PROTOCOL

Murine IFN-γ / IL-2 Double-Color Enzymatic ELISPOT Assay

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

PROCEDURE

DAY 0 — STERILE CONDITIONS

- Prepare *Murine IFN-γ/IL-2 Capture Solution* and prepare 70% ethanol (see Solutions).
- Remove plate underdrain, pipette 15µl of 70% ethanol into each well quickly. 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. Wash ethanol off as quickly as possible following activation.
- Replace underdrain and immediately (before plate dries) pipette 80µl/well *Murine IFN-γ/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 (and provided stimulatory reagent Anti-*CD28,* 0.1µg/ml, if desired), 100µl/well. Ensure the pH and temperature are ideal for cells by placing the plate containing antigens into a 37°C incubator, 5-9% CO_2 if it will be more than 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 IFN-γ/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 IFN-γ/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 and offers a trial version of ImmunoSpot® Software with the purchase of any kit. Email kitscanningservices@immunospot.com.) **Note:** Fluorescent signals must be read with compatible light source(s) and filter sets. The optimized settings differ depending on the model of instrument used. Please consult with Technical Support for assistance at +1 216-791-5084.

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 IFN-γ* and *Murine IL-2 Capture Antibodies* in *Diluent A*. For one plate, add 60µl of *Murine IFN-γ* and 80µl of *Murine IL-2 Capture Antibodies* to 10ml of *Diluent A*.
- *Detection Solution:* Dilute *Anti-murine IFN-γ (Biotin)* and *Anti-murine IL-2 (FITC) Detection Antibodies* in *Diluent B*. For one plate, add 10µl of *Anti-murine IFN-γ (Biotin)* and 10µl of *Anti-murine IL-2 (FITC) Detection Antibodies* to 10ml of *Diluent B* and filter through a 0.1µm low protein binding filter.
- *Tertiary Solution:* Dilute *Strep-AP* 1:1000 and *FITC-HRP* 1:1000 in *Diluent C*. For one plate, add 10µl of *FITC-HRP* and 10µl of *Strep-AP* to 10ml of *Diluent C*.
- *Blue Developer Solution:* To develop *IFN-γ* 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, IFN-γ spots will be blue IL-2 spots will be red.
- CTL highly recommends doing single-color, positive control wells for color compensation during analysis.
- To maximize the use of each non-precoated plate, an adhesive plate-sealing sheet has been included that can be adhered to the top of the plate to cover unused wells for use in subsequent assays. Use your thumbs to firmly adhere the sheet to the plate and a razor blade to cut the sheet to expose only the necessary wells.
- 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.
- Low protein binding PVDF syringe filters (Millipore catalog #SLVV033RS) should be used for filtration of the Detection Solution to avoid loss of protein content.
- 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).
- To avoid damage to the PVDF membrane in the wells, do not touch the membrane with pipette tips or with the plate washer. The PVDF membrane is permeable and protected by an underdrain. Avoid direct contact between the well bottom and wet surfaces, including paper towels or any other materials that will absorb liquid.
- While processing plates, the PVDF membrane at the bottom of the wells must remain wet.
- After completion of the experiment, do not dry the ELISPOT assay plates at temperatures exceeding 37°C as this may cause the membrane to crack.
- Spots may not be readily visible while the membrane is still wet. Scan and count plates only after membranes have completely dried.
- Higher background appearing in the control wells can be potentially overcome using the following steps:
  - When working with precultured cells, wash the cells thoroughly in CTL-Wash™ prior to the experiment in order to avoid carryover of cytokines and other substances; use CTL-Test™ for testing cells.
  - The SmartCount™ module of the ImmunoSpot® counting software automatically recognizes spots over high background or uneven background, correcting background deviations. The Autogating™ module will help discern between T cell-derived and background spots. The CTL technical support team will gladly assist you with using the ImmunoSpot® Software for the analysis of complicated test results.
- 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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