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 Neon® Transfection System

Prepare CRISPR reagents

Resuspend your oligos in Nuclease-Free IDTE, pH 7.5.

Component	Final concentration
Alt-R crRNA with tracrRNA or Alt-R sgRNA	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 at www.idtdna.com/SciTools.

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 a 4 µL volume.
- The Megamer dose may need to be optimized for your cell type. (See the protocol Homology-directed repair using the Alt-R CRISPR-Cas9 System and Megamer ssDNA Fragments).

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 toform 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
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:

Component	Amount (µL)
PBS	1.8
gRNA (50 µM)	0.7 (36 pmol)
Alt-R Cas9 enzyme (61 µM)	0.5 (30 pmol)
Total volume	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

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 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 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.

6. After electroporation, transfer cells to wells containing 190 µL of 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.

See Homology-directed repair using the Alt-R CRISPR-Cas9 System and Megamer ssDNA Fragments for the comprehensive protocol.

Technical support: applicationsupport@idtdna.com

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