ELISPOT

Euroclone Elispot System allows evaluation of the frequency of Cytokines and Soluble Molecule producing cells.
Kits are available in 5,10,15 and 20 x 96 well plate formats, either based on PVDF or Agarose Gel Overlay Systems.

Single Color Elispot

Dual Color Elispot

Antibody Pair

Human Cytokines

ELISPOT

human IL-1β

Cat. No. :

DSH100	(5x96 wells)	Agarose Gel Overlay System
DSH100-10	(10x96 wells)	Agarose Gel Overlay System
DSH100-15	(15x96 wells)	Agarose Gel Overlay System
DSH100-20	(20x96 wells)	Agarose Gel Overlay System
DSH101	(5x96 wells)	PVDF System
DSH101-10	(10x96 wells)	PVDF System
DSH101-15	(15x96 wells)	PVDF System
DSH101-20	(20x96 wells)	PVDF System

human IL-2

Cat. No. :

DSH000	(5x96 wells)	Agarose Gel Overlay System
DSH000-10	(10x96 wells)	Agarose Gel Overlay System
DSH000-15	(15x96 wells)	Agarose Gel Overlay System
DSH000-20	(20x96 wells)	Agarose Gel Overlay System
DSH001	(5x96 wells)	PVDF System
DSH001-10	(10x96 wells)	PVDF System
DSH001-15	(15x96 wells)	PVDF System
DSH001-20	(20x96 wells)	PVDF System

human IL-4

Cat. No. :

DSH010	(5x96 wells)	Agarose Gel Overlay System
DSH010-10	(10x96 wells)	Agarose Gel Overlay System
DSH010-15	(15x96 wells)	Agarose Gel Overlay System
DSH010-20	(20x96 wells)	Agarose Gel Overlay System
DSH011	(5x96 wells)	PVDF System
DSH011-10	(10x96 wells)	PVDF System
DSH011-15	(15x96 wells)	PVDF System
DSH011-20	(20x96 wells)	PVDF System

human IL-5

Cat. No. :

DSH010	(5x96 wells)	Agarose Gel Overlay System
DSH010-10	(10x96 wells)	Agarose Gel Overlay System
DSH010-15	(15x96 wells)	Agarose Gel Overlay System
DSH010-20	(20x96 wells)	Agarose Gel Overlay System
DSH011	(5x96 wells)	PVDF System
DSH011-10	(10x96 wells)	PVDF System
DSH011-15	(15x96 wells)	PVDF System
DSH011-20	(20x96 wells)	PVDF System

human IL-6

Cat. No. :

DSH020	(5x96 wells)	Agarose Gel Overlay System
DSH020-10	(10x96 wells)	Agarose Gel Overlay System
DSH020-15	(15x96 wells)	Agarose Gel Overlay System
DSH020-20	(20x96 wells)	Agarose Gel Overlay System
DSH021	(5x96 wells)	PVDF System
DSH021-10	(10x96 wells)	PVDF System
DSH021-15	(15x96 wells)	PVDF System
DSH021-20	(20x96 wells)	PVDF System

Agarose Gel Overlay Elispot Kit

contents

- Capture and Detection Antibodies
- Alkaline Phosphatase Streptavidin
- Low-Temperature Gelling Agarose
- Stabilizers 1 and 2
- BSA
- 10x PBS; 200x Wash Buffer
- Adhesive Coverslips

PVDF Elispot Kit

contents

- PVDF-backed 96 wells plate
- Capture and Detection Antibodies
- Alkaline Phosphatase Streptavidin
- BSA
- Skimmed Dry Milk
- BCIP/NTB Substrate Buffer
- 10x PBS; 200x Wash Buffer

"WITHOUT PLATES OPTION"

All PVDF-designed Elispot Kits are available without plates for those reasearchers who want to use their own 96 microwell system.

human IL-10

Cat. No. :

DSH030	(5x96 wells) Agarose Gel Overlay System
DSH030-10	(10x96 wells) Agarose Gel Overlay System
DSH030-15	(15x96 wells) Agarose Gel Overlay System
DSH030-20	(20x96 wells) Agarose Gel Overlay System
DSH031	(5x96 wells) PVDF System
DSH031-10	(10x96 wells) PVDF System
DSH031-15	(15x96 wells) PVDF System
DSH031-20	(20x96 wells) PVDF System

human IL-12

Cat. No. :

DSH080	(5x96 wells) Agarose Gel Overlay System
DSH080-10	(10x96 wells) Agarose Gel Overlay System
DSH080-15	(15x96 wells) Agarose Gel Overlay System
DSH080-20	(20x96 wells) Agarose Gel Overlay System
DSH081	(5x96 wells) PVDF System
DSH081-10	(10x96 wells) PVDF System
DSH081-15	(15x96 wells) PVDF System
DSH081-20	(20x96 wells) PVDF System

human TNF-α

Cat. No. :

DSH040	(5x96 wells) Agarose Gel Overlay System
DSH040-10	(10x96 wells) Agarose Gel Overlay System
DSH040-15	(15x96 wells) Agarose Gel Overlay System
DSH040-20	(20x96 wells) Agarose Gel Overlay System
DSH041	(5x96 wells) PVDF System
DSH041-10	(10x96 wells) PVDF System
DSH041-15	(15x96 wells) PVDF System
DSH041-20	(20x96 wells) PVDF System

human IFN-γ

Cat. No. :

DSH050	(5x96 wells) Agarose Gel Overlay System
DSH050-10	(10x96 wells) Agarose Gel Overlay System
DSH050-15	(15x96 wells) Agarose Gel Overlay System
DSH050-20	(20x96 wells) Agarose Gel Overlay System
DSH051	(5x96 wells) PVDF System
DSH051-10	(10x96 wells) PVDF System
DSH051-15	(15x96 wells) PVDF System
DSH051-20	(20x96 wells) PVDF System

human s CD138 / SYNDECAM-1

Cat. No. :

DSH070	(5x96 wells) Agarose Gel Overlay System
DSH070-10	(10x96 wells) Agarose Gel Overlay System
DSH070-15	(15x96 wells) Agarose Gel Overlay System
DSH070-20	(20x96 wells) Agarose Gel Overlay System
DSH071	(5x96 wells) PVDF System
DSH071-10	(10x96 wells) PVDF System
DSH071-15	(15x96 wells) PVDF System
DSH071-20	(20x96 wells) PVDF System

Human Cytokines
One Plate Elispot Kit

ELISPOT

human IL-1β

Cat. No. DSH060-1 (1x96 wells) PVDF System

human IFN-γ

Cat. No. DSH040-1 (1x96 wells) PVDF System

human IL-4

Cat. No. DSH010-1 (1x96 wells) PVDF System

human IL-5

Cat. No. DSH070-1 (1x96 wells) PVDF System

human IL-6

Cat. No. DSH020-1 (1x96 wells) PVDF System

human IL-10

Cat. No. DSH030-1 (1x96 wells) PVDF System

human TNF-α

Cat. No. DSH050-1 (1x96 wells) PVDF System

PVDF Elispot Kit
contents

- PVDF-backed 96 wells plate
- Capture and Detection Antibodies
- Alkaline Phosphatase Streptavidin
- BSA
- Skimmed Dry Milk
- BCIP/NTB Substrate Buffer
- 10x PBS; 200x Wash Buffer

Human Cytokines
Dual Colors Elispot

ELISPOT

Evaluate Two Cytokines simultaneously in the same well

human IL-2 / IFN-γ

Cat. No. DSH4040 (5x96 wells) PVDF System
DSH4040-10 (10x96 wells) PVDF System
DSH4040-15 (15x96 wells) PVDF System
DSH4040-20 (20x96 wells) PVDF System

human IL-4 / IFN-γ

Cat. No. DSH060 (5x96 wells) PVDF System
DSH060-10 (10x96 wells) PVDF System
DSH060-15 (15x96 wells) PVDF System
DSH060-20 (20x96 wells) PVDF System

human IL-5 / IFN-γ

Cat. No. DSH4020 (5x96 wells) PVDF System
DSH4020-10 (10x96 wells) PVDF System
DSH4020-15 (15x96 wells) PVDF System
DSH4020-20 (20x96 wells) PVDF System

human IL-10/ IFN-γ

Cat. No. DSH070 (5x96 wells) PVDF System
DSH070-10 (10x96 wells) PVDF System
DSH070-15 (15x96 wells) PVDF System
DSH070-20 (20x96 wells) PVDF System

human IL-4 / IL-10

Cat. No. DSH4030 (5x96 wells) PVDF System
DSH4030-10 (10x96 wells) PVDF System
DSH4030-15 (15x96 wells) PVDF System
DSH4030-20 (20x96 wells) PVDF System

Dual Colors Kit
contents

- Capture and Detection Antibodies (Citokine 1)
- Alkaline Phosphatase Conjugate
- Skimmed Dry Milk
- Ready to use BCIP and NTB Substrate Buffer
- Capture and Detection Antibodies (Citokine 2)
- Peroxidase Conjugate
- BSA
- Ready to use AEC Substrate Buffer

"WITHOUT PLATES OPTION"

All PVDF-designed Elispot Kits are available without plates for those reasearchers who want to use their own 96 microwell system.

We recommend the use of an Elispot Reader for the interpretation of the Dual Colors Elispot

Human Cytokines
Matched Antibody Pairs

ELISPOT

human IL-1β

Cat. No. DSH100 (10x96 tests)

human IL-2

Cat. No. DSH900 (10x96 tests)

human IL-4

Cat. No. DSH910 (10x96 tests)

human IL-5

Cat. No. DSH110 (10x96 tests)

human IL-6

Cat. No. DSH920 (10x96 tests)

human IL-10

Cat. No. DSH930 (10x96 tests)

human IL-12

Cat. No. DSH0940 (10x96 tests)

human TNF-α

Cat. No. DSH950 (10x96 tests)

human IFN-γ

Cat. No. DSH960 (10x96 wells)

human sCD138 /SYNDECAM-1

Cat. No. DSH970 (10x96 tests)

Euroclone offers Matched Antibody Pairs to be used by those researchers which have developed their own Elispot protocol, but who wish to have the Euroclone Certified Quality. The Antibodies provided are identical to those included in the Elispot kits, especially formulated and tested for the Elispot Immunoassay .

Murine Cytokines

murine IL-2

Cat. No. :		
	DSM000	(5x96 wells) Agarose Gel Overlay System
	DSM000-10	(10x96 wells) Agarose Gel Overlay System
	DSM000-15	(15x96 wells) Agarose Gel Overlay System
	DSH000-20	(20x96 wells) Agarose Gel Overlay System
	DSM001	(5x96 wells) PVDF System
	DSM001-10	(10x96 wells) PVDF System
	DSM001-15	(15x96 wells) PVDF System
	DSM001-20	(20x96 wells) PVDF System

murine IL-4

Cat. No. :		
	DSM010	(5x96 wells) Agarose Gel Overlay System
	DSM010-10	(10x96 wells) Agarose Gel Overlay System
	DSM010-15	(15x96 wells) Agarose Gel Overlay System
	DSH010-20	(20x96 wells) Agarose Gel Overlay System
	DSM011	(5x96 wells) PVDF System
	DSM011-10	(10x96 wells) PVDF System
	DSM011-15	(15x96 wells) PVDF System
	DSM011-20	(20x96 wells) PVDF System

murine IL-10

Cat. No. :		
	DSM020	(5x96 wells) Agarose Gel Overlay System
	DSM020-10	(10x96 wells) Agarose Gel Overlay System
	DSM020-15	(15x96 wells) Agarose Gel Overlay System
	DSH020-20	(20x96 wells) Agarose Gel Overlay System
	DSM021	(5x96 wells) PVDF System
	DSM021-10	(10x96 wells) PVDF System
	DSM021-15	(15x96 wells) PVDF System
	DSM021-20	(20x96 wells) PVDF System

murine IFN-γ

Cat. No. :		
	DSM030	(5x96 wells) Agarose Gel Overlay System
	DSM030-10	(10x96 wells) Agarose Gel Overlay System
	DSM030-15	(15x96 wells) Agarose Gel Overlay System
	DSH030-20	(20x96 wells) Agarose Gel Overlay System
	DSM031	(5x96 wells) PVDF System
	DSM031-10	(10x96 wells) PVDF System
	DSM031-15	(15x96 wells) PVDF System
	DSM031-20	(20x96 wells) PVDF System

ELISPOT

Agarose Gel Overlay Elispot Kit

contents

- Capture and Detection Antibodies
- Alkaline Phosphatase Conjugate
- Low-Temperature Gelling Agarose
- Stabilizers 1 and 2
- BSA
- PBS
- Wash Buffer

PVDF Elispot Kit

contents

- PVDF-backed 96 wells plate
- Capture and Detection Antibodies
- Alkaline Phosphatase Streptavidin
- BSA
- Skimmed Dry Milk
- BCIP/NTB Substrate Buffer
- 10x PBS; 200x Wash Buffer

"WITHOUT PLATES OPTION"

All PVDF-designed Elispot Kits are available without plates for those reasearchers who want to use their own 96 microwell system.

Murine Cytokines
Matched Antibody Pairs

ELISPOT

murine IL-2

Cat. No. DSM900 (10x96 tests)

murine IL-4

Cat. No. DSM910 (10x96 tests)

murine IL-6

Cat. No. DSM920 (10x96 tests)

murine IL-10

Cat. No. DSM930 (10x96 tests)

murine IFN-γ

Cat. No. DSM950 (10x96 wells)

Euroclone offers Matched Antibody Pairs to be used by those researchers which have developed their own Elispot protocol, but who wish to have the Euroclone Certified Quality. The Antibodies provided are identical to those included in the Elispot kits, especially formulated and tested for the Elispot Immunoassay .

Rat Cytokines

rat IL-4

Cat. No. :	DSR000	(5x96 wells) Agarose Gel Overlay System
	DSR000-10	(10x96 wells) Agarose Gel Overlay System
	DSR000-15	(15x96 wells) Agarose Gel Overlay System
	DSR000-20	(20x96 wells) Agarose Gel Overlay System
	DSR001	(5x96 wells) PVDF System
	DSR001-10	(10x96 wells) PVDF System
	DSR001-15	(15x96 wells) PVDF System
	DSR001-20	(20x96 wells) PVDF System

rat IFN-γ

Cat. No. :	DSR010	(5x96 wells) Agarose Gel Overlay System
	DSR010-10	(10x96 wells) Agarose Gel Overlay System
	DSR010-15	(15x96 wells) Agarose Gel Overlay System
	DSR010-20	(20x96 wells) Agarose Gel Overlay System
	DSR011	(5x96 wells) PVDF System
	DSR011-10	(10x96 wells) PVDF System
	DSR011-15	(15x96 wells) PVDF System
	DSR011-20	(20x96 wells) PVDF System

rat TNF-α

Cat. No. :	DSR020	(5x96 wells) Agarose Gel Overlay System
	DSR020-10	(10x96 wells) Agarose Gel Overlay System
	DSR020-15	(15x96 wells) Agarose Gel Overlay System
	DSR020-20	(20x96 wells) Agarose Gel Overlay System
	DSR021	(5x96 wells) PVDF System
	DSR021-10	(10x96 wells) PVDF System
	DSR021-15	(15x96 wells) PVDF System
	DSR021-20	(20x96 wells) PVDF System

Agarose Gel Overlay Elispot Kit

contents

- Capture and Detection Antibodies
- Alkaline Phosphatase Conjugate
- Low-Temperature Gelling Agarose
- Stabilizers 1 and 2
- BSA
- PBS
- Wash Buffer

PVDF Elispot Kit

contents

- PVDF-backed 96 wells plate
- Capture and Detection Antibodies
- Alkaline Phosphatase Streptavidin
- BSA
- Skimmed Dry Milk
- BCIP/NTB Substrate Buffer
- 10x PBS; 200x Wash Buffer

ELISPOT

Matched Antibody Pairs

rat IL-4

Cat. No. DSR910 (10x96 tests)

rat IFN-γ

Cat. No. DSR920 (10x96 tests)

rat TNF-α

Cat. No. DSR930 (10x96 tests)

"WITHOUT PLATES OPTION"

All PVDF-designed Elispot Kits are available without plates for those reasearchers who want to use their own 96 microwell system.

Technical Notes

Elispot Immunoassay

Introduction

The Elispot assay for detecting individual cytokine-secreting cells is based on the use of highly specific monoclonal and polyclonal antibodies to cytokines and soluble molecules. The procedure is based on the Elispot assay first described to detect individual B cells secreting immunoglobulins. Since that time, various modifications and applications of this technique have been described. The Elispot technique can be utilized in almost all systems where ELISA measurements can be made, although the information obtained from Elispot assay differs from that obtained in ELISA. First the elispot is 20-to200-fold more sensitive than ELISA in that cells secreting a particular cytokine protein cannot be measured in cell-free-supernantants. Moreover, this technique gives additional information about the frequency of cytokine-secreting cell populations that is not obtainable from conventional ELISA or bioassays. When used in parallel with ELISA, the Elispot technique can provide an estimate of the amount of cytokine protein produced on a per-cell basis. When coupled with cell separation techniques, the frequency of leucocyte subpopulations secreting a given cytokine can also be determined. The enumeration of cytokine producing cells by Elispot provides a unique method for measuring the effector response of a cell population to a specific antigen. Although this has been extensively and most successfully used for T cell subpopulation obtained from peripheral blood mononuclear cells (PBMCs); it has also been applied to purified monocyte and granulocyte populations. Using cloned cell lines stably transfected with specific cytokines genes, this technique has an efficiency that reaches 100%, i.e. every plated cells can be detected. Nevertheless, it is likely that, even when maximally stimulated, some cells purified from peripheral blood, spleen, tonsils or lymph nodes may produce cytokines in concentrations too low to be detected by Elispot. This Technique has been proved useful in determining the frequency of cytokine-producing cells in normal and disease state. These frequencies are strictly dependent upon the cytokine being studied, the cell subpopulation being analyzed, and the presence of the absence of exogenous stimuli.

Critical Parameters

The major factor in successful frequency analysis for cytokines using Elispot is the availability of high-affinity purified anti-cytokines antibodies that are directed against different epitopes on the cytokine molecule but especially tested for their properties to work on solid phases. Nevertheless, because many cytokines are not constitutively expressed, it is important to determine the optimal type, strength, and duration of the stimulus to be applied before measuring cytokine release. With every set of stimuli, it is very important that a set of unstimulated cultures are used as control to identify the spontaneous level of cytokine production.

When few or no Elispots are detected among cells that have been appropriately stimulated, as verified by other technique, the problem usually lies in the selection of the anti-cytokine antibodies, and most commonly the coating antibody. To avoid this major problem it is extremely important that antibodies that have been specifically selected to be used within this technique are used. One useful approach to solving technical problems with cytokine Elispot assay is to always include positive control cells on each plate, i.e. cells that constitutively produce the cytokine under investigation.

Two types of background problems can be seen in an elispot assay. The first is a diffuse darkening and lack of clear spots appearance. This is most commonly observed when large numbers of cytokines-secreting cells per well are studied, and reflects the accumulation of secreted cytokine in the culture supernantant with a subsequent bind or diffusion within the assay solid phase (either membrane or agarose gel). The diffuse background can be eliminated either by titrating the number of cells studied or by reducing the time of cells incubation prior to assay.

The second type of background is the appearance of very small and dark spots that do not reflect secreted cytokine. User should be trained to discriminate between real elispot and these artifacts; real spots have dark center and light outer edges formed by the diffusion of the cytokine outward from the secreting cell. False spots are smaller and not uniform in their intensity. This spots sometimes, appear when antibodies used in the assay tend to aggregate, but more frequently when cells are not adequately removed from plates during washes. Some cells have endogenous phosphatase activity, or aspecifically bind biotin-labeled antibodies, leading to the formation of these artifacts. This problem can generally be prevented by washing the plates at least once in distilled water.

ELISPOT Protocol: General Procedure

Coating-Blocking

- Coat plate with 100 ∝l/well capture antibody reconstituted with PBS and incubate 1 hr at 37°C or overnight at 4°C.
- Discard coating antibody and wash wells three times with washing buffer. Add 200 ∝l/well Blocking Buffer (2% BSA in PBS) and incubate 1 hr at 37°C. Dry wells by flicking the plate over the sink and pat bottom of the plate with dry absorbent paper.

At this stage the plate is ready for use and can be stored at 4°C in a sealed pouch with a drying bag for several weeks.

Preparation of effector cells/target cells

- Purify immune cells of interest. Wash cells in complete tissue culture medium (TCM), count and resuspend them at a final concentration of $2x10^6$ cells/ml TCM .
- Prepare target cells by washing 2 times with TCM. Pulse with antigen/peptide at appropriate concentration if necessary, and irradiate. Resuspend target cells in TCM at defined concentration.

Plate out assay

- Add 100 ∝l/well RPMI -10% FCS (or any other complete medium appropriate for a given cell type) and incubate 10 min at room temperature (RT). Discard liquid.
- Serially dilute and immediately after plate out purified immune cells in 50 ∝l/well TCM. Allow cells to settle down on plate for 1 hr at 37°C. Carefully add target cells in 50 ∝l/well TCM.

Alternatively, gently plate immune cells suspension containing mitogens or other stimuli in a final volume of 100 ∝l/well TCM. Always maintain unstimulated cells-containing wells (medium alone) to be used to define background cytokine production and in vivo activation.

- Cover plate with conventional tissue culture microtiter plates plastic lids and incubate at 37°C, 5% CO2, for the appropriate length of time. Do not move or shake plates during the incubation.

Discard cells

- Flick plate over the sink to remove all medium, place on an ice bath and fill wells with 200 ∝l ice cold distilled water. Let the plate sit for 15 min. Proceed with extensive washes with washing buffer (~10 times).

Detection spot-forming cells

- Add 100 ∝l/well biotinylated detection antibody reconstituted at the appropriate concentration in PBS containing 1% BSA. Seal the plate and incubate for at least 2 hours at 37°C.
- Discard the antibody and wash plate 3-6 times with washing buffer. Pat plate dry.
- Add 100 ul of Streptavidin-alkaline phosphatase/ well, appropriately diluted in PBS-1%BSA. Incubate for 1 hr at 37°C.
- Discard Streptavidin and wash 3 times with washing buffer. Remove all residual buffer by repeated tapping on absorbent paper.
- Dispense 100 ∝l/well BCIP/NTB substrate solution and incubate 5-30 minutes at RT until color (blue spots) develops. Appropriately discard and dispose BCIP/NTB as potentially carcinogenic solution.
- Wash troughly with distilled water and air dry. Store the plate upside down so no left liquid will go back to the membrane. Read and quantitate the spots under a dissecting microscope, once dried. Note that the spot may become sharper after one night incubation at 4°C. Plate can be further stored in the dark at RT.

Depending on the method used, proceed considering specific notes about the selected protocol: either PVDF or Agarose Gel Overlay Systems.

- Notes on PVDF System

All Euroclone Elispot-PVDF kits include PVDF-bottomed 96 wells plate, for those who want to develop their own assay using matched antibody pairs we recommend the use of Millipore Multi-screen plates (cat.# MAIPN4510).
Incubate PVDF-bottomed well plates with 100 µl/well 70% ethanol for 10 min at RT, empty wells and wash three times with PBS prior to proceed with coating step.
During incubation steps, reagents are leaking through the PVDF membrane by capillary action. If the liquid is not properly removed during washes, this might increase the background signal. To avoid this, it is strongly recommended to peel off the membrane after incubation with detection antibody, and to wash the membrane reverse side. After removing plate bottom, we suggest to put the plate on top of an empty 96-well plate to be used as a tray for liquid collection.

- Notes on Agarose Gel Overlay System

For optimal coating efficiency we recommend the use of high binding Corning plates (cat. # 9018).
During the last 20 minutes of Streptavidin-AP incubation time start making the Substrate Solution as follows: in a glass beaker, weight 42 mg agarose; add 5 ml distilled water and dissolve the agarose on a heating plate or in a boiling waterbath. Make sure that the agarose is completely dissolved by gentle swirling. The solution is then cooled down and kept to 42-43°C in a waterbath.
Following Streptavidin-AP incubation, wash the plate and put it on ice.
Prewarm 1 ml of stabilizer solution R1 and 1 ml stabilizer solution R2 to 42-43°C and add them sequentially to the gel solution. Just before dispensing agarose into the wells add the BCIP previously warmed at RT. Gently mix and immediately dispense 50 µl gel into each well. Make sure that no air bubble are trapped between plastic and overlay while filling wells with the agarose/substrate.
Let the agarose solidify at RT for 15-20 min without moving or shaking the plate. Tightly seal the plate, wrap with aluminium foil and incubate 2-4 hrs at 37°C, subsequently move to 4°C. If spots are rapidly appearing, put the plate at 4°C, this will give sharper spots. Finally proceed numerating spots under dissecting microscope.

- Helpful Hints

Effector cells should be plated as soon as they are ready. In the case of adherent cells that must be harvested prior to use, it is not necessary to let them rest before use. When using PBMCs, always perform a RBCs lysis step. Lysis of red blood cells might not be required when using murine spleen cells. Typically, 10^6 cells/well is a good starting concentration, especially for cells that have never been tested before, test at least two-to-four-fold additional dilutions. Run each condition at least in triplicate to ensure well-to-well consistency. If available, a cytokine-secreting cell line can be plated as positive control.
Cells can be stimulated directly in the antibody coated wells (direct Elispot) or, first stimulated and then harvested and plated into the coated wells (indirect Elispot): the method should be chosen on the basis of the cell type and the expected frequency. When a low number of cytokine-producing cells are expected, it is advised to test them with the direct method, when the number expected is particularly high the indirect method is suggested. If there is concern about the "de-novo" cytokine production as opposed to the release of preformed cytokine, a protein inhibitor such as cycloheximide (100 ∝g/ml) can be used as a control. The length of incubation depends on the cell type and the nature of applied stimulus. Each cytokine has its own specific kinetic, therefore appropriate conditions must be experimentally determined performing kinetic assay.

Removing the cells prior to detect the spots, is the most critical step. If the cells are left behind because of inappropriate washing, a high background will result. Using distilled water in the first wash might increase the efficiency as its hypotonicity will lyse all the cells.

Although spots can develop in a few minutes, they become more distinct after 24 hr and do not fade over time, Spots can be counted using 10X or 30X magnification any time after they have developed. Quantitation can be made more sophisticated by image analysis using a video camera technology or a simple photograph of each well.

Dual Color ELISPOT Protocol

Principle of the Method

The Dual color Elispot allows the monitoring of the production of two cytokines simultaneously in the same well. After cell stimulation, locally produced cytokines are captured by two different specific coating monoclonal antibodies. After cell lysis, trapped cytokines molecules are revealed by a secondary anti cytokine antibody FITC-.conjugated and a biotinylated one. Those are in turn revealed by anti FITC-HRP and streptavidin-AP conjugates. PVDF-bottomed well plates are then incubated first with AEC substrate, washed and subsequently incubate with BCIP/NTB substrate. Colored red/brownish and blue/purple spots will reveal production of the first and the second cytokine respectively.

Dual Color Elispot Procedure

- Incubate PVDF-bottomed plates with 100 µl/well 70% ethanol for 10 min at room temperature

- Empty wells and wash 3 times with 100 µl/wel PBS

- Pipette 100 µl/wel appropriately diluted capture antibody A and B mix
- Cover and incubate over night (ON) at 4°C

- Empty wells and wash once with 100 µl/wel PBS

- Dispense 100 µl/wel 2% skimmed milk in PBS
- Cover and incubate for 2 hrs at room temperature

- Empty the wells and pat dry on absorbent paper and wash once with 100 µl/wel PBS

- Dispense 100 µl/wel cell suspension containing the appropriate number of effector cells and stimulators
- Cover plate with a standard 96-well plate plastic lid and incubate at 37°C. 5%CO2 for the appropriate length of time to be experimentally determined. Do not move or shake the plate during this incubation time.
- Empty wells and pat dry on absorbent paper

- Dispense 100 µl/well 0.1%tween20 in PBS and incubate 10 min at 4°C
- Wash three times with 0.1%tween20 in PBS

- Dispense 100 µl/wel of premixed and appropriately diluted anti-FITC-HRP and streptavidin-AP
- Seal the plate and incubate 1 hr at 37°C
- Empty wells and wash three times with 0.1%tween20 in PBS. Remove all residual buffer by repeated tapping on absorbent paper

- Dispense 100 µl/wel AEC buffer, let the color reaction proceed for 5-15 min at room temperature. When the spots have developed empty the plate and discard the substrate solution appropriately as potentially carcinogenic
- Wash thoroughly both sides of the PVDF membrane with distilled water. Remove all residual water by repeated tapping on absorbent paper

- Dispense 100 µl/well BCIP/NTB buffer, let the color reaction proceed for 5-15 min at Room temperature. When the spots have developed empty the plate and discard the substrate solution appropriately as potentially carcinogenic
- Wash thoroughly both sides of the PVDF membrane with distilled water. Remove all residual water by repeated tapping on absorbent paper
- Dry the membrane by repeatedly tapping the plate on absorbent paper. Store the plate upside down in order to prevent any residual liquid going back on the membrane. Enumerate the spots once dried. Note that spots may become sharper after ON incubation at 45°C.
- Store plates at Room temperature away from direct light

ELISPOT References

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