ELISpot

Primate IFN-γ

Catalog Number EL961

For the quantitative determination of the frequency of cells releasing primate IFN-γ.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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INTRODUCTION

Interferon-gamma (IFN-γ, also known as type II interferon) is an important immunoregulatory cytokine that was originally identified through its anti-viral activity (1). It plays key roles in host defense by exerting antiviral, antiproliferative and immunoregulatroy activities (2-5). On many cell types, IFN-γ induces the production of cytokines and upregulates the expression of various membrane proteins including class I and II MHC antigens, Fc receptors, leukocyte adhesion molecules and B7 family antigens. IFN-γ is a potent activator of macrophage effector functions. It potentiates the secretion of immunoglobulins by B cells and directs the synthesis of IgG. IFN-γ also influences T-helper cell phenotype development by inhibiting Th2 differentiation and stimulating Th1 development (2-5).

IFN-γ is produced by a number of cell types, including dendritic epidermal/γδ T cells (6), keratinocytes (7), peripheral blood γδ T cells (8), mast cells (9), neurons (10), $CD8^+$ T cells (11), macrophages (12), B cells (13), neutrophils (14), NK cells (15), $CD4^+$ T cells (16) and testicular spermatids (17). The production of IFN-γ is upregulated synergistically by IL-12, IL-18, and IL-23 (18-21). Rhesus monkey IFN-γ cDNA encodes a 165 amino acid (aa) residue precursor protein that contains a 20 aa signal sequence that is cleaved to generate a 145 aa residue mature monkey IFN-γ (22). Rhesus monkey IFN-γ is presumably a noncovalent homodimer (3). In the mature segment, it shares 94%, 88%, 100%, 100%, 98%, and 94% aa sequence identity with human (23, 24), squirrel monkey (25), pigtailed macaque (22), cynomolgus monkey (26), sooty mangabey (22), and gorilla (27) IFN-γ, respectively (22). By contrast, mature human IFN-γ shares 94%, 90%, 94%, 94%, 93%, and 100% aa sequence identity with rhesus monkey (22), squirrel monkey (25), pigtailed macaque (22), cynomolgus monkey (26), sooty mangabey (22), and gorilla (27) IFN-γ, respectively.

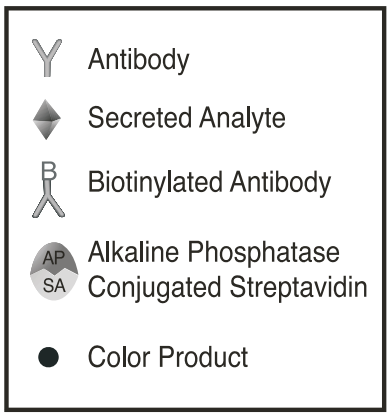
The functional IFN-γ receptor complex consists of two distinct subunits: the alpha-subunit (IFN-γ R1) that binds IFN-γ with high affinity and species specificity, and the beta-subunit (IFN-γ R2, also known as AF-1 or accessory factor-1) that interacts with the IFN-γ occupied α-subunit in a species-specific manner (28). For most IFN-γ induced responses, signal transduction via the JAK-STAT pathway is initiated by the formation of the multimeric receptor complex, composed of one IFN-γ homodimer, two α chains and two β chains (28, 29). Additional, as yet unidentified (30), components for the receptor complex may exist for some IFN-γ induced responses (31).

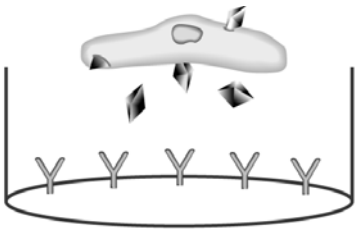
The Primate IFN-γ ELISpot assay is designed for the detection of IFN-γ secreting cells at the single cell level, and it can be used to quantify the frequency of primate IFN-γ secreting cells. ELISpot assays are well suited for monitoring immune responses to various stimuli, treatments and therapies, and they have been used for the quantitation of antigen-specific CD4 and/or CD8 T cell responses. Other methods for the assessment of antigen-specific T cell responses, such as the chromium release assay with quantitation by limiting dilution, are tedious and require previous *in vitro* expansion of T cells for several days. These assays typically are not suitable for measuring infrequent T cell responses that occur at less than 1 in 1000. ELISpot assays are highly reproducible and sensitive and can be used to measure responses with frequencies well below 1 in 100,000. ELISpot assays do not require prior *in vitro* expansion of T cells, and they are suitable for high-throughput analysis using only small volumes of primary cells. As such, ELISpot assays are useful tools for research in areas as diverse as antigen recognition, vaccine development, cytokine secretion and the monitoring of various clinical trials.

PRINCIPLE OF THE ASSAY

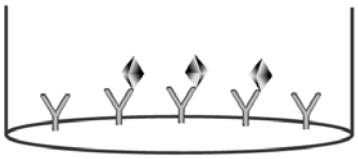
The enzyme-linked immunospot (ELISpot) assay was originally developed for the detection of individual B cells secreting antigen-specific antibodies (32, 33). This method has since been adapted for the detection of individual cells secreting specific cytokines or other antigens (34, 35). ELISpot assays employ the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique. An anti-human IFN-γ monoclonal antibody has been pre-coated onto a PVDF (polyvinylidene difluoride)-backed microplate. Appropriately stimulated cells are pipetted into the wells and the microplate is placed into a humidified 37° C CO_2 incubator for a specified period of time. During this incubation period, the immobilized antibody in the immediate vicinity of the secreting cells binds secreted IFN-γ. After washing away any cells and unbound substances, a biotinylated, anti-rhesus macaque IFN-γ polyclonal antibody is added to the wells. Following a wash to remove any unbound biotinylated antibody, alkaline-phosphatase conjugated to streptavidin is added. Unbound enzyme is subsequently removed by washing and a substrate solution (BCIP/NBT) is added. A blue-black colored precipitate forms and appears as spots at the sites of cytokine localization, with each individual spot representing an individual IFN-γ secreting cell. The spots can be counted with an automated ELISpot reader system or manually using a stereomicroscope.

ELISpot SCHEMATIC

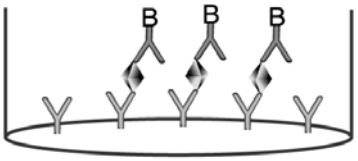




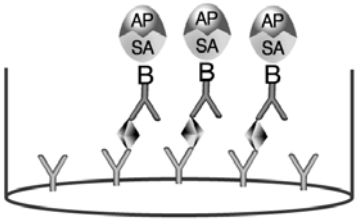
Incubate IFN-γ-secreting cells in an antibody-coated well.



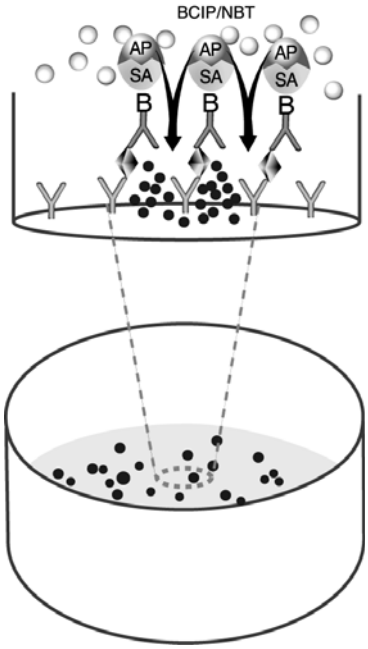
Remove cells by washing. Secreted IFN-γ is captured by the immobilized antibody.



Incubate with biotinylated anti-IFN-γ antibody.



Incubate with alkaline phosphatase conjugated streptavidin.



Add substrate and monitor the formation of colored spots.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Any variation in pipetting and washing techniques, incubation time or temperature, or kit age can cause variation in density of spots, intensity of specific staining and background levels.

PRECAUTIONS

Some components of this kit contain sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

Do not use reagents from this kit with components from other R&D Systems' ELISpot or ELISA kits and/or components manufactured by other vendors.

Do not remove the flexible plastic underdrain on the bottom of the microplate before or during incubation and development since it may damage the PVDF membrane filters. The underdrain cover may be removed only after completing the incubation with BCIP/NBT chromogen.

Although the toxicity of the chromogenic substrate BCIP/NBT is not currently known, wear gloves to avoid contact with skin. Follow local, state and federal regulations to dispose of used BCIP/NBT.

MATERIALS PROVIDED

Primate IFN-γ Microplate (Part 892394) - One 96-well PVDF-backed microplate coated with anti-human IFN-γ monoclonal antibody.

Detection Antibody Concentrate (Part 892395) - 150 µL of a 120X concentrated solution of biotinylated anti-rhesus macaque IFN-γ polyclonal antibody with preservatives.

Streptavidin-AP Concentrate A (Part 895358) - 150 µL of a 120X concentrated solution of Streptavidin conjugated to Alkaline Phosphatase with preservatives.

Dilution Buffer 1 (Part 895307) - 12 mL of a buffer for diluting Detection Antibody Concentrate with preservatives.

Dilution Buffer 2 (Part 895354) - 12 mL of a buffer for diluting Streptavidin-AP Concentrate A with preservatives.

Wash Buffer Concentrate (Part 895308) - 50 mL of a 10X concentrated solution of a buffered surfactant with preservative.

BCIP/NBT Chromogen (Part 895867) - 12 mL of a stabilized mixture of 5-Bromo-4-Chloro-3′ Indolylphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tetrazolium Chloride (NBT).

Primate IFN-γ Positive Control (Part 892396) - 1 vial (2 ng) of recombinant rhesus macaque IFN-γ; lyophilized.

STORAGE

Store the unopened kit at 2 - 8° C. Do not use beyond the kit expiration date. This kit is validated for single use only. Results obtained with opened/reconstituted reagents at a later date may not be reliable.

OTHER SUPPLIES REQUIRED

- Pipettes and pipette tips
- Deionized or distilled water
- Multi-channel pipette, squirt bottle, manifold dispenser, or automated microplate washer
- 500 mL graduated cylinder
- 37° C CO_2 incubator
- Sterile culture media
- Dissection microscope or an automated ELISpot reader

TECHNICAL HINTS

- To minimize edge effect, place the microplate (bottom down) onto a piece of soft aluminum foil (about 4 x 6 inches). Add cells, cover the microplate with the lid and shape the foil around the edges of the microplate. The foil may be left on the microplate for the rest of the experimental procedure and removed after the BCIP/NBT has been washed off.
- Do not touch PVDF membrane filters with pipette tips when pipetting cells and reagents to avoid damage to the membrane.
- After completion of the experiment, do not dry the microplate at a temperature higher than 37° C since it may cause cracking of the PVDF membrane filters.
- The 96-well microplate provided in the kit is not sterile. However, due to the short incubation period and presence of antibiotics in the culture media, microbial contamination has not been a problem during the ELISpot procedure.
- The kit is designed for single use only. The layout of the assay should be carefully planned to maximize the use of the plate and reagents provided.
- The controls listed are recommended for each ELISpot experiment.

  Positive Control - Use recombinant rhesus macaque IFN-γ.

  Unstimulated/Negative Control - Use the same number of unstimulated cells as stimulated cells.

  Background Control - Use sterile culture media.

  Detection Antibody Control - Substitute phosphate buffered saline for Detection Antibody.

REAGENT PREPARATION

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare Wash Buffer, add 50 mL of Wash Buffer Concentrate to 450 mL of deionized water and mix well.

Primate IFN-γ Positive Control - Reconstitute lyophilized rhesus macaque IFN-γ with 250 µL of culture medium that is used to incubate cells.

Detection Antibody - Tap or vortex the vial to release reagent collected in the cap. Transfer 100 µL of Detection Antibody Concentrate into the vial labeled Dilution Buffer 1 and mix well. **For optimal performance, prepare Detection Antibody immediately before use.**

Streptavidin-AP - Tap or vortex the vial to release reagent collected in the cap. Transfer 100 µL of Streptavidin-AP Concentrate A into the vial labeled Dilution Buffer 2 and mix well. **For optimal performance, prepare Streptavidin-AP immediately before use.**

SAMPLE PREPARATION

The types of effector and responder cells used, method of cell separation, mode of stimulation, and length of incubation are to be determined by each investigator.

ASSAY PROCEDURE

Bring all reagents to room temperature, except the Detection Antibody Concentrate and Dilution Buffer 1, which should remain at 2 - 8° C. All samples and controls should be assayed at least in duplicate. An Assay Record Template is provided at the back of this insert to record controls and samples assayed.

1. Fill all wells in the microplate with 200 µL of sterile culture media and incubate for approximately 20 minutes at room temperature.
2. When cells are ready to be plated, aspirate the culture media from the wells. Immediately add 100 µL of the appropriate cells or controls to each well (see Technical Hints for appropriate controls).
3. Incubate cells in a humidified 37° C CO_2 incubator. Optimal incubation time for each stimuli should be determined by the investigator. **Do not disturb the cells during the incubation period.**
4. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (250 - 300 µL) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
   Note: Adjust the height of the prongs of the manifold dispenser or autowasher to prevent damage to the membranes.
5. Add 100 µL of diluted Detection Antibody into each well and incubate at 2 - 8° C overnight.
6. Repeat step 4.
7. Add 100 µL of diluted Streptavidin-AP into each well and incubate for 2 hours at room temperature.
8. Repeat step 4.
9. Add 100 µL of BCIP/NBT Chromogen into each well and incubate for 1 hour at room temperature. **Protect from light**.
10. Discard the chromogen solution from the microplate and rinse the microplate with deionized water. Invert the microplate and tap to remove excess water. Remove the flexible plastic underdrain from the bottom of the microplate, wipe the bottom of the plate thoroughly with paper towels and dry completely either at room temperature (60 - 90 minutes) or 37° C (15 - 30 minutes).

CALCULATION OF RESULTS

The developed microplate can be analyzed by counting spots either manually using a dissection microscope or by using a specialized automated ELISpot reader. Specific spots are round and have a dark center with slightly fuzzy edges. Quantitation of results can be done, for example, by calculating the number of spot forming cells (SFC) per number of cells added into the well.

REPRODUCIBILITY DATA

Peripheral blood mononuclear cells from five different non-human primates were stimulated with 50 ng/mL of phorbol 12-myristate-13-acetate and 0.5 μg/mL calcium ionomycin overnight at 37° C in a 5% CO_2 incubator. The sample was assayed in five wells according to the procedure and analyzed with a dissection microscope. The species and cell concentration used are given above each table.

Rhesus Monkey - 5 x 10^4 cells/mL

Well	Number of Spots Counted
1	159
2	161
3	154
4	155
5	164

Chimpanzee - 1 x 10^5 cells/mL

Well	Number of Spots Counted
1	56
2	58
3	58
4	74
5	68

Pigtail Monkey - 5 x 10^4 cells/mL

Well	Number of Spots Counted
1	148
2	172
3	144
4	161
5	121

Baboon - 1 x 10^5 cells/mL

Well	Number of Spots Counted
1	74
2	54
3	63
4	68
5	74

Cynomolgus Monkey - 5 x 10^4 cells/mL

Well	Number of Spots Counted
1	43
2	44
3	50
4	40
5	45

TROUBLESHOOTING GUIDE

Observation	Problem	Corrective Action
Following the incubation with BCIP/NBT chromogen and rinsing the microplate with deionized water, the dark-blue background color of filter membrane attenuates visualization and quantitation of spots.	Wet membrane	Microplates cannot be analyzed accurately until PVDF filter membranes are completely dry. Wait until membrane becomes dry, usually 15 - 30 minutes at 37° C or 60 - 90 minutes at room temperature.
The number of spots in the wells that contained the cells is high but their contrast as well as intensity of staining in the Positive Control wells is low.	Underdevelopment - perhaps the result of using Streptavidin-AP and/or BCIP/NBT solutions that have not been adjusted to room temperature	Adjust the temperature of the reagents to room temperature before adding to the wells.
The number of spots in the wells that contained cells is lower than expected whereas Positive Control wells turned black-blue.	Cell stimulation problem	Ensure that reagents used to stimulate the cytokine release from the cells retained their biological activity. One way to check is to perform immunocytochemistry on fixed cells after stimulation.
	Too few cells added to the wells	Increase the number of cells added per well.
Following incubation with BCIP/NBT and drying the microplate, the density of the spots makes it difficult to quantify them.	Too many cells were added to the wells	Make dilutions of cells (*i.e.,* 1×10^6, 5×10^5, 1×10^5, 5×10^4, 1×10^4 cells per well) to determine the optimal number of cells that will result in formation of distinct spots.

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ASSAY RECORD TEMPLATE

This template may be used as a record of samples and controls run in an assay.