Achieve functional knockout in up to 90% of human primary T cells

Introduction

The powerful CRISPR gene editing technology has the potential to transform research at an astonishing rate. The rate of success is dependent upon many factors, such as gRNA design, Cas9 efficiency, and delivery conditions. Through years of experience, we've refined and optimized each factor to help ensure maximum editing efficiency across a broad spectrum of cell types, including iPSCs and primary cells. Here we demonstrate an end-to-end workflow, from T cell isolation and activation using Invitrogen™ Dynabeads™ bead-based kits to gene editing, using award-winning Invitrogen™ TrueCut™ Cas9 Protein v2, TrueGuide™ Modified Synthetic sgRNA, and the Neon™ Transfection System. TrueCut Cas9 Protein v2 is a next-generation, ready-to-transfect Cas9 protein manufactured under strict ISO 13485 quality standards and engineered to deliver maximum editing efficiency. TrueGuide Synthetic sgRNA is ready-to-transfect single guide RNA (sgRNA) designed and validated to work with the Invitrogen™ suite of genome editing tools to provide consistent, high-efficiency editing.

High editing efficiency is especially important when working with difficult-to-edit and valuable cells such as primary cells, immune cells, and stem cells. By combining the power of our high-quality and efficient CRISPR-Cas9 RNP system along with the protocols described here for T cell isolation and gene editing, we were able to demonstrate over 90% functional knockout of the T cell receptor in donor-derived primary T cell populations (Figure 1).

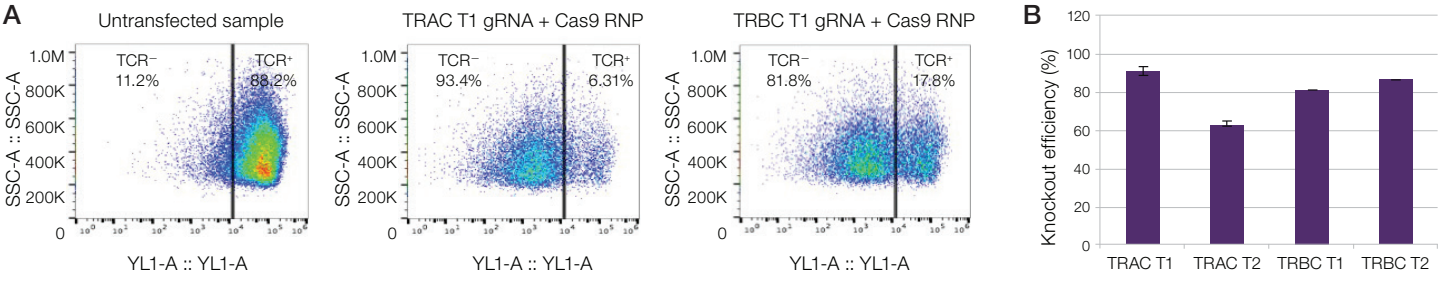

Figure 1. High-efficiency functional knockout in T cells. T cells were isolated from PBMCs (from a healthy donor) using Dynabeads magnetic beads, and then transfected with TrueCut Cas9 Protein v2 and TrueGuide Modified Synthetic sgRNAs targeting T cell receptor alpha (TRAC) or beta (TRBC) regions using the Neon Transfection System. **(A)** Analysis by flow cytometry following binding with antibody specific to the T cell receptor (TCR) shows >90% functional knockdown of the receptor. For both TRAC and TRBC, gRNAs specific for two different genomic DNA targets (T1 and T2) were tested, and results are shown only for the T1 target in each case. **(B)** Summary of NGS-based analysis of cleavage efficiency at two different genomic DNA targets (T1 and T2) for both TRAC and TRBC loci.

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Workflow

The workflow for high-efficiency gene editing in primary T cells is shown in Figure 2.

T cell receptor–specific gRNA design

In humans, the T cell receptor (TCR) is composed of an alpha (α) chain and a beta (β) chain encoded by the *TRA* and *TRB* genes, respectively. For the TCR knockout application data described here, we chose two targets within the TCR alpha or beta constant regions (TRAC or TRBC) that have been described in previous publications [1]. The TRAC- or TRBC-specific target sequences are also listed in Table 1. The TrueGuide Modified Synthetic sgRNAs were ordered through **thermofisher.com/trueguide.**

TrueGuide Modified Synthetic sgRNA working stock

TrueGuide Modified Synthetic sgRNAs are shipped as dried-down RNA oligos. Working solutions of gRNA need to be made before delivery into cells. For a detailed protocol, please refer to the TrueGuide Synthetic gRNA user guide at **thermofisher.com/trueguide**. The TrueGuide Modified Synthetic sgRNAs were resuspended in 1X Invitrogen™ TE buffer, pH 8.0 (Cat. No. AM9849), to obtain a 100 µM stock, and were placed at –20°C for long-term storage. On the day of transfection, an aliquot of the resuspended stock was then further diluted to a final concentration of 20 µM with 1X TE buffer, pH 8.0.

Isolation of T cells

T cells were isolated from peripheral blood mononuclear cells (PBMCs) derived from a healthy donor, using the Invitrogen™ Dynabeads™ Untouched™ Human T Cells Kit (Cat. No. 11344D). Please refer to the Dynabeads Untouched Human T Cells Kit user manual for a detailed protocol, including PBMC media preparation and T cell isolation [2]. Note: Gibco™ OpTmizer™ CTS™ T Cell Expansion Basal Medium requires supplementation with OpTmizer™ CTS™ T Cell Expansion Supplement and Gibco™ GlutaMAX™ Supplement. Please refer to the manual for additional details [3].

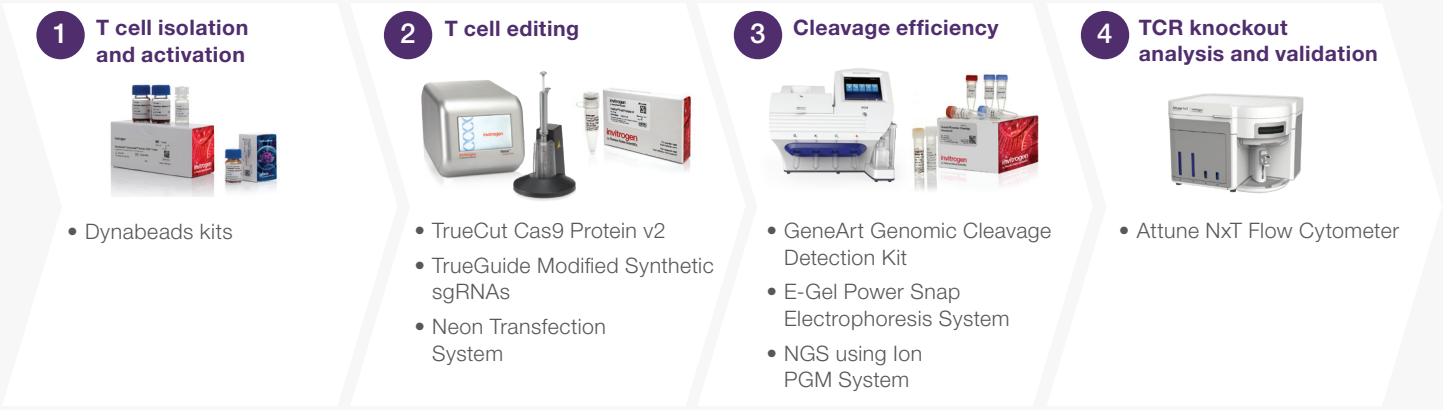

Figure 2. An end-to-end workflow solution for primary T cell editing.

Table 1. gRNA target sequences and primers for next-generation sequencing (NGS) assay.

Target name	Target sequence	NGS forward primer	NGS reverse primer
TRAC T1	AGAGTCTCTCAGCTGGTACA	GTGACTTGCCAGCCCCAC	GTTGCTCCAGGCCACAGC
TRAC T2	TGTGCTAGACATGAGGTCTA	CCTGATCCTCTTGTCCCACAG	CTGCGAAGGCACCAAAGCTG
TRBC T1	GCAGTATCTGGAGTCATTGA	GCCACACTGGTGTGCCTG	CGATCTGGGTGACAGGTTTG
TRBC T2	GGAGAATGACGAGTGGACCC	GTCAGCACAGACCCGCAG	CTAGTCTTGTCCGCTACCTGG

Prepare a PBMC suspension according to the "General Guidelines" in reference 2. Resuspend the cells at 1 x 10^8 cells/mL in Isolation Buffer. The PBMC suspension can be cryopreserved and stored in a liquid nitrogen tank if not proceeding to the isolation procedure. This isolation procedure is for 5 x 10^7 PBMCs.

1. Transfer 500 µL (5 x 10^7) of PBMCs in Isolation Buffer to a sterile tube.
2. Add 100 µL of 25% human serum albumin (HSA).
3. Add 100 µL of Antibody Mix.
4. Mix well and incubate for 20 min at 2°C to 8°C with gentle tilting and rotation.
5. Wash the cells by adding 4 mL Isolation Buffer. Mix well by tilting the tube several times, and centrifuge at 250 x *g* for 11 min (or 1,100 rpm for 10 min) at ambient temperature (20°C). Discard the supernatant.
6. Resuspend the cells in 500 µL Isolation Buffer.
7. Add 500 µL prewashed Dynabeads magnetic beads.
8. Incubate for 15 min at 18°C to 25°C with gentle tilting and rotation.
9. Add 4 mL Isolation Buffer. (Note: When working with lower cell volumes, never use less than 1 mL Isolation Buffer.)
10. Thoroughly mix the suspension of beads and cells by pipetting >10 times using a pipette with a narrow tip opening. Avoid foaming.
11. Place the tube in the Invitrogen™ DynaMag™ magnet for 3 min. Transfer the supernatant containing the untouched human T cells to a new, larger tube.
12. Add 4 mL Isolation Buffer to the tube containing the Dynabeads magnetic beads and resuspend the bead-bound cells by pipetting as described in step 10.
13. Place the tube in the magnet for 3 min.
14. Combine the two supernatants that contain the T cells.

The purified T cells can be used directly for activation, or cryopreserved (500 µL per vial) and stored in a liquid nitrogen tank.

Activation of T cells

Post-isolation T cells were activated with Invitrogen™ Dynabeads™ Human T-Expander CD3/CD28 (Cat. No. 11141D) for 72 hours. Please refer to the Dynabeads Human T-Expander CD3/CD28 user manual for a detailed protocol [4].

1. Thaw previously purified T cells and slowly add the cells to Incomplete Medium (15–20 million cells per 5 mL medium).
2. Count cells and spin down the cells at 1,500 rpm for 5 min at room temperature (RT). Resuspend the cells in Complete Medium to a final concentration of 1 x 10^6 cells/mL.
3. Add washed Dynabeads Human T-Expander CD3/CD28 at a 3:1 ratio of beads to $CD3^+$ T cells.
   Note: The ratio of beads to $CD3^+$ T cells can be dropped to as low as 1:1 when the starting T cell pool is more prone to activation-induced cell death or when short-term activation is desired.
4. Plate 5 x 10^6 $CD3^+$ cells in a total volume of 5 mL in a T-25 flask. Place in a humidified incubator at 37°C and 5% CO_2 for 3 days.
5. At the end of culture (after 72 hours, and the day of electroporation using the Neon Transfection System [5]), count the cells, remove the beads with a magnet, and proceed to electroporation.

Transfection of Cas9 RNP in T cells

To maximize transfection and editing efficiency in T cells, we recommend using the Neon Transfection System. In this work, 250 ng (7.5 pmol) of TrueGuide Modified Synthetic sgRNA and 1,250 ng of TrueCut Cas9 Protein v2 were delivered into activated T cells in 24-well format using the Invitrogen™ Neon™ Transfection System 10 µL Kit with Resuspension Buffer R and protocol 24 (1,600 V, 10 ms, 3 pulses). Transfected cells were then incubated at 37°C in a humidified CO_2 incubator for 48 hours. For a detailed Cas9–gRNA delivery protocol using the Neon Transfection System, please refer to the TrueGuide Synthetic gRNA or TrueCut Cas9 Protein v2 user manual [6,7].

1. On the day of transfection using the Neon Transfection System, add 1,000 µL of Gibco™ OpTmizer™ CTS™ T Cell Expansion SFM into each well of a 24-well plate and place it in the 37°C incubator to prewarm.

2. Prepare the Cas9 RNP complex in Resuspension Buffer R as follows, and always include extra volume to avoid pipetting errors and creating bubbles:

   a. Mix the TrueCut Cas9 Protein v2, TrueGuide Modified Synthetic sgRNA, and Resuspension Buffer R in an RNase-free microcentrifuge tube according to the following table. Mix well.
   Important: Maintain the molar ratio of Cas9 protein to sgRNA at 1:1.

Reagent	Quantity
TrueCut Cas9 Protein v2*	1,250 ng (7.5 pmol)
TrueGuide Synthetic gRNA (crRNA:tracrRNA duplex or sgRNA)*	250 ng (7.5 pmol)
Resuspension Buffer R	5.0 µL

   * Use high-concentration Cas9 protein, and ensure that the total volume of the Cas9 RNP complex (Cas9 protein plus gRNA) does not exceed 1/10 of the total reaction volume (e.g., 1 µL of Cas9 protein plus gRNA in 10 µL total reaction volume).

   b. Incubate the mixture at room temperature for 5–20 min.

3. Collect T cells and determine cell density. Resuspend the cells in Resuspension Buffer R at a density of 4 x 10^7 cells/mL. To avoid pipetting errors and bubbles, we recommend preparing an extra amount of cells (1.5–2x more cells).

4. Pipette the cell suspension up and down to resuspend any cells that might have settled at the bottom of the tube. For each reaction, add 5 µL of the cell suspension to the Cas9–gRNA mixture in Resuspension Buffer R (from step 2).

5. Using the 10 µL Neon™ tip, aspirate the 10 µL of cells mixed with Cas9–gRNA complex in Resuspension Buffer R, then electroporate with optimization protocol 24 (1,600 V, 10 ms, 3 pulses).
   Important: Avoid creating bubbles that can hinder electroporation.

After electroporation, immediately transfer the contents of the Neon tip into one well of the 24-well culture plate containing 1,000 µL of prewarmed growth medium (from step 1). After all electroporated cells are transferred into their respective wells, incubate the plate at 37°C in a humidified CO_2 incubator for 48 hours.

Validation of T cell receptor knockout

After 48 hours of incubation, a portion of the cells were stained with PE Anti–Human α/β TCR Antibody (BioLegend, Cat. No. 306708) for flow cytometry analysis. The Invitrogen™ Attune™ NxT Flow Cytometer was used with the following protocol to quantify TCR knockout efficiency by measuring the percentage of TCR-negative cells in the population.

1. For each transfected sample, add 200 µL of T cells to a 5 mL flow tube.

2. Add 5 µL of PE Anti–Human α/β TCR Antibody to each tube.

3. Incubate the T cells with the antibody for 20 min at RT.

4. Wash the cells with PBS and centrifuge at 1,500 rpm for 5 min at RT. Resuspend cells in 500 µL PBS.

5. Run samples on the Attune NxT Flow Cytometer to quantify the percentage of TCR-negative cells.

Measurement of genome editing efficiency by NGS
The editing efficiencies at targeted loci in T cells were measured by NGS using the Ion PGM™ System. Primers for NGS are listed in Table 1.

For each sample, the remaining cells were collected into a 1.5 mL microcentrifuge tube. A total of 100 µL lysis buffer containing Proteinase K was added to each tube, and the mixture was pipetted up and down to resuspend the cells and transferred to a 0.2 mL PCR tube. Note: In this case we used lysis buffer from the Invitrogen™ GeneArt™ Genomic Cleavage Detection Kit and added Proteinase K as recommended.

The PCR tubes were then placed in a thermal cycler and the following program was run: 68°C for 15 min, 98°C for 10 min, 4°C hold. The samples were then prepared as an amplicon library for Ion Torrent™ NGS sequencing using the Ion Xpress™ Barcode Adapters 1-96 Kit (Cat. No. 4474517) combined with the Ion Xpress™ Plus Fragment Library Kit (Cat. No. 4471269) and Ion PGM™ Hi-Q™ Chef Kit (Cat. No. A25948). For a detailed protocol, please refer to the user manuals [8,9].

Results

Using the protocols described here for TrueCut Cas9 Protein v2 and TrueGuide Modified Synthetic sgRNA, we were able to achieve over 90% knockout of the T cell receptor.

Validation of T cell receptor knockout
CRISPR-Cas9 RNP targeting TRAC or TRBC cleaves DNA at the target site and makes double-stranded breaks. These breaks can then be repaired via nonhomologous end joining (NHEJ), which introduces small insertions or deletions (indels) at the cleavage site and knocks out TCR function. The PE Anti–Human α/β TCR Antibody reacts with a monomorphic determinant of the α and β subunits of the TCR. Therefore, following antibody staining and flow cytometry, cells expressing the TCR can be distinguished from those that do not express the TCR. Flow cytometric analysis showed that 92% of untransfected cells expressed the TCR (Figure 1A). For both TRAC- and TRBC-targeted samples, one out of the two gRNAs resulted in >90% TCR-negative cells. The TCR knockout efficiency data for all 4 targets were normalized and compared to the untransfected samples (Figure 1B).

Editing efficiency of CRISPR-Cas9 RNP for TCR gene knockout
The results from NGS assays indicated high editing efficiency using our CRISPR-Cas9 system in primary T cells. The indels mostly occurred proximal to the protospacer adjacent motif (PAM) sequence. The percentages of indels are summarized and shown in Figure 1B.

Conclusions

In this work, we showed that our CRISPR-Cas9 RNP system comprising TrueCut Cas9 Protein v2 and TrueGuide Modified Synthetic sgRNA can be efficiently delivered into primary T cells using the Neon Transfection System, and resulted in high genomic DNA cleavage efficiency and functional knockout. In both the TRAC and TRBC regions, at least 1 out of the 2 target-specific gRNAs effected >90% functional knockout. The suite of reagents and protocols described here enables a streamlined, end-to-end solution for maximizing editing efficiency in primary T cells.

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Ordering information

Product	Cat. No.
TrueGuide Modified Synthetic sgRNA	A35511
TrueCut Cas9 Protein v2	A36499
TE, pH 8.0	AM9849
Dynabeads Untouched Human T Cells Kit	11344D
OpTmizer CTS T Cell Expansion SFM	A1048501
Dynabeads Human T-Expander CD3/CD28	11141D
Ion Xpress Barcode Adapters 1-96 Kit	4474517
Ion Xpress Plus Fragment Library Kit	4471269
Ion PGM Hi-Q View Chef Kit	A29902
Additional products for T cells	thermofisher.com/cts

References

1. Ren J, Liu X, Fang C et al. (2017) Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition. *Clin Cancer Res* 23(9):2255-2266.
2. Dynabeads Untouched Human T Cells Kit (user manual), Pub. No. SPEC-06428.
3. OpTmizer CTS T Cell Expansion SFM (user manual), Pub. No. MAN0007325.
4. Dynabeads Human T-Expander CD3/CD28 (user manual), Pub. No. SPEC-06245.
5. Neon Transfection System (user guide), Pub. No. MAN0001557.
6. TrueGuide Synthetic gRNA (user guide), Pub. No. MAN0017058.
7. TrueCut Cas9 Protein v2 (user guide), Pub. No. MAN0017066.
8. Prepare Amplicon Libraries without Fragmentation Using the Ion Plus Fragment Library Kit (user bulletin), Pub. No. MAN0006846.
9. Ion PGM Hi-Q Chef Kit (user guide), Pub. No. MAN0010919.

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