IMMUNOSPOT®
High content ELISPOT

January 2024

PROTOCOL

Human IFN-γ Single-Color Enzymatic ImmunoSpot® Rapid

CONTENTS:

- Anti-human IFN-γ AP Detection Ab
- CTL-Test™ Medium
- Diluent B
- Diluent Blue
- S1 (Blue substrate component 1)
- S2 (Blue substrate component 2)
- S3 (Blue substrate component 3)
- Pre-coated Anti-human IFN-γ plates: 96-well, high-protein-binding, PVDF filter plates
- Protocol

PROCEDURE

DAY 1: CELL CULTURE (STERILE CONDITIONS)

- **Prepare media and antigen solutions:** Prepare CTL-Test™ Medium or desired assay medium (see Solutions).
- Plate antigen/mitogen solutions 100µl/well. Ensure the pH and temperature are ideal for cells by placing the plate containing antigens into a 37°C humid incubator, 5-10% CO_2 if it will be more than 10-20 minutes before plating cells.
- Adjust PBMC or other cell-type to desired concentration in CTL-Test™ Medium, e.g.: 3 million/ml corresponding to 300,000 cells/well (cell numbers can be adjusted according to expected spot counts since 100,000-800,000 cells/well will provide linear results). Keep processed cells at 37°C in humidified incubator, 5-10% CO_2 until plating.
- Plate cells, 100µl/well, using large orifice tips. Once completed, gently tap the sides of the plate and immediately place into a 37°C humidified incubator, 5-10% CO_2.
- Incubate plate overnight;18-24 hours. Incubation times can vary but innate and TH1 cytokines are typically 24 hour, TH2 cytokines are typically 48-72 hour incubations and cytotoxic mediators like Granzyme B and Perforin will need a 72 hour pre-stimulation prior to the ELISPOT assay if the cells have not had a recent exposure to the antigen.
- Do not stack plates. Avoid shaking plates by carefully opening and closing incubator door. Do not touch plates during incubation.

DAY 2: DETECTION

- **Prepare wash solutions:** Prepare Wash Buffers: PBS, distilled water, and Tween-PBS (see Solutions).
- **Detection:** Prepare Anti-human IFN-γ Detection Solution (see Solutions).
- After the completion of assay incubation, decant assay medium containing cells and wash plate twice with 150µl/well of PBS.
- Wash plate two times with 0.05% Tween-PBS, 150µl/well each time. Decant final wash, turn plate over and blot the extra wash solution out on paper towels without allowing the membrane to dry.
- Add 80µl/well of Anti-human IFN-γ (AP) Detection Solution. Incubate at room temperature, in the dark, for two hours.
- Prepare Substrate Solution (See Solutions).
- Decant and wash plate three times with 150µl/well of distilled water. Decant final wash and blot the extra wash solution out on paper towels without allowing the membrane to dry.

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DAY 2 CONTINUED

- Add 80µl/well of Substrate Solution. Incubate at RT for 10 minutes.
- Decant and remove underdrain. Wash both sides of the plate gently under the faucet several times with tap water. Decant final wash and blot the extra wash solution out on paper towels.
- Dry plate in a running laminar flow hood for 15 minutes or for 24 hours face down on paper towels in the dark.
- Scan and count plate. CTL has scanning and analysis services available. Email kitscanningservices@immunospot.com to inquire.

SOLUTIONS

- CTL-Test™ Medium: Prepare medium by adding fresh 2mM L-glutamine, (1% v/v final). The volume of medium needed will depend on many assay design variables but will be no less than 20ml for one full plate; warm to 37°C before use.
- Detection Solution: Dilute Anti-human IFN-γ Detection Antibody in Diluent B. For one plate, add 10µl of Anti-human IFN-γ AP Detection Antibody to 10ml of Diluent B.
- Substrate Solutions: Dilute substrate components by adding reagents in sequential steps.
  CTL- TrueBlue- For one plate: Step 1 – Aliquot 10ml of Diluent Blue
  Step 2 – Add 160µl of S1. Mix well!
  Step 3 – Add 160µl of S2. Mix well!
  Step 4 – Add 92µl of S3. Mix well!

*It is recommended to make the Substrate Solution within ten minutes of use and to keep it protected from direct light.

- Wash Buffers (not included) For each plate prepare: 200ml distilled water, 200ml PBS and 200ml 0.05% Tween-PBS: 100µl Tween-20 in 200ml PBS.

*Our team will gladly assist you with data analysis and troubleshooting, as well as customizing ELISPOT assays to suit your needs. Please contact us at **kits@immunospot.com**.*
*Visit our YouTube channel for several helpful videos on working with ELISPOT assays and PBMC: **www.youtube.com/user/ImmunoSpot**.*
*The CTL Thawing Protocol for Cryopreserved Human PBMC and mouse splenocytes are available at **www.immunospot.com**.*

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TECHNICAL TIPS

- Upon successful completion of the assay, IFN-γ spots will appear blue.
- It is recommended to use CTL Serum-Free™ Media for freezing, washing, and testing PBMC and many other cell types. Even brief exposure to a mitogenic serum can cause high background while other sera can have suppressive effects. CTL recommends using the CTL-LDC™ Kit for accurate live/dead cell counts.
- If culturing cells prior to plating the cells in an ELISPOT assay in serum-containing media, higher viability is often seen when the cells are transferred into the same formulation of culture medium for the ELISPOT assay (minus IL-2 for ELISPOT assay). Pre- cultured cells are often constitutively secreting cytokine; at these times, a blocking step is recommended.
- Resting cells overnight is typically not needed. It can lead to a higher percentage of functioning cells; however, this may result in significant cell loss and can often be mitigated by increasing the number of cells plated after isolation.
- Deviations from specified temperatures, timing requirements, number of washing steps, and specified reagent preparation volumes may alter the performance of the assay. (Longer substrate incubation times will result in darker spots. Lighter spots have larger differences between the RGB values making it easier to separate color during analysis; therefore, shorter substrate incubation times are generally encouraged for double-color enzymatic assays.)
- Anti-CD28 reagent is provided in kits that capture both IFN-γ and IL-2 in order to help mitigate the effects of the captured IL-2 on the release of IFN-γ. When Anti-CD28 is omitted in the multi-color ELISPOT/ FluoroSpot assays, a reduction in IFN-γ spots would be expected.
- When coating ELISPOT/ FluoroSpot plates, the plates need to be stored overnight at 4°-8°C at a minimum, but they can remain at 4°-8°C in a humid chamber for up to one week.
- The PVDF membrane must remain wet during the detection steps. Therefore, when blotting the plate on paper towels, the goal is to eliminate excess wash buffer volume without completely drying the membrane.
- An initial cell concentration 1-5 x 10^5 cells/well is usually required. A serial titration of cells starting at a maximum of 1 x 10^6 cells/well is recommended in order to determine the optimal cell concentration.
- To avoid aggregates CTL recommends using syringe filters for detection antibodies and for FluoroSpot kit tertiaries. CTL recommends using Millipore syringe filter catalog number SLVVR33RS or SLVVM33RS.
- To avoid damage to the PVDF membrane in the wells, do not touch the membrane with pipette tips, with a plate washer or the underdrain. The PVDF membrane is permeable and protected by an underdrain. Do not push the underdrain too far up on the plate after the pre-wetting step, or if it falls off, as this will cause leaking of the membrane. Pressing down on the plate frame or the lid rather than the underdrain itself is preferred. It is OK if the underdrain falls off after the cell incubation; just avoid direct contact of the membrane with your hand, wet surfaces or absorbent materials.
- ELISPOT plates are stable for years and they can be scanned weeks to months later without any loss of signal when stored away from direct light. FluoroSpot plates should be kept in the dark and should be scanned within one week for optimal signal. Storing the fluorescent plate at 4°C can help maintain signal stregnth.
- Scan and count plates only after membranes have completely dried. FluoroSpot assay plates can have decreased membrane auto-fluorescence if left overnight to dry in the dark if a vacuum manifold is not accessible.
- Optimal removal of fiber and debris is achieved by removing the underdrain and filtering the final wash on a vacuum manifold upside down or "back to front" (CTL recommends Millipore vacuum manifold catalog number MSVMHTS00).
- Plates may be washed manually or with a suitable automated plate washer with adjusted pin length and flow rate so membranes and spots are not damaged (CTL recommends the CTL 405LSR).
- Data analysis: The CTL ImmunoSpot® Analyzers along with the ImmunoSpot® Software have advanced features that permit automated, objective recognition of spots, gating and counting.
- Double-color enzymatic ELISPOT assays should have single color control wells within the plate. This is best achieved by eliminating one detection antibody but using double color reagents for all other steps in single-color control wells.

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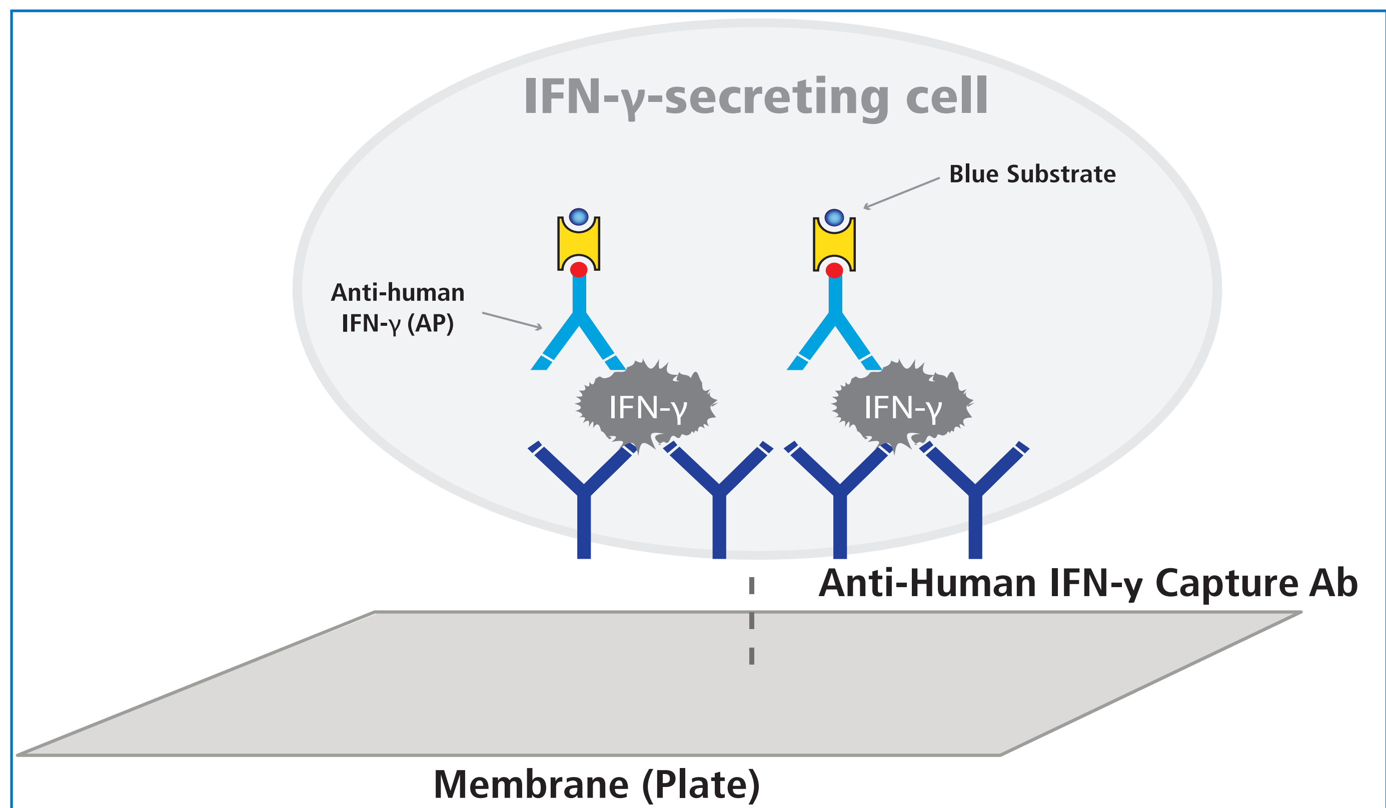
T Cell ELISPOT Assay

The CTL ImmunoSpot® Platform permits maximized, scientifically-validated, single-cell ELISPOT analysis. At the unprecedented resolution of up to 1 in 800,000 cells, ImmunoSpot® Assays measure effector molecule secretion by individual T cells or other cell type that have been stimulated by an antigen. ImmunoSpot® Assays therefore provide information on the numbers of the antigen-specific cells present in a test cell population, typically PBMC or splenocytes. The frequency of the antigen-specific cells reflects the magnitude of the individual's cellular immunity. Spot sizes record the amount of cytokine produced by the individual cells. Spot sizes can be relatively larger sized for polyfunctional T cells and for recently activated T cells and can be relatively smaller sized in states of anergy or immune suppression. Measuring different molecules that T cells secrete provides information on the effector lineage of T cell immunity.

Principle of the Test

Specifically designed 96-well PVDF membrane plates are coated with a monoclonal Capture Antibody specific for human IFN-γ. Freshly-isolated PBMC or other cell types that have been frozen and thawed following optimized protocols are plated together with the antigens of interest and incubated to allow for the activation of the antigen-specific cells and the subsequent release of cytokines. The activated immune cells secrete molecules that are captured by the membrane-bound antibody.

For the best results in an ELISPOT assay, the cells should be cultured with antigen for a time period that induces maximal secretion of cytokine (or other analyte) production; for this assay that is 24 hours or overnight. After the activation culture, the cells are discarded (or can be transferred for further characterization/ propagation), leaving the antibody-bound analyte on the PVDF membrane at the bottom of the well. A detection antibody that is specific for a different determinant of the analyte is added to the plate; in this assay it is the Alkaline Phosphatase (AP) Anti-human IFN-γ Detection Antibody. Subsequently, this complex is visualized by adding a chromogenic substrate (CTL-TrueBlue). This enzyme-catalyzed reaction results in the spot development on the PVDF membrane. Spot numbers therefore denote the accurate frequency of the antigen-specific immune cells among the plated cells; spot sizes and morphology provide additional information on the magnitude and kinetics of the cells' secretory activity.



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