Notices

Limitations of use

For research use only. Not for use in clinical or 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 or online at https://www.idtdna.com/pages/support/safety-data-sheets.

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.

Version history

Version	Release date	Description of changes
1	August 2025	*Initial release*

Contents

Technical support

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

Introduction

Alt-R™ HDR Enhancer Protein is an engineered ubiquitin variant designed to increase rates of homology-directed repair (HDR) during genome editing in a variety of cell types using a variety of donor template types, including ssDNA, linear dsDNA, plasmid DNA, and AAV template. HDR Enhancer Protein is intended to be delivered simultaneously with other genome editing reagents, including RNP complex or Cas nuclease mRNA and gRNA, HDR donor, and optional Alt-R™ Electroporation Enhancer, and can be used in combination with the Alt-R™ HDR Enhancer V2.

HDR Enhancer Protein promotes cellular HDR through 53BP1 inhibition. HDR Enhancer Protein binds to 53BP1 to prevent 53BP1 recruitment to double-strand breaks and thereby promotes end resection, a critical step in HDR. HDR Enhancer Protein can further increase rates of HDR even when non-homologous end joining (NHEJ) is strongly repressed using small molecule inhibitors of NHEJ.

HDR Enhancer Protein is distinct from the Alt-R™ HDR Enhancer V2, which is a small molecule that promotes cellular HDR via NHEJ inhibition. The two products are delivered to cells at different steps during the CRISPR editing workflow and have demonstrated an additive increase in HDR when used in combination.

This protocol is designed for cell and reagent preparation and precise genome editing in HEK293 cells using a Lonza Nucleofector. Instructions include an option for a 25 µM final concentration of HDR Enhancer Protein or an option for concentration optimization, and an option to include the Alt-R HDR Enhancer V2. Final concentration of HDR Enhancer Protein varies by cell type. IDT recommends testing a range of concentrations from 1.5 to 50 µM.

For additional guidance, email our Scientific Applications Support team at amr-applicationsupport@idtdna.com.

Alt-R™ HDR Enhancer Protein

The Alt-R™ HDR Enhancer Protein is provided in storage buffer (20 mM Tris-HCL, 300 mM NaCl, 0.1 mM EDTA, 50 % glycerol). Recommended storage is -20ºC.

Materials Supplied		
Description	**Part Number**	**Quantity**
Alt-R™ HDR Enhancer Protein	10029790	500 ug, 35 mg/mL, 4.1 mM, 14.3 uL storage buffer
Alt-R™ HDR Enhancer Protein	10029801	5 mg, 35 mg/mL, 4.1 mM, 142.8 uL storage buffer

Before Getting Started

Materials required but not supplied. Use of other materials have not been tested by IDT.

Materials Required, but not Supplied		
Description	**Supplier**	**Part Number**
Alt-R™ CRISPR-Cas9 tracrRNA (optional with crRNA)	IDT	1072532, 1072533, 1072534
Alt-R™ CRISPR-Cas9 gRNA (crRNA or sgRNA)	IDT	IDT predesigned and custom gRNA*: www.idtdna.com/CRISPR-Cas9
Alt-R™ CRISPR Cas9 Nuclease	IDT	1081058, 1081059, 10000735
Alt-R™ HDR Donor, or other HDR template	IDT	https://www.idtdna.com/pages/products/crispr-genome-editing/alt-r-hdr-donor-oligos
Alt-R™ Electroporation Enhancer (optional)	IDT	1075915, 1075916, 10007805
Alt-R™ HDR Enhancer v2 (optional)	IDT	10007910, 10007921
IDTE pH 7.5	IDT	11-01-02-02
Nuclease-free water	IDT	11-04-02-01
DMSO (molecular biology grade) with optional HDR Enhancer V2	General laboratory supplier	
1X Phosphate buffered saline, pH 7.4	General laboratory supplier	
Cell growth media	Varies	
P3 Primary Cell 96-well Nucleofector Kit	Lonza	V4SC-2096
4D-Nucleofector System	Lonza	AAF-1002B with AAF-1002X
96-well Shuttle System	Lonza	AAM-1001S

Protocol

Prepare CRISPR reagents

Resuspend oligonucleotides in Nuclease-Free IDTE Buffer, pH 7.5. Resuspend dsDNA template in nuclease-free water, IDTE, or an appropriate buffer for your application.

Reagent	Final concentration (µM)
Option 1 (2-part) Alt-R™ Cas9 crRNA Alt-R™ Cas9 tracrRNA	 200 200
Option 2 (sgRNA) Alt-R™ Cas9 sgRNA	100
Alt-R™ HDR Donor Oligo	100 or user optimized concentration
Alt-R™ Electroporation Enhancer (recommended when delivering RNP and donor template separately, e.g., for AAV donor)	100

For assistance, use the IDT Resuspension Calculator at www.idtdna.com/SciTools.

Note: Always store CRISPR reagents at -20ºC.

Prepare the gRNA complex

Annealing

Prepare a two-part gRNA complex by combining and annealing crRNA and tracrRNA.

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

1. Combine the following components to make the gRNA complex at a final concentration of 100 µM.

Reagent	Amount (µL)
200 µM Alt-R™ Cas9 crRNA	5
200 µM Alt-R™ Cas9 tracrRNA	5
Total volume	**10**

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

Stopping point (optional): gRNA complexes can be stored at -20ºC up to 1 year.

Prepare the RNP complex

Combining the gRNA and Cas9 nuclease allows an RNP complex to form. IDT recommends a 1:1.2 molar ratio of Cas9 protein to gRNA.

Note: You can optimize the final RNP concentration for each guide. In general, a 1–4 µM RNP concentration allows for maximal editing.

1. Combine the following components:

Reagent	Amount per reaction (µL), 21.8 µL final reaction volume	Amount per reaction (µL), 24.0 µL final reaction volume
Alt-R™ Cas9 Nuclease (62 µM stock*)	0.71	0.78
sgRNA (100 µM)	0.52	0.58
Total volume per reaction	**1.23**	**1.36**

* Alt-R™ S.p. Cas9 nucleases and nickases are provided at a stock concentration of 62 µM (10 mg/mL). Cas9-GFP and Cas9-RFP are provided at 52 µM (10 mg/mL).

Note: 24 µL reaction volume is recommended for HDR Enhancer Protein concentration optimization experiments.

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

3. Place tube or plate back on ice.

Stopping point (optional): RNP complexes can be stored at 4ºC up to 1 month, or at -80ºC up to 2 years in single-use aliquots.

Prepare cell culture media

If using Alt-R™ HDR Enhancer V2: Optimal final concentration is typically 1–2 µM and can be optimized for each cell type. The following prepares a 1.0 µM final concentration stock solution of HDR Enhancer V2.

Cell culture media with or without the optional Alt-R™ HDR Enhancer V2 should be prepared and pre-warmed to 37ºC for use after electroporation. Alt-R™ HDR Enhancer V2 is a small molecule that works to promote HDR via a different mechanism and is added to the cell media used to incubate cells after treatment with CRISPR reagents.

1. Pre-warm 180 µL per nucleofection sample.

   Note: This media will be added to cells in the 96-well Nucleocuvette plate following nucleofection.

2. If using Alt-R™ HDR Enhancer V2, add 1.74 µL per 1.0 mL of total media (1.2 µM). Addition of cells brings to a final concentration of 1 µM.

   Note: Media with DMSO (1.74 µL per 1.0 mL) is recommended as a control.

3. Add 125 µL of media per well to plate. If desired, plate triplicate wells for each nucleofection reaction. After nucleofection, 25 µL of cells in recovery media will be added for a final volume of 150 µL per well for incubation.

Prepare CRISPR reagent mix

IDT recommends testing varying concentrations of HDR Enhancer Protein from 1.5 to 50 µM to optimize your specific application. Dilute the HDR Enhancer Protein stock (4.1 mM) with electroporation buffer or 1X PBS to 600 µM and perform a series of five 2-fold dilutions with a buffer-only control.

Note: Minimizing non-electroporation buffer components in the final mix may improve editing rates. If additional volume is needed (e.g., for HDR Enhancer Protein optimization), we recommend comparing dilution with 1X PBS or electroporation buffer.

1. Combine all gene editing components to add to cells. The following outlines the preparation of a final concentration of 2 µM RNP, 2 µM ssODN donor, and 25 µM HDR Enhancer Protein, or a second option for HDR Enhancer Protein concentration optimization. We recommend testing a range of doses from 1.56 to 50 µM.

Component	Final Concentration (µM)	Amount *per reaction* (µL) for 21.8 µL final reaction volume	Amount *per reaction* (µL) for 24.0 µL final reaction volume
RNP complex	2	1.231	1.355
HDR Enhancer Protein (35 mg/ml, 4.1 mM stock)	25	0.133	2 (from dilution series)
ssODN donor (100 µM)	2	0.436	0.48
1X PBS or Electroporation buffer (optional diluent)	-	-	0.165
Total CRISPR mix volume	-	**1.8**	**4**

Note: Volumes above are *per reaction*. 24 µL reaction volume is recommended for HDR Enhancer Protein concentration optimization experiments.

2. Mix by repeated pipetting.

3. Transfer the CRISPR mix into a well of a 96-well low-bind plate for each reaction.

4. Keep plate on ice.

Note: If optimizing HDR Enhancer Protein using a dilution series, transfer 2 µL of RNP+donor+buffer (without Enhancer) into wells then mix in 2 µL of HDR Enhancer Protein from a dilution series.

Transfect cells by Nucleofection

The following outlines the delivery of CRISPR reagent mix into HEK293 cells at 2 x 10^5 cells in 20 µL per nucleofection reaction. Cell density and electroporation conditions should be optimized for the cell type and application.

Prepare cells as you would for a standard CRISPR Cas9 nucleofection experiment, make sure that the cells are washed with PBS before nucleofection to remove any residual media/nucleases.

Note: To achieve 2 x 10^5 cells per reaction, adjust cell concentration such that when 20 µL of cells are transferred to the nucleofector plate, it contains 2 x 10^5 cells.

1. Suspend cells in appropriate electroporation buffer (Solution SF for HEK293 cells) at 20 µL per reaction.

   Note: Lonza recommends minimizing the time cells are in electroporation buffer. The following steps should be completed without pause.

2. Transfer 20 µL cells to the wells of the plate containing the CRISPR reagent mix.

3. Mix by repeated pipetting.

4. Transfer 20 µL to the wells of a 96-well Nucleocuvette module. Gently tap to ensure no air bubbles are present.

   Note: Electroporate using desired method. For HEK293 cells, we recommend pulse code DS-150.

5. Recover cells by adding 180 µL pre-warmed cell media to each well in the Nucleocuvette.

6. Transfer 25 µL of cells (2.5 x 10^4 cells) to pre-warmed culture plate containing 125 µL cell media.

7. Incubate cells at 37ºC with 5% CO_2.

   Note: *If using HDR Enhancer V2*, change media to fresh media without HDR Enhancer V2 12-24 hours after electroporation.

8. Incubate cells for a total of 48 hours after RNP complex delivery before assessing for editing/HDR. If delivering Cas9 as mRNA or plasmid, incubate cells for 72 hours.

Appendix

Equation 1. Determination of Final Reaction Volume

$$\text{Final Volume} = \frac{\text{Volume of cells + Volume of Diluent*}}{1 - \left(\frac{[\text{Cas9}]_{\text{Final}}}{[\text{Cas9}]_{\text{Stock}}} + \frac{[\text{gRNA}]_{\text{Final}}}{[\text{gRNA}]_{\text{Stock}}} + \frac{[\text{Donor}]_{\text{Final}}}{[\text{Donor}]_{\text{Stock}}} + \frac{[\text{HDR Enhancer Protein}]_{\text{Final}}}{[\text{HDR Enhancer Protein}]_{\text{Stock}}}\right)}$$

*Recommended diluents are 1X PBS or electroporation buffer

Equation 2. Determination of Component Volumes

$$\text{Volume of Each Component} = \frac{\text{Final Volume} \times [\text{Component}]_{\text{Final}}}{[\text{Component}]_{\text{Stock}}}$$

Where the components are Cas9, gRNA, donor template, and HDR Enhancer Protein