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TITLE:

Analysis of Somatic Hypermutation in the JH4 intron of Germinal Center B cells from Mouse Peyer's Patches

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SUMMARY:

Presented here is an assay to quantify somatic hypermutation within the immunoglobulin heavy chain gene locus using germinal center B cells from mouse Peyer's patches.

ABSTRACT:

Within the germinal centers of lymphoid organs, mature B cells alter their expressed immunoglobulin (Ig) by introducing untemplated mutations into the variable coding exons of the Ig heavy and light chain gene loci. This process of somatic hypermutation (SHM) requires the enzyme activation-induced cytidine deaminase (AID), which converts deoxycytidines (C), into deoxyuridines (U). Processing the AID-generated U:G mismatches into mutations by the base excision and mismatch repair pathways introduces new Ig coding sequences that may produce a higher affinity Ig. Mutations in *AID* or DNA repair genes can block or significantly alter the types of mutations observed in the Ig loci. We describe a protocol to quantify JH4 intron mutations that uses fluorescence activated cell sorting (FACS), PCR, and Sanger sequencing. Although this assay does not directly measure Ig affinity maturation, it is indicative of mutations in Ig variable coding sequences. Additionally, the methods utilize common molecular biological techniques and analyzes mutations in Ig sequences of multiple B cell clones. Thus, this assay is an invaluable tool in the study of SHM and Ig diversification.

INTRODUCTION:

B cells, members of adaptive immune system, recognize and eliminate antigens by producing

antibodies, also known as immunoglobulins (Ig). Each Ig is composed of two heavy (IgH) and two light (IgL) chain polypeptides, which are held together by disulfide bonds to form the characteristic "Y" shape structure of the Ig[1]. The N-termini of IgH and IgL comprise the variable (V) region of each polypeptide and together they form the antigen binding site of the Ig, whereas the constant region of IgH imparts the effector function of the Ig. Developing B cells in the bone marrow rearrange the V coding exons of IgH and IgL in a process known as V(D)J recombination[2-4]. Transcription of the recombined V exons, coupled with the respective constant region exons, forms the mRNA that is translated into the Ig.

Mature B cells expressing a membrane bound Ig, also known as a B cell receptor (BCR), circulate to secondary lymphoid organs, such as the spleen, lymph node, or Peyer's patches, where they survey the environment for antigens and interact with other cells of the immune system[1]. Within the germinal centers (GC) of secondary lymphoid organs, B cells that recognize antigen through the BCR become activated. Aided by follicular dendritic cells and follicular helper T cells, activated B cells can then proliferate and differentiate into plasma and memory cells, which are important effectors of a robust immune response[5-9]. Additionally, these activated B cells can undergo secondary Ig gene diversification processes - class switch recombination (CSR) and somatic hypermutation (SHM). During CSR, B cells exchange the default μ constant region of the IgH polypeptide with another constant region (γ, α, ε) through a DNA deletional-recombination reaction (**Figure 1**). This allows for the expression of a different constant exon and translation of a new Ig. The B cell will switch from expressing IgM to another isotype (IgG, IgA, IgE). CSR changes the effector function of the Ig without altering its antigen specificity[10-12]. However, during SHM, B cells mutate the V coding regions of IgH and IgL to enable the production and selection of higher affinity Igs, which can more effectively eliminate an antigen[13-15] (**Figure 1**). Importantly, both CSR and SHM depend on the function of one enzyme: activation-induced cytidine deaminase (AID)[16-18]. Humans and mice deficient in AID cannot complete CSR or SHM and present with elevated IgM serum titers or Hyper-IgM[17,19].

In CSR, AID deaminates deoxycytidines (C) in the repetitive switch regions that precede each constant coding exons, converting them into deoxyuridines (U)[20,21], which creates mismatched base pairing between deoxyuridines and deoxyguanosines (U:G). These U:G mismatches are converted into the double-stranded DNA breaks, which are required for DNA recombination, by either the base excision repair (BER) or mismatch repair (MMR) pathway[22-29]. In SHM, AID deaminates C within the V coding exons. Replication across the U:G mismatch generates C:G to T:A transition mutations, whereas removal of the uracil base by the BER protein, uracil DNA glycosylase (UNG), prior to DNA replication produces both transition and transversion mutations[16]. Null mutations in *UNG* significantly increase C:G to T:A transition mutations[21,22]. Similar to CSR, SHM requires the complementary roles of MMR and BER. During SHM, MMR generates mutations at A:T base pairs. Inactivating mutations in MutS homology 2 (*MSH2*) or DNA polymerase η (*Polη*) significantly reduces mutations at A:T bases and compound mutations in *MSH2* and *Polη* virtually abolishes mutations at A:T bases[21,30,31]. Consistent with the critical role for BER and MMR in converting AID-generated U into transition or transversion mutations, mice deficient for both MSH2 and UNG (*MSH2*$^{-/-}$*UNG*$^{-/-}$) display only C:G to T:A transition mutations resulting from replication across the U:G mismatch[21].

The analysis of SHM in V coding regions remains complicated because developing B cells can recombine any of the V(D)J coding exons in the *IgH* and *IgL* loci[1,2,4]. Accurate analysis of these uniquely recombined and somatically mutated V regions requires the identification and isolation of clones of B cells or the Ig mRNA[11,13]. The JH4 intron, which is 3' of the last J coding exon in the IgH locus, harbors somatic mutations due to the spreading of mutations 3' of the V promoter[32-34] and therefore is frequently used as a surrogate marker for SHM in V regions[31, 35] (**Figure 1).** To experimentally elucidate how specific genes or genetic mutations alter SHM patterns or rates, the JH4 intron can be sequenced from Peyer's patches (PP) germinal center B cells (GCBCs), which undergo high rates of SHM[36-38]. GCBCs can be readily identified and isolated with fluorescently conjugated antibodies against cell surface markers ($B220^{+}PNA^{HI}$)[17,39].

In this manuscript, a detailed protocol is presented to characterize JH4 intron mutations in PP GCBCs from mice using a combination of FACS (fluorescence activated cell sorting), PCR, and Sanger sequencing (**Figure 2**).

PROTOCOL:

All mutant mice were maintained on a C57BL/6 background. Age-matched (2-5 months old) male and female mice were used for all experiments. Husbandry of and experiments with mice were conducted according to protocols approved by The City College of New York Institutional Animal Care and Use Committee.

1. Dissection of Peyer's patches

1.1. Euthanize the mouse with 100% CO_2 at 3 L/min for 5 min followed by cervical dislocation to confirm death. Sterilize dissection tools (scissors, forceps, fine forceps) and gloved hands with 70% ethanol.

1.2. Lay the mouse on the dissection pad with the abdomen exposed. Generously spray the body of the mouse with 70% ethanol prior to making any incisions to sterilize the dissection area.

1.3. Make an incision into the skin across the abdomen and remove the skin from the abdomen by pulling simultaneously on both sides of the incision towards the head and tail using forceps (or sterilized, gloved hands).

1.4. Pin down the fore and hind limbs of the mouse.

1.5. Cut the peritoneal cavity with scissors to expose the internal organs.

1.6. Locate the small intestine between the stomach and caecum ("J" shaped structure near the colon). Remove the small intestine by cutting below the stomach and above the caecum.

1.7. Remove any connective tissue and fat linking the folds of the small intestine together.

NOTE: Fat will have a distinctive white color, unlike the pink color of the small intestine.

1.8. Examine the external surface of the small intestine for the Peyer's patches (PPs), which are small (~1 mm), oval-shaped structures that appear white below a thin layer of translucent epithelial cells.

1.9. Carefully excise all visible PP with scissors.

NOTE: One C57BL/6 wild-type (WT) mouse can yield 4-8 PPs, whereas an *AID*$^{-/-}$ mouse will have 6-10 PPs.

1.10. Collect the PPs into a 1.5 mL microcentrifuge tube containing 1 mL of FACS buffer on ice.

NOTE: The PP should sink, whereas fat will float to the surface and can be removed.

2. Cell isolation for FACS

2.1. Place a 40 µm filter in a 6-well dish with 1 mL of cold (4 ˚C) FACS buffer.

2.2 Pour the PPs from the 1.5 mL tube onto the filter.

2.3. Wash PPs with 1 mL of cold FACS buffer, making sure that they are always in liquid and on ice.

2.4. Use the flat end of the plunger from a 1 mL syringe as a pestle to crush the PPs on the filter until only connective tissue remains on the filter.

2.5. Wash the filter and plunger with 1 mL of cold FACS buffer to release the cells into the 6 well dish.

2.6. Collect the ~4 mL of cells in cold FACS buffer and filter them through a 40 µm strainer cap FACS tube.

2.7. Wash the strainer cap with 1 mL of cold FACS buffer.

2.8. Pellet the cells at 600 *x g* at 4 ˚C for 5 min in a swinging bucket centrifuge.

2.9. Decant the supernatant.

2.10. Resuspend the cells in 0.4 mL of cold FACS buffer.

2.11. Remove 10 µL for cell counting to verify yield (expect ~5 x 10^6 cells/mouse, see **Figure 3A**)

2.12. Filter the remaining cells through a 40 µm strainer cap into a FACS tube and proceed to

staining for FACS.

3. Staining GCBCs for FACS

3.1. Add 1 µL Fc block (unlabeled anti-mouse CD16/CD32) to the 400 µL cell suspension and place the cells on ice for 15 min.

3.2. Add 2 mL of cold FACS buffer to wash the cells.

3.2.1. Pellet cells at 600 *x g* at 4 ˚C for 5 min and discard the supernatant.

3.3. Resuspend the cells in 80 µL of cold FACS buffer.

3.4. Remove 10 µL of cells from the WT PP for each staining control (4 in total, including 3 single stain controls and 1 unstained control). Leave 40 µL of the WT PP for the next step. Alternatively, use compensation beads for the staining controls.

3.5. Stain each of the experimental samples (e.g., WT and *AID*$^{-/-}$) in 500 µL of cold FACS buffer with 2.5 µL of peanut agglutinin (PNA)-biotin for 15 min on ice.

3.6. Add 2 mL of cold FACS buffer to wash the cells.

3.6.1. Pellet cells at 600 *x g* at 4 ˚C for 5 min and discard the supernatant.

3.7. Stain each experimental sample with 500 µL of the cocktail in the dark, on ice, for 15 min **(Table 1).** Ensure the cells are fully resuspended in the staining cocktail.

3.8. Prepare single stain controls for the compensation matrix.

3.8.1. Stain the cells in 500 µL of cold FACS buffer using the dilutions specified in **Table 2**.

3.8.2. Incubate the staining controls in the dark, on ice, for 15 min.

3.9. Add 2 mL cold FACS buffer to all the tubes in steps 3.7 and 3.8, pellet the cells, and discard the supernatant to wash off unbound antibodies or DAPI.

3.10. Resuspend the cells in 500 µL of cold FACS buffer and place on ice.

3.11. Using a cell sorter, collect B220^{+}PNA^{HI} from each stained experimental sample. **Figure 3B** shows the typical percentages of B220^{+} PNA^{HI} obtained from WT and *AID*$^{-/-}$ PPs. **Figure 3C** displays the FACS gating strategy.

4. DNA extraction from GCBCs

4.1. Pellet sorted cells at 600 *x g* at 4 ˚C for 5 min and discard the supernatant.

4.2. Resuspend the cells in 1 mL of cold FACS buffer and transfer the cells into a 1.5 mL microcentrifuge tube.

4.2.1 Pellet the cells at 600 *x g* at 4 °C for 5 min and discard the supernatant.

4.3. Resuspend the cells in 500 µL of DNA extraction buffer and 5 µL of 20 mg/mL Proteinase K.

4.4. Incubate at 56 °C overnight.

4.5. Precipitate DNA with 500 µL isopropanol and 1 µL of 20 mg/mL glycogen. Mix the tube thoroughly by inverting 5-6x.

4.6. Incubate at room temperature for 10 min.

4.7. Centrifuge in a microcentrifuge for 15 min at 25 ˚C at 21,000 *x g*.

4.7.1. Discard the supernatant and retain the pellet, which contains the precipitated DNA and glycogen.

4.8. Wash the DNA pellet with 1 mL of 70% ethanol.

4.8.1. Pellet the DNA in a microcentrifuge for 10 min at 25 ˚C at 21,000 *x g.*

4.8.2. Remove the 70% ethanol and air-dry the DNA pellet for 5-10 min.

NOTE: Avoid over-drying as the DNA may not rehydrate completely.

4.9. Resuspend the DNA in 30 µL TE buffer and incubate overnight at 56 °C.

5. JH4 intron sequence amplification and analysis

5.1. Quantify DNA by measuring the absorbance at a wavelength of 260 nm (A260).

NOTE: The typical concentration of DNA recovered from sorted $B220^{+}PNA^{HI}$ GCBCs of a C57BL/6 mouse is 20-40 ng/µL.

5.2. Perform the nested PCR for the JH4 intron (**Table 3, Table 4**). Normalize the total amount of genomic DNA used in the first PCR to the least concentrated sample. (e.g., if the least concentrated sample is 5 ng/µL, use 58.75 ng of DNA for all the samples in the maximum volume of water (11.75 µL) in PCR #1).

5.3. Resolve the PCR product on a 1.5% agarose gel at 200 V for 20 min. The expected amplicon

size is 580 bp.

5.4. Excise the amplicon from the gel and extract the DNA using a gel extraction kit according to manufacturer's instructions (see **Supplementary Figure 1**).

5.4.1. Elute the DNA with 30 µL of water and quantify the amount of DNA by measuring the absorbance at O.D.260.

NOTE: The typical concentration of the purified PCR product is 3-10 ng/µL.

5.5. Ligate the purified PCR product into a plasmid with blunt ends. Standardize the total amount of PCR product used in every ligation reaction (**Table 5**).

5.5.1. Incubate the ligation reaction at room temperature for 5 min or overnight at 16 °C.

5.6. Transform electrocompetent bacterial cells with 2 µL of the ligation reaction.

5.6.1. Electroporate at 1.65 kV.

5.6.2. Rescue in 600 µL of SOC media for 1 h at 37 °C in a shaking incubator at 225 rpm.

5.6.3. Plate 100 µL of transformed bacteria onto LB supplemented with ampicillin (100 µg/mL) agar plates and incubate overnight at 37 °C.

5.7. Submit the plate of bacterial colonies for Sanger sequencing using the T7 forward primer. Alternatively, grow overnight cultures of each bacterial colony and perform a plasmid purification.

5.7.1. If necessary, repeat the PCR, ligation, and/or transformation to optimize the yield of bacterial colonies

NOTE: A minimum of 30 colonies should be picked from each plate.

5.8. Standardize the sequence data in the .txt files

5.8.1. Delete the plasmid sequence.

5.8.2. Ensure every sequence is oriented 5' to 3' according to the JH4 intron reference sequence (NG_005838). Generate the reverse complement of any sequence, as necessary.

5.9. Align the sequences obtained for each PCR against the JH4 intron reference sequence (NG_005838) using a Clustal Omega software (**Figure 4A**).

5.9.1. Identify differences from the reference sequence as mutations

5.9.2. Verify that all mutations are true point mutations by examining the electropherogram of the Sanger sequencing. Repeat sequencing if necessary. (**Figures 4B,C**).

5.10. Tabulate and quantify unique mutations in the JH4 intron for each genotype (**Figure 5**).

5.10.1. Count sequences with identical mutations only once

NOTE: It is not possible to determine if the identical sequences were generated during the PCR or identical SHM events in different B cells.

5.10.2. Count every instance of WT germline JH4 intron sequences (i.e., those with no mutations) as a unique sequence.

REPRESENTATIVE RESULTS:

Flow cytometry

Mature B cells circulate to germinal centers where they undergo affinity maturation, clonal expansion, and differentiation into plasma or memory cells[40-44]. These GCBCs can be identified by numerous cell surface markers, including high expression of the CD45R/B220 receptor and binding of peanut agglutinin (PNA)[45,46]. To isolate activated GCBCs, PP cells were stained with anti-B220 antibodies conjugated to phycoerythrin (PE) and biotinylated-PNA, followed by streptavidin conjugated to APC-eFluor780. Dead cells were eliminated using the fluorescent 4',6-Diamidino-2-Phenylindole (DAPI) dye, which stains the nucleic acid of dying or dead cells[47,48]. The stained cells were subsequently analyzed and sorted via flow cytometry. The PPs consisted of ~80% $B220^+$ cells[49,50]. WT PPs contain on an average 4 x 10^6 cells per mouse **(Figure 3A)**. Approximately 8% of the WT PP cells were $B220^+PNA^{HI}$, which is half the number observed in *AID*$^{-/-}$ (**Figure 3B**). Thus, 0.3-0.6 x 10^6 $B220^+PNA^{HI}$ GCBCs were obtained after sorting, which were sufficient to analyze mutations in the JH4 intron.

JH4 Sequence Analysis

The JH4 intron was amplified by a nested PCR using common VHJ558 family primers (J558FR3Fw and VHJ558.2) followed by JH4 intron spanning primers VHJ558.3 and VHJ558.4[35,37]. Of the 105 unique sequences obtained from WT GCBCs, a total of 226 mutations were found (**Figure 5A**). Analysis of the GCBC mutation spectrum in the WT mice showed a range of transitions and transversions at a rate of 4 x 10^{-3} mutations/bp, which was calculated by dividing the total number of mutated bases by the total number of bases that were sequenced[32,36-38]. Additionally, each JH4 PCR product from WT GCBCs contained 1-25 mutations (**Figure 5B**), where multiple mutations were frequently found on one sequence[33, 36]. Only two mutations were identified in 113 *AID*$^{-/-}$ sequences (**Figure 5A**). *AID*$^{-/-}$ B cells exhibited 1.66 x 10^{-5} mutations/bp, which was significantly lower than WT B cells ($p < 0.05$)[36] and compares to the error rate of the high fidelity polymerase (5.3 x 10^{-7} sub/base/doubling)[51,52] . Thus, *AID*$^{-/-}$ B cells served as a useful negative control for this assay.

FIGURE LEGENDS:

Figure 1: Schematic of the *IgH* gene locus and the regions targeted by AID during CSR and SHM. The red bar indicates the 580 bp JH4 intron that is 3' of VDJH4 rearrangements and was analyzed in this protocol. In CSR, AID-dependent deamination of intronic switch regions (Sμ and Sε) promotes DSB formation that allows for deletional-recombination and the expression of a new antibody isotype (IgM to IgE). During SHM, V regions (grey boxes) accumulate mutations (blue lines) that may lead to higher affinity Ig.

Figure 2: Workflow to analyze SHM of the JH4 intron in GCBCs isolated from PPs.

Figure 3: Characterization of PP GCBCs. (**A**) Total number of PP cells from WT and *AID*$^{-/-}$ mice (n = 4 per genotype). Error bars represent standard deviation from the mean. (**B**) Percentage of B220^{+}PNA^{HI} GCBCs obtained from PPs of WT and *AID*$^{-/-}$ mice (n = 4 per genotype)[36]. Error bars represent standard deviation from the mean, *p<0.05 using student's t-test. (**C**) Representative FACS plots to sort B220^{+}PNA^{HI} GCBCs from PPs.

Figure 4: Analysis of JH4 Sanger sequence data. (**A**) Sample sequence alignments of Sanger sequence data of the JH4 PCR product from WT (top) and *AID*$^{-/-}$ (bottom) GCBCs to the reference genomic sequence (NG_005838), which is the sequence immediately below the numbered tick marks. Alignments were generated using Clustal Omega. (**B**) Electropherogram of high-quality Sanger sequence data, which displayed distinct peaks for each base. (**C**) Electropherogram of low-quality sequence data, which showed ambiguous peaks and unspecified bases (N). The sequence text file (shown in red) must be manually annotated.

Figure 5: Analysis of mutations in the JH4 intron in WT and *AID*$^{-/-}$ GCBCs. (**A**) The total number of transition (red) and transversion (blue) mutations at A, C, G, and T bases for each genotype is summarized in the tables. The total number of sequences analyzed is indicated the table. (**B**) The number of mutations per PCR amplicon for each genotype is depicted in the pie charts. This figure has been modified from Choi et al.[36] Copyright 2020. The American Association of Immunologists, Inc.

Table 1: Staining cocktails for GBC. Cocktail of the indicated antibodies or dye (indicated in italics) at the specified dilutions were used to stain PP cells in 500 μL for flow cytometry.

Table 2: Single stain for compensation. B220 antibodies conjugated to the indicated fluorophores were used for single stain controls to compensate for spectral overlap.

Table 3: **Nested PCR of the JH4 intron.** PCR components and thermocycler conditions for the first amplification reaction. Dilute the first PCR product 1:5 with water and use 1 μL of this dilution for the second PCR.

Table 4: PCR components and thermocycler conditions for the second PCR.

Table 5: Ligation reaction. Components for the ligation of the purified JH4 intron PCR product into the plasmid.

Table 6: Buffer recipes.

Table 7: Oligonucleotides used in the assay.

Supplementary Figure 1: Representative agarose gel image after completion of step 5.4. The JH4 intron nested PCR product was resolved on a 1.5% agarose gel and the 580 bp amplicon was excised. WT PP indicates that WT PP GCBC genomic DNA was used as a template for the first PCR and AID PP indicates that *AID*$^{-/-}$ PP GCBC genomic DNA was used as a template for the first PCR. ϕ indicates the no template PCR control and - indicates nothing was loaded into the well of the agarose gel. The last lane shows a 100 bp DNA ladder.

DISCUSSION:

Characterizing SHM within the *IgH* and *IgL* V coding sequences of a heterogenous B cell population presents a challenge, given that each B cell uniquely reorganizes V coding segments during V(D)J recombination[34]. In this paper, we describe a method to identify mutations in the JH4 intron of GCBCs. The JH4 intron, which is located 3' of the last J coding segment in the *IgH* locus, is used as a surrogate for SHM of V regions (**Figure 1**)[31, 33-35]. To catalog these JH4 intron mutations and assess how specific genes affect the production or pattern of mutations, PP GCBCs are specifically analyzed. These cells accumulate JH4 intron mutations as a result of chronic stimulation by intestinal microbiota[53]. Furthermore, the B220^{+}PNA^{HI} GCBCs from the PPs of unimmunized mice have a mutation spectra that compares to splenic GCBCs from immunized animals[54, 55]. However, mutations in the JH4 intron cannot be correlated to Ig affinity maturation because these mutations are non-coding.

To determine whether SHM alters Ig affinity, mice should be immunized intraperitoneally with an antigen, such as NP (4-hydroxy-3-nitrophenylacetyl) conjugated to CGG (chicken gamma globulin) or KLH (keyhole limpet hemocyanin)[56]. Subsequently, mRNA can be purified from splenic B220^{+}PNA^{HI} GCBCs to examine SHM within V_H186.2, the V coding exon that most frequently recognizes NP and is mutated following NP-CGG or NP-KLH immunization[31,57-60]. Mutation of tryptophan-33 to a leucine in V_H186.2 has been characterized to increase Ig affinity up to 10-fold[59,60] and is, therefore, one indicator that SHM and clonal selection has generated high affinity Ig. Measuring NP7- and NP20-specific serum Ig titers by ELISA and calculating the Ig-specific NP7/NP20 ratio during the course of the immunization also documents Ig affinity maturation resulting from SHM of V regions[17,21,36]. Both these assays can be used to correlate SHM within the V_H186.2 coding sequences with changes in NP-specific Ig affinity maturation.

Whether immunized or unimmunized animals are used to analyze SHM of V_H186.2 or the JH4 intron, GCBCs must be accurately identified. We present a FACS based approach to isolate B220^{+}PNA^{HI} GCBCs. Alternatively, Fas and non-sulfated α2-6-sialyl-LacNAc antigen, which is recognized by the GL7 antibody[61-64], can also be used to isolate GCBCs, which are identified as B220^{+}Fas^{+}GL7^{+}[65] or CD19^{+}Fas^{+}GL7^{+}[37]. GL7 expression closely mirrors PNA in activated GCBCs of the lymph nodes[64-66]. In addition to using antibody markers specific for GCBCs, staining cocktails should maximize the excitation of a fluorophore and detection of a biomarker while minimizing spectral overlap of fluorescence emission. Antigens expressed at low levels should be detected

with an antibody that is conjugated to a fluorophore with a robust emission fluorescence[67]. The recommended staining protocol was optimized for analysis on a cell sorter equipped with four lasers (405nm, 488nm, 561nm, 633nm) and 12 filters; however, filter configurations and laser availability vary between cytometers. To amend the protocol according to reagent and equipment availability, the reader is referred to additional resources, online spectrum viewers and published literature[67-73]. The multi-color staining protocol described herein requires compensation of spectral overlap to ensure that the sorted cell populations are GCBCs rather than inaccurate detection of fluorescence emission. B220 serves as a useful staining control for the described FACS (**Table 1B**) because PPs will have distinctive B220 negative and positive populations (**Figure 3C**), which allows for appropriate compensation of spectral overlap. The gating strategy presented in **Figure 3C** should be used as a guideline. The flow cytometry plots may vary depending on the staining conditions and cytometer settings. Nevertheless, 4-10% of the live cells should be $B220^{+}PNA^{HI}$ [35, 52].

All mutations within the JH4 intron of PP GCBCs must be validated to ensure that the observed mutations are truly reflective of SHM and not an artefact of PCR or sequencing. *AID*$^{-/-}$ B cells can serve as a useful negative control when examining the SHM phenotype in other mutant mouse models because these cells cannot complete SHM[17,19]. The JH4 intron mutation rate in the of *AID*$^{-/-}$ GCBCs ($1.66x10^{-5}$ mutations/bp)[20,21,36-38,50,74] is comparable to the error rate of the high fidelity polymerase ($5.3x10^{-7}$ sub/base/doubling)[51, 52] that is used to amplify the DNA in the nested PCR. If *AID*$^{-/-}$ mice are not available, compare the observed mutation pattern and frequency to the published literature. Ig V regions accumulate 10^{-3}-10^{-4} mutations per base pair division, which is approximately 10^{6}-fold higher than the mutation rate of other gene loci[73,75]. Results may vary with the age of the animal[76]. Alternatively, $B220^{+}PNA^{LO}$ cells, which marks non-GCBCs, may be used as a negative control in the absence of *AID*$^{-/-}$ mice[52]. If the mutation frequency in WT GCBCs is lower than expected, the WT germline JH4 intronic sequence may be disproportionately represented. In this case, ensure that GCBCs were stained and sorted appropriately and PCRs are free from WT germline JH4 intron contamination. Additionally, raw sequencing data in electropherograms should be analyzed thoroughly to ensure that mutations in the sequence text data are not artefacts of sequencing errors. For example, poor Sanger sequencing results may reduce the reliability of the sequence data (**Figure 4**). This quality control of the Sanger sequence data will increase the accuracy and reproducibility of the JH4 intron mutation analysis.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

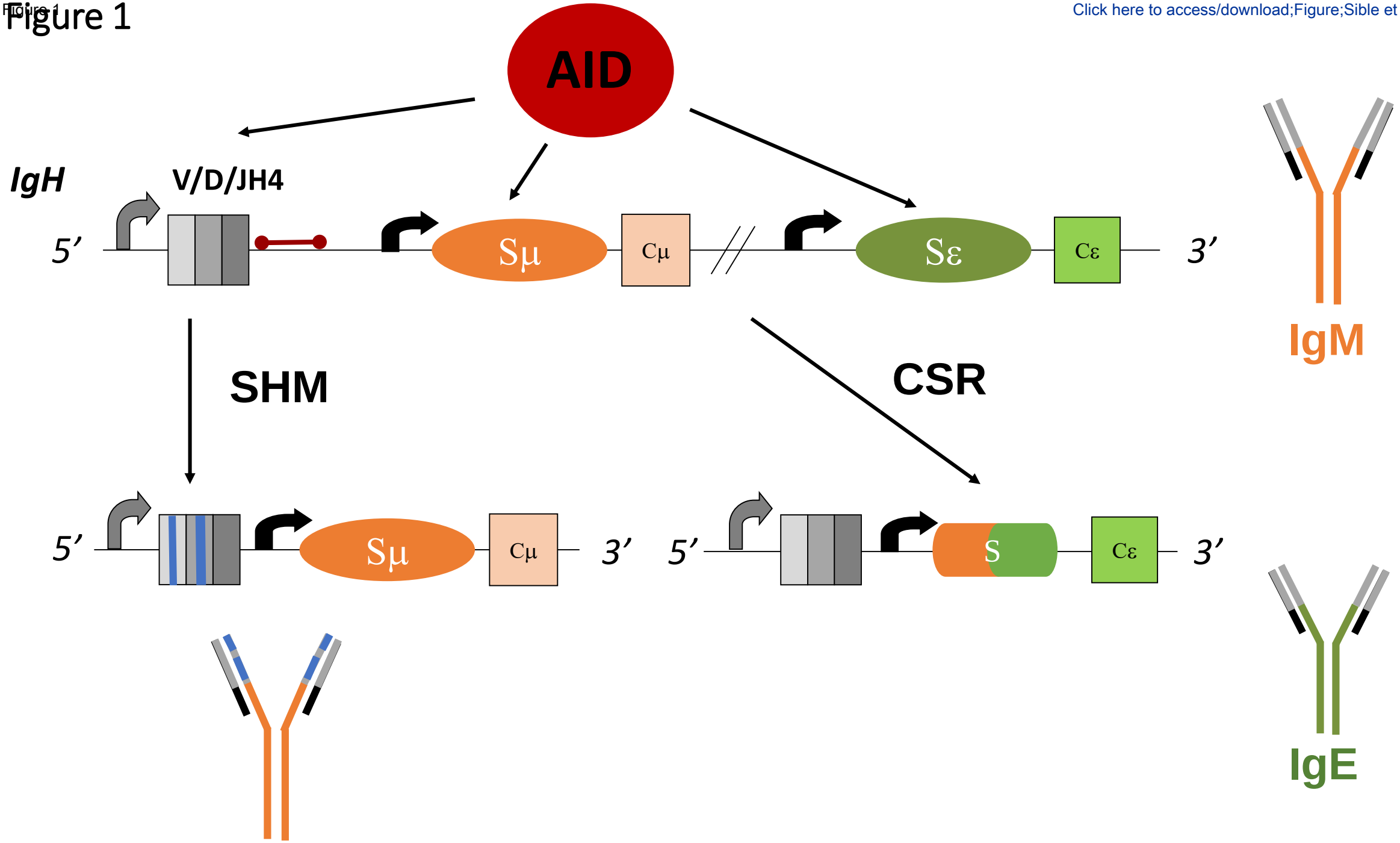


Figure 2

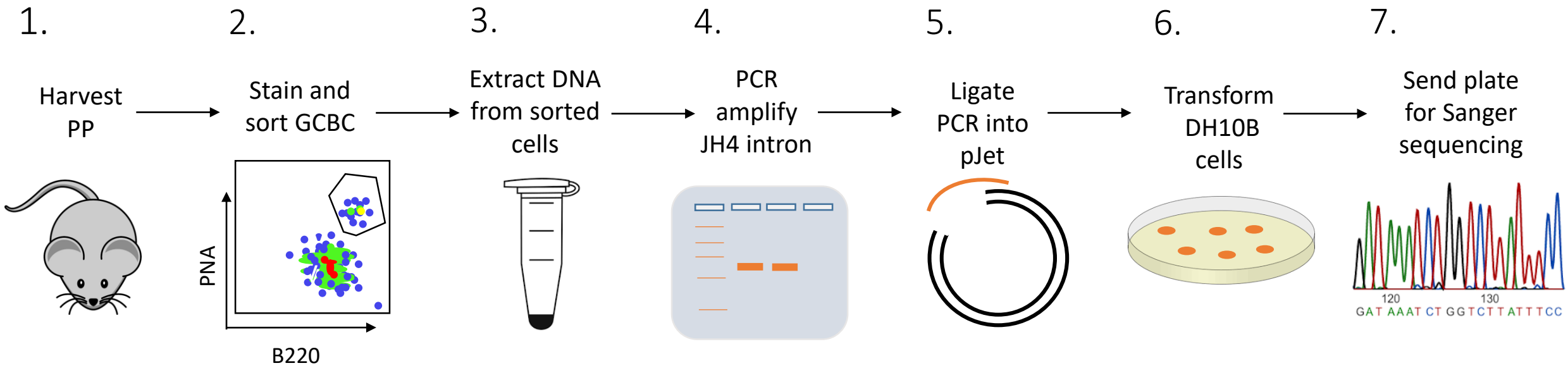
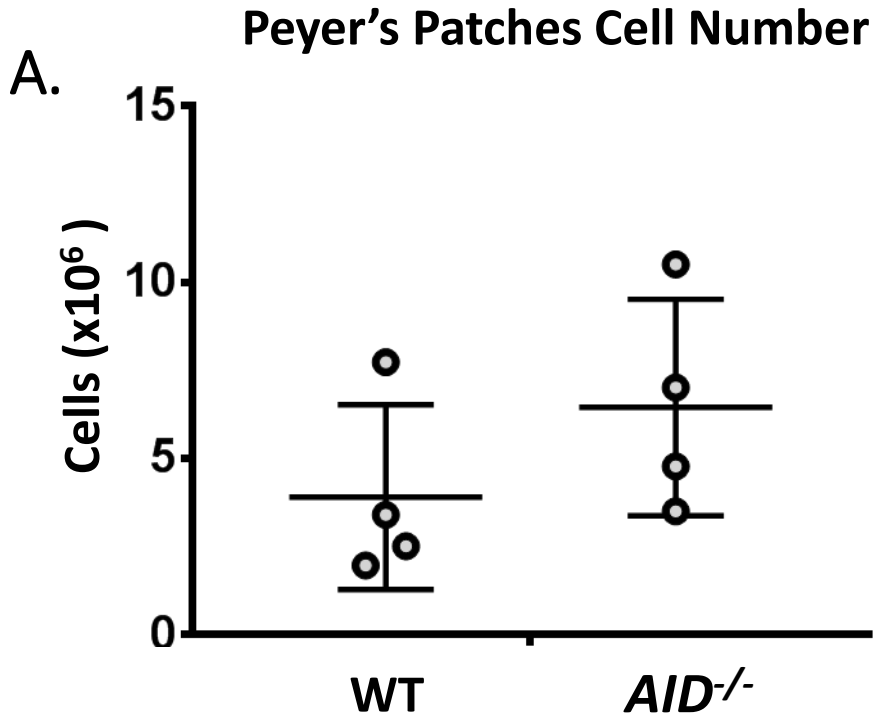
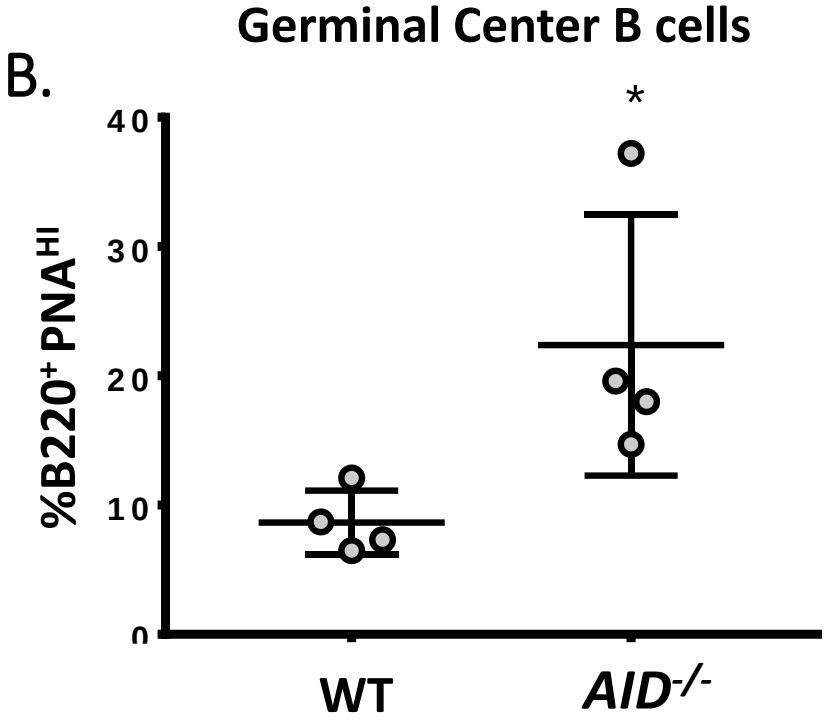


Figure 3





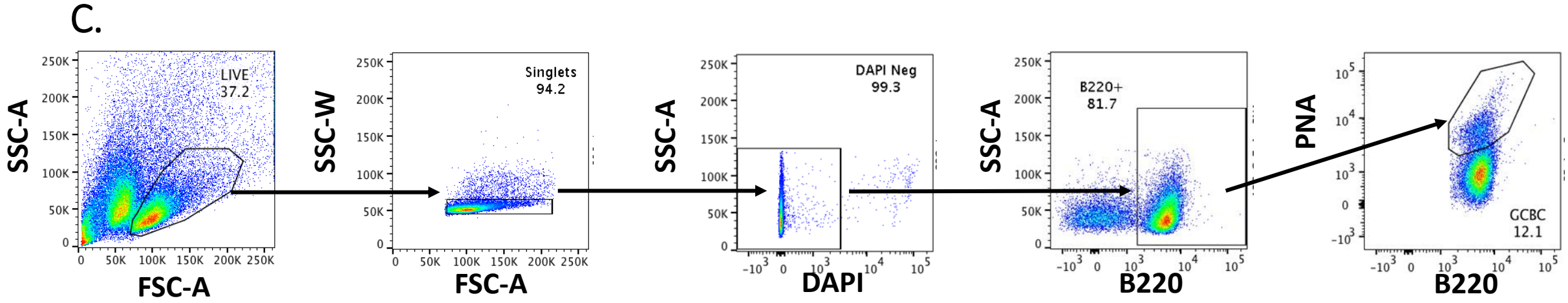
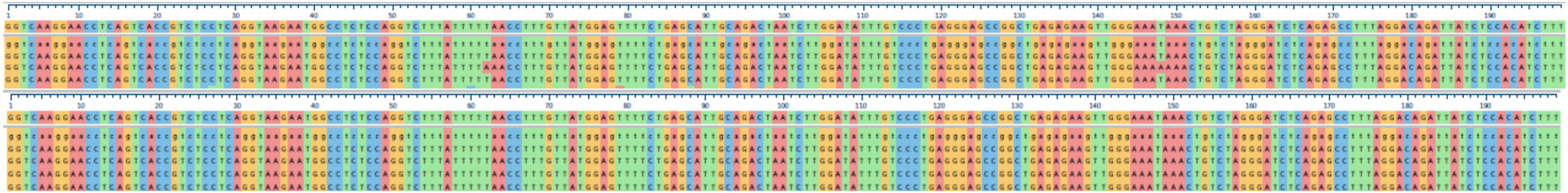


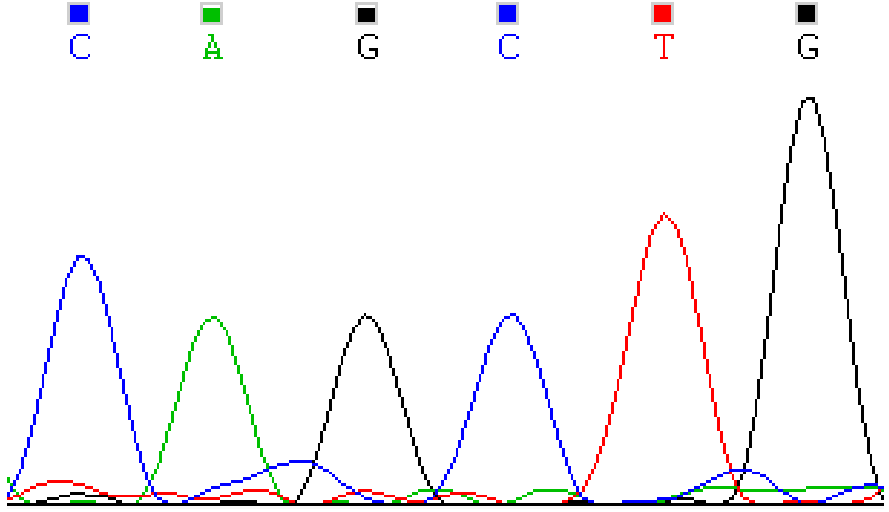
Figure 4

A.



B.

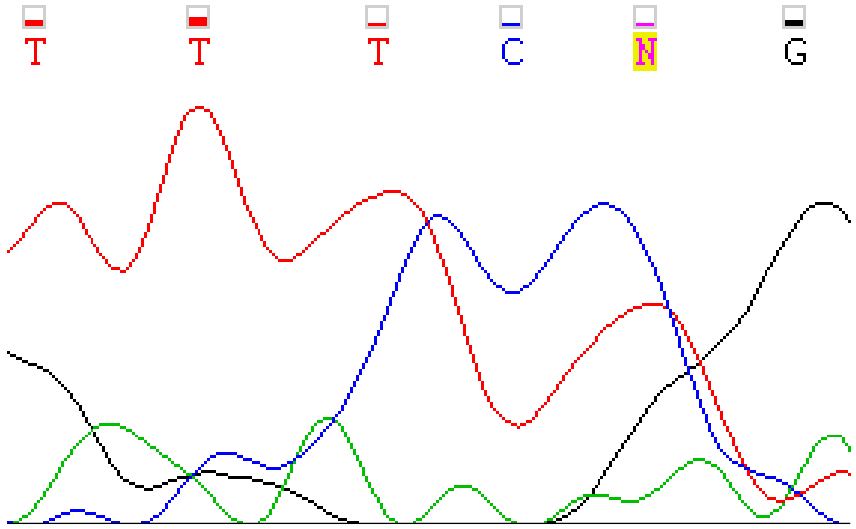
High Quality Sequence



Sequence:
5’- CAGCTG-3’

C.

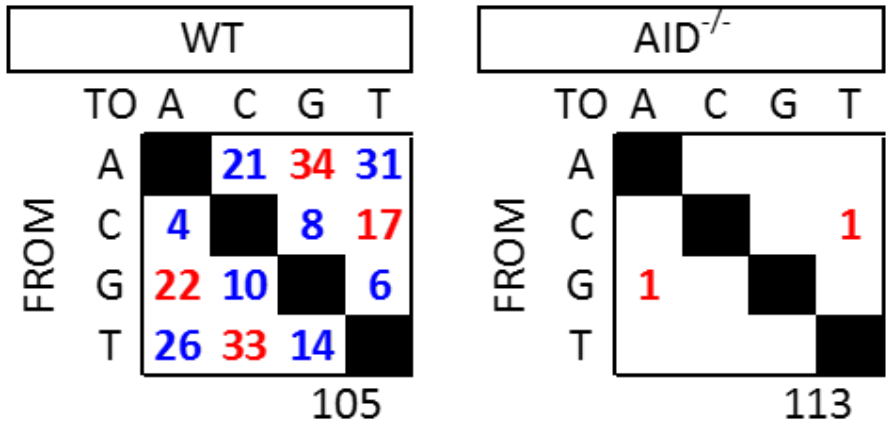
Low Quality Sequence



Sequence:
5’-TTTCCG-3’

Figure 5

A.



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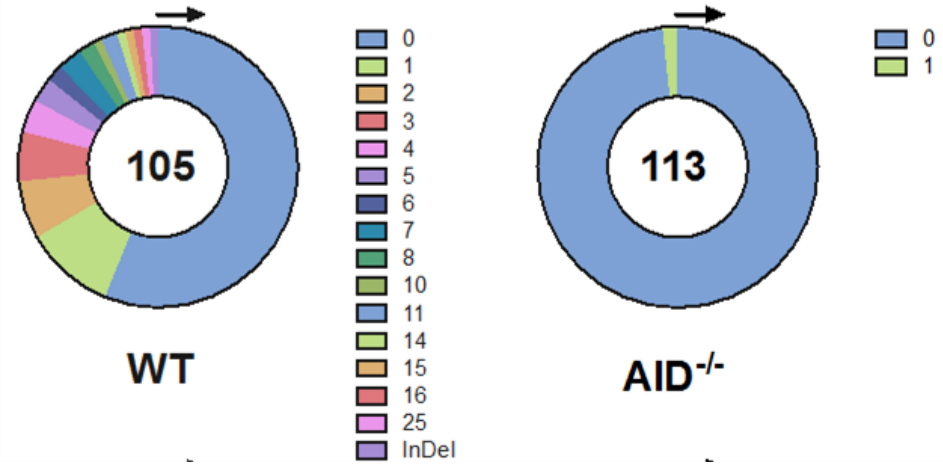


Table 1

Staining Cocktail for GCBCs		Volume: 500 µL	
Antibody or *Dye*	**Fluorophore**	**Dilution**	**µL**
B220	PE	1000	0.5
Streptavidin	APC-eFluor780	500	1
DAPI	*N/A*	500	1

Table 2

Single Stains for Compensation		Volume: 500 µL	
Antibody or *Dye*	**Fluorophore**	**Dilution**	**µL**
B220	PE	1000	0.5
B220	APC-eFluor780	750	0.67
DAPI	*N/A*	500	1

Table 3 Click here to access/download;Table;Table 3.xlsx

PCR #1				
Reagent	**Volume**	**Thermocycler Conditions**		
5x Buffer	4 µL	1	95 °C	3 min
10 mM dNTP	2 µL	2	94 °C	30 sec
10 µM J558FR3Fw	1 µL	3	55 °C	30 sec
10 µM VHJ558.2	1 µL	4	72 °C	1:30 min
High Fidelity DNA polymerase	0.25 µL	Cycle 2-4 9x		
DNA	x (standardize to least concentrated sample)			
H_2O	to 20 µL	5	72 °C	5 min
Dilute PCR product 1:5 in H_2O before proceeding to PCR #2				

Table 4

PCR #2				
Reagent	**Volume**	**Thermocycler Conditions #2**		
5x Buffer	4 µL	1	94 °C	3 min
10 mM dNTP	2 µL	2	94 °C	30 sec
10 µM VHJ558.3	1 µL	3	55 °C	30 sec
10 µM VHJ558.4	1 µL	4	72 °C	30 sec
h Fidelity DNA polymer	0.25 µL	Cycle 2-4 21x		
Diluted PCR#1	1 µL			
H_2O	to 20 µL	5	72 °C	5 min

Table 5 Click here to access/download;Table;Table 5.xlsx

Reagent	Volume
2x Buffer	10 µL
Purified PCR	x (standardize to least concentrated sample)
Plasmid with blunt ends	1 µL
T4 DNA Ligase	1 µL
H_2O	to 20 µL

Incubate at room temp for 5 min or overnight at 16 ºC

Buffers Preparation
FACS Buffer
Heat inactivate FBS at 56 ˚C for one hour prior to use. Supplement PBS, pH 7.4 (Gibco, #10010049) with 2.5% (v/v) of heat-inactivated FBS. Store at 4˚C.
DNA Extraction Buffer (100 mM Tris pH 8.0, 0.1 M EDTA, 0.5% (w/v) SDS)
Add 50 mL of 1 M Tris pH 8.0, 100mL of 0.5 M EDTA, and 12.5 mL of 20% SDS. Add distilled water to 500 mL. Store at room temperature.
TE Buffer (10 mM Tris pH 8.0, 1 mM EDTA)
Add 2.5 mL of 1 M Tris pH 8.0, and 500 mL of 0.5 M EDTA. Add distilled water to 250 mL. Store at room temperature.

Oligonucleotides List

J558FR3Fw	5’-GCCTGACATCTGAGGACTCTGC-3’
VHJ558.2	5’-CTGGACTTTCGGTTTGGTG-3’
VHJ558.3	5’-GGTCAAGGAACCTCAGTCA-3’
VHJ558.4	5’-TCTCTAGACAGCAACTAC-3’

Table of Materials

Click here to access/download;Table of Materials;Materials List.xlsx

Name of Material/ Equipment
0.2 ml PCR 8-tube FLEX-FREE strip, attached clear flat caps, mixed
Ampicillin sodium salt
APC-eFluor780 anti-CD45R/B220

BD FACSAria II

BD slip tip 1mL syringe
Biotinylated peanut agglutinin (PNA)
C57BL/6J mice
Corning Falcon test tube with cell strainer snap cap
DAPI (4',6-Diamidino-2-Phenylindole, dihydrochloride)
dNTP
ElectroMAX DH10B competent cells
Falcon cell strainer 40mm
Falcon round-bottom polystyrene tubes (FACS tubes)
Falcon round-bottom polystyrene tubes (capped)
Fetal bovine serum
Gibco phosphate buffered saline PBS pH 7.4
Glycogen
Lasergene Molecular Biology (MegAlign Pro)
PE anti-CD45R/B220
Proteinase K
Q5 High-Fidelity DNA Polymerase
QIAquick Gel Extraction Kit
Seal-Rite 1.5mL microcentrifuge tubes
Streptavidin APC-eFluor 780 Conjugate
T4 DNA ligase
Thermo Scientific CloneJET PCR Cloning Kit
Tissue culture plate 6 well
Unlabeled anti-mouse CD16/CD32 (Fc block), BD

Company	Catalog Number
USA Scientific	1402-4708
Fisher	BP1760-5
eBioscience	47-0452-80
BD	643186
Fisher	14-823-434
Vector Labs	B-1075-5
Jackson Laboratories	664
Fisher	08-771-23
Fisher	D1306
NEB	N0447L
Fisher	18-290-015
Fisher	08-771-1
Fisher	14-959-5
Fisher	149591A
R&D Systems (Atlanta Biologicals)	S11150
Fisher	10-010-049
Sigma	10901393001
DNA Star	
BD	553090
Fisher	BP1700-100
NEB	M0491L
Qiagen	28706
USA Scientific	1615-5500
eBioscience	47-4317-82
NEB	M020L
ThermoFisher	FERK1231
Fisher	08-772-1B
Fisher	BDB553142

Comments/Description
clone RA3-6B2
four lasers (405nm, 488nm, 561nm, 633nm) and 12 filters (PacBlue (450/50), AmCyan (502LP; 530/30), SSC (488/10), FITC (502LP; 530/30), PerCP-Cy5.5 (655LP; 695/40), PE (585/15), PE-Texas Red (600LP; 610/20), PE-Cy5 (630LP; 670/14), PE-Cy7 (735LP; 780/60), APC (660/20), Alexa700 (710LP; 730/45), APC-Cy7 (755LP; 780/60))
sterile
0.5 mg/ml
10 mM
version 15
clone RA3-6B2
sterile
Clone 2.4G2

The City College of New York

Department of Biology

May 4, 2020

Dear editor,

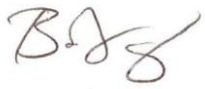
Please find enclosed our revised manuscript entitled “Analysis of Somatic Hypermutation in the JH4 Intron of Germinal Center B cells from Mouse Peyer’s Patches” for your consideration to publish in *The Journal of Visual Experimentation*. In the revised manuscript, we have edited and/or corrected content that was noted by the reviewers. We have appended a point-by-point rebuttal of the reviewers’ comments to this letter.

Please note that we did not track the changes to the revisions because of the significant formatting changes that were requested in the editorial review. We made minor edits to the text, some of which were requested by the reviewers. In addition, we uploaded the figures individually as .pdf files, which was specified in the editorial reviewer’s comments.

We think your publication of this visual protocol would be of interest to scientists who study the molecular pathways regulating immunoglobulin gene diversification or DNA repair and recombination. Additionally, those who seek to understand how the immune system produces antibodies to pathogens or commensal microbes may also find this protocol useful for their research.

We thank you and the external reviewers for taking the time to consider our manuscript.

Sincerely,



Bao Q. Vuong, PhD
Assistant Professor

Marshak Science Building, Room 526 160 Convent Avenue New York NY 10031 Office 212-650-6800 Fax 212-650-8585

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Department of Biology

Sible et al JoVE – Response to reviewers' comments

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We confirm that we have proofread the manuscript for spelling and grammatical errors.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

We made the suggested edits and reformatted the text accordingly.

3. Please provide at least 6 keywords or phrases.

We included 6 keywords or phrases.

4. Please ensure all abbreviations are defined during the first time use.

We defined all abbreviations in the first use.

5. Please ensure that the long Abstract is within 150-300 word limit and clearly states the goal of the protocol.

The abstract is 175 words and states the goal of the protocol, which is to analyze somatic hypermutation and immunoglobulin diversification.

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Eppendorf, BD FACSAria II, ElectroMAX, etc.

We removed commercial language from the manuscript.

7. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

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CUNY

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Department of Biology

We added an ethics statement before the numbered protocol.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

We revised the protocol to the imperative tense.

9. The Protocol should contain only action items that direct the reader to do something.

The protocol only contains steps that direct the reader to do something. Notes are included in parentheses.

10. Please do not include all the buffer preparations and oligos as a part of the protocol. These can be uploaded as a table separately to your editorial manager account as .xlsx file.

We removed the buffer preparations and oligos from the manuscript and created separate Excel files for both.

11. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

We verified that each step of the protocol has at most 3 sentences.

12. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

We added details to our protocol steps to ensure that the reader is able to perform the necessary steps.

13. 1: How do you perform euthanasia in your experiment? Age, sex, strain of the mouse used?

We added details regarding the euthanasia of the mouse in step 1.1. The age, sex, and strain of the mice are specified immediately prior to protocol.

14. 1.8: How do you examine for PP presence? Any visual cues to look for?

The visual description of the PP is detailed in step 1.8 in the revised manuscript.

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15. 2: Do you perform any washing step before this?

No, there is no washing step prior to beginning Part 2 of the protocol. The Peyer's patches are stored in 1 mL of FACS buffer, as specified in step 1.10. The washing steps for the cells are outlined in Part 2 of the protocol.

16. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The protocol is 5 pages. We highlighted approximately 2.5 pages of the protocol that will be included in the video.

17. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We obtained permission to reproduce Figure 5 from the American Association of Immunologists, Inc. (AAI). The email from the Director of Publications, Catherine Wattenberg, has been uploaded as a .docx file. The legend for Figure 5 has been edited according to the requests by JoVE and AAI.

18. Please discuss all figures in the Representative Results in order (Figure 4 should be discussed before figure 5). However, for figures showing the experimental set-up, please reference them in the Protocol.

We revised the discussion in the Representative Results to follow the order of the Figures.

19. Please ensure that the result are described with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.

We described the results with respect to the protocol, mouse genotypes, and title of the manuscript.

20. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

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a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We edited the Discussion to cover the points outlined above in 4 paragraphs.

21. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

We moved the Figure Legends to follow the Representative Results.

22. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text

We removed the Tables from the Figures .pptx file and uploaded each table as a separate .xlsx file. Each Table has a title and description that follows the Representative Results.

23. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

We included a Disclosures section.

24. Please do not abbreviate the journal titles in the reference section.

We expanded the journal titles in the References section.

25. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file. Please combine all panels of one figure into a single image file.
26. Please upload each table separately as .xlsx file. Please do not make panels in the table. For table of materials please sort the table in alphabetical order.

We uploaded each Figure as a .pdf or .tiff file.

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Reviewers' comments:
Reviewer #1:
This manuscript provides a detailed protocol for analyzing mutation frequency in the JH4 intron of germinal center B cells from mouse Peyer's patches. It is clearly written and is a frequently used and useful method to study SHM.
Some suggestions are listed as following:
1. Line 97, Could the authors specify the ages of the mice used?

We thank the reviewer for noting our omission. We specified the ages of the mice in line 97 of the revised manuscript.

2. Line 137, Were the cells kept at 4 degree? Did the authors use cold FACS buffer?

We thank the reviewer for the comment. The cells were kept at 4˚C. We have revised the manuscript to specify the use of cold (4˚C) FACS buffer.

3. Line 146, Is the cocktail specified in Table 1A? To my understanding, the cocktail should contain only PE-B220 antibody, DAPI and APC-dFluor780-Steptavidin which recognizes biotinylated PNA. Since cells are treated with biotinylated PNA in step 3.5 before adding the cocktail, why do you need to add biotinylated PNA in the cocktail?

We thank the reviewer for noting our error. We have revised Table 1A to include only PE-B220 antibody, APC-eFluor780-Streptavidin, and DAPI.

4. Line 149, Why do the authors use 500ul FACS buffer for staining controls but 200ul FACS buffer for experimental samples? Besides, "B220" in Table 1B is duplicated. I guess the second one should be Streptavidin.

We thank the reviewer for the comment. We revised the protocol to stain the controls and experimental samples in 500ul of FACS buffer to simplify the protocol. For Table 1B, we use B220 directly conjugated to APC-eFluor780 as a staining control to reduce the number of steps that are required to prepare the staining control. With this staining control, we are better able to compensate for spectral overlap because the B220 antigen is more highly expressed on germinal center cells than the T-antigen, which is recognized by PNA. We have included a portion of this justification in the Discussion (lines 425-427 of the revised manuscript).

5. Line 151, Does "steps 29 and 30" mean steps 3.7 and 3.8?

We thank the reviewer for noting our error. “Steps 29 and 30” refer to steps 3.7 and 3.8. We have revised the manuscript accordingly.

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6. Line 185, I wonder if the author could provide a representative picture of a DNA gel to show if the PCR product is specific?

We thank the reviewer for the suggestion. We have included an image of the agarose gel as Supplementary Figure 1 to show the expected size of the PCR product.

7. Line 199 and 200, How many clones were picked for sequencing?

We thank the reviewer for noting our omission. We picked 30 clones for sequencing. We have added this to line 277 in the revised manuscript.

8. In the Representative Results section, it seems odd that the authors describe the results of flow cytometry in the order of Figure 3C, Figure 3B and then Figure 3A.

We thank the reviewer for the comment. We have revised the manuscript to reference the figures chronologically.

Reviewer #2:
Manuscript Summary:
The mutation frequency at JH4 intron is a widely used assay in the field to assess the somatic hypermutation in germinal center B cells. In this manuscript, Vuong and colleagues offer a detailed protocol including using fluorescence activated cell sorting (FACS) to separate GC B cells from Peyer's patches, and amplifying V(D)J regions, selecting the V(D)JH4 regions, and Sanger-sequencing to determine the mutation frequency. The protocol should be a great resource to the field.

Major Concerns:
NO major concerns. This reviewer only have some minor comments.

Minor Concerns:
Specific minor comments:
1. In line 126, the word "buffer" is missed for "FACS buffer".

We thank the reviewer for noting our error. We have revised the manuscript accordingly.

2. In line 151, "…all the tubes in steps 29 and 30", there are no steps 29 and 30.

We thank the reviewer for noting our error. “Steps 29 and 30” refer to steps 3.7 and 3.8. We have revised the manuscript accordingly.

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3. In addition to AID-/- B cells, B220+PNAlow cells of the GC B cells should be considered as a useful negative control for people who do not have access to the AID KO mice.

We thank the reviewer for the suggestion. We have included B220+PNAlow cells as a negative control in lines 441-442 of the revised manuscript.

4. A better discussion or description of the primers and the amplified V(D)J exons should be helpful.

We thank the reviewer for the suggestion. We have included a brief description of the primers in lines 319-320 of the revised manuscript.

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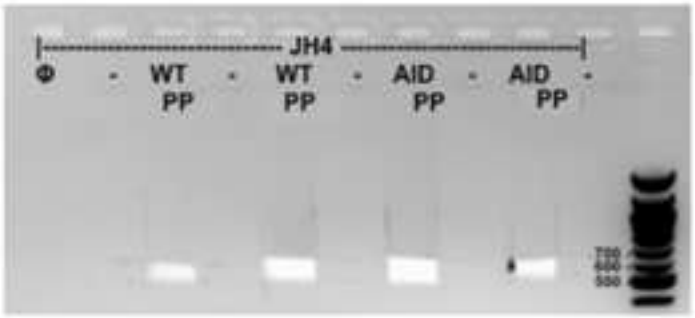
Supplementary Figure 1



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Sent: Monday, April 13, 2020 12:17 PM
To: Bao Vuong
Subject: [EXTERNAL] Vuong Permission Request

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* Provide a brief explanation of how the material will be used	We would like to use these figures to write a methods paper that details how to analyze somatic hypermutation of the JH4 intron.