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ELISPOT Ready-SET-Go! Protocols

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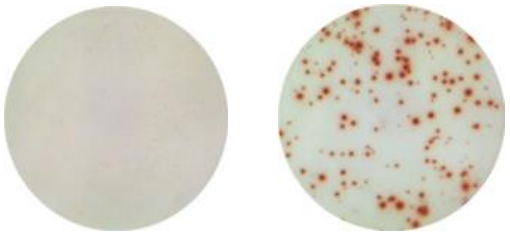
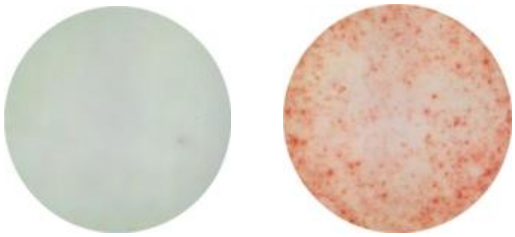
Introduction

Enzyme-linked immunosorbent spot (ELISPOT) assays were originally developed to enumerate B cells secreting antigen-specific antibodies, but subsequently the assay has been adapted for identification and enumeration of cytokine-producing cells at the single cell level. The method employs the sandwich assay approach of the enzyme-linked immunosorbent assay (ELISA), with some variations. The ELISPOT capture antibody is coated aseptically onto a polyvinylidene difluoride (PVDF)-backed microwell plate. After blocking the plate with serum proteins, cells of interest are plated out at varying densities, along with antigen or mitogen, and then incubated at 37°C. Cytokine secreted by activated cells is captured locally by the coated antibody on the high surface area PVDF membrane. The wells are washed to remove cells, debris, and media components and the final steps do not have to be performed in an aseptic environment. A second antibody (biotinylated) reactive with a distinct epitope of the target cytokine is employed to detect the captured cytokine. The detected cytokine is then visualized using avidin-HRP and a precipitating substrate (e.g., AEC). The colored end product (spot) represents an individual cytokine-producing cell. The spots can be counted manually (e.g., with a dissecting microscope) or using an automated reader to capture the microwell images and to analyze spot number and size.

The ELISPOT assay enables analysis of activated or responding cells at the single cell level. The sensitivity of the ELISPOT assay allows for frequency analysis of rare cell populations (e.g., antigen-specific responses) that is not possible using bulk assay methods. By virtue of exquisite sensitivity of the ELISPOT assay, frequency analysis of rare cell populations (e.g., antigen-specific responses) are possible. Limits of detection are below 1/100,000 rendering the assay useful for monitoring antigen-specific responses applicable to a wide range of areas of immunology research, including cancer, transplantation, infectious disease, and vaccine development.



Human IL-17 ELISPOT:
Left: Human PBMCs cultured for 24 hrs (no mitogen).
Right: Human PBMCs activated with PMA/Iono for 24 hrs



Human Granzyme B ELISPOT:
Left: Human PBMCs cultured for 24 hrs (no mitogen)
Right: Human PBMCs activated with PMA/Iono for 24 hrs

General Notes

Precautions:

1. To ensure optimal results, only use the components included in the particular set. Exchanging of components is not recommended, as changes in performance may occur.
2. Do not use components past expiration date. Refer to the Certificate of Analysis included with each set.
3. Be certain that the buffers used to dilute reagents do not contain sodium azide, as this will inactivate the HRP enzyme.

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Experimental Procedure

Materials

- Please refer to the Certificate of Analysis (C of A) for components

Other Materials Required (See buffer recipes below)

- 96-Well PVDF membrane ELISPOT plates (e.g., Millipore MultiScreen-IP Cat. No. MAIPS4510)
- AEC (3-amino-9-ethyl-carbazole) substrate (Sigma, Cat. No. A-5754)
- 0.1 M acetate solution
- ELISPOT wash buffer (PBS + 0.05% Tween-20) (or ELISA Wash Buffer Cat. No. 00-0400)
- PBS
- Complete tissue culture medium (e.g., RPMI-1640 + 10% FBS and other supplements)
- Cell stimulant (e.g., PMA + Ionomycin) (or Cell Stimulation Cocktail Cat. No. 00-4970)

Instruments

- Pipettes and pipettors
- Refrigerator
- Incubator
- Laminar flow hood
- ELISPOT plate reader or microscope

Experiment Duration

- 1 overnight incubation to coat plate
- 24-48 hour cell activation
- 3-5 hour incubations for detection antibody, enzyme, and substrate

Experimental Procedure

Aseptic Procedures: Use sterile buffers and perform in a laminar flow hood

1. Dilute coating buffer to 1X with reagent-grade sterile water.
2. Dilute Functional Grade capture antibody in sterile 1X ELISPOT coating buffer as noted on Certificate of Analysis included in the reagent set. Coat ELISPOT plate with 100 µL/well of antibody, cover, and incubate overnight at 4°C.
   ***Note**: Filter plates are not pre-wet prior to coating with capture antibody in coating buffer.*
3. Decant or aspirate antibody from plate. Wash plate 2 times with 200 µl/well of 1X ELISPOT coating buffer.
4. Block plate with 200 µL/well of tissue culture medium, cover, and incubate for 1 hour at room temperature.
5. Decant or aspirate medium from plate.

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6. Aliquot mitogen, antigen, or controls diluted in tissue culture medium to the appropriate wells on the plate. Add prepared cells diluted to desired densities (e.g. $1x10^5$ – $2x10^6$/mL) at 100 µL/well and incubate for 24-48 hours in a 37°C incubator.
   ***Note**: Kinetics and cell densities vary with target cytokine, treatment, and cell type, and must be empirically determined. Cells can be diluted in a sterile 96-well tissue culture plate starting at $2x10^6$/mL in triplicate wells with a series of 3- or 4-fold serial dilutions down the plate and then transferred to the ELISPOT plate.*

Non-Aseptic Procedures: The following steps do not need to be performed under sterile conditions

1. Decant cells and medium from the plate and wash 3 times with ELISPOT wash buffer. Decanting can be done by flicking the solution out of the plate.
2. Dilute biotinylated detection antibody in 1X ELISA/ELISPOT Diluent according to the instructions in the Certificate of Analysis. Add 100 µL/well to the plate, cover, and incubate for 2 hours at room temperature or overnight at 4°C.
3. Decant antibody solution. Wash plate 4 times with ELISPOT wash buffer, allowing the buffer to soak for 1 minute in between each wash.
4. Dilute Avidin-HRP in 1X ELISA/ELISPOT diluent according to the instructions in the Certificate of Analysis. Add 100 µL/well, cover, and incubate at room temperature for 45 minutes.
5. Decant Avidin-HRP solution. Wash plate 3 times with ELISPOT wash buffer, and then 2 times with 1X PBS without Tween-20.
6. Add 100 µL/well of freshly-prepared AEC substrate solution and develop at room temperature for 10-60 minutes.
7. When spots have reached the desired intensity, stop the reaction by washing the plate 3 times with 200 µL/well reagent grade water.
8. Allow plate to air-dry overnight. Count spots using a microscope or automated ELISPOT reader. Store plate in the dark prior to reading to prevent the fading of spots.

Buffer Recipes

1X PBS: Add the following reagents to 1 L of DI H_2O and adjust pH to 7.0

- 80.0 g NaCl
- 11.6 g Na_2HPO_2
- 2.0 g KH_2PO_4
- 2.0 g KCl

ELISPOT Wash Buffer:

- 1 L of 1X PBS
- 0.5 mL Tween-20
- Alternately, powdered ELISA/ELISPOT wash buffer can be purchased (Cat. No. 00-0400) and reconstituted with 1 L DI H_2O

5X ELISA/ELISPOT Diluent:

- Dilute 5X solution to 1X with DI H_2O

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Complete RPMI-1640:

- RPMI-1640 with 10% FBS and 1% Pen/Strep/L-Glu

AEC (3-amino-9-ethyl-carbazole) Substrate Solution:

- Prepare AEC stock solution by dissolving 100 mg of AEC in 10 ml of N,N Dimethylformamide (DMF)
- Add 333 µL of AEC stock solution to 10 mL of 0.1 M Acetate Solution (see below for recipe). Allow to stand 5-10 minutes, and then filter with a 0.45 µM filter to remove precipitate.
- Just before use, add 5 µL of 30% H_2O_2. Mix and use immediately.

0.1 M Acetate Solution, pH 5.0:

- Prepare 0.2 M acetic acid by combining 11.55 mL glacial acetic acid with 1 L DI H_2O
- Prepare 0.2 M sodium acetate by adding 27.2 g sodium acetate to 1 L DI H_2O
- Combine 148 mL 0.2 M acetic acid with 352 mL 0.2 M sodium acetate.
- Adjust volume to 1 L with DI H_2O and pH to 5.0

References

Gebauer BS, et al. 2002. Evolution of the enzyme-linked immunosorbent spot assay for post-transplant alloreactivity as a potentially useful immune monitoring tool. Am. J. Transplant. 9: 857-866.

Guerkov RE, et al. 2003. Detection of low-frequency antigen-specifi c IL-10-producing CD4(+) T cells via ELISPOT in PBMC: cognate vs. nonspecifi c production of the cytokine. J. Immunol. Methods. 279: 111-121.

Kreher CR, et al. 2003. CD4+ and CD8+ cells in cryopreserved human PBMC maintain full functionality in cytokine ELISPOT assays. J. Immunol. Methods. 278: 79-93.

Ott PA, et al. 2004. CD28 costimulation enhances the sensitivity of the ELISPOT assay for detection of antigen-specifi c memory effector CD4 and CD8 cell populations in human diseases. J. Immunol. Methods. 285: 223-235.

Smith JG, et al. 2001. Development and validation of a gamma interferon ELISPOT assay for quantitation of cellular immune responses to varicella-zoster virus. Clin. Diag. Lab. Immunol. 8: 871-879.

Shafer-Weaver K, et al. 2003. The Granzyme B ELISPOT assay: an alternative to the 51Cr-release assay for monitoring cell-mediated cytotoxicity. J. Translational. Med. 1: 14.

Rininsland F, et al. 2000. Granzyme B ELISPOT assay for ex vivo measurements of T cell immunity. J. Immunol. Meth. 240:143-155.

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