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Qualification of an IFN-γ ELISpot Assay for Monitoring Antigen-Specific T-Cell Responses in Clinical Trials of T-cell-inducing Vaccines

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Abstract

The IFN-γ ELISpot assay is a sensitive, widely used platform for quantifying antigen-specific T-cell responses and serves as a benchmark assay for immunomonitoring in vaccine trials. Despite its broad adoption, clinical use requires formal qualification to ensure reproducibility, reliability, and fitness for use. However, standardised, qualified ELISpot assays remain scarce for clinical trials in Africa. We present an IFN-γ ELISpot assay for measuring antigen-specific T-cell responses in human PBMCs qualified under Good Clinical Laboratory Practice (GCLP) standards. Assay performance was evaluated using predefined parameters, including linearity, precision, specificity, detection capacity, robustness, and reportable range, across multiple PBMC stimulation conditions. Linearity was assessed across six different cell inputs per well using the CEFSX stimulation. Precision was assessed via the predefined coefficient of variation (CV) threshold of ≤ 35% across the evaluated stimulation conditions. Robustness was assessed using a one-factor-at-a-time approach across different post-thawing and incubation times for the three stimulations. Strong linearity was observed across the donor samples (R^2=0.90-0.98). All samples met the precision acceptance criteria across all tested conditions, demonstrating performance consistent with accepted standards in the field. Intermediate precision was similarly robust, with minimal operator-dependent variability and 94.4% of replicate values falling within the predefined acceptance limits. There was no significant effect of different post-thawing and incubation times on the assay output (p-value >0.05), and the assay achieved 100% specificity. Overall, the qualified assay demonstrated strong analytical performance and readiness for immunomonitoring in clinical vaccine trials. Importantly, this work establishes our lab as a GCLP-certified African site for clinical vaccine trial immunomonitoring of IFN-γ ELISpot assays.

Keywords: ELISpot, Cytokine, Antigens, Africans, GCLP

Abbreviations

ELISpot	–	Enzyme-linked ImmunoSpot
GCLP	–	Good Clinical Laboratory Practice
CEFSX	–	Cytomegalovirus Epstein-Bar virus Influenza A virus (Flu) SARS-CoV-2 Extended
PHA	–	Phytohemagglutinin
SFU	–	Spot Forming Unit
NC	–	Negative Control
IFN-γ	–	Interferon-gamma
LoQ	–	Limit of Quantification
LLoQ	–	Lower limit of Quantification
ULoQ	–	Upper limit of Quantification
LoB	–	Limit of Blank
LoD	–	Limit of Detection
PLWoH	–	People living without HIV
PLWH	–	People living with HIV
PBMC	–	Peripheral Blood Mononuclear Cell
CV	–	Coefficient of Variation

1. Introduction

One of the most widely used functional immunological assays for quantifying antigen-specific T-cell responses in human clinical samples is the interferon-gamma enzyme-linked Immuno-Spot assay (IFN-γ ELISpot). The assay provides a sensitive measure of antigen-responsive T-cells by detecting cytokine secretion following stimulation with cognate peptide pools, proteins, viral lysates or other immune stimuli (1, 2). This makes the IFN-γ ELISpot assay particularly important in vaccine clinical trials, where robust data on key parameters, such as the reproducibility and specificity of immune responses, are essential for evaluating whether a vaccine can reliably elicit T-cell responses (1-7).

Although the IFN-γ ELISpot assay is sensitive and has been broadly utilized in several studies, its performance can be affected by analytical and operational factors, such as input sample cell viability due to poor cryopreservation and thawing procedures, incorrect cell input number, antigen concentration, incubation time, plate development, spot-counting techniques, operator effect, reagent lots, and laboratory-specific procedures (8-10). Therefore, the assay's qualification provides documented evidence that the method and data generated are accurate, reproducible, reliable, and fit for purpose under defined conditions (1, 4, 8, 10). Furthermore, the need for assay qualification is reinforced by regulatory and quality frameworks governing clinical trial laboratories. Good Clinical Laboratory Practice (GCLP) provides a quality framework for laboratories generating data from human clinical trial specimens and emphasizes reliable, auditable, and reproducible laboratory results (11).

Africa bears a disproportionate burden of infectious diseases, including HIV, tuberculosis and emerging viral infections, for which T-cell immunity may be critical (12-16). Although many vaccine and therapeutic trials are conducted on the continent, most sample analyses are performed in laboratories in the Global North (17, 18). Factors such as limited laboratory infrastructure, shortages of trained personnel, and gaps in quality management systems have historically constrained African vaccine trials. Work by the International AIDS Vaccine Initiative (IAVI) at clinical research centres has demonstrated that targeted capacity

building can establish accredited laboratories, train personnel, and implement qualified assays that generate reliable clinical trial data (3). Therefore, African scientists must also contribute substantially to assay development, qualification and interpretation in vaccine clinical trials and designs. Documenting African scientist-led assay qualification will therefore promote locally validated immunomonitoring tools, strengthen African scientific leadership, address gaps in vaccine-trial evaluation, and support more equitable vaccine development.

In this paper, we present the qualification of an IFN-γ ELISpot assay for measuring antigen-specific T-cell responses in human clinical samples for use in vaccine trial immunomonitoring in African settings, with the specific objective of evaluating the performance parameters such as linearity, precision, specificity, detection capacity, robustness, and reportable range of the IFN-γ ELISpot assay.

2. Materials and Methods

2.1 Experimental Design, Procedure, Test and Control Materials

2.1.1 Study Participants and PBMC Samples

A total of 21 peripheral blood mononuclear cell (PBMC) samples obtained from people living with HIV (PLWH) and people living without HIV (PLWoH) were used for the assay qualification. These samples were obtained from a well-characterised cohort (19), collected in accordance with approved ethical procedures, processed and cryopreserved using established biorepository protocols for PBMC isolation and cryopreservation.

2.1.2 *Cell Thawing, Counting and Resting*

Cryopreserved PBMCs were retrieved from the liquid nitrogen biorepository and transported to the tissue culture laboratory on dry ice. Cells were rapidly thawed by holding the lower half of each cryovial in a 37°C Labotec Scientific-103 water bath for 30-45 seconds with intermittent gentle flicking, consistent with PBMC thawing protocols (20, 21). Immediately after thawing, the cell suspension was transferred into a 15 mL Falcon tube containing 9 mL of pre-warmed serum-free CTL Test Medium (Cellular Technology Limited; catalogue no. CTLT-005). The tube was gently inverted 3-4 times and centrifuged at 754 x g for 6 minutes. The supernatant was discarded, and the pellet was gently resuspended in 1 mL of CTL-Test complete medium, then brought to a final volume of 10 mL with cell culture medium. The tube was gently inverted 3–4 times and centrifuged again at 754 × g for 6 minutes. This second wash step was important to remove any excess DMSO. Following centrifugation, the supernatant was discarded, and the pellet was resuspended in 10 mL of medium. Cell viability was assessed by mixing 10 µL of the well-mixed PBMC suspension with 90 µL of 0.4% trypan blue stain (Gibco; catalogue no. 15250-061) to obtain a 1:10 dilution. After brief vortexing, 10 µL of the mixture was loaded into a haemocytometer in duplicate (Kova Plastics- Alltrista, Fisher Scientific, U.S.A.), avoiding air bubbles. Viable cells were manually counted in three haemocytometer squares per chamber using a ZEISS counting microscope, typically with a 10x or 40x objective, consistent with the trypan blue dye-exclusion principle (22). Viability was calculated as (viable cells/total

cells counted) × 100, and a post-thaw viability of ≥ 70% was required for assay use. The use of viable PBMCs is essential in IFN-γ ELISpot assays because assay performance depends on the functional capacity of antigen-responsive T-cells to secrete cytokine following stimulation (23). The remaining PBMC suspension was rested for 2 hours at 37°C in a 5% CO_2 incubator before ELISpot assay setup, to maximize PBMC recovery and viability under controlled conditions (20).

2.1.3 *Peptide Stimulations*

The PBMC samples were stimulated with two peptide pools, namely: Optimal (9-12mer HIV-Gag optimal peptide pools) and PepMix CEFSX Ultra SuperStim peptide pools. The HIV optimal peptide pool was the HIV-specific test stimulus. The PepMix CEFSX Ultra SuperStim pool (JPT Peptide Technologies; catalogue no. PM-CEFSX-2), comprised of 202 known viral peptide epitopes from cytomegalovirus, Epstein–Barr virus, influenza virus and other common infectious agents, served as the recall-antigen positive control and is widely used in T-cell immunomonitoring assays to assess functional antigen responsiveness in human PBMCs (9, 24). Both peptide pools were used at a final assay concentration of 1 µg/mL per peptide. Peptides were prepared using appropriate solvents and diluted in assay medium immediately prior to use. To preserve peptide stability and minimize freeze-thaw-related degradation, peptide aliquots were stored according to the manufacturer's instructions and thawed before use.

2.1.4 Positive Control

Phytohemagglutinin (PHA; Merck; catalogue no. 431784-5MG) was used as the positive control at a final concentration of 0.05 mg/mL and was tested in triplicate for each PBMC sample on each plate. PHA is a mitogenic stimulant used to confirm the functional integrity of PBMC samples and the capacity of viable lymphocytes to mount a robust cytokine-secreting response. The inclusion of a positive control is an established component of ELISpot assay

design and supports the interpretation of negative or low antigen-specific responses by confirming that the test cells are functionally viable (25).

2.1.5 Negative Control

Unstimulated wells containing PBMCs in assay medium only served as the negative control and were tested in triplicate for each sample. These wells were included to measure background IFN-γ spot formation, estimate baseline assay noise, and support calculation of net antigen-specific responses. Negative or no-antigen control wells are recommended in IFN-γ ELISpot assay protocols because background cytokine secretion can vary among PBMC donors and may influence interpretation of responses (26).

2.1.6 Media, Supplements, Plate Layout and Replicates

Serum-free CTL Test Medium (Cellular Technology Limited; catalogue no. CTLT-005), supplemented with L-glutamine (Gibco; catalogue no. 25030024) and penicillin–streptomycin (Gibco; catalogue no. 15140-122), was used as the assay medium. The medium was prepared fresh prior to use. Sterility, storage conditions, and expiration dates were verified and noted before each experiment. A predefined, consistent plate layout was used across all experiments to minimize positional bias and improve comparability between plates. The arrangement of test stimuli, positive controls, negative controls, and cell-density conditions was maintained throughout the experiments. Harmonized ELISpot assay procedures and standardized plate layouts are important for reducing inter-plate variability and improving reproducibility in immune-monitoring studies (9). PBMCs were seeded at 100,000 cells/well for all qualification experiments except linearity for which cell densities of 25,000, 50,000, 75,000, 100,000, 150,000 and 200,000 cells/well were used. Plates were incubated for 18–24 hours at 37°C in 5% CO_2. Each test and control condition was plated in triplicate. The triplicate wells were used to assess intra-assay variability, background consistency, and precision across assay conditions.

2.1.7 IFN-γ ELISpot Assay

The IFN-γ ELISpot assay was used to quantify antigen-specific T-cell responses in human PBMCs following stimulation with the CEFSX Ultra SuperStim and HIV optimal peptide pools. IFN-γ ELISpot assays were performed using the T Cell Single Color Enzymatic Human IFN-γ ImmunoSpot kit (Cellular Technology Limited, USA; catalogue no. hIFNgp-1M/5). The kit provided white 96-well PVDF-bottomed plates (pre-coated with human IFN-γ capture antibody), biotinylated anti-human IFN-γ detection antibody, streptavidin–alkaline phosphatase and blue substrate components.

2.1.8 Assay Plate Washing, Detection and Development

Following stimulation, plates were washed twice with PBS, then twice with PBS containing 0.05% Tween-20 (200 µL/well per wash). Biotinylated anti-human IFN-γ detection solution (20 µL antibody in 10 mL Diluent B) was added at 80 µL/well and incubated for 2 hours at room temperature. After incubation, the plate was washed three times with 0.05% Tween-20/PBS, and 80 µL/well of streptavidin–alkaline phosphatase solution (5 µL in 10 mL of Diluent C) was added, followed by incubation for 30 minutes at room temperature. Plates were washed twice with 0.05% Tween-20 in PBS and twice with distilled water. Following the wash steps, blue developer (160 µL S1, 160 µL S2, and 92 µL S3 in 10 mL Diluent Blue) was added to each well at 80 µL/well and incubated for 5 minutes at room temperature. Development was stopped by gently rinsing the plate with tap water three times. Notably, the plates were blot-dried after each wash step. The plates were air-dried overnight according to the manufacturer's instructions.

2.1.9 ELISpot Plate Imaging and SFU Enumeration

After overnight air-drying, each IFN-γ-secreting cell was visualized as an individual spot. ELISpot plate reading and spot enumeration were performed using an ImmunoSpot S6 Analyzer (Cellular Technology Limited; S6 Ultimate, model S6ULM2-009013) with ImmunoCapture software version 7.0.21.7, and exported SFU values were used for downstream statistical analysis. The primary assay readout is the spot-forming units (SFU),

expressed either as raw SFU per well or normalized as SFU per 10^6 PBMCs, depending on the qualification objective.

2.2 Statistical Analyses

The ELISpot assay performance was evaluated using background-subtracted Net SFU per 10^6 PBMC under HIV optimal, CEFSX, and PHA stimulation conditions. **Linearity**, defined as the ability of the assay response to increase proportionally with cell input, was assessed in seven CEFSX-stimulated samples tested in triplicate across six cell concentrations (25,000, 50,000, 75,000, 100,000, 150,000, and 200,000 cells per well) using linear regression, with a coefficient of determination of $R^2 \geq 0.90$ considered acceptable. **Accuracy**, which is defined as the closeness of observed SFU to predicted SFU, was evaluated using percentage recovery and bias, with 80 – 120% recovery and absolute bias $\leq$ 20% required (27, 28). **Precision**, defined as agreement among repeated SFU measurements, included repeatability, intermediate precision across operators and samples, and replicate agreement, with %CV $\leq$ 35% and at least 90% of replicates meeting the three-fold criterion (29). **Variability** attributable to sample, operator and residual effects was estimated using mixed-effects models and likelihood-ratio testing (LRT). **Specificity**, defined as the assay's ability to correctly classify samples from PLWoH, was assessed using HIV optimal SFU responses from six HIV-negative and eight HIV-positive donors (26, 30). **The limit of detection (LoD)**, defined as the lowest response distinguishable from background, was set equal to the limit of blank (LoB), defined as the highest apparent response expected from assay background, estimated as the empirical 95th percentile of all the negative-control wells. **The limit of quantification (LoQ)**, defined as the response interval measurable with acceptable linearity, precision, and accuracy, was determined from the CEFSX cell input experiments, with the LLoQ and ULoQ representing the lowest and highest concentrations meeting all criteria. **Robustness**, defined as the ability of the assay to remain reliable following small deliberate procedural changes, was assessed by comparing 2-hour versus 6-hour post-thaw resting times and 18-hour versus

24-hour incubation periods. Analyses were performed using R (version 4.5.2) and GraphPad Prism 11 with SFU values generated by the CTL ImmunoSpot reader.

3. Results

3.1 Linearity

To evaluate linearity, six cell input concentrations were assessed using the CEFSX peptide pool. The test was performed by a single operator, who evaluated the serial dilutions across 7 samples, each plated in triplicate. An ordinary least squares (OLS) regression model of raw SFU was fitted to cell input. R^2, correlation coefficient (r), slope (with 95% CI), lack-of-fit, residual diagnostics, and 95% prediction interval bands for visualization were assessed. All seven samples met the predefined acceptance criterion of $R^2 \geq 0.90$, a threshold previously applied in the validation of a human IFN-γ ELISpot assay (31). The R^2 values across all 7 samples ranged from 0.92 to 0.98, and the experimental plate layout and linear regression outputs are shown in **Figure 1A-B**. These values indicate that approximately 92%-98% of the observed variation in ELISpot responses across cell inputs was explained by the linear regression models, demonstrating a strong linear relationship between cell input number and SFU response.

3.2 Repeatability

In this ELISpot assay report, repeatability was assessed using the coefficient of variation (CV) of SFU values obtained when the same sample was plated in triplicate wells on two different plates by the same operator. Five different samples were used to assess repeatability across the three conditions: HIV optimal, CEFSX, and PHA, with each condition plated in triplicate on two plates (n = 6). All five samples had CV values below the predefined 35% acceptance threshold (9, 32). This indicates that replicate measurements exhibited limited within-run variability and supports the assay's consistency under the same operating conditions. **Figure 2** provides the results of the samples assessed for repeatability for all three conditions.

3.3 Limit of Quantification (LoQ) and Range Analysis

Precision was assessed across serial cell input levels for each of the seven samples using the CEFSX condition. For each cell concentration, the mean observed SFU, predicted SFU (from the linear regression model), %Recovery, %Bias, and %CV were calculated (**Table 1)**. The LoQ was defined as the lowest and highest cell input levels at which the Linearity ($R^2 \geq 0.90$), Precision (%CV < 35%), and Accuracy (%|Bias|<20%) criteria were met. **Figure 3** below shows the LoQ results for each cell input.

Using the ≤ 20% bias and ≤ 35% CV criteria, the only cell-input range where all seven donor samples simultaneously met both requirements was 150,000–200,000 cells. However, when examining samples individually, six of seven samples met both criteria at 100k cells, with one exception (Sample 7), which showed a 36.2% bias. The assay range was assessed using the same dataset obtained from the linearity and LoQ analyses (experiments), respectively. Based on this dataset, the ELISpot assay demonstrated consistent linearity, precision, and accuracy between 100,000 and 200,000 cells per well, within which the measured performance met the predefined thresholds of %CV ≤ 35% and absolute bias ≤ 20%, with a linearity of $R^2 \approx 0.93$. The 35% CV limit was selected as a study-specific precision criterion guided by similar studies on IFN-γ ELISpot assay qualification to impose a tighter numerical threshold than the published precision benchmark of CV ≤ 40% (8, 9). This interval therefore represents the validated analytical range, bounded by the lower and upper working-cell inputs. A summary of the 100,000–200,000 cell inputs that determine the analytical range is presented in **Table 2** below. The R^2 in the table indicates that the assay remains linear over the 100,000 to 200,000 cell input range.

3.4 Robustness

The robustness of this ELISpot assay was assessed by varying one factor (or parameter) at a time (OFAT). Two assay parameters previously identified as critical (9) were evaluated: Post-thaw cell resting time and plate incubation time. Resting times of 2 and 6 hours, and plate incubation times of 18 and 24 hours (representing the lower and upper bounds of the typical

operational ranges) were assessed. Three samples were assessed for post-thaw cell resting time, and 4 samples were used to assess plate incubation time. **Tables 3** and **4** summarise the SFU per 10^6 PBMCs across 3 replicates and the mean for each condition across samples. **Figures 4** and **5** display the %CV for each sample across the two resting and incubation time conditions for all stimulation conditions, providing a direct assessment of assay precision. The %CV threshold of < 35%, previously used to define the quantifiable range, was also applied here to assess whether variation remained within control limits for each condition. In addition, **Figure 6** presents the results of the student's t-test comparing mean SFU values between the two resting/incubation times to evaluate for statistical significance. The lack of significant differences ($p>0.05$), together with acceptable %CV values, collectively support the robustness of the assay to the tested variations in resting and incubation times (31, 33, 34).

As shown in **Figure 6A**, extending post-thaw resting from 2 to 6 hours did not significantly affect assay output. Similarly, **Figure 6B** confirms that SFU responses remain stable whether plates are incubated for 18 or 24 hours.

3.5 Intermediate Precision (Inter-operator variability)

For intermediate precision testing (intra-operator variability), six samples across the three antigen conditions were plated in triplicate by 2 operators. The samples were blinded to the operators. Two different operators in the lab performed these assays on two different days. Using the 3-fold-of-the-mean criterion, the acceptance criteria from optimization experiments indicated that the net SFU should be within 3-fold of the mean, with a 10% error rate (29). Replicate was assessed using a threefold outlier criterion, whereby an individual SFU value was considered discordant if it was more than threefold higher than or less than one-third of the mean of the replicate wells (29). **Table 5** below summarizes the SFU response of the intermediate precision experiment. The results in **Table 6** show that 94.4% of the Net SFU were within 3-fold of the mean, with an acceptable error rate of 5.6%.

A linear mixed-effects model (LMM), which partitions variance into fixed and random components to account for both operator and sample variability, was used to assess operator differences within each condition (**Table 5**). Operator effect was evaluated using a likelihood ratio test (LRT) by comparing a model with sample as the only random effect to another one that included both sample and operator as random effects. The two nested models were compared using the LRT to evaluate whether the inclusion of operator significantly improved model fit (35, 36). For HIV optimal and CEFSX responses, the operator effect was not statistically significant ($p = 0.125$ and $p = 0.240$, respectively), indicating no meaningful operator-driven variability. In contrast, for the PHA condition, the LRT indicated a statistically significant operator effect ($p = 0.033$), suggesting that operator contributed measurably to the variability in PHA-induced responses. Although the PHA condition showed a statistically significant operator effect, this may be due to PHA's strong stimulatory response, in which high spot counts make small technical fluctuations statistically detectable without reflecting true biological differences. Supporting this interpretation, operator-related %CV and variance contributions were negligible, while sample variance dominated, reflecting biological differences attributable to factors such as HIV status and individual immune responses (**Table 7)**. Additionally, PHA Net SFU replicates remained within the 3-fold-of-mean acceptance threshold for both operators (**Table 6**). Overall, the findings demonstrate minimal operator-related impact across all assay conditions.

3.6 Limit of Detection (LoD)

The LoD was determined following the principles outlined in the CLSI EP17-A2 statistical guidelines (37). First, the limit of blank (LoB) was established by analyzing a large number of negative control (NC) wells to accurately characterize the background signal distribution (38). All triplicate SFU values from NC wells across multiple experiments were pooled and included in the LoD assessment. As shown in **Figure 7**, the distribution of NC SFU values exhibited noticeable skewness. Therefore, in accordance with CLSI recommendations for non-Gaussian data, the non-parametric LoB calculation was applied (i.e., using the empirical 95th percentile

of the NC distribution). The LoD was subsequently derived from this non-parametric LoB, ensuring a robust, distribution-appropriate estimate of the assay's detection capability.

The limit of blank (LoB), defined as the 95th percentile of responses in negative control (NC) wells, was estimated from the NC wells tested across plates, operators, and assay conditions, thereby providing an overall estimate of assay background. Because suitable low-positive responders were unavailable, an independent limit of detection (LoD) could not be determined, therefore, the LoB was provisionally used as the operational LoD. This yielded a value of 8 spot-forming units (SFU)/well, equivalent to 80 SFU per 10^6 PBMC (29). This LoD should be distinguished from the positivity threshold of 4 SFU/well established during the specificity assessment, because the two thresholds were derived using different criteria and serve different analytical purposes.

3.7 Specificity (SPE)

In this qualification, specificity was proven by testing HIV-negative donor PBMCs (PLWoH) and people living with HIV (PLWH). Samples were stimulated with the HIV optimal peptide pool. Fourteen samples (6 PLWoH and 8 PLWH) were used to evaluate specificity in this assay qualification. All 6 PLWoH samples showed low SFU responses per well (0-2), consistent with the expected absence of HIV-specific T-cell activity (**Figure 8**). The Net SFU values were used to define the upper limit of the negative response range, also known as the limit of blank (LOB), from which the positivity threshold was derived. Using this negative population, the assay's positivity threshold was set at the 95th percentile of HIV-specific responses. Then, the minimum number of spots that can be reliably distinguished from the lowest number at a 95% confidence level was determined using the power-definition approach (assuming a Poisson distribution). The positivity threshold was set at 4 SFU per well (or 40 SFU per 10^6 PBMCs).

The specificity results in **Table 8** above indicate that the assay is 100% specific, with a false positive rate of 0%. This approach ensures that the positivity cut-off is data-driven,

biologically justified, and based on a well-characterized negative population, thereby strengthening the specificity assessment of the ELISpot assay.

In summary, the method-performance qualification results (**Table 9**) demonstrated that the IFN-γ ELISpot assay achieved strong linearity, acceptable accuracy and precision, high specificity, reliable detection capability, and robust performance within the established analytical range. Published qualification and validation studies provide a methodological basis for evaluating the analytical performance of our IFN-γ ELISpot assays. In a study of an IFN-γ ELISpot assay to evaluate cell-mediated immunity against severe acute respiratory syndrome coronavirus 2, strong linearity (R^2 = 0.98–0.99) and a mean inter-assay CV of 25.4% were reported for linearity and precision, respectively (39). Similarly, a qualification study of an ELISpot assay to measure influenza-specific cell-mediated immunity within the FLUCOP consortium demonstrated a mean inter-assay CV of 20% above the quantification limit and a linear range of 120,000–360,000 cells/well (9). In addition, a 2007 study to detect HIV vaccine-induced cell-mediated immunity in HIV-seronegative clinical trial participants established positivity criteria associated with a false-positive rate of ≤1% (40), while Patton and colleagues reported an inter-assay CV below 31% in an IFN-γ ELISpot assay for detecting human Respiratory Syncytial Virus and also reported detection and quantification limits of 21 and 63 SFC/10^6 PBMC (41). Together, these findings support the assessment of precision, specificity, linearity, and detection limits under clearly defined ELISpot assay conditions. Collectively, our findings confirm that the assay is reliable, reproducible, and fit for purpose for measuring antigen-specific T-cell responses in cryopreserved human PBMCs during vaccine clinical trials.

4. Discussion

We conducted a rigorous IFN-γ ELISpot assay qualification guided by published ELISpot validation recommendations and Good Clinical Laboratory Practice principles for assays used in human vaccine clinical trials (8, 11). We now have a standardised, robust and precise ELISpot assay protocol that delivers reliable and highly reproducible results for testing

antigen-specific T-cell responses in PBMCs. The data reported here across all study parameters, including linearity, precision, sensitivity, specificity, robustness, and operator variability, support the assay's suitability for high-quality immune monitoring and downstream clinical or vaccine-related applications.

One of the analytical performance and quantitative reliability findings from this ELISpot qualification analysis is a strong positive linear relationship between cell input and spot-forming units (SFUs). The coefficient of determination R^2 confirms that the assay behaves predictably across serial dilutions and supports proportional quantification of antigen-specific responses. Importantly, the preservation of linearity within the operational range indicates that assay performance is within realistic laboratory workflows (1, 42, 43). Repeatability analysis further supported the reliability of the ELISpot assay, as all evaluated stimulation conditions met the acceptance criterion, which is consistent with analytical validation principles that use CV to quantify within-assay precision and with ELISpot validation studies showing that replicate SFU responses can remain acceptably precise despite expected donor-to-donor biological variability (1, 32, 43, 44).

Furthermore, the broader performance assessment of the cell input range is consistent with CLSI EP17-A2 and ICH M10 principles, which support defining assay performance based on integrated evidence and overall method reliability rather than absolute uniformity across all individual specimens (37, 45).

Evidence from the robustness testing showed that moderate variations in post-thaw resting times and plate incubation durations did not significantly affect the ELISpot assay responses. In the present qualification dataset, responses remained statistically comparable across the tested conditions and replicate variability stayed within acceptable limits, indicating that the assay maintained stable performance despite operational variations. This is consistent with bioanalytical validation principles that robust methods are those capable of producing reliable and consistent results under minor procedural variations and that PBMC handling and resting conditions should therefore be evaluated during assay qualification (44-46). These

findings highlight the assay's potential for reliable implementation in multicentre clinical studies, where minor operational and procedural variations are inevitable.

The intermediate precision analyses revealed minimal inter-operator variability. The linear mixed-effects modelling further confirmed that sample-to-sample biological differences were the dominant source of variability, accounting for the vast majority of total variance, whereas operator effects were negligible for the HIV optimal and CEFSX conditions. However, a statistically significant operator effect was detected for PHA stimulation, which reflects the high signal intensity associated with mitogenic activation rather than meaningful analytical inconsistency. In high-response conditions such as PHA, small technical differences can become statistically detectable without being biologically relevant (32, 47). The low operator-associated %CV values and maintained acceptance thresholds reinforce this interpretation and indicate that assay reproducibility remains strong across personnel.

The detection capability analyses (LoB and LoD) ensure a statistically grounded threshold for detecting positive responses above background noise. Similarly, the specificity analysis demonstrates clear discrimination between samples from PLWH and PLWoH using the HIV-optimal peptide pool. The data-driven positivity threshold yielded an excellent specificity in the evaluated cohort. This approach strengthens confidence that detected responses represent genuine antigen-specific activity rather than background. This is a critical consideration for immunogenicity studies and vaccine trials.

Limitations and future considerations

Although the qualification results collectively position the assay as a reliable tool for quantifying antigen-specific T-cell responses in settings where precision and reproducibility are essential, some limitations should be acknowledged. The sample size for certain qualification parameters such as robustness and specificity was modest, and broader validation across additional donor populations may further refine thresholds. Additionally, while the assay performed strongly under controlled conditions, future multicentre validation would be valuable

to confirm transferability across different laboratory environments. Future work may also benefit from integrating longitudinal approaches to better characterize biological variability over time and to distinguish technical from immunological drift in large clinical studies.

Conclusion

The results from this qualification study collectively support our IFN-γ ELISpot assay method as a reliable platform for quantifying antigen-specific T-cell responses in settings where precision, reproducibility, and biological interpretability are essential. Overall, the qualification findings demonstrate that the assay exhibits strong linearity, acceptable precision, robust operational performance, high specificity, and reliable detection capacity within a clearly defined analytical range that overlaps those reported for other qualified IFN-γ ELISpot assays (9, 32, 43, 48). Successful qualification of the IFN-γ ELISpot assay under GCLP standards, with certification by the Quality Assurance for Duke Vaccine Immunology Programs (QADVIP), establishes the Africa Health Research Institute (AHRI) as a qualified site for ELISpot-based immunomonitoring in vaccine clinical trials. This achievement expands regional capacity to generate reproducible, high-quality, and regulatory-aligned cellular immunology data, strengthening Africa's ability to contribute directly to vaccine evaluation and development (3).

Declarations

Ethics approval and consent to participate: This study was approved by the Biomedical Research and Ethics Committee (BREC), UKZN Ethics Review Board with study number BREC/00006306/2023. Participation in the study was voluntary, and participants provided both verbal and signed informed consent.

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References

1. Carreto-Binaghi LE, Nieto-Ponce M, Palencia-Reyes A, Chávez-Domínguez RL, Blancas-Zaragoza J, Franco-Mendoza P, et al. Validation of the Enzyme-Linked ImmunoSpot Analytic Method for the Detection of Human IFN-γ from Peripheral Blood Mononuclear Cells in Response to the SARS-CoV-2 Spike Protein. Biomolecules. 2024;14(10):1286.

2. Lin H, Zhang J, Dong S, Liu Y, Liu P, Gao GF, et al. An adjusted ELISpot-based immunoassay for evaluation of SARS-CoV-2-specific T-cell responses. Biosafety and Health. 2022;4(3):179-85.

3. Langat RK, Farah B, Indangasi J, Ogola S, Omosa-Manyonyi G, Anzala O, et al. Performance of International AIDS Vaccine Initiative African clinical research laboratories in standardised ELISpot and peripheral blood mononuclear cell processing in support of HIV vaccine clinical trials. 2021. 2021;10(1).

4. Streeck H, Frahm N, Walker BD. The role of IFN-γ Elispot assay in HIV vaccine research. Nature Protocols. 2009;4(4):461-9.

5. Girlanda S, Mantegani P, Baldissera E, Aiello P, Ratti M, Sabbadini MG, et al. ELISPOT-IFN-γ assay instead of tuberculin skin test for detecting latent Mycobacterium tuberculosis infection in rheumatic patients candidate to anti-TNF-α treatment. Clinical Rheumatology. 2010;29(10):1135-41.

6. Gideon HP, Hamilton MS, Wood K, Pepper D, Oni T, Seldon R, et al. Impairment of IFN-Gamma Response to Synthetic Peptides of Mycobacterium tuberculosis in a 7-Day Whole Blood Assay. PLOS ONE. 2013;8(8):e71351.

7. Latorre I, Martínez-Lacasa X, Font R, Lacoma A, Puig J, Tural C, et al. IFN-γ response on T-cell based assays in HIV-infected patients for detection of tuberculosis infection. BMC Infectious Diseases. 2010;10(1):348.

8. Islam R, Vance J, Poirier M, Zimmer J, Khadang A, Williams D, et al. Recommendations on ELISpot Assay Validation by the GCC. Bioanalysis. 2022;14(4):187-93.

9. Waerlop G, Leroux-Roels G, Lambe T, Bellamy D, Medaglini D, Pettini E, et al. Harmonization and qualification of an IFN-γ Enzyme-Linked ImmunoSpot assay (ELISPOT) to measure influenza-specific cell-mediated immunity within the FLUCOP consortium. Frontiers in Immunology. 2022;Volume 13 - 2022.

10. Janetzki S. Important Considerations for ELISpot Validation. In: Kalyuzhny AE, editor. Handbook of ELISPOT : Methods and Protocols. New York, NY: Springer US; 2024. p. 1-13.

11. Ezzelle J, Rodriguez-Chavez IR, Darden JM, Stirewalt M, Kunwar N, Hitchcock R, et al. Guidelines on good clinical laboratory practice: Bridging operations between research and clinical research laboratories. Journal of Pharmaceutical and Biomedical Analysis. 2008;46(1):18-29.

12. de Oliveira T, Baxter C. Investing in Africa's scientific future. Science. 2024;383(6678):eadn4168.

13. Naghavi M, Mestrovic T, Gray A, Gershberg Hayoon A, Swetschinski LR, Robles Aguilar G, et al. Global burden associated with 85 pathogens in 2019: a systematic analysis for the Global Burden of Disease Study 2019. The Lancet Infectious Diseases. 2024;24(8):868-95.

14. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, et al. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. Journal of Virology. 1994;68(7):4650-5.

15. Lin PL, Rutledge T, Green AM, Bigbee M, Fuhrman C, Klein E, et al. CD4 T Cell Depletion Exacerbates Acute Mycobacterium tuberculosis While Reactivation of Latent Infection Is Dependent on Severity of Tissue Depletion in Cynomolgus Macaques. AIDS Research and Human Retroviruses. 2012;28(12):1693-702.

16. Sullivan NJ, Hensley L, Asiedu C, Geisbert TW, Stanley D, Johnson J, et al. CD8+ cellular immunity mediates rAd5 vaccine protection against Ebola virus infection of nonhuman primates. Nature Medicine. 2011;17(9):1128-31.

17. Langat RK, Farah B, Indangasi J, Ogola S, Omosa-Manyonyi G, Anzala O, et al. Performance of International AIDS Vaccine Initiative African clinical research laboratories in

546 standardised ELISpot and peripheral blood mononuclear cell processing in support of HIV
547 vaccine clinical trials. African Journal of Laboratory Medicine; Vol 10, No 1 (2021)DO -
548 104102/ajlmv10i11056. 2021.
549 18. Kaguthi G, Nduba V, Rabuogi P, Okelloh D, Ouma SG, Blatner G, et al. Development
550 of a TB vaccine trial site in Africa and lessons from the Ebola experience. BMC Public Health.
551 2020;20(1):999.
552 19. Banjoko AW, Ng'uni T, Naidoo N, Ramsuran V, Hyrien O, Ndhlovu ZM. High resolution
553 class I HLA-A, -B, and -C diversity in Eastern and Southern African populations. Scientific
554 Reports. 2025;15(1):23667.
555 20. Hønge BL, Petersen MS, Olesen R, Møller BK, Erikstrup C. Optimizing recovery of
556 frozen human peripheral blood mononuclear cells for flow cytometry. PLOS ONE.
557 2017;12(11):e0187440.
558 21. Weinberg A, Song L-Y, Wilkening C, Sevin A, Blais B, Louzao R, et al. Optimization
559 and Limitations of Use of Cryopreserved Peripheral Blood Mononuclear Cells for Functional
560 and Phenotypic T-Cell Characterization. Clinical and Vaccine Immunology. 2009;16(8):1176-
561 86.
562 22. Strober W. Trypan Blue Exclusion Test of Cell Viability. Current Protocols in
563 Immunology. 2015;111(1):A3.B.1-A3.B.
564 23. Cedrone E, Neun BW, Dobrovolskaia MA. Detection of Interferon gamma (IFNγ) by
565 Enzyme-Linked Immunosorbent Spot (ELISpot) Assay: Version 1. National Cancer Institute's
566 Nanotechnology Characterization Laboratory Assay Cascade Protocols. Bethesda (MD):
567 National Cancer Institute (US); 2005.
568 24. Munseri PJ, Kroidl A, Nilsson C, Joachim A, Geldmacher C, Mann P, et al. Priming with
569 a Simplified Intradermal HIV-1 DNA Vaccine Regimen followed by Boosting with Recombinant
570 HIV-1 MVA Vaccine Is Safe and Immunogenic: A Phase IIa Randomized Clinical Trial. PLOS
571 ONE. 2015;10(4):e0119629.

572 25. Gill DK, Huang Y, Levine GL, Sambor A, Carter DK, Sato A, et al. Equivalence of
573 ELISpot Assays Demonstrated between Major HIV Network Laboratories. PLOS ONE.
574 2010;5(12):e14330.
575 26. Liu AY, De Rosa SC, Guthrie BL, Choi RY, Kerubo-Bosire R, Richardson BA, et al. High
576 background in ELISpot assays is associated with elevated levels of immune activation in HIV-
577 1-seronegative individuals in Nairobi. Immunity, Inflammation and Disease. 2018;6(3):392-
578 401.
579 27. Tagliaferro S, Adani M, Pepe N, Briganti G, D'Isidoro M, Bonini M, et al. The impact of
580 the spatial resolution of vegetation cover on the prediction of airborne pollen concentrations
581 over northern Italy. Agricultural and Forest Meteorology. 2024;355:110153.
582 28. Zartarian V, Xue J, Tornero-Velez R, Brown J. Children's Lead Exposure: A Multimedia
583 Modeling Analysis to Guide Public Health Decision-Making. Environ Health Perspect.
584 2017;125(9):097009.
585 29. Sarzotti-Kelsoe M, Daniell X, Todd CA, Bilska M, Martelli A, LaBranche C, et al.
586 Optimization and validation of a neutralizing antibody assay for HIV-1 in A3R5 cells. Journal
587 of Immunological Methods. 2014;409:147-60.
588 30. Cosma A, Nagaraj R, Bühler S, Hinkula J, Busch DH, Sutter G, et al. Therapeutic
589 vaccination with MVA-HIV-1 nef elicits Nef-specific T-helper cell responses in chronically HIV-
590 1 infected individuals. Vaccine. 2003;22(1):21-9.
591 31. Patton KS, Harrison MT, Long BR, Lau K, Holcomb J, Owen R, et al. Monitoring cell-
592 mediated immune responses in AAV gene therapy clinical trials using a validated IFN-γ
593 ELISpot method. Molecular Therapy Methods & Clinical Development. 2021;22:183-95.
594 32. Barabas S, Spindler T, Kiener R, Tonar C, Lugner T, Batzilla J, et al. An optimized IFN-
595 γ ELISpot assay for the sensitive and standardized monitoring of CMV protein-reactive effector
596 cells of cell-mediated immunity. BMC Immunology. 2017;18(1):14.
597 33. Dapporto F, De Tommaso D, Marrocco C, Piu P, Semplici C, Fantoni G, et al. Validation
598 of a double-color ELISpot assay of IFN-γ and IL-4 production in human peripheral blood
599 mononuclear cells. Journal of Immunological Methods. 2024;524:113588.

600 34. Ermer J. ICH Q2(R2): Validation of Analytical Procedures. Method Validation in
601 Pharmaceutical Analysis2025. p. 351-72.
602 35. Carnoli AJ, Lohuis Po, Buydens LMC, Tinnevelt GH, Jansen JJ. Linear Mixed-Effects
603 Models in chemistry: A tutorial. Analytica Chimica Acta. 2024;1304:342444.
604 36. Dowdy A, Prime K, Peltier C. Generalized Linear Mixed Effects Modeling (GLMM) of
605 Functional Analysis Graphical Construction Elements on Visual Analysis. Perspectives on
606 Behavior Science. 2024;47(2):499-521.
607 37. CSLI. Evaluation of Detection Capability for Clinical Laboratory Measurement
608 Procedures; Approved Guideline—Second Edition. CLSI document EP17-A22012.
609 38. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. Clin
610 Biochem Rev. 2008;29 Suppl 1(Suppl 1):S49-52.
611 39. Kim Y, Jang E-J, Hwang J-Y, Lee K-M, Xayaheuang S, Choi S-T, et al. Validation of an
612 interferon-gamma enzyme-linked immunosorbent spot assay to evaluate cell-mediated
613 immunity against severe acute respiratory syndrome coronavirus 2. Human Vaccines &
614 Immunotherapeutics. 2024;20(1):2428516.
615 40. Dubey S, Clair J, Fu T-M, Guan L, Long R, Mogg R, et al. Detection of HIV Vaccine-
616 Induced Cell-Mediated Immunity in HIV-Seronegative Clinical Trial Participants Using an
617 Optimized and Validated Enzyme-Linked Immunospot Assay. JAIDS Journal of Acquired
618 Immune Deficiency Syndromes. 2007;45(1):20-7.
619 41. Patton K, Aslam S, Lin J, Yu L, Lambert S, Dawes G, et al. Enzyme-Linked Immunospot
620 Assay for Detection of Human Respiratory Syncytial Virus F Protein-Specific Gamma
621 Interferon-Producing T Cells. Clinical and Vaccine Immunology. 2014;21(5):628-35.
622 42. Maecker HT, Hassler J, Payne JK, Summers A, Comatas K, Ghanayem M, et al.
623 Precision and linearity targets for validation of an IFNγ ELISPOT, cytokine flow cytometry, and
624 tetramer assay using CMV peptides. BMC Immunology. 2008;9(1):9.
625 43. Yang F, Patton K, Kasprzyk T, Long B, Gupta S, Zoog SJ, et al. Validation of an IFN-
626 gamma ELISpot assay to measure cellular immune responses against viral antigens in non-
627 human primates. Gene Therapy. 2022;29(1):41-54.

628 44. Robert J. McEnroe APD, Marc D. Goldford, Marina V. Kondratovich, Samir Lababidi,
629 Robert Magari,, Jonathan Guy Middle JFP-P, Jeffrey E. Vaks. EP05-A3: Evaluation of
630 Precision of Quantitative Measurement Procedures; Approved Guideline. Third ed. document
631 C, editor. Clinical and Laboratory Standards Institute: CLSI; 2014.
632 45. Vazvaei-Smith F, Wickremsinhe E, Woolf E, Yu C. ICH M10 Bioanalytical Method
633 Validation Guideline-1 year Later. The AAPS Journal. 2024;26(5):103.
634 46. Santos R, Buying A, Sabri N, Yu J, Gringeri A, Bender J, et al. Improvement of IFNg
635 ELISPOT Performance Following Overnight Resting of Frozen PBMC Samples Confirmed
636 Through Rigorous Statistical Analysis. Cells. 2015;4(1):1-18.
637 47. Smith JG, Joseph HR, Green T, Field JA, Wooters M, Kaufhold RM, et al. Establishing
638 acceptance criteria for cell-mediated-immunity assays using frozen peripheral blood
639 mononuclear cells stored under optimal and suboptimal conditions. Clin Vaccine Immunol.
640 2007;14(5):527-37.
641 48. Walsh PN, Friedrich DP, Williams JA, Smith RJ, Stewart TL, Carter DK, et al.
642 Optimization and qualification of a memory B-cell ELISpot for the detection of vaccine-induced
643 memory responses in HIV vaccine trials. Journal of Immunological Methods. 2013;394(1):84-
644 93.

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648 **Table 1. Summary of recovery, bias, and CV at different cell inputs across all seven**
649 **samples.** [a]Recovery is obtained by dividing the mean SFU (predicted) by the mean SFU
650 (actual) and multiplying by 100.

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Sample ID	Cell Input	Mean SFU (Actual)	SD SFU (Actual)	Mean SFU (Predicted)	% Recovery[a]	%|Bias|	%CV
Sample 1	25,000	546.7	171.6	-64.6	-11.8	111.8	31.4
	50,000	1400.0	314.8	1324.7	94.6	5.4	22.5
	75,000	1843.3	222.8	2714.0	147.2	47.2	12.1

	100,000	4186.7	393.1	4103.3	98.0	2.0	9.4
	150,000	6526.7	535.4	6882.0	105.4	5.4	8.2
	200,000	10116.7	173.9	9660.6	95.5	4.5	1.7
Sample 2	25,000	119.0	70.0	-75.3	-63.3	163.3	58.8
	50,000	422.3	30.6	463.9	109.8	9.8	7.2
	75,000	872.3	47.3	1003.1	115.0	15.0	5.4
	100,000	1349.0	289.3	1542.3	114.3	14.3	21.5
	150,000	2779.0	216.6	2620.8	94.3	5.7	7.8
	200,000	3712.3	66.6	3699.2	99.6	0.4	1.8
Sample 3	25,000	523.3	40.4	179.8	34.4	65.6	7.7
	50,000	1546.7	238.6	1409.3	91.1	8.9	15.4
	75,000	2300.0	375.1	2638.8	114.7	14.7	16.3
	100,000	3716.7	155.3	3868.3	104.1	4.1	4.2
	150,000	5863.3	869.6	6327.4	107.9	7.9	14.8
	200,000	9260.0	338.7	8786.4	94.9	5.1	3.7
Sample 4	25,000	860.0	145.3	412.2	47.9	52.1	16.9
	50,000	1786.7	37.9	1820.9	101.9	1.9	2.1
	75,000	2603.3	340.2	3229.6	124.1	24.1	13.1
	100,000	5016.7	235.0	4638.3	92.5	7.5	4.7
	150,000	6800.0	697.6	7455.8	109.6	9.6	10.3
	200,000	10763.3	675.5	10273.2	95.4	4.6	6.3
Sample 5	25,000	265.7	70.2	215.8	81.2	18.8	26.4
	50,000	769.0	320.5	861.7	112.1	12.1	41.7
	75,000	1205.7	100.2	1507.6	125.0	25.0	8.3
	100,000	2505.7	327.5	2153.4	85.9	14.1	13.1
	150,000	3599.0	380.0	3445.2	95.7	4.3	10.6
	200,000	4575.7	326.2	4737.0	103.5	3.5	7.1
Sample 6	25,000	29.3	25.9	-12.7	-43.4	143.4	88.3
	50,000	265.7	83.3	251.8	94.8	5.2	31.3
	75,000	489.0	70.0	516.2	105.6	5.6	14.3
	100,000	779.0	174.4	780.7	100.2	0.2	22.4
	150,000	1192.3	25.2	1309.7	109.8	9.8	2.1
	200,000	1929.0	165.2	1838.6	95.3	4.7	8.6
Sample 7	25,000	19.7	34.1	-51.8	-263.2	363.2	173.2
	50,000	289.0	253.6	201.1	69.6	30.4	87.7
	75,000	399.0	87.2	454.0	113.8	13.8	21.9
	100,000	519.0	85.4	706.9	136.2	36.2	16.5
	150,000	1212.3	332.9	1212.7	100.0	0.0	27.5
	200,000	1802.3	142.2	1718.4	95.3	4.7	7.9

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654 **Table 2. Summary of Range Analysis.** The R^2 was determined via a simple linear regression
655 model using only CEFSX SFU per 10^6 PBMC data that fall within the 100,000 to 200,000 cell
656 input range. We conclude that under the CEFSX condition, the 100,000 to 200,000 cell input
657 range corresponds to a mean LLOQ of at least 2,582 SFU per 10^6 PBMC and a mean upper
658 limit of quantification (ULOQ) of at least 6,023 SFU per 10^6 PBMC.

Sample	**R^2**
Sample 1	0.97
Sample 2	0.96
Sample 3	0.95

Sample 4	0.92
Sample 5	0.90
Sample 6	0.92
Sample 7	0.90
Average	**0.93**

Table 3. Summary of SFU/10^6 PBMC for different post-thawing resting times across samples. Peptide pools are as follows: HIV optimal, CEFSX and PHA

Sample	Condition	2 hrs				6 hrs			
		Replicates			Mean	Replicates			Mean
		1	2	3		1	2	3	
Sample 16	HIV optimal	0	0	0	0.0	0	0	0	0.0
	CEFX	390	440	480	436.7	609	749	589	649.0
	PHA	7940	8190	7220	7783.3	8149	8909	8859	8639.0
Sample 4	HIV optimal	496	546	596	546.0	349	519	629	499.0
	CEFX	4406	3946	3656	4002.7	4269	4149	4289	4235.7

	PHA	12376	12476	12686	12512.7	13999	13769	14009	13925.7
Sample 5	HIV optimal	0	0	0	0.0	0	0	0	0.0
	CEFX	529	719	509	585.7	550	630	630	603.3
	PHA	10079	9959	9379	9805.7	12220	10330	12470	11673.3

Table 4. Summary of SFU/10^6 PBMC for different plate incubation times across samples. Peptide pools are as follows: HIV optimal, CEFSX and PHA

Sample	Condition	18 hrs				24 hrs			
		Replicates			Mean	Replicates			Mean
		1	2	3		1	2	3	
Sample 4	HIV optimal	0	0	0	0.0	0	0	0	0.0
	CEFX	409	719	489	539.0	770	840	690	766.7
	PHA	5519	9089	6829	7145.7	11590	11250	9280	10706.7
Sample 3	HIV optimal	680	680	770	710.0	780	1000	760	846.7

	CEFX	410	480	340	410.0	280	330	180	263.3
	PHA	9730	9740	11170	10213.3	13420	14040	10570	12676.7
Sample 2	HIV optimal	188	258	268	238.0	179	219	129	175.7
	CEFX	4098	5788	3908	4598.0	4579	5669	6449	5565.7
	PHA	9268	10518	8638	9474.7	9859	9689	9639	9729.0
Sample 7	HIV optimal	970	1000	1030	1000.0	709	899	709	772.3
	CEFX	2530	2410	1930	2290.0	1439	1929	2049	1805.7
	PHA	6370	7090	8100	7186.7	7309	5089	6369	6255.7

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Table 5. Summary of SFU/10^6 PBMC for the intermediate experiments of each condition across samples.

Day	Plate	Operator	Sample	Replicate	HIV optimal	CEFSX	PHA
Day 1	Plate 1	1	Sample 17	1	392	4312	14042
				2	562	4192	13812
				3	672	4332	14052
			Sample 16	1	0	150	13140
				2	0	70	13340
				3	20	130	13300
			Sample 5	1	0	390	7940
				2	0	440	8190
				3	0	480	7220
		2	Sample 17	1	448	3338	12908
				2	568	4248	13068
				3	568	3838	12228
			Sample 16	1	29	99	12659
				2	79	149	12999
				3	39	89	12809
			Sample 3	1	0	506	6476
				2	0	416	8106
				3	0	476	7746
Day 2	Plate 2	1	Sample 10	1	1831	0	12281
				2	1761	0	12481
				3	1911	0	12441
			Sample 7	1	0	90	17520
				2	0	50	17470
				3	0	140	16820
			Sample 13	1	849	1929	6029
				2	1169	2159	7389
				3	889	2399	7179
		2	Sample 10	1	1479	19	11569
				2	1599	0	12289
				3	1269	0	11909
			Sample 7	1	0	90	16830
				2	0	130	17070
				3	10	110	17810
			Sample 13	1	709	1439	7309
				2	899	1929	5089
				3	709	2049	6369

Table 6. Intermediate precision for each condition across samples using SFU per 10^6 PBMC. *p-value = 0.9. The p-value of 0.90 was obtained using a student's t-test on pooled SFU values across samples and conditions. Only 6 of 108 observations fell outside the 3-fold mean boundary, confirming no significant operator differences.

Condition	Operator	Sample	Replicate	Mean Net SFU	3-fold boundary	Outside boundary
HIV optimal	1	Sample 17	3	542	(180.7, 1626)	0
		Sample 16	3	6.7	(2.2, 20.1)	2
		Sample 5	3	0	(0, 0)	0
		Sample 10	3	1834.3	(611.4, 5502.9)	0
		Sample 7	3	0	(0, 0)	0
		Sample 13	3	969	(323, 2907)	0
	2	Sample 17	3	528	(176, 1584)	0
		Sample 16	3	49	(16.3, 147)	0
		Sample 5	3	0	(0, 0)	0
		Sample 10	3	1449	(483, 4347)	0
		Sample 7	3	3.3	(1.1, 9.9)	2
		Sample 13	3	772.3	(257.4, 2316.9)	0
CEFSX	1	Sample 17	3	4278.7	(1426.2, 12836.1)	0
		Sample 16	3	116.7	(38.9, 350.1)	0
		Sample 5	3	436.7	(145.6, 1310.1)	0
		Sample 10	3	0	(0, 0)	0
		Sample 7	3	93.3	(31.1, 279.9)	0
		Sample 13	3	2162.3	(720.8, 6486.9)	0
	2	Sample 17	3	3808	(1269.3, 11424)	0
		Sample 16	3	112.3	(37.4, 336.9)	0
		Sample 5	3	466	(155.3, 1398)	0
		Sample 10	3	6.3	(2.1, 18.9)	2
		Sample 7	3	110	(36.7, 330)	0
		Sample 13	3	1805.7	(601.9, 5417.1)	0
PHA	1	Sample 17	3	13968.7	(4656.2, 41906.1)	0
		Sample 16	3	13260	(4420, 39780)	0
		Sample 5	3	7783.3	(2594.4, 23349.9)	0
		Sample 10	3	12401	(4133.7, 37203)	0
		Sample 7	3	17270	(5756.7, 51810)	0
		Sample 13	3	6865.7	(2288.6, 20597.1)	0
	2	Sample 17	3	12734.7	(4244.9, 38204.1)	0
		Sample 16	3	12822.3	(4274.1, 38466.9)	0
		Sample 5	3	7442.7	(2480.9, 22328.1)	0
		Sample 10	3	11922.3	(3974.1, 35766.9)	0
		Sample 7	3	17236.7	(5745.6, 51710.1)	0
		Sample 13	3	6255.7	(2085.2, 18767.1)	0
Total			**108**			**6***

Table 7. Linear Mixed Effect Model (LMM) summary across conditions. The Residual source represents the portion of variability in the observed data that is not explained by operator or sample source effects. The Grand mean is the average SFU across conditions. **Abbreviations:** Var: variance, and SD: Standard Deviation.

Source	HIV optimal			
	Var	**% Var**	**SD**	**%CV**
Operator	3419.0	0.8	58.5	11.4
Sample	430100.1	96.1	655.8	127.8
Residual	14175.5	3.2	119.1	23.2
Total	447694.5			
Grand Mean	512.8			
	CEFSX			
	Var	**% Var**	**SD**	**%CV**
Operator	6255.1	0.2	79.1	7.1
Sample	2600410.3	98.3	1612.6	144.5
Residual	39247.3	1.5	198.1	17.7
Total	2645912.7			
Grand Mean	1116.3			
	PHA			
	Var	**% Var**	**SD**	**%CV**
Operator	120456.8	0.7	347.1	3.0
Sample	15689588.7	97.5	3961.0	33.9
Residual	287801.0	1.8	536.5	4.6
Total	16097846.5			
Grand Mean	11663.58			

Table 8. Classification summary of the assay's specificity analysis. The actual response refers to the true HIV status of each sample, while the assay response is the classification assigned by the assay using the positivity threshold. The diagonal values for 8 samples from PLWH and 6 from PLWoH indicate the number of correctly classified samples at the assay threshold, and the zeros indicate the number of misclassified samples.

		Assay Response		
		PLWH (HIV^+)	PLWoH (HIV^-)	**Total**
Actual/True Response	PLWH (HIV^+)	8	0	**8**
	PLWoH (HIV^-)	0	6	**6**
	Total	**8**	**6**	**14**

754 **Table 9: Summary of the Human IFN- γ ELISpot assay validation**

Assay parameter	Acceptance criteria	Validation results
Linearity	Linear regression of responses shows an $R^2 \geq 0.90$.	0.92 – 0.98
Precision	Repeatability and Inter-operator variability : % Coefficient of variation (CV) < 35%.	All the samples have %CV < 35%
	At least 90% of the well SFU for the HIV optimal, CEFSX and PHA conditions must fall within the 3-fold boundaries with error rate of 10% maximum.	94.4% of the Net SFU were within 3-fold of the mean, with an error rate of 5.6%
Robustness	A Student's t-test p-value > 0.05 were required for the HIV optimal, CEFSX, and PHA conditions for both post-thaw resting (2 to 6 hrs) and plate incubation times (18 to 24 hrs).	P-values for both post-thaw resting and incubation times were greater than 0.05 with %CV < 35%
Specificity	Samples that are truly negative must remain negative when tested with the HIV optimal pool.	Assay correctly show negative result for 6 of 6 individuals (PLWOH) who truly have no antigen-specific T-cell responses
LLoQ	Using the CEFSX condition, the lowest peptide SFU response at which the Linearity ($R^2 \geq 0.90$), Precision (%CV < 35%), and Accuracy (%|Bias|<20%) criteria were met.	2,582 SFU per 10^6 PBMCs
ULoQ	Using the CEFSX condition, the highest peptide SFU response at which the Linearity ($R^2 \geq 0.90$), Precision (%CV < 35%), and Accuracy (%|Bias|<20%) criteria were met.	6,023 SFU per 10^6 PBMCs
LoD	The LOD is calculated using the 95th percentile of all the negative-control wells.	LoD = 80 SFU per 10^6 PBMCs
Cell input Range	Using the ≤20% bias and ≤35% CV criteria in the CEFSX condition.	100,000 – 200,000 cell per well

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Figure Legends

Figure 1. Linearity test and experimental plate output for ELISpot assay. (A). Experimental plate output for the ELISpot assay across the seven samples. NC and PHA indicate negative controls and Phytohemagglutinin wells, respectively. 25k, 50k, 75k, 100k, 150k and 200k indicate 25,000, 50,000, 75,000, 100 000, 150,000 and 200,000 cells per well. All wells are plated in triplicate. **(B)** Test assessment **r**esults are shown for assays performed with CEFSX with different cell input dilutions for seven samples.

Figure 2. Repeatability plot. All five samples show repeatability results with %CV < 35% threshold. The CV for Sample 3 under the HIV optimal condition is reported as NA because all replicate SFU values were zero, resulting in a mean and standard deviation of zero. Since CV requires division by the mean, it cannot be calculated in this case.

Figure 3. Plot showing the LLOQ and ULOQ cell inputs. For each sample, the lowest and highest cell inputs that simultaneously satisfied the acceptance criteria of %CV ≤ 35% and %Bias ≤ 20% were designated as the lowest limit of quantification (LLOQ) and upper limit of quantification (ULOQ) regions, respectively. The purple box highlights the cell concentrations where both criteria are met. Concentrations falling outside this range demonstrated unacceptable precision or accuracy and were excluded from the quantifiable range.

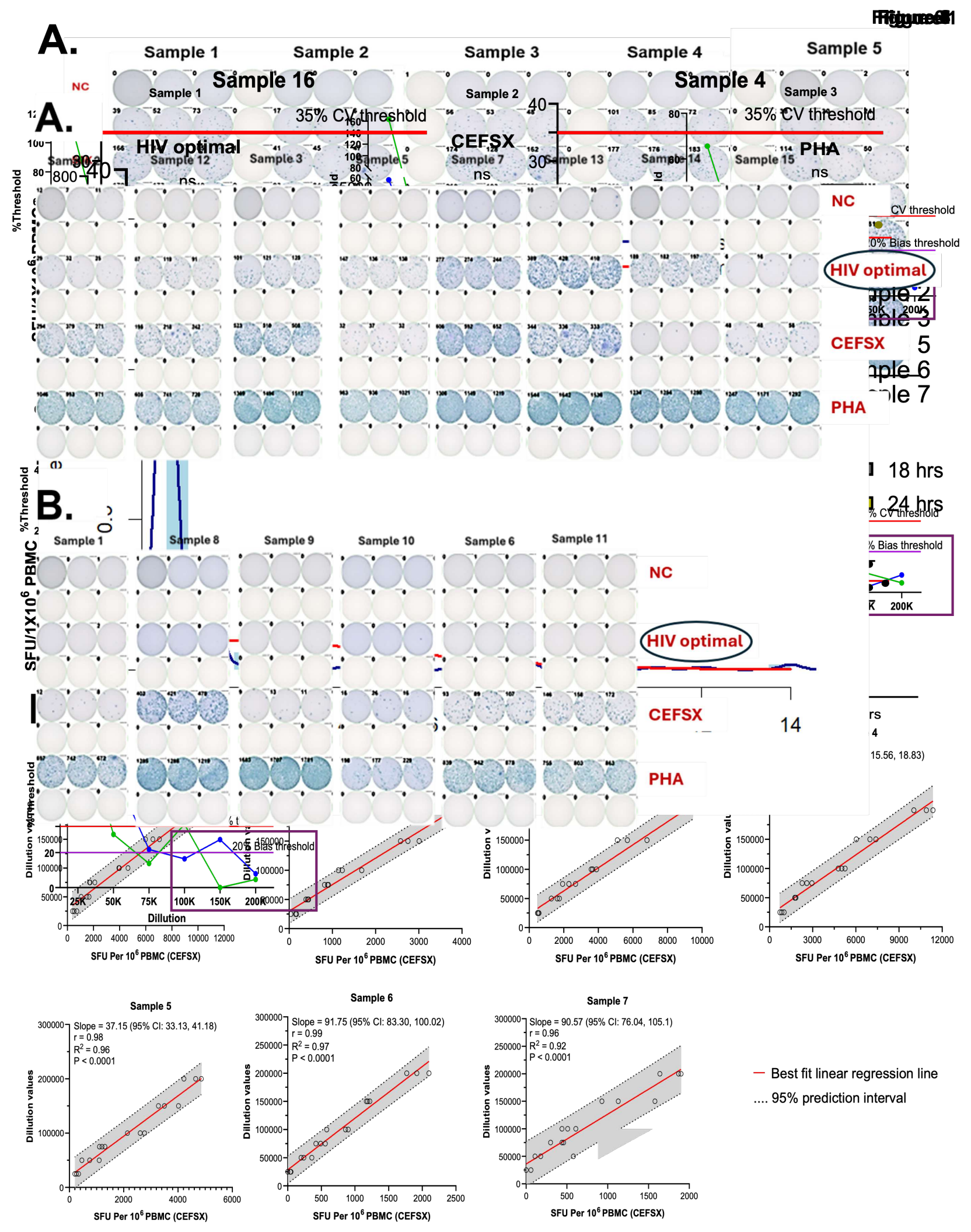
Figure 4. Plots of CV for post-thawing resting time robustness parameter. All the samples show CV<35% CV acceptance criteria for HIV optimal, CEFSX and PHA. The CV for Samples 4 and 16 under the HIV optimal condition is reported as NA because all replicate SFU values were zero, resulting in a mean and standard deviation of zero. Since CV requires division by the mean, it cannot be calculated in this case.

Figure 5. Plots of CV for incubation time robustness parameter. All the samples show CV<35% CV acceptance criteria for HIV optimal, CEFSX, and PHA. The CV for Sample 4 under the HIV optimal condition is reported as NA because all replicate SFU values were zero, resulting in a mean and standard deviation of zero. Since CV requires division by the mean, it cannot be calculated in this case.

Figure 6. Plots of statistical differences between two means. Student's t-test was used for testing differences in robustness parameters across assay conditions. **(A)** post-thawing resting time and **(B)** Plate incubation time. The red horizontal line shows the mean SFU, and the black vertical lines represent the 95% CI of the mean. Each dot represents the SFU replicate for each sample.

Figure 7. Density plot of the NC SFU. The plot shows the skewness of the SFU (Spot Forming Unit) values.

Figure 8. ELISpot assay experimental plate output for the specificity test across different samples. Samples were stimulated with the HIV optimal peptide pool. **(A)** 8 samples from PLWH and **(B)** 6 samples from PLWoH were used to evaluate specificity in this assay qualification.


A.
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
NC
HIV optimal
CEFSX
PHA
35% CV threshold
ns
B.
SFU/1X10^6 PBMC
%Threshold
18 hrs
24 hrs
20% Bias threshold
Dilution
SFU Per 10^6 PBMC (CEFSX)
Sample 5
Sample 6
Sample 7
Slope = 37.15 (95% CI: 33.13, 41.18)
r = 0.98
R^2 = 0.96
P < 0.0001
Slope = 91.75 (95% CI: 83.30, 100.02)
r = 0.99
R^2 = 0.97
P < 0.0001
Slope = 90.57 (95% CI: 76.04, 105.1)
r = 0.96
R^2 = 0.92
P < 0.0001
Dilution values
Best fit linear regression line
95% prediction interval