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

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 an 8 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 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 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.

Safe stop (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 these components per each electroporation well:

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

* This represents the concentration of the CRISPR-Cas9 enzyme; Cas9-GFP and Cas9-RFP are provided at 52 uM (10 mg/mL). Adjust your volume accordingly.

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

Safe stop (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 Alt-R HDR Enhancer V2 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 enhancer, mix Alt-R HDR Enhancer V2 with cell culture media, then aliquot in a 96-well plate.

   Tip: To support reduction in sample-to-sample variability, a stock solution of cell culture media with Alt-R HDR Enhancer V2 may be made and, then aliquoted 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 to prepare each working solution, for a final concentration of 1.0 µM Alt-R HDR Enhancer V2. Each well will require 175 µL after the nucleofected cells are added. If needed, scale up according to the number of samples you have.

Component	Sample (µL)	Negative control, no enhancer (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 Alt-R 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, ensuring the cells are washed with PBS before nucleofection to remove potential 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.
5. After electroporation, add 75 µL of prewarmed culture media (without Alt-R HDR Enhancer V2) 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 Alt-R 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 Alt-R HDR Enhancer V2.

Isolate gDNA

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

Safe stop (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.

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