ImmunoSpot®

THE ELISPOT Source

PROTOCOL

Murine TNF-α / IL-2 Double-Color Enzymatic ELISPOT Assay

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YouTube

Visit our YouTube channel for several helpful videos on working with ELISPOT and FluoroSpot assays: **www.youtube.com/user/ImmunoSpot.**

Protocols and technical resources available at **www.immunospot.com.**

PROCEDURE

DAY 0 — STERILE CONDITIONS

- Prepare *Murine TNF-α/IL-2 Capture Solution* and prepare 70% ethanol (see Solutions).
- Remove plate underdrain, pipette 15µl of 70% ethanol into each well and incubate for less than one minute. Add 150µl of PBS, decant, and wash with 150µl of PBS two more times. (If using strip plates, there is no underdrain to remove before prewetting.) **Note:** Activitation of the membrane with ethanol is instantaneous and can be seen visually as a graying of the membrane. Ethanol should be washed off as quickly as possible following activation.
- Replace underdrain and immediately (before plate dries) pipette 80µl/well *Murine TNF-α/IL-2 Capture Solution.* Seal plate with parafilm and incubate at 4°C overnight.

DAY 1 — STERILE CONDITIONS

- Prepare *CTL-Test™ Medium* (see Solutions).
- Prepare antigen/mitogen solutions at two times final concentration in *CTL-Test™ Medium.*
- Decant plate containing *Capture Solution* from Day 0 and wash one time with 150µl PBS.
- Plate antigen/mitogen solutions. Ensure the pH and temperature are ideal for cells by placing the plate containing antigens into a 37°C incubator for 10-20 minutes before plating cells.
- Adjust cells to desired concentration in *CTL-Test™ Medium*, e.g.: 3 million/ml corresponding to 300,000 cells/well (cell numbers can be adjusted according to expected spot counts since 100,000-800,000 cells/well will provide linear results). Keep cells at 37°C in humidified incubator, 5-9% CO_2 while processing cells and until plating.
- Plate cells 100µl/well using large orifice tips. Once completed, gently tap the sides of the plate and immediately place into a 37°C humidified incubator, 5-9% CO_2.
- Incubate for 24 hours. Do not stack plates. Avoid shaking plates by carefully opening and closing incubator door. Do not touch plates during incubation.

DAY 2

- Prepare Wash Buffers for the day: PBS, distilled water and Tween-PBS.
- Prepare *Anti-murine TNF-α/IL-2 Detection Solution* (see Solutions).
- Wash plate two times with PBS and then two times with 0.05% Tween-PBS, 200µl/well each time.
- Add 80µl/well *Anti-murine TNF-α/IL-2 Detection Solution.* Incubate at room temperature, two hours.
- Prepare *Tertiary Solution* (see Solutions).
- Wash plate three times with 0.05% Tween-PBS, 200µl/well.
- Add 80µl/well of *Tertiary Solution.* Incubate at room temperature, one hour.
- During incubation, prepare *Blue* and *Red Developer Solutions* (see Solutions).
- Wash plate two times with 0.05% Tween-PBS, and then two times with distilled water, 200µl/well each time.
- Add *Blue Developer Solution,* 80µl/well. Incubate at room temperature, 15 minutes.
- Stop reaction by gently rinsing membrane with tap water, decant, and repeat three times.
- Decant plate with tap water and wash once more with distilled water, 200µl/well.
- Add *Red Developer Solution,* 80µl/well. Incubate at room temperature, 5-10 minutes.
- Stop reaction by gently rinsing membrane with tap water, decant, and repeat three times.
- Remove protective underdrain from the plate and rinse back of plate with tap water.
- Air-dry plate for two hours in running laminar flow hood or for 24 hours face down on paper towels on bench top.
- Scan and count plate. (CTL has scanning and analysis services available with the purchase of any kit. Email kitscanningservices@immunospot.com for more info.)

See other side for Solutions and Technical Tips.

SOLUTIONS

All solutions should be freshly-made prior to use. It is important to quick-spin the vials before use to ensure content volumes.

- 70% Ethanol (not included): Dilute 190-200 proof Ethanol. For 10ml, add 7ml of ethanol to 3ml of distilled water.
- *CTL-Test™ Medium:* Prepare medium by adding 1% fresh L-glutamine. The amount of medium needed will depend on variables such as cell yield and number of samples tested but will be no less than 20ml for one full plate. Warm to 37°C before using.
- *Capture Solution:* Dilute *Murine TNF-α* and *Murine IL-2 Capture Antibodies* in *Diluent A.* For one plate, add 160µl of *Murine TNF-α* and 80µl of *Murine IL-2 Capture Antibodies* to 10ml of *Diluent A.*
- *Detection Solution:* Dilute *Anti-murine TNF-α (Biotin)* and *Anti-murine IL-2 (FITC) Detection Antibodies* in *Diluent B.* For one plate, add 10µl of *Anti-murine TNF-α(Biotin)* and 10µl of *Anti-murine IL-2 (FITC) Detection Antibodies* to 10ml of *Diluent B.*
- *Tertiary Solution:* Dilute *Strep-AP* 1:1000 and *FITC-HRP* 1:500 in *Diluent C.* For one plate, add 10µl of *Strep-AP* and 20µl of *FITC-HRP* to 10ml of *Diluent C.*
- *Blue Developer Solution:* To develop *TNF-α* spots, add the *Substrate Solutions* in sequential steps to 10ml of *Diluent Blue.*

  For one plate:

  Step 1 – Add 160µl of *S1* to 10ml of *Diluent Blue.* Mix well!
  Step 2 – Add 160µl of *S2.* Mix well!
  Step 3 – Add 92µl of *S3.* Mix well!
- *Red Developer Solution:* To develop *IL-2* spots, add the *Substrate Solutions* in sequential steps to 10ml of *Diluent Red.*

  For one plate:

  Step 1 – Add 180µl of *R1* to 10ml of *Diluent Red.* Mix well!
  Step 2 – Add 160µl of *R2.* Mix well!

  It is recommended to make the Blue and Red Developer Solutions within ten minutes of use and to keep them protected from direct light.

Wash Buffers (not included)

For each plate prepare:

- 0.05% Tween-PBS: 100µl Tween-20 in 200ml PBS
- PBS, sterile, 100ml
- Distilled water, 100ml

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TECHNICAL TIPS

- Upon successful completion of the assay, TNF-α spots will be blue and IL-2 spots will be red.
- CTL highly recommends doing single-color, positive control wells for color compensation during analysis.
- We highly recommend the use of CTL Serum-free Media for freezing, washing, and testing cells. Even brief exposure to a mitogenic serum can cause high background while other sera can have suppressive effects. CTL also recommends using the CTL-LDC™ Kit for accurate live/dead cell counts.
- Deviations from specified temperatures, timing requirements, number of washing steps, and specified reagent preparation volumes may alter the performance of the assay.
- Plates may be washed manually or with a suitable automated plate washer with adjusted pin length and flow rate so membranes and spots are not damaged (CTL recommends the CTL 405LSR).
- When coating ELISPOT/ FluoroSpot plates, the plates need to be stored overnight at 4°-8°C at a minimum they can remain in at 4°-8°C in a humid chamber for up to one week.
- If culturing cells prior to plating the cells in an ELISPOT assay in serum-containing media, higher viability is often seen when the cells are transferred into the same formulation of culture medium (minus IL-2) for the ELISPOT assay. Pre-cutured cells are often constitutively secreting cytokine; at these times, a blocking step, with assay medium for one hour at room temperature, is recommended.
- An initial cell concentration 1-5 x 10^5 cells/well may be required. A serial titration of cells starting at a maximum of 1 x 10^6 cells/well is recommended in order to determine the optimal cell concentration.
- To avoid damage to the PVDF membrane in the wells, do not touch the membrane with pipette tips, with a plate washer or the underdrain. The PVDF membrane is permeable and protected by an underdrain. Do not push the underdrain too far up on the plate after the pre-wetting step or if it falls off as this will cause leaking of the membrane. Pressing down on the plate frame or the lid rather than the underdrain itself is preferred. It is OK if the underdrain falls off after the cell incubation, just avoid direct contact of the membrane with your hand, wet surfaces or absorbant materials.
- While processing plates, the PVDF membrane must remain wet. Therefore, when blotting the plate on paper towels, the goal is to eliminate excess wash buffer volume without completely drying the membrane.
- ELISPOT plates are stable for years and they can be scanned weeks to months later without any loss of signal when stored away from direct light. FluoroSpot plates should be kept in the dark and should be scanned within one week for optimal signal.
- Spots may not be readily visible while the membrane is still wet. Scan and count plates only after membranes have completely dried.
- Data analysis: The CTL ImmunoSpot® Analyzers along with the ImmunoSpot® Software have advanced features that permit automated, objective recognition of spots, gating and counting. An ELISPOT data management tool, SpotMap®, is also available to facilitate high-throughput ELISPOT work.

The CTL team will gladly assist you with data analysis and troubleshooting, as well as in customizing ELISPOT assays to suit your needs. Please contact us at kits@immunospot.com.

See other side for Contents and Procedure.

For laboratory research use only. Not for use in diagnostic or therapeutic procedures.

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