Human IL-17A/IL-4 Dual FluoroSpot

Instructions for use

Catalogue Numbers:

	Without Plates	With non-Sterile Plates	With sterile Plates
1x96 tests	EA102121	EA102122	EA102123
5x96 tests	EA102124	EA102125	EA102126
10x96 tests	EA102127	EA102128	EA102129
15x96 tests	EA102130	EA102131	EA102132
20x96 tests	EA102133	EA102134	EA102135

For research use only

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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

IL-17A (1-10)

Classically following antigenic stimulation and regulation by specific co-stimulatory molecules Naïve CD4+ T-cells where known to differentiate into Th1 and Th2 cells. However in recent years the identification of IL-17 and IL-23 has led to the classification of a third subset of the Th cell family, Th17 cells. These cells are classified on their ability to secrete IL-17A but not IFNγ and IL-4 the main effector cytokines of Th1 and Th2 cells.

IL-17A, was originally identified as a transcript from a rodent T-cell hybridoma by Rouvier et al. in 1993 and also called CTLA-8. IL-17 A is a homodimeric glycoprotein consisting of 155 amino acids and has a molecular weight of 35kDa.

IL-17A links innate and adaptive immunity and has both beneficial and pathological effects on the immune system. IL-17A is involved in inducing and mediating proinflammatory responses, commonly associated with allergic responses and induces the production of many other cytokines (such as IL-6, G-CSF, GM-CSF, IL-1β, TGFβ, TNFα), chemokines (including IL-8, GRO-α and MCP-1) and prostaglandins (e.g. PGE2) from many cell types (fibroblasts, endothelial cells, epithelial cells, keratinocytes and macrophages. In-vivo studies have now indicated that IL-17A is an especially potent activator of neutrophils. IL-17A has been shown to play an important role in the host immune response to various infection and disease states, including bacterial, fungal and viral infections, autoimmune disease including psoriasis, rheumatoid arthritis (increased levels in the synovial fluid) and multiple sclerosis as well as inflammatory conditions such as Crohns disease.

IL-4 (11-17)

IL-4 is a lymphokine that co-stimulates the proliferation of activated B- and T-cells, augments the cytotoxic activity of lymphocytes and monocytes and enhances the functional activity of myeloid cells. Produced by mast cells, T-cells and bone marrow stromal cells, IL-4 regulates the differentiation of naive CD4+ cells into helper Th2 cells characterized by their cytokine secretion-profile that includes secretion of IL-4, IL-5, IL-6, IL-10 and IL-13 which favour a humoral immune response. In addition, IL-4 can inhibit the proliferation of TNF, IL-1 and IL-6 by macrophages.

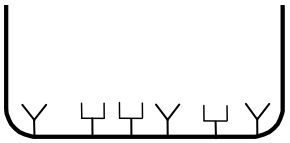
IL-4 induces B-cell class switching to IgE and IgG1 isotypes, and up regulates MHC Class II production and CD23 expression.

IL-4 is a 15kDa globular glycoprotein containing 129 amino acid residues. The non-glycosylated form of the protein is fully biologically active.

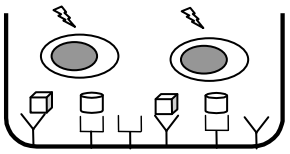
2.2. Principle of the method

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.

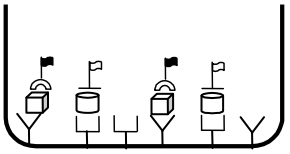
1. 96 PVDF-bottomed-well plates are first treated with 35% ethanol and then coated with anti-IL-17A and anti-IL-4 capture antibodies



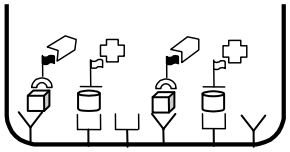
2. Cells are incubated in the presence of the antigen. Upon stimulation they release cytokines which bind to the capture antibodies.



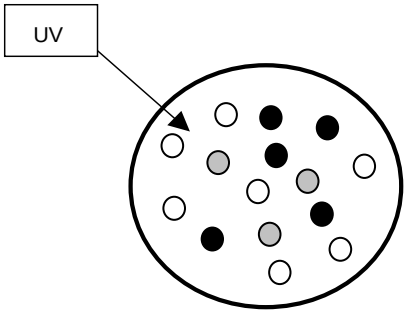
3. Anti-IL-17A-FITC and anti-IL-4-biotin detection antibodies are added and bind to the captured cytokines



4. Detection antibodies are in turn bound by anti-FITC-Green-Fluorescence for IL-17A and Streptavidin-PE for IL-4.



5. Finally fluorescent spots are visualised under a UV light beam. Cells producing IL-17A give green spots while those producing IL-4 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 IL-17A (0.5ml supplied sterile)
- Capture Antibody for human IL-4 (0.5ml supplied sterile)
- FITC conjugated detection antibody for IL-17A (lyophilised, resuspend in 0.55ml)
- Biotinylated detection antibody for IL-4 (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°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, IL-17A /IL-4 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

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