

CRISPR/Cas9-mediated Gene Knockout Followed by Negative Selection Leads to a Complete TCR Depletion in *ortho*CAR19 T Cells

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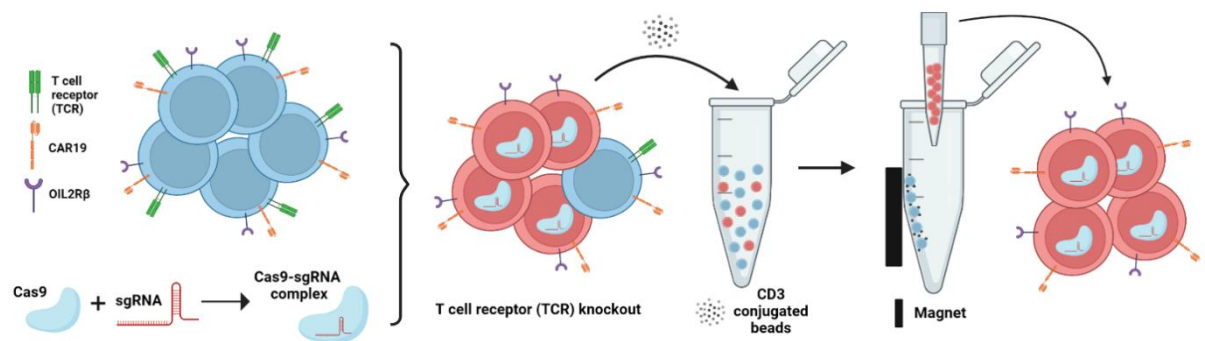
Abstract

Genome-editing technologies, especially CRISPR (clustered regularly interspaced short palindrome repeats)/Cas9 (CRISPR-associated protein 9), endows researchers the ability to make *efficient*, *simple*, and *precise genomic* DNA changes in many eukaryotic cell types. CRISPR/Cas9-mediated efficient gene knockout holds huge potential to improve the efficacy and safety of chimeric antigen receptor (CAR) T cell-based immunotherapies. Here, we describe an optimized approach for a complete loss of endogenous T cell receptor (TCR) protein expression, by CRISPR/Cas9-mediated TCR α constant (TRAC) and TCR β constant (TRBC) gene knockout, followed by subsequent CD3 negative selection in engineered human *ortho*CAR19 T cells. We believe this method can be expanded beyond CAR T cell application, and target other cell surface receptors.

Keywords: CRISPR-Cas9, TCR knockout, CD3 negative selection, *ortho*CAR T cells, TRAC/TRBC guide RNA, 4D-Nucleofector Lonza Electroporator

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Graphical abstract:



Schematic overview of the two-step process of endogenous TCR depletion in engineered human *ortho*CAR19 T cells using (1) CRISPR/Cas9-mediated gene knockout followed by (2) CD3 negative selection.

Background

CRISPR-Cas9 technology enables gene knockouts (KO) and complete loss of protein translation in human cells, especially in primary cells (Seki and Rutz, 2018). In [recently published research](#), we showed that combining an orthogonal human IL-2 and IL-2Rβ (*ortho*-hIL-2/*ortho*-hIL-2Rβ) system with CAR19 T cell therapy markedly enhanced the efficacy of the CAR T cell antitumor activity, in a well-established preclinical model of acute lymphoblastic leukemia (ALL) (Zhang *et al.*, 2021). However, the unexpected deaths observed with high-dose *ortho*-hIL-2 led us to investigate mechanisms of toxicity. To explore whether T cell-mediated toxicity is related to acute xenogeneic graft-versus-host disease (GVHD) mediated through the T cell receptor (TCR), we knocked out the endogenous TCR of CAR T cells co-expressing *ortho*-hIL-2Rβ, using a CRISPR-Cas9 gene KO approach. We use guide RNAs (gRNA) targeting the TCR α constant (TRAC) and TCR β constant (TRBC) loci, followed by CD3 negative selection, resulting in TCR negative *ortho*CAR19 T cells.

Gene silencing is a powerful approach to study gene function and to discern molecular mechanisms underlying complex human diseases. Previous studies have utilized RNA interference (RNAi), small interfering RNA (siRNA), or short hairpin RNA (shRNA) technologies to effectively knock down genes of interest (Berns *et al.*, 2004; O’Keefe, 2013). However, these knockdown approaches do not result in complete loss of gene/protein expression or KO and can often result in off-target effects (Jackson *et al.*, 2006).

The CRISPR-Cas9 genome editing system consists of two components: a “guide” RNA (gRNA) and a Cas9. The Cas9 protein is an endonuclease that uses gRNA to form base pairs with DNA target sequences, enabling Cas9 to introduce a site-specific double-stranded break in the DNA. Through RNA-directed Cas9 nucleases, the CRISPR-Cas9 system can modify DNA with greater precision than existing technologies, such as transcription activator-like endonuclease (TALEN), and zinc-finger nuclease (ZFN) (Conant *et al.*, 2022). While both TALEN and ZFN are artificial structures formed by joining restriction endonucleases with DNA-binding protein domains, CRISPR-Cas9 technology takes advantage of the antiviral defense mechanism of prokaryotes to perform genome editing at the targeted DNA sequence (Khan, 2019). Recent work done by Cui *et al.* revealed that both TALEN and ZFN inevitably generated massive off-target effects when targeting human papillomavirus 16 (HPV16), while the CRISPR-Cas9 system was shown to be more efficient and specific (Cui *et al.*, 2021). Therefore, CRISPR-Cas9 technology shows better efficiency, feasibility, and practicality in multi-role clinical applications, as compared to TALEN and ZFN. Inference of CRISPR Edits (ICE) was used to analyze CRISPR editing data in this study. ICE is a free and easy-to-use software tool that offers fast and reliable analysis of CRISPR experiments, highly comparable to that of next-generation sequencing (NGS) data. CRISPR editing can be assessed and analyzed by uploading the Sanger sequencing data and specifying a guide sequence(s).

A knockout score will be generated, which represents the proportion of cells that have either a frameshift or insertion-deletion mutation (indels). This score is a useful means to quantify the number of contributing indels that are likely to result in a functional KO of the targeted gene.

Our results showed that the CRISPR-Cas9 system is easy to operate, has a short cycle time, low cost, *higher accuracy*, and is more controllable in targeting TCR α and TCR β genes together than existing technologies such as TALEN and ZFN. The combination of CRISPR/Cas9 with CD3 negative selection leads to a complete TCR depletion in *ortho*CAR19 T cells.

Materials and Reagents

1. Tissue culture plates, flasks, and tubes
 - a. 24 well cell tissue culture plates (Corning, catalog number: 3524)
 - b. T25 cell culture flasks (Thermo Fisher, catalog number: 156367)
 - c. T75 cell culture flasks (Thermo Fisher, catalog number: 156499)
 - d. 15 mL centrifuge tubes (Corning, catalog number: 352095)
 - e. 50 mL centrifuge tubes (Corning, catalog number: 430829)
2. Phosphate Buffered Saline solution (PBS) (Corning, catalog number: 21-031-CM)
3. RPMI 1640 medium (Gibco, catalog number: 11875085)
4. Bovine Calf Serum (Sigma, catalog number: 12306C-500ml)
5. Penicillin/Streptomycin (Thermo Fisher, catalog number: 10378016)
6. L-glutamine (Thermo Fisher, catalog number: A2916801)
7. Recombinant human Interleukin-2 (IL2) (R&D System, catalog number: 202-IL-050)
8. APC anti-human TCR α/β antibody (Biolegend, catalog number: 306718)
9. Dynabeads™ CD3 (Thermo Fisher, catalog number: 11151D)
10. TrueCut™ Cas9 Protein v2 (Invitrogen, catalog number: A36497)
11. EasySet Magnet (Thermo Fisher, catalog number: NC9284020)
12. DNeasy Blood & Tissue Kit (Qiagen, catalog number: 69504)
13. Q5 Hot Start High-Fidelity DNA Polymerase Kit (BioLabs, catalog number: E0555S)
14. Trypan Blue Solution, 0.4% (Thermo Fisher, catalog number: 15250061)
15. QIAquick PCR Purification Kit (Qiagen, catalog number: 28106)
16. 4D-Nucleofector™ X Kit (Lonza, catalog number: V4XC-2032)
17. Cell culture media (see Recipes)
18. Wash buffer for cells (see Recipes)
19. gRNA (see Recipes)

Equipment

1. 4D-Nucleofector™ X Unit (Lonza, catalog number: AAF-1002X)
2. Flow Cytometer LSRFortessa (BD Biosciences, catalog number: 647800L6)
3. Eppendorf™ 5424 Microcentrifuges (Fisher, catalog number: 05-400-005)
4. Cell culture hood HeraSafe KS (Thermo Fisher, catalog number: 10110910)
5. CO2 incubator HeraCell VIOS 160i (Thermo Fisher, catalog number: 15381075)
6. VeritiPro™ 96-well Thermal Cycler (Applied Biosystems, catalog number: A47394)

Software

1. Genewiz (Azenta Life Sciences, <https://www.genewiz.com/en>)

2. ICE analysis (Synthego, <https://ice.synthego.com/#/>)
3. CRISPOR (Tefor, <http://crispor.tefor.net/>)
4. FlowJo (Tree Star Inc., version 10.1)
5. Prism 9 for macOS V.9.3.1(350) (<https://www.graphpad.com/scientific-software/prism/>)
6. BioRender (<https://biorender.com/>)

Procedure

A general workflow for the overall experimental procedures can be found in Figure 1.

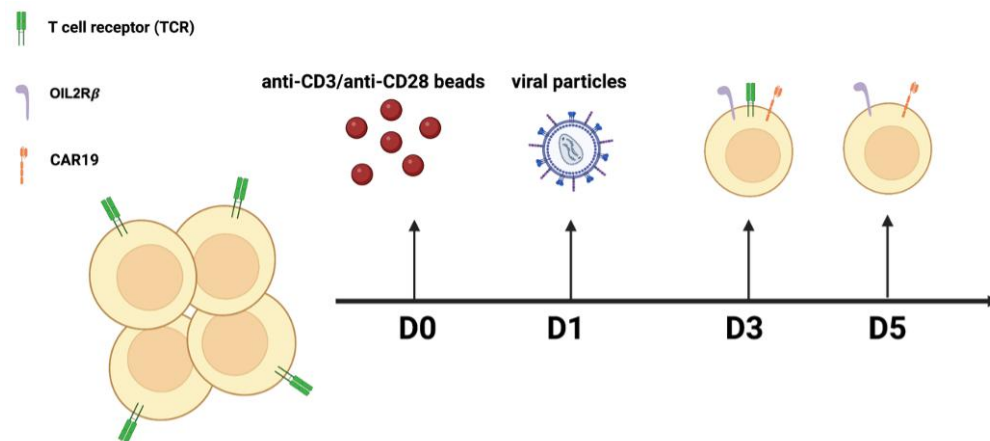


Figure 1. General workflow of the overall experimental procedures.

CRISPR/Cas9-mediated gene knockout followed by CD3 negative selection results in a complete depletion of TCR in *ortho*CAR19 T cells.

A. Preparation of human *ortho*CAR19 T cells

1. Primary human total T cells were purchased from the Immunology Core of University of Pennsylvania.
2. Count cells and adjust to 1×10^6 cells/mL in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100-U/mL penicillin, 100-g/mL streptomycin sulfate, and 10-mM HEPES. Assess cell viability using the Trypan Blue solution (0.4%, Thermo Fisher) as a cell stain for the dye exclusion test.
3. Wash the magnetic beads coated with anti-CD3/anti-CD28 three times with PBS, and once with cell culture media.
4. Mix T cells with the washed beads at a ratio of 1:3, for 16–24 h.
5. Transduce activated T cells with viral particles encoding *ortho*CAR19, as described by our previous work (Zhang *et al.*, 2021). Briefly, both CD19BBz and *ortho*-hIL-2Rβ sequences were cloned in a p-TRPE lentiviral backbone, in frame with the restriction site XbaI and SalI for CD19BBz, *ortho*-hIL-2Rβ-T2A-mCherry, and CD19-*ortho*-hIL-2Rβ. Lentivirus was produced by transfecting HEK293T cells with a combination of the above transfer plasmid and three lentiviral package plasmids, pCI VSV-G, pRSV Rev, and pMDL gag/pol.RRE (Addgene).
6. Culture cells with 100 IU/mL human recombinant IL2, and feed them every day for three days, in preparation for CRISPR/Cas9-mediated TCR KO.

B. Preparation of the CRISPR-Cas9 complex

1. TCRα and TCRβ gRNA sequence (TRAC gRNA sequence: TGTGCTAGACATGAGGTCTA; TRBC

gRNA sequence: GGAGAATGACGAGTGGACCC) were designed by using CRISPOR, an online program that helps design, evaluate, and clone guide sequences for the CRISPR/Cas9 system. The main considerations regarding potential gRNA candidates are for finding the gRNAs that (1) efficiently KO the gene close to ATG (translation start site) for maximum loss of function, or at the location of functional domain to disrupt the target function; (2) have no or less off-target genes.

The single human genomic sequence used in the TRAC and TRBC gRNA design was CTTCTTTGTCCCAATCTGGGCGCGCGCCGGCGCCCCCTGGCGGCCTAAGGACTCGGCGCGCCGGAAGTGGCCAGGGCGGGGGCGACCTCGGCTCACAGCGCGCCCGGCTATTCTCGCAGCTCACCATGGATGATGATATCGCCGCGCTCGTCGTCGACAACGGCTCCGGCATGTGCAAGGCCGCTTCGCGGGCGACGATGCCCCCGGGCCGTCTTCCCCTCCATCGTGGGGCGCC, with a protospacer adjacent motif (PAM) sequence of 20bp-NGG-Sp Cas9, SpCas9-HF1, eSpCas9 1.1.

2. Dissolve TCR α and TCR β gRNAs (Agilent) in 1 \times TE buffer (IDT) at 10 μ g/ μ L, and mix them together in equimolar concentrations.
3. Combine 1 μ L of gRNA mixture, including 5 μ g TCR α and 5 μ g TCR β , with 2 μ L (5 μ g/ μ L) of Cas9 (Invitrogen).
4. Mix the gRNAs-Cas9 complex mixture by gently pipetting up and down twice; avoid creating air bubbles in the tube.
5. Incubate the complex mix at room temperature (RT: 15–25 °C) for 10 min.

C. Preparation of T cell suspension for electroporation

1. For each electroporation condition (including positive and negative controls), prepare 5 mL of cell expansion medium supplemented with 10% FBS, 2 mM L-glutamine, 100-U/mL penicillin, 100-g/mL streptomycin sulfate, 10-mM HEPES, and 100U IL2.
2. For each condition, add 2 mL of supplemented medium (prepared in Procedure A) to a 12-well plate (for 100- μ L tip reactions), and place in a 37°C incubator.
3. Transfer 1.5×10^6 cells (for 100- μ L cuvette reactions) from the *ortho*CAR19 T cells to a 15-mL tube. Centrifuge at 300 \times g and RT for 5 min.

D. Electroporation of T cells with gRNA-Cas9 complex using the Lonza 4D system

A demonstration of how to perform electroporation using Lonza 4D system can be found in Figure 2.

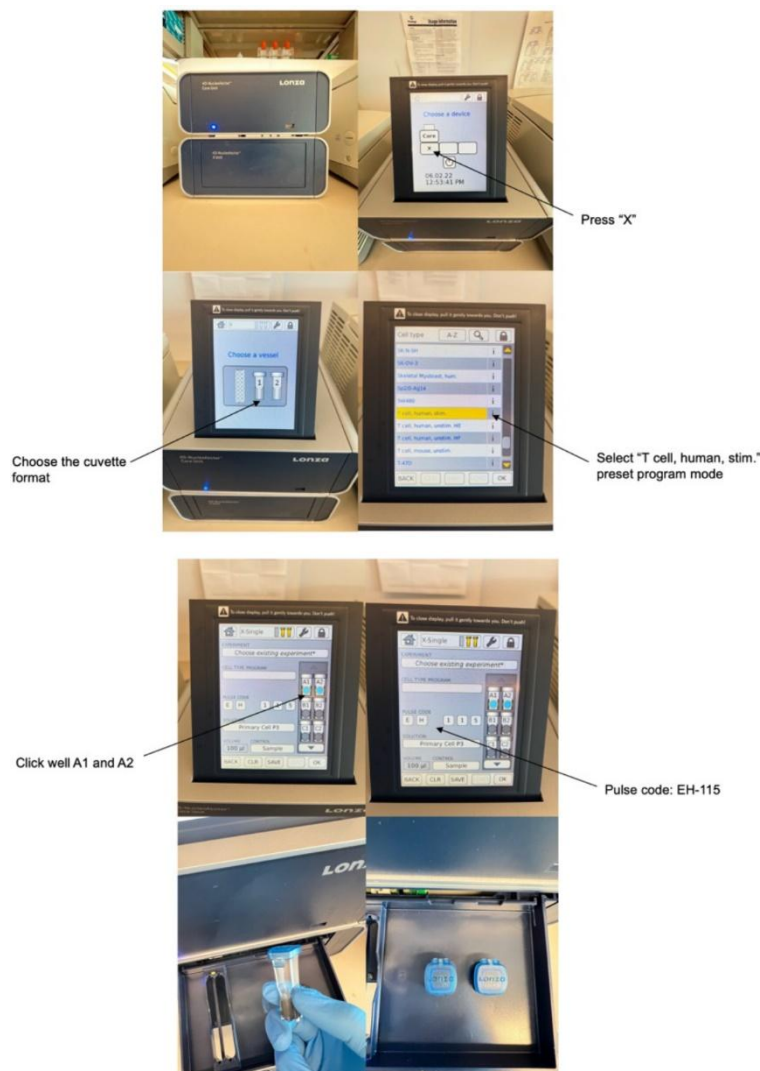


Figure 2. A demonstration of how to perform electroporation using Lonza 4D system.

1. Aspirate the supernatant from the cell pellet (prepared in Procedure C). Resuspend cells in 100 μ L of P3 buffer from the 4D-Nucleofector X Kit and pipette up and down to mix.
2. Using sterile 200- μ L PCR tubes for each condition, combine the gRNA-Cas9 complex mixture (prepared in Procedure B) with cells in P3 buffer.
3. Mix gently by pipetting up and down carefully twice; avoid creating bubbles in the tube.
4. Transfer cells with the gRNA-Cas9 complex mixture into a cuvette from the 4D-Nucleofector X Kit, making sure that there are no air bubbles. Place the cuvette into the electroporation chamber.
5. Electroporate the cell mixture using the "human stim T-cell" preset program and a pulse code of EH-115.

E. Post-knockout culture

1. Immediately add culture media into the cuvette containing the electroporated cells, and incubate at 37 $^{\circ}$ C for 15 min.
2. Count cells, and adjust cell density to 0.8×10^6 cells/mL, by adding IL2-supplemented cell culture media.
3. Incubate at 37 $^{\circ}$ C for 72 h, for genome editing to occur.
4. Harvest cells for assessment of genome editing efficiency. Isolate and amplify the genomic DNA using

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primers that flank the target region. Sequence the PCR product.

PCR kit used: Q5 Hot Start High-Fidelity DNA Polymerase Kit

5. Harvest cells and check for TCR protein expression with flow cytometry, using APC human anti-TCR α/β .

F. Isolation of CD3 negative T cells

1. Negative selection of CD3⁺ cells is performed using Dynabeads CD3 (Thermo Fisher Scientific).
2. Wash the Beads
 - a. Resuspend the beads in the vial.
 - b. Transfer the desired volume of beads to a 5-mL tube.
 - c. Add the same volume of Isolation Buffer (PBS supplemented 0.1% BSA).
 - d. Place the tube in a magnet for 1 min, and discard the supernatant.
 - e. Remove the tube from the magnet and resuspend the washed beads in the same volume of Isolation Buffer as the initial volume of beads.
3. Prepare Cells
 - a. Prepare TCR KO T cells to 1×10^7 cells/mL in Isolation Buffer.
 - b. Transfer 1 mL of cells (1×10^7) to a tube, and add 25 μ L of pre-washed and resuspended beads.
 - c. Incubate with gentle tilting and rotation at 2–8 °C for 30 min (depletion).
 - d. Place the tube in a DynabeadsTM magnetic rack for 2 min.
4. Transfer the supernatant to a new tube for further use, and discard the beads.

G. Flow cytometry

1. Wash the CD3 negative T cells twice with PBS, and stain with APC anti-human TCR α/β antibody (1:50, Biolegend) for 30 min, followed by two washes with PBS.
2. Quantify CD3 negative T cells using a BD LSRFortessa flow cytometer.
3. Perform analysis of T cell CD3 expression, using FlowJo software (Tree Star Inc., version 10.1).

Data analysis

The expression of CAR19 and orthogonal IL-2R β was confirmed by flow cytometry before performing TCR KO (Figure 3). Flow cytometry data were analyzed using the FlowJo software. For direct estimation of the efficiency and the type of mutations introduced by genome editing, genomic DNA was extracted from edited and non-edited T cells. PCR reactions were performed with primers surrounding the guide RNA, and the PCR products were visualized in 1.0% gel (Figure 4), and purified for standard Sanger sequencing (Genewiz). Once Sanger sequencing of the DNA was complete, the sequence traces were analyzed using ICE (Figures 5 and 6A-D). To completely deplete the unedited TCR positive cells, CD3 negative selection was performed using CD3 DynabeadsTM. The purity of the TCR α/β depletion was *assessed by flow cytometry* (Figure 6E); a demonstration of the flow cytometry gating strategy used in this study can be found in Video 1.

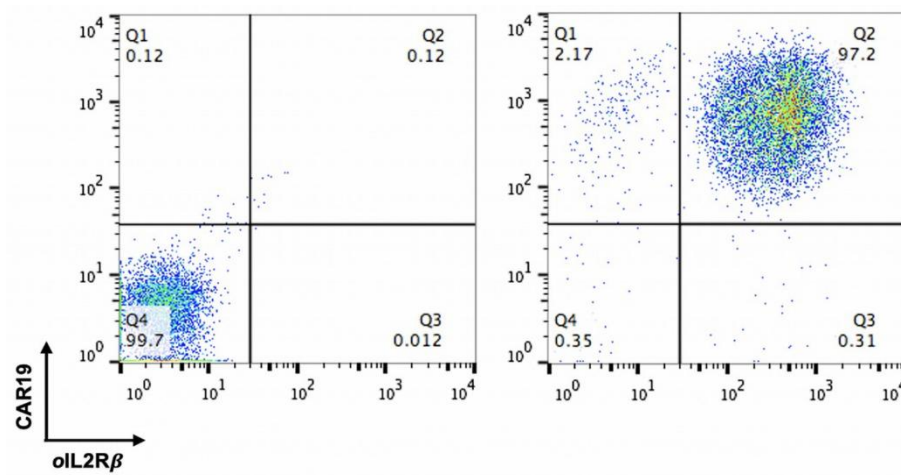


Figure 3. FACS analysis of CAR19 and *ortho*-IL2R β expression in T cells.

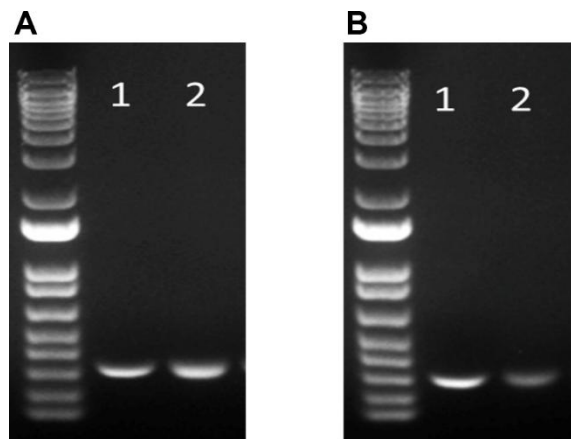
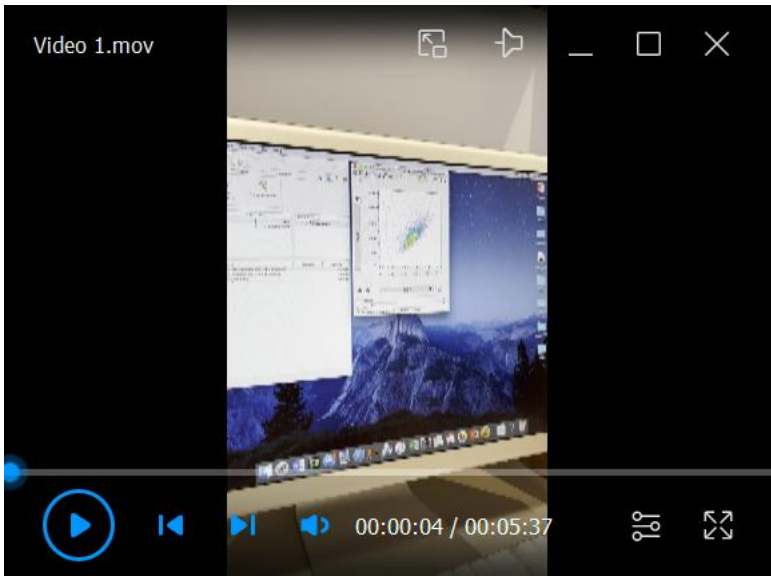


Figure 4. Agarose gel electrophoresis results for the analysis of PCR products.

(A) TCR α . (B) TCR β . Lane 1: Control (without TCR gRNA) Lane 2: TCR KO + CD3 negative selection.



Video 1. Demonstration of the flow cytometry gating strategy for the confirmation of complete TCR depletion.



Figure 5. Purification and sequencing of PCR products with a quantitative assessment of genome editing of TCRα/β knockout by using Synthego's Inference of CRISPR Edits (ICE).



Figure 6. Sequencing results of TCR α/β KO visualized using ICE and flow cytometric assessment of

TCR α / β depletion on day 4 after gene editing followed by CD3 negative selection.

Samples of TCR α and TCR β KO were successfully analyzed with multiple parameters shown, including sample label, ICE score, R² score, knockout score, guide sequence, and PAM sequence. (A–B) The “contributions” tab shows the inferred sequences of TCR α (A) and TCR β (B) that are present in an edited population, as well as their relative representation in the edited pool. The black vertical dotted line represents the cut site. (C–D) Sequencing chromatograms of TCR α KO vs. TCR α wild type (WT) (C) and TCR β KO vs. TCR β WT (D). (E) FACS analysis was used to confirm complete TCR α / β depletion after CD3 negative selection.

Recipes

1. Cell culture media

RPMI supplemented with 10% heat-inactivated FBS, 1% L-glutamine, 1% HEPES, 1% penicillin, and streptomycin.

2. Wash buffer for cells

PBS supplemented with 2% BSA

3. gRNA resuspended in TE buffer at 5 μ g/ μ L

Acknowledgments

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Competing interests

The authors have no financial and/or non-financial competing interests to disclose.

Ethics

T cells were collected under a University Institutional Review Board-approved protocol, and written informed consent was obtained from each healthy donor.

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