

Alt-R[®] CRISPR-Cas9 System:

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



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Introduction

This protocol describes the delivery of a CRISPR-Cas9 ribonucleoprotein (RNP) complex, containing Alt-R® CRISPR-Cas9 crRNA:tracrRNA 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 (Support 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 typeOptimal 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 crRNA, 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. 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).

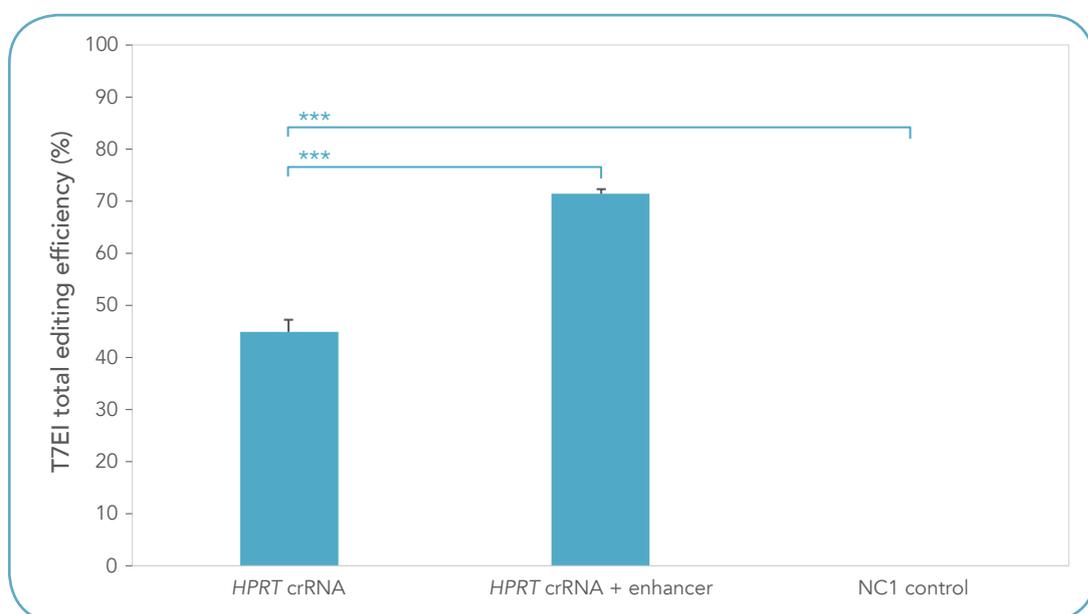


Figure 1. Alt-R® Cas9 Electroporation Enhancer increases CRISPR editing efficiency in ribonucleoprotein (RNP) electroporation experiments. An RNP complex that included Alt-R CRISPR-Cas9 *HPRT* Positive Control crRNA (Human), tracrRNA, and Cas9 nuclease was delivered into HEK-293 cells by electroporation. 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
Alt-R® CRISPR-Cas9 crRNA	IDT 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)
(Recommended)	IDT (Cat # 1072554 [human], 1072555 [mouse], or 1072556 [rat])
Alt-R® CRISPR-Cas9 Control Kit	
Alt-R® S.p. Cas9 Nuclease 3NLS*	IDT (Cat # 1074181, 1074182)
Alternatives:	
Alt-R® S.p. HiFi Cas9 Nuclease	IDT (Cat # 1078727, 1078728)
Alt-R® S.p. Cas9 D10A Nickase 3NLS	IDT (Cat # 1078729, 1078730)
Alt-R® S.p. Cas9 H840A Nickase 3NLS	IDT (Cat # 1078731, 1078732)
	IDT (Cat # 1075915, 1075916)
(Optional, but recommended)	Sequence (100 nt):
Alt-R® Cas9 Electroporation Enhancer†	TTAGCTCTGTTTACGTCCCAGCGGGCATGAGAGTAACA AGAGGGTGTGGTAATATTACGGTACCGAGCACTATCGA TACAATATGTGCATACGGACACG
Nuclease-Free IDTE, pH 7.5 (1X TE solution)	IDT (Cat # 11-01-02-02)

* Alt-R® S.p. Cas9 Nuclease 3NLS is suitable for most genome editing studies. However, some experiments may benefit from use of Alt-R S.p. HiFi Cas9 Nuclease, which has been engineered for to reduce of 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 crRNAs, off-target effects are reduced, and homology-directed repair can be 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 $\leq 90\%$.

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. Form the crRNA:tracrRNA duplex

1. Resuspend each RNA oligo (Alt-R CRISPR-Cas9 crRNA and tracrRNA) in IDTE Buffer to final concentrations of 200 μM . For assistance, use the IDT Resuspension Calculator at www.idtdna.com/scitools.

Store resuspended RNAs at -20°C .

2. Mix the two RNA 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

3. Heat at 95°C for 5 min.
4. 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 crRNA:tracrRNA duplex (from **Step B4**) and Cas9 enzyme components in PBS, gently swirling the pipet tip while pipetting:

Component	Amount (μL)
PBS	2.1
crRNA:tracrRNA duplex (from Step B4)	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].

† This 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.

Note: To save time, the RNP can be prepared 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 on 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 μ M 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 μ L of culture media (DMEM, 10% FBS).
 - b. Store in a tissue culture incubator (37°C, 5% CO₂).
4. Prepare an additional aliquot of 75 μ L/well of culture media (DMEM, 10% FBS) and pre-warm to 37°C.
 5. Harvest cells in 150 cm² 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 the cells and incubate at 37°C until the cells just release from flask. It is important to avoid over trypsinizing the 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, $2\text{--}5 \times 10^5$ cells per well is the optimal range of HEK-293 cells for Nucleofection. To develop this protocol, we used 3.5×10^5 HEK-293 cells per Nucleofection. Scale up for the appropriate number of wells.

8. Centrifuge the cells at 200 rpm for 10 min at room temperature.
9. Remove as much supernatant as possible without disturbing the pellet.
10.  **Important:** Wash cells in 5 mL of 1X PBS.

Note: 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 is to use 'enzyme-free' dissociation media.

11. Centrifuge at 200 rpm for 10 min at room temperature.
12. Remove as much supernatant as possible without disturbing the pellet.
13. Resuspend cells by adding 20 μL of Nucleofactor Solution SF (from **Step E2**) per 3.5×10^5 cells.
14. Pipet 20 μL of cell suspension into each well of a V-bottom plate.
15. To each well, add 5 μL of the crRNA:tracrRNA:Cas9 RNP (from **Step C2**) and 1 μL of 100 μM Alt-R Cas9 Electroporation Enhancer (from **Step E1**).

Note: The total volume is 26 μL , containing 4.6 μM crRNA:tracrRNA, 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. 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.
17. Gently tap the Nucleocuvette module to make sure no air bubbles are present.
18. Place Nucleocuvette module in Shuttle device, and select **Upload and start**.

Note: You will be asked to save the file before nucleofection occurs.

19. After electroporation, remove the Nucleocuvette module from the instrument.

20. Add 75 μL of pre-warmed culture media (from **Step E4**) per well and resuspend cells by gently pipetting up and down.
21. Transfer 25 μL of resuspended cells to the 175 μL of culture media (DMEM, 10% FBS) from **Step E3**, in triplicate.
22. Incubate cells in a tissue culture incubator (37°C, 5% CO₂) for 48 hr.

To detect on-target mutations with the Alt-R Genome Editing Detection Kit (T7E1 mismatch assay), use the protocol described in Part 2 of *Alt-R® CRISPR-Cas9 System: User guide for cationic lipid delivery of CRISPR-Cas9 ribonucleoprotein into mammalian cells* [2].



References

1. (2009) Amaxa™ 96-well Shuttle™ Protocol for HEK-293 (ATCC®). Lonza. Available at http://bio.lonza.com/fileadmin/groups/marketing/Downloads/Protocols/Generated/Optimized_Protocol_181.pdf. (Accessed July 28, 2017.)
2. (2017) Alt-R® CRISPR-Cas9 System: User Guide for cationic lipid delivery of CRISPR-Cas9 ribonucleoprotein into mammalian cells. Integrated DNA Technologies. Available at www.idtdna.com/pages/docs/user-guide_lipofection_of_cas9_ribonucleoprotein.pdf. (Accessed July 28, 2017.)
3. Yan S, Schubert M, et al. (2017) Applications of Cas9 nickases for genome engineering. Integrated DNA Technologies. Available at www.idtdna.com/CRISPR-Cas9/#tab3. (Accessed August, 2017.)



Revision history

Version	Date released	Description of changes
2.1	August 2017	Updated with 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	October 2016	Original protocol

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