

IFNG/GZMB

Human Interferon gamma/Granzyme B Dual Color ELISPOT Kit—Fluorescent Detection

Cat. No:	<u>Without Plates</u>	<u>With sterile plates</u>	Size:
	874.062.005	874.062.005P	5 x 96 wells
	874.062.010	874.062.010P	10 x 96 wells
	874.062.015	874.062.015P	15 x 96 wells
	874.062.020	874.062.020P	20 x 96 wells

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. Cell Sciences 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.

Content of the Kit for 5x96 wells

- Capture antibody for IFN-gamma (0.50 ml). Supplied sterile.
- Capture antibody for Granzyme B (0.50 ml). Supplied sterile.
- FITC conjugated detection antibody for IFN-gamma (lyophilized; resuspend in 0.55ml).
- Biotinylated detection antibody for Granzyme B (lyophilized; resuspend in 0.55ml).
- Anti-FITC antibody green fluorescence conjugate
- Streptavidin-phycoerythrin conjugate
- Bovine Serum albumin (1g)
- Dry skimmed milk (1g) (non-sterile ELISPOT) or Liquid sterile milk (sterile ELISPOT)
- 96 PVDF-bottomed-well plates (5 if ordered).

Store all reagents at 2-4°C. Plates should be stored at room temperature.

Materials / Reagents not provided

- 96 PVDF-bottomed-well plates (if not ordered). We recommend Millipore MultiScreen plates Cat. No. MSIPN4510 or Cat. No. MSIPS4510.



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- Cell culture media
- CO₂ incubator
- 70% ethanol
- Tween-20
- Phosphate buffered saline
- ELISPOT reading system

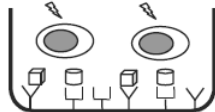
Principle of the Method

After cell stimulation, locally produced cytokines are captured by IFN-gamma and Granzyme B specific monoclonal antibodies. After cell lysis, trapped cytokine molecules are revealed by a secondary anti-IFN-gamma FITC conjugated antibody and a biotinylated anti-Granzyme B antibody. Those are in turn recognized by anti-FITC green fluorescent dye and streptavidin-phycoerythrin conjugates. PVDF-bottomed-well plates are then read under a UV light beam. Green fluorescent spots indicate IFN-gamma production while Granzyme B is revealed by red spots. Yellow spots will indicate dual cytokine producing cells.

Procedure Summary



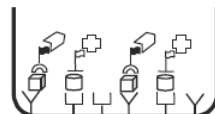
96 PVDF-bottomed-well plates are first treated with 70% ethanol and then coated with anti-IFN-gamma and anti-Granzyme B capture antibodies.



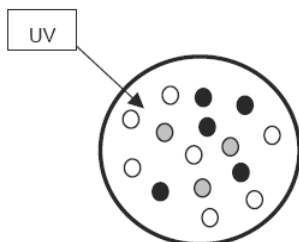
Cells are incubated in the presence of the antigen. Upon stimulation they release cytokines molecules which bind to the capture antibodies.



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



Detection antibodies are in turn bound by anti-FITC-green fluorescent dye for IFN-gamma and streptavidin-phycoerythrin for Granzyme B.



Finally fluorescent spots are visualised under a UV light beam. Cells producing IFN-gamma give green spots while those producing Granzyme B give red spots. Dual cytokine producing cells give yellow spots.



Assay Control

IFN-gamma/Granzyme B production by PBMC upon stimulation by PMA and Ionomycin. This protocol is given as a suggestion.

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 from 1.10^5 to $2.5 \cdot 10^4$ cells in antibody coated PVDF-bottomed-wells and incubate for 15-20 hours in an incubator.

For other stimulators incubation times may vary, depending on the frequency of cytokine producing cells, and should be optimized in each situation.

Reagent Preparation

Detection antibodies

- Reconstitute each vial with 0.55 ml of distilled water. Gently mix the solution and wait until all the lyophilized material is reconstituted.

If not used within a short period of time, reconstituted detection antibody should be aliquotted and stored at -20°C. In these conditions the reagent is stable for at least one year.

Anti-FITC green fluorescence conjugate/Streptavidin-phycoerythrin

- Dilute each reagent with the volume indicated on each vial in 10 ml of PBS 1% BSA.
- For 1 plate, prepare 10 ml of anti FITC-green fluorescence conjugate / Streptavidin-phycoerythrin solution.

PREPARATION IMMEDIATELY BEFORE USE IS RECOMMENDED.

Phosphate buffered saline (10X Concentrate solution).

- For 1 liter weigh: 80 g NaCl ; 2 g KH_2PO_4 ; 14.4 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$. Add distilled water to 1 liter. Check that pH is comprised between 7.4 ± 0.1 . **This solution should be diluted to 1X before use.**

2% skimmed dry milk in PBS

- For one plate dissolve 0.2 g of powder in 10 ml of 1X diluted PBS.

1% BSA in PBS

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

0.1% Tween in PBS

- For one plate dissolve 70 μl of Tween 20 in 70 ml of 1X diluted PBS.

70% ethanol in water

- For one plate mix 7 ml of ethanol with 3 ml of distilled water.



ELISPOT Procedure

1. Incubate PVDF-bottomed-well plates with 25 μ l of 70% ethanol for 30 seconds at room temperature.
2. Empty wells and wash three times with 100 μ l/well of PBS.
3. Pipette 100 μ l of IFN-gamma capture antibody and 100 μ l of Granzyme B capture antibody in 10 ml of PBS. Mix and dispense 100 μ l into each well, cover the plate and incubate overnight at 2-4°C.
4. Empty wells and wash once with 100 μ l of PBS.
5. Dispense 100 μ l/well of 2% dry skimmed milk in PBS into wells, cover and incubate for 2 hours at room temperature.
6. Empty wells by flicking the plate over a sink and tapping it on absorbent paper.
7. Wash plate once with PBS.
8. Dispense into wells 100 μ l/well of cell suspension containing the appropriate number of cells and appropriate concentration of stimulator. Cells may have been previously *in vitro* stimulated (Indirect ELISPOT). Cover the plate with a standard 96-well plate plastic lid and incubate cells at 37°C in a CO₂ incubator for an appropriate length of time (15-20 hours). **During this period do not disturb the plate.**
9. Empty wells by flicking the plate over a sink and gently tapping it on absorbent paper.
10. Dispense 100 μ l of PBS-0.1% Tween 20 into wells and incubate for 10 minutes at 2-4°C.
11. Wash wells three times with PBS-0.1% Tween 20.
12. For 1 plate dilute 100 μ l of reconstituted IFN-gamma detection antibody and 100 μ l of reconstituted Granzyme B detection antibody into 10 ml of PBS containing 1% BSA. Dispense 100 μ l into wells, cover the plate and incubate 1 hour 30 minutes at room temperature.
13. Empty wells and wash three times with PBS-0.1% Tween 20.
14. Distribute 100 μ l of anti FITC-green fluorescence conjugate / Streptavidin-phycoerythrin solution (see reagent preparation) in each well. Seal the plate and incubate for 1 hour at room temperature.
15. Empty wells and wash three times with PBS-0.1% Tween 20.
16. Peel off the plate bottom and wash three times both sides of the membrane under running distilled water. Remove all residual buffer by repeated tapping on absorbent paper.
17. Dry wells away from light.
18. Read spots on an ELISPOT reader under a UV light source.
19. Store the plate at 2-4°C away from light.

Note

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).



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The method used is dependent on 1) the type of cell assayed and 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 procedure beyond the stimulation step is the same whatever the method (direct/indirect) chosen.

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



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