virax biolabs

ELISpot Human IFN-γ Kit

Instructions for use

Catalogue Numbers:

2091-01	**Pre-coated 96-well plate – 1 plate**
2091-05	**Pre-coated 96-well plate – 5 plates**
2291-01	**Pre-coated 12x8 well strip – 1 plate**
2291-05	**Pre-coated 12x8 well strip – 5 plates**

Note: This protocol (version 1.1) is given as a general procedure to assist when using **Virax Biolabs** Capture and Detection antibodies for ELISpot testing. Optimal dilutions of all reagents, samples and controls as well as the incubation times should be determined by each laboratory for every application.

For research use only

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INTENDED USE

The Human IFN-γ Enzyme-Linked ImmunoSpot (ELISpot) assay is a highly sensitive method used to detect and quantify individual cells that secrete specific cytokines or other soluble molecules. This assay is utilized in immunology to monitor cellular immune responses at the single-cell level and reliably detects and measures human IFN-γ secretion by stimulated effector cells.

INTRODUCTION

Description

IFN-γ is a dimerized soluble cytokine involved in both innate and adaptive immunity. It is produced primarily by Natural Killer cells during the innate immune response, and by $CD4^+$ Th1 cells and $CD8^+$ T cells during antigen-specific immune responses.

As a proinflammatory cytokine, IFN-γ plays a significant role in activating macrophages and endothelial cells, enhancing their pathogen-killing capabilities. It regulates immune responses by modulating the activity of antigen-presenting cells and T cells. IFN-γ is critical for both innate and adaptive immunity against viral, bacterial, and protozoal infections due to its ability to inhibit viral replication directly and through its immunostimulatory and immunomodulatory effects. One of its key functions is the induction of MHC class II molecule expression, which improves antigen presentation and immune recognition.

This kit is for research use only and is not to be used in diagnostic procedures.

STORAGE

Product is shipped at ambient temperature. Store product contents; antibodies, enzyme conjugate, substrate and plates at 2-8°C upon receipt. Expiry of the kit and reagents is stated on the box label. Required storage conditions should be maintained to ensure the stated expiry of the kit components.
Return reagents immediately to 2-8°C after use. Handle reagents under sterile conditions. Any contamination of the reagents or wrong storage conditions will invalidate the shelf life of the product.

CONTENTS

Plates	96 well ELISpot plate precoated with IFN-γ capture antibody
Detection mAb	Biotinylated IFN-γ antibody
Enzyme conjugate	Streptavidin-ALP (Alkaline Phosphatase)
Substrate	BCIP/NBT Ready-to-use solution
Blocking agent	Bovine Serum Albumin (BSA)

REAGENTS AND MATERIALS REQUIRED BUT NOT SUPPLIED

- Various sterile laboratory consumables
- Cell culture reagents (e.g. RPMI-1640, L-glutamine, FCS)
- Cell stimulation reagents (e.g. PMA, Ionomycin, peptide pool, CD3, CD28)
- CO_2 incubator
- Phosphate Buffered Saline (PBS) / Dulbecco's phosphate buffer saline (DPBS)

REAGENTS PREPARATION

a) Blocking Buffer

Cell culture medium containing 10% FCS or serum-free medium containing 10% BSA. Use same cell culture medium as used for the cell suspension.

b) Dilution Buffer

Add 1g of BSA into 100mL of sterile PBS/DPBS to prepare 1% BSA solution. To ensure buffer sterility, it is recommended to filter 1% BSA solution through a 0.2µm filter.

c) Detection Antibody

⚠ **Prepare this reagent immediately prior to use.**

For one plate, dilute 100µl of antibody into 10mL of Dilution Buffer and mix well. It is recommended to filter the detection antibody through a 0.2µm. Any unused stock antibody can be kept at -20°C for long term. It is recommended to aliquot the reagent prior to storage.

d) Streptavidin-Alkaline Phosphatase (ALP) conjugate

⚠ **Prepare this reagent immediately prior to use.**

For one plate, dilute 10µL of Streptavidin-ALP conjugate into 10mL of Dilution Buffer and mix well. It is recommended to filter the streptavidin-ALP conjugate through a 0.2µm filter to reduce the non-specific background.

e) BCIP/NBT

Normal appearance of this ready-to-use reagent is clear to pale yellow. Crystalline precipitate can occur in the product sometimes. In this case, filter the reagent through a 0.2µm filter. Discard if solution is turbid or purple.

ELISPOT PROTOCOL

a) Plate Preparation and Blocking

⚠ **Work under sterile conditions.**

1. Remove plate from its sealed package.
2. Wash the plate three times with 200µL of sterile PBS.
3. Add 100µL of blocking buffer.
4. Cover the plate and incubate at room temperature for at least 30 minutes.

Do not tap the plate. Empty the wells by flicking.

b) Cell Incubation

⚠ **Work under sterile conditions.**

At this step, either frozen or freshly isolated cells can be used. While working with frozen samples, it is suggested that to leave thawed cells at 37°C for at least one hour in order to remove cell debris.

1. Remove blocking buffer.
2. Pipette 50µL of stimulant and 50µL of cell suspension. Preferably stimulant and cell suspension can be mixed prior to plating. Cell suspension should not be added first.
3. Place the plate in a CO_2 and temperature controlled humidified incubator (37°C and 5% CO_2) with lid on.
4. Incubate the plate for desire length of time depending on the stimulant and cell type i.e. 18-48 hours.

At this step, plate can also be wrapped using aluminium foil to prevent evaporation and allow equal heat distribution. Do not stack plates in order to maintain continuous and equal flow. Do not move plate during incubation to avoid cytokine "trailing" or streaks and ensure accurate spot formation.

c) Detection Antibody

1. Remove cells and wash the plate 5 times with 200µL of PBS.
2. Pipette 100µL of detection antibody to every well.
3. Cover the plate and incubate at room temperature for 1 hour 30 minutes.
4. Empty the wells and wash the plate 5 times with 200µL of PBS.
5. Pipette 100µL of diluted streptavidin-ALP conjugate to every well.
6. Cover the plate and incubate at room temperature for 1 hour.
7. Empty the wells and wash the plate 5 times with 200µL of PBS.
8. Add 100µl of ready-to-use BCIP/NBT substrate to every well, and incubate in the dark.
9. Observe coloured spot development until distinct spots form.
10. To stop the spot development, wash the wells thoroughly with running water.
11. Tap dry the plate on lint free absorbent paper.
12. Air-dry the plate at room temperature then read the wells in an ELISpot reader.

Make sure wells are completely dry before spot counting.
Wet membrane increases background noise and interferes with spot detection and clarity.

Store plate in the dark at room temperature.

USEFUL INFORMATION

a) Plate Handling and Washing

ELISpot membrane is delicate. Handle the plate with extra care during pipetting and washing as the PVDF membrane may easily be damaged i.e. puncture on the membrane.

It is also important not to allow membrane to dry for the duration of the assay.

Automated plate washers or squirt bottle with a wide spout can be used for the washing steps that do not require sterile conditions. However, vigorous dispensing for both manual and automated washing should be avoided to prevent damaging the PVDF membrane and dislodging antibodies from the well surface. This could lead to inaccurate results and poor reproducibility.

Wash efficiency can be improved by allowing wash buffer to wet the wells for a short duration i.e. 1 minute.

Following the detection antibody incubation, Section c), step 4, removing the underdrain and washing both sides of the membrane can help reducing the background.

b) Buffer

To ensure sterile conditions during washing and blocking steps, use sterilized PBS or filter through a 0.2µm filter to avoid background.

c) Serum

FCS is recommended to use for conditioning the wells. Prepare 10% FCS in cell culture medium for cell blocking. However, some batches of FCS may non-specifically activate the cells and can therefore be the cause of non-specific background. Test different batches of FCS to assess its low background reactivity and high antigen-specific responses before use.

Non-human serum i.e. primate or rodent serum can be used as alternative for FCS.

d) Sample

Fresh or frozen PMBCs can be used as samples. While working with frozen cells, it is recommended to rest the cells at 37°C for at least an hour to remove the cell debris.

It is recommended to use between 150000 and 250000 viable cells per well for the assay. However, cell number should be optimised while using polyclonal activators as higher cell concentration can lead to confluent spot formation and overlapping spots.

e) Stimuli

Sample can be stimulated using antigen of interest i.e. infected cells, vaccine, virus, and peptide pool. If the cell type and/or cytokine/protein of interest requires longer incubation, then a preincubation step can be added.

However, prolonged cell incubation will lead to excessive cytokine/protein secretion. This will cause the larger spots to start merging and become indistinguishable. Therefore, incubation time and the amount of stimulant may require optimization depending on the cell type and antigen of interest.

f) Assay Controls

For positive control wells, anti-CD3/CD28 antibodies, phytohemagglutinin (PHA), and concanavalin A (ConA) are the commonly used T cell activators. Negative control wells should contain only unstimulated cell and cell culture medium. However, if anti-CD28 is used as a stimulant in the other wells, it should also be added to the negative control to help identifying the response is solely due to the stimulant i.e. peptide pool.

g) Detection Antibody and Streptavidin-ALP Conjugate

It is recommended to filter the detection antibody and the conjugate through a 0.2µm filter to reduce background noise.

h) Spot Development

It is recommended to allow 5-30 minutes to develop spots. However, it is crucial to optimise spot development time as overdevelopment leads to increased background. Wash wells extensively under running water to stop spot development.

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