

Human IL-1 β ELISpot Kit – Pre-coated

Instructions for use

Catalogue Numbers:

	Pre-coated
1x96 tests	856.101.001PC
5x96 tests	856.101.005PC

For research use only

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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 5x96 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).....	6
7.2.	1% BSA PBS Solution (Dilution Buffer)	6
7.3.	0.05% PBS-T Solution (Wash Buffer).....	6
7.4.	Detection Antibody	6
7.5.	Streptavidin – AP Conjugate	6
8.	Sample And Control Preparation.....	7
8.1.	Cell Stimulation	7
8.2.	Positive Assay Control, IL-1 β 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

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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-1 Beta (IL-1 β) is a member of the interleukin-1 family. This family contains three structurally related polypeptides. The first two are IL-1 α and IL-1 β , each of which has a broad spectrum of both beneficial and harmful biologic actions, and the third is IL-1-receptor antagonist, which inhibits the activities of interleukin-1.

IL-1 α and β present approximately 25% homology at the amino acid level, but the difference is in their tri dimensional structure. Two distinct receptor types have been isolated, that bind both forms. IL-1 β is synthesized as a large precursor, with a molecular weight of 31 kDa. The molecular weight of the mature form is 17.5 kDa. Unlike IL-1 α , the IL-1 β precursor shows little or no biological activity in comparison to the mature form.

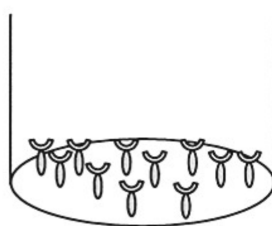
IL-1 is primarily an inflammatory cytokine. It belongs to a group of cytokines with overlapping biologic properties (TNF α and IL-6). IL-1, TNF and IL-6 share the ability to stimulate T and B lymphocytes, increase cell proliferation, and initiate or suppress gene expression for several proteins exerting their effects by binding to specific receptors.


IL-1 (α and β) have similar biological properties, among them, the ability to induce fever, sleep, anorexia and hypotension. IL-1 stimulates the release of pituitary hormones, increases the synthesis of collagenases, resulting in the destruction of cartilage, and stimulates the production of prostaglandins, leading to decrease in the pain threshold. In addition IL-1 has some host defence properties. However, whereas IL-1 β is a secreted cytokine, IL-1 α is predominantly a cell-associated cytokine. IL-1 has also been implicated in the destruction of beta cells of the islets of Langerhans, the growth of myelogenous leukaemia cells, and the development of atherosclerotic plaques. It is described in several diseases: sepsis syndrome, rheumatoid arthritis, inflammatory Bowel disease, acute and chronic myelogenous leukaemia, insulin dependent diabetes mellitus and atherosclerosis.

2.2. Principle of the method

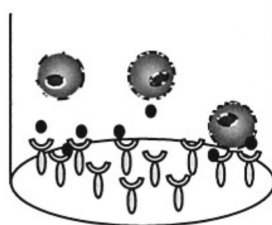
A capture antibody highly specific for the analyte of interest is coated to the wells of a PVDF bottomed 96 well microtitre plate either during kit manufacture or in the laboratory. The plate is then blocked to minimise any non-antibody dependent unspecific binding and washed. Cell suspension and stimulant 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




 Capture antibody

2. Incubation of cells in the coated microwell



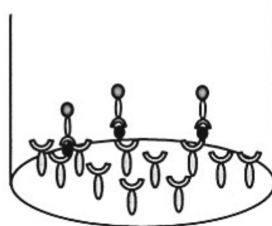
● Antigen / Mitogen

 Biotinylated detection antibody

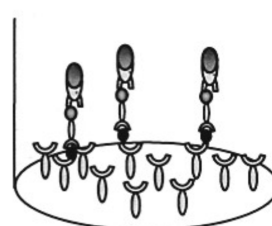
 Streptavidin - alkaline phosphatase conjugated

 Substrate product

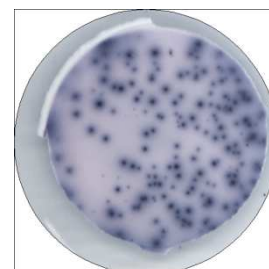
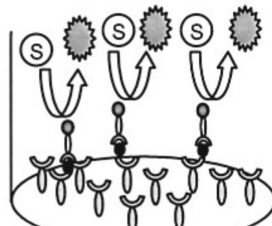
3. Cell removal by washing. Incubation with biotinylated antibody



4. Incubation with streptavidin – alkaline phosphatase conjugated



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



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

- Pre-coated 96 well PDVF bottomed plates (5)
- Biotinylated detection antibody (lyophilised, resuspend in 0.55ml)
- Streptavidin-Alkaline Phosphatase conjugate (50µl)
- Bovine Serum Albumin (BSA)
- Ready to use BCIP/NBT substrate buffer (50ml)

Please note for 1 x 96 demo kits Biotinylated detection antibody is provided in liquid form.

4. Materials/Reagents required but not provided

- Miscellaneous laboratory plastic and/or glass, if possible sterile
- Cell culture reagents (e.g. RPMI-1640, L-glutamine, FCS)
- Cell stimulation reagents (PMA, Ionomycin)
- CO₂ incubator
- Tween 20
- Phosphate Buffered Saline (PBS)

5. Storage Instructions

Store kit reagents between 2 and 8°C. 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 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)

For 1 litre of 10X PBS weigh-out: 80g NaCl
 2g KH₂PO₄
 14.4g Na₂HPO₄ · 2H₂O.

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

7.4. 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.

7.5. Streptavidin – AP conjugate

For optimal performance prepare the Streptavidin-AP dilution immediately prior to use.

For 1 plate dilute 10µl of Streptavidin-AP conjugate into 10 mL Dilution Buffer and mix well.

Do not keep this solution for further experiments.

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-1 β production

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

Dilute PBMC in culture media (e.g. RPMI 1640 supplemented with 2mM L-glutamine and 10% heat inactivated fetal calf serum) containing 1 μ g/ml LPS (Sigma, Saint Louis, MO). Distribute 1×10^4 to 2.5×10^4 cells per 100 μ l in required wells of an antibody coated 96-well PVDF plates and incubate for 15-20 hours in an incubator.

Note that as IL-1 β is mainly produced by monocytes and macrophages PBMC population. Spontaneous release of IL-1 β can occur when the cells adhere to the PVDF membrane

For antigen specific stimulation, the optimal concentration of the antigen and the optimal concentration of number of cells have to be determined experimentally, as it is depending on the frequency of cytokine producing cells

8.3. Negative Assay Control

Dilute PBMC in culture media to give an appropriate cell number (same number of unstimulated cells as stimulated sample cells) per 100 μ l with no stimulation.

8.4. Sample

Dilute PBMC in culture medium and stimulator of interest (i.e. Sample, Vaccine, Peptide pool or infected cells) to give an appropriate cell number per 100 μ l.

Optimal assay performances are observed between 2.5×10^4 and 1×10^5 cells per 100 μ l.

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 100µl of 1X PBS to every well
2.	Incubation	Incubate plate at room temperature (RT) for 10 min
3.	Wash	Empty the wells by flicking the plate over a sink & gently tapping on absorbent paper.
4.	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.)
5.	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
6.	Addition	Empty the wells and remove excess solution then add 100µl of PBS-T to every well
7.	Incubation	Incubate the plate at 4°C for 10 min
8.	Wash	Empty the wells as previous and wash the plate 3x with 100µl of PBS-T
9.	Addition	Add 100µl of diluted detection antibody to every well
10.	Incubation	Cover the plate and incubate at RT for 1 hour 30 min
11.	wash	Empty the wells as previous and wash the plate 3x with 100µl of PBS-T
12.	Addition	Add 100µl of diluted Streptavidin-AP conjugate to every well
13.	Incubation	Cover the plate and incubate at RT for 1 hour
14.	Wash	Empty the wells and wash the plate 3x with 100µl of PBS-T
15.	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.
16.	Addition	Add 100µl of ready-to-use BCIP/NBT buffer to every well
17.	Development	Incubate the plate for 5-15 min monitoring spot formation visually throughout the incubation period to assess sufficient colour development
18.	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
<p>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.</p> <p><i>Note: spots may become sharper after overnight incubation at 4°C</i></p>		

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-1 β .

To define specificity, several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested (IL-1 α , IL-2, IL-10, IL-12, IL-17A, IL-23, IFN γ , Gp130, IL-33, TNF α) This testing was performed using the equivalent human IL-1 β 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 (LPS) of 5 different PBMC cell concentrations, 12 repetitions in 1 batch. The data shows the mean spot number, range and CV for the five cell concentrations.

Cells / well	n	Mean number of spots per well	Min	Max	CV%
25000 recommended	12	360	327	398	6.6%
12500 recommended	12	266	234	328	9.2%
6250	12	164	143	205	10.4%
3125	12	94	70	112	12.6%
1560	12	50	34	58	14.2%

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