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

Simultaneous delivery of RNP complexes and ssODN repair templates using the Nucleofector™ System

For use with:

- HDR Enhancer V2
- Cas9 Electroporation Enhancer
- Cas9 Nuclease
- gRNA: crRNA + tracrRNA or sgRNA
- HDR Donor Oligos (specialized Ultramer™ ssDNA Oligos)

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REVISION HISTORY

Version	Release date	Description of change
4	February 2022	Updated to inlcude new products (labeled tracrRNAs and fluorescent Cas9 enzymes).
3	August 2021	Updated to include additional information on new Alt-R HDR Enhancer V2.
2	November 2019	Added information on selecting a guide RNA using the Alt-R HDR design tool and designing donor DNA templates for HDR experiments. Included guidelines on how to maximize use of Alt-R HDR Enhancer.
1	October 2018	Initial release

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INTRODUCTION

This protocol is designed for homology-directed repair (HDR) using CRISPR-Cas9 genome editing in cultured cells for research applications. The protocol involves the codelivery of an HDR donor oligo [single-stranded oligodeoxynucleotide (ssODN)] and a CRISPR-Cas9 ribonucleoprotein (RNP) complex using electroporation with the Nucleofector™ System (Lonza).

Important! This protocol is for use with Alt-R HDR Donor Oligos that are synthesized as specialized Ultramer DNA Oligos and should be designed using the **Alt-R HDR design tool**. If you are using longer donor templates based on **Megamer™ DNA Fragments**, use this protocol: **Homology-directed repair using the Alt-R CRISPR-Cas9 System and Megamer ssDNA Fragments**. For more information about designing Megamer DNA Fragments as HDR donor templates, read this **guide**.

For primary or difficult-to-transfect cells, we recommend using the Alt-R Cas9 Electroporation Enhancer to improve delivery. For HDR experiments, we recommend using the Alt-R HDR Enhancer V2, which is a small molecule compound that can increase the rate of HDR. Alt-R HDR Enhancer V2 is a new and improved version, and is active at much lower concentrations. While the efficiency of HDR and relative improvement in HDR rates vary by cell line, editing site, and the desired insert, we offer guidelines and suggestions that maximize HDR potential while limiting cytotoxicity often associated with the delivery of HDR Enhancer V2 and genome editing reagents into cells.

Workflow

1	Prepare the gRNA complex	Total time: 15 minutes
2	Prepare the RNP complex	Total time: 20 minutes
3	Prepare the cell culture media	Total time: 10 minutes
4	Transfect cells of interest	Total time: 30 minutes
5	Change media	Total time: 15 minutes
6	Isolate gDNA and detect mutations	Total time: Varies

Optional stopping point

GUIDELINES

Optimize CRISPR editing

Confirm guide RNA (gRNA) efficiency before using in HDR experiments. Take the following factors into consideration:

- Low Cas9 editing efficiency will negatively impact HDR rates. (See our DECODED article for more detailed information: Applications of CRISPR: Tips for designing CRISPR-Cas9 mediated HDR experiments.)
- If using more than one gRNA (e.g., with Cas9 Nickase D10A), assemble the RNP complexes in individual tubes, then combine only prior to delivery.
- IDT recommends the use of proper controls in your experiment, such as the appropriate Alt-R CRISPR-Cas9 Control Kit for studies in human, mouse, or rat cells.

Design an ssODN donor template

The template sequence for ssDNA HDR donors can be designed to contain either the targeting or non-targeting strand of the genomic DNA. Homology arms (the donor template sequence elements that match either side of a cut site) must be included for HDR experiments. We recommend using the Alt-R HDR design tool for assistance in selecting a guide RNA and designing donor DNA templates for HDR experiments.

Generally, we observed higher HDR efficiency when the following conditions were met:

- **Adding chemical modifications on each end of an ssODN template to help stabilize the donor oligo.** Alt-R HDR Donor Oligos contain chemical modifications optimized for improved HDR rates; modifications present include two phosphorothioate (PS) bonds between the first and last three bases and a proprietary end-blocking group on both the 5′ and 3′ end of ssODN templates.
- **Keeping homology arms between 30–50 nt long.** Longer homology arms may be beneficial in cell types with high nuclease environments or when stabilizing modifications are not included in the donor oligo.
- **Incorporating silent mutations within the donor oligo to prevent Cas9 from re-cutting the target after the desired edit has been made.**

Note: See our application note Optimizing for CRISPR-Cas9 homology-directed repair using Ultramer Oligonucleotides for more information on template design.

Minimize cytotoxicity when using Alt-R HDR Enhancer V2

The Alt-R HDR Enhancer V2 is provided as a 0.69 mM concentrated solution in dimethyl sulfoxide (DMSO). Use of both DMSO and the HDR Enhancer V2 can be toxic to cells—the toxicity of DMSO is noticeable when used at high doses, while the toxicity of HDR Enhancer V2 is noticeable at high doses or for long periods of exposure. Therefore, we recommend using the following:

- A maximum of 1% by volume DMSO in the final media.
- A control sample with DMSO, but no HDR Enhancer V2, in the final media to monitor toxicity.
- A concentration of 1 µM of HDR Enhancer V2 in the final media.
- A change to growth media without HDR Enhancer V2 12–24 hours after electroporation.

Important! The optimal concentration for Alt-R HDR Enhancer V2 will be cell type dependent and may require a dose titration. IDT uses 1 µM in immortalized cell lines. Toxicity should be monitored closely when used at concentrations higher than 2 µM.

Use HDR Enhancer V2 with other genome editing reagents

This protocol describes the use of HDR Enhancer V2 with the following components:

- HDR Donor Oligo
- Wild-type Cas9 Nuclease

If your experiment requires other HDR donor formats (e.g., Megamer Single-Stranded Gene Fragments) and CRISPR nuclease variants (e.g., HiFi Cas9 Nuclease, Cas9 D10A Nickase, or Cas12a/Cpf1 Nuclease), deliver the genome editing reagents by following their standard protocols. Then, simply add HDR Enhancer V2 to the final incubation media at a concentration of 1.0 µM, and importantly, change to media without HDR Enhancer V2 12–24 hours after electroporation for optimal cell response.

CONSUMABLES AND EQUIPMENT

Consumables from IDT

Item	Ordering information
Guide RNA choice:	
• Alt-R CRISPR-Cas9 crRNA	IDT predesigned and custom crRNA*: www.idtdna.com/CRISPR-Cas9
• Alt-R CRISPR-Cas9 tracrRNA	IDT (Cat # 1072532, 1072533, 1072534)
Alternatives:	
• Alt-R CRISPR-Cas9 tracrRNA–ATTO™ 488	IDT (Cat # 10007810)
• Alt-R CRISPR-Cas9 tracrRNA–ATTO 550	IDT (Cat # 1075927, 1075928)
• Alt-R CRISPR-Cas9 tracrRNA–ATTO 647	IDT (Cat # 10007853)
• Alt-R CRISPR-Cas9 crRNA	IDT predesigned and custom crRNA*: www.idtdna.com/CRISPR-Cas9
Alternative:	
• Alt-R CRISPR-Cas9 sgRNA	IDT predesigned and custom sgRNA*: www.idtdna.com/CRISPR-Cas9
Donor template (ssODN): Alt-R HDR Donor Oligo	www.idtdna.com/hdrdonoroligos
(Recommended) Alt-R CRISPR-Cas9 Control Kit	IDT (Cat # 1072554 [human] or 1072555 [mouse])
Alt-R *S.p.* Cas9 Nuclease V3†	IDT (Cat # 1081058, 1081059, 10000735)
Alternatives:	
• Alt-R *S.p.* HiFi Cas9 Nuclease V3	IDT (Cat # 1081060, 1081061, 10007803)
• Alt-R *S.p.* Cas9 V3, glycerol-free	IDT (Cat# 10007806, 10007807, 10007808)
• Alt-R *S.p.*Cas9-GFP V3	IDT (Cat# 10008100, 10008161)
• Alt-R *S.p.*Cas9-RFP V3	IDT (Cat# 10008162, 10008163)
• Alt-R *S.p.* Cas9 D10A Nickase V3	IDT (Cat # 1081062, 1081063)
(Optional, but recommended)	IDT (Cat # 1075915, 1075916, 10007805)
Alt-R Cas9 Electroporation Enhancer‡	Sequence (100 nt): TTAGCTCTGTTTACGTCCCAGCGGGCATGAGAGTAACA AGAGGGTGTGGTAATATTACGGTACCGAGCACTATCGA TACAATATGTGTCATACGGACACG
Alt-R HDR Enhancer V2	IDT (Cat # 10007910, 10007921)
Nuclease-Free IDTE, pH 7.5 (1X TE solution)	IDT (Cat # 11-01-02-02)
Nuclease-Free Duplex Buffer	IDT (Cat # 11-01-03-01)

* We guarantee that predesigned Alt-R CRISPR-Cas9 guide RNAs will provide successful editing at the target site, when delivered as a ribonucleoprotein complex as described in the Alt-R User Guides, using Alt-R CRISPR-Cas9 guide RNAs (crRNA:tracrRNA duplex or sgRNA) and either Alt-R *S.p.* Cas9 nuclease or Alt-R *S.p.* HiFi Cas9 nuclease. Analysis of editing must be at the DNA level, such as with the Alt-R Genome Editing Detection Kit or DNA sequencing. If successful editing is not observed for a predesigned guide RNA while an appropriate positive control is successful, a one-time "no-cost" replacement of the predesigned Alt-R CRISPR-Cas9 guide RNA will be approved, upon discussion with our Scientific Applications Support team (applicationsupport@idtdna.com). This guarantee does not extend to any replacement product, or to any other incurred or incidental costs or expenses. For other species, we recommend using our proprietary algorithms to enable customers to design custom guide RNAs. If you have protospacer designs of your own or from publications, use our design checker tool to assess their on- and off-targeting potential before ordering guide RNAs that are synthesized using our Alt-R gRNA modifications. For details about the predesigned gRNA guarantee, see www.idtdna.com/CRISPR-Cas9.

† Alt-R *S.p.* Cas9 Nuclease V3 is suitable for most genome editing studies. However, some experiments may benefit from the use of Alt-R *S.p.* HiFi Cas9 Nuclease, which has been engineered to reduce off-target effects, while retaining the on-target potency of wild-type Cas9. Alt-R Cas9 nickases create single-strand breaks. When a nickase variant is used with 2 gRNAs, off-target effects are reduced, and homology-directed repair can be promoted.

‡ The Electroporation Enhancer is designed to avoid homology to the human, mouse, or rat genomes, and has been tested as carrier DNA in multiple cell lines, including HEK-293, Jurkat, and K562. Before use in other species, verify that the Electroporation Enhancer does not have similarity to your host cell genome to limit participation in the double-stranded DNA break repair process.

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Consumables from other suppliers

Item	Supplier	Catalog #
96-well Nucleofector™ Kit	Lonza	V4SC-2096
Appropriate growth media for cells	Varies	Varies
1X Phosphate buffered saline (PBS)	General laboratory supplier	
DMSO (molecular biology grade)	General laboratory supplier	

Equipment

Item	Supplier	Catalog #
4D-Nucleofector™ System	Lonza	AAF-1002B with AAF-1002X
96-well Shuttle™ System		AAM-1001S

PROTOCOL

Prepare CRISPR reagents

Resuspend your oligos in Nuclease-Free IDTE.

Guide RNA	Final concentration (µM)
Alt-R crRNA and tracrRNA or sgRNA (if not using a two-part system)	100
HDR Donor Oligo	100 or the optimized concentration for your cell line
Alt-R Cas9 Electroporation Enhancer	100

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

Note: Always store CRISPR reagents at –20°C.

Prepare the gRNA complex

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

Annealing

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
Duplex Buffer (to final volume)	As needed
Total volume	10

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

Stopping point (optional): gRNA complexes can be stored at –20°C up to 1 year.

Prepare the RNP complex

Combining gRNA and Cas9 Nuclease allows an RNP complex to form. In this electroporation mix, the final Cas9:gRNA RNP concentration is 4:4.8 µM.

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 per each electroporation well:

Component	Amount
gRNA (50 µM)*	3.0 µL (150 pmol)
Alt-R Cas9 enzyme (62 µM)†	2.0 µL (125 pmol)
PBS (to final volume)	As needed
Total volume	5 µL

* If working with Cas9-GFP or Cas9-RFP, we recommend using a 1:1.2 ratio of Cas9:gRNA, instead of a 1:1 ratio.

† Alt-R S.p. Cas9 nucleases 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).

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

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

Prepare the cell culture media

Cell culture media with and without HDR Enhancer V2 must be prepared and prewarmed to 37°C for use after electroporation.

1. Prewarm 75 µL of cell culture media per electroporation sample.

Note: This media will be added to cells in the 96-well Nucleocuvette™ module following electroporation.

2. If using Alt-R HDR Enhancer V2, mix it with cell culture media, then aliquot in a 96-well plate.

Tip: To reduce sample-to-sample variability, we recommend making a stock solution of cell culture media with HDR Enhancer V2, then aliquoting to the final culture plate.

3. Prewarm the plate in a tissue culture incubator.

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

The following table lists the amounts needed to prepare each working solution for a final concentration of 1.0 µM HDR Enhancer V2. Add 175 µL working solution per well after the electroporated cells are added. If needed, scale up according to the number of samples you have.

Component	Sample (µL)	Negative control, no HDR Enhancer V2 (µL)	Negative control, DMSO only (µL)
0.69 mM Alt-R HDR Enhancer V2	1.7*	—	—
DMSO	—	—	1.7
Cell culture media	1000	1000	1000
Total volume	~1000	1000	1000

* The final concentration of the HDR Enhancer V2 may need to be optimized for your cell type (see Minimize cytotoxicity when using Alt-R HDR Enhancer V2).

Transfect cells by Nucleofection™

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

1. Suspend cells in 20 µL of the required Nucleofection™ Buffer.
2. Make the final transfection mix by combining the following components:

Component	Amount (µL)
RNP complex	5
100 µM HDR Donor Oligo	1.2
100 µM Alt-R Cas9 Electroporation Enhancer*	1.2
Cell suspension	20
PBS (to final volume)	2.6
Total volume	**30**

* Alt-R Cas9 Electroporation Enhancer is recommended to improve editing efficiency.

Note: You can optimize the final ssODN template concentration for each site.

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 [1].
5. After electroporation, add 75 µL of prewarmed culture media (without 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 containing HDR Enhancer V2.
7. Incubate cells in a tissue culture incubator.

Change media

After 12–24 hours, remove the media from the cells, and replace with fresh media *without* HDR Enhancer H2.

Isolate gDNA

You can perform genomic DNA isolation and detect mutations 48–72 hours after electroporation.

Stopping point (optional): gDNA can be stored for future use by following the recommendations of the gDNA isolation protocol used.

REFERENCES

1. Lonza. (2009) Amaxa 96-well Shuttle Protocol for HEK-293 (ATCC®). [Online] Basel, Switzerland, Lonza, Ltd. [Accessed 5 June, 2019]

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