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ImmunoSpot®
THE ELISPOT Source

PROTOCOL

Human IFN-γ / TNF-α / Granzyme B Three-Color FluoroSpot Assay

CONTENTS

Human IFN-γ/TNF-α/ Granzyme B Capture Kit:

- *Human IFN-γ Capture Ab*
- *Human TNF-α Capture Ab*
- *Human GrzB Capture Ab*
- *CTL-Test™ Medium*
- *Diluent A*
- *Diluent B*
- *Diluent C*
- *Plates: 96-well, low auto-fluorescent, high-protein-binding, PVDF filter plates*
- *Adhesive plate sealing sheet*
- *Protocol*

Human IFN-γ Detection Kit:

- *Anti-human IFN-γ (FITC) Detection Ab*
- *Anti-FITC Alexa Fluor® 488*

Human TNF-α Detection Kit:

- *Anti-human TNF-α (Hapten1) Detection Ab*
- *Anti-Hapten1 CTL-Yellow™*

Human Granzyme B Detection Kit:

- *Anti-human GrzB (Biotin) Detection Ab*
- *Strep CTL-Red™*

YouTube

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

The CTL Thawing Protocol for Cryopreserved Human PBMC is available at **www.immunospot.com.**

PROCEDURE (If using precoated plates, start at Day 1)

DAY 0 — STERILE CONDITIONS

- Prepare *Human IFN-γ/TNF-α/Granzyme B 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. **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 *Human IFN-γ/TNF-α/Granzyme B 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, 100µl/well. Ensure the pH and temperature are ideal for cells by placing the plate containing antigens into a 37°C, 5-9% CO_2 incubator if it will be more than 10-20 minutes before plating cells.
- Adjust PBMC 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 PBMC and until plating.
- Plate PBMC, 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. (Prestimulation of cells with an antigen is often needed to elicit a Granzyme B response. The duration of this prestimulation is antigen-dependent and can last up to one week. Alternatively, PHA is an excellent positive control and is only a 24-hour assay.) Do not stack plates. Avoid shaking plates by carefully opening and closing incubator door. Do not touch plates during incubation.

DAY 2

- Prepare Buffer Solutions: PBS, distilled water and Tween-PBS (see Wash Buffers).
- Prepare *Anti-human IFN-γ/TNF-α/Granzyme B 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-human IFN-γ/TNF-α/Granzyme B 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.
- Decant and wash plate three times with distilled water, 200µl/well. (Optimal results can be seen when the last water wash is filtered through with a vacuum manifold to get rid of any unbound tertiary.)
- Remove protective underdrain from the plate and rinse back of plate with sterile 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.

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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. 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 *Human IFN-γ*, *TNF-α*, and *Granzyme B, Capture Antibodies* in *Diluent A*. For one plate, add 40µl of *Human IFN-γ*, 80µl of *Human TNF-α,* and 80µl of *Human Granzyme B Capture Antibodies* to 10ml of *Diluent A.*
- ***Detection Solution:*** Dilute *Anti-human IFN-γ (FITC), Anti-human TNF-α (Hapten1),* and *Anti-human Granzyme B (Biotin) Detection Antibodies* in *Diluent B* and filter. For one plate, add 20µl of *Anti-human IFN-γ (FITC),* 30µl of *Anti-human TNF-α (Hapten1),* and 40µl of *Anti-human Granzyme B (Biotin) Detection Antibodies* to 10ml of *Diluent B* and filter through a 0.22µm filter.
- ***Tertiary Solution:*** Dilute *Anti-FITC Alexa Fluor® 488* (visualizes *IFN-γ*), *Anti-Hapten1 CTL-Yellow™* (visualizes *TNF-α*), and *Strep CTL-Red™* (visualizes *Granzyme B*) in *Diluent C* and filter. For one plate, add 25µl of *Anti-FITC Alexa Fluor® 488,* 25µl of *Anti-Hapten1 CTL-Yellow™*, and 50µl of *Strep CTL-Red™* in 10ml of *Diluent C* and filter through a 0.22µm filter.

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 and proper excitation, IFN-γ spots will fluoresce green, TNF-α spots will fluoresce yellow, and Granzyme B spots will fluoresce red.
- To maximize the use of each 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 PBMC. 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).
- 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.
- When underdrain and gloves are wet, the underdrain may be slippery and difficult to remove. Wipe gloves and underdrain with paper towel before removing.
- 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 PBMC.
  - 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.

C.T.L.

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