Alt-R™ CRISPR-Cpf1 System:

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

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Introduction

This protocol describes the delivery of a CRISPR-Cpf1 ribonucleoprotein (RNP) complex, containing Alt-R™ CRISPR-Cpf1 crRNA and Alt-R A.s. Cpf1 Nuclease 2NLS, into HEK-293 cells using electroporation with the Amaxa® Nucleofector® system (Lonza) [1]. To detect on-target mutations and estimate editing efficiency, we recommend using the Alt-R Genome Editing Detection Kit [2].

The CRISPR-Cpf1 system is distinct from the more commonly used CRISPR-Cas9 system. For example, Cpf1 nuclease does not require a tracrRNA; recognizes a T-rich, protospacer-adjacent motif (PAM: TTTV, where V is an A, C, or G base); and creates a staggered double-stranded DNA cut with a 5′ overhang. For additional information, visit www.idtdna.com/CRISPR-Cpf1.

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 critical CRISPR RNA components. Therefore, **it is crucial to wash the cells with PBS** to remove any trypsin or FBS-containing media. Alternatively, use "enzyme-free" dissociation media instead of trypsin.

3. **Alt-R Cpf1 Electroporation Enhancer, a non-targeting, single-stranded carrier DNA, is required for successful electroporation.** See the Performance section at www.idtdna.com/CRISPR-Cpf1 for supporting data.

4. **Always include appropriate controls in your experiment.** We recommend using an HPRT-specific, positive control crRNA and a non-targeting negative control. For suggested sequences for studies in human, mouse, or rat cells, see page 4.

   To detect on-target editing of the suggested HPRT control crRNA, use Alt-R HPRT PCR Primer Mix (available for human, mouse, or rat) with the Alt-R Genome Editing Detection Kit (T7 endonuclease I assay).

Required materials

Instruments, 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
Penicillin-streptomycin (Pen-Strep)	General laboratory supplier
1X Phosphate buffered saline (PBS)	General laboratory supplier
Alt-R™ CRISPR-Cpf1 crRNA	IDT custom crRNA (www.idtdna.com/CRISPR-Cpf1)
Alt-R™ A.s. Cpf1 Nuclease 2NLS	IDT (Cat # 1076158)
Alt-R™ Cpf1 Electroporation Enhancer	IDT (Cat # 1076300) **Note:** The electroporation enhancer does not have significant homology to the human, mouse, or rat genomes, and has been tested as carrier DNA in multiple cell lines, including HEK-293, Jurkat, and HeLa.
Nuclease-Free IDTE, pH 7.5 (1X TE solution)	IDT (Cat # 11-01-02-02)

To order control crRNAs, enter the appropriate sequence into the Cpf1 crRNA ordering tool (accessible at www.idtdna.com/CRISPR-Cpf1). These sequences are available online for copying and pasting into the ordering tool.

Recommended control crRNAs	Sequence
Positive control Cpf1 crRNA, Human HPRT1	GGTTAAAGATGGTTAAATGAT
Positive control Cpf1 crRNA, Mouse Hprt	GGATGTTAAGAGTCCCTATCT
Positive control Cpf1 crRNA, Rat Hprt1	ATGCTTAAGAGGTATTTGTTA
Negative control Cpf1 crRNA #1	CGTTAATCGCGTATAATACGG
Negative control Cpf1 crRNA #2	CATATTGCGCGTATAGTCGCG
Negative control Cpf1 crRNA #3	GGCGCGTATAGTCGCGCGTAT

Protocol

A. Culture cells [1]

1. Do not use freshly thawed cells for electroporation: passage your cells 1X after thawing, and verify that they grow well and look healthy.
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. Form the RNP complex

1. Resuspend Alt-R CRISPR-Cpf1 crRNA to 100 µM in IDTE Buffer. For assistance, use the IDT Resuspension Calculator at www.idtdna.com/scitools.

   Store resuspended RNA at –20°C.

2. For each well undergoing electroporation, dilute the crRNA (from **Step B1**) and Cpf1 protein in PBS, gently swirling the pipet tip while pipetting:

Component	Amount
PBS	1.4 µL
Alt-R™ CRISPR-Cpf1 crRNA (from **Step B1**)	1.6 µL (160 pmol)
Alt-R™ A.s. Cpf1 Nuclease 2NLS*	2 µL (126 pmol)
Total volume	**5 µL†**

* Alt-R™ A.s. Cpf1 Nuclease 2NLS is provided at a stock concentration of 63 µM.
† The 5 µL total volume is for a single Nucleofection reaction; scale up as necessary for your experiment.

3. 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 D8 and D11 below.

C. 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 (for example, **96-DS-150** program for HEK-293 cells).

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

1. Prepare the Alt-R Cpf1 Electroporation Enhancer. For assistance, use the Resuspension Calculator at www.idtdna.com/scitools.
   a. At first use, resuspend the Alt-R Cpf1 Electroporation Enhancer to 100 µM in IDTE to create a stock solution.
   b. For each set of experiments, dilute stock to 78 µM (working solution). You will need 1 µL of working solution for each electroporation.
2. Add the entire Supplement to the Nucleofection Solution SF before first use, as directed by the manufacturer.
3. Prepare 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 150 cm^2 flask by trypsinization.
   a. Aspirate media from cultured cells and wash 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 to 5) x 10^5 cells per well is the optimal range of HEK-293 cells for Nucleofection. We used 3.5 x 10^5 HEK-293 cells per Nucleofection to develop this protocol. Scale up for the appropriate number of wells.

8. Centrifuge the cells at 200 rpm (Beckman GH-3.8 rotor: 6 x g) 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 (Beckman GH-3.8 rotor: 6 x g) 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 Nucleofector Solution SF (from **Step D2**) per 3.5 x 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 Cpf1 RNP (from **Step B3**) and 1 µL of 78 µM Alt-R Cpf1 Electroporation Enhancer (from **Step D1b**).
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:** Before the nucleofection takes place, you will be asked to save the file.

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

20. Add 75 µL of pre-warmed culture media per well (from **Step D4**) 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 D3**, in triplicate.

22. Incubate cells in a tissue culture incubator (37°C, 5% CO_2) for 48 hr.

To detect on-target mutations with the Alt-R Genome Editing Detection Kit (T7EI mismatch assay), follow the protocol [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 May 19, 2017)

2. (2017) Alt-R™ Genome Editing Detection Kit protocol. Integrated DNA Technologies. Available at www.idtdna.com/pages/docs/default-source/user-guides-and-protocols/altr-genome-editing-detection-kit-protocol.pdf. (Accessed May 19, 2017)

Revision history

Version	Date released	Description of changes
2	May 2017	• Updated Cpf1 PAM sequence from TTTN to TTTV, based on new IDT research data (see www.idtdna.com/CRISPR-Cpf1/#tab4). • Updated instructions for crRNA preparation. • Updated control PCR primer ordering information.
1	January 2017	Original protocol