PROTOCOL

Human TNF-α / IgA / IgG Three-Color FluoroSpot Assay

CONTENTS:

Human Ig Capture Kit:

- *CTL-Test™ Medium*
- *CTL B-Poly-S™ Polyclonal B Cell Activator*
- *Human Ig Capture Ab*
- *Human TNF-α Capture Ab*
- *Diluent A*
- *Diluent B*
- *Diluent C*
- *Plates: 96-well, low auto-fluorescent, high-protein-binding PVDF filter plates*
- *Adhesive plate sealing sheet*
- *Protocol*

Human TNF-α Blue Detection Kit:

- *Anti-human TNF-α (Biotin) Ab*
- *SA-eFluor® 450*

Human IgA Red Detection Kit:

- *Anti-human IgA (Hapten2) Ab*
- *Anti-Hapten2 CTL-Red™*

Human IgG Yellow Detection Kit:

- *Anti-human IgG (Hapten1) Ab*
- *Anti-Hapten1 CTL-Yellow™*

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

PROCEDURE

DAY -3: Prestimulate B Cells — STERILE CONDITIONS

- Prepare *CTL-Test™ Culture Medium.*
- Adjust PBMC (freshly-isolated from blood or thawed from cryopreserved PBMC) in *CTL-Test™* to 4 million/ml.
- Add *B-Poly-S™* reagent to PBMC in *CTL-Test™* 1:1000 (see Solutions) and additional antigen for cytokine prestimulation, if necessary.
- Culture cells in a 37°C humidified incubator 5-9% CO_2 for 72-96 hours.

DAY 0: Coat the ELISPOT Plate — STERILE CONDITIONS

- Prepare *Capture Solution* containing the *Human Ig* and *Human TNF-α Capture Abs;* also prepare 70% ethanol (see Solutions).
- Remove plate underdrain, pipette 15µl of 70% ethanol in each well and incubate for less than one minute. 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. Ethanol should be washed off as quickly as possible following activation.
- Replace underdrain and immediately (before plate dries) pipette 80µl/well of the *Capture Solution* into the PVDF plate provided.
- Seal plate in parafilm and incubate at 4°C overnight.

DAY 1: Cell Harvest and Plating — STERILE CONDITIONS

- Prepare *CTL-Test™ Medium* (see Solutions).
- Prepare antigen/mitogen solutions at two times final concentration in *CTL-Test™ Medium* for *TNF-α* secretion.
- Decant plate containing *Capture Solution* from Day 0 and wash one time with 150µl PBS.
- 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).
- 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 6-24 hours. Do not stack plates. Avoid shaking plates by carefully opening and closing incubator door. Do not touch plates during incubation.

DAY 2

- Prepare Buffer Solutions: PBS, distilled water and Tween-PBS (see Wash Buffers).
- Prepare *Anti-human TNF-α/IgA/IgG 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 TNF-α/IgA/IgG 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 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 in the dark.
- 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 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™ Cell Culture 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.
- *CTL B-Poly-S™ Polyclonal B Cell Activator:* Add 10µl *B-Poly-S™* reagent to PBMC in 10ml *CTL-Test™ Medium* (1:1000) for polyclonal prestimulation.
- *Capture Solution:* Dilute *Human TNF-α* and *Human Ig Capture Antibodies* in *Diluent A*. For one plate, add 80µl of *Human TNF-α* and 200µl of *Human Ig Capture Antibodies* to 10ml of *Diluent A*.
- *Anti-human TNF-α/IgA/IgG Detection Solution:* Dilute the *Anti-human TNF-α (Biotin), IgA (Hapten2),* and *IgG (Hapten1) Detection Antibodies* in *Diluent B*. For one plate, add 20µl of the *Anti-human TNF-α (Biotin),* 25µl of the *Anti-human IgA (Hapten2),* and 25µl *Anti-human IgG (Hapten1) Detection Ab* to 10ml of *Diluent B* and filter through a 0.22µm filter.
- *Tertiary Solution:* Dilute *SA-eFluor® 450* (Blue), *Anti-Hapten2 CTL-Red™*, add *Anti-Hapten1 CTL-Yellow™* in *Diluent C* and filter. For one plate, add 10µl of *SA-eFluor® 450* (Blue), 25µl of *Anti-Hapten2 CTL-Red™*, and 25µl of *Anti-Hapten1 CTL-Yellow™* to 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, TNF-α spots will fluoresce blue, IgA spots will fluoresce red, and IgG spots will fluoresce yellow.
- Three-to-four day prestimulation cultures are recommended for most in vitro antibody-secreting cell measurements. Adhere strictly to sterile conditions for prestimulation culture. The *B-Poly-S™* provided with the Kit is sufficient for stimulation of 320 million PBMC at 4 million cells/ml. Additional *B-Poly-S™* can be purchased separately, Cat.# CTL-hBPOLYS-200.
- Isolations of B cells from PBMC are most successful when done after the three-to-four day prestimulation.
- High background can result from antibody carryover from the prestimulation culture. To avoid it, wash the cells thoroughly with medium prior to the experiment.
- 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 (Cat.# CTL-LDC-100) 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.
- If cell preparation prior to incubation will result in a long day, it is acceptable to incubate the detection solution overnight at 4°C.
- 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.
- 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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