Dec 07, 2019

ELISPOT Protocol V.3

Sam Li[1]

[1]BioLegend

1 Works for me dx.doi.org/10.17504/protocols.io.98yh9xw

BioLegend

Sam Li
BioLegend

EXTERNAL LINK

https://www.biolegend.com/protocols/elispot-protocol/4249/

GUIDELINES

Notes: Do not use sodium azide in any buffers or solutions as sodium azide inactivates the horseradish-peroxidase (HRP) enzyme.

MATERIALS TEXT

- **Phosphate Buffered Saline (PBS):** 80.0g NaCl
  14.4g Na_2HPO_4, 2.4g KH_2PO_4, 2.0g KCl
  Add ddH_2O up to 10L; pH to 7.2 with HCl
- **Coating Buffer:** Can use either Sterile PBS or
  Sterile Carbonate Buffer (per ELISA protocol)
  8.4g Na HCO_3, 3.56g Na_2CO_3, add ddH_2O up to 1.0L, pH to 9.5.
- **PBS-Tween:**
  0.05% Tween-20 in PBA (500µl 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.
- **Substrate Solution:**
  800µl AEC solution in 24ml AEC buffer
  Filter with 0.45µm filter and add 12µl 30% H_2O_2. Use immediately.

Prepare the Plate:

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.

2 Wash thoroughly with PBS to remove any residual ethanol.Note: Ethanol can negatively affect cell viability and antibody binding.

Coat the plate:

3 Dilute Low-Endotoxin/Azide-Free sterile unlabeled capture antibody to a final concentration of 0.5-4µg/ml in sterile Coating Buffer and transfer 100µl/well to the prepared ELISPOT plate.Note: BioLegend's LEAF™ and Ultra-LEAF™ format antibodies are specifically designed for this assay.

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

Block the Plate:

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.

5.1 Since ELISPOT plates are more delicate than ELISA plates, they should be gently tapped and washed manually.

5.2 Do not use an automatic plate washer, since this could compromise the integrity of the PVDF membrane.

6 Add 200µl/well of sterile Blocking Buffer.

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

8 Repeat step 5.

Set-Up Tissue Culture and Add Antigen or Mitogen:

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

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

10.1 The minimum number of cells should be determined in preliminary experiments.

10.2 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.

10.3 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.

11 Seal the plate and incubate at 37°C with 5% CO_2 in a humidified atmosphere for the optimal stimulation period.

11.1 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.

11.2 Do not shake or move the plates while the cells are culturing. This will lead to spots that are not well-defined.

11.3 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 plate.

Add Detection Antibody:

12 Wash plate 3 times with 200µl/well of PBS.

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

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

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

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

Add Avidin-Horseradish Peroxidase (Av-HRP):

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

17 Add 100µl/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 dilution).

18 Seal the plate and incubate at room temperature for 1-2 hours.

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

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.

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

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

Add Substrate:

21 Add 200µl/well of fresh Substrate solution.

22 Monitor spot/color development at room temperature and stop the reaction by rinsing the plate with tap water and vigorously flicking the plate over a waste container or the sink, followed by blotting on clean paper towels or other absorbent materials.

23 Air dry the plate overnight, or until it is completely dry.

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

24 Count spots manually with a dissecting microscope, or using an automated image acquisition/analysis unit.

Plates can be stored and analyzed for up to 3 months.