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Human IFN-gamma/IL-5 Dual Color ELISPOT Kit
PVDF Format

Catalog No: CDK179A	**Quantity:** 5 x 96 tests	With Sterile Plates
Catalog No: CDK179B	**Quantity:** 10 x 96 tests	With Sterile Plates
Catalog No: CDK179C	**Quantity:** 15 x 96 tests	With Sterile Plates
Catalog No: CDK179D	**Quantity:** 20 x 96 tests	With Sterile Plates

PRODUCT SPECIFICATIONS :

Specificity : Recognizes both natural and recombinant human IFN-γ and IL-5

Incubation : 3 hr 30 min after cell stimulation procedure

Kit Content : Capture and Detection antibody for cytokine 1, Alkaline phosphatase conjugate, Ready-to-use BCIP/NBT substrate buffer, Capture and Detection for cytokine 2, Peroxidase conjugate, BSA, Ready-to-use AEC substrate buffer.

Intended Use

The ELISPOT assay is designed to enumerate cytokine producing cells in a single cell suspension. This method has the advantage of requiring a minimum of *in-vitro* manipulations allowing cytokine production analysis as close as possible to *in-vivo* conditions in a highly specific way. This technique is designed to determine the frequency of cytokine producing cells under a given stimulation, and the follow-up of such frequency during a treatment and/or a pathological state. ELISPOT assay constitutes an ideal tool in the Th1 / Th2 response, vaccine development, viral infection monitoring and treatment, cancerology, infectious diseases, autoimmune diseases and transplantation.

The ELISPOT assay is based on sandwich immuno-enzyme technology. 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. The dual color ELISPOT allows you to monitor the production of two cytokines simultaneously in the same well.

Content of the Kit for 5 x 96 wells

- 96 PVDF-bottomed-well plates.
- Capture antibody for IFNγ (0.50 ml). Supplied sterile
- Capture antibody for IL-5 (0.50 ml). Supplied sterile
- FITC conjugated detection antibody for IFNγ (lyophilized, resuspend in 0.55 ml).
- Biotinylated detection antibody for IL-5 (lyophilized, resuspend in 0.55 ml).
- Anti-FITC antibody HRP conjugate (100 µl).
- Streptavidin Alkaline Phosphatase conjugate (50 µl).
- Bovine Serum albumin
- 50 x concentrate AEC substrate buffer (1 ml).
- 10 x concentrate buffer for the preparation of AEC buffer (5 ml).
- Ready-to-use BCIP/NBTsubstrate buffer (50 ml).

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Materials / Reagents not provided

- Cell culture media.
- CO_2 incubator.
- 70% ethanol.
- Tween 20.
- Phosphate buffered saline.
- ELISPOT reading system.

Safety & Precautions for use

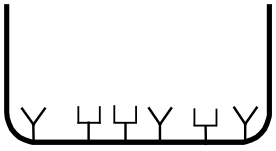
- For **research use only** not to be used as a diagnostic test
- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g. CDC/NIH Health manual: " Biosafety in Microbiological and Biomedical Laboratories" 1984
- Do not eat, drink, smoke or apply cosmetics 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
- **AEC and BCIP/NBT buffers** are potentially carcinogenic and should be disposed of appropriately, caution should be taken when handling these reagent's, always wear gloves.
- Follow incubation times described in the assay procedure.

Principle of the Method

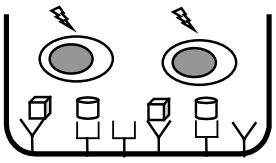
Capture antibodies highly specific for the analytes of interest are coated to the wells of a PVDF bottomed 96 well microtiter plate either during kit manufacture or in the laboratory. The plate is then blocked to minimize any non-antibody dependent unspecific binding and finally washed before adding the cells to be investigated. Cell suspension and stimulant are added to the coated and blocked microtiter plate and the plate incubated allowing the specific antibodies to bind any analytes produced. Biotinylated and FITC detection antibodies are then added which bind to the previously captured analyte. HRP conjugated anti-FITC antibodies and Streptavidin Alkaline Phosphatase are added binding to the detection antibodies. Any

excess unbound analyte and antibodies are removed by careful washing. Color substrate is then applied to the wells resulting in colored spots which can be quantified using appropriate analysis software or manually using microscopes.

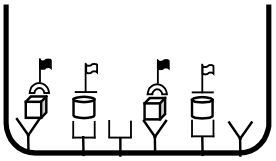
Procedure Summary



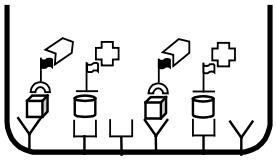
96 PVDF-bottomed-well plates are first treated with 35% ethanol and then coated with anti-IFN-gamma and anti-IL-5 capture antibodies



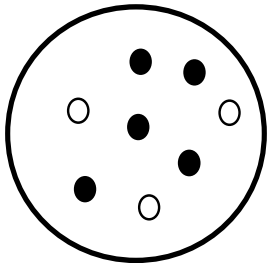
Cells are incubated in the presence of the antigen. Upon stimulation they release cytokines molecules which bind to the capture antibodies.



Cells are lysed. Anti-IFN-gamma-FITC and anti-IL-5 biotin detection antibodies are added and bind to the captured cytokines.



Detection antibodies are in turn bound by anti-FITC-HRP for IFN-gamma and biotin-AP for IL-5.



Finally colored spots are developed by separate incubations with first AEC and then BCIP/NBT substrate buffers. Cells producing IFN-gamma give red/brownish spots while those producing IL-5 give blue/purple spots.

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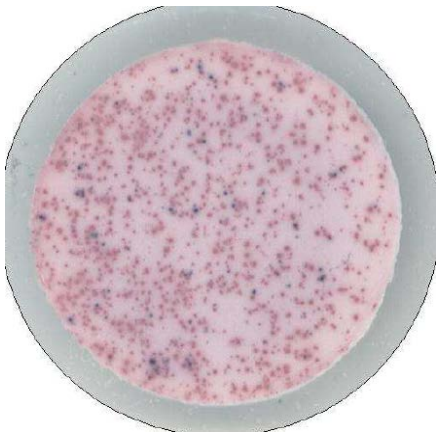

ELISPOT well showing numerous IFN-gamma red spots and fewer IL-5 blue spots.

Reagent Preparation

- **Phosphate buffered saline (10X Concentrate solution).**

For 1 liter weigh out: 80 g NaCl; 2 g KH_2PO_4; 14.4 g Na_2HPO_4-$2H_2O$. Add distilled water to 1 liter. Adjust the pH of the solution to 7.4 +/- 0.1 when required. **This solution should be diluted to 1X before use.**

- **1% BSA in PBS (Dilution Buffer)**

For one plate dissolve 0.2 g of BSA in 20 mL of 1X diluted PBS.

- **0.05% Tween in PBS**

For one plate dissolve 50 µl of Tween 20 in 100 ml of 1X diluted PBS.

- **35% ethanol in water (PVDF Membrane Activation Reagent)**

For one plate mix 3.5 ml of ethanol with 6.5 ml of distilled water.

- **Capture Antibodies**

These reagents are supplied sterile once opened keep the vials sterile or aliquot and store at -20oC. For optimal performance prepare the Capture Antibodies dilution immediately before use.

Dilute 100 µl of each capture antibody in 10 ml of 1X PBS and mix well.

- **Detection antibodies**

Reconstitute the lyophilized antibody with 0.55 ml of distilled water. Gently mix the solution and wait until all the lyophilized 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 each antibody into 10ml of Dilution Buffer and mix well.

- **Streptavidin-AP conjugate and Anti-FITC antibody-HRP conjugate (Diluted conjugates)**

For optimal performance prepare the dilution immediately prior to use.

For one plate, dilute 10 µl of Streptavidin-AP conjugate and 20 µl of anti-FITC antibody HRP conjugate in 10 ml of Dilution Buffer. Mix well.

DO NOT KEEP THE DILUTIONS FOR FURTHER EXPERIMENTS

- **AEC Substrate**

For one plate, mix 1 ml of AEC Buffer A 10X with 9 ml of distilled water. Then add 200 µl of AEC Substrate B 50X.

Reagent Storage

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). Expiration of the kit and reagents is stated on box front labels. The expiration date 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.

Assay Control:

Positive Control: Positive Assay Control, IFNγ / IL-5 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 2 mM L-glutamine and 10% heat inactivated fetal calf serum) containing 1 ng/ml PMA and 500 ng/ml ionomycin (Sigma, Saint Louis, MO). Distribute $1x10^5$ to $2.5x10^5$ cells per 100µl in required wells of an antibody coated 96-well PVDF plates and incubate for 15-20 hours in an incubator.

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 of the frequency of cytokine producing cells.

Negative Control

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

Samples

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.

Method

Prepare all reagents as described above

Assay Step		Details
1.	Addition	Add 25µl 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 3x with 100µl of 1X PBS per well
4.	Addition	Add 100µl of diluted **capture antibodies** 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 100µl of 1X PBS per well
7.	Addition	Add 100µl 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 three times with 100µl of 1X PBS per well
10.	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.)
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 100µl 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 100µl of **Wash Buffer**
15.	Addition	Add 100µl of diluted **detection antibodies** 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 100µl of **Wash Buffer**
18.	Addition	Add 100 µl per wells of **diluted HRP and AP conjugates**
19.	Incubation	Cover the plate and incubate at RT for 1 hour
20.	Wash	Empty the wells and wash the plate 3x with 100µl of **Wash Buffer**

21.	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.
22.	Addition	Add 100µl of **prepared AEC substrate** to every well
23.	Development	Incubate the plate for **5-20 min** 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
25.	Addition	Add 100µl of **ready to use BCIP/NBT** buffer to every well
26.	Development	Incubate the plate for **5-15 min** monitoring spot formation visually throughout the incubation period to assess sufficient colour development
27.	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*		

Plate should be stored at RT away from direct light. Color may fade over prolonged periods of time so please read as soon as possible, optimally within 24 hours.

NOT FOR HUMAN USE. FOR RESEARCH ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE.

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