ELISPOT Protocol

Coat the Plate:

1. Dilute Low-Endotoxin/Azide-Free sterile unlabeled capture antibody (BioLegend's LEAF™ format antibodies are specifically designed for this assay) to a final concentration of 0.5–4 µg/ml in sterile Coating Buffer and transfer 100 µl/well to a high affinity binding PVDF membrane ELISPOT plate (e.g., Millipore; Cat. No. MAIPS-4510).
2. Store plates overnight in humidified box at 4°C or at 37°C for ≥ 4 hours in humidified atmosphere.

Block the Plate:

3. Wash plate 3 times with sterile PBS, 200 µl/well.
4. Add 200 µl/well of sterile Blocking Buffer.
5. Seal plate and incubate at room temperature for ≥ 1 hour.
6. Wash plate 3 times with sterile PBS, 200 µl/well.

Set-Up Tissue Culture and Add Antigen or Mitogen:

7. Add appropriate sterile antigen or mitogen solution diluted in appropriate sterile tissue culture medium (TC) to ELISPOT plate, 100 µl/well.
8. Add cells diluted in sterile TC medium, 100 µl/well. Use 50,000-500,000 cells/well (the minimum number of cells should be determined in preliminary experiments).
9. Seal plate and incubate at 37°C 5% CO_2 in humidified atmosphere for the optimum stimulation period. BioLegend recommends a 24 hour incubation for IFN-γ, IL-2, and TNF-α, and a 48 hour incubation period for IL-4, IL-5, and IL-10 for most activation conditions.

Add Detection Antibody:

10. Wash plate 3 times with PBS, 200 µl/well.
11. Wash plate 3 times with PBS-Tween, 200 µl/well.
12. Add 100 µl/well of diluted biotinylated detection antibody at 0.25-2 µg/ml in PBS-Tween-BSA.
13. Seal the plate and incubate at 4°C overnight, or 2 hr at room temperature.

Add Avidin-Horseradish Peroxidase (Av-HRP):

14. Wash plate 4 times with PBS-Tween, 200 µl/well.
15. Add 100 µl per well of the Av-HRP conjugate (Cat. No. 405103) or other enzyme conjugate diluted to its pre-determined optimal concentration in PBS-Tween-BSA (usually between 1/500 – 1/2000).

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16. Seal the plate and incubate at room temperature for 1 – 2 hours.
17. Wash plate 3 times with PBS-Tween, 200 µl/well.
18. Wash plate 3 times with PBS, 200 µl/well.

Add Substrate:

19. Add 200 µl/well of fresh Substrate solution.
20. Monitor spot/color development at room temperature and stop reaction by rinsing plate with tap water and vigorously flicking plate over a waste container or sink, followed by blotting on paper towels or other absorbent materials.
21. Air dry plate overnight, until it is completely dry.
22. Count spots manually with a dissecting microscope or using an automated image acquisition/analysis unit (plates can be analyzed for up to 3 months).

SOLUTIONS & BUFFERS:

Note: Do not use sodium azide in any buffers or solutions as sodium azide inactivates the horseradish-peroxidase enzyme.

Phosphate Buffered Saline (PBS):

80.0 g NaCl

14.4 g Na_2HPO_4

2.4 g KH_2PO_4

2.0 g KCl

Add ddH_2O up to 10 L; pH to 7.2 with HCl

Coating Buffer:

Can use either Sterile PBS or

Sterile Carbonate Buffer (per ELISA protocol)

8.4 g Na HCO_3

3.56 g Na_2CO_3

Add ddH_2O up to 1.0 L, pH to 9.5.

PBS-Tween:

0.05% Tween-20 in PBS

(500 µl Tween-20 in 1L PBS)

Blocking Buffer (PBS-BSA):

1% BSA in PBS

(10 g BSA-Fraction V in 1L PBS)

PBS-Tween-BSA:

1% BSA in PBS-Tween

(10 g BSA-Fraction V in 1L PBS-Tween)

Tissue Culture (TC) Medium:

As appropriate for cells being analyzed

AEC Solution:

100 mg AEC (3-amino-9-ethyl-carbazole) in 10 ml DMF
(N,N, Dimethylformamide)

Solution should be prepared in a glass tube in a fume hood.

AEC Buffer:

(0.1 M Acetate): 148 ml 0.2 M acetic acid (11.55 ml glacial acetic acid per liter of water) and 352 ml of 0.2 M sodium acetate (27.2 g per liter of water)

Bring up to 1L with water and adjust to pH 5.0 if required

Substrate Solution:

800 µl AEC solution in 24 ml AEC buffer

Filter with 0.45 µm filter and add 12 µl 30% H_2O_2

Use immediately

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