

ELISPOT Protocol

The **Enzyme-linked immunosorbent spot (ELISPOT)** assay is a common method for monitoring immune responses in humans and animals. It was developed by Cecil Czerkinsky in 1983. The ELISPOT assay is based on, and was developed from a modified version of the ELISA immunoassay. ELISPOT assays were originally developed to enumerate B cells secreting antigen-specific antibodies, and have subsequently been adapted for various tasks, especially the identification and enumeration of cytokine-producing cells at the single cell level. At appropriate conditions the ELISPOT assay allows visualization of the secretory product of individual activated or responding cells. Each spot that develops in the assay represents a single reactive cell. Thus, the ELISPOT assay provides both qualitative (type of immune protein) and quantitative (number of responding cells) information.

Protocol:

NB: Antibody concentrations should be optimized by titrating various combinations by each end user as conditions will vary. The protocol below is to be used as a guideline only.

Day 1:

1. Soak each well with 15 ul of 35% ethanol and an equal volume of sterile water for one minute. Rinse with 150 ul sterile PBS buffer three times.

Note: Once the membrane/wells are soaked with alcohol, care should be taken to not allow membrane to become dry for the duration of this assay. Do not exceed the recommended volume of 15 ul of ethanol.

2. Coat the supplied IP plates with 100 ul primary antibody in sterile PBS buffer at a concentration of 10 ug/ml (or recommended concentration). Incubate plates overnight at 4 deg C.
3. The following control wells are recommended for inclusion in the assay:
 - Well containing no cells
 - Well containing no primary antibody
 - Well containing a positive control

Day 2:

1. Remove excess antibody solution from wells and wash unbound antibody with 150 ul of sterile water per well, remove and repeat.
2. Block non-specific binding of the membrane using 150 ul per well of the following cell medium solution:

RPMI-1640 medium with 10% fetal bovine serum (FBS), 1% nonessential amino acids, glutamine, penicillin and streptomycin for at least 2 hours at 37 deg C.
3. Human peripheral blood mononuclear cells (HPBMC) should be purified using an appropriate density gradient separation from freshly drawn whole blood. An appropriate anti-coagulant such as acid citrate dextrose should be added to the blood sample.

4. Following purification, HPBMC cells should be washed once in cold, sterile PBS buffer and resuspended following counting at a concentration of 0.25 to 2×10^6 cells/ml

Note: The final concentration in step 4 is dependent on the expected immune response, if the expected response is not known beforehand; it is recommended that a series of cell concentration levels is tested in a serial dilution to determine the optimum concentration.

5. Remove excess blocking solution from wells.
6. Carefully seed wells with HPBMC in 100 ul of cell medium, do not seed wells in excess of 200,000 cells per well as excess amounts will interfere with the assay.
7. Incubate seeded wells @ 37 deg C for between 18 and 48 hours in 5% CO₂ and 95% humidity.

Day 3:

1. Prior to addition of secondary antibody, remove excess medium containing HPBMC cells and thoroughly wash the wells 6 times with a solution of PBS buffer containing 0.01% Tween 20.
2. Dilute the biotinylated secondary antibody to a concentration of 2 ug/ml with a solution of PBS buffer and 0.5% BSA. Filter with an appropriate 0.22 um system and add 100 ul of the filtered antibody solution to each well.
3. Incubate wells @ 37 deg C for 2 hours in 5% CO₂ and 95% humidity.
4. Following incubation once again thoroughly wash the wells 6 times with a solution of PBS buffer containing 0.01% Tween 20.
5. Dilute the streptavidin-alkaline phosphatase conjugate to a final dilution of 1:1000 in sterile PBS buffer.
6. Add 100 ul of the streptavidin-alkaline phosphatase conjugate to each well and incubate for 45 minutes at room temp.
7. Following incubation, remove excess streptavidin-alkaline phosphatase conjugate and wash the wells 3 times with PBS buffer containing 0.01% Tween 20.
8. Wash wells a further 3 times with sterile PBS.
9. Add 100 ul substrate to each well and incubate for 5 minutes at room temp.
10. Do not incubate excessively at this step as it will increase background signal, terminate spot development under running water and wash thoroughly with PBS buffer. Remove the underdrain from the plate while continuing to wash wells.
11. Blot plate to remove excess liquid, an absorbent wipe can also be used to dry the back of the wells to ensure complete removal of any excess substrate from the membrane.
12. Allow the plate to air dry overnight in a dark area. Avoid excessive exposure to light at this stage as this can reduce the intensity of any spots which have developed.

Day 4:

1. Analyze the plate using an appropriate imaging system.

Membranes can optionally be removed from the wells by covering wells in sealing tape and using a blunt tool and gentle pressure to force each one out of the well where it then attaches to the sealing tape.