Alt-R™ CRISPR-Cas9 System

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

<table>
<thead>
<tr>
<th>Version</th>
<th>Date released</th>
<th>Description of changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>June 2021</td>
<td>Updated to include additional information on new products.</td>
</tr>
<tr>
<td>3.1</td>
<td>August 2019</td>
<td>Specified unit of measure for centrifuge from 600 rpm to 150 x g.</td>
</tr>
<tr>
<td>3</td>
<td>July 2018</td>
<td>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.</td>
</tr>
<tr>
<td>2.3</td>
<td>May 2018</td>
<td>Added note about use of improved Alt-R enzymes (V3): direct substitution in protocol of V3 enzymes for original enzymes (3NLS).</td>
</tr>
<tr>
<td>2.2</td>
<td>October 2017</td>
<td>Added information about new IDT crRNA design tools.</td>
</tr>
<tr>
<td>2.1</td>
<td>August 2017</td>
<td>Added information about new IDT products (Alt-R Cas9 variants).</td>
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<tr>
<td>2</td>
<td>January 2017</td>
<td>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.</td>
</tr>
<tr>
<td>1</td>
<td>November 2016</td>
<td>Original protocol.</td>
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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 [2] and the IDT Alt-R CRISPR-Cas9 System User Guide [3].

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 GFP-Cas9) 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 [1].

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

<table>
<thead>
<tr>
<th>Option, 2-part guide RNA (crRNA + tracrRNA):</th>
<th>Ordering information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alt-R CRISPR-Cas9 crRNA</td>
<td>IDT predesigned and custom crRNA*: <a href="http://www.idtdna.com/CRISPR-Cas9">www.idtdna.com/CRISPR-Cas9</a></td>
</tr>
<tr>
<td>or Alt-R CRISPR-Cas9 crRNA XT</td>
<td></td>
</tr>
<tr>
<td>Alt-R CRISPR-Cas9 tracrRNA</td>
<td>IDT (cat # 1072532, 1072533, 1072534)</td>
</tr>
<tr>
<td>or Alt-R CRISPR-Cas9 tracrRNA – ATTO 550</td>
<td>IDT (cat # 1075927, 1075928)</td>
</tr>
<tr>
<td>or Alt-R CRISPR-Cas9 tracrRNA – ATTO 488</td>
<td>IDT (cat # 10007810)</td>
</tr>
<tr>
<td>or Alt-R CRISPR-Cas9 tracrRNA – ATTO 647</td>
<td>IDT (cat # 10007853)</td>
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<table>
<thead>
<tr>
<th>Option 2, single guide RNA (sgRNA):</th>
<th>Ordering information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alt-R CRISPR-Cas9 sgRNA</td>
<td>IDT predesigned and custom sgRNA*: <a href="http://www.idtdna.com/CRISPR-Cas9">www.idtdna.com/CRISPR-Cas9</a></td>
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(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          | IDT (cat # 1081060, 1081061, 10007803) |
| Alt-R S.p. Cas9 D10A Nickase V3           | IDT (cat # 1081062, 1081063) |
| Alt-R S.p. Cas9 H840A Nickase V3          | IDT (cat # 1081064, 1081065) |
| Alt-R S.p. Cas9 V3, glycerol-free         | IDT (cat# 10007806, 10007807, 10007808) |
| Alt-R S.p. Cas9-GFP V3                    | IDT (cat# 10008100, 10008161) |

(Optional, but recommended)

| Alt-R Cas9 Electroporation Enhancer‡       | IDT (cat # 1075915, 1075916, 10007805) |
| Sequence (100 nt):                         |                                      |
| TTAGCTCTGTTTACGTTCCAGGGCATGAGATTCAGGACGTAAT |                                      |
| CAAAGAGGGTGTTGGTAATATTACGGTGACCAGGACTAGCAG |                                      |
| TCGATACAAATGTGTCATACGGACAGCG               |                                      |

| 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](http://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

<table>
<thead>
<tr>
<th>Kits and reagents</th>
<th>Ordering information</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF Cell Line 96-well Nucleofector™ Kit</td>
<td>Lonza (cat # V4SC-2096)</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle’s Medium (DMEM)</td>
<td>ATCC (cat # 30-2002)</td>
</tr>
<tr>
<td>Fetal bovine serum (FBS)</td>
<td>General laboratory supplier</td>
</tr>
<tr>
<td>Trypsin</td>
<td>General laboratory supplier</td>
</tr>
<tr>
<td>1X Phosphate buffered saline (PBS)</td>
<td>General laboratory supplier</td>
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## Equipment

<table>
<thead>
<tr>
<th>Kits and reagents</th>
<th>Ordering information</th>
</tr>
</thead>
<tbody>
<tr>
<td>4D-Nucleofector™ System</td>
<td>Lonza (cat # AAF-1002B with AAF-1002X)</td>
</tr>
<tr>
<td>96-well Shuttle™ System</td>
<td>Lonza (cat # AAM-1001S)</td>
</tr>
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</table>
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 [1].
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


   **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:

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

* All Alt-R S.p. Cas9 nucleases and nickases are provided at a stock concentration of 61 µM (10 mg/mL). The GFP-tagged Cas9 is 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, steps 8 and 11 below.

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% CO₂).
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 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 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 [1]. 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 1) 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% CO₂) 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


Alt-R CRISPR-Cas9 System: Delivery of ribonucleoprotein complexes into HEK-293 cells using the Lonza® Nucleofector™ System

Technical support: applicationsupport@idtdna.com

For more than 30 years, IDT’s innovative tools and solutions for genomics applications have been driving advances that inspire scientists to dream big and achieve their next breakthroughs. IDT develops, manufactures, and markets nucleic acid products that support the life sciences industry in the areas of academic and commercial research, agriculture, medical diagnostics, and pharmaceutical development. We have a global reach with personalized customer service.

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