Human IFNγ ELISpot Kit – Pre-coated

Instructions for use

Catalogue Numbers:

Regular	Pre-coated plates
1x96 tests	856.051.001PC
2x96 tests	856.051.002PC
5x96 tests	856.051.005PC

EasySplit	Pre-coated strip plates
1x96 tests	856.051.001PCS
2x96 tests	856.051.002PCS
5x96 tests	856.051.005PCS

For research use only

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Table of Contents

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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 transplantation.

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. IFNγ production is a key function of Th1, $CD8^+$ CTLs and also NK cells. IFNγ 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. IFNγ is the hallmark effector cytokine of Th1 and therefore is an excellent marker for identifying a host response to intracellular pathogens.

IFNγ 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 IFNγ, whereas Th2 cells mainly produce IL-3, IL-4, IL-5, and IL-10, but little or no IFNγ (*1*). IFNγ preferentially inhibits the proliferation of Th2 but not Th1 cells, indicating that the presence of IFNγ during an immune response will result in the preferential proliferation of Th1 cells (*2*).

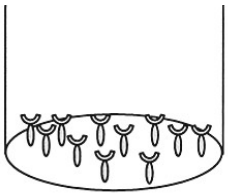
In addition, IFNγ has several properties related to immunoregulation. IFNγ is a potent activator of mononuclear phagocytes (*3*), and activates macrophages to kill tumor cells by releasing reactive oxygen intermediates and TNFα (*4*). IFNγ induces or augments the expression of MHC antigens on macrophages, T and B cells and some tumor cell lines (*5*). On T and B cells IFNγ 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 IFNγ 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*).

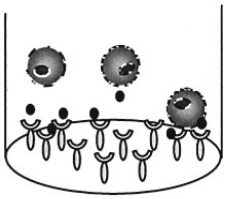
2.2. Principle of the method

A capture antibody highly specific for the analyte of interest is 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 washed. Cell suspension and stimulant are added and the plate incubated allowing the specific antibodies to bind any analytes produced. Cells are then removed by washing prior to the addition of Biotinylated detection antibodies which bind to the previously captured analyte. Enzyme conjugated streptavidin is then added binding to the detection antibodies. Following incubation and washing substrate is then applied to the wells resulting in coloured spots which can be quantified using appropriate analysis software or manually using a microscope.

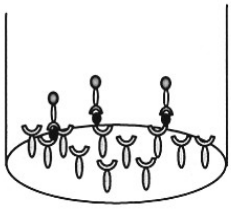
1. 96-PVDF bottomed-well plates are first treated with 35% ethanol and then coated with capture antibody.

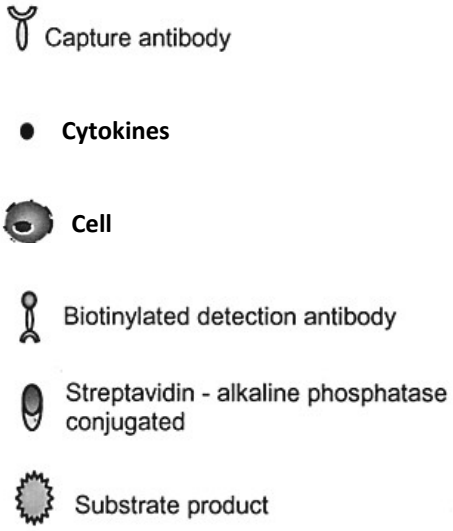


2. Cells are incubated in the presence of the stimulating agent. Upon stimulation they release cytokines which bind to the capture antibodies.

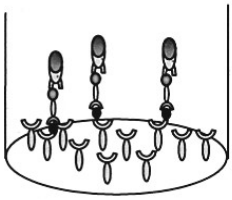


3. Cell removal by washing. Incubation with biotinylated detection antibody.





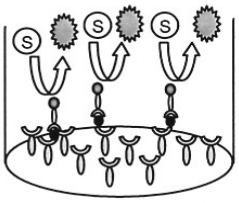
4. Any excess unbound detection antibodies is removed by washing. Incubation with streptavidin – alkaline phosphatase conjugate.

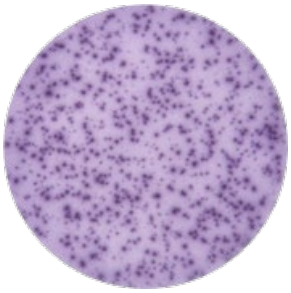


5. Any excess unbound Strep-AP is removed by washing. Incubation with BCIP/NBT.

Finally BCIP/NBT reduction by alkaline phosphatase give a precipitated product which give blue/purple spots.

One spot correspond to one single producing cell.





3. Reagents provided

Reagents	.001*	.002*	.005	Reconstitution
Pre-coated PVDF plates	1	2	5	Wash with 100 µl of PBS1X Saturate with blocking buffer (see section 9)
Biotinylated Detection Antibody	1 (100 µl)	1 (200 µl)	1	Reconstitute with 0.55 ml of distilled water Dilute prior to use (see Detection Antibody, section 7.5)
Streptavidin-Alkaline Phosphatase Conjugate	1 (10 µl)	1 (20 µl)	1 (50 µl)	Dilute prior to use (see Streptavidin-AP Conjugate, section 7.6)
Bovine Serum Albumin (BSA) – 2 g	1	1	1	Dissolve to prepare dilution buffer (see 1%BSA PBS solution, section 7.4)
Ready to use BCIP/NBT - (Substrate Buffer)	1 (11 ml)	1 (25 ml)	2 (25 ml)	Ready to use

***Please note for 001PC and 002PC kits : detection antibody is provided in liquid form.**

4. Materials/Reagents required but not provided

- Miscellaneous laboratory plastic and/or glass, if possible sterile
- Cell culture reagents (e.g. RPMI-1640, L-glutamine, FCS)
- Cell stimulation reagents (e.g. PMA, Ionomycin)
- CO_2 incubator
- Phosphate Buffered Saline (PBS)

5. Storage Instructions

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2 to 8°C). 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, blood specimens, PBMC, human cell lines 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 substrate** may cause an allergic skin reaction, 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)

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.

Dilute the solution to 1X before use.
Check the pH of the 1X solution and adjust to required pH : 7.4 +/- 0.1.

7.2. Cell culture medium + 10% Serum (Blocking Buffer)

For one plate, add 1 ml Serum (e.g. FCS) to 9 ml of culture medium.
Use same cell culture medium as used to derive the cell suspension.

7.3. 1% BSA PBS Solution (Dilution Buffer)

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

7.4. Detection Antibody

Reconstitute the lyophilised antibody with 0.55 ml of distilled water. Gently mix the solution and wait until all the lyophilised material is back into solution.
Please note for 001PC and 002PC kits, detection antibody is provided in liquid form.

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

For one plate, dilute 100 µl of antibody into 10 ml of Dilution Buffer and mix well.

To avoid nonspecific background, it is recommended to filter the working solution using a disposable syringe and a 0.2µm filter disc.

7.5. Streptavidin – AP conjugate

For optimal performance, prepare the Streptavidin-AP dilution immediately prior to use.
It is recommended to centrifuge the vial for a few seconds to collect all the volume at the bottom.

For one plate, dilute 10 µl of Streptavidin-AP conjugate into 10 ml of Dilution Buffer and mix well.

Do not keep this solution for further experiments.
To avoid nonspecific background, it is recommended to filter the working solution using a disposable syringe and a 0.2µm filter disc.

7.6. BCIP/NBT

The reagent is ready-to-use.

It should be clear to pale yellow. If precipitates occur, filter the solution using a disposable syringe and a 0.2µm filter disc.

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, IFNγ production

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

Dilute PBMC in culture medium (e.g. RPMI 1640 supplemented with 2mM L-glutamine and 10% heat inactivated fetal calf serum) containing 1 ng/ml PMA and 500 ng/ml ionomycin (Sigma, Saint Louis, MO). Distribute $1x10^4$ to $2x10^4$ cells per 100 µl in required wells of an antibody coated 96-well PVDF plates and incubate for 15-20 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 PBMC in culture medium to give an appropriate cell number (same number of unstimulated cells as stimulated sample cells) per 100 µl with no stimulation.

8.4. Sample

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

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

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.	Wash	Wash the plate 3x with 100 µl of PBS 1X. Empty the wells by flicking the plate over a sink & gently tapping on absorbent paper
2.	Addition / Incubation	Add 100 µl of **blocking buffer** to every well. Incubate plate at room temperature (RT) for 2 hours
3.	Wash	Empty the wells by flicking the plate over a sink & gently tapping on absorbent paper
4.	Addition	Add 100 µl 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.)
5.	Incubation	Cover the plate and incubate at 37°C in a CO_2 incubator for an appropriate length of time (15-20 hours) ***Note: do not agitate or move the plate during this incubation***
6.	Addition	Empty the wells and remove excess solution then add 200 µl of PBS 1X to every well
7.	Incubation	Incubate the plate at 4°C for 10 min
8.	Wash	Empty the wells as previous and wash the plate 3x with 200 µl of PBS 1X
9.	Addition	Add 100 µl of diluted **detection antibody** to every well
10.	Incubation	Cover the plate and incubate at RT for 1 hour 30 min
11.	Wash	Empty the wells as previous and wash the plate 3x with 200 µl of PBS 1X
12.	Addition	Add 100 µl of diluted **Streptavidin-AP conjugate** to every well
13.	Incubation	Cover the plate and incubate at RT for 1 hour
14.	Wash	Empty the wells and wash the plate 3x with 200 µl of PBS 1X
15.	Wash	Peel off 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
16.	Addition	Add 100 µl of ready-to-use **BCIP/NBT buffer** to every well
17.	Development	Incubate the plate for **5-15 min** monitoring spot formation visually throughout the incubation period to assess sufficient colour development
18.	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°C in the dark*		

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

10. Performance Characteristics

10.1. Specificity

The assay recognizes natural human IFNγ.

To define specificity of this human IFNγ antibody pair, several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested (IL-1α, IL-1β, IL-10, IL-12, IL-4, IL-6, TNFα, IL-8 and IL-13). This testing was performed using the equivalent human IFNγ antibody pair in an ELISA assay.

10.2. Reproducibility and Linearity

Intra-assay reproducibility and linearity were evaluated by measuring the spot development following the stimulation (PMA / Ionomycin) of :

- 4 different PBMC cell concentrations, 12 repetitions, 1 batch. The data show the mean spot number, range and CV for the 4 cell concentrations.

Cells / well	n	Mean number of spots per well	Min	Max	CV%
10000	12	324	295	350	5.0
5000	12	271	244	303	5.6
2500	12	163	138	181	8.5
1250	12	91	80	108	8.7

- 2 different PBMC cell concentrations, 96 repetitions, 1 batch. The data show the mean spot number, range and CV for the 2 cell concentrations :

Cells / well	n	Mean number of spots per well	Min	Max	CV%
2500	96	184	158	219	6.8
1250	96	99	79	119	8.6

Inter-batch reproducibility and linearity were evaluated by measuring the spot development following the stimulation (PMA / Ionomycin) of 5 different PBMC cell concentrations, 2 donors, 6 repetitions per batch, 2 different batches tested. The data show the mean spot number, range and CV for the 5 cell concentrations obtained with the 2 batches :

- Donor 1 :

Cells / well	n	Mean number of spots per well	Min	Max	CV%
10000	6	280	253	307	1
5000	6	207	180	240	3
2500	6	125	117	142	4
1250	6	62	51	74	9
625	6	35	21	47	2

- Donor 2 :

Cells / well	n	Mean number of spots per well	Min	Max	CV%
10000	6	249	210	300	10
5000	6	238	203	260	1
2500	6	204	186	217	1
1250	6	149	116	166	3
625	6	90	71	113	7

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