



NCL Method ITA-41

Detection of Interferon gamma (IFN γ) by Enzyme-Linked Immunosorbent Spot (ELISpot) Assay

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1. Introduction

Interferon gamma (IFN- γ) is a type II interferon produced by activated T cells, particularly CD8⁺ T cells and Th1 CD4⁺ T cells [1]. IFN γ is a well-established biomarker of T cell activation [2]. IFN γ produced by T-lymphocytes in response to immunotherapies and vaccines informs about the desired efficacy of these products because this cytokine plays a key role in the immune defense against cancer and infections [3]. In contrast, IFN γ produced as a result of undesirable T cell activation may inform about product safety, as overt T-lymphocyte activation is associated with T cell-mediated hypersensitivity and autoimmune reactions [4, 5]. Therefore, monitoring IFN γ secretion by immune cells exposed to drugs, immunotherapy, or vaccine products aids in preclinical efficacy and safety studies.

IFN γ can be detected using traditional single-plex enzyme-linked immunosorbent assays (ELISA) as well as multiplex ELISAs, in which IFN γ can be one of several other cytokines, and Enzyme-Linked Immunosorbent Spot assays (ELISpot). The ELISpot format is helpful to verify the results of traditional and multiplex ELISAs. It can also be used when IFN γ is the primary cytokine of interest. Advantages and limitations of the ELISpot assay vs. multiplex ELISA have been discussed elsewhere [6]. This document describes a protocol for IFN γ detection using the ELISpot assay.

2. Principles

In this protocol, human peripheral blood mononuclear cells (PBMCs) from healthy donor volunteers are exposed to controls and test samples for 24 hours on 96-well PVDF-backed ELISpot plates coated with IFN γ capture antibodies. At the end of the incubation, the cells are washed away, and IFN γ captured by the antibodies immobilized on the ELISpot plate is detected using biotinylated IFN γ detection antibody, alkaline-phosphatase conjugated streptavidin, and a BCIP/NBT substrate, which forms a blue-black colored precipitate. The precipitate co-localizes with IFN γ -producing cells, which appear as spots on the plate. These spots are next analyzed using the ELISpot reader, which produces two readouts: the number of spots and activity. The number of spots reflects the number of cells that produce IFN γ . The activity readout reflects the brightness of the blue-black color and, therefore, indicates the quantity of IFN γ produced by a single cell. This protocol utilizes freshly isolated PBMCs, the IFN γ ELISpot kit from R&D

Systems, and the AID multispot reader for plate analysis. Experimental steps involving instrument settings, plate reading, and analysis are based on a series of technical documents by R&D Systems [7] and Autoimmun Diagnostika GmbH [8-11]. When other cells, kits, and instruments are used for this analysis, the assay principle remains unchanged; however, some experimental parameters, such as seeding cell density, incubation time, and spot analysis algorithm, may need to be adjusted.

3. Reagents, Materials, Cell Lines, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol, and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo name changes due to various factors. Brands and part numbers typically remain consistent but may also change over time.

3.1 Reagents

- 3.1.1 Human IFN γ Kit (R&D Systems, EL285)
- 3.1.2 RPMI-1640 (Cytiva, HyClone, SH30096.01)
- 3.1.3 Penicillin streptomycin solution (Cytiva, Hyclone, SV30010)
- 3.1.4 L-glutamine (Cytiva, Hyclone, SH30034.01)
- 3.1.5 Fetal bovine serum (FBS) (Cytiva, Hyclone, SH30070.03)
- 3.1.6 Phosphate-buffered saline (PBS) (Cytiva, SH30256.01)
- 3.1.7 Trypan Blue solution (Gibco, 15250-061)
- 3.1.8 Phytohemagglutinin (PHA-M) (Sigma, L8902)
- 3.1.9 Sterile distilled water

3.2 Materials

- 3.2.1 Pipettes covering the range of 0.05 to 10 mL
- 3.2.2 Polypropylene tubes, 50 and 15 mL
- 3.2.3 Reagent reservoirs

3.3 Equipment

- 3.3.1 Centrifuge
- 3.3.2 Refrigerator, 2–8°C

- 3.3.3 Freezer, -20°C
- 3.3.4 Cell culture incubator with 5% CO₂ and 95% humidity
- 3.3.5 37°C water bath
- 3.3.6 Biohazard safety cabinet approved for level II handling of biological material
- 3.3.7 Inverted microscope
- 3.3.8 Vortex
- 3.3.9 Hemocytometer or automated cell counter
- 3.3.10 AID multispot reader

4. Preparation of Reagents and Controls for Cell Culture

4.1 Complete RPMI-1640 Medium

10% FBS (heat-inactivated)

2 mM L-glutamine

100 U/mL penicillin

100 µg/mL streptomycin sulfate

Store at 2–8°C protected from light for no longer than 1 month. Before use, warm in a water bath.

4.2 Heat-inactivated Fetal Bovine Serum

Thaw a bottle of FBS at room temperature, or overnight at 2–8°C, and allow to equilibrate to room temperature. Incubate for 30 minutes at 56°C in a water bath, mixing every 5 minutes. Fifty (50) mL single-use aliquots may be stored at 2–8°C for up to one month or at a nominal temperature of -20°C indefinitely.

4.3 Negative Control

Use PBS as a negative control. Process it the same way as your study samples.

4.4 Positive Control

PHA-M at a final concentration of 10 µg/mL is used as a positive control in this assay. The stock with a concentration of 1 mg/mL can be prepared in PBS or medium and stored in small, single-use aliquots at -20°C.

Note: Other T cell agonists, such as concanavalin A, PMA/Ca²⁺ ionophore, and anti-CD3 antibody alone or in combination with anti-CD28 can also be used at relevant concentrations.

4.5 Vehicle Control

Vehicle control is the buffer or media used to formulate test nanomaterials.

Common excipients used in nanoformulations are trehalose, sucrose, and albumin.

However, other reagents and materials are also used alone or in combination.

Vehicle control should match the formulation buffer of the test nanomaterial in both composition and concentration. This control can be skipped if nanoparticles are stored in PBS.

5. Preparation of Study Samples

The required amount of nanoparticles will depend on the number of concentrations tested, the number of replicates, and the nanoparticle stock concentration. The minimum required dilution (MRD) of this assay is 2; therefore, the working stock concentration of the test sample should be 2-times higher than the final concentration desired for analysis in this assay. For example, if one desires to test nanoparticles at 1 mg/mL, the working stock concentration should be 2 mg/mL. The recommended minimum volume of the working stock is 450 µL for 3 donors (50 µL per well, in duplicate, plus extra to prepare dilutions and account for dead volume of pipette tips and sample tubes). A strategy for estimating nanoparticle test concentrations for in vitro testing has been discussed elsewhere [12].

6. PBMC Preparation

- 6.1 Place freshly drawn blood into 15- or 50-mL conical centrifuge tubes. Add an equal volume of room-temperature PBS and mix well.
- 6.2 Slowly layer the Ficoll-Paque solution underneath the blood/PBS mixture by placing the tip of the pipette containing Ficoll-Paque at the bottom of the blood sample tube. Alternatively, the blood/PBS mixture may be slowly layered over the Ficoll-Paque solution. Use 3 mL of Ficoll-Paque solution per 4 mL of blood/PBS mixture. For example, 15 mL Ficoll-Paque per 20 mL of diluted blood in a 50 mL tube.

Note: To maintain Ficoll-blood interface it is helpful to hold the tube at a 45° angle.

- 6.3 Centrifuge 30 min at 900xg, 18–20°C, without brake.

Note: Depending on the type of centrifuge, one may also need to set the acceleration speed to a minimum.

- 6.4 Using a sterile pipette, remove the upper layer containing plasma and platelets and discard.

- 6.5 Using a fresh sterile pipette, transfer the mononuclear cell layer into a new centrifuge tube.

- 6.6 Wash cells by adding an excess of HBSS and centrifuge for 10 min at 400xg, 18–20°C. The HBSS volume should be ~3 times the volume of the mononuclear layer.

Note: Typically, 4 mL of blood/PBS mixture results in ~ 2 mL of mononuclear layer and requires at least 6 mL of HBSS for the wash step. We use 10 mL of HBSS for each 2 mL of cells.

- 6.7 Discard supernatant and repeat the wash step once more.

- 6.8 Re-suspend cells in complete RPMI-1640 medium. Dilute cells 1:5 or 1:10 with trypan blue, count cells, and determine viability using trypan blue exclusion. If viability is at least 90%, proceed to step 6.9.

- 6.9 Dilute cells to a concentration of 1.0×10^6 viable cells per 1 mL, so that each 50 µL aliquot of this cell suspension, when plated per well on the ELISpot plate, contains 50,000 cells per well.

7. Set-up ELISpot Plate

- 7.1 Fill all wells of the ELISpot plate with 200 µL of sterile culture medium and incubate for 20 minutes at room temperature.

Note: This step is intended for wetting the PVDF membrane; the medium will be discarded later, in step 7.2, before loading the treatments and PBMC cells.

- 7.2 Aspirate 200 µL of the culture medium from the ELISpot plate prepared in step 7.1.

- 7.3 Add 50 µL of controls and treatments to the appropriate wells of the plate.

Note: It is advisable to use positive control samples in wells A1, A12, and H12 to facilitate stage calibration. Please consider the plate map in the Appendix of this protocol as an example of plate setup.

- 7.4 Seed 50 μ L of cells from step 6.9 into the appropriate wells of the plate prepared in step 7.3.

- 7.5 Incubate at 37°C, 5% CO₂, and 90–95% humidity for 20–24 hours.

Note: During the incubation, it is important to avoid disturbing the plate.

- 7.6 At the end of the incubation, remove the plate from the incubator and continue to detect IFN γ using the IFN γ ELISpot kit according to the manufacturer's instructions.

8. IFN γ Detection (R&D Human IFN γ Kit)

Note: The procedure described in this step is specific to the R&D Systems kit. When kits from other manufacturers are used, the steps outlined below may vary.

8.1 ELISpot Reagent Preparation

- 8.1.1 Warm all reagents to room temperature except for the Human IFN- γ detection antibody and Dilution Buffer 1.

- 8.1.2 Wash Buffer: Make sure any visible crystals have been dissolved by gentle mixing. Add 50 mL of wash buffer to 450 mL of deionized water.

- 8.1.3 Human IFN- γ Positive Control: Reconstitute with 250 μ L of cell culture medium.

- 8.1.4 Detection Antibody Mixture: Immediately before use, transfer 100 μ L of human IFN- γ detection antibody to the Dilution Buffer 1 vial and mix well.

- 8.1.5 Streptavidin-AP Concentrate A: Immediately before use, transfer 100 μ L of Streptavidin-AP Concentrate A to the Dilution Buffer 2 vial and mix well.

8.2 ELISpot Procedure for IFN γ Detection

- 8.2.1 Wash the plate from step 7.5 with 250–300 μ L of the previously prepared wash buffer. Repeat three times for a total of four washes.

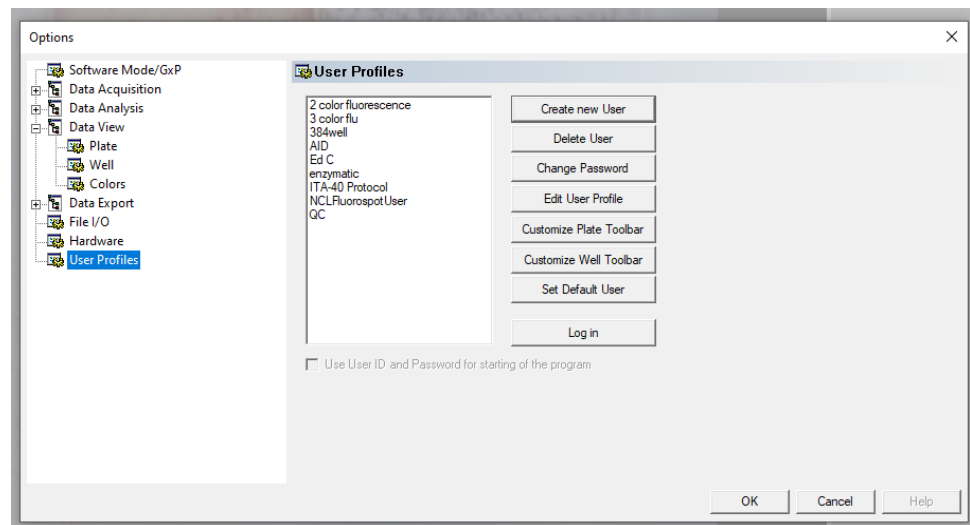
-
- The screenshot shows the Multispot software interface with five panels arranged horizontally. Each panel has a title and a corresponding image:
- HEp-2**: A green fluorescence image showing numerous bright green spots.
 - Terasaki**: A dark image with many small, multi-colored spots (red, green, yellow).
 - Cell counter**: A dark image with a few scattered small, multi-colored spots.
 - Area scanner**: A dark image with a few scattered small, multi-colored spots.
 - EliSpot**: A light-colored image showing many small, dark spots.

-

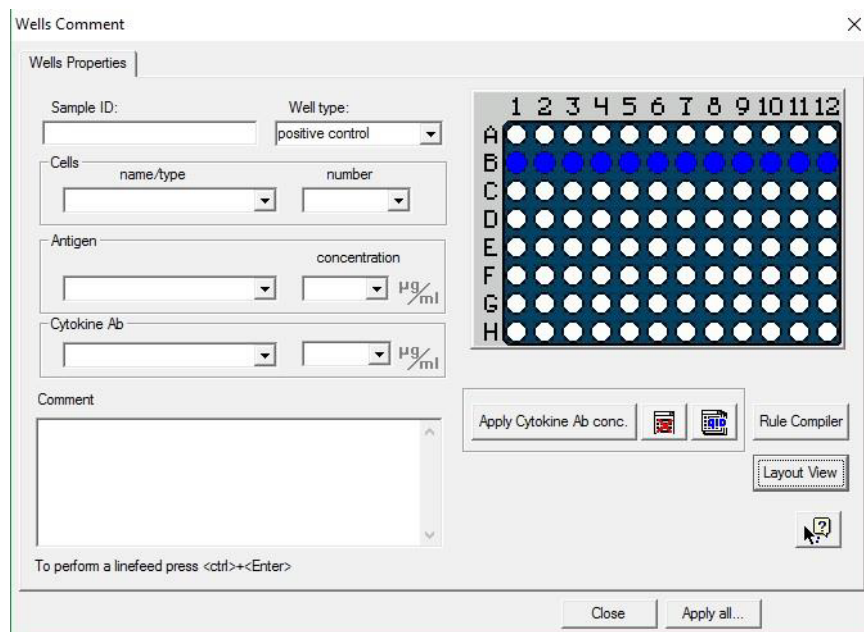
- 8.3.4 In the user profile window, highlight **IFN- γ Enzymatic ELISpot Protocol**, click **Log In**, enter the user password, and then click **OK**.

Note: Each user should have their own password to operate this software on their computer.

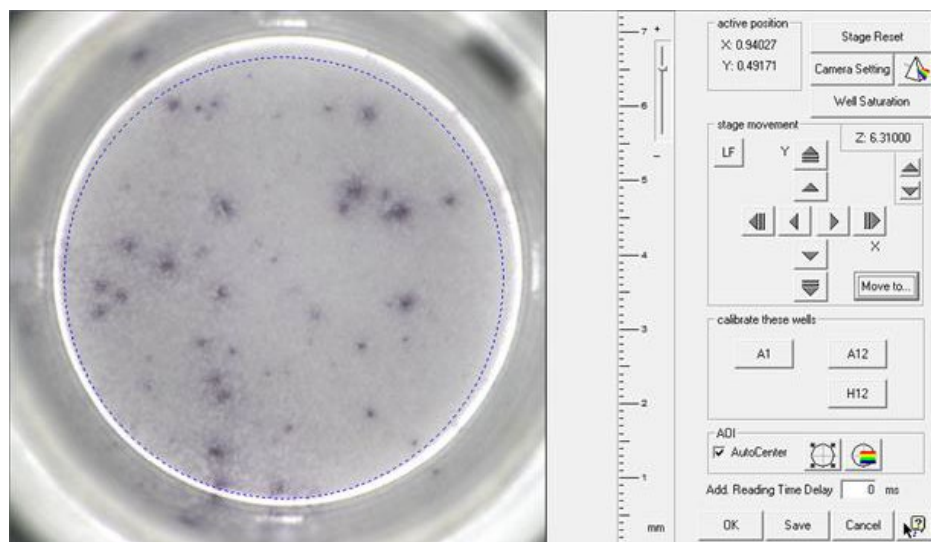
- 8.3.5 Click **OK** in the Options window to close.



- 8.3.6 Under the **File** menu, click **New**. This should open a plate template with the ITA-41 settings. Remember to save the file under a new name.
- 8.3.7 To add information on cell lines used, cells/well, and treatments, click on the **Tools** menu, click **Plate Layout**, then click **Edit Layout**. This will open the **Wells Properties** window.



8.3.8 Under the **Tools** menu, choose **Calibrate Stage** and choose well **A1**; center and adjust the imaging ring to encompass most of the well. Do the same for wells **A12** and **H12**. Click **Save** and **OK** to save the stage calibration.



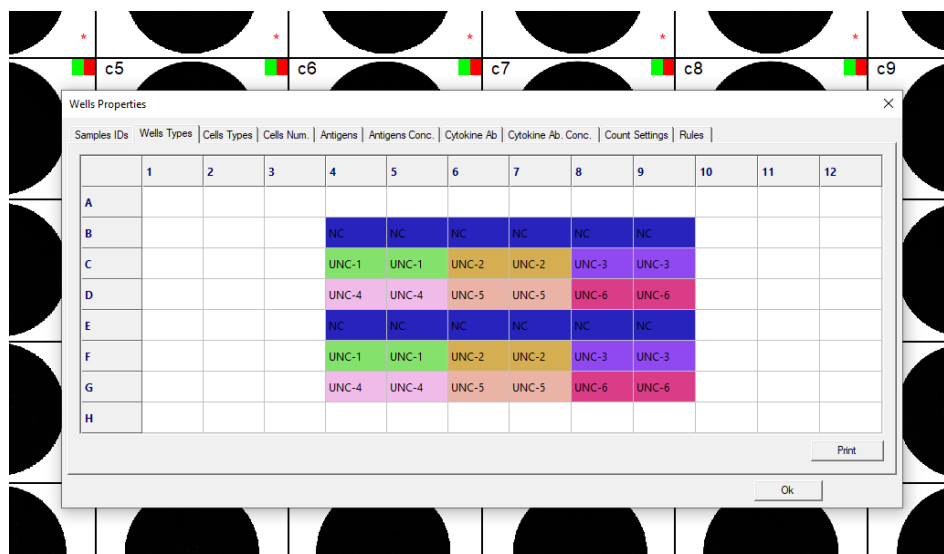
8.3.9 On the plate map, use the mouse to select the wells to be read, then right-click and highlight **Read, Count & Analyze** on the drop-down menu.

8.3.10 After the plate has been read, click the **Save** button.

9. Results and Analysis

If necessary, the analysis algorithms and count settings may be changed from the AID default. This may be done before or after the plate is read.

- 9.1 Once the plate has been read and analyzed, click the **Excel** button on the menu bar. This will open an Excel file with the spot counts and activities for each of the selected wells.
- 9.2 Images of the plate and individual wells may be selected and captured using the **PowerPoint** button on the menu bar or by using the Print Screen function.
- 9.3 Plate and well information may be captured by clicking **Tools, Plate Layout**, then **View Layout**.



10. References

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11. Abbreviations

AID	Advanced Imaging Devices (Autoimmun Diagnostika)
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium
CF	cell-free
FBS	fetal bovine serum
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	positive control
PHA-M	Phytohemagglutinin M
RT	room temperature
T	test sample

12. Appendix

Example Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	Cell-free	CF PC	CF T1	CF T2	CF T3	CF T4					PC
B	Untreated	Untreated	PC	PC	T1	T1	T2	T2	T3	T3	T4	T4
C	Untreated	Untreated	PC	PC	T1	T1	T2	T2	T3	T3	T4	T4
D	Untreated	Untreated	PC	PC	T1	T1	T2	T2	T3	T3	T4	T4
E	Untreated	Untreated	PC	PC	T1	T1	T2	T2	T3	T3	T4	T4
F	Untreated	Untreated	PC	PC	T1	T1	T2	T2	T3	T3	T4	T4
G	Untreated	Untreated	PC	PC	T1	T1	T2	T2	T3	T3	T4	T4
H		Cell-free	CF PC	CF T1	CF T2	CF T3	CF T4					PC

Wells A1, A12, H12: PBMC + PC for stage calibration

Rows A, H: Cell-free (CF) controls

Rows B, C: Donor 1

Rows D, E: Donor 2

Rows F, G: Donor 3

PC, positive control; CF, cell-free well;

T, test sample (at 4 different concentrations, i.e., T1, T2, T3, T4)