Journal of Visualized Experiments

Genome Engineering of Primary Human B Cells Using CRISPR/Cas9

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61855R1
Full Title:	Genome Engineering of Primary Human B Cells Using CRISPR/Cas9
Corresponding Author:	Branden Moriarity, Ph.D. University of Minnesota Minneapolis, Minnesota UNITED STATES
Corresponding Author's Institution:	University of Minnesota
Corresponding Author E-Mail:	mori0164@umn.edu
Order of Authors:	Branden Moriarity, Ph.D.
	Kanut Laoharawee
	Matthew Johnson
	Walker Lahr
	Joseph Peterson
	Beau Webber
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Minneapolis, MN, USA
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please specify the section of the submitted manuscript.	Bioengineering
Please provide any comments to the journal here.	

TITLE:

Genome Engineering of Primary Human B Cells Using CRISPR/Cas9

Kanut Loaharawee[1,2,3], Matthew J. Johnson[1,2,3], Walker S. Lahr[1,2,3], Joseph S. Peterson[1,2,3], Beau R. Webber[1,2,3], Branden S. Moriarity[1,2,3]

[1]Department of Pediatrics, University of Minnesota, Minneapolis MN, USA
[2]Center for Genomic Engineering, University of Minnesota, Minneapolis MN, USA
[3]Masonic Cancer Center, University of Minnesota, Minneapolis MN, USA

Corresponding Author:
Branden S. Moriarity (mori0164@umn.edu)

Co-Authors:
Kanut Laoharawee (laoha002@umn.edu)
Matthew J. Johnson (joh02946@umn.edu)
Walker S. Lahr (wslahr@umn.edu)
Joseph J. Peterson (pet01059@umn.edu)
Beau R. Webber (webb0178@umn.edu)

KEYWORDS:

CRISPR/Cas9, genome engineering, recombinant AAV, gene editing, primary human B cells

SUMMARY:

Here we provide a detailed, step-by-step protocol for CRISPR/Cas9-based genome engineering of primary human B cells for gene knockout (KO) and knock-in (KI) to study biological functions of genes in B cells and the development of B-cell therapeutics.

ABSTRACT:

B cells are lymphocytes derived from hematopoietic stem cells and are a key component of the humoral arm of the adaptive immune system. They make attractive candidates for cell-based therapies because of their ease of isolation from peripheral blood, their ability to expand in vitro, and their longevity in vivo. Additionally, their normal biological function—to produce large amounts of antibodies—can be utilized to express very large amounts of a therapeutic protein, such as a recombinant antibody to fight infection, or an enzyme for the treatment of enzymopathies. Here, we provide detailed methods for isolating primary human B cells from peripheral blood mononuclear cells (PBMCs) and activating/expanding isolated B cells in vitro. We then demonstrate the steps involved in using the CRISPR/Cas9 system for site-specific KO of endogenous genes in B cells. This method allows for efficient KO of various genes, which can be used to study the biological functions of genes of interest. We then demonstrate the steps for using the CRISPR/Cas9 system together with a recombinant, adeno-associated, viral (rAAV)

vector for efficient site-specific integration of a transgene expression cassette in B cells. Together, this protocol provides a step-by-step engineering platform that can be used in primary human B cells to study biological functions of genes as well as for the development of B-cell therapeutics.

INTRODUCTION:

B cells are a subgroup of the lymphocyte lineage derived from hematopoietic stem cells. They perform a critical role in the adaptive humoral immune system by producing large amounts of antibodies in response to immune challenges[1]. B cells are also precursors of memory B cells and the terminally differentiated, long-lived plasma cells, thereby providing lasting humoral immunity[2]. Plasma cells, in particular, are unique among immune cells in their ability to produce large amounts of a specific antibody while surviving for years or decades[3]. Additionally, the ease of isolation from peripheral blood makes the B-cell lineage an excellent candidate for novel cell-based therapies[4].

Previously, random integration methods, such as those using lentiviral vectors or a *Sleeping Beauty* transposon, have been used to engineer B cells for transgene delivery and expression[5–8]. However, the non-specific nature of these approaches makes it difficult to study the biological functions of a specific gene in the B cells and carries an inherent risk of insertional mutagenesis and variable transgene expression and/or silencing in the therapeutic setting.

The CRISPR/Cas9 system is a powerful genome engineering tool that allows researchers to precisely edit the genome of various cells in numerous species. Recently, two groups, including our own, have successfully developed methods for ex vivo expansion and targeted genome engineering of primary human B cells[9,10]. We will describe the process of purifying primary human B cells from a leukaphoresis sample. After that, we will describe our updated protocol for B-cell expansion and activation of isolated B cells. We will then describe a process for knocking out cluster of differentiation 19 (CD19), a specific B-cell receptor and a hallmark of B cells, by electroporation to introduce CRISPR/Cas9 mRNA together with CD19 sgRNA into activated B cells.

Cas9 mRNA gets translated and binds to the CD19 sgRNA to form a CRISPR/Cas9-sgRNA ribonucleoprotein complex (RNP). Subsequently, sgRNA in the complex leads Cas9 to create double-strand break (DSB) at the target sequence on exon 2 of the gene. The cells will repair the DSB by "non-homologous end joining" by introducing or deleting nucleotides, leading to frameshift mutation and causing the gene to be knocked out. We will then measure the loss of CD19 by flow cytometry and analyze indel formation by tracking of indels by decomposition (TIDE) analysis.

We will then describe the process of using CRISPR/Cas9 together with a recombinant AAV6 vector (rAAV6, a donor template for homology-directed repair (HDR)) to mediate site-specific insertion of enhanced green fluorescent protein (*EGFP)* at the adeno-associated virus integration site 1 (AAVS1) gene. The AAVS1 gene is an active locus without known biological functions and an AAV viral integration site on the human genome; therefore, it is considered a "safe harbor" for genome engineering. Here, we report that expansion and activation of B cells allowed up to 44-

fold expansion in 7 days of culture (**Figure 1**). Electroporation of B cells showed a slight reduction of overall cell health (**Figure 2A**) at 24 h post-transfection. Scatter plot analysis of the CD19 marker (**Figure 2B**) showed up to 83% reduction in the edited cells (**Figure 2C**).

TIDE analysis of the chromatographs (**Figure 3A**) revealed that the % indels was similar to the % protein loss by flow cytometry (**Figure 3B**). Flow cytometry analysis of the KI experiment showed that the cells that received AAV vector (**Figure 4**), together with RNP, expressed up to 64% EGFP-positive cells (**Figure 5A**) and later displayed successful integration by junction polymerase chain reaction (PCR) (**Figure 5B**). Cell counts showed that all samples quickly recovered within 3 days post-engineering (**Figure 5C**).

PROTOCOL:

Leukapheresis samples from healthy donors were obtained from a local blood bank. All experiments described here were determined to be exempt for research by the Institutional Review Board (IRB) and were approved by the Institutional Biosafety Committee (IBC) at the University of Minnesota.

NOTE: All experiments were performed in compliance with the universal precaution for bloodborne pathogens, with sterile/aseptic techniques and proper biosafety level-2 equipment.

1. Prepare supplements for B-cell expansion medium

1.1. Reconstitute CpG oligonucleotide to a concentration of 1 mg/mL.

1.2. Reconstitute CD40 ligand (CD40L) to a concentration of 100 μg/mL.

1.3. Reconstitute recombinant human IL-10 (rhIL-10) to a concentration of 50 μg/mL.

1.4. Reconstitute recombinant human IL-15 (rhIL-15) to a concentration of 10 μg/mL.

NOTE: Keep each supplement in small aliquots at -20 °C to -80 °C for up to 6 months.

2. Prepare basal medium

2.1. Combine B-cell basal medium with 5% (v/v) media supplement for in vitro immune cell expansion (e.g., CTS Immune Cell SR) and 1% (v/v) penicillin and streptomycin.

2.2. Filter-sterilize the basal medium using a 0.22 μm filter adaptor into a sterilized bottle.

2.3. Keep the basal medium at 4 °C for up to 1 month.

3. Prepare B-cell expansion medium

3.1. Transfer the required amount of the basal medium into a sterile container to culture the B cells at 5×10^5 cells/mL.

3.2. Supplement the basal medium with 1 μg/mL CpG, 100 ng/mL CD40L, 50 ng/mL rhIL-10, and 10 ng/mL rhIL-15.

3.3. Filter the B-cell expansion medium using a 0.22 μm filter.

3.4. Equilibrate the B-cell expansion medium in the tissue culture incubator at 37 °C, 5% CO_2 with humidity for at least 30 min before use.

NOTE: Prepare fresh B-cell expansion medium to use for one day. **Do not** prepare the B-cell expansion medium to use for multiple days. This media recipe encourages proliferation of naïve B cells.

4. Human B-cell purification and expansion

NOTE: Add 99–100% isopropyl alcohol to a temperature-controlled freezing container, following the manufacturer's instruction, and chill the freezing container at 4 °C before starting step 4.1.

4.1. Isolate PBMCs from a leukaphoresis sample

4.1.1. Transfer a leukaphoresis sample (approximate 8–10 mL) to a sterile 50 mL conical tube.

4.1.2. Bring up the volume to 35 mL with sterile 1x phosphate-buffered saline (PBS).

4.1.3. Carefully layer a 35 mL leukaphoresis sample on 15 mL of density-gradient medium.

4.1.4. Centrifuge at 500 × *g* for 25 min without brake, remove the plasma layer without disturbing the buffy coat (PBMC layer), collect the PBMCs from the interface, and transfer to a new sterile 50 mL conical tube.

4.1.5. Bring up the PBMCs to 50 mL with 1x PBS.

4.1.6. Centrifuge at 500 × *g* for 5 min without brake. Remove the supernatant without disturbing the PBMC pellet, which may appear red.

4.1.7. Add 7 mL of ammonium-chloride-potassium lysis buffer, pipette 3 times to mix well, and incubate at room temperature (RT) for 3 min.

4.1.8. Bring up the volume to 50 mL with 1x PBS.

4.1.9. Centrifuge at 400 × *g* for 5 min with low-resistance brake. Remove the supernatant without disturbing the pellet. The pellet should look pinkish or white.

NOTE: To continue to culture the freshly isolated B cells, prepare the B-cell expansion medium before starting B-cell isolation (step 4.2).

4.2. B-cell isolation from PBMCs using human primary B-cell negative isolation kit

4.2.1. Resuspend PBMCs in the isolation buffer to a concentration of 5×10^7 cells/mL.

NOTE: If the total number of PBMCs is less than 5×10^7 cells, scale down the volume of isolation buffer to maintain 5×10^7 cells/mL. Minimum and maximum volumes of cell suspension are 0.25 mL and 8 mL, respectively.

4.2.2. Transfer up to 8 mL (5×10^7 cells/mL) to a sterile, polypropylene, round-bottom tube with cap.

4.2.3. Add 50 μL/mL of Cocktail Enhancer to the PBMCs.

4.2.4. Add 50 μL/mL of Isolation Cocktail to the PBMCs, cap the tube, and invert 2–3 times to mix.

4.2.5. Incubate at RT for 5 min; at the 4^{th} minute of incubation, vortex magnetic microbeads for at least 30 s.

4.2.6. Transfer 50 μL of magnetic microbeads per 1 mL of PBMCs, cap the tube, and invert 2–3 times to mix.

4.2.7. Top up to 10 mL with isolation buffer and gently pipette up and down 2–3 times.

4.2.8. Place the tube in a magnetic station and incubate at RT for 3 min.

4.2.9. Hold the magnet and tube together, and in one motion, invert the magnet and tube together to pour the cell suspension into the new tube. Discard the old tube.

4.2.10. Repeat step 4.2.8 (reduce the incubation time to 2 min) and pour the B-cell suspension into a clean conical tube.

4.2.11. The enriched B cells are ready to use. If cells will be used immediately, continue to section 4.3 (Human B-cell expansion). Check the purity of the isolated B cells by flow cytometry (optional). If cells are to be frozen before use, continue to step 4.2.12.

4.2.12. To freeze the B cells, centrifuge at $400 \times g$ for 5 min, and discard the supernatant without disturbing the pellet.

4.2.13. Resuspend the cells in freezing medium at 10^7 cells/mL, and aliquot 1 mL/cryovial.

4.2.14. Place the cryovial in the chilled freezing container, and store at -80 °C overnight; then, transfer the frozen cryovial to a liquid nitrogen tank; keep frozen cells up to 1 year.

NOTE: Expected yield of isolated B cells is 2%–8% of the total PBMCs, with 95%–99% viability.

4.3. Human B-cell expansion

NOTE: If using freshly isolated B cells, skip steps 4.3.1–4.3.5. Count the cells and transfer the required number of cells into a sterile conical tube and continue with step 4.3.6.

4.3.1. Pre-warm fetal bovine serum (FBS) in a water bath prior to thawing the B cells. Prepare 20 mL of B-cell expansion medium, transfer to a T25 flask, and pre-equilibrate the medium in a tissue-culture incubator (at 37 °C, 5% CO_2, with humidity) at least 15 min before use.

4.3.2. Thaw B cells in a 37 °C water bath. While waiting, transfer 2 mL of pre-warmed FBS into a sterile 15 mL conical tube.

4.3.3. After the B cells are completely thawed, immediately add 1 mL of pre-warmed FBS, drop-wise, into the sample. Incubate at RT for 1 min.

4.3.4. Gently pipette to resuspend the sample and transfer the whole volume, dropwise, into a conical tube containing 2 mL of pre-warmed FBS.

4.3.5. Bring up the volume to 15 mL with sterile 1x PBS, cap, and invert the tube gently 2–3 times.

4.3.6. Centrifuge at 400 × *g* for 5 min.

4.3.7. Discard the supernatant without disturbing the cell pellet, resuspend the cell pellet with 1 mL of pre-equilibrated B-cell expansion medium, and count the cells. The total cell number should be approximately 10^7 cells.

4.3.8. Transfer the cells into a flask containing 20 mL of the pre-equilibrated B-cell expansion medium. The final concentration of the cells should be approximately 5 x 10^5 cells/mL.

4.3.9. Incubate the flask vertically in a tissue-culture incubator.

4.3.10. Refresh the expansion medium completely every 2 days by transferring the whole volume of B cells in to a sterile conical tube and repeat steps 4.3.6–4.3.9.

NOTE: T25 flask can hold 10–20 mL of medium; T75 flask can hold up to 20–60 mL of medium.

5. Primary human B-cell engineering

5.1. Engineer B cells at 48 ± 2 h after expansion/activation for optimal results. Prepare the B-cell

expansion medium, aliquot 1 mL of the medium into a 48-well tissue-culture plate, and pre-equilibrate in a tissue-culture incubator at least 15 min prior to use.

NOTE: When designing a CRISPR/Cas9 sgRNA for a gene of interest, follow the steps outlined below.

- Design sgRNAs using an online tool[11].
- Design sgRNAs on an exon that is common to all isoforms of the protein.
- Order chemically modified sgRNA from reputable companies.
- sgRNA usually comes in lyophilized form; reconstitute sgRNA in sterile DNase/RNase-free Tris-EDTA (TE) buffer to a concentration of 1 μg/μL .

5.2. Prepare CRISPR/Cas9 transfecting substrate by mixing 1 μL (1 μg/μL) of chemically modified sgRNA with 1.5 μL (1 μg/μL) of chemically modified *Streptococcus pyogenes* Cas 9 (S.p. Cas9) nuclease per transfection reaction. For control, use 1 μL of TE buffer instead of sgRNA.

NOTE: When CD19 sgRNA is used for a gene KO experiment, see **Figure 2** for results. When AAVS1 sgRNA is used for a gene KI experiment, see **Figure 5** for results.

5.2.1. Keep all the components on ice.

5.3. Mix gently and transfer 2.5 μL of CRISPR/Cas9 transfecting substrate per reaction into a tube of a 0.2 mL 8-tube strip; set aside at RT.

5.4. Turn on a nucleofector (electroporator), and prepare transfection reagents as shown in **Table 1**.

NOTE: This is a good step for a pause, if necessary, by putting all reagents on ice. Remove all reagents from ice when ready to resume the experiment. When using S.p. Cas9 protein, the investigator **MUST** pre-complex CRISPR/Cas9-sgRNA ribonucleoprotein by mixing 1 μg sgRNA with 5 μg of S.p. Cas9 protein, avoiding any bubbles, and incubating the mixture at RT for at least 20 min before use for optimal results.

5.5. Count and transfer 10^6 B cells per transfection reaction into a sterile conical tube.

5.6. Bring up the volume to 15 mL with sterile 1x PBS, and centrifuge at 400 × *g* for 5 min. While waiting, prepare the primary cell transfection reagent (**Table 2**) and set aside at RT.

5.7. Discard the supernatant without disturbing the cell pellet.

5.8. Resuspend the cell pellet with 10 mL of sterile 1x PBS and centrifuge at 400 × *g* for 5 min.

5.9. Discard the supernatant completely without disturbing the cell pellet.

5.10. Transfer 0.5 μg of chemically modified GFP mRNA (as a transfection reporter) per 10^6 B cells to the cell pellet (optional).

5.11. Resuspend the cell pellet with 20 μL primary cell transfection reagent per 10^6 B cells; mix gently by pipetting 5–6 times. Transfer 20.5 μL per transfection reaction into the 0.2 mL tube of the 8-tube strip containing 2.5 μL of the CRISPR/Cas9 transfection substrate.

5.12. Pipette up and down **once** to mix and transfer the entire volume (23 μL) into a transfection cuvette. Cap and tap the cuvette on the bench gently to ensure that the liquid covers the bottom of the cuvette.

5.13. Use human primary B-cell protocol on the nucleofector for transfection.

NOTE: The nucleofector (electroporator) can be placed and be used outside the tissue-culture hood. Cap the cuvette to ensure sterility.

5.14. Rest the electroporated cells in the cuvette at RT for 15 min.

5.15. Transfer 80 μL of the pre-equilibrated B-cell expansion medium from the tissue culture plate into the transfection reaction in the cuvette. Place the cuvette in the tissue culture incubator for 30 min.

5.16. Gently pipette a couple times to mix and transfer the whole volume of the sample from the cuvette to an appropriate well of a 48-well tissue-culture plate containing 1 mL of the B-cell expansion medium. The final concentration of the cells should be 10^6 cells/mL.

5.17. **If performing a gene KI experiment,** transfer rAAV6 vector at 500,000 multiplicity of infection into the appropriate well containing electroporated cells (approximately 45 min post-electroporation). See example rAAV6 vector construct in **Figure 4A**.

NOTE: For example: A control sample will be electroporated without CRISPR/Cas9 or the rAAV6 vector. A vector-only sample will be electroporated without CRISPR/Cas9 and then be transduced with the rAAV6 vector. A KI sample will be electroporated with CRISPR/Cas9 and then be transduced with the rAAV6 vector. rAAV6 must contain homology arms up- and downstream of the targeted DSB site for HDR.

5.18. Place the plate in a tissue culture incubator at 37 °C and 5% CO_2 with humidity.

5.19. Count the cells and record viability at day 1 post-engineering.

5.20. Refresh the B-cell expansion medium every 2 days by counting the cells and then transferring the whole volume of the cells into a clean 1.5 mL microcentrifuge tube. Centrifuge at 400 × *g* for 5 min, and discard the supernatant without disturbing the pellet. Resuspend the

cells with 100 µL of fresh B-cell expansion medium, and transfer to a well of a 24-well tissue culture plate. Bring up the medium volume to achieve a final cell concentration at 5×10^5 cells/mL.

NOTE: A 48-well plate can hold up to 1 mL medium/well; a 24-well plate can hold up to 2 mL medium/well; a 12-well plate can hold up to 4 mL medium/well, and a 6-well plate can hold up to 8 mL medium/well.

5.21. Allow the engineered cells to expand for at least 5 days before performing downstream analyses such as for flow cytometry analysis, TIDE analysis, and junction PCR.

REPRESENTATIVE RESULTS:

The updated expansion and activation protocol enabled the rapid expansion of B cells up to 44-fold in 7 days (**Figure 1**; n =3 donors). In the KO experiment, the B-cell count using Trypan blue staining showed more than 80% viable cells with a slight reduction in cell recovery in both the control and the CD19 KO samples at 24 h post-electroporation (**Figure 2A**; $p \geq 0.05$, n = 3 donors). This result indicates that electroporation slightly affected overall B-cell health. B cells were collected on day 5 post-transfection for flow cytometry and TIDE analyses. Representative scatter plots of the control and KO sample showed 14% and 95% CD19-negative cells, respectively (**Figure 2B**). Quantitation of the flow plots showed significant reduction in CD19 expression in the KO samples when compared to the control (**Figure 2B**; $p \leq 0.0001$, n = 3 donors). Chromatograms of genomic sequencing (see primer sequences in **Table 3**) showed double peaks in the CD19 KO B cells, indicating insertions/deletions of nucleotides post-CRISPR/Cas9-mediated DSB, whereas single peaks were observed in the control, indicating no DSB occurred in this sample (**Figure 3A**). Indel analysis of the chromatographs (using a free online TIDE analysis tool) of the KO samples showed high % of indel formation (>90%) at the CD19 locus, which is consistent with % CD19 protein loss detected by flow cytometry (**Figure 3C**; $p \geq 0.05$, n = 3 donors). These results indicate that CRISPR/Cas9 efficiently generated a CD19 KO in B cells. B cells from the KI experiment were collected on day 12 post-engineering for flow cytometry and junction PCR analyses (**Table 4**). Scatter plots showed 64% of EGFP-positive cells in the sample that received the rAAV6 vector (**Figure 4**) together with RNP, whereas no EGFP-positive cell was observed in the control; minimal EGFP-positivity was observed in the sample that received AAV vector only (**Figure 5A**). A junction PCR amplification (see primer sequences in **Table 3**) showed 1.5 Kbps amplicons in the KI sample (**Figure 5B**), whereas no PCR product was observed in either the control or vector-only sample. Cell counts showed that the engineering process affects cell recovery in the KI sample more than the control or the vector-only samples (**Figure 5B**). However, all samples quickly rebounded within 3 days after engineering (**Figure 5B**). Together, these results indicate that successful integration of *EGFP* at the AAVS1 locus leads to stable expression of EGFP at least 12 days post-engineering.

FIGURE AND TABLE LEGENDS:

Figure 1. B cell expansion *in vitro*. B cells were seeded at 1×10^6 cells on day 0 (zero) at a density of 5×10^5 per mL and expanded 44-fold in 7 days (n=3 independent donors).

Figure 2. CRISPR/Cas9-mediated CD19 knockout (KO) in B cells. (**A**) Bar graph shows >70% cell recovery (left panel) and >80% viability (right panel) of cells post transfection were observed in both the control and the CD19 KO samples at 24 hours post electroporation. (**B**) Representative flow plots of CD19 gating of live cells shows 84.3% and 3.43% CD19-positive cells in the control sample and the CD19 KO sample, respectively. (**C**) Bar graph shows significant reduction of CD19 in the CRISPR/Cas9-mediated CD19 KO group ($p \leq 0.0001$).

Figure 3. CD19 protein loss vs indel formation. (**A**) Chromatograms depict sequencing peaks of the control and CD19 knockout (KO) sample. The gray box on the control peaks highlights the target sequence of CD19 gRNA with the predicted cut site indicated by an arrow. CD19 KO showed "double peaks" sequencing around the predicted cut site, indicating insertion/deletions of nucleotides after the double-stranded break. (**B**) Bar graph showing consistent results between % CD19 protein loss and % indel formation at the CD19 locus ($p \geq 0.05$).

Figure 4. rAAV6 AAVS1 MND-GFP vector construct. Expression cassette contains a strong synthetic promoter (MND) sequence, immediately followed by an enhanced green fluorescence protein (EGFP) coding sequence and poly adenylation (Poly A) sequence. AAVS1 homology arms flank upstream of the MND promoter and downstream of the poly A sequences. EGFP will be expressed under the regulation of the MND promoter. Sequence lengths are indicated above each component of the construct.

Figure 5. CRISPR/Cas9- and rAAV6-mediated site-specific integration of the EGFP reporter cassette in B cells at day 12 post-engineering. (**A**) Representative flow plot shows no EGFP-positive B cells in either the control or the vector-only sample versus 64.4% of the EGFP-positive B cells from the knockin (KI) sample. (**B**) Junction polymerase chain reaction of KI sample shows the predicted 1.5 Kbps band; no band was found in the control or vector-only sample. Water was used to ensure no contamination during the PCR process. (**C**) Bar graph depicts cell growth of the control, the vector-only, and the EGFP-KI samples over a period of 3 days after engineering. Broken line indicates the 1×10^6 cell input.

Table 1. gRNA sequences

Table 2. Preparation of Nucleofection reagent mix

Table 3. Primers used

Table 4. Flow cytometry antibody and viability dye

DISCUSSION:

Precise genome engineering in primary human B cells has been challenging until recently[9-10]. We had previously published protocols using CRISPR/Cas9 to engineer primary human B cells[9]. Here, we outline improved protocols for B-cell isolation, expansion, and engineering to allow for efficient KO of CD19 or for knocking-in *EGFP*.

Here we demonstrate that our expansion protocol allows the rapid expansion of B cells in culture for up to 44-fold expansion in 7 days (**Figure 1**). This protocol showed a faster expansion rate than those reported by Johnson et al.[9] and Hung et al.[10] The critical steps to ensure healthy and rapid expansion of primary B cells are replenishing the medium with fresh activation factors every two days and ensuring that the number of total B cells in culture does not exceed 2×10^6 cell/mL.

We also found that our improved protocol for transfecting the CRISPR/Cas9 system for CD19 KO resulted in high CD19 KO efficiency (**Figure 2** and **Figure 3**). Similar to a previous study[9], we observed a slight reduction in the cell recovery and viability post-electroporation at 24 h. This indicates that electroporation affects the overall cell health of primary B cells; however, the cells eventually rebound within 48 h (data not shown).

The benefit of using this electroporator protocol is that we can electroporate samples at a much faster rate (16 reactions in less than 1 min), whereas previous protocols[9,10] take approximately 10–12 min for 16 reactions. In addition, this transfection system eliminates the potential electric arc of the electroporator observed in previous studies[9,10]. Furthermore, this engineering method can be scaled up for larger numbers of B cells using larger, commercially available cuvettes (data not shown).

A few critical steps to ensure optimal overall cell health and KO efficiencies: First, ensure that the B-cell expansion medium is prepared and pre-equilibrated for at least 15 min before use. Second, the total volume of transfection substrate should not exceed 20% (v/v) of the nucleofection reagent. Third, the cells must be expanded/activated for 48 ± 2 h for optimal results. Fourth, tap the electroporation cuvette gently before placing in the electroporator to ensure that the transfection reaction solution covers the bottom of the cuvette. Fifth, be sure to rest the electroporated cells in the cuvette for only 15 min at RT; leaving cells in the nucleofection reagent after electroporation for too long can harm the overall cell survival. Sixth, be sure to incubate the cells with media in the cuvette for no longer than 30 min. Seventh, placing 10^6 electroporated cells in 1 mL for the first 48 h tends to help cells recover more quickly than culturing them at lower density (data not shown). Lastly, using either Cas9 mRNA or Cas9 protein will result in similar editing efficiencies (data not shown); however, we used Cas9 mRNA for convenience and cost.

Two major concerns when using CRISPR/Cas9 are off-target effects and chromosomal translocations. Off-target effects caused by mismatched base pairs of sgRNA to the target sequence, lead to numerous possible binding sites on the genome and create multiple, unwanted gene KOs. Therefore, the predicted off-target score should be taken into consideration along with the on-target score to minimize this issue. Chromosomal translocation can occur due to off-target effects or when knocking out multiple genes. This can cause catastrophic events experimentally and clinically. Base editors[12] alter a single nucleotide (two classes: cytosine base editor and adenine base editor) to disrupt the splicing element, to create a premature stop codon, or to create a point mutation, leading to target gene KO without DSB (reviewed extensively

elsewhere[12]). Thus, the base-editing approach can be used for single or multiple-gene KOs to circumvent chromosomal translocations.

We also demonstrate that CRISPR/Cas9, together with rAAV6, can be used to efficiently mediate site-specific KI and expression of *EGFP* at the AAVS1 locus. We observed EGFP expression for at least 12 days post-engineering. We also observed two EGFP-positive populations: a high- and an intermediate-EGFP expression in the KI sample on day 12 (**Figure 5A**), whereas the vector-only sample showed a minimal percentage of cells with intermediate EGFP expression. We speculate that this "high and intermediate populations" phenomenon is due to biallelic integration of the vector. Further investigation using spanning PCR[13] of the intermediate- and high-EGFP cells can be done to confirm this hypothesis. Two caveats on using AAV as a vector: First, AAV vectors have a small cargo capacity, up to 4.7 kilobases, causing issues when knocking in a large gene. Reducing the size of homology arms will allow accommodation of a larger gene, which in turn will reduce KI efficiency (data not shown). Alternatively, simultaneous or sequential integration of multiple gene-loading vectors can be used[14,15]. Studies have reported an immune response and a clearance of AAV-transduced cells in immunocompetent animal models[16,17]. Alternatively, a non-viral-based donor HDR template can be explored to circumvent this issue.

In summary, we have demonstrated comprehensive, step-by-step processes of isolation, expansion, and engineering of B cells that resulted in high gene modification efficiencies. This engineering method can be used for gene KO and to study the functions of genes in B cells. In addition, this method can be used to engineer B cells to express a recombinant antibody to fight against infection. Lastly, this method can be applied to engineer B cells to express and secrete therapeutic enzymes that can be used as an autologous cell-based therapy to treat enzymopathies.

ACKNOWLEDGMENTS:

This work was funded by the Children's Cancer Research Fund (CCRF) and NIH R01 AI146009 to B.S.M.

DISCLOSURES:

A patent has been filed on the methods of making and using genome- edited B cells with M.J.J, K.L., and B.S.M. as inventors. B.S.M is a consultant for and owns stock in Immusoft Inc. Immusoft Inc has sponsored research in the lab of B.S.M.

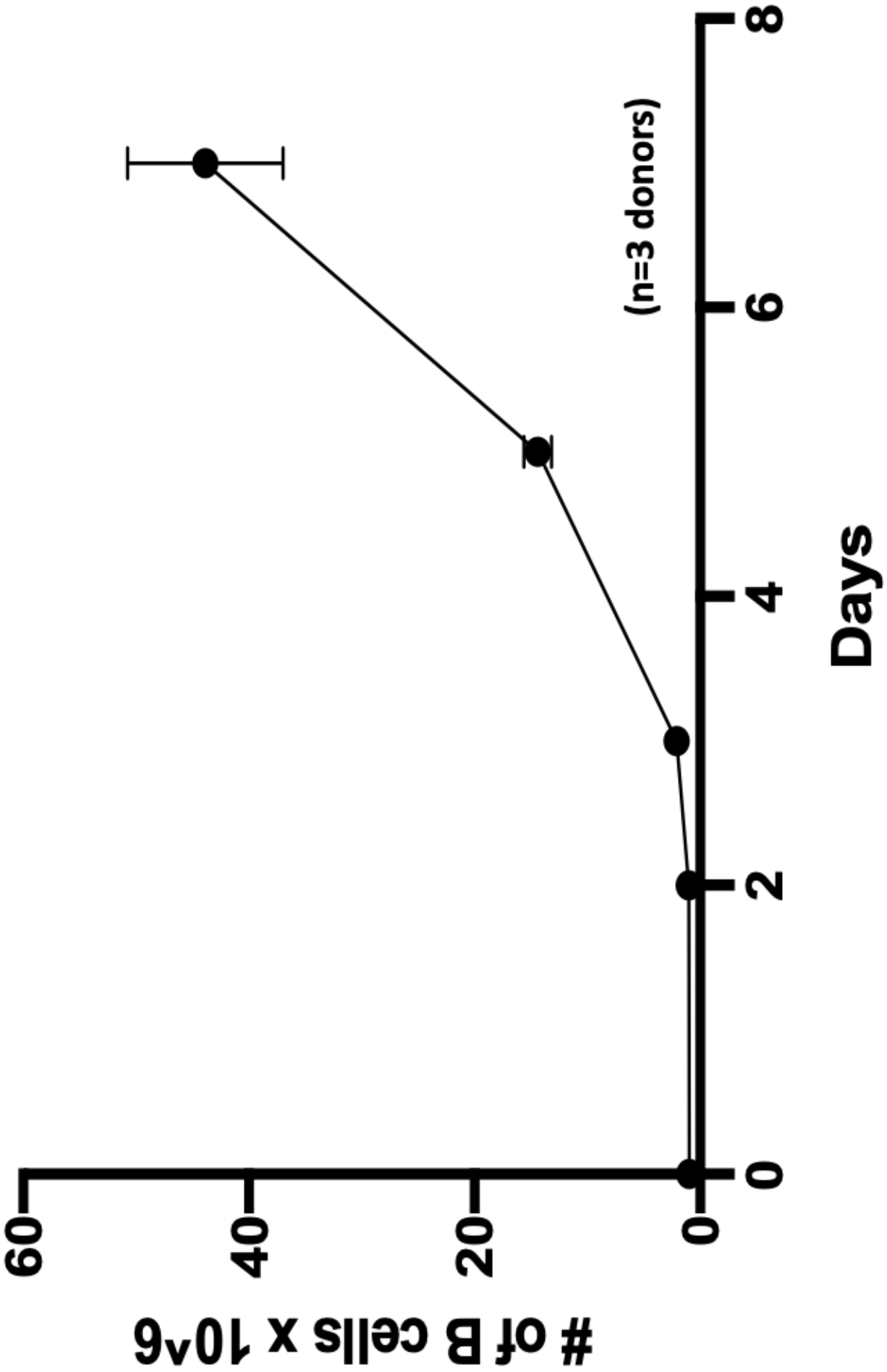
REFERENCES:

1. LeBien, T. W., Thomas, T. F. B lymphocytes: how they develop and function. *Blood*. **112** (5), 1570–1580 (2008).
2. Nutt, S. L., Hodgkin, P. D., Tarlinton, D. M., Corcoran, L. M. The generation of antibody-secreting plasma cells. *Nature Reviews Immunology.* **15**, 160–171 (2015).
3. Slifka, M. K., Antia, R., Whitmire, J. K., Ahmed, R. Humoral immunity due to long-lived plasma cells. *Immunity.* **8** (3), 363–372 (1998).
4. Spriggs, M. K. et al. Recombinant human CD40 ligand stimulates B cell proliferation and

immunoglobulin E secretion. *Journal of Experimental Medicines.* **176** (6), 1543–1550 (1992).
5. Fusil, F. et al. A lentiviral vector allowing physiologically regulated membrane-anchored and secreted antibody expression depending on B-cell maturation status. *Molecular Therapy.* **23** (11), 1734–1747 (2015).
6. Luo, X. M. et al. Engineering human hematopoietic stem/progenitor cells to produce a broadly neutralizing anti-HIV antibody after in vitro maturation to human B lymphocytes. *Blood.* **113** (7), 1422–1431 (2008).
7. Mock, U., Thiele, R., Uhde, A., Fehse, B., Horn, S. Efficient lentiviral transduction and transgene expression in primary human B cells. *Human Gene Therapy Methods.* **23** (6), 408–415 (2012).
8. Heltemes-Harris, L. M. et al. Sleeping Beauty transposon screen identifies signaling modules that cooperate with STAT5 activation to induce B-cell acute lymphoblastic leukemia. *Oncogene.* **35** (26), 3454–3464 (2016).
9. Johnson, M. J., Laoharawee, K., Lahr, W. S., Webber, B. R., Moriarity, B. S. Engineering of primary human B cells with CRISPR/Cas9 targeted nuclease. *Scientific Reports.* **8**, 12144 (2018).
10. Hung, K. L. et al. Engineering protein-secreting plasma cells by homology-directed repair in primary human B cells. *Molecular Therapy.* **26** (2), 456–467 (2018).
11. Cui, Y., Xu, J., Cheng, M., Liao, X., Peng, S. Review of CRISPR/Cas9 sgRNA design tools. *Interdisciplinary Sciences.* **10** (2), 455–465 (2018).
12. Rees, H. A., Liu, D. R. Base editing: precision chemistry on the genome and transcriptome of living cells. *Nature Reviews Genetics.* **19** (12), 770–788 (2018).
13. Spiegel, A., Bachmann, M., Jurado Jiménez, G., Sarov, M. CRISPR/Cas9-based knockout pipeline for reverse genetics in mammalian cell culture. *Methods.* **164–165**, 49–58 (2019).
14. Suzuki, T., Kazuki, Y., Oshimura, M., Hara, T. A novel system for simultaneous or sequential integration of multiple gene-loading vectors into a defined site of a human artificial chromosome. *PLoS One* **9**, e110404 (2014).
15. Bak, R. O., Porteus, M. H. CRISPR-mediated integration of large gene cassettes using AAV donor vectors. *Cell Reports* **20** (3), 750–756 (2017).
16. Shirley, J. L., de Jong, Y. P., Terhorst, C., Herzog, R. W. Immune responses to viral gene therapy vectors. *Molecular Therapy.* **28** (3), 709–722 (2020).
17. Martino, A. T., Markusic, D. M. Immune response mechanisms against AAV vectors in animal models. *Molecular Therapy - Methods and Clinical Development.* **17**, 198–208 (2020).

Figure

Click here to access/download;Figure;Figure 1.eps

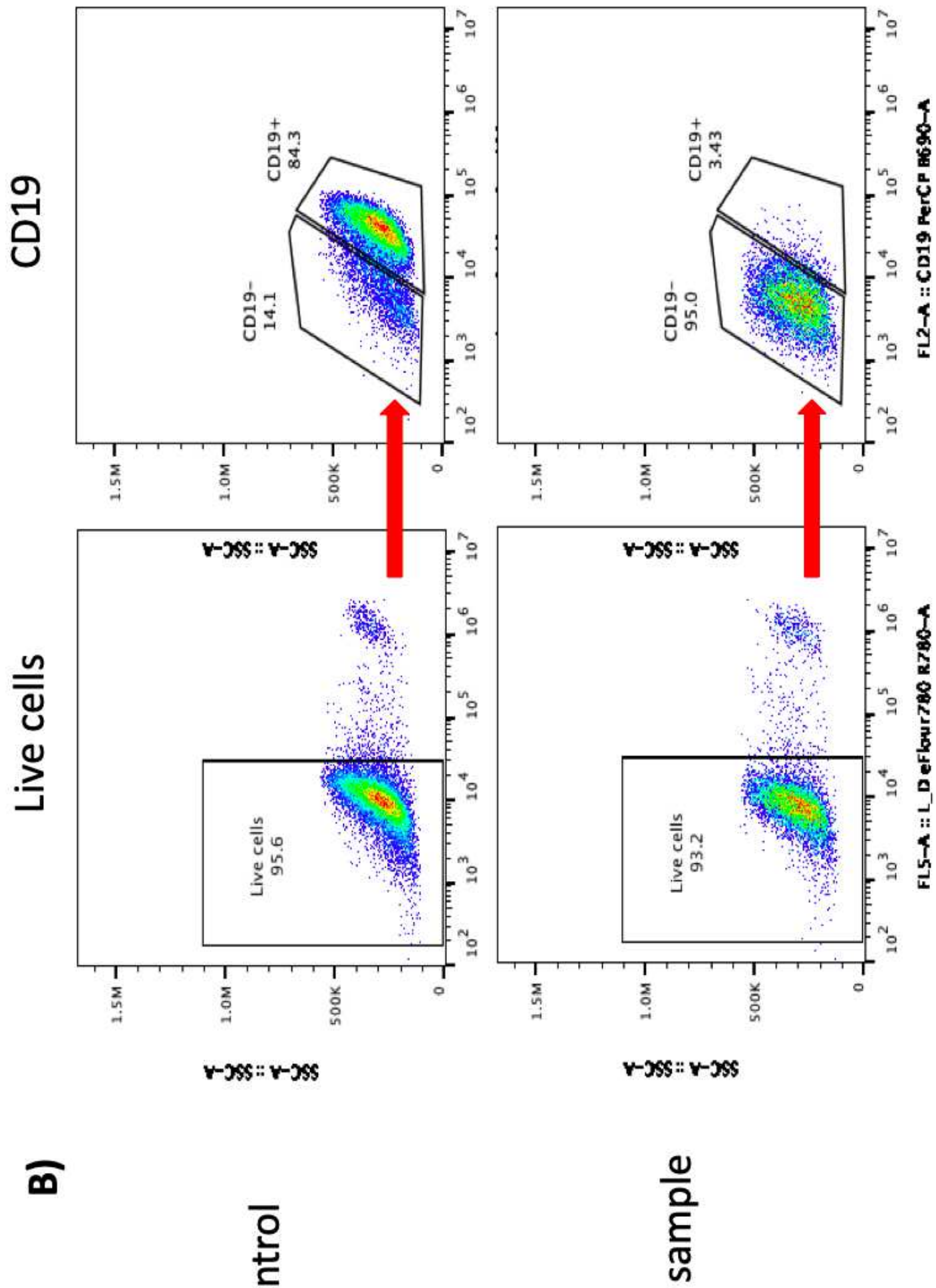


Figure

Click here to access/download;Figure;Figure 2A.eps

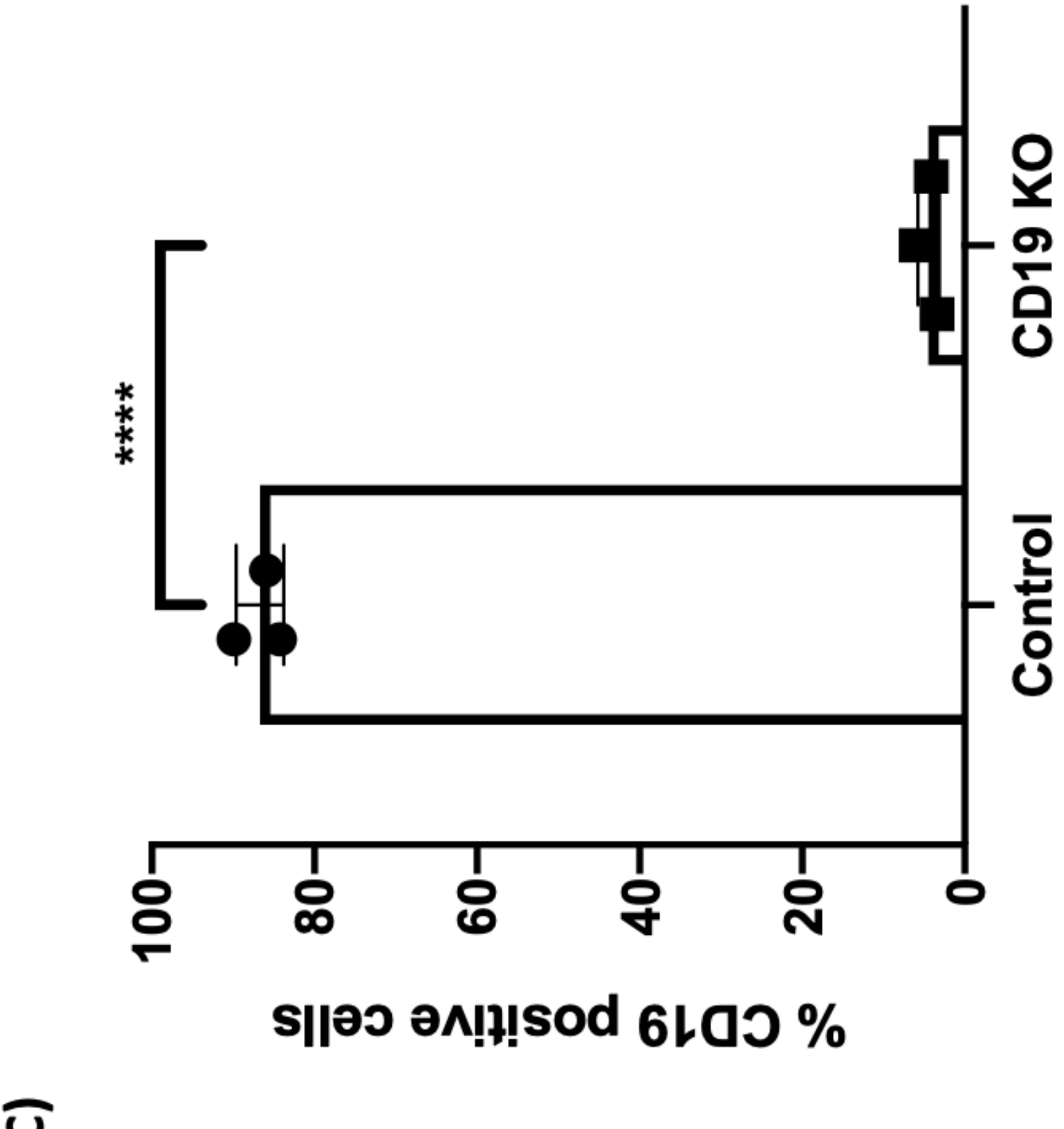
Figure

Click here to access/download;Figure;Figure 2B.eps



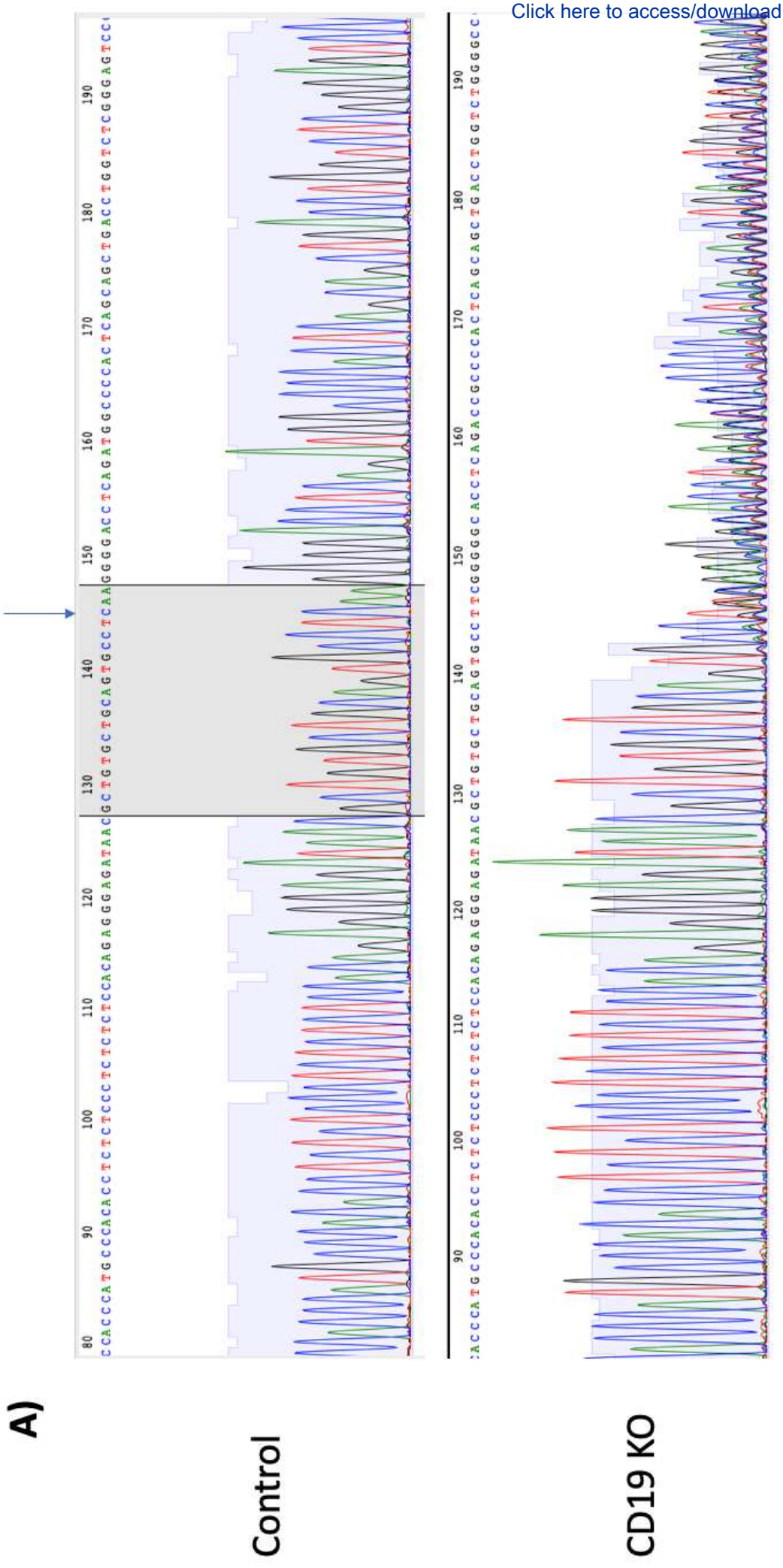
Figure

Click here to access/download;Figure;Figure 2C.eps



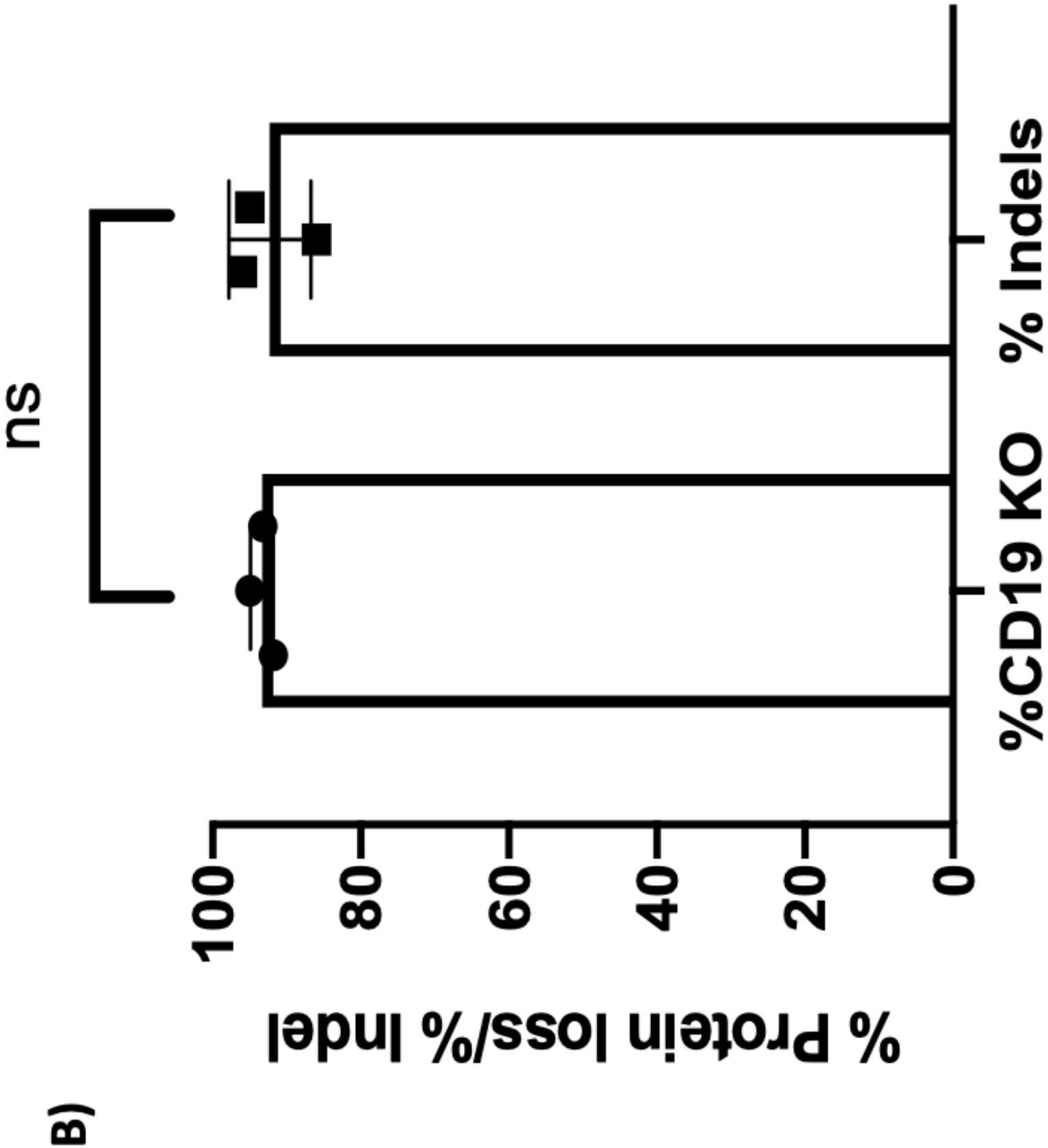
Figure

Click here to access/download;Figure;FIgure 3A.eps



Figure

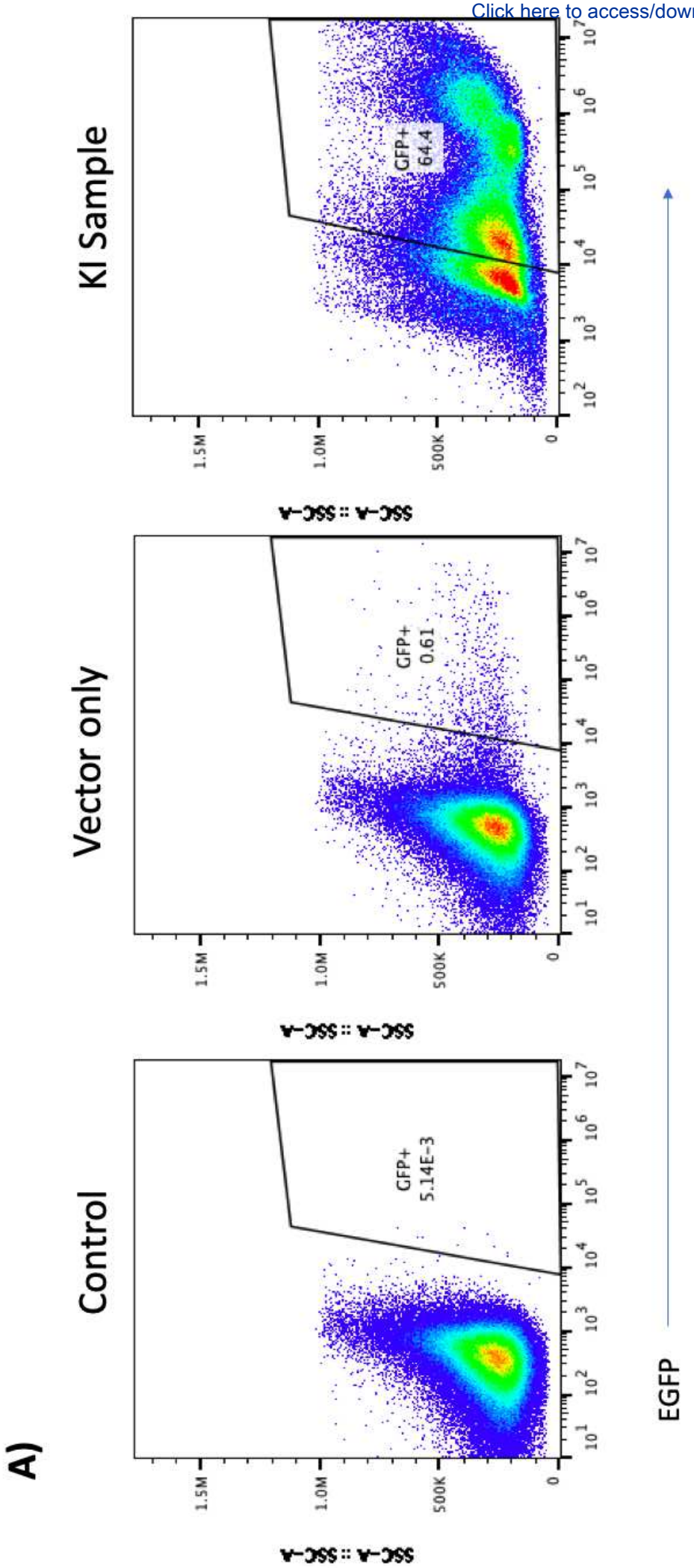
Click here to access/download;Figure;Figure 3B.eps



Figure

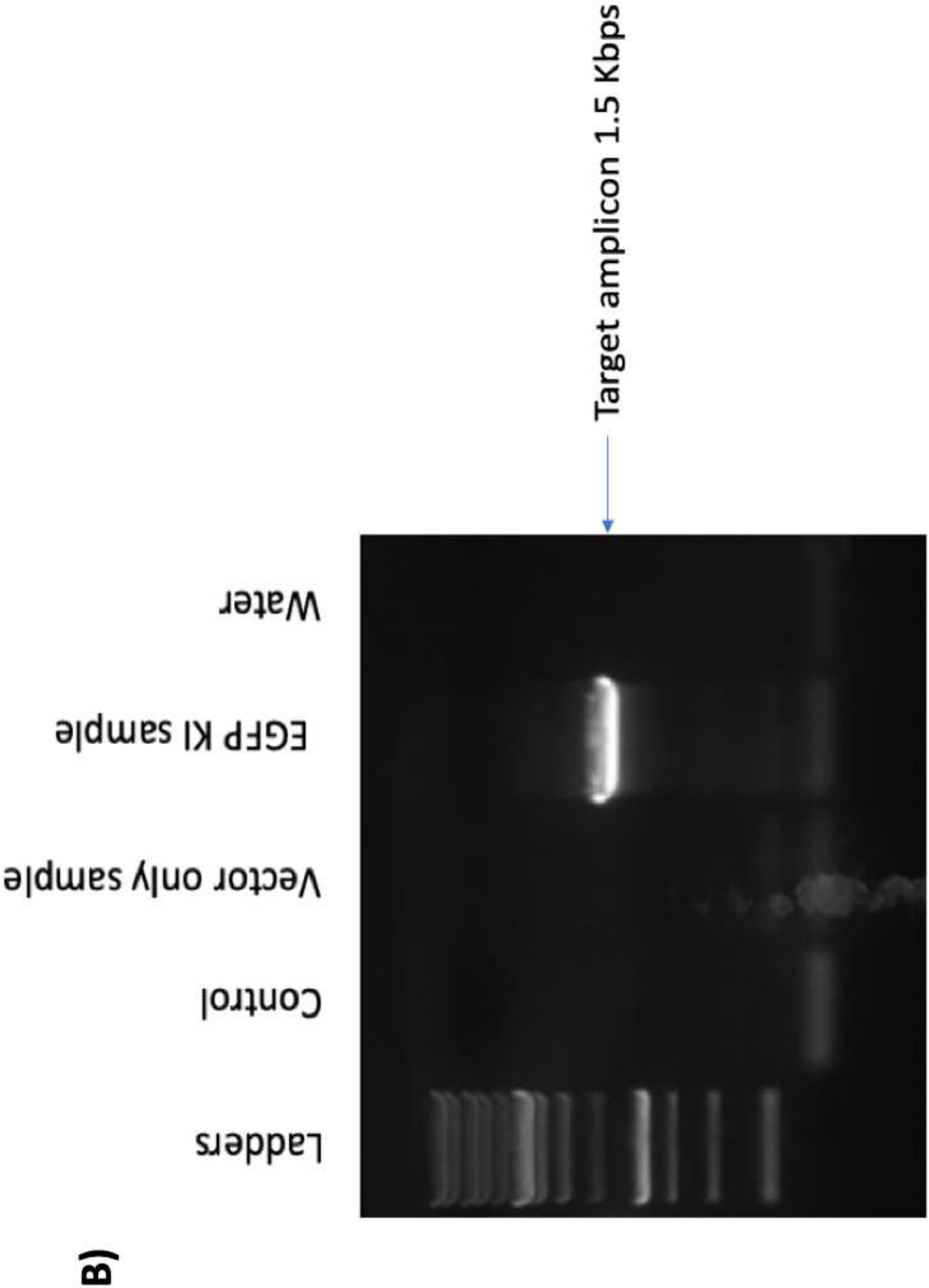
Figure

Click here to access/download;Figure;Figure 5A.eps



Figure

Click here to access/download;Figure;Figure 5B.eps



Figure

Click here to access/download;Figure;Figure 5C.eps

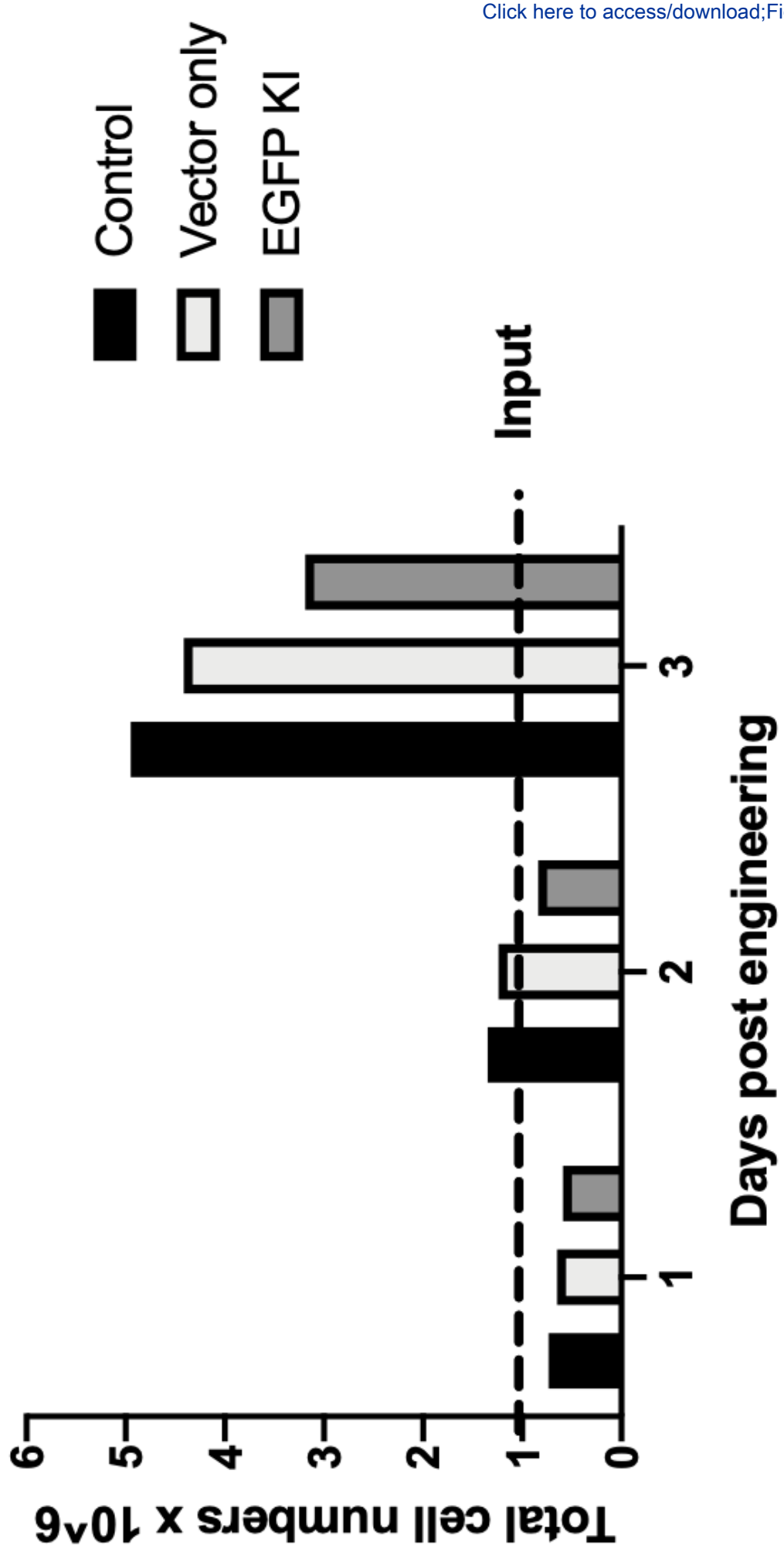


Table 1

Name	gRNA sequence
CD19	5’-GCTGTGCTGCAGTGCCTCAA-3’
AAVS1	5’-GTCACCAATCCTGTCCCTAG-3’

Table 2

Reagents
P3 Primary Cell solution
Supplement 1
Total

Volume per 1 reaction
16.4 mL
3.6 mL
20 mL

Table 3

Description	Sequence
CD19 forward primers	5’-AAATTCAGGAAAGGGTTGGAAG-3’
CD19 Reverse primer	5’-GCGGACCTCTTCTGTCCATG-3’
Junction PCR forward primer	5’-GGACGAGCTGTACAAGTAACG-3’
Junction PCR Reverse primer	5’-GAGACAGTGACCAACCATCC-3

Purpose
Amplify the CD19 locus for Indel analysis
Amplify the CD19 locus for Indel analysis
Junction PCR
Junction PCR

Table 4

Description	Fluorophore	Clone
Anti-human CD19	PerCP	HIB19
Viability Dye	eFlour 780	-

Name of Material/Equipment	Company	Catalog Number
Alt-R S.p. Cas9 Nuclease V3 protein, 500 ug	IDT	1081059
Amaxa P3 primary cell 4D- Nucleofector X Kit S (32 RCT)	Lonza	V4XP-3032
Ammonium-chloride-potassium (ACK) lysing buffer	Quality Biological	118-156-101
CleanCap chemically modified Cas9 mRNA	Trilink Biotechnology	L-7206-1000
CpG ODN 2006 (ODN 7909) 5 mg	Invivogen	TLRL-2006-5
Cryostor CS10, 100 mL	STEMCELL Technology	7930
CTS Immune Cell SR	Thermo Fisher Scientific	A2596101
EasySep human B cells isolation kit	STEMCELL Technology	17954
eBioscience fixable viability dye eFlour 780	eBiosciences	65-0865-14
Excellerate B cell media, Xeno-free	R&D Systems	CCM031
Falcon 14 mL Polypropylene Round-bottom Tube	Corning	352059
Fetal Bovine Serum (FBS)	R&D Systems	S11550
Ficoll-Paque Plus	GE Healthcare	17-1440-03
GeneMate SnapStrip® 8-Strip 0.2 mL PCR Tubes with Individual Attached Dome Caps	BioExpress	T-3035-1 / 490003-692
Hyclone 0.0067M PBS (No Ca, No Mg) or 1x PBS	GE lifesciences	SH30256.01
Lonza 4D Nucleofector core unit	Lonza	AAF-1002B
Lonza 4D Nucleofector X unit	Lonza	AAF-1002X
Mega CD40 Ligand	Enzo Life Sciences	ALX-522-110-C010
Mr. Frosty	Sigma-Aldrich	C1562-1EA
Pen/Strep 100X	Sigma-Aldrich	TMS-AB2-C
PerCP anti-human CD19 clone HIB19	biolegend	302228
rAAV6 SA-GFP pakaging (with our SA-GFP cassette see Figure 4.)	Vigene Biosciences	N/A
Recombinant human IL-10 protein 250 ug	R&D Systems	217-IL-250

Recombinant human IL-15 protein 25 ug	R&D Systems	247-ILB-025
The Big Easy EasySep Magnet	STEMCELL Technology	18001
Tris-EDTA (TE) buffer	Fisher Scientific	BP2476100

	Comments/Description
	smaller size is also available
	different sizes available
	B-cell basal medium
	For thawing B cells only
	For freezing cells
	smaller size is also available
	large scale packaging, 1e13 GC/mL, 500 mL
	different sizes available

different sizes available

different sizes available

Dear Editor and reviewers,

Thank you so much for taking the time to review my submission; your feedback and comments were both insightful and helpful. I have addressed each comment in my revised manuscript and have noted my changes and responses below (in red font). With these changes, I'm hoping you will feel that my manuscript meets the high standards for which JoVE is known.

I'd be happy to take additional feedback and/or questions to ensure that my manuscript is as complete, informative and useful for other researchers.

Kind regards,

Kanut

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. I have addressed this issue accordingly.
2. Please cite figures in order (in the introduction, you have cited Fig 1, then Fig 2, Fig 2C, and then Fig 5A. I have addressed this issue accordingly
3. Some additional protocol details are needed:

Please specify the volumes of all solutions and medium used throughout. How much medium is prepared? I have addressed this issue throughout the manuscript accordingly.

In the note after 5.1, do you want viewers to perform all three steps (design sgRNAs using the online tool and on the exon as well as ordering chemically modified sgRNA)? I have changed this to instruct the investigator to follow the steps in the note after 5.1 to order sgRNA.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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. I have addressed this issue accordingly.

For example: Amaxa 4D nuclefector (Lonza 4D electroporator), Lonza 4D cuvette, Mr. Frosty", "Big Easy" Magnetic Station, and Cryostor CS10 freezing medium….

5. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. Currently, there is about 3 pages of protocol text highlighted but without the one line space. I've addressed this issue accordingly.
6. In Representative Results, please explain why double peaks are expected in the CD19 KO B cells and not in control cells and the difference in EGFP-positivity between AAVS1+rAAV6 and control. I explained this in the result section.
7. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

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

I have addressed these issues accordingly.

8. Please write journal names fully in the reference list. I have addressed this issue accordingly.
9. Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.). Please remove the titles and Figure Legends from the uploaded figures. Please add the Figure Legends after the Representative Results as indicated in the Information for authors. I have addressed this issue accordingly.
9. Fig.1: Please add the number of independent donors. I have addressed this issue accordingly.
10. Please sort the Materials Table alphabetically by the name of the material. I have addressed this issue accordingly.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Loaharawee et al. have provided protocols for the purification and culture of primary human B cells as well as for performing, gene knock out and targeted knock in. KO of CD19 and KI of eGFP via AAV6 into a CRISPR cut site at the AAV1 locus are the examples used. The protocols are quite straight forward and will be useful to the community of scientists entering into this field. We do have some suggestions for improving the manuscript for clarity and readability.

Major Concerns:

1. My opinion is that the introduction should be a little more pedagogical. It should more clearly describe what you will be doing in the paper. The last paragraph is good but it would be helpful to have more of a layout and rational for what is to follow rather than just launching into detailed protocols. For example: (…we first describe how to purify B cells from….. We will then describe how to knock out a gene using the example of CD19, a B cell surface receptor. We will do this by introducing a CRISPRcas9-gRNA ribonucleoprotein (RNP) complex into activated cells using electroporation. We designed several gRNAs that will guide the RNP to make dsDNA cuts with the ? exon of CD19. Cells will repair these cuts by 'non-homologous end joining' which introduces or deletes nucleotides disrupting the reading frame of this gene. We then measure loss of CD19 protein in targeted cells by FACS and analyze indels in the exon using TIDE analysis….). I have addressed this concern in the introduction section as suggested.
2. It is easier to follow protocols when they have a list of required equipment and reagents at the beginning of the section (with catalogue numbers) but just a suggestion. The required equipment and reagents are listed in the table of materials according to

the journal's instruction.
3. The discussion does not really follow the Jove format and should be re-written accordingly… I have re-written according to the JoVE format.
DISCUSSION: (3-6 paragraphs)
JoVE is a methods journal. Thus, the Discussion section of the article should be focused on the Protocol. Discuss the following with citations:
Critical steps in the protocol
Modifications and troubleshooting of the method
Limitations of the method
The significance of the method with respect to existing/alternative methods
Importance and potential applications of the method in specific research areas

Minor Concerns:
1. In the abstract… 'can be utilized to express very large amounts of recombinant protein in the form of recombinant antibody or enzyme' is awkward sounding…maybe say 'therapeutic protein in the form of recombinant antibody…' etc. I have re-written this part accordingly.
2. Check for grammar: ..In the introduction, third paragraph 'allows researchers to precisely editing' should be 'to precisely edit'…Summary, should say 'and to develop B cell therapeutics' or something like this. etc. Thanks for your advice; I have addressed this issue accordingly.
3. Should make a note about how purification from B cells might be different compared with a leukophoresis, expected yield ranges would be nice. I added the expected yield of the isolated B cells at the end of the B-cell purification process.

Reviewer #2:
Manuscript Summary:
In this manuscript, the Authors described a method to perform site specific gene editing of human B cells. The protocol is based on the use of Amaxa 4D nucleofector and rAAV6 vector for the delivery of CRISPR/Cas9 RNAs and donor DNA template, respectively, and allows efficient site-specific gene disruption or in situ targeted gene addition on in vitro stimulated B cells.
The described method harnesses the momentum seen in the past couple of years exploiting the straightforward genetic manipulation of B cells for therapeutic potential and thus it is expected that it will be well received by the experts in the field. The protocol is well presented and sufficiently detailed to be repeated by personnel with basic expertise on laboratory techniques.

Major Concerns:
Since the presented protocol is an optimization of a previously published method, it would be better to have more details in the representative results in order to better appreciate the expected differences respect to the reference paper. In particular:
- it would be better to add Fig.1B the growth curve of the cells treated for editing (CRISPR/Cas9 only and plus rAAV6) to have an indication of the overall cell yield after

editing. I have added Figure 5C to show a representative growth curve of control, vector-only, and KI samples over a period of 3 days after engineering.

- Please add some comment on why in Fig.5A two populations of GFP cells, with high or intermediate GFP expression, are present. Is this due to biallelic integration or is the intermediate GFP expression derived from unintegrated rAAV6? Yes, we speculated that this is due to biallelic integrations; further investigation using spanning PCR can be done in the intermediate- and high EGFP-expressing cells to address this issue. Is the overall percentage of GFP+ cell stable also in long term culture? We only expanded them to 12 days post engineering; we are in the process of engrafting the engineered B cells in NSG mice to investigate the long-term survival of the engineered cells in vivo.

Minor Concerns:
- Point 5.3: in the note, please specify that 5 ug of Cas9 is mRNA and not recombinant protein. I'm sorry for the confusion; my note suggests that if the investigator preferred to use Cas9 protein, the amount needs to be 5 ug in order to have optimal results.
- Point 5.16: check the number of cells and concentration after electroporation. The transfer of 10*6 cells in 1ml of pre-warmed medium will lead to a concentration of 10*6/ml, not 5x10*5/ml. I fixed the number accordingly.
- Point 5.17: please specify if the rAAV is added just upon electroporation or after the cells are plated in the well (45 minutes after editing?) I specified in the protocol accordingly.
- Representative results: "Representative flow plots of the CD19 KO sample and control showed 95% and 11% CD19 negative cells, respectively (Figure2A)". The mentioned results are reported in Figure 2B not Figure 2A. Please correct. I corrected it accordingly.
- Figure 1B: please add the number of replicates since the graph reports SD. I added the numbers of replicates accordingly.
- Figure 2A: In the Figure1 authors show that cells continue to growth from day 2 (day of editing) up to day 7. Assuming that cells reported in Figure 1B are untreated cells (please specify), why control cells reported in Figure 2A decrease as compared to the input 24 hours after editing procedure? If the "control cells" undergo some treatment that explain their reduction in number (i.e. electroporation only without Cas9 or gRNA) please specify. I have now addressed this question in the results section. The electroporation protocol seems to cause some cell death within 24 hours; however, they quickly rebounded (data not shown) – as also observed in Figure 5C.
- Increase the font size in the figures. I have addressed this issue accordingly.