Notices

Limitations of use

For research use only. Not for use in diagnostic procedures. Unless otherwise agreed to in writing, IDT does not intend these products to be used in clinical applications and does not warrant their fitness or suitability for any clinical diagnostic use. Purchaser is solely responsible for all decisions regarding the use of these products and any associated regulatory or legal obligations.

Safety data sheets pertaining to this product are available upon request.

Safety notices

Reminder symbols call attention to minor details that may be easily overlooked and compromise the procedure resulting in decreased assay performance.

Caution symbols denote critical steps in the procedure where risk of protocol failure or damage to the product itself could occur if not carefully observed.

Stop symbols indicate where this procedure may be safely suspended and resumed at a later time without risk of compromised assay performance. Make note of these steps and plan your workflow accordingly.

Revision history

Version	Release date	Description of changes
1	July 2023	Initial Release (Doc ID: RUO23-2040_001)
2	October 2025	New Doc ID: RA-DOC-622 REV01. Under “Prepare the cell culture dish and media”: Updated wording on Prepared Media and Prepared Media with HDR Enhancer V2 for clarity. Under “Transfect cells”: Updated Note on cell count for transfection. Throughout protocol: Minor changes to fix typos and improve clarity.

Contents

Technical support

Visit https://www.idtdna.com/pages/support for a helpful answers to frequently asked questions or contact us directly at applicationsupport@idtdna.com.

Overview

Product Description

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 Available from IDT	
Description	**Part Number**
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 similar results.

Recommended iPSC Reagents – Other Suppliers		
Description	**Supplier**	**Part Number**
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

*Suggested products

Consumables – Other Suppliers		
Description	**Supplier**	**Part Number**
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 an optimal 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.

Reagent	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 bench top.

Safe Stop: (optional) gRNA complexes can be stored at -20°C 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 components per each electroporation well:

Reagent	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. Incubate at room temperature for 10-20 min.

Tip: Editing 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 reaction (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 target site and total (insert and homology arm) 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 following manufacturer's protocol.
2. Prepare appropriate iPSC culture media (for example, complete mTeSR™ Plus) with preferred ROCKi at desired concentration (for example, CloneR™2 at 1X final concentration). iPSC culture media with ROCKi will be referred to as Prepared Media henceforth.
    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 Prepared Media for the nucleofection sample and plate(s) with Prepared Media in a tissue culture incubator.
3. For conditions with HDR Enhancer V2, add the Enhancer to 175 µL Prepared Media per nucleofection sample at desired concentration and prewarm to 37°C for use after nucleofection, similar to the previous step.

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, it is recommended to include negative controls of DMSO only and untreated media. For the DMSO only control, simply add 0.7 µL of DMSO per 1 ML of media.

Note: This media will be used for culturing cells for 12–24 hr after nucleofection. If necessary, 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 clumps of cells into single cells.

1. Suspend cells in 20 µL of Nucleofection Buffer.

Note: For a 96-well plate Lonza Nucleocuvette module, the recommended total cell count per nucleofection well is 1 to 2 x 10^5 cells. The recommended buffer kit is iPSC is P3.

2. Make the final transection mix by combining the following components.

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

*Although not required for HDR experiments, if Alt-R Electroporation Enhancer is also used, the transfection volume can be increased up to 30 µL. Recommended final concentration of Alt-R Electroporation Enhancer is 4µM.

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 Prepared Media with or without HDR Enhancer V2, as applicable.
7. Incubate cells in a tissue culture incubator at 37°C and 5 % CO_2.

Change media

1. Change media in the plate according to the manufacturer's protocol and/or iPSC workflow, replacing with fresh iPSC culture media *without* ROCKi after 12-24 hours.
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* HDR Enhancer V2.

Isolate gDNA

You can perform genomic DNA isolation using the desired method at 48–72 hours post-electroporation. However, if confluency is low, cells may be 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 and add 50 µL QuickExtract solution per well of a 96-well plate and follow manufacturer's protocol for downstream steps.

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 of 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.