Recommendations for the ELISPOT Assay

Enrique Iglesias

División de Vacunas, Centro de Ingenieria Genética y Biotecnología
AP 6162, CP 10600, Ciudad de La Habana, Cuba.
E-mail: enrique.iglesias@cigb.edu.cu

ABSTRACT

The ELISPOT assay is the widest used technique for measuring T cell responses. Because is not too expensive and easy to perform it is very useful for testing samples in clinical trials. Nevertheless, it is necessary to establish standardized protocols to make feasible comparisons among results obtained in different labs. A lot of data has been accumulated these years of hard work. Then, some recommendations based on the experience might be of value for those who are starting doing ELISPOT. Following them the researcher may find the best benefice-cost relationship to get high quality results at the less delay possible.

Keywords: assay, ELISPOT, T-cell, technique

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RESUMEN

Recomendaciones para el ensayo ELISPOT. El ensayo ELISPOT es la técnica más usada para medir la respuesta de células T. Por no ser muy caro y fácil de llevar a cabo es muy útil para estudiar muestras en ensayos clínicos. Sin embargo, es necesario establecer protocolos estándares para que sea posible comparar resultados obtenidos en diferentes laboratorios. Gran cantidad de datos se ha acumulado durante estos años de duro trabajo. Por eso algunas recomendaciones basadas en la experiencia podrían ser de valor para aquellos que comienzan a hacer ELISPOT. Siguiendo estas recomendaciones el investigador encontrará la mejor relación beneficio-costo para lograr resultados de alta calidad en el menor tiempo posible.

Palabras claves: células T, ELISPOT, ensayo, técnica

*I*ntroduction

The ELISPOT (enzyme linked immunospot) assay is a 19 years old technique [1]. It was developed to assess the frequency of specific B-lymphocytes. Nowadays it is the widest used technique for measuring T cell responses allowing the quantification of specific-cytokine secreting cells. Nonetheless, results depend on scientist's experience, good working procedures, protocols, antibodies and materials.

It is a very sensitive assay being possible to detect 1 specific cell in 100 000 [2, 3]. Because of that it is feasible to do *ex vivo* ELISPOT analysis lasting no more than 24 h. Working so close to the real situation *in vivo*—in experimental conditions where the samples do not suffer many manipulations—the assay fairly reflect the immune status of the patient or animal under investigation. The ELISPOT is a functional assay allowing mainly the quantification of cytokine-secreting cells after stimulation with antigens. Several protocols have been developed allowing the use of recombinant vaccinia virus (rVV), peptides or transfected cell lines for antigen presentation in the assay. It is a very consistent test with less than 20% inter-lab variability [2]. On the other side the result using frozen or fresh cells did not differ [3, 4]. Thus, retrospective analyses are feasible which is very important in the conduction of phase II and III clinical trials where many subject are enrolled and shipping of samples to other countries is necessary.

*T*he Assay Step by Step

In principle it is an adaptation of a sandwich ELISA (Figure 1). The plates are coated with a MAb specific for a particular cytokine. After blocking, effector cells and antigen presenting cell (APC) loaded with a particular peptide or infected with a rVV for a particular antigen (a transfected cell line can be used as APC too) are incubated between 16-24 h in a CO_2 atmosphere at 37 ºC. Then cells are removed, a second biotynilated-MAb is added and afterwards streptavidine coupled to peroxidase or alkaline-phosphatase. When substrates are transformed to produce a colored precipitate spots become visible. By

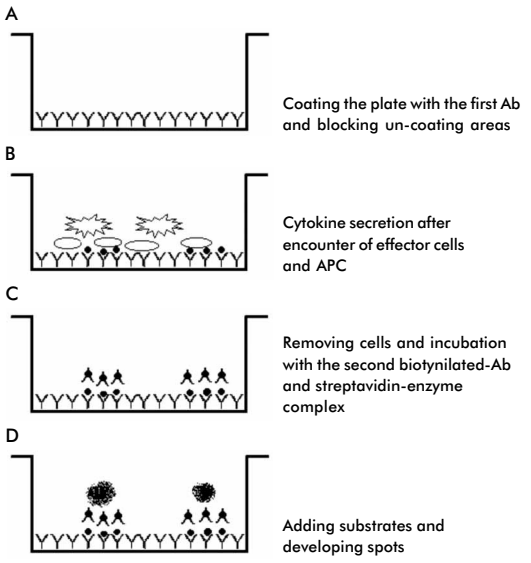

Figure 1. The ELISPOT assay step by step.

scoring spots is possible to calculate the frequency of responding cells because each one corresponds to a single secreting cell.

Coating plates

There are certified plates for ELISPOT from commercial sources. In our hands plates from Millipore are the best. These 96-well plates have a membrane at the bottom of wells. At this moment is still possible to buy plates having HATF (mixed cellulose esters) or IPVH (Immobilon-P) membranes. However, comparisons between both materials have shown that Immobilon-P behaves better than cellulose. In general, working with the last one higher backgrounds are attained and spots have a poor definition.

In addition, antibody pairs for an increasing number of cytokines for humans, monkeys and mice are available in the market. It is possible to measure the frequency of cell secreting any of them after antigen stimulation using ELISPOT. Most of the companies provide some information regarding the best condition for coating the plates for the ELISPOT application. However, it should be taken into consideration that big and fuzzy spots may indicate insufficient coating (Figure 2).

Blocking

Ten percent serum in culture medium is effective to block unspecific binding. Nevertheless, a serum from the same species of tested cells might increase the background if molecules of the particular cytokine are in it. To avoid that, use serum from another species. It is also important to heat inactivate the serum to eliminate complement activity.

Adding cells and antigens to wells

Depending on the protocol it may be necessary to add APCs. If it is the case they should be carefully prepared to avoid any interference in the assay. In particular, it is important to prevent any trace of mitomycin C if it is used because it can impair T cell responses.

In general, several protocols can be used to obtain effector cells depending on the organ source, etc. Sometimes previous manipulations are required to enrich a particular population. In any case which is important for the ELISPOT assay is to add first the effectors cells in a number not higher than 200 000 cells per well. An increased number will promote stacking of cells. In such situation not all the effectors will be in direct contact with the coating antibody. If cells are not in direct contact with the bottom of the well when they become activated their cytokine secretion will be diluted in the medium. Because of that in the worst scenario no spots will be scored and the response would be underestimated. In the best scenario big, fuzzy spots would be found. But, if too many cells (more than 500 000) are added to the well a very strong response may be archieved even in wells with effector cells alone, incubated with irrelevant peptides or without peptide. It is a consequence of the high density of cells promoting unspecific secretion of cytokines.

Developing spots

There are many substrates in the market for peroxidase and phosphatase enzymes. Experiments comparing several of them have shown their influence in the number of spots (Janetzki, S. 2002. Personal communication) (Table 1). It could be the result of the influence of many parameters such as enzyme activity, affinity for the substrate, etc. But what real matters here is: once you select a particular substrate do not change it. Our advice is to use 3-amino-9-ethylcarbazole (AEC) for peroxidase and 4-nitro-blue tetrazolium chloride salt (NTB)/5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP) for phosphatase. Avoid the use of 3, 3´-diaminobenzidine (3, 3´, 4, 4´-tetraaminobiphenyl) tetrahydrochloride (DAB) because it gives a lot of background and the poorest spot definition.

Controls in the Assay

There are some controls you should never avoid no matter the goals of the ELISPOT: 1, medium alone; 2, target cells (APCs) alone; 3, target cells presenting a non-related peptide (or infected with wild type vaccinia virus (wtVV)) + effector cells; 4, positive control. The first control is for ruling out any problem related to the blocking step and infection of the medium. Appearance of spots in the second control may indicate secretion of cytokines by the APCs, which is a real situation mainly for cells coming from the linage of immune cells. The third one is very important when working with VV because you get an idea about the specific response to the virus allowing calculating the specific response to the antigen. A non-related peptide is necessary to rule out any unspecific activation in the effectors cells or cross-reaction (in a less sense) letting to spots. Positive control using mitogens like phytohemagglutinin from *Phaseolus vulgaris* (PHA) or concanavalin A from *Canavalia ensiformis* (ConA) are commonly used. They give a rough idea about the immune-status of the cells; tell us a lot about the quality of reagents and our manipulations. Nevertheless, some researchers are proposing a panel of peptides belonging to common pathogens like influenza virus, cytomegalovirus and Epstein Barr virus to be use in a pool as positive control for ELISPOTs [5]. The rationally is based on the fact that those peptides are epitopes recognized by CD8 positive T cells and presented by 11 class I HLA-A and HLA-B alleles whose cumulative frequencies represent >100% of Caucasian individuals. So, most of the persons will score positive for the pool, and because in fact they are recall antigens, it gives us a better idea about the patient's immune status.

There are other controls you may include in an ELISPOT assay but we let the decision to yourself.

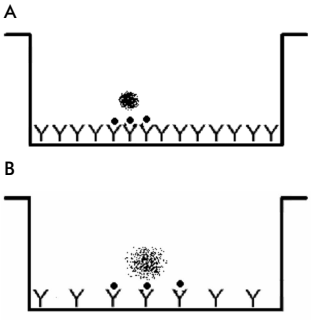

Figure 2. Influence of antibody concentrations on spots. A, good coating; B, bad coating.

Table 1. Substrate effect on the number of spots. Data are expressed as percentage taking the spot numbers for NTB/BCIP as 100%.

Substrates	Spots referred to NTB/BCIP (%)
AEC	120
TMB Green	115
TMB Blue	190
Vector Red	60
Vector Blue	50
BCIP Blue	80
BCIP Plus	160

Among them: 1, internal control of the assay, consistent in an already tested sample for which the number of spots is known for a particular antigenic stimulation; 2, effector cells alone; 3, effector cells plus APCs without antigen.

Reading Plates

Counting spots under the stereomicroscope is a very hard task. The effectiveness of the scoring relies too much on the experience of the scientist and the average number of spots per well. We know that people tend to count only quite well defined spots in those wells with many of them letting to an underestimation of the response. Because of all these problems different automatic readers have been developed. Though all of them have their own characteristics using them the human subjectivity is eliminated and the variability reduced. Unfortunately, prices are still out of the possibility of many labs. In view of that we are going to discuss a little on how to recognize real spots.

The architecture of spots is defined by the speed and the amount of secretion. Shape, size, color and color distribution help to identify them. All spots have a similar circular shape and fuzzy borders. In a single well spots having different sizes is a normal situation. Not all the small spots are artifacts. They may correspond to a poor secretion. The color depends on the substrate and buffer used during the enzymatic reaction. In real spots the color distribution follows a pattern. They are dark at the center and become progressively lighter to the border of the circle.

Criteria for Choosing a Cut-off Value

It is a very important issue; however, there is not a consensus up to now. Many labs select *a priori* the cut-off value based on their own experience doing the assay. Thus, several criteria have been published [6, 7]. But depending on the approach scientists use, sometimes, some positives responses are lost or, on the contrary, some negative values are considered as positive. Because of that researchers are working on a statistical solution [8, 9].

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