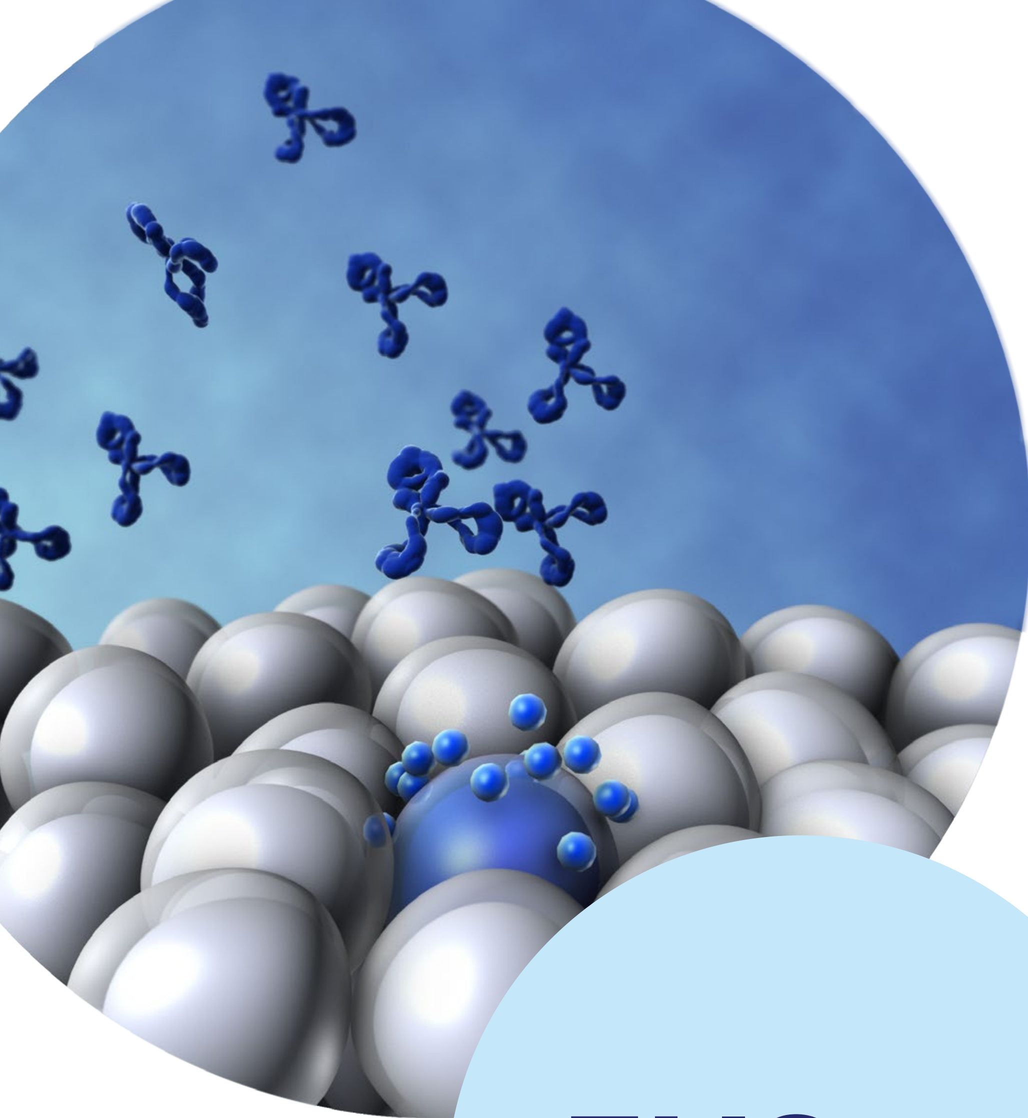


ELISpot

MABTECH

The Enzyme-Linked ImmunoSpot (ELISpot) assay is a very sensitive immunoassay, which allows for the detection of secreted analyte at the single cell level[1,2,3] (see Fig. 1).

With detection levels as low as one cell in 100,000, the ELISpot is one of the most sensitive cellular assays available. Depending on the cytokine/factor analyzed, it is between 20 and 200 times more sensitive than a conventional ELISA. In fact, the ELISpot displays similar sensitivity to RT-PCR analysis, but detects secreted protein instead of mRNA. For cytokine analyses this is advantageous, since many cytokines are translationally regulated. ELISpot analyses are also less impaired by binding proteins and protease activity, since the analyte is bound to the capture antibody immediately after secretion. Due to its high sensitivity, the ELISpot has proven particularly useful when studying small populations of active cells such as those regularly found in specific immune responses.

Applications

The cytokine ELISpot assay has been widely applied to investigate specific immune responses in infections, cancer, allergies and autoimmune diseases. It has also become a standard tool in the development and monitoring of new vaccines and vaccine candidates. Depending on the cytokines analyzed, the ELISpot assay can be used to discriminate between different subsets of activated T cells. For example, T-helper (Th) 1 cells are characterized by their production of IFN-γ, IL-2 and TNF-α, whereas other cytokines like IL-4, IL-5 and IL-13 are typically produced by Th2 cells. The ELISpot assay is suitable for performing individual tests as well as large-scale trials. In contrast to tetramer analysis (an excellent method for extracting detailed information on CTL responses), screening of large sample numbers by ELISpot does not require prior identification of epitopes and their MHC class-I restriction. With a limited need for advanced

1) Cytokine-specific monoclonal antibodies (mAb) are immobilized on a 96-well ELISpot plate.
2) Cells are added in the presence or absence of specific stimulus.
3) During incubation, cells become activated by the stimulus and start to produce and secrete cytokine that binds to the capture mAb.
4) Cells are removed and a detection mAb, which may be directly conjugated with enzyme or biotinylated, is added.
5) If using biotinylated detection mAb, a Streptavidin-enzyme conjugate is added.
6) Finally, a substrate will form a colored spot at the location of the secreting cell.
7) By counting the number of spots in stimulated cultures and controls without stimulus, the frequency of responding cells is determined.

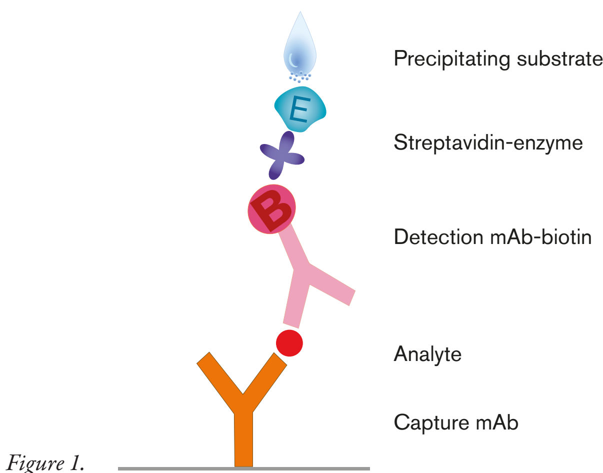


Figure 1.

laboratory equipment, it has also proven to be well suited for field studies. Diagnostic tests based on the ELISpot platform are now available. These include an approved test which detects patients with active or latent tuberculosis infection by measuring IFN-γ secretion from T cells responding to defined antigens from *Mycobacterium tuberculosis*. It replaces the commonly used skin test and adds both greater sensitivity and specificity and is, unlike the skin test, not confounded by prior vaccination with BCG[4-5]. Finally, the ELISpot technique may be applied to a variety of species. In addition to its use with human, monkey, mouse and rat cells, reagents are available for veterinary applications, enabling assessment of T-cell responses in samples of bovine, ovine, equine and porcine origin.

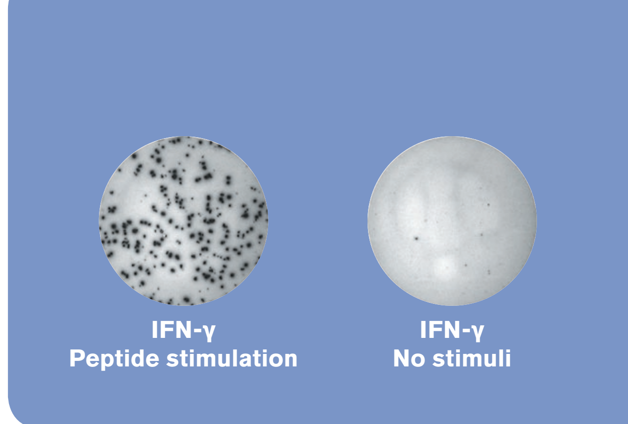


Figure 2. Human IFN-γ ELISpot analysis of peptide-reactive T cells. $CD8^+$ T cells (200,000 cells/well) were stimulated with a pool of virus-derived MHC class I-restricted peptides[6] (available from Mabtech as "CEF peptide pool") for 16 hours. Cells cultured in medium alone (no stimuli) are shown as a negative control.

Vaccine development

Interferon-gamma (IFN-γ) has often been the analyte of choice serving as an immunocorrelate for $CD8^+$ cytotoxic T-cell (CTL) responses (see Fig. 2). Today, the IFN-γ ELISpot is used in many large-scale trials to monitor new HIV vaccine candidates as well as in the evaluation of vaccines and vaccine strategies for other pathogens and also tumors. The ELISpot assay is used to define vaccine efficacy by measuring the capacity to elicit potent T-cell responses. This information can then be used to evaluate suitable strategies for vaccine design as well as routes of administration. In addition to measurements of IFN-γ, ELISpot assays for other key factors in cytotoxicity, such as Perforin and Granzyme B, have been developed to facilitate analysis of further aspects of CTL activity[7].

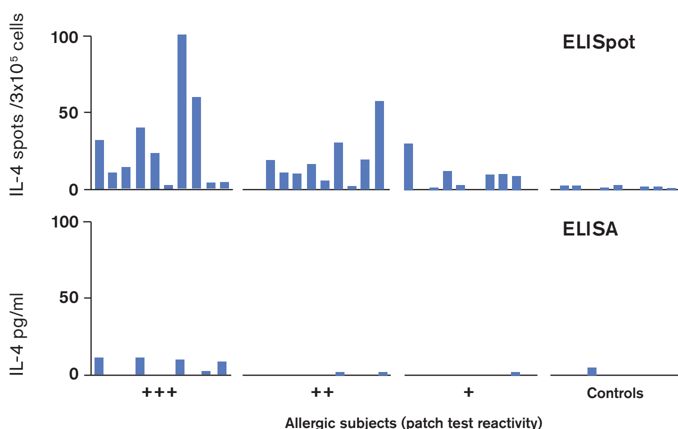


Figure 3. PBMC from subjects with or without contact allergy to nickel were tested for in vitro reactivity to nickel. The subjects displayed +++ (n=10), ++ (n=11), + (n=10) or no (controls; n=10) patch test reactivity to nickel. PBMC were incubated with or without 50 µM $NiCl_2$ for 40 h and IL-4 responses were assessed in ELISpot and in ELISA (using cell culture supernatants). Detectable responses to nickel, above cut-off levels, were seen in 23 of the 31 allergic individuals when tested with ELISpot whereas only 4 were defined as positive in the ELISA.

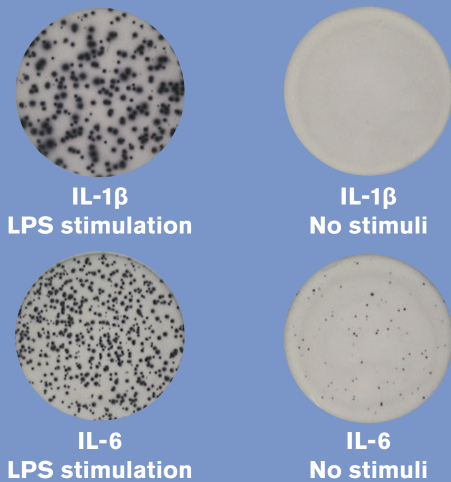


Figure 4. ELISpot analysis of LPS-induced IL-1β and IL-6 secretion by purified human PBMC. Cells (5,000 cells/well) were incubated for 20 hours in the presence or absence of LPS (100 ng/ml).

ELISpot in allergy

Determination of Th2 type cytokines by ELISpot is of particular interest in allergy research, where Th2 responses are of major importance but most often occur at low frequencies, requiring highly sensitive methods for successful detection. Applications of relevance include monitoring of T-cell reactivity in specific immunotherapy against atopic allergies and development of potential *in vitro* diagnostic tests for cell-mediated contact allergies[8]. ELISpot detection of IL-4, the principal cytokine of Th2 type responses, is especially interesting since other methods such as flow cytometry and ELISA often display insufficient sensitivity, resulting in a failure to detect allergen-specific responses[9] (see Fig. 3).

Analysis of other cell types

Initially the ELISpot assay was used for enumeration of specific antibody-secreting cells and in the last few years there has been a revival for the B-cell ELISpot as a powerful tool to detect B-cell responses to natural infections and those elicited by vaccination.

Thus, while excellent for evaluating specific immune responses by both B and T cells, the ELISpot technique can theoretically be applied to any system where one wants to investigate protein secretion at the single cell level. The method is especially attractive whenever enumeration of the producing cells is essential or of biological interest, as exemplified in Figure 4 with the quantification of IL-1β and IL-6 secreting monocytes in lipopolysaccharide (LPS)-stimulated PBMC. It is also ideal in situations where the producing cells constitute a small minority of the cells, like plasmacytoid dendritic cells, which occur at very low frequency in the blood but are easily detected in the ELISpot based on their production of IFN-α.

Technical aspects

Optimization of the protocol

Several steps of the ELISpot assay protocol are critical in order to achieve optimal detection of spots. The choice of highly specific antibodies is of utmost importance and sets the basis for other aspects of optimization. Other crucial parameters include the choice of ELISpot plates and the coating procedure. The use of PVDF membrane plates enables efficient binding of high amounts of capture antibody, provided that the PVDF membrane is treated with ethanol (EtOH) prior to coating. Empirically, different types of plates have been found to react differently to the EtOH treatment and it is critical to use the appropriate protocol (see Table 1).

After proper EtOH treatment, the spots obtained will be of significantly better quality and spot numbers may often be higher compared to what is seen in non-EtOH treated plates. It is important to note that over-treatment with larger volumes of EtOH or longer treatment times than recommended can reduce the performance of the assay (see Fig. 5).

For adsorption of the capture antibody, we generally recommend the use of 15 µg/ml of capture antibody (100 µl/well), based

on the fact that spot quality declines as the capture antibody is further diluted. Although ELISpot protocols using lower capture antibody concentrations can be used following proper validation, the spots obtained tend to become more diffuse and less focused making the evaluation more difficult. In many cytokine ELISpot assays, the use of less capture antibody may also result in a significant loss of detectable spots. These aspects need to be taken into account when designing and validating new assay protocols, especially when monitoring cell populations in which the frequency of producing cells or the amount of produced cytokine is low.

For the final detection of spots a precipitating substrate is used. This will vary depending on which enzyme (ALP or HRP) is used in the previous step. As different substrates not only produce different colors (see Fig. 7) but may also yield different sensitivities, the choice of substrate is not trivial. We normally recommend BCIP/NBT for ALP and TMB for HRP, as both show excellent sensitivity and give distinct spots that are easy to evaluate.

Precoated plates and one-step detection reagents (ELISpot^{PRO}) represent further streamlining that help to facilitate experimental and clinical studies as well as diagnostic applications based on ELISpot.

Positive controls

When measuring antigen-specific T-cell responses in ELISpot, relevant controls are crucial. In addition to the negative control, which normally consists of cells in medium without antigen or other stimuli, the inclusion of a polyclonal T-cell activator can serve as a positive control both for cell viability and the functionality of the immunoassay.

For polyclonal activation, certain antibodies specific for CD3 are ideally used. A multitude of T-cell cytokines including IFN-γ, IL-2, IL-4, IL-5, IL-10 and IL-13 are elicited by this stimu-

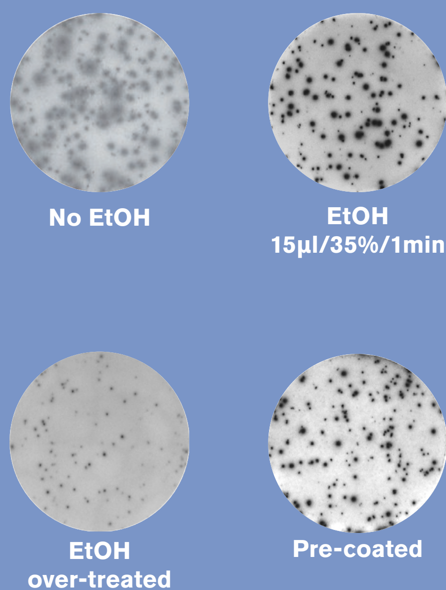


Figure 5. Influence of ethanol (EtOH) treatment prior to the coating step when using MSIP plates. A pre-coated plate from Mabtech is shown for comparison. Human PBMC were stimulated with CEF peptide pool and assessed for IFN-γ production (250,000 cells/well).

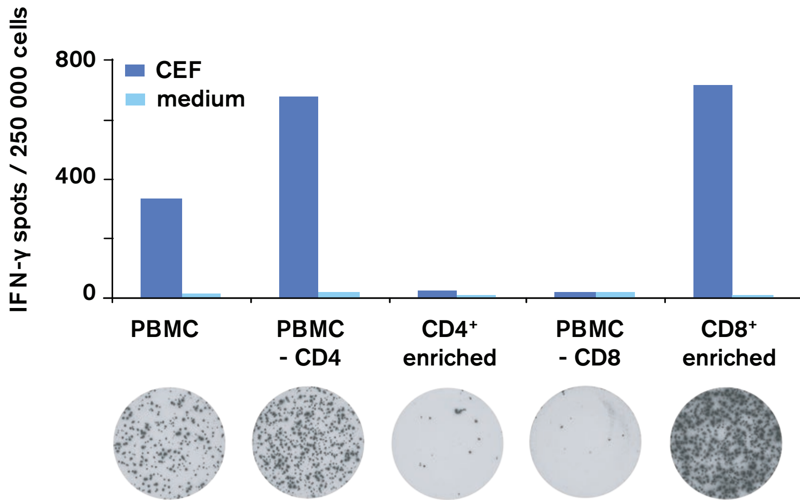
Recommended EtOH treatment of PVDF plates

Plate types (Millipore) MSIPS4510 and MSIPS4W10
Add 15 µl of 35% EtOH per well and incubate ≤ 1 min.

Plate type (Millipore) MAIPSWU10
Add 50 µl of 70% EtOH per well and incubate ≤ 2 min.

The EtOH treatment is followed by washing with sterile water before addition of capture antibody.

Table 1.



lation, and this control can thus be used in a variety of ELISpot assays. Other commonly used stimulators include phytohemagglutinin (PHA) and Concanavalin A (ConA), which are both potent T-cell activators and can be used in a similar way as anti-CD3 antibodies for activation of $CD4^+$ and $CD8^+$ T cells.

Another useful positive control is based on the addition of antigens to which most people have specific T cells. As IFN-γ production by antigen-specific human $CD8^+$ T cells is a major area of focus for ELISpot, a positive antigen-specific control, the CEF peptide pool, has been developed particularly for this application. This pool contains 23 different class-I restricted peptides; all defined as common $CD8^+$ T-cell epitopes derived from cytomegalovirus, Epstein-Barr virus and influenza virus[7]. It efficiently induces IFN-γ production by virus-specific $CD8^+$ T cells in almost 90% of Caucasians and also elicits Perforin, Granzyme B and MIP-1β responses in many individuals. In addition to serving as a positive control, the CEF peptide pool is useful for standardization procedures (see Fig. 6).

Figure 6. Positive control in ELISpot. Human PBMC were depleted or enriched for either $CD4^+$ or $CD8^+$ T cells. Cells (250,000/well) were stimulated using CEF peptide pool available from Mabtech.

Detection of multiple protein secretion

The growing interest in sensitive cellular assays for the identification and enumeration of cells secreting multiple cytokines resulted in the development of dual color ELISpot. Based on the parallel use of two detection systems utilizing different enzymes and

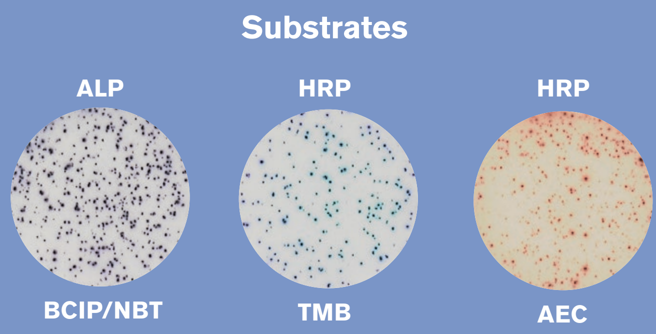


Figure 7. Common substrates in ELISpot. Different enzymes (ALP or HRP) in the ELISpot can be combined with different substrates. This is illustrated by using IFN-γ ELISpot with substrate BCIP/NBT for alkaline phosphatase (ALP) and with TMB and AEC for horseradish peroxidase (HRP).

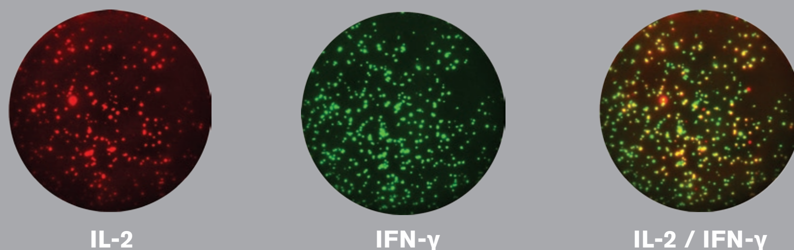


Figure 8. Staining for human IL-2 and IFN-γ. An image overlay creates yellow spots corresponding to cells secreting both cytokines.

substrates, this method has the capacity to differentiate between cells secreting either of two cytokines or both. However, due to the difficulties in analyzing enzymatic double-stained spots in an objective manner, and with a functional limitation of analyzing only two cytokines in parallel, the need for a more reliable and versatile method is evident.

This has led to the development of the FluoroSpot technique which is identical in principle to ELISpot, but based on fluorescent detection[10-11]. The assay has so far been used to simultaneously look at the secretion of two cytokines and has allowed for the analysis of different functional T cells in an antigen-specific context[12].

Plates are analyzed in a FluoroSpot/ELISpot reader equipped with separate fluorophore filters that enable easy distinction between cytokines and identification of single- and double-stained spots (see Fig 8). This is of great value when studying cellular co-expression, poly-functional T cells, and when cell numbers are limited. With separate filters for each color, we now have the ability to analyze more than two cytokines in one well.

Mabtech has worked extensively for more than 20 years to optimize the ELISpot protocol and ensure delivery of the most accurate analytical performance. If you have further questions or wish to discuss applications or optimization strategies of the ELISpot or FluoroSpot technique, please contact us directly (see back cover for contact details).

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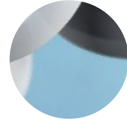

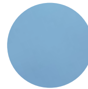

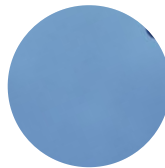

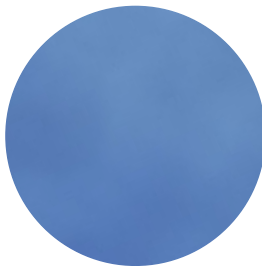

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