Video Article

Enzyme-linked Immunospot Assay (ELISPOT): Quantification of Th-1 Cellular Immune Responses Against Microbial Antigens

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Abstract

Adaptive immunity is an important component to clearance of intracellular pathogens. The ability to detect and quantify these responses in humans is an important diagnostic tool. The enzyme-linked immunospot assay (ELISPOT) is gaining popularity for its ability to identify cellular immune responses against microbial antigens, including immunosuppressed populations such as those with HIV infection, transplantation, and steroid use. This assay has the capacity to quantify the immune responses against specific microbial antigens, as well as distinguish if these responses are Th1 or Th2 in character. ELISPOT is not limited to the site of inflammation. It is versatile in its ability to assess for immune responses within peripheral blood, as well as sites of active involvement such as bronchoalveolar lavage, cerebral spinal fluid, and ascites. Detection of immune responses against a single or multiple antigens is possible, as well as specific epitopes within microbial proteins. This assay facilitates detection of immune responses over time, as well as distinctions in antigens recognized by host T cells. Dual color ELISPOT assays are available for detection of simultaneous expression of two cytokines. Recent applications for this technique include diagnosis of extrapulmonary tuberculosis, as well as investigation of the contribution of infectious antigens to autoimmune diseases.

Video Link

The video component of this article can be found at https://www.jove.com/video/2221/

Protocol

For the following protocol, we use peripheral blood mononuclear cells (PBMC) as the cells of interest. However, this protocol may be used with other cell types. Perform the assay in a tissue culture hood using sterile technique.

Day 1: Preparation of Plate and Cells

1. Take out an ELISPOT plate and open it in the hood.
2. Wash the plate 3 times with 150 µL of 1X PBS using a multichannel pipettor, if available, otherwise single well pipetting is acceptable.
3. Take out a package of reservoirs and open it in the hood.
4. Add 2 µg/mL of the capture antibody, anti-human Interferon-γ, to 11 mLs of 1X PBS. Vortex well. Transfer antibody mix to a reservoir.
5. Place 100 µL of the coating solution into each well using the multichannel.
6. Wrap the plate in parafilm.
7. Place the plate in the refrigerator overnight. The plates are good for weeks. The rule of thumb is that if there is still liquid left in the wells, the plate can be used.

Cell Preparation

If the PBMCs are fresh then count the cells and incubate overnight at 37°C with 5% CO_2. For PBMC stored in liquid nitrogen, thawing of cells is necessary. The protocol is as follows:

1. Thaw the cryovial of cells in 37°C water bath until almost completely thawed.
2. Immediately resuspend in 5 mL of R10 media.
3. Spin for 5 minutes at 225 x g.
4. Resuspend again in 5 mL of R10 media and count the viable cells using trypan blue.
5. Bring concentration to 2 x 10^6 cells/mL in R10 media.
6. Rest overnight at 37°C with 5% CO_2.

Day 2: Setting-Up the Plate

1. Label plate lid with specimen identification number, well conditions, and date.
2. Wash the plate 6 times with 150 µL of sterile 1x PBS using the dump and blot method. Be careful not to splash the plate while dumping. This could cause the wells of the plate to turn purple.
3. Add 100 µL of R20 to each well.
4. Incubate the plate at 37°C for 1 hour.
5. While the plate is incubating, count the cells that were rested overnight.
6. Spin the cell suspension at 1500 rpm for 5 minutes. Decant the supernatant and resuspend the pellet so that you have a final concentration of $1X10^6$ cells per 1mL in R10 media.
7. After the 1 hour incubation, remove the R20 using the dump and blot method. Be careful not to splash the plate while dumping.
8. Add 100 µL of cell solution (10^5 cells) to each well. Add appropriate peptides and/or antigens to the corresponding wells in duplicate. Use a new pipette tip every time you go into the peptide working solution. Normal peptide concentration is 10-40 µg/mL. Since peptides vary, each investigator should titrate peptides to obtain optimal results.
9. In the negative control wells, only add cells (no peptides)
10. In the positive control wells, add phytohemagglutinin (PHA; 10 µg/mL; Sigma).
11. Incubate overnight at 37°C with 5% CO_2 (~18 hours).

Day 3: Developing the Plate

1. Wash the plate 6 times with 150 µL 1xPBS using the dump and blot method.
2. Add 100 µL PBS to each well of the plate using the multichannel pipettor.
3. Put the plate in the refrigerator for 15 minutes.
4. Take the plate out of the refrigerator and place it in the tissue culture hood.
5. Prepare the biotin antibody solution
    - Add 0.5 µg/mL of biotin antibody (mAB 7-B6-1) to 11mL PBS.
    - Vortex the solution to mix well.
    - If planning to add the solution using a multichannel, pour it into a reservoir.
6. Discard the PBS by flicking it into the wastebasket. Be careful not to splash the plate while dumping.
7. Place 100 µL of the solution into each well.
8. Incubate the plate in the tissue culture hood at room temperature for one hour.
9. Wash plate 6 times with 150µL 1xPBS using the dump and blot method. On the 6^{th} wash, leave the PBS on the plate until you have prepared the Streptavidin antibody.
10. Prepare the Streptavidin antibody.
    - Add 5.5 µ l Streptavidin antibody (Streptavidin-ALP) to 11mL PBS (1:2000 dilution).
    - Vortex the solution to mix well.
    - If planning to add the solution using a multichannel, pour it into a reservoir.
11. Discard the PBS remaining in the wells and blot the plate.
12. Add 100 µL of Streptavidin solution to each well.
13. Incubate the plate in the tissue culture hood at room temperature for 1 hour.
14. Wash plates 6 times with PBS using the dump and blot method. On the 6^{th} wash, leave the PBS on the plate until you have made the color solution.
15. Work in the dark. Prepare the alkaline phosphatase substrate solution (Alkaline Phosphatase Substrate Kit IV BCIP/NBT):
    - Add 4 drops of substrate reagent 1 to 11 mL Tris buffer, vortex well. Add 4 drops of substrate reagent 2 to Tris buffer, vortex well. Add 4 drops of substrate reagent 3 to Tris buffer, vortex well.
16. Discard the remaining PBS and blot the plate.
17. Add 100 µL of the color solution to each well of the plate using the multichannel.
18. Turn on the light after 5 to 10 minutes of color developing.
19. Allow the color reagents to remain on the plate until the spots begin to turn purple and are quite dark. This should take anywhere from 5 to 20 minutes. Be careful not to leave the substrate solution on too long because doing so could cause high background.
20. Wash the ELISPOT plate 3 times with tap water.
21. Leave to dry for about an hour or overnight.
22. Read plate either manually or with ELISPOT plate reader.
23. Store the plate in a box away from light exposure.

Representative Results:

ELISPOT is designed to quantitate immune responses against microbial antigens. Each spot indicates a single responsive cell, expressing the cytokine of interest. The spots can be counted manually or using an ELISPOT plate reader.

Assessment that the ELISPOT has been performed correctly should begin with investigation of the negative control for background production of the cytokine of interest. In the negative control wells one would expect to see no spots, although a little background (purple color) may be present. Typically, there should be less than an average of five spots per well. The positive control wells confirm that the reaction has been performed correctly, and that the cells are responsive to antigenic stimulation. In the positive control wells you would expect to see a deep purple background due to a confluence of spots (Fig1). There should be little to no white background. If the positive control and negative control

samples are appropriate, one can then count the wells containing the antigen of interest. A "suboptimal" result would be reflected by high background (the wells are purple), no spots or very few spots, blank center, poorly defined spots or confluent spots. A list of troubleshooting tips is provided (Table 1).

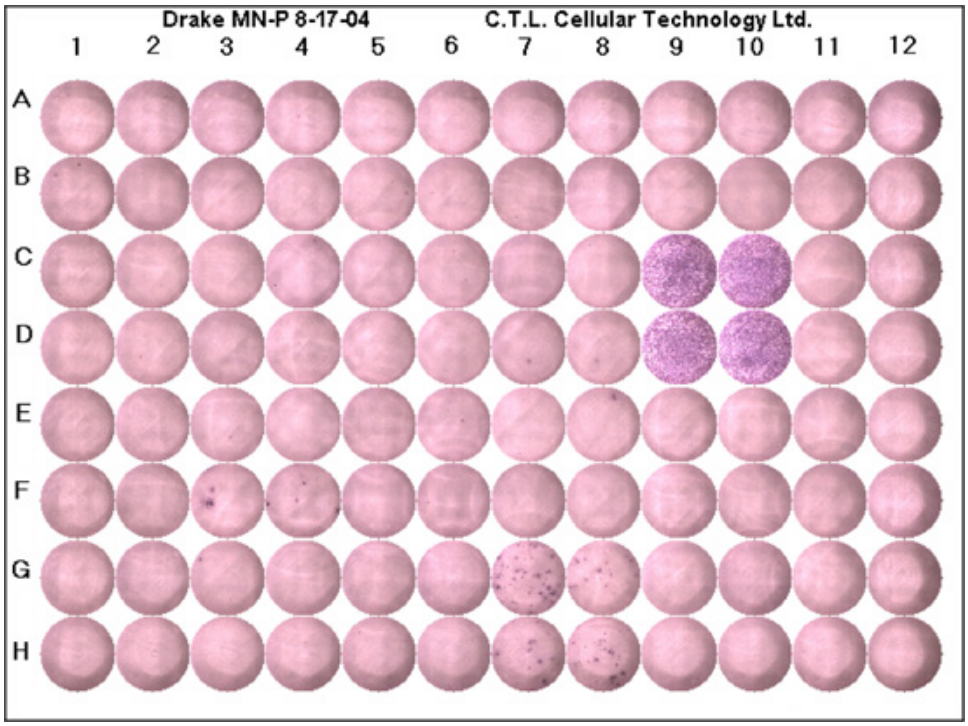

Figure 1. A representative of ELISPOT plate. Each plate should contain a positive control, negative control and peptide of interest conducted at the minimum in two wells. C9, C10, D9 and D10 are the positive control wells, as you can see there is a deep purple color, confluent cells and hardly any white background. The negative control wells are G11, G12, H11 and H12. These wells have no purple background and no spots as expected. The spots G7, G8, H7 and H8, illustrate cellular responses to microbial peptides; the purple spots represent a single responsive cell expressing the cytokine of interest.

Table 1

Observation	Possible Problem	Possible Solution
High background	1. Both sides of membrane not washed properly 2. Too many cells secreting cytokine 3. Plate not dried properly 4. Over developed plate	1. Wash both sides of the membrane with distilled water before and after color development. Reagents may leak through the membrane into the base of the plate, and these can cause high background if not washed away. 2. Reduce the number of cells per well, this will require optimization 3. Dry the plate longer before reading 4. Reduce developing time
No spots/Very few Spots	1. Not enough cells secreting cytokine/protein of interest 2. Ensure the cells are stimulated correctly 3. Cells not incubated for long enough or may take time to respond to stimulant 4. Inadequate color development 5. Not enough primary or secondary antibody	1. Increase the number of cells. This will require optimization 2. Also use a positive stimulation control - a stimulant that you know will induce expression of your cytokine/protein of interest 3. Increase the cell incubation time or use indirect method (pre-treat cells with stimulant) 4. Monitor color development with an overhead microscope and ensure that the developing reagents have been stored correctly and have not lost activity 5. Concentration of the primary and/or secondary antibody will need to be increased. This will require optimization.
Blank Areas	1. Membrane not pre-treated 2. Membrane has dried out at some stage 3. Cells unevenly distributed	1. Ensure membrane is adequately pretreated with 70% ethanol (all membranes don't need

		this so check with your supplier). Wash well with PBS 3X afterwards 2. Ensure membrane does not dry 3. Ensure you mix the cells gently to have a good homologous cell suspension before pipetting out into the wells
Blank Center	1. Damage from washing	1. Flow rate on automated washer (or pipetting) may be too high. Need a more gentle washing procedure
False Positives	1. Secondary antibody aggregates 2. Cells still on the membrane 3. Cell culture contamination	1. Filter the secondary antibody 2. Ensure all the cells are washed from the membrane with PBS Tween 20 before secondary antibody incubation. Cells left on the membrane will give irregular shaped spots 3. Keep reagents as sterile and clean as possible. Ensure your cell culture technique is aseptic. Check for false positives by running a media negative control. For plate movement, see poorly defined spots
Confluent Spots	1. Poor coating, too much antibody 2. Prolonged cell culture 3. Cells over-stimulated	1. Reduce primary antibody concentration 2. The longer the cells are incubated, the more cytokine/protein they will secrete. This will result in larger spots that will start to merge and become indistinguishable. 3. Reduce cell culture step incubation time. Over-stimulation will result in a lot of cytokine/protein being secreted by the cell. This will produce spots that will start to merge and become indistinguishable. Reduce the amount of stimulant in the culture media or culture for a shorter amount of time
Poorly Defined Spots	1. Membrane not pre-treated (if your membrane requires this, check with supplier) 2. Plate movement during cell incubation	1. The membrane must be pre-treated with ethanol or this can result in fuzzy, poorly defined spots. It will be difficult for the reader to distinguish these 2. Do not allow the plate to move during cell incubation as cells that have moved will create more than one spot. If possible use a dedicated incubator that will not be opened during the incubation. Do not tap the plate after adding cells
Positive Control wells is low	1. Underdevelopment - may be a result of using Streptavidin-ALP and/or BCIP/NBT solutions that have not been brought to room temperature	1. Bring reagents to room temperature before adding to the wells
Density of the spots makes it difficult to quantify them	1. Too many cells were added to the wells	1. Make dilutions of cells (i.e., 1 x 10^6, 5 x 10^5, 1 x 10^5, 5 x 10^4, 1 x 10^4 cells per well) to determine the optimal number of cells that will result in formation of distinct spots

Discussion

The ELISPOT assay has emerged as one of the most important and widely used assays to monitor immune responses in humans and a variety of other species. With the ELISPOT assay, immune cell frequencies can be measured at the single cell level without expansion or manipulation of cell populations. ELISPOT assay has been widely applied to investigate antigen specific immune responses in various diseases including infections, cancers, allergies and autoimmune diseases. The application of this assay has been largely applied to studies where the quantification of the antigen-specific cells is critical or comparison of the immune response to specific antigen in various disease states, such as tuberculosis before or after antibiotic therapy [1]. However, recent reports have shown that this assay can be used to detect frequencies of antigen-specific T cells within isolated cell material such as the ascitic fluid, cerebral spinal fluid or bronchoalveolar lavage. Sung-Han Kim et. al. has shown that by using the ELISPOT assay on PBMCs from patients suspected of abdominal tuberculosis, there is a sensitivity of 89% for diagnosing active abdominal TB [2]. It has also been shown that the T-cell based ELISPOT assay had a 91% sensitivity for diagnosis of active TB in patients with suspected of central nervous system involvement by tuberculosis using peripheral blood and cerebrospinal fluid mononuclear cells [3]. Our laboratory utilizes the ELISPOT assay to assess cellular recognition of mycobacterial antigen ESAT-6 in PBMC from sarcoidosis patients. This report is important because it shows how the ELISPOT assay can be used also as a diagnostic method to delineate a role for infectious agents in idiopathic diseases [4,5]. Another benefit of this assay is it also allows the identification of tuberculosis in patients who have

undergone BCG vaccination. The assay is not limited by immunosuppressive conditions such as steroid use and HIV infection [6]. It has also facilitated quantification and characterization of the human immunodeficiency virus (HIV)-specific CD8+ T cell responses [7]. Responses can even be detected among healthy subjects to microbial antigens, such as *Aspergillus fumigatus* [8]. ELISPOT has also been used to identify immunodominant antigens from survivors of Hantavirus cardiopulmonary syndrome [9], as well as to assess T cell function during chronic HIV infection [10]. 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. This summary provides only a limited view of the vast possibilities of the relatively simple, inexpensive assay. The possible applications of this assay continue to expand.

Disclosures

No conflicts of interest declared.

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