ORIGENE

Human IFNγ /IL-10 Dual FluoroSpot

Instructions for use

Catalogue Numbers:

	Without Plates	With non-Sterile Plates	With sterile Plates
1x96 tests	EA101911	EA101912	EA101913
5x96 tests	EA101914	EA101915	EA101916
10x96 tests	EA101917	EA101918	EA101919
15x96 tests	EA101920	EA101921	EA101922
20x96 tests	EA101923	EA101924	EA101925

For research use only

Fast Track Your Research………….

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1. Intended use

OriGene**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, OriGene 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 Dual Colour ELISpot kit allows you to analysis the production of two cytokines simultaneously in the same well.

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

2. Introduction

2.1. Summary

IFNγ (1–21)

IFNγ, also called Type II interferon, is a homodimeric glycoprotein containing approximately 21 to 24 kD subunits. The human IFNγ gene, situated on chromosome 12, contains three introns; the four exons code for a polypeptide of 166 amino acids, 20 of which constitute the signal peptide (11). In contrast to IFNα and IFNβ synthesis, which can occur in any cell, production of IFNγ is a function of T cells and NK cells. All IFNγ inducers activate T cells either in a polyclonal (mitogens or antibodies) or in a clonally restricted, antigen-specific, manner. 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 main produce IL-3, IL-4, IL-5, and IL-10, but little or no IFNγ (9). 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 Th1cells (7).

Type II IFN or IFNγ is a lymphokine that displays no molecular homology with type I IFN, but shares some important biologic activities. Specifically, IFNγ induces an anti-viral state and is anti-proliferative. In addition, IFNγ has several properties related to immunoregulation. **1)** IFNγ is a potent activator of mononuclear phagocytes, e.g. IFNγ stimulates the expression of Mac-1, augments endocytosis and phagocytosis by monocytes (15), and activates macrophages to kill tumor cells by releasing reactive oxygen intermediates and TNFα (21). **2)** IFNγ induces or augments the expression of MHC antigens on macrophages , T and B cells and some tumor cell lines (3). **3)** 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. (8,13). IFNγ is one of the natural B-cell differentiation factors (17). **4)** Finally, IFNγ activates neutrophils, NK cells and vascular endothelial cells (6).

The role of IFNγ as a disease marker has been demonstrated for a number of different pathological situations:

- *Infections*: IFNγ is produced during viral infections (4). IFNγ is a diagnostic tool for distinguishing tuberculous from other nontuberculous ascites (5,18). IFNγ values in pleural fluid are significantly higher in tuberculous pleuritis patients than those in non- tuberculous pleuritis patients, with a sensitivity and a specificity of 100% (1, 2). Therapy-induced (treatment with thalidomide) alleviation of clinical symptoms of erythema nodosum leprosum correlates with IFNγ and TNFα levels (14). Tuberculoid leprosy patients show increased lymphocyte proliferation and IFNγ production in response to stimulation with Mycobacterium leprae as compared to lepromatous leprosy patients and healthy individuals (16).

- *Autoimmune diseases*: Accurate measurements of cellular production of cytokines, e.g. IFNγ is important in the design and monitoring of immunotherapy of multiple sclerosis (12).

- *Transplant rejection*: Intragraft IFNγ mRNA expression occurs in active acute transplant rejection preceding clinical transplant rejection, thus offering an early diagnostic tool for detection of transplant rejection (10).

- *Allergy*: IFNγ production by isolated lymphocytes is not detectable in patients with cow's milk allergy as compared to control individuals (19). Infants who develop atopy produce significantly less IFNγ at birth compared to infants who do not develop atopy (20).

- *Diabetes*: Peripheral blood lymphomononuclear cells from newly diagnosed type I diabetes produce significantly less IFNγ in comparison to controls and long standing diabetes (4).

IL-10 (22-36)

Interleukin-10 is a pleiotropic cytokine playing an important role as a regulator of lymphoid and myeloid cell function. Due to the ability of IL-10 to block cytokine synthesis and several accessory cell functions of macrophages this cytokine is a potent suppressor of the effector functions of macrophages, T-cells and NK cells. In addition, IL-10 participates in regulating proliferation and differentiation of B-cells, mast cells and thymocytes (30). The primary structure of human IL-10 has been determined by cloning the cDNA encoding the cytokine (36). The corresponding protein exists at 160 amino acids with a predicted molecular mass of 18.5 kDa (29, 36). Based on its primary structure, IL-10 is a member of the four -helix bundle family of cytokines (33). In solution human IL-10 is a homodimer with an apparent molecular mass of 39 kDa (35). Although it contains an N-linked glycosylation site, it lacks detectable carbohydrates (36). Recombinant protein expressed in E. coli thus retains all known biological activities.The human IL-10 gene is located on chromosome 1 and is present as a single copy in the genome (27). The human IL-10 exhibits strong DNA and amino acid sequence homology to the murine IL-10 and an open reading frame in the Epstein- Barr virus genome, BCRF1 (22, 29, 36) which shares many of the cellular cytokine's biological activities and may therefore play a role in the host- virus interaction. The immunosuppressive properties of IL-10 (25) suggest a possible clinical use of IL-10 in suppressing rejections of grafts after organ transplantations. IL-10 can furthermore exert strong anti-inflammatory activities (25).

IL-10 in disease

IL-10 expression was shown to be elevated in parasite infections like in Schistosoma mansoni (28), Leishmania (26), Toxoplasma gondii (33) and Trypanosoma (34) infection.
Furthermore, high IL-10 expression was detected in mycobacterial infections as shown for Mycobacterium leprae (24), Mycobacterium tuberculosis (23) and Mycobacterium avium infections.
High expression levels of IL-10 are also found in retroviral infections inducing immunodeficiency (31).

2.2. Principle of the method

Capture antibodies highly specific for the analytes of interest are 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 finally washed before adding the cells to be investigated. Cell suspension and stimulant are added to the coated and blocked microtiter plate and the plate incubated allowing the specific antibodies to bind any analytes produced. Biotinylated and FITC detection antibodies are then added which bind to the previously captured analyte. Green fluorescent conjugated anti-FITC antibodies and Streptavidin Phycoerythrine are added binding to the detection antibodies. Any excess unbound analyte and antibodies are removed by careful washing. PVDF-bottom-well plates are then read under a UV light beam. Green fluorescent spots indicate IFNγ production while granzyme B is revealed by red spots. Yellow spots indicate dual cytokine producing cells.

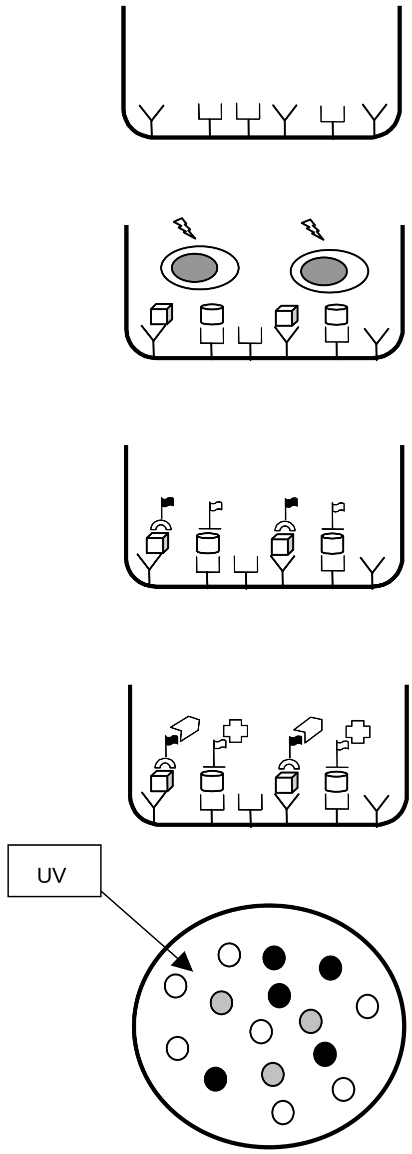
1. 96 PVDF-bottomed-well plates are first treated with 35% ethanol and then coated with anti-IFNγ and anti-IL-10 capture antibodies

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

3. Anti-IFNγ-FITC and anti-IL-10-biotin detection antibodies are added and bind to the captured cytokines

4. Detection antibodies are in turn bound by anti-FITC-Green-Fluorescence for IFNγ and Streptavidin-PE for IL-10.

5. Finally fluorescent spots are visualised under a UV light beam. Cells producing IFNγ give green spots while those producing IL-10 give red spots. Dual cytokine producing cells give yellow spots.



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

- 96 well PVDF bottomed plates (5 if ordered)
- Capture Antibody for human IFNγ (0.5ml supplied sterile)
- Capture Antibody for human IL-10 (0.5ml supplied sterile)
- FITC conjugated detection antibody for IFNγ (lyophilised, resuspend in 0.55ml)
- Biotinylated detection antibody for IL-10 (lyophilised, resuspend in 0.55ml)
- Anti-FITC antibody green fluorescent conjugate
- Streptavidin-phycoerythrin conjugate
- Bovine Serum Albumin (BSA)
- Fluorescence Buffer (2.5ml)

Please note for 1x96 demo kits, detection antibodies are 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 (e.g. PMA and 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)
- FluoroSpot reader

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, 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
- **Fluorescence buffer**is potentially carcinogenic and should be disposed of appropriately, caution should be taken when handling these 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 $Na_2HPO_{42}H_2O$.

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. 1% BSA PBS Solution (Dilution Buffer)

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

7.3. 0.05% PBS-T Solution (Wash Buffer)

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

7.4. 35% Ethanol (PVDF Membrane Activation Buffer)

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

7.5. Capture Antibodies

These reagentsare supplied sterile once opened keep the vials sterile or aliquot and store at -20^{o}C. For optimal performance prepare the Capture Antibodies dilution immediately before use.

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

7.6. Detection Antibodies

Reconstitute the lyophilised antibodies 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°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.

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

Please note for 1x96 demo kits, detection antibodies are provided in liquid form.

7.7. Streptavidin-PEconjugate and Anti-FITC antibody-Green Fluorescence conjugate (diluted conjugates)

For optimal performance prepare the solution immediately prior to use.

Add the volume indicated on each vial of Streptavidin-PE conjugate and anti-FITC antibody Green Fluorescence conjugate to 10mlof Dilution Buffer.
10ml of diluted conjugates is required for one plate.Mix well.

DO NOT KEEP THE DILUTED SOLUTION FOR FURTHER EXPERIMENTS

The quantity of anti FITC-green fluorescence and Strepatvidin-PE conjugates may need adjustements depending on the cell types and on the stimulating antigen studied.
The balance of the 2 different cytokines secreted varies with the cells stimulation. Conjugates dilutions advised in this protocol have been optimised for best results in the suggested protocol (polyclonal activation).

7.8. Fluorescence Buffer

For one plate, dissolve0.5 ml of Fluorescence bufferin10 ml of PBS 1X.

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)

8.2. Positive Assay Control, IFNγ /IL-10 production

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

Dilute CD4+ T cells 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 $1x10^5$ to $2.5x10^4$ cells per 100µl 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 CD4+ T cells 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 CD4+ T cells 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 $5x10^4$ and $2\ x10^5$ 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.

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 antibodies** 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 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 three times 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°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 100µl 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 100µl of **Wash buffer**
15.	Addition	Add 100µl of diluted **detection antibodies** to every well
16.	Incubation	Cover the plate and incubate at RT for 1 hour 30 min away from light.
17.	wash	Empty the wells as previous and wash the plate 3x with 100µl of **Wash buffer**
18.	Addition	Add 100 µl per wells of **Diluted Green FluorescentandPE conjugates**
19.	Incubation	Cover the plate and incubate at RT for 1 hour away from light.
20.	Wash	Empty the wells and wash the plate 3x with 100µl 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.
Read Spots: allow the wells to dry and then read results. The frequency of the resulting fluorescent spots corresponding to the cytokine producing cells can be determined using an appropriate FluoroSpot reader and analysis software or manually using a microscope.		
Optional Steps		**Using Fluorescence buffer** : This buffer will help to increase the fluorescent signal. It can be useful in case of weak fluorescence intensity observed. Please follow the procedure detailed below
21.	Addition	Add 100µl of **Fluorescence buffer** to every well
22.	Incubation	Incubate the plate for **15 min**away from light
23.	Wash	Empty the wells Peel off the plate bottom, remove any excess solution by repeated tapping on absorbent paper.
Read Spots: allow the wells to dry and then read results. The frequency of the resulting fluorescent spots corresponding to the cytokine producing cells can be determined using an appropriate FluoroSpot reader and analysis software or manually using a microscope.		

Plate should be stored at +4°C away from direct light.

10. Bibliography

10.1. IFNγ

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TECHNICAL CONSULTATION

OriGene Technologies, Inc.
9620 Medical Center Dr., Suite 200
Rockville, MD 20850

Phone: 1.888.267.4436
Fax: 301-340-9254
Email: techsupport@origene.com
Web: www.origene.com