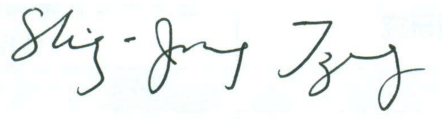
July 19, 2016

Dear Editors,
I am submitting a revised manuscript (JoVE54582R1) entitled: “The isolation, differentiation and quantification of human antibody-secreting B cells from blood: ELISpot as a functional readout of humoral immunity” by Tzeng S-J for revision. Dr. Nam Nguyen has assisted me in the submission process.

I have addressed the editorial and reviewers’ comments point by point (please see below). With the reviewers’ comments, I believe the contents in this manuscript will be very useful for researchers, who work in the fields of human B-cell biology and who would like to measure vaccine-induced B cell response.

Best Regards,



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Editorial comments:

1. Grammar:
-Please copyedit the manuscript for numerous grammatical errors. This editing is required prior to acceptance and should be performed by a native English speaker. In particular, many articles (a, an, the) are either misused or missing.
Reply: In addition to previous two Americans, the manuscript has been edited by a

native English teacher in my school.

-1.2.1 – Please correct the grammar in this step. The step should begin “To determine whether RBCs have been lysed…”
Reply: I have deleted the sentence and rephrased it, **“**NOTE: The appearance of light transmission through tube indicates completion of RBC lysis.”(line 139)

-1.15 – Should be “to the tube wall next to the magnet”.
Reply: I have corrected it (line 181).

-1.19 note – “Typically, can isolate”
Reply: I have corrected it, “Typically, “we” can isolate …”. (line 193)

-5.18 – Should be “to air dry”
Reply: I have corrected it (line 334).

-5.17 – “after completion of developing all spots”
Reply: I have rephrased it, “after completing the development of all spots.” (line 332).

2. Formatting: Please define all abbreviations at first occurrence (Abs, Ab, ELISpot, etc.). This should not be done in the keywords section, but rather in the main text.
Reply: I have corrected it.

3. Additional detail is required:
-1.2.1 – Please clarify “place the tube toward a bench daylight lamp.”
Reply: I have deleted the sentence (line 139).

-5.2 – Please clarify “Avoid touching the membrane in wells any time.”
Reply: I rephrase it as “When pipetting, avoid touching the membrane in wells at all times (line 283)”.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
In this manuscript, authors try to describe a method for total IgG/IgM/IgA ELISPOT following polyclonal activation of peripheral blood B cells. They describe a negative

selection method for B cells, mention several different polyclonal activation cocktails and then provide only a couple of representative ELISPOT pictures for some Ig isotypes. They do not give any information on how many samples they have tested with which culture condition and what results they have obtained upon different stimuli for total Ig spot production.

Major Concerns:

The journal clearly states that their main interest is not the novelty of a method but more sharing and teaching highly reproducible techniques. To achieve that even for a very straightforward method such as total Ig ELISPOT assay, the manuscript needs to be very much improved.

General remarks:

-Punctuation and grammar mistakes need to be corrected.

-References should be in order of appearance.

-In introduction, authors do not do not mention what type of an ELISPOT assay protocol (total Ig ELISPOT or antigen-specific ELISPOT) they will explain in the following sections.

-At least a model antigen should be used to show the applicability of the ELISPOT assay (such as tetanus toxoid). A total Ig ELISPOT is quite useless in a clinical setting. Furthermore, the authors use so many isolation steps of cells, how representative are their results for the in vivo situation?

Reply: This scope of this work was to describe how to perform the ELISpot assay. In the Discussion section, I have mentioned the application of antigen-specific ELISpot (lines 519 and 555) and its clinical applications.

-An overview of the method before getting into the protocol section should be given. Each step regarding incubation times, number of cells, type of cells, culture cocktail used should be written precisely and remain consistent throughout the whole manuscript.

Reply: An overview on the basics and advantages of the ELISpot was described in Introduction (lines 95-119).

-A detailed result section is missing. How many samples are tested, what are the overall findings for all the samples tested?

Reply: Since I only showed ELISpot data of CpG differentiated PCs in Figure 1, I moved the entire non-CpG parts (line 270) in the original Protocol section to the Discussion section (lines 467-481).

-Informed consent information should be complete. Just mentioning one reference number of the review board approval is not sufficient.
Reply: The reference number was actually an “approved protocol number” (lines 126-128).

Other remarks:
-Line 50: It is important to mention in introduction that memory B cells/plasma cells develop after germinal center reaction. Furthermore, short-lived plasma blasts are not PB precursors, as written in the introduction
Reply: I have deleted the sentence (line 51).

-Line 78-79: What do the authors want to tell about ELISA in this sentence? It needs rephrasing.
Reply: It was a general description about ELISA. To be more specific, I add “the titers of” serum Ag-specific Abs… (line 83).

-Line 98-99-100: Authors mention that there is a positive correlation between serum ab levels by ELISA and ASC numbers by ELISPOT. Is this always the case? References for this and contra statements should be added.
Reply: I have rephrased the sentence (lines 105-106). Consistent with our experiences, references 19 and 20 show a correlation between ELISA and ELISpot.

-Line 161: Authors isolate B cells from peripheral blood by negative selection. Do they use a commercial kit, if yes they should specify, or if they make their own anti-human Ab cocktail specific for blood cells what are these antibodies directed at?
Reply: We did use a commercial kit. Since JoVE does not allow commercial trademark or language in the text, I put the kit info in the Reagent list.

-Line 167: Which microbeads are used?
Reply: JoVE does not allow commercial trademark or language in the text. The info is in the Reagent list.

-Line 187: The authors state that typically, 1-3x10^6 B cells can be isolated from 10 mL of peripheral blood. This is impossible. Even in their own example from flow cytometry only 6.14% of lymphoctes are B cells
Reply: I have made a correction, “1-5x10^5 B cells” can be isolated (line 194). Based on the reference 23, it is estimated 1.6x10^6 B cells in 10 ml blood.

-Line 209: Brownish microbeads? Beads are either brown or they are not
Reply: At line 180, “brownish” has been corrected to “brown”.

-Line 216 and 222: references are jumping from 10 to 21 and then to 15.
Reply: I have arranged the references in order.

-Line 215: add ''what?'' to the tube?
Reply: transfer the supernatant “fraction” into a new sterile tube (line 219).

-Line 250-251-252: What do authors do with these cells? Do they culture them like the other cells or not?
Reply: I added “Note: Sorted naïve and memory B cells can be cultured as described in section 4.” at line 261.

-Line 270: Authors mention a culture frame of 3-6 days. How many days of culture exactly do they recommend for each activation condition they used? What condition should be used when? What is the most optimal condition? Why is R848 not mentioned? Any differences in memory vs. naive B cells activation?
Reply: We cultured cells in the presence of CpG for 5 days (line 271). I have added R848 in Discussion section (lines 478 and 501) and added references 32-33.

-Line 272: It would be better to use the term ''harvest the cells' instead of ''take the culture medium".
Reply: I have corrected it (line 275).

-Line 291: Do authors have any data comparing the spot count and intensity between previously coated (1week old) and freshly coated (overnight) ELISPOT plates?
Reply: I deleted the sentence.

-Line 307: Authors mention cells from section 4.7, however, there is no section number like that. Are cells from section 3.10 added to ELISPOT plates without being activated? How many cells do they exactly plate in ELISPOT wells and what is the range for cell titration they make?
Reply: I have made a correction; section 4.7 should be section 4.6 (line 310). I added a note, “NOTE: Sorted naïve and memory B cells can be cultured as described in section 4.” (line 260). Step 5.10 describes the cell number put into the ELISpot plate (line 309).

-Line 333: Which of these tools did authors use to count the spots?
Reply: We used CTL ImmunoSpot analyzer and this info is in the reagent list. JoVE does not allow commercial language in the text.

-Line 343**:** Authors mention a very high percentage of memory B cells in PBMC? Which part of figure 1 shows that 83% of CD19+ B cells is CD27+?
Reply: Base on references 25 and 26, the percentage of CD27+ memory B cells in PBMCs is 30-60% (line 228). The new Figure 1 shows about 50% of CD27+ memory B cells (line 351).

-Line 368: Here, authors mention a 6 hr to overnight incubation whereas in line 312, 8-14hr incubation is mentioned? For how long exactly, do they incubate the cells in ELISPOT plates?
Reply: Cells were cultured overnight (~14 hr, line 395)". Cells were counted in the end of CpG stimulation (step 4.6) before culturing in the ELISpot plate.

-Line 375: Where do these cell numbers come from? These cell counts seem to very high to plate for a total Ig ELISPOT assay? How do authors explain the presence of IgG spots at day 0 for CD19+ B cells and why is there no spots in well #2, if the cells plated here are half of the well# 1? Authors coat and detect the plates also with IgA abs however, do not show any representative pictures for IgA.
Reply: Yes, circulating PBs are rare (line 458). We use a new Figure 3 (line 392). Thank you for your comments. We don't routinely do IgA ELISpot though and it is not the scope of this work.

-Line 388: This paragraph does not belong to discussion since it is an alternative method to remove RBC and platelets. It should be integrated into the protocol section.
Reply: It was in the Protocol section, but I was suggested to move it to the Discussion section because it was too long as a note.

-Line 396: in this and the next paragraph, the authors discuss how to best isolate (memory/naive) B cells. They have not considered activating whole PBMC with a R848 based strategy, and optionally perform isotype ELISPOT to determine memory vs. naive.
Reply: I have added R848 (line 478) as a differentiation agent and described the use of PBMCs for the ELISpot assay (lines 470 and 539).

-Line 440: In this paragraph of the discussion, authors give an overview of the literature on different polyclonal activation cocktails for B cells. However, they do not comment on their own results about several different cocktails they mention in their protocol section. In fact, they do not show their own results regarding these cocktails.

Reply: Except for the R848, our experiences on different polyclonal activation cocktails for B cells can be found in the reference 11. Other researchers' experiences, including on the R848, can be found in the references 12-15, 18, 31-33.

-Line 464: Agents to promote Ig class switching are mentioned: this you don't want in an ELISPOT pre-culture system. In fact, the authors should rule out class switching in their pre-culture.

Reply: In vitro differentiated PCs, whether class switched or not, can be used to study the physiology of PCs. It is then up to the researchers to decide the use of agents to promote Ig class switching. Regarding your concern in vaccine studies, I have added a comment, "Since the addition of a differentiation agent in culture promotes the generation of switched B cells, it is not advised when pre-existing PBs/PCs or unswitched memory B cells are to be measured." (lines 507-510)

-Line 481: Antigen can also be used a detection agent, with the benefit of requiring less antigen. This should be mentioned (Karahan et al., Hum Immunol 2015)

Reply: Thank you for the great suggestion. I have described this at lines 555-558 and added Karahan's paper as the reference 36.

-Line 502: Authors recommend shaking of ELISPOT plates when cells are added before placing in the incubator. Doesn't this act also cause moving of the cells and produce spots with tails since these cells are activated and already producing antibodies?

Reply: I have deleted the sentence.

-Line 508: The ballpark of 6hr to overnight? Please. Keep this scientific.

Reply: I have corrected it to "the range of 8 hr to overnight" (line 547).

Minor Concerns:

N/A

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

This protocol focuses on the ELISpot technique applied to the analysis of human humoral immune response. The author also describes how to purify and stimulate subsets of human B cells from peripheral blood.

The manuscript suffers from several approximations and mistakes that preclude its publication as it stands.

Two PhD students from my lab who performed this experiment routinely helped for the reviewing of this manuscript.

Our comments are listed below.

General comment:

The English shall be corrected throughout the manuscript.

There are several occasions when a range of volume or concentration is given without clear indication of how/when to choose between them.

Reply: In the Protocol section, I have minimized the description of “a range of volume and concentration”.

Specific comments:

Introduction:

- It is unclear for us what the author means by "Since naïve B cells by nature do not experience Ags, they tend to be homogeneous. In contrast, memory B cells and PBs/PCs are polyclonal and heterogeneous". The naïve B cells are even more heterogeneous than activated B cells in terms of immunoglobulin repertoire.

Reply: Thank you for the comment and I have rephrased it at lines 62-65.

- page 2 line 91: the sentence "ELISpot is a technique that employs a sandwich ELISA…" is not appropriate and could be replaced by "ELISpot is a technique related to a sandwich ELISA..".

Reply: I have corrected it as you suggested (line 96).

Protocol:

- 1.1: Blood is almost never draw from radial artery. Most blood samples are collected by venous puncture.

Reply: Thanks for the suggestion. Indeed, most people draw blood from the median

cubital vein now. I have made correction at lines 131-132 (step 1.1).

-1.3: Two rounds of RBC lysis may affect surface epitopes and lead to a very high mortality and shall be avoided
Reply: I have deleted the sentence (step 1.2).

-1.5: The macrophage elimination does not seem necessary if B cells are then purified. This part of the protocol shall be optional.
Reply: We routinely perform steps 1.5 to 1.7 to enhance the efficiency of microbead-based B cell isolation.

-1.6: The centrifuge step is detailed in 1.7 and shall then be removed from 1.6.
Reply: I have deleted the sentence (line 152).

-1.9: "centrifuge as step 1.6" shall be replaced by "centrifuge as step 1.7"
Reply: I have corrected it (line 162).

-1.10: The composition of the anti-human Ab cocktail should be given as Ab from other companies could be used instead of this kit.
Reply: I have added “NOTE: Anti-human Ab cocktail should at least include Abs specific to CD2 (or CD3), CD14 and CD16 at line 168.

-1.15: The reference of the magnetic stand should be given.
Reply: In steps 1.15 and 1.16, I have added reference 22 of the magnetic stand.

-1.17: "repeat step 1.13 to 1.15" shall be replaced by "repeat step 1.12 to 1.16".
Reply: I have made correction. It should be “steps 1.14 to 1.16. (line 187)

-1.19: It is unclear in which conditions the cells shall be resuspended in PBS rather than RPMI medium. Moreover, keeping the cells in PBS only for some period of time could lead to increased cell mortality. The same applies to 2.12.
Reply: I have deleted “PBS”.

-2.1: A centrifugation step seems to be missing between 2.1 and 2.2.
Reply: I have added the centrifugation step (step 2.2, line 201).

-2.2: In which case shall the cells be resuspended in 50ul rather than 100ul of cold PBS buffer?

Reply: The 100 ul has been deleted (line 201).

-2.3: The composition of the PBS Buffer has already been given and does not need to be repeated. The same applies to 3.2.
Reply: I have deleted them at steps 2.4 and 3.2 (lines 205 and 235).

-4.2: Which volume is added per well depending on the type of plates used?
Reply: The sentence is rephrased; “Resuspend cells with RPMI 1640 medium at a concentration of 1-10 x 10^5 per mL and aliquot them into wells of a 12-well plate.” (lines 268-269).

-4.5: This sentence is unclear and should be rephrased.
Reply: I have rephrased the sentence; “Harvest the cells from each well, place them into 15 mL tubes separately, add 5 mL of PBS to each tube and centrifuge at 600 x g at RT for 5 min.” (lines 275-276)

-5.1: Ethanol treatment is not necessary for all ELISpot plates. This should be optional.
Reply: We follow the manufacturer’s instructions. Nevertheless, I have added a note; “NOTE: Steps 5.1 to 5.3 may be optional, depending on the manufacture of plates.” (line 291)

-5.6: 0.5% of BSA is not enough to saturate the wells. It is more efficient to saturate the wells with RPMI medium supplemented with FCS at 37°C for 2 hours. Thus step 5.6 could be suppressed providing that step 5.8 (incubation with RPMI 1640 at 37°C) is performed for 2 hours.
Reply: It should be 5% of BSA and it works fine in our hands. I added RPMI medium as an alternative (line 302)

-5.10: "4.7" is actually "4.6".
Reply: I have corrected it.

-5.10: The number of cells indicated here does not correspond to what is indicated in the legend of figure 3.
Reply: They are corresponded.

-5.10: It is good practice to do triplicates or at minima duplicates of all conditions.
Reply: Thank you for your suggestion, but this requires enough cells to do so. I have

described the method of normalization to enhance accuracy in the Discussion section (lines 570-582).

-5.15: A wash step is missing between 5.14 and 5.15.
Reply: I have added the wash step (line 330).

Representative results and figure legends:
There are some issues with figure 1 and figure 3.
Figure 1: the B cell subsets from peripheral blood are not gated properly. Naïve B cells are CD19+ CD27-CD38+, plasma cells are CD19lowCD27highCD38high.
Usually, all CD19+ B cells are first gated from the lymphocyte gate. On the B cell gate, naïve B cells are gated as CD27-IgD+, memory B cells are gated as CD27+ (IgD-: switched memory, IgD+:IgM memory) and plasma cells are gated as CD27highCD38high.
Accordingly, the % for the different subsets given in "representative results" are not right.
Reply: The percentage of CD27+ memory B cells is 30-60% (lines 228, ref. 7, 25 and 26). The new Figure 1 showed ~50% of B cells are CD27+.

Figure 3: It is highly surprising to observe IgG+ spots at day 0 with both total CD19+ cells and memory B cells. One possibility could be that some plasma cells are still present in the purified populations. However, based on the literature, the frequency of IgG+ and IgM+ plasma cells in the peripheral blood is equivalent. It is thus unexpected to detect spots only for the IgG ELISpot. That should be discussed.
Another surprising point is the absence of clear differences between the conditions 1 and 2 considering that twice as many cells are plated in condition 1 compared to condition 2. Altogether this suggests that the wells depicted are not representative of the experiment described.
Reply: Thank you for the comment. Please see the Figure 3 (lines 392-399).

Discussion:
In the second paragraph of the discussion, the author seems to describe the realization of a Ficoll gradient. However, a sentence must be missing as Ficoll is never mentioned. If it is indeed the description of PBMCs enrichment by Ficoll gradient, a subsequent red blood cell lysis step is normally not required.
Reply: I have deleted the Ficoll gradient.

List of Material/Equipment:

It is not clear at which step of the protocol the Biotinylated CD27 Ab-conjugated microbeads are used. The anti-human CD19-APC from Biolegend is indicated twice.
Reply: I have deleted the biotinylated CD27 Ab-conjugated microbeads and the duplicated anti human CD19-APC.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
The submitted MS describes a useful and adaptable method for the isolation, differentiation and quantification of human ASC cells using ELISpot. Acceptance for publication is recommended with the following changes.

Major Concerns:
Flow cytometry gating strategy for memory, plasmablasts, and naïve B cells:

a. An excellent example of plasmablast gating exists in both, Smith, K. et al, Nat Protoc. 2009; 4(3): 372-384 and Caraux, A. et al, Haematologica, 2010;95:1016-1020. The former also describes another important utilization of the author's proposed protocol - following ASC responses to vaccines. Please include these references in the appropriate sections of the paper.

Reply: I have added these two references (ref. 30 and 31).

b. Figure 1 comments: There are several problems with the flow cytometric gating and labels. First, please add instructions for a doublet discrimination gating.

Reply: I have made correction and added a new Figure 1 (lines 368-377).

c. The protocol indicates that the cells to be sorted by flow cytometry were from the "…cells purified from section 1." The cells purified in section 1 are untouched, negatively selected B cells. Therefore, most of these cells will be CD19+ (low to high). The second flow diagram (CD27 x CD19) should therefore

represent a gradient of CD19+ cells, and not a positive and negative population of CD19. It appears possible that the authors have gated on CD19hi cells and gated out many CD19low/int cells, which should have been included in the analysis. It is hard to tell without seeing the flow gating controls that were used. See your reference #2: Jackson, S.J., et al (2008), Table 5.2.

Reply: I used unpurified PMBCs in Figure 1 (lines 368-377).

d. More terminally differentiated "plasma cells" express CD138 and are generally located in the bone marrow. The flow diagram indicating, Q2 - "Plasma cells" should be labled as "plasmablasts" without this discriminating marker. Please correct this in the manuscript as well.

Reply: I have deleted plasma cells (PCs) and used PBs (lines 369 and 68).

e. Furthermore, circulating plasmablasts will be rare in healthy individuals (2/ul blood, see Caraux, A. et al, Haematologica, 2010;95:1016-1020) and are normally able to be sorted only after an immunization (or during infection or disease state), which gives rise to a flush of newly-generated plasmablasts. While the plasmablast population can be found increased without immunization in some disease states, such as SLE (Potter, K.N., et al Lupus, 2002;11, 872-877), it is very unlikely that sorting this population would be possible in the peripheral blood of healthy, unimmunized subjects - especially at the numbers needed for sorting. This should be mentioned.

Reply: I have mentioned this at lines 55 and 458.

f. The gating for the Q2 plasmablast population shown in the flow diagram of this manuscript is most certainly incorrect at 64%. This is a result of incorrect gating.

Reply: The new Figure 1 gated the CD19+CD27+/hiCD38+ as the PBs (lines 368-377).

g. Assuming plasmablasts are present in the blood of represented sample, they will have begun to downregulate CD19 (see Jackson SJ, et al,2008, your ref #2). Therefore, be sure to include the entire CD19+ subset in analysis so the cells nearing plasma cell stage will be included. It is suggested that CD20 could be added to help discriminate the plasmablast population. Also, the plasmablast gate should not include all positive CD27 and CD38 cells, but be limited to the CD27+/high/CD38high cells (CD19+CD20-/loCD27+/hiCD38hi).

Reply: I have gated the CD19+CD27+/hiCD38+ as the PBs in Figure 1 and comment on the use of CD20 (lines 368-377, 460).

g. Naïve cells: In a study of 106 healthy donors, Caraux, A, et al, 2010, demonstrated that their naïve cell population represented 60-70% of the peripheral CD19+ population. The author's gating scheme results in a naïve population of 17% (Q4). It is unclear if this is a percentage of the parent, CD19+, or FSC/SSC gate. Regardless, excluding CD38+ cells from a CD19+CD20+CD27- naïve population may result in the exclusion of a substantial population of activated naïve B cells (see Potter, K.N., et al Lupus, 2002;11, 872-877).

Reply: The percentage of CD27- naïve B cells in the new Figure 1 is ~50% (lines 368-377).

Figure 2 Comments:

i. It appears that D (top panel) is color-coded incorrectly for the secreted antibody (should be yellow, but was changed to purple). Actually, the entire top-down view (A-E, top panel) is confusing and unnecessary. Please remove.

Reply: I have removed the top-down view in the Figure 2.

ELISpot dilutions and normalization.

j. Figure 3, panels A & B, Day 5 (CpG): The 1:2 dilutions of IgM and IgG CD19+ and memory B cells are not apparent. It is also unlikely at even the lower dilution that these could be quantified consistently and accurately at this density. To these figures, please include the ELISpot image from (at least) the third dilution described in section 5.10. If the dilution is still not apparent at the third dilution, include additional dilutions. See Smith, et al, 2009, fig. 1 for example of proper titrations for quantitation.

Reply: A new Figure 3 is presented.

k. In discussion section, please include discussion on ELISpot normalization procedures for inter-assay and longitudinal studies including commentary on plate reading linearity and accuracy. Please reference: Smith, S.G., et al, (2009) Identification of Major Factors Influencing ELISpot-Based Monitoring of Cellular Responses to Antigens from Mycobacterium tuberculosis. PLoS One 4(11):e7972. Doi:10.1371/journal.pone.0007972.

Reply: The ELISpot normalization procedures have been discussed at lines 570-582. Reference 41 is Smith SG's.

Minor Concerns:

1) Page 2, lines 60-62: The sentence, "Since naïve B cells by nature….." is unclear. Please expand on what is meant by "homogeneous" and "heterogenous."

Reply: I have rephrased it," Although naïve B cells are homogeneous in expressing BCR (B-cell antigen receptor)-associated molecules - e.g., CD19, CD20 and CD22 - they are heterogeneous in immunoglobulin repertoire (ref. 5)". (line 63).

2) Please provide reference for Page 2, line 69-71, "As a matter of fact, ASCs derived from in vitro……"

Reply: I added the Ref. 6 (line 76).

3) Page 2, line 85-86; Remove statement, "Moreover, the amount of Abs secreting into culture medium by ASCs is far below the sensitivity of detection by ELISA." This is not supported by many studies. Three are cited here for example: Immunology and Cell Biology (2003) 81, 305-310; doi:10.1046/j.1440-1711.2003.t01-1-01173.x AND: Clin Vaccine Immunol, May 2001 vol. 8 no. 3 482-488 doi: 10.1128/CDLI.8.3.482-488.2001 AND: Infect. Immun. January 2010 vol. 78 no. 1 253-259 doi: 10.1128/IAI.00868-09.

Reply: Thank you for the comment. I have removed the statement.

4) Page 2, line 91: (grammar) Change "sample" to "samples."

Reply: I have corrected it (line 96).

5) Please include reference for Page 3, lines 98-100, "In the measurement of humoral immune function…"

Reply: I have added references 19-20 (lines 105-106).

5) Page 3, line 93: (grammar) Remove "In a way" and start the sentence with "The ELISpot assay…."

Reply: I have corrected it (line 96).

6) Page 3, line 96: (grammar) Insert "an" before "immune response."

Reply: I have corrected it (line 101).

7) Page 3, line 101: (grammar) Replace "out of" with "from."

Reply: I have corrected it (line 105).

8) Page 3, line 107: Remove "ng/mL range" and replace with language such as, "optimized assay dependent concentrations," as many commercial ELISA kits

can detect in the pg/mL range.

Reply: I have corrected it (line 112).

9) Page 3, line 117: Please replace "was obtained" with "must be obtained" for consistency in present tense use in Protocol section.

Reply: I have corrected it (line 122).

10) Page 4, line 146: (grammar) Remove "in" before "into."

Reply: I have corrected it (line 151).

11) Page 4, line 161: Replace "(exclude B-cell specific Abs)" with "(for negative selection of B-cells).

Reply: I have corrected it (line 165).

12) Page 4, line 162: Should this read "….per 1 x 10^6 cells…," instead of "…to 10^6 cells…" What if the starting number of cells exceeded 1 x 10^6? Could the volume be increased in the same incubation tube?

Reply: Yes, “per 1 x 10^6 cells” was what I meant (line 166). Thank you.

13) Page 4, line 180: The authors instruct reader to Repeat steps 1.13 to 1.15, and combine supernatants. It is unclear what fractions are being combined. Please clarify.

Reply: I have rephrased it as **“**Repeat steps 1.13 to 1.16, and combine the two supernatants” **(**line 187).

14) Page 6, Sec. 4.1, line 256: Instructions for counting isolated cell fractions by various purification steps. Please include "or section 3.10 isolated fractions."

Reply: I have corrected it (line 265).

15) Page 7, Sec. 5.2, line 281: (grammar) insert "at" between "wells" and "any" in sentence.

Reply: I have corrected it (line 284).

16) Page 7, Sec 5.10, line 307: reference to section 4.7 should be replaced with "section 4.6."

Reply: I have corrected it (line 313).

17) Page 7, Sec 5.13, line 317: Reword this sentence to make clear that it is a "wash

step." Add instructions to invert plate to empty wash buffer between each of the 5 washes. This should be evident to one that does this assay regularly, but should be written more precisely for a reader that has no experience with the assay.

Reply: Thank you for the suggestion. I have added it at line 324.

18) Page 10, line 424: (grammar) Change "do an equally good block" to "block equally well."

Reply: I have corrected it (line 445).

19) Page 10, line 454: Change "…million" to scientific notation, as used in the rest of the MS ("1 x 10^6").

Reply: I have corrected it (lines 445 and 490).

20) Page 11, line 464: (grammar) Insert "is" between "cells" and "often."

Reply: I have corrected it (line 500).

22) Page 11, line 465: (grammar) Change "combines" to "combined."

Reply: I have corrected it (line 501).

23) Page 11, line 469: (grammar) Change "mention" to "mentioning."

Reply: I have corrected it (line 506).

24) Page 11, line 474: (grammar) Insert "The" before "ELISpot assay."

Reply: I have corrected it (line 514).

25) Page 11, line 476: Also reference Leehan, KM, Methods Mol Biol. 2015;1312:427-34. doi: 10.1007/978-1-4939-2694-7_43.

Reply: Thank you for the great suggestion. I have added it as Ref. 36 (line 515).

26) Page 11, line 478: (grammar) Change from "…of ELISpot…" to "…in ELISpot…"

Reply: I have corrected it (line 515).

27) Page 11, line 495: Remove reference to step 4.7 and replace with step 4.6 (step 4.7 does not exist).

Reply: I have corrected it (line 534).

28) Page 12, line 508: (grammar) The use of idioms in scientific writing should be

avoided, as they are phrases that have a figurative meaning that may not be understood by all readers. Please remove the idiom, "in the ballpark," and replace with more formal language.
Reply: I have corrected it to “in the range” (line 547).

29) Page 12, line 527: Include reference, Smith, K. et al, Nat Protoc. 2009; 4(3): 372-384, along with your reference #13.
Reply: I have added the reference as ref. 30 (line 539).

30) Reference number 1 is incorrectly shown as "Benmark." Please correct to "Bemark."
Reply: I have corrected it (line 597).

Additional Comments to Authors:
N/A