Alt-R™ CRISPR-Cas9 System

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

REVISION HISTORY

Version	Date released	Description of changes
5	February 2022	Added information about Cas9 enzyme to gRNA ratio when using Cas9-GFP or Cas9-RFP.
4	June 2021	Updated to include additional information on new products.
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 Nucleofector™ System (Lonza) and is based on 2 protocols: the Lonza 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, 488, and 647, as well as Cas9-GFP or Cas9-RFP) to investigate 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 help 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

     Note: Optimal confluency for HEK-293 cells is 70–85% at the time of nucleofection. Higher cell densities are likely to reduce viability and electroporation efficiency [3].
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. We recommend to 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. When using crRNA:tracrRNA duplexes, we recommend using the appropriate Alt-R CRISPR-Cas9 Control Kit for studies in human or mouse cells.

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

CONSUMABLES AND EQUIPMENT

Consumables from IDT

Kits and reagents	Ordering information
Option 1, 2-part guide RNA (crRNA + tracrRNA):	
Alt-R CRISPR-Cas9 crRNA or Alt-R CRISPR-Cas9 crRNA XT	IDT predesigned and custom crRNA*: www.idtdna.com/CRISPR-Cas9
Alt-R CRISPR-Cas9 tracrRNA or Alt-R CRISPR-Cas9 tracrRNA – ATTO 550 or Alt-R CRISPR-Cas9 tracrRNA – ATTO 488 or Alt-R CRISPR-Cas9 tracrRNA – ATTO 647	IDT (cat # 1072532, 1072533, 1072534) IDT (cat # 1075927, 1075928) IDT (cat # 10007810) IDT (cat # 10007853)
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] or 1072555 [mouse])
Alt-R *S.p.* Cas9 Nuclease V3†	IDT (cat # 1081058, 1081059, 10000735)
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 Alt-R *S.p.* Cas9 V3, glycerol-free Alt-R *S.p.* Cas9-GFP V3	 IDT (cat # 1081060, 1081061, 10007803) IDT (cat # 1081062, 1081063) IDT (cat # 1081064, 1081065) IDT (cat# 10007806, 10007807, 10007808) IDT (cat# 10008100, 10008161)
(Optional, but recommended) Alt-R Cas9 Electroporation Enhancer‡	IDT (cat # 1075915, 1075916, 10007805) 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 *C. elegans* 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. Alt-R™ *S.p.* Cas9 V3, glycerol-free may be of interest when working with samples or systems where the presence of glycerol may interfere, such as primary cell cultures or high-throughput instruments with sensitive fluidics.

‡ 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.

Consumables from other suppliers

Kits and reagents	Ordering information
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

Equipment

Kits and reagents	Ordering information
4D-Nucleofector™ System	Lonza (cat # AAF-1002B with AAF-1002X)
96-well Shuttle™ System	Lonza (cat # AAM-1001S)

PROTOCOL

Culture cells

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 70–85%. Higher cell densities may reduce electroporation efficiency [3].

5. Subculture cells for a minimum of 2–3 days before electroporation, and visually inspect the cells with a microscope to ensure healthy appearance.

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 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.

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 or sgRNA from step 1)	1.2 (120 pmol)
Alt-R Cas9 enzyme (62 µM stock)*	1.7 (104 pmol)
Total volume	5†

* Alt-R *S.p.* Cas9 nucleases and nickases are provided at a stock concentration of 62 µM (10 mg/mL). Cas9-GFP and Cas9-RFP provided at 52 µM (10 mg/mL). Refer to the Application note for tips for using the nickases [4].

† 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 Perform electroporation of cells with Nucleofector™ system.

Prepare the Nucleofector™ System

1. Turn on the 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.

Perform electroporation of cells with the Nucleofector™ System

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.

   Important: Avoid over-trypsinizing cells while making sure that you achieve a single-cell suspension.

   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 [3]. 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 may contain RNAse activity. Therefore, after neutralizing trypsin with FBS-containing media, we recommend washing the cells with PBS. A good alternative would be to use "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 Perform electroporation of cells with Nucleofector™ system, step 2) 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 Form the RNP complex, step 2) and 1 µL of 100 µM Alt-R Cas9 Electroporation Enhancer (from Perform electroporation of cells with Nucleofector™ system, step 1).
15. Pipet to mix, then 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 Perform electroporation of cells with Nucleofector™ system, step 4) 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 Perform electroporation of cells with Nucleofector™ system, step 3 in triplicate.
21. Incubate cells in a tissue culture incubator (37°C, 5% CO_2) for 48 hr.

To investigate on-target mutations with the mismatch endonuclease T7EI, use the Alt-R Genome Editing Detection Kit (cat # 1075931, 1075932, 1075933) [5].

REFERENCES

1. Lonza. (2009) Amaxa 96-well Shuttle Protocol for HEK-293 (ATCC®). [Online] Basel, Switzerland, Lonza, Ltd. [Accessed 6 Jun 2021]
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 6 Jun 2021
3. Lonza. (2010) Amaxa™ 4D-Nucleofector™ Optimization Protocol for Cell Lines [Online] Basel, Switzerland, Lonza, Ltd. [Accessed 27 Aug 2021]
4. Yan S, Schubert M, et al. (2017) Applications of Cas9 nickases for genome engineering. [Online] Coralville, IA, Integrated DNA Technologies, Inc. [Accessed 6 Jun 2021]
5. Integrated DNA Technologies. (2017) Alt-R Genome Editing Detection Kit protocol. [Online] Coralville, IA, Integrated DNA Technologies, Inc. [Accessed 6 Jun 2021]

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Technical support: applicationsupport@idtdna.com

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