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ELISPOT Assay: Detection of IFN- γ Secreting Splenocytes

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Overview

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ELISPOT is a standardized, reproducible assay used to detect cellular immune responses. The assay utilizes an enzyme-linked immunosorbent assay (ELISA)- based method to detect single-cell immune responses which can be visualized by spots, hence the name ELISPOT. ELISPOT was first described in 1983, by Czerkinsky, as a method of enumerating the number of B cell hybridomas producing antigen-specific immunoglobulins (1). The same group further developed the assay to measure the frequency of cytokine producing T lymphocytes. Now ELISPOT has become a gold standard for measuring antigen-specific T cell immunity in clinical trials and vaccine candidates. For example, after vaccination or during an infection, plasma cells and memory B cells secrete antibodies that provide protection. Typically, these B cell responses are assessed by measuring serum titers of antigen-specific antibodies. However, this type of analysis, typically measured by ELISA, may not include memory B cells, which can be present even in the absence of detectable serum antibody levels. Furthermore, it has been well-established that circulating memory B cells are important for the rapid and protective antibody response observed following pathogen re-exposure, thus it is critical to be able to detect these cells. Therefore, to clearly assess antigen-specific memory B-cell responses, both ELISA and ELISPOT should be used (2).

ELISPOT assay uses a plate containing membrane-lined wells that are coated with antibodies in order to capture secreted proteins of interest. Then, the plate is loaded with cells and stimuli to induce protein production. The secreted proteins are captured by the antibodies coated on the surface. After appropriate incubation time, cells are removed and the secreted molecule is detected by using a biotinylated antibody that is specific for a different epitope, as compared to the capture antibody. Next, streptavidin peroxidase is added, followed by the addition of a substrate that allows the detection of the spots (Figure 1). The strength of this assay is that it allows one to quantitate the number of cells producing the protein of interest. Importantly, one can assess if there are changes in the total number of cells producing a specific protein or if individual cells within a population are producing more protein. Moreover, it can provide information regarding kinetics and can be used to assess overall immune activation (mitogen stimulation) relative to antigen-specific responses (antigen simulation). The ELISPOT assay will permit the detection one activated cell amongst 300,000 cells following mitogenic or antigen-specific activation.

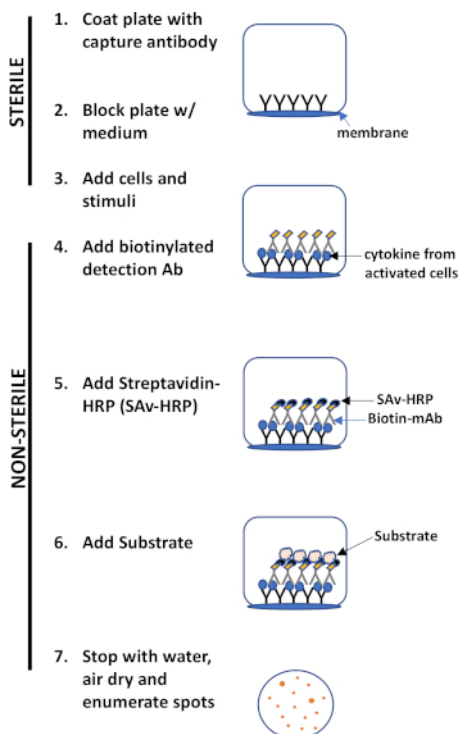


Figure 1: ELISPOT protocol overview.

The major advantages of this assay are its- a. Simplicity- the protocol is relatively simple and straightforward. It does not require technical expertise, b. Sensitivity- it permits the detection of immune cells at the single cell level and requires very few cells compared to other methods such as flow cytometry, c. Functionality- it provides quantitative data regarding immune function.

This lab exercise demonstrates the ELISPOT protocol for detection of IFN- γ secreting-splenocytes, but as mentioned above this assay can also be used to assess antibody secretion by B cells (3).

Procedure

1. Set-up

Buffers and Reagents

1. Sterile phosphate-buffered saline (PBS) without calcium or magnesium
2. Coating buffer- either sterile PBS or carbonate buffer
3. Assay diluent- 10% fetal bovine serum (FBS) in PBS
4. Cell culture medium- RPMI 1640 with 10% FBS, penicillin/streptomycin, & L-glutamine
5. Wash buffer- PBS containing 0.05% Tween20
6. Double distilled water (ddH₂O)
7. Detection substrate- 100 mg AEC (3-amino-9-ethyl-carbazole) in 10 mL DMF (N,N, Dimethylformamide).

Equipment

8. Laminar flow hood
9. Humidified incubator (set at 37°C, 5% CO₂)
10. Automated ELISPOT Reader or dissecting microscope

Materials

11. ELISPOT plates
12. Sterile and nonsterile reservoirs
13. Pipettors and tips
14. Sterile serological pipettes
15. Sterile, conical polypropylene tubes
16. Two squeeze bottles for plate washing

Assay Specific Reagents

17. Cells- primary cells or cell lines (here, splenocytes from C57BL/6 mice were used)
18. Stimulants- mitogen or antigen (here, phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) and ionomycin (1 μ M) were used)
19. Primary antibody- biotinylated anti-cytokine detecting antibody (diluted to 2 μ g/mL in assay diluent)
20. Secondary antibody- streptavidin-horseradish peroxidase (SAv-HRP)

2. Procedure

Coating

1. Keeping the conditions sterile and inside a laminar flow hood, dilute the purified anti-cytokine capture antibody to a final concentration of 0.5-4.0 μ g/mL in sterile coating buffer. (Note: for IFN- γ and IL-6 use 5 μ g/mL).
2. Transfer the capture antibody solution, 100 μ L/well, to the ELISPOT plate.
3. Cover the plate with a plate cover and seal it to prevent evaporation.
4. Incubate the plate overnight at 4°C.

Blocking

5. The next day, uncover the ELISPOT plate in the laminar flow hood. Quickly invert the plate onto sterile wipes to remove the capture antibody solution from each well.
6. Then, add 200 μ L of cell culture medium to each well. This step will block non-specific binding during the assay.
7. Replace the plate cover and incubate the plate for 2 hours at 37°C.

Plating and Activating Cells

8. While the plate is incubating, prepare a 2X mitogen solution containing 50 ng/mL PMA and 1 μ M ionomycin in cell culture medium.
9. Then, prepare target cellular suspensions to a stock concentration of 2×10^6 cells/mL.
10. After the incubation is complete, remove the cell culture medium from each well by quickly inverting the plate onto sterile wipes inside the laminar flow hood.
11. Next, generate a 2X serial dilution of the stock cell suspension solution. To do so, first add 200 μ L of the prepared cellular suspension stock solution into the wells in the top row of the ELISPOT plate.
12. Then, add 100 μ L of plain cell culture medium to the next five rows of the plate below the rows containing cellular stock solution.
13. After that, perform a 2X serial dilution by pipetting 100 μ L of the cellular suspension from the top row into the row directly below. Ensure proper mixing by gently pipetting this solution up and down to ensure even distribution the cells.
14. Repeat this process for the remaining four rows.
15. Leave the sixth row with the culture medium only. It will serve as experimental control.

16. Next, add 100 μL of the prepared mitogen solution to the experimental wells of the first five rows of the plate. In the control wells and the sixth row, add 100 μL of the cell culture media without mitogen.
17. Replace the plate cover and incubate plate at 37°C, 5% CO₂ in an incubator for 20-48 hours. (Note: 20-24 hours is typically sufficient for detecting IL-2 and TNF- α , whereas 48 hours is optimal for IL-4 and IFN- γ).

Detection

Primary antibody

18. Prepare the biotinylated anti-cytokine detecting antibody to a concentration of 2 $\mu\text{g}/\text{mL}$ in assay diluent.
19. Prepare 20-25 mL of wash buffer at this time by mixing 0.05% Tween-20 in PBS.
20. After the incubation is complete, uncap the plate and quickly invert it over a sink to remove all liquid from the wells. (Note: After this point the plate no longer has to be kept sterile).
21. Then, wash the plate by adding \sim 200 μL wash buffer to each well. Expel this liquid by quickly inverting and flicking the plate over a sink. Repeat this process for a total of five washes.
22. Next, add 100 μL of the diluted biotinylated anti-cytokine detecting antibody solution to each well. Incubate at room temperature for 2 hours at room temperature or overnight at 4°C.

Secondary antibody

23. After incubation is complete, expel the detection antibody by inverting and flicking the plate over the sink.
24. As before, wash the plate 5 times with \sim 200 μL wash buffer, expelling the liquid between each wash.
25. Next add 100 μL of diluted streptavidin-horseradish peroxidase solution to each well (diluted to its pre-determined optimal concentration in assay diluent).
26. Replace the plate cover and incubate at room temperature for 1.5-2 hours at 37°C.

Substrate

27. After the incubation, no more than 15 minutes before use, first activate AEC substrate solution according to manufacturer's instructions.
28. Next, discard the contents of the wells and wash the plate five times with wash buffer, as before.
29. Then, immediately add 100 μL of prepared AEC substrate solution into each well.
30. Incubate the plate at room temperature for \sim 10-20 minutes while monitoring the spot development.
31. Stop the reaction by rinsing the plate with water and flicking the plate over the sink.
32. Blot the plate on paper towels and allow the plate to air dry overnight or until it is completely dry. Removing the plastic tray under the plate will facilitate drying.

3. Data Acquisition and Analysis

1. After drying, the spots are ready to be counted with an automated plate reader. Here, the CTL Immunospot reader is used, but this protocol can be adapted for any reader.
2. First turn on the instrument, then the computer. Then, open the CTL program and click "scan count."
3. Push "eject" for the tray to extend from the machine. Then, remove the plastic adaptor and align row "A" on the ELISPOT plate and adaptor.
4. Choose a file name and location for the file to be saved and load the plate onto the tray.
5. Then, click "load" on the software and close the door on the side of the machine.
6. Press "start- after counting." Ensure that the file is saved, and then open the quality control "QC" software to analyze the data and count the number of spots.

Notes:

1. *The minimum number of cells should be determined in preliminary experiments. The optimal number of spots is \sim 50/well. If too many cells are loaded, it will be hard to detect distinct spots. Additionally, the cells will overlap and may not form a monolayer on the membrane, thus the level of detection may be reduced.*
2. *When optimizing the experiment, consider the expected expression level of the target protein. The lower the expression, the higher the number of cells required per well.*
3. *Unlike ELISA, it is better to hand wash the plate rather than use a plate washer. ELISPOT plates are more delicate and one should avoid puncturing the PVDF membrane.*
4. *One should limit movement of the plate during the incubation period as it can cause the spots to smudge.*
5. *Plates should be stored in the dark, as exposure to direct light causes spots to fade.*

Results

In this ELISPOT assay, splenic leukocytes from wildtype and tumor-bearing mice were analyzed for IFN- γ . Figure 2 A shows the visual image of the assay result. The numbers in the green color indicate the number of spots per well (TNTC indicates "too numerous to count"). Notice that the number of spots decreases with decreasing cell concentration.

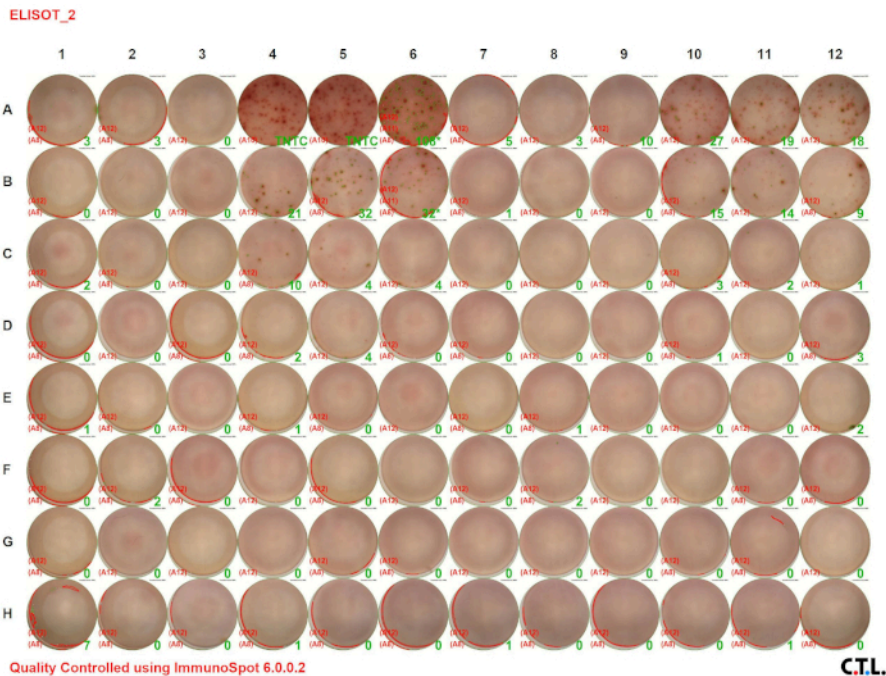


Figure 2A: Decreased immune responses in tumor-bearing mice. Please click here to view a larger version of this figure.

Typically, ELISPOT data are presented as the number of spot counts per number of cells plated. In Figure 2 B the number of spots is displayed in a bar graph, with each respective cellular concentration listed on the X-axis. For graphing purposes, 150 was used to indicate the maximum number of spots. The number of IFN- γ producing murine splenic leukocytes in tumor-bearing animals is lower than the wild type ones.

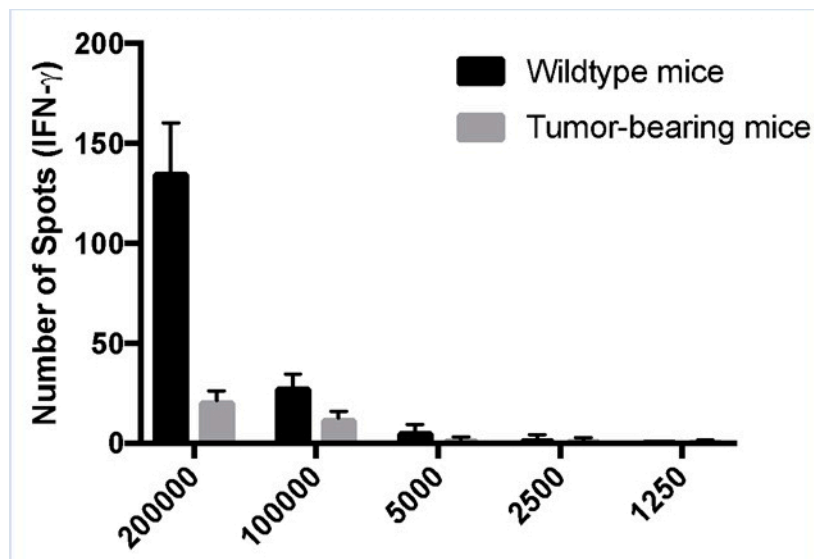


Figure 2B: Decreased immune responses in tumor-bearing mice. Splenocytes were harvested from control C57BL/6 (wildtype) and tumor-bearing mice and stimulated with PMA/ionomycin for 48 hours. ELISPOT assays were used to quantitate the number of IFN- γ -producing splenic leukocytes. (A) Visual and (B) graphical representation of the data. TNTC indicates too numerous to count. For graphing purposes, 150 was used to indicate the maximum number of spots. The green numbers indicate the number of spots counted per well. The red numbers indicate the reference wells that were used to determine which spots were cells and which spots were debris, artifacts, or edge effects and should be excluded from the analysis.

Applications and Summary

The ELISPOT assay allows one to assess immune cell activation by determining the number of cells secreting a specific analyte. The size and intensity of the spots provides information regarding the amount of analyte being produced by each cell. The protocol outlined above detailed the detection of a single cytokine. However, recent developments have enhanced the utility of this assay. Currently, one can use fluorescent detection dyes in order to detect multiple analytes within a well. This permits the detection of different subpopulations of cells secreting either one or both analytes.

References

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