Protocol

RNP-based editing of primary CD4+ T cells with Edit-R™ pre-designed synthetic single guide RNAs targeting T cell receptor constant regions

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For research only. Not for use in diagnostic procedures.

Introduction

Gene editing using CRISPR technologies has revolutionized functional genomics by enabling site-specific genomic changes such as knock-out by indel induced frameshift, knock-in by homologous directed repair, and swaps with prime or base editing. Through years of experience, we have refined the design and synthesis of high-quality synthetic RNAs. Now, we introduce algorithmically ranked and functionally validated Edit-R™ single synthetic RNAs (sgRNAs) targeting the TRAC, TRBC, and TRDC constant regions for robust knock-out phenotype of the T cell receptor (TCR). These synthetic sgRNAs knock-out the endogenous TCR at high-efficiency acting as invaluable controls for screening the specificity of engineered chimeric antigen receptors (CARs) to an antigen of interest.

When working with primary, isolated immune cells and other resilient cell types, such as induced pluripotent stem cells (iPSCs) and γδ T cells, high levels of viability and editing efficiency are essential. Here we provide experimental guidelines for high efficiency TCR knock-out, implementing the pre-designed Edit-R sgRNAs individually, to edit primary $CD4^+$ T cells.

Although this protocol provides an example of RNP-based editing using TRAC and TRBC pre-designed single sgRNAs, this is a protocol for RNP delivery of any other pre-designed Edit-R sgRNAs (located here).

Workflow

Generate high-efficiency TCR-negative primary cells

Here, we have 13 validated Edit-R single sgRNAs capable of efficiently targeting the TRAC, TRBC, or TRDC constant regions and knocking out the TCR α/β or TCR γ/δ (Table1). These sgRNAs have been tested in primary $CD4^+$ T cells from two individual donors.

Table 1: **Edit-R sgRNAs targeting TCR constant regions.**
4 TRAC, 5 TRBC, and 4 TRDC validated sgRNAs are listed. Genomic location, orientation of target sequence, exon of target sequence, and spacer sequence are provided in the subsequent table columns. The far right two columns are primer pairs that can be used to generate amplicons around the sgRNA target site.

Gene Target	Edit-R™ sgRNA	Orientation of sgRNA		Exon	Location	sgRNA sequence	Amplicon Primer Set (T7EI/TIDE)	
TRAC	sgRNA1	-	antisense	3	chr14:22550626	CGTCATGAGCAGATTAAACC	GGAACTCACTAAGGGGCCCA	GAGCCGTACTGGGGAGAGAG
	sgRNA2	-	antisense	3	chr14:22550612	TAAACCCGGCCACTTTCAGG	GGAACTCACTAAGGGGCCCA	GAGCCGTACTGGGGAGAGAG
	sgRNA3	-	antisense	1	chr14:22547533	AGAGTCTCTCAGCTGGTACA	CCATCACGAGCAGCTGGTTT	AAGGCCGAGACCACCAATCA
	sgRNA4	+	sense	2	chr14:22549656	AAGTTCCTGTGATGTCAAGC	CCAGGCAAGAGCAGGGTCTA	TGAGTTCCGCTACCACAGGA
TRBC	sgRNA1	-	antisense	1	chr7:142791815	CCCACCAGCTCAGCTCCACG	CCTCTTCCCTTTCCAGAGGAC	GCTACCTGGATCTTTCCATTTTCCC
	sgRNA2	-	antisense	1/Intron1	chr7:142792072	CCACTCACCTGCTCTACCCC	CCTCTTCCCTTTCCAGAGGAC	GCTACCTGGATCTTTCCATTTTCCC
	sgRNA3	+	sense	1	chr7:142792021	GGAGAATGACGAGTGGACCC	CCTCTTCCCTTTCCAGAGGAC	GCTACCTGGATCTTTCCATTTTCCC
	sgRNA4	+	sense	1	chr7:142792014	GGCTCTCGGAGAATGACGAG	CCTCTTCCCTTTCCAGAGGAC	GCTACCTGGATCTTTCCATTTTCCC
	sgRNA5	+	sense	1	chr7:142792027	TGACGAGTGGACCCAGGATA	CCTCTTCCCTTTCCAGAGGAC	GCTACCTGGATCTTTCCATTTTCCC
TRDC	sgRNA1	-	antisense	3	chr14:22464243	TCGTAGCCCAAGCACTGTGA	AACTAGGCAGACTTGGGGCA	GGAGAAGCCTCACACGCAAA
	sgRNA2	+	sense	3	chr14:22464219	AGCCATAGTTCATACCGAGA	AACTAGGCAGACTTGGGGCA	GGAGAAGCCTCACACGCAAA
	sgRNA3	-	antisense	1	chr14:22463090	ACAGCATTGTACTTCCCACT	TCCCCTTGCCTGTCCTTTCA	AGCACTGGATTAGAATGCACGA
	sgRNA4	+	sense	3	chr14:22464248	TGATGTCCCTCACAGTGCTT	AACTAGGCAGACTTGGGGCA	GGAGAAGCCTCACACGCAAA

Make TCR-targeting Edit-R sgRNA working stocks

Edit-R sgRNAs are shipped as lyophilized dried powder and must be reconstituted prior to complexing with Cas9 nuclease protein. To reconstitute the sgRNAs, resuspend sgRNAs in 10 mM Tris-HCl buffer pH 7.4 (Cat #B-006000-100) at a recommended concentration for electroporation. For more detail, see Dharmacon™ Edit-R synthetic guide RNA resuspension protocol. To make a working stock to use for primary cells, TCR-targeting Edit-R sgRNAs were diluted to an 80 µM working concentration.

Note: Typically, we recommend testing three to five guides RNA designs per gene of interest to identify the guide RNA with the highest editing efficiency that also results in complete knockout of functional protein in your primary cell of interest.

$CD4^+$ T cell maintenance prior to electroporation

$CD4^+$ T cells are isolated from whole blood (see Materials for recommended isolation kit) and cultured in complete RPMI 1640 medium (10% FBS, 2mM L-glutamine, 1 mM sodium pyruvate, 10mM HEPES, 10 mM nonessential amino acids) supplemented with 50 UI IL-2/mL.

1. 24 hours after isolation the cells are stimulated with Human T-Activator CD3/CD28 Gibco™ Dynabeads + 200 UI/mL IL-2 + 15 ng/mL IL-15 in complete RPMI 1640 medium.

2. After 72 hours of stimulation, magnetic beads are removed with recommended magnet. The culture is maintained between $0.5x10^5$ and $2.0x10^6$ cells/mL in complete RMPI supplemented with fresh 50 UI IL-2/mL until expanded to appropriate cell numbers.

Note: Every 7 to 10 days, as well as 72 hours prior to nucleofection, primary CD4+ T cells should be re-stimulated/activated with Human T-Activator CD3/CD28 Gibco Dynabeads (or equivalent) + 200 UI/mL IL-2 + 15 ng/mL IL-15 in complete RPMI 1640 medium.

Electroporation of activated $CD4^+$ T cells with TCR-targeting Edit-R sgRNAs

The following example is for knockout of the TCR α/β in primary $CD4^+$ T cells. RNP nucleofections (in 16-well Nucleocuvette® strips) are performed to achieve efficient delivery of the TCR-targeting (TRAC or TRBC) Edit-R sgRNAs or Edit-R sgRNA controls (a positive control such as PPIB and a non-targeting control [NTC]). The protocol below describes the delivery conditions for the Lonza Bioscience P3 Primary Cell 96-well Nucleofector Kit and program EO-115.

1. Culture primary $CD4^+$ T cells to reach appropriate cell numbers for nucleofection experiment (250,000/Nucleocuvette well).

2. Stimulate/active the $CD4^+$ T cells using the recommended protocol for Human T-Activator CD3/CD28 Gibco Dynabeads + 200 UI/mL IL-2 + 15 ng/mL IL-15 in complete RPMI 1640 medium (10% FBS, 2mM L-glutamine, 1 mM sodium pyruvate, 10mM HEPES, 10 mM nonessential amino acids) for 72h prior to electroporation.

3. Mix individual working stocks of TCR-targeting Edit-R sgRNAs with Cas9 nuclease protein NLS (CAS12206, 500 µg at 61.8 µM) at a 2:1 complex ratio. Complexes formed for 10-15 minutes at room temperature in a V-bottom 96-well sterile microtiter plate.

Note: To deliver three replicates of 50 pmol RNP complexes to 250,000 $CD4^+$ T cells/well, 4.5 µL 80 µM sgRNA was mixed with 4.5 µL 40 µM Cas9-NLS protein to generate RNP complexes (20 pmol/µL).

4. While RNP complexes are forming, prepare primary $CD4^+$ T cells for nucleofection by counting $CD4^+$ T cell culture concentration and viability using an instrument such as the Cellometer®K2 or Cellometer® Auto T4.

5. Wash the total number of $CD4^+$ T cells for the experiment (250,000 cells/Nucleocuvette well) with sterile DPBS in a table-top centrifuge (300-500xg for 5 minutes at 25°C) and resuspend in Lonza P3 buffer (20 µL/well).

Note: Set the $CD4^+$ T cells on ice as a pellet and only resuspend cells in P3 buffer just prior to aliquoting into the Nucleocuvette wells, when RNP complexation is complete.

6. Pre-warm a 96-well tissue culture plate with 100 µL supplement RPMI 1640 per 96-well to transfer electroporated cells in the incubator (37 °C, 5% CO_2).

7. After complexes form, aliquot 2.5 µL of RNP complex (50 pmol) to three wells of sterile 96w V- or U-bottom plate for three replicates.

8. Add 20 µL of $CD4^+$ T cells in Lonza P3 buffer to the 2.5 µL RNP sample solution and carefully mix by pipetting.

9. Pipette the $CD4^+$ T cell/RNP solution into individual Nucleocuvette wells and place Nucleocuvette strip cover (lid) over the wells. Load the 16-well Nucleocuvette strip into the 4D-Nucleofector Unit™ X being careful not to form bubbles in the wells. Run the EO-115 nucleofector protocol.

10. Add 80 µL of pre-warmed supplement RPMI 1640 (with fresh 50 UI/mL IL-2) to the $CD4^+$ T cells.

11. Let electroporated cells rest for 5-20 min in the incubator (37 °C, 5% CO_2).

12. Transfer the electroporated $CD4^+$ T cells to the prepared 96-well tissue culture plate of 100 µL RPMI 1640 supplemented with fresh 50 UI/ml IL-2 using a pipette. Return plate to the incubator for 72 hours.

Note: Cells were assessed for TCR knock-out phenotype by flow cytometry (e.g., TRAC- and TRBC-targeting sgRNAs) at 72 hours, however editing by indel formation (T7EI, TIDE, or NGS) can be evaluated as early as 48 hours post electroporation.

Measure TCR knockout on primary $CD4^+$ T cells by flow cytometry

72 hours after electroporation, primary $CD4^+$ T cells edited with TRAC or TRBC targeting Edit-R sgRNA RNPs can be assessed for TCR α/β knock-out by flow cytometry using BioLegend APC anti-human TCR α/β antibody.

In addition to a phenotypic read-out by flow cytometry, between 48–72-hours post RNP-based editing, indel formation can be measured by T7EI/TIDE. Using the primers listed per validated sgRNA in Table 1, follow the direct cell lysis and PCR conditions from Dharmacon™ Edit-R™ synthetic gRNA positive control kits protocol. To measure indel formation by T7EI endonuclease, complete the above listed protocol and use analysis software. To measure indel formation by tracking of indels by decomposition (TIDE) analysis, send resulting PCR amplicon for Sanger sequencing and use webtool such as http://shinyapps.datacurators.nl/tide/.

The protocol below describes the staining conditions used for assessing phenotypic knockout of TCR α/β from primary $CD4^+$ T cells by flow cytometry.

1. Pellet $CD4^+$ T cells electroporated with PPIB, NTC2, TRAC, or TRBC targeting RNPs by centrifugation (5 minutes at 300-500xg at 25°C) in a V-bottom 96-well plate. Wash cells in 100 µL fluorescent activated sorting (FACS) buffer by gently pipetting to resuspend pellet followed by centrifugation (300-500xg at 25°C for 5 minutes).

Note: Remove wash supernatant by quickly flicking the plate over sink (or another appropriate receptacle) or use a multi-channel pipette to carefully remove all supernatant.

2. Resuspend the washed $CD4^+$ T cells in 50 µL of 1:400 dilution of Zombie Green™ Viability Dye (lyophilized powder resuspended in DMSO per BioLegend instructions) to DPBS. Incubate at room temperature in the dark for 15-30 minutes.

3. Quinch permeabilizing dye and wash with 100 µL BioLegend Cell Staining Buffer (Cat No. 420201) or equivalent FACS staining buffer containing serum or BSA. Pellet cells by centrifugation (300-500xg at 25°C for 5 minutes) and remove wash supernatant.

4. Resuspend by gently pipetting cells in 50 µL with a 1:20 dilution of Human TruStain FcX™ in FACS staining buffer. Incubate at room temperature for 5-10 minutes.

Note: Fc receptor (FcR) blocking solution reduces false positive and false negative immunostaining that results from FcR expression on a variety of immune cell types.

5. Pellet cells (washing is not necessary) and resuspend in 50 µL of 1:25 dilution APC anti-human TCR α/β antibody FACS staining buffer. Incubate on ice, in the dark, for 20-30 minutes.

Note: Determining the correct concentration of antibody for your cell type of interest, the fluorophore you choose, and flow cytometer. This is important.

6. Bring the 50 µL of cell stain solution up to 150 µL with FACS buffer to wash. Pellet cells by centrifugation (300-500xg at 25°C for 5 minutes) and remove wash supernatant.

7. Wash cells a second time by gently pipetting to resuspend pellet with 150 µL of FACS buffer. Pellet cells by centrifugation (300-500xg at 25°C for 5 minutes) and remove wash supernatant.

8. Resuspend in a final volume of FACS buffer that works with your flow cytometer and plate type (i.e., 96-well plate or individual flow tubes) for collection/analysis.

Note: It is important to determine the correct concentration of TCR α/β antibody for your flow cytometer by performing an antibody titration experiment. In addition, determine whether compensation is required to co-stain with Zombie Green or other viability dye of choice.

9. Run samples on flow cytometer and analyze with software such as FlowJo or FCS Express™, to determine the TCR knockout phenotype per sample.

Materials

Edit-R CRISPR-Cas9 materials for gene editing can be ordered at horizondiscovery.com

Cells maintenance:

Primary isolated $CD4^+$ T cells
Recommended isolation kit: MojoSort™ Human CD4 T Cell Isolation Kit from Biolegend

Complete and supplemented RPMI (10% FBS, 2mM L-glutamine, 1 mM sodium pyruvate, 10mM HEPES, 10 mM nonessential amino acids)

Cell counting instrument
Recommended: Cellometer®K2 or Cellometer® Auto T4

Human Recombinant IL-2

Human Recombinant IL-15

Human T-Activator CD3/CD28 Gibco Dynabeads

RNP-complex formation and electroporation:

Cas9 nuclease protein NLS

Edit-R synthetic sgRNA non-targeting control

Edit-R synthetic sgRNA PPIB positive control

Edit-R pre-designed synthetic sgRNAs

10 mM Tris-HCl buffer pH 7.4 (Cat #B-006000-100)

Lonza Bioscience 4D-Nucleofector® Unit X

P3 Primary Cell 96-well Nucleofector Kit

V-bottom microtiter plate

96-well tissue culture plate

Measure edit rate by flow cytometry:

FACS buffer: DPBS supplemented with 2% FBS and 1 mM EDTA

FACS staining solution: DPBS supplemented with 2% FBS, 1 mM EDTA, and 0.5% BSA (bovine serum albumin)

BioLegend APC anti-human TCR α/β antibody (clone IP26)

BioLegend APC Mouse IgG1, κ Isotype Ctrl (clone MOPC-21)

BioLegend Zombie Green Fixable Viability Kit

BioLegend Human TruStain FcX (Fc receptor blocking solution)

Dulbecco's Phosphate-Buffered Saline (PBS)

Flow cytometer (Attune NxT)

For more information:
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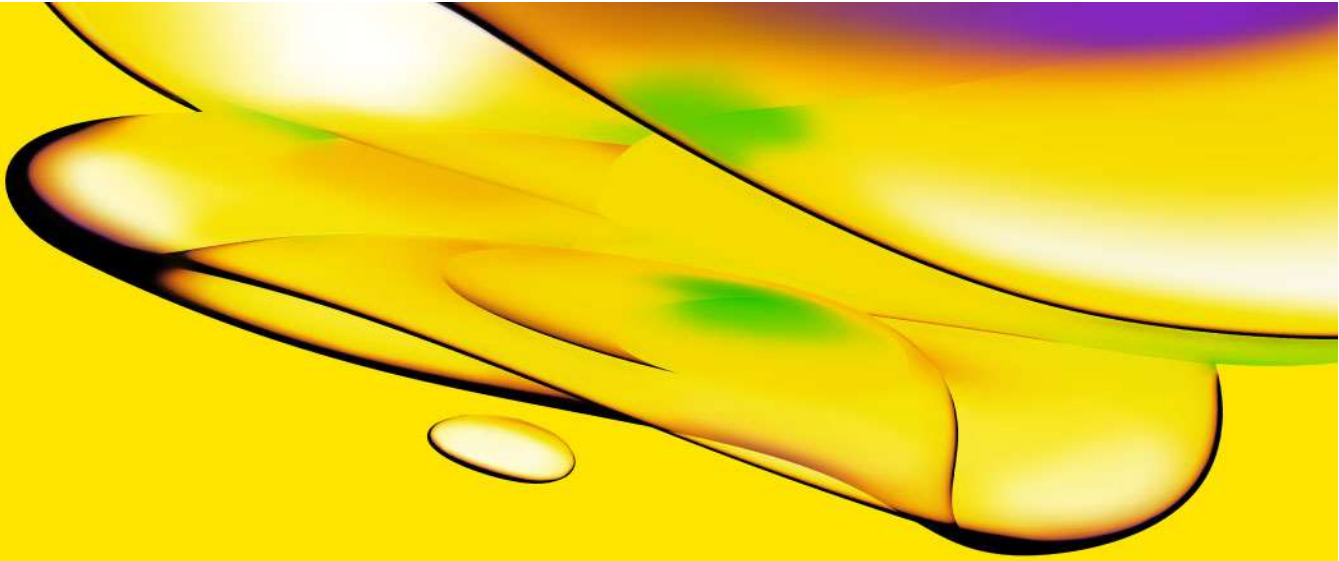

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