Human IL-17A / IL-4
Dual FluoroSpot Set

Instructions for use

Catalogue Numbers:

	Without Plates	With non-Sterile Plates	With Sterile Plates
1x96 tests	874.092.001	874.092.001P	874.092.001S
5x96 tests	874.092.005	874.092.005P	874.092.005S
10x96 tests	874.092.010	874.092.010P	874.092.010S
15x96 tests	874.092.015	874.092.015P	874.092.015S
20x96 tests	874.092.020	874.092.020P	874.092.020S

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.

Fast Track Your Research…….

Table of Contents

Human IL-17A / IL-4 Dual FluoroSpot Set

1. Intended use

Diaclone **FluoroSpot** 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 FluoroSpot 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-assay technology, Diaclone FluoroSpot 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 FluoroSpot kit allows you to analyze 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-17A is a homodimeric glycoprotein consisting of 155 amino acids and has a molecular weight of 35 kDa.

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.

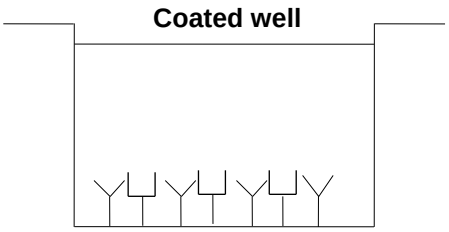
IL-4 is a 15kDa globular glycoprotein containing 129 amino acid residues. The non-glycosylated form of the protein is fully biologically active.

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. Anti-FITC Green Fluorescent conjugate and Streptavidin Phycoerythrin are added binding to the detection antibodies. Any excess unbound analyte and antibodies are removed by careful washing. PVDF-bottom-well plate is then read under a UV light beam.

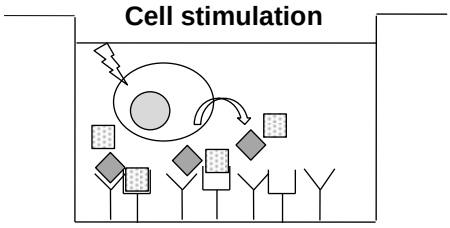
1. 96 PVDF-bottomed-well plates are first treated with 35% ethanol and then coated with anti-IL-17A and anti-IL-4 capture antibodies.

Figure 1



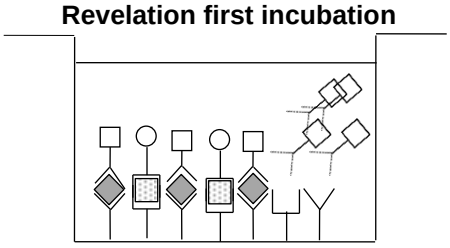
2. Cells are incubated in the presence of the antigen. Upon stimulation they release cytokines which bind to the capture antibodies.

Figure 2



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

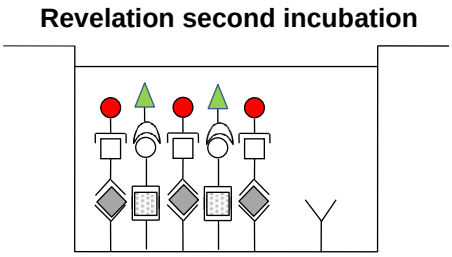
Figure 3

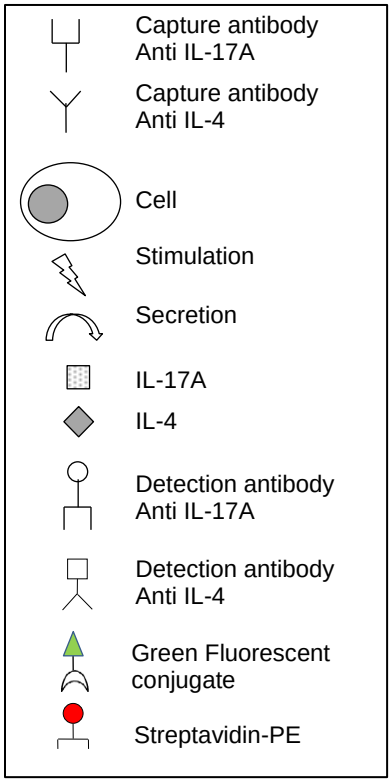


4. Any excess unbound detection antibodies is removed and wells are washed.

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

Figure 4





5. Any excess of Green Fluorescent and PE conjugate is removed and wells are washed.

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. Double producing cells give yellow spots.

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)
Capture Antibody anti hIL-17A	1 (0.1 ml)	1 (0.5 ml)	2 (0.5 ml)	3 (0.5 ml)	4 (0.5ml)	Sterile, dilute prior to use (see Capture antibodies, section 7.6)
Capture Antibody anti hIL-4	1 (0.1 ml)	1 (0.5 ml)	2 (0.5 ml)	3 (0.5 ml)	4 (0.5ml)	
FITC conjugated detection antibody anti hIL-17A	1 (100 µl)	1	2	3	4	Reconstitute with 0.55 ml of distilled water Dilute prior to use (see Detection Antibodies, section 7.7)
Biotinylated detection antibody anti hIL-4	1 (100 µl)	1	2	3	4	
Anti-FITC Green Fluorescent conjugate	1	1	1	1	1	Dilute prior to use as indicated on the vial (see diluted conjugates, section 7.8)
Streptavidin-Phycoerythrin conjugate	1	1	1	1	1	
Bovine Serum Albumin (BSA) – 2 g	1	1	2	3	4	Dissolve to prepare dilution buffer (see 1%BSA PBS solution, section 7.4)

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

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

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

7.4. 1% BSA PBS Solution (Dilution Buffer)

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

7.5. 0.05% Tween PBS Solution (Wash Buffer)

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

7.6. Capture Antibodies

These reagents are 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.

For one plate, add 100 µl of each capture antibody in a same tube in 10 ml of PBS 1X. Mix well.

7.7. Detection Antibodies

Reconstitute each lyophilised antibody with 0.55 ml of distilled water. Gently mix the solutions and wait until all the lyophilised material is back into solution.
Please note for 1x96 demo kits, detection antibodies are provided in liquid form.

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

For one plate, add in a same tube 100 µl of each detection antibody in 10 ml of Dilution Buffer. 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. Diluted PE and Green Fluorescent conjugates

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

For one plate, add in the same tube Streptavidin-PE conjugate and anti-FITC Green Fluorescent conjugate at the volume indicated on each vial to 10 ml of Dilution Buffer. Mix well.

DO NOT KEEP THE DILUTIONS FOR FURTHER EXPERIMENTS.

The quantity of anti-FITC Green Fluorescent and Streptavidin-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).

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

Optimal assay performances are observed between $0.5x10^5$ and $2\ x10^5$ 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 PE and Green Fluorescent conjugates 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 3x with 100 µl of PBS 1X per well
4.	Addition	Add 100 µl of the diluted mixture of **capture antibodies** 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 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^{o}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^{o}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 the diluted mixture of **detection antibodies** 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 **PE and Green Fluorescent conjugates** to every well
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 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.
Read Spots: allow the wells to dry away from light 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 2-8°C away from direct light, but please note spot intensity may fade over prolonged periods so read results within 24 hours.

10. Performance Characteristics

10.1. Specificity

The assay recognizes natural human IL-17A and human IL-4.

To define specificity of the IL-17A antibody pair, several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested: IL-1β, IL-6, IL-23, IFNγ, IL-17B, IL-17D, IL-17E, IL-17F, IL-17A/F and murine IL-17A. This testing was performed using the equivalent human IL-17A antibody pair in an ELISA assay.

To define specificity of the IL-4 antibody pair, several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested: IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, IL-13, IFNγ and TNFα. This testing was performed using the equivalent human IL-4 antibody pair in an ELISA assay.

11. Bibliography

IL-17A

1. Rouvier et.al. CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene. J Immunol. 1993;150(12):5445-56.
2. F. Y. Cheung P, K. Wong C, W. K. Lam C, Molecular Mechanisms of Cytokine and Chemokine Release from Eosinophils Activated by IL-17A, IL-17F, and IL-23 : Implication for Th17 Lymphocytes-Mediated Allergic Inflammation. J Immunol 2008 ; 180 : 5625–5635.
3. Kawaguchi M, Adachi M, Oda N, Kokubu F, Shau-Ku Huang, IL-17 cytokine family. J Allergy Clin Immunol 2004 ; 114 : 1265-73
4. Ouyang W, K. Kolls J, Zheng Y, The Biological Functions of T Helper 17 Cell Effector Cytokines in Inflammation. Immunity 2008 ; 28 : 454-467.
5. Shen F, L. Gaffen S, Structure–function relationships in the IL-17 receptor: Implications for signal transduction and therapy. Cytokine 2008 ; 41 : 92–104.
6. Yoshihara K, Yamada H, Hori A, Yajima T, Kubo C, Yoshikai Y, IL-15 exacerbates collagen-induced arthritis with an enhanced CD4+ T cell response to produce IL-17. Eur. J. Immunol. 2007 ; 37: 2744–2752.
7. Bettelli et al: Th17: The third member of the effector T cell Trilogy. Curr Opin Immunol. 2007 Dec;19(6):652-7. Review.
8. Gaffen S; An overview of IL-17 function and signalling. Cytokine. 2008 Sep;43(3):402-7. Review.
9. Crome et al: Translational mini-review series on Th17 cells: function and regulation of human T helper 17 cells in health and disease. Clin Exp Immunol. 2010 Feb;159(2):109-19. Review.
10. Kolls et.al: Interleukin 17 family members and inflammation. Immunity. 21: 467-476. 2004

IL-4

11. Howard M., Paul W.E. (1982) lymphokine res. (1-4)
12. Paul W. E. , and R. A. Seder. (1994) Cell 76, 241-251.
13. Hart P.H., G. F. Vitti, D. R. Burgess, G. A. Whitty, D. S. Piccoli, and J. A. Hamilton. (1989) Proc. Natl. Acad. Sci, USA 86, 3803-3807.
14. Lee J. D. , S. G. Swisher, E. H. Minehart, W. H. McBride, and J. S. Economou. (1990) J. Leukocyte Biol. 47, 475-479.
15. Coffman R. L., J. Ohara, M. W. Bond, J. Carty, A. Zlotnik, and W. E. Paul. (1986) J. Immunol.141.504-501
16. Sideras P. , S. Bergstedt-Lindqvist, H. R. MacDonald, and e. Severison. (1985) Eur. J. Immunol. 15, 586-596.
17. Paul, W. E. (1991) Blood 77, 1859-1830.

Notes

Products Manufactured and Distributed by:

Diaclone SAS
6 Rue Docteur Jean-François-Xavier Girod
BP 1985
25020 Besançon Cedex
France
Tel +33 (0)3 81 41 38 38
Fax +33 (0)3 81 41 36 36
Email: info@diaclone.com
www.diaclone.com