

PROTOCOL

Human IFN- γ / TNF- α / IL-4 Three-Color FluoroSpot Assay

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Human IFN- γ /TNF- α /IL-4 Capture Kit:

- Human IFN- γ Capture Ab
- Human TNF- α Capture Ab
- Human IL-4 Capture Ab
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- Diluent A
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- Diluent C
- Plates: 96-well, low auto-fluorescent, high-protein-binding, PVDF filter plates
- 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 IL-4 Detection Kit:

- Anti-human IL-4 (Biotin) Detection Ab
- Strep CTL-Red™

PROCEDURE (If using pre-coated plates, start at Day 1)

DAY 0 — STERILE CONDITIONS

- Prepare Human IFN- γ /TNF- α /IL-4 Capture Solution and prepare 70% ethanol (see Solutions).
- Remove plate underdrain, pipette 15 μ l of 70% ethanol into each well quickly. Add 150 μ l of PBS water, decant, and wash with 150 μ l of PBS two more times. **Note:** Activation 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 80 μ l/well Human IFN- γ /TNF- α /IL-4 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 150 μ l PBS.
- Plate antigen/mitogen solutions 100 μ l/well. Ensure the pH and temperature are ideal for cells by placing the plate containing antigens into a 37°C incubator, 5-9% CO₂ 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% CO₂ while processing PBMC and until plating.
- Plate PBMC, 100 μ l/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% CO₂.
- Incubate for 24-72 hours (pre-stimulating cells with an antigen for 24-48 hours in a culture flask or round bottom 96-well plate at 37°C 5-9% CO₂ then plating desired cell concentration and incubating for an additional 24 hours at 37°C can have optimal results for this assay). Do not stack plates. Avoid shaking plates by carefully opening and closing incubator door. Do not touch plates during incubation.

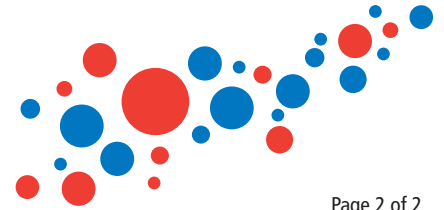
DAY 2

- Prepare Buffer Solutions: PBS, distilled water and Tween-PBS (see Wash Buffers).
- Prepare Anti-human IFN- γ /TNF- α /IL-4 Detection Solution (see Solutions).
- Wash plate two times with PBS and then two times with 0.05% Tween-PBS, 200 μ l/well each time.
- Add 80 μ l/well Anti-human IFN- γ /TNF- α /IL-4 Detection Solution. Incubate at room temperature, two hours.
- Prepare Tertiary Solution (see Solutions).
- Wash plate three times with 0.05% Tween-PBS, 200 μ l/well.
- Add 80 μ l/well of Tertiary Solution. Incubate at room temperature, one hour.
- Decant and wash plate three times with distilled water, 200 μ l/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. 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.

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

www.immunospot.com.

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



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 , Human TNF- α , and Human IL-4 Capture Antibodies in Diluent A. For one plate, add 40 μ l of Human IFN- γ , 80 μ l of Human TNF- α , and 80 μ l of Human IL-4 Capture Antibodies to 10ml of Diluent A.
- **Detection Solution:** Dilute Anti-human IFN- γ (FITC), Anti-human TNF- α (Hapten1), and Anti-human IL-4 (Biotin) Detection Antibodies in Diluent B and filter. For one plate, add 20 μ l of Anti-human IFN- γ (FITC), 30 μ l of Anti-human TNF- α (Hapten1), and 30 μ l of Anti-human IL-4 (Biotin) Detection Antibodies to 10ml of Diluent B and filter through a 0.22 μ m filter.
- **Tertiary Solution:** Dilute Anti-FITC Alexa Fluor® 488 (visualizes IFN- γ), Anti-Hapten1 CTL-Yellow™ (visualizes TNF- α), and Strep CTL-Red™ (visualizes IL-4) in Diluent C and filter. For one plate, add 25 μ l of Anti-FITC Alexa Fluor® 488, 25 μ l of Anti-Hapten1 CTL-Yellow™, and 50 μ l of Strep CTL-Red™ in 10ml of Diluent C and filter through a 0.22 μ m filter.

Wash Buffers (not included)

For each plate prepare:

- 0.05% Tween-PBS: 100 μ l 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 IL-4 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.
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