Chapter 8

Detecting all Immunoglobulin Classes and Subclasses in a Multiplex 7 Color ImmunoSpot® Assay

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Abstract

Antibody molecules in peripheral blood have a relatively short half-life of roughly 20 days, and therefore their persistence in the serum depends on continuous replenishment by plasma cells. Serum antibody titers are thus indirect and unreliable indicators of immunological memory. In contrast, memory B cells persist in peripheral blood for decades, and enumerating these cells provides direct evidence of having developed an immune response to a given antigen. ELISPOT is an ideal research tool for enumerating antigen-specific memory B cells. Traditionally, B cell ELISPOT assays have been performed for detecting a single class of immunoglobulin (Ig), using a single colorimetric substrate. For comprehensive monitoring of B cell memory, however, all immunoglobulin classes and subclasses need to be assessed. Thus, seven single color assays would need to be performed to measure the numbers of antigen-specific B cells producing IgM, IgA, IgE, IgG_1, IgG_2, IgG_3, and IgG_4. We report here the development of a multiplex seven color B cell ImmunoSpot® assay in which the number of antigen-specific B cells can be established simultaneously for all major antibody classes and subclasses, requiring the PBMC, antigen, and labor corresponding to a single color assay.

Key words Immune monitoring, B cell, Humoral immunity, Antibody, ELISPOT, Fluorospot, Multiplexing

1 Introduction

For over a century, the detection of serum antibodies has served as the gold standard for the assessment of exposure to, and development of, immunity to an antigen. Indeed, in many instances serum antibodies can be detected even decades after an infection, as seen for example with influenza [1]. In other cases however, antibody titers wane over time, and booster immunizations are required to maintain protective antibody titers, as exemplified by tetanus, and many other vaccines [2]. An increasing number of recent findings show that seronegative individuals can develop strong T-cell immunity, as was seen in subsets of HIV- and HCMV-exposed individuals [3–7]. In the majority of multiple sclerosis patients, neuroantigen-specific memory B cells were detected in peripheral blood, demonstrating that an

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autoimmune response had occurred, while titers of serum antibodies specific for neuroantigens were not elevated [8]. Limitations of serologic tests are also well known in other fields. Thus, the interpretation of serological testing results remains inconclusive for subjects who have received transfusions of blood or blood products due to the transfer of donor antibodies. This also applies to children less than 12 months of age, while the mother's antibodies continue to be detectable [9, 10].

The reason for the above limitations of serodiagnostics becomes evident by taking a closer look at the relevant immunobiology. Antibody molecules in serum have a relatively short half-life, in the range of days to weeks, and therefore their presence in serum depends on ongoing production by B cell-derived plasma cells [11]. During the course of an immune response, naïve antigen-specific B-cells become activated by the antigen and through interactions with antigen-specific CD4 T-helper cells. As a consequence of activation, these B cells differentiate into plasma cells that produce antibodies. At the same time, long-lived memory B-cells also develop. These memory cells can give rise to new generations of plasma cells in the presence of persisting/reappearing antigen and T-cell-help, or in the absence of antigen, long-lived plasma cells can continue to spontaneously secrete antibody [1]. In either case, the presence of antibodies in serum of individuals results from an active, ongoing antibody synthesis process, which may or may not truthfully reflect previous antigen exposure. As antigen itself rarely persists to drive the continuous production of antibodies, it is now assumed that non-antigen-specific polyclonal stimuli maintain serological memory [12].

Serum antibodies are, therefore, indirect indicators of immunity that arise from complex and sometimes even random cellular reactions. Subsequently, it has become a mainstream effort to go directly to the source, and assess B cell memory itself. Memory B cells are long lived and persist for decades while recirculating in the blood [13]. Unlike antigen-specific T cells that can be stained with antigen-embedding multimers of MHC molecules (tetramers, pentamers, dextramers) [14], efforts to directly stain B cells with labeled antigen so far have failed in large. Instead, the detection of antigen-specific B cells is enabled by the ELISPOT technique [15]. In brief, 96 well plates with a special PVDF membrane on the bottom of each well are coated with the test antigen. When PBMC (which contain B cells) or purified B cells are plated into such wells, antibodies produced by the B cells that are specific for the test antigen will be captured on the membrane around the secreting B cell. These plate-bound antibodies can then be visualized by adding labeled detection antibodies. In this way, each antigen-specific B cell will produce a "spot," and counting the numbers of these Spot Forming Units (SFU) permits one to establish the frequency of antigen-specific B cells among all cells plated. Increased numbers of

antigen-specific B cells detected in PBMC ex vivo prove that these B cells have undergone clonal expansions in vivo. Importantly, antigen-specific B cell ELISPOT assays also provide insight into the affinity of the antibody that each B cell produces. Antibodies secreted by B cells that have high affinity for antigen will bind faster and stronger to the antigen on the membrane than low affinity antibodies, resulting in dense ELISPOTs for high affinity B cells, and diffuse spots for low affinity B cells [16]. By studying the morphology of spots produced by a high number of antigen-specific B cells within a test subject, one therefore can gain insights into the affinity distribution of the B cell/antibody repertoire within that individual (*see* **Note 1**).

Antibodies occur in different classes and subclasses, and each differ in their ability to precipitate antigen, neutralize, activate complement, promote phagocytosis, migrate to mucosal surfaces, or sensitize mast cells (reviewed in [17]). All B cells initially produce IgM, but in the course of an immune response, undergo class switching to secrete other types of immunoglobulins, whereby the mature plasma B cells will each secrete only one class/subclass of antibody. Immunoglobulin class switching is under tight control by CD4 T cells and cytokines, as the engagement of the appropriate class and subclass of antibody critically defines successful immune defense [17]. Therefore, if ex vivo antigen-specific B cells produce antibody classes/subclasses other than IgM, this shows that the memory cells have undergone immunoglobulin class switching in vivo [18], and the detection of a particular immunoglobulin class/subclass in this ELISPOT format establishes the quality of B cell/antibody memory.

Comprehensive immune monitoring, therefore, requires identifying the numbers and ratios of antigen-specific B cells producing antibodies of all classes and subclasses to a given antigen, i.e., IgM, IgG, IgA, and IgE, and the four subclasses of IgG: IgG_1, IgG_2, IgG_3, and IgG_4. To accomplish this goal, previously eight single color B cell ELISPOT assays would need to be run in parallel, each detecting one of the classes or subclasses. Here we report and communicate the protocol for combining these assays into a single seven color multiplex assay. Figure 1a shows a representative example of such a seven color assay. Of note, the assay was designed such that each analyte can be detected in a separate fluorescent channel or combination of channels without the leakage of signal (*see* **Note 2**). Panels b–g of Fig. 1 show the individual analyte planes for the seven color B cell assay described here. Analysis therefore can be done without the need for compensation, monochromatically for each of the analytes. In this chapter, we describe the seven color assay itself; additionally, we contributed other dedicated chapters on the experimental validation of multi-color analysis [19], as well as to the step by step process to its implementation [20].

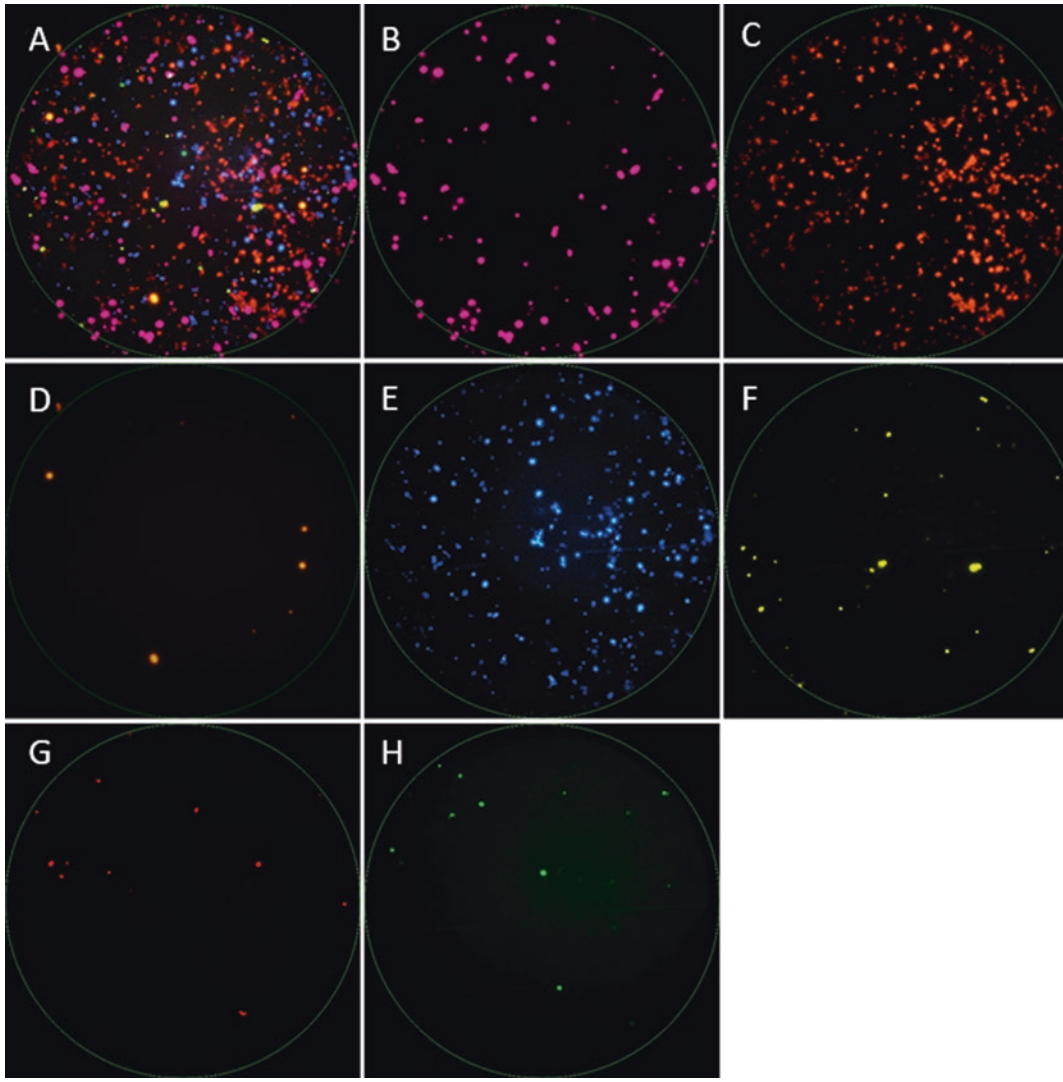

Fig. 1 Reperesentative image of a seven color B cell ImmunoSpot® well. The assay was performed as described in this chapter. For analysis, each color is detected in a dedicated color plane (*see* **Note 2**). Each color plane is shown in one of the panels: IgA in Panel **b**, IgM in Panel **c**, IgE in Panel **d**, IgG_1 in Panel **e**, IgG_2 in Panel **f**, IgG_3 in Panel **g**, and IgG_4 in Panel **h**, respectively. Panel **a** shows the overlay of these images (*see* **Note 17**)

2 Materials

2.1 Isolation of PBMC from Whole Blood

1. Green vacutainer tubes with sodium heparin.
2. Ficoll-Paque™ Plus.
3. Ca^{2+}, Mg^{2+} free phosphate buffered saline (PBS) pH 7.2.
4. CTL-Wash™ Supplement 10× (Cellular Technology Ltd., CTL, Shaker Hts, OH, Cat# CTLW-010).
5. Centrifuge capable of spinning 50 mL conical tubes at 740 × *g*.

2.2 Thawing of Cryopreserved PBMC

1. Bead bath (set to 37 °C).
2. CTL-Test™ B Culture Medium (CTL, Cat# CTLTB-010), supplemented with 1% L-Glutamine.
3. Centrifuge capable of spinning 50 mL conical tubes at 330 × *g*.

2.3 Counting of PBMC

1. ImmunoSpot® Series 6 Ultimate Analyzer (CTL, Cat# S6UTM12), running LD Software for live/dead cell counting (CTL, Cat# LDA01).
2. CTL-LDC™ Live/Dead Cell Counting Kit (CTL, Cat# CTL-LDC-100-2).

2.4 Polyclonal Stimulation of Memory B Cells from PBMC

1. Cryopreserved or freshly isolated PBMC.
2. B-Poly-SE™ (CTL, Cat# hBPOLYSE-200).
3. Culture flask or plate.
4. Humidified CO_2 incubator set for 37 °C, 9% CO_2.
5. CTL-Test™ B supplemented with 1% L-Glutamine (CTL, Cat# CTLTB-010).
6. Centrifuge capable of spinning 50 mL conical tubes at 330 × *g*.

2.5 Elispot Assay

1. Commercially available, seven Color Fluorospot B Cell kit (CTL, Cat# hB7F) optimized to detect human IgM, IgA, IgE, IgG_1, IgG_2, IgG_3, and IgG_4 (*see* **Note 3**).
2. 0.05% Tween-PBS: 100 µL Tween-20 in 200 mL PBS (per plate).
3. PBS, sterile, 100 mL (per plate).
4. Distilled water, 100 mL (per plate).
5. 70% EtOH.
6. 0.1 µm syringe-driven filter.
7. Plate washer (CTL, Cat# 405LSR) (*see* **Note 4**).
8. Vacuum manifold (Millipore, USA).
9. ImmunoSpot® S6 Ultimate 5LED Analyzer (CTL, Cat# S6UTM12), running ImmunoSpot® 7.0 Software (CTL, Cat# IPS01).

3 Methods

3.1 Isolation of PBMC from Whole Blood

1. Obtain blood samples according to institution's IRB protocol.
2. Keeping donors' samples separate, pool each donor's blood into appropriately labeled 50 mL conical tubes. Rinse each vacutainer tube with PBS and add to blood.
3. Measure blood volume and dilute 1:1 with PBS.

4. Layer diluted blood slowly over Ficoll® taking care not to disrupt the interface (*see* **Note 5**).
5. Centrifuge balanced tubes at 740 × *g* for 30 min with the centrifuge brake off.
6. Identify the cloudy interface between the Ficoll® and the plasma layer. Remove this interface with a serological pipette and transfer to a fresh 50 mL conical tube (*see* **Note 6**).
7. Dilute cells 1:2 with warm CTL-Wash™ Medium (for example, 15 mL cells + 30 mL medium) and spin at 330 × *g* for 10 min with centrifuge brake on.
8. Decant supernatant and re-suspend cells in warm CTL-Wash™ Medium at approximately 1×10^6 cells/mL and take a sample for cell counting (*see* **Note 7**).

3.2 Thawing of Cryopreserved PBMC

1. Warm CTL-Test B™ Medium to 37 °C.
2. Place frozen cryovials (containing $>1 \times 10^7$ cells per vial) in a 37 °C metal bead bath for 15 min.
3. Flip the cryovial twice 180° to re-suspend the cells.
4. Aspirate all liquid within cryovial using a serological pipette and transfer into a 50 mL conical tube.
5. Wash cryovial with 1 mL of fresh CTL-Test™ B Medium and add slowly to the rest of the cells.
6. Slowly add 8 mL of warm CTL-Test™ B Medium in the following manner: while swirling sample tube, add first 3 mL at the approximate rate of 1 mL/10 s, and the remaining 5 mL can be added progressively faster.
7. Centrifuge at 330 × *g* for 10 min.
8. Re-suspend cells at approximately 1×10^6 cells/mL and take a sample for counting (*see* **Note 7**).

3.3 Counting of PBMC

1. For each sample to be counted, mix 50 µL CTL-LDC™ Reagent with 50 µL of the cell suspension, pipetting up and down three times.
2. Aspirate 10 µL of the stained cells and load into the hemocytometer chamber—the liquid will fill the chamber by capillary action and any excess will be collected in the overflow reservoir.
3. Count cells (*see* **Note 8**).

3.4 Polyclonal Stimulation of Memory B Cells from PBMC (See Note 9)

1. Use warm (37 °C) CTL-Test™ B Medium.
2. Adjust PBMC (freshly isolated from blood or thawed from cryopreserved PBMC) in CTL-Test™ B Medium to 4 million/mL.
3. Add B-Poly-SE™ reagent to PBMC in CTL-Test B™ 1:200 (e.g., 50 µL B-Poly-SE™ to 10 mL CTL-Test™ B).

4. Culture cells in a 37 °C humidified incubator at 9% CO_2 for 4–7 days (*see* **Notes 10** and **11**).

3.5 The seven Color ImmunoSpot® B Cell Assay

1. One day before plating cells, prepare 70% EtOH, Capture Antibody or Antigen working solutions.
2. Remove plate underdrain, pipette 15 μL of 70% ethanol into each well, and incubate for less than 1 min. Add 150 μL of PBS, decant, and wash with 150 μL of PBS two more times.
3. Replace underdrain and immediately (before plate dries) pipette 80 μL/well of the Capture Solution into the low-autofluorescence PVDF plate provided with the kit.
4. Seal plate with parafilm and incubate at 4 °C overnight.
5. The next day, count PBMC and centrifuge at 330 × *g* for 10 min, decant supernatant, then re-suspend cells to a concentration of 2×10^6 cells/mL.
6. Plate PBMC in serial dilution into the PVDF plate (*see* **Note 12**).
7. Incubate cells on plate overnight at 37 °C (*see* **Note 13**).
8. Prepare Detection Antibody working solution by following kit protocol.
9. After incubation, decant cells and wash plate two times with PBS and two times with 0.05% Tween-PBS, 200 μL/well each time (*see* **Notes 4** and **14**).
10. Decant wash buffer and add 80 μL/well of Detection Solution to plate, incubate 2 h at 4 °C.
11. Prepare a working Tertiary Solution containing the fluorescent tags by following kit protocol.
12. Wash plate three times with 0.05% Tween PBS, 200 μL/well.
13. Decant wash buffer and add 80 μL/well of Tertiary Solution to plate. Incubate at room temperature for 1 h.
14. Wash plate two times with distilled water, 200 μL/well each time.
15. Rinse membrane with tap water, decant, and repeat three times.
16. Remove protective underdrain, place plate face down on vacuum manifold and completely fill the backside of plate with water, then vacuum water through the membrane.
17. Let plate dry completely, protected from light (*see* **Note 15**).
18. Scan and count plate with compatible analyzer (*see* **Note 16**).

4 Notes

1. The affinity distribution of antigen-specific B cells can be readily studied using the ImmunoSpot® software: images of wells containing antigen-specific B cell ELISPOTs are converted

into FCS files, and these are plotted in a spot size vs. density diagram.

2. The detection of individual analytes using fluorochromes requires an analyzer configured to ensure that the signal from the dyes do not cross-bleed between the detection channels. In this way, each analyte is detected in a separate analyte plane. Each channel is defined by a narrow excitation wavelength in conjunction with a narrow emission filter and can be selected from an array of channels preinstalled on CTL fluorescent analyzers.
3. Kit is suited for detecting either antibody secreting cells that have been immunized/activated in vivo, or memory B cells that have been polyclonally stimulated in vitro to secrete antibody. Each kit contains capture and detection antibodies, fluorescent detection reagents, diluent buffers, serum-free B cell assay medium, low autofluorescence PVDF-membrane plates, and a polyclonal B cell activator.
4. Plate washes may also be performed manually, but for automated washing, the pin length and flow rate need to be customized so membranes and spots are not damaged, as has been done for the CTL 405LSR plate washer.
5. Alternately, the diluted blood can be added first, and the Ficoll® gently underlaid with a serological pipette.
6. While collecting the cells, be sure to aspirate as little Ficoll® as possible. At this point, interphase cells from two 50 mL tubes can be combined into one tube. If the proportion of Ficoll® is too high (>5 mL), a significant cell loss will occur.
7. As a point of reference, keep in mind that 1 mL of fresh blood should yield approximately 1×10^6 PBMC.
8. Live cells will fluoresce green (480/525) and dead cells will fluoresce red (570/620). Cells can be counted with either a fluorescence-capable microscope or using the LDA software of the ImmunoSpot® Analyzer.
9. Resting memory B cells do not secrete antibodies, and they can be detected in ELISPOT assays only after polyclonal stimulation. In contrast, the spontaneous production of antibodies by B cells in freshly isolated blood signifies recent and ongoing antigen stimulation in vivo.
10. For detection of all antibody classes except IgE, 4 day stimulation with CTL B-Poly-S™ is suitable. However IgE producing memory B cells occur in very low frequency. IgE class switching can be induced in vitro by culturing cells for 7 days with CD40L and IL-4.
11. If a 24-well plate is used, plate 1 mL of the cell suspension per well. For larger scale polyclonal stimulation cultures, traditional culture flasks can be used. Suggested conditions for culture

flasks are approximately 3 mL of 4 million/mL cell suspension per cm^2. For example, in a T-25 flask, we recommend 8 mL of total cell suspension, for a T-75 flask, 25 mL.

12. After in vitro stimulation, B cells secreting certain antibody classes and subclasses (such as IgA or IgG_1) can be rather high, whereas in vivo stimulated, spontaneously secreting B cells tend to occur in rather low frequencies. Therefore it is important to establish the frequency range for the test groups and antigens of interest, and then cover this range in serial dilutions. For the detection of low-frequency Ig-producing cells, we recommend plating cells at 1×10^6, 5×10^5, 2.5×10^5, and 1.25×10^5 cells per well. In the case of very rare Ig-secreting cells, magnetic bead-based enrichment of B cells is recommended. For high-frequency Ig-secreting cells, continue dilution of cells down to 15,000, 10,000, and 5000 cells per well. Keep the diluted PBMC in a CO_2 incubator with lid open until pipetting into the assay.
13. Do not stack plates in the incubator. Avoid disturbing incubator or plates during incubation so cells do not roll during the assay.
14. The membrane must not dry at any time during the analyte detection process.
15. To completely dry plate, place in running laminar flow hood for 2 h or on the bench top for 24 h at a 45° angle on paper towels. Do not dry the ELISPOT assay plates at temperatures exceeding 37 °C as this may cause the membrane to warp or crack. Spots may not be readily visible while the membrane is still wet and the background fluorescence may be elevated. Scan and count plates only after membranes have completely dried.
16. The multicolor analysis process is described, step by step, in a dedicated chapter of this book [19]. The experimental validation of multi-color analysis is described in another chapter of this book [20]. CTL has scanning and analysis services available, and offers a trial version of ImmunoSpot® Software with the purchase of a kit.
17. A comparison of the individual panels in Fig. 1b–h shows that spots present in one channel are not visible in any of the other channels, i.e., the individual colors are being selectively detected without cross-bleeding. Double positive spots, thus, are neither seen, nor expected to be seen, as B cells can produce only one class or subclass of immunoglobulin at each differentiation state.

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