Human IL-12p70 ELISpot Kit

SKU: HUDC0149

Instructions for use

	Pre-coated Plates
1x96 tests	HUDC0149
2x96 tests	HUDC0149-2
5x96 tests	HUDC0149-5

For research use only

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Human IL-12p70 ELISpot Kit – Pre-coated

1. Intended use

Assay Genie **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, Assay Genie 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

IL-12 is a potent regulator of cell mediated immune response produced by activated monocytes / macrophages cells, B lymphocytes and connective tissue type mast cells. The biologically active form of IL12 is a 70 kDa heterodimeric glycoprotein consisting of disulfide-linked 35 kDa (p35) light chain and 40 kDa (p40) heavy chain subunits. The two subunits are genetically unrelated.

The p70 form is the only biologically active form of IL-12.

The p35 subunit has homology to IL-6, while p40 has homology with IL-23. IL-12 has been found to bind to IL-12R. IL-12R has been reported to be present on IL-2 activated CD4+, CD8+ and CD56+ cells. IL-12 exerts a variety of biological effects on human T and NK cells. IL-12 induces an IFN gamma production and other cytokines from peripheral blood T and NK cells. Its role is directing development and proliferation of Th1 cells. Thus IL-12 is linked with autoimmunity, high level have also been reported for chronic inflammatory reactions, bacterial and viral infection.

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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.

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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.

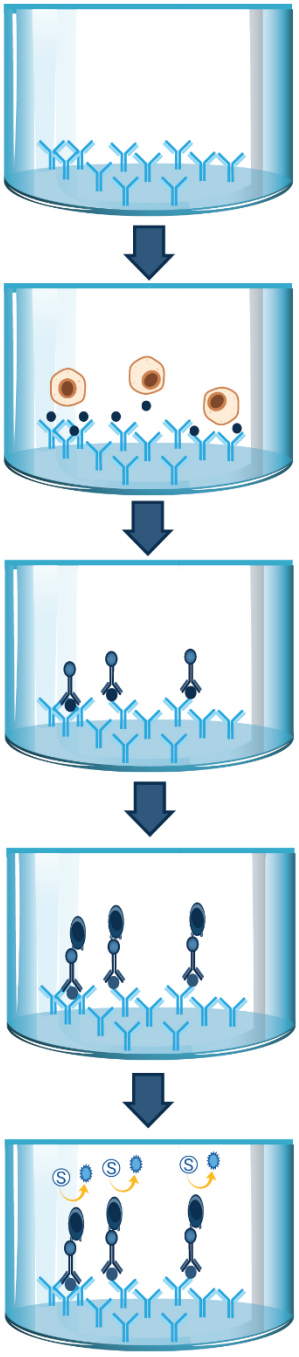
3. Cells are removed by washing. Detection antibody is added.

4. Any excess unbound detection antibodies are removed by washing. Streptavidin-alkaline phosphatase is added.

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

BCIP/NBT is reduced by alkaline phosphatase to produce a precipitate observed as blue/purple spots.

One spot corresponds to one single producing cell.



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3. Reagents provided

Reagents	HUDC0149	HUDC0149-2	HUDC0149-5	Reconstitution
Pre-coated 96-well PVDF bottom plates	2	2	5	Rehydrate with 100 µl of PBS1X (see section 9)
Biotinylated Detection antibody	1 (100 µl)	1 (200 µl)	1	Reconstitute with 0.55 ml of distilled water Dilute prior to use (see Detection Antibody, section 7.4)
Streptavidin-Alkaline Phosphatase conjugate	1 (10 µl)	1 (20 µl)	1 (50 µl)	Dilute prior to use (see Streptavidin-AP conjugate, section 7.5)
Bovine Serum Albumin (BSA) – 2 g	1	1	1	Dissolve to prepare dilution buffer (see 1%BSA PBS solution, section 7.3)
Ready to use BCIP/NBT - (Substrate buffer)	1 (11 ml)	1 (25 ml)	2 (25 ml)	Ready to use

***Please note for HUDC0149 and HUDC0149-2: detection antibody is provided in liquid form.**
For HUDC0149: 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
- Cell culture reagents (e.g. RPMI-1640, L-glutamine, FCS)
- Cell stimulation reagents (e.g. PMA, Ionomycin)
- CO_2 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 if in the case of repeated use of one component, the reagent is not contaminated by the first handling.

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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.

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7. Reagent Preparation

7.1. 1X Phosphate Buffered Saline (PBS)

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. 0.05% Tween PBS Solution (Wash Buffer)

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

7.3. 1% BSA PBS Solution (Dilution Buffer)

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

7.4. 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 HUDC0149 and HUDC0149-2 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.

7.5. 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.

7.6. BCIP/NBT

The reagent is ready-to-use.

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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-12 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 100 ng/ml IFN□. Incubate overnight. Take off non adherent materials and harvest adherent cells with a cell scraper. Wash cells once. Dilute cells in culture medium supplemented with 1 µg/ml LPS and distribute $1x10^5$ to $2.5x10^5$ cells in antibody coated PVDF-bottomedwells and incubate overnight 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 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 $1x10^5$ 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.

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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 100 µl of PBS 1X 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 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.)
5.	Incubation	Cover the plate and incubate at 37ºC 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 100 µl of Wash Buffer to every well
7.	Incubation	Incubate the plate at 4ºC for 10 min
8.	Wash	Empty the wells as previous and wash the plate 3x with 100 µl of Wash Buffer
9.	Addition	Add 100 µl 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 100 µl of Wash Buffer
12.	Addition	Add 100 µl 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 100 µl of Wash Buffer
15.	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
16.	Addition	Add 100 µl 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
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 4ºC 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.

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10. Performance Characteristics

10.1. Specificity

The assay recognizes natural Human IL-12p70.

To define specificity of this IL-12p70 antibody pair, several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested (IL-1□, IL-1□, IL-10, IL-12p40, IFN□, IL-4, IL-6, TNF□, IL-8 and IL-13). This testing was performed using the equivalent Human IL-12p70 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%
200000 recommended	12	323	317	328	1.4
100000 recommended	12	221	203	234	7.1
50000	12	142	124	160	10.5
25000	12	83	70	93	11.6
12500	12	35	33	38	11.5

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