

Dual ELISPOT staining provides the opportunity to detect two target proteins at the same time in the ELISPOT cell assay. An advantage of this is that it enables the researcher to detect a marker protein to determine which cell type is secreting the protein, as well as the secreted protein of interest.

The following dual ELISPOT procedure describes a protocol using an FITC-conjugated primary antibody and a biotinylated primary antibody. Those are in turn recognized by anti-FITC HRP and streptavidin-AP conjugates. PVDF-bottomed well plates are then incubated first with AEC substrate buffer, washed and subsequently incubated with BCIP/NBT. The colored spots will either be red/brownish blue/purple so the two target proteins can be distinguished.

Materials and reagents

1. Detection antibody
2. Streptavidin alkaline phosphatase conjugate
3. Phosphate buffered saline (10 x concentrate solution)
For 1 liter weight : 80g NaCl ; 2g KH₂PO₄; 14.4g Na₂HPO₄·2H₂O. Add distilled water to 1 liter. Check that pH is 7.4 +/- 0.1. Dilute the solution to 1X before use.
4. 2% dry skimmed milk in PBS
For one plate dissolve 0.2 g of powder in 10 mL of 1X diluted PBS.
5. 1% BSA in PBS
For one plate dissolve 0.2 g of BSA in 20 mL of 1X diluted PBS.
6. 0.1% Tween in PBS
For one plate dissolve 100µl of Tween 20 in 100 ml of 1X diluted PBS.
7. 70% ethanol in water
For one plate mix 7 ml of ethanol with 3 ml of distilled water.
8. AEC Buffer
For one plate mix 1 ml of ACE Buffer A with 9ml of distilled water. Then add 200µl of ACE Buffer B.
9. 4. ELISPOT 96 well plates
(Provided with Abcam ELISPOT kits).

General dual ELISPOT procedure

1. Prepare PVDF membranes in the 96-well plates by incubating in 70% ethanol for 30 seconds.
2. Wash the plates 3 times with PBS.
3. Coat 96-well plate with an optimized concentration of both the required primary capture antibodies (diluted in PBS). Incubate at 4°C overnight.

If not using antibodies supplied in a dual ELISPOT kit, use antibodies from different host species so they can be distinguished using conjugated secondary antibodies specific for those species.



4. Empty the wells, tapping them dry, and wash with PBS.

ELISPOT plates should be handled more carefully than ELISA plates. When tapping dry, tap gently. Do NOT use plate washer at this stage.



5. Block the plates by adding 100 ul per well 2% dry skim milk. Incubate 2 hours at room temperature.

6. Wash wells in PBS once. If necessary, the plates can be stored at this stage. Wash twice in PBS and leave to dry. Store at 4°C for not more than 2 weeks.
7. Prepare peripheral blood Monocytes (PBMC's) from fresh blood on a Ficoll gradient. Count the cells (make sure they are over 95% viable). Dilute cells to the required concentration and add cell suspension to wells. If optimizing the assay for cell number, dilute 1:2 down the plate as in an ELISA. DO NOT SHAKE THE PLATES.

The number of cells will require optimization. For example, if a low percentage of cells are expected to secrete the target cytokine, a larger cell number will be required. If using a kit, please refer to specific target kit protocols for recommendations on assay controls and cell number per well. Cell numbers should usually range between 1×10^5 to 2×10^5 cells per well.

8. Culture overnight 37°C in CO₂ incubator. DO NOT SHAKE THE PLATES
*If cells take time to respond to stimulation, please see indirect method below.

- a. *Don't move the plates while the cells are culturing. This will lead to 'snail trail' spots that will not be well defined* 
- b. *Don't stack the plates if you have more than one. The temperature will not even out across the well and each not be at the same temperature, giving an edging effect as cells will respond less well in the centre of the plate where it is cooler.*

9. During the overnight incubation the cells will secrete cytokine (or other protein), which will become bound to the primary antibody.
10. Wash cells and unbound cytokine away by incubating with PBS 0.1% Tween 20 for 10 minutes. Then 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.



11. Detect by adding the two conjugated secondary antibodies:
In general, the two secondary antibodies can be co-incubated in the plate. Prepare a solution from stock in PBS 1% BSA that contains both secondary antibodies at their working concentrations. Dispense 100 µl of the solution into the wells. Seal the plate and incubate for 1 hour at 37°C.

If not using antibodies supplied in a dual ELISPOT kit, use secondary antibodies with different conjugates to enable the two detected proteins to be distinguished.



- a. *The base should be taken off the bottom of the plates, washing between substrate and developing solution (e.g., the wash between Streptavidin alkaline phosphatase conjugate and BICP/NBT development). 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.*
- b. *Monitor spot formation visually. As soon as the membranes look optimally developed, stop the reaction immediately by gently washing the plate. It is not a good idea to walk away while the spots are developing and the timing for optimal results will be slightly different each time.*
12. Empty wells and wash three times with PBS-0.1% Tween 20. Remove all residual buffer by repeated tapping on absorbent paper.
13. Prepare AEC buffer, or other required development buffer. Dispense 100µl of solution in wells.
14. Let the color reaction proceed for 5-15 min at room temperature. When the spots have developed empty the buffer into an appropriate tray.
15. Wash thoroughly both sides of the membrane with distilled water. Remove residual water by tapping the plate on absorbent paper.
16. Dispense 100µl of BCIP/NBT (or other development buffer depending on conjugates used) buffer in wells.
17. Let the reaction go for about 2-10 mins at room temperature. When spots have developed, empty the buffer into an appropriate tray.

18. Wash thoroughly both sides of the membrane with distilled water.
19. Dry the plates and let membranes dry completely
20. *Spots may become sharper if stored overnight at 4°C at this stage before reading. Membranes can be wrapped and stored at 4°C in the dark.*



21. Punch the membranes out of the wells onto a sticky plastic sheet. This step will depend on your plate reader's requirements. Consult plate reader manual.
22. Scan the sheet and analyse the membrane circles analysed on the computer. Set parameters using the software so computer can distinguish spots:
 - Size/spot diameter
 - Intensity/saturation
 - Circularity/shape
 - Spot development/slope

These parameters can be set and saved, then used for repeated experiments for standardized results.

* Indirect ELISPOT:

If the cells take some time to respond to stimulation, then they will need to be pretreated with the stimulant for an optimized amount of time (e.g. 24 hours) in a separate 96 well culture dish. They can then be transferred to the ELISPOT 96 well assay plate.

Positive control stimulation

Experiments to detect cytokines using ELISPOT will require use of a 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 and to compare as a positive control to other stimulant treatments used.

Ensure you are stimulating the PBMC's with the correct stimulant for detection of your target cytokine.



LPS

Activates Macrophages via the Toll receptor 4/complement pathways
E.g. Stimulation of IL1 beta, IL-6

PMA and Ionomycin. Cytokine lymphotoxins
E.g. Stimulation of IL-2, IL-4