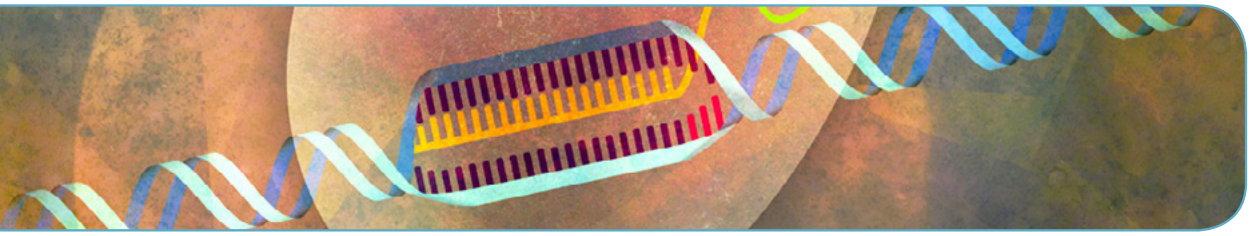
Alt-R® CRISPR-Cas9 System:

Cationic lipid delivery of CRISPR ribonucleoprotein complex into mammalian cells



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IDT® INTEGRATED DNA TECHNOLOGIES

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Overview

For the most efficient genome editing, we recommend using a ribonucleoprotein (RNP) consisting of Alt-R S.p. Cas9 nuclease (or nickase) in complex with Alt-R CRISPR-Cas9 crRNA and tracrRNA. Using this combination provides very high editing efficiency across most target sites and addresses issues (e.g., inconsistent Cas9 expression levels and incorporation of DNA expression constructs) that can be problematic with other CRISPR-Cas9 editing methods [1–3].

This user guide discusses formation of an RNP using Alt-R CRISPR-Cas9 System components and lipofection of the RNP into HEK-293 cells. General guidance for optimization of delivery into other cell types is provided (see page 22). The guide also describes use of Alt-R CRISPR-Cas9 System controls and the Alt-R Genome Editing Detection Kit.

What do I need for CRISPR-Cas9 genome editing?

Healthy cells	Low passage-number cells are essential for lipofection delivery. You can monitor delivery of CRISPR RNAs into your cells using fluorescently labeled Alt-R CRISPR-Cas9 tracrRNA – ATTO™ 550.
Alt-R CRISPR-Cas9 crRNA	This crRNA is chemically modified to provide increased nuclease resistance in cells. It contains a target-specific 20 nt protospacer domain and a 16 nt sequence that is complementary to the tracrRNA. Our online design tools allow you to select predesigned crRNA, generate a custom crRNA, or analyze the on- and off-target potential of your designs. The crRNA and tracrRNA must be complexed to form the bimolecular RNA trigger (guide RNA) recognized by *S. pyogenes* Cas9.
Alt-R CRISPR-Cas9 tracrRNA	This tracrRNA is a conserved, 67 nt RNA sequence required for complexing to the crRNA to form the guide RNA recognized by *S. pyogenes* Cas9. This RNA molecule is chemically modified to provide increased nuclease resistance in cells and is offered unlabeled or labeled with a fluorescent dye. The fluorescent label is useful for optimizing transfection conditions or for enriching for transfected cells using fluorescence-activated cell sorting (FACS). The tracrRNA is HPLC-purified to ensure >70% full length purity as verified by electrospray ionization-mass spectrometry (ESI-MS).
Alt-R S.p. Cas9 nuclease or nickase	Alt-R Cas9 endonucleases are derived from *S. pyogenes* and contain 1 N-terminal nuclear localization sequence (NLS), 2 C-terminal NLSs, and a C-terminal 6-His tag. They recognize and cleave double-stranded DNA in the presence of target-specific crRNA:tracrRNA complexes. • Alt-R S.p. Cas9 Nuclease 3NLS—suitable for most genome editing studies • Alt-R S.p. HiFi Cas9 Nuclease 3NLS—engineered for reduced off-target effects, while retaining similar on-target potency to wild-type Cas9 • Alt-R S.p. Cas9 D10A and H840A Nickase 3NLS—create single-stranded breaks (When a nickase variant is used with 2 crRNAs, off-target effects are reduced, and homology-directed repair can be promoted.)

Transfection reagent	We currently recommend Lipofectamine® RNAiMAX or CRISPRMAX™ reagent (Thermo Fisher). Other RNA-specific reagents may work as well. However, in our research, most classical plasmid and small RNA delivery reagents perform poorly with RNPs.
Alt-R Genome Editing Detection Kit	The T7EI mismatch cleavage assay can be used to detect genome editing and estimate editing efficiency. You will need to design a PCR assay that is specific for your CRISPR target region. Cleavage of reannealed PCR products by T7EI into predicted sizes indicates successful CRISPR editing.

This table presents the main components necessary for genome editing experiments with the CRISPR-Cas9 system. Additional reagents are described in the **Required materials** section of this user guide.

Introduction

Use of the CRISPR (clustered regularly interspaced short palindromic repeats) system for genome editing has been a major technological breakthrough, making genome modification in cells or organisms faster, more efficient, and more robust than previous genome editing methods.

The native bacterial CRISPR system in *S. pyogenes* requires 2 short RNA molecules—a sequence-specific CRISPR RNA (crRNA) and a conserved, transactivating crRNA (tracrRNA)—that interact through partial homology to form a crRNA:tracrRNA complex. This complex is often called the guide RNA, because it guides and activates Cas9 to cleave double-stranded DNA targets, activating the non-homologous end joining (NHEJ) system or generating a potential insertion site for exogenous donor DNA by homology-directed repair (HDR).

Alt-R CRISPR-Cas9 RNAs and enzymes

The Alt-R CRISPR-Cas9 System improves the efficiency of CRISPR genome editing through the use of a ribonucleoprotein (RNP), consisting of experimentally optimized Alt-R CRISPR-Cas9 crRNA and tracrRNA, as well as Alt-R S.p. Cas9 nucleases or nickases. Along with improved efficiency, the system saves time by providing ready-to-use RNA reagents, and reduces or eliminates activation of the cellular immune response observed with *in vitro* transcribed RNAs (such as single guide RNAs and mRNAs). For more product information, see the sidebar, **Essential Alt-R CRISPR-Cas9 components** (page 5). For information about design, ordering, and experimental set up, see **Appendices A and B** (starting on pages 15 and 18, respectively).

Alt-R CRISPR-Cas9 kits and controls

Multiple variables affect CRISPR genome editing performance. The Alt-R CRISPR-Cas9 Control Kits will give you the confidence of validated reagents to help optimize and troubleshoot your experiments. Kits are available for targeting the *HPRT* gene in human, mouse, or rat cells.

Additional advice for setting up controls can be found on page 21. IDT application specialists can also help, and providing them with data from your experiments using the control kits will allow them to give you the most accurate support. For assistance, email us at **applicationsupport@idtdna.com**.

Mutation detection: Alt-R Genome Editing Detection Kit

This guide also describes the use of the Alt-R Genome Editing Detection Kit to estimate editing efficiency using T7 endonuclease I (T7EI). In the T7EI assay,

Essential Alt-R CRISPR-Cas9 components

The combination of Alt-R Cas9 protein with Alt-R RNAs provides the lowest off-target editing performance available.

Alt-R CRISPR-Cas9 RNAs with increased nuclease resistance

Based on extensive research, Alt-R CRISPR-Cas9 crRNA and tracrRNA have been optimized by the addition of proprietary chemical modifications that protect the RNA oligos from degradation by cellular RNases and further improve on-target editing performance.

Alt-R guide RNA design tools

Use our design tools to find or generate potent crRNAs or assess the targeting potential of your own designs. We guarantee that each Alt-R Predesigned CRISPR-Cas9 crRNA will edit the target gene by 40% or more when transfected by lipofection into HEK-293 cells.*

Alt-R CRISPR-Cas9 tracrRNA labeled with fluorescent dye

Fluorescently labeled tracrRNAs provide the same function as their unlabeled counterparts. However, the fluorescent dye allows you to:

- Monitor transfection or electroporation efficiency during optimzation.
- Enrich cells via FACS (fluorescence-activated cell sorting) analysis to simplify screening for cells with CRISPR events.

For tips on using the labeled tracrRNA, see **www.idtdna.com/CRISPR-Cas9** (**Resources** section, Application notes).

Alt-R S.p. Cas9 nucleases and nickases

Recombinant *S. pyogenes* Cas9 enzymes are purified from an *E. coli* strain expressing a codon-optimized Cas9. Product specifications are as follows:

- Amount provided: 100 µg (611 pmol) or 500 µg (3054 pmol)
- Molecular weight: 163,700 g/mol
- Concentration: 10 µg/µL in 50% glycerol [61 µM]
- Endotoxin tested: <2 EU/mg
- Shipping conditions: dry ice
- Storage conditions: –20°C
- Heat inactivation: 20 min at 65°C

* See www.idtdna.com/CRISPR-Cas9 for details.

target genomic regions from CRISPR-modified cells are amplified by PCR. The PCR products are denatured and reannealed to allow heteroduplex formation between wild-type DNA and CRISPR-mutated DNA. Mutations are then detected using T7EI, which recognizes and cleaves mismatched DNA heteroduplexes. T7EI assay results are analyzed by visualizing cleavage

Important Alt-R CRISPR-Cas9 controls

Alt-R CRISPR-Cas9 Control Kits target *HPRT* in human (gene ID: 3251), mouse (gene ID: 15452), and rat (gene ID: 24465) cells and contain the following:

Components*	Amount
Alt-R CRISPR-Cas9 tracrRNA	5 nmol
Alt-R CRISPR-Cas9 HPRT Positive Control crRNA (human, mouse, or rat, depending on kit)	2 nmol
Alt-R CRISPR-Cas9 Negative Control crRNA #1 (contains a 20 nt protospacer sequence that is computationally designed to be non-targeting in human, mouse, and rat reference genomes)	2 nmol
Alt-R HPRT PCR Primer Mix (human, mouse, or rat, depending on kit)	2 nmol (each primer)
Nuclease-Free Duplex Buffer	2 mL

* Kit components are also available separately. Cas9 enzyme and PCR master mix are not included in the kits.

Estimating CRISPR-Cas9 editing efficiency

Alt-R Genome Editing Detection Kits contain the following:

Components*	25 rxn kit	100 rxn kit	1000 rxn kit
T7 endonuclease I (T7EI; 1 U/µL)	50 µL	200 µL	2000 µL
T7EI Reaction Buffer (10X)	50 µL	200 µL	2000 µL
Alt-R Control A (template/primer mix)	20 µL	20 µL	20 µL
Alt-R Control B (template/primer mix)	20 µL	20 µL	20 µL

* PCR master mix and target-specific primers/probe sets are not included in the kits.

Alt-R Controls A and B are positive controls that each contain template and PCR primers for monitoring the function of the T7EI assay. Full-length PCR products from Controls A and B differ by a 6 bp deletion in Control B. Reanneal and digest PCR products from Control A as a homoduplex control (no T7EI cleavage expected), and reanneal and digest PCR products from Control A and B as a heteroduplex control (T7EI cleavage expected).

products and full-length amplicons by gel or capillary electrophoresis. See protocol in part 2 (page 11).

Note that T7EI activity is sensitive to the DNA:enzyme ratio, as well as incubation temperature and time [4]. T7EI recognizes insertions and deletions of ≥2 bases that are generated by NHEJ activity in CRISPR experiments [5]. Because T7EI does not recognize 1 bp indels, T7EI underrepresents the total editing. See representative data at **www.idtdna.com/CRISPR-Cas9** (**Performance** section).

For a quick, robust assessment of editing events in CRISPR experiments, we recommend using the T7EI assay instead of alternative methods such as Surveyor® mismatch endonuclease assays and Sanger sequencing. The T7EI method is simple and fast, and provides clean electrophoresis results. T7EI is also compatible with a broad range of PCR buffers, and does not usually require purification of PCR products before digestion.

Required materials

Kits and reagents	Ordering information*
Nuclease-Free IDTE	IDT (Cat # 11-01-02-02)
Nuclease-Free Water	IDT (Cat # 11-04-02-01)
Phosphate buffered saline (PBS)	General laboratory supplier
Reagents for transfection	
Alt-R CRISPR-Cas9 tracrRNA Alternative: Alt-R CRISPR-Cas9 tracrRNA – ATTO 550	IDT (Cat # 1072532, 1072533, 1072534) IDT (Cat # 1075927, 1075928)
Alt-R CRISPR-Cas9 crRNA	IDT predesigned or custom crRNA: **www.idtdna.com/CRISPR-Cas9**
(Recommended) Alt-R CRISPR-Cas9 HPRT Positive Control crRNA	IDT (Cat # 1072541 [human], 1072542 [mouse], or 1072571 [rat])
(Recommended) Alt-R CRISPR-Cas9 Negative Control crRNA	IDT (Cat # 1072544 [#1], 1072545 [#2], or 1072546 [#3])
Alt-R S.p. Cas9 Nuclease 3NLS†	IDT (Cat # 1074181, 1074182)
Alternatives: Alt-R S.p. HiFi Cas9 Nuclease Alt-R S.p. Cas9 D10A Nickase 3NLS Alt-R S.p. Cas9 H840A Nickase 3NLS	 IDT (Cat # 1078727, 1078728) IDT (Cat # 1078729, 1078730) IDT (Cat # 1078731, 1078732)
Nuclease-Free Duplex Buffer‡	IDT (Cat # 11-01-03-01)
Option for Cas9 dilution, if not using PBS or Opti-MEM® media: HEPES KCL	 General laboratory supplier General laboratory supplier

Reagents for transfection (continued)	
Trypsin	General laboratory supplier
Opti-MEM Media	Thermo Fisher (Cat # 51985091)
Alternatives: Lipofectamine RNAiMAX Transfection Reagent Lipofectamine CRISPRMAX Transfection Reagent	 Thermo Fisher (Cat # 13778100) Thermo Fisher (Cat # CMAX00008)
Reagents for mutation detection	
Alt-R Genome Editing Detection Kit	IDT (Cat # 1075931, 1075932, 1075933)
(Optional) Alt-R HPRT PCR Primer Mix (2 nmol each)§	IDT (Cat # 1072551 [human], 1072552 [mouse], or 1072553 [rat])
KAPA HiFi HotStart PCR Kit	Kapa Biosystems (Cat # KK2501)
QuickExtract™ DNA Extraction Solution	Epicentre (Cat # QE09050)
(Option 1) Agarose	General laboratory supplier
(Option 2) Mutation Discovery Kit‖	Advanced Analytical Technologies, Inc. (Cat # DNF-910-K1000T)

* These are suggested sources for reagents used by the IDT R&D team when this protocol was written. Individual components (e.g., the polymerase and buffer from the PCR kit) may be substituted with some optimization.

† Alt-R S.p. Cas9 Nuclease 3NLS is suitable for most genome editing studies. However, some experiments may benefit from use of Alt-R S.p. HiFi Cas9 Nuclease, 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 crRNAs, off-target effects are reduced, and homology-directed repair can be promoted.

‡ Nuclease-Free Duplex Buffer is provided with the Alt-R CRISPR-Cas9 tracrRNA or may be ordered separately.

§ For use with positive control experiments that used Alt-R CRISPR-Cas9 HPRT Postitive Control crRNA.

‖ For use on a Fragment Analyzer™ system (Advanced Analytical Technologies, Inc.)

Go to **www.idtdna.com** for safety data sheets (SDSs) and certificates of analysis (COAs) for IDT products.

Protocol

Part 1: Transfection of Cas9:crRNA:tracrRNA ribonucleoprotein (RNP) complex

We recommend including wells for independent transfections of positive and negative controls (see page 21). If you are targeting >1 PAM site in each sample (e.g., in nickase experiments), use separate reactions to form RNPs with each guide RNA before transfection.

A. Assemble the RNP complex.

1. Prepare Cas9 enzyme working buffer (20 mM HEPES; 150 mM KCl, pH 7.5). Depending on your cell type, Opti-MEM media or 1X PBS can be used instead.

2. Resuspend each RNA oligo (Alt-R CRISPR-Cas9 crRNA and tracrRNA) in Nuclease-Free IDTE Buffer or Nuclease-Free Duplex Buffer.

   - We suggest resuspending the RNA oligos to 100 µM stock concentrations, using the volumes in the following table:

Normalized amount delivered (nmol)*	Volume of resuspension buffer (µL)
2	20
5	50
10	100
20	200
100	1000

* Prepare positive and negative controls using the same methods as the experimental complexes—ideally using the same lots of buffers. Alt-R CRISPR-Cas9 HPRT Positive Control crRNAs and Alt-R CRISPR-Cas9 Negative Control crRNAs are available at 2 nmol scale. Custom Alt-R CRISPR-Cas9 crRNAs are available at 2 and 10 nmol scales. Alt-R CRISPR-Cas9 tracrRNA is available at 5, 20, and 100 nmol scales.

   - To calculate your own dilutions, use the molecular weight information provided on page 20. The estimated number of reactions that can be completed using this protocol is shown on page 19, which also includes various plate sizes and scales of Alt-R CRISPR oligos.

   Note: Store resuspended RNA oligos at –20°C.

3. Mix the two RNA oligos in equimolar concentrations in a sterile microcentrifuge tube. For example, create a final duplex concentration of 1 µM using the following table:

   Note: For smaller volumes, such as for 384-well plates, a higher concentration may be necessary.

Component	Amount (µL)
100 µM Alt-R CRISPR-Cas9 crRNA	1
100 µM Alt-R CRISPR-Cas9 tracrRNA	1
Nuclease-Free Duplex Buffer	98
Total volume	**100**

4. Heat at 95°C for 5 min.
5. Remove from heat and allow to cool to room temperature (20–25°C) on your bench top.

6. If needed, dilute the complexed RNA to a working concentration (for example, 1 µM) in Nuclease-Free Duplex Buffer or IDTE Buffer.

   Note: The crRNA:tracrRNA guide complexes are stable for at least 6 months with no loss in activity when stored at –20°C at a concentration of ≥1 µM.

7. Before use, thoroughly mix the stock Alt-R S.p. Cas9 enzyme by inverting the tube several times, and briefly centrifuge the tube.

8. Dilute Alt-R S.p. Cas9 enzyme to a working concentration (for example, 1 µM) in Cas9 working buffer, OptiMEM, or 1X PBS (**part 1, step A1**).

   Note: The molecular weight of Alt-R S.p. Cas9 enzymes is 163,700 g/mol. All Alt-R S.p. Cas9 enzymes are provided at a stock concentration of 61 µM. Refer to the Application note [6] for tips for using the nickases.

9. To produce the RNP for each well in the 96-well plate, combine the following:

Component	RNAiMAX reagent Volume per well (µL)	CRISPRMAX reagent Volume per well (µL)
Complexed crRNA:tracrRNA oligos [1 µM] (**part 1, step A6**)	1.5	1.5
Diluted Cas9 enzyme [1 µM] (**part 1, step A8**)	1.5	1.5
Cas9 PLUS™ Reagent (from CRISPRMAX kit)	—	0.6
Opti-MEM Media	22.0	21.4
Total volume	**25**	**25**

10. Incubate at room temperature for 5 min to assemble the RNP complexes.

    Note: The RNP complex can be stored for up to 4 weeks at 4°C or for up to 6 months at –80°C.

B. Reverse transfect the RNP complex in a 96-well plate.

1. For each well of a 96-well plate, combine the following, and incubate at room temperature (20–25°C) for 20 min to form transfection complexes:

Component	Amount (µL)
RNP (**part 1, step A10**)	25
RNAiMAX or CRISPRMAX transfection reagent	1.2
Opti-MEM Media	23.8
Total volume	**50**

2. During incubation, wash cells with PBS and trypsinize.
3. Dilute cells to 400,000 cells/mL using complete media without antibiotics.
4. When incubation is complete, add 50 µL of transfection complexes (**part 1, step B1**) to the wells of a 96-well tissue culture plate.

   Note: Make sure to include wells for replicates of positive and negative controls.
5. Add 100 µL of diluted cells (**part 1, step B3**) to the transfection complexes in the wells of the 96-well tissue culture plate (40,000 cells/well; final concentration of RNP will be 10 nM).
6. Incubate the plate containing the transfection complexes and cells in a tissue culture incubator (37°C, 5% CO_2) for 48 hr.

Part 2: Mutation detection with T7EI

Design PCR primers that amplify your experimental target site and adjacent sequence. We recommend using a 600–1000 bp PCR amplicon with >100 bp flanking the CRISPR cut site and with the CRISPR cut site off-center to allow fragment resolution by gel analysis or capillary electrophoresis.

This protocol includes use of the polymerase and buffer from a Kapa Biosystems Kit; however, other polymerases and buffers can also be used.

Validate the PCR assay to determine the optimal annealing temperature to use with your samples and to verify that only the expected PCR product is synthesized. You can design the PCR assays using the PrimerQuest® Tool at **www.idtdna.com/primerquest**. Calculate the T_m of your primers at **www.idtdna.com/oligoanalyzer**.

A. Process CRISPR-Cas9–edited genomic DNA from cultured cells.

The volumes in **part 2, steps A2** and **A5** are optimized for confluent samples in 96-well plates. Some cell types and samples in larger wells will require larger volumes.

1. Wash CRISPR-Cas9–treated cells with 100 µL of PBS.
2. Lyse cells by adding 50 µL of QuickExtract DNA Extraction Solution.
3. Transfer cell lysate to appropriate PCR tubes or plate.
4. Vortex and heat in a thermal cycler at 65°C for 10 min, followed by 98°C for 5 min.
5. Add 100 µL of Nuclease-Free Water to dilute the genomic DNA.
6. Vortex and spin down.

B. Amplify genomic DNA and detect mutations.

1. Set up PCR using the amounts of template, primers, and components of the Alt-R Genome Editing Detection Kit and KAPA HiFi HotStart PCR Kit as follows:

Component	Sample reaction	Alt-R Control A reaction	Alt-R Control B reaction
Genomic DNA (**part 2, step A6**)	4 µL (~40 ng)	—	—
Forward primer	300 nM	—	—
Reverse primer	300 nM	—	—
Alt-R Control A (template/primer mix)	—	1 µL	—
Alt-R Control B (template/primer mix)	—	—	1 µL
KAPA HiFi Fidelity Buffer (5X)	5 µL (1X)	5 µL (1X)	5 µL (1X)
dNTPs	1.2 mM (0.3 mM each)	1.2 mM (0.3 mM each)	1.2 mM (0.3 mM each)
KAPA HiFi HotStart DNA Polymerase (1 U/µL)	0.5 U	0.5 U	0.5 U
Total volume	**25 µL**	**25 µL**	**25 µL**

2. Run the PCR using the following conditions:

Step	Temperature (°C)	Time (min:sec)	Cycles
Denature	95	5:00	1
Denature	98	0:20	30
Anneal	Variable (see next table)	0:15	
Extend	72	0:30	
Extend	72	2:00	1

Note: These optimal annealing temperatures for the Alt-R PCR Primer Mixes and Controls have been determined using KAPA HiFi HotStart DNA Polymerase. You may need to optimize for other polymerases.

PCR primer mix	Annealing temperature (°C)
Alt-R HPRT PCR Primer Mix, Human and Mouse	67
Alt-R HPRT PCR Primer Mix, Rat	64
Alt-R Control A and B	64–67

C. Form heteroduplexes for T7EI digestion.

1. Combine the following volumes of reagents in an appropriate PCR tube:

Component	Sample reaction	Homoduplex control reaction	Heteroduplex control reaction
PCR (from **part 2, step B2**)	10 µL experimental target or Alt-R HPRT control	10 µL Control A	5 µL Control A 5 µL Control B
T7EI Reaction Buffer (10X)	2 µL	2 µL	2 µL
Nuclease-Free Water	6 µL	6 µL	6 µL
Total volume	**18 µL**	**18 µL**	**18 µL**

2. Heat and cool PCR products in a thermal cycler to form heteroduplexes:

Step	Temperature (°C)	Time
Denature	95	10 min
Ramp 1	95–85	Ramp rate –2°C/sec
Ramp 2	85–25	Ramp rate –0.3°C/sec

3. Combine the following in a microcentrifuge tube for the T7EI digestion:

Component	Amount (µL)
PCR heteroduplexes **(from part 2, step C2)**	18
T7 endonuclease I (1 U/µL)	2
Total volume	**20**

4. Incubate the T7EI reaction at 37°C for 60 min.

D. Visualize T7EI mismatch detection results.

Visualize the digestion using one of the following methods:

- Use agarose gels.
- Dilute digestion with 150 µL of 0.1X IDTE, and run on a Fragment Analyzer system with the Mutation Discovery Kit. See Figure 1 for representative results.

The expected amplicon and digested product sizes for Alt-R Controls A/B and the Alt-R CRISPR-Cas9 HPRT Positive Controls are shown in Figures 1 and 2, respectively.

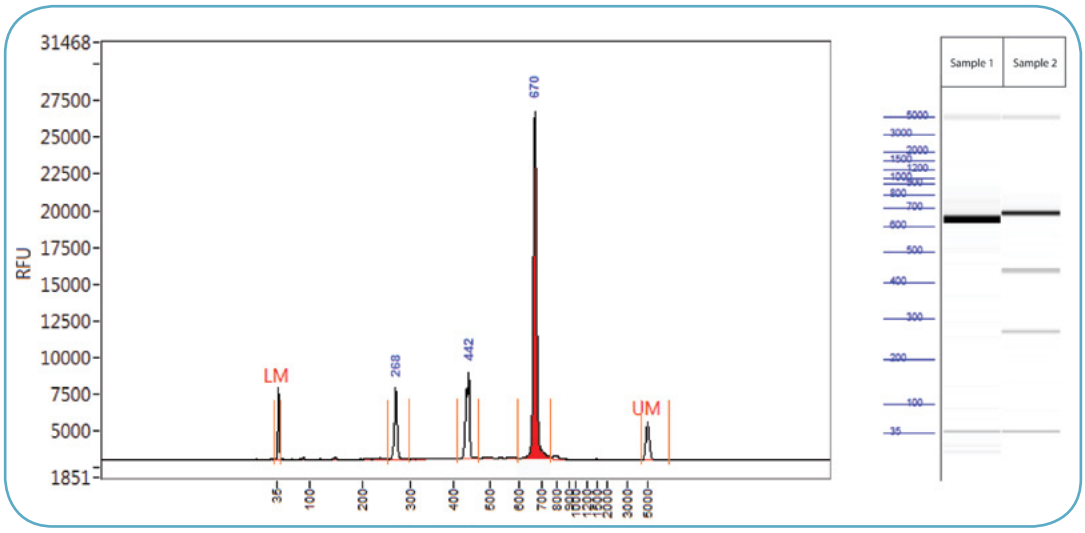
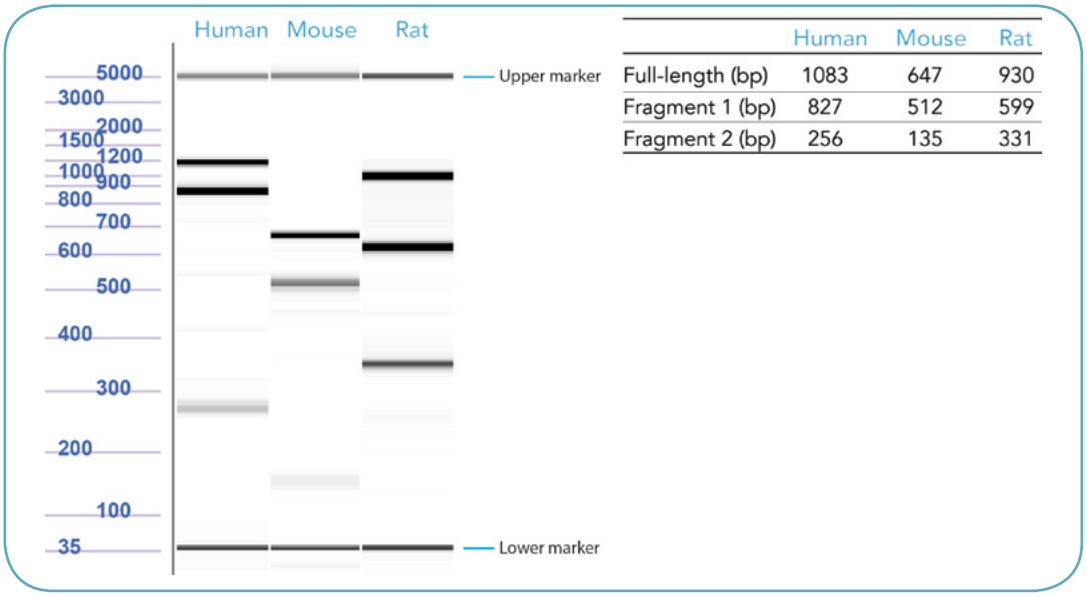


Figure 1. Sample data from Fragment Analyzer system—T7EI digestion containing PCR products from Alt-R Controls A and B. PCR using template and primers in Controls A and B (Alt-R Genome Editing Detection Kit) were run using KAPA HiFi HotStart DNA Polymerase (Kapa Biosystems). Cycling conditions were 5 min. 95°C; 30 x (20 sec. 98°C, 15 sec. 64°C, 30 sec. 72°C); 2 min. 72°C. PCR products were denatured and reannealed in a thermal cycler [10 min. 95°C; 95–85°C (ramp rate: –2°C/sec); 85–25°C (ramp rate: –0.3°C/sec)]. Sample 1 contains homoduplexes of Control A PCR products, while Sample 2 contains homoduplexes and heteroduplexes of Control A and B PCR products. Reannealed PCR products were digested with 2 U of T7EI for 60 min at 37°C. Digestion reactions were analyzed on a Fragment Analyzer system (Advanced Analytical Technologies, Inc.). Trace (left) shows results from Sample 2. Gel image (right) shows results for Samples 1 and 2. LM = lower marker; UM = upper marker.



	Human	Mouse	Rat
Full-length (bp)	1083	647	930
Fragment 1 (bp)	827	512	599
Fragment 2 (bp)	256	135	331

Figure 2. Sample data from T7EI digestion of Alt-R CRISPR-Cas9 HPRT Positive Controls. Genomic DNA from CRISPR-Cas9–edited human, mouse, and rat HPRT controls were PCR amplified, digested using T7 endonuclease I, and run on the Fragment Analyzer system. Reference standard bands at 5000 bp (upper marker) and 35 bp (lower marker) are used to align the gel and analyze the results. Estimated band sizes for the cut and uncut positive control amplicons are listed in the table. Cell lines used were HEK-293 (human), Hepa1-6 (mouse), and RG2 (rat).

Appendix A

This appendix provides background data supporting the strong performance of the core Alt-R CRISPR-Cas9 products (i.e., the RNP components), as well as tips for designing and ordering your target-specific crRNA.

Choosing the best CRISPR-Cas9 guide RNA format

We have found that shortening the crRNA and the tracrRNA, compared to the natural 42 nt crRNA and 89 nt tracrRNA, significantly increases the potency of the triggers for gene editing (Figure A1). For best editing performance, we recommend Alt-R CRISPR-Cas9 crRNA, which is an optimized, chemically modified 35–36 nt crRNA that contains a custom 19 or 20 nt protospacer domain (20 nt recommended) and a fixed 16 nt tracrRNA interaction domain. This Alt-R CRISPR-Cas9 crRNA should be paired with the optimized, chemically modified, 67 nt Alt-R CRISPR-Cas9 tracrRNA (or the labeled Alt-R CRISPR-Cas9 tracrRNA—ATTO 550).

These short RNA oligonucleotides are easily manufactured as high quality, synthetic RNA oligos, which allow for chemical modifications that further improve performance and stability in the presence of cellular RNases. Importantly, in our research, the Alt-R CRISPR-Cas9 crRNA and tracrRNA do not show toxicity or activation of the cellular immune response.

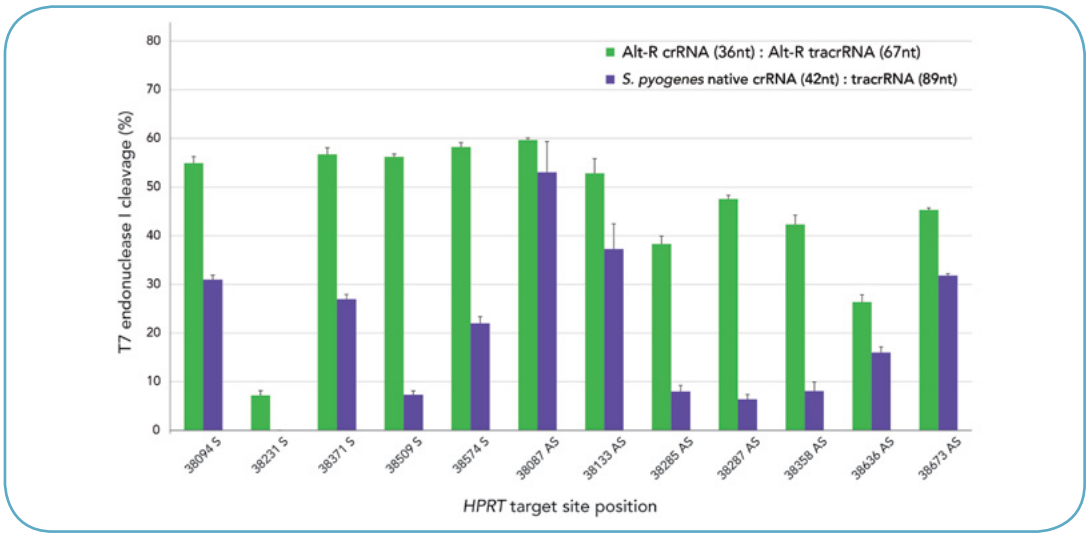


Figure A1. Optimized Alt-R CRISPR-Cas9 RNAs improve Cas9 editing efficiency compared to longer CRISPR RNAs. Alt-R CRISPR-Cas9 crRNA and longer, native crRNAs were designed to recognize 12 sites in the human *HPRT* gene, adjacent to required NGG PAM motifs on both the sense (S) and antisense (AS) strands. Alt-R CRISPR-Cas9 crRNAs were complexed with the optimized Alt-R CRISPR-Cas9 tracrRNA, and the native crRNA was complexed with a long, native tracrRNA. The complexes were reverse transfected using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher) into HEK-293 cells that constitutively express *S. pyogenes* Cas9. Editing was measured by PCR amplification of target sites, followed by cleavage with T7EI and analysis using the Fragment Analyzer system (Advanced Analytical).

Designing and ordering Alt-R CRISPR-Cas9 crRNAs

We guarantee the performance of our predesigned crRNAs targeting human, mouse, rat, zebrafish, or nematode genes. For details about the predesigned crRNA guarantee, see www.idtdna.com/CRISPR-Cas9.

For other species or to target intergenic regions, you may use our proprietary algorithms to design custom crRNAs.

If you have crRNA protospacer designs of your own or from publications, use our design checker tool to assess their on- and off-targeting potential before ordering crRNAs that are synthesized incorporating Alt-R crRNA modifications.

Alt-R design tool	URL
Predesigned crRNA selection	www.idtdna.com/Cas9Predesigned
Custom crRNA design	www.idtdna.com/Cas9Custom
User-defined crRNA design checker	www.idtdna.com/Cas9Checker

Alt-R S.p. Cas9 enzymes

Several methods have been developed for the delivery of the Cas9 endonuclease into mammalian cells. Endonuclease delivery is commonly done by lipid transfection of DNA expression constructs. However, this exogenous double-stranded DNA can be randomly incorporated into genomic DNA, potentially altering endogenous genes [3]. In addition, transfection efficiency varies from one reaction to the next due, in part, to the difficulty in consistently delivering the very large Cas9 expression construct. Furthermore, delivering Cas9 as a DNA expression construct has been shown to produce very high levels of Cas9 enzyme that accumulate over time, thereby increasing the risk of off-target effects [9].

In applications where protein transfections are possible, we recommend using an Alt-R RNP complex to achieve high editing efficiency (Figure A4). Using a Cas9 RNP, instead of *Cas9* mRNA or DNA expression constructs, has been shown to resolve some problems associated with other methods [1,2]. Using the Alt-R S.p. Cas9 enzymes allows control of exactly how much Cas9 is introduced. More importantly, the Alt-R S.p. Cas9 enzyme is non-renewable, limiting the duration of Cas9 activity in cells. These factors help to reduce off-target effects and provide more consistent editing. In addition, use of the RNP eliminates issues of genomic incorporation from DNA constructs and the toxicity associated with transfecting long mRNA.

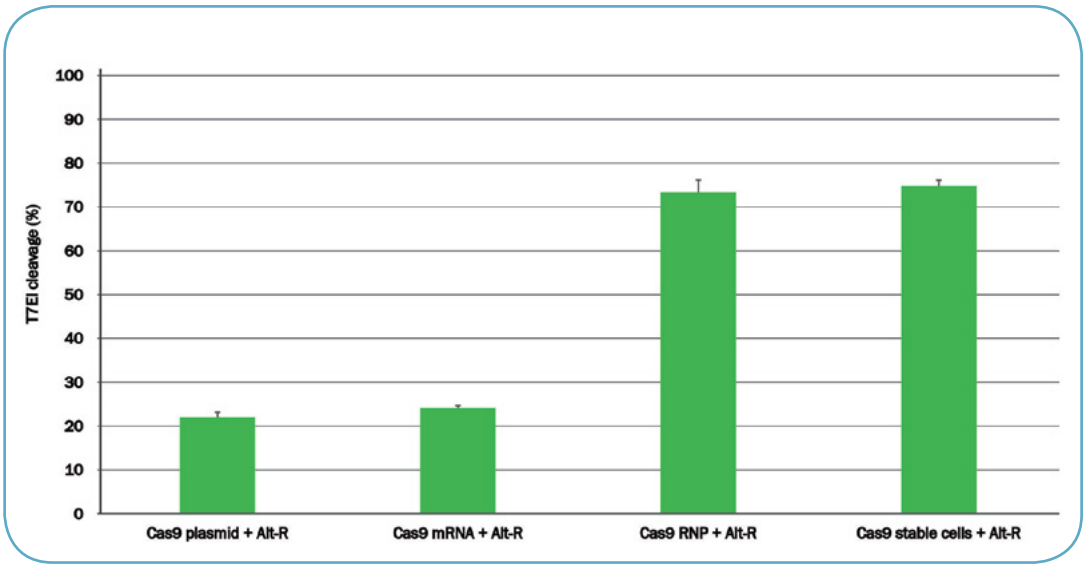


Figure A4. Lipofection of ribonucleoprotein generated using Alt-R CRISPR-Cas9 System components outperforms other transient CRISPR transfection methods. Alt-R CRISPR HPRT Control crRNA:tracrRNA complexes were co-transfected with Cas9 expression plasmid, Cas9 mRNA, or as part of Cas9 ribonucleoprotein (RNP, with crRNA:tracrRNA complex incorporated before transfection) into HEK-293 cells. The RNP outperformed the other transient Cas9 expression methods and performed similar to HEK-293 cells that stably express Cas9. Similar results were confirmed for Alt-R CRISPR HPRT Controls for mouse and rat. Each type of component was transfected using its optimal reagent (i.e., Cas9 plasmid was delivered using TransIT-X2® system [Mirus Bio], Cas9 mRNA was delivered using MessengerMAX® reagent [Thermo Fisher], and the RNP and crRNA:tracrRNA complexes were delivered using Lipofectamine RNAiMAX Reagent [Thermo Fisher]). Editing was measured by PCR amplification of target sites, followed by cleavage with T7EI and analysis using the Fragment Analyzer system (Advanced Analytical).

Appendix B

This appendix provides helpful information for planning, optimizing, and setting up your CRISPR-Cas9 experiments.

Guidance for Alt-R CRISPR-Cas9 RNA and enzyme usage

Tables B1 and B2 are provided to help you determine how much Alt-R CRISPR-Cas9 crRNA and tracrRNA you will need, depending on the number and size of the transfections you require. The provided numbers are only estimates to help you plan experiments. The estimates are based on our experimental observations and assume no wasted material or instrument variation for pipetting volumes.

How much Alt-R CRISPR-Cas9 crRNA, tracrRNA, and S.p. Cas9 enzyme are used in each transfection?

Table B1 shows the estimated amounts of the major transfection components that will be used **per well** for lipofection when the Alt-R CRISPR-Cas9 crRNA and tracrRNA are pre-complexed with recombinant Alt-R S.p. Cas9 enzymes to form the RNP, as described in this guide.

Table B1. Lipofection* of Alt-R CRISPR-Cas9 crRNA and tracRNA pre-complexed with recombinant Alt-R S.p. Cas9 enzymes.

Plate format	crRNA (pmol)	tracrRNA (pmol)	Cas9 enzyme (pmol)	Lipofection complex volume (µL)	Resuspended cell volume (µL)	Total volume (µL)
6-well plate	24	24	24	800	1600	**2400**
12-well plate	12	12	12	400	800	**1200**
24-well plate	6	6	6	200	400	**600**
48-well plate	3	3	3	100	200	**300**
96-well plate	1.5	1.5	1.5	50	100	**150**

* Listed conditions were optimized for transfection using RNAiMAX and 10 nM of Alt-R CRISPR-Cas9 crRNA, tracrRNA, and S.p. Cas9 enzyme. Further optimization may be required for alternative transfection reagents.

How many Alt-R CRISPR-Cas9 System reactions are provided?

Table B2 shows the estimated number of reactions that can be performed for the available scales of Alt-R CRISPR-Cas9 crRNA and tracrRNA, when they are pre-complexed with an Alt-R S.p. Cas9 enzyme, prior to lipofection. The pmol amounts of crRNA and tracrRNA used to estimate the total number of reactions can be found in Table B1.

Table B2. Number of lipofections* using Alt-R CRISPR-Cas9 crRNA and tracrRNA pre-complexed with recombinant Alt-R S.p. Cas9 enzyme by product scale.

Plate format	crRNA, 2 nmol	crRNA, 10 nmol	tracrRNA, 5 nmol	tracrRNA, 20 nmol	tracrRNA, 100 nmol	Cas9 enzyme, (100 µg)	Cas9 enzyme, (500 µg)
6-well plate	82	410	205	820	4100	25	125
12-well plate	166	830	415	1660	8300	50	250
24-well plate	332	1660	830	3320	16,600	100	500
48-well plate	666	3330	1665	6660	33,300	200	1000
96-well plate	1332	6660	3330	13,320	66,600	400	2000

* Listed conditions were optimized for transfection using RNAiMAX and 10 nM concentrations of Alt-R CRISPR-Cas9 crRNA, tracrRNA, and S.p. Cas9 enzyme. Further optimization may be required for alternative transfection reagents.

Resuspension of Alt-R CRISPR-Cas9 crRNA Controls and tracrRNA

For this protocol, we suggest that you resuspend Alt-R CRISPR-Cas9 crRNA Controls and tracrRNA in IDTE buffer or Nuclease-Free Duplex Buffer to 100 µM stock concentrations, and prepare 1 µM working concentrations from those stock resuspensions. Additional instructions for resuspension and dilution can be found in *Part 1: Transfection of Cas9:crRNA:tracrRNA ribonucleoprotein (RNP) complex* (page 8).

Your unique application may require dilution of the crRNA and tracrRNA oligonucleotides to different concentrations. IDT offers the free Resuspension Calculator at **www.idtdna.com/scitools** to help calculate other concentrations. For calculating dilutions of Alt-R CRISPR-Cas9 crRNA Controls and Alt-R CRISPR-Cas9 tracrRNA, the molecular weights are provided in Table B3. The molecular weights have been updated to reflect the modified Alt-R CRISPR-Cas9 oligos.

Store resuspended RNAs at –20°C.

Table B3. Molecular weights and extinction coefficients.

Product	Molecular weight (g/mol)	Extinction coefficient [L/(mol x cm)]
Alt-R S.p. Cas9 Nuclease 3NLS Alt-R S.p. HiFi Cas9 Nuclease 3NLS Alt-R S.p. Cas9 D10A Nickase 3NLS Alt-R S.p. Cas9 H840A Nickase 3NLS	163,700	—
Alt-R CRISPR-Cas9 tracrRNA	22,182	687,200
Alt-R CRISPR-Cas9 tracrRNA—ATTO 550	22,937.4	718,600
Alt-R CRISPR-Cas9 Human HPRT Positive Control crRNA	11,854	378,000
Alt-R CRISPR-Cas9 Mouse HPRT Positive Control crRNA	11,750	364,400
Alt-R CRISPR-Cas9 Rat HPRT Positive Control crRNA	11,892.1	374,600
Alt-R CRISPR-Cas9 Negative Control crRNA #1	11,750	365,800
Alt-R CRISPR-Cas9 Negative Control crRNA #2	11,741.9	356,700
Alt-R CRISPR-Cas9 Negative Control crRNA #3	11,836	356,100

Setting up controls

Setting up control experiments (Table B4) is important for publication and provides useful information if you need to troubleshoot your experiments. It is also good experimental practice to perform technical and biological replicates.

Table B4. The importance of recommended control reactions.

Control reaction	Details
HPRT positive control crRNA	Shows that Cas9 editing is functional in your cells • Complex the Alt-R CRISPR-Cas9 HPRT Positive Control crRNA (available for human, mouse, and rat) with the Alt-R CRISPR-Cas9 tracrRNA and Alt-R S.p. Cas9 enzyme. Transfect the resulting RNP complexes into cells. • Use the Alt-R HPRT PCR Primer Mix in the T7EI assay to confirm on-target editing. **Note:** The expected amplicon and digested-product sizes for analyzing the Alt-R CRISPR-Cas9 HPRT Positive Controls using the T7EI assay are shown in Figure 2 (page 14).
Negative control crRNA	Shows that transfection of RNP complex is not responsible for observed phenotypes • Complex the Alt-R CRISPR-Cas9 Negative Control (verified negative for human, mouse, and rat) with the Alt-R CRISPR-Cas9 tracrRNA and Alt-R S.p. Cas9 enzyme. Transfect the resulting RNP complexes into cells. • Amplification of DNA from these negative controls with your experimental primers and cycling conditions should result in only full-length products in the T7EI assay. **Note:** This does not rule out off-target effects of your experimental crRNA.
No RNA negative control	Used primarily as a toxicity control for transfection conditions • No crRNA or tracrRNA is used in this control • Amplification of DNA from these negative controls with your experimental primers and cycling conditions should result in only full-length products in the T7EI assay.

Optimization of lipofection protocol for other cell types

This guide describes optimized conditions for lipofection of the CRISPR-Cas9 RNP into HEK-293 cells. The protocol can be used for other adherent, immortalized, eukaryotic cell lines, but may require further optimization. Conditions that demonstrate maximal editing efficiency and minimal cell toxicity are considered optimal. For difficult-to-transfect cells or non-dividing cells, another delivery method, such as electroporation, may be required (see User guides under Resources, **www.idtdna.com/CRISPR-Cas9**).

We recommend that you conduct a pilot experiment—preferably, including one of the validated Alt-R CRISPR-Cas9 HPRT Positive Controls for human, mouse, or rat—with the current protocol.

For optimization of this protocol for your cell type, the following factors have the greatest impact on transfection efficiency and need to be optimized:

1. Poor cell health and high passage number cells can negatively affect lipofections. Use the lowest passage number possible for your cells, especially if you observe significant cell death during your transfections.
2. Lipofection requires dividing cells. Cell density at time of transfection should be sub-confluent, typically 60–75%. Low confluency tends to lead to higher cell death, and high confluency may affect transfection efficiency.
3. Reagent used: We currently recommend Lipofectamine RNAiMAX or Lipofectamine CRISPRMAX (Thermo Fisher). Other RNA-specific reagents may work as well. However, in our research, most of the available plasmid and small RNA delivery reagents perform poorly with RNPs.
4. Amount and relative ratio of cationic lipid and RNP cargo: For optimization of the 96-well plate transfections described in this guide, we recommend varying the ranges of the following components:

Component	Optimization range
CRISPR-Cas9 RNP complex	3, 10, and 30 nM
RNAiMAX or CRISPRMAX reagent	0.2–2.0 µL

5. Other consideration for transfection conditions:

   The protocol in this user guide describes lipofection using a reverse transfection protocol. This means that the transfection complexes are added to the plate before the appropriate number of resuspended cells are added to the plate. This is different from forward transfection protocols, where the adherent cells are plated first, often the day before, and transfection complexes are added to the cells. In our experiments, the reverse transfection method we describe is more efficient for most common cell types. However, a few cell types perform better with forward transfection.

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Revision history

Version	Date released	Description of changes
3	October 2017	Added information about new IDT design tools. Added additional transfection reagent option.
2.1	August 2017	Updated with 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 general T7EI assay reagents and instructions with those for the Alt-R Genome Editing Detection Kit. Added ordering information and references for the fluorescently labeled Alt-R CRISPR-Cas9 tracrRNA – ATTO 550. Reorganized and updated some information in the introduction and appendices. Updated IDT user guide template.
1	July 2016	Original protocol.

user guide

genome editing

Alt-R CRISPR-Cas9 System

Cationic lipid delivery of CRISPR ribonucleoprotein complex into mammalian cells

Technical support:

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