TECHNICAL BULLETIN

Genome Editing of Human Primary T Cells Using the ArciTect™ CRISPR-Cas9 System and ImmunoCult™

Introduction

Through targeted modification of specific genes or regulatory regions with CRISPR genome-editing technology, researchers can now rapidly generate precise genetic models to study normal and diseased cell physiology. Beyond genetic manipulation for research purposes, CRISPR-Cas9 genome editing also holds great potential for therapeutic applications, including immunotherapy and regenerative medicine. Early attempts to apply CRISPR-Cas9 for genome editing in human primary T cells used either viral vectors[1,2] or plasmids[3,4] for Cas9 and gRNA expression, resulting in low targeting efficiency and high toxicity. More recently, ribonucleoprotein (RNP)-based CRISPR-Cas9 expression systems have achieved high efficacy across a number of targets.[5-7]

The following document provides instructions for genome editing of human primary T cells using an RNP-based CRISPR-Cas9 system, including optimization of pre- and post-editing culture conditions and methods to evaluate genome editing efficiency. The protocol details instructions for isolation and activation of primary human T cells, preparation of a CRISPR-Cas9 RNP complex using sgRNA or crRNA:tracrRNA duplexes, and delivery of RNP complex into activated primary T cells using electroporation (Figure 1). We describe one strategy to generate knockout primary T cells with high efficiency, and present a case study and data on CRISPR-Cas9 genome editing of the TCRαβ locus across a number of activation conditions; see the discussion section of this document for alternative strategies and expected outcomes, as well as protocol modifications.

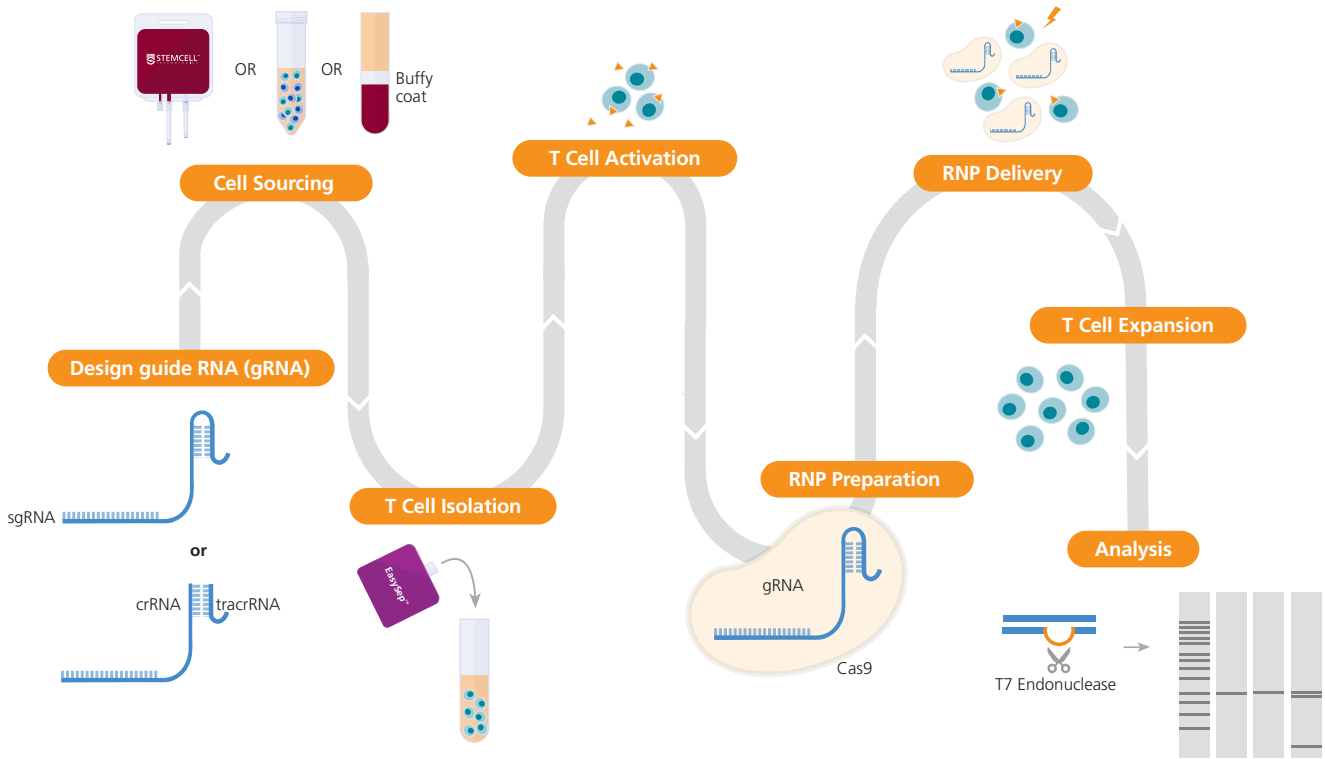


Figure 1. Experimental Workflow for T Cell Genome Editing

The ArciTect™ sgRNA (single guide RNA) or ArciTect™ crRNA (CRISPR RNA) sequences can be designed using the CRISPR Design Tool once a target locus for editing is identified. Human primary T cells* can be sourced from STEMCELL or isolated from a number of sources, including whole blood, buffy coat, washed leukapheresis samples, or peripheral blood mononuclear cells (PBMCs), using STEMCELL's column-free cell separation technology, including EasySep™. Next, T cells are activated with either ImmunoCult™ CD3/CD28 T Cell Activator or ImmunoCult™ CD3/CD28/CD2 T Cell Activator. The ArciTect™ CRISPR-Cas9 RNP is then prepared and delivered into T cells using electroporation, and cells are expanded in ImmunoCult™-XF T cell expansion Medium. Editing efficiency can be analyzed using the ArciTect™ T7 Endonuclease I Kit or flow cytometry, if the experimental design permits. Orange triangles indicate ImmunoCult™ Human T Cell Activator.

*Certain products are only available in select territories. Please contact your local Sales representative or the Product & Scientific Support team at techsupport@stemcell.com for further information.

Background

The mechanism of CRISPR-Cas9 genome editing is shown in Figure 1. The CRISPR-Cas9 system is composed of two key components: a guide RNA (gRNA) and a CRISPR-associated endonuclease (Cas) protein. Briefly, Cas9 endonuclease is targeted to the desired genomic locus through complementary base pairing of the associated single guide RNA (sgRNA) or CRISPR RNA (crRNA) at a site 5′ to a protospacer adjacent motif (PAM) sequence (NGG for Streptococcus pyogenes Cas9). The crRNA partners with a trans-acting CRISPR RNA (tracrRNA), which acts as a scaffold for the interaction with Cas9, to form the guide RNA (gRNA). Once complexed with the gRNA, Cas9 is targeted to the desired genomic locus and will generate a DNA break 3 to 4 bases upstream of the PAM sequence. Endogenous DNA repair systems then function to repair the DNA break. Non-homologous end joining (NHEJ) mediates direct ligation of the broken DNA molecule, and due to the error-prone nature of NHEJ, this can result in the formation of insertion or deletion (INDEL) mutations. If a donor DNA sequence template is provided, knock-in of precise DNA sequences can be accomplished through the homology-directed repair (HDR) pathway (Figure 2).

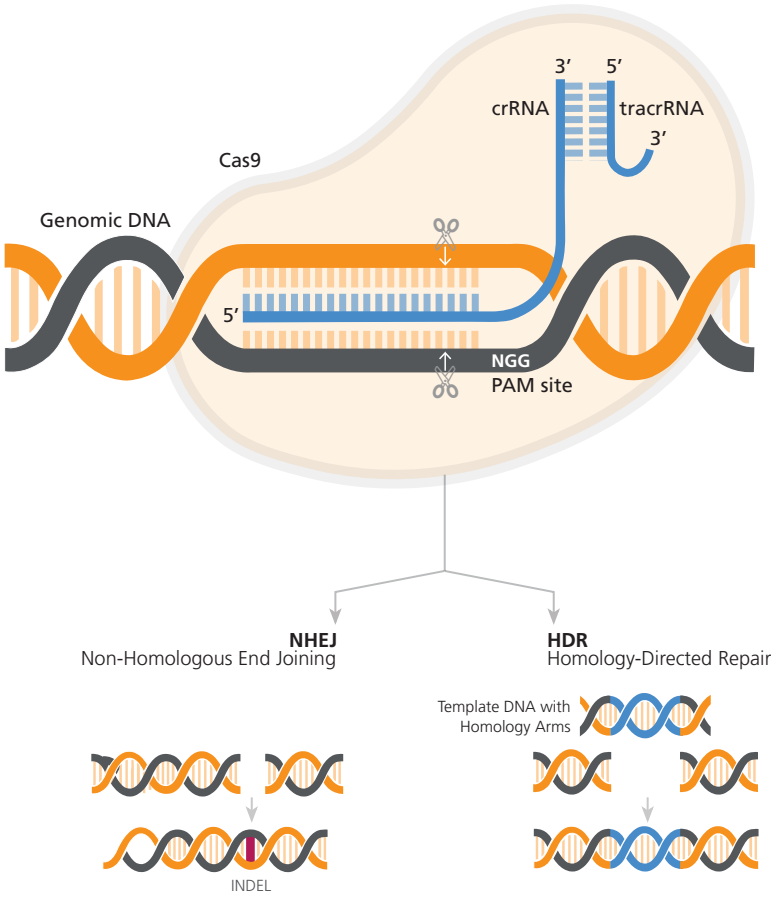


Figure 2. General Mechanism of CRISPR-Cas9 Genome Editing

The RNP complex (Cas9 partnered with gRNA) orientation with respect to target and PAM sites. Cas9 generates a DNA break 3 - 4 bp upstream of the PAM sequence, triggering endogenous DNA repair pathways. This can result in the formation of INDELs if repair occurs via the NHEJ pathway or knock-in of precise sequence changes via the HDR pathway, if template DNA is included in experimental design.

While this technology has been successfully applied in numerous cell lines, its application in primary human immune cells has been hampered by challenges in efficient delivery and expression of CRISPR-Cas9 components. As mentioned before, early attempts to apply CRISPR-Cas9 for genome editing in primary human T cells used either viral vectors[1,2] or plasmids[3,4] for Cas9 and gRNA expression, which resulted in low targeting efficiency and high toxicity. These expression systems also pose concerns about safety in clinical translation due to the risk of unwanted genetic mutations and immunogenicity.[5] More recently, electroporation of activated T cells with ribonucleoprotein (RNP) complexes made from recombinant Cas9 protein and in vitro-transcribed (IVT) or synthetic gRNAs has achieved high efficacy across a number of targets.[6-10] In many cell types, including human T cells, IVT gRNAs cause high cytotoxicity due to the presence of a 5' triphosphate single-stranded RNA (ssRNA) that activates a type I interferon-mediated immune response.[11] Synthetic gRNAs are therefore a preferred format for the RNP-based method. ArciTect™ is an RNP-based CRISPR-Cas9 expression system that includes custom synthetic gRNA (sgRNA and crRNA) and is designed to fully support genome editing of human primary T cells.

Beyond CRISPR-Cas9 expression methods, the culture systems for expansion and activation of primary human T cells also represent critical elements for successful genome editing, with cell activation being required in most experimental contexts.[12] While T cells can be isolated from a number of sources using a variety of isolation techniques, to date most genome editing studies involving T cells have used primary human T cells isolated from peripheral blood mononuclear cells (PBMCs) by immunomagnetic selection.[7, 12-14]

With improved expression and delivery methods, CRISPR-Cas9 genome editing is now rapidly being incorporated into the development of next-generation immunotherapies. One example is chimeric antigen receptor (CAR) T cell therapy, wherein CRISPR-Cas9 enables generation of allogeneic immune effector cells that are compatible for patient infusion.[15] For this purpose, T cells can be engineered to escape immune rejection through deletion of endogenous T cell receptors (TCRs) and human leukocyte antigen (HLA) class I molecules, which shows high efficacy when combined with deletion of inhibitory receptors such as programmed death-1 (PD-1) or cytotoxic T-lymphocyte antigen protein 4 (CTLA-1).[9-10,16-17] This strategy can then be coupled with targeting CAR constructs to the endogenous TCR alpha constant (TRAC) locus to generate universal allogeneic CAR-T cells.[18]

CRISPR-Cas9 Genome Editing in Primary Human T Cells

Materials Required

PRODUCT	CATALOG #
ArciTect™ sgRNA† or ArciTect™ crRNA† ArciTect™ tracrRNA Kit • ArciTect™ tracrRNA • ArciTect™ Annealing Buffer (5X)	200-0013 or 76010 / 76011 / 76012 76016 / 76017 / 76018
ArciTect™ Cas9 Nuclease	76002 / 76004
Nuclease-Free Water	79001
EasySep™ Human T Cell Isolation Kit	17951
ImmunoCult™-XF T Cell Expansion Medium	10981
ImmunoCult™ Human CD3/CD28 T Cell Activator or ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator	10971 or 10970
Human Recombinant IL-2	e.g. 78036
L-Glutamine	07100
Gentamicin	---
Neon® Transfection System 100 µL Kit or 10 µL Kit • Resuspension Buffer T • Electrolytic Buffer E2 • 10 µL Neon® pipette tips or P3 Primary Cell 4D-Nucleofector™ X Kit S • 16-well Nucleocuvette™ Strips • P3 Primary Cell Nucleofector™ Solution • Supplement 1	ThermoFisher MPK10025 or Lonza V4XP-3032
24-well tissue culture-treated plate	e.g. 38017
15 mL conical tubes	e.g. 38009
DNase- and RNase-free microcentrifuge tubes	e.g. 38038
Heating block or thermocycler	---

†ArciTect™ sgRNA is only available in Australia, Austria, Belgium, Canada, China, Denmark, Finland, France, Germany, Iceland, Ireland, Luxembourg, the Netherlands, New Zealand, Norway, Poland, Portugal, Singapore, Spain, Sweden, Switzerland, the United Kingdom, and the United States.

A. T Cell Isolation and Activation

1. Isolate human T cells from peripheral blood (e.g. Catalog #70500) using EasySep™ Human T Cell Isolation Kit. Refer to the Product Information Sheet (Document #DX20004) for details.

   Optional: Source frozen primary T cells (e.g. Catalog #70024). Refer to the Product Information Sheet (Document #DX27569) for details.

2. Count cells and adjust to 1 x 10^6 cells/mL in ImmunoCult™-XF T Cell Expansion Medium supplemented with 2 mM L-Glutamine, 50 µg/mL gentamicin, and 10 ng/mL (130 IU/mL for the specific lot*) Human Recombinant IL-2.

   *International Units (IU) need to be calculated per lot. For more information visit www.stemcell.com/international-units-conversion-data-for-cytokines.

3. Activate human T cells by adding 25 µL/mL of ImmunoCult™ Human CD3/CD28 T Cell Activator. Incubate cell suspension at 37°C and 5% CO_2 for 72 hours.

   Optional: Assess T cell activation by binding the activation marker CD25 with Anti-Human CD25 Antibody, Clone BC96 (Catalog #60158) and performing flow cytometry.

B. Preparation of ArciTect™ sgRNA working solution and ArciTect™ crRNA and ArciTect™ tracrRNA stock Solutions

1. Briefly centrifuge the vials before opening.
2. Add nuclease-free water to each vial to give a final concentration of 200 µM (crRNA and tracrRNA) or 100µM (sgRNA), as indicated in Table 1 and Table 2, respectively

Table 1. Preparation of 200 µM* ArciTect™ crRNA or ArciTect™ tracrRNA

ArciTect™ crRNA OR ArciTect™ tracrRNA	VOLUME OF NUCLEASE-FREE WATER (µL)
2 nmol	10
5 nmol	50
10 nmol	50
20 nmol	100

*200 µM is equal to 200 pmol/µL

Table 2. Resuspension Volume for 100 µM* ArciTect™ sgRNA

ArciTect™ sgRNA	VOLUME OF NUCLEASE-FREE WATER (µL)
4 nmol	40

*100 µM is equal to 100 pmol/µL

3. Mix thoroughly. If not used immediately, aliquot and store at -20°C for up to 6 months or at -80°C for longer than 6 months. After thawing the aliquots, use immediately. Do not refreeze.

C. Annealing of crRNA:tracrRNA Duplexes

RNP complexes can be prepared using either the two-part crRNA:tracrRNA gRNA, which requires pre-annealing (see below), or sgRNA. If working with sgRNA, proceed directly to section D.

1. Prepare a 60 µM crRNA:tracrRNA solution by combining components in a DNase- and RNase-free microcentrifuge tube as indicated in Table 3. Volumes are sufficient for a single electroporation reaction, adjust as required. Mix thoroughly.

Table 3. Preparation of 60 µM (60 pmol/µL) crRNA:tracrRNA Duplex

COMPONENT	VOLUME PER ELECTROPORATION (µL)
Nuclease-free water	2
ArciTect™ Annealing Buffer (5X)	2
200 µM ArciTect™ crRNA	3
200 µM ArciTect™ tracrRNA	3
Total volume	**10**

2. In a thermocycler or heating block, incubate crRNA:tracrRNA mixture at 95°C for 5 minutes followed by 60°C for 1 minute. Cool to room temperature (15 - 25°C) and place on ice. If not used immediately, store at -80°C for up to 1 month.

D. Preparation of ArciTect™ CRISPR-Cas9 RNP Complex Mix

1. To prepare the RNP Complex Mix, combine the components in the order listed in Table 4 in a microcentrifuge tube. Adjust component volumes according to the desired number of transfections.
2. Mix thoroughly.

Table 4. Preparation of RNP Complex Mixture for Electroporation

COMPONENT	sgRNA		crRNA:tracrRNA	
	Neon® Electroporation (100 µL Kit)	Neon® Electroporation (10 µL Kit)	Neon® Electroporation (100 µL Kit)	Neon® Electroporation (10 µL Kit)
	Volume per reaction (µL)			
Resuspension Buffer T	8.0	6.0	6.8	6.04
ArciTect™ Cas9 Nuclease (4 µg/µL)*	7.2	0.9	7.2	0.90
100 µM sgRNA	4.8	0.6	--	--
60 µM crRNA:tracrRNA		--	6.0	0.56
Total volume	**20.0**	**7.5**	**20.0**	**7.50**

3. Mix the RNP Complex Mixture by gently pipetting up and down 2 times; avoid creating air bubbles or foam in the tube.
4. Incubate the RNP Complex Mixture at room temperature (15 - 25°C) for 10 - 15 minutes.

E. Preparation of T Cell Suspension for Electroporation

1. For each electroporation condition (including positive and negative controls), prepare 2 mL of ImmunoCult™-XF T Cell Expansion Medium supplemented with 2 mM L-Glutamine, 50 µg/mL gentamicin, and 10 ng/mL Human Recombinant IL-2.
2. For each condition, add 2 mL of supplemented medium (prepared in step 1) to 1 well of a 6-well plate (for 100 µL tip reactions) or 1 mL of supplemented medium to 1 well of a 12-well plate (for 10 µL tip reactions) and place in a 37°C incubator. Store the remainder of the supplemented medium at 2 - 8°C.
3. Transfer 1.2 x 10^6 cells (for 100 µL tip reactions) or 1.5 x 10^5 cells (for 10 µL tip reactions) from the activated T cell suspension (prepared in section A) to a 15 mL conical tube. Centrifuge at 300 x *g* for 5 minutes at room temperature.

F. Electroporation of T cells with RNP Complex Using Neon® Transfection System

1. Aspirate supernatant from cell pellet (prepared in section E) and Resuspend cells in 100 µL of Resuspension Buffer T if using 100 µL tips or 7.5 µL of Resuspension Buffer T if using 10 µL tips and pipette up and down to mix.
2. Using individual RNase-free microcentrifuge tubes for each condition, combine RNP Complex Mixture (prepared in section D) with activated T cells as indicated in Table 5.

Table 5. Mixture of RNP Complex and T Cells for Electroporation

COMPONENT	VOLUME PER ELECTROPORATION (µL)	
	Neon® Electroporation (10 µL Kit)	Neon® Electroporation (100 µL Kit)
RNP Complex	7.5	20
Activated T Cell Suspensionw	7.5	100
Total volume	**15**	**120**

3. Mix gently by pipetting up and down carefully 2 times; avoid creating air bubbles or foam in the tube.

   Note: If air bubbles are present in the tip when the cells are electroporated, cell viability and transfection efficiency will be significantly reduced.

4. Use a 100 µL Neon® pipette tip to draw up 100 µL of the mixture and place into the electroporation chamber containing 3 mL of Electrolytic Buffer E2.
5. Electroporate mixture according to conditions indicated in Table 6.

   Note: Refer to the manufacturer's instructions for electroporation. Electroporation conditions may require optimization for different cell types.

Table 6. Recommended Electroporation Conditions for Human T Cells Using a Neon® Electroporation Device

ELECTROPORATION PARAMETER	
Electrical potential	1400V
Pulse width	30 milliseconds
Number of pulses	1

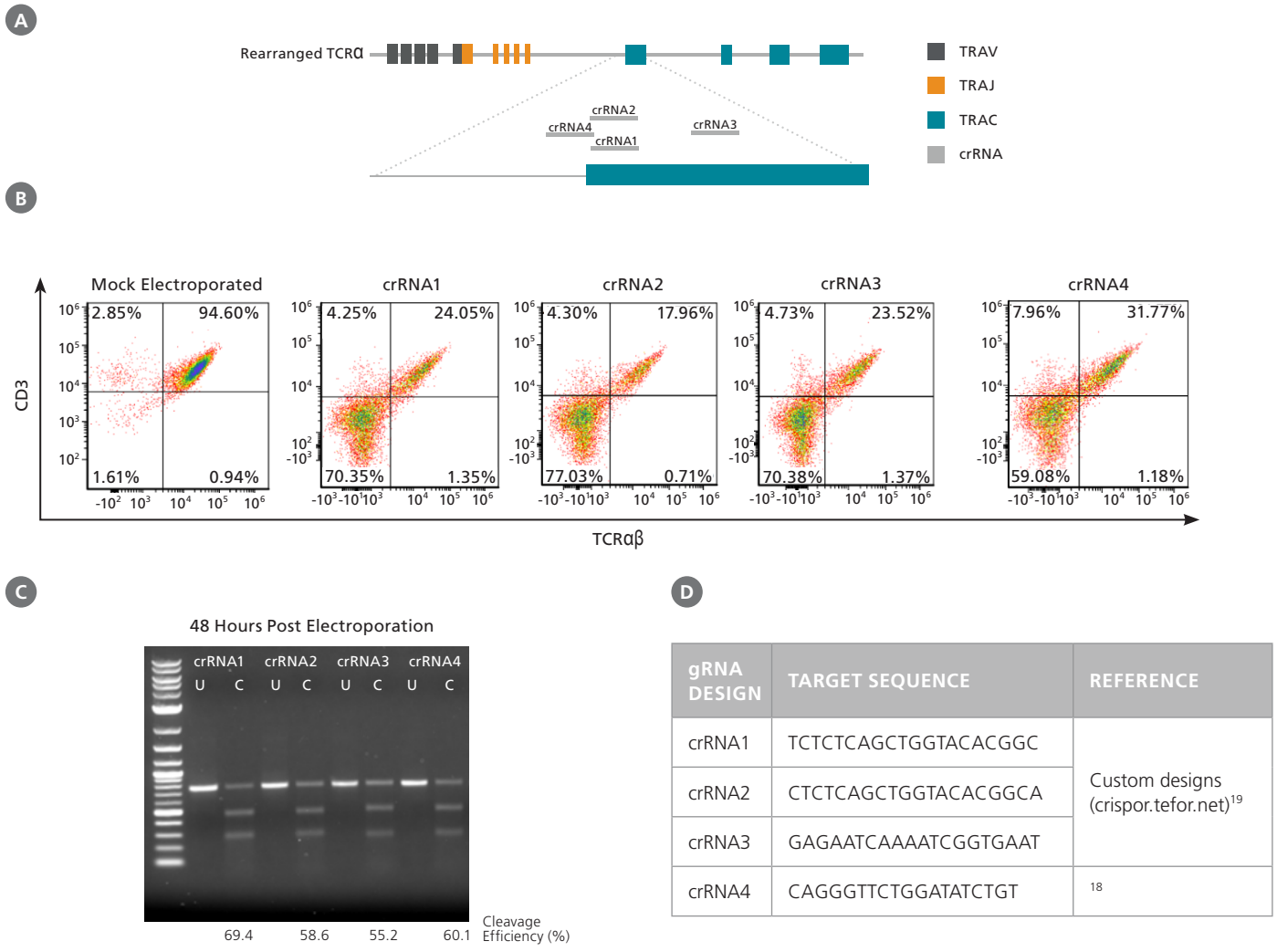
G. Post-Editing Culture

1. Immediately add the electroporated cells to 1 well of the 6- or 12-well plate prepared in Section E step 2. Incubate at 37°C and 5% CO_2 for 2 - 3 hours.
2. Count cells and adjust cell density to 2.5 x 10^5 viable cells/mL by adding IL-2-supplemented ImmunoCult™-XF T Cell Expansion Medium (prepared in step 1).
3. Optional: If using Cas9-eGFP nuclease, assess electroporation efficiency 12 - 24 hours after electroporation by flow cytometry.
4. Incubate at 37°C and 5% CO_2 for 48 - 72 hours for genome editing to occur. Harvest cells for assessment of genome editing efficiency, genomic DNA can be amplified by PCR using primers flanking the target region and ArciTect™ High-Fidelity DNA Polymerase Kit (Catalog #76026), followed by sequencing of PCR products. Alternatively, ArciTect™ T7 Endonuclease Kit (Catalog #76021) can be used to assess editing efficiency (% INDEL formation) following PCR amplification. For further information, refer to the Technical Bulletin: Evaluation of Genome Editing (Document #27126), available at www.stemcell.com or contact us to request a copy.

Case Study: Evaluation of Optimal Culture Methods For High Efficiency TRAC Knockout in Human Primary T Cells

To demonstrate genetic knockout with the ArciTect™ CRISPR-Cas9 system, we targeted the TRAC locus using the protocol described in this document. In addition, we tested multiple T cell activation reagents and dynamics to identify a condition with the highest knockout efficiency. Loss of function due to INDELs at this locus is readily identifiable by the lack of TCR expression on the cell surface. The practical importance of targeting this locus lies in the generation of universal allogeneic T cells for CAR T cell therapies.[18]

We first designed multiple crRNAs (Figure 3A, D), as different crRNA sequences can exhibit varying efficiency at the target site (see Technical Bulletin: Genome Editing with Direct Cas9 RNP Delivery Design Considerations, Document #27083). We tested the efficacy of the four crRNAs in activated human T cells, with TRAC knockout efficiency detected by flow cytometry, and INDEL generation detected by the ArciTect™ T7 Endonuclease I Kit. All four crRNAs significantly reduced the frequency of TCRαβ$^+$ cells (Figure 3B) and resulted in cut bands in the T7 endonuclease I assay (Figure 3C), confirming the presence of genomic mismatches that are indicative of INDELs. Based on these results, we proceeded with crRNA2, as it showed the highest knockdown efficiency by flow cytometry.



gRNA DESIGN	TARGET SEQUENCE	REFERENCE
crRNA1	TCTCTCAGCTGGTACACGGC	Custom designs (crispor.tefor.net)[19]
crRNA2	CTCTCAGCTGGTACACGGCA	
crRNA3	GAGAATCAAAATCGGTGAAT	
crRNA4	CAGGGTTCTGGATATCTGT	[18]

Figure 3. TRAC gRNA Design and Evaluation

(A) Schematic of the TRAC locus with crRNA sequences 1 - 4 aligned to the TRAC first exon. (B) Representative TCRαβ/CD3 flow cytometry results at 48 hours post RNP complex electroporation (containing the corresponding crRNA1 - 4) into activated T cells. (C) Genome editing (cleavage) efficiency was assessed at 48 hours post electroporation using the ArciTect™ T7 Endonuclease I Kit. U: Uncut; C: Cut: (D) gRNA target sequences for TRAC knockout.

We then used the validated crRNA2 to compare knockout efficiency in a variety of T cell activation conditions. First, T cells were isolated from the human peripheral blood of four independent donors. Cells were cultured in Immunocult™-XF T Cell Expansion Medium supplemented as described with either ImmunoCult™ Human CD3/CD28 T Cell Activator or CD3/CD28/CD2 T Cell Activator for 2 or 3 days, for a total of 4 test conditions. On day 0, approximately 10 - 20% of cells expressed the activation marker CD25 (data not shown), which increased to over 80% after 2 or 3 days in the presence of the activators (Figure 4). T cell activation was greater when cells were incubated with the CD3/CD28/CD2 T Cell Activator compared to the CD3/CD28 T Cell Activator (Figure 4). The percentage of activated cells was also greater when cells were incubated with either activator for 3 days compared to 2 days (Figure 4). For more information about optimization of T cell activation and expansion conditions, refer to the Technical Bulletin: Optimization of Human T Cell Expansion Protocol: Effects of Early Cell Dilution (Document #27143), available at **www.stemcell.com** or contact us to request a copy.

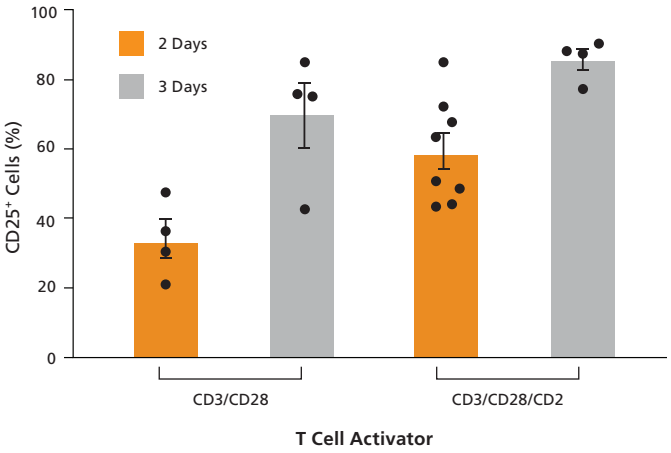


Figure 4. T Cells Express the CD25 Activation Marker After Treatment with ImmunoCult™ Human T Cell Activators

Isolated human T cells were activated with either ImmunoCult™ Human CD3/CD28 or CD3/CD28/CD2 T Cell Activator for 2 or 3 days. Activation status was assessed by CD25 flow cytometry. Each data point per condition represents an individual donor; n = 4 - 8 donors. Error bars represent standard error of the mean.

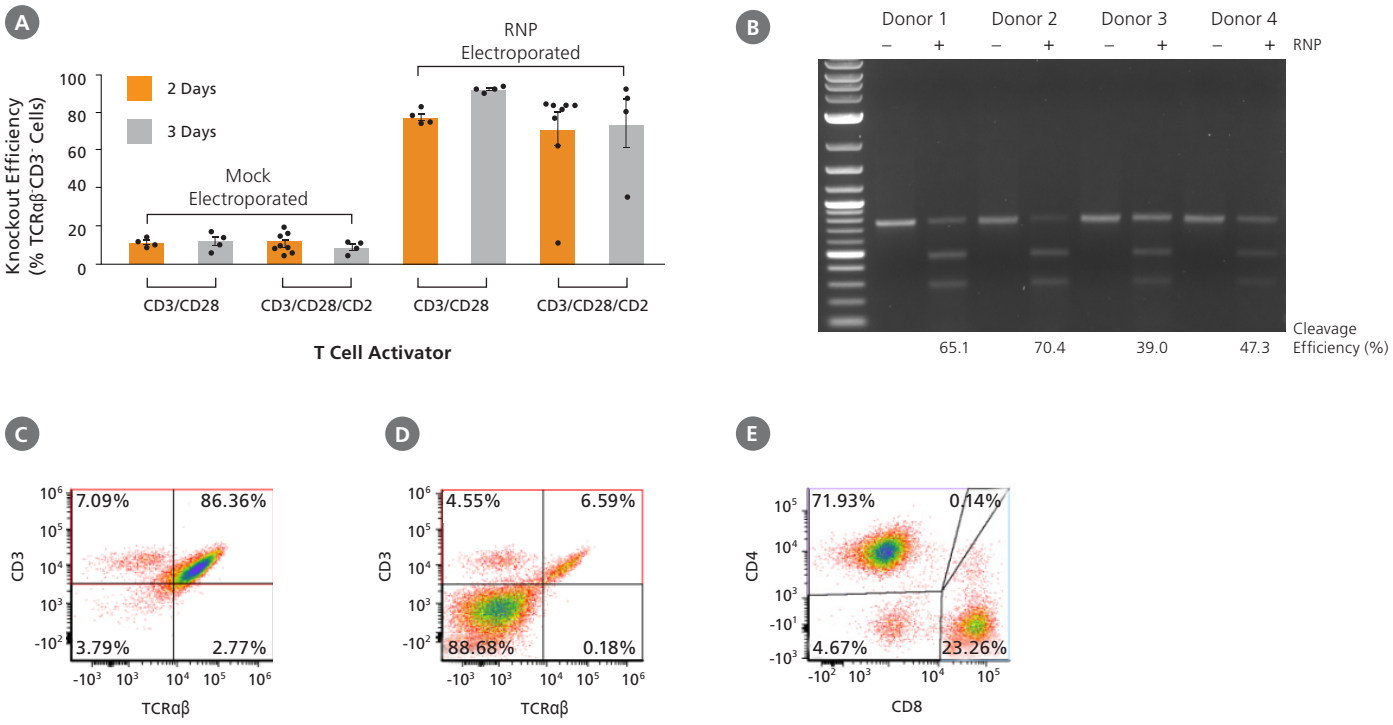


Figure 5. High Efficiency TRAC Knockout Across Activation Conditions and Dynamics

(A) TRAC knockout efficiency in human T cells activated with either ImmunoCult™ Human CD3/CD28 or CD3/CD28/CD2 T Cell Activator for 2 or 3 days was assessed by binding the TCRαβ and CD3 receptors with antibodies followed by flow cytometry analysis. Each data point per condition represents an individual donor; n = 4 - 8 donors. Error bars represent standard error of the mean. (B) Genome editing (cleavage) efficiency was assessed at 48 hours post electroporation in human T cells activated with ImmunoCult™ Human CD3/CD28 T Cell Activator for 3 days using the ArciTect™ T7 Endonuclease I Kit. Mock electroporated: - RNP; RNP electroporated: + RNP. (C - D) Representative dot plots of TCRαβ and CD3 flow cytometry analysis from (C) mock electroporated and (D) RNP electroporated human T cells activated with ImmunoCult™ Human CD3/CD28 T Cell Activator for 3 days. (E) Representative dot plot of CD4 and CD8 flow cytometry analysis of human T cells activated with ImmunoCult™ Human CD3/CD28 T Cell Activator for 3 days.

Following activation, T cells were electroporated with RNP complexes containing TRAC crRNA2. Viability ranged from 52 - 82% (data not shown) immediately after electroporation, independent of activation condition. Knockout efficiency was measured 3 days after electroporation (day 5 or 6 from start of activation), and we observed up to 90% increase in knockout efficiency (TCRαβ$^-$ cells; Figure 5A). The highest consistent knockout efficiencies were observed when cells were electroporated after 3 days of activation, particularly when the ImmunoCult™ Human CD3/CD28 T Cell Activator was used (Figure 5A). Moreover, INDEL formation at the target locus was confirmed by the T7 endonuclease I assay in treated samples from all 4 donors (Figure 5B). Results were more variable among donors when cultured with the ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator over 2 days, with one non-responsive sample. We therefore tested an additional 4 donors with that condition and observed more consistent knockout in all additional samples. As TCRαβ forms a complex with CD3, the knockout was confirmed by concomitant loss of CD3 expression on the cell surface (Figure 5A, C, D), while T cell markers CD4 and CD8 continued to be highly expressed (Figure 5E).

Human primary T cells were then electroporated with RNP complexes containing sgRNA or crRNA:tracrRNA duplexes targeting *TRAC* (Figure 6A) or a second target, β2 microglobulin (*B2M*; Figure 6B), to test knockout efficiency with different gRNA formats. Since expression of *B2M* is required for surface expression of major histocompatibility class I (MHC-I) molecules, this strategy enabled quantitative readout of editing efficiency by flow cytometry to detect MHC-I expression.[20,21] Both gRNA formats resulted in similar knockout efficiencies at either target, suggesting that the ArciTect™ CRISPR-Cas9 system exhibits high editing efficiency in human primary T cells regardless of gRNA formats. Both formats enable high editing efficiency.

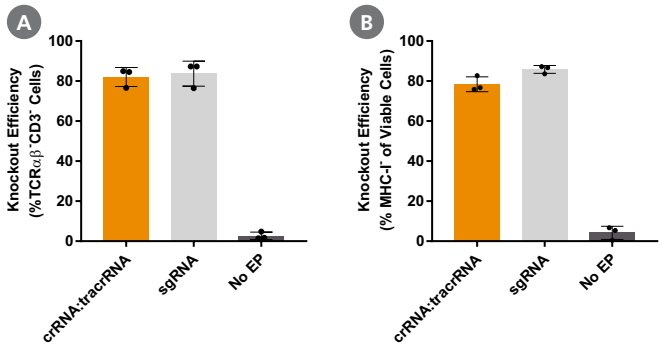


Figure 6. Comparison of *TRAC* and *B2M* Knockout Editing Efficiency Using Either ArciTect™ sgRNA or crRNA:tracrRNA

Human T cells were activated with ImmunoCult™ Human CD3/CD28 T Cell Activator for 3 days and electroporated with CRISPR-Cas9 RNP complexes containing ArciTect™ Cas9 Nuclease and either ArciTect™ crRNA:tracrRNA duplexes or sgRNA targeting (A) *TRAC* or (B) *B2M*. Knockout efficiency was measured by flow cytometry 3 days after electroporation. Each data point per condition represents an individual donor; n = 3 donors. No EP: non-electroporated cells. Error bars represent standard deviation.

Following CRISPR-Cas9 genome editing, cells were cultured an additional 10 days by adding in fresh Immunocult™-XF T Cell Expansion Medium supplemented with IL-2 every 2 - 3 days. TCRαβ- cells persisted over the course of expansion (data not shown). Cells treated with ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator prior to electroporation exhibited a higher degree of expansion post gene editing compared to those treated with ImmunoCult™ Human CD3/CD28 T Cell Activator. Based on the fold expansion, calculated cell yields ranged from 4.1 x 10^7 to 3.8 x 10^8 cells after 7 days of incubation post-electroporation.

TCRαβ-knockout efficiency typically ranged from 60 - 90% (Figure 5A). There has been a general interest in targeting TRAC to deplete TCRαβ-expressing cells for the generation of allogeneic immune effector cells18, the success of which will require highly pure populations of TCRαβ- cells.. Figure 7 shows an example where the EasySep™ Human TCR Alpha/Beta Depletion Kit (Catalog #17847) was used to reduce the frequency of CD3+TCRαβ+ cells from 19.1% to 0.1%.

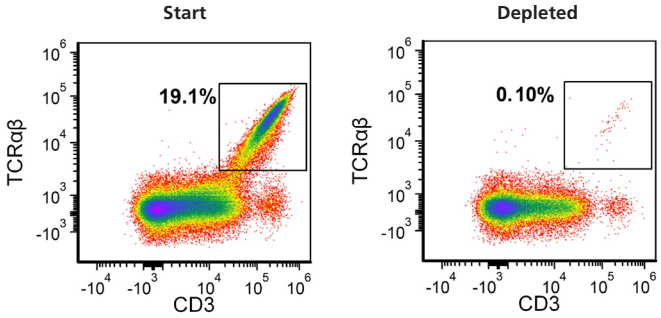


Figure 7. Residual TCRαβ+ Cells can be Removed From Expanded TRAC Knockout Cell Populations Using EasySep™ Human TCRαβ Depletion Kit

Gene-edited TCRαβ knockout T cells were expanded for 7 - 10 days, harvested and resuspended in EasySep™ Buffer at 5 x 10^7 cells/mL. Following a 13-minute EasySep™ TCRαβ depletion protocol, flow cytometry was performed to assess the depletion of TCRαβ$^+$ cells. Representative data plots are shown for the frequency of TCRαβ cells in the expanded TRAC kncockoutcell population before and after EasySep™ depletion.

Discussion

In genome editing primary human T cells, there exists a delicate balance between cell exhaustion, cell viability, and gene editing efficiency. We found that stimulation with ImmunoCult™ Human CD3/CD28 T Cell Activator for 3 days prior to electroporation resulted in the highest and most consistent editing efficiencies. Both crRNA:tracrRNA duplexes and sgRNA formats resulted in similar knockout efficiencies, suggesting that the ArciTect™ CRISPR-Cas9 system exhibits high editing efficiency in human primary T cells regardless of gRNA formats. To deplete the remaining TCRαß+ cells, the EasySep™ Human TCR Alpha/Beta Depletion Kit can be used. This is important for development of next-generation cellular therapeutics that rely on TCRαß- status for efficacy. Greater cell expansion post editing was observed for cells stimulated with ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator; consider the output cell number required for downstream applications when choosing a protocol. If a greater number of edited cells are required for downstream applications, one can also scale up with more cells per electroporation or with more wells. In general, two wells are recommended for positive (e.g. ArciTect™ Human HPRT Positive Control Kit, Catalog #76013) and negative (non-electroporated) controls. The total number of wells should be determined before starting, to calculate the total amount of materials required. Optimization of conditions such as cell density or Cas9:gRNA ratio may be necessary, or multiple donor samples may be required (e.g. diseased and healthy).

The genome editing strategy outlined in this document is typical for most genetic knockout applications. Application-specific protocol modifications, not thoroughly detailed here, might include: use of Cas9-eGFP Nuclease for visualization of positive transfectants; use of the single-strand endonuclease Cas9 Nickase with two flanking gRNAs; or the addition of a DNA donor template for homology-directed repair (HDR)-mediated genetic knock-in

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