ELISPOT Protocol

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Abstract

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Guidelines

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 Na2HPO4
2.4 g KH2PO4
2.0 g KCl
Add ddH2O 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 HCO3
3.56 g Na2CO3
Add ddH2O 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

Protocol

Coat the Plate

Step 1.
Dilute Low-Endotoxin/Azide-Free sterile unlabeled capture antibody (BioLegend'sLEAF™ format antibodies are specifically designed for this assay) to afinal concentration of 0.5–4 µg/ml in sterile Coating Buffer and transfer 100µl/well to a high affinity binding PVDF membrane ELISPOT plate (e.g., Millipore;Cat. No. MAIPS-4510).

Coat the Plate

Step 2.
Store plates overnight in humidified box at 4°C or at 37°C for ≥ 4 hours inhumidified atmosphere.

Block the Plate:

Step 3.
Wash plate 3 times with sterile PBS, 200 µl/well.

Block the Plate:

Step 4.

Add 200 µl/well of sterile Blocking Buffer.

Block the Plate:

Step 5.

Seal plate and incubate at room temperature for ≥ 1 hour.

Block the Plate:

Step 6.

Wash plate 3 times with sterile PBS, 200 µl/well.

Set-Up Tissue Culture and Add Antigen or Mitogen

Step 7.

Add appropriate sterile antigen or mitogen solution diluted in appropriatesterile tissue culture medium (TC) to ELISPOT plate, 100 µl/well.

Set-Up Tissue Culture and Add Antigen or Mitogen

Step 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 preliminaryexperiments).

Set-Up Tissue Culture and Add Antigen or Mitogen

Step 9.

Seal plate and incubate at 37°C 5% CO2 in humidified atmosphere for theoptimum stimulation period. BioLegend recommends a 24 hour incubationfor IFN-γ, IL-2, and TNF-α, and a 48 hour incubation period for IL-4, IL-5, andIL-10 for most activation conditions.

Add Detection Antibody

Step 10.

Wash plate 3 times with PBS, 200 µl/well.

Add Detection Antibody

Step 11.

Wash plate 3 times with PBS-Tween, 200 µl/well.

Add Detection Antibody

Step 12.

Add 100 µl/well of diluted biotinylated detection antibody at 0.25-2 µg/ml inPBS-Tween-BSA.

Add Detection Antibody

Step 13.

Seal the plate and incubate at 4°C overnight, or 2 hr at room temperature.

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 14.

Wash plate 4 times with PBS-Tween, 200 µl/well.

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 15.
Add 100 µl per well of the Av-HRP conjugate (Cat. No. 405103) or otherenzyme conjugate diluted to its pre-determined optimal concentration inPBS-Tween-BSA (usually between 1/500 – 1/2000).

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 16.
Seal the plate and incubate at room temperature for 1 – 2 hours.

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 17.

Wash plate 3 times with PBS-Tween, 200 µl/well.

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 18.
Wash plate 3 times with PBS, 200 µl/well.

Add Substrate

Step 19.

Add 200 µl/well of fresh Substrate solution.

Add Substrate

Step 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.

Add Substrate

Step 21.
Air dry plate overnight, until it is completely dry.

Add Substrate

Step 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).