Alt-R™ CRISPR-Cas9 System

Cationic lipid delivery of CRISPR ribonucleoprotein complexes into mammalian cells

REVISION HISTORY

Version	Release date	Description of changes
5.1	August 2022	Updated for internal MAPSS compliance
5	January 2022	Added information for Cas9 fusions Removed references to rat kit since kit was discontinued Added information for ATTO dyes Updated format to protocol style guide
4	July 2018	Added instructions for using Alt-R CRISRP-Cas9 sgRNA Removed information about guide RNA format (most recent information is available at www.idtdna.com/CRISPR-Cas9) Updated names and catalog numbers for Alt-R enzymes (V3)
3.1	May 2018	Added note about use of improved Alt-R enzymes (V3): direct substitution in protocol of V3 enzymes for original enzymes (3NLS)
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

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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 guide RNA (crRNA:tracrRNA duplex or sgRNA). 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 protocol discusses formation of an RNP using Alt-R CRISPR-Cas9 System components and lipofection of the RNP into HEK-293 cells. General guidance for optimizing delivery into other cell types is provided (see Table B2). This 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	Cells with a low passage number are essential for lipofection delivery. You can monitor delivery of CRISPR RNAs into your cells using fluorescently labeled Alt-R CRISPR-Cas9 tracrRNA, as well as Cas9-GFP and Cas9-RFP fusion proteins.
Option 1 (2-part guide RNA): Alt-R CRISPR-Cas9 crRNA with Alt-R CRISPR-Cas9 tracrRNA	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 duplexed to form the biomolecular RNA trigger (guide RNA) recognized by *S. pyogenes* Cas9. When using 2-part gRNAs under challenging experimental conditions (e.g., high nuclease environments or with Cas9 mRNA), use Alt-R CRISPR-Cas9 crRNA XT, which have additional chemical modifications for the highest level of stability and performance. 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).
Option 2 (single guide RNA): Alt-R CRISPR-Cas9 sgRNA	This sgRNA (single guide RNA) is an alternative to using crRNA:tracrRNA duplexes. Under challenging experimental conditions (e.g., high nuclease environments or with Cas9 mRNA), Alt-R CRISPR-Cas9 sgRNAs may provide increased editing potency. The sgRNA is a long RNA molecule containing the target-specific 20 nt protospacer domain and other crRNA and tracrRNA segments required for forming an active RNP. The Alt-R CRISPR-Cas9 sgRNA is chemically modified to provide increased nuclease resistance in cells. If a fluorescent marker is required, Cas9 sgRNA can be used with Cas9-GFP or Cas9-RFP fusion proteins.

Alt-R *S.p.* Cas9 nuclease or nickase	Alt-R Cas9 endonucleases are derived from *S. pyogenes* and contain nuclear localization sequences (NLSs) and C-terminal 6-His tags. The enzymes recognize and cleave double-stranded DNA in the presence of target-specific guide RNAs (gRNAs; crRNA:tracrRNA duplex or sgRNA): • **Alt-R *S.p.* Cas9 Nuclease V3 (wild-type)**—suitable for most genome editing studies • **Alt-R *S.p.* HiFi Cas9 Nuclease V3**—engineered for reduced off-target effects, while retaining similar on-target potency to wild-type Cas9 • **Alt-R *S.p.* Cas9 fusions** • **Alt-R *S.p.* Cas9-GFP (or RFP) V3 Nuclease**—IDT's Alt-R *S.p.* Cas9 Nuclease fused with either eGFP or RFP, allowing for fluorescent visualization or sorting by FACS • **Alt-R *S.p.* Cas9 V3, glycerol-free**—IDT's Alt-R *S.p.* Cas9 Nuclease in a buffer formulated without glycerol; suitable for applications that may be sensitive to the presence of glycerol • **D10A and H840A Nickase V3**—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
Transfection reagent	We currently recommend Lipofectamine® RNAiMAX or CRISPRMAX™ reagent (Thermo Fisher Scientific). 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	This T7EI mismatch cleavage assay can be used to identify 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 listed in the Consumables section of this protocol.

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 duplex that is often called the guide RNA (gRNA). The gRNA guides and triggers Cas9 to cleave double-stranded DNA targets, activating the non-homologous end joining (NHEJ) system or generating a site for potential insertion of exogenous donor DNA by homology-directed repair (HDR).

Alt-R CRISPR-Cas9 RNAs and enzymes

The Alt-R CRISPR-Cas9 System improves efficiency of CRISPR genome editing through use of a ribonucleoprotein (RNP), consisting of experimentally optimized Alt-R CRISPR-Cas9 gRNA (crRNA:tracrRNA duplex or sgRNA), as well as Alt-R *S.p.* Cas9 nucleases or nickases. In addition, 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 gRNAs and Cas9 mRNA. For more product information, see the sidebar, Essential Alt-R CRISPR-Cas9 components (on the next page). For information about design, ordering, and experimental set up, see Appendix A and B.

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 confirmed reagents to help optimize and troubleshoot your experiments involving crRNA:tracrRNA duplexes. Kits are available for targeting the HPRT gene in human or mouse cells.

Additional advice for setting up controls can be found on Table B4. IDT application specialists can also help, and providing them with data from experiments using the control kits will allow them to give you the most accurate support. For assistance or information about sgRNA controls, Contact us.

Mutation identification: 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, 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 identified using T7EI, which recognizes and cleaves mismatched DNA heteroduplexes. T7EI assay results are analyzed by visualizing cleavage products and full-length amplicons by gel or capillary electrophoresis. See Part 2: Mutation identification with T7EI in this protocol.

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 Alt-R Genome Editing Detection Kit page (Product data section).

Essential Alt-R CRISPR-Cas9 components

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

Alt-R CRISPR-Cas9 gRNAs with increased nuclease resistance

Based on extensive research, all Alt-R CRISPR-Cas9 gRNAs 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 gRNAs or assess the targeting potential of your own designs. We guarantee that each predesigned Alt-R CRISPR-Cas9 gRNA 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. Additionally, the fluorescent dye allows you to:

- Monitor transfection or electroporation efficiency during optimization.
- 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, 500 µg, or 5 mg for select products
- Concentration: 10 µg/µL
- Shipping conditions: dry ice
- Storage conditions: –20°C
- Heat inactivation: 20 min at 65°C

* See www.idtdna.com/CRISPR-Cas9 for details about the predesigned gRNA guarantee.

For a quick assessment of editing events in CRISPR experiments, we recommend using the T7EI assay instead of alternative methods such as 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.

Important Alt-R CRISPR-Cas9 controls

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

Components*	Amount
Alt-R CRISPR-Cas9 tracrRNA	5 nmol
Alt-R CRISPR-Cas9 HPRT Positive Control crRNA (human or mouse 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 or mouse reference genomes)	2 nmol
Alt-R HPRT PCR Primer Mix (human or mouse, 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).

CONSUMABLES

Consumables—IDT

Item	Catalog #*
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	1072532, 1072533, 1072534
• Alt-R CRISPR-Cas9 tracrRNA—ATTO 550	1075927, 1075928
• Alt-R CRISPR-Cas9 tracrRNA—ATTO 488	10007810
• Alt-R CRISPR-Cas9 tracrRNA—ATTO 647	10007853
Option 2, single guide RNA (sgRNA): Alt-R CRISPR-Cas9 sgRNA	Predesigned and custom sgRNA: www.idtdna.com/CRISPR-Cas9
(Recommended for option 1, 2-part guide RNA) Alt-R CRISPR-Cas9 Control Kit	1072554 [human], 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
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 Genome Editing Detection Kit	1075931, 1075932
(Optional) Alt-R HPRT PCR Primer Mix (2 nmol each)§	1072551 [human] or 1072552 [mouse]
Nuclease-Free Duplex Buffer‡	11-01-03-01
Nuclease-Free IDTE	11-01-02-02
Nuclease-Free Water	11-04-02-01

* 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 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 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 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.

Consumables—Other suppliers

Item	Supplier	Catalog #
Phosphate buffer saline (PBS)	General laboratory supplier	Varies
Option for Cas9 dilution, if not using PBS or Opti-MEM® media:		
HEPES	General laboratory supplier	Varies
KCL	General laboratory supplier	Varies
Trypsin	General laboratory supplier	Varies
Opti-MEM Media	Thermo Fisher Scientific	51985091
Transfection reagent:		
Lipofectamine RNAiMAX Transfection Reagent	Thermo Fisher Scientific	13778100
or		
Lipofectamine CRISPRMAX Transfection Reagent	Thermo Fisher Scientific	CMAX00008
KAPA HiFi HotStart PCR Kit	Kapa Biosystems	KK2501
QuickExtract™ DNA Extraction Solution	Epicentre	QE09050
Digestion product analysis:		
Agarose	General laboratory supplier	Varies
or		
Mutation Discovery Kit[II]	Advanced Analytical Technologies	DNF-910-K1000T

II 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:gRNA ribonucleoprotein (RNP) complex

We recommend including wells for independent transfections of positive and negative controls (see Appendix B: Guidance for Alt-R CRISPR-Cas9 RNA and enzyme usage). 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. Prepare RNA oligos

1. Resuspend each RNA oligo (Alt-R CRISPR-Cas9 crRNA, tracrRNA, sgRNA) 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 and sgRNA are available at 2, 10, 50, and 100 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 in Table B3. The estimated number of reactions that can be completed using this protocol is shown on Table B2, which also indicates various plate sizes and scales of Alt-R CRISPR oligos.

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

2. If using sgRNA, proceed to step 1A6.
3. Mix the crRNA and tracrRNA 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 volumne	**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 or sgRNA to a working concentration (for example, 1 µM) in Nuclease-Free Duplex Buffer or IDTE Buffer.

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

B. Form the RNP complex

1. Prepare Cas9 working buffer (20 mM HEPES; 150 mM KCl, pH 7.5).

   Note: Depending on your cell type, you may use Opti-MEM media or 1X PBS instead of Cas9 working buffer.

   Note: Alt-R *S.p.* Cas9 V3, glycerol-free should not be diluted directly into media, such as Opti-MEM, and should instead be diluted in Cas9 working buffer or in 1X PBS.

2. Before use, thoroughly mix the stock Alt-R *S.p.* Cas9 enzyme by inverting the tube several times, then briefly centrifuge the tube.
3. Dilute Alt-R *S.p.* Cas9 enzyme to a working concentration (for example, 1 µM) in Cas9 working buffer (from step 1B1), OptiMEM, or 1X PBS.

   Note: The molecular weight of Alt-R *S.p.* Cas9 enzyme is 162,200 g/mol. Alt-R *S.p.* Cas9 enzymes are provided at a stock concentration of 62 µM (10 mg/mL). The molecular weight of Cas9-GFP and Cas9-RFP is 189,200 g/mol and these are provided at 52 µM (10 mg/mL). Refer to the Application note [6] for tips on using the nickases.

4. 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)
Guide RNA oligos [1 µM] (step 1A6)*	1.5	1.5
Diluted Cas9 enzyme [1 µM] (step 1B3)	1.5	1.5
Cas9 PLUS™ Reagent (from CRISPRMAX kit)	–	0.6
Opti-MEM Media	22.0	21.4
Total Volume	**25**	**25**

* 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.

5. 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.

C. 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 roomtemperature (20–25°C) for 20 min to form transfection complexes:

Component	Amount (µL)
RNP (step 1B5)	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 (from step 1C1) 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 (from step 1C3) 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 identification 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 steps 2A2 and 2A5 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 briefly centrifuge.

B. Amplify genomic DNA and identify 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 (from **step 2A6**)	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	Number of Cycles	Temperature (°C)	Time
Activation	1	95	5 minutes
Denature	30	98	20 seconds
Anneal		Variable (see next table)	15 seconds
Extend		72	30 seconds
Final extension	1	72	2 minutes

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 products (from **step 2B2**)	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 **step 2C2**)	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 identification 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 Figure 1.

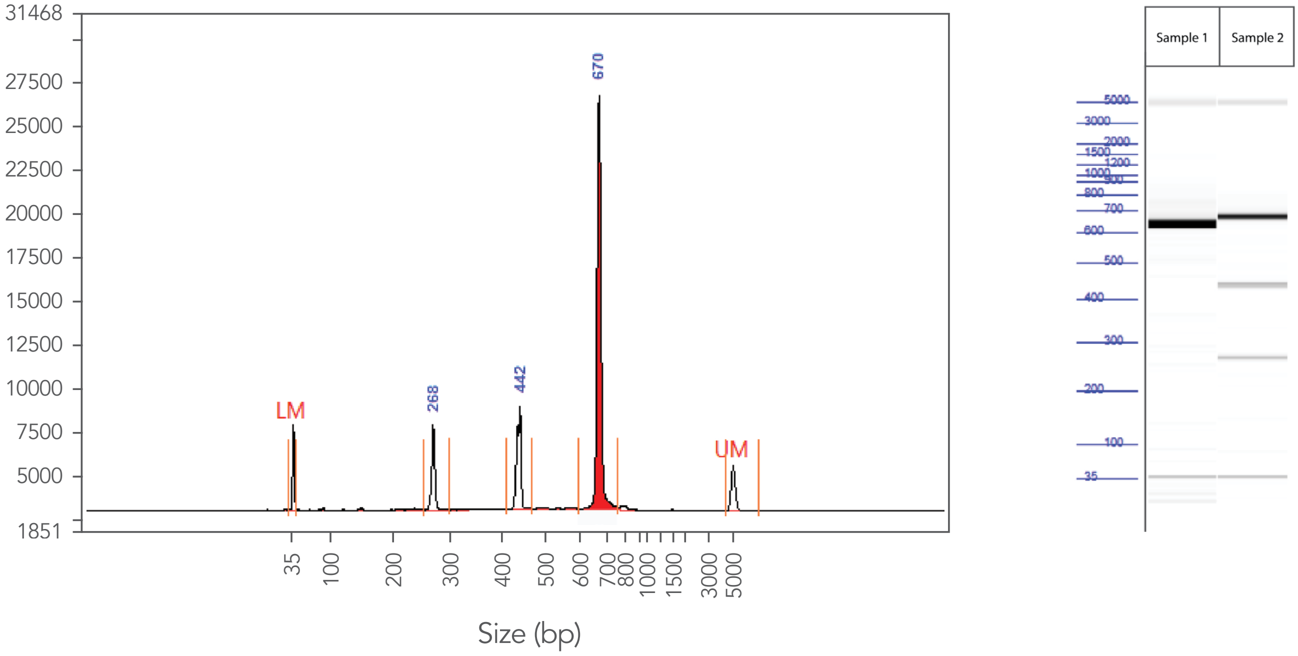

Figure 1. Sample data from Fragment Analyzer system—T7EI digestion containing PCR products from Alt-R Controls A and B. PCR using template and primers in Controls A and B (Alt-R Genome Editing Detection Kit) were run using KAPA HiFi HotStart DNA Polymerase (Kapa Biosystems). Cycling conditions were 5 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.

APPENDIX A: DESIGNING AND ORDERING Alt-R CRISPR-Cas9 gRNAs

Tips for designing

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

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

For other species or to target intergenic regions, 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 with Alt-R gRNA modifications.

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

Positive and negative control crRNAs are available at www.idtdna.com/CRISPR-Cas9. For assistance with designs for sgRNA controls, contact us.

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 A1). 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.

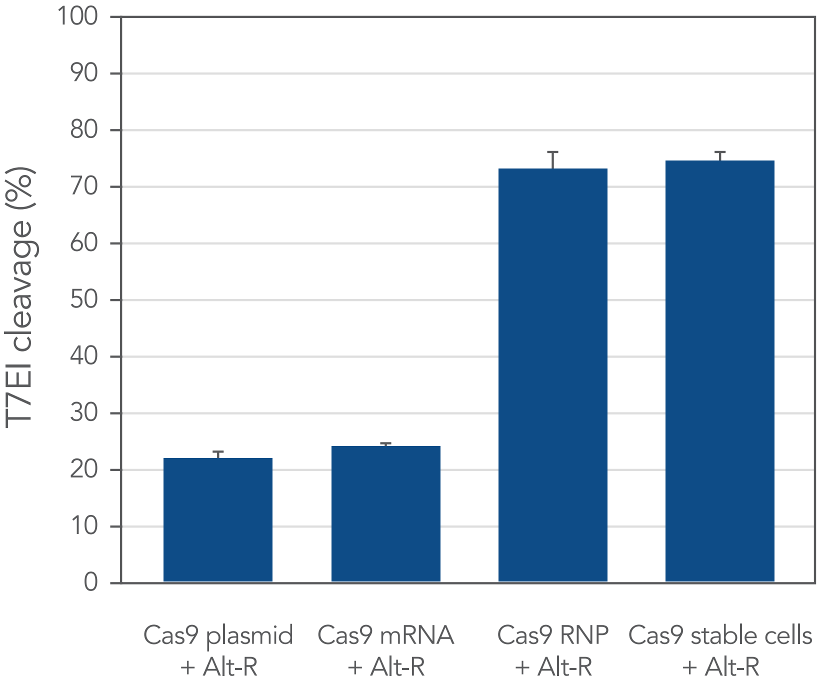

Figure A1. Lipofection of ribonucleoprotein generated using Alt-R CRISPR-Cas9 System components outperforms other transient CRISPR transfection methods. Alt-R CRISPR HPRT Control crRNA:tracrRNA duplexes were co-transfected with Cas9 expression plasmid, Cas9 mRNA, or as part of Cas9 ribonucleoprotein (RNP, with crRNA:tracrRNA duplex incorporated before transfection) into HEK-293 cells (n = 3). 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. 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 Scientific], and the RNP and crRNA:tracrRNA duplexes were delivered using Lipofectamine RNAiMAX Reagent [Thermo Fisher Scientific]). 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: GUIDANCE FOR Alt-R CRISPR-Cas9 RNA AND ENZYME USAGE

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

Tables B1 and B2 are provided to help you determine how much Alt-R CRISPR-Cas9 gRNA (crRNA:tracrRNA duplex or sgRNA) 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 gRNA 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:tracrRNA duplex or sgRNA 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:tracRNA or sgRNA pre-complexed with recombinant Alt-R *S.p.* Cas9 enzymes.

Plate format	crRNA and tracrRNA (pmol each)	sgRNA (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**

* Shown conditions were optimized for transfection using RNAiMAX reagent (Thermo Fisher Scientific) 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, tracrRNA, and sgRNA, when crRNA:tracrRNA duplex or sgRNA are pre-complexed with an Alt-R *S.p.* Cas9 enzyme, before lipofection. The pmol amounts of crRNA, tracrRNA, and sgRNA 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:tracrRNA duplex or sgRNA pre-complexed with recombinant Alt-R *S.p.* Cas9 enzyme by product scale.

Plate format	sgRNA or crRNA		tracrRNA			Cas9 enzyme	
	2 nmol	10 nmol	5 nmol	20 nmol	100 nmol	100 µg	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

* Shown conditions were optimized for transfection using RNAiMAX reagent (Thermo Fisher Scientific) and 10 nM 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 gRNA Controls and tracrRNA

For this protocol, we suggest that you resuspend Alt-R CRISPR-Cas9 crRNA (or sgRNA) 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:gRNA ribonucleoprotein (RNP) complex.

Your unique application may require dilution of the crRNA and tracrRNA (or sgRNA) to other concentrations. IDT offers a 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.

Note: 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 V3 Alt-R *S.p.* HiFi Cas9 Nuclease V3 Alt-R *S.p.* Cas9 D10A Nickase V3 Alt-R *S.p.* Cas9 H840A Nickase V3	162,200	—
Alt-R *S.p.* Cas9-GFP V3 Alt-R *S.p.* Cas9-RFP V3	189,200	—
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 tracrRNA–ATTO 448	22,932.4	699,800
Alt-R CRISPR-Cas9 tracrRNA–ATTO 647	22,989.4	705,900
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 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 and mouse) 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 toconfirm on-target editing. • The expected amplicon and T7EI digested fragment sizes for the human and mouse HPRT controls are below: **Human:** • Full-length (bp): 1083 • Fragment 1 (bp): 827 • Fragment 2 (bp): 256 **Mouse:** • Full-length (bp): 647 • Fragment 1 (bp): 512 • Fragment 2 (bp): 135
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 and mouse) 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.
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 conditions for lipofection of Alt-R 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 other protocols listed under **Resources**, www.idtdna.com/CRISPR-Cas9).

We recommend that you conduct a pilot experiment—preferably, including one of the Alt-R CRISPR-Cas9 HPRT Positive Controls for human or mouse 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 lipofection.** 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 reagent (Thermo Fisher Scientific). 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. **Another consideration for transfection conditions:** The protocol outlined here describes lipofection using a reverse transfection protocol—meaning 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. Still, a few cell types will perform better with forward transfection.

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Alt-R CRISPR-Cas9 System—Cationic lipid delivery of CRISPR ribonucleoprotein complex into mammalian cells

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