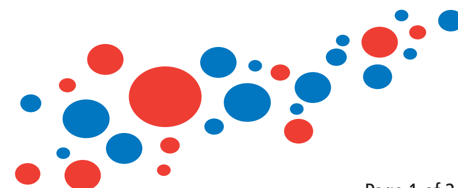
IMMUNOSPOT®
by C.T.L.



PROTOCOL

Murine IFN-γ Single-Color Enzymatic ImmunoSpot®

CONTENTS

- *Murine IFN-γ Capture Ab*
- *Anti-murine IFN-γ (Biotin) Detection Ab*
- *Strep-AP*
- *CTL-Test™ Medium*
- *Diluent A*
- *Diluent B*
- *Diluent C*
- *Diluent Blue*
- *S1 (Blue substrate component 1)*
- *S2 (Blue substrate component 2)*
- *S3 (Blue substrate component 3)*
- *Plates: 96-well, high-protein-binding, PVDF filter plates*
- *Protocol*

Visit our Video Library for several helpful videos on working with ELISPOT/ FluoroSpot assays, PBMC and splenocytes:

www.youtube.com/user/ImmunoSpot.

PROCEDURE

DAY 0: COAT THE ELISPOT PLATE (STERILE CONDITIONS)

- Prepare *Capture Solution* and prepare 70% ethanol (see Solutions).
- **Pre-wetting:** Remove underdrain from the PVDF plate and set aside. Pipette 15µl of 70% ethanol into each well and then immediately add 150µl/well of PBS. Decant and wash two more times with 150µl/well of PBS.

  Note: Activation of the membrane with ethanol is instantaneous and can be seen visually as a graying of the membrane. It is important to be sure that the ethanol solution has wicked across the entire membrane of all wells before adding the first PBS wash.
- **Coating:** Decant PBS from plate, blot excess on paper towels and replace the underdrain. Do not press directly on the bottom of the underdrain. Instead, line up the underdrain, and put pressure on the plate frame to spread force evenly across the entire plate. Quickly pipette 80µl of the Capture Solution in each well.
- Seal plate in parafilm or place it in a humid chamber and incubate overnight at 4°-8°C.

DAY 1: CELL CULTURE (STERILE CONDITIONS)

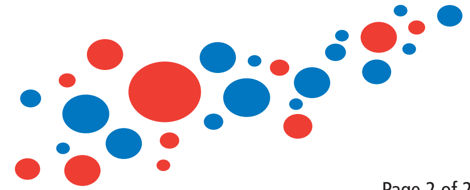
- **Prepare media and antigen solutions:** Prepare CTL-Test™ Medium (see Solutions).
- Prepare antigen/mitogen solutions at two times final concentration in CTL-Test™ Medium.
- **Culture:** Decant plate containing Capture Solution from Day 0, wash one time with 150µl PBS and blot on paper towel to remove excess wash. **If using pre-coated plates, begin after this wash step.**
- Plate antigen/mitogen solutions, 100µl/well. If it will be more than 10-20 minutes before plating cells, place the plate containing antigens into a 37°C humid incubator, 5-10% CO_2 to ensure the pH and temperature are ideal for cells.
- Adjust splenocyte or other cell type 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 processed cells at 37°C in humidified incubator, 5-10% CO_2 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, 5-10% CO_2.
- Incubate plate overnight, 18-24 hours. Incubation times can vary but most mouse cytokines are secreted within 24 hours. When low frequency responses are expected, incubations up to 48 hours can be useful.
- Do not stack plates. Avoid shaking plates by carefully opening and closing incubator door. Do not touch plates during incubation.

DAY 2: DETECTION

- **Prepare wash solutions:** Prepare Wash Buffers: PBS, distilled water, and Tween-PBS (see Solutions).
- **Detection:** Prepare Detection Solution (see Solutions).
- After the completion of assay incubation, decant and wash plate twice with 150µl/well of PBS.
- Wash plate two times with 0.05% Tween-PBS, 150µl/well. Decant final wash and blot excess wash solution out on paper towels without allowing the membrane to dry.
- Add 80µl/well of Detection Solution. Incubate at room temperature, in the dark, for two hours.
- Prepare Tertiary Solution (See Solutions).
- Wash plate three times with 0.05% Tween-PBS, 150µl/well each time. Decant final wash and blot excess wash solution out on paper towels without allowing the membrane to dry.
- Add 80µl/well of Tertiary Solution. Incubate at RT, in the dark, for 30 minutes.
- Prepare Substrate Solution (See Solutions).
- Decant and wash plate three times with 150µl/well of distilled water. Decant final wash and blot excess wash solution out on paper towels without allowing the membrane to dry.
- Add 80µl/well of CTL TrueBlue Substrate Solution. Incubate at RT for 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 water.
- Air-dry plate in running laminar flow hood or overnight face down on paper towels on bench top. Scan and count dry plate. CTL has scanning and analysis services available. Email kitscanningservices@immunospot.com to inquire.

See other side for Solutions and Technical Tips.

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



SOLUTIONS

Quick Spin all vials to ensure contents prior to using. When using partial plates or strip plates, it is advised to calculate the volumes of each Reagent Solution at 80ul per well to ensure adequate volumes of all Reagents.

- 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 *Capture Antibody* in *Diluent A.* For one plate, add 60µl of *Murine IFN-γ Capture Antibody* to 10ml of *Diluent A.*
- *Detection Solution:* Dilute *Detection Antibody* in *Diluent B.* For one plate, add 50µl of *Anti-murine IFN-γ (Biotin) Detection Antibody* to 10ml of *Diluent B* (see Technical Tips).
- *Tertiary Solution:* Dilute enzymatic *Tertiary* in *Diluent C.* For one plate, add 10µl of *Strep-AP* to 10ml of *Diluent C* (see Technical Tips).
- *Blue Developer Solution:* Add individual components of *CTL TrueBlue Substrate Solution* 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!

  It is recommended to make the Blue Developer Solution within ten minutes of use and keep it 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

Upon successful completion of the assay, IFN-γ spots will appear blue.

Protocols and technical resources are available at
www.immunospot.com
For questions, contact us at **kits@immunospot.com**

ELISPOT/ FLUOROSPOT TECHNICAL TIPS

- It is recommended to use CTL Serum-Free™ Media for freezing, washing, and testing PBMC and many other cell types. Even brief exposure to a mitogenic serum can cause high background while other sera can have suppressive effects. CTL recommends using the CTL-LDC™ Kit for accurate live/dead cell counts.
- CTL does not recommend resting cells overnight after isolation because it is typically not needed. Resting may lead to a higher percentage of functioning cells; however, this may also result in significant cell loss overall and can often be mitigated by increasing the number of cells plated initially.
- It is never recommended to use an RBC lysis buffer for cells used for an ELISPOT assay as it leads to impaired cellular functionality.
- If culturing/ proliferating cells prior to plating them in an ELISPOT assay, higher viability is often seen when the cells are transferred into the same formulation of culture medium for the ELISPOT assay (minus IL-2 for ELISPOT assay, if using) rather than switching to CTL-Test media. Pre-cultured cells are often constitutively secreting cytokines; at these times, a blocking step is recommended with the fresh culture media for 1 hour at room temperature.
- Deviations from specified temperatures, timing requirements, number of washing steps, and specified reagent preparation volumes may alter the performance of the assay. (Longer substrate incubation times will result in darker spots. Lighter spots have larger differences between the RGB values making it easier to separate color during analysis; therefore, shorter substrate incubation times are generally encouraged for double-color enzymatic assays.)
- Double-color enzymatic ELISPOT assays should have single-color control wells within the plate. This is best achieved by eliminating one detection antibody but using double-color reagents for all other steps in single-color control wells.
- The Anti-CD28 reagent is only provided in kits that capture both IFN-γ and IL-2 and triple-color mouse kits with IL-10 or IL-17 to help mitigate the effects of the loss of the captured cytokines in the supernatant. When Anti-CD28 is omitted in these multi-color ELISPOT/ FluoroSpot assays, a reduction in spots should be expected.
- When coating ELISPOT/ FluoroSpot plates, the plates need to be stored overnight at 4°-8°C at a minimum, but they can remain at 4°-8°C in a humid chamber for up to one week.
- To avoid aggregates, CTL recommends using syringe filters for Detection Solutions and for Tertiary Solutions. CTL recommends using a 0.1µm low-protein binding filter (Millipore syringe filter catalog number SLVVM33RS or SLVVR33RS).
- While processing plates, the PVDF membrane must remain wet. To avoid drying of the membrane, leave the final wash of each step on the plate until the next reagent is prepared. In addition, when blotting the plate on paper towels, the goal is to eliminate excess wash buffer volume without completely drying the membrane.
- 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 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. Aligning the underdrain and then 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; avoid direct contact of the membrane with your hand, wet surfaces or absorbent materials.
- 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, 4°-8°C once dry, and should be scanned within one week for optimal signal.
- Scan and count plates only after membranes have completely dried. FluoroSpot assay plates can have decreased membrane auto-fluorescence if the plate is flipped face-down and distilled water is filtered through the plate using a vacuum manifold (Millipore MSVMTOO). Alternatively, plates can be left overnight to dry in the dark.
- When using strip plates, liquid can slip between the strips when decanting and pool in the underdrain. It is advised to double-check the underdrain prior to adding reagent to the wells and to wick any liquid seen in the underdrain.
- Data analysis: The CTL ImmunoSpot® Analyzers along with the ImmunoSpot® Software have advanced features that permit automated, objective recognition of spots, gating and counting.

See other side for Contents and Procedure.
For laboratory research use only. Not for use in diagnostic or therapeutic procedures.

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