Human IL-10 ELISpot Pair

Instructions for use

Catalogue Number : 10x96 tests: EA101813

For research use only

Fast Track Your Research……

Table of Contents

1. Intended Use 2
2. Introduction 2
2.1. Summary 2
2.2. Principle Of The Method 3
3. Reagents Provided (Contents Shown For 10x96 Test Format) 4
4. Materials/Reagents Required But Not Provided 4
5. Storage Instructions 4
6. Safety & Precautions For Use 5
7. Reagent Preparation 6
7.1. 1x Phosphate Buffered Saline (PBS) (Coating Buffer) 6
7.2. Cell Culture Media + 10% Serum (Blocking Buffer) 6
7.3. 1% BSA PBS Solution (Dilution Buffer) 6
7.4. 0.05% PBS-T Solution (Wash Buffer) 6
7.5. 35% Ethanol (PVDF Membrane Activation Buffer) 6
7.6. Capture Antibody 6
7.7. Detection Antibody 6
7.8. Streptavidin – AP Conjugate 6
8. Sample And Control Preparation 7
8.1. Cell Stimulation 7
8.2. Positive Assay Control, IL-10 Production 7
8.3. Negative Assay Control 7
8.4. Sample 7
9. Method 8
10. Performance Characteristics 9
10.1. Specificity 9
10.2. Reproducibility And Linearity 9
11. Bibliography 10
12. OriGene IL-10 Elispot References 11

Human IL-10 ELISpot Pair

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 tranplantation.

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

2. Introduction

2.1. Summary

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 (9). The primary structure of human IL-10 has been determined by cloning the cDNA encoding the cytokine (15). The corresponding protein exists at 160 amino acids with a predicted molecular mass of 18.5 kDa (8, 15). Based on its primary structure, IL-10 is a member of the four -helix bundle family of cytokines (11). In solution human IL-10 is a homodimer with an apparent molecular mass of 39 kDa (14). Although it contains an N-linked glycosylation site, it lacks detectable carbohydrates (15). 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 (6). 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 (1, 8, 15) 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 (4) 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 (4).

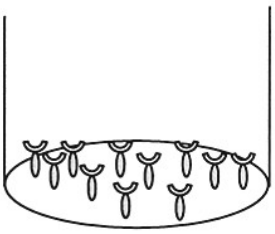
IL-10 in disease

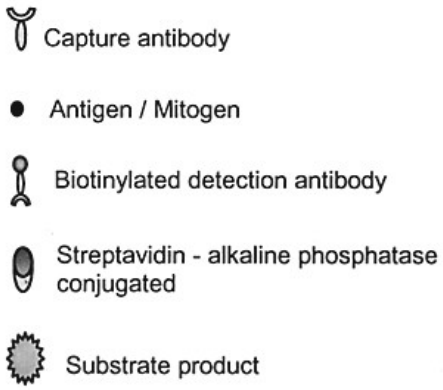
IL-10 expression was shown to be elevated in parasite infections like in Schistosoma mansoni (7), Leishmania (5), Toxoplasma gondii (12) and Trypanosoma (13) infection.
Furthermore, high IL-10 expression was detected in mycobacterial infections as shown for Mycobacterium leprae (3), Mycobacterium tuberculosis (2) and Mycobacterium avium infections.
High expression levels of IL-10 are also found in retroviral infections inducing immunodeficiency (10).

2.2. Principle of the method

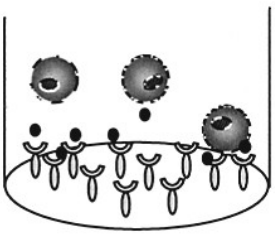
Capture antibodies highly specific for the analyte of interest are coated to the wells of a PVDF bottomed 96 well microtitre plate either during kit manufacture or in the laboratory. The plate is then blocked to minimise any non-antibody dependent unspecific binding and finally washed before adding the cells to be investigated. Cell suspension and stimulant are added to the coated and blocked microtitre plate and the plate incubated allowing the specific antibodies to bind any analytes produced. Biotinylated detection antibodies are then added which bind to the previously captured analyte. Enzyme conjugated streptavidin is added binding to the detection antibodies. Any excess unbound analyte and antibodies are removed by careful washing. Colour substrate is then applied to the wells resulting in coloured spots which can be quantified using appropriate analysis software or manually using microscopes.

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

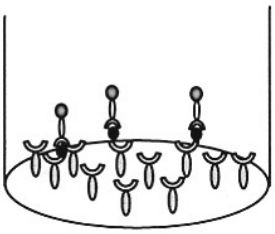




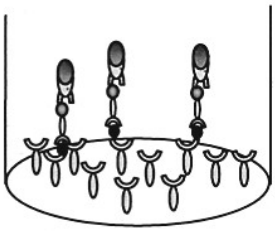
2. Incubation of cells in the coated microwell



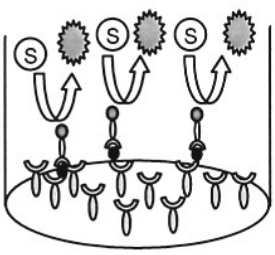
3. Cell removal by washing. Incubation with biotinylated antibody



4. Incubation with streptavidin – alkaline phosphatise conjugated



5. Addition of substrate BCIP/NBT and monitoring of spot formation.



3. Reagents provided (Contents shown for 10x96 test format)

- Capture Antibody (2 vials of 0.5ml). The antibody is supplied sterile and does not contain preservative. We strongly advise sterile pipetting.
- Biotinylated detection antibody (2 vials, lyophilised)

4. Materials/Reagents required but not provided

- Miscellaneous laboratory plastic and/or glass, if possible sterile
- Streptavidin-Alkaline Phosphatase conjugated
- Bovine Serum Albumin (BSA)
- Substrate solution (BCIP/NBT)
- Ethanol
- Cell culture reagents (e.g. RPMI-1640, L-glutamine, FCS)
- Cell stimulation reagents (PMA, Ionomycin)
- CO_2 incubator
- Tween 20
- Phosphate Buffered Saline (PBS)
- 96 well PVDF bottomed plates (we recommended Millipore plates catalogue # MSIPN4510, MSIPS4510 and M8IPS4510)

5. Storage Instructions

Store reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2 to 8°C). Expiry of the components is stated on box front label and 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
- **BCIP/NBT buffer** is potentially carcinogenic and should be disposed of appropriately, 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 $Na_2HPO_4\ _2H_2O$.

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

For one plate add 1ml Serum (e.g. FCS) to 9ml of culture media (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 1X PBS.

7.4. 0.05% PBS-T Solution (Wash Buffer)

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

7.5. 35% Ethanol (PVDF Membrane Activation Buffer)

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

7.6. Capture Antibody

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

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

7.7. Detection Antibody

Reconstitute the lyophilised antibody 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 antibody into 10ml Dilution Buffer and mix well.

7.8. Streptavidin – AP conjugate

Dilute in Dilution buffer according to the instructions of the supplier.

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, IL-10 production

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

Dilute CD4+ 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 $2.5x10^4$ to $5x10^4$ 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+ 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+ 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 $1x10^5$ and $2.5x10^5$ 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.
Note: For optimal performance prepare the Streptavidin-AP dilution immediately prior to use

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 antibody** 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 100µl of 1X PBS per well
7.	Addition	Add 100µl of **culture media with 10% serum** 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 once 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^{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 100µl of PBS-T 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 100µl of PBS-T
15.	Addition	Add 100µl 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 100µl of PBS-T
18.	Addition	Add 100µl of diluted **Streptavidin-AP conjugate** to every well
19.	Incubation	Cover the plate and incubate at RT following the supplier 'instructions
20.	Wash	Empty the wells and wash the plate 3x with 100µl of PBS-T
21.	Wash	Peel of 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 100µl of ready-to-use **BCIP/NBT buffer** to every well
23.	Development	Incubate the plate for **5-15 min** 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
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^{o}C*		

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

10. Performance Characteristics

10.1. Specificity

The assay recognizes natural human IL-10.

To define specificity of this ELISpot, several proteins were tested for cross reactivity. There was no cross reactivity observed for these proteins tested IL-1β, IL-12, IFNγ, IL-4, IL-6, TNFα, IL-8, IL-2 and IL-13. This testing was performed using the equivalent IL-10 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 6 different CD4+ cell concentrations, 12 repetitions in 1 batch. The data shows the mean spot number, range and CV for the six cell concentrations.

Cells / well	n	Mean number of spots per well	Min	Max	CV%
100000	12	771	637	851	9
50000 recommended	12	606	564	636	4
25000 recommended	12	349	287	380	8
12500	12	165	142	177	7
6250	12	64	51	78	12
3125	12	29	21	35	17

11. Bibliography

1. Baer R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, P. S. Tuffnell, and B. G. Barrell. (1984). DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature 310, 207-211.
2. Barnes P. F., D. Chatterjee, J. S. Abrams, S. Lu, E. Wang, M. Yamamura, P. J. Brennan, and R. L. Modlin. (1992). Cytokine production induced by Mycobacterium tuberculosis lipoarabinomannan. Relationship to chemical structure. J. Immunol. 149, 541-547.
3. Bloom B. R., and V. Mehra. (1984). Immunological unrespon-siveness in leprosy. Immunol. Rev. 80, 5-28.
4. De Waal Malefyt R., J. Abrams, B. Bennett, C. G. Figdor, and J. E. de Vries. (1991). Interleukin-10 inhibits cytokine synthesis by human monocytes - an autoregulatory role of IL-10 produced by monocytes. J. Exp. Med. 174, 1209-1220.
5. Heinzel F. P., M. D. Sadick, S. S. Mutha, and R. M. Locksley. (1991). Production of interferon gamma, interleukin 2, interleukin 4, and interleukin 10 by CD4 positive lymphocytes in-vivo during healing and progressive murine leishmaniasis. Proc. Natl. Acad. Sci., USA 88, 7011-7015.
6. Kim J. M., C. I. Brannan, N. G. Copeland, N. A. Jenkins, T. A. Khan, and K. W. Moore. (1992). Structure of the mouse IL-10 gene and chromosomal localization of the mouse and human genes. J. Immunol. 148, 3618-3623.
7. Kullberg M. C., E. J. Pearce, S. E. Hieny, A. Sher, and J. A. Berzofsky. (1992). Infection with Schistosoma mansoni alters Th1/Th2 cytokine responses to a non-parasite antigen. J. Immunol. 148, 3264-3270.
8. Moore K. W., P. Vieira, D. F. Fiorentino, M. L. Trounstine, T. A. Khan, and T. R. Mosmann. (1990). Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein Barr Virus gene BCRF1. Science 248, 1230-1234.
9. Moore K. W., A. O'Garra, R. de Waal Malefyt, P. Vieira, and T. R. Mosmann. (1993). Interleukin-10. Ann. Rev. Immunol. 11, 165-190.
10. Mosier D. E., R. A. Yetter, and H. C. Morse III. (1985). Retroviral induction of acute lymphoproliferative disease and profound immunosuppression in adult C57 BI/6 mice. J. Exp. Med. 161, 766-784.
11. Shanafelt A. B., A. Miyajima, T. Kitamura, and R. A. Katelein. (1991). The amino -terminal helix of GM-CSF and IL-5 governs high-affinity binding to their receptors. EMBO J. 10, 4105-4112.
12. Sher A., R. T. Gazzinelli, I. P. Oswald, M. Clerici, M. Kullberg, E. J. Pearce, J. A. Berzofsky, T. R. Mosmann, S. L. James, H. C. Morse III, and G. M. Shearer. (1992). Role of T-cell derived cytokines in the downregulation of immune responses in parasitic and retroviral infection. Immunol. Rev. 127, 183-204.
13. Silva, J. S., P. J. Morrissey, K. H. Grabstein, K. M. Mohler, D. Anderson, and S. G. Reed. (1992). Interleukin 10 and interferon gamma regulation of experimental trypanosoma cruzi infection. J. Exp. Med. 175, 169-174.
14. Spits H., and R. de Waal Malefyt. (1992). Functional characterization of human IL-10. Int. Arch. Allergy Immunol. 99, 8-15.
15. Vieira P., R. de Waal Malefyt, M. N. Dang, K. E. Johnson, R. Kastelein, D. F. Fiorentino, J. E. de Vries, M. G. Roncarolo, T. R. Mosmann, and K. W. Moore, (1991). Isolation and expression of human cytokine synthesis inhibitory factor (CSIF/IL-10) cDNA clones: homology to Epstein-Barr virus open reading frame BCRF1. Proc. Natl. Acad. Sci. USA 88, 1172-1176.

12. OriGene IL-10 ELISpot references

1. Bain C. et al., J. Virol., Memory T-Cell-Mediated Immune Responses Specific to an Alternative Core Protein in Hepatitis C Virus Infection, 2004; 78(19): 10460 – 10469.

2. Hudak S. et al., J. Immuno., Immune surveillance and effector functions of CCR10(+) skin homing T cells, 2002; 169 : 1189 - 1196

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

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES