

Human Dual IFN γ /Granzyme B Elispot

PVDF - Enzymatic



Without plates

- 874.060.005 5x96 wells
- 874.060.010 10x96 wells
- 874.060.015 15x96 wells
- 874.060.020 20x96 wells

With non-sterile plates

- 874.060.005 P 5x96 wells
- 874.060.010 P 10x96 wells
- 874.060.015 P 15x96 wells
- 874.060.020 P 20x96 wells

With sterile plates

- 874.060.005 S 5x96 wells
- 874.060.010 S 10x96 wells
- 874.060.015 S 15x96 wells
- 874.060.020 S 20x96 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.

Diaclone 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 colour Elispot allows you to monitor the production of two cytokines simultaneously in the same well.

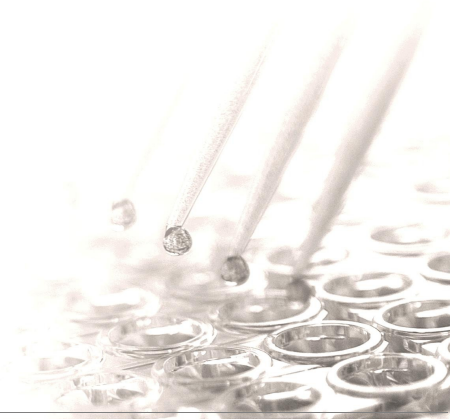
Content of the Kit for 5x96 wells

- Capture antibody for IFN γ (0.50 mL). Supplied sterile
- Capture antibody for Granzyme B (0.50 mL). Supplied sterile
- FITC conjugated detection antibody for IFN γ (lyophilised, resuspend in 0.55mL).
- Biotinylated detection antibody for Granzyme B (lyophilised, resuspend in 0.55mL).
- Anti-FITC antibody HRP conjugate (100 μ L).
- Streptavidin Alkaline Phosphatase conjugate (50 μ L).
- Bovine Serum albumin (1g).
- Dry skimmed milk (1 g): non sterile Elispot / Liquid sterile milk: sterile Elispot
- 50 x concentrate AEC substrate buffer (1mL).
- 10 x concentrate buffer for the preparation of AEC buffer (5mL).
- Ready-to-use BCIP/NBT substrate buffer (50mL).
- 96 PVDF-bottomed-well plates (5 if ordered).

Store all reagents at 4°C except plates which should be stored at room temperature.

Materials / Reagents not provided

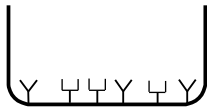
- 96 PVDF-bottomed-well plates. We recommend Millipore MultiScreen plates cat # MSIPN4510 or cat # MSIPS4510
- Cell culture media
- CO₂ incubator
- 70% ethanol
- Tween 20
- Phosphate buffered saline.
- ELISPOT reading system.



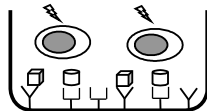
Principle of the method

After cell stimulation, locally produced cytokines are captured by IFN γ and Granzyme B specific monoclonal antibodies. After cell lysis, trapped cytokine molecules are revealed by a secondary anti-IFN γ FITC conjugated antibody and a biotinylated anti-Granzyme B antibody. Those are in turn recognised by anti-FITC HRP and streptavidin-AP conjugates. PVDF-bottomed-well plates are then incubated first with AEC substrate buffer, washed and subsequently incubated with BCIP/NBT. Coloured red/brownish spots indicate IFN γ production while Granzyme B is revealed by blue/purple spots.

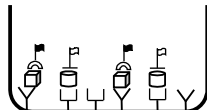
Procedure Summary



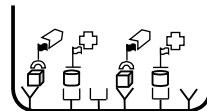
96 PVDF-bottomed-well plates are first treated with 70% ethanol and then coated with anti-IFN γ and anti-Granzyme B capture antibodies



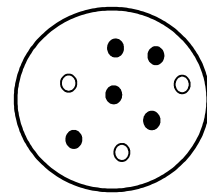
Cells are incubated in the presence of the antigen. Upon stimulation they release cytokines molecules which bind to the capture antibodies.



Cells are lysed. Anti-IFN γ -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-HRP for IFN γ and streptavidin-AP for Granzyme B.



Finally coloured spots are developed by separate incubations with first AEC and then BCIP/NBT substrate buffers. Cells producing IFN γ give red/brownish spots while those producing Granzyme B give blue/purple spots.

Assay control

IFN γ / 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 2mM L-glutamine and 10% heat inactivated foetal calf serum) containing 1ng/ml PMA and 500ng/ml ionomycin (Sigma, Saint Louis, MO). Distribute from $1 \cdot 10^5$ 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 optimised in each situation.

Reagent Preparation

▪ Detection antibodies

Reconstitute the lyophilised antibody with 0.55mL of distilled water. Gently mix the solution and wait until all the lyophilised material is back into solution.

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

▪ Streptavidin alkaline phosphatase conjugate

Dilute 1/5000 in PBS 1% BSA. We recommend to proceed in two steps:

1: Pre-dilution 1/100: 5 μ l of streptavidin alkaline phosphatase in 495 μ l of PBS 1% BSA.

2: See step 14 Elispot Procedure.

DO NOT KEEP THE DILUTIONS FOR FURTHER EXPERIMENTS

▪ Phosphate buffered saline (10X Concentrate solution).

For 1 litre weigh : 80g NaCl ; 2g KH₂PO₄ ; 14.4g Na₂HPO₄ 2H₂O. Add distilled water to 1 litre. Check that pH is comprised between 7.4 +/- 0.1. **This solution should be diluted to 1X before use.**

▪ 2% dry skimmed 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 μ l 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.

▪ AEC buffer

For one plate mix 1 ml of AEC buffer A with 9 ml of distilled water. Then add 200 μ l of AEC buffer B.

Elispot Procedure

1. Incubate PVDF-bottomed-well plates with 25 μ l / well of 70% ethanol for 30 sec at room temperature.
2. Empty wells and wash three times with 100 μ l / well of PBS.
3. Pipette 100 μ l of IFN γ capture antibody and 100 μ l of Granzyme B capture antibody in 10 mL of plain PBS. Mix and dispense 100 μ l into each well, cover the plate and incubate overnight at +4°C.
4. Empty wells and wash once with 100 μ l of PBS.
5. Dispense 100 μ l 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 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 min at +4°C.
11. Wash wells three times with PBS-0.1% tween 20.
12. For 1 plate dilute 100 μ l of reconstituted IFN γ 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 min at room temperature.
13. Empty wells and wash three times with PBS-0.1% tween 20.

14. For 1 plate dilute 20 μ l of anti-FITC HRP and 200 μ l of pre-dilute (1/100) streptavidin-Alkaline phosphatase conjugates into 9.8 mL of PBS-1% BSA. Dispense 100 μ l of the dilution into wells. 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; wash three times both sides of the membrane under running distilled water. Remove all residual buffer by repeated tapping on absorbent paper.
17. Prepare AEC buffer (see reagents preparation). Dispense 100 μ l of solution in wells.
18. Let the colour reaction proceed for 5-15 min at room temperature. When the spots have developed empty the buffer into an appropriate tray.
19. Wash three times both sides of the membrane with distilled water. Remove residual water by tapping the plate on absorbent paper.
20. Dispense 100 μ l of ready-to-use BCIP/NBT buffer into wells.
21. Let the colour reaction proceed for about 2-10 min at room temperature. When spots have developed, empty the buffer into an appropriate tray.
22. Wash thoroughly both sides of the membrane with distilled water
23. Dry the membrane by repeatedly tapping the plate on absorbent paper. Store the plate upside down so no remaining liquid will go back on the membrane. Read spots once the membrane is dried. Note that spots may become sharper after one night at +4°C.

Store the plate at +4°C away from direct light.

Notes and recommendations

Cell stimulation

1. 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 procedure beyond the stimulation step is the same whatever the method (direct/indirect) chosen.

Substrate

2. AEC and BCIP/NBT buffers are potentially carcinogenic and should be disposed off appropriately. Caution should be taken while handling those reagents. Always wear gloves.