

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

**STANDARD OPERATING PROCEDURE: Detection of Human Cytokine Response by Elispot in Human peripheral blood mononuclear cells following stimulation with antigen.**

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Approval:

### 1. Purpose:

The response of peripheral blood lymphocytes to antigen stimulation is measured by the capture of secreted cytokines with specific monoclonal antibodies bound to nitrocellulose filter microtiter plates by Elispot.

### 2. Principle:

The frequency of cytokine secreting cells in a lymphocyte population can be enumerated in Elispot. Frequencies are most often determined following antigenic stimulation *in vivo* or *in vitro* with antigen. A single anti-cytokine antibody diluted in PBS is allowed to bind to the wells of a polyvinylidene difluoride (PVDF) Elispot microtiter plate for a minimum of one hour at room temperature. The plates are washed with RPMI medium containing 8% fetal bovine serum/antibiotics/antimycotics before adding peripheral blood mononuclear cells (PBMC) at 1 to 5 X 10<sup>5</sup> cells per well and allowing antigen presenting cells to adhere to the well for 2 hours at 37° C. Either the non-adherent cells are removed (by washing 3X with RPMI with 8% serum) and CD4<sup>+</sup> or CD8<sup>+</sup> cells are selected to add back to the well or endogenous T cells from the PBMC are incubated with antigen/peptide for 24 hours to 48 hours. After incubation at 37° C the stimulated cells are removed and the plates are washed extensively with PBS-Tween. Biotinylated anti-cytokine antibody is added to the washed wells and incubated for 2 hours at room temperature. A streptavidin-alkaline phosphatase reagent is added for 30 minutes to 2 hours to detect biotinylated anti-cytokine antibody. Substrate is added to develop spots before reading on a CTL Elispot Analyzer with ImmunoSpot software.

### 3. Materials and Reagents:

96 well PVDF Elispot plates, Millipore, Catalog # MSIPN4W50

Human peripheral blood mononuclear cells

Specific anti-cytokine antibody or monoclonal antibody for coating (See Appendix A for specifics)

Recall Antigens (See Appendix B for specifics)

Paired biotinylated detection antibody (See Appendix A for specifics)

Phosphate buffered saline (Dulbecco's), Gibco Catalog # 14190-144

Fetal Bovine Serum, Hyclone, Catalog # SH30396.03

Bovine Serum Albumin(BSA), Bovuminar® Reagent Pure pH 7.0, Intergen, Cat # 3160-60

Digital 20-200µL Multichannel Pipettor, VWR, Catalog # 40000-294

Finnpipette 5-50µL single channel pipet, Thermo Electron, Cat # 4600220

Finnpipette 1-10µL single channel pipet, Thermo Electron, Cat # 4600180

Finnpipette 30-300µL single channel pipet, Thermo Electron, Cat # 4600240

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Sterile 20-200 $\mu$ L disposable pipet tips, Eppendorf, Catalog # 2249193-8  
Sterile 30-300 $\mu$ L disposable pipet tips, Eppendorf, Catalog # 2249194-6  
Sterile 0.1-20 $\mu$ L disposable pipet tips, Eppendorf, Catalog # 2249113-0  
1 mL sterile disposable serological pipets, Costar, Catalog # 4012  
5 mL sterile disposable serological pipets, Costar, Catalog # 4051  
10 mL sterile disposable serological pipets, Costar, Catalog # 4101  
25 mL sterile disposable serological pipets, Costar, Catalog # 4251  
Pipet Aid, Drummond, Catalog # 4-000-101  
Tween 20 detergent, Sigma, catalog # P1379  
15 mL sterile polypropylene conical tubes, Falcon, Catalog # 35-2096  
50 mL sterile polypropylene conical tubes, Falcon, Catalog # 35-2070  
12x75mm culture tubes with closures, VWR Cat # 60818-500  
Sterile reagent reservoirs, Costar, Catalog # 4871  
37 <sup>0</sup> C incubator with 5% CO<sub>2</sub> , Binder, Model # 9040-0030  
Microplate washer, Biotek, Model ELx405 Select CW  
CTL reader, Cellular Technology, LTD, Model ImmunoSpot® Series 3A Analyzer  
Streptavidin-alkaline phosphatase conjugate, Jackson, Catalog # 016-050-084  
Vector AP substrate kit, Catalog # SK-5300  
Household bleach for decontamination, VWR, Catalog # 37001-056  
Biological safety cabinet, Class II, Baker, Model # SG403  
10X phosphate buffered saline (Dulbecco's), Gibco, Catalog # 14200-075  
5 mL syringe, VWR, Catalog # BD301603  
10 mL syringe, VWR, Catalog # BD301604  
Filter for syringe, Corning, Catalog #431215  
Antibiotic/Antimycotic 100X Sigma Cat #A5955  
ImmunoSpot® Software, Cellular Technology, LTD  
RPMI 1640 medium, Cellgro, Catalog # 10-040-CV  
RPMI 1640 with 8% FBS/Antibiotics/Antimycotics (R8A<sup>2</sup>)  
PBS- 0.1% Tween 20  
PBS-0.1TWEEN-2% BSA  
100mM Tris-HCl Buffer pH 8.2  
10% hypochlorite solution  
Distilled water

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### 3.1 Reagent preparation:

- 3.1.1 Coating buffer: Dulbecco's Phosphate Buffered Saline: 8 g Na Cl; 0.2 g KCl; 2.16 g  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.2 g  $\text{KH}_2\text{PO}_4$ ; add  $\text{dH}_2\text{O}$  to 1 liter. pH 7.2 sterile filter or autoclave. Store at 4<sup>o</sup> C. Expires 30 days after opening.
- 3.1.2 10X Wash buffer: 10 X PBS: Dulbecco's Phosphate Buffered Saline: 80 g Na Cl; 2 g KCl; 21.6 g  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ; 2 g  $\text{KH}_2\text{PO}_4$ ; add  $\text{dH}_2\text{O}$  to 1 liter. pH 7.2 . Store at room temperature. Expires after 3 months if not sterile.
- 3.1.3 Wash buffer: Phosphate Buffered Saline/0.1% Tween-20: Add 500mL of 10 X PBS to carboy. Add 5.0 mL of Tween-20. QS to 5 liters with distilled water. pH 7.2. Expires within two weeks.
- 3.1.4 Assay Buffer: Dulbecco's Phosphate Buffered Saline/0.1% Tween-20/2%BSA: Add 100mL of 10 X PBS to clean beaker. Add 1 mL of Tween-20. Add 20 g BSA. QS to 1 liter with distilled water. Mix for 15 minutes on stir plate. pH 7.2. Sterile filter using a 0.45 micron filter into a sterile bottle. Expires within 24 hours if not sterile.
- 3.1.5 Media: RPMI/8%FBS: In hood, remove 40 mL RPMI from bottle and place in another sterile tube. Add 40 mL FBS. Add 5 mL antibiotics/antimycotics Mix. Store at 4°C.
- 3.1.6 Substrate Buffer: 100mM Tris-HCl pH 8.2: Add 12.114 g Tris to beaker. QS to 1 liter with Di water and mix. pH to 8.2 using HCl dropwise. Sterile filter using a 0.45 micron filter into sterile bottle. Store at room temperature. Expires after 3 months.

### 4. Procedure:

#### 4.1. Prepare fresh or frozen Peripheral blood mononuclear cells as described below:

- 4.1.1. PBMC are isolated from fresh blood using ficoll hypaque separation or BD CPT tubes and spun twice at 1000 rpm for 10 minutes to remove platelets.
- 4.2. Dilute the sterile anti-cytokine antibody or Mab to the optimal concentration into sterile Dulbecco's PBS. (See appendix A for Ab/MAB concentrations). Add 35  $\mu\text{L}$  per well being careful not to touch the pipet tips to the filter. Tap the plate to wet the wells and verify that the wells are entirely coated. Incubate the Elispot plate wells for 1 to 3 hours at room temperature or overnight at 4°C to coat the filter with antibody.
- 4.3. Add 100  $\mu\text{L}$  R8A<sup>2</sup> to each well in the plate using a multichannel pipet and aspirate using a sterile aspirator being careful not to touch the pipet tips to the filter. Repeat two times.

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- 4.4. Add 50  $\mu\text{L}$  of antigen diluted to the appropriate concentration in R8A<sup>2</sup> to the appropriate wells of the plate and then add 50  $\mu\text{L}$  per well of the cell suspension in R8A<sup>2</sup> that has been gently and thoroughly mixed being careful not to touch the pipet tips to the filter (Reference Appendix B). Cover microtiter plate and place in 37°C incubator with 5 % CO<sub>2</sub> for 4 to 48 hours depending on the cytokine to be detected. Reference appendix C. Do not stack plates. Be careful not to tip or shake when handling. (For IL-4 and IL-5, the volume is 100  $\mu\text{L}$  per well for a total volume of 200  $\mu\text{L}$ )
- 4.5. Inspect plate visually to determine if any contamination is present. Wells with discolored media may be an indication of contamination.
- 4.6. Wash the plate on the microtiter plate washer three times with 250 $\mu\text{L}$  per well of PBS- 0.1% tween-20 soaking for 5 seconds between each wash. Repeat wash step.
- 4.7. Add 30  $\mu\text{L}$  per well of the optimal concentration (Reference Appendix A) of the (sterile filtered) biotinylated detection antibody in PBS-0.1%Tween with 2% BSA being careful not to touch the pipet tips to the filter and incubate for 2 hours at room temperature. Wash 3 times as in step 4.6.
- 4.8. Add 30  $\mu\text{L}$  per well of streptavidin-alkaline phosphatase at a 1:1000 dilution in PBS-0.1%Tween with 2% BSA and incubate at r.t. for 1 hour. Wash 3 times as in step 4.6.
- 4.9. Soak the plates in PBS-0.1%Tween for 1 hour at room temperature after removing the back of the Elispot plate. After 1 hour, dry plate off by blotting dry onto absorbent paper.
- 4.10. Develop by adding 100  $\mu\text{L}$  per well being careful not to touch the pipet tips to the filter of Vector AP substrate kit III at room temperature in the dark from 3 to 120 minutes. Aspirate out the substrate using the plate washer or multichannel pipett being careful not to touch the pipet tips to the filter. Wash the plate on a microtiter plate washer as indicated in 4.6 using distilled water. Allow plates dry overnight.
- 4.11. After the plates have dried, read on CTL reader.

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## Appendix A: Coating and Detection Antibody Concentration

Cytokine	Species	Coat Source Catalog number	ELISpot Coat Concentration	Coat Clone	Detection Source Catalog number	Detection concentration	Detection Clone
IFN $\gamma$	human	Mab tech 3420-3-1000	10 $\mu$ g/mL	1-D1K	Mab tech 3420-6-1000	1 $\mu$ g/mL	7-B6-1
IL-2	human	Mab tech 3440-3-1000	10 $\mu$ g/mL	IL2-I	Mab tech 3440-6-1000	1 $\mu$ g/mL	IL2-II
IL-4	human	Mab tech 3410-3-18	15 $\mu$ g/mL	TL4-I (82.4)	Mab tech 3410-6-11	2 $\mu$ g/mL	IL4-II (12.1)
IL-5	human	Mab tech 3490-3-1000	10 $\mu$ g/mL	TRFK5	Mab tech 3490-6-1000	1 $\mu$ g/mL	5A10

**NOTE: Alternative antibodies for IL-2 can be obtain from R&D Systems, Catalog number SEL-202 and catalog number SEL202. Alternative antibodies for IL-5 can be obtained from BD Pharmingen, catalog #551085(coating and detection antibody included).**

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Appendix B: Recall Antigen Concentrations

<b>Recall Antigen</b>	<b>Source</b>	<b>Concentration</b>
Tetanus Toxoid	Calbiochem	1/500
Inactivated Flu 4907151	Charles River SPAFAS	5µg/mL
RSV	U of R	1/4

Appendix C: Incubation time

<b>Cytokine</b>	<b>Minimum Incubation Time (hours)</b>
IFNγ	18
IL-2	18
IL-4	40
IL-5	40

**Revision History**

<b>Version</b>	<b>Change</b>	<b>Impact</b>	<b>Justification</b>	<b>Change Date:</b>
HIC-1-0002	New		Control document management system	03/04/05
HIC-1-0002.1	Addition of Specific Antibody Information	Information available for future ordering	Customer Requests for Information	9/7/05
HIC-1-0002.2	Addition of Revision History	Change Tracking Record in SOP	Control document management	1/4/07