ELISPOT Protocol Version 2

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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

Prepare the Plate

Step 1.

Prepare the PVDF membrane 96-well ELISPOT plates (e.g., Millipore Cat. No. MAIPS-4510) by soaking them in 35% ethanol for 30 seconds.

DURATION
00:00:30

Prepare the Plate

Step 2.

Wash thoroughly with PBS to remove any residual ethanol.

Note: Ethanol can negatively affect cell viability and antibody binding.

Coat the Plate

Step 3.

Dilute Low-Endotoxin/Azide-Free sterile unlabeled capture antibody (BioLegend'sLEAF™ format antibodies are specifically designed for this assay) to a final 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).

Note: BioLegend's LEAF™ and Ultra-LEAF™ format antibodies are specifically designed for this assay

Coat the Plate

Step 4.

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

Block the Plate:

Step 5.

Wash the plate 3 times with 200 µl/well of sterile PBS, gently tapping plates dry on a clean paper towel between each wash.

NOTES

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1. Since ELISPOT plates are more delicate than ELISA plates, they should be gently tapped and washed manually.
2. Do not use an automatic plate washer, since this could compromise the integrity of the PVDF membrane.

Block the Plate:

Step 6.

Add 200 µl/well of sterile Blocking Buffer.

Block the Plate:

Step 7.

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

Block the Plate:

Step 8.

Repeat step 5

Set-Up Tissue Culture and Add Antigen or Mitogen

Step 9.

Add 100µl/well of appropriate sterile antigen or mitogen solution diluted in appropriate sterile tissue culture (TC) medium.

Set-Up Tissue Culture and Add Antigen or Mitogen

Step 10.

Add 100µl/well of cells diluted in sterile TC medium. Use 5 x 10^4 to 5 x 10^5cells/well

NOTES

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1. The minimum number of cells should be determined in preliminary experiments.
2. When determining the optimal number of cells to use, keep in mind the expected levels of expression of the target protein. If the expression is expected to be low, use a higher number of cells.
3. If the cells can withstand the environment, use a serum-free media. Serum contains proteins that can affect results or give a high background or nonspecific signal.

Set-Up Tissue Culture and Add Antigen or Mitogen

Step 11.

Seal plate and incubate at 37°C 5% CO_2 in humidified atmosphere for the optimum stimulation period.

NOTES

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1. BioLegend recommends a 24 hour incubation for IFNγ, IL-2, and TNFα; and a 48 hour incubation for IL-4, IL-5, and IL-10 for most activation conditions.
2. Do not shake or move the plates while the cells are culturing. This will lead to spots that are not well-defined.
3. If your cells take more than 48 hours to respond to stimulation, they can be treated with the stimulant in a separate 96-well plate prior to transferring to the ELISPOT plat

Add Detection Antibody

Step 12.

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

Add Detection Antibody

Step 13.

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

Note: Tween-20 is included in the wash buffer to aid detachment of any cells that have attached during overnight cell culture.

Add Detection Antibody

Step 14.

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

Add Detection Antibody

Step 15.

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

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 16.
Wash plate 4 times with PBS-Tween, 200 µl/well.

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 17.
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 18.
Seal the plate and incubate at room temperature for 1 – 2 hours.

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 19.

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

NOTES
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Take the base off of the bottom of the plate to ensure it is thoroughly washed. This will help prevent high background, since some reagents can leak through the PVDF membrane and stick to the base or bottom of the plate

Add Avidin-Horseradish Peroxidase (Av-HRP)

Step 20.
Wash plate 3 times with PBS, 200 µl/well.

NOTES
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- When you are done washing, be sure to replace the base to the bottom of the plate.

Add Substrate

Step 21.

Add 200 µl/well of fresh Substrate solution.

Add Substrate

Step 22.

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 23.

Air dry plate overnight, until it is completely dry.

NOTES

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- Spots could become sharper if the plates are stored overnight at 4°C. Wrap plates in foil prior to storing.

Add Substrate

Step 24.

Count spots manually with a dissecting microscope or using an automated image acquisition/analysis unit (plates can be analyzed for up to 3 months).