（FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSTICS !）

Catalog No: ESP-H0007

Product size: 96T/96T*5

Elabscience® Human IL-4 (Interleukin 4) ELISPOT Kit

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help (info in the header of each page).

Tel: 1-832-243-6086
Fax: 1-832-243-6017
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please refer to specific expiry date from label outside of box.

Please kindly provide us with the lot number (on the outside of the box) of the kit for more efficient service.

Intended use

This ELISPOT kit is designed for the detection of the frequency of Human IL-4 secreting cells.

ELISPOT is a highly specific immunological analysis method used to analyze the production and secretion of cytokines and other soluble molecules by T/B cells at the single-cell level. The assay mimics the physiological environment closely and requires minimal cell manipulation. This immunological analysis technique aims to determine the frequency of cells that produce cytokines in response to a given stimulus and compare this frequency with specific treatments or pathological conditions. ELISPOT detection is an ideal tool for studying T/B cell functionality, autoimmune testing, vaccine development, organ transplantation, oncology, infectious diseases, virus infection monitoring, and therapy, among others.

Character

Item	
Specificity	This kit recognizes Human IL-4 in samples. No significant cross-reactivity or interference between Human IL-4 and analogues was observed
Repeatability	Coefficient of variation is < 10%

Test principle

This assay kit uses the Sandwich-ELISA principle. The PVDF membrane at the bottom of the micro ELISPOT plate has been re-coated with anti-Human IL-4 antibodies. During the experiment, cell suspension and stimulants are added first, and IL-4 secreted by the cells during the incubation process will bind to the coated antibodies. Then wash away the cells, add biotinylated anti-Human IL-4 antibodies and alkaline phosphatase conjugated avidin sequentially. The biotinylated anti-Human IL-4 antibodies will bind specifically to the IL-4 which has already bounded, then avidin will bind specifically to biotin, together forming the complex. After free components are washed away, add chromogenic substrate (BCIP/NBT), BCIP undergoes hydrolysis under the catalysis of alkaline phosphatase and reacts with NBT, forming insoluble spots with color dark blue to blue-purple. Professional ELISPOT plate reader software can be used to analyze the result, calculating the frequency of Human IL-4 secreting cells.

Kit components & Storage

An unopened kit can be stored at 2-8℃ for 1 year. After opening, store the items separately according to the following conditions

Item	Specifications	Storage
ELISPOT Plate (Strip)	96T: 8 wells×12strips 96T*5: 5 plates, 96T	2-8°C, 1 year
Positive Control：Anti-CD3 mAb (500×)	96T: 1 vial 100μL 96T*5: 5 vials 100μL	-20℃, up to expiry date (1 year)
Concentrated Biotinylated Detection Ab (100×)	96T: 1 vial 70μL 96T*5: 5 vials 70μL	
Concentrated ALP Conjugate(100×)	96T: 1 vial 70μL 96T*5: 5 vials 70μL	
Biotinylated Detection Ab Diluent	96T: 1 vial 10mL 96T*5: 5 vials 10mL	2-8°C, 1 year
ALP Conjugate Diluent	96T: 1 vial 10mL 96T*5: 5 vials 10mL	
Wash Buffer 1 (Ready to use, sterile)	96T: 2 vials, 25mL (sterile) 96T*5: 10 vials, 25mL (sterile)	
Concentrated Wash Buffer 2 （10×）	96T: 2 vials, 25mL 96T*5: 10 vials, 25mL	
Substrate Reagent	96T: 1 vial 7mL 96T*5: 5 vials 7mL	2-8°C, protect from light, 1 year
Product Description	1 copy	

Note: All reagent bottle caps must be tightened to prevent evaporation and microbial pollution. The volume of reagents in partial shipments is a little more than the volume marked on the label, please use accurate measuring equipment instead of directly pouring into the vial(s).

Other supplies required

1. Laminar flow cabinet
2. High-precision pipette, EP tubes and disposable pipette tips: 0.5-10μL, 2-20μL, 20-200μL, 200-1000μL
3. Loading slot
4. General purpose centrifuge
5. Automated cell counter
6. Sterile culture media
7. CO2 cell incubator
8. Incubator capable of maintaining 37°C
9. Deionized or distilled water
10. ELISPOT reader

Note

1) For research use only. Not for use in diagnostic procedures.
2) Please wear lab coats, eye protection and latex gloves for protection. Please perform the experiment following the national security protocols of biological laboratories when handling reagents, blood specimens, PBMC, and human cell lines.
3) Return the unused wells to the foil pouch and store according to the conditions suggested in the above table.
4) Do not reuse the reconstituted biotinylated detection Ab and ALP conjugate working solution. The unspent Detection Ab (100×), ALP Conjugate (100×), ELISPOT Plate and other stock solutions should be stored according to the storage conditions in the table above.
5) All EP tubes and pipette tips used in the experiment are disposable; mixing or reuse is strictly prohibited.
6) BCIP/NBT substrate is toxic and may cause severe skin allergic reactions. Handle the reagents with care, and always wear gloves.
7) Do not mix or substitute reagents from other batches or sources with the reagents included in this assay kit.。
8) The kit should not be used beyond the expiration date on the kit label.
9) Follow the incubation times specified in the experimental procedures.

Reagent preparation

1. If the kit will not be used up in one assay, please only take out the necessary strips and reagents for present experiment, and store the remaining strips and reagents at required condition.
2. **Wash Buffer 1：** Ready to use. Note: concentrated Wash Buffer 1 removed from the refrigerator may contain crystals, which is a normal phenomenon. Note: if crystals have formed inthe concentrate, warm it in a 40℃ water bath and mix it gently until the crystals have completely dissolved. (under ultra-clean table conditions)
3. **Wash Buffer 2：** Dilute Concentrated Wash Buffer 2 with deionized or distilled water at a ratio of 1:9. Note: concentrated Wash Buffer 1 removed from the refrigerator may contain crystals, which is a normal phenomenon. Allow the crystals to completely dissolve by micro-heating in a 40°C water bath (under ultra-clean table conditions) prior to preparation.
4. **Biotinylated Detection Ab Working Solution**: Calculate the required amount before the experiment (50μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the Concentrated Biotinylated Detection Ab at 800×g for 1 min, then dilute the 100× Concentrated Biotinylated Detection Ab to 1× working solution with Biotinylated Detection Ab Diluent(Concentrated Biotinylated Detection Ab: Biotinylated Detection Ab Diluent = 1:99). The working solution should be prepared just before use.
5. **ALP Conjugate working solution**: ALP Conjugate is ALP conjugated avidin. Calculate the required amount before the experiment (50μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the Concentrated ALP Conjugate at 800×g for 1 min, then dilute the 100× Concentrated ALP Conjugate to 1× working solution with ALP Conjugate Diluent(Concentrated ALP Conjugate: ALP Conjugate Diluent= 1: 99).The working solution should be prepared just before use.
6. **BCIP/NBT Substrate Reagent**: The reagent is ready-to-use and appears clear to pale yellow. If there is any precipitation, filter the reagent using a disposable syringe and 0.2μm membrane filter.

Sample preparation

1. Dilute primary cells (immune cells) or cell lines with the sterile culture medium to an appropriate number, and add a stimulant (stimulants, vaccines, peptide pools, or infected cells, as recommended in the kit's "typical data", etc.). The types of effector and responder cells used, method of cell separation, mode of stimulation, and length of incubation are to be determined by each investigator.
2. Once the suitable cell number for the experiment is determined, plate the cells and stimulate them. In addition to the conditions of interest, we recommend setting three control conditions for each sample (blank control can be shared):

Control	Condition	Purpose
Positive Control	Cells cultured with validated specific antigens or polyclonal stimulants	Assesse cell functionality and experiment efficacy Reveal the false-negative result
Negative Control	Cells cultured without any stimulation	Confirm the number of spontaneously secreting cells Reveal the false-positive result
Blank Control	Culture medium with no cell cultured	Check whether reagents or cell culture medium generate false spots Reveal the false-positive result

Note:

1) Freshly prepared cells are recommended for use in ELISPOT experiments. Frozen cells may be used but should be handled with caution due to potential changes in cell viability or function.
2) This kit is suitable for detection of primary cells and cell lines cultured in suspension, and has not been validated for adherent cells.
3) Before the formal experiment, it is recommended to conduct a pre-test to determine the optimal stimulation condition and cell concentration, to avoid spot aggregation or unclear color development.
4) The Anti-CD3 mAb in this kit is only effective for PBMC and has not been verified for other cells.
5) In the formal ELISPOT experiment, it is advisable to include three replicate wells for each test sample to ensure data reliability and reproducibility.
6) Achieving sufficient stimulation requires a certain number of cells. The relationship between the number of spots generated in the experiment and the number of cultured cells is not linear
7) The number of cells added into the ELISPOT plate should not exceed 4×10^5 cells per well, typically adding $0.5\sim2.5\times10^5$ cells per well for assessing the antigen-specific response. Higher cell concentration may lead to the formation of multilayers, resulting in poor spot formation. For polyclonal

stimulation, it might need to reduce cell numbers to avoid the formation of conglomerate spots.

Assay procedure

1. Plate Washing: Remove the ELISPOT plate from its sealed packaging and wash it 2 times with Wash Buffer 1 (200μL/well).
2. Sample Addition: Decant the liquid from each well, add 100μL of the prepared cell suspension to each well (set positive control, negative control, and blank control for each sample), and cover the plate with the plate lid.
3. Incubation: Place the plate in an incubator, 37°C 5% CO2 for 18-48 hours. The specific incubation time depends on cell type, cell status, cell number, the protein being detected, protein release kinetics, and whether the experimental process includes pre-stimulation. (Note: Do not move the ELISPOT plate during the incubation period)
4. Plate Washing: Decant the liquid from each well, Add 200 μL of Wash Buffer 2 to each well, aspirate or decant the solution from each well, and repeat 5 times.
5. Biotinylated Detection Antibody Addition: Add 50μL of Biotinylated Detection Ab working solution to each well and incubate at 37°C for 2 hours.
6. Plate Washing: Decant the liquid from each well, Repeat the wash process for 5 times as conducted in step 4.
7. ALP Conjugate Addition: Add 50μL of ALP Conjugate working solution to each well and incubate at 37°C for 1 hour.
8. Plate Washing: Decant the liquid from each well, Repeat the wash process for 5 times as conducted in step 4.
9. Substrate Addition: Add 50μL of BCIP/NBT Substrate Reagent to each well and incubate at 37°C until evident spots appear, usually for 5-25 minutes.
10. Reaction Termination: Decant the liquid from each well, remove the outer wrap from the bottom of the plate frame, and thoroughly rinse both sides of the PVDF membrane with deionized water. (The inside membrane surface can be washed by filling the well with deionized water and then decanting the liquid, repeat for at least 10 times; directly rinse the outside membrane surface 3 times with deionized water)
11. Drying: Air dry the plate at room temperature. (inverted and light-protected).
12. Reading：Use an ELISPOT reader to count the spots.

Note:

1) No need to slap the plate face-down to thoroughly remove the liquid in the well after washing.
2) Manual plate washing is recommended. If using an automated plate washer, be cautious of the probe height, ensuring it does not touch the bottom of the micro plate.
3) Avoid the contact between the pipette tip and the bottom of the micro plate through the entire experiment to prevent piercing the membrane on the bottom of the micro plate.

Calculation of results

The ELISPOT plate is analyzed by counting the spots using an ELISPOT reader. The spots are circular and have a typical "nuclear halo" structure: the center is darker and the edge is slightly blurred. The results can be quantified, for example, by calculating the frequency of cells secreting a certain protein or antibody.

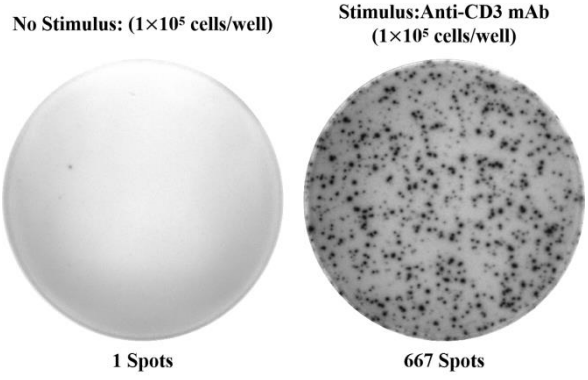
Technology resources

If you have any technical problems, please feel free to contact our technical support (it is recommended to take pictures and save the experimental data in time. Keep the used plate and remaining reagents).

Wechat of technical support

Typical data

The following data and spot images only for reference

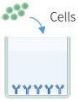


Incubate PBMC (1*10^5 cells/well) under the condition with or without the stimulus Anti-CD3 mAb (1:500) for 20 hours. The spot count represents the number of cells secreting human IL-4.

Assay Procedure Summary



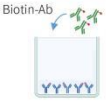
1. Remove the ELISPOT plate and wash each well twice with wash buffer 1 (200 µL/well)



2. Decant the liquid, add 100 µL of pre-treated cell suspension sample to each well. Incubate the plate at 37°C in a 5% CO_2 incubator for 18-48 hours



3. Decant the liquid from each well, wash the plate 5 times with wash buffer 2 (200 µL/well)



4. Add 50µL of Biotinylated Detection Ab working solution to each well and incubate at 37°C for 2 hours



5. Decant the liquid from each well, wash the plate 5 times with wash buffer 2 (200 µL/well)



6. Add 50µL of ALP Conjugate working solution to each well and incubate at 37°C for 1 hour. Decant the liquid, wash the plate 5 times with wash buffer 2 (200 µL/well)



7. Add 50 µL of BCIP/NBT substrate solution to each well, incubate at 37°C for 5-25 minutes until distinct spots appear



8. Terminate the reaction by thoroughly rinsing the PVDF membrane (both sides) with deionized water. Air-dry and analyze spots with ELISPOT reader



Declaration

1. Limited by current conditions and scientific technology, we can't conduct comprehensive identification and analysis on all the raw material provided. So there might be some qualitative and technical risks for users using the kit.
2. This assay is designed to eliminate interference by factors present in biological samples. Until all factors have been tested in the ELISA immunoassay, the possibility of interference cannot be excluded.
3. The final experimental results will be closely related to the validity of products, operational skills of the operators, the experimental environments and so on. We are only responsible for the kit itself, but not for the samples consumed during the assay. The users should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
4. To get the best results, please only use the reagents supplied by the manufacturer and strictly comply with the instructions.
5. Even the same operator might get different results in two separate experiments. In order to get reproducible results, the operation of every step in the assay should be controlled.
6. Every kit has strictly passed QC test. However, results from end users might be inconsistent with our data due to some variables such as transportation conditions, different lab equipment, and so on. Intra-assay variance among kits from different batches might arise from the above reasons too.
7. The kit is designed for research use only, we will not be responsible for any issues if the kit is applied in clinical diagnosis or any other related procedures.