Murine IFNγ ELISpot Kit

Instructions for use

Catalogue Numbers:

	Without Plates	With non-Sterile Plates	With sterile Plates
1x96 tests	-	862.031.001	862.031.111
5x96 tests	862.031.005	862.031.005P	862.031.005S
10x96 tests	862.031.010	862.031.010P	862.031.010S
15x96 tests	862.031.015	862.031.015P	862.031.015S
20x96 tests	862.031.020	862.031.020P	862.031.020S

For research use only

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1. Intended use

Diaclone **ELISpot** is a highly specific immunoassay for the analysis of cytokine and other soluble molecule production and secretion from T-cells at a single cell level in conditions closely comparable to the *in-vivo* environment with minimal cell manipulation. This technique is designed to determine the frequency of cytokine producing cells under a given stimulation and the comparison of such frequency against a specific treatment or pathological state. The ELISpot assay constitutes an ideal tool in the investigation of Th1 / Th2 responses, vaccine development, viral infection monitoring and treatment, cancerology, infectious disease, autoimmune diseases and tranplantation.

Utilising sandwich immuno-enzyme technology, Diaclone ELISpot assays can detect both secreted cytokines and single cells that simultaneously produce multiple cytokines. Cell secreted cytokines or soluble molecules are captured by coated antibodies avoiding diffusion in supernatant, protease degradation or binding on soluble membrane receptors. After cell removal, the captured cytokines are revealed by tracer antibodies and appropriate conjugates.

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

2. Introduction

2.1. Summary

Different populations of T-cells secrete differing patterns of cytokines that ultimately lead to different immune responses. IFN-γ production is a key function of Th1, $CD8^+$ CTLs and also NK cells. IFN-γ is a cytokine critical for cell mediated immunity against viral and intracellular bacterial infections and is involved in the inflammatory response following secretion via macrophage activation and stimulation of antibody secretion. IFN-γ is the hallmark effector cytokine of Th1 and therefore is an excellent marker for identifying a host response to intracellular pathogens.

IFN-γ is produced during infection by T cells of the cytotoxic/suppressor phenotype (CD8) and by a subtype of helper T cells, the Th1 cells. Th1 cells secrete IL-2, IL-3, TNFα and IFN-γ, whereas Th2 cells mainly produce IL-3, IL-4, IL-5, and IL-10, but little or no IFN-γ (*1*). IFN-γ preferentially inhibits the proliferation of Th2 but not Th1 cells, indicating that the presence of IFN-γ during an immune response will result in the preferential proliferation of Th1 cells (*2*).

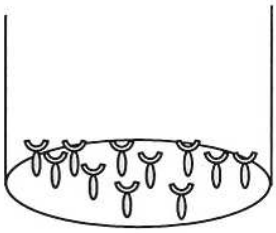
In addition, IFN-γ has several properties related to immunoregulation. IFN-γ is a potent activator of mononuclear phagocytes(*3*), and activates macrophages to kill tumor cells by releasing reactive oxygen intermediates and TNFα (*4*). IFN-γ induces or augments the expression of MHC antigens on macrophages, T and B cells and some tumor cell lines (*5*). On T and B cells IFN-γ promotes differentiation. It enhances proliferation of activated B cells and can act synergistically with IL-2 to increase immunoglobulin light-chain synthesis (*6*,*7*).

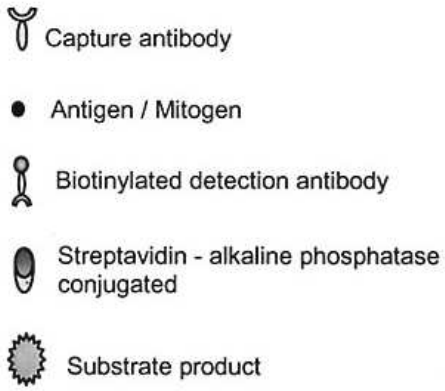
The role of IFN-γ as a disease marker has been demonstrated for a number of different pathological situations including, viral infection (*8*), Autoimmune disease (*9*), transplant rejection (*10*), Diabetes (*5*) and allergy (*11*).

2.2. Principle of the method

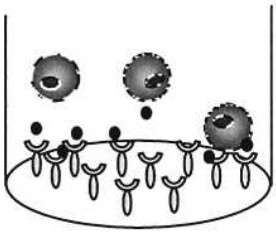
Capture antibodies highly specific for the analyte of interest are coated to the wells of a PVDF bottomed 96 well microtitre plate either during kit manufacture or in the laboratory. The plate is then blocked to minimise any non-antibody dependent unspecific binding and finally washed before adding the cells to be investigated. Cell suspension and stimulant are added to the coated and blocked microtitre plate and the plate incubated allowing the specific antibodies to bind any analytes produced. Biotinylated detection antibodies are then added which bind to the previously captured analyte. Enzyme conjugated Streptavidin is added binding to the detection antibodies. Any excess unbound analyte and antibodies are removed by careful washing. Colour substrate is then applied to the wells resulting in coloured spots which can be quantified using appropriate analysis software or manually using microscopes.

1. 96-PVDF bottomed-well plates are first treated with 35% ethanol and then coated with capture antibody

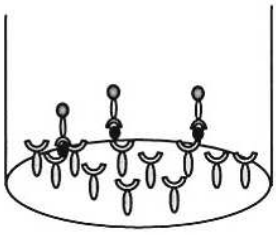




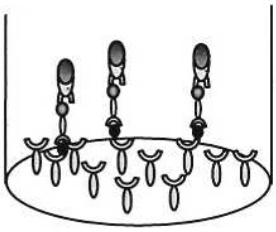
2. Incubation of cells in the coated microwell



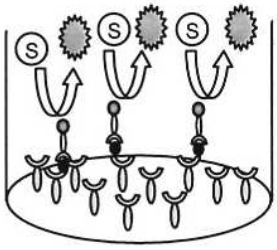
3. Cell removal by washing. Incubation with biotinylated antibody

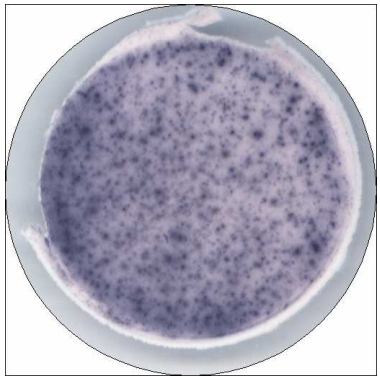


4. Incubation with streptavidin – alkaline phosphatise conjugated



5. Addition of substrate BCIP/NBT and monitoring of spot formation.





3. Reagents provided (Contents shown for 5x96 test format)

- 96 well PVDF bottomed plates (5 if ordered)
- Capture Antibody for murine IFNγ (0.5ml supplied sterile)
- Biotinylated detection antibody (lyophilised, resuspend in 0.55ml)
- Streptavidin-Alkaline Phosphatase conjugate (50µl)
- Bovine Serum Albumin (BSA)
- Blocking reagent
- Ready to use BCIP/NBT substrate buffer (50ml)

Please note for 1x96 demo kits, Biotinylated detection antibody is provided in liquid form.

4. Materials/Reagents required but not provided

- Miscellaneous laboratory plastic and/or glass, if possible sterile
- Ethanol
- Cell culture reagents (e.g. RPMI-1640, L-glutamine, FCS)
- Cell stimulation reagents (PMA, Ionomycin)
- CO_2 incubator
- Tween 20
- Phosphate Buffered Saline (PBS)
- 96 well PVDF bottomed plates if not ordered (we recommended Millipore plates catalogue # MSIPN4510, MSIPS4510 and M8IPS4510)

5. Storage Instructions

Store kit reagents between 2 and 8℃ except uncoated plates which should be stored at RT. Immediately after use remaining reagents should be returned to cold storage (2 to 8℃). Expiry of the kit and reagents is stated on box front labels. The expiry of the kit components can only be guaranteed if the components are stored properly, and if in the case of repeated use of one component, the reagent is not contaminated by the first handling.

6. Safety & Precautions for use

- For **research use only** not to be used as a diagnostic test
- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures , e.g.CDC/NIH Health manual : " Biosafety in Microbiological and Biomedical Laboratories" 1984
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used
- Do not pipette by mouth
- When not in use, kit components should be stored refrigerated or frozen as indicated on vials or bottles labels
- All reagents should be warmed to room temperature before use.
- Cover or cap all reagents when not in use
- Do not mix or interchange reagents between different lots
- Do not use reagents beyond the expiration date of the kit
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination
- Use a clean plastic container to prepare the washing solution
- Thoroughly mix the reagents and samples before use by agitation or swirling
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells
- **BCIP/NBT buffer** is potentially carcinogenic and should be disposed of appropriately, caution should be taken when handling this reagent, always wear gloves
- Follow incubation times described in the assay procedure

7. Reagent Preparation

7.1. 1X Phosphate Buffered Saline (PBS) (Coating Buffer)

For 1 litre of 10X PBS weigh-out: 80g NaCl
2g KH_2PO_4
14.4g $Na_2HPO_4\ _2H_2O$.

Add distilled water to 1 litre. Adjust the pH of the solution to 7.4 +/- 0.1 were required.

Dilute the solution to 1X before use.

7.2. Skimmed milk in 1X PBS (Blocking Buffer)

For one non-sterile plate dissolve 0.2g of powder in 10mL of 1X PBS

For one sterile plate dilute 5ml of liquid milk in 5ml of 1X PBS

Please note liquid milk has a shorter expiration date than the other reagents of the kit (indicated on the vial).
The use of expired milk can lead to unspecific stimulation.
Use any fresh semi skimmed milk (UHT) if the one provided has expired.

7.3. 1% BSA PBS Solution (Dilution Buffer)

For one plate dissolve 0.2 g of BSA in 20 ml of 1X PBS.

7.4. 0.05% PBS-T Solution (Wash Buffer)

For one plate dissolve 50µl of Tween 20 in 100 ml of 1X PBS.

7.5. 35% Ethanol (PVDF Membrane Activation Buffer)

For one plate mix 3.5 ml of ethanol with 6.5 ml of distilled water.

7.6. Capture Antibody

This reagent is supplied sterile once opened keep the vial sterile or aliquot and store at -20^{o}C. For optimal performance prepare the Capture Antibody dilution immediately before use.

Dilute 100µl of capture antibody in 10 mL of 1X PBS and mix well.

7.7. Detection Antibody

Reconstitute the lyophilised antibody with 0.55mL of distilled water. Gently mix the solution and wait until all the lyophilised material is back into solution.

If not used within a short period of time, reconstituted Detection Antibody should be aliquoted and stored at -20℃. In these conditions the reagent is stable fo r at least one year. For optimal performance prepare the reconstituted antibody dilution immediately prior to use.

Dilute 100µl of antibody into 10ml Dilution Buffer and mix well.

Please note for 1x96 demo kits, Biotinylated detection antibody is provided in liquid form.

7.8. Streptavidin – AP conjugate

For optimal performance prepare the Streptavidin-AP dilution immediately prior to use

For 1 plate dilute 10µl of Streptavidin-AP conjugate into 10 mL Dilution Buffer and mix well.

Do not keep this solution for further experiments.

8. Sample and Control Preparation

8.1. Cell Stimulation

Cells can either be stimulated directly in the antibody coated wells (Direct) or, first stimulated in 24 well plates or flask, harvested, and then plated into the coated wells (Indirect).

The method used is dependent on 1) the type of cell assayed 2) the expected cell frequency. When a low number of cytokine producing cells are expected it is also advised to test them with the direct method, however, when this number is particularly high it is better to use the indirect ELISpot method.

All the method steps following stimulation of the cells are the same whatever the method (direct/indirect) chosen.

8.2. Positive Assay Control, m IFNγ production

We recommend using the following polyclonal activation as a positive control in your assay.

Dilute mouse splenocytes in culture media (e.g. RPMI 1640 supplemented with 2mM L-glutamine and 10% heat inactivated fetal calf serum) containing 1ng/ml PMA and 500ng/ml ionomycin (Sigma, Saint Louis, MO). Distribute $2x10^4$ to $5x10^4$ cells per 100µl in required wells of an antibody coated 96-well PVDF plates and incubate for 10-15 hours in an incubator.

For other stimulators incubation times may vary, depending on the frequency of cytokine producing cells, and should be optimised in each situation.

8.3. Negative Assay Control

Dilute mouse splenocytes in culture media to give an appropriate cell number (same number of unstimulated cells as stimulated sample cells) per 100µl with no stimulation.

8.4. Sample

Dilute mouse splenocytes in culture medium and stimulator of interest (i.e. Sample, Vaccine, Peptide pool or infected cells) to give an appropriate cell number per 100µl.

Optimal assay performances are observed between $1x10^5$ and $2.5x10^5$ cells per 100µl.

Stimulators and incubation times can be varied depending on the frequency of cytokine producing cells and therefore should be optimised by the testing laboratory.

9. Method

Prepare all reagents as shown in section 7 and 8.
Note: For optimal performance prepare the Streptavidin-AP dilution immediately prior to use

Assay Step		Details
1.	Addition	Add 25µl of 35% ethanol to every well
2.	Incubation	Incubate plate at room temperature (RT) for 30 seconds
3.	Wash	Empty the wells by flicking the plate over a sink & gently tapping on absorbent paper. Thoroughly wash the plate 3x with 100µl of 1X PBS per well
4.	Addition	Add 100µl of diluted **capture antibody** to every well
5.	Incubation	Cover the plate and incubate at 4^{o}C overnight
6.	Wash	Empty the wells as previous and wash the plate once with 100µl of 1X PBS per well
7.	Addition	Add 100µl of **Blocking buffer** to every well
8.	Incubation	Cover the plate and incubate at RT for 2 hours
9.	Wash	Empty the wells as previous and thoroughly wash once with 100µl of 1X PBS per well
10.	Addition	Add 100µl of **sample, positive and negative controls** cell suspension to appropriate wells providing the required concentration of cells and stimulant (cells may have been previously stimulated see section 8.)
11.	Incubation	Cover the plate and incubate at 37^{o}C in a CO_2 incubator for an appropriate length of time (10-15 hours). ***Note: do not agitate or move the plate during this incubation***
12.	Addition	Empty the wells and remove excess solution then add 100µl of PBS-T to every well
13.	Incubation	Incubate the plate at 4^{o}C for 10 min
14.	Wash	Empty the wells as previous and wash the plate 3x with 100µl of PBS-T
15.	Addition	Add 100µl of diluted **detection antibody** to every well
16.	Incubation	Cover the plate and incubate at RT for 1 hour 30 min
17.	wash	Empty the wells as previous and wash the plate 3x with 100µl of PBS-T
18.	Addition	Add 100µl of diluted **Streptavidin-AP conjugate** to every well
19.	Incubation	Cover the plate and incubate at RT for 1 hour
20.	Wash	Empty the wells and wash the plate 3x with 100µl of PBS-T
21.	Wash	Peel of the plate bottom and wash both sides of the membrane 3x under running distilled water, once washing complete remove any excess solution by repeated tapping on absorbent paper.
22.	Addition	Add 100µl of ready-to-use **BCIP/NBT buffer** to every well
23.	Development	Incubate the plate for **5-15 min** monitoring spot formation visually throughout the incubation period to assess sufficient colour development
24.	Wash	Empty the wells and rinse both sides of the membrane 3x under running distilled water. Completely remove any excess solution by gentle repeated tapping on absorbent paper
	Read Spots: allow the wells to dry and then read results. The frequency of the resulting coloured spots corresponding to the cytokine producing cells can be determined using an appropriate ELISpot reader and analysis software or manually using a microscope. *Note: spots may become sharper after overnight incubation at 4^{o}C*	

Plate should be stored at RT away from direct light, but please note colour may fade over prolonged periods so read results within 24 hours.

10. Performance Characteristics

10.1. Reproducibility and Linearity

Intra-assay reproducibility and linearity were evaluated by measuring the spot development following the stimulation (PMA / Ionomycin) of 6 different mouse splenocytes concentrations, 12 repetitions in 1 batch. The data shows the mean spot number, range and CV for the six cell concentrations.

Cells / well	n	Mean number of spots per well	Min	Max	CV%
100000	12	429	364	474	7
50000 recommended	12	541	504	601	6
25000 recommended	12	597	506	703	12
12500	12	478	375	617	17
6250	12	390	258	487	16
3125	12	259	163	296	13

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