

Rat IFN γ ELISpot Set

Instructions for use

Catalogue Numbers:

	Without Plates	With non-Sterile Plates	With Sterile Plates
1x96 tests	867.011.001	867.011.001P	867.011.001S
5x96 tests	867.011.005	867.011.005P	867.011.005S
10x96 tests	867.011.010	867.011.010P	867.011.010S
15x96 tests	867.011.015	867.011.015P	867.011.015S
20x96 tests	867.011.020	867.011.020P	867.011.020S

For research use only

As a material condition to Diaclone providing its Products to Purchaser, Purchaser agrees that the end user shall not, directly or indirectly, attempt to reverse engineer, disassemble, or otherwise perform any compositional, structural, functional or other analyses directed to learning the methodology, components, formulae, processes, make-up, or production of any Product or any portion thereof.

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Rat IFN γ ELISpot Set

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. 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, TNF α 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).

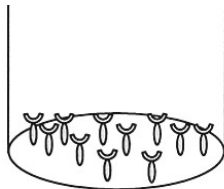
In addition, IFN γ has several properties related to immunoregulation. IFN γ is a potent activator of mononuclear phagocytes (3), and activates macrophages to kill tumor cells by releasing reactive oxygen intermediates and TNF α (4). IFN γ induces or augments the expression of MHC antigens on macrophages, 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).

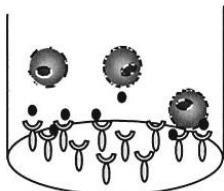
2.2. Principle of the method

A capture antibody highly specific for the analyte of interest is coated to the wells of a PVDF bottomed 96 well microtiter 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.

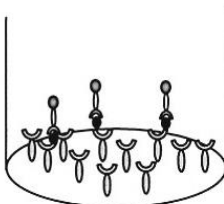
1. 96-PVDF bottomed-well plates are first treated with 35% ethanol and then coated with capture antibody.



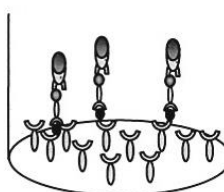
2. Cells are incubated in the presence of the stimulating agent. Upon stimulation they release cytokines which bind to the capture antibodies.



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



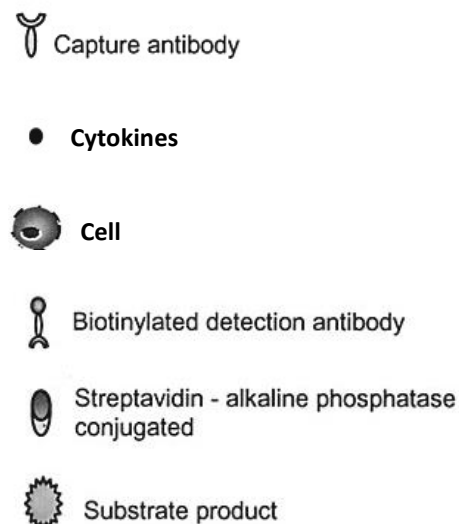
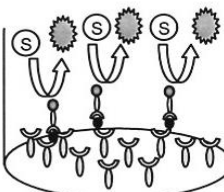
4. Any excess unbound detection antibodies is removed by washing. Incubation with streptavidin – alkaline phosphatase conjugate.



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	Set 001*	Set 005	Set 010	Set 015	Set 020	Reconstitution
96-well PVDF bottomed plates (if ordered)	2	5	10	15	20	Ethanol treatment (see section 9)
Blocking reagent Dry skimmed milk – 2 g Or liquid skimmed milk – 25 ml	1	1	2	3	4	Dissolve or dilute prior to use (see skimmed milk in PBS, section 7.3)
Capture Antibody	1 (0.1 ml)	1 (0.5 ml)	2 (0.5 ml)	3 (0.5 ml)	4 (0.5 ml)	Sterile, dilute prior to use (see Capture Antibody, section 7.6)
Biotinylated Detection Antibody	1 (100 µl)	1	2	3	4	Reconstitute with 0.55 ml of distilled water. Dilute prior to use (see Detection Antibody, section 7.7)
Streptavidin-Alkaline Phosphatase Conjugate	1 (10 µl)	1 (50 µl)	2 (50 µl)	3 (50 µl)	4 (50 µl)	Dilute prior to use (see Streptavidin- AP conjugate, section 7.8)
Bovine Serum Albumin (BSA) – 2 g	1	1	2	3	4	Dissolve to prepare dilution buffer (see 1%BSA PBS solution, section 7.5)
Ready to use BCIP/NBT - Substrate buffer	1 (11 ml)	2 (25 ml)	4 (25 ml)	6 (25 ml)	8 (25 ml)	Ready to use

*Please note for discovery set 001 : detection antibody is provided in liquid form.

Volumes of reagents are sufficient for a total of 96 tests but 2 plates are provided to allow to run 2*48 tests.

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)
- CO₂ 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°C except uncoated plates which should be stored at RT. 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) (Coating Buffer)

For 1 litre of 10X PBS, weigh-out: 80g NaCl
2g KH_2PO_4
14.4g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$.

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. 35% Ethanol (PVDF Membrane Activation Buffer)

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

7.3. Skimmed milk in PBS 1X solution (Blocking Buffer)

For one non-sterile plate, dissolve 0.2 g of dry skimmed milk in 10 ml of PBS 1X.

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

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 nonspecific stimulation. Use any fresh semi skimmed milk (UHT) if the one provided has expired.

7.4. 0.05% Tween PBS Solution (Wash Buffer)

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

7.5. 1% BSA PBS Solution (Dilution Buffer)

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

7.6. Capture Antibody

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

For one plate, dilute 100 µl of capture antibody in 10 ml of PBS 1X and mix well.

7.7. 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 1x96 demo 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.

7.8. 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.

7.9. 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, IFN γ production

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

Isolate splenocytes 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 1×10^5 to 2.5×10^5 cells per 100 μ l in required wells of an antibody coated 96-well PVDF plate 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 splenocytes in culture medium to give an appropriate cell number (same number of unstimulated cells as stimulated sample cells) per 100 μ l with no stimulation.

8.4. Sample

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

Optimal assay performances are observed between 1×10^5 and 2.5×10^5 cells per 100 μ l.

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 25 µl 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 5x with 250 µl of PBS 1X per well
4.	Addition	Add 100 µl of diluted capture antibody to every well
5.	Incubation	Cover the plate and incubate at 4°C overnight
6.	Wash	Empty the wells as previous and wash the plate once with 100 µl of PBS 1X per well
7.	Addition	Add 100 µl 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 3x with 100 µl of PBS 1X per well
10.	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.)
11.	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
12.	Addition	Empty the wells and remove excess solution then add 100 µl of Wash Buffer to every well
13.	Incubation	Incubate the plate at 4°C for 10 min
14.	Wash	Empty the wells as previous and wash the plate 3x with 100 µl of Wash Buffer
15.	Addition	Add 100 µl 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 100 µl of Wash Buffer
18.	Addition	Add 100 µl 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 100 µl of Wash Buffer
21.	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.
22.	Addition	Add 100 µl of ready-to-use BCIP/NBT substrate to every well
23.	Development	Incubate the plate for 5-15 min protected from light, 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
<p>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.</p> <p><i>Note: spots may become sharper after overnight incubation at 4°C in the dark</i></p>		

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 Rat IFN γ .

10.2. Reproducibility and Linearity

Intra-assay reproducibility and linearity were evaluated by measuring the spot development following the stimulation (PMA / Ionomycin) of 5 different splenocytes concentrations, 3 repetitions. The data show the mean spot number, range and CV for the five cell concentrations.

Cells / well	n	Mean number of spots per well	Min	Max	CV%
100000 recommended	3	366	354	388	5.3%
50000	3	280	264	302	7.1%
25000	3	172	159	181	6.8%
12500	3	87	78	105	17.5%
6250	3	38	31	50	28.4%

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12. Diaclone Rat IFN γ ELISpot References

Ginzkey, C. et al., *Acta Neurochir (Wien)*, 2013; 155(1): 51-8; discussion 59

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