ≤ LOD on stimulation with medium or positive control; IL-4 spot counts ≤ LOD on stimulation with medium and ≥ LLOQ on stimulation with positive control.

Table 5
SEB-stimulated and unstimulated IFN-γ and IL-4 repeatability results. Using the 36 biological replicates obtained from neat sample tested in linearity assay, arithmetic mean was calculated per operator and replicate, obtaining 6 reportable values (O1-R1, O1-R2, O1-R3, O2-R1, O2-R2, O2-R3). CV% for repeatability was calculated as the ratio between the standard deviation and mean value.

Cytokine	Treatment	CV%
IFN-γ	Unstimulated	N/D
IFN-γ	SEB 1:1	0.67%
IL-4	Unstimulated	N/D
IL-4	SEB 1:1	5.47%

Table 6
SEB-stimulated and unstimulated IFN-γ and IL-4 intermediate precision results. Using the 36 biological replicates obtained from neat sample tested in linearity assay, arithmetic mean was calculated to assess intermediate precision. CV% for intermediate precision was calculated as the ratio between the standard deviation and mean value.

Cytokine	Treatment	CV%
IFN-γ	Unstimulated	N/D
IFN-γ	SEB 1:1	1.45%
IL-4	Unstimulated	N/D
IL-4	SEB 1:1	17.78%

- CONDITION 7/8: 300,000 hPBMCs stimulated with medium or positive control were processed with all the reagents necessary for the IFN-γ and IL-4 ELISpot assay, except for the detection reagent for IL-4; acceptability criteria were: IFN-γ spot counts ≤ LOD on stimulation with medium and ≥ LLOQ on stimulation with positive control; IL-4 spot counts ≤ LOD on stimulation both with medium and positive control.
- CONDITION 9/10: 300,000 hPBMCs stimulated with medium or positive control were processed with all the reagents necessary for the IFN-γ and IL-4 ELISpot assay; acceptability criteria were: IFN-γ spot counts ≤ LOD on stimulation with medium and ≥ LLOQ on stimulation with positive control; IL-4 spot counts ≤ LOD on stimulation with medium and ≥ LLOQ on stimulation with positive control.

As shown in Table 7, all tested conditions met the defined acceptance criteria for Specificity for both IFN-γ and IL-4.

3.7. Robustness

Robustness measures the capacity of an analytical procedure to be unaffected by minor variations in operating conditions. To assess robustness, we considered the impact of two independent conditions on the results: variability among hPBMCs (three different lots were compared) and incubation time of hPBMCs with stimuli (standard incubation time is 44 h; 44 ± 2 h conditions were also tested) (Table 7S and 8S, supplementary materials). The robustness of the assay (Table 8) was evaluated by calculating the arithmetic mean of 4 values obtained from same lot, operator, and replicate in two different plates and in two different days. The %CV was calculated on 9 averages and acceptability criteria were set as follows: Not Determined (N/D) when the number of spots per well was <50 or above the ULOQ (TNTC); ≤ 50% CV when the number of spots per well was between 50 and 150; ≤ 25% CV for a number of spots >150/well.

As shown in Table 8, SEB-stimulated and unstimulated hPBMCs met the assay acceptance criteria for Robustness for the two variables analyzed (hPBMC lots and incubation time) in both IFN-γ and IL-4 ELISpot.

4. Discussion

Enzyme-Linked ImmunoSpot (ELISpot) is an antigen-specific T-cell functional assay that can measure the proportion of T cells producing a specific cytokine (Slota et al., 2011). It is highly sensitive and has become routinely used for the immunologic monitoring both of specific T-cell responses to infectious diseases and of therapy-induced immune responses (Slota et al., 2011). Previously, ELISpot testing has been extensively performed in exploratory research, in which no rigorous assay performance validation is necessary.

Unlike immunogenicity assays that detect total or neutralizing antibodies, or ligand-binding assays that quantify drug and biomarker concentrations, there are no universally accepted procedures for validating ELISpot assays for regulated use in clinical trials to evaluate vaccine immunogenicity (Slota et al., 2011; Yang et al., 2022). This makes it difficult to establish LOD, cut-off points and acceptability criteria for the evaluation of assay performance. Here, we suggest a method of validating a double-color IFN-γ and IL-4 ELISpot assay by using uncharacterized hPBMCs from healthy donors and the superantigen SEB as a positive stimulus.

ELISpot is regarded as a highly sensitive assay, and its limit of detection (LOD) can be defined as the lowest number of spots that are clearly distinguishable from those seen in an unstimulated control well (O): LOD = O + 2SD of O. The Lower Limit Of Quantitation (LLOQ) can be defined as LOD*3.3. However, an excessively low LOD value may not be an optimal cut-off in a clinical study, as the samples used may display variable T cell responses. Indeed, hPBMCs may be sourced from healthy donors, vaccinees or convalescents affected by a specific pathogen. In these two latter groups, the immune system might be 'activated', resulting in higher spot counts than in unstimulated healthy donors. Hence, as healthy donors were used in our validation experiments, the LOD identified cannot properly be applied in clinical studies. For this reason, a theoretical LOD of 15 spots/well (Waerlop et al., 2022) has been set for validation experiments, and was applied in the analysis of clinical samples, with LLOQ = 50 spots/well. Mathematically, as the number of spots approaches zero, the %CV will increase dramatically. Maecker et al. (2008) carefully evaluated the precision of ELISpot and found that, below 50 spots per well (number of spots revised according to the number of cells seeded during the validation assays: 300,000 cells/well plated), %CV was not an effective measure of precision. The target precision is ≤50% CV when the number of spots per well is between 50 and 150 and, for donors with a mean number of spots >150 spots/well, a % CV ≤ 25% is then used.

The assay displayed linearity in the detection of both IFN-γ and IL-4, as a linear relationship between analyte (hPBMCs) concentration and response (spots/well) was found across the working range of the analytical procedure; this confirmed the suitability of the procedure for the intended use. In this kind of analysis, a plot of the data, the correlation coefficient or coefficient of determination, the y-intercept and the slope of the regression line should be provided. In our analysis of linearity, we tested three different lots of hPBMCs in order to take into consideration the high biological variability of hPBMC samples analyzed by means of ELISpot; indeed, each subject has a different immune system and, consequently, a different basal activity. The results obtained demonstrate that this technique allows a linear decay of the signal despite the variability of the cells examined. Obtaining good accuracy is essential not only to the use of the ELISpot assay in clinical practice but also to successful validation, as linearity can only be calculated on

Table 7
SEB-stimulated and unstimulated IFN-γ and IL-4 specificity results. Ten different conditions were tested, counts of SFU/well for SEB-stimulated and Unstimulated hPBMCs are reported as Geometric Means of 12 biological replicates obtained from four independent tests performed by two operators in two different days using one lot of hPBMCs. Each condition was tested in triplicate.

Cytokine	Condition	SEB-stimulated	Unstimulated
IFN-γ	(1/2) No hPBMCs	0.167	0.000
IFN-γ	(9/10) All reagents + hPBMCs	TNTC	2.750
IFN-γ	(3/4) No detection reagent	0.000	0.250
IFN-γ	(7/8) hPBMCs + det IFN-γ	TNTC	2.833
IFN-γ	(5/6) hPBMCs + det IL-4	0.000	0.667
IL-4	(1/2) No hPBMCs	0.000	0.000
IL-4	(9/10) All reagents + hPBMCs	520.833	0.250
IL-4	(3/4) No detection reagent	0.000	0.000
IL-4	(7/8) hPBMCs + det IFN-γ	0.000	0.000
IL-4	(5/6) hPBMCs + det IL-4	522.000	0.000

Table 8
SEB-stimulated and unstimulated IFN-γ and IL-4 robustness results. Two independent conditions were tested: three different lots of hPBMCs and three different incubation time. Arithmetic mean of 4 values obtained from same lot, operator, and replicate in two different plates and in two different days was calculated. CV% for robustness was calculated as the ratio between the standard deviation and mean value among 9 replicates.

Cytokine	Parameter	Treatment	CV%
IFN-γ	LOT	Medium	N/D
IFN-γ	LOT	SEB	5.12%
IFN-γ	TIME	Medium	N/D
IFN-γ	TIME	SEB	N/D
IL-4	LOT	Medium	N/D
IL-4	LOT	SEB	14.69%
IL-4	TIME	Medium	N/D
IL-4	TIME	SEB	11.77%

dilution points that are accurate. The choice of the dilution factor used is also essential, in order to have a high number of points for the calculation of the parameters; indeed, the higher the spot count, the more accurate the point will be, and, above all, the more dilution points will be above the LLOQ (50 spots/well). Our results are in line with this concept. Indeed, in the case of IFN-γ, which displayed a greater number of spots per well, accuracy was maintained at seven dilution points. By contrast, IL-4 showed much lower values and displayed accuracy at only five dilution points; nevertheless, this was sufficient to establish the linearity of the assay.

We demonstrated that the analytical procedure used for validation was precise, as all acceptability criteria established for repeatability, intra-assay precision and intermediate precision were met. To investigate potential sources of variability, we considered different days, different lots of hPBMCs and different operators. In both unstimulated and SEB-stimulated samples, the assay proved consistent and accurate for both cytokines analyzed (IFN-γ and IL-4) in the three lots of hPBMCs, over two different days of testing and independently from the operator performing the test; these findings provide evidence of its precision.

With regard to the specificity of this analytical method, 10 different conditions were tested in order to ascertain that no unspecific spot counts were generated by extraneous substances, or the reagents used during the assay procedure. This approach reveals possible non-specific colorimetric signals (false-positive spots) due to the absence of cells or detection substrate in the wells. The results obtained demonstrated the specificity of the spots in each stimulated well, for both cytokines under analysis.

The robustness of an analytical procedure is a measure of its capacity to meet the expected performance requirements during normal use. We assessed the impact of varying independent assay parameters on the results by testing three different lots of hPBMCs for a standard incubation period (44 h) ±2 h. The results obtained revealed that, for both IFN-γ and IL-4, the number of spots developed after stimulation with SEB showed a low coefficient of variability. This confirmed the robustness of the assay and demonstrated that perturbing the system, whether by changing the batch under analysis or changing the incubation time, does not alter the reliability of the ELISpot technique.

5. Conclusions

T cells play a key role in fighting bacterial or viral infection and in the generation of an immunological memory after recovery from many infectious diseases. An increasing number of techniques, including ELISpot, are therefore being used to study cell-mediated immunity. The increased use of ELISpot highlights the need for a recognized method of validation that can be applied to clinical studies that assess T cell activity.

IFN-γ and IL-4 are commonly studied in order to evaluate immune system skewing towards T helper 1 or 2 phenotype, respectively. We implemented a validation protocol for the analysis of these two cytokines by means of the ELISpot technique. Within this validation, the classical parameters of analytical procedures were evaluated: detection and quantitation limits (LOD, LLOQ, ULOQ), Linearity, Relative Accuracy, Repeatability, Intermediate precision, Specificity and Robustness.

All the results obtained in this validation study met the established acceptability criteria. Overall, the assay proved to be Linear, Accurate, Repeatable, Precise, Specific and Robust. We can conclude that the ELISpot assay is able to reliably detect and quantify IFN-γ and IL-4 expression in hPBMCs after stimulation with stimulating agents. It may therefore constitute a powerful analytical tool for assessing T cell responses towards different antigens of interest.

Throughout the recent COVID-19 pandemic, immunological data from both convalescent patients and vaccinated subjects highlighted the key role played by T cell immune responses to SARS-CoV-2 and their contribution to protection from disease progression (Zhang et al., 2022). In this context, validated methods of assessing T cell functionality, such as ELISpot, and also flow cytometry, may constitute a powerful tool for the development of new drugs and the evaluation of novel vaccines against infectious diseases (Zhao et al., 2021).

Funding

This research received no external funding.

Declaration of Competing Interest

FD, DDT, CM, PP, CS, GF, IF, FV, IR, GP, MM and AM are employed by VisMederi Srl; EM is founder and Chief Scientific Officer of VisMederi Srl and VisMederi Research Srl; IM is employed by University of Siena.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2023.113588.

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