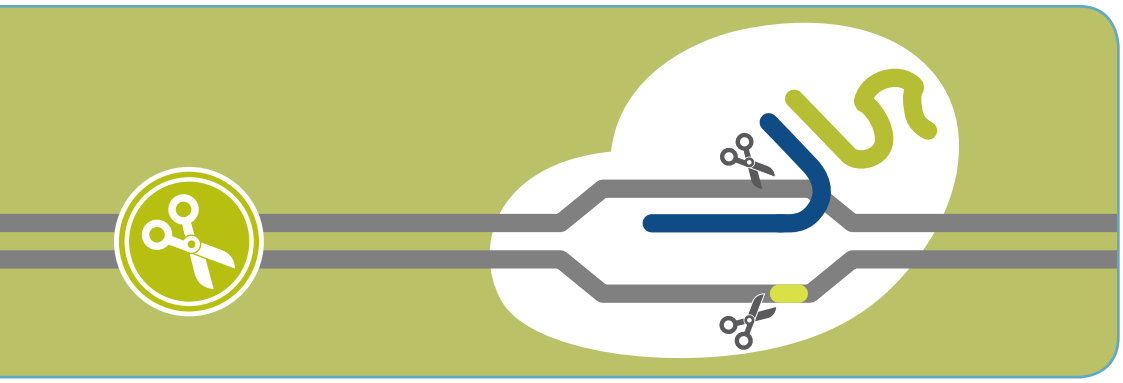
Homology-directed repair using the Alt-R CRISPR-Cas9 System and Megamer ssDNA Fragments

Simultaneous delivery of RNP complexes and ssDNA repair templates using the Nucleofector™ System or Neon® Transfection System



For use with:

- HDR Enhancer
- Cas9 Electroporation Enhancer
- Cas9 nuclease
- gRNA—crRNA + tracrRNA or sgRNA
- Megamer ssDNA fragments

Table of contents

Introduction

This protocol is designed for homology-directed repair (HDR) using CRISPR-Cas9 genome editing in cultured cells. The protocol involves the codelivery of a Megamer single-stranded DNA fragment and a CRISPR-Cas9 ribonucleoprotein (RNP) complex using electroporation with the Nucleofector System (Lonza) or the Neon Transfection System (Thermo Fisher).

Important! This protocol is for use with long Alt-R HDR donor templates based on Megamer DNA fragments. For more information about designing Megamer DNA Fragments as HDR donor templates, read this guide. If you are using Alt-R HDR donor oligos that are synthesized as specialized Ultramer DNA Oligos (with the Alt-R HDR design tool), use this protocol: 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, which is a small molecule compound that has demonstrated an ability to increase the rate of HDR. While the efficiency of HDR and relative improvement in HDR rates varies 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 and genome editing reagents into cells.

Workflow

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
Alternative:	
Alt-R CRISPR-Cas9 tracrRNA–ATTO™ 550	1075927, 1075928
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 (ssODN): Megamer ssDNA Fragment	www.idtdna.com/Megamer
Alt-R CRISPR-Cas9 Control Kit	1072554 (human), 1072555 (mouse), or 1072556 (rat)
Alt-R S.p. Cas9 Nuclease V3†	1081058, 1081059
Alternatives:	
Alt-R S.p. HiFi Cas9 Nuclease V3	1081060, 1081061
Alt-R S.p. Cas9 D10A Nickase V3	1081062, 1081063
(Optional) Alt-R Cas9 Electroporation Enhancer‡	1075915, 1075916 Sequence (100 nt): TTAGCTCTGTTTACGTCCCAGCGGGCATGAGAGTAACA AGAGGGTGTGGTAATATTACGGTACCGAGCACTATCGA TACAATATGTGTCATACGGACACG
(Optional) Alt-R HDR Enhancer	1081072, 1081073
Nuclease-Free IDTE, pH 7.5 (IX TE solution)	11-01-01-01
Nuclease-Free Duplex Buffer	11-01-03-01

* We guarantee the performance of our predesigned gRNAs targeting human, mouse, rat, zebrafish, or nematode genes. For other species, you may use our proprietary algorithms to design custom gRNAs. If you have gRNAs protospacer designs of your own or from publications, use our design checker tool to assess their on- and off-targeting potential before ordering gRNAs 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-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
Neon Transfection System Kit	Neon	MPK1096
or		
96-well Nucleofector Kit	Lonza	V4SC-2096

Equipment

Item	Supplier	Catalog #
4D-Nucleofector System	Lonza	AAF-1002B with AAF-1002X
96-well Shuttle™ System		AAM-1001S
or		
Neon Transfection System	Thermo Fisher Scientific	MPK5000
Neon Reagent kit		MPK2096

Guidelines

Optimize CRISPR editing

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

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

Design an ssDNA 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.

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

- Keep homology arms between 100–500 nt long. Longer homology arms may be beneficial in cell types with high nuclease environments.
- Incorporate silent mutations within the donor oligo to prevent Cas9 from re-cutting the template after the desired edit has been made.

Minimize cytotoxicity when delivering Megamer ssDNA donor templates

Cytotoxicity can vary with Megamer length and cell line. To protect cell viability following electroporation, do not exceed certain µg doses depending on the Megamer length and electroporation system used (see Table 1 below).

Table 1. Recommended maximum dose when delivering Megamer ssDNA donor templates.

	Maximum dose	
Megamer length (nt)	Nucleofection System	Neon Transfection System
200–1000	4 µg	1.5 µg
1000–1500	3 µg	1.0 µg
1500–2000	2 µg	0.5 µg

Notes:

- These recommendations are based on data from HEK-293 and Jurkat cell lines.
- Toxicity may vary with other cell lines; therefore, optimize each cell line you use for cytotoxicity.

Generally, we recommend using Alt-R Cas9 Electroporation Enhancer (EE) at the same molar concentration as the RNP complex (1–4 µM). However, while this non-targeting carrier DNA improves electroporation efficiency, in some cell lines it can result in increased toxicity when used with high µg amounts of Megamer ssDNA donor template (see Table 2).

Important! To avoid increased toxicity, test every cell line for Megamer delivery *with* and *without* Electroporation Enhancer to optimize for cytotoxicity and overall editing.

Table 2. Example guidelines for the use of Alt-R Cas9 Electroporation Enhancer (EE) with Megamer ssDNA donors.

Delivery method	Cell line	Megamer dose	Is Alt-R Cas9 EE recommended?*
Nucleofector System	HEK-293	<1.0 µg	Yes
		≥1.0 µg	No
	Jurkat	Any dose	Yes
Neon Transfection System	HEK-293	<0.5 µg	Yes
		≥0.5 µg	No
	Jurkat	<1.5 µg	Yes
		≥1.5 µg	No

* Cas9 Electroporation Enhancer can result in increased toxicity when used with Megamer ssDNA donor template, especially at high doses and longer Megamer lengths.

For more information on the importance of the Electroporation Enhancer used in this protocol, see the article Successful CRISPR genome editing in hard-to-transfect cells [5].

Minimize cytotoxicity when using Alt-R HDR Enhancer

The Alt-R HDR Enhancer is provided as a 3 mM concentrated solution in dimethyl sulfoxide (DMSO). Use of both DMSO and the HDR Enhancer can be toxic to cells—the toxicity of DMSO is noticeable when used at high doses, while the toxicity of HDR Enhancer is noticeable at high doses or for long periods of exposure. Because of this, we recommend the following guidelines:

- Use a maximum of 1% by volume DMSO in the final media.
- Use a control sample with DMSO, but no HDR Enhancer, in the final media to monitor toxicity.
- Use a concentration of 20–30 µM of HDR Enhancer in the final media.
- Change to growth media without HDR Enhancer 12–24 hours after electroporation.

Important! The optimal concentration for Alt-R HDR Enhancer is cell type dependent and may require a dose titration. Toxicity should be monitored closely when used at concentrations higher than 30 µM.

Use HDR Enhancer with other genome editing reagents

This protocol describes the use of HDR Enhancer with these components:

- Megamer single-stranded DNA donor
- Wild-type Cas9 Nuclease

If your experiment requires other HDR donor formats (e.g., Ultramer DNA Oligonucleotides) and CRISPR nuclease variants (i.e., 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 to the final incubation media at the recommended concentration of 30 µM, and importantly, change to media without HDR Enhancer 12–24 hours after electroporation for optimal cell response.

Protocol

Prepare CRISPR reagents

Resuspend your oligos in Nuclease-Free IDTE.

Guide RNA	Final concentration
Alt-R crRNA and tracrRNA or sgRNA (if not using a two-part system)	100 µM
Alt-R Cas9 Electroporation Enhancer	100 µM
Megamer ssDNA fragment	0.5 µg/µL, or an optimal concentration for your planned experiment (See **Tips** below)

Note: For assistance, use the IDT Resuspension Calculator.

Tips:

- Always store CRISPR reagents at –20°C.
- When preparing your Megamer donor, dilute donor in nuclease-free IDTE, or water, so that your desired dose is delivered in an 8 µL volume (Nucleofector System) or a 4 µL volume (Neon Transfection System).
- The Megamer dose may need to be optimized for your cell type. (See Minimize cytotoxicity when using Megamer ssDNA donor templates).

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 crRNA	5
IDT 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 the gRNA and Cas9 Nuclease allows an RNP complex to form. Prepare the delivery mix to yield a Cas9:gRNA RNP final concentration of 2:2.4 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:

	Amount (µL)	
Megamer length (nt)	**Nucleofector System**	**Neon Transfection System**
PBS	0.6	1.8
gRNA (50 µM)	1.4 (72 pmol)	0.7 (36 pmol)
Alt-R Cas9 enzyme (61 µM)	1.0 (60 pmol)	0.5 (30 pmol)
Total volume	**3**	**3**

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

   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.

For delivery by nucleofection, use Part A—Delivery by the Nucleofector System; for delivery by electroporation, use Part B—Delivery by the Neon Transfection System.

Part A—Delivery by the Nucleofector System

Prepare the cell culture media

Prepare cell culture media with and without HDR Enhancer and prewarm to 37°C for use after nucleofection.

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

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

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

   Tip: To reduce sample-to-sample variability, make a stock solution of cell culture media with HDR Enhancer, then aliquot 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 nucleofection. If desired, plate triplicate wells for each nucleofection sample.

   The following table demonstrates the amounts needed per well, for a final concentration of 30 µM HDR Enhancer after the nucleofected cells are added. If needed, scale up according to the number of samples you have.

Component	Sample (µL)	Negative control, no HDR Enhancer (µL)	Negative control, DMSO only (µL)
3 mM Alt-R HDR Enhancer	2*	—	—
DMSO	—	—	2
Cell culture media	173	175	173
Total volume	**175**	**175**	**175**

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

Transfect cells

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

1. Suspend cells in 18 µL of the required Nucleofection Buffer.
2. Make the final transfection mix by combining the following components:

Component	Amount (µL)
RNP complex	3
Megamer ssDNA donor	8
100 µM Alt-R Cas9 Electroporation Enhancer or Nucleofection Buffer*	0.6
Cell suspension	18
PBS (to final volume)	0.4
Total volume	**30**

* The final concentration of Alt-R Cas9 Electroporation Enhancer may need to be optimized for your cell type (see Minimize cytotoxicity when using Megamer ssDNA donor templates).

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) 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 (if applicable).
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.

Isolate gDNA

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

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

Part B—Delivery by the Neon Transfection System

Prepare the cell culture media

Prepare cell culture media with and without HDR Enhancer and prewarm to 37°C for use after electroporation.

1. Prewarm 190 µL of cell culture media per electroporation sample.

Note: Cells will be added to this media for recovery after electroporation.

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

Tip: To reduce sample-to-sample variability, make a stock solution of cell culture media with HDR Enhancer, then aliquot to the final culture plate.

3. Prewarm the plate in a tissue culture incubator.

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 demonstrates the amounts needed per well, for a final concentration of 30 µM HDR Enhancer 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 (µL)	Negative control, DMSO only (µL)
3 mM Alt-R HDR Enhancer	2*	—	—
DMSO	—	—	2
Cell culture media	148	150	148
Total volume	**150**	**150**	**150**

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

Transfect cells

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

1. Suspend cells in 6 µL of the required Neon Electroporation Buffer (e.g., R or T).
2. Dilute the Alt-R Cas9 Electroporation Enhancer to a final concentration of 15 µM (if applicable).
3. Make the final transfection mix by combining the following components:

Component	Amount (µL)
RNP complex	3
Megamer ssDNA donor	4
15 µM Alt-R Cas9 Electroporation Enhancer or Nucleofection Buffer*	2
Cell suspension	6
Total volume	**15**

* The final concentration of Alt-R Cas9 Electroporation Enhancer may need to be optimized for your cell type (see Minimize cytotoxicity when using Alt-R HDR Enhancer).

4. After mixing the transfection mix, gently pipette 10 µL into a Neon Tip that has been inserted into the Neon Pipette, being careful to avoid air bubbles.
5. Transfect cells according to the manufacturer's specifications. [3]
6. After electroporation, transfer cells to wells containing 190 µL prewarmed culture media (without HDR Enhancer) per well and gently resuspend cells.
7. Transfer 50 µL of resuspended cells to the culture plates containing the prewarmed 150 µL of culture media containing HDR Enhancer (if applicable).
8. 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.

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

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