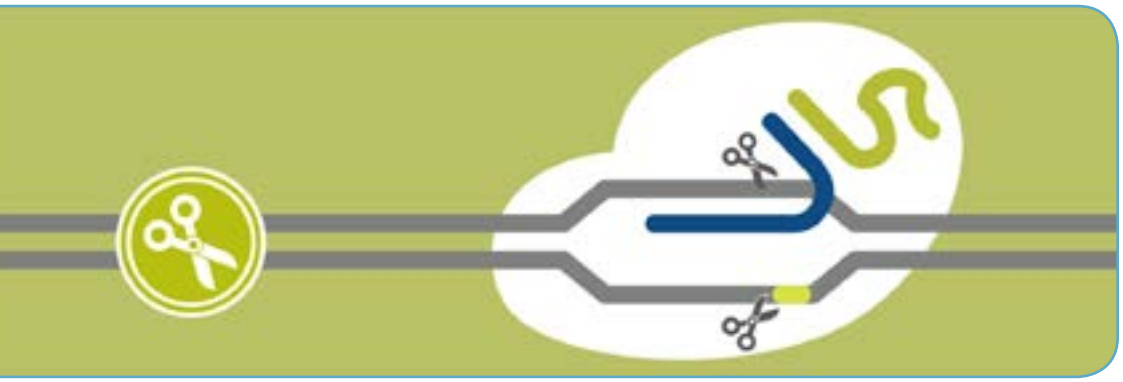
Alt-R CRISPR-Cas9 System:

Delivery of ribonucleoprotein complexes into Jurkat T cells using the Neon® Transfection System



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.
1	November 2016	Original protocol.

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Introduction

This protocol describes the delivery of a Cas9 ribonucleoprotein (RNP) complex, containing an Alt-R CRISPR-Cas9 guide RNA (crRNA:tracrRNA duplex or sgRNA) and a Cas9 enzyme (nuclease or nickase) into Jurkat cells using the Neon Transfection System (Thermo Fisher). The methods reference two protocols: the Neon Transfection user guide [1], and the 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 – 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 do the following:
    - 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
2. **Wash the cells.** FBS may contain RNase activity that can quickly degrade the critical CRISPR RNA components. Therefore, it is crucial to wash the cells with PBS to remove any FBS-containing media.
3. **Assemble RNPs individually.** Use separate reactions for each guide RNA (gRNA), if targeting multiple sites per sample (e.g., in nickase experiments).
4. **Include Alt-R Cas9 Electroporation Enhancer in the electroporation.** This protocol recommends the use of this non-targeting carrier DNA to improve electroporation efficiency. We recommend using the same molar concentration of the electroporation enhancer as ribonucleoprotein complex. For more information on the importance of the electroporation enhancer in this protocol, see the article Successful CRISPR genome editing in hard-to-transfect cells [3].
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 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).

Required materials

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

Kits and reagents	Ordering information
Neon Transfection System	Thermo Fisher Scientific (cat # MPK5000)
Neon Transfection System 10 µL Kit	Thermo Fisher Scientific (cat # MPK1096)
RPMI-1640 Medium (RPMI)	ATCC (cat # 30-2001)
Fetal bovine serum (FBS)	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 RNAs) 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)
Nuclease-Free IDTE, pH 7.5 (1X TE solution)	IDT (cat # 11-01-02-02)
(Optional, but recommended) Alt-R Cas9 Electroporation Enhancer‡	IDT (cat # 1075915, 1075916) Sequence (100 nt): TTAGCTCTGTTTACGTCCCAGCGGGCATGAGAGTAA CAAGAGGGTGTGGTAATATTACGGTACCGAGCACTA TCGATACAATATGTGTCATACGGACACG

* We guarantee the performance of our predesigned gRNAs targeting human, mouse, rat, zebrafish, or nematode genes. For other species, you may 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 thepredesigned 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 for reduction of off-target effects, while retaining on-target potency of Alt-R S.p. Cas9 Nuclease V3. Also, 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 can be promoted.

‡ The enhancer is designed to avoid homology to 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 this oligo does not have similarity to your host cell genome to limit participation of the oligo in the double-stranded DNA break repair process.

Protocol

A. Prepare cell cultures for electroporation

1. Do not use freshly thawed cells for electroporation.
2. Use cells with the lowest passage number possible.
3. Change the cell culture media on the cells 1 day before electroporation.
4. Split cells, if necessary, to obtain optimal confluency for electroporation.

Note: For Jurkat cells, optimal cell density is between 1 x 10^5 and 1 x 10^6 cells/mL at the time of transfection.

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	44

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

Important! 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 44 µM. The following table shows an example for a 10 µL final volume:

Component	Amount (µL)
200 µM Alt-R CRISPR-Cas9 crRNA	2.2
200 µM Alt-R CRISPR-Cas9 tracrRNA	2.2
Nuclease-Free IDTE Buffer	5.6
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 Alt-R Cas9 enzyme to 36 µM by combining the following:

Component	Amount (µL)
Alt-R Cas9 enzyme (62 µM stock)*	0.3
Resuspension Buffer R (from Neon System Kit)	0.2
Total volume	**0.5**

* All Alt-R S.p. Cas9 nucleases and nickases are provided at a stock concentration of 62 µM. Refer to the application note [4] for tips on using the nickases.

2. For each well undergoing electroporation, combine the guide RNA and Cas9 enzyme, gently swirling the pipet tip while pipetting:

Component	Amount
Alt-R guide RNA (crRNA:tracrRNA duplex from step B5 or sgRNA from step B2)	0.5 µL (22 pmol)
Diluted Alt-R Cas9 enzyme (from step C1)	0.5 µL (18 pmol)
Total volume	**1.0***

* This 1 µL volume is for 1 electroporation reaction; scale up as necessary for your experiment—we recommend making 1.2X volume needed to correct for pipetting errors.

3. Incubate the mixture at room temperature for 10–20 min.

Tip: To save time, prepare the RNP during the 2 x 10 min centrifugation in steps E6 and E8 below.

D. Prepare Neon Transfection System

1. Turn on the Neon system.
2. Enter electroporation settings, or choose setting from the optimization protocol.

Note: In our experiments, the optimum settings for Jurkat cells was found to be 1600 V, 10 ms pulse width, 3 pulses [3].

3. Set up the Neon Pipette Station by filling the Neon Tube with Electrolytic Buffer (included in the Neon Transfection System Kit) and insert into the station.

E. Perform electroporation of cells

1. Prepare the Alt-R Cas9 Electroporation Enhancer. For assistance, use the Resuspension Calculator at www.idtdna.com/SciTools.
   a. At first use, resuspend the Alt-R Cas9 Electroporation Enhancer to 100 µM in IDTE to create a stock solution.
   b. For each set of experiments, dilute stock to 10.8 µM (working solution). You will need 2 µL of working solution for each electroporation.

2. Prepare culture plate to receive cells following electroporation. Fill necessary wells with 190 µL culture media (RPMI, 10% FBS) to resuspend the cells, and pipette 150 µL of culture media (RPMI, 10% FBS) into 3 additional wells for triplicate growth. Store in a tissue culture incubator (37°C, 5% CO_2).

3. Pipette cells up and down to dissociate cell clumps.

4. Count the cells in the suspension culture.

5. Determine the total number of cells necessary for your experiment.

   Note: For Jurkat cells, we use 5 x 10^5 cells per electroporation.

6. Centrifuge the required number of cells for all electroporation samples at 150 x *g* for 10 min at room temperature.

7. Remove as much supernatant as possible without disturbing the pellet.

8. Wash cells in 5 mL of 1X PBS, and then centrifuge at 150 x *g* for 10 min at room temperature.

9. Remove as much supernatant as possible without disturbing the pellet.

10. Resuspend cells by adding 9 µL of Resuspension Buffer R per electroporation.

11. For each electroporation, add the following to a 200 µL PCR tube:

Component	Amount (µL)*
RNP complex (from step C3)	1
Cell suspension (from step E10)	9
10.8 µM Alt-R Cas9 Electroporation Enhancer (from step E1b)	2
Total volume	**12**

* The final concentration for each electroporation is 1.8 µM gRNA, 1.5 µM Cas9 nuclease, and 1.8 µM Cas9 electroporation enhancer.

12. Insert a Neon Tip into the Neon Pipette.

13. Pipette 10 µL of cell/RNP complex mixture (from step E11) into the Neon Tip, avoiding air bubbles.

14. Insert the Neon Pipette and Tip into the Pipette Station. Verify the presence of Electrolytic Buffer in the Neon Tube.

15. Press **Start.**

16. After electroporation, transfer cells to wells containing 190 µL of pre-warmed culture media (RPMI, 10% FBS) (from step E2) and slowly resuspend.

17. Transfer 50 µL of resuspended cells in triplicate to the wells containing 150 µL of culture media (RPMI, 10% FBS) (from step E2).

18. Incubate cells in a tissue culture incubator (37°C, 5% CO_2) for 72 hr.

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

References

1. Thermo Fisher Scientific. (2014) Neon Transfection System for transfecting mammalian cells, including primary and stem cells, with high transfection efficiency. [Online] Waltham, MA, Thermo Fisher Scientific, Inc. [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. Turk R and Prediger E. (2016) Successful CRISPR genome editing in hard-to-transfect cells. [Online] Coralville, IA, Integrated DNA Technologies, Inc. [Accessed 26 Jun 2018]
4. 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]
5. 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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