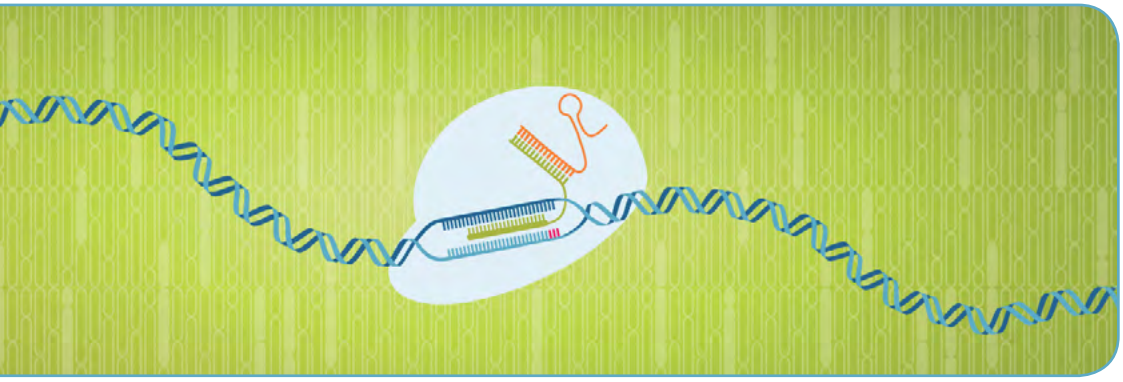
Homology-directed repair using Alt-R CRISPR-Cas9 System and Ultramer Oligos

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



For use with:

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

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Introduction

This protocol is designed for homology-directed repair (HDR) using CRISPR-Cas9 genome editing in cultured cells. The protocol involves the codelivery of an Ultramer single-stranded oligodeoxynucleotide (ssODN) and a CRISPR-Cas9 ribonucleoprotein (RNP) complex using electroporation with the Amaxa Nucleofector system (Lonza).

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, 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.

Guidelines

Optimize CRISPR editing

Validate guide RNA (gRNA) efficiency before using them 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: CRISPR-Cas9 mediated HDR: Tips for successful experimental design.)
- 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. (See our application note Applications of Cas9 nickases for genome engineering for more information.)
- 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 ssODN donor template

The template sequence for ssDNA HDR donors can be designed to bind to either the targeting or non-targeting strand. Homology arms (the donor template sequence elements that match either side of a cut site) must be included for HDR experiments. We observed robust HDR efficiency when the following conditions were met:

- Generally, using an ssDNA donor sequence with homology arms identical to the non-targeting strand (i.e., the strand containing the PAM sequence)
- Keeping homology arms between 30 and 50 nt long
- Using phosphorothioate (PS) bonds on each end of an ssODN template

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

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. Therefore, we recommend the following:

- 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 will be 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 the following components:

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

If your experiment requires other HDR donor formats (e.g., Megamer Single-Stranded Gene Fragments) 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.

Workflow

1	Prepare gRNAs (15 minutes) ⛔	Optional STOP—gRNA complexes can be stored at –20°C for up to 1 year.
2	Prepare RNP complexes (20 minutes) ⛔	Optional STOP—RNP complexes can be stored at 4°C for up to 1 month or –80°C for up to 2 years in single use aliquots.
3	Prepare cell culture media with and without Alt-R HDR Enhancer (10 minutes)	
4	Transfect cells of interest by nucleofection (30 minutes)	
5	Change media 12–24 hours after nucleofection to non-HDR Enhancer containing media (15 minutes)	
6	Isolate gDNA and detect mutations (est. time varies) ⛔	Optional STOP—gDNA can be stored for future use following the recommendations of the gDNA isolation protocol used.

Consumables

Kits and reagents	Ordering information
96-well Nucleofector Kit	Lonza (Cat # V4SC-2096)
Appropriate growth media for cells	Varies
1X Phosphate buffered saline (PBS)	General laboratory supplier
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)
Alternative:	
• Alt-R CRISPR-Cas9 tracrRNA – ATTO 550	IDT (Cat # 1075927, 1075928)
• 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): Ultramer DNA Oligo	www.idtdna.com/Ultramer
(Recommended) Alt-R CRISPR-Cas9 Control Kit	IDT (Cat # 1072554 [human], 1072555 [mouse], or 1072556 [rat])
Alt-R S.p. Cas9 Nuclease V3†	IDT (Cat # 1081058, 1081059)
Alternatives:	
• Alt-R S.p. HiFi Cas9 Nuclease V3	IDT (Cat # 1081060, 1081061)
• Alt-R S.p. Cas9 D10A Nickase V3	IDT (Cat # 1081062, 1081063)
(Optional, but recommended) Alt-R Cas9 Electroporation Enhancer‡	IDT (Cat # 1075915, 1075916) Sequence (100 nt): TTAGCTCTGTTTACGTCCCAGCGGGCATGAGAGTAACA AGAGGGTGTGGTAATATTACGGTACCGAGCACTATCGA TACAATATGTGTCATACGGACACG
Alt-R HDR Enhancer	IDT (Cat # 1081072, 1081073)
Nuclease-Free IDTE, pH 7.5 (1X TE solution)	IDT (Cat # 11-01-02-02)
DMSO (molecular biology grade)	General lab supplier
Nuclease-Free Duplex Buffer	IDT (Cat # 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.

Equipment

Required equipment	Ordering information
4D-Nucleofector System	Lonza (Cat # AAF-1002B with AAF-1002X)
96-well Shuttle™ System	Lonza (Cat # AAM-1001S)

Protocol

Prepare CRISPR reagents before transfection

Resuspend your RNA 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
Ultramer HDR donor	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

When preparing a two-part gRNA complex (which combines crRNA and tracrRNA), the oligos must be annealed 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)
Alt-R CRISPR-Cas9 crRNA (100 µM)	5
Alt-R CRISPR-Cas9 tracrRNA (100 µM)	5
Duplex Buffer (to final volume)	As needed
Total volume	**10**

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

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

Prepare the RNP complex

Combining gRNA and Cas9 Nuclease allows an RNP complex to form. In this nucleofection 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 (61 µM)	2.0 µL (125 pmol)
PBS (to final volume)	As needed
Total volume	**5 (µL)**

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

Prepare the cell culture media

Cell culture media with and without HDR Enhancer must be prepared and prewarmed 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 following nucleofection.

2. Mix HDR Enhancer with cell culture media, then aliquot in a 96-well plate. Prewarm the plate in a tissue culture incubator.

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

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

   Note: 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 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, page 4).

Transfect cells by nucleofection

Prepare cells as you would for a standard CRISPR-Cas9 nucleofection experiment, ensuring the cells are washed with PBS prior to nucleofection 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 Ultramer ssODN donor	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 concentration for each site. Generally, 1–4 µM ssODN concentration allows for maximal HDR.

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.
7. Incubate cells in a tissue culture incubator.
8. After 12–24 hours, remove the media from the cells, and replace with fresh media without HDR Enhancer.

Note: Genomic DNA isolation and detection of mutations can be performed 48–72 hours after nucleofection.

References

1. Lonza. (2009) Amaxa 96-well Shuttle Protocol for HEK-293 (ATCC®). [Online] Basel, Switzerland, Lonza, Ltd. [Accessed 12 Sept, 2018]

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