HOMOLOGY-DIRECTED REPAIR (HDR) USING THE ALT-R™ CRISPR-Cas9 SYSTEM AND HDR DONOR OLIGOS

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

Prepare CRISPR reagents

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

Component	Final concentration (µM)
Alt-R crRNA with tracrRNA, or Alt-R sgRNA	100
Alt-R HDR Donor Oligos	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

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
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 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

* This represents the concentration of the CRISPR-Cas9 enzyme; if you are using fusion proteins, i.e., Cas9-GFP or Cas9-RFP, the concentration will differ. Adjust your volume accordingly.

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

Cell culture media with and without Alt-R HDR Enhancer V2 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 (Lonza) following nucleofection.

2. If using Alt-R HDR Enhancer V2, mix it with cell culture media, then aliquot in a 96-well plate.

   Tip: To reduce sample-to-sample variability, we recommend making a stock solution of cell culture media with HDR Enhancer V2, then aliquoting 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 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 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 by nucleofection

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 20 µL of the Nucleofection Buffer.
2. Make the final transfection mix by combining the following components:

Component	Amount (µL)
RNP complex	5
100 µM Alt-R HDR Donor Oligos	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 template concentration for each site. Generally, 1–4 µM ssODN template 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.
5. After electroporation, add 75 µL of prewarmed culture media (without 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 HDR Enhancer V2.
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.

See Homology-directed repair using the Alt-R CRISPR-Cas9 System and HDR Donor Oligos for the comprehensive protocol.

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