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Theoretical and practical considerations for validating antigen-specific B cell ImmunoSpot assays

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ABSTRACT

Owing to their ability to reliably detect even very rare antigen-specific B cells in cellular isolates such as peripheral blood mononuclear cells (PBMC), and doing so robustly in a high throughput-compatible manner, B cell ELISPOT/FluoroSpot (collectively "B cell ImmunoSpot") tests have become increasingly attractive for immune monitoring in regulated settings. Presently, there are no guidelines for the qualification and validation of B cell ImmunoSpot assay results. Here, we propose such guidelines, building on the experience acquired from T cell ImmunoSpot testing in an environment adhering to the requirements of regulatory bodies yet taking the unique features of B cell assays into account. A streamlined protocol is proposed that permits the performance of all tests needed for the formal validation of an antigen-specific B cell ImmunoSpot assay in only three experiments, utilizing 2.2×10^7 PBMC per donor. Subsequently, utilizing only $1–2 \times 10^6$ PBMC per sample (obtainable from 1 to 2 mL of blood), a validated multiplexed assay enables accurate quantification of the frequency of antigen-specific memory B cell-derived blasts secreting IgM, IgG, IgA or IgE antibodies. Collectively, such multiplexed B cell ImmunoSpot assays offer immense value for B cell immune monitoring programs due to their ease of implementation, scalability, applicability to essentially any antigenic system, economy of PBMC utilization, and last but not least, the high content information gained.

1. Introduction

Despite the fact that the ELISPOT assay system was first developed for quantifying the abundance of antigen-specific antibody-secreting B cells (ASC) (Czerkinsky et al., 1983; Sedgwick and Holt, 1983), it is the T cell ImmunoSpot assay (Note 1; a link to all "Notes" can be found at the end of this paper, under "Appendix") which were introduced much later (Czerkinsky et al., 1988) that has become a mainstay for T cell immune monitoring (Korber et al., 2016). This happened after modification of the original T cell ELISPOT protocol enabled the assay to reliably reveal secretory footprints of individual antigen-specific T cells ((Lehmann, 1995; Hesse et al., 2001), and Note 2), and the development of automated, objective, and validated machine reading approaches rendered the assay suitable for high throughput analysis and compliant with regulatory guidelines (Forsthuber et al., 1996; Zhang and Lehmann, 2012) (Note 3). The urgent need for measuring the contribution of the T cell compartment to immune responses, coupled with the ease of standardization, high sensitivity, and high throughput capacity together rendered T cell ImmunoSpot assays an attractive alternative to flow cytometry-based analysis (both being used initially for basic discovery, and now increasingly for clinical analysis) (Slota et al., 2011; Shafer-Weaver et al., 2006; Pass et al., 1998; Malyguine et al., 2007; Ranieri et al., 2014).

In contrast to T cell assays, to date, B cell ImmunoSpot assays have not been intensively exploited in either the basic research or clinical arenas. Perhaps the main technical reason for this is that the original B cell ImmunoSpot protocol was suitable for only a limited set of antigens. However, recently, this problem has been overcome with the implementation of an affinity capture coating approach (Koppert et al., 2021), which enables rapid development of ImmunoSpot assays for essentially any desired antigen. On the other hand, it is also likely that there was a major conceptual reason that B cell ImmunoSpot assays were not exploited: there has been the widely-held belief that sufficient information on B cell immunity could be obtained by simple measurements of

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serum antibodies, a notion that rendered it unnecessary to struggle with complex, fragile, and more difficult to preserve cell samples. However, this view is no longer tenable since we have learned that detection of memory B cells (B_{mem}) reflects the existence of antigen-specific immunity in infectious- (Terlutter et al., 2018; Wolf et al., 2022) and autoimmune- (Kuerten et al., 2014) settings much more reliably than measurements of serum antibodies. Moreover, with serum antibodies possessing a half-life of only weeks (Note 4), and the plasma cells that replenish them apparently also having shorter lifespans than originally believed (Robinson et al., 2023), we now understand that in some instances serum antibodies only provide evidence of quite recent immune encounters (Long et al., 2020; Gaebler et al., 2021; Seow et al., 2020; Xiang et al., 2021). In contrast, B_{mem} are long-lived and thus not only enable a rapid antibody response upon antigen reencounter, but they can also undergo further refinement of their fine specificity to accelerate the response to new antigenic challenges, e.g., when encountering variant viruses (reviewed in (Akkaya et al., 2020)). Newly developed modifications of B cell ImmunoSpot assays also permit the assessment of B_{mem} cross-reactivity at single-cell resolution (Lehmann et al., 2024), as well as the detailed investigation of the affinity distribution/maturation of an antigen-specific B_{mem} repertoire (Becza et al., 2023). Although immune monitoring assays that enable the characterization of antigen-specific B cells, whether based on flow cytometry or ImmunoSpot assays, have already been available for some time in the research arena, adapting such complex assays for use in the clinical realm has been challenging at several levels. In particular, although guidelines for compliance with industry standards in a regulated environment have been developed for measurements of soluble analytes in liquids (Administration, F.a.D, 2018), they require adaptation for cell-based test systems. This has already been accomplished for T cell ImmunoSpot assays (Korber et al., 2016; Tary-Lehmann et al., 2008) that enumerate antigen-specific T cells within PBMC by detecting cytokines secreted upon specific antigen encounter. Subsequently, T cell ImmunoSpot testing has become an assay of choice for evaluating cell-mediated immune responses in many clinical trials (Slota et al., 2011; Shafer-Weaver et al., 2006; Pass et al., 1998; Malyguine et al., 2007; Ranieri et al., 2014); however, such guidelines do not yet exist for B cell ImmunoSpot assays.

Supplemental Fig. 1 (Suppl. Fig. 1) depicts distinct types of B cell ImmunoSpot assays used for immune monitoring purposes (a link to all "Supplemental Figures" can be found at the end of this paper, under "Appendix"). Because these assays differ fundamentally from T cell ImmunoSpot tests in many aspects, here we describe B cell ImmunoSpot assay development, qualification, and validation recommendations that build on those developed for T cell ImmunoSpot assays but take into consideration the distinguishing features of B cell biology. In doing so, we first outline these distinguishing features; then, following the definitions and experimental templates provided by Bauer et al. (Korber et al., 2016) for T cell ImmunoSpot assays, we provide recommendations for B cell ImmunoSpot assay protocols. As a model for B cell ImmunoSpot assay development, qualification, and validation (Note 5), we leveraged the SARS-CoV-2 Spike (*S*)-antigen-specific B cell response elicited following SARS-CoV-2 infection and/or COVID-19 mRNA vaccination of "naïve" subjects.

2. Theoretical considerations for the verification and validation of antigen-specific B cell ImmunoSpot assays

2.1. B cell biology-dictated implications for verification/validation

There is a considerable gap between the terminology used by basic scientists (in whose realm antigen-specific B cells assays are primarily used presently, and from where knowledge gained thus far needs to be transferred) and the regulated world (that increasingly attempts to adopt such cellular assays for clinical trial monitoring and diagnostics). Therefore, in this communication we will always begin with defining the terminology used in the regulated space so that it also becomes transparent to basic scientists as well.

B cells are the only cell type capable of secreting antibody (immunoglobulin, Ig). Moreover, those B cells that have responded in a T helper cell-dependent manner can switch from secreting IgM molecules (produced by naïve B cells) to the production of the other Ig classes: namely, IgA (subclasses IgA1 and IgA2), IgE, and IgG (subclasses IgG1-IgG4) (Note 6). Importantly, B_{mem} are resting lymphocytes that do not spontaneously secrete antibodies but can do so rapidly following (re-) activation by antigen. This is typically also dependent on antigen-specific, cognate T cell help in vivo but in addition it can be induced in vitro following stimulation with certain mitogens capable of activating all B cells polyclonally (i.e., regardless of their intrinsic antigen specificity). Such polyclonal stimulation is required to detect resting B_{mem} by ImmunoSpot ex vivo (Note 7). Under these conditions, B_{mem} transition, over the course of several days (optimally 5 days, Suppl. Fig. 2 A-F), into ASC. Consequently, one can safely conclude that detecting any number of (even a single) antigen-specific ASC that produced class-switched Ig following polyclonal stimulation of PBMC identifies the presence of antigen-specific B_{mem}. Even if cross-reactive, such B_{mem} can give rise to a rapid-onset secondary immune response. The immune system operates very efficiently with low numbers of memory lymphocytes (Note 8).

As resting B_{mem} do not spontaneously secrete antibody, antigen-specific ASC are rarely detectable in freshly isolated blood (except a few weeks after initiation of an immune response, see below). This is the case even if antigen-specific B_{mem} are found to be abundant in that sample after in vitro mitogenic stimulation (i.e., after the B_{mem} have been polyclonally activated to become ASC, as illustrated on a sample isolated >125 days following recovery from SARS-CoV-2 infection in Suppl. Fig. 3 A vs. 3B). However, ~1 week after antigen exposure in vivo, ASC can be detected directly ex vivo in the blood (Suppl. Fig. 3C and 3D) (Note 9). Such ASC are not B_{mem} cells; they are plasmablasts that appear in the blood as they migrate from the lymphoid tissue in which they became activated to the bone marrow and other immunological niches where they may become resident as plasma cells (PC). Such ASC can readily be detected in ImmunoSpot assays by seeding the PBMC into the test system without prior in vitro polyclonal stimulation. Of note, most of these plasmablasts are specific for the antigen that triggered the immune response in vivo (Suppl. Fig. 3C and 3D).

Detecting such spontaneously antibody-secreting antigen-specific plasmablasts has considerable and thus far largely unexploited immunodiagnostic potential. In the case of B cell-mediated autoimmune conditions; for example, the appearance of these cells can reveal a flare of the disease (Kuerten et al., 2014). Similarly, it is likely to reflect on the reactivation of latent viruses in an immune compromised state (e.g., Cytomegalovirus or Epstein-Barr virus) or might indicate the initiation of transplant rejection episodes. While the appearance of peripheral blood ASC secreting Ig classes/subclasses other than IgA or IgM (Note 10) by itself already documents an ongoing immune response, knowledge of the specificity of these ASC offers so far underutilized opportunities for identifying the antigen(s) that trigger(s) untoward immune responses. Examples are identifying the autoantigens in autoimmune diseases, environmental agents causing allergic reactions, and those responsible for flares of inflammation due to reactivation of persisting infections. Inherent challenges with such assessments are the transient appearance of such antibody-secreting plasmablasts in the blood, as well as the possibility that ASC in some conditions may reside predominantly in the tissue and therefore would not be present in the blood.

Antigen-specific B cell ImmunoSpot assays for detecting such circulating plasmablasts or B_{mem} differ only in the nature of the PBMC (or other cell isolate) to be tested; for the former, the cells are seeded into the ImmunoSpot test plates without any additional treatment, whereas for the latter, the cells are plated after 5 days of in vitro polyclonal stimulation. In both cases, the scope of the assay is the same: to determine unambiguously the number/frequency of antigen-specific ASC

producing a certain Ig class/subclass within either all cells plated, or within all B cells secreting that Ig class/subclass (Note 11).

2.2. Assay optimization for detecting antigen-specific ASC at single cell resolution

The overall scope for optimizing B cell ImmunoSpot assays can be broken down into two separate components. The first can be readily accomplished: detecting plate-bound antibody secretory footprints generated by individual ASC once the capture conditions for these antibodies have been optimized. The second is more challenging: the coating of the antigen itself to the membrane at a sufficiently high density for the antigen-specific Ig released by ASC to be efficiently captured on the membrane. Fulfilling this latter requirement has caused considerable challenges in the past, but these have now been overcome by the technique of affinity coating of the antigen (Koppert et al., 2021). (See also Suppl. Fig. 1 A vs. 1B).

Suppl. Fig. 4 A shows secretory footprints that were generated by a murine B cell hybridoma when incubated on a membrane coated with high-affinity anti-mouse Igκ capture antibody (of goat origin; for a description of this type of assay refer to Suppl. Fig. 1C). When high-affinity anti-mouse IgG detection antibodies are used to visualize the plate-bound murine (B cell hybridoma-derived) antibody, pristine spot-forming units (SFU) become visible. Plating the hybridoma cells in serial dilution and counting the resulting SFU using a suitable reader reveals a near perfect linear relationship between the number of hybridoma cells plated per well and the number of SFU detected per well within a defined cell input range, revealing that 56 % of the hybridoma cells were capable of generating a secretory footprint during the assay incubation period (Suppl. Fig. 4C, Notes 12 and 13). Suppl. Fig. 4D shows secretory footprints generated by B cell blasts (following in vitro polyclonal stimulation of PBMC) detected in an analogous manner using species-specific detection reagents; with these too, cell numbers plated and SFU counts detected precisely follow a linear function (Suppl. Fig. 4F). In both instances, the spot morphologies originating from the hybridomas or the B cell blasts exhibit very similar profiles in their size, shape, and density. Such data establish that the per cell antibody productivity of the hybridomas and that of B cell blasts during the analyte capture phase of the assay were both clearly detectable. Therefore, the verification that secretory footprints originating from single hybridoma cells are being visualized implies the same for the detection of human ASC. In other words, these types of tests verify that the detection reagents used indeed detect secretory footprints of individual ASC. To control for the quality of the detection reagents' performance, we recommend the inclusion of positive controls in which polyclonally stimulated PBMC (because of the high inter-individual variability among donors, preferably pre-characterized PBMC) reference samples (Tary-Lehmann et al., 2008) are tested in serial dilution after pan Ig capture for Ig class/subclass-producing ASC (Suppl. Fig. 5).

The real challenge for antigen-specific B cell ImmunoSpot assay development has been efficient coating of the membrane with the antigen of interest. For detecting antigen-specific ASC, the membrane must be densely coated with antigen. The membrane best suited for ImmunoSpot analysis is a PVDF fibered filter (Forsthuber et al., 1996) that, due to its hydrophobicity and fractal surface structure, excels in binding proteins (Weiss, 2012), or to be more precise in this context, to bind proteins with sufficient hydrophobic regions, such as Ig. For such hydrophobic proteins, the classic, direct membrane-absorption-based coating strategy suffices for developing antigen-specific B cell ImmunoSpot assays. However, such proteins are more the exception than the rule among antigens of interest; and, for the remainder, affinity capture of the antigen greatly reduces the quantity of protein required to achieve the necessary coating density. This can be accomplished by coating the membrane first with an Ig (Koppert et al., 2021; Terlutter et al., 2018; Bisceglia et al., 2023) or with an alternative capture protein that is sufficiently hydrophobic (see Suppl. Fig. 1B). This specific "antigen-capture reagent" will then bind with high affinity to the soluble antigen in solution (Note 14). For example, recombinant proteins are commonly engineered to contain a short amino acid sequence tag (e.g., His-tag) that facilitates their purification via affinity chromatography; the same tags can be used to achieve high density protein coating via plate-bound anti-tag capture antibodies (Koppert et al., 2021). Notably, the anti-tag capture approach is far more universal than direct antigen coating as it enables the high affinity capture of different unrelated proteins that need only to share a common affinity tag. Therefore, the recent introduction of affinity capture coating has made detection of antigen-specific ASC via B cell ImmunoSpot analysis a generalizable immune monitoring tool (Note 15); failed past attempts with direct coating are likely one reason that this simple and powerful B cell ImmunoSpot technology did not find its place in the standard immune monitoring repertoire until now.

As a rule of thumb, coating the PVDF membrane with 10 μg/mL of antigen-capture antibody, and then adding the antigen itself at 10 μg/mL will result in high density antigen coating. However, as both reagents are unique to the type of (or even batch of) antibody and antigen used, titrating both during the assay development phase is highly recommended for obtaining optimal antigen coating while also minimizing the amount of antigen-capture antibody and antigen required. Suppl. Fig. 6 shows spot morphologies seen with suboptimal vs. optimal coating. Of note, not only does the spot morphology deteriorate with suboptimal coating (to the point of becoming vague or even undetectable), but, as the antigen-density on the membrane decreases, just the high affinity end of the antigen-specific B cell repertoire becomes detectable (Becza et al., 2023). When testing primary cell material containing B cells with different affinities for the antigen, coating can be considered optimal if a full spectrum of spot morphologies is detected, ranging from dense and sharp spots (produced by the high affinity end of the B cell repertoire) to small and faint, often also diffuse spots representing the low affinity end of the repertoire. (See also Suppl. Fig. 7 and Notes 16 and 17).

For optimizing antigen coating conditions, reference PBMC are well-suited (Tary-Lehmann et al., 2008) if available (Note 18). If unavailable, or when B cell reactivity to the antigen in question needs to be verified (as for autoimmunity or cancer, see Note 19), B cell hybridomas specific for that antigen can be used for antigen coating optimization. In case such hybridomas are not available, immunizing mice with the antigen of interest and testing the spleen cells of the immunized mice in a murine B cell ImmunoSpot assay is another option (Note 20). In either case, antigen coating optimization can be considered complete only if pristine spots are detected covering a wide range of sizes and morphologies (Note 21).

We strongly advise principal investigators, study directors, auditors, and reviewers not to accept mere SFU counts as B cell ImmunoSpot data reports without visually verifying the quality of spots evaluated. Suitable analyzers will automatically retain raw well images and the results of the analysis as count overlays in a tamper-proof fashion, providing ease of access and transparent audit trails (Zhang and Lehmann, 2012).

2.3. Count optimization for detecting secretory footprints of individual cells

2.3.1. Counting parameters for machine reading

Once assay development for visualization of secretory footprints generated by individual antibody-secreting B cells has been accomplished, the next step is counting them by machine reading. These secretory footprints (which we also refer to as SFU) are readily countable by eye under a dissection microscope (ELISPOT) or a fluorescent microscope (FluoroSpot), but visual counting is, with these assays being high throughput and ideally high content in nature, tedious, imprecise, and leaves no audit trails. Based on theoretical modeling (Karulin and Lehmann, 2012) and as experimentally verified (Becza et al., 2023), machine reading of direct B cell assays is needed to precisely resolve four distinct spot morphologies at the extremes and all transitions in between

(see Suppl. Fig. 7D and (Karulin et al., 2024)). Regarding spot intensity (the first dimension), the brightest spots originate from ASC that produced high affinity antibodies for the antigen of interest, whereas faint or dim spots are generated by ASC secreting lower affinity antibodies. In the second dimension, spot sizes reflect per cell productivity (Note 22). Suitable software can accurately assess spot sizes and intensities (Suppl. Fig. 7B—D) and thus permit not only precise SFU counting, but also high content analysis for assessing the affinity spectrum of an antigen-specific B_{mem}-derived ASC repertoire (Becza et al., 2023) (Note 23).

Due to clonal diversity of the naïve antigen-specific B cell repertoire originally engaged, and the subsequent emergence of subclones with diverse affinities for the antigen generated through the process of affinity maturation (Note 24), the antigen-specific B_{mem} repertoire in most instances will include a wide spectrum of affinities. This can range from B cell receptors (BCR)/secreted antibodies with weak to very weak binding ($K_D > 10^{-4}$) to very strong ($K_D \langle 10^{-11}$) (Wysocki et al., 1986; Wrammert et al., 2008; Padlan et al., 1989; Moulana et al., 2023; Dzimianski et al., 2023; Di Niro et al., 2015; Dal Porto et al., 2002; Liao et al., 2013), encompassing greater than a 10-million-fold potential difference in the binding strength of the BCR/antibody to antigen (Note 25). Overall, antibody affinity/avidity for antigen is (in addition to the Ig class/subclass) the critical determining factor for their biological effector potential (Note 26). As all B_{mem} have passed an affinity-based selection criterion when the parental (germline) naïve B cell was first engaged in the immune response, and as the hypermutated subclones of those parental cells have in most cases undergone additional rounds of affinity-based positive selection (Note 24), the B_{mem} repertoire secreting IgG, IgA, and IgE antibodies consists primarily of cells secreting Ig towards the higher affinity end of the spectrum. For optimized antigen-specific IgG (and IgA) assays, there are relatively few "ambiguous" spots observed in assays detecting B_{mem}-derived ASC producing these class-switched antibodies in naïve vs immunized individuals (Suppl. Table 1 and Suppl. Fig. 8 A) reflecting the individual's antigen-primed status with near perfect accuracy. The analysis of IgM antibodies produced by broadly-reactive naïve B cells after in vitro polyclonal stimulation, however, provides a different level of conceptual and technical challenge (Note 27).

2.4. Accurate ASC frequency assessment

When measuring frequencies of B_{mem}-derived antigen-specific ASC using the direct assay, we need to keep in mind that not only do the frequencies of ASC producing different Ig classes (and subclasses) span orders of magnitude, and additionally exhibit considerable variation among individuals (Wolf et al., 2022) and Suppl. Fig. 5), but also the number of antigen-specific B cells within each class/subclass spans a similarly wide range. A representative illustration of frequency variations for B_{mem}–derived IgG^+ ASC specific for different antigens in a cohort of healthy subjects is provided in Suppl. Fig. 9. The data for this figure were obtained by seeding polyclonally stimulated PBMC at a fixed number of 3×10^5 PBMC per well for screening purposes. When analyzed in the context of Suppl. Fig. 10 (which shows that SFU counts >100 per well underestimate the actual number of ASC present in a sample), it becomes clear that testing samples at a single PBMC concentration precludes accurate frequency assessments; many of the data points obtained are either over or under the reliable quantification limit of the assay when a single cell concentration (e.g. 2×10^5 per well) is used for testing.

2.4.1. Upper quantification limit

The upper quantification limit of B cell ImmunoSpot assays is caused by overcrowding of the spots. While the individual ASC secretory footprints can be clearly visualized when there is sufficient space for each to be captured without overlap, this does not start to occur for antigen-specific assays until around 100 SFU per well (examples of which are shown in Suppl. Fig. 10) and for pan Ig assays (that display tighter footprints) at ~300 SFU per well (see Suppl. Fig. 4, and Note 28). In addition, as the number of ASC per well increases, the "ELISA effect" increases in magnitude. Specifically, the ELISA effect occurs due to an increased concentration of ASC-derived antigen-specific antibody in the supernatant that is captured on the antigen-coated membrane independent of the original cellular source. Consequently, the ELISA effect elevates the overall background staining of the assay membrane and interferes with the detection not only of faint spots, but also the outlines that define individual ASC-derived secretory footprints (Note 29). Defining the upper limit of quantification (ULOQ) as the maximal number of ASC which can be detected in the assay well, is assay-dependent at ~100 SFU per well for antigen-specific, and ~ 300 SFU per well for pan Ig tests. However, the cells can be sufficiently diluted, as we suggest here – i.e. plating them in serial dilution as a simple and necessary solution to extend frequency measurements for PBMC samples without an upper quantification limit (Note 30). When testing PBMC for B_{mem}-derived antigen-specific ASC, we recommend starting at 2×10^5 cells per well and progressing in a 1 + 1 dilution series (see Suppl. Fig. 5 A). While SFU crowding may occur at the highest inputs of the cell titration for some test samples, in such cases the lower cell inputs will still follow a linear relationship between cell numbers plated and SFU counts. Using such a serial dilution approach also permits an accurate assessment of ASC frequency in the sample by averaging the frequencies calculated from each dilution point in the linear range (Suppl. Fig. 10) (Note 31).

2.4.2. Lower detection limit and lower limit of quantification

With regard to interpreting antigen-specific B cell ImmunoSpot test results, there are two basic questions to be answered. The first is qualitative: are there primed B_{mem} present in an individual? The second is quantitative: what is the precise frequency of B_{mem}-derived ASC within the PBMC test sample? Regarding the immunodiagnostic significance of the lower detection limit, it can be assumed that, if antigen-specific B_{mem} are present, even at very low numbers, this can have a major impact for the individual host (Note 8). As antigen-specific B (and T) cells, can occur at low frequencies in antigen-primed individuals, often the aim is to lower the detection limit for antigen-specific ASC as much as possible.

Despite not yet having studied this issue in depth, our experience gained so far indicates that if a single ASC producing antigen-specific IgG is plated, that this ASC will be reliably detected on the basis of its secretory footprint. Therefore, while the lower limit of quantification will depend on the number of PBMC assessed, and is thus specific for a particular assay protocol, there is no inherent lower limit of detection for such tests. If, for example, 10^6 PBMC are seeded per well into 100 replicate wells, the detection limit can be extended to be, in this case, 1 in 10^8 PBMC, provided there is no background noise.

The general rule in the T cell field for detecting weak antigen-specific responses is that the mean number of antigen-specific SFU (established in 3–4 replicate wells at a single cell input) should be more than 1.65-fold the standard deviation of the SFU count in the control wells (established in 3–4 replicate wells at the same single cell input). As B cells are the only cell type capable of producing antibody, in antigen-specific B cell assays every spot counts. In future studies aiming to extend the lower detection threshold, it should be kept in mind that (unlike for T cell assays), in B cell assays, the Poisson distribution applies when analyzing low frequency ASC events (Suppl. Fig. 11 and Suppl. Fig. 12). As to the lower limit of quantification for B cell ImmunoSpot assays in general, Poisson rules also apply (Suppl. Fig. 11 and Suppl. Fig. 12). Here too, increasing the number of PBMC plated per well, along with the number of replicate wells, will increase the accuracy of frequencies determined in the very low frequency range.

As frequencies of antigen-specific B_{mem}-derived ASC span a wide range (Suppl. Fig. 9 and (Wolf et al., 2022; Lehmann et al., 2024), it is recommended to test the PBMC first in a serial dilution, starting at 2×10^5 per well (Suppl. Fig. 5 A); or, if the frequencies are anticipated to be lower, we recommend to start the serial dilution at 1×10^6 PBMC per

well (cell material permitting) (Note 32). Should samples be outside of the range covered by this first approach and if extending the lower limit of detection is necessary for such samples, additional aliquots can be thawed (Note 33) in which the aforementioned testing of replicate wells with high cell numbers is applied to extend the lower limit of detection/qualification.

2.4.3. Interpreting B_{mem} presence and frequencies

As a first approximation, therefore, one could expect the frequency of B_{mem} to be proportional to the magnitude of secondary antibody responses that these cells will generate upon antigen reencounter (Note 34). Similarly, even if an individual has not been exposed to a specific antigen (e.g., a new virus variant that emerges, a so-called heterotypic virus) but possesses cross-reactive B_{mem} (e.g., induced by the original homotypic virus), such B_{mem} can be expected to convey an elevated level of immune protection through their ability to rapidly differentiate into ASC and raise the level of specific antibody. Confirming this notion, lower B_{mem} frequencies may correlate with the risk for vaccine breakthrough infection caused by the SARS-CoV-2 variants. Therefore, detecting any number of antigen-specific B_{mem}-derived ASC (as identified by their production of class-switched antibodies), whether primed by the nominal antigen or a cross-reactive antigen, should be of immune diagnostic significance (Note 35). Refer to (Lehmann et al., 2024) for measurements of B_{mem} cross-reactivity at single-cell resolution using the B cell ImmunoSpot approach, and to an in-depth description of preformed antibody (the "first wall of B cell defense") and B_{mem}-mediated immunity (the "second wall of B cell defense") as it relates to homo- vs. heterotypic immune defense (Akkaya et al., 2020).

2.5. Accurate detection of Ig class

In addition to establishing the mere number of B_{mem} present in the body, which reveals the existence and magnitude of immunological memory, it is also essential to define the Ig class (and/or subclass) these B_{mem} will produce upon antigen encounter or reencounter. This is because immunoglobins of different Ig classes (and subclasses) mediate fundamentally different defense reactions, primarily due to their differential binding to Fc receptors and complement-activating properties (Note 36).

Upon antigen (re-)encounter, and subsequent re-activation, B_{mem} can rapidly differentiate into PC that secrete the same Ig class/subclass expressed by the parental B_{mem}. Therefore, detecting the Ig class that B_{mem}-derived ASC produce in ImmunoSpot assays permits the specific type(s) of antibody that will be produced following the next antigen encounter to be predicted. Knowledge of the full spectrum of Ig classes that the antigen-specific B_{mem} repertoire will produce is essential for predicting the protective efficacy of future antibody responses to a "specific" antigen. Moreover, such B cell ImmunoSpot assays may also predict the likelihood of antibody-mediated complications occurring upon antigen re-exposure (e.g., causing allergic reactions or antibody-dependent enhancement leading to exacerbation of clinical disease (Wang et al., 2017). Importantly, the frequencies of antigen-specific B_{mem} capable of secreting different Ig classes differ by orders of magnitude (Wolf et al., 2022; Yao et al., 2024). Thus, it is not only recommended to assess all antigen-specific Ig classes, but also to do so over a wide frequency range for each. The latter can readily be accomplished by combining the serial dilution strategy with 4-color ImmunoSpot analysis (Yao et al., 2024) (Suppl. Fig. 5 and Suppl. Fig. 13).

2.5.1. Four-color B cell ImmunoSpot analysis

Individual B cells can only express a single Ig class/subclass (Stavnezer et al., 2008). Therefore (unlike T cell ImmunoSpot analysis), standard multi-color B cell ImmunoSpot analysis does not need to account for the co-expression of the different analytes (Ig classes or subclasses) studied. This basic biologic fact greatly facilitates objective identification of secretory footprints in multiplexed B cell assays, provided that the fluorochromes used only yield a signal in the corresponding detection plane and do not cross-bleed into the others (Suppl. Fig. 13 and Note 37). Such fluorochromes, which possess non-overlapping spectra, are used in CTL's multi-color detection systems (Karulin et al., 2018). Of note, the sensitivity of the enzymatic (single- or double-color) ELISPOT and of the (up to four-color) FluoroSpot modalities for quantifying individual B cell-derived Ig secretory footprints are similar (Yao et al., 2024), see also Suppl. Fig. 14, and Note 38). For the comprehensive assessment of the antigen-specific B cell repertoire, we recommend performing a four-color assay to determine the major classes of secreted Ig (MAGE), and, in parallel, a four-color assay measuring individual IgG subclasses if cell material and coating antigen(s) are not limiting.

2.6. Optimization of PBMC utilization

The volume of blood available for cellular immune monitoring is often the limiting factor in clinical trials, particularly when it comes to obtaining PBMC from children and critically ill patients. Thus, devising the most efficient PBMC utilization protocols primarily defined our approach for comprehensive assessment of the antigen-specific B cell repertoire. Conventionally, cellular assays, including T cell ImmunoSpot tests, are performed using replicates, typically 3–4 replicate wells per test condition, from which means and standard deviations (SD) are established with the required level of precision. In the T cell assay, to become activated, very rare antigen-specific T cells must interact with antigen-presenting cells within the PBMC monolayer on the well bottom, triggering antigen-induced cytokine production. In contrast, in B cell ImmunoSpot tests, individual ASC are detected that are already producing Ig "spontaneously", in a cell autonomous manner (no matter whether these B cells became activated in vivo, or whether resting B_{mem} were subjected to in vitro polyclonal stimulation prior to testing in a B cell ImmunoSpot assay).

Because pre-activated B cells do not require cell-cell contacts for secreting antibodies, the PBMC test samples can be diluted much further than in T cell assays (that strictly depend on T cell and antigen-presenting cell contacts) to obtain optimal (20−100) SFU counts. For frequency calculations, it is plausible that testing PBMC in serial dilution will provide similarly accurate ASC frequency measurements when done with a single well per cell input by calculating the frequency by averaging the results from several dilution points compared to establishing frequencies from the mean of replicate wells at each cell input. Extensive comparisons confirmed this assumption (Suppl. Fig. 15). Testing PBMC in single well serial dilutions for the initial frequency assessment in yet uncharacterized samples, therefore, allows the investigator to reduce the number of cells needed to 4.4×10^5 (with safety margin 5×10^5) cells per antigen (when the serial dilution is started at 2×10^5 PBMC per well in a four-color IgM, IgA, IgG and IgE detection test). An illustration of a recommended well layout for such a B cell ImmunoSpot test is provided in Suppl. Fig. 5 A. Note 39 provides suggestions for how to set up such serial dilutions in a streamlined manner. PBMC can be cryopreserved while maintaining full B cell functionality (Fecher et al., 2018), with 80–90 % of the cells typically recovered after thawing. If the PBMC are to be tested for B_{mem}-derived ASC reactivity after in vitro polyclonal stimulation, up to 50 % cell loss can be expected (Suppl. Fig. 2H). Therefore, when working with cryopreserved cell material to detect B_{mem}-derived ASC after 5 days of in vitro polyclonal stimulation (or plasmablasts directly ex vivo), cryopreserving cells at $1–2 \times 10^6$ PBMC per aliquot is minimally recommended per antigen (Note 40) – ideally, several such aliquots. The first aliquot will suffice for assessing the frequency of B_{mem}-derived antigen-specific ASC producing one of the four Ig classes, including defining the overall numbers of ASC producing these Ig classes irrespective of their antigen specificity. Additional aliquots may be used for extending the lower detection limits, if that would be needed. Alternatively, affinity- (Becza et al., 2023) or cross-reactivity (Lehmann et al., 2024) assessments of the antigen-specific ASC

repertoire could be performed using the remaining cell material, if desired. It should also be noted here that ASC survive the ImmunoSpot testing unharmed, and instead of discarding them at the end of the ImmunoSpot assay, they can be utilized for additional functional or genetic analyses.

3. Validation of B cell ImmunoSpot tests

According to the accepted definition, method validation is an evaluation process of the performance characteristics of an established analytical procedure through laboratory studies with all performance characteristics meeting the requirements of the intended analytical applications. In other words, an analytical method should be examined from a variety of aspects to prove that the test results can be trusted and appropriately applied to the intended quality objective.

Following the guidelines for T cell ImmunoSpot assay validation (Korber et al., 2016; Tary-Lehmann et al., 2008), modified to accommodate the divergent features of B cell assays described above, we determined the (1) Specificity, (2) Accuracy, (3) Precision, (4) Detection Limits, (5) Quantification Limits, and (6) Linearity of our SARS-CoV-2 S-antigen-specific, 4-color Ig class (IgM/IgA/IgG/IgE, "MAGE") B cell ImmunoSpot assay using the serial dilution variant. As B cell ImmunoSpot assays lend themselves to high throughput testing (Notes 41–44), in the following we will recommend a workflow and experimental design that permits the extraction of all the raw data needed for a full validation of the antigen-specific B cell ImmunoSpot test by performing only three relatively simple tests, requiring in total 2.2×10^7 PBMC; as outlined in Note 43 (see also Note 44).

3.1. Specificity

3.1.1. Immunological specificity

Establishing antigen-specificity requires testing PBMC from negative control individuals (who have not been exposed yet to the antigen of interest) as compared to positive controls (those known to have been exposed). In the case of our example, the negative control tested the presence of B_{mem}-derived ASC with reactivity for the S-antigen (representing the prototype Wuhan-Hu-1 strain of SARS-CoV-2 virus) in PBMC collected prior to the onset of the COVID pandemic (pre-COVID era). This was compared with the results of testing PBMC from individuals with PCR-verified SARS-CoV-2 infection before vaccines became available. We also tested PBMC collected from individuals 6 months after receiving two doses of COVID-19 mRNA vaccine (that encoded the Wuhan-Hu-1 S-antigen) before infections with SARS-CoV-2 variants became widespread. The non-infected status of these latter donors was verified by testing for SARS-CoV-2 Nucleocapsid (NCAP) antigen reactivity. Suppl. Table 1 shows the frequencies of Wuhan S-antigen-reactive B_{mem}-derived ASC detected in these three cohorts. The data indicate that 100 % of the vaccinated individuals and 100 % of the subjects that recovered from PCR-verified SARS-CoV-2 infection, but none of the pre-COVID era samples, possessed B_{mem}-derived IgG^+ ASC reactivity against the S-antigen at high or relatively high frequencies. Of note, the frequencies of pan IgG^+ (irrespective of antigen specificity) ASC measured in parallel were similar in the three cohorts, documenting that neither cryopreservation nor the duration of storage could account for the observed differences in S-antigen-specific IgG^+ ASC detected. In contrast, S-antigen "specific" IgM^+ ASC reactivity following polyclonal stimulation was detected in the positive and negative control cohorts alike (Suppl. Fig. 8B), implying broad reactivity and lack of specificity for in vitro induced IgM^+ ASC.

3.1.2. Diagnostic specificity

The data in Suppl. Table 1 also establish the 100 % diagnostic specificity of this B cell ImmunoSpot test for identifying individuals that received a COVID-19 mRNA vaccine or developed B cell memory following infection. While it may be more the exception than the rule, the S-antigen example we have selected here perfectly illustrates the immunological and diagnostic specificity of the IgG^+ B cell ImmunoSpot approach. Notably, in spite of some sequence conservation between the S-antigen encoded by SARS-CoV-2 and other coronaviruses causing common colds, we failed to detect B_{mem}-derived ASC reactivity against the S-antigen of SARS-CoV-2 in any of the pre-COVID era samples. In contrast, B_{mem}-derived IgG^+ ASC reactivity against recombinant hemagglutinin (rHA) antigens representing seasonal influenza vaccine strains were clearly detectable in these same assays in all cohorts (data not shown).

Fast forwarding to the present, because most individuals have since either received multiple COVID-19 vaccinations and/or have been infected with one or more variant SARS-CoV-2 viruses, identifying an individual that is immunologically naïve to the prototype Wuhan-Hu-1 S-antigen has become quite rare in the population (Kirchenbaum, manuscript in preparation). As such, B_{mem}-derived ASC reactivity against the S-antigen can potentially serve as a universal positive control for B cell ImmunoSpot testing, alongside influenza antigens, and probably many other antigens that originate from other viruses causing ubiquitous infections.

3.1.3. Immunological interpretation of B_{mem} or plasmablast specificity

The clarity of the data shown in Suppl. Table 1 (i.e., none of the SARS-CoV-2-naïve, but all the convalescent or vaccinated individuals display vigorous S-antigen-specific B cell memory) would imply a tremendous potential for B_{mem} monitoring in immunodiagnostics. But to what extent are these findings likely to apply to antigens other than S-antigen of the prototype Wuhan-Hu-1 strain, where a clearly naïve vs. exposed state can be defined? And even when it comes to the S-antigen, does the marked difference seen between the naïve and antigen-primed state hold up for later time points when B cell memory has established itself, for example when desiring to monitor the impact of booster vaccinations? While these are the most fundamental questions for the immunodiagnostic interpretation of B cell ImmunoSpot results, the answers are still awaited. However, the observations we have already made anticipate some of these answers, as discussed in the following.

Better understanding of B cell cross-reactivity in vivo, including affinity cut-offs, will be an issue. The B cell system has evolved to achieve remarkable specificity for the selective recognition of any given antigen. This is mediated through the enormous clonal diversity present in the naïve B cell repertoire which encodes $\sim 10^{12}$ BCR from which a small number of cells endowed with BCR specific for the antigen of interest (and with a sufficient affinity to be activated) are recruited into an immune response. Subsequently, the antigen-specificity of the B cell response is further improved by somatic hypermutation of the BCR expressed by the positively selected B cells participating in the germinal center reaction, which is then followed by further rounds of hypermutation and positive selection of the subclones which express BCR with the highest affinity for the antigen (a process termed "affinity maturation") (Victora and Nussenzweig, 2022). While such highly specific B cells can immediately contribute to immune defense by differentiating into ASC and rapidly increasing the abundance of specific antibody (constituting the so-called "first wall" of B cell-mediated immunity (Akkaya et al., 2020), we also need to keep in mind that the B cell system is adaptive and has evolved the ability to confer cross-reactive protection against antigenically-related pathogens in order to provide increased responsiveness to variant viruses (the so-called "second wall" of B cell-mediated immunity (Akkaya et al., 2020).

For example, as discussed above, we did not detect B_{mem}-derived IgG^+ ASC with reactivity against the SARS-CoV-2 S-antigen in pre-COVID era PBMC samples (Suppl. Table 1), despite the fact that most likely everyone has been infected with related coronaviruses responsible for causing common colds (Nickbakhsh et al., 2020), and therefore could be expected to possess cross-reactive B_{mem}. However, we found that ~40 % of B_{mem}-derived IgG^+ ASC with reactivity against the receptor binding domain (RBD) of the S-antigen expressed by the prototype

Wuhan-Hu-1 strain, elicited by infection early in the COVID pandemic, cross-reacted with the RBD probe representing the BA.1 Omicron variant (Lehmann et al., 2024). Such PBMC samples were collected long before the emergence of the Omicron variant, which caused widespread infections, so this finding implies that some S-antigen epitopes targeted by such "cross-reactive" ASC remained conserved on the variant (BA.1) strain. Studies of pre-Omicron and post-Omicron PBMC using the BA.1 RBD probe are therefore not likely to provide the same clear-cut discrimination between Omicron-naïve and Omicron-exposed individuals due to the pre-existence of such cross-reactive B_{mem}. Importantly, however, these pre-existing cross-reactive B_{mem} also were capable of contributing to protective responses, as evidenced by reduced disease severity in cases of breakthrough Omicron infection. Therefore, while blurring the diagnostic specificity of comparisons between PBMC samples collected before or after emergence of SARS-CoV-2 variants of concern, the detection of cross-reactive B_{mem}-derived ASC still provides valuable immune diagnostic information on the pre-existence of a primed, clonally expanded and IgG class-switched B_{mem} repertoire reactive with the antigen being studied. What percentages of such pre-existing, cross-reactive B_{mem} are recruited into such a (secondary) B cell response, including to what extent they undergo further somatic mutation and affinity maturation, and clonal expansions, remains an open question.

Additionally, to what extent increases of B_{mem} frequencies reveal renewed antigen exposures is also an open question. In our preliminary studies on this issue, we found evidence for clonal expansions after each COVID-19 mRNA vaccination. In paired PBMC samples collected prior to and 14 days following the first COVID-19 mRNA vaccination, frequencies of S-antigen-reactive B_{mem}-derived IgG^+ ASC went from undetectably low to an average of 20 IgG^+ ASC per 10^6 PBMC (Suppl. Fig. 8 A). In a separate cohort, paired PBMC collected ~1 month following the second and third COVID-19 mRNA vaccinations also evidenced an increase in the frequency of S-antigen-reactive B_{mem}-derived IgG^+ ASC following each COVID-19 mRNA vaccination. Following the second COVID-19 mRNA vaccination the average frequency of S-antigen-reactive B_{mem}-derived IgG^+ ASC was ~527 IgG^+ ASC per 10^6 PBMC and it increased to ~3822 IgG^+ ASC per 10^6 PBMC following the third dose (~7.5-fold increase). Detailed assessment of PBMC samples collected following additional COVID-19 mRNA vaccinations and/or breakthrough infection with SARS-CoV-2 variants is ongoing in our group and seeks to define whether S-antigen-reactive B_{mem}-derived ASC frequencies plateau after repeated antigen encounters. Therefore, increases in B_{mem}-derived ASC frequencies may no longer be a reliable marker for diagnosing new antigen encounters once the memory B cell repertoire has been established.

When frequency increases do not reveal antigen exposures, what other measures could be applied to better understand whether a new wave of B cell responses has been initiated, and whether this may have medical implications? For example, this is highly relevant to the important question of whether there is a benefit from repeated booster vaccinations, or is this merely a misinterpretation of Burnett's clonal selection theory? While the answer to this question is still unknown, we can make some predictions, as follows.

We must keep in mind that not all antigen-specific B_{mem} are equal, even if they produce the same IgG class/subclass. Specifically, for example, owing to differences in the affinity of secreted antibody, a one million times higher concentration of low affinity ($K_D = 10^{-5}$) antibody would be required to achieve the same degree of antigen binding as a high affinity ($K_D = 10^{-11}$) antibody (Notes 25 and 26). Moreover, it is known that affinity maturation is reengaged with each repeated antigen encounter (Victora and Nussenzweig, 2022). Therefore, studying changes within the affinity distribution of the antigen-specific B_{mem} repertoire (that also can readily be measured by B cell ImmunoSpot (Lehmann et al., 2024)) might shed light on the beneficial effects of booster vaccinations when mere frequency increases do not provide conclusive information.

Studying the appearance of spontaneously Ig-secreting antigen-specific plasmablasts, which appear in the blood shortly after the (re-) engagement of an immune response (Note 9), may also hold great immunodiagnostic potential when simply measuring frequency increases of antigen-specific B_{mem} no longer provides adequate resolution for detecting the induction of an immune response. Shortly following an antigen encounter, e.g. after SARS-CoV-2 mRNA vaccination, descendants of antigen-specific B cells that were engaged in the immune response appear in the blood (Suppl. Fig. 3C and 3D). Representing an alternative B cell fate than B_{mem}, such plasmablasts are in the process of migrating via the bloodstream to immunological niches, such as the bone marrow, where they may take up residence and become long-lived plasma cells that contribute to maintaining antibody titers long-term. Similar to B_{mem}, plasmablasts can also be cryopreserved while retaining their full functional activity (Fecher et al., 2018). Importantly, plasmablasts in blood can be detected using the same direct B cell ImmunoSpot approach as described here for B_{mem}-derived ASC, but without the need for prior in vitro polyclonal stimulation, by simply plating the PBMC as isolated (or after the thawing of a cryopreserved sample).

A fascinating, but still completely underdeveloped field is the study of the B_{mem} specific for autoantigens (including tumor-associated antigens). The following fundamental questions will need to be answered before the field of B (or T) cell immune diagnostics can progress with autoimmune diseases or cancers. In healthy individuals, and if so, to what extent, does the presence of the autoantigen (and which of the diverse autoantigens present on the target cells of interest) result in the spontaneous priming of autoantigen-specific B_{mem}, and lead to their activation, deletion or induction of unresponsiveness? What is the situation with those tumor-associated antigens that are in fact overexpressed autoantigens? As autoantibodies specific for such autoantigens can be frequently detected in healthy individuals (Nagele et al., 2013), it might not come as a surprise if B_{mem} will also be detected. How does this autoantigen-specific B_{mem} repertoire change during an exacerbation of the autoimmune response in an autoimmune disease and/or cancer? Will studies of the resting B_{mem} repertoire, or that of plasmablasts provide more insights into the underlying autoimmune responses? Owing to their exquisite sensitivity, if properly developed and validated, B cell ImmunoSpot assays are presently best positioned to provide answers to these fundamental questions. In these settings, however, a simple comparison of autoantigen/tumor associated antigen-reactive B_{mem} frequencies between a "healthy" cohort vs. patients with the "autoimmune disease/cancer" may not always provide the expected diagnostic specificity information (primarily, if B_{mem} are present in healthy donors), even if the antigen-specificity of the B cell ImmunoSpot test system is exquisite.

3.1.4. *Traditional definition of specificity in terms used by regulatory bodies*

In bioanalysis, specificity refers to the ability to detect an analyte in the presence of potentially interfering substances. In the context of B cell ImmunoSpot assays, this interpretation of specificity translates into assessing whether the reliable detection of individual antigen-specific ASC in PBMC is compromised by other components present in the test sample. This could be due to absorption of antibodies by bystander cells, for example, or through restricted access to the antigen-coated membrane and lack of secretory footprint formation when large numbers of cells are plated in the assay well.

To test the hypothesis that such interference might occur, we evaluated PBMC containing S-antigen-specific, B_{mem}-derived IgG^+ ASC following polyclonal stimulation with or without the addition of autologous non-stimulated PBMC from the same blood draw (while the latter contain S-antigen-specific B_{mem}, these cells are not actively secreting antibody). The results are shown in Suppl. Table 2, revealing negligible interference following addition of high numbers (5×10^5) of resting autologous PBMC. Furthermore, the addition of unstimulated autologous PBMC did not appreciable alter the morphology of S-antigen-

specific secretory footprints (Suppl. Fig. 16).

As resting PBMC (that do not contain spontaneous ASC) were used in the above experiment, we also tested whether the addition of polyclonally activated PBMC (that contained an abundance of ASC but which lack specificity for the S-antigen) would interfere with the test results. To be able to do so, we needed to select donors for whom we had pre-COVID era PBMC cryopreserved (as the latter do not contain S-antigen-specific ASC); the addition of such autologous polyclonally stimulated pre-COVID PBMC to PBMC collected following COVID-19 mRNA vaccination resulted in an increased number of pan IgG^+ ASC. However, the number of S-antigen-specific B_{mem}-derived IgG^+ ASC measured in the assay was not altered (Notes 45–47). The results shown in Suppl. Fig. 17 suggest that increasing the number of third-party antigen-specific ASC in the test sample does not interfere with the detection of antigen-specific ASC.

3.2. Accuracy

Accuracy is defined as closeness of agreement to a verifiably true value. For antigen-specific ImmunoSpot assays the true value that the assay aims to reveal is the number/frequency of antigen-specific, functional B or T cells in a test sample (Notes 48 and 49). However, ImmunoSpot assay accuracy cannot be determined due to the lack of a reference standard or an alternative test that could provide an exact measurement of functional antigen-specific B or T cells in a given sample (except highly developed ImmunoSpot measurements, themselves). However, evaluating B cell hybridomas in such B cell ImmunoSpot tests, as shown in Suppl. Fig. 4, comes close to this requirement. For validated T cell ImmunoSpot assays, therefore, the emphasis has shifted towards obtaining data on the accuracy of a laboratory's performance by participation in proficiency panels that yield information on a laboratory's performance relative to the other laboratories testing the same reference samples (Note 50). For B cell ImmunoSpot assays, such proficiency panels do not exist yet.

While the proficiency panel approach is certainly a work-around to the intended accuracy mandate, it is, for obvious reasons, a rather questionable one. As such proficiency panels neither require, nor encourage, the participating laboratories to harmonize through adopting standardized (best performing) protocols, reagents, or instruments, the participants, randomly utilizing a wide variety of such approaches, are likely to underperform on average. At the same time, the average itself sets the acceptance criterion for passing the accuracy assessment, which can even cause the better performing laboratories to fail (Note 51). The authors of the present communication strongly believe that expert laboratories, i.e. those that have committed extensive effort to developing in-depth expertise refining such assays, should set the standard for the performance of participating laboratories providing pre-characterized reference PBMC so other laboratories can verify their proficiency by reproducing the pre-established test results.

3.3. Precision

Precision in validation is defined as determining the variability of the assay results. Precision testing has to evaluate intra-assay and inter-assay, including inter-operator, and day-to-day, variability.

3.3.1. Intra-assay precision

Intra-assay precision in regulatory language is also called "repeatability". In T cell ImmunoSpot assays, it is measured by testing three replicates of the same PBMC sample in the same plate, whereby the PBMC samples from different donors are plated at a fixed "ideal" cell number per well that best measures a range of antigen-specific T cell frequencies (Note 52) (Korber et al., 2016). This approach is valid for B cell ImmunoSpot assays only if the PBMC are seeded in numbers such that the frequencies of antigen-specific SFU are <100 per well (or pan Ig SFU are <300 per well), that is, before spot crowding and ELISA effects interfere with accurate SFU counting (see above, and Suppl. Fig. 4 and Suppl. Fig. 10). Owing to the highly variable frequency of antigen-specific B cells in different individuals' PBMC, there are two solutions for establishing intra-assay precision. If the numbers of antigen-specific ASC are still unknown for the samples, testing PBMC at serial dilutions with 3–4 replicate wells for each dilution step (starting e.g. at 3×10^5 PBMC per well, and progressing in a $1 + 1$ dilution series) may suffice. In this case, the replicate wells containing <100 SFU and > 20 SFU should be compared. Wells within this SFU count range provide the most accurate information on intra-assay precision, whereas lower spot counts exhibit increased variation due to the onset of Poisson noise (Suppl. Fig. 11 and Suppl. Fig. 12). Results for such a S-antigen-specific ImmunoSpot test are shown in Suppl. Table 3.

If the frequencies of antigen-specific ASC have already been pre-established for the PBMC samples, the cells can be seeded in replicates at a single cell concentration, in numbers that are expected to produce ~50–75 ASC per well. Fewer PBMC are needed for this second variant of intra-assay precision testing.

3.3.2. Inter-assay precision

Inter-assay precision is also designated "intermediate precision", or "plate-to-plate variability" in regulatory language. For T cell ImmunoSpot validation, it is typically established by testing two (or cells permitting, more) plates in parallel (Korber et al., 2016), and this approach can also be adopted for B cell ImmunoSpot validation. However, as outlined above for assessing intra-assay variation, for the same reasons, PBMC have to be plated in serial dilution, and only the SFU counts in the upper linear range should be accepted.

For establishing inter-assay precision, one approach is to seed pre-characterized PBMC in replicate wells at the fixed cell number that is expected to result in ~50–75 SFU per well. In this case, the coefficient of variation (CV) can be calculated for the corresponding wells. As a second option, frequency extrapolations for serially diluted PBMC for the two (or more) plates may be compared (Note 53). Results for the latter type of plate-to-plate variability testing are shown in Suppl. Table 4. For a suggestion on how to combine establishing the inter assay-precision with testing for day-to-day variability and specificity, see Note 43, and below.

Because in the proposed inter-assay design the cells are tested in replicate wells in serial dilution, the results obtained will also permit the verification of the frequency calculations established from the means of the replicates vs. those obtained from individual wells (see Suppl. Fig. 15).

3.3.3. Inter-investigator precision

Inter-investigator precision is one criterion to establish Reproducibility. It requires that the same cell samples are tested by three different investigators in parallel (Note 54). PBMC are plated at serial dilutions and frequencies are established from the linear range of the results. Suppl. Table 5 shows inter-investigator precision data for S antigen-specific B_{mem}-derived IgG^+ ASC measurements. For a suggestion on how to consolidate establishing the inter-investigator precision testing with testing for day-to-day variability and specificity, see Note 43, and below.

3.3.4. Day-to-day variability

Day-to-day (also called inter-assay) variability testing includes establishing the reproducibility of results obtained when the test is done on at least 3 different days by the same investigator(s). Without having to perform a separate series of tests involving extra cells, these data will be obtained as part of the 3 assay runs proposed in Note 43 for a streamlined validation effort, the only requirement being that at least one of the investigators tests the same PBMC samples (at least 3 of them) during the three occasions. Frequency extrapolations for each of the PBMC should be compared for the three independent tests.

Of note, the day-to-day variability in such a B cell ImmunoSpot assay

is expected to be substantially higher than for all the other variability tests due to the inclusion of additional variables before the actual assay is performed. These include possible variability among individual aliquots of cryopreserved PBMC, any day-to-day differences in the thawing and washing process, the subsequent cell counting for polyclonal B cell stimulation cultures, the culture conditions themselves, and the ensuing washing steps and counting of the polyclonally stimulated cells for adjusting their concentration prior to plating – all these variables and more can contribute to the degree of day-to-day reproducibility of the result. Despite all these possible sources of variability, and despite the fact that these tests have been performed in an R&D setting, in our hands, antigen-specific B cell ImmunoSpot assays yield data within <20 % CV (Suppl. Table 6). In the example shown here, the frequency of S-antigen-specific B_{mem}-derived IgG^+ ASC was established as the percentage of pan IgG^+ ASC present in each sample. With maximally 23 % CV, the day-to-day variability was much lower than the 50 % acceptability for cellular assays (Korber et al., 2016).

4. Detection limits

4.1. Limit of blank

In analogy to T cell ImmunoSpot tests (Korber et al., 2016), and also applicable for B cell ImmunoSpot assays, the limit of blank (LOB) is determined by evaluating wells that are treated identically to actual test wells but which were not seeded with PBMC (i.e., the membrane is coated with the antigen, but instead of PBMC, culture medium is added for the same culture period, then the Ig class-detecting antibodies are added, and visualized with the corresponding detection reagents (see Suppl. Fig. 1). Two wells per PBMC sample are reserved in the serial dilution series for the LOB (Suppl. Fig. 5 A). As typically a multitude of PBMC samples are tested in B cell ImmunoSpot assays, the number of LOB wells will add up to a significant number. By definition, the LOB must be zero. If it is not, aggregated reagents are likely the cause of background noise. This can be remedied by filtering the reagents, or better, by subjecting the detection reagents to high-speed centrifugation (Note 55). Also, the recommended back-to-front final washing step helps to eliminate non-specific retention of reagents on the membrane (Note 56).

4.2. Lower limit of detection

In T cell ImmunoSpot assays, the lower limit of detection (LOD) is generally set at 1.6 (conservatively 2–3)-fold the median SFU count for the background wells for each PBMC sample tested in replicate (in that case, the number of cytokine-producing cells present in the well detected if PBMC are plated in the absence of antigen). In the case of T cell assays, such a definition is appropriate because cells of the innate immune system can also secrete the measured cytokine and generate background "noise" above which the antigen-induced cytokine production by antigen-specific T cells needs to be detected as the specific signal. This definition cannot be applied to B cell ImmunoSpot assays, however, because only B cells produce antibody. While B cell ImmunoSpot assays can detect a single SFU in a cell input of up to 1×10^6, this only indicates a secretory footprint was generated. Without putting an equal number of cells in an alternatively coated well for comparison, this one single spot is evidence of "reactivity" but is not necessarily "specific" for the coated antigen of interest. B cell assays – in theory at least – need to account for "chance" recognition of elements coated on the plate (antigen itself, but also potentially the anti-affinity tag capture reagent, an affinity tag if present on the antigen of interest, any components of the assay medium that could absorb to the assay membrane, and any antigens (secreted IgG included) present in the PBMC that could absorb to the membrane during the assay incubation). When frequencies of antigen-specific ASC are relatively high, such negative controls will be less necessary (and the LOB serves its function). However, when studying very rare events using high cell inputs ($>5 \times 10^5$ PBMC per well) specificity controls are needed to dissect antigen-specific SFU from such background "noise" (which is typically donor intrinsic). In our experience gained so far, such "background noise" was either entirely absent in most samples, or, when present, was often <2 SFU per 2×10^5 PBMC. Therefore, while further lowering the LOD is one area of ongoing investigation in our group, we presently believe, without extensive additional controls being involved, it is safe to set the LOD of standard B cell ImmunoSpot assays at 10 per 10^6 PBMC.

4.3. Upper detection limit

When performed using a serial dilution approach, antigen-specific B cell ImmunoSpot assays do not have an inherent upper detection limit: if SFU counts exceed the linear range even at the lowest cell dilutions, re-testing the sample starting with a lower cell input and/or performing larger serial dilutions (e.g. 3–5-fold) will increase the upper detection limit. This can be as high as detecting a single secretory footprint when B cell hybridomas are tested (data not shown). Thus, the upper detection limit of a B cell ImmunoSpot assay is merely a function of the cell numbers plated.

5. Quantification limit

5.1. Lowest limit of quantification

The lower limit of quantification (LLOQ) is the lowest concentration/frequency of antigen-specific ASC within PBMC that can be defined with the minimal required level of accuracy and precision (usually 10 % or 20 %). For T cell ImmunoSpot assays the term LLOQ is rarely used due to the mathematical considerations of a high %CV at low spot numbers per well (Korber et al., 2016), and because the background noise and T cell/APC contacts required for T cell activation to occur prevent Poisson's rule from applying for the precise evaluation of rare events.

For B cell ImmunoSpot measurements, the LLOQ depends on the actual test variant performed. If lowering the LLOQ is desired, plating the cells in multiple replicate wells at high cell numbers per well is the assay approach of choice (see Section 2.4.2 above). When frequencies are measured in screening tests by serial dilution, the initial cell input and obtaining a SFU count >5 at the third dilution point will set the LLOQ. In the type of single well serial dilution tests we show here, when starting at 3×10^5 PBMC, the LLOQ is 67 SFU per 10^6. Due to the onset of Poisson noise that occurs with low frequency SFU counts, increasing the number of replicate wells in such tests will further improve the LLOQ (Suppl. Fig. 12).

5.2. Upper limit of quantification

For T cell assays, the upper limit of quantification (ULOQ) has been defined as the maximum number of individual spots per well the plate reader software can discriminate. Typically, this is achieved by counting spots from PBMC samples treated with a mitogen (or peptide for a donor with a very strong peptide response) and by analyzing the linearity of SFU counts per well and cell numbers plated per well. Applying this approach for S-antigen-specific B cell ImmunoSpot analysis, the ULOQ would be reached at ~100 SFU per well for antigen-specific assays (see Suppl. Fig. 10). It should be noted, however, that owing to the smaller and tighter spot morphologies observed in pan Ig assays, the ULOQ is ~300 SFU per well. Of note, the ULOQ is also affected by the software's ability to distinguish individual SFU over elevated background membrane staining caused by the "ELISA effect" (in which antibody captured distally from the source ASC inevitably accumulates in wells as the number of antigen-specific ASC per well increases, as noted above).

Because antigen-specific B cell ImmunoSpot testing should always rely on PBMC tested by serial dilution to accommodate the wide range of antigen-specific B_{mem} frequencies (Suppl. Fig. 9), the above definition of

ULOQ is not sufficient. Performing the assay with serially diluted samples, this definition of ULOQ applies only for establishing the linear range between cell numbers plated and SFU counts obtained, from which the actual frequencies are calculated by extrapolation. That number will define the ULOQ of the assay performed. For B cell ImmunoSpot tests performed with serial dilutions, the ULOQ is merely a function of the cell numbers plated; by their very nature, such B cell assays' inherent ULOQ is 100 % of all cells tested.

5.3. Linearity

Linearity for B cell ImmunoSpot assays requires that the numbers of PBMC plated, and the number of SFU counted per well follows a linear function. Such linearity is inherent for detection of individual ASC in solution but will only be observed in a rather narrow range in B cell ImmunoSpot tests between 100 SFU for the antigen-specific test at the high end, and around 10 SFU per well on the low end (under which the Poisson noise comes into play, and only measurements done in increasing numbers of replicate wells can reveal the precise frequency. B cell ImmunoSpot assays with serial dilutions will reveal this linear range for each PBMC sample, from which SFU frequencies are to be calculated with the R^2 value revealing the precision for the goodness of fit. By the very nature of the test, any significant deviation from linearity in the 10 to <100 SFU per well range can only result (a) from imprecise pipetting while performing the cell dilution series, (b) from damaging the cells through applying vigorous sheer forces while making the dilution series and pipetting, and/or (c), by damaging the membrane during cell transfers (see Note 39), or by counting parameters set incorrectly on the reader (both of the latter being verifiable by visual inspection of the deviant wells in QC mode). While in the 10 to <100 SFU per well range, measurements of single well serial dilutions will enable accurate frequency calculations based on linearity fits (Suppl. Fig. 15), at <20 SFU counts the Poisson noise increasingly affects the precision of linearity/frequency calculations. Increasing the number of replicate wells can increase the precision of linearity in this low count range.

6. Precision across different types of assays

Our analysis of SARS-CoV-2 S-antigen-specific B_{mem} reactivity so far, whether assessed in vaccinated individuals, in unvaccinated infected people, or in those both vaccinated and infected, showed that only IgG and IgA class-determinations provided readily interpretable results (Suppl. Fig. 8, and work in progress). In polyclonally-stimulated PBMC, IgM^+ S-antigen-reactive SFU were detectable in naïve, pre-pandemic PBMC samples at frequencies similar to verifiably exposed individuals (Suppl. Fig. 8B), a finding we attribute to the presence of low affinity, broadly cross-reactive naïve B cells. Therefore, S-antigen-reactive IgM^+ ASC in polyclonally stimulated PBMC lack diagnostic specificity. In contrast, S-antigen-specific IgM^+ ASC can be detected in PBMC collected acutely following initiation of a COVID-19 mRNA-elicited immune response, identifying in vivo-induced not (yet) class-switched B cells (Note 57) (Pape et al., 2021). Although our four-color multiplexed assay has been optimized for detecting IgE^+ ASC (Yao et al., 2024; Franke et al., 2020), thus far we have not detected any IgE^+ ASC following polyclonal (TLR7/8 + rIL-2, B-Poly-S) stimulation of PBMC isolated from healthy (non-allergic) individuals (Suppl. Fig. 5B and Note 40). Therefore, one might argue that a two-color IgA/IgG ELISPOT or FluoroSpot assay would suffice for monitoring the S-antigen-specific B_{mem} compartment.

For pan Ig detection, we have established that single-color enzymatic, and single- or multicolor fluorescent B cell ImmunoSpot assays yield nearly identical SFU counts (Suppl. Fig. 14, and (Yao et al., 2024)). However, such polyclonal assays fundamentally differ from antigen-specific B cell ImmunoSpot detection. In the former, the ASC-derived Ig is captured by high affinity antibodies, irrespective of the ASC-derived antibody affinity for the antigen, i.e., only the amount of Ig secreted (productivity) per ASC will define the quantity of ASC-derived Ig retained on the membrane (see Suppl. Fig. 1C). For antigen-specific assays, in contrast, the antibodies' affinity for the antigen will decisively define their binding to the coated membrane (see Suppl. Fig. 1 A and 1B). It could be expected that, due to enzymatic amplification, ELISPOT assays will be more sensitive in detecting "faint" secretory footprints originating from low affinity ASC than when these are detected via immunofluorescence, without amplification. Without carrying out a full validation effort, we performed preliminary assays to test the precision across these assays. Cryopreserved PBMC from an individual known to possess B_{mem}-derived ASC secreting either IgG or IgA (at different frequencies) following in vitro polyclonal stimulation were re-tested in S-antigen-specific assays for secretory footprints detected in two-color enzymatic (DCE), two-color fluorescent (DCF) assays detecting IgA^+ and IgG^+ ASC, or in a four-color MAGE assay. The results are shown in Suppl. Fig. 18, revealing no major differences in SFU counts.

7. Pre-analytical considerations

Pre-analytical considerations refer to the (many) variables that can be present between blood collection and the actual analysis of the PBMC in the antigen-specific B cell ImmunoSpot assay. These are similar to those that can affect T cell ImmunoSpot test results, but, while they have been extensively studied for T cells, no such systematic studies exist to date on B cells. Here we also need to distinguish between circulating antigen-specific B cell plasmablasts, that are metabolically highly active cells, and resting B_{mem}. Most of our experience has been gained studying the latter, and they are also the main focus of this paper. While working towards reliable and consistent detection of underlying B_{mem}, thus far we have adhered to pre-analytical considerations established for detecting resting memory cells in T cell ImmunoSpot assays. Nonetheless, we have gained the overall impression that the lessons learned in the T cell field will also apply to the pre-analytical considerations for B_{mem} testing.

When the blood is drawn, it is important to avoid unnecessary sheer forces to prevent hemolysis or damage to the white blood cells (e.g. by using a 21G needle to avoid forceful aspiration) and using heparin as the anticoagulant. Until cryopreservation or testing, the cells should be kept in whole blood, rather than as separated PBMC, and at ambient temperature (25-35 °C) in the dark (never chilled or kept on ice). Our preliminary results obtained so far showed that, stored (or shipped) in this way, B_{mem} preserve their full functionality for at least 24 h (Yao, work in progress). Accordingly, overnight shipment of blood (at controlled ambient temperature) from clinical sites to central test facilities is not expected to interfere with the test results. For the isolation of PBMC from the blood, density gradient centrifugation over a medium with a density of 1.077 g/mL (e.g. Lymphoprep™ or Ficoll-Paque™) is the method of choice for T cell assays, and also works well for B cells. In fact, B cell assays are likely to be less sensitive to the PBMC separation method, but this still needs to be confirmed. Cells should continue to be kept at ambient temperature during isolation, and for short-term storage of the PBMC after isolation until initiation of the testing, or cryopreservation.

PBMC can be cryopreserved to maintain full T- (Kreher et al., 2003) and B memory cell activity (Fecher et al., 2018) in serum-free DMSO containing freezing medium (Notes 58 and 59). Starting the freezing process by adding DMSO-containing cryopreservation medium that is warmed to the same ambient temperature as the cells is key to success (Note 60). For the actual cryopreservation, using a rate-controlled freezer is preferable, but in our hands has no detectable advantage over freezing in Mr. Frosty™ freezing containers, or even just using the "low tech" approach in which two suitable styrofoam racks are sandwiched together in a loose plastic bag wrap (containing plenty of air) prior to placing the cells in the -80 °C freezer short-term. Cryopreserved cells should be transferred to liquid nitrogen within 24 h; storing them longer in the -80 °C freezer should be avoided. Partial warming up of cryovials during storage should be avoided to prevent "glass phase"

transitions from occurring, which can damage cryopreserved cells (Smith et al., 2007). Even before thawing, cryovials should not be kept in the -80 °C freezer for longer than 24 h (Wunsch et al., 2015).

For thawing the PBMC, follow the protocol described in (Ramachandran et al., 2012). Here too, bringing the contents of the cryovial up to 37 °C by placing it in a water bath (or better for sterility, in a glass bead bath) before dilution of the DMSO is advisable. The cells can be maintained at 37 °C, in 10 % (*v*/v) DMSO-containing cryopreservation medium for >30 min without appreciable differences in functionality. Therefore, many cryovials can be thawed simultaneously to facilitate high throughput testing. The addition of warm washing medium containing Benzonase (Note 61) is required for obtaining the best result (definitively better results than following the classical protocol that suggests thawing cryovials one by one until the last ice crystal is visible in the cryovial, followed by immediate washing with ice-cold medium (Kreher et al., 2003).

For subsequent polyclonal B cell stimulation, fetal bovine serum (FBS)-containing culture medium is required. The serum-free media that work so well for T cell assays, and are ideal for standardization of assay conditions for T cell ImmunoSpot (Janetzki et al., 2010), do not contain the necessary growth factors required to support the transition of resting B_{mem} into ASC during the 5 days of in vitro stimulation (Notes 62 and 63). Unlike T cell assays, in which different FBS batches can have profound effects on assay performance (Janetzki et al., 2010), in our preliminary assessments so far, we did not find FBS to be a critical assay variable (Suppl. Fig. 19), but this notion needs additional robust verification.

A combination of the TLR7/8 ligand R848 and recombinant human interleukin 2 (rIL-2) is commonly used to achieve potent polyclonal B cell stimulation for downstream assessment in B cell ImmunoSpot tests (Jahnmatz et al., 2013; Pinna et al., 2009). It is also ideal for studies of Ig class and subclass expression by B_{mem} in vivo, because it does not provide signals that promote Ig class switching during the in vitro stimulation culture period (Note 64). Owing to the potential variable that different batches of polyclonal stimulation reagent(s) may possess different potencies, we recommend to always include a positive control PBMC sample alongside the test sample(s) to verify the potency of the polyclonal stimulation culture by establishing in a pan Ig assay that the maximal numbers of Ig-producing ASC have indeed been induced (Note 65). Suppl. Fig. 2 A-C shows that maximal IgM^+, IgA^+ and IgG^+ ASC activity following R848 + rIL-2 (B-Poly-S) stimulation occurred after 5 days of in vitro stimulation for most donors tested. Of paramount importance for performing B cell ImmunoSpot tests following in vitro stimulation of the test samples is the need to thoroughly wash the cells to remove any "specific" Ig present in the culture supernatant before plating into the actual assay, as this can increase the background staining of the membrane and interfere with detection of antigen-specific ASC.

8. Concluding remarks

In Note 43 we have provided an experimental plan for how - using only 2.2×10^7 PBMC in total – to generate all data required for the validation of an antigen-specific B cell ImmunoSpot assay. The plate layout shown in Suppl. Fig. 5 A permits the subsequent high throughput testing of clinical samples requiring only $1–2 \times 10^6$ PBMC per test subject to determine the frequency of antigen-specific B_{mem}-derived ASC secreting all four classes of Ig. Detailed protocols (a link to Supplemental Materials and Methods can be found at the end of this paper, under "Appendix") and in-depth technical and theoretical comments provided above should help laboratories interested in B cell immune monitoring adopt this technique to develop and validate such assays for their antigens of interest.

The above considerations apply for testing frequencies of resting, antigen-specific B_{mem} cells. While dramatic frequency increases are seen following primary and secondary antigen challenge, frequency changes after additional antigen encounters may no longer be as notable after B cell memory has been established. In such cases, the elicitation of a plasmablast response (Note 9) or changes in the affinity of antigen-specific ASC may provide evidence for the re-activation of a B cell response. As indicated previously above, the detection of such antigen-specific plasmablasts follows the same approach as described for B_{mem} except that the actual B cell ImmunoSpot testing is done with freshly isolated PBMC (or after such cells are cryopreserved and thawed), without the requirement for prior polyclonal stimulation (Note 66). For in-depth studies of affinity distributions within the antigen-specific B cell repertoire, an approach described in (Becza et al., 2024) is best suited.

In addition to assessing the frequency of antigen-specific B_{mem}-derived ASC (or plasmablasts), four-color ImmunoSpot assays described here are also uniquely suited to studying additional Ig class (and IgG subclass) switching that can occur with repeated antigen encounters. Recently, for example, attention has been drawn to the change within the S-antigen-specific IgG response after repeated booster immunizations: an initially IgG1/IgG3 polarized B cell response switching to a mixed IgG1/IgG4 composition (Irrgang et al., 2023), a finding we have also confirmed performing four-color S-antigen-specific IgG subclass analysis. Such shifts are of major clinical significance because IgG1 and IgG4 have antagonistic immuno-biological effects (Note 67). IgG subclass-detecting four-color assays follow the same protocols described above, except that the detection reagents used are IgG subclass-, instead of Ig class-specific.

All of these recent developments in B cell immune monitoring abilities have opened a new window onto the thus far hidden universe of B_{mem} responses by employing B cell ImmunoSpot® analysis, greatly supplementing what can be discerned by the present, limited, techniques deployed for immune monitoring of humoral immunity.

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CRediT authorship contribution statement

Paul V. Lehmann: Supervision, Project administration, Methodology, Conceptualization, Writing – review & editing, Writing – original draft. **Alexey Y. Karulin:** Visualization, Software, Resources, Formal analysis, Writing – review & editing. **Noémi Becza:** Visualization, Methodology, Investigation, Formal analysis. **Lingling Yao:** Visualization, Investigation, Formal analysis. **Zhigang Liu:** Resources, Methodology, Investigation. **Jack Chepke:** Visualization, Resources, Investigation, Formal analysis. **Andrea Maul-Pavicic:** Investigation. **Carla Wolf:** Investigation, Formal analysis. **Sebastian Köppert:** Investigation, Formal analysis. **Alexis V. Valente:** Investigation. **Anton V. Gorbachev:** Project administration, Investigation, Formal analysis, Data curation. **Magdalena Tary-Lehmann:** Supervision, Writing – review & editing. **Greg A. Kirchenbaum:** Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Writing – review & editing, Writing – original draft.

Declaration of competing interest

All authors are employees of CTL and may hold stock.

Data availability

Qualified researchers may request access to test subject-related data and related documents. Such data will be anonymized, and study documents will be redacted to protect the privacy of trial participants.

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Appendix A. Supplementary data

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