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

Simultaneous delivery of RNP complexes and dsDNA repair templates using the Lonza 96-well Shuttle Nucleofector™ System

For use with:

- Alt-R HDR Donor Blocks
- Cas9 nuclease
- gRNA—crRNA + tracrRNA or sgRNA
- HDR Enhancer V2 (optional)
- Cas9 Electroporation Enhancer (optional)

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REVISION HISTORY

Version	Release date	Description of changes
2.1	July 2024	Update to the part number for the IDTE pH 7.5 reagent.
2	February 2022	Updated product names
1	January 2022	Original protocol

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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 co-delivery of an Alt-R HDR Donor Block and a CRISPR-Cas9 ribonucleoprotein (RNP) complex using electroporation with the Nucleofector™ System (Lonza).

Important: This protocol describes delivering long donor sequences (200 bp–3 kb) using IDT Alt-R HDR Donor Blocks. If you are using short Alt-R HDR donor oligos (specialized Ultramer™ DNA oligos, up to 200 nt), use this protocol instead: Homology-directed repair using the Alt-R CRISPR-Cas9 System and HDR Donor Oligos.

For HDR experiments, we recommend using the Alt-R HDR Enhancer V2, which is a small molecule compound that has shown an ability to increase the rate of HDR. While HDR efficiency and any improvements vary by cell line, editing site, and the desired insert, here we offer guidelines and suggestions that maximize HDR potential and help to limit cytotoxicity that is often associated with the delivery of 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

CONSUMABLES AND EQUIPMENT

Consumables from IDT

Item	Ordering information
Guide RNA choice:	
Alt-R CRISPR-Cas9 crRNA	Predesigned and custom crRNA*: www.idtdna.com/CRISPR-Cas9
Alt-R CRISPR-Cas9 tracrRNA	1072532, 1072533, 1072534
Alternatives:	
Alt-R CRISPR-Cas9 tracrRNA–ATTO™ 550 (ATTO-Tec GmbH)	1075927, 1075928
Alt-R CRISPR-Cas9 tracrRNA–ATTO 488	10007810
Alt-R CRISPR-Cas9 tracrRNA–ATTO 647	10007853
Alternative:	
Alt-R CRISPR-Cas9 crRNA XT	Predesigned and custom crRNA*: www.idtdna.com/CRISPR-Cas9
Alternative:	
Alt-R CRISPR-Cas9 sgRNA (no tracrRNA required)	Predesigned and custom sgRNA*: www.idtdna.com/CRISPR-Cas9
Donor template (dsDNA): Alt-R HDR Donor Block	www.idtdna.com/HDRDonorBlocks
Alt-R CRISPR-Cas9 Control Kit	1072554 (human) or 1072555 (mouse)
Alt-R *S.p.* Cas9 Nuclease V3†	1081058, 1081059, 10000735
Alternatives:	
Alt-R *S.p.* HiFi Cas9 Nuclease V3	1081060, 1081061, 10007803
Alt-R *S.p.* Cas9 D10A Nickase V3	1081062, 1081063
Alt-R *S.p.* Cas9 V3, glycerol-free	10007806, 10007807, 10007808
Alt-R *S.p.* Cas9-GFP V3	10008100, 10008161
Alt-R *S.p.* Cas9-RFP V3	10008162, 10008163
(Optional) Alt-R Cas9 Electroporation Enhancer‡	1075915, 1075916, 10007805 Sequence (100 nt): TTAGCTCTGTTTACGTCCCAGCGGGCATGAGAGTAACA AGAGGGTGTGGTAATATTACGGTACCGAGCACTATCGA TACAATATGTGTCATACGGACACG
(Optional) Alt-R HDR Enhancer V2	10007910, 10007921
Nuclease-Free IDTE, pH 7.5 (IX TE solution)	11-01-02-02

* 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.

† 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-stranded 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.

Consumables from other suppliers

Item	Supplier	Catalog #
1X Phosphate buffered saline (PBS)	General laboratory supplier	Varies
Appropriate growth media for cells	General laboratory supplier	Varies
DMSO (molecular biology grade)	General laboratory supplier	Varies
96-well Nucleofector Kit	Lonza	V4SC-2096, or appropriate kit for cell line of choice

Equipment

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

GUIDELINES

Optimize CRISPR editing

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

- High Cas9 editing efficiency maximizes the potential for HDR. When possible, avoid use of low activity guides as these will limit HDR rates. See our application note **Homology-directed repair using the Alt-R™ CRISPR-Cas9 System and HDR Donor Oligos** for more information.
- When confirming guide activity, IDT recommends the use of proper controls in your experiment.
- When selecting guides, it may be helpful first to optimize HDR efficiency with short insertions at your desired edit location (for example, by introducing a novel restriction site using ssODNs such as Alt-R HDR Donor Oligos). IDT recommends the use of proper controls in your experiment, such as the appropriate Alt-R CRISPR-Cas9 Control Kit for studies in human or mouse cells. See **Using CRISPR-Cas9 HDR positive controls** for further details.

Design a dsDNA donor template

The template sequence for dsDNA HDR donors should be designed to include the desired insert sequence flanked by homology arms (sequence elements that match either side of a cut site). We recommend using the **Alt-R HDR Design Tool** for assistance in selecting a guide RNA and designing donor DNA templates. Sequence complexities present within your insert or genomic region of interest (such as extreme GC content, homopolymeric runs, and hairpin or repeat elements) may pose a manufacturing challenge. For design assistance in such cases, we encourage you to reach out to **applicationsupport@idtdna.com**.

Alt-R HDR Donor Blocks contain additional universal sequences located outside the homology arms. These 30 bp segments contain proprietary modifications that help improve HDR rates by both stabilizing the donor and reducing unwanted homology-independent integration of the donor. See our **DECODED article on HDR Donor Blocks** for more information.

We observed better HDR efficiency when the following conditions were met:

- **Keep homology arm lengths between 100–500 bp.** We recommend starting with a homology arm length of 200 bp, but longer homology arms may be beneficial in cell types with high nuclease environments.
- **Incorporate silent mutations within the donor sequence when the desired HDR edit does not disrupt Cas9 binding.** This is particularly important with dsDNA templates, as the silent mutations will prevent Cas9 from degrading the donor template and from re-cutting genomic DNA after the desired edit has been made.

Optimize the concentration of dsDNA donor templates

Due to recognition of cytosolic dsDNA by innate immune response pathways, careful consideration of the final Alt-R HDR Donor Block concentration is necessary to avoid cytotoxicity. We recommend testing for optimal concentrations for each cell line you use, as sensitivity to dsDNA may vary. Here, we present recommendations for Alt-R HDR Donor Block concentrations based on usage in immortalized cell lines. Cytotoxicity was observed when the dsDNA template amount exceeded 2 µg. As such, we recommend testing within the range of 0.5–2 µg amounts for initial optimization.

In Table 1 below, we provide a conversion between µg amounts and the nM concentration in the final transfection mixture. Values within the table indicate the µg amount corresponding to a given final nM concentration and template sequence length. The recommended range for initial optimization is indicated in blue.

Table 1. Conversion between µg amount of Alt-R HDR Donor Block and final concentration

		Concentration in final transfection mixture					
		10 nM	25 nM	50 nM	100 nM	200 nM	500 nM
Donor Length	200 bp	0.04	0.1	0.2	0.4	0.8	2
	500 bp	0.1	0.25	0.5	1	2	5
	1000 bp	0.2	0.5	1	2	4	10
	2000 bp	0.4	1	2	4	8	20
	3000 bp	0.6	1.5	3	6	12	30

Note: Values within the table indicate µg amounts. These conversions are based on a 30 µL volume for electroporation using the Nucleofector™ System (Lonza).

We generally do not recommend use of the Alt-R Cas9 Electroporation Enhancer when using Alt-R HDR Donor Blocks. The DNA template is typically sufficient to act as a carrier for improved Cas9 delivery, and the addition of Electroporation Enhancer may increase cytotoxicity. For more information, see the DECODED article, Successful CRISPR genome editing in hard-to-transfect cells.

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 HDR Enhancer V2 can be toxic to cells; the toxicity of DMSO is noticeable when used at high concentrations, while the toxicity of HDR Enhancer V2 is noticeable at high concentrations or for long periods of exposure. Due to the increased potency of the HDR Enhancer V2, the enhancer solution should be used at lower concentrations than our original solution, to result in improved cell viability. Because of this, we recommend using:

- 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 within the range of 1–2 µM of HDR Enhancer V2 in the final media.
- A switch to growth media without HDR Enhancer V2 12–24 hours after electroporation.

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

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.

Component	Final concentration
Alt-R crRNA and tracrRNA, or sgRNA	100 µM
Alt-R Cas9 Electroporation Enhancer (optional)	100 µM
Alt-R HDR Donor Block	0.5 µg/µL, 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.
- When resuspending your dsDNA template at a high concentration (≥ 0.5 µg/µL), vortex well to ensure the DNA pellet is fully dissolved. A 10-minute incubation at 50°C may improve the resuspension efficiency.

Prepare the gRNA complex

Note: If you are preparing an sgRNA, an annealing step is not required. Simply dilute the sgRNA to the desired concentration in Nuclease-Free IDTE and skip this section.

To anneal the oligos and form a guide complex, prepare a two-part gRNA complex that combines crRNA and tracrRNA.

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
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.

Stopping point (optional): gRNA complexes can be stored at –20°C up to 2 years.

Prepare the RNP complex

Combining the gRNA and Cas9 Nuclease allows an RNP complex to form. Prepare the RNP complex to yield 2 µM Cas9 protein and 2.4 µM gRNA in the final delivery mixture.

Important: You can optimize the final RNP concentration for each guide, but maintaining a Cas9:gRNA ratio of 1:1.2 is recommended.

1. Combine the following components in each electroporation well:

Component	Amount (µL)
gRNA (50 µM)*	1.4 (72 pmol)
Alt-R Cas9 enzyme (62 µM) †	1.0 (60 pmol)
PBS (to final volume)	0.6
Total volume	3

* 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 conentration 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 2 months, or at –20°C or –80°C up to 1 year in single-use aliquots.

Prepare the HDR donor

When preparing your Alt-R HDR Donor Block, dilute your template in the nuclease-free IDTE, water, or an appropriate buffer so that your desired amount is delivered in a 6 µL volume (Table 2).

Table 2. Example: Dilution of a 1000 bp template for a final 50 nM concentration

Component	Amount (µL)
Alt-R HDR Donor Block stock at 500 ng/µL	2 (1 µg)
IDTE, water, or appropriate buffer	4
Total volume	6

Note: The optimal concentration of your Alt-R HDR Donor Block will vary with the sequence length and may need to be optimized for your cell type (see the earlier section Optimize the concentration of dsDNA donor templates).

Prepare the cell culture media

Prepare cell culture media with and without HDR Enhancer V2 and prewarm to 37°C for use after Lonza Nucleofection™.

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

   Note: This media will be added to cells in the 96-well Lonza Nucleocuvette™ module after Nucleofection™.

2. If using Alt-R HDR Enhancer V2, mix the enhancer with cell culture media. For a final concentration of 1 µM HDR Enhancer V2 after the transfected cells are added, add 1.7 µL of the HDR Enhancer V2 stock solution (690 µM) per 1 mL of media. If needed, scale up according to the number of samples you have.

   Tip: When monitoring toxicity, including negative controls of DMSO only and untreated media is recommended. For the DMSO-only control, simply add 1.7 µL of DMSO per 1 mL of media.

   Note: 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).

3. Aliquot 175 µL of the prepared media into a 96-well plate. If not using HDR Enhancer V2, use standard cell culture media.

   Note: 25 µL of transfected cells will be added for a final volume of 200 µL per well.

4. Prewarm the plate in a tissue culture incubator.

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

Transfect cells

Prepare cells as you would for a standard CRISPR-Cas9 Nucleofection™ experiment, ensuring the cells are washed with PBS prior to transfection to remove any residual nucleases.

1. Suspend cells in 21 µL of the appropriate Nucleofector™ Solution.
2. Make the final transfection mix by combining the following components:

Component	Amount (µL)
RNP complex	3
Alt-R HDR Donor Block	6
Cell suspension*	21
Total volume	30

* If you are using Alt-R Electroporation Enhancer, reduce the volume of the cell suspension to 20.4 µL and add 0.6 µL of 100 µM Alt-R Electroporation Enhancer.

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) to each 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 HDR Enhancer V2 (if applicable).
7. Incubate cells in a tissue culture incubator.

Change media

If using HDR Enhancer V2: After 12–24 hours, carefully remove the media from the cells, and replace with fresh media without HDR Enhancer V2.

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. (2009) Amaxa™ 96-well Shuttle™ Protocol for HEK-293 (ATCC®). Lonza. Available at http://lonza.picturepark.com/Website/?Action=downloadAsset&AssetId=21182 (Accessed March 23, 2021).

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