Human IL-10 ELISpot Set

Instructions for use

Catalogue Numbers:

	Without Plates	With non-Sterile Plates	With Sterile Plates
1x96 tests	856.031.001	856.031.001P	856.031.001S
5x96 tests	856.031.005	856.031.005P	856.031.005S
10x96 tests	856.031.010	856.031.010P	856.031.010S
15x96 tests	856.031.015	856.031.015P	856.031.015S
20x96 tests	856.031.020	856.031.020P	856.031.020S

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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Human IL-10 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

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).

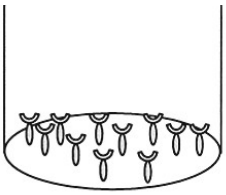
IL-10 in disease

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).

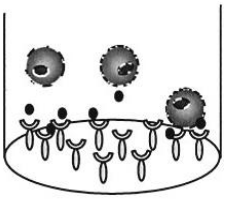
2.2. Principle of the method

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.

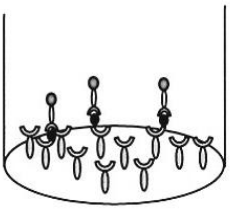
1. 96-PVDF bottomed-well plates are first treated with 35% ethanol and then coated with capture antibody.

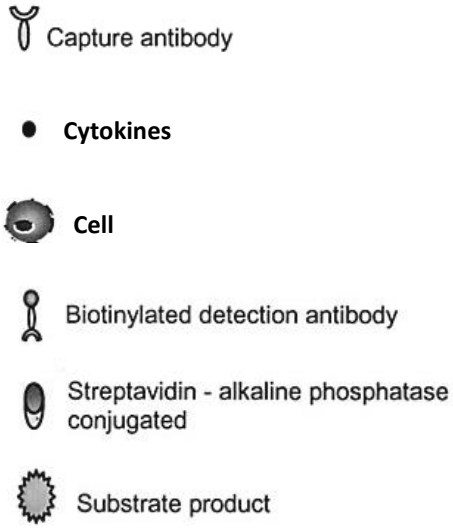


2. Cells are incubated in the presence of the stimulating agent. Upon stimulation they release cytokines which bind to the capture antibodies.

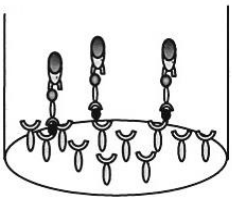


3. Cell removal by washing. Incubation with biotinylated detection antibody.





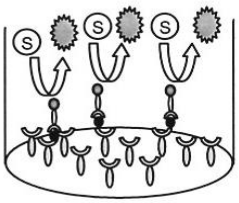
4. Any excess unbound detection antibodies is removed by washing. Incubation with streptavidin – alkaline phosphatase conjugate.

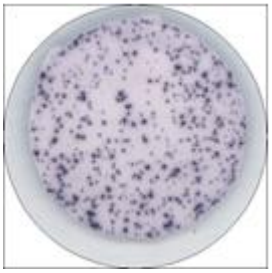


5. Any excess unbound Strep-AP is removed by washing. Incubation with BCIP/NBT.

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)
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 vial (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)
- 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)

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 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. 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, IL-10 production

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

Dilute CD4+ 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 $5x10^4$ 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+ 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+ 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 $1x10^5$ and $2.5x10^5$ 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 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 once 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
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°C in the dark*		

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 Human IL-10.

To define specificity of this IL-10 antibody pair, several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein 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 Human 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. The data show 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

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