

Human Immunology Center Core Laboratory
David H. Smith Center for Vaccine Biology and Immunology
Aab Institute of Biomedical Sciences

STANDARD OPERATING PROCEDURE: Detection of Multiple Human Cytokine responses by Fluorispot in Human peripheral blood mononuclear cells following stimulation with antigen.

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1.0 Purpose:

Cytokine secretion by peripheral blood lymphocytes is measured on a per cell basis by the capture of multiple (two to three) cytokines using cytokine-specific monoclonal antibodies bound to polyvinylidene (PVDF) filters in microwell plates by Fluorispot.

2.0 Principle:

A multicolor fluorescent Elispot assay (Fluorispot) detects the secretion of multiple cytokines by a single cell (up to 3 cytokines) while measuring the cytokine-secreting cell number in a population. A microwell plate with a polyvinylidene difluoride membrane is coated with two to three anti-cytokine antibodies in phosphate buffered saline (PBS) for 3 hours at room temperature. The plates are washed with RPMI medium containing 8% fetal bovine serum before adding peripheral blood mononuclear cells (PBMC) at varying concentrations from 1×10^5 to 5×10^5 per well. PBMC are incubated with allogeneic cells or with antigen/peptide for 24 to 40 hours at 37°C . The cells are removed and the plates are washed with PBS-Tween. Fluorochrome or biotinylated labeled anti-cytokine antibody is added sequentially to the well with washing between each step. A streptavidin fluorochrome conjugate is used to detect biotinylated anti-cytokine antibody. After removal of the streptavidin, the plastic backing is removed and plates are washed with PBS/Tween followed by tap water then dried and analyzed under a fluorescent microscope. The fluorescent image is digitally captured via camera and can be enumerated by color using (MATLAB) and Cell Quest software.

3.0 Materials and Reagents:

96 well PVDF microwell plates, Millipore Multicsreen HTC clear plates Catalog # MSIPN4550
Human peripheral blood mononuclear cells, thawed or fresh
Specific anti-cytokine antibody or monoclonal antibody for coating
For IFN- γ : Mouse monoclonal anti-human IFN- γ clone 7-B6-1 (Mabtech Catalog # 4320-5-250)
For IL-2: Mouse monoclonal anti-human IL-2 clone BD Bioscience Cat # 51-2516-KZ and biotinylated detection antibody BD Bioscience Cat # 51-2311-KZ (catalog #551884 pair)
Rabbit anti-human IFN- γ (Biosource Catalog # AHC4734)
Streptavidin-alkaline phosphatase fluorochrome conjugate or tertiary antibody fluorochrome conjugate
-Cychrome 3 goat anti-rabbit (IgG heavy & light chain specificity?) Jackson Lab Cat # 111-165-003

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-Streptavidin-Alexa 488 (Molecular Probes Cat # S-11223)
1 to 50 and 10 to 300 μ L air displacement pipets
disposable plastic pipet tips
1, 5, 10, 25 mL sterile disposable serological pipets
Tween 20 detergent
5, 15 and 50 mL plastic tubes, sterile
sterile reagent reservoirs
37 °C incubator with 5% CO₂
microplate washer
10% hypochlorite solution or 1% Vesphene
household bleach
Vesphene concentrate
Biological safety cabinet, Class II A
500 mL bottle Phosphate buffered saline 10X, Cellgro Mediatech Cat # 20-031-cv
Fetal Bovine Serum Hyclone or equivalent
Bovine Serum Albumin, Fraction V
RPMI 1640 medium
RPMI 1640 with 8% FBS =R8
10X PBS, Cellgro Mediatech Cat # 20-031-cv
25X phosphate buffered saline (PBS)
PBS- 0.1% Tween 20 (PBS-T) $pH\ 7.2 \pm 0.2$
PBS-0.1% Tween 20- 2% BSA solution (PBS-TB) $pH\ 7.2 \pm 0.2$
PBS-0.1% Tween 20- 2% BSA- 1% d-biotin
d-biotin, Sigma Cat.# B-4501
Perkin-Elmer TopSeal-A: 96-well microplate sealers
ZellNet Eli-puncher
See Appendix A for additional antibody reagents

3.1 Reagent preparation:

Coating buffer: Add 100 mL of 10X Phosphate Buffered Saline solution to a sterile 1L bottle and then add pyrogen free water sterile H₂O to 1 liter or use commercial 1X PBS. Check pH and adjust to pH 7.2 prior to sterile filtering or autoclaving. Store at r.t. Handle using sterile technique.

25X PBS preparation: 4000g NaCl, 100g KCL, 720g Na₂HPO₄, 120g KH₂PO₄, qs to 20 L with diH₂O. Take 1 mL and dilute with 24 mL of diH₂O. Check pH. Discard if pH is not 7.2 ± 0.2 units.

Wash buffer: PBS-T: 1600 mL of 25X PBS, 40 mL of Tween 20, qs to 40L with dH₂O. Check pH to meet $pH\ 7.2 \pm 0.2$.

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PBS-TB preparation: Add 20 mg of BSA per mL of PBS-T. Sterile filter and store at 4 degrees C.

PBS-TBB preparation: Add 10 mg d-biotin per mL of PBS-TB required for experiment + 5 mL extra. Prepare fresh daily and discard if not used.

4.0 Procedure:

- 4.1 Make the desired amount of sterile coating solution by diluting the anti-IFN γ Mab to 5 μ g/mL and the anti-IL-2 Mab to 10 μ g/mL in sterile coating buffer (PBS). (For anti-IFN γ Mab at 1 mg/mL use 5 μ L/mL.) (For anti-IL-2 Mab at 1 mg/mL use 10 μ L/mL.) Use polypropylene tubes, not polystyrene. Don't vortex. Mix thoroughly but minimally. Use immediately after mixing. Add 35 μ L/well coating solution with Mabs to a fluorispot microtiter plate, being careful not to touch the pipet tips to the filter. Cover with a plate lid. Tap the plate to wet the wells or place in the microplate reader on the shake cycle for 30 seconds. Cover with a plate lid. Incubate the Fluorispot plate for 3 hours at room temperature to coat the filter with antibodies.
- 4.2 Prepare fresh or frozen Peripheral blood mononuclear cells as described below:
 - 4.2.1. PBMC are isolated from fresh blood using ficoll hypaque separation or BD CPT tubes. Spin the PBMC twice in 10 mL of R8 at 400 g (1300 rpm) to remove platelets.
 - 4.2.2. PBMC frozen in LN $_2$ are rapidly thawed with shaking in 37 $^{\circ}$ C water bath until last ice crystal remains. The cells are immediately added to an empty 15 mL tube, and then cold R8 is added dropwise with gentle mixing. After about 2mls R8 have been added, the rate of addition can be increased. The diluted cell suspension is spun at 1000 RPM for 5 minutes at 4 $^{\circ}$ C to remove DMSO. The supernatant is removed by pouring into a waste container through gauze and into disinfectant solution. Tap tubes gently with finger to resuspend cells and repeat wash once. with 10 mL of cold R8 medium. Do not vortex or rub tube roughly along a tube rack. Resuspend cells in the required amount of R8 to a concentration of desired concentration (i.e. 2 X 10 6 cells/mL) Prepare a 1:10 dilution with 0.4 % trypan blue exclusion dye in saline (i.e. 5 μ L into 45 μ L). Count cells and record number. Adjust cell concentration to desired concentration.

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- 4.3 After coating the Fluorispot plate, add 200 μ L RPMI medium with 8% FBS (R8) to each well in the plate using a multichannel pipet. Aspirate the R8 with Costar aspirator with 8 channel adaptor and sterile 300 μ L tips. Immediately add the next wash to avoid wells drying out – never leave wells empty for more than one minute. Repeat the wash and aspirate two times. Do not aspirate the final R8 wash until the cell suspension is ready to immediately be added to the wells – leave the final wash for at least five minutes.
- 4.4 Gently and thoroughly mixed the cell suspension by drawing up and dispensing 3 times with a serological pipet. Add 100 μ L of cell suspension in RPMI with 8% FBS to the appropriate wells of the plate. Next add either 100 μ L of antigen diluted to the optimal concentration in R8 or R8 only to the appropriate wells of the fluorispot plate. All wells should contain a final of 200 μ L. After all cells and other components are added, mix all wells carefully with a multichannel pipettor set for 100 μ L. Each well should be mixed five times, starting at the four points of the compass, and moving through a 90° arc for each mix, and ending with one mix in the middle. This should ensure thorough suspension of all cells. Change tips for each row. Do not touch the bottom membrane, and do not blow bubbles. Cover the microtiter plate with the lid and place in a 37 °C incubator with 5 % CO₂ for 21 to 24 hours. to allow antigen stimulation of the cells. Record time, be specific, accurate to the hour. Do not stack plates, do not place too close to the (heated) door. Be careful not to tip or shake when handling. Close incubator door gently to avoid any vibration in the incubator.

Alternatively, assemble all reagents and cells in a round-bottom 96-well tray, and then use clean pipette tips to transfer the entire well contents (e.g. set pipettor for 210 μ L to transfer 200 μ L) to the coated Elispot tray.

- 4.5 Visually verify the plate is not contaminated as they are taken out of the incubator. (Optional - Wash the plate the first time by hand, pipetting the supernatant five times around the perimeter of the well, as well as in the center, to ensure all cells and debris are resuspended. Transfer the cell suspensions to a clean 96-well tray to assess cell viability and contamination.) Wash the plate on the microtiter plate washer six times with 250 μ L per well of PBS- 0.1% tween-20 soaking for 5 seconds between each wash.
- 4.6 Forcefully blot plate on towels to remove all supernatant. Add 35 μ L per well of PBS-TB containing 2.5 μ g/mL of biotinylated anti-IL-2 Mab and 1 μ g/mL Rabbit anti-IFN γ detection antibody. (i.e. 5 μ L/mL of biotinylated anti-IL-2 Mab and 2

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$\mu\text{L}/\text{mL}$ rabbit anti- $\text{IFN}\gamma$, filtered). Incubate for 2 hours at room temperature. Wash plate 3 times as in step 4.5.

4.7 Add 35 μL per well of a solution containing streptavidin-Alexa 488 at 5 $\mu\text{g}/\text{mL}$ and Goat anti-Rabbit IgG-Cy3 at 1 $\mu\text{g}/\text{mL}$ in PBS-TB. (i.e. SA-Alexa488 at 5 $\mu\text{L}/\text{mL}$ and G anti-Rab IgG-Cy3 at 2 $\mu\text{L}/\text{mL}$) Incubate the plates at room temperature in the dark (e.g. covered by foil, in a hood with the hood lights off) for 45 minutes. Wash 3 times as in step 4.5.

4.8 Remove the backing from the plate and soak the plate in a plastic container with PBS-T to cover for 1 hour at room temperature. Remove from the plate from the container and forcefully blot on absorbent paper toweling without touching the back membrane. Wash with eight changes of tap water and forcefully blot again. Dry plate in the dark in a biological safety cabinet with the hood on for 30 to 60 minutes.

4.9 To prepare for reading on a fluorescent microscope, use a plastic membrane punch to punch each well onto the sticky side of a microplate sealer. Read with fluorescent microscope and analyze with Matlab software program.

Appendix A contains information on alternate antibodies and fluorochrome systems that can be used when designing a fluorispot assay. The reagents expand to the use to IL-4 and IL-5 fluorispot.

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Appendix A: Coating and Detection Antibody Concentration

Cytokine	Species	<u>Coat Source Catalog number</u>	<u>ELISpot Coat Concentration</u>	<u>Coat Clone</u>	<u>Detection Source Catalog number</u>	<u>Detection concentration</u>	<u>Detection Clone</u>
IFN γ	human	Mab tech 3420-3-1000	10 μ g/mL	1-D1K	Mab tech Special order anti-IFN γ -FITC* Or Biosource AHC4734 Rabbit anti-hu IFN γ **	1 μ g/mL 1 μ g/mL	7-B6-1
IL-2	human	Mab tech 3440-3-1000 or BD 51-2516KZ	10 μ g/mL 10 μ L/mL	IL2-I	Mab tech 3440-6-1000*** or BD 51-2311KZ***	1 μ g/mL 10 μ L/mL	IL2-II biotin
IL-4	human	Mab tech 3410-3-1000	15 μ g/mL	TL4-I (82.4)	Mab tech 3410-6-1000 or BD554484 rat anti-hu IL4-FITC*	2 μ g/mL	IL4-II (12.1) MP4-25D2
IL-5	human	Mab tech 3490-3-1000 or any vendor for TRFK5	10 μ g/mL	TRFK5	Mab tech 3490-6-1000 (biotin) or BD 5544491	1 μ g/mL 2 μ g/mL	5A10 rat anti-hu IL-5

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* Amplified using Goat anti-FITC-Alexa488 InVitrogen Cat# A11096 as tertiary fluorochrome (2 μ L/mL).

** Goat anti-rabbit IgG heavy and light chain-Cychrome 3 Jackson Lab Cat# 111-165-003 (1 μ g/mL)

*** Streptavidin-Cychrome 2, 3 or 5 Jackson (1 μ L/mL) or SA-Alexa488

[Cychrome 2 is in same wavelength as FITC and Alexa488. Cychrome 3 or 5 should be used opposite these fluorochromes in the Fluorispot.]

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Revision History

Version	Change	Impact	Justification	Change Date:
HIC-1-0003	New		Control document management system	4/25/05
HIC-1-0003.1	Addition of Appendix with additional Specific Antibody Information	Information available for future ordering	Customer Requests for Information	4/5/07