# The Isolation, Differentiation, and Quantification of Human Antibody-secreting B Cells from Blood: ELISpot as a Functional Readout of Humoral Immunity

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## Abstract

The hallmark of humoral immunity is to generate functional ASCs, which synthesize and secrete Abs specific to an antigen (Ag), such as a pathogen, and are used for host defense. For the quantitative determination of the functional status of the humoral immune response of an individual, both serum Abs and circulating ASCs are commonly measured as functional readouts. In humans, peripheral blood is the most convenient and readily accessible sample that can be used for the determination of the humoral immune response elicited by host B cells. Distinct B-cell subsets, including ASCs, can be isolated directly from peripheral blood via selection with lineage-specific Ab-conjugated microbeads or via cell sorting with flow cytometry. Moreover, purified naïve and memory B cells can be activated and differentiated into ASCs in culture. The functional activities of ASCs to contribute to Ab secretion can be quantified by ELISpot, which is an assay that converges enzyme-linked immunoabsorbance assay (ELISA) and western blotting technologies to enable the enumeration of individual ASCs at the single-cell level. In practice, the ELISpot assay has been increasingly used to evaluate vaccine efficacy because of the ease of handling of a large number of blood samples. The methods of isolating human B cells from peripheral blood, the differentiation of B cells into ASCs *in vitro*, and the employment of ELISpot for the quantification of total IgM- and IgG-ASCs will be described here.

## Video Link

The video component of this article can be found at https://www.jove.com/video/54582/

## Introduction

B cells play a central role in the development of humoral immunity. They initially develop in the bone marrow and enter the blood stream as naïve B cells, which can migrate into the lymphoid tissues, such as the spleen, lymph nodes, and tonsils, for further development. Upon Ag encounter, some naïve B cells migrate into lymphoid follicles, where germinal center B cells can differentiate into memory B cells and plasmablasts (PBs)/plasma cells (PCs). While most PBs/PCs egress into the blood stream, a few eventually reside in the bone marrow to undergo terminal differentiation into long-lived PCs[1]. B cells in circulation are heterogeneous, and at steady state, PBs/PCs are rare in peripheral blood[2]. As a result of the availability of lineage-specific surface markers, flow cytometry has become a popular method for the identification and characterization of the B-cell subsets in peripheral blood. An extended application of flow cytometry is the addition of a cell sorter function, which permits the separation and isolation of individual subsets of B cells with high purity. Based on the expression of specific surface receptors at different developmental stages, human circulating B cells are generally classified into three main subpopulations: naïve B cells ($CD19^+CD27^-CD38^-$), memory B cells ($CD19^+CD27^+CD38^-$), and PBs/PCs ($CD19^+CD27^+CD38^+$)[3-4] (**Figure 1**). Naïve B cells by nature have not encountered Ags. However, they can be differentiated into $IgM^+CD27^+$ memory B cells. Although naïve B cells are homogeneous in expressing B-cell antigen receptor (BCR)-associated molecules (*e.g.,* CD19, CD20 and CD22) they are heterogeneous in their immunoglobulin repertoire[5]. The majority of $CD27^+$ memory B cells can be differentiated into $CD27^{+/hi}CD38^+$ PBs/PCs[6]. In addition, memory B cells and PBs/PCs are polyclonal and exhibit developmental and functional heterogeneity[4-7]. PBs/PCs in circulation are normally short-lived and do not express CD138, but those made to settle down in the bone marrow will terminally differentiate and become long-lived. Terminally differentiated PCs express CD138 and down-regulate CD27 molecules on their surfaces[8]. Since both PBs and PCs are capable of secreting Abs, in many occasions they are collectively denoted as ASCs. In contrast, neither naïve B cells nor memory B cells can produce appreciable amounts of Abs[9-10]. Nevertheless, when isolated, both naïve and memory B cells can be differentiated into ASCs in 3 - 10 days when placed in the proper culture conditions[6, 11-15]. In fact, ASCs derived from *in vitro* differentiation share similar surface expressions of CD27 and CD38 with those directly isolated from peripheral blood[6]. In addition, the ASCs differentiated *in vitro* express a low level of surface CD20, similar that of circulating PBs/PCs[6]. Although the culture-derived ASCs are all short-lived, they can secrete Abs, indicating that they are functionally competent and able to contribute to the humoral immunity.

Both ELISA and ELISpot are by far the most commonly applied methods with which to obtain functional information on the humoral immune response. ELISA is a 96-well plate-based assay, and it is frequently used to measure the titers of serum Ag-specific Abs and other analytes (*e.g.,* cytokines). It is convenient and scalable. ELISA is designed to use a solid-phase enzyme assay to detect the presence of Abs or other substances, such as serum, in a liquid sample[16]. The readouts from serum ELISAs have been widely used to represent the immune response

of the body. A tool necessary for the acquisition of readouts from ELISA assays is a spectrophotometric microplate reader. The reader can determine the optical density (O.D.) of the end products typically resulting from the reaction of horseradish peroxidase (HRP)-conjugated detection Abs and their specific substrates[17]. With regard to reporting the humoral immune response, serum Ab levels determined by ELISA denote the collective, but not individual, performance of ASCs in the body. In addition, ELISA fails to take into account the participation by memory B cells, which do not secrete Abs.

Like ELISA, ELISpot is a widely used method for detecting and monitoring the immune response in peripheral blood samples[17-18]. ELISpot is a technique related to a sandwich ELISA. In it, cells are placed into the polyvinylidene difluoride (PVDF) membrane-backed wells of 96-well microplates for a short-term culture. The ELISpot assay is analogous to performing western blotting on a microplate and developing the spots on the PVDF membrane in each well. An automated ELISpot reader system or a stereomicroscope for manual counting is required. The main advantage of ELISpot in detecting an immune response is its superb sensitivity in the quantification of ASCs and cytokine-secreting cells. It reports their functional activities in humoral and cellular immunity, respectively. In the measurement of humoral immune function, serum Ab levels determined by ELISA and the number of ASCs enumerated by ELISpot are often correlated, but the data readouts from these two assays have some differences in functional implications[19-20]. The main advantage of ELISpot is its sensitivity of method. The level of serum Ab titers as reported by ELISA is presented semi-quantitatively as O.D. readouts, denoting the relative Ab level, or more quantitatively, as concentration readouts when a known amount of the proper isotypes of Abs is included for reference. In contrast, the results of ELISpot are presented as the absolute number of ASCs in a cell pool of interest (*e.g.,* unfractionated peripheral blood mononuclear cells (PBMCs) and purified B cells from PBMCs). ELISpot can detect a single ASC, but ELISA requires Ab amounts from ASCs to reach optimized assay-dependent concentrations prior to measurement. Hence, ELISpot is obviously superior to ELISA in sensitivity of quantification. Moreover, ELISpot is also suitable for quantifying the *in vitro* differentiated ASCs from activated memory B cells. Memory B cells do not secrete Abs but can differentiate into ASCs upon activation; they therefore have no contribution to serum Abs detected by ELISA. Thus, ELISpot is the method of choice in the measurement of the immune response of circulating memory B cells after activation in culture. It allows for the monitoring of the maintenance of long-term humoral immunity.

## Protocol

Human peripheral blood must be obtained from healthy donors under informed consent, and the use of blood samples must conform to the approved guidelines established by individual institutional review boards. In this study, the protocol to use human blood in a demonstration of the results of flow cytometry (**Figure 1**) and ELISpot assays (**Figure 3**) was approved by the Internal Review Board of National Taiwan University Hospital (protocol number 201307019RINB).

## 1. Isolation and Purification of Human Peripheral Blood B Cells

1.  Draw ~ 10 mL of blood from the median cubital vein (in the cubital fossa anterior to the elbow) into a 15-mL tube containing $K_2$EDTA (1.5 to 2.0 mg/mL blood) and immediately invert the tube several times to prevent clot formation.
2.  Add 35 mL of autoclaved (121 °C, 15 min) red blood cell (RBC) lysis buffer (150 mM $NH_4Cl$, 10 mM $KHCO_3$, and 1 mM EDTA; pH 7.4) to the tube containing the fresh blood sample (≥ 3:1 vol/vol) and incubate at room temperature (RT) for no longer than 5 min.
    NOTE: The appearance of light transmission through the tube indicates the completion of RBC lysis.
3.  Centrifuge at 600 x g at RT for 5 min. Ensure that the pellet is white in color.
4.  Resuspend the pellet with 10 mL of autoclaved phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, and 1.47 mM $KH_2PO_4$; pH 7.4) and centrifuge as in step 1.3.
5.  Discard the supernatant, resuspend the pellet with 10 mL of RPMI 1640 medium (supplements: 10% fetal calf serum, 100 U/mL penicillin/ streptomycin, 0.25 µg/mL amphotericin B, and 2 mM L-glutamine), and then plate the cells into a 10-cm culture dish (10 mL of blood per dish). Place the dish in a 37 °C incubator with 5% $CO_2$ for 30 min.
6.  Gently swirl the culture dish a few times and place the culture medium (suspension cells) into 15-mL plastic conical tubes. Discard the adherent cells (mostly macrophages) on the culture dishes.
7.  Centrifuge at 600 x g at RT for 5 min. Discard the supernatant.
8.  Resuspend the pellet with 1 mL of RPMI 1640 medium. Count the cell number with a hemocytometer or an automated cell counter.
    NOTE: The viability of isolated leukocytes is normally greater than 90% by trypan blue exclusion.
9.  Centrifuge as in step 1.7 and resuspend the cells in ~ 200 µL of cold PBS buffer (0.5% bovine serum albumin (BSA) and 2 mM EDTA) at the concentration of 5 - 10 x $10^6$ cells/mL.
10. Add 5 µL of biotinylated anti-human Ab cocktail specific to blood cells (for the negative selection of B cells) per $10^6$ cells and incubate on ice for 30 min[21].
    NOTE: The anti-human Ab cocktail should at least include Abs specific to CD2 (or CD3), CD14, and CD16.
11. Add a 10-fold excess volume of sterile PBS to the cells, centrifuge at 600 x g for 5 min, and discard the supernatant.
12. Add equal amounts of streptavidin-conjugated microbeads (5 µL per $10^6$ cells) to the pellet and mix thoroughly.
13. Incubate on ice for 30 min in a 15-mL plastic conical tube.
14. Add 2 mL of PBS buffer into the tube.
15. Place the tube into a magnetic stand and incubate at RT for 8 min. The brown microbeads will gradually attach to the tube wall next to the magnet[22].
16. With the tube remaining in the magnetic stand, carefully transfer the supernatant into a new sterile 15-mL tube[22].
17. Repeat steps 1.14 to 1.16, and combine the two supernatants that contain the untouched B cells. Discard the microbeads.
18. Centrifuge at 600 x g at RT for 5 min. Discard the supernatant.
19. Resuspend the pellet in RPMI 1640 medium for downstream experiments.
    NOTE: Typically, 1 - 5 x $10^5$ B cells with a purity greater than 95% from 10 mL of peripheral blood can be isolated[23].

## 2. Purification and Separation of Memory and Naïve B Cells from Isolated B Cells

1. Use cells purified from step 1 and determine the cell number using a hemocytometer or an automatic cell counter.
2. Resuspend the cells in 100 µL of cold PBS buffer after centrifugation at 600 x g and RT for 5 min.
3. Add 1 - 2 µg of biotinylated CD27 mAb per $10^6$ cells and incubate on ice for 30 min.
4. Add 10 mL of PBS buffer to the tube and centrifuge at 600 x g for 5 min.
5. Discard the supernatant and resuspend the cells in 50 µL of PBS buffer.
6. Add equal amounts of streptavidin magnetic microbeads (1 - 2 µg/$10^6$ cells) to cells in a 15-mL plastic conical tube.
7. Gently mix well and incubate on ice for 30 min.
8. Add 2 - 3 mL of PBS buffer to the tube.
9. Place the tube into a magnetic stand and incubate at RT for 8 min to allow the brown microbeads to attach to the side closest to the magnet.
10. With the tube in the magnetic stand, carefully transfer the supernatant fraction into a new sterile tube. This fraction contains the enriched CD27^{-} naïve B cells.
11. Add 5 mL to the tube containing the microbeads and gently resuspend the microbeads (*i.e.,* the enriched fraction of CD27^{+} memory B cells)[24-25].
12. Centrifuge at 600 x g at RT for 5 min. Resuspend the pellets with RPMI 1640 medium for the downstream experiments.
    NOTE: Typically, ~ 30 - 60% of B cells can be purified as CD27^{+} memory cells from the PBMCs of a healthy donor[7, 25-26].

## 3. Cell Sorting for the Collection of Naïve B Cells, Memory B Cells, and PBs/PCs

1. Using the cells purified from step 1, determine the cell number using a hemocytometer or an automatic cell counter.
2. Resuspend the cells in cold PBS buffer at the concentration of $10^7$ per mL in a 5-mL polystyrene tube.
3. Add 1 - 2 µg of human IgG per $10^6$ cells and incubate on ice for 10 min for the Fc block.
4. Add 1 µg each of anti-CD19-APC (clone: HIB19), anti-CD27-eFluor450 (clone: O323), and anti-CD38-PE (clone: HIT2) per $10^6$ cells; mix well and incubate on ice for 30 min[4, 27].
5. In the last 5 min in step 3.4, add 5 µL of the commercial 7-aminoactinomycin D (7-AAD).
6. Add 2 mL of PBS to the tube, vortex, and centrifuge at 600 x g for 5 min.
7. Resuspend the cells in sorting buffer (sterile PBS with 2% BSA and 2 mM EDTA) at a concentration of 1 - 5 x $10^7$ cells per mL in a 15-mL tube.
8. Filter the cells through a nylon mesh cell strainer (40 µm pore size) to eliminate cell clumps.
9. Separate the cells with a flow cytometric sorter equipped with three lasers: violet (405 nm), blue (488 nm), and red (640 nm).
    NOTE: The blue laser alone is sufficient for 3-color flow cytometry[27-28].
10. Sort the cells into three 15-mL tubes (containing 5 mL of RPMI medium) for the simultaneous collection of naïve B cells (CD19^{+}CD27^{-}), memory B cells (CD19^{+}CD27^{+}), and PBs/PCs (CD19^{+}CD27[+/hi]CD38^{+})[3-4, 28].
    NOTE: Sorted naïve and memory B cells can be cultured as described in step 4.

## 4. *In Vitro* Differentiation of Isolated Human CD19^{+} B Cells, CD19^{+}CD27^{+} Memory B Cells, and CD19^{+}CD27^{-} Naïve B Cells

1. Using the cells purified in steps 1.19, 2.10, 2.11 and 3.10, determine the cell number with a hemocytometer or an automatic cell counter.
2. Resuspend the cells with RPMI 1640 medium at a concentration of 1 - 10 x $10^5$ per mL and aliquot them into the wells of a 12-well plate.
3. Add CpG (ODN 2006) at 5 µg/$10^6$ cells/mL[18, 29].
4. Culture the cells in a 37 °C incubator with 5% $CO_2$ for 5 days.
5. Harvest the cells from each well, place them separately into 15-mL tubes, add 5 mL of PBS to each tube, and centrifuge them at 600 x g and RT for 5 min.
6. Count the cells using a hemocytometer or an automatic cell counter. Resuspend the cells at a concentration of 1 - 10 x $10^5$ per mL with RPMI 1640 medium.

## 5. ELISpot Assay

1. Add 30 µL of 35% ethanol in distilled water to each well of the ELISpot plates for 30 s.
    NOTE: When pipetting, avoid touching the membrane in the wells at all times.
2. Invert the ELISpot plates to remove the ethanol.
3. Put 150 µL of autoclaved ddH$_2$O into each well and incubate them at RT for 5 min to flush off the residual ethanol; follow with a wash of sterile PBS at RT for 3 min.
    NOTE: Steps 5.1 to 5.3 may be optional, depending on the manufacture of the plates.
4. Put 50 µL of 5 µg/mL polyclonal F(ab')$_2$ fragment of anti-human Ig (IgG + IgM + IgA) (in PBS) into each well of the ELISpot plates and incubate them at 4 °C overnight (preferred) or 37 °C for 2 h[11].
    NOTE: Seal the edges of plates with Parafilm until use.
5. Invert the plates to remove unbound Abs, add 200 µL of PBS to each well, and incubate them twice at RT for 3 min each time.
6. Add 200 µL of PBS with 5% BSA (or RPMI 1640 medium) to each well for blocking, and incubate them at RT for 2 h.
7. Invert the plates to remove the blocking buffer. Wash each well twice with 200 µL of PBS, as in step 5.5.
8. Add 100 µL of RPMI 1640 medium to each well and incubate them at 37 °C.
9. When ready to seed the cells, invert the plates to remove the RPMI 1640 medium.
10. Seed 100 µL (5 x $10^4$), 50 µL (2.5 x $10^4$), and 25 µL (1.25 x $10^4$) of the cells (from steps 1.19, 2.10, 2.11, 3.10, and 4.6) into the wells of an ELISpot plate. With RPMI 1640 medium, bring the volume to 150 µL/well.
    NOTE: A minimum of one or two 2-fold serial dilutions for plating the cells is recommended.

11. Incubate the plates in a 37 °C incubator with 5% $CO_2$ for 8 - 14 h[18]. Avoid moving the plates during incubation.
12. Invert the plates to remove the cells and RPMI 1640 medium.
13. Add 200 µL/well of PBS-T (PBS with 0.05% Tween 20) and incubate them 5 times at RT, each time for 3 min. Invert the plate to remove the wash buffer between each of the 5 washes.
14. Add either goat anti-human IgG-alkaline phosphatase (AP), Fcγ-specific Abs (for IgG detection, 1:5,000 in PBS-T) or goat anti-human IgM-AP, Fcμ fragment-specific Abs (for IgM detection, 1:5,000 in PBS-T) into the designated wells and incubate at RT for 2 h in the dark.
15. Wash each well twice with 200 µL of PBS, as in step 5.5.
16. Add 50 µL/well of bromochloroindolyl phosphate-nitro blue tetrazolium (BCIP/NBT) substrate solution. Purple-colored spots normally appear in 5 - 15 min.
17. Add 100 µL/well of $ddH_2O$ to prevent the over-development of spots.
18. Rinse the plates with running tap water after the complete development of all spots.
19. Remove the underdrain of the plates and allow them to air dry in the dark.
20. Count the spots using an automated plate reader with an image acquisition/analysis unit (*e.g.,* an automatic scanner or manually via a dissecting microscope).
    NOTE: The plates can be stored at RT in the dark and analyzed later.

## Representative Results

PBMCs were depleted of RBCs and adherent cells (steps 1.2 to 1.7). An aliquot (2 x 10^{6}) of cells were subjected to a flow cytometric analysis to illustrate the populations of naïve B cells, memory B cells, and PBs/PCs in peripheral blood (**Figure 1**). In this donor's PBMCs, about 10% of the lymphocytes were CD19^{+} B cells. In the B-cell compartment, the percentage of CD19^{+}CD27^{-} naïve B cells was around 50%. On the other hand, about 50% of the CD19^{+} B cells were CD27^{+} memory B cells[7, 25-26]. Of note, CD27^{+} memory B cells can be further separated with the inclusion of IgD[4]. The phenotype of circulating PBs can be better defined with low or no surface expression of CD20 (CD19^{+}CD27[+/hi]CD38^{+}CD20^{lo/-})[2,30-31]. To exclude the dead cells, the 7-AAD can be included in cell staining (step 3.5), and a plot for FSC *versus* 7-AAD allows for gating the population of live cells (7-AAD^{-}).

The key steps of the ELISpot assay are depicted in **Figure 2**. Both purified human B cells and CD19^{+}CD27^{+} memory B cells were cultured in the presence of CpG with RPMI 1640 medium for 5 days. Cells were harvested for the ELISpot assay for quantifying the newly emerged ASCs. If present, the pre-existing PBs isolated from the peripheral blood will die out in 48 h[11]. The ELISpot assay was performed, and the spot images acquired by an automatic plate scanner are demonstrated in **Figure 3**. The results of ELISpot typically will show 50 - 200 IgM-ASCs from 10[4] B cells treated with CpG for 5 days, whereas the number of IgG-ASCs is 10 - 50 in 10[4] cells[11].
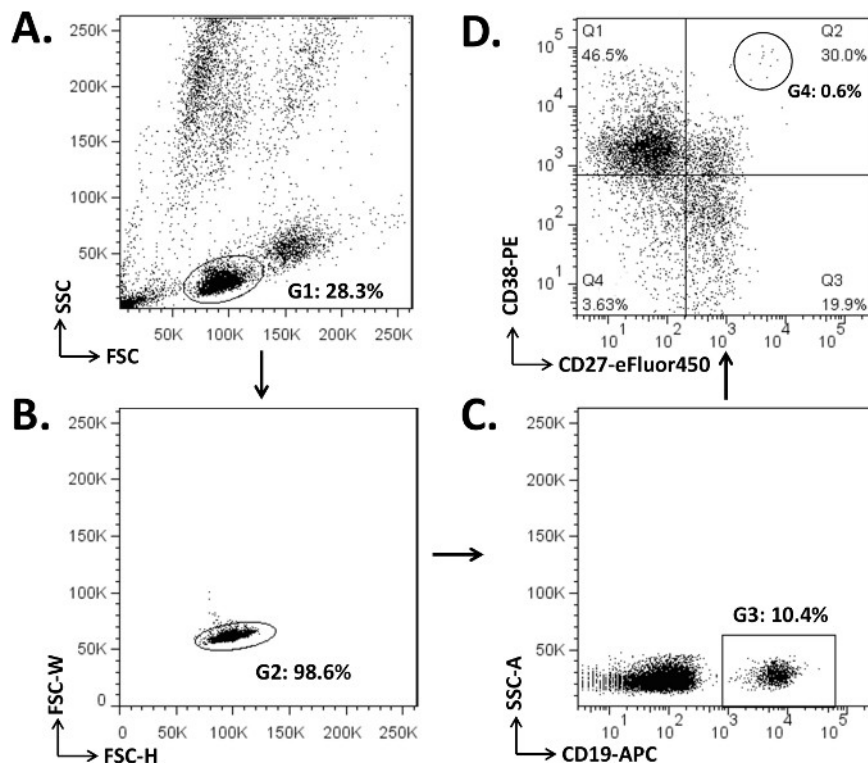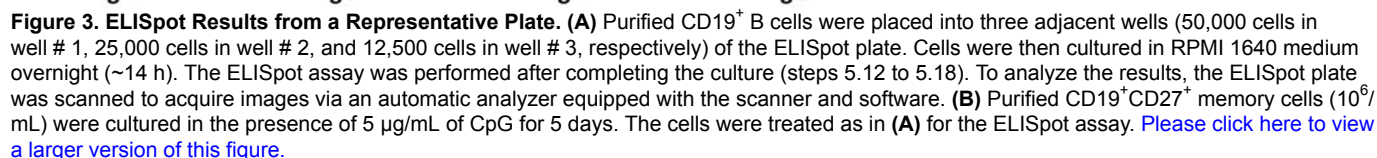
**Figure 1. Illustration of the Gating Strategy to Separate the Naïve B cells, Memory B cells, and PBs in the PBMCs. (A)** Cells ($2 \times 10^6$) were stained with 2 µg each of CD19-APC, CD27-eFluor450, and CD38-PE mAbs (step 3.4). The lymphocyte compartment was gated (G1) on the dot plot for forward scattering (FSC) versus side scattering (SSC). **(B)** Cell doublets, which are formed by two cells stuck together, were excluded (those outside G2) on the plot for FSC-W (width) versus FSC-H (height). **(C)** CD19$^+$ B cells were gated as G3 on the plot for SSC-A (area) and CD19-APC. **(D)** Of the CD19$^+$ cells, the dot plot of CD27 and CD38 parameters showed naïve B cells (CD19$^+$CD27$^-$; Q1 and Q4), memory B cells (CD19$^+$CD27$^+$; Q2 and Q3), and PBs (CD19$^+$CD27$^{+/hi}$CD38$^+$; G4, 0.6% of Q2). Please click here to view a larger version of this figure.
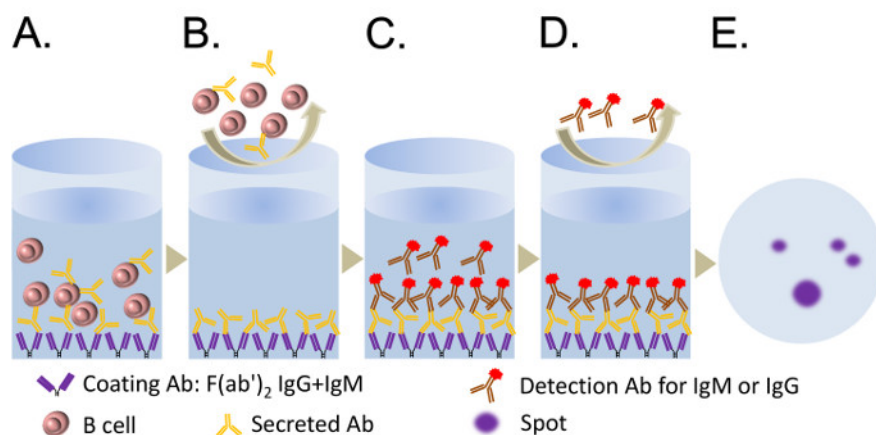


**Figure 2. Illustrations of the Key Steps of the ELISpot Assay. (A)** Put 50 µL/well (5 µg/mL) of F(ab')$_2$ fragment of anti-human Ig (IgG+IgM +IgA) into an ELISpot plate for 2 h at 37 °C or overnight at 4 °C (preferred) (step 5.4). Seed the cells into the wells of the ELISpot plate. Allow the ASCs to attach to the PVDF membrane and to secrete Abs for 8 - 14 h (step 5.11**). (B)** Wash off the cells with PBS (with 0.05% Tween 20). **(C)** Add the AP- or HRP-conjugated detection Abs specific to either IgG or IgM. **(D)** Wash off the detection Abs that are unbound. **(E)** Develop the spots with a substrate solution of AP or HRP to match the detection Abs. Please click here to view a larger version of this figure.

**Figure 3. ELISpot Results from a Representative Plate. (A)** Purified CD19^{+} B cells were placed into three adjacent wells (50,000 cells in well # 1, 25,000 cells in well # 2, and 12,500 cells in well # 3, respectively) of the ELISpot plate. Cells were then cultured in RPMI 1640 medium overnight (~14 h). The ELISpot assay was performed after completing the culture (steps 5.12 to 5.18). To analyze the results, the ELISpot plate was scanned to acquire images via an automatic analyzer equipped with the scanner and software. **(B)** Purified CD19^{+}CD27^{+} memory cells (10[6]/ mL) were cultured in the presence of 5 µg/mL of CpG for 5 days. The cells were treated as in **(A)** for the ELISpot assay. Please click here to view a larger version of this figure.

## Discussion

### Isolation and Purification of Human Peripheral Blood B Cells

Normally, RBCs can be efficiently ruptured and cleared by lysis buffer (step 1.2). It is important not to incubate PBMCs with the RBC lysis buffer longer than 5 min, as cell viability might be affected by the ammonium chloride. Alternatively, RBCs and platelets can be simultaneously removed by the following protocol.

Mix fresh whole blood with acid-citrate-dextrose (ACD) buffer (39 mM citric acid, 75 mM sodium citrate, and 135 mM dextrose; pH 7.4) in a volume ratio of 9:1, blood-to-buffer. Centrifuge at 250 x g for 10 min at RT. Pay attention to the formation of layers, indicative of separation. Aspirate and discard the upper layer, which contains platelets and platelet-rich plasma (yellow color). Remove the thin middle layer at the interface, which contains PBMCs (*i.e.*, the buffy coat). Avoid the contamination of RBCs in the bottom layer (dark red color). Add lysis buffer to the PBMCs to remove the residual RBCs, and then follow steps 1.2 to 1.19.

Two approaches can be used to isolate B cells from PBMCs: positive and negative selection[21]. The method of negative selection (steps 1.1 to 1.19) is used to obtain untouched, functional B cells, with no bound Abs or microbeads, for downstream experiments. It is crucial to take this into account when activation and/or differentiation of purified B cells occurs in culture. The concern is that cells purified by positive selection might be inadvertently influenced by the mAb-conjugated microbeads (0.2 to 5 µm in diameter). mAbs can bind to specific receptors on B cells and therefore might crosslink receptors for activation. Moreover, microbeads can be endocytosed by B cells through bound receptors. Although biodegradable, the microbeads can stay inside cells for longer than a week. Whether the retention of microbeads will influence experimental results needs to be empirically determined. In the purification of human B cells *via* positive selection, anti-CD19 (clones: 4G7 or HIB19) microbeads are commonly used because CD19 is widely expressed throughout the developmental stages of B cells. The purity of B cells after separation can be determined by flow cytometry with anti-CD19 mAbs (clones: SJ25C1 or LT19), which recognize distinct epitopes from anti-CD19 microbeads.

### Purification and Separation of Memory and Naïve B Cells from Isolated B Cells

CD27 is a widely accepted surface marker for human memory and naïve B cell distinction[24]. The CD27 molecule belongs to the tumor necrosis factor receptor (TNFR) family. Because CD27 is expressed on most human memory B cells, it is commonly used to discriminate them from CD27^{-} naïve B cells. However, because CD27 is also expressed on T cells and NK cells, the use of anti-CD27 microbeads (clone: O323) in positively selecting memory B cells requires prior purification of B cells (steps 1.1 to 1.19). For a purity check after separation, mAbs specific for human CD27 (clones: L128 or LG.3A10) are recommended (step 2.12). Because CD27^{+} memory B cells are heterogeneous, additional surface markers, such as IgD and FCRL4, can be considered for further separation in cell sorting[4, 7].

### Cell Sorting to Obtain Naïve B Cells, Memory B Cells, and PBs/PCs

Because B cells express FcγRIIB, an Fc block (step 3.3) is recommended prior to staining the cells with mAbs for flow cytometry. The Fc fragments of the human IgG alone can block equally well as the whole human IgG at 1 µg/10[6] cells. Anti-human CD32 mAb (clones: 3D3 or AT10) is another option for the Fc block. Of note, if FcγRIIB is a surface marker to be stained in purified B cells, the fluorophore-conjugated anti-CD32 mAb should be added to waive the Fc block, followed by staining with other mAbs without a PBS wash.

For three-color flow cytometry, CD38-FITC (Fluorescein), CD27-PE (Phycoerythrin), and CD19-APC (Allophycocyanin) are a typical combination of fluorophores. Nevertheless, if the flow cytometric sorter is equipped with three lasers, such as those of 405 nm, 488 nm and 640 nm, the researchers then have the luxury to choose fluorophores excited by different lasers for the cell sorting. As illustrated in **Figure1**, cells were stained with CD19-APC (clone: HIB19), CD27-eFluor450 (equivalent to pacific blue, clone: O323), and CD38-PE (clone: HB7) to separate the naïve B cells, memory B cells, and PBs. Of note, some researchers prefer the inclusion of CD45 (clone: HI30) as a leukocyte lineage marker and

gate B cells as a CD45^{+}CD19^{+} population. Because the detection of PBs in circulation at steady state is a rare event in healthy individuals, low or even no surface expression of CD20 on PBs (CD19^{+}CD27[+/hi]CD38^{+}CD20^{lo/-}) substantiates their bona fide existence[2,30-31]. This is useful when the quantification results of PBs by surface phenotypes and ASCs by ELISpot assays are being compared. During the period prior to cell-sorting, 7-AAD is preferred to propidium iodide for the exclusion of dead cells, due to its narrower emission spectrum and lower spillover in multicolor flow cytometry.

### *In Vitro* Stimulation and Differentiation of Isolated Human B Cells

The type B CpG (ODN 2006) alone can induce limited differentiation of both IgM- and IgG-ASCs from memory B cells. Moreover, naïve B cells are only weakly responsive to CpG and mainly give rise to IgM-ASCs[6, 11]. The concentration of CpG in culture is in the range of 2.5 - 5 µg/mL per 10[6] B cells or PBMCs[11, 18]. Other than CpG, purified B cells and PBMCs can be cultured in the presence of combinations of CpG with mitogens, cytokines, and BCR agonists, to differentiate human B cells into ASCs in culture (step 4.3). For instance, commonly used recipes for 10[6] cells per mL include (a) CpG (5 µg/mL), recombinant human IL-2 (10 ng/mL), and recombinant human IL-10 (10 ng/mL)[15, 34]; (b) CpG (5 µg/mL) and F(ab')$_2$ fragments of anti-human Ig (IgG+IgM+IgA) (20 µg/mL)[11]; (c) CpG (5 µg/mL), protein A from *S. aureus* Cowan (SAC) (1:10,000 dilution), and Pokeweed mitogen (PWM) (1:100,000 dilution)[18]; (d) recombinant human IL-21 (100 ng/mL) and recombinant sCD40L (1 µg/mL)[12, 15, 33]; and (e) recombinant human IL-2 (10 ng/mL) and R848 (1 µg/mL), a TLR7 agonist[33-34]. Although B cells can be differentiated into ASCs in three days[11], cells are generally pre-stimulated for 5 to 6 days prior to being added to the ELISpot plate for the quantification of ASCs[11, 18]. During the culture period, there is usually no replenishment of the starting differentiation agents.

The addition of IL-2 and IL-10 to CpG-containing cultures can significantly increase the number of differentiated IgM- and IgG-ASCs[15, 35]. The presence of IL-2 in culture can provide mitogenic stimulation to facilitate the expansion of differentiated B cells by CpG. IL-10 in the range of 10-25 ng/mL can greatly increase the production of ASCs (~ 3- to 10-fold) in the presence of CpG[35]. BCR agonists, including anti-BCR and SAC, and PWM can also stimulate proliferation of primary human B cells[18]. Incubating memory B cells with CpG and polyclonal anti-BCR mainly results in an increase in IgM-ASCs but not in IgG-ASCs[28]. For the prevention of the inhibition of signaling through BCR by FcγRIIB, a F(ab')$_2$ fragment of anti-Ig (10 - 20 µg/mL per 10[6] cells) is recommended when crosslinking BCR[11]. Instead of polyclonal anti-Ig, anti-BCR mAbs specific to either IgM[+] or IgG^{+} B cells should be considered to target isotype-specific B-cell subsets for differentiation into ASCs. A combination of CpG, SAC (1:10,000 dilution), and PWM (1:100,000 dilution) can efficiently activate memory B cells to differentiate into IgM- and IgG-ASCs[18, 24]. Moreover, CpG can moderately induce class switching of Ig *in vitro*[28-29]. In contrast, IL-21 (100 ng/mL) and sCD40L (1 µg/mL) effectively induce memory B cell differentiation exclusively to switched IgG-ASCs[12, 30]. The sCD40L can activate B cells by mimicking T-cell help through CD40 on B cells, influencing their differentiation into PCs *in vivo*. Of note, anti-CD40 (clones: 89 or G28.5) can substitute for sCD40L in culture[15]. However, neither sCD40L nor anti-CD40 alone is able to induce the differentiation of memory B cells. Thus, the CD40 activation of cultured B cells is often combined with a differentiation agent, such as IL-4, CpG, R848, or IL-21, any of which can promote Ig class switching. IL-4 plays a key role in the differentiation of Th2 CD4 cells, allowing the induction of most IgM^{+} B cells to switch to IgG1^{+} B cells in culture. Similarly, in the presence of IL-21, both memory B cells and naïve B cells differentiate exclusively into class-switched ASCs[12, 32]. Like CpG, R848 can induce a marginal class switch of B cells[34]. Finally, it is worth mentioning that although lipopolysaccharide (LPS) can effectively induce differentiation of mouse B cells into ASCs, human B cells express low levels of TLR4 and CD14[29]. Because the addition of a differentiation agent in culture promotes the generation of switched B cells, this addition should be avoided when pre-existing PBs/PCs or unswitched memory B cells are to be measured.

### ELISpot Assay

The ELISpot assay combines plate-based ELISAs with membrane-based western blotting technologies, allowing for the detection of Abs secreted by a single B cell[18]. The ELISpot has also been adapted to quantify Ag-specific T cells that secrete cytokines[17, 36]. In an ELISpot assay, either purified cells or unfractionated PBMCs (free of RBCs) can be used. However, ~ 10-fold more PBMCs are required than purified cells to obtain consistent results in ELISpot assays. A good starting point is 1 - 2 x 10^{5} cells per well for detecting the circulating ASCs in PBMCs. In the detection of Ag-specific ASCs, anti-Ig is usually replaced by Ag (5 - 10 µg/mL in PBS) to capture Ag-specific ACSs (step 5.4). The use of isotype-specific detection Abs permits the distinction between Ag-specific IgM-ASCs and IgG isotype-ASCs (step 5.14). Of note, regardless of whether anti-Ig or Ag are used to capture ASCs, the detection Abs used for ELISA might not always be suitable for ELISpot. Similarly, not all color substrates for ELISA function properly in ELISpot. AP- and HRP-conjugated Abs are most commonly used for spot detection in ELISpot. The BCIP/NBT substrate solution is a popular choice for AP-based detection, whereas 3,3',5,5'-tetramethyl-benzidine (TMB) and 3-amino-9-ethylcarbazole (AEC) solutions are common substrates for HRP in ELISpot. If the AP or HRP is directly conjugated to the detection Abs, only one step is needed for detection, as described in the protocol (step 5.14). In contrast, the detection Abs are biotinylated and require ensuing incubation with AP- or HRP-conjugated streptavidin before reacting with the substrates in a two-step method. Both one-step and two-step methods work efficiently to detect spots in ELISpot.

The ELISpot assay can quantify not only circulating but also differentiated ASCs in culture (step 1.19 versus 4.6). If present, circulating ASCs, which normally live no longer than two days in culture, can be directly cultured in the ELISpot plate, with or without purification from PBMCs[11]. In contrast, the differentiation of memory B cells requires 5 days. Hence, direct culturing in ELISpot plates after cell isolation (step 1.19) is not recommended; the dead cell debris resulting from the extended culture period can increase the background in spot detection. Instead, both purified B cells and total PBMCs can first be cultured in a 6-well or 12-well plate in the presence of mitogens and differentiation agents (step 4.3). During the culture process, the repeated addition of stimulation agents is unnecessary (step 4.3). While plates are in the incubator, the door of the incubator should be gently opened and closed to prevent seeded ASCs from moving, for they can diffuse secreted Abs along the moving tracks, generating spots with tails (the so-called "comet-like" spots) (step 5.11). The incubation period in ELISpot plates is determined by the time needed for the cells to secrete detectable amounts of Abs without allowing the cells to divide, which would create difficulties in spot counting. Thus, cells are usually cultured in the range of 8 h to overnight (step 5.11).

The ELISpot assay is the most widely used method for quantifying memory B cell responses. It has become a popular, independent readout of humoral immune response and immunological memory. In practice, the preceding mitogenic culture is required to activate memory B cells for differentiation into ASCs (step 4.3). However, the frequency at which memory B cells expand and differentiate into ASCs varies in different culture milieu, as described above. Although without consensus, most researchers adopt the induction condition that can maximize the production of ASCs. In terms of the agent used for spot detection, a soluble biotinylated Ag can replace the unsatisfactory detection Ab without compromising the sensitivity[37]. This is of particular importance when the availability of Ag is limited because a much lower quantity

of Ag is required for detection than for coating the plates. Lastly, ELISpot does not allow phenotypic analysis of cellular heterogeneity, and thereby requires prior fractionation of cell subpopulations. Recently, a multicolor ELISpot assay, in which detection Abs are labeled with different fluorophores, has been developed to detect multiple types of cytokine-secreting cells in a single well[38-39]. This new technology will be of particular interest for the detection of low-frequency cells in PBMCs and for a large-scale analysis of vaccine-induced B-cell responses.

Because the ELISpot assay is fast and efficient in quantifying ASCs and cytokine-secreting cells, it has been extended to measure the functionality of not only circulating lymphocytes, but also tissue immune cells such as intrahepatic leukocytes[40]. Because of its sensitivity reaching the level of detecting even a single ASC, the ELISpot assay has been increasingly used to measure human immune responses to vaccine efficacies against existing and emerging pathogens. Although the high-throughput performance and robustness of ELISpot assay are superb for large-scale assessments of immune responses using PBMCs, the results of the assays can be influenced by various factors, such as the processing delay of samples, whether cells are fresh or frozen, serum components in the culture medium, and the number of cells added to the ELISpot plate[41]. Thus, normalization procedures should be included in the ELISpot protocol to minimize intra- and inter-assay variations in large-scale and longitudinal follow-up studies (*e.g.,* vaccine trials). The normalization method of plate reading is also critical for linearity, accuracy, and consistency in the assay results[41-42]. One way to perform plate reading normalization is to extend the procedure of the serial dilution of cells seeded to ELISpot plates (step 5.10). By creating a reference plate, more serial dilutions should demonstrate a linear relationship between the cell numbers plated per well and the spot counts per well. Repeated scans of the ELISpot plate can be used to validate the template of spot counting, both automatically and manually. Minimal intra-well variability in spot counts is anticipated after these normalization procedures[43]. Lastly, the application of ELISpot can be extended in many ways, such as to the monitoring of the treatment responses of patients with infections and taking cancer immunotherapies, as well as to the evaluation of immunotoxicity (*e.g.,* immunosuppression and drug-induced autoimmunity).

## Disclosures

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