TITLE: DETECTION OF IFN-γ and IL-10 SECRETING CELLS BY ELISPOT
Modified Format March, 2002

Standard Operating Procedure

Document Number

P1-RD-011
Revision: 000

	Signature	Date
Originator/Reviser:		
Research and Development:		
Quality Assurance:		

APPROVAL STAMPS

TITLE : DETECTION OF IFN-γ and IL-10 SECRETING CELLS BY ELISPOT

1. PURPOSE

The purpose of this standard operating procedure is to detect and analyze IFN-γ and IL-10 cytokine producing cells in MS patients.

2. SCOPE

The number of IFN-γ and IL-10 secreting cells in PBMC of all the patients enrolled in the clinical trial will be determined prior to and post treatment.

3. REFERENCE DOCUMENTS

3.1. P1-RD-001 PBMC Isolation from Peripheral Blood
3.2. P1-RD-002 Limiting Dilution Assay

4. DEFINITIONS

4.1. ELISPOT = Enzyme-linked immunospot assay
4.2. PT = PBS and 0.05% Tween
4.3. PTB = PBS, 0.05% Tween, and 1% BSA
4.4. Cocktail peptides = (Y49T)BV5S2-38-58, BV6S5-38-58, BV13S1-38-58.
4.5. IL-10 inducing peptides = BV10S1P, BV11S1A1T, BV12S2A2T, BV13S7, BV19S2O
4.6. PBS = NaCl, Na_2HPO_4, KH_2PO_4, pH 7.6
4.7. PBMC = Peripheral blood mononuclear cells
4.8. FBS = Fetal Bovine Serum
4.9. RPMI = RPMI-1640

5. MATERIALS/SUPPLIES

5.1. PBS (Sigma)
5.2. Tween20 (Sigma, Catalogue # P1379)
5.3. BSA (Sigma, Catalogue # A-7030)
5.4. Con A (Sigma)
5.5. Peptides (Y49T)BV5S2-38-58,BV10S1P, BV11S1A1T, BV12S2A2T, BV13S7, BV19S2O were supplied by Multiple Peptide Systems (San Diego CA), and diluted to 500 ug/ml in RPMI
5.6. Peptides BV6S5 and BV13S1 were supplied by The Immune Response Corp., and diluted to 500ug/ml in RPMI.

5.7. Peptide cocktail consists of all three peptides diluted to 1500ug/ml in RPMI (500ug/ml of each peptide)

5.8. IFN-γ capture and detection antibody (Mabtech, Mab1-D1K)
5.9. IL-10 capture antibody (BD Pharmingen, 554497)
5.10. IL-10 detection antibody (BD Pharmingen, 554499)
5.11. FBS (Summit Biotechnology)
5.12. Strepavidin AP (DAKO Corporation, D0396)
5.13. BCIP/NBT Substrate (Kirkegaard and Perry Lab, Catalogue # 50-81-08)
5.14. Unifilter plates (Whatman, Catalogue # 7770-0001)

6. EQUIPMENT

	EQUIPMENT	Suggested Vendor
6.1.	Immunospot Reader	Cellular Technologies Limited
6.2.	Class II Biosafety Hood	Forma Scientific
6.3.	CO_2 Water Jacketed Incubator	Forma Scientific

7. SAFETY PRECAUTIONS

7.1. Blood-borne Pathogen Warning: All specimens and any materials in contact with the specimen should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Since no test method can offer complete assurance that laboratory specimens do not contain HIV, hepatitis B virus, or other infectious agents, the specimen should be handled and disposed of at the Health and Safety Level (BSL) 2 as recommended for any potentially infectious human serum or blood specimen.

7.2. Perform all cell plating work in a Class II Biosafety hood.

8. PROCEDURE

The steps 8.1, 8.2, and 8.3 are done in a biosafety hood under sterile conditions.

8.1. Coating Plate with Primary (capture) Antibody
Note: This step is done the day prior to the arrival of the blood.
8.1.1 Dilute IFN-γ specific primary capture antibody to 10 ug/ml and IL-10 specific primary antibody to 4 ug/ml in sterile PBS.
8.1.2. Add 100 ul of the dilute antibody into each well.
8.1.3. Incubate overnight in humidifying chamber at 4C.

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8.2. Blocking Plate
- 8.2.1. Wash 3 times with sterile PBS.
- 8.2.2. Add 200 ul/ well of 10% FCS/RPMI.
- 8.2.3. Store in the dark at room temperature for at least 1 hour.
- 8.2.4. Wash 3 times with sterile PBS.

8.3. Cell and Antigen Addition

Note: Cells are obtained using P1-RD-001 protocol.
- 8.3.1. 0.2 x 10^6 cells/well are added to a 96 well ELISpot plate for a final volume of 200 ul/well, according to the chart below.
- 8.3.2. Add 10 ul of 500 ug/ml stock of peptide in each well (1500 ug/ml of cocktail stock) according to the charts below. Add 10 ul of 100ug/ml stock of ConA to positive control wells. The final volume in each well will be 210 ul. Each patient requires one half of an ELISpot plate for the IL-10 assay, and one half of an ELISpot plate for the IFN-γ assay. Note that more antigens will be tested for IL-10 production than for IFN-γ production, and so the templates for each assay are different from each other. Both templates are shown below. If two patients are drawn on the same day, their assays can be run side by side on each plate.

IL-10 ELISPOT Layout for One-Half Plate:

<table>
<tr><td></td><td colspan="6">0.2x10^6 cells/well</td></tr>
<tr><td></td><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td></tr>
<tr><td>A</td><td rowspan="4">Negative Control (no antigen)</td><td rowspan="4">BV13S1</td><td rowspan="4">BV5S2 (Y49T)</td><td rowspan="4">BV11S1A1T</td><td rowspan="4">BV13S7</td><td></td></tr>
<tr><td>B</td><td></td></tr>
<tr><td>C</td><td rowspan="2">CONA</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td><td rowspan="4">Vaccine Cocktail</td><td rowspan="4">BV6S5</td><td rowspan="4">BV10S1P</td><td rowspan="4">BV12S2A2T</td><td rowspan="4">BV19S2O</td><td rowspan="4">Negative Control (no antigen)</td></tr>
<tr><td>F</td></tr>
<tr><td>G</td></tr>
<tr><td>H</td></tr>
</table>

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IFN-γ ELISPOT Layout for One-Half Plate:

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</table>

8.3.3. Store in 37C/7% CO_2 incubator for required time (24 hours for IFN-γ; 48 hours for IL-10)

8.4.A Detection of IFN-γ Spot-forming Cells
- 8.4.1. Wash 3 times with PBS
- 8.4.2. Wash 3 times with PBS/Tween
- 8.4.3. Dilute IFN-γ detection antibody to 1 ug/ml in PTB solution.
- 8.4.4. Add 100 ul of the appropriate antibody into each well
- 8.4.5. Store in the dark at room temperature for 90 minutes.

8.4.B Detection of IL-10 Spot-forming Cells
- 8.4.1. Wash 3 times with PBS
- 8.4.2. Wash 3 times with PBS/Tween
- 8.4.3. Dilute IL-10 detection antibody to 2 ug/ml in PTB solution.
- 8.4.4. Add 100 ul of the appropriate antibody into each well
- 8.4.5. Store in the dark at room temperature for 90 minutes.

8.5. Visualization (Same procedure for both IFN- γ and IL-10 plates)
- 8.5.1. Wash 4 times with PBS/Tween
- 8.5.2. Dilute Strepavidin-AP 1:1000 in PTB and add 100 ul/well.
- 8.5.3. Store at room temperature for 45 minutes.
- 8.5.4. Wash 4 times in PT.
- 8.5.5. Wash 6 times in PBS with one minute in between each wash.
- 8.5.6. Add 100 ul of BCIP/NBT per well and watch 2-5 minutes for spot development.
- 8.5.7. Rinse with distilled water 3 times to stop the reaction.
- 8.5.8. Dry upside down on a clean paper towel.

9. RECORD KEEPING

9.1. Results are logged in laboratory notebook.
9.2. A copy of results is submitted to laboratory manager.
9.3. All data will be reviewed and approved by the associate performing the procedure and the laboratory manager.

10. DATA TREATMENT/REPORTING

10.1. A digital image of the wells are scanned by the ImmunoSpot reader. The image is stored on a CD-ROM. The spots in the individual wells are analyzed manually by a qualified laboratory technician and then counted by ImmunoSpot Software.