Homology-directed repair using the Alt-R™ CRISPR-Cas9 System and Alt-R HDR Donor Oligos in induced pluripotent stem cells (iPSCs)

REVISION HISTORY

Version	Release date	Description of changes
1	July 2023	Initital release

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INTRODUCTION

This protocol explains how to perform HDR in iPSCs using the Alt-R CRISPR Cas9 System with Alt-R HDR Donor Oligos.

CONSUMABLES

Gene editing reagents—IDT

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

* This protocol has been developed with the Alt-R *S.p.* Cas9 Nuclease V3, however you could substitute our Alt-R *S.p.* HiFi Cas9 Nuclease V3 as well for the same result.

Recommended iPSC reagents—Other suppliers

Item	Supplier	Catalog #
iPSC from Fibroblast	Coriell Institute	GM23338
mTeSR™ Plus Kit, cGMP	STEMCELL Technologies Inc.	100-0276
Vitronectin XF™	STEMCELL Technologies Inc.	07180
CellAdhere™ Dilution Buffer	STEMCELL Technologies Inc.	07183
CloneR™2	STEMCELL Technologies Inc.	100-0691
ReLeSR™	STEMCELL Technologies Inc.	100-0483

Consumables—Other suppliers

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

PROTOCOL

Prepare CRISPR reagents

Resuspend your oligos in nuclease-free IDTE. Resuspend your dsDNA template in Nuclease-Free Water, IDTE, or an appropriate buffer for your use case.

Reagent	Final concentration
Alt-R crRNA and tracrRNA or sgRNA	100 µM
Alt-R Cas9 Electroporation Enhancer (optional)	100 µM
Alt-R HDR Donor Oligo	100 µM, or the right concentration for your planned experiment

Note: For assistance, use the IDT Resuspension Calculator.

Tips:

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

Prepare the gRNA complex

Note: If you are preparing a 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 complex.

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

Component	Amount (µL)
100 µM Alt-R CRISPR-Cas9 crRNA	5
100 µM Alt-R CRISPR-Cas9 tracrRNA	5
IDT 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 benchtop.

Safe Stop: (optional) gRNA complexes can be stored at –20°C for up to 1 year.

Prepare the RNP complex

An RNP complex forms when the gRNA and Cas9 Nuclease are combined. Prepare the RNP complex to yield 4 µM Cas9 protein and 4.8 µM gRNA in the final delivery mixture.

Note: You can fine tune the final RNP concentration for each guide. In general, a 2–4 µM RNP concentration allows for maximal editing in iPSC.

1. Combine the following reagents per each electroporation well:

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

2. Incubate at room temperature for 10–20 min.

Tip: Editing capabilities in iPSC can be improved with the use of Alt-R Electroporation Enhancer. Recommended final concentration of Alt-R Electroporation Enhancer is 4 µM. Alternatively, the Cas9:gRNA ratio of 1:1.2 can be increased to 1:2.5 with or without the addition of Alt-R Electroporation Enhancer.

Safe Stop: (optional) Cas9 RNP complexes are stable up to 1 year at -80°C and up to 2 months at 4°C.

Prepare the HDR donor oligo

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

Example: Dilution for a 4 µM dose of an 86 nt oligo for one transfection sample (25 µL volume). Scale up as needed.

Reagent	Amount (µL)
Alt-R HDR oligo at 100 µM	1
IDTE, water, or appropriate buffer	0
Total volume	1

Note: The actual dose of your Alt-R HDR Donor Oligo will vary with the sequence length and may need to be fine-tuned. In general, a 2–4 µM concentration allows for maximal HDR in iPSC.

Prepare the cell culture dish and media

1. Prepare non-treated 96-well plate(s) with desired matrix (feeder-cell free method). For example, use Vitronectin XF™ to coat wells according to the manufacturer's protocol.
2. Prepare appropriate iPSC culture media (for example, complete mTeSR™ Plus) with preferred ROCKi (for example, CloneR™2 at 1X final concentration) at desired concentration. Prepared media is iPSC culture media with ROCKi.
   a. Prewarm 75 µL of the prepared media per nucleofection sample. This media will be added to cells in the 96-well Nucleocuvette module after nucleofection.

b. Aliquot 175 µL of the prepared media per nucleofection sample into a 96-well plate according to the number of samples being nucleofected. Following nucleofection, 25 µL of nucleofected cells will be added for a final volume of 200 µL per well.

c. Prewarm the plate(s) in a tissue culture incubator.

3. Prepare iPSC culture media with ROCKi and Alt-R HDR Enhancer V2, then prewarm to 37°C for use after nucleofection.

a. Mix the Alt-R HDR Enhancer V2 with prepared media to the desired concentration.

Note: See Minimize cytotoxicity when using Alt-R HDR Enhancer V2. Recommended concentration of Alt-R HDR Enhancer V2 for iPSC is 0.5 µM. To achieve a final concentration of 0.5 µM Alt-R HDR Enhancer V2 after the nucleofected iPSC are added, add 0.7 µL of the Alt-R HDR Enhancer V2 stock solution (690 µM) per 1 mL of media. If needed, scale up according to the number of samples.

Tip: When monitoring toxicity, include negative controls of DMSO only and note it is recommended to use untreated media. For the DMSO only control, simply add 0.7 µL of DMSO per 1 mL of media.

b. Aliquot 175 µL of the prepared media into a 96-well plate. For conditions without Alt-R HDR Enhancer V2, use prepared media. Following nucleofection, 25 µL of nucleofected cells will be added for a final volume of 200 µL per well.

c. Prewarm the plate in a tissue culture incubator.

Note: This media will be used for culturing cells for 12–24 hr after nucleofection. If wanted, plate triplicate wells for each nucleofection sample.

Transfect cells

Prepare iPSC for a standard nucleofection experiment, making sure the cells are washed with PBS before nucleofection to remove any residual media components. For example, use ReLeSR™ to detach cells according to the manufacturer's protocol. Gently break up cells into single cells.

1. Suspend cells in 20 µL of Nucleofection Buffer.

Note: For a 96-well plate Nucleocuvette module, recommended cell density per nucleofection well is 1 to 2 x 10^6. Recommended buffer kit for iPSC is P3.

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

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

* When using Alt-R Electroporation Enhancer, the transfection volume can be increased up to 30 µL. Recommended final concentration of Alt-R Electroporation Enhancer is 4 µM.

3. After mixing the transfection mix, transfer 20 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.

Tip: The recommended zap code for iPSC is CA-137.

5. After electroporation, add 75 µL of prewarmed prepared media (without Alt-R HDR Enhancer V2) per well and gently resuspend cells.
6. Transfer 25 µL of resuspended cells to the culture plates containing the prewarmed 175 µL of culture media with or without Alt-R HDR Enhancer V2, as applicable.
7. Incubate cells in a tissue culture incubator.

Change media

1. Change iPSC culture media in the plate according to the manufacturer's protocol and/or the iPSC workflow.
2. **When using Alt-R HDR Enhancer V2:** After 12–24 hours, carefully remove the media from the cells, and replace with fresh iPSC culture media *without* Alt-R HDR Enhancer V2.

Isolate gDNA

You can perform genomic DNA (gDNA) isolation using the desired method at 48–72 hours post electroporation. However, if confluency is low, cells maybe grown for up to 4 or 5 days before genomic DNA isolation. For example, to isolate genomic DNA using QuickExtract™ DNA Extraction Solution, wash cell with PBS, then add 50 µL QuickExtract solution per well in 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).

Important: 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 the Alt-R HDR Enhancer V2 is noticeable at high doses, or for long periods of exposure. Follow the guidelines below to avoid or minimize the risk of cell toxicity.

Because of the increased potency of the Alt-R HDR Enhancer V2, use these guidelines for best results and improved cell viability:

- 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 for most cell lines. For iPSC, the recommended dose is 0.5 µM.
- Change to growth media *without* Alt-R HDR Enhancer V2 12–24 hours after electroporation.

  Important: The right concentration for Alt-R HDR Enhancer V2 is cell type dependent and may require a dose titration. Toxicity should be closely monitored when used at concentrations higher than 1 µM.

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For more information, go to: www.idtdna.com/ContactUs

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