

Evaluation protocol for CRISPR/Cas9-mediated CD19 knockout GM24385 cells by flow cytometry and Sanger sequencing

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ABSTRACT

Although several genome editing options are available, CRISPR/Cas9 is one of the most commonly used systems for protein and advanced therapies. There are some long-term data regarding genomic and phenotypic stability, however, information is sparse. Flow cytometry can offer a method to characterize these edited cells for longitudinal studies. The objective of this work is to describe a protocol for using flow cytometry to measure the edits from CRISPR/Cas9 on a well-characterized B-lymphoblast cell line, GM24385, with the goal of supporting safe and effective CRISPR/Cas9-engineered therapies.

METHOD SUMMARY

The objective of this work is to describe a protocol for using flow cytometry to measure the edits from CRISPR/Cas9 on a well-characterized B-lymphoblast cell line, GM24385, with the goal of longitudinal monitoring to support safe and effective CRISPR/Cas9-engineered therapies. The protocol methods include cell culture, flow cytometry, CRISPR/Cas9 editing and Sanger sequencing.

KEYWORDS:

CD19 • CRISPR/Cas9 • flow cytometry • genome editing • GM24385 cell line

The CRISPR/Cas9 system is naturally found in bacterial and archaeal immune systems [1]; however, it has been adapted for use in eukaryotes as a Nobel prize-winning genome editing system [2,3]. The CRISPR/Cas9 system uses gRNA to direct the Cas9 nuclease to a specific target near an NGG protospacer adjacent motif sequence. The Cas9 then cleaves the DNA and the cell's native DNA damage repair machinery repairs the double-strand break.

The two main repair pathways leveraged for knockout and modification of target genes during CRISPR/Cas9 editing are the non-homologous end joining (NHEJ) and homology-directed repair pathways. NHEJ is the primary choice for repairing double-strand breaks when the donor DNA template is not available, making it a good option for CRISPR/Cas9 knockout experiments. Loss and gain of nucleotides are common occurrences during NHEJ-mediated repair, and a knockout comes from a shift in the coding sequence. Homology-directed repair is based on availability of the donor DNA template and is mainly used for custom gene modifications in coding or non-coding sequences [4,5].

The CRISPR/Cas9 system is being used more and more often for advanced therapies, including cell and gene therapies, with great promise (e.g., CRISPR test for treating sickle cell disease) [6,7]. Many commercial products for enabling CRISPR/Cas9 nuclease RNA-guided genome editing are available in a variety of delivery methods. These products include DNA encoded in viruses, plasmids, mRNA and RNPs. Additionally, CRISPR gRNA can be delivered in two parts, crRNA and tracrRNA, or as an sgRNA. Multiple bacterial species offer CAS protein options; a commonly used one is *Streptococcus pyogenes* Cas9. Similarly, there are many options for how these molecules can be delivered into cells, including viral vectors, such as lentiviral and adeno-associated viral vectors, and chemical methods, such as lipids, injection and electroporation [8,9]. Although CRISPR/Cas9 is an exciting genome editing tool, there are limited longitudinal data on genomic and phenotypic stability after using CRISPR/Cas9 to edit cells. There are now several reports providing evidence that there is also the potential for additional unintended long-term changes [10–12] associated with CRISPR/Cas9 editing. In addition, genetic bottlenecks can arise during selection of edited cell subpopulations. Hence, it is essential to characterize the edited cells intended for use in advanced therapies such as cell and gene therapies, where the therapy often involves administering populations of edited cells to the patient [13].

Table 1. Flow cytometry reagents used for cell analysis.

B-cell panel						
Marker	Clone	Fluorophore	Company	Product no.	Dilution volume per 0.5 ⁶ cells, μ l	B-cell subtype expression indicator
Live/Dead		Aqua	Thermo Fisher Scientific	L34957	0.5	Dead cells
CD19	J3-119	PE	Beckman Coulter	IM1285U	0.5	All B-cell lineages
CD27	1A4CD27	PE-Cy7	Beckman Coulter	A54823	7.5	Memory B cells and short-lived plasma cells
CD20	B9E9 (HRC20)	Pacific Blue	Beckman Coulter	A74777	7.5	Mature B cells and memory B cells
CD24	ALB9	APC	Beckman Coulter	A87785	7.5	All B cells
CD38	LS198.4.3	APC-AF700	Beckman Coulter	B23489	7.5	Regulatory B cells
IgD	IA6-2	FITC	Beckman Coulter	B306527	30	Naive B cells
CD138	MI14	BV711	BD Biosciences	563184	7.5	Bright plasma B cells
Compensation						
Marker	Clone	Fluorophore	Company	Product no.	Dilution volume per 0.5 ⁶ cells, μ l	
CD19	SJ24C1	PE-Cy7	BD Biosciences	557835	5	
CD19	HIB19	APC	BioLegend	302212	5	
CD19	J3-119	APC-AF700	Beckman Coulter	A78837	5	
CD19	HIB19	FITC	BioLegend	302206	5	
CD19	SJ25C1	BV711	BD Biosciences	563036	5	
Intracellular						
Marker	Clone	Fluorophore	Company	Product no.	Dilution volume per 0.5 ⁶ cells, μ l	
GAPDH	14C10	AF647	Cell Signaling Technology	3907S	5	

AF647: Alexa Fluor 647; APC: Allophycocyanin; APC-AF700: Allophycocyanin–Alexa Fluor 700; BV711: Brilliant Violet 711; FITC: Fluorescein isothiocyanate; PE: Phycoerythrin; PE-Cy7: Phycoerythrin–Cyanine7.

In biomanufacturing, mammalian cells are often engineered to produce protein products. Common mammalian cell lines for protein expression include Chinese hamster ovary, mouse myeloma (NS0) and human embryonic kidney 293 cells [14,15]. However, for cell and gene therapies, a variety of primary human cell types, including B cells and fibroblast cells, are modified [16,17].

In cell therapies, the final therapeutic edited cell product may be a pool of cells or cells that have been sorted for specific characteristics. Since the therapeutic value is often reliant on DNA sequence changes, it is imperative to determine if the edits are persistent [17]. Although it is expected that different cell types may have varying responses to CRISPR/Cas9 editing, there may also be some similar underlying long-term trends that have yet to be elucidated.

The Genome in a Bottle Consortium has put forth a significant amount of effort in characterizing and developing National Institute of Standards and Technology reference materials for the genomes of several cell lines [18]. For this reason, one of the Genome in a Bottle B-lymphoblast cell lines, GM24385, has become a good candidate for characterizing the products of genome editing. A well-known B-lymphocyte antigen, CD19, is expressed on the surface of human B cells [19] from early stages to terminally differentiated plasma cells, making it a good target for editing a B-lymphoblast cell line.

The objective of this protocol is to enable study of the longitudinal genotypic and phenotypic stability of CRISPR/Cas9 using genomics and flow cytometry. By evaluating the stability of CRISPR/Cas9 genome editing, we will have a better understanding of its long-term effects on the cell. This will help to further characterize the safety and effectiveness of therapies produced using the CRISPR/Cas9 system.

Protocol

Cells & media

The authors used the GM24385 B-lymphoblast cell line (Coriell Institute for Medical Research, NJ, USA), Roswell Park Memorial Institute (RPMI) 1640 medium with 2 mM L-glutamine (30-2001; American Type Culture Collection, VA, USA), fetal bovine serum (FBS) (16140071; Thermo Fisher Scientific, MA, USA) and T flasks or cell culture plates.

Flow cytometry

The authors used an Attune NxT flow cytometer (A24858; Thermo Fisher Scientific), antibodies for B-cell panel (Table 1), phosphate-buffered saline (PBS) (10010049; Thermo Fisher Scientific), FBS, FIX & PERM Cell Fixation and Cell Permeabilization Kit (GAS003; Thermo Fisher Scientific), Attune performance check beads (4449754; Thermo Fisher Scientific), Attune focusing fluid (100085929CST; Thermo Fisher Scientific), centrifuge and tubes (1.5, 5 and 15 ml).

Guide	Sequence	Cut site, hg19	Exon	Binds to
1	CGCUGUGCUGCAGUGCCUCA	28,932,373	2	Sense
2	GCGUGUGCUGCAGUGCCCAA	28,932,374	2	Sense
3	UUCCCAGGCCUGGCAGCCCC	28,932,458	2	Antisense
4	UUCAACGUCUCUCAACAGAU	28,932,527	2	Sense

Direction	Sequence
Forward	GGGTGTCCTTGGCTGAGTAA
Reverse	CCTCTCTCCAGCTCCATTGT

Transfection reagents & instrumentation

The authors used 4D-Nucleofector (AAF-1003B; Lonza, NJ, USA), SF Cell Line Kit (V4XC-2032 or V4XC-2012; Lonza), sgRNA designed to target (Synthego, CA, USA) (Table 2), *S. pyogenes* Cas9 (9212-5MG; Aldevron, ND, USA) and tissue culture plates.

Sanger sequencing

The authors used a Veriti thermocycler (A48141; Thermo Fisher Scientific), NanoDrop (ND-ONE-W; Thermo Fisher Scientific), Quick-DNA Miniprep Plus Kit (D4069; Zymo Research Corporation, CA, USA), Q5 Hot Start High-Fidelity 2× Master Mix (M0494L; New England Biolabs, MA, USA), PCR primers (Table 3) and DNA Clean & Concentrator kit (D4014; Zymo Research Corporation).

Data analysis

The authors used FlowJo (Becton, Dickinson and Company, NJ, USA) and the inference of CRISPR Edits analysis tool (Synthego) for statistical analysis (alternative instruments, kits and software may have similar results).

Methods

Cell culture

The Genome in a Bottle B-lymphoblast cell line, GM24385, was selected as the cell line of choice as a result of its extensive use in genome sequencing [20]. GM24385 cells are anchorage-dependent cells and loosely aggregated when cultured in upright T flasks or plates. Low passage cells are recommended because growth rate decreases after a number of passages [21]. It should be noted that one should always be extremely careful to avoid contamination.

The authors maintained cells in RPMI 1640 medium supplemented with 15% FBS. Cells were cultured in a humidified incubator set at 5% CO₂ and 37°C. Cells were passaged when the density reached approximately 1 × 10⁶ cells/ml (a split ratio of 1:4 is recommended). Prior to passage, cells were dissociated by pipetting. If cells had not reached the splittable density in 3–4 days, the medium was changed by centrifuging cells (200 × g for 10 min at 25°C), gently aspirating the supernatant and resuspending cells in prewarmed fresh medium.

CRISPR/Cas9 editing for CD19 knockout

CRISPR/Cas9 is a popular method for creating a DNA double-strand break. When a repair template is unavailable, the cell repairs double-strand breaks using the NHEJ pathway, during which several nucleotides may be lost at the repair sites, leading to knockout of gene expression. Of the many delivery methods available, transfection of an RNP complex by electroporation is popular because of its transient nature, which works for many cell types [22]. In this experiment, because of the possibility of different guide efficiencies, four gRNAs were tested. To knock out CD19, four gRNAs targeting human *CD19* (Table 2) were purchased as modified sgRNAs in a Synthego gene knockout kit. sgRNAs were selected based on location in the coding region of the gene, location in an exon found in most transcripts, activity score and base mismatches. sgRNAs were used following the manufacturer's instructions with the kit-supplied *S. pyogenes* Cas9 2NLS nuclease and transfected using 4D-Nucleofector and an SF Cell Line Kit. Prior to scaling up to cuvettes, samples were tested in nucleofection strips.

When designing transfection experiments, the authors recommend controls such as electroporation only, Cas9 and electroporation with no gRNA as well as a positive control such as a gRNA targeting the *RELA* gene with a known knockout effect. Because of the nature of nucleofection, many cells die during this step, and it takes time for the surviving cells to stabilize and replicate to a sufficient quantity to analyze. It is important to begin with healthy cells and handle them gently to minimize cell death.

The authors resuspended gRNA sterile nuclease-free water to make a 30-μM stock solution, used the stock solution to form RNP complexes with 20 μM of Cas9 and nucleofector reagent at a ratio of 9:1 for sgRNA and Cas9 and incubated for 10 min at room temperature. Depending on the size of the cuvette to be used, 1 × 10⁶ cells per cuvette or 2 × 10⁵ cells per well of a 16-well strip cuvette needed to be prepared. Cells were centrifuged at 100 × g for 10 min at 25°C and then resuspended in nucleofector reagent to the appropriate

Table 4. Laser and antibody matrix for flow cytometry analysis.

Attune channel	440/50	512/25	710/50	530/30	585/30	780/60	670/14	720/30
Channel label	VL1	VL2	VL4	BL1	YL1	YL5	RL1	RL2
Fluorophore	Pacific Blue	Aqua	BV711	FITC	PE	PE-Cy7	APC	APC-AF700
Comp_unstained								
Comp_Pac Blue	CD20							
Comp_Aqua		Live/Dead						
Comp_BV711			CD19					
Comp_FITC				CD19				
Comp_PE					CD19			
Comp_PC7						CD19		
Comp_APC							CD19	
Comp_APC-AF700								CD19
FM01		Live/Dead			CD19			
FM02	CD20	Live/Dead	CD138	IgD	CD19		CD24	
FM03	CD20	Live/Dead			CD19	CD27		CD38
Samples	CD20	Live/Dead	CD138	IgD	CD19	CD138	CD27	CD24

APC: Allophycocyanin; APC-AF700: Allophycocyanin–Alexa Fluor 700; BV711: Brilliant Violet 711; Comp: Compensation; FITC: Fluorescein isothiocyanate; FMO: Fluorescence minus one; Pac Blue: Pacific Blue; PC7: Phycoerythrin–Cyanine7; PE: Phycoerythrin; PE-Cy7: Phycoerythrin–Cyanine7.

Spillover values are read across rows

Spillover	BL1-A	RL1-A	RL2-A	VL1-A	VL2-A	VL4-A	YL1-A	YL4-A
BL1-A	100.00	0.17	0.06	0.10	0.31	0.24	0.18	0.06
RL1-A	0.19	100.00	7.83	0.00	0.00	3.10	0.00	2.18
RL2-A	0.40	35.31	100.00	0.35	0.12	24.99	0.43	27.82
VL1-A	0.20	0.10	0.00	100.00	19.23	0.64	0.21	0.07
VL2-A	6.82	4.64	0.50	15.55	100.00	16.68	3.13	0.60
VL4-A	0.12	2.05	14.37	2.37	0.04	100.00	0.13	0.80
YL1-A	0.61	0.00	0.00	0.03	0.00	0.43	100.00	0.42
YL4-A	0.49	0.05	0.03	0.00	0.09	0.13	3.03	100.00

Figure 1. Example compensation matrix for flow cytometry analysis. A compensation matrix was used to ensure proper separation of the fluorescence signals for cell marker staining.

volume (5 μ l of cells at 3×10^4 cells/ml per well). Cells were then transferred to a nucleofector cuvette, with RNP complexes or controls added to the cells in the cuvettes and wells to a total of 100 μ l per cuvette or 30 μ l per well of solution. Next, cells were nucleofected using the SF Cell Line and DN-100 in 4D-Nucleofector. Prewarmed medium was then added to the cuvettes and wells and incubated at 37°C for 30 min. Finally, cells were transferred to a prewarmed six-well plate and incubated at 37°C for 72 h until harvesting.

B-cell panel with flow cytometry

To measure the phenotypic effect of knocking out CD19, the authors established a panel for measuring differences and a baseline phenotype. B lymphoblasts that are immortalized with Epstein–Barr virus express CD19 on the cell surface, so this was used as the test marker for knockout [23–25]. A panel containing several surface markers common to B cells and a viability marker was designed based on the expression levels of surface markers and availability of lasers and fluorescence channels (Tables 1 & 4). This panel was initially measured to create a baseline from the parent GM24385 cells. The panel was used with the parent GM24385 cells to set the gates (Figures 1 & 2) [26]. The steps for performing the flow cytometry analysis are discussed in the following sections. This analysis was periodically applied to the edited cells.

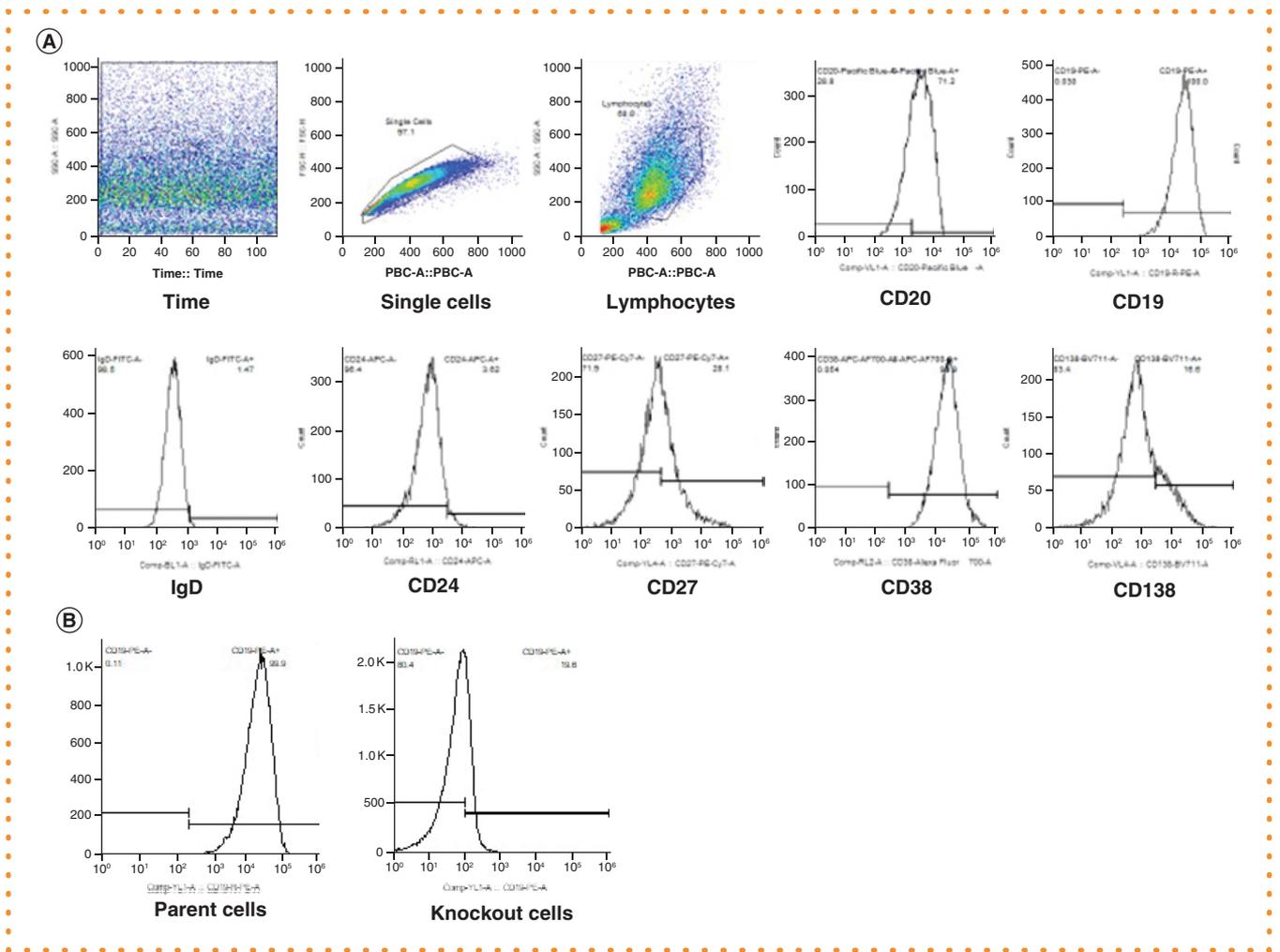


Figure 2. Representative gating strategy for flow cytometry analysis. (A) Representative gating strategy for flow cytometry analysis based on gates set using FMO controls. Time versus SSC was used to gate the steady-state population. Single cells were then gated using the FSC-A versus FSC-H plot. A lymphocyte gate was created using the FSC versus SSC plot. The remaining plots are histograms of the respective markers (CD20, CD19, IgD, CD24, CD27, CD38 and CD138). (B) Example CD19 histogram plot comparing parent cells with knockout cells demonstrating a count of CD19+ cells in the knockout group.

FMO: Fluorescence minus one; FSC: Forward scatter; FSC-A: Forward scatter area; FSC-H: Forward scatter height; SSC: Side scatter.

Because of the influence of cell passaging time in cell culture, it is important to be consistent with timing of the sample collection between experiments (e.g., collecting cells of the same passage). Therefore, to minimize variations due to sample preparation and cell behavior, measurements should be made at the same time point.

For cell sample preparation, cells were divided into three groups: live/dead compensation samples; single fluorophore-conjugated antibody-stained samples for compensation; and sample cells, including the controls. Additionally, an intracellular staining process was used with CD19 antibody to determine whether any CD19 proteins were expressed but not translocated on the cell surface. Three groups of samples were stained in parallel. Approximately 5×10^5 cells were needed for each sample, and this was determined using the flow cytometry analysis laser and antibody matrix as a guideline for staining (Table 4). It should be noted that the parent cells were used for compensation and fluorescence minus one controls. Compensation beads can alternatively be used, but they are not the same size as stained cells. Compensation is a technique used in multifluorophore panels to correct for fluorescence spillover between detectors. Fluorescence minus one controls are used to identify positive and negative cells to appropriately place gates for marker identification.

With regard to staining cell samples, the aqua compensation tube should possess dead cells to stain for compensation. To obtain dead cells, cells were placed in a tube and heated to 60°C for 20 min. Cells were stained with the Live/Dead stain after washing with PBS or alternatively stained with the compensation stain using PBS containing 2% FBS. The authors added 1 ml of PBS containing Live/Dead stain and incubated in the dark at room temperature for 30 min. To obtain a compensation matrix, the authors stained one tube for each

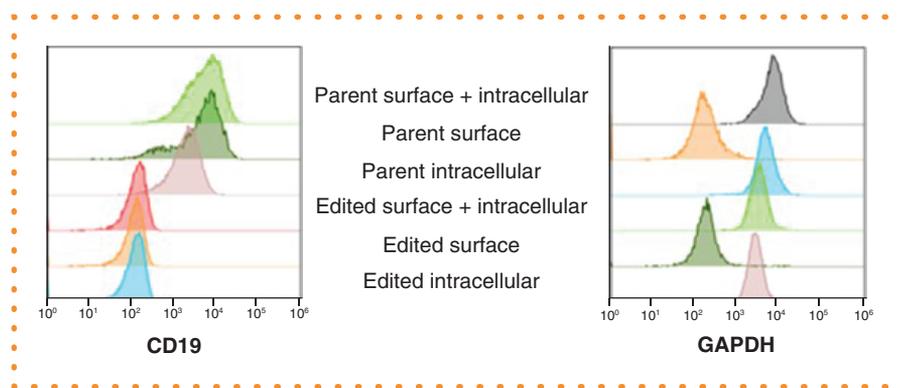


Figure 3. Sample histograms from intracellular staining. An overlay plot of CD19 histograms comparing the knockout and parental cell lines with surface staining only, internal staining only and both surface and internal staining shows less CD19 in the knockout cell lines. Using GAPDH as a control for the staining protocol shows a lack of GAPDH in the surface staining-only cell lines.

of the fluorophores used (in this case with CD19 to get a sharp peak). After coating the tube with PBS containing FBS, the stain was added to the cells and incubated for 30 min at room temperature.

To remove extra fluorophores, the authors washed cells with PBS containing 2% FBS and then placed the compensation tubes in a refrigerator until use. Next, antibodies were added for additional markers and incubated for 30 min at room temperature. For intracellular staining, cells were split into three groups: cells that were stained with both surface and intracellular antibodies, cells that were stained with only surface antibodies and cells that were stained with only intracellular antibodies (Figure 3). Cells were then washed with PBS and stained with Live/Dead as previously described. Next, cells were washed with PBS containing 2% FBS and stained with a cocktail of CD19 antibodies and GAPDH antibodies as a control for 30 min at 4°C. Finally, cells were washed with PBS with 2% FBS.

The authors fixed and permeabilized the cells using the FIX & PERM Cell Fixation and Cell Permeabilization Kit following the manufacturer's instructions. Briefly, FIX & PERM buffer A was added to the cells and incubated at room temperature for 15 min. Cells were washed with PBS containing 5% FBS and 0.1% sodium azide. FIX & PERM buffer B was added to cells with intracellular antibodies, CD19 and GAPDH and incubated at room temperature for 15 min. Excess fluorophores were washed and all cells were then resuspended in PBS containing 2% FBS.

To measure samples, the authors used a panel designed for the Attune NxT flow cytometer. The steps used included checking performance and generating a compensation matrix and then measuring the samples. First, a startup and performance check was done. Following the manufacturer's instructions, the authors started the instrument, computer and software. Next, three drops of performance beads were added to 2 ml of PBS, vortexing before and after, and a performance check was run.

For compensation samples, the lasers to be used were selected in the compensation window (Table 4). Using a small sample size and slow rate, a small amount of each compensation sample was sampled to make sure the voltages were correct (highest peaks for the positive sample with good separation of positive and negative peaks). After voltages were set, compensation samples were measured at a sample rate of 100 μ l/min, and the authors collected 100,000 lymphocyte events. Finally, a compensation matrix was applied (Figure 1). With regard to samples, the authors set up plots for single cell, live/dead and lymphocyte (one for the knockout marker is also recommended). Samples were measured at a sample rate of 200 μ l/min, and 200,000 lymphocyte events were collected.

Although several data analysis options are available for flow cytometry data, the authors used FlowJo software. The authors created a group for compensation and a group for the samples and added the respective flow cytometry standard files. The authors used the compensation wizard to create a compensation matrix and applied it to the samples. The authors then set up the gates for the samples using the fluorescence minus one control samples (Figure 2) and used the layout and table editors to generate plots and tables for the samples.

DNA sequencing

To determine the effect of CRISPR/Cas9 editing on the genome, sequencing analysis of the target site was performed after 72 h and then at periodic time points after cell harvest. Cells were harvested by collecting up to 1.5 ml of cell culture by centrifuging at 100 \times g and washing cells with PBS. The remaining cells were maintained in the incubator for flow cytometry analysis as described earlier and for future sequencing. The authors extracted gDNA using a Quick-DNA Miniprep Plus Kit following the manufacturer's protocol and measured DNA concentration using a NanoDrop. The authors used PCR to amplify the CRISPR/Cas9-edited *CD19* locus using the primers listed in Table 3 and Q5 Hot Start High-Fidelity 2 \times Master Mix; the authors followed the manufacturer's instructions for preparing PCR reaction samples. A total of 500 ng of DNA, the Q5 Hot Start High-Fidelity 2 \times Master Mix, 10 μ M of each primer and nuclease-free water were combined for a 50- μ l reaction.

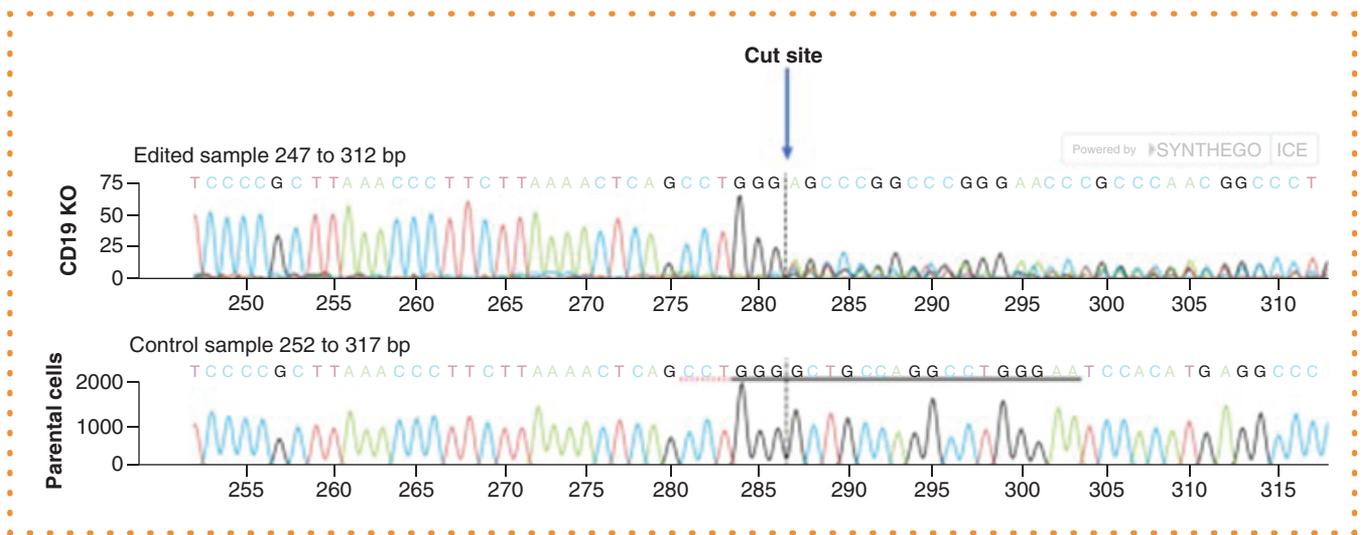


Figure 4. Example Inference of CRISPR Edits analysis result demonstrating edit near the cut site. The defined peaks prior to the targeted CRISPR/Cas9 DNA cut site in the knockout cell line match the parental cell line. At the cut site, the lower-amplitude peaks demonstrate a mixed population of edited cells.

The following conditions were used for PCR in a Veriti thermocycler: denaturation at 95°C for 30 s followed by 35 cycles at 95°C for 5 s, 67°C for 30 s and 72°C for 30 s and a final extension at 72°C for 2 min. The PCR products were cleaned using the DNA Clean & Concentrator kit according to the manufacturer’s instructions and eluted into 25 µl of ultrapure water. The final PCR concentration was determined using a NanoDrop. Clean PCR products were submitted to Psomagen (MD, USA) for Sanger sequencing using their protocol for difficult-to-sequence DNA products. DNA sequences were returned as .ab1 files and analyzed using the Synthego Inference of CRISPR Edits tool to determine editing efficiency (Figure 4). The expected outcome for Sanger sequencing on a mixed population of edited cells is a lower-amplitude signal and mixed signal near the expected CRISPR/Cas9 DNA cut site. Analytics such as the Synthego Inference of CRISPR Edits tool may enable disambiguation of mixed Sanger sequence signal around the cut site.

Additional analysis

For long-term stability analysis, Sanger sequencing and flow cytometry are used at multiple time points following editing. To further analyze the effects of CRISPR/Cas9 editing, transcriptomics, next-generation DNA sequencing and single-cell sequencing should be considered as follow-ups for stability analysis. To ensure that the cell population contains a 100% knockout, cell sorting can be used to isolate knockout cells. Ideally, this would occur over a significant period of time, but culture limitations and cell exhaustion will likely impact this time frame.

Author contributions

S Inwood, with assistance from L Wang and S Maragh, formulated the idea and direction for the manuscript. L Tian and K Parratt worked with S Inwood to complete supporting experiments.

Disclaimer

To specify an experimental procedure as completely as possible, certain commercial materials, instruments and equipment are identified in this manuscript. In no case does identification of the manufacturer of particular equipment or materials imply a recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials, instruments and equipment identified are necessarily the best available for the purpose.

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