Chapter 15

Four-Color ImmunoSpot® Assays Requiring Only 1–3 mL of Blood Permit Precise Frequency Measurements of Antigen-Specific B Cells-Secreting Immunoglobulins of All Four Classes and Subclasses

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Abstract

The B lymphocyte response can encompass four immunoglobulin (Ig) classes and four IgG subclasses, each contributing fundamentally different effector functions. Production of the appropriate Ig class/subclass is critical for both successful host defense and avoidance of immunopathology. The assessment of an antigen-specific B cell response, including its magnitude and Ig class/subclass composition, is most often confined to the antibodies present in serum and other biological fluids and neglects monitoring of the memory B cell (B_{mem}) compartment capable of mounting a faster and more efficient antibody response following antigen reencounter. Here, we describe how the frequency and Ig class and IgG subclass use of an antigen-specific B_{mem} repertoire can be determined with relatively little labor and cost, requiring only 8×10^5 freshly isolated peripheral blood mononuclear cells (PBMC), or if additional cryopreservation and polyclonal stimulation is necessary, 3×10^6 PBMC per antigen. To experimentally validate such cell saving assays, we have documented that frequency measurements of antibody-secreting cells (ASC) yield results indistinguishable from those of enzymatic (ELISPOT) or fluorescent (FluoroSpot) versions of the ImmunoSpot® assay, including when the latter are detected in alternative fluorescent channels. Moreover, we have shown that frequency calculations that are based on linear regression analysis of serial PBMC dilutions using a single well per dilution step are as accurate as those performed using replicate wells. Collectively, our data highlight the capacity of multiplexed B cell FluoroSpot assays in conjunction with serial dilutions to significantly reduce the PBMC requirement for detailed assessment of antigen-specific B cells. The protocols presented here allow GLP-compliant high-throughput measurements which should help to introduce high-dimensional B_{mem} characterization into the standard immune monitoring repertoire.

Key words ELISPOT, FluoroSpot, B cells, Immune monitoring, Immune memory, Ig class, IgG subclass, High throughput, Antibody-secreting cell

Alexander E. Kalyuzhny (ed.), *Handbook of ELISPOT: Methods and Protocols*, Methods in Molecular Biology, vol. 2768, https://doi.org/10.1007/978-1-0716-3690-9_15,

1 Introduction

One might ask why bother with detecting antigen-specific memory B cells (B_{mem}), fragile live cells that need to be processed within a short time window after they have been collected from the body when simple serum antibody measurements provide the sought-after information? The answer is simple: B_{mem} measurements can provide insights into immune responsiveness that serum antibodies cannot. Because another chapter in this volume [1] is dedicated to this issue, here we will just touch on the major points. Antigen-specific plasma cells (PC) and B_{mem} both arise during an immune response triggered by antigen encounter, but the generation of these two daughter cell lineages follows different fate-decision pathways. Precursors of both cell types, germinal center B cells (GCB), undergo somatic hypermutations (SHM) that result in the generation of subclones with slightly modified B cell antigen receptors (BCR). From this repertoire of daughter cells, subclones that have an increased affinity for the antigen are positively selected to undergo further rounds of proliferation and SHM, and eventually differentiate into PC. Contrary to the previously held notion, PC are not necessarily long-lived (*see* **Note 1**) and neither are the Ig molecules they secrete (*see* **Note 2**). In contrast, GCB progeny endowed with lower affinity BCR for the antigen can still join the long-lived B_{mem} compartment. Hence, PC and B_{mem} fulfill different roles in maintaining host immune defense.

The antibodies produced by PC constitute the first wall of acquired humoral immune defense [2]. They provide immediate protection by preventing the re-entry of the antigen, or, if it enters, by neutralizing it and/or facilitating its elimination by phagocytes via immune complex formation (precipitation), opsonization, and complement fixation. As evidenced during the recent COVID-19 pandemic, and previously with seasonal influenza, the first wall of adaptive humoral defense may fail to prevent (re)-infection when antibody titers drop below protective levels, or upon emergence of viral escape mutants capable of evading the neutralizing activity of antibodies elicited by the original (homotype) virus strain. In such cases, B_{mem} provide the second wall of adaptive humoral host defense [2]. Owing to their increased frequencies compared to antigen-specific naive B cells, and having already switched from IgM to the expression of specialized Ig classes and subclasses (*see* **Note 3**), B_{mem} not only mediate a faster and more efficient "secondary" antibody response against the same (homotypic) virus but also against antigenically-related viral escape mutants (heterotypes). This is because, within the antigen-specific B cell repertoire that was clonally expanded by the homotypic virus during the primary response, there will be B_{mem} that joined the memory compartment with mutated BCR that have affinity for the heterotype as well.

Because such cross-reactive B_{mem} occur in increased frequencies compared to naive B cells and have already undergone Ig class switching, this population is poised to engage in a quasi-secondary B cell response if infection with a variant virus occurs.

From the above, it follows that measurements of serum antibodies provide insights only about the fading first wall of immune protection. Measurements of B_{mem}, in contrast, provide insights into the cellular basis of long-term immunity. Through measuring the frequency of antigen-specific B_{mem} within all peripheral blood mononuclear cells (PBMC) (*see* **Note 4**), the magnitude of the existing memory compartment within an individual can be directly quantified. Such information sheds light on the vigor of future secondary antibody responses upon antigen encounter. Moreover, by establishing the Ig class/subclass utilization of the B_{mem} compartment, one can also predict the type of antibody that will be produced upon antigen encounter (*see* **Note 5**).

There are few techniques capable of detecting rare antigen-specific B_{mem} while also providing information regarding their relative abundance, Ig class/subclass usage, and functional affinity (*see* **Note 6**). B cell ImmunoSpot® assays are ideally suited for this purpose as they enable the detection of Ig molecules secreted by individual antibody-secreting cells (ASC). While resting B_{mem} do not secrete antibodies, such cells can readily be differentiated into ASC following a simple in vitro stimulation protocol (*see* **Note 7**). The ImmunoSpot® assay principle for detecting ASC, irrespective of their antigen specificity, is described in Fig. 1a, and its variant for detecting antigen-specific ASC in Fig. 1b.

In this chapter, we share our expertise on how to best establish the frequency of antigen-specific, B_{mem}-derived ASC in human PBMC, including their Ig class and subclass usage, and how to do so with the least labor, and the lowest number of PBMC possible (*see* **Note 4**). The type of testing described here is also essential for determining the so-called “Goldilocks” number of PBMC to be seeded into subsequent ImmunoSpot® assays aimed at evaluating the affinity distribution present among antigen-specific ASC (see the chapter by Bezca et al. in this volume, [3]), or the cross-reactivity of homotype antigen-primed ASC with heterotypic antigens (see the chapter by Lehmann et al., also in this volume [1]).

Owing to the requirement to detect individual ASC-derived secretory footprints to accurately determine the frequency of antigen-specific B cells, an ImmunoSpot® assay-related challenge to overcome is that B_{mem}-derived ASC producing different classes and subclasses of Ig span orders of magnitude [4]. Importantly, this problem is readily overcome by seeding PBMC (or other single-cell suspensions) in serial dilutions. For establishing the frequencies of antigen-specific ASC following in vitro polyclonal stimulation of PBMC, we recommend starting at 2×10^5 PBMC per well and progressing in a 1 + 1 (two-fold dilution series) down

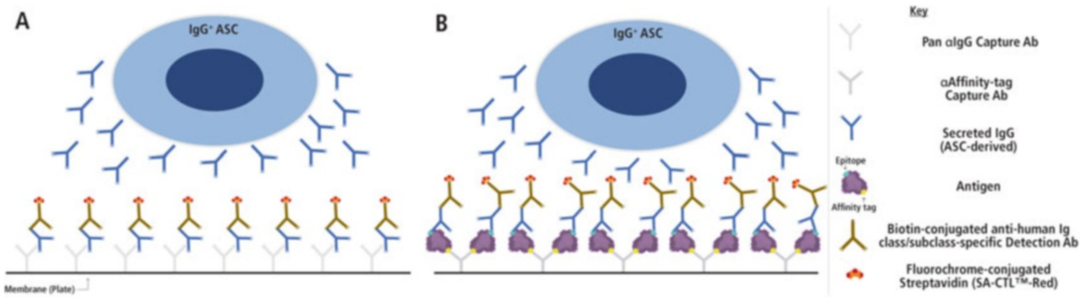

Fig. 1 Schematic representation of (**a**) the pan (total), or (**b**) the antigen-specific, direct ImmunoSpot® assays (the latter, using the affinity capture coating variant). For (**a**), the PVDF membrane on the bottom of a 96-well plate is densely coated with a pan anti-Ig capture antibody that will bind the ASC-secreted Ig(G) with high-affinity irrespective of the ASC's antigen specificity. In (**b**), the membrane is coated first with an anti-affinity tag-specific antibody (in this example anti-His) that captures the (His)-tagged antigen with high affinity. In this way, a dense coating of the membrane with the antigen is accomplished. As the next step in both assay variants, the PBMC containing the ASC are plated. In (**a**), an ASC-secreted Ig(G) antibody is captured around each ASC that is secreting Ig(G), and results in the formation of individual secretory footprints. In (**b**), the antibody produced by antigen-specific ASC is captured only on the lawn of the antigen. The subsequent steps are similar for both assay variants. After removal of the cells, the membrane-associated antibody is visualized using biotinylated anti-human Ig class/subclass-specific detection antibody reagents that subsequently are revealed by the addition of a fluorescently conjugated streptavidin (FluoroSpot, as shown) or via an enzymatic reaction (ELISPOT, not shown). Counting the spot-forming units (SFU) per well reveals the number of (**a**) total Ig (G) or (**b**) antigen-specific, Ig(G)-producing ASC within the PBMC plated. The spot morphologies in (**b**) also provide insights into the functional affinities of the antibody secreted by the individual ASC for the antigen, a topic covered in detail in the chapter by Becza et al., in this issue [3]

the 96-well plate to generate 8 additional data points. Similarly, for establishing the frequency of all ASC-producing IgM, IgG, or IgA, irrespective of their antigen-specificity, we recommend starting at 2×10^4 and performing a similar two-fold dilution series down the 96-well plate for 8 points of cell titration (*see* **Note 10**).

Four-color ImmunoSpot® assays are suited to generate maximal data while saving on cells (*see* **Note 11**). Figures 2 and 3 show that such fluorescence-based tests detect the secretory footprints of individual B_{mem} with the same efficacy as single-color enzymatic assays. As can be seen for higher cell numbers in Fig. 3, the confluence of secretory footprints and the resulting ELISA effect interfere with the accurate recognition and counting of individual spot-forming units (SFU). At SFU counts lower than 100 SFU per well, however, a close to perfect linear relationship exists between the number of PBMC seeded per well and the number of SFU per well, from which, by linear regression, the frequency of SFU within all PBMC plated can be accurately extrapolated. A fully automated software module built into the ImmunoSpot® software permits the identification of the linear range of SFU counts, the calculation of means of replicates, and the frequency extrapolation (see the chapter by Karulin et al. on this issue in this volume [5]).

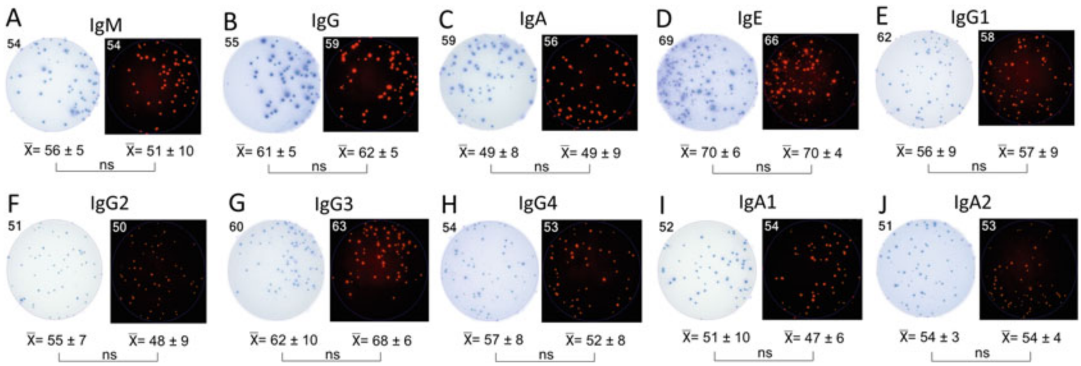

Fig. 2 Single-color ELISPOT and FluoroSpot assays have similar sensitivities for detecting ASC-derived secretory footprints. Peripheral blood mononuclear cells (PBMC) were polyclonally stimulated with B-Poly-S (or B-Poly-SE for detection of IgE$^+$ ASC in panel D) for 5 days in vitro (*see* **Notes 7** and **10**), washed, and seeded into single-color ELISPOT or FluoroSpot assays in parallel, detecting the immunoglobulin (Ig) class/ subclass specified in panels A–J, respectively; the type of assay shown in Fig. 1a was performed. Since antibody-secreting cells (ASC) producing the different Ig classes or subclasses occur in vastly different frequencies in PBMC following in vitro polyclonal stimulation (as detailed in the text), the assays were performed by plating PBMC in a 1 + 1 serial dilution series, starting at 2×10^5, in 4 replicates per cell dilution. Images of representative wells are shown for the dilution step in which the individual secretory footprints were clearly discernable; between 50 and 70 SFU/well. The cell inputs for panels A-J were: 781, 391, 3125, 200,000, 781, 6250, 6250, 25,000, 3125, and 6250 per well, respectively. The number of secretory footprints (spot forming units, SFU) measured using ImmunoSpot® software is reported in the left upper corner of the corresponding well images, and means ± SD of the four replicate wells are specified under each pair of wells. Statistical analysis (unpaired Student's *t*-test) indicated no significant difference ("ns") between the ELISPOT and corresponding FluoroSpot counts between the replicates for each condition. The presented results are representative of 4 independent experiments using different PBMC donors, leading to the same conclusion

Our in-depth studies of such regression analysis-based frequency calculations showed that performing serial dilution experiments involving replicates offers only a negligible advantage in precision over performing the assay with single wells (Fig. 4 and Becza et al., manuscript in preparation). Doing such serial dilution-based frequency measurements in four-color reduces the required number of PBMC by four-fold compared to performing 4 independent single-color assays, and doing so using a single well serial dilution approach permits another four-fold reduction in cell material compared to testing each cell input in quadruplicate (as was done in the data presented in Figs. 2 and 3).

Figure 5 depicts the results obtained from a typical serial dilution-based four-color ImmunoSpot® test detecting all four antibody classes (IgM, IgG, IgA, and IgE) of SARS-CoV-2 Spike-specific ASC in a convalescent individual with PCR-verified infection. Similarly, the IgG subclass usage of Spike-specific ASC was determined in parallel using an IgG1/IgG2/IgG3/IgG4 four-color assay. The abundance of all ASC in the test sample producing each Ig class or IgG subclass was also established to permit

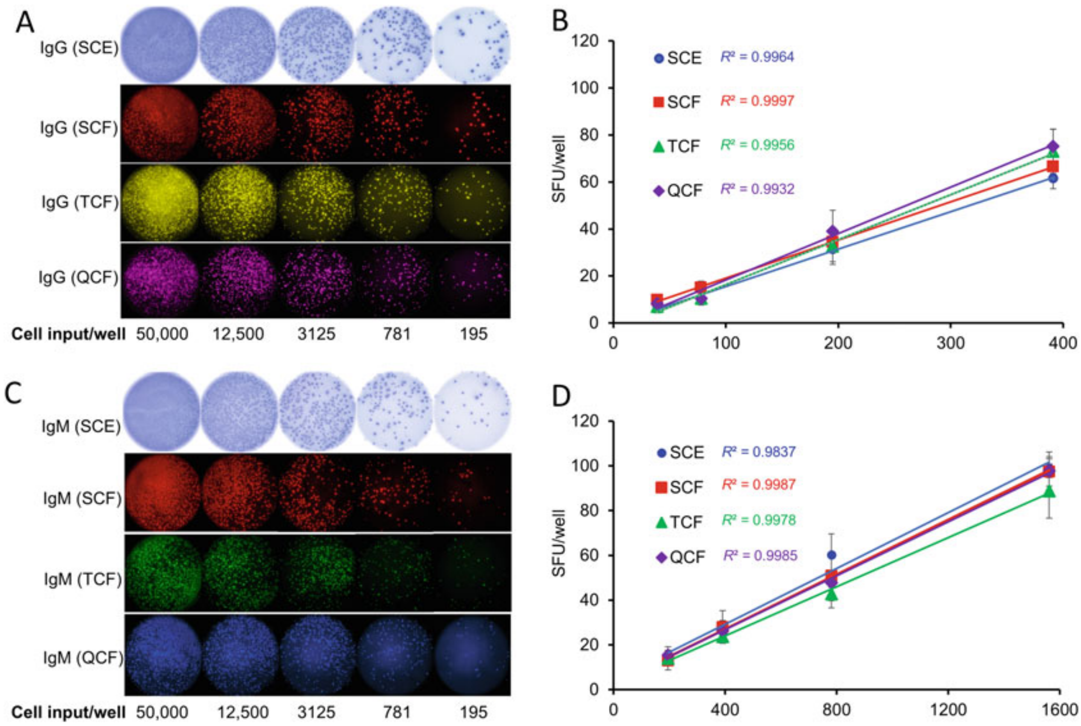

Fig. 3 Single-color ELISPOT and multi-color FluoroSpot assays have similar sensitivities for detecting ASC-derived secretory footprints. Peripheral blood mononuclear cells (PBMC) were polyclonally stimulated with B-Poly-S for 5 days in vitro, washed, and then seeded into single-color enzymatic (SCE) ELISPOT, single-color FluoroSpot (SCF), three-color FluoroSpot (TCF), or four- (quadruple) color FluoroSpot (QCF) assays in parallel, diluting the cell inputs two-fold per well, detecting pan IgG^+ (**a**, **b**) or IgM^+ ASC (**c**, **d**) in four replicate wells, irrespective of antigen specificity; the type of assay illustrated in Fig. 1a was performed. In panels (**a**) and (**c**), representative images (one of the four replicates tested) depicting secretory footprints visualized using anti-IgG or anti-IgM detection reagents are shown, respectively, with the corresponding cell input specified. ELISA effects are evident at high cell inputs. In panels (**b**) and (**d**), the spot-forming unit (SFU) counts for the four replicate wells for each condition are shown as means ± SD (y-axis) at the corresponding cell inputs (x-axis). The detection modalities are color-coded, as specified, including the matching trend lines calculated through linear regression analysis also denoting R^2 values

calculation of the frequency of antigen-specific ASC among all ASC, for each Ig class and subclass (*see* **Notes 12** and **13**). As seen for this individual, and consistent with the majority of convalescent COVID-19 donors we tested thus far (Kirchenbaum, unpublished observation), the SARS-CoV-2 Spike-specific B_{mem} primarily secrete IgG, and IgG1 in particular. Notably, although our IgA class-specific detection system works perfectly well for the detection of total IgA^+ ASC activity (see Fig. 2c), very few Spike-specific IgA^+ B_{mem}-derived ASC were detectable in this individual. Moreover, a small population of Spike-specific $IgG3^+$ B_{mem}-derived ASC was also detectable in this donor. Importantly, for the precise enumeration of the latter very rare antigen-specific ASC, higher cell

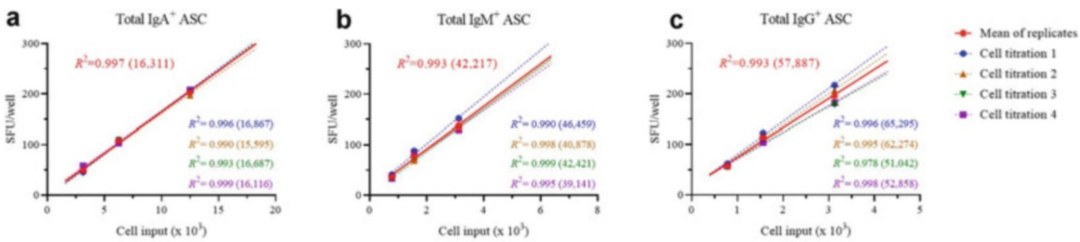

Fig. 4 Linear regression analysis performed using single wells in serial dilution experiments yields similar accuracy for determining the frequency of ASC compared to that calculated from the mean of 4 replicate wells at each cell input. Peripheral blood mononuclear cells (PBMC) were polyclonally stimulated with B-Poly-S for 5 days in vitro, washed, and then serially diluted two-fold, with four replicate wells for each cell input, in a four-color FluoroSpot assay to determine the frequency of antibody-secreting cells (ASC) producing IgA (**a**), IgM (**b**), or IgG (**c**). The type of assay shown in Fig. 1a was performed, detecting ASC irrespective of their antigen specificity. Red symbols denote means ± SD of SFU counts in the four replicate wells, at each of the cell inputs, respectively; the solid red trend line was calculated by linear regression analysis of these means with the R^2 value specified. The respective frequencies as calculated by extrapolating the linear regression line to a 10^6 PBMC input are given in parentheses. The color-coded "Cell titration 1-4" data with the corresponding dashed trend lines, R^2 values, and frequencies were obtained by independent single-well analysis of the four replicates. The results are representative of 5 independent experiments using different PBMC donors leading to the same conclusion

inputs would be required, inputs that would be too high for determining the frequency of antigen-specific ASC producing IgG/IgG1 (highlighting the importance of testing samples across multiple cell inputs through serial dilutions and the value of multicolor analysis). Of note, the large number of IgM^+ SFU detected in Spike antigen-coated wells does not appear to be "specific" since comparable numbers of SFU were also present in negative control wells. Such IgM^+ SFU likely originate from naive B cells possessing broadly reactive BCR specificities, and which differentiated into ASC following in vitro polyclonal stimulation. This lack of "specificity" exhibited by IgM^+ ASC following polyclonal stimulation of human PBMC has also been reported previously [6] and reiterates the importance of including negative controls in such B cell ImmunoSpot® assays. In contrast, while IgG^+ Spike- or NCAP-antigen-specific ASC were absent in all PBMC collected in the pre-COVID era, abundant such IgG^+ ASC were detected in individuals with PCR-verified SARS-CoV-2 infection [4]. Therefore, unlike IgM, detecting antigen-specific IgG (and IgG subclass)-producing ASC in PBMC following short-term in vitro polyclonal stimulation signifies in vivo primed and class-switched memory B cells. Antigen-specific, IgM^+ ASC can be detected, however, when PBMC are studied acutely (5–9 days) after induction of a primary B cell response.

Performing the above test required 8×10^5 PBMC to be seeded into the antigen-specific assay, plus we added an optional pan Ig class/subclass assay requiring an additional 8×10^4 PBMC (*see*

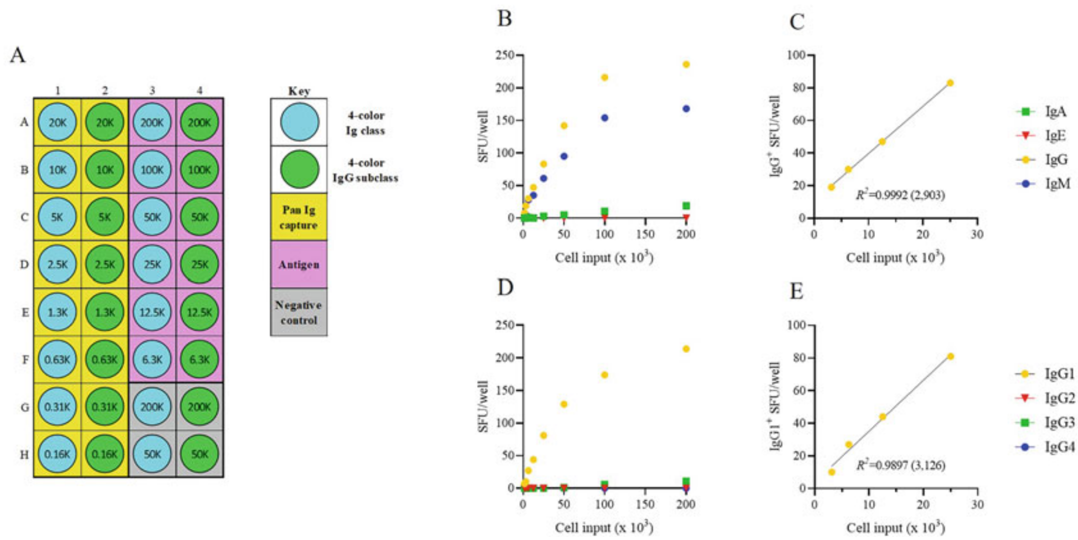

Fig. 5 Four-color ImmunoSpot® assays permit assessment of SARS-CoV-2 Spike-specific ASC frequency, plus the frequency of all ASC producing different Ig classes and IgG subclasses using 8.8×10^5 PBMC (*see* **Note 4**). Cryopreserved peripheral blood mononuclear cells (PBMC) from a convalescent donor with PCR-verified SARS-CoV-2 infection were polyclonally stimulated with B-Poly-S for 5 days in vitro, washed, and then evaluated in four-color ImmunoSpot® assays. (**a**) Recommended plate layout for determining ASC frequencies for each Ig class and IgG subclass using a serial dilution approach: columns 1 and 2 measure pan (total) Ig class and subclass usage are illustrated in Fig. 1a; columns 3 and 4 for measuring the antigen-specific ASC. (**b**) Spike-specific SFU counts were measured using the four-color Ig class detection system over the entire PBMC range plated, and (**d**) for the IgG subclass system with Ig class/subclass specified by color. Note deviation from linearity at high cell inputs/SFU counts. (**c**) Trend line for Spike-specific IgG^+ ASC calculated by linear regression analysis of the wells containing SFU < 100/well with the R^2 value; in parenthesis the extrapolated frequency per 10^6 PBMC. (**d**) The results for $IgG1^+$ ASC are represented in (**e**)

Fig. 5a and **Note 4**). As PBMC can be cryopreserved without loss of B cell functionality [7], samples can be run in batches instead of testing them one by one as soon as the blood is drawn (*see* **Note 13**). During freeze-thawing up to 30% of the cells may be lost, but the functionality of the recovered B cells will be unaltered compared to freshly isolated PBMC ([7] and Becza et al., manuscript in preparation). If an additional polyclonal stimulation is needed prior to the actual ImmunoSpot® test to convert resting B_{mem} into ASC, approximately 50% of the frozen PBMC will be recovered after thawing and performing the 5 day in vitro polyclonal stimulation protocol (N. Becza, manuscript in preparation). Therefore, only 8.8×10^5 PBMC are needed for the type of test shown in Fig. 5, and allowing for a safety margin in cell recovery, we suggest that $2–3 \times 10^6$ PBMC should be cryopreserved per aliquot to perform such a test. Furthermore, we recommend freezing several aliquots of PBMC (or other single-cell suspensions) to permit subsequent tests using higher cell inputs and/or additional replicates in scenarios where antigen-specific ASC frequencies are very low (*see* **Note**

15). Furthermore, the availability of additional aliquots of cell material permits further in-depth characterization of affinity distributions or heterotypic cross-reactivity within the B_{mem}-derived ASC repertoire (*see* **Notes 16** and **17**) and references [1, 3].

In the following, we provide detailed protocols for cryopreservation of PBMC to maintain their full functionality, subsequent polyclonal stimulation of these PBMC to differentiate resting B_{mem} into ASC, and four-color ImmunoSpot® assays for defining the Ig class and IgG subclass usage of antigen-specific ASC.

2 Materials

2.1 Isolation and Cryopreservation of PBMC from Whole Blood

1. Class II biosafety cabinet (BSC)
2. Green vacutainer tubes containing sodium heparin
3. Lymphoprep™
4. 15 or 50 mL conical tubes
5. Centrifuge capable of spinning tubes at 800 × *g* (temperature set to 25 °C)
6. Sterile transfer pipette
7. Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS), pH 7.2 (room temperature)
8. Parafilm
9. CTL-LDC™ counting kit
10. CTL-Cryo™ ABC media kit
11. ImmunoSpot® S6 Ultimate 4 LED analyzer, or a suitable instrument equipped with the appropriate detection channels, running CTL's live/dead cell counting suite software
12. DMSO
13. Bead bath (set to 37 °C)
14. 1.8 mL Cryovials (with internal thread and silicone washer seal)
15. Mr. Frosty™ freezing container, or controlled rate freezer.
16. Isopropyl alcohol
17. −80 °C freezer
18. Liquid nitrogen tank

2.2 Thawing of Cryopreserved PBMC

1. Cryopreserved PBMC sample(s) (*see* **Note 14**)
2. 70% EtOH
3. DNase-containing washing medium (pre-warmed to 37 °C) (*see* **Note 18**)
4. Complete B cell medium (BCM) (pre-warmed to 37 °C) (*see* **Note 19**)

2.3 In Vitro Polyclonal Stimulation of B Cells in PBMC

1. B-Poly-S
2. Tissue culture plate (48 or 24-well) 25 cm^2 sterile culture flask
3. Humidified incubator set at 37 °C, 5% CO_2

2.4 Single-Color ELISPOT Assay

1. Commercially available, single-color human Ig class (IgA, IgE, IgG, or IgM) or subclass (IgA1, IgA2, IgG1, IgG2, IgG3, or IgG4) ELISPOT kit (*see* **Note 20**)
2. 190 proof (95%) EtOH
3. Cell culture-grade water
4. 96-well, round bottom dilution plate
5. 0.05% Tween-PBS wash solution
6. 0.1 μm low-protein binding syringe filter
7. Plate washer
8. ImmunoSpot® S6 Ultimate 4 LED analyzer, or a suitable instrument equipped with the appropriate detection channels, running CTL's ImmunoSpot® UV

2.5 Single-, Three- or Four-Color FluoroSpot Assays

Commercially available, single-color human Ig class (IgA, IgE, IgG, or IgM) or subclass (IgA1, IgA2, IgG1, IgG2, IgG3, or IgG4) FluoroSpot kit (*see* **Note 21**).

1. Commercially available, three-color human Ig class (IgA, IgG, and IgM) FluoroSpot kit (*see* **Note 21**)
2. Commercially available, four-color human Ig class (IgA, IgE, IgG, and IgM) FluoroSpot kit (*see* **Note 21**)
3. Vacuum manifold

2.6 Four-Color Antigen-Specific FluoroSpot Assay (Affinity Capture Coating)

1. Commercially available, four-color human Ig class (IgA, IgE, IgG, and IgM) affinity capture (His) FluoroSpot kit (*see* **Note 22**)
2. Commercially available, four-color human IgG subclass affinity capture (His) FluoroSpot kit (*see* **Note 22**)
3. The His-tagged recombinant protein (*see* **Notes 23** and **24**)

3 Methods

3.1 Isolation and Cryopreservation of PBMC from Whole Blood (Sterile Conditions)

1. Obtain blood samples according to IRB-approved protocol. Keep blood at ambient temperature, do not refrigerate.
2. Keeping donors' material separate, inside a class II BSC, pool each donor's blood into labeled conical tubes. Rinse each vacutainer tube with PBS at ambient temperature and combine it with whole blood.

3. Measure blood volume and dilute 1:1 with PBS. If the volume of whole blood to be processed is ≥20 mL, transfer half of the volume into another labeled 50 mL tube prior to diluting with PBS.
4. Layer diluted blood at ambient temperature slowly over Lymphoprep™, also at ambient temperature taking care not to disrupt the interface.
5. Centrifuge balanced tubes at 800 × *g* for 20 min with the centrifuge brake off, non-refrigerated.
6. Identify the buffy coat at the interface between Lymphoprep™ and diluted plasma layers. Carefully remove the cells at this interface and transfer them to a fresh conical tube (*see* **Note 25**).
7. Wash the harvested PBMC by adding additional PBS at RT and pellet cells by spinning at 330 × *g* for 10 min with centrifuge brake on, non-refrigerated.
8. Decant supernatant and resuspend cell pellet(s) using PBS at RT. If appropriate, pool cell pellets from a single donor into one conical tube. Centrifuge balanced tubes at 300 × *g* for 15 min with centrifuge brake on, non-refrigerated.
9. Decant supernatant and resuspend the cell pellet(s) using PBS to achieve a cell density of ~2–5 × 10^6 cells/mL.
10. Pipet 15 μL of live/dead cell counting dye onto a piece of parafilm to form a droplet.
11. Remove 15 μL of cell suspension and combine with a droplet of live/dead cell counting dye. Pipet up and down three to five times to mix the sample while avoiding the formation of bubbles.
12. Transfer 15 μL of the cell and dye suspension into each chamber of a hemacytometer.
13. Determine live cell count and viability using CTL's live/dead cell counting suite.
14. Increase volume of cell suspension(s) with additional PBS and centrifuge balanced tubes at 330× *g* for 10 min with centrifuge brake on, non-refrigerated.
15. Decant supernatant and gently resuspend the cell pellet (s) using pre-warmed CTL-Cryo™ medium at a cell density of 6–10 × 10^6 cells/mL to generate aliquots containing 3–5 × 10^6 PBMC/vial, respectively.
16. Double the volume of the sample(s) by dropwise addition of pre-warmed CTL-Cryo™ medium containing 20% v/v DMSO while gently swirling the tube to ensure adequate mixing.

17. Immediately transfer sample(s) into labeled 1.2 mL cryovials and place into Mr. Frosty™ freezing containers and transfer into −80 °C freezer (*see* **Note 26**), or use a controlled rate freezer.

3.2 Thawing of Cryopreserved PBMC (Sterile Conditions)

1. Place cryovial(s) into a 37 °C bead bath, or better, glass bead bath, for 8 min to thaw.
2. Remove cryovial(s) and wipe with 70% EtOH inside the BSC before unscrewing the cap(s).
3. Using a sterile pipette, transfer contents of cryovial(s) into a labeled conical tube (if applicable, up to 5 vials of the same donor's cell material can be pooled in one conical tube).
4. Rinse each of the cryovials with 1 mL of warm anti-aggregate solution. Add the warm rinse solution to the conical tube dropwise while swirling the tube to ensure its adequate mixing with the cells in the thawing medium.
5. Double the volume of the cell suspension by dropwise addition of warm anti-aggregate solution while swirling the tube to ensure adequate mixing of the cells and thawing medium.
6. Continue doubling the volume of the cell suspension by dropwise addition of warm anti-aggregate solution while swirling the tube until the cryopreserved cell material has been diluted ten-fold. If multiple cryovials are pooled, calculate using 1 mL of cryopreserved cell suspension + 9 mL of anti-aggregate solution to determine the necessary final resuspension volume.
7. Centrifuge balanced tubes at 330 × *g* for 10 min with the centrifuge brake on, non-frigerated.
8. Decant supernatant and resuspend the cell pellet(s) using pre-warmed B cell medium (BCM) to achieve a cell density of ~2–5 × 10^6 cells/mL. You may estimate this number assuming a typical recovery of 70–80% of the frozen PBMC.
9. Pipet 15 μL of live/dead cell counting dye onto a piece of parafilm to form a droplet.
10. Remove 15 μL of cell suspension and combine with a droplet of live/dead cell counting dye. Pipet up and down three to five times to mix the sample while avoiding the formation of bubbles.
11. Transfer 15 μL of the cell and dye suspension into each chamber of a hemacytometer.
12. Determine live cell count and viability using CTL's live/dead cell counting suite.
13. Increase volume of cell suspension(s) with additional sterile PBS and centrifuge-balanced tubes at 330 × *g* for 10 min with a centrifuge, non-refrigerated, brake on.

14. Decant supernatant and gently resuspend the cell pellet (s) using pre-warmed BCM at a cell density of ~2–4 × 10^6 cells/mL.

3.3 Polyclonal In Vitro Stimulation of B Cells in PBMC (Sterile Conditions)

1. Dilute CTL's B-Poly-S polyclonal stimulation reagent 1:500 into prewarmed BCM to achieve a final concentration of 2X. Into labeled sterile culture vessels add 50% vol of BCM containing 2X concentration of B-Poly-S.
2. Add the same volume of cell suspension at ~2–4 × 10^6 cells/mL to achieve a final culture at ~1–2 × 10^6 cells/mL with 1X potency of CTL's B-Poly-S polyclonal stimulation reagent (*see* **Note 27**).
3. Transfer culture vessels (flasks or plates) into a humidified incubator set at 37 °C, 5% CO_2 for 4–6 days (96–144 h).

3.4 Four-Color, Antigen-Specific FluoroSpot Assay (Affinity Capture Coating)

1. Two days before plating cells (Day 2), prepare 70% EtOH and anti-His affinity capture antibody solutions.
2. Remove underdrain and pipet 15 μL of 70% EtOH solution into the center of each well (or designated wells) of the assay plate. Immediately after the addition of the 70% EtOH solution to the entire plate (or designated wells) add 180 μL/well of PBS. Decant and wash wells again with 180 μL/well of PBS.
3. Decant the assay plate, replace underdrain, and immediately add 80 μL/well of the anti-His affinity capture antibody solution into each well (or designated wells, **Note 28**) of the low autofluorescence PVDF-membrane plate provided with the kit.
4. Incubate the plate overnight at 4 °C in a humified chamber.
5. The following day (Day 1) dilute the His-tagged protein (s) into diluent A (provided with the kit) to the previously determined optimal concentration (*see* **Note 24**).
6. Decant the assay plate and wash wells with 180 μL/well of warm PBS. Immediately, add 80 μL/well of the corresponding His-tagged protein-coating solution(s) into the designated wells (*see* **Note 29**).
7. Incubate the plate overnight at 4 °C in a humidified chamber.
8. On the day of the assay (Day 0), decant the assay plate and wash wells with 180 μL/well of warm PBS. Next, decant the plate and add 150 μL/well of pre-warmed BCM to block the plate (≥1 h at RT).
9. If using PBMC following in vitro polyclonal activation, collect the cell suspension(s) and transfer it into labeled conical tube (s). Keep the cells warm during processing. Wash the culture vessel's interior with sterile warm PBS to collect residual PBMC and transfer it into the corresponding conical tube(s). Increase the volume to fill the tube with additional warm PBS

and then centrifuge balanced tubes at 330 × *g* for 10 min non-refrigerated, centrifuge with the brake on. Alternatively, follow the procedures detailed above to obtain freshly isolated PBMC, or to thaw PBMC that were previously cryopreserved, if prior in vitro stimulation is not required to elicit antigen-specific ASC activity in the sample(s).

10. Decant supernatant and resuspend the cell pellet(s) using pre-warmed BCM to achieve a cell density of ~2–5 × 10^6 cells/mL (the cell number recovered at this point can be estimated to be 50% of the number of cells frozen).
11. Pipet 15 μL of live/dead cell counting dye onto a piece of parafilm to form a droplet.
12. Remove 15 μL of cell suspension and combine with a droplet of live/dead cell counting dye. Pipet up and down three to five times to mix the sample while avoiding the formation of bubbles.
13. Transfer 15 μL of the cell and dye suspension into each chamber of a hemacytometer.
14. Determine live cell count and viability using CTL's live/dead cell counting suite.
15. Increase volume of cell suspension(s) with additional sterile warm PBS and centrifuge balanced tubes at 330 × *g* for 10 min with centrifuge brake on, non-refrigerated (*see* **Note 30**).
16. Decant supernatant and resuspend the cell pellet(s) using pre-warmed BCM at 2 × 10^6 PBMC/mL (*see* **Note 31**).
17. Decant the BCM used for blocking the ImmunoSpot® assay plate and replace it with 100 μL/well of pre-warmed BCM.
18. Prepare PBMC serial dilution series in a round bottom 96-well polystyrene plate to match the plate layout shown in Fig. 5a (*see* **Note 32**). For this, we recommend the following procedure. Into the round bottom 96 well dilution plate, add 10 μL of pre-warmed BCM into all wells, except for row A of columns 1 and 2 for the pan-Ig assay and columns 3 and 4 for the antigen-specific assay. Into wells A3 and A4 add 200 μL/well each of the 2 × 10^6 PBMC stock (for the pan-Ig assays), dilute 40 μL of the 2 × 10^6 PBMC stock with 360 μL of warm BCM to obtain 400 μL of cell suspension at 2 × 10^5 PBMC/mL, of which plate 200 μL each into wells A1 and A2. Using a multichannel pipettor, perform a two-fold dilution series of the PBMC by transferring 100 μL from each row to the next, diluting the cells by gently aspirating and ejecting twice at each dilution step. Once the cell dilution in the round bottom plate is complete, using a multichannel pipettor and fresh tips, transfer 100 μL of the serially diluted cells from the dilution plate into the actual ImmunoSpot® test plate.

19. Incubate cells in the ImmunoSpot® assay plate for 16–18 h at 37 °C, 5% CO_2.
20. After completion of the assay incubation period, decant (or reutilize) cells and wash the plate two times with warm PBS (200 μL/well), followed by two additional washing steps with 0.05% Tween-PBS wash solution (*see* **Note 33**).
21. Prepare anti-Ig class/subclass-specific detection antibody solution(s) according to kit protocol and pass through a 0.1 μm low-protein binding syringe filter to remove any protein aggregates.
22. Decant 0.05% Tween-PBS wash solution, add 80 μL/well of the anti-Ig class/subclass-specific detection antibody solution into designated wells, and incubate for 2 h at RT (protected from light).
23. Wash plate(s) two times with 0.05% Tween-PBS wash solution.
24. Prepare a tertiary solution by following kit protocol and pass through a 0.1 μm low-protein binding syringe filter to remove any aggregates.
25. Decant 0.05% Tween-PBS wash solution, add 80 μL/well of tertiary solution into designated wells, and incubate for 1 h at RT (protected from light).
26. Wash plates(s) twice with distilled water.
27. Remove the protective underdrain and place the plate face down on the vacuum manifold. Completely fill the backside of the plate with distilled water and apply a vacuum to draw water through the membrane ("back to front") (*see* **Note 34**).
28. Allow the plate to dry completely, protected from light (*see* **Note 38**).
29. Scan and count plate(s) with a suitable analyzer equipped with the appropriate detection channels (*see* **Note 35**).

4 Notes

1. While plasma cells (PC) elicited during the primary immune response can secrete large amounts of antibodies, their lifespans are heterogeneous and likely fall on a continuum [8, 9].
2. The half-life of different Ig classes, and IgG subclasses, is variable and relatively short in vivo. The half-life of IgG1, IgG2, and IgG4 in humans is 21–28 days, whereas for IgG3 it is ~1 week [10]. For IgA and IgM, their half-lives are even shorter (3–7 days) [11, 12] and IgE has the shortest half-life in serum, ~2–3 days [13].

3. A hallmark of immunological memory is the rapid increase in the level of class-switched, antigen-specific IgG and IgA.
4. To establish the frequency, and Ig class or IgG subclass usage of B_{mem}-derived ASC for one antigen using a direct ImmunoSpot® assay approach, a typical serial dilution experiment requires 8.8×10^5 PBMC to be seeded into the assay – see the plate layout shown in Fig. 5a. Therefore, if freshly isolated PBMC are to be tested on day 5–9 post onset of the B cell response when spontaneously Ig-secreting, antigen-specific plasmablasts are present in the blood, 1 mL of blood should suffice to complete such a test. If the PBMC from such blood is to be cryopreserved before testing, $1.5–2 \times 10^6$ PBMC should be frozen to obtain, with a safety margin, 8.8×10^5 viable and fully functional PBMC after thawing [7]. If resting memory B cells in PBMC are to be assessed, however, the PBMC need first to be subject to a 5 day in vitro stimulation culture [15], after which about 50% of the (fresh or thawed) PBMC are recovered (Becza et al., manuscript in preparation). Thus, for working with thawed PBMC and after polyclonal stimulation, freezing 3 (with added safety, 4) $\times 10^6$ PBMC is required to end up with 8.8×10^5 PBMC on the day of the test. Any number of freshly isolated PBMC between 1 and 10×10^6 per vial can be cryopreserved recovering the proportional number of fully functional PBMC after thawing (Becza et al., manuscript in preparation).
5. Stimulating optimal Ig class usage during an infection or following vaccination is vital to successful host defense and the avoidance of collateral immune-mediated pathology (reviewed in [14]).
6. Flow cytometry does not reliably reveal the class/subclass of Ig produced by the individual B cell because surface BCR expression can be highly variable and this is an underappreciated complexity of probe staining. In particular, in the case of IgG^+ ASC, they express little if any surface BCR and this undermines the assessment of their antigen specificity and subclass usage by traditional surface staining approaches. Consequently, fixation and intracellular staining are required to define the IgG subclass usage of these cells (a procedure that results in substantial cell loss in the sample).
7. Memory B cells exist in a quiescent state in the absence of recent antigen encounter and do not secrete their individual BCR as soluble antibodies. To overcome this obstacle for detecting them in ImmunoSpot® assays, in vitro polyclonal stimulation protocols can be used to trigger the antigen-independent activation of resting memory B cells into ASC [15].

8. Spontaneous ASC activity can also be evaluated directly ex vivo (e.g. plasmablasts that occur in PBMC 5–9 days after onset of a B cell response, or PC residing in the bone marrow).
9. If testing PBMC in the absence of prior stimulation (e.g. to measure plasmablasts), or other samples for spontaneous ASC activity directly ex vivo, the optimal cell inputs for establishing the frequency of all ASC-producing IgM, IgG, and IgA will be much higher.
10. IgE^+ ASC are quite rare and consequently, ImmunoSpot® assays that aim to determine their relative frequency require high initial starting cell inputs. Despite testing numerous human PBMC samples (>50) of healthy, non-allergic individuals following in vitro stimulation with CTL's B-Poly-S, we have not detected IgE^+ ASC activity in any of these samples thus far. IgE^+ ASC can be detected in PBMC of such individuals, however, following polyclonal stimulation with IL-21 in conjunction with anti-CD40 and IL-4 [15] that mimics T cell help (suggesting class switching to IgE during the in vitro cell culture period using this particular polyclonal stimulation protocol).
11. Cell material is most often the limiting component for immune monitoring.
12. B cell ImmunoSpot® data can be expressed as SFU per cell input per well to determine the frequency of antigen-specific cells. However, owing to the variable abundance of pan IgG^+ ASC in test samples following polyclonal stimulation, some prefer to report data as the frequency of antigen-specific B cells secreting a given Ig class/subclass among all B cells secreting that Ig class/subclass.
13. Each investigator in our laboratory can routinely test, following the protocol outlined in this chapter, in a single experiment, 10–20 PBMC samples for reactivity against a panel of antigens, assessing the frequency of ASC producing each of the Ig classes and IgG subclasses. With additional logistical refinements, this throughput is readily upward scalable.
14. If a special protocol is followed, PBMC can be frozen without impairing the B cells' functionality ([7] and N. Becza manuscript in preparation). Thus, by freezing B cells of a sample in several aliquots, the same PBMC can be tested repeatedly, reproducing the results of the previous experiment with high accuracy [4], or extending those studies. Of note, when planning the numbers of PBMC to be frozen per cryovial, as a rule of thumb, one can anticipate recovery of ~50% of PBMC initially frozen after these cells are thawed and have undergone 5 days of polyclonal stimulation to promote terminal differentiation of resting B_{mem} into ASC (see also **Note 4**). It is also

important to know that any number of PBMC between 1 and 10×10^6 can be frozen per cryovial permitting the optimization of PBMC utilization when planning experiments (N. Becza, manuscript in preparation).

15. In ImmunoSpot® assays, there is no inherent lower limit of detection. If, e.g., 3×10^6 PBMC are plated at 3×10^5 PBMC across 10 replicate wells, 1 in 3×10^6 is the detection limit, etc. Importantly, however, owing to increased Poisson noise occurring with such low-frequency measurements, the number of replicate wells evaluated needs to be increased accordingly to obtain accurate low-frequency measurements.
16. The so-called "Goldilocks" number is defined as the maximal number of cells that can be plated in an assay well while still being able to discern clearly individual secretory footprints derived from antigen-specific ASC. As it is assay-dependent, it needs to be experimentally established, but 50 SFU/well is a safe estimate.
17. Once the so-called "Goldilocks" number has been established, using a serial dilution approach as illustrated in Fig. 5, in a second experiment, the cell number(s) can be chosen accordingly for generating replicates containing ≥300 individual ASC-derived footprints for studying the functional affinity distribution (see also the chapter in this volume by Becza et al. [3]). Obviously, the higher the assay-specific Goldilocks number, the fewer replicates are needed to obtain ≥300 cumulative secretory footprints.
18. Thawing of cryopreserved cells causes a fraction of the cells (up to 30%) to die, and the DNA released from such cells can cause clumping of the thawed cell material. This cell clumping can be reduced, if not completely eliminated, by including an immunologically neutral endonuclease, Benzonase. Ready-to-use Benzonase-containing, serum-free wash solutions are available: CTL anti-aggregate Wash™ 20X solution.
19. A suitable assay medium for use in B cell ImmunoSpot® is RPMI 1640 with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 8 mM HEPES, and 50 μM 2-mercaptoethanol.
20. Kit is suited for detecting all antibody-secreting cells (ASC) producing a given Ig class or Ig subclass, irrespective of antigen-specificity, that differentiated in vivo, or following an in vitro polyclonal stimulation protocol to promote their transition to ASC. Each kit contains pan anti-Ig capture antibody, Ig class or Ig subclass-specific detection reagents, diluent buffers, PVDF-membrane plates, development substrate solutions, and polyclonal B cell activator (B-Poly-S or B-Poly-

SE). Of note, B-Poly-SE is capable of stimulating ASC that produce one of the distinct Ig classes/subclasses; including IgE (see also **Note 10**).

21. Kit is suited for detecting all antibody-secreting cells (ASC) producing a given Ig class or Ig subclass, irrespective of antigen-specificity, that differentiated in vivo, or following an in vitro polyclonal stimulation protocol to promote their transition to ASC. Each kit contains pan anti-Ig capture antibody, Ig class or Ig subclass-specific detection reagents, diluent buffers, low autofluorescence PVDF-membrane plates, and polyclonal B cell activator (B-Poly-S or B-Poly-SE). Importantly, Ig class-specific and/or Ig subclass-specific detection reagents can be combined to generate multiplexed detection systems enabling two-, three, or four-color B cell ImmunoSpot® assays.
22. Kit is suited for detecting either antigen-specific antibody-secreting cells (ASC) that differentiated in vivo, or antigen-specific B_{mem} that have been polyclonally stimulated in vitro to promote their transition to ASC. Each kit contains anti-His capture antibody, Ig class-specific (IgA, IgE, IgG, and IgM) detection reagents, diluent buffers, low autofluorescence PVDF-membrane plates, and polyclonal B cell activator (B-Poly-S). Alternatively, IgG class-specific (IgG1, IgG2, IgG3, and IgG4) detection reagents can be substituted in the context of such four-color B cell ImmunoSpot® assays.
23. Traditional B cell ELISPOT assays have been performed by direct coating of the assay membrane with the antigen of interest. However, many (in fact, most) antigens do not adsorb sufficiently to the membrane to enable reliable detection of ASC-derived secretory footprints. We have overcome this limitation by introducing an affinity coating approach for achieving high-density antigen absorption to the assay membrane [16].
24. We recommend optimizing the concentration of His-tagged protein(s) used for affinity capture coating. A concentration of 10 μg/mL His-tagged protein has yielded well-formed secretory footprints for most antigens, but increased concentrations of the anti-His affinity capture antibody and/or His-tagged protein may be required to achieve optimal assay performance [16].
25. Take care to collect as little Lymphoprep™ as possible. At this point, the interphase of two conical tubes can be combined into one tube. If the proportion of Lymphoprep™ is too high (≥ 10% v/v), significant cell loss may occur.
26. A cooling rate of 1 °C/min is optimal for cell cryopreservation. Be sure to fill the lower compartment of the Mr. Frosty™ Freezing container with 100% isopropyl alcohol and replace it after five cryopreservation cycles. After approximately 20 h,

and no more than 2 days, transfer cryopreserved PBMC from the −80 °C freezer into liquid nitrogen for long-term storage.

27. The volume of in vitro stimulation cultures can be scaled up or down accordingly, but we recommend keeping the cell density of PBMC at approximately 1–2 × 10^6 cells/mL. If larger numbers of in vitro stimulated PBMC are required for downstream ImmunoSpot® assays, tissue culture flasks should be used; store flat or standing such the height of the cell suspension in the flask is between 0.5 and 1 cm. Smaller in vitro stimulation cultures can be initiated in 48- or 24-well plates with a final volume of 1–2 mL, respectively. Be sure to fill empty wells in tissue culture plates with sterile PBS to avoid dehydration of cell cultures.
28. If the entire plate will not be coated with the anti-His affinity capture antibody solution, the remainder of the EtOH pre-wet wells should receive 80 μL/well of PBS.
29. If performing both an antigen-specific affinity capture and total ASC assay on the same plate, we recommend adding the pan anti-Ig capture antibody at this stage.
30. If the cells are not washed thoroughly, contaminating antibodies in the cell suspension(s) can compete for binding of the affinity captured antigen and may also result in elevated membrane staining that interferes with accurate enumeration of individual antigen-specific ASC.
31. This starting cell input was used to generate the data presented in Figs. 2, 3, 4 and 5 and to highlight the vastly different frequencies of ASC that produce each Ig class or subclass following in vitro polyclonal stimulation.
32. Do NOT do serial dilutions in the actual ImmunoSpot® plates because the pipet tips can easily damage the membrane. For multiple PBMC, these cell transfers can readily be done simultaneously using a multichannel pipettor.
33. Plate washes may also be performed manually. For automated washing, the pin height and flow rate should be customized to avoid damaging the assay membranes, which is the case for the CTL 405LSR plate washer.
34. Optimal removal of background staining, fibers, and other debris, along with reduction of "hot spots" in the center of the assay wells, is achieved through performing the "back to front" water filtration technique.
35. The chapter by Karulin et al. in this volume [5] introduces artificial intelligence-based SFU analysis that can partially compensate for ELISA effects and SFU crowding, thus extending the linear range of accurate quantification for cell numbers plated per well and SFU detected.

Acknowledgments

We wish to thank the R&D and the Software Development teams at CTL for their continued support and technological innovation that made our B cell ImmunoSpot® endeavor possible. We thank Drs. Alexey Y. Karulin and Graham Pawelec for in-depth discussions of the subject matter, Diana Roen for carefully proofreading the manuscript, and Gregory Kovacs for his support in the generation of graphic illustrations. All efforts were funded from CTL's research budget.

Conflicts of Interest P.V.L. is Founder, President, and CEO of CTL, a company that specializes in immune monitoring by ImmunoSpot®. L.Y., N.B., A.M.P., J.C., and G.A.K. are employees of CTL.

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