Alt-R™ CRISPR-Cas9 System

Delivery of ribonucleoprotein complexes into Jurkat T cells using the Bio-Rad Gene Pulser® Xcell™ Electroporation System

REVISION HISTORY

Version	Release date	Description of changes
2.2	July 2024	Update to the part number for the IDTE pH 7.5 reagent.
2.1	April 2024	Description of changes Table on page 8 volumes were updated to reflect suggested reagent ratios.
2	February 2022	Updated protocol to include these new products: Cas9 V3 glycerol-free, Cas9-GFP, and Cas9-RFP, as well as new predesigned gRNAs.
1.2	July 2018	Added instructions for using Alt-R CRISPR-Cas9 sgRNA. Updated names and catalog numbers for Alt-R enzymes (V3).
1.1	May 2018	Added note about use of improved Alt-R enzymes (V3): direct substitution in protocol of V3 enzymes for original enzymes (3NLS).
1	November 2017	Original protocol.

Table of contents

INTRODUCTION

This protocol describes the delivery of a CRISPR-Cas9 ribonucleoprotein (RNP) complex, containing Alt-R CRISPR-Cas9 crRNA:tracrRNA and a Cas9 endonuclease, into Jurkat T cells using electroporation with the Bio-Rad Gene Pulser Xcell Electroporation System.

Important considerations

1. Use low-passage, healthy Jurkat T 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
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 crRNA, if targeting multiple sites per sample.
4. Include Alt-R Cas9 Electroporation Enhancer in the electroporation. To improve electroporation efficiency, we recommend using this non-targeting carrier DNA at a fixed dose of 4.8 µM.
5. Always include proper controls in your experiment. We recommend using the appropriate Alt-R CRISPR-Cas9 control kit for studies in human or mouse cells.

   Note: For more information on the importance of the electroporation enhancer in this protocol, see the DECODED article: Successful CRISPR genome editing in hard-to-transfect 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 the 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
Alt-R CRISPR-Cas9 crRNA	Predesigned and custom crRNA*: www.idtdna.com/CRISPR-Cas9
Alt-R CRISPR-Cas9 tracrRNA	1072532, 1072533, 1072534
Alternatives:	
Alt-R CRISPR-Cas9 tracrRNA–ATTO™ 488	10007810
Alt-R CRISPR-Cas9 tracrRNA–ATTO™ 550	1075927, 1075928
Alt-R CRISPR-Cas9 tracrRNA–ATTO™ 647	10007853
(Optional/Recommended) 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 V3, glycerol-free	10007806, 10007807, 10007808
Alt-R *S.p.* Cas9-GFP V3	10008100, 10008161
Alt-R *S.p.* Cas9-RFP V3	10008162, 10008163
Alt-R *S.p.* Cas9 D10A Nickase V3	1081062, 1081063
Nuclease-Free IDTE, pH 7.5 (1X TE solution)	11-01-02-02
(Optional, but recommended) Alt-R Cas9 Electroporation Enhancer‡	1075915, 1075916, 10007805 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 crRNAs. If you have gRNA 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 the use of Alt-R *S.p.* HiFi Cas9 Nuclease V3, which has been engineered to reduce off-target effects, while retaining 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 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 repair process of double-stranded DNA breaks.

Recent product improvements

The Alt-R Cas9 enzymes have recently been further improved. The latest versions (V3) can be directly substituted into this protocol in place of earlier Alt-R Cas9 enzymes.

Consumables—Other suppliers

Item	Supplier	Catalogue #
Gene Pulser Xcell Electroporation System	Bio-Rad	Varies
Gene Pulser/MicroPulser™ Electroporation Cuvettes, 0.2 cm gap	Bio-Rad	1652082
RPMI-1640 Medium (RPMI)	ATCC	302001
Fetal bovine serum (FBS)	Varies	Varies
1X phosphate buffered saline (PBS)	Varies	Varies

PROTOCOL

A. Prepare cell cultures before electroporation

Note: Do not use freshly thawed cells for electroporation; use cells with the lowest passage number possible.

1. Split cells, if necessary, to obtain optimal confluency for electroporation.
2. Change the cell culture media on the cells 1 day before electroporation.
3. Prepare the culture plate to receive cells following electroporation by filling the applicable wells of a 12–well plate with 2 mL of culture media (RPMI, 10% FBS).
4. Preheat the plate in a tissue culture incubator at 37°C, 5% CO_2.

B. Form the crRNA:tracrRNA duplex

1. Resuspend each RNA oligo (Alt-R CRISPR-Cas9 crRNA and Alt-R CRISPR-Cas9 tracrRNA) in IDTE buffer to the final concentration of 200 µM.

   Note: You can use the IDT resuspension calculator at www.idtdna.com/scitools. Always store resuspended RNAs at –20°C.

2. Mix the two RNA oligos in equimolar concentrations in a single microcentrifuge tube to a final duplex concentration of 100 µM.

   The following table shows an example of a 20 µL final volume:

Component	Amount (µL)
200 µM Alt-R CRISPR-Cas9 crRNA	10
200 µM Alt-R CRISPR-Cas9 tracrRNA	10
Total volume	20

3. Heat at 95°C for 5 min.
4. Remove the duplex from the heat and allow to cool to room temperature (15–25°C).

C. Form the RNP complex

1. Mix the Cas9 enzyme and crRNA:tracrRNA duplex components at 1:1.2 molar ratio in PBS.

   Note: We observe reliable editing efficiency in Jurkat T cells when the concentration of Cas9 RNP ranges from 1–4 µM in the final 100 µL mixture for electroporation (see Figure 1). The following table shows how to create a 4 µM final concentration to use in step E14. Reactions at lower concentrations can be performed by diluting the mixture.

Component	Amount (µL)
Alt-R crRNA:tracrRNA duplex (100 µM) (from **step B4**)[1]	12
Alt-R Cas9 enzyme (62 µM stock)[2]	16.1
PBS	21.9
Total volume[3]	50

1 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.

2 The Alt-R *S.p.* Cas9 enzyme is provided at a stock concentration of 62 µM (10 mg/mL). The Alt-R Cas9-GFP and Cas9-RFP enzymes are provided at 52 µM (10 mg/mL). Cas9 RNP complexes can be made in PBS or in Cas9 dilution buffer (30 mM HEPES, 150 mM KCl, pH 7.5).

3 In the 50 µL mixture, the concentration for Cas9 enzyme is 20 µM, while the concentration for duplexed RNA is 24 µM.

2. Incubate at room temperature for 20 minutes to allow formation of the RNP complex.
3. After the RNP complex has formed, keep the mixture on ice until electroporation.

D. Prepare the electroporation station

1. Keep cuvettes at 4°C. If any condensation is present, wipe it away before using.
2. Enter these electroporation settings:
   - 250 V
   - 2 ms pulse width
   - 1 pulse
   - Unipolar polarity

   Note: We have found these settings to be optimal for Jurkat T cells. Other cell lines may require different parameters.

E. Perform electroporation of cells

1. Create a working solution by resuspending the Alt-R Electroporation Enhancer to 96 µM in IDTE.
2. Take the cells subject to electroporation and resuspend them in culture media (RPMI, 10% FBS). Pipette mix to dissociate any cell clumps.
3. Count the cells in the suspension culture.
4. Determine the total number of cells for each experiment. In this example, we will use 1 x 10^7 cells which is enough for 10 electroporations.

   Note: For Jurkat cells, we recommend using 1 x 10^6 cells per electroporation.

5. Dilute 1 x 10^7 cells to a final volume of 40 mL using PBS.

6. Centrifuge the required number of cells for all electroporation samples at 200 x g for 5 min at room temperature (15–25°C).
7. Remove as much of the supernatant as possible without disturbing the pellet.
8. Wash cells in 5 mL of PBS.
9. Centrifuge at 200 x g for 5 min at room temperature.
10. Remove as much of the supernatant as possible without disturbing the pellet.
11. Resuspend the cells by adding 750 µL PBS, which results in a cell density of 1 x 10^6/75 µL.
12. Aliquot 75 µL of the resuspended cells for each electroporation in 1.5 mL microcentrifuge tubes.
13. Keep the resuspended cells on ice for at least 5 min before starting electroporation.
14. For each electroporation, combine the following components into a sterile microcentrifuge tube:

Component	Amount (µL)
Alt-R RNP complex (from **step C3**)	20
96 µM Alt-R Electroporation Enhancer (from **step E1**)	5
Aliquoted cell resuspension (from **step E12**)	75
Total volume*	100

* The final concentration for each electroporation in the 100 µL total volume is 4 µM Cas9 nuclease—4.8 µM guide RNA, and 4.8 µM electroporation enhancer.

15. Immediately transfer the mixture to cooled cuvettes (from step D1) and start electroporation.
16. After electroporation, transfer cells to the preheated wells containing 2 mL of culture media (RPMI, 10% FBS) on the 12-well tissue culture plate (from step A4).
17. Incubate cells in a tissue culture incubator at 37°C, 5% CO_2 for 72 hr.

Note: To detect on-target mutations with the mismatch endonuclease T7E1, use the protocol described in Part 2 of Alt-R CRISPR-Cas9 System: User guide for cationic lipid delivery of CRISPR-Cas9 ribonucleoprotein into mammalian cells.

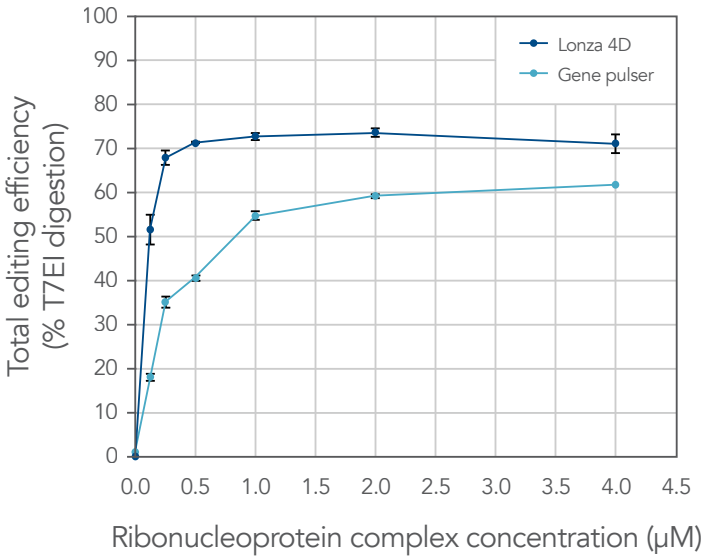

Figure 1. Robust genome editing achieved using electroporation with the Bio-Rad Gene Pulser® Xcell™ system. Jurkat T cells were transfected with 0.125–4 µM RNP (Alt-R *S.p.* Nuclease V3 complexed with Alt-R CRISPR-Cas9 crRNA and tracrRNA) in the presence of Alt-R Cas9 Electroporation Enhancer, on the Lonza 4D and Bio-Rad Gene Pulser® platforms. Genomic DNA was isolated 72 hr after transfection. Total editing effi ciency was determined using the Alt-R Genome Editing Detection Kit (T7 endonuclease I assay). N=3.

Alt-R™ CRISPR-Cas9 System

Delivery of ribonucleoprotein complexes into Jurkat T cells using the Bio-Rad Gene Pulser® Xcell™ Electroporation System

For more information, go to: www.idtdna.com/ContactUs

Doc ID: RUO22-0678_001.2 07/24