

Mouse IFN-gamma Eli-spot

Catalog No. 862.031.005

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. 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.

Principle of the method

After cell stimulation, locally produced cytokines are captured by a specific monoclonal antibody. After cell lysis, trapped cytokine molecules are revealed by a secondary biotinylated detection antibody, which is in turn recognised by streptavidin conjugated to alkaline phosphatase. PVDF-bottomed-well plates are then incubated with BCIP/NTB substrate. Colored "purple" spots indicate cytokine production by individual cells.

Content of the Kit for 5x96 wells

- Capture antibody (0.52 mL). Store at +4°C.
- Biotinylated detection antibody (lyophilised, resuspend in 0.55mL).
- Streptavidin Alkaline Phosphatase conjugate. Store at +4°C.
- Bovine Serum albumin (3.5 g). Store at +4°C.
- Ready-to-use substrate buffer (50mL). Store at +4°C.

Materials / Reagents not provided

- 96 PVDF-bottomed-well plates. We recommend Millipore MultiScreen plates cat # MAIPN4510
- Cell culture media.
- CO₂ incubator.
- 70% ethanol.
- Tween 20.
- Phosphate buffered saline.

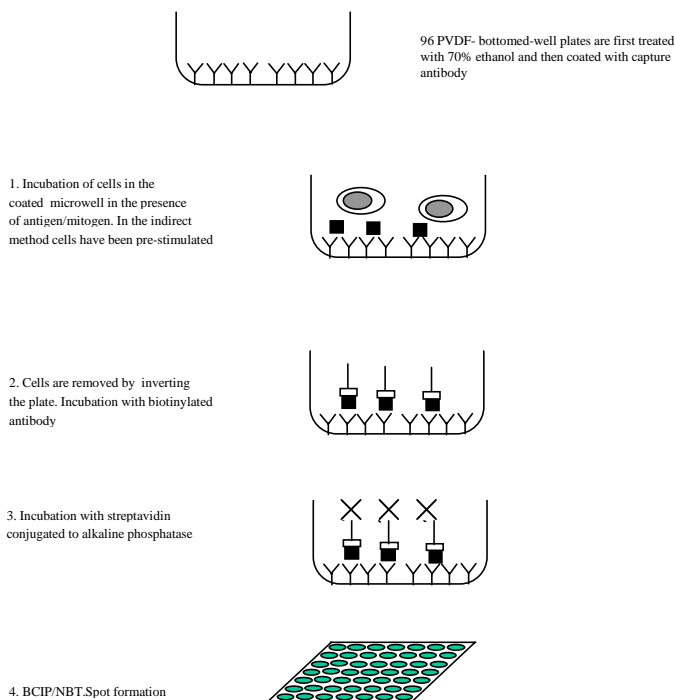


Direct versus Indirect Eli-spot

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 Eli-spot method. All the procedure beyond the stimulation step is the same whatever the method (direct/indirect) chosen.

Procedure Summary



Stimulation protocol : IFN γ 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 fetal calf serum) containing 1ng/ml PMA and 500ng/ml ionomycin (Sigma, Saint Louis, MO). Distribute 2.10^4 to 5.10^4 cells in antibody coated PVDF-bottomed-wells and incubate for 10-15 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 antibody**
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.
- **Streptavidin alkaline phosphatase**
Dilute 1/1000 in PBS-1% BSA
- **Phosphate buffered saline (10X Concentrate solution).**
For 1 liter weight : 80g NaCl ; 2g KH $_2$ PO $_4$; 14.4g Na $_2$ HPO $_4$ 2H $_2$ O and 2g KCl. Add distilled water to 1 liter. Check that pH is comprised between 7.2 and 7.4. **This solution should be diluted to 1X before use.**
- **5% BSA in PBS**
For one plate dissolve 0.5 g of BSA 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.

Reagent Storage

- a) 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.
- b) substrate buffer shall be stored at +4°C.
- c) Streptavidin-Alkaline phosphatase shall be stored at +4°C.



Eli-spot PROCEDURE

1. Incubate PVDF-bottomed-well plates with 100µl of 70% ethanol for 10 min at room temperature.
2. Wash wells three times with 100µl of PBS.
3. Pipette 100µl of capture antibody in 10 mL of PBS. Mix and dispense 100 µl into each well, cover the plate and incubate overnight at +4°C.
4. Wash wells three times with 100 µl of PBS.
5. Dispense 100µl of 5% BSA in PBS into wells, cover and incubate for 2 hour at 37°C. Empty wells by flicking the plate over a sink and tapping it on absorbent paper. **Do not wash wells.**
6. Dispense into wells 100 µl of cell suspension containing the appropriate number of cells and adequate 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. During this period do not agitate or move the plate.
7. Wash wells three times with PBS-0.1% Tween 20.
8. Distribute 100µl of PBS-0.1% Tween 20 in wells and let sit for 10 min at room temperature.
9. Wash again three times with PBS-0.1% Tween 20.
10. For 1 plate dilute 100µl of reconstituted detection antibody into 10 mL of PBS containing 1% BSA. Distribute 100µl in wells, cover the plate and incubate 1 hour 30 min at 37°C and then 1 hour 30 min at room temperature.
11. Wash wells three times with PBS.
12. For 1 plate dilute 10µl of streptavidin-Alkaline phosphatase conjugate into 10 mL of PBS-1% BSA. Distribute 100µl of the dilution in wells. Seal the plate and incubate for 45 min at 37°C.
13. After three washes with PBS, empty wells by repeated tapping on absorbent paper. At this stage it is important to remove all residual buffer.
14. Distribute 100µl of ready-to-use BCIP/NTB buffer in wells.
15. Let the reaction go for no longer than 3-5 min at room temperature.
16. Rinse wells three times with distilled water.
17. Dry wells. Read spots the day after.
18. Store the plate at room temperature away from direct light.

