Cancer Immune Monitoring and Analysis Center
Dana-Farber Cancer Institute
Harvard Medical School

IFNγ ELISpot Assay — Analytical Performance Report, Version 1.2

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1. Purpose of the Assay

Interferon-gamma (IFNγ) is a key inflammatory cytokine produced by antigen-specific T cells in response to antigen stimulation. The IFNγ ELISpot assay is a clinically accepted, highly reproducible (1) , quantitative, and functional assay for measuring antigen-specific T-cell immune responses (2). At the DFCI CIMAC, this well-established assay is used to quantify responses following *ex vivo* stimulation of cryopreserved peripheral blood mononuclear cells (PBMCs), in the context of cancer immunotherapy, vaccine immunogenicity (3–8), and infectious disease studies (9–12).

Table 1. Summary of analytical performance characteristics for IFNγ ELISpot assay

Characteristic	Finding
Accuracy	The assay is capable of detecting low frequencies of antigen-specific T cells. Published inter-laboratory studies have demonstrated strong concordance of ELISpot measurements across laboratories when standardized procedures are followed. Responses are reported as spot-forming cells (SFC) per 10^6 PBMC; the practical detection limit depends on assay background and replicate variability. A commonly used reference threshold is >55 SFC/10^6 PBMC or mean + 3 SD of negative control wells, whichever is higher; however, this threshold is not used alone to define positivity, and lower responses may be considered when supported by orthogonal assays (e.g., ICS, tetramer staining, or flow cytometry)(4,13).
Precision	Performed in triplicate wells per condition. Intra-assay variability is assessed by comparing spot counts across replicate wells. Variability is generally low and typically within acceptable ranges for ELISpot assays (commonly <20% coefficient of variation [CV]). Wells with spot counts near background may be excluded from CV calculations, as very low absolute counts can disproportionately inflate variability. Consistent spot counts across triplicate wells indicate reliable assay performance.
Analytical Sensitivity	For *ex vivo* PBMC assays, cell densities typically range from 0.5×10^5 to 3×10^5 cells/well depending on the expected frequency of antigen-specific T cells and experimental design. Under these conditions the assay can detect low-frequency responses; sensitivity is further dependent on assay background and replicate variability. Practical sensitivity is influenced by PBMC viability, background cytokine secretion, and peptide stimulation conditions.
Analytical Specificity	Specificity ensured by paired comparison with negative control wells (DMSO vehicle; HIV-GAG irrelevant peptide at 10 µg/mL). Potential sources of non-specific signal: PBMC viability <70%, suboptimal cryopreservation, peptide impurities, or DMSO >1%. PBMC samples used in the assay are cryopreserved prior to testing, and synthetic peptides are typically ≥90% purity.
Reportable Range	SFC per 10^6 PBMC (normalized to cells plated). Assay- and antigen-dependent. Several predefined criteria may be used to define a positive response for a given antigen-stimulation condition, depending on study design and the prespecified

Characteristic	Finding
	analysis plan: (i) mean spot count ≥ 2.5 SD above the mean of the assay negative control (DMSO) (8); or (ii) mean spot count > 6 (LOD: 3× the median DMSO), ≥ 3× matched DMSO control, and statistically significant by distribution-free resampling [DFR(eq), $p < 0.05$] (14). Lower values may be reported when supported by orthogonal evidence (ICS, tetramer, flow cytometry).
Reference Interval	Not applicable. Responses are assay- and antigen-dependent; no universal reference interval is defined.
Standardization / Reproducibility	Assay has been in use for over 20 years. Reproducibility demonstrated across laboratories when standardized procedures and appropriate controls are applied.
Quality Control	Each plate includes: negative control wells (DMSO; HIV-GAG 10 µg/mL), CEF viral peptide pool (Mabtech #3618, 6.4 µg/µL) to confirm memory T-cell function, and PHA mitogen (Gibco #10576015, 3 µL/mL) as non-specific T-cell activation control. Assay runs are considered valid only if positive control wells produce robust responses and negative control wells remain near background levels.
Other Performance Data	Not applicable.

2. Materials and Methods

Thawing of cryopreserved PBMC samples

Frozen PBMC vials are thawed in a bead bath at 37°C for 3–5 minutes until a small ice fragment remains. Add 1 mL wash medium (DMEM [Life Technologies #11965092] + 1% Pen-Strep [Life Technologies #15140122]) to each cryovial, transfer to 15 mL conicals, fill to 10 mL, centrifuge 1,500 rpm × 5 min. Resuspend at 5-10 × 10^6 cells/mL in rest medium (AIM V [Thermo Fisher #12055083] + 10% CTS Immune Cell SR [Life Technologies #A2596101] + 0.01 M HEPES [#15630080] + 1 mM sodium pyruvate [#11360070] + 1× MEM NEAA [#11140050] + 0.1 µg/mL human IL-7 [PeproTech #200-07]). Plate in 24-well plates and rest overnight at 37°C.

Technical note: PBMC viability must be ≥70% (Trypan blue exclusion) before proceeding. Overnight resting significantly improves assay performance post-cryopreservation (15,16).

Peptide preparation

After overnight rest, wash cells to remove IL-7 medium. Stimulate at 1–10 µg/mL per peptide, or 1–2 µg/mL per peptide in pools. Pool sizes vary by experimental context: pools of 8–10 peptides are common in cancer immunotherapy studies, while substantially larger pools have been successfully employed in HIV and infectious disease research - including pools of 50 or more peptides used in HIV immunology studies (17) - provided peptide concentrations, purity, and final DMSO are controlled. Peptides are ≥90% purity, reconstituted in hybridoma-grade DMSO (Sigma #D2650) at 10–20 mg/mL. Final DMSO in assay wells must be ≤1%. Crude peptide preparations should be avoided.

Peptide stimuli and assay controls

Negative controls: (1) DMSO vehicle at equivalent concentration; (2) HIV-GAG irrelevant peptide (SLYNTVATL, HLA-A*02-restricted) at 10 µg/mL.

Positive controls: (1) CEF peptide pool (Mabtech #3618, 6.4 µg/µL) - 32 dominant CMV/EBV/influenza epitopes recognized by most human donors; (2) PHA (Gibco #10576015, 3 µL/mL) - non-specific mitogen confirming T-cell viability.

Test antigens: Peptide pools derived from tumor-associated antigens, patient-specific neoantigens (16–32 aa), or vaccine antigens, as defined by study protocol.

Preparation and coating of ELISpot plates

Pre-wet MilliporeSigma 96-well PVDF plates (Fisher Scientific #MSIPS4W10) with 15 µL/well 35% ethanol; wash immediately 6× with distilled water. Coat with 2 µg/mL anti-IFN-γ capture antibody 1-D1K (Mabtech #3420-3-250) in PBS. Seal with Parafilm; store at 4°C ≥24 h before use.

Cell plating

Wash coated plates 6× with PBS. Block with 50 µL/well AIM V; incubate at 37°C while preparing cells. For *ex vivo* detection of antigen-specific T-cell responses, PBMCs are typically plated at densities ranging from 0.5×10^5 to 3×10^5 cells/well (200 µL AIM V), depending on the expected frequency of antigen-specific T cells and experimental design. Higher densities can increase background and risk CD8 T-cell fratricide; lower densities may be appropriate when antigen-specific frequencies are expected to be high. Add peptides and controls at concentrations described above. For in vitro-expanded T-cell lines with high antigen-specific frequencies, as few as 10,000 cells/well may suffice.

Detection and development

Incubate 18–24 h at 37°C. Wash 6× PBS, pat dry. Add 100 µL/well biotinylated anti-IFN-γ detection antibody 7-B6-1 at 1 µg/mL (Mabtech #3420-6-250); incubate 1 h, dark, room temperature. Wash 6×, pat dry. Add 100 µL/well streptavidin-HRP (Mabtech #3310-9-1000, 1:1,000); incubate 1 h, dark, room temperature. Wash 6×, pat dry. Add 0.45 µm-filtered TMB substrate (Mabtech #3651-10); develop 2–6 min. Stop with distilled water wash. Dry completely before image acquisition.

Plate scanning and data processing

Fully dried plates are scanned using the CTL ImmunoSpot Analyzer ("Basic Count" program). Spot detection parameters optimized on positive and negative control wells. Spot size and sensitivity thresholds are kept constant within an experiment to ensure comparability across plates. Counts reported as SFC/well and normalized to SFC per 10^6 PBMC. Automated ELISpot counting follows published guidelines (13).

Statistical analysis

Raw spot counts from triplicate wells are summarized per stimulation condition. Background is estimated from DMSO negative control wells and subtracted from antigen-stimulated wells prior to analysis.

Well-level positivity criteria. Several predefined criteria may be used to define a positive response for a given antigen-stimulation condition, depending on study design and the prespecified analysis plan: (i) mean spot count ≥ 2.5 SD above the mean of the assay negative control (DMSO) (8); or (ii) mean spot count > 6 (LOD: 3× the median DMSO), ≥ 3× matched DMSO control, and statistically significant by distribution-free resampling [DFR(eq), $p < 0.05$] (14). The criterion applied is specified at the study level.

Group-level comparative analysis. For comparisons across timepoints or cohorts, the statistical approach is selected according to study design and specified at the study level. For longitudinal studies with repeated measures across multiple timepoints and peptide pools, data may be log2-transformed and analyzed using repeated-measures random-effects models with an unstructured covariance structure; comparisons between antigen-stimulated conditions and DMSO are conducted using contrasts within the model, with false discovery rate (FDR) controlled using the Benjamini-Krieger-Yekutieli procedure (8). For simpler designs with a limited number of peptide pools and serial timepoints, such as the representative example presented in Section 3, two-way mixed ANOVA with post-hoc multiple-comparison correction (e.g., Benjamini-Hochberg or Benjamini-Krieger-Yekutieli) may also be applied. Alternative statistical models may be used for paired pre-post or unpaired cross-cohort comparisons, depending on study design and sample availability.

3. Representative Results

Figure 1 shows representative IFN-γ ELISpot plate images from a neoantigen vaccine study (NCT02287428), comparing PBMCs at Week 0 (pre-vaccine, Figure 1A) and Week 16 post-vaccination (Figure 1B). At Week 0, neoantigen pools generate minimal responses comparable to negative controls; the CEF positive control yields a mean of 89 spots, confirming pre-existing viral memory T-cell responses. At Week 16, all four neoantigen pools show 10- to 25-fold increases over DMSO background. HIV-GAG negative control remains negative. CEF yields a mean of 123 spots at Week 16, consistent with expected inter-individual variability in viral memory responses. Quantitative spot counts and standard deviations per triplicate well are summarized in Table 2. The statistical comparison of neoantigen pool responses between Week 0 and Week 16 is shown in Figure 2; all post-vaccine pools reached statistical significance (two-way mixed ANOVA, Benjamini-Hochberg correction, $p<0.0001$).

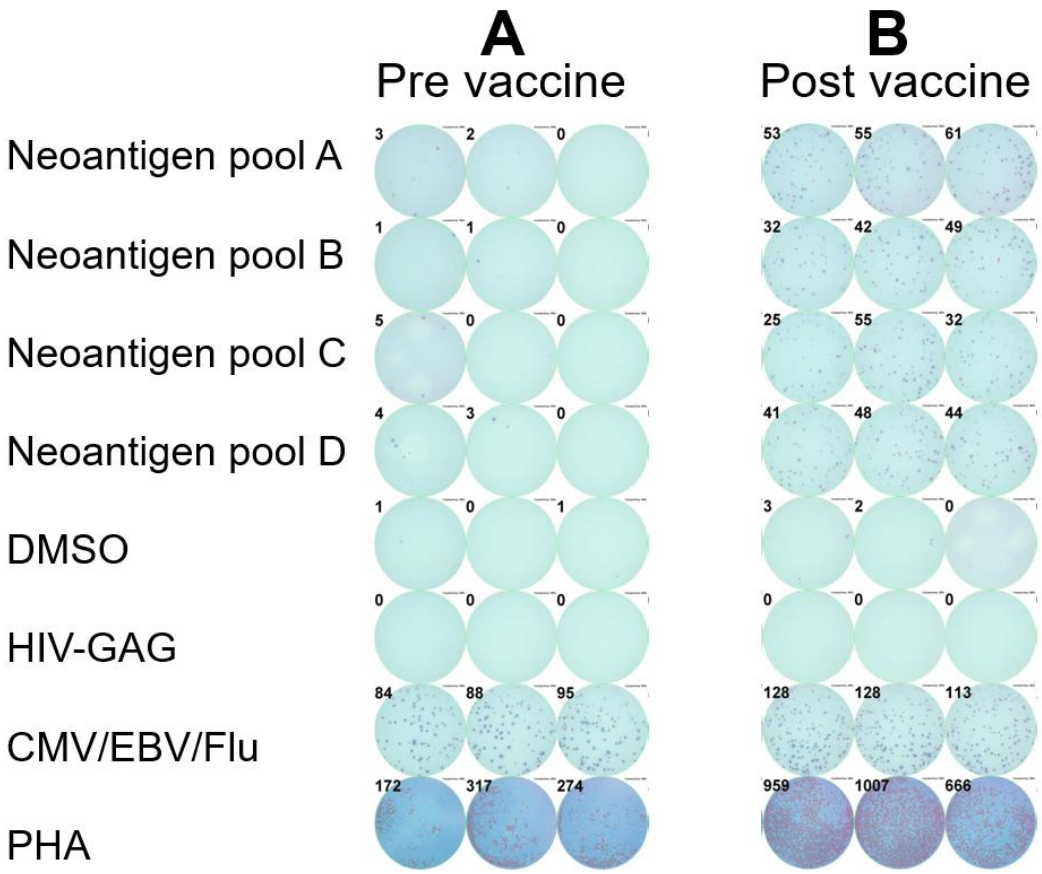

Figure 1. *Representative IFN-γ ELISpot plate images from a personalized neoantigen vaccine study (NCT02287428). Panel A: pre-vaccination (Week 0); Panel B: post-vaccination (Week 16). Neoantigen peptide pools (A–D), negative controls (DMSO, HIV-GAG), and positive controls (CEF, PHA) are shown.*

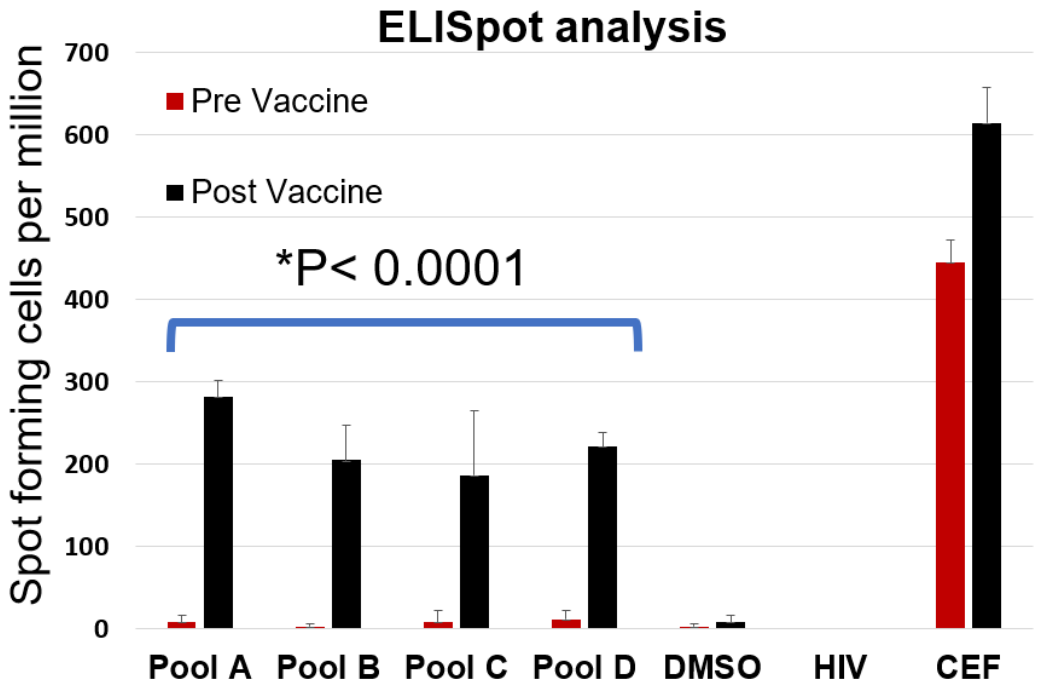

Figure 2. *Quantitative comparison of IFN-γ ELISpot responses to neoantigen peptide pools at Week 0 vs. Week 16 post-vaccination (NCT02287428). Two-way mixed ANOVA with Benjamini-Hochberg correction; all post-vaccine pools p<0.0001.*

	AVG Pre Vaccine	Post Vaccine		STDEV Pre Vaccine	Post Vaccine
Pool A	1.666667	56.33333	Pool A	1.527525	4.163332
Pool B	0.666667	41	Pool B	0.57735	8.544004
Pool C	1.666667	37.33333	Pool C	2.886751	15.69501
Pool D	2.333333	44.33333	Pool D	2.081666	3.511885
DMSO	0.666667	1.666667	DMSO	0.57735	1.527525
HIV	0	0	HIV	0	0
CEF	89	123	CEF	5.567764	8.660254

Table 2. *Mean spot-forming cell counts (± SD) per stimulation condition at Week 0 and Week 16 post-vaccination (NCT02287428).*

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