Human IL-1β ELISpot Set

Instructions for use

Catalogue Numbers:

	Without Plates	With non-Sterile Plates	With Sterile Plates
1x96 tests	856.101.001	856.101.001P	856.101.001S
5x96 tests	856.101.005	856.101.005P	856.101.005S

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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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-1β) is a member of the interleukin-1 family. This family contains three structurally related polypeptides. The first two are IL-1α and IL-1β, 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-1α 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-1β 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-1α, the IL-1β precursor shows little or no biological activity in comparison to the mature form.

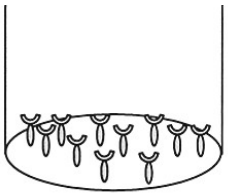
IL-1 is primarily an inflammatory cytokine. It belongs to a group of cytokines with overlapping biologic properties (TNFα and IL-6). IL-1, TNF and IL-6 share the ability to stimulate T and B lymphocytes, increase cell proliferation, and initiate or suppress gene expression for several proteins exerting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

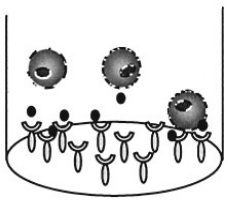
2.2. Principle of the method

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.

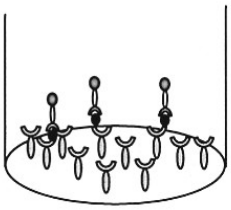
1. 96-PVDF bottomed-well plates are first treated with 35% ethanol and then coated with capture antibody.

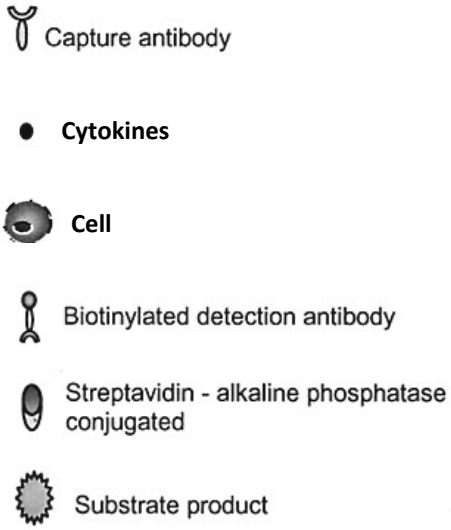


2. Cells are incubated in the presence of the stimulating agent. Upon stimulation they release cytokines which bind to the capture antibodies.

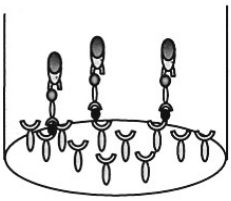


3. Cell removal by washing. Incubation with biotinylated detection antibody.





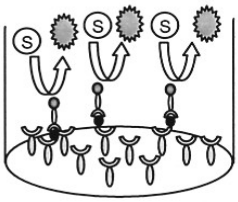
4. Any excess unbound detection antibodies is removed by washing. Incubation with streptavidin – alkaline phosphatase conjugate.

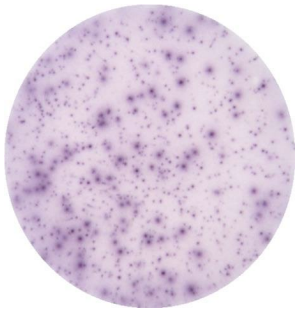


5. Any excess unbound Strep-AP is removed by washing. Incubation with BCIP/NBT.

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	Reconstitution
96-well PVDF bottomed plates (if ordered)	2	5	Ethanol treatment (see section 9)
Capture Antibody	1 (0.1 ml)	1 (0.5 ml)	Sterile, dilute prior to use (see Capture Antibody, section 7.6)
Biotinylated Detection Antibody	1 vial (100 µl)	1	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)	Dilute prior to use (see Streptavidin- AP conjugate, section 7.8)
Bovine Serum Albumin (BSA) – 2 g	1	1	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)	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, LPS)
- CO_2 incubator
- 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 & Wash step)

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. 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.

7.6. 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.

7.7. 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.

7.8. 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-1β production

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

Dilute PBMC in culture medium (e.g. RPMI 1640 supplemented with 2mM L-glutamine and 10% heat inactivated fetal calf serum) containing 1 µg/ml LPS (Sigma, Saint Louis, MO). Distribute $1x10^4$ to $2.5x10^4$ cells per 100 µl in required wells of an antibody coated 96-well PVDF plate and incubate for 15-20 hours in an incubator.
Note that as IL-1β is mainly produced by monocytes and macrophages PBMC population. Spontaneous release of IL-1β can occur when the cells adhere to the PVDF membrane.

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 PBMC 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 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 µl.

Optimal assay performances are observed between $2.5x10^4$ and $1x10^5$ 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 200 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 200 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 200 µl of PBS 1X to every well
13.	Incubation	Incubate the plate at 4°C for 10 min
14.	Wash	Empty the wells and wash the plate 3x with 200 µl of PBS 1X
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 and wash the plate 3x with 200 µl of PBS 1X
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 200 µl of PBS 1X
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-1β.

To define specificity of this IL-1β antibody pair, several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested (IL-1α, IL-2, IL-10, IL-12, IL-17A, IL-23, IFNγ, Gp130, TNFα and IL-33). This testing was performed using the equivalent human IL-1β 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. The data show 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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