Homology-directed repair using the Alt-R™ CRISPR-Cas9 System and HDR Donor Oligos in primary T cells

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REVISION HISTORY

Version	Release date	Description of changes
1	July 2023	Initial release

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INTRODUCTION

CRISPR editing in primary T lymphocytes has distinct challenges compared to genome editing in immortalized cell lines. Based on our research findings, we have developed this optimized protocol for editing primary T cells. The protocol provides guidance on the preparation of T cells for editing, precise genome editing using Alt-R HDR Donor Oligos, and minimizing cytotoxicity when using the Alt-R HDR Enhancer V2.

CONSUMABLES & EQUIPMENT

Consumables—Kit contents

Gene editing reagents

Item	Catalog #
Alt-R CRISPR-Cas9 tracrRNA, 5 nmol	1072532
Alt-R CRISPR-Cas9 crRNA	Website
Nuclease Free Duplex Buffer	11-01-03-01
Alt-R CRISPR-Cas9 sgRNA	Website
Alt-R S.p. Cas9 Nuclease V3, 100 µg	1081058
Alt-R Cas9 Electroporation Enhancer, 2 nmol	1075915
Alt-R HDR Enhancer V2, 30 µL	10007910
IDTE pH 7.5 (1X TE Solution)	11-01-02-02
Nuclease Free Water	11-04-02-01

Consumables—Other suppliers

Recommended primary T cell culture reagents

Item	Supplier	Catalog #
Human PB Pan-T, Cryo, 2x10e7	STEMCELL Technologies, Inc.	70024
ImmunoCult™ Human CD3/CD28 T Cell Activator, 10 mL	STEMCELL Technologies Inc.	10991
ImmunoCult-XF T Cell Expansion Medium, 500 mL	STEMCELL Technologies Inc.	10981
Human Recombinant IL-2, ACF	STEMCELL Technologies Inc.	78145

Other reagents

Item	Supplier	Catalog #
QuickExtract™ DNA Extraction Solution	LGC Biosearch Technologies	QE0901L or SS000035-D2
PBS, pH 7.4	Thermo Fisher Scientific	10010023
P3 Primary Cell 96-well Nucleofector™ Kit	Lonza	V4SP-3096

PROTOCOL

Prepare CRISPR reagents

Resuspend your RNA oligos in nuclease-free IDTE Buffer.

Item	Final concentration (µM)
Option 1 • Alt-R CRISPR-Cas9 crRNA + Alt-R CRISPR-Cas9 tracrRNA Option 2 • Alt-R CRISPR-Cas9 sgRNA	100
Alt-R Cas9 Electroporation Enhancer (optional)	100
Alt-R HDR Donor Oligo	100, or the adjusted concentration for your planned experiment

Note: For assistance, use the IDT Resuspension Calculator.

Tips:

- Always store CRISPR reagents at –20°C.
- Always centrifuge tubes prior to resuspension.

Prepare the gRNA complex

Note: If you are preparing an sgRNA, no annealing step is required. Simply dilute the sgRNA to the desired concentration in nuclease-free IDTE.

Prepare a two-part gRNA complex (combining crRNA and tracrRNA), which anneals the oligos to form a guide RNA complex.

1. Combine the following components to make the gRNA complex at a final concentration of 50 µM.

Item	Amount (µL)
100 µM Alt-R CRISPR-Cas9 crRNA	5
100 µM Alt-R CRISPR-Cas9 tracrRNA	5
Nuclease Free Duplex Buffer (to final volume)	As needed
Total volume	10

2. Heat the mixture at 95°C for 5 min.
3. Cool to room temperature (15–25°C) on the bench top.

Safe Stop (optional): Store gRNA complexes at –20°C up to 1 year.

Prepare the RNP complex

Combining the gRNA and Cas9 nuclease allows an RNP complex to form. Prepare the RNP complex to yield 4 µM Cas9 protein and 4.8 µM gRNA in the final delivery mixture.

Note: You can optimize the final RNP concentration for each guide. In general, a 2 to 4 µM RNP concentration allows for maximal editing in primary T cells.

1. Combine the following components per each electroporation well:

Item	Amount (µL)
gRNA (50 µM)	2.4
Alt-R Cas9 enzyme (61 µM)	1.6
PBS (to final volume)	As needed
Total volume	4

2. Vortex and incubate at room temperature for 10–20 min.

Tip: Editing in primary T cells can be improved with the use of Alt-R Electroporation Enhancer. Recommended final concentration of Alt-R Electroporation Enhancer is 3 µM.

Safe Stop (optional): Store RNP complexes at 4°C up to 1 month, or at –80°C up to 2 years in single-use aliquots.

Prepare the Alt-R HDR Donor Oligos

When preparing your Alt-R HDR Donor Oligos, dilute your template in nuclease-free IDTE, water, or an appropriate buffer such that your desired dose is delivered in a 1 µL volume.

Example: Dilution for a 3 µM dose of an 86 nt oligo for 1 reaction. Scale up as needed.

Component	Amount (µL)
Alt-R HDR Donor Oligos at 100 µM	0.75
IDTE, water, or appropriate buffer	0.25
Total volume	1

Note: The right dose of your Alt-R HDR Donor Oligos will vary with the sequence length and may need to be customized. In general, a 2 to 4 µM concentration allows for maximal HDR in primary T cells.

Prepare the cell culture dish and media

1. Thaw and activate primary T cells according to supplier's directions. For example, thaw frozen cells and add contents of tube to a 15 mL conical with 10 mL ImmunoCult expansion medium. Pellet cells at 300 x *g* for 10 minutes, gently remove supernatant, and gently resuspend cell pellet in 10 mL expansion medium with IL-2 at 10 ng/mL and 25 µL/mL ImmunoCult T cell activator. Transfer cells to a 100 mm dish. Incubate and allow activation 24–48 hrs prior to transfection.
2. Prepare media for transfection:
   a. Prepare complete media as desired (for example, ImmunoCult expansion medium with 10 ng/mL IL-2) per nucleofection sample.
   b. Prewarm 75 µL per nucleofection of complete media in a conical tube. This recovery media will be added to cells in the 96-well Nucleocuvette™ plate immediately after nucleofection.
   c. Aliquot 175 µL of complete media per nucleofection sample into a 96-well plate. If desired, plate triplicate wells for each nucleofection sample. 25 µL of nucleofected cells in recovery media will be added for a final volume of 200 µL per well.
   d. Prewarm the recovery media and final incubation plate(s) in a 37°C tissue culture incubator.
3. (Optional) Prepare complete media with Alt-R HDR Enhancer V2 and prewarm to 37°C for use after nucleofection.
   a. Mix the Alt-R HDR Enhancer V2 with prepared media at desired concentration of enhancer.

   Note: Review section Minimize cytotoxicity when using Alt-R HDR Enhancer V2 in this protocol. Recommended concentration of Alt-R HDR Enhancer V2 for primary T cells is 1 µM. To reach a final concentration of 1 µM Alt-R HDR Enhancer V2 after the nucleofected cells are added, add 1.4 µL of the Alt-R HDR Enhancer V2 stock solution (690 µM) per 1 mL of media. Scale according to the number of samples.

   Tip: When monitoring toxicity, we recommend including negative controls of DMSO only and untreated media. For the DMSO only control, add 1.4 µL of DMSO per 1 mL of media.

Transfect cells

Prepare primary T cells for a standard nucleofection experiment and make sure the cells are washed with PBS prior to nucleofection to remove any residual media components.

For example, gently mix cell suspension to break up cell clumps. Transfer cells to a sterile conical tube and count density using a hemacytometer. Aliquot desired number of cells to a new conical. Pellet cells at 300 x *g* for 10 minutes, then gently remove supernatant without disturbing the cell pellet. Add 10 mL 1X PBS and gently mix to wash cells. Pellet cells again at 300 x *g* for 10 minutes, then carefully remove supernatant without disturbing the cell pellet.

1. Suspend cells in P3 Buffer at 20 µL per nucleofection.

   Note: For a 96-well plate Nucleocuvette module, we recommended that the cell density per nucleofection well is 1 x 10^6.

2. Make the final transfection mix by combining the following components:

Item	Amount (µL)
RNP complex	4
Alt-R HDR Donor Oligos	1
Cell suspension*	20
Total volume	**25**

* If using Alt-R Electroporation Enhancer, transfection volume can be increased up to 30 µL. Recommended final concentration of Alt-R Electroporation Enhancer is 3 µM.

3. After mixing the transfection mix, transfer 25 µL to a 96-well Nucleocuvette module. Gently tap to remove any air bubbles that may be present.
4. Transfect cells according to the manufacturer's specifications.

   Note: Recommended zap code for primary T cells is 96-EH-140.

5. After electroporation, add 75 µL of prewarmed recovery media (without Alt-R HDR Enhancer V2) per well and gently mix cells.
6. Transfer 25 µL of resuspended cells to the culture plates containing the prewarmed 175 µL of culture media with and without Alt-R HDR Enhancer V2, as applicable.
7. Incubate cells in a tissue culture incubator until desired confluency is reached, 48–72 hours.
8. If using Alt-R HDR Enhancer V2, change media:

   After 12–24 hours, carefully remove the media from the cells, and replace with fresh primary T cell culture media without Alt-R HDR Enhancer V2.

Isolate gDNA

Perform genomic DNA isolation using the desired method at 48–72 hours post electroporation.

For example, to isolate genomic DNA using QuickExtract DNA Extraction Solution, wash cells with PBS (optional), then add 50 µL QuickExtract solution per well of a 96-well plate.

Minimize cytotoxicity when using Alt-R HDR Enhancer V2

The Alt-R HDR Enhancer V2 is provided as a 690 µM concentrated solution in dimethyl sulfoxide (DMSO). Use of both DMSO and the Alt-R HDR Enhancer V2 can be toxic to cells—the toxicity of DMSO is noticeable when used at high doses, while the toxicity of Alt-R HDR Enhancer V2 is noticeable at high doses, or for long periods of exposure. Due to the increased potency of the Alt-R HDR Enhancer V2, the enhancer solution can be used at lower doses than Alt-R HDR Enhancer V1 and results in improved cell viability. Because of this, we recommend the following guidelines:

- Use a maximum of 1% by volume DMSO in the final media.
- Use a control sample with DMSO (but no Alt-R HDR Enhancer V2) in the final media to monitor toxicity.
- Use a concentration within the range of 1–2 µM of Alt-R HDR Enhancer V2 in the final media.
- Change to growth media without Alt-R HDR Enhancer V2 12–24 hours after electroporation.

Important: The best concentration for Alt-R HDR Enhancer V2 is cell type dependent and may require a titration. Toxicity should be monitored closely when used at concentrations higher than 1 µM. See Improving efficiency of homology-directed repair (HDR) for more information.

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