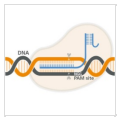

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CRISPR-Cas9 Genome Editing in Human Primary T Cells

STEMCELL Technologies[1]

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ABSTRACT

CRISPR-Cas9, an RNA-guided genome editing technology, is revolutionizing cell biology due to the ease and efficiency by which it enables genetic manipulation of mammalian cells. Through targeted modification of specific genes or regulatory regions, 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. This protocol provides instructions for CRISPR-Cas9 genome editing of human primary T cells, including the isolation and activation of primary human T cells, preparation of a CRISPR-Cas9 RNP complex, delivery of an RNP complex into activated primary T cells using electroporation, and methods to evaluate genome editing efficiency.

For a discussion of alternative strategies and expected outcomes, and protocol modifications, please see: Technical Bulletin: Genome Editing of Human Primary T Cells.

EXTERNAL LINK

https://www.stemcell.com/genome-editing-of-human-primary-t-cells-using-the-arcitect-crispr-cas9-system.html?utm_source=protocolsio&utm_medium=referral

GUIDELINES

ArciTect™

CRISPR-Cas9 Genome Editing Precision for Cell Biologists

The ArciTect™ family of products for CRISPR-Cas9 genome editing provides you with a rapid, flexible and precise system to modify the genome as you see fit. The guide RNA (gRNA) complex, consisting of crRNA and tracrRNA, can be customized using the online order tool. A protocol for the efficient genome editing of human embryonic stem (hES) cells or human induced pluripotent stem (hiPS) cells has been optimized using the ArciTect™ product family.

Reduce Off-Target Effects

The ArciTect™ product family is a ribonucleoprotein (RNP)-based Cas9 genome editing system. Unlike previous CRISPR technologies which utilize plasmid or mRNA-based systems, the ArciTect™ system shows timely degradation of the RNP complex to minimize cleavage of off-target regions.

Why use ArciTect™?

- **CUSTOMIZABLE**. Design crRNA to target your sequence of interest.
- **FLEXIBLE**. Multiple variations of Cas9 to suit your specific genome editing needs.
- **RAPID**. No need for transcription and translation.
- **REDUCED OFF-TARGET EFFECTS**. Timely degradation of the RNP complex to minimize potential off-target cutting.

MATERIALS

NAME	CATALOG #	VENDOR
Gentamicin	15750037	Gibco - Thermo Fischer

NAME	CATALOG #	VENDOR
Human Peripheral Blood Leuko Pak, Fresh	70500	Stemcell Technologies
EasySep™ Human T Cell Isolation Kit	17951	Stemcell Technologies
ImmunoCult™-XF T Cell Expansion Medium	10981	Stemcell Technologies
L-Glutamine	07100	Stemcell Technologies
Human Recombinant IL-2 (CHO-expressed)	78036	Stemcell Technologies
ImmunoCult™ Human CD3/CD28 T Cell Activator	10971	Stemcell Technologies
ArciTect™ crRNA	View	Stemcell Technologies
ArciTect™ tracrRNA Kit	View	Stemcell Technologies
Nuclease-Free Water	79001	Stemcell Technologies
Costar® 24-Well Flat-Bottom Plate Tissue Culture-Treated	38017	Stemcell Technologies
Falcon® Conical Tubes 15 mL	38009	Stemcell Technologies
Costar® Microcentrifuge Tubes	38038	Stemcell Technologies
Neon™ Transfection System 100 µL Kit	MPK10025	Thermo Fisher Scientific
ArciTect™ Cas9 Nuclease	76002	Stemcell Technologies
ArciTect™ Cas9-eGFP Nuclease	76006	Stemcell Technologies
ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator	10970	Stemcell Technologies
ArciTect™ High-Fidelity DNA Polymerase Kit	76026	Stemcell Technologies
ArciTect™ T7 Endonuclease I Kit	76021	Stemcell Technologies

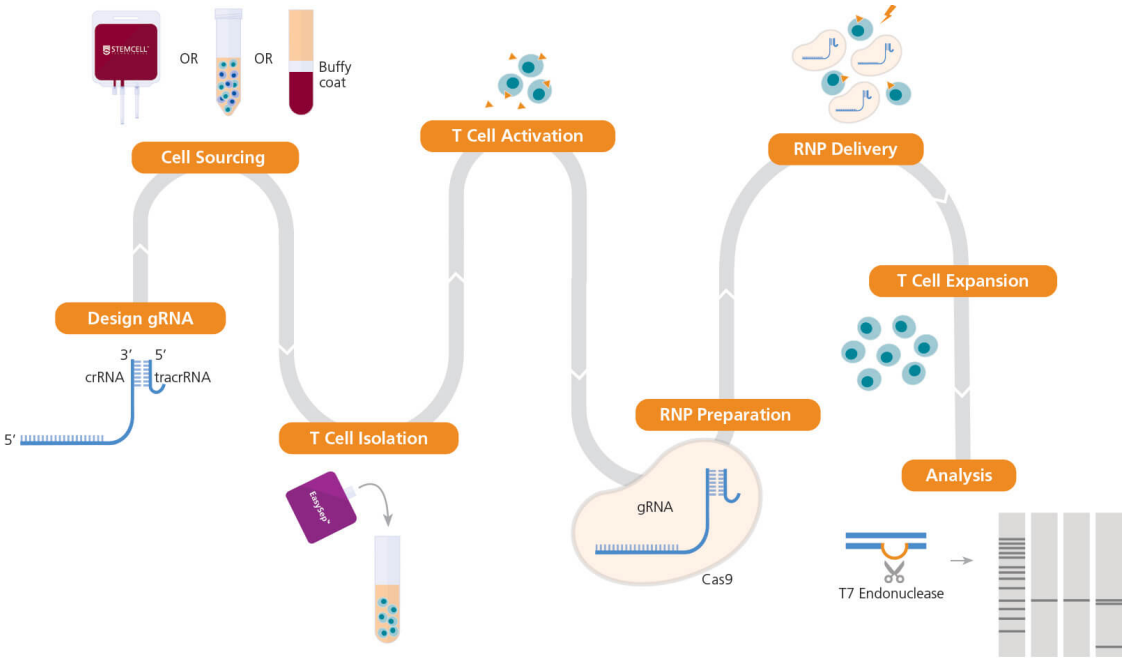
MATERIALS TEXT

Optional: Source frozen primary T cells in Step 1.

Human Peripheral Blood Pan-T Cells, Frozen
by Stemcell Technologies
Catalog #: 70024

BEFORE STARTING

This protocol describes the following workflow for T cell genome editing.



The guide RNA (gRNA) complex, consisting of crRNA and tracrRNA, can be designed and customized using our online order tool.

T Cell Isolation and Activation

1 Isolate human T cells from peripheral blood using EasySep™ Human T Cell Isolation Kit. Refer to the Product Information Sheet 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
- 10 ng/mL Human Recombinant IL-2

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. 72h

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

Preparation of Stock Solutions

4 Briefly centrifuge the vials of ArciTect™ crRNA and ArciTect™ tracrRNA before opening.

5 Depending on the size of the ArciTect™ crRNA and ArciTect™ tracrRNA vials being used, add nuclease-free water to each vial to give a final concentration of 200 µM, as indicated in Table 1.

ArciTect™ crRNA OR ArciTect™ tracrRNA Vial Size	Catalog #	Volume of Nuclease-Free Water (µL)
2 nmol	76010/76016	10
10 nmol	76011/76017	50
20 nmol	76012/76018	100

Table 1. Resuspension volumes for 200 µM* ArciTect™ crRNA or ArciTect™ tracrRNA.
*200 µM is equal to 200 pmol/µL

6 Mix thoroughly. If not used immediately, aliquot and store at -80°C for up to 1 month. After thawing the aliquots, use immediately. Do not re-freeze.

Preparation of ArciTect™ CRISPR-Cas9 RNP Complex

7 Prepare a 60 µM guide RNA solution by combining components in an RNase-free microcentrifuge tube as indicated below in Table 2. Volumes are sufficient for a single electroporation reaction (10 µL/electroporation reaction); adjust as required. Mix thoroughly.

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**

Table 2. Preparation of 60 µM Guide RNA solution per electroporation reaction.

Note: ArciTect™ Annealing Buffer (5X) and ArciTect™ tracrRNA are both included in the ArciTect™ tracrRNA kit.

8 In a thermocycler or heating block, incubate guide RNA solution at 95°C for 5 minutes followed by 60°C for 1 minute. Cool to room temperature and place on ice. 6m

Note: If not used immediately, store guide RNA solution at -80ºC for up to 1 month.

9 To prepare the ribonucleoprotein (RNP) complex, combine 60 µM guide RNA (prepared in step 8) and ArciTect™ Cas9 Nuclease (or ArciTect™ Cas9-eGFP) in an RNase-free microcentrifuge tube as indicated below in Table 3. Volumes are sufficient for a single electroporation; adjust as required for the desired number of wells.

Component	Volume per Electroporation (µL)
Resuspension Buffer T	6.8
4 µg/µL ArciTect™ Cas9 Nuclease*	7.2
60 µM guide RNA	6
Total volume	**10**

Table 3. Preparation of RNP Complex Mixture.

***Note:** If using 3 µg/µL ArciTect™ Cas9-eGFP Nuclease, add 11.4 µL of Cas9-eGFP to 6 µL of 60 µM guide RNA and 2.6 µL of Resuspension Buffer T for a total volume of 20 µL. Note that 1 µg/µL ArciTect™ Cas9 should not be used for Neon® electroporation, as the total Resuspension buffer volume should represent ~90% of the total reaction volume.

Note: Resuspension Buffer T is included in the Neon™ Transfection System 100 µL Kit.

10 Mix the RNP Complex Mixture by gently pipetting up and down 2 times; avoid creating air bubbles or foam in the tube.

11 Incubate the RNP Complex Mixture at room temperature (15 - 25°C) for 10 - 15 minutes. 15m

Electroporation of RNP Complex into T Cells

12 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
- 10 ng/mL Human Recombinant IL-2.

13 For each condition, add 2 ml of supplemented medium (prepared in step 12) to 1 well of a 6-well plate and place in a 37°C incubator. Store the remainder of the supplemented medium at 2 - 8°C.

14 Transfer 1.2 x 10^6 cells 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.

15 Aspirate medium and resuspend cells in 100 µL of Resuspension Buffer T.

16 Using individual RNase-free microcentrifuge tubes for each condition, combine RNP Complex Mixture (prepared in steps 7 - 11) with activated T cells as indicated in Table 4.

Component	Volume Per Electroporation (µL)
RNP Complex	20
Activated T cell suspension	100
Total Volume	**120**

Table 4. Mixture of RNP Complex and T Cells for Electroporation

17 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.

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

18 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. Electroporate mixture according to conditions indicated in Table 5.

Electroporation Parameter	
Electrical potential	1400V
Pulse width	30 milliseconds
Number of pulses	1

Table 5. Recommended Electroporation Conditions for Human T Cells Using a Neon® Electroporation Device

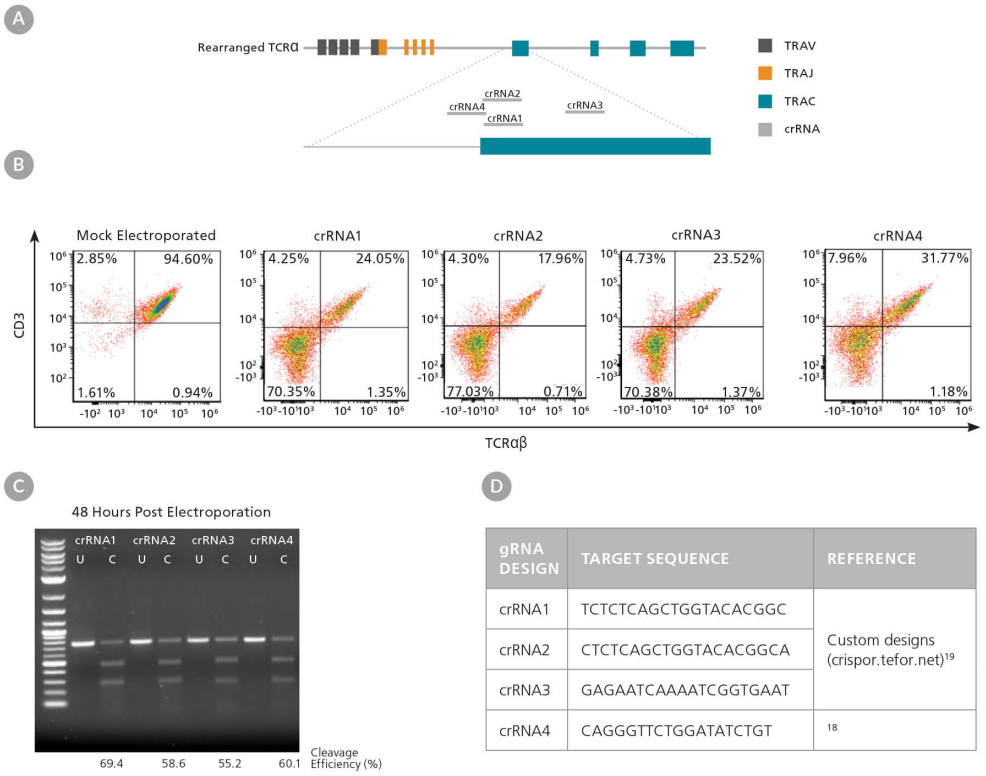
19 Immediately add the electroporated cells to 1 well of the 6-well plate prepared in step 2. Incubate at 37°C and 5% CO_2 for 2 - 3 hours. 3h

20 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 12).

Optional: If using Cas9-eGFP nuclease, assess electroporation efficiency 12 - 24 hours after electroporation by flow cytometry.

21 72h

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, followed by sequencing of PCR products. Alternatively, ArciTect™ T7 Endonuclease Kit can be used to assess editing efficiency (% INDEL formation) following PCR amplification.



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

Figure 1. 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.

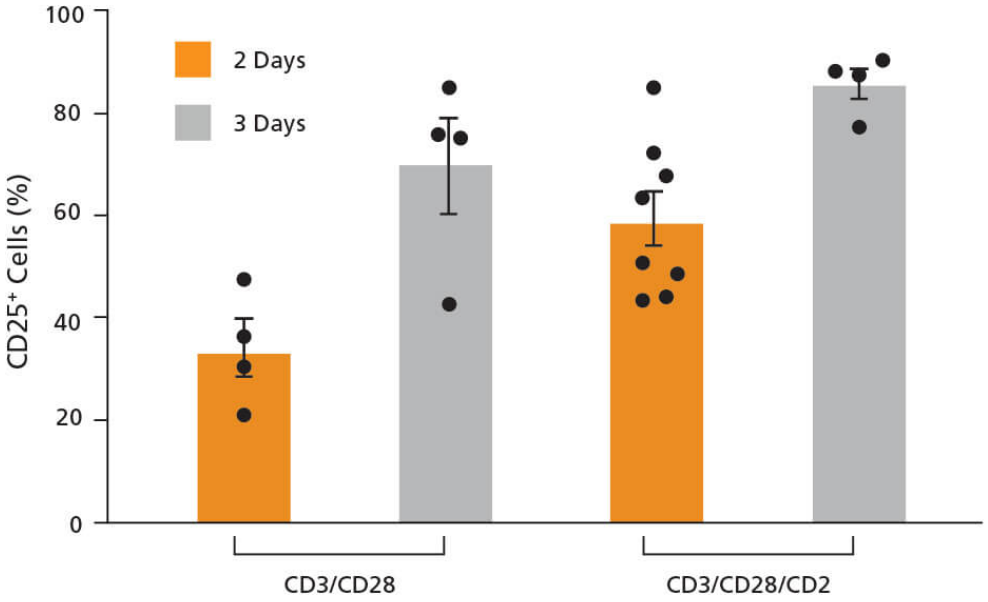


Figure 2. T Cells express the CD25 activation marker after treatment with ImmunoCult™ Human T Cell Activators.
Isolated human T cells were activated with either ImmunoCult™ Human CD3/ CD28 or CD3/CD28/CD2 T Cell Activator for 2 or 3 days. Activation status was assessed by CD25 flow cytometry. Each data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

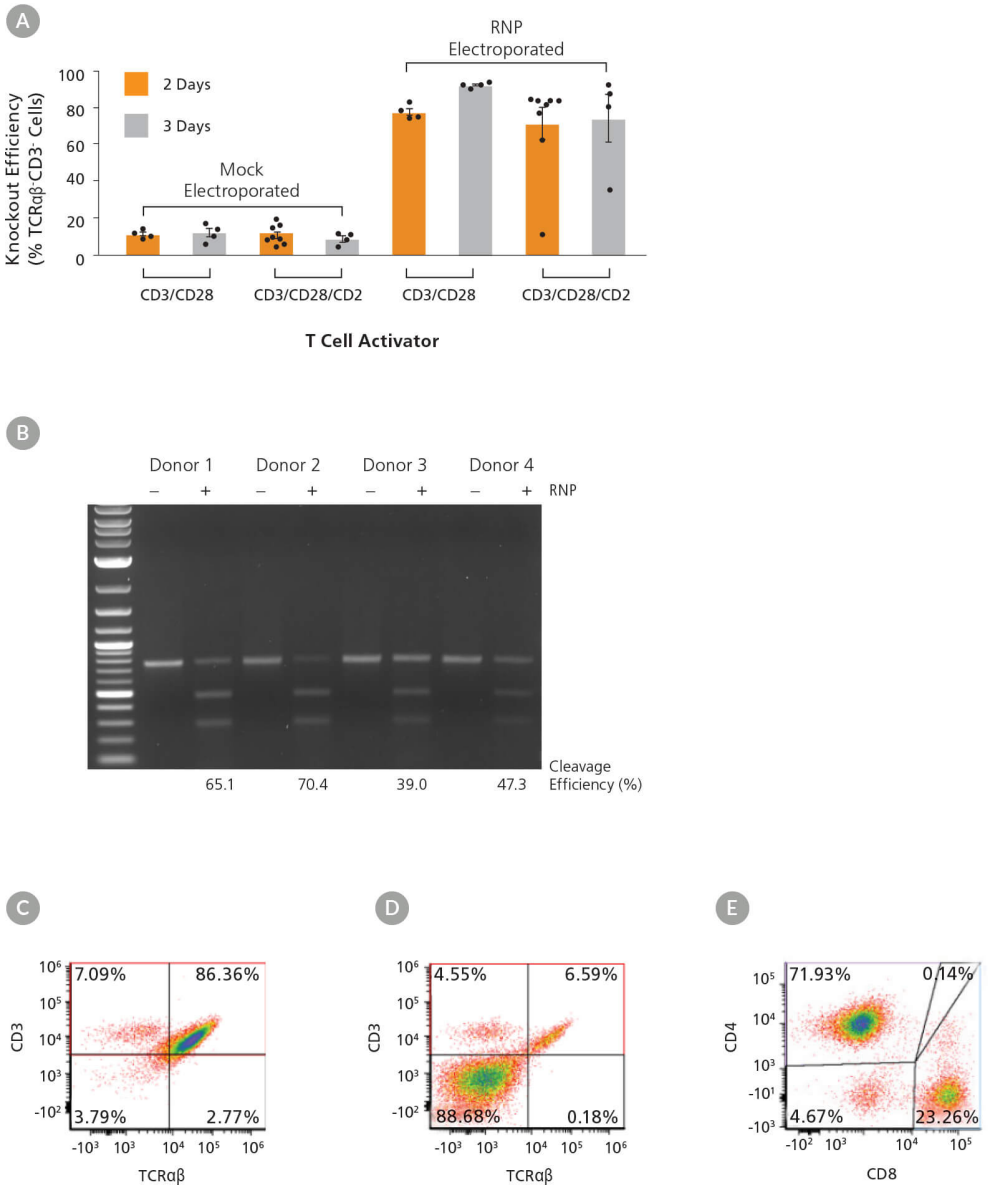


Figure 3. High efficiency TRAC knockout across activation conditions and dynamics.
(A) TRAC knockout efficiency in human T cells activated with either ImmunoCult™ Human CD3/CD28 or CD3/CD28/CD2 T Cell Activator for 2 or 3 days was assessed by binding the TCRαβ and CD3 receptors with antibodies followed 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.