Human Dual IFNγ/Perforin Elispot

PVDF - Enzymatic

Without plates

- ❒ 874.070.005 5x96 wells
- ❒ 874.070.010 10x96 wells
- ❒ 874.070.015 15x96 wells
- ❒ 874.070.020 20x96 wells

With non-sterile plates

- ❒ 874.070.001 1x96 wells
- ❒ 874.070.005 P 5x96 wells
- ❒ 874.070.010 P 10x96 wells
- ❒ 874.070.015 P 15x96 wells
- ❒ 874.070.020 P 20x96 wells

With sterile plates

- ❒ 874.070.111 1x96 wells
- ❒ 874.070.005 S 5x96 wells
- ❒ 874.070.010 S 10x96 wells
- ❒ 874.070.015 S 15x96 wells
- ❒ 874.070.020 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.

Reagents provided (Contents shown for 5x96 wells)

- Capture antibody for IFNγ (0.50 mL). Supplied sterile
- Capture antibody for Perforin (0.50 mL). Supplied sterile
- FITC conjugated detection antibody for IFNγ (lyophilised, resuspend in 0.55mL).
- Biotinylated detection antibody for Perforin (lyophilised, resuspend in 0.55mL).
- Anti-FITC antibody HRP conjugate (100 µL).
- Streptavidin Alkaline Phosphatase conjugate (50 µL).
- Bovine Serum albumin (1g).
- 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).

Please note for 1x96 demo kits, detection antibodies are provided in liquid form.
Store all reagents at 4°C except plates which should be stored at room temperature.

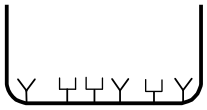
Materials / Reagents not provided

- 96 PVDF-bottomed-well plates. We recommend Millipore MultiScreen plates cat # MSIPN4510 or cat # MSIPS4510
- Cell culture media
- CO_2 incubator
- 70% ethanol
- Tween 20
- Phosphate buffered saline.
- ELISPOT reading system.

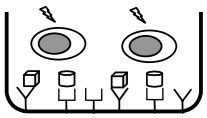
Principle of the method

After cell stimulation, locally produced cytokines are captured by IFNγ and Perforin specific monoclonal antibodies. After cell lysis, trapped cytokine molecules are revealed by a secondary anti-IFNγ FITC conjugated antibody and a biotinylated anti-Perforin 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 IFNγ production while Perforin is revealed by blue/purple spots.

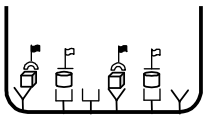
Procedure Summary



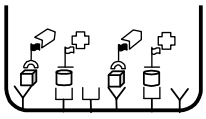
96 PVDF-bottomed-well plates are first treated with 35% ethanol and then coated with anti-IFNγ and anti-Perforin capture antibodies



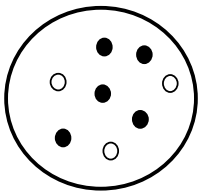
Cells are incubated in the presence of the antigen. Upon stimulation they release cytokines molecules which bind to the capture antibodies.



Cells are lysed. Anti-IFNγ-FITC and anti-Perforin biotin detection antibodies are added and bind to the captured cytokines.



Detection antibodies are in turn bound by anti-FITC-HRP for IFNγ and strepavidin-AP for Perforin.



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

Assay control

IFNγ / Perforin 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 1.10^5 to $2.5\ 10^4$ 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.

Please note for the 1X96 wells, detection antibodies are provided in liquid form.

- **Streptavidin alkaline phosphatase conjugate**

Dilute 1/1000 in PBS 1% BSA.

DO NOT KEEP THE DILUTIONS FOR FURTHER EXPERIMENTS

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

For 1 litre weigh : 80g NaCl ; 2g KH_2PO_4 ; 14.4g Na_2HPO_4 $2H_2O$. 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.**

- **1% BSA in PBS**

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 50µl of Tween 20 in 100 ml of 1X diluted PBS.

- **35% ethanol in water**

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

- **AEC buffer**

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

Elispot Procedure

1. Incubate PVDF-bottomed-well plates with 25µl / well of 35% ethanol for 30 sec at room temperature.
2. Empty wells and wash three times with 100µl / well of PBS.
3. Pipette 100µl of IFNγ capture antibody and 100µl of Perforin capture antibody in 10 mL of plain PBS. Mix and dispense 100 µl into each well, cover the plate and incubate overnight at +4°C.
4. Empty wells and wash once with 100 µl of PBS.
5. Dispense 100 µl/well of RPMI 10% FCS 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 µl 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 CO_2 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 100µl of PBS-0.05% tween 20 into wells and incubate for 10 min at +4°C.
11. Wash wells three times with PBS-0.05% tween 20.
12. For 1 plate dilute 100µl of reconstituted IFNγ detection antibody and 100µl of reconstituted Perforin detection antibody into 10 mL of PBS containing 1% BSA. Dispense 100µl into wells, cover the plate and incubate 1 hour 30 min at room temperature.
13. Empty wells and wash three times with PBS-0.05% tween 20.

14. For 1 plate dilute 20µl of anti-FITC HRP and 10 µl of streptavidin-Alkaline phosphatase conjugates into 10 mL of PBS-1% BSA. Dispense 100µl 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-0.05% 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. Prepare AEC buffer (see reagents preparation). Dispense 100µl of solution in wells.
18. Let the colour reaction proceed for 5-20 min at room temperature. Monitor spot formation visually. When the spots have developed empty the buffer into an appropriate tray.
19. Wash three times both sides of the membrane with running distilled water. Remove residual water by tapping the plate on absorbent paper.
20. Dispense 100µl of ready-to-use BCIP/NBT buffer into wells.
21. Let the colour reaction proceed for about 5-20 min at room temperature. Monitor spot formation visually. When spots have developed, empty the buffer into an appropriate tray.
22. Wash three times both sides of the membrane with running distilled water.
23. Dry the membrane by repeated 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.