Human Immunology Center Core Laboratory
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STANDARD OPERATING PROCEDURE: Detection of IgG and IgM Secreting B cells in human peripheral blood mononuclear cells by Elispot.

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1. Purpose:

   To enumerate the total number of IgG or IgM secreting plasma B cells in human peripheral blood mononuclear cells.

2. Principle:

   The frequency of immunoglobulin G or M secreting plasma cells in the circulating peripheral mononuclear cell population can be enumerated in Elispot. Frequencies can be determined without stimulation or following antigenic stimulation in vivo or in vitro with antigen. Anti-immunoglobulin G or M 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 1×10^5 to 3×10^4 cells per well in triplicate. The cells are incubated at 37^0 C for 16 to 18 hours in the anti-Ig coated plates before aspirating the cells and washing extensively with PBS-Tween. Alkaline phosphatase labeled anti human IgG or IgM antibody is added to the washed wells and incubated for 2 hours at room temperature. The secondary is washed from the plates and the plate is soaked for one hour before substrate is added to develop spots. The spots can be counted 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-human IgG or IgM antibody for coating (See Appendix A for specifics)
   Alkaline phosphatase conjugated anti-human IgG and IgM detection antibodies (See Appendix A for specifics)
   Phosphate buffered saline (Dulbecco's), Gibco Catalog # 14190-144 or equivalent
   Fetal Bovine Serum, Hyclone, Catalog # SH30396.03 or equivalent
   Bovine Serum Albumin(BSA), Bovuminar® Reagent Pure pH 7.0, Intergen, Cat # 3160-60 or equivalent
   Digital 20-200µL Multichannel Pipettor, VWR, Catalog # 40000-294 or equivalent
   Finnpipette 5-50µL single channel pipet, Thermo Electron, Cat # 4600220 or equivalent
   Finnpipette 1-10µL single channel pipet, Thermo Electron, Cat # 4600180 or equivalent
   Finnpipette 30-300µL single channel pipet, Thermo Electron, Cat # 4600240 or equivalent
   Sterile 20-200µL disposable pipet tips, Eppendorf, Catalog # 2249193-8 or equivalent
   Sterile 30-300µL disposable pipet tips, Eppendorf, Catalog # 2249194-6 or equivalent

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Sterile 0.1-20μL disposable pipet tips, Eppendorf, Catalog # 2249113-0 or equivalent
1 mL sterile disposable serological pipets, Costar, Catalog # 4012 or equivalent
5 mL sterile disposable serological pipets, Costar, Catalog # 4051 or equivalent
10 mL sterile disposable serological pipets, Costar, Catalog # 4101 or equivalent
25 mL sterile disposable serological pipets, Costar, Catalog # 4251 or equivalent
Pipet Aid, Drummond, Catalog # 4-000-101 or equivalent
Tween 20 detergent, Sigma, catalog # P1379 or equivalent
15 mL sterile polypropylene conical tubes, Falcon, Catalog # 35-2096 or equivalent
50 mL sterile polypropylene conical tubes, Falcon, Catalog # 35-2070 or equivalent
12x75mm culture tubes with closures, VWR Cat # 60818-500 or equivalent
Sterile reagent reservoirs, Costar, Catalog # 4871 or equivalent
37 ^{0} C incubator with 5% CO_2, Binder, Model # 9040-0030 or equivalent
Microplate washer, Biotek, Model ELx405 Select CW or equivalent
CTL reader, Cellular Technology, LTD, Model ImmunoSpot® Series 3A Analyzer or equivalent
Vector AP substrate kit, Catalog # SK-5300, or equivalent
Household bleach for decontamination, VWR, Catalog # 37001-056 or equivalent
Biological safety cabinet, Class II, Baker, Model # SG403, or equivalent
10X phosphate buffered saline (Dulbecco's), Gibco, Catalog # 14200-075 or equivalent
5 mL syringe, VWR, Catalog # BD301603 or equivalent
10 mL syringe, VWR, Catalog # BD301604 or equivalent
0.2 micron filter for syringe, Corning, Catalog #431215 or equivalent
Antibiotic/Antimycotic 100X Sigma Cat #A5955 or equivalent
ImmunoSpot® Software, Cellular Technology, LTD
RPMI 1640 medium, Cellgro, Catalog # 10-040-CV or equivalent
RPMI 1640 with 8% FBS/Antibiotics/Antimycotics ($R8A^2$)
PBS- 0.1% Tween 20
PBS-0.1TWEEN-2% BSA
100mM Tris-HCl Buffer pH 8.2
10% hypochlorite solution
Distilled water
8-channel aspirator for microplates

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 $Na_2HPO_47H_2O$; 0.2 g KH_2PO_4; add dH_2O to 1 liter. pH 7.2 sterile filter or autoclave. Or alternately purchase equivalent sterile reagent. Store at 4^{O} C. Expires 30 days after opening.

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3.1.2 10X Wash buffer: 10 X PBS: Dulbecco's Phosphate Buffered Saline: 80 g Na Cl; 2 g KCl; 21.6 g $Na_2HPO_47H_2O$; 2 g KH_2PO_4; add dH_2O 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 after 2 weeks.

3.1.4 Assay Buffer: Dulbecco's Phosphate Buffered Saline/0.1% Tween-20/2%BSA: Add 100mL of 10 X PBS to bottle. Add 1 mL of Tween-20. Add 20g 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. Store at 4°C Expires within 24 hours if not sterile.

3.1.5 Media: RPMI/8%FBS: In hood, remove 45 mL RPMI from a 500 mL bottle of medium and place in another sterile tube. Add 40 mL FBS and 5 mL 100X antibiotics/antimycotics and 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 distilled pyrogen free 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 PBMC are isolated from fresh blood using ficoll hypaque separation or BD CPT tubes and washed three times with HBSS and spun at 1000 rpm (300 X g) for 10 minutes to remove platelets and reduce residual serum containing Ig.

4.2 Dilute the sterile anti-IgG or IgM antibody to the optimal (10 μg/mL) concentration in sterile Dulbecco's PBS. (See appendix A for Ab concentrations). Add 50 μL per well being careful not to touch the pipet tips to the filter. Tap the plate to wet the wells and verify by visual inspection that the wells are entirely coated. Cover with a plate cover and incubate the Elispot wells for 1 to 3 hours at room temperature and then overnight at 4°C to coat the filter with antibody.

4.3 Aspirate off the Ab coating solution. Proceed to sterilely wash three times with 100 μL $R8A^2$ per well using a multichannel pipet and aspirate the block using a sterile aspirator being careful not to touch the pipet tips to the filter. Block the plate before use by adding 100 μL $R8A^2$ medium to each well in the plate. Place at 37^0 C in a CO_2 incubator for one hour. Proceed directly to adding cells to wells.

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4.4 Add 100 μL per well of the cell suspension in $R8A^2$ at three fold concentrations from 1 X 10^5 to 1 X 10^4 PBMC/mL to the 100 μL $R8A^2$ in the wells. Cover the microtiter plate and place in 37°C incubator with 5 % CO_2 for 16 to 18 hours. Do not stack plates. Be careful not to tip or shake when handling. Plates must be on a level shelf in the incubator.

4.5 Inspect plate visually to determine if any contamination is present. Wells with discolored media may be an indication of contamination. Note any contaminated wells in notebook/protocol record.

4.6 Wash the plate on the microtiter plate washer three times with 250μL per well of PBS-0.1% tween-20 soaking for 5 seconds between each wash. Repeat wash step for a total of 6 washes (2 wash cycles). Blot plate on absorbent toweling after washing. Only wash one plate at a time if doing multiple plates to avoid drying of plate.

4.7 Prepare the dilution of enzyme conjugate in PBS-0.1%Tween with 2% BSAaccording to Appendix A for detection of antibody binding and sterile filter using a 0.2 micron acrodisk filter and a 5 or 10 mL syringe. Add 50 μL per well of the optimal concentration (Reference Appendix A) of the (sterile filtered) alkaline phosphatase labeled detection antibody being careful not to touch the pipet tips to the filter and incubate for 2 hours at room temperature. Wash 3 times (one wash cycle) using a plate washer.

4.8 Soak the plates in a plastic container containing enough PBS-0.1%Tween to cover 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.

4.9 Develop by adding 100 μL per well of Vector AP substrate kit III at room temperature in the dark being careful not to touch the pipet tips to the filter for 1 to 30 minutes depending on color development. Stop the reaction by adding 100 μL of water or immediately dunk in a container filled with tap water. Repeat washing with tap water 6 to 8 times. Blot plate and place in dark drawer or in dark biological cabinet on the grill to dry. Allowing plates to dry overnight before reading is preferred.

4.10 After the plates have dried, read on CTL reader.

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Appendix A: Coating and Detection Antibody Concentration

Isotype	**Antibody/ AB-conjugate**	**Source & Catalog number and concentration**	**Elispot Coating**	**Detection concentration**	**Development time**
IgG	Goat anti-human IgG	Biosource AHI0301, 1 mg/mL	10μg/mL		
IgG	Goat anti-human IgG Fc-alkaline phosphatase	Bethyl A80-104AP 1.0 mg/mL		1 μg/mL (1:1000)	@ 15 minutes
IgM	Goat anti-humanIgM	Biosource AHI0601 1 mg/mL	10 μg/mL		
IgM	Goat anti-human IgM-Alkaline phosphatase	Biosource AHI 10605 0.85 mg/mL		1μg/mL	@ 20 minutes

Revision History

Version	Change	Impact	Justification	Change Date:
HIC-1-1009	new	none		10-04-05