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# Mouse IFN-γ ELISPOT Kit w/ PVDF Plates

**Catalog No:** CKM038A **Size:** 2 x 96 tests

## INTENDED USE

The cytokine ELISPOT (Enzyme-Linked ImmunoSPOT) assay is designed to enumerate cytokine-secreting cells in single cell suspensions of lymphoid tissue, central nerve system (CNS) tissue, bone marrow or preparations of peripheral blood mononuclear cells (PBMCs). The assay has the advantage of detecting only activated/memory T cells and has the ability to detect cytokine release in response to antigen by a single cell thereby permitting direct calculation of responder T cell frequencies. The high sensitivity and easy performance, allowing the determination of peptide-reactive T cells without prior in vitro expansion, makes the ELISPOT assay eminently well suited to monitor T cell responses. The higher sensitivity of ELISPOT in comparison to that of ELISA or intracellular staining is due to the plate-bound antibodies directly capturing the cytokine released by the cell before it is diluted in the supernatant, trapped by high-affinity receptors or degraded by proteases. The sensitivity of the assay lends itself to measurement of very low frequencies of cytokine-secreting cells (1/300,000).

Background

IFN-γ, the Type II Interferon, is a cytokine critical for cell mediated immunity against viral and intracellular bacterial infections. Its production is a key function of a subset of CD4+ helper T cells known as Th1 cells, as well as by CD8+ CTL and NK cells. IFN-γ is an excellent marker for identifying a host response to intracellular pathogens, and is the hallmark effector cytokine of Th1 cells, whereas the Th2 subset produces little or no IFN-γ. Additionally, IFN-γ preferentially inhibits the proliferation of Th2 but not Th1 cells, resulting in preferential proliferation of Th1 cells.

The active IFN-γ protein is a homodimer that binds to IFN-γ receptor, triggering its many immunoregulatory properties. The results of IFN-γ ligation include upregulation of effector molecules and functions such as: macrophage activation; promotion/differentiation of NK, Th1 and CTL cells; activation/release of reactive oxygen intermediates; upregulation of antigen presentation and cell adhesion molecules; antiproliferative effects on transformed cells; and potentiation of antiviral and antitumor effects of the type I interferons. Mutations in the IFN-γ gene are associated with an increased susceptibility to viral, bacterial and parasitic infections and to several autoimmune diseases.

Brief Description

Cells are incubated in the wells of the ELISPOT plate pre-coated with a high-affinity monoclonal antibody to which the cytokine, produced during incubation, will bind. Subsequently, cells are washed away. Areas in which the cytokine has been bound are detected with a combination of biotinylated anti-cytokine detection antibodies and Streptavidin-horseradish peroxidase [Streptavidin-HRP]. The last step in the assay is the addition of AEC (3-amino-9-ethylcarbazole) yielding a colored ('spot'). This zone reveals the site of cytokine secretion.



**Cell Sciences**
65 Parker Street, Unit 11
Newburyport, MA 01950

Toll Free: 888 769-1246
Phone: 978-572-1070
Fax: 978-992-0298

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Web Site: www.cellsciences.com

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**Reagents Included:**

| Items | Quantity (2 plates) |
|---|---|
| Coating antibodies (lyophilized) | 1 vial |
| Biotinylated detection antibodies (lyophilized) | 1 vial |
| Streptavidin-HRP conjugate (lyophilized) | 1 vial |
| AEC* coloring system:<br>I. AEC stock solution<br>II. Substrate buffer capsules | <br>4 ml<br>2 |
| Blocking stock solution R (10x) | 4 ml |
| Dilution buffer R (10x) | 4 ml |
| Tween-20 | 5 ml |
| PVDF plates | 2 |
| Adhesive Cover Slip | 5 |

**Hazard Information**

**AEC (3-Amino-9-ethylcarbazole) stock solution is classified as harmful according to Directive 1999/45/EC and its amendments. Risk phrases: R22/R36 Safety phrases: S26/S39/S45.**

**The AEC stock solution should be handled only in a chemical fume hood.**

**In case of contact with skin, wash with soap and water and remove contaminated clothing and shoes. Upon ingestion or contact with eyes, rinse mouth (if person is conscious) or eyes with copious amounts of water for at least 15 minutes. Assure adequate flushing by separating the eyelids. Seek medical advice immediately.**

**Other kit components are not classified as dangerous according to Directive 67/548/EC or 1999/45/EC and their amendments.**

**Storage of Kit Reagents:**

- Store lyophilized coating antibodies and biotinylated detection antibodies at 2-8°C until kit expiration date. After reconstitution, the antibodies are stable for at least 6 months at 2-8°C, when kept sterile, or for 1 year when stored in working aliquots at -20 to -80°C.
- Store lyophilized Streptavidin-HRP conjugate at -20 to -80°C until the kit expiration date. After reconstitution, the conjugate is stable for at least 2 months at 2-8°C, when kept sterile. It is strongly recommended to store the conjugate in working aliquots at -20 to -80°C, stable for at least one year.
- Store AEC stock solution at -20 to -80°C for at least 1 year. Protect from light. It is recommended to store the AEC in single use aliquots in polypropylene vials.
- Store substrate buffer capsules at room temperature in a dry place until the kit expiration date.
- Store Blocking stock solution R (10x) and Dilution buffer R (10x) at 2-8°C until the kit expiration date. After opening, these solutions are stable for at least 6 months, when kept sterile.
- Store Tween-20 at room temperature until kit expiration date.



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**Materials/reagents required but not provided:**

- Sterile distilled water
- 70% Ethanol
- Phosphate Buffered Saline (PBS): home-made, filter-sterilize or autoclave. For washing purposes only.
- Wash Buffer: PBS containing 0.05% Tween-20
- Sterile and pyrogen free PBS (PBS-I)
- Culture Medium: see Addendum**
- Cell Stimuli: see Addendum**
- Pipetting devices
- Squirt (wash or squeeze) bottle with wide spout for washing, see Addendum**
- $CO_2$-incubator (37°C, 100% humidity, 5% $CO_2$)
- Tissue culture plates for pre-stimulation (optional)
- A dissecting microscope or an immunospot image analyzer for spot counting

** Addendum to this ELISPOT manual contains guidelines and troubleshooting.

**Preparation of Reagents:**

*Prepare reagents under aseptic conditions (e.g. Laminar Flow Hood).*

1. Coating antibodies
   Reconstitute the lyophilized contents by injecting the volume of sterile distilled water indicated on vial. Mix the solution gently and allow it to stand for 2 minutes at room temperature. Avoid vigorous shaking. For one ELISPOT plate (from 2-plate kit), mix 100 µl (aseptically pipetted out of the vial) 5 ml PBS-I.
2. Blocking Buffer R (1x)
   Dilute Blocking stock solution R (10x) in PBS-I. For one ELISPOT plate, mix 2 ml with 18 ml PBS-I.
3. Dilution Buffer R (1x)
   Dilute Dilution Buffer R (10x) in PBS-I. For one ELISPOT plate, mix 2 ml with 18 ml PBS-I.
4. Biotinylated detection antibodies
   Reconstitute the lyophilized contents by injecting the volume of sterile distilled water indicated on vial. Mix the solution gently and allow it to stand for 2 minutes at room temperature. Avoid vigorous shaking. For one ELISPOT plate, mix 100 µl (aseptically pipetted out of the vial) 10 ml Dilution Buffer R (1x).
5. Streptavidin-HRP conjugate
   Reconstitute the lyophilized contents by injecting the volume of sterile distilled water indicated on vial. Mix the solution gently and allow it to stand for 2 minutes at room temperature. Avoid vigorous shaking. For one ELISPOT plate, mix 100 µl (aseptically pipetted out of the vial) 10 ml Dilution Buffer R (1x).
6. AEC coloring system
   The AEC coloring system includes: 1) a concentrated AEC stock solution* and 2) a substrate buffer capsule. To prepare AEC reagents for one ELISPOT plate, completely dissolve the contents of 1 substrate buffer capsule in 57 ml water. Add 43 ml 70% ethanol for a final concentration of 30% ethanol. Thoroughly mix 10 ml of this solution with 330 µl AEC stock solution*. After mixing, the solution should be clear. Use within 30 minutes after preparation.

   ***Note: AEC solution is toxic.** Use a fume hood for dispensing and mixing solution. Do not bring AEC stock solution into contact with polystyrene pipettes, vials, etc.



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**ELISPOT method:**

*Use ELISPOT plates and reagents under aseptic conditions (e.g. Laminar Flow Hood) for steps 1 to 6.*

1. Pre-wet the PVDF-membranes by adding 25 µl of 70% ethanol to each well. Incubate for 1 minute at room temperature.
2. Aspirate or "flick" to remove ethanol. Immediately wash the wells 2x with PBS-I, then empty and tap the plate on tissue paper.
3. Add 50 µl of diluted coating antibodies to each well. Cover the plate with a lid and incubate overnight at 4°C.
4. Decant solution from wells. Wash wells 3x with 200 µl PBS-I/well. Subsequently, add 200 µl Blocking Buffer R (1x) to each well. Cover the plate with lid and incubate for 1 hour at 37°C.
5. Decant solution from wells. ***Do not wash the wells.*** Dilute the cells in Culture medium containing an appropriate stimulus (polyclonal stimulus or antigen). Add cells to the wells of the ELISPOT plate, in 100 µl/well.

   Triplicates of $3x10^6$ cells/ml are often used to assess antigen-specific responses. For polyclonal stimuli, the cell number may have to be altered. No more than $3x10^5$ cells/well should be suspended in the ELISPOT plate. See Addendum.
6. Cover ELISPOT plate with lid and incubate at 37°C, 5% $CO_2$, and 100% humidity. The incubation time can vary from 5 to 24 hours. Specific activation conditions will vary, depending on cell type, cytokine of interest, kinetics of cytokine release and whether a pre-incubation step was included in the procedure. See Addendum.
7. Remove the bulk of cells with a firm shake-out action and wash 2x with room temperature PBS (200 µl/well). Wash wells 5x with 250 µl Wash Buffer/well. See Addendum.
8. Discard wash buffer and add 100 µl of diluted biotinylated detection antibodies to each well. Seal the plate with an adhesive cover slip and incubate 1 hour at 37°C or overnight at 4°C.
9. Decant solution from wells. Remove and discard the underdrain from the bottom of the plate and wash both sides of the membrane 5x with Wash Buffer. Add 100 µl of diluted Streptavidin-HRP conjugate solution to each well. Seal the plate with an adhesive cover slip and incubate 1 hour at 37°C.
10. Decant solution from wells. Wash both sides of the membrane 5x with Wash Buffer.
11. Add 100 µl/well AEC substrate solution. Cover the plate with a lid and incubate for 25 minutes at room temperature in the dark.
12. Stop the reaction by thoroughly rinsing both sides of the PVDF membrane with de-mineralized water.
13. Air-dry plate at room temperature and count spots by use of a dissecting microscope or an immunospot image analyzer. The quality of the spots is preserved for several months when the plate is stored at a dry place in the dark (to prevent bleaching of spots).



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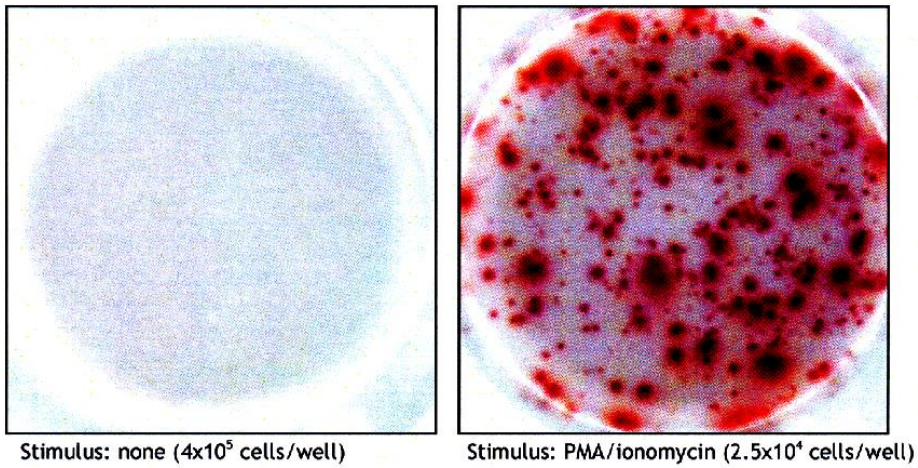
# Mouse IFN-γ ELISPOT Kit w/ PVDF Plates

**Typical Data:**

Example of IFN-gamma specific spots produced by mouse (Balb/c) spenocytes. Preincubation: 42 hours, incubation ELISPOT: 20 hours.



Stimulus: none ($4x10^5$ cells/well)

Stimulus: PMA/ionomycin ($2.5x10^4$ cells/well)

**NOT FOR HUMAN USE. FOR RESEARCH ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE.**



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