
Technical Note

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Title: IFN- γ Elispot Assays on MultiScreen[®] HA

INTRODUCTION

The Elispot (Enzyme Linked Immuno-Spot) assay provides an effective method of measuring the antibody or cytokine production of immune cells on the single cell level. The popularity of this assay has seen resurgence in recent years as researchers attempt to gain a better understanding of immune responses in a variety of applications. The following protocol is an example of a typical Elispot assay for quantifying the number cells producing interferon-gamma (IFN- γ) in response to antigen or non-specific activation using phytohemagglutinin (PHA). It may be optimized as necessary for other applications.

PROTOCOL

Day 1

Coat Primary Antibody

1. Coat MultiScreen HA plates with 100 μ L (10 μ g/mL) anti-IFN- γ antibody in sterile PBS (sPBS). Incubate overnight at 4 °C.
2. Millipore recommends the following control wells be incorporated into the assay:
 - a. No cells
 - b. No primary antibody
 - c. No antigen stimulation
 - d. Positive control with PHA or phorbol ester such as PMA

Day 2

Block membrane

1. Decant primary antibody solution.
2. Wash off unbound antibody with 150 μ L sterile Milli-Q[®] water per well; decant wash and repeat.
3. Block membrane with 150 μ L per well of cell medium (RPMI-1640, 10% fetal bovine serum, 1% non-essential amino acids, penicillin, streptomycin, glutamine) for at least 2 hours at 37 °C.

Prepare HPBMC

4. Purify human peripheral blood mononuclear cells (HPBMC) using a Ficoll[™] density gradient separation from freshly drawn whole blood anti-coagulated with acid citrate dextrose.
5. Wash cells in cold sPBS, count and resuspend at a final concentration of 0.25 to 2 x 10⁶ cells/mL in cell medium.

NOTE: The desired cell concentration is dependent on the intensity of the immune response. If the expected response is not known, a serial dilution of cell concentrations is recommended.

6. Stimulate cells with desired antigen. (for example with PHA-L or CEF peptide)

Plate out assay

7. Decant blocking medium.
8. Gently plate HPBMC in 100 μ L cell medium per well.

NOTE: To minimize over seeding of the wells, Millipore recommends adding no greater than 200,000 cells/well.

9. Incubate for 18 to 48 hours at 37 °C, 5% CO₂, and 95% humidity.

Day 3

Secondary Antibody

1. Decant cells.
2. Wash plate 6 times with PBS/0.01% Tween[®] 20. A squeeze bottle can be utilized to ensure adequate washing.

NOTE: If using a plate washer, increase number of wash cycles to 1.5 times the recommended number of manual cycles. It is important not to exceed 0.01% Tween[®] 20 to prevent the possibility of leakage.

3. Dilute biotinylated anti-IFN- γ antibody to 2 μ g/mL in PBS/0.5% BSA. . Filter with a Steriflip[®]-GP (Millipore, #SCGP00525) or Millex[®]-GP (Millipore, # SLGP033RS). Add 100 μ L/well.

NOTE: Failure to filter secondary antibody may results in non-specific spot formation due to protein aggregates.

4. Incubate for 2 hours at 37 °C, 5% CO₂, and 95% humidity.
5. Wash plate 6 times with PBS/0.01% Tween 20.

Enzyme Conjugate and Substrate Development

6. Prepare streptavidin-alkaline phosphatase enzyme conjugate 1:1000 dilution in sPBS.
7. Add 100 μ L per well of streptavidin-alkaline phosphatase. Incubate for 45 minutes at room temperature.

NOTE: Exceeding 1 hour incubation with enzyme conjugate will result in increased background color.

8. Decant streptavidin, wash 3 times with PBS/0.01% Tween 20, followed by 3 washes with PBS.

NOTE: The final washes with PBS only are important as Tween[®] 20 will interfere with the spot development.

9. Add 100 μ L/well BCIP/NBT plus substrate. Incubate for 5 minutes.

NOTE: Optimization of substrate development time is critical. Development times may vary. Over-development will result in increased background.

10. Stop spot development under running water and wash extensively. While washing, remove underdrain and continue rinsing.

NOTE: To facilitate the removal of the underdrain a pair of needle nose pliers can be used. Millipore offers such a tool for this need. The part number is MSPLRS096

11. Blot plate to remove excess liquid and dry back of wells thoroughly with an absorbent wipe. This will ensure that the substrate has been completely removed from the membrane.

12. Let plate dry overnight in the dark. Spot intensity may decrease with exposure to light.

Day 4

Analyze plate using imaging system.

Membrane Removal (optional)

Although many ELISpot reading systems allow for in-well counting, the unique design of the MultiScreen plate makes it possible to perform the ELISpot assay in the 96-well filter plate and subsequently remove each individual filter disc for counting. The removal of the membranes from the bottom of the MultiScreen plate is possible to facilitate reading and storage of the filter discs.

Membrane Removal Protocol: ELI-Puncher Kit (Millipore, # MELIPUNCH)

1. Attach the sealing tape to the bottom of the plate and ensure that the tape sticks to the edge of each well by applying pressure to the tape with a blunt or curved implement such as the handle of a spatula.
2. Place the plate on the blue ELISpot transfer pad with the membrane and tape down.
3. Insert the black punch-out device into the well, punch out the membrane by applying light pressure. A faint click sound indicates that the membrane has transferred to the sealing tape. Repeat for all wells.
4. Total time for removing all membranes is approx. 2-3 min.
5. Store tape in a plastic sheet protector along with protocol and evaluation results.

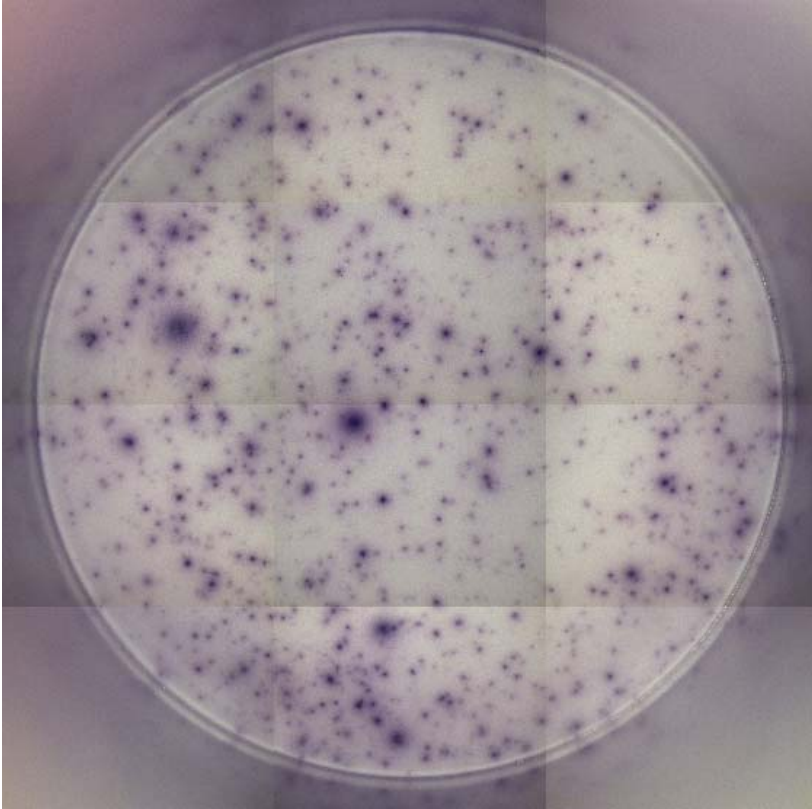


Image of a typical Elispot well, note that the spots are clear, focused and easy to distinguish. The image represents the number of cells secreting IFN- γ in response to PHA-L stimulation of HPBMC. The assay was performed using Millipore MultiScreen nitrocellulose plates (MSHAS4510), antibodies and enzyme conjugate from Mabtech AB (part number 3420-2A) and substrate from Moss, Inc. (part number NBTH-100). Wells were imaged using the Zeiss KS Elispot imaging system.

FOR ADDITIONAL INFORMATION: We suggest visiting Millipore's web page www.millipore.com/elispot or the Elispot Resource Group web page www.elispotresource.com

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