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 V2
- Cas9 Electroporation Enhancer
- Cas9 Nuclease
- gRNA—crRNA + tracrRNA or sgRNA
- Megamer ssDNA Fragments

REVISION HISTORY

Version	Release date	Description of changes
2	March 2024	Updated references
1	February 2022	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 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 Scientific).

Important: This protocol is for use with long Alt-R HDR donor templates based on Megamer DNA Fragments. 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 V2, which is a small molecule compound that has shown an ability to increase the rate of HDR. 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

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	1075927, 1075928
or Alt-R CRISPR-Cas9 tracrRNA - ATTO 488	10007810
or 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 (ssODN): Megamer ssDNA Fragment	www.idtdna.com/Megamer
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 (1X 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	Thermo Fisher Scientific	AAM-1001S
or		
Neon® Transfection System		MPK5000
Neon® Reagent kit		MPK2096

GUIDELINES

Optimize CRISPR editing

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

- Low Cas9 editing efficiency will negatively impact HDR rates. See our DECODED article Improving efficicency of homology-directed repair (HDR).
- 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 or mouse 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 higher 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 donor length and cell line. To protect cell viability following electroporation, do not exceed certain µg levels depending on the Megamer donor length and electroporation system used (see Table 1).

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

	Maximum amount	
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 donor 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 (µg)	Is Alt-R Cas9 EE recommended?*
Nucleofector™ System	HEK-293	<1.0	Yes
		≥1.0	No
	Jurkat	Any dose	Yes
Neon® Transfection System	HEK-293	<0.5	Yes
		≥0.5	No
	Jurkat	<1.5	Yes
		≥1.5	No

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

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 concentrations, while the toxicity of HDR Enhancer V2 is noticeable at high concentrations or for long periods of exposure. 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 of 0.5–2 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 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 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 (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.

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 dose is delivered in either an 8 µL volume (Nucleofector™ System), or a 4 µL volume (Neon® Transfection System).
- The Megamer donor 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 sgRNA, an annealing step is not required. Simply dilute the sgRNA to a concentration of 50 µM 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. First, 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
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 (62 µM) †	1.0 (60 pmol)	0.5 (30 pmol)
Total volume	**3**	**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 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 minutes.

Stopping point (optional): RNP complexes can be stored at 4°C up to 2 months, or at –80°C up to 1 year or more 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 V2 and prewarm to 37°C for use after transfection.

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 after electroporation.

2. If using Alt-R HDR Enhancer V2, mix HDR Enhancer V2 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 V2, 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 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 to each 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

Prepare cells as you would for a standard CRISPR-Cas9 Nucleofection™ experiment, then make sure that the cells are washed with PBS before transfection 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 V2 (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 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.

Part B—Delivery by the Neon® Transfection System

Prepare the cell culture media

Prepare cell culture media with and without HDR Enhancer V2 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 V2, mix HDR Enhancer V2 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 V2, 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 lists the amounts needed to prepare each working solution for a final concentration of 1.0 µM HDR Enhancer V2. Add 150 µL working solution 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

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 electroporation 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 using a fresh tip, while 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 V2) 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 V2 (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 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

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For more information, go to: **www.idtdna.com/ContactUs**

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