ELISpot

Mouse IFN-γ Kit

Catalog Number EL485

For the quantitative determination of the frequency of cells releasing mouse Interferon gamma (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 anti-viral, anti-proliferative and immunoregulatory activities (2-4). 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 antigen. IFN-γ is a potent activator of macrophage effector functions. It potentiates the secretion of immunoglobulins by B cells and affects isotype switching. IFN-γ also influences T-helper cell phenotype determination by inhibiting Th2 differentiation and stabilizing Th1 cells (2-4).

IFN-γ is produced primarily by activated NK cells, activated Th1 cells and activated $CD8^+$ cytotoxic cells (2-4). Additional cell types that produce IFN-γ include macrophages (5), dendritic cells (6, 7) and mast cells (8). The production of IFN-γ is upregulated synergistically by IL-12 and IL-18 (9-11). Mouse IFN-γ cDNA encodes a 155 amino acid (aa) residue precursor protein containing a 22 aa residue predicted signal peptide that is cleaved to generate the 133 aa residue mature mouse IFN-γ (12, 13). In solution, mouse IFN-γ exists exclusively as a noncovalent homodimer (4). Mouse IFN-γ shares approximately 40% aa sequence identity with human IFN-γ and does not have cross–species activity (2-4).

The functional IFN-γ receptor complex consists of two distinct subunits (14). The α subunit (IFN-γ R1) binds IFN-γ with high-affinity and species-specificity. The β subunit (IFN-γ R2, also referred to as the accessory factor 1, AF-1) interacts with the IFN-γ-occupied α subunit in a species-specific manner and is required for signal transduction via the JAK-STAT pathway. Both the α and the β subunits are type I membrane proteins. Whereas the α subunit is expressed constitutively at low levels on many cell types, the cellular expression of the β subunit correlates with the IFN-γ responsive state and is tightly-regulated.

The Mouse IFN-γ ELISpot assay is designed for the detection of mouse IFN-γ secreting cells at the individual single cell level and can be used to quantitate the frequency of mouse IFN-γ secreting cells. ELISpot assays are well suited for monitoring immune responses to various treatments and therapies and have been used for the quantitation of antigen-specific $CD4^+$ and/or $CD8^+$ T cells responses. Other methods for assessment of antigen-specific T cells responses, such as chromium release assays 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 are suitable for high-throughput analysis using only small volumes of primary cells. As such, ELISpot assays are useful tools for research in vaccine development and for 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 (15, 16). This method has since been adapted for the detection of individual cells secreting specific cytokines or other antigens (17, 18). ELISpot assays employ the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique.

A monoclonal antibody specific for mouse IFN-γ 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 monoclonal antibody specific for mouse IFN-γ 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 at the sites of cytokine localization and appear as spots, with each individual spot representing an individual IFN-γ secreting cell. The spots can be counted with an ELISpot reader system or using a stereomicroscope.

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.
- Do not mix or substitute reagents with those from other lots or sources.
- Any variation in pipetting and washing techniques, incubation time or temperature, or kit age can cause variation in the density of spots, intensity of specific staining, and background levels.

TECHNICAL HINTS

- To minimize edge effect, place the microplate (bottom down) onto a piece of 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 remove the flexible plastic underdrain on the bottom of the microplate before or during incubation and development. It may damage the PVDF membrane filter. The underdrain cover may be removed only after completing the incubation with BCIP/NBT Substrate.
- Do not touch PVDF membrane filters with pipette tips when pipetting cells and reagents to avoid damage to the membrane.
- Upon completing the experiment, do not dry the microplate at a temperature above 37 °C. It may cause the PVDF membrane filters to crack.
- The 96-well microplate provided in this kit is not sterile. Due to the short incubation period and the presence of antibiotics in the culture media, microbial contamination has not been shown to be an issue with this ELISpot procedure.
- This kit is designed for single use only. The layout of the assay should be carefully planned to maximize the use of the provided microplate and reagents.
- The controls listed are recommended for each ELISpot experiment:

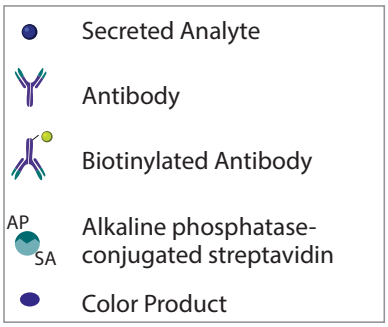
  Positive Control - Use recombinant mouse IFN-γ.

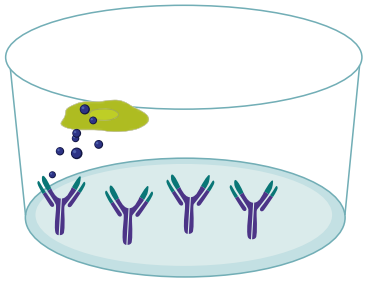
  Unstimulated/Negative Control - Use the same number of unstimulated cells as stimulated cells.

  Background Control - Use sterile culture media.

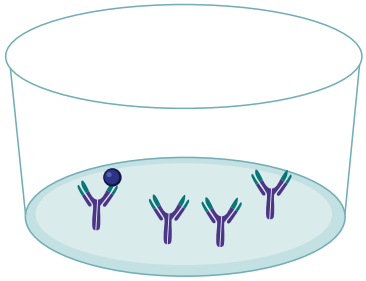
  Detection Antibody Control - Substitute phosphate buffered saline for detection antibody.

ELISpot SCHEMATIC

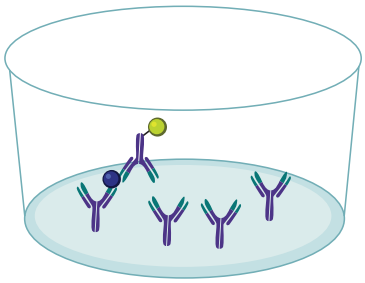




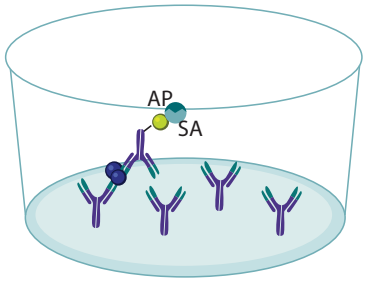
Incubate IFN-γ-secreting cells in an antibody-coated well.



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

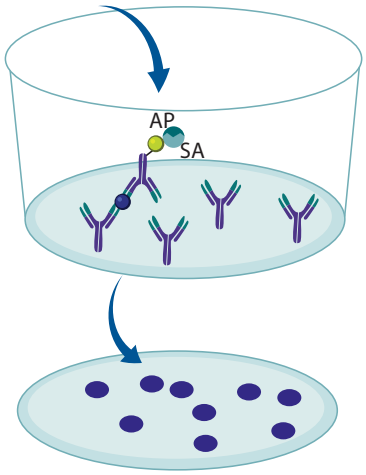


Incubate with biotinylated anti-IFN-γ antibody.



Incubate with alkaline phosphatase conjugated streptavidin.

Add BCIP/NBT Substrate



Add substrate and monitor the formation of colored spots.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.
This kit is validated for single use only.

Note: *Results obtained using previously opened or reconstituted reagents may not be reliable.*

PART	PART #	DESCRIPTION
Mouse IFN-γ Microplate	890894	96-well PVDF-backed microplate coated with a monoclonal antibody specific for mouse IFN-γ.
Detection Antibody Concentrate	890895	150 µL of a 120X concentrated solution of biotinylated monoclonal antibody specific for mouse IFN-γ with preservatives.
Streptavidin-AP Concentrate A	895358	150 µL of a 120X concentrated solution of Streptavidin conjugated to Alkaline Phosphatase with preservatives.
Dilution Buffer 1	895307	12 mL of a buffer for diluting Detection Antibody Concentrate with preservatives.
Dilution Buffer 2	895354	12 mL of a buffer for diluting Streptavidin-AP Concentrate A with preservatives.
Wash Buffer Concentrate	895308	50 mL of a 10X concentrated solution of a buffered surfactant with preservative.
BCIP/NBT Substrate	895867	12 mL of a stabilized mixture of 5-Bromo-4-Chloro-3' Indolylphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tetrazolium Chloride (NBT).
Mouse IFN-γ Positive Control	890896	8 ng of recombinant mouse IFN-γ with preservatives; lyophilized.

OTHER SUPPLIES REQUIRED

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

PRECAUTIONS

BCIP/NBT is toxic if swallowed, in contact with skin, or if inhaled. It is a highly flammable liquid and vapor may cause serious irritation and damage to organs. Do not eat, drink, or smoke when using this product. Do not breathe fumes. Use only in a well-ventilated area. Keep away from heat, sparks, open flames, and hot surfaces. Keep the container tightly closed.

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.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

REAGENT PREPARATION

Bring all reagents to room temperature, except the Detection Antibody Concentrate and Dilution Buffer 1, which should remain at 2-8 °C.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 50 mL of Wash Buffer Concentrate to 450 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Mouse IFN-γ Positive Control - Reconstitute the lyophilized mouse IFN-γ control 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 the Detection Antibody mixture immediately before use.**

Streptavidin-AP Concentrate A - **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 the 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 diluted Detection Antibody mixture and Dilution Buffer 1, which should remain at 2-8 °C. All samples and controls should be assayed at least in duplicate.

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 stimulus 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, 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 mixture into each well, and incubate overnight at 2-8 °C. Alternatively, incubation with detection antibodies can be done for 2 hours at room temperature on a rocking platform.
6. Repeat the wash procedure described in step 4.
7. Add 100 µL of diluted Streptavidin-AP Concentrate A into each well, and incubate for 2 hours at room temperature.
8. Repeat the wash procedure described in step 4.
9. Add 100 µL of BCIP/NBT Substrate into each well, and incubate for 1 hour at room temperature. **Protect from light.**
10. Decant the BCIP/NBT Substrate 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 using either a dissection microscope or 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 to the well.

REPRODUCIBILITY DATA

Splenocytes from a C57BL mouse (5 x 10^5 cells/mL) 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 eight wells according to the procedure and analyzed with a dissection microscope.

Well	Number of Spots Counted
1	410
2	419
3	414
4	406
5	420
6	385
7	398
8	408

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 the filter membrane attenuates visualization and quantitation of spots.	The membrane is wet.	Microplates cannot be analyzed accurately until the PVDF filter membranes are completely dry. Wait until the membrane becomes dry (typically 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 brought to room temperature.	Warm the appropriate reagents to room temperature before adding them to the wells.
The number of spots in the wells that contained cells is lower than expected whereas Positive Control wells turned blue-black.	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 were 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 them difficult to quantify.	Too many cells were added to the wells.	Make dilutions of cells (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.

REFERENCES

1. Wheelock, E.F. (1965) Science **146**:310.
2. Billiau, A. (1996) Adv. Immunol. **62**:61.
3. Boehm, U. *et al.* (1997) Annu. Rev. Immunol. **15**:749.
4. Farrar, M.A. and R.D. Schreiber (1993) Annu. Rev. Immunol. **11**:571.
5. Puddu, P. *et al.* (1997) J. Immunol. **159**:3490.
6. Sugaya, M. *et al.* (1999) J. Invest. Dermatol. **113**:350.
7. Ohteki, T. *et al.* (1999) J. Exp. Med. **189**:1981.
8. Gupta, A.A. *et al.* (1997) J. Immunol. **157**:2123.
9. Lebel-Binay, S. *et al.* (2000) Eur. Cytokine Netw. **11**:15.
10. Tominaga, K. *et al.* (2000) Int. Immunol. **12**:151.
11. Trinchieri, G. and F. Gerosa (1996) J. Leukoc. Biol. **59**:505.
12. Gray, P.W. *et al.* (1982) Nature **295**:503.
13. Jarpe, M.A. and H.M. Johnson (1990) J. Immunol. **145**:3304.
14. Bach, E.A. *et al.* (1997) Annu. Rev. Immunol. **15**:563.
15. Czerkinsky, C.C. *et al.* (1983) J. Immunol. Methods **65**:109.
16. Sedgwick, J.D. and P.G. Holt (1983) J. Immunol. Methods **57**:301.
17. Czerkinsky, C.C. *et al.* (1984) J. Immunol. Methods **72**:489.
18. Helms, T. *et al.* (2000) J. Immunol. **164**:3723.