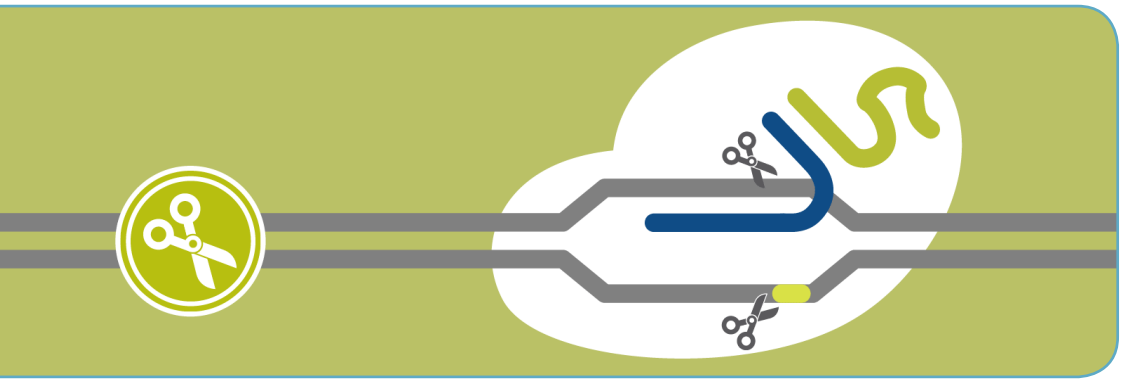
Alt-R CRISPR-Cas9 System:

Delivery of ribonucleoprotein complexes into HEK-293 cells using the Amaxa® Nucleofector® System



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Revision history

Version	Date released	Description of changes
3.1	August 2019	Specified unit of measure for centrifuge from 600 rpm to 150 x *g*.
3	July 2018	Added instructions for using Alt-R CRISPR-Cas9 sgRNA. Updated names and catalog numbers for Alt-R enzymes (V3). Updated to current IDT styles and formatting.
2.3	May 2018	Added note about use of improved Alt-R enzymes (V3): direct substitution in protocol of V3 enzymes for original enzymes (3NLS).
2.2	October 2017	Added information about new IDT crRNA design tools.
2.1	August 2017	Added information about new IDT products (Alt-R Cas9 variants).
2	January 2017	Updated product names to specify CRISPR-Cas9 system to differentiate these from CRISPR-Cpf1 system reagents. Replaced custom Ultramer oligo with the Alt-R Cas9 Electroporation Enhancer. Added ordering information and references for the fluorescently labeled tracrRNA, Alt-R CRISPR-Cas9 tracrRNA – ATTO 550. Corrected typographical errors.
1	November 2016	Original protocol.

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Introduction

This protocol describes the delivery of a CRISPR-Cas9 ribonucleoprotein (RNP) complex, containing Alt-R CRISPR-Cas9 guide RNA (crRNA:tracrRNA duplex or sgRNA) and a Cas9 enzyme (nuclease or nickase), into HEK-293 cells using electroporation with the Amaxa Nucleofector system (Lonza) and is based on 2 protocols: the Lonza Amaxa 96-well Shuttle Protocol for HEK-293 [1] and the IDT Alt-R CRISPR-Cas9 System user guide [2].

Go to www.idtdna.com/CRISPR-Cas9 (Resources section, Application notes), for tips on using the following:

- Fluorescently labeled tracrRNA (Alt-R Cas9 tracrRNA – 5′ ATTO™ 550) to monitor electroporation efficiency or to select for transfected cells via cell sorting.
- Nickases (Alt-R S.p. Cas9 D10A Nickase or Alt-R S.p. Cas9 H840A Nickase) to reduce off-target effects and promote homology-directed repair.

Important considerations

1. Use low-passage, healthy cells. A critical factor affecting the success of electroporation is the health of the cells. It is important to:

   - Use the lowest passage number cells available
   - Subculture cells for at least 2–3 days before the electroporation procedure
   - Replace the media the day before electroporation
   - Determine the optimal confluency for your cell type

   Optimal confluency for HEK-293 cells is 80–90% at the time of Nucleofection. Higher cell densities are likely to reduce viability and electroporation efficiency.

2. Wash cells after trypsinization. Trypsin and FBS may contain RNase activity that can quickly degrade the critical CRISPR RNA components. Therefore, after neutralizing the trypsin with FBS-containing media, it is crucial to wash the cells with PBS. Alternatively, use "enzyme-free" dissociation media, instead of trypsin.

3. Assemble RNPs individually. For example, use separate reactions for each guide RNA, if targeting multiple sites per sample (e.g., in nickase experiments).

4. Include the Alt-R Cas9 Electroporation Enhancer in the electroporation. This protocol recommends the use of this non-targeting carrier DNA to improve electroporation efficiency (Figure 1). Use the same molar concentration of the electroporation enhancer as ribonucleoprotein complex.

5. Always include proper controls in your experiment. When using crRNA:tracrRNA duplexes, we recommend using the appropriate Alt-R CRISPR-Cas9 Control Kit for studies in human, mouse, or rat cells.

The control kits include an Alt-R CRISPR-Cas9 HPRT Positive Control crRNA targeting the HPRT gene and a computationally validated Alt-R CRISPR-Cas9 Negative Control crRNA. The kits also include the Alt-R CRISPR-Cas9 tracrRNA for complexing with the crRNA controls, Nuclease-Free Duplex Buffer, and validated PCR primers for amplifying the targeted HPRT region in the selected organism. The inclusion of the PCR assay makes the kits ideal for verification of HPRT gene editing using the Alt-R Genome Editing Detection Kit (T7 endonuclease I assay).

For assistance with control sgRNAs, contact applicationsupport@idtdna.com.

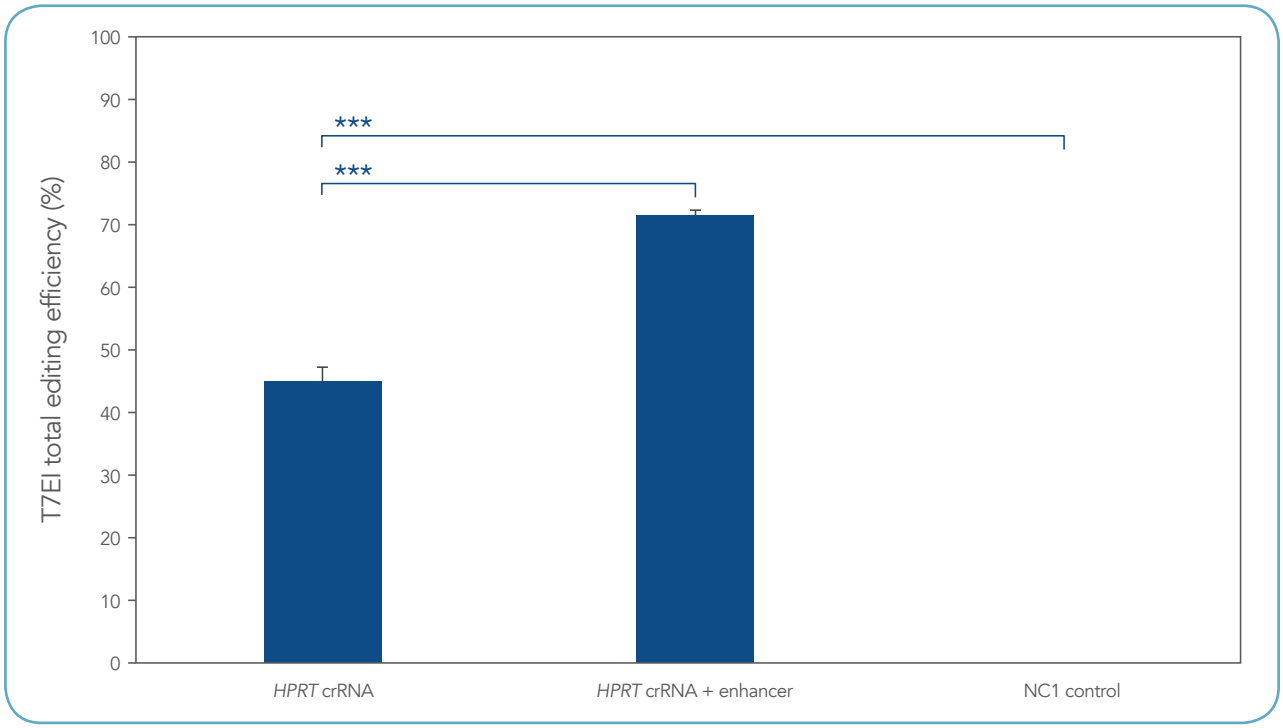

Figure 1. Alt-R Cas9 Electroporation Enhancer increases CRISPR editing efficiency in ribonucleoprotein (RNP) electroporation experiments. An RNP complex that included Alt-R CRISPR-Cas9 HPRT Positive Control crRNA (Human), tracrRNA, and Cas9 nuclease was delivered into HEK-293 cells by electroporation. The RNP was delivered with or without Alt-R Cas9 Electroporation Enhancer (carrier DNA), as described in this protocol. The electroporation enhancer significantly improved editing efficiency as measured by the Alt-R Genome Editing Detection Kit (T7EI assay). The Alt-R CRISPR-Cas9 Negative Control crRNA #1 (NC1) is bioinformatically determined to have no known targets in human, mouse, or rat genomes. Error bars represent standard deviation; $p<0.01$ (***).

Required materials

Kits and reagents	Ordering information
4D-Nucleofector System	Lonza (cat # AAF-1002B with AAF-1002X)
96-well Shuttle™ System	Lonza (cat # AAM-1001S)
SF Cell Line 96-well Nucleofector Kit	Lonza (cat # V4SC-2096)
Dulbecco's Modified Eagle's Medium (DMEM)	ATCC (cat # 30-2002)
Fetal bovine serum (FBS)	General laboratory supplier
Trypsin	General laboratory supplier
1X Phosphate buffered saline (PBS)	General laboratory supplier
Option 1, 2-part guide RNA (crRNA + tracrRNA): • Alt-R CRISPR-Cas9 crRNA or Alt-R CRISPR-Cas9 crRNA XT • Alt-R CRISPR-Cas9 tracrRNA or Alt-R CRISPR-Cas9 tracrRNA – ATTO 550	IDT predesigned and custom crRNA*: www.idtdna.com/CRISPR-Cas9 IDT (cat # 1072532, 1072533, 1072534) IDT (cat # 1075927, 1075928)
Option 2, single guide RNA (sgRNA): Alt-R CRISPR-Cas9 sgRNA	IDT predesigned and custom sgRNA*: www.idtdna.com/CRISPR-Cas9
(Recommended for option 1, 2-part guide RNA) Alt-R CRISPR-Cas9 Control Kit	IDT (cat # 1072554 [human], 1072555 [mouse], or 1072556 [rat])
Alt-R S.p. Cas9 Nuclease V3† Alternatives: Alt-R S.p. HiFi Cas9 Nuclease V3 Alt-R S.p. Cas9 D10A Nickase V3 Alt-R S.p. Cas9 H840A Nickase V3	IDT (cat # 1081058, 1081059) IDT (cat # 1081060, 1081061) IDT (cat # 1081062, 1081063) IDT (cat # 1081064, 1081065)
(Optional, but recommended) Alt-R Cas9 Electroporation Enhancer‡	IDT (cat # 1075915, 1075916) Sequence (100 nt): TTAGCTCTGTTTACGTCCCAGCGGGCATGAGAGTAA CAAGAGGGTGTGGTAATATTACGGTACCGAGCACTA TCGATACAATATGTGTCATACGGACACG
Nuclease-Free IDTE, pH 7.5 (1X TE solution)	IDT (cat # 11-01-02-02)

* We guarantee the performance of our predesigned gRNAs targeting human, mouse, rat, zebrafish, or nematode genes. For other species, use our proprietary algorithms to design custom gRNAs. If you have 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 (wild-type) is suitable for most genome editing studies. However, some experiments may benefit from use of Alt-R S.p. HiFi Cas9 Nuclease V3, 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 is promoted.

‡ The 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.

Protocol

A. Culture cells [1]

1. Do not use freshly thawed cells for electroporation.
2. Use cells with the lowest passage number possible. Lonza recommends not using HEK-293 cells after passage 20.
3. Replace cell culture media every 2–3 days. For stable cell lines, make sure to include appropriate selection antibiotic.
4. Split cells to maintain confluency ≤90%.

   Note: Optimal confluency for electroporation of HEK-293 cells with the Nucleofector system is 80–90%. Higher cell densities may reduce electroporation efficiency.
5. Subculture cells for a minimum of 2–3 days before electroporation, and visually inspect the cells with a microscope to ensure healthy appearance.

B. Prepare RNA

1. Resuspend your RNA oligos in IDTE Buffer.

Guide RNA	Final concentration (µM)
Option 1	
Alt-R CRISPR-Cas9 crRNA	200
Alt-R CRISPR-Cas9 tracrRNA	200
Option 2	
Alt-R CRISPR-Cas9 sgRNA	100

   For assistance, use the IDT Resuspension Calculator at www.idtdna.com/SciTools.

   Note: Store resuspended RNAs at –20°C.
2. If using sgRNA, proceed to step C (Form the RNP complex).
3. Mix the crRNA and tracrRNA oligos in equimolar concentrations in a sterile microcentrifuge tube to a final duplex concentration of 100 µM. The following table shows an example for a 10 µL final volume:

Component	Amount (µL)
200 µM Alt-R CRISPR-Cas9 crRNA	5
200 µM Alt-R CRISPR-Cas9 tracrRNA	5
Total volume	**10**

4. Heat at 95°C for 5 min.
5. Remove from heat and allow to cool to room temperature (15–25°C) on the bench top.

C. Form the RNP complex

1. For each well undergoing electroporation, dilute the guide RNA and Cas9 enzyme in PBS, gently swirling the pipet tip while pipetting:

Component	Amount (µL)
PBS	2.1
Alt-R guide RNA (crRNA:tracrRNA duplex from step B5 or sgRNA from step B2)	1.2 (120 pmol)
Alt-R Cas9 enzyme (61 µM stock)*	1.7 (104 pmol)
Total volume	**5†**

* All Alt-R S.p. Cas9 nucleases and nickases are provided at a stock concentration of 61 µM. Refer to the Application note for tips for using the nickases [3].

† The 5 µL reaction volume is for a single Nucleofection reaction; scale up as necessary for your experiment.

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

Tip: To save time, prepare the RNP during the 2 x 10 min centrifugation in **steps E8** and **E11** below.

D. Prepare Nucleofector system

1. Turn on Nucleofector system and Shuttle device. Open software and log in. Make sure the software connects to the device.
2. Open new parameter file.
3. Select wells of the 96-well plate; then select the appropriate Nucleofector program for your cell line.

E. Perform electroporation of cells with Nucleofector system [1]

1. Resuspend the Alt-R Cas9 Electroporation Enhancer to 100 µM in IDTE. For assistance, use the Resuspension Calculator at www.idtdna.com/SciTools.
2. Add the entire Supplement to the Nucleofection Solution SF before first use, as directed by the manufacturer.
3. Prepare a 96-well culture plate to receive cells following Nucleofection.

Note: We recommend dividing each Nucleofection into 3 replicate wells.

   a. Fill necessary wells with 175 µL of culture media (DMEM, 10% FBS).

   b. Store in a tissue culture incubator (37°C, 5% CO_2).

4. Prepare an additional aliquot of 75 µL/well of culture media (DMEM, 10% FBS) and pre-warm to 37°C.

5. Harvest cells in a 150 cm^2 flask by trypsinization.
   a. Aspirate media from cultured cells and wash once with 10 mL of 1X PBS.
   b. Add 4 mL of 1X trypsin solution to cells and incubate at 37°C until cells just release from flask. **It is important to avoid over trypsinizing cells, while making sure a single-cell suspension is achieved.**
   c. Neutralize trypsin by adding 16 mL of culture media (DMEM, 10% FBS).
6. Count the trypsinized cells.
7. Transfer the total number of cells you need for your experiment to a sterile, 15 mL tube.

   Note: Typically, 2 x 10^5–5 x 10^5 cells per well is the optimal range of HEK-293 cells for Nucleofection. In developing this protocol we used 3.5 x 10^5 HEK-293 cells per Nucleofection; scale up for the appropriate number of wells.
8. Centrifuge the cells at 30 x *g* for 10 min at room temperature.
9. Remove as much supernatant as possible without disturbing the pellet.

   Important! Wash cells in 5 mL of 1X PBS.

   Trypsin and FBS commonly contain RNase activity. Therefore, after neutralizing trypsin with FBS-containing media, it is crucial to wash the cells with PBS. A good alternative to use is "enzyme-free" dissociation media.
10. Centrifuge at 30 x *g* for 10 min at room temperature.
11. Remove as much supernatant as possible without disturbing the pellet.
12. Resuspend cells by adding 20 µL of supplemented Nucleofector Solution SF (from **step E2**) per 3.5 x 10^5 cells.
13. Pipet 20 µL of cell suspension into each well of a V-bottom plate.
14. To each well, add 5 µL of the RNP (from **step C2**) and 1 µL of 100 µM Alt-R Cas9 Electroporation Enhancer (from **step E1**).
15. Pipet up and down 2 times, and transfer 25 µL of the cell:RNP complex mixture to the wells of the 96-well Nucleocuvette module.

    Note: The total volume is 26 µL, containing 4.6 µM guide RNA, 4 µM Cas9 nuclease, and 4 µM Cas9 electroporation enhancer. The total volume allows for easy pipetting of the 25 µL required volume in the electroporation cuvette, and prevents the formation of air bubbles.
16. Gently tap the Nucleocuvette module to make sure no air bubbles are present.
17. Place Nucleocuvette module in Shuttle device, and select **Upload and start**.
18. After electroporation, remove the Nucleocuvette module from the instrument.

    Note: Be sure to save the file before nucleofection occurs.

19. Add 75 µL of pre-warmed culture media (from **step E4**) per well and resuspend cells by gently pipetting up and down.
20. Transfer 25 µL of resuspended cells to the 175 µL of culture media (DMEM, 10% FBS) from **step E3**, in triplicate.
21. Incubate cells in a tissue culture incubator (37°C, 5% CO_2) for 48 hr.

To detect on-target mutations with the mismatch endonuclease T7EI, use the Alt-R Genome Editing Detection Kit (cat # 1075931, 1075932, 1075933) [4].

References

1. Lonza. (2009) Amaxa 96-well Shuttle Protocol for HEK-293 (ATCC®). [Online] Basel, Switzerland, Lonza, Ltd. [Accessed 26 Jun 2018]
2. Integrated DNA Technologies. (2017) Alt-R CRISPR-Cas9 System: User Guide for cationic lipid delivery of CRISPR-Cas9 ribonucleoprotein into mammalian cells. [Online] Coralville, IA, Integrated DNA Technologies, Inc. [Accessed 26 Jun 2018]
3. Yan S, Schubert M, et al. (2017) Applications of Cas9 nickases for genome engineering. [Online] Coralville, IA, Integrated DNA Technologies, Inc. [Accessed 26 Jun 2018]
4. Integrated DNA Technologies. (2017) Alt-R Genome Editing Detection Kit protocol. [Online] Coralville, IA, Integrated DNA Technologies, Inc. [Accessed 26 Jun 2018]

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