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
4	February 2022	Included product and catalog numbers for Alt-R Cas9 tracrRNA-ATTO 488 and 647, and for Alt-R *S.p.* Cas 9 V3. Nuclease (glycerol free, GFP and RFP).
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 Scientific). 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 (Resource section, Application notes) for applications that use:

- **Fluorescently labeled tracrRNA** (Alt-R Cas9 tracrRNA – ATTO™ 550, 488, and 647), **Cas9-GFP** and **Cas9-RFP** 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 consider the following recommendations:
   - 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 confirmed 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 confirmed PCR primers for amplifying the targeted *HPRT* region in the selected organism. The inclusion of the PCR assay makes the kits ideal for confirmation of *HPRT* gene editing using the Alt-R Genome Editing Detection Kit (T7 endonuclease I assay).

CONSUMABLES

Consumables—IDT

Item	Ordering information
Option 1, 2-part guide RNA (crRNA +tracrRNA):	
• Alt-R CRISPR-Cas9 crRNA or Alt-R CRISPR-Cas9 crRNA XT	Predesigned and custom crRNA*: www.idtdna.com/CRISPR-Cas9
• Alt-R CRISPR-Cas9 tracrRNA or	1072532, 1072533, 1072534
Alt-R CRISPR-Cas9 tracrRNA – ATTO™ 488	10007810
or Alt-R CRISPR-Cas9 tracrRNA–ATTO 550	1075927, 1075928
or Alt-R CRISPR-Cas9 tracrRNA–ATTO 647	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 RNAs):	
• Alt-R CRISPR-Cas9 Control Kit	1072554 (human) or 1072555 (mouse)
• Alt-R *S.p.* Cas9 Nuclease V3†	1081058, 1081059, 10000735
Alternatives:	
Alt-R *S.p.* HiFi Cas9 Nuclease V3	1081060, 1081061, 10007803
Alt-R *S.p.* Cas9 D10A Nickase V3	1081062, 1081063
Alt-R *S.p.* Cas9 H840A Nickase V3	1081064, 1081065
Glycerol-free and GFP/RFP:	
Alt-R *S.p.* Cas9 V3, glycerol-free	10007806, 10007807, 10007808
Alt-R *S.p.* Cas9-GFP V3	10008100, 10008161
Alt-R *S.p.* Cas9-RFP V3	10008162, 10008163
Nuclease-Free IDTE, pH 7.5 (1X TE solution)	11010202
(Optional, but recommended) Alt-R Cas9 Electroporation Enhancer‡	1075915, 1075916, 10007805 Sequence (100 nt): TTAGCTCTGTTTACGTCCCAGCGGGCATGAGAGTAA CAAGAGGGTGTGGTAATATTACGGTACCGAGCACTA TCGATACAATATGTGTCATACGGACACG

* We guarantee that predesigned Alt-R CRISPR-Cas9 guide RNAs will provide successful editing at the target site, when delivered as a ribonucleoprotein complex as described in the Alt-R User Guides, using Alt-R CRISPR-Cas9 guide RNAs (crRNA:tracrRNA duplex or sgRNA) and either Alt-R *S.p.* Cas9 nuclease or Alt-R *S.p.* HiFi Cas9 nuclease. Analysis of editing must be at the DNA level, such as with the Alt-R Genome Editing Detection Kit or DNA sequencing. If successful editing is not observed for a predesigned guide RNA while an appropriate positive control is successful, a one-time "no-cost" replacement of the predesigned Alt-R CRISPR-Cas9 guide RNA will be approved, upon discussion with our Scientific Applications Support team (applicationsupport@idtdna.com). This guarantee does not extend to any replacement product, or to any other incurred or incidental costs or expenses.

† 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.

Consumables—Other suppliers

Item	Supplier	Catalogue #
Neon™ Transfection System	Thermo Fisher Scientific	MPK5000
Neon™ Transfection System 10 µL Kit	Thermo Fisher Scientific	MPK1096
RPMI-1640 Medium (RPMI)	ATCC	302001
Fetal bovine serum (FBS)	Varies	Varies
1X phosphate buffered saline (PBS)	Varies	Varies

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 minutes.
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**

* 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 are provided at 52 µM (10 mg/mL). 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 by gently swirling the pipette tip while pipetting:

Component	Amount
Alt-R guide RNA (crRNA:tracrRNA duplex from step B3 or sgRNA)	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. If working with Cas9-GFP or Cas9-RFP, we recommend using a 1:1.2 ratio of Cas9:gRNA, instead of a 1:1 ratio. See Bio-Rad Gene Pulser Xcell Electroporation System protocol note for reference.

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

Tip: To save time, prepare the RNP during the 2 x 10 minute 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, then 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 minutes 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 hrs.

    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]
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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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