



ANIARA

Manufactured By: U-CyTech Biosciences



Do You Like Spots?

Form AU02
4-2007

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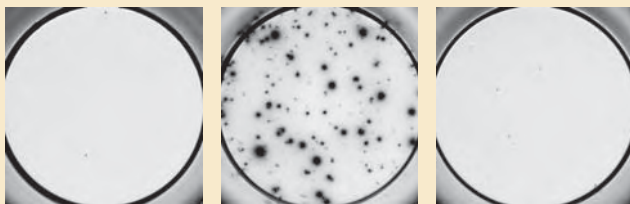


What is a cytokine ELISPOT assay?

The cytokine enzyme-linked immunospot (ELISPOT) assay is a unique method for the *ex-vivo* quantification of cytokine secreting T cells after stimulation with an antigen *in vitro*.

An ELISPOT assay is very similar to an ELISA and is based on the same immunochemical 'sandwich' principle. The major difference is that an ELISPOT is a combination of both an immunoassay and bioassay because living cells are cultured directly in the ELISPOT plate.

Example of human IFN- γ ELISPOT results:



2×10^5 human PBMC/well

Left: no stimulus; *Leishmania* patient

Middle: 0.1 $\mu\text{g/ml}$ L. major antigen; *Leishmania* patient

Right: 0.1 $\mu\text{g/ml}$ L. major antigen; healthy individual

Why an ELISPOT assay?

ELISPOT assays are among the most-sensitive methods available for cytokine research allowing the detection of a single cell out of a million.

Major advantages of the ELISPOT assay are its relatively easy performance, its potential for high throughput screening and no requirement for expensive instruments. Because of the short-term *in vitro* culture, the measured response closely mirrors the *in vivo* T cell frequency.

The ELISPOT assay has the lowest detection threshold among the 'standard' T cell assays such as the lymphoproliferation assay (LPA) and cytotoxic T lymphocyte (CTL) assay. Drawbacks of the LPA and CTL assay are the use of radioactivity, low throughput screening and technical burden.

What is the difference between an ELISPOT assay and an ELISA?

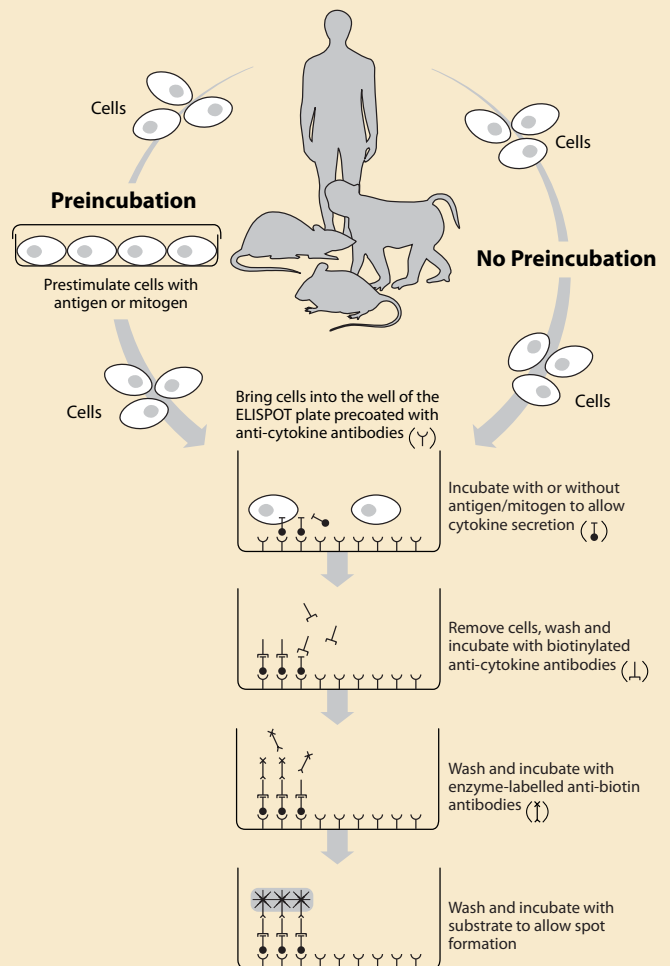
An ELISA determines the total concentration of the secreted cytokine, whereas an ELISPOT enumerates cytokine secreting cells answering the question: '*what is the frequency of secreting cells?*'. Therefore an ELISPOT should be used not 'instead of' but rather 'in addition to' an ELISA.

An ELISPOT assay can be up to 400 times more sensitive than a conventional ELISA because the cytokine is captured directly onto a solid phase before having the chance to be diluted in the culture supernatant, degraded by proteases or captured by cytokine receptors on adjacent cells.

What type of information can be obtained from an ELISPOT assay?

One key piece of information that can be obtained is the frequency of antigen-specific T cells within a pool of different cell types. This frequency reflects the clonal size of the antigen-specific T cells and, therefore, the magnitude of T cell immunity.

Flow diagram ELISPOT



In which area of research can an ELISPOT assay be used?

- vaccine development
- cancer research
- monitoring infectious diseases
- autoimmune disease studies
- allergy research
- organ transplantation research



What about spots?

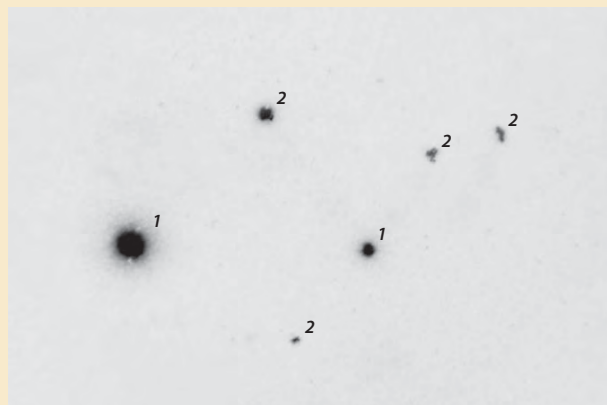
Each spot within an ELISPOT well is the 'footprint' of a single cytokine secreting cell. True spots have a dense center with a light outer ring caused by the diffusion of the cytokine from the producing cell.

The color depth and the size of spots depends on the amount of secreted cytokines. Only strong and well-defined spots should be counted; any irregular or faint spot is likely to be an artifact and should be ignored.

Artificial spots can be caused by the aggregation of antibodies used in the processes of coating and detection or by incomplete removal of cells from the plate after incubation. A simple measure to minimize false spot formation is to thoroughly wash the plate between all incubation steps and after completion of the coloring reaction.

The average size of a spot is 30-150 μm , although this varies depending on incubation time, antibody concentration and other materials used in the assay as well as the functional state of the cytokine-secreting cells.

Example of true and artifactual spots:

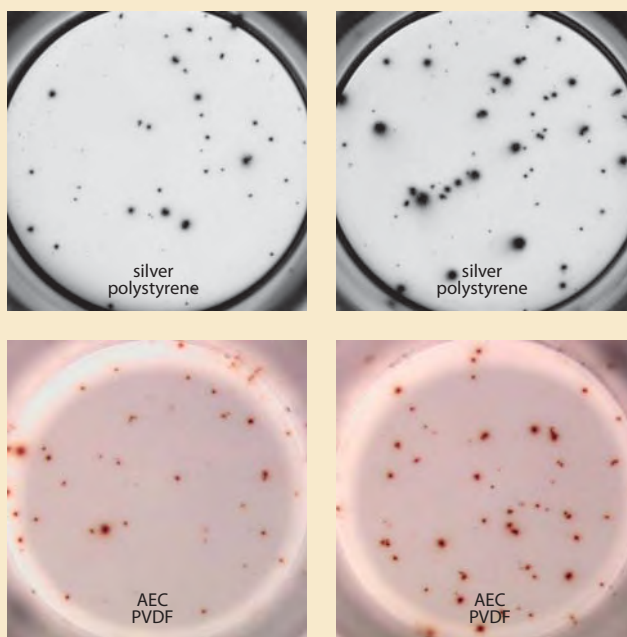


Human IFN- γ spots (magnification: 140 \times)
1 = True spots / 2 = Artifactual spots

Culture medium

For the 'direct' ELISPOT assay (no preincubation step), serum-free medium (AIM-V) has proven to be the best choice for obtaining an optimal ELISPOT response. On the other hand, the 'indirect' assay (with preincubation step) requires medium supplemented with 10% fetal calf serum (FCS) or human serum. AIM-V medium is far less effective in the 'indirect' assay. Because some batches of FCS or human serum may non-specifically activate the cells, different batches of FCS and human serum should therefore be pre-tested before using it in the ELISPOT assay.

Effect of culture medium in the direct human IFN- γ ELISPOT assay:



Left: RPMI with 10% FCS
2 \times 10⁵ human PBMC/well; stimulus: peptide pool
Right: AIM-V

Critical aspects of the ELISPOT assay

Several aspects of the ELISPOT assay are crucial for the sensitive detection of antigen-specific T cells including a cell preincubation step and the monocyte concentration.

A preincubation step at high cell density (>10⁶ cells/well/ml) is required when proteins or peptides (>15 mer) are used for re-stimulation. These antigens must first be internalized, processed and presented by antigen-presenting cells (APC) before they can stimulate cytokine release by T cells. Omitting this step leads to a significant lower frequency of spot forming cells (SFC).

Monocytes are critical as APC and a low number of monocytes may also cause a decreased number of SFC. Particularly cryopreserved samples may be short of APCs.

Cells

The efficacy of spot formation depends for a major part on the quality of cultured cells. A high number of dead cells (30-50% and more) is a reason for high background staining and even lack of specific spots.

Cryopreserved peripheral blood mononuclear cells (PBMC) may be more active in secreting some cytokines which is thought to be caused by elimination of inhibitory platelets which do not withstand freezing.

The integrity of PBMCs is critical for success. If performed correctly, the separation process yields a pure population of mononuclear cells consisting of monocytes and lymphocytes with high viability and minimal red blood cell and platelet contamination.



Antibodies

The best antibody pairs (coating and detection) are selected by the manufacturer on basis of extensive research and should be used as a matched antibody pair for the ELISPOT.

One of the most important parameters to standardize the assay is the total amount of antibody used for coating. A general guideline is that approx 0.5-1 µg coating antibody per well result in well-defined spots. Coating concentrations that are too low result in fainter but also in fewer detectable spots.

Spot detection system

Horseshradish peroxidase (HRP), alkaline phosphatase (AP) or gold particles can be used as streptavidin conjugates. The advantage of using HRP is its fast turnover rate (spots develop fast), whereas the drawback is increased background. The HRP substrate AEC (3-amino-9-ethylcarbazole) forms intense red colored spots. However, AEC is unstable and spots will bleach in a short period of time.

AP has a linear reaction rate with little risk of high background. However spot development is slow and bleaching of spots is a problem.

A simple and effective way of spot staining can be achieved with gold-labeled streptavidin using silver precipitation for spot visualization. Because of the high stability of silver, spots do not fade and ELISPOT plates can be reanalyzed after being stored for several years at room temperature.

Washing

Washing of ELISPOT plates is critical and necessary to remove cells, residual biotinylated antibody and staining reagents. Washing can be performed in several ways: manually (multi-channel pipet or squirt bottle) or (semi)automatically using a multichannel washer or an ELISA microplate autowasher.

Since damaging of the membrane is a serious risk and the fact that both sides of the membrane need to be washed, a squirt bottle is the best choice for effective washing of membrane-bottomed plates. On the other hand, an automatic plate washer is highly convenient and effective for polystyrene-bottomed plates, but may cause problems in case the probes are placed too far or too close to the bottom of the wells generating a poor wash or well-washed concentric areas in the center of the well. Automated washers also require consistent decontamination and cleaning procedures, otherwise accumulation of debris in the probes will create high and irregular background staining and slow flow.

Plates

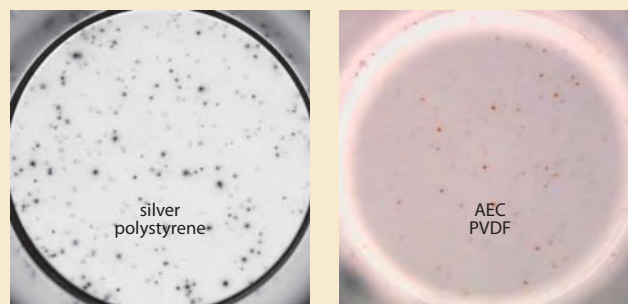
ELISPOT assays can be performed using either 96-well polystyrene- or PVDF membrane-bottomed plates.

Although PVDF has much higher protein retaining capacity than polystyrene, it is only the top layer of the membrane that is involved in protein binding implicating that both plates have a similar surface area for antibody-cell contact. On the other hand, there are only a very few polystyrene-bottomed plates with a comparable antibody binding activity as PVDF membranes.

A disadvantage of using PVDF membrane-bottomed plates is that for optimal binding of the coating antibody a prewetting step with 70% ethanol is required. This prewetting step creates the risk of liquid collecting under the membrane. This liquid cannot be washed out effectively and may create serious problems later in the ELISPOT assay such as high and irregular background staining. Polystyrene plates do not have such problems.

Because the polystyrene plates are far more cost-effective than PVDF plates and the fact that polystyrene plates in combination with silver staining show a similar or even higher sensitivity, it might be desirable to switch from PVDF to polystyrene plates.

Differences in sensitivity of two spot detection systems:



*α-CD3-triggered IL-10 spot formation
(5×10⁴ human PBMC/well)*

