Technical Note

IFN-γ ELISpot Assays on MultiScreen® IP

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. Pre-wet each well with 15 µL of 35% ethanol (v/v in Milli-Q® water) for one minute. Rinse with 150 µL sterile phosphate buffered saline (PBS) three times before the ethanol evaporates.

CAUTION: Ethanol volumes greater than 15 µL are not recommended. Once the membrane is pre-wet with alcohol, do not allow membrane to dry for the duration of the assay.

2. Coat MultiScreen® IP plates with 100 µL (10 µg/mL) anti-IFN-gamma antibody in sterile PBS (sPBS). Incubate overnight at 4 °C.
3. The following control wells are recommended for incorporation 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 µL sterile Milli-Q® purified water per well; decant wash and repeat.
3. Block membrane with 150 µL 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 × 10^6 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 µL cell medium per well.

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

9. Incubate for 18 to 48 hours at 37 °C, 5% CO_2, and 95% humidity.

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Day 3

Secondary antibody

1. Decant cells.
2. Wash plate 6 times with PBS/0.01% Tween® 20 detergent.
   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 detergent to prevent the possibility of leakage.

3. Dilute biotinylated anti-IFN-γ antibody to 2 µg/mL in PBS/0.5% BSA. Filter with a Steriflip®-GP Filter (Cat.#SCGP00525) or Millex®-GP Syringe Filter Unit (Cat.# SLGP033RS). Add 100 µL/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% CO_2, and 95% humidity.
5. Wash plate 6 times with PBS/0.01% Tween® 20 detergent.

Enzyme Conjugate and Substrate Development

6. Prepare streptavidin-alkaline phosphatase enzyme conjugate 1:1000 dilution in sPBS.
7. Add 100 µL 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 µL/well BCIP/NBTplus 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.

11. Blot plate to remove excel 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.

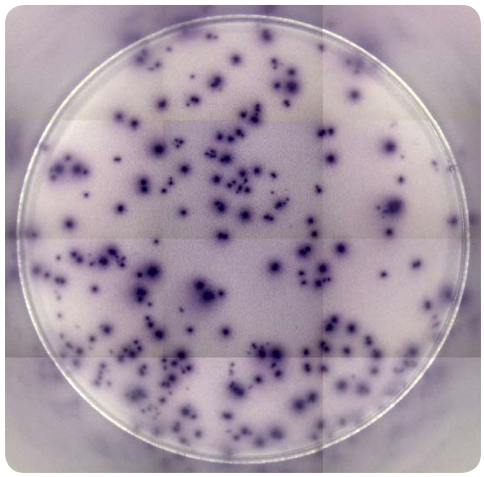

Image of a typical ELISpot well, note that the spots are clear, focused and easy to distinguish. The image represents the number of cells secreting IFN-γ in response to CEF peptide pool stimulation of HPBMC. The assay was performed using MultiScreen® Immobilon®-P plates (MSIPS4W10), antibodies and enzyme 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 more information on ELISpot, visit our learning center at:
emdmillipore.com/elispot

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