ELISPOT protocol

Detailed procedures for Enzyme-linked immunospot (ELISPOT) and introduction to how ELISPOT works

ELISPOT is a technique that was developed for the detection of secreted proteins, such as cytokines and growth factors. It is most commonly used in immunology research. For example, ELISPOT can be used in to understand the biological effects involved in:

- Transplantation
- Vaccine development (IFNy)
- Th1/Th2, T cell regulation
- Monocyte and dendritic cell analysis
- Autoimmune disease
- Cancer
- Allergies
- Viral infection monitoring and treatment

Description of ELISPOT

ELISPOT is performed in PVDF or nitrocellulose membrane 96-well plate, in which the membrane has been pre-coated with an antibody specific to the secreted protein. Cells are added to the plate and attach to the coated membrane. Cells are then stimulated and the secreted protein binds to the antibody. Next, a detection antibody is added that binds specifically to the bound protein.

The resulting antibody complex can be detected either through enzymatic action to produce a colored substrate or with fluorescent tags. An advantage to using fluorescence is the ability to identify more than one secreted protein at a time.

The membrane can be analyzed by manually counting the spots or with an automated reader designed for this purpose. Each secreting cell appears as a spot of color or fluorescence, thus this is a quantitative method for evaluating protein secretion.

General ELISPOT procedure

1. Prepare PVDF membranes in the 96-well plates by incubating in 35% ethanol for 30 s.
   Ensure the ethanol is completely removed by washing thoroughly with PBS. Any remaining ethanol can affect cell viability as well as antibody binding.

2. Coat 96-well plate with capture antibody diluted in phosphate buffered saline (PBS). Incubate overnight at 4°C.
   Approximately 0.5–1 µg per well of antibody should be used for well-defined spots. Kits are optimized with capture concentrations for best performance (100 µL per well).

3. Empty the wells, tapping them dry, and wash with PBS.
   ELISPOT plates are more delicate than ELISA plates and should be handled with care. When tapping dry, do this gently. Do not use a plate washer at this stage.

4. Add 100 µL per well 2% dry skimmed milk to block non-specific binding to the membrane. Incubate the plate for 2 h at room temperature.

5. Wash the plate 3 times in PBS and leave to dry. If necessary, the plates can be stored at this stage. Store at 4°C for not more than 2 weeks, in a sealed plastic pouch with desiccant.

6. Prepare peripheral blood mononuclear cells (PBMCs) from fresh blood using a Ficoll™ gradient. Count the cells using a viability dye like trypan blue. They should be over 95% viable. Dilute cells to the required concentration and add the cell suspension to wells. If optimizing the assay for cell number, use a 1:2 dilution series. Do not shake the plates.

   The number of cells per well should be optimized. For example, use more cells if a low percentage of cells are expected to secrete the target cytokine. Refer to the specific target kit protocols for recommendations on assay controls and cell number per well. Typically cell numbers should usually range from between 2 X 10^5 to 4 X 10^5 PBMC cells per well.

   If possible, use serum-free media as serum contains proteins which could affect the results. Alternatively, several batches of serum can be tested to find one with optimal response to noise ratio. This batch can then be stored and used in subsequent experiments.

7. Culture overnight at 37°C in a CO_2 incubator. Do not shake the plates.
   Do not move the plates while the cells are culturing. This will lead to 'snail trail' spots that will not be well defined. Do not stack plates if you have more than one to prevent edge effects.

8. During the overnight incubation the cells will secrete cytokine, which will bind to the primary antibody.
   If cells take time to respond to stimulation, please see indirect method below.

9. Wash away the cells and the unbound cytokine away by incubating with PBS 0.1% Tween 20 for 10 min. Wash the plates 3 times with PBS 0.1% Tween 20.
   Ensure you include Tween 20 in the wash buffer. Some cells will have started attaching after culture overnight (e.g. some stem cells are known to do this). Tween 20 will help wash these off the membrane. Do not use a plate washer at this stage.

10. Dilute the conjugated detection antibody in PBS 1% BSA. Add to wells and incubate for 1–2 h at room temperature (the incubation time may require optimization). Wash plate 3 times with PBS 0.1% Tween 20 to remove non-specific detection antibody binding.
    For our ELISPOT kits, detection antibody concentrations have been optimized for best results.

11. Add the conjugated detection antibody. Dilute the antibody in PBS 1% BSA. Incubate for 1 to 2 h at room temperature. (The incubation time may require optimization). Wash plate 3 times with PBS 0.1% Tween 20 to remove non-specific detection antibody binding.
    For our ELISPOT kits, detection antibody concentrations have been optimized for best results.

12. Add the enzyme substrate solution to each well.
    For enzymatic detection protocols, the base should be taken off the bottom of the plate to enable thorough washing of the membrane before adding substrate/chromogen. For example, after incubation with the streptavidin alkaline phosphatase conjugate, remove the base and wash both sides of the membrane under running distilled water. This helps to prevent high background as some reagents can leak through the membrane into the bottom tray of the plate.

13. After replacing the base and adding the substrate, carefully monitor spot formation. Stop the reaction by gently washing the plate with PBS 0.1% Tween 20 once development appears to slow. Take the base off the plates and wash both sides of the membrane with distilled water to stop the spot formation.

14. Dry the plates and allow the membranes to dry at room temperature.
    Spots may become sharper if membranes are stored overnight at 4°C, and may continue to improve

for up to 2 weeks. If storing, wrap membranes can be wrapped in foil keep at 4°C.

15. Punch the membranes out of the wells onto a sticky plastic sheet. This step will depend on your reader's requirements. Consult the reader manual.

16. Measure the sheet and analyze the membrane circles.

In the analysis software, set the following parameters for measurement:

- Size/spot diameter
- Intensity/saturation
- Circularity/shape
- Spot development/slope

These parameters can be saved and used for subsequent experiments for standardized results.

We recommend reading each plate 3 times and averaging the results in order to minimize error in the measurements.

Indirect ELISPOT

If the cells take some time to respond to stimulation, they may require pretreatment with the stimulant in a separate 96-well culture dish before transferring to the ELISPOT plate.

Positive control stimulation

Experiments to detect cytokines using ELISPOT will require use of positive control. In these positive control wells, the cells should be stimulated with an agent known to induce expression of the cytokine being detected. This can then be used to compare to the negative control (no treatment or stimulation of a different secreted protein).

Ensure you are stimulating the PBMCs with the correct stimulant for detection of your target cytokine:

Typical stimulates include:

LPS to stimulate IL1β, IL6 secretion

PMA and Ionomycin stimulate IL2, IL4 secretion

PHA, 10 µg/ml for IFN gamma

Anti-CD3/CD28 antibodies for IFN gamma, IL4, IL10 and Granzyme B.