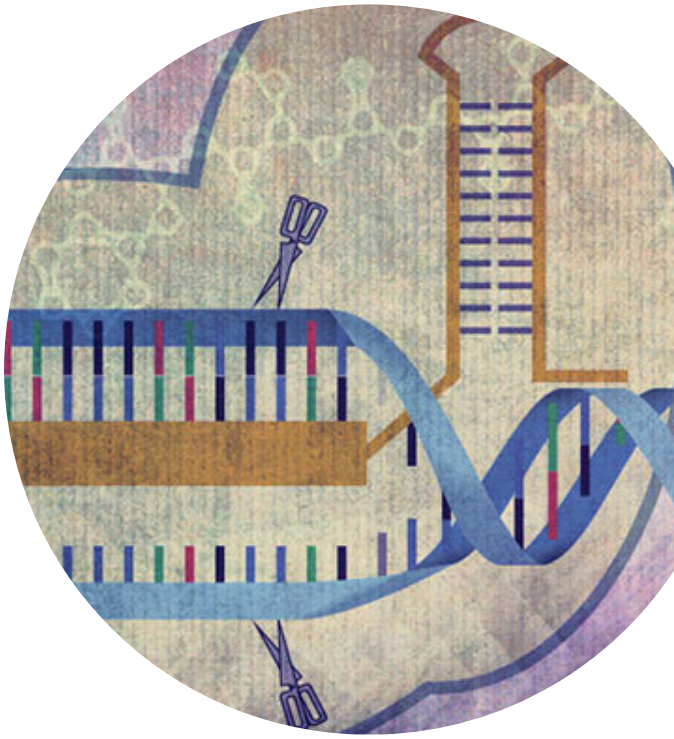
genome editing

user guide

Alt-R™ CRISPR-Cas9 System



For use with:

- Alt-R™ CRISPR crRNA
- Alt-R™ CRISPR tracrRNA
- Alt-R™ S.p. Cas9 Expression Plasmid
- Alt-R™ CRISPR HPRT Positive Controls
- Alt-R™ CRISPR Negative Controls

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Alt-R™ CRISPR-Cas9 System

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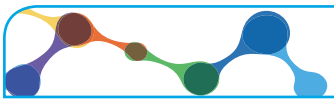


Introduction

The Alt-R™ CRISPR-Cas9 System improves the efficiency of CRISPR (clustered regularly interspaced short palindromic repeats) genome editing performance through use of experimentally optimized Alt-R CRISPR crRNA and Alt-R CRISPR tracrRNA designs. Along with improved efficiency, the system saves time by providing ready-to-use RNA reagents that reduce or eliminate activation of the cellular immune response observed with *in vitro* transcribed RNAs.

Use of the CRISPR-Cas9 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 crRNA and a conserved, transactivating RNA (tracrRNA)—that interact through partial homology to form a crRNA:tracrRNA complex. The crRNA:tracrRNA complex guides and activates Cas9 to cleave double-stranded DNA targets. Double-stranded cleavage of the target DNA by Cas9 then activates the non-homologous end joining (NHEJ) system, or generates a potential insertion site for exogenous donor DNA by homology directed repair (HDR).

It has been shown that the crRNAs and tracrRNAs can also be fused into a chimeric single-guide RNA (sgRNA) [1,2]. This is convenient when expressing the trigger RNA in target cells using a plasmid. However, manufacturing of sgRNAs as high quality RNA molecules by chemical synthesis is difficult and expensive because of their length. Also, sgRNAs obtained by *in vitro* transcription are expensive and can be cytotoxic, activating the innate immune system of many cells. We found it preferable to use a 2-part crRNA:tracrRNA complex to drive Cas9, as those are easily manufactured in high quality as synthetic RNA oligonucleotides and show no such toxicity.

For best editing performance, we recommend our Alt-R CRISPR crRNA, which is an optimized 35–36 nt crRNA that contains a custom 19 or 20 nt protospacer domain and fixed 16 nt tracrRNA interaction domain. This Alt-R CRISPR crRNA should be paired with the 67 nt, Alt-R CRISPR tracrRNA. We found that shortening the crRNA and the tracrRNA significantly increased the potency of the triggers for gene editing compared to the natural 42 nt crRNA and 89 nt tracrRNA (Figure 1).

Alt-R™ CRISPR-Cas9 System

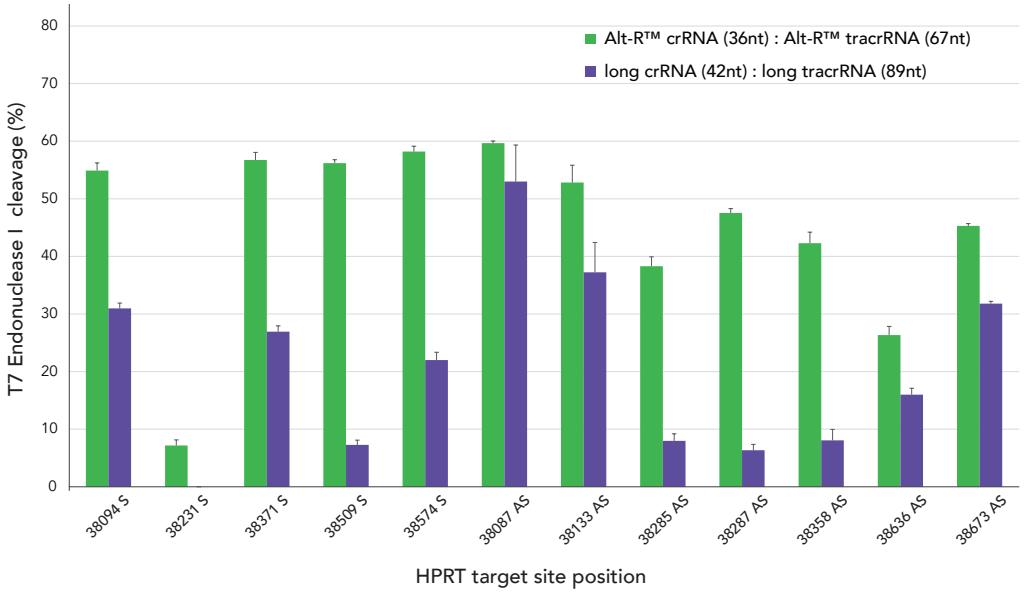


Figure 1. Optimized Alt-R™ CRISPR RNAs improve Cas9 editing efficiency over longer CRISPR RNAs. Alt-R CRISPR crRNA and longer, native crRNAs were designed to recognize 12 sites in the human *HPRT* gene, adjacent to required NGG PAM motifs on both the sense (S) and antisense (AS) strands. Alt-R CRISPR crRNAs were complexed with the optimized Alt-R CRISPR 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 HEK293 cells that constitutively express *S. Pyogenes* Cas9. Editing was measured by PCR amplification of target sites, followed by cleavage with T7EI mismatch endonuclease and by analysis using the Fragment Analyzer™ system (Advanced Analytical).

What you will need

Alt-R CRISPR crRNA	Contains a target-specific 19 or 20 nt protospacer domain and a 16 nt sequence that is complementary to the tracrRNA. The crRNA and tracrRNA must be complexed to form the bimolecular RNA trigger recognized by *S. pyogenes* Cas9. This RNA molecule is chemically modified to provide increased nuclease resistance in cells.
Alt-R CRISPR tracrRNA	Conserved, 67 nt RNA sequence that is required for complexing to the crRNA to form the bimolecular RNA trigger recognized by *S. pyogenes* Cas9. This RNA molecule is chemically modified to provide increased nuclease resistance in cells.
***S. pyogenes* Cas9 endonuclease**	Endonuclease derived from *S. pyogenes* that recognizes and cleaves double-stranded DNA in the presence of target-specific crRNA:tracrRNA complexes.
PAM site information	Protospacer adjacent motif for *S. pyogenes* Cas9 is defined as NGG. Cas9 cuts 3–4 bases upstream of this sequence when directed by the crRNA:tracrRNA complex. The PAM site is adjacent to, but not part of, the crRNA protospacer domain.

Designing/Ordering Alt-R CRISPR products

Designing Alt-R CRISPR crRNAs

There are multiple open source CRISPR design tools available. We found that most of the tools do not consistently predict crRNA potency, and some do not predict potency at all. The crRNA:tracrRNA complex is very effective, and we routinely find that >80% of target sites work well (i.e., >20% by T7EI cleavage as shown in Figure 2, or >40% as indicated by Sanger sequencing). If your goal is complete disruption of a target gene, you may choose to select target sites that are adjacent to PAM sites located towards the 5′ end of your gene (close to the ATG). We recommend that you select and test 2–3 target sites to ensure that at least one will give >20% on-target editing, measured by the T7EI assay.

Our research also suggests that, rather than trying to identify the most potent crRNA, a better design strategy might be to focus on reducing the risk of off-target effects (disruption/modification of unintended genomic sites). The Broad Institute's CRISPR Design tool (**http://crispr.mit.edu/**) selects guide RNAs that are predicted to have minimal off-target effects in the genome; the tool does not provide information about on-target potency.

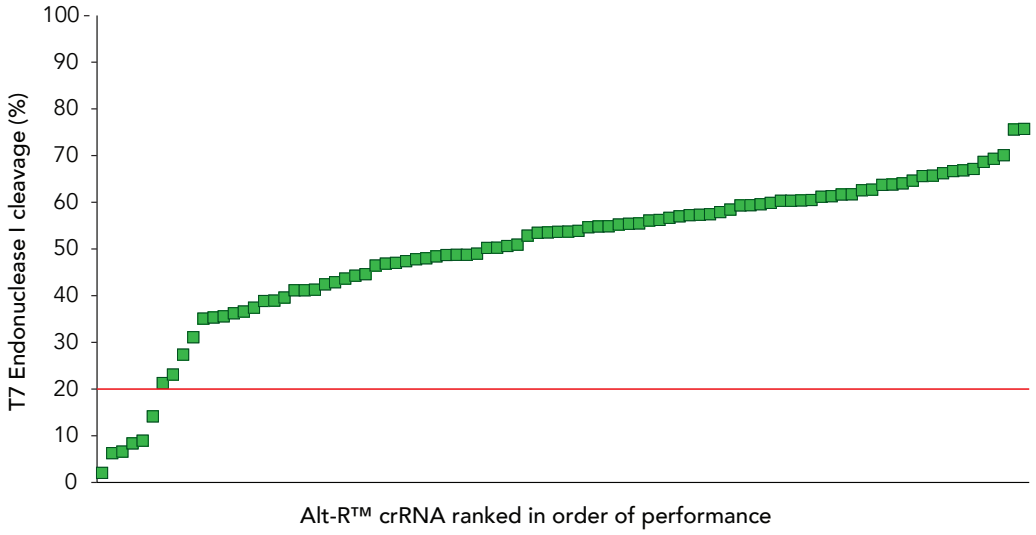


Figure 2. Potent editing efficiency of Alt-R™ CRISPR crRNA designs for 92 PAM sites in the STAT3 gene. 92 protospacer adjacent motifs were identified, and the 20 bases upstream of each site were used to generate Alt-R CRISPR crRNA oligos. The crRNAs were used to modify sites in HEK293-Cas9 stable cell lines, and the results were measured by T7EI cleavage. 93% of the crRNAs showed good to excellent performance with editing efficiency >20%.

Note: T7EI does not detect single base deletions or insertions, and underestimates actual editing efficiency compared to Sanger sequencing.

Alt-R™ CRISPR-Cas9 System

Ordering Alt-R CRISPR crRNAs

Enter the 19 or 20 nt protospacer domain directly into the CRISPR Oligo Entry web page. The crRNA protospacer bases must be entered as the 19–20 bases, upstream of the PAM site, in the forward orientation as shown in Figure 3. The ordering system will automatically append a 16 nt tracr interaction domain and manufacture as synthetic RNA. High quality manufacturing and standard desalting will ensure >80% full length crRNAs as verified by ESI-MS, our standard QC method.

Figure 3. How to enter your crRNA target sequence. Because the crRNA recognizes and binds 20 bases on the DNA strand opposite from the NGG sequence of the PAM site, order your crRNA by entering the 20 bases upstream of the PAM site, in the forward orientation as shown. If you are pasting your CRISPR target site from an online design tool, make sure you verify the correct strand orientation. Do not include the PAM site. Common incorrect design examples are shown in red.

Ordering Alt-R CRISPR tracrRNA

Order Alt-R tracrRNAs from the Alt-R CRISPR web page. Alt-R tracrRNAs are HPLC-purified to ensure >70% full length purity as verified by ESI-MS.

Important: You will need to use equimolar ratios of the Alt-R tracrRNA and your Alt-R crRNA to form the RNA trigger. Nuclease-Free Duplex Buffer is included with your order of Alt-R CRISPR tracrRNA.

Alt-R CRISPR Control Kits

There are multiple variables required for CRISPR genome editing. The Alt-R CRISPR Control Kits will give you what you need to properly analyze your results or to optimize and troubleshoot your experiments, if necessary. IDT application specialists can help you with your experiments, and having data from the control experiments will allow us to give you the most accurate support. For support, email us at **applicationsupport@idtdna.com**.

Validated Alt-R CRISPR Control Kits are available for targeting the *HPRT1* gene in human, mouse, or rat cells.

Kit contents

Components	Amount
Alt-R CRISPR tracrRNA	5 nmol
Alt-R CRISPR HPRT Positive Control crRNA (Human, mouse, or rat, depending on kit)	2 nmol
Alt-R CRISPR 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

Alt-R S.p. Cas9 Expression Plasmid

The best editing performance is obtained using the Alt-R CRISPR RNAs with cells that stably express *S. pyogenes* Cas9. Stably transfected Cas9 cell lines eliminate the experiment-to-experiment variability of transiently transfecting Cas9 into the cells. Unfortunately, this is not always possible or practical.

Alternatively, Cas9 can be introduced into the cells using a Cas9 expression plasmid, Cas9 mRNA, or the Cas9 protein. The crRNA:tracrRNA complexes formed from Alt-R CRISPR RNAs will function with these sources of Cas9.

The Alt-R S.p. Cas9 Expression Plasmid, which is 7.3 kb, allows for easier transfection into mammalian cells compared to larger Cas9 expression plasmids or Cas9/sgRNA dual-expression plasmids. Order the Alt-R S.p. Cas9 Expression Plasmid from the product menu on the Alt-R CRISPR web page.

Important: When using a plasmid to express Cas9, transfect the cells with the plasmid at least 24 hr before introducing the crRNA:tracrRNA. Co-transfection of the plasmid and the crRNA:tracrRNA complex may result in reduced efficiency as some of the RNA complexes may be degraded by endogenous nucleases before the Cas9 protein is expressed.

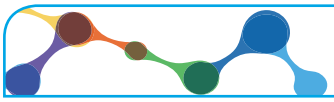
Alt-R™ CRISPR-Cas9 System

Mutation detection with T7EI mismatch endonuclease

This protocol uses T7 endonuclease I (T7EI, New England Biolabs) to determine the success and efficiency of CRISPR gene disruptions. In the T7EI assay, genomic DNA from CRISPR-Cas9–modified cells is amplified by PCR. The PCR products are denatured and reannealed to allow heteroduplex formation between wild-type DNA and CRISPR-Cas9–mutated DNA. Mutations are detected using T7EI, which recognizes and cleaves mismatched DNA, to digest heteroduplexes. The resulting cleaved and full-length PCR products are visualized by gel or capillary electrophoresis.

We currently recommend using T7EI instead of the Surveyor® mismatch endonuclease for CRISPR mutation detection. The T7EI method is simple and provides clean electrophoresis results. T7EI is also compatible with a broader range of PCR buffers and does not usually require purification of the PCR product prior to digestion. Note that T7EI activity is sensitive to the DNA:enzyme ratio, as well as incubation temperature and time [4]. T7EI is able to recognize insertions and deletions of ≥2 bases that are generated by non-homologous end joining (NHEJ) activity in CRISPR experiments [3].

Important: The T7EI enzyme does not detect single-base changes [3]. Therefore, the assay always underestimates editing efficiency as determined by Sanger sequencing.



Protocol

Setting up controls

Setting up control experiments is important for publication and provide useful information if you need to troubleshoot your experiments. It is also good experimental practice to perform technical replicates along with your biological replicates.

For each experiment described in the protocol below, include the following control reactions. Please have data from the control reactions available if you would like troubleshooting assistance from **applicationsupport@idtdna.com**. Also, the expected amplicon and digested product sizes for analyzing the Alt-R CRISPR HPRT Positive Controls using the T7E1 assay are shown in Figure 5 (page 17).

Control reaction in Cas9-expressing cells	Details
HPRT positive control	• Complex the Alt-R CRISPR HPRT Positive Control crRNA (available for human, mouse, and rat) with the Alt-R CRISPR tracrRNA and transfect into cells expressing S. pyogenes Cas9. • Used to show that Cas9 editing is functional in your cells with the positive HPRT control.
Negative control RNA	• Complex the Alt-R™ CRISPR Negative Control (verified negative for human, mouse, and rat) with Alt-R CRISPR tracrRNA and transfect into cells expressing S. pyogenes Cas9. • Used to show that transfection of the RNA complex is not responsible for observed phenotypes. • Note: this does not rule out off-target effects of your experimental crRNA.
No RNA negative control	• No crRNA or tracrRNA is used in this control. • This is primarily a toxicity control for the transfection conditions.

Alt-R™ CRISPR-Cas9 System

Part 1: Delivery of Alt-R S.p. Cas9 Expression Plasmid

In addition to transfection of the Alt-R S.p. Cas9 Expression Plasmid, we recommend performing a separate transfection using a standard fluorescent reporter plasmid (not provided), such as GFP, as a transfection control. The expressed fluorescent protein control can be used to quickly verify that the transfection is successful and to estimate and optimize transfection efficiency for your cells.

Required materials

Kits and reagents	Ordering information*
Alt-R™ S.p. Cas9 Expression Plasmid (1 µg)	IDT (Cat # 1072566)
Nuclease-Free Water	IDT (Cat # 11-04-02-01)
Competent *E. coli*	General laboratory supplier
Opti-MEM® Media	Thermo Fisher (Cat # 51985091)
Plasmid preparation reagents	General laboratory supplier
Trypsin	General laboratory supplier
TransIT-X2® Dynamic Delivery System	Mirus (Cat # MIR 603 or MIR 604)
100 mm tissue culture treated dish	General laboratory supplier

* These are suggested sources for reagents used by the IDT R&D team when this protocol was written. Individual components may be substituted with some optimization. Go to www.idtdna.com for safety data sheets (SDSs) and certificates of analysis (COAs) for IDT products.

A. Propagate the Cas9 expression plasmid in competent *E. coli*

1. Transform competent *E. coli* with the Alt-R S.p. Cas9 Expression Plasmid.

   Note: The Alt-R S.p. Cas9 Expression Plasmid expresses the beta-lactamase gene for ampicillin selection during standard subcloning protocols.

2. Prepare additional Cas9 plasmid from your transformed cells using your method of choice.

B. Transfect cells with the Cas9 expression plasmid

1. Trypsinize and plate sufficient cells in a 100 mm dish to obtain 70–80% confluency after 24 hr incubation.
2. Incubate cells in a tissue culture incubator (37°C, 5% CO_2) for 24 hr.
3. After the 24 hr incubation, prepare the working stock of Alt-R S.p. Cas9 plasmid:
   a. Add 20 µL of Nuclease-Free Water to 20 µg of Alt-R S.p. Cas9 plasmid (from Part 1, Step A2) in a 1.5 mL microcentifuge tube (final concentration of 1 µg/µL).
   b. Mix well and centrifuge to collect the contents at the bottom of the tube.

4. Combine the following and incubate at room temperature (20–25°C) for 20 min to form transfection complexes:

Component	Amount required per transfection
1 µg/µL Alt-R™ S.p. Cas9 Expression Plasmid	15 µL (15 µg)
Opti-MEM® Media	1440 µL
TransIT-X2® Dynamic Delivery System	45 µL
Total volume	**1.5 mL**

5. During incubation of the transfection complexes, replace the media in the plated cells (from Part 1, Step B2) with 15.5 mL of fresh complete media without antibiotics.
6. When incubation is complete, add 1.5 mL of transfection complex (from Part 1, Step B4) to the plate.
7. Remove media after 6 hr and replace with 20 mL of fresh complete media.
8. Incubate the plate containing the transfection complexes and cells in a tissue culture incubator (37°C, 5% CO_2) for 24 hr.

Part 2: Delivery of Alt-R CRISPR RNAs

The Alt-R CRISPR crRNA and tracrRNA are compatible with *S. pyogenes* Cas 9 from any source.

When preparing for delivery of your experimental crRNA:tracrRNA complex, make sure to include wells in your experimental design for separate transfections of appropriate positive and negative controls (described at the start of this protocol, page 9). IDT offers the Alt-R CRISPR HPRT Positive Control and three Alt-R CRISPR Negative Control crRNAs for human, mouse, and rat.

Important: For best results when using any Cas9 expression construct, including the Alt-R S.p. Cas9 Expression Plasmid or mRNA, we recommend that you transfect the Cas9 construct into your cells at least 24 hr before transfecting your crRNA:tracrRNA complexes. Co-transfecting the Cas9 construct and crRNA:tracrRNA complexes may allow cellular RNases to degrade your RNA complexes before active Cas9-editing complexes can form. However, Cas9 protein and crRNA:tracrRNA complexes can be delivered in one experiment as a ribonucleoprotein complex.

Required materials

Kits and reagents	Ordering information*
5 nmol Alt-R™ CRISPR tracrRNA	IDT (Cat # 1072532)
Alt-R CRISPR crRNA Alt-R CRISPR HPRT Positive Control crRNA Alt-R CRISPR Negative Control	IDT custom crRNA www.idtdna.com/CRISPR
IDTE, pH 7.5 (1X TE Solution)	IDT (Cat # 11-01-02-02)
Nuclease-Free Duplex Buffer	IDT (Cat # 11-01-03-01)
Nuclease-Free Water	IDT (Cat # 11-04-02-01)
Lipofectamine® RNAiMAX Transfection Reagent	Thermo Fisher (Cat # 13778100)
Opti-MEM® Media	Thermo Fisher (Cat # 51985091)
Phosphate buffered saline	General laboratory supplier
Trypsin	General laboratory supplier
96-well tissue culture treated plates	General laboratory supplier

* Visit www.idtdna.com for additional sizes of IDT products. These are suggested sources for reagents used by the IDT R&D team when this protocol was written. Individual components may be substituted with some optimization.

A. Form the crRNA:tracrRNA complex

1. Resuspend each RNA oligo (crRNA and tracrRNA) in nuclease-free IDTE, pH 7.5 (1X TE solution).

   We suggest that you use 100 µM final concentrations for the crRNA and tracrRNA oligonucleotides. Store resuspended RNAs at –20°C.

   Alt-R CRISPR HPRT Positive Control crRNAs and Alt-R CRISPR Negative Control crRNAs are delivered normalized to 2 nmol. Custom Alt-R CRISPR crRNAs are available in 2 and 10 nmol scale. The Alt-R CRISPR tracrRNA is available in 5, 20, and 100 nmol scales. Use the volumes in following table to resuspend your dry CRISPR RNAs to 100 µM stock solutions.

   To calculate your own dilutions, you can use molecular weight information provided in Appendix A. The estimated number of reactions that can be completed using this protocol is shown in Appendix B. In addition, Appendix B shows numbers of reactions for other plate sizes, and various scales of Alt-R CRISPR oligos.

Normalized amount delivered (nmol)	IDTE resuspension volume (µL)
2	20
5	50
10	100
20	200
100	1000

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

Component	Amount
100 µM Alt-R™ crRNA	3 µL
100 µM Alt-R tracrRNA	3 µL
Nuclease-Free Duplex Buffer	94 µL
Total volume	**100 µL**

B. Reverse transfect the crRNA:tracrRNA complex in a 96-well plate

1. If necessary, dilute Alt-R CRISPR RNAs to a final concentration of 3 µM with Nuclease-Free Duplex Buffer.
2. Incubate the following at room temperature (20–25°C) for 20 min to form transfection complexes:

Component	Amount
3 µM crRNA:tracrRNA complex	1.5 µL
Lipofectamine® RNAiMAX Transfection Reagent	0.75 µL
Opti-MEM® Media	47.75 µL
Total volume	**50 µL**

3. During incubation (Part 2, Step B2), wash cells expressing Cas9 (from Part 1, plasmid transfection) with PBS and trypsinize. Dilute using complete media without antibiotics to obtain 75% confluency in a 96-well plate.
4. When incubation is complete, add 50 µL of transfection complexes (from Part 2, Step B2) to a 96-well tissue culture plate.
5. Add 100 µL of diluted cells (from Part 2, Step B3) to the 96-well tissue culture plate (final concentration of Alt-R CRISPR RNAs will be 30 nM).
6. Incubate the plate containing the transfection complexes and cells in a tissue culture incubator (37°C, 5% CO_2) for 48 hr.

Alt-R™ CRISPR-Cas9 System

Part 3: Mutation detection with T7EI mismatch endonuclease

We recommend that you generate a 600–1000 bp PCR amplicon with >100 bp flanking the CRISPR cut site. PCR conditions should be carefully optimized, and a single PCR product should be confirmed by electrophoresis. You can design the PCR assays using the PrimerQuest® Tool at **www.idtdna.com/primerquest**.

Required materials

Kits and reagents	Ordering information*
IDTE, pH 7.5 (1X TE Solution)	IDT (Cat # 11-01-02-02)
KAPA HiFi HotStart PCR Kit	Kapa Biosystems (Cat # KK2501)
Nuclease-Free Water	IDT (Cat # 11-04-02-01)
Phosphate buffered saline	General laboratory supplier
QuickExtract™ DNA Extraction Solution	Epicentre (Cat # QE09050)
T7 Endonuclease I	New England Biolabs (Cat # M03025)
Reagents for mutation detection:	
(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 may be substituted with some optimization.

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

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

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 at 65°C for 15 min in a thermal cycler.
5. Vortex and heat at 95°C for 15 min in a thermal cycler.
6. Add 100 µL of Nuclease-Free Water to dilute the genomic DNA.

B. Amplify genomic DNA and detect mutations

Design your PCR assay at **www.idtdna.com/primerquest**. 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. Calculate the T_m of your primers at **www.idtdna.com/oligoanalyzer**.

1. Set up the PCR using template, primers, and components of the KAPA HiFi HotStart PCR Kit as follows:

Component	Amount
Genomic DNA	1.5 µL (~5 ng)
Forward primer	300 nM
Reverse primer	300 nM
KAPA HiFi Fidelity Buffer (5X)	2 µL (1X)
dNTPs	1.2 mM (0.3 mM each)
KAPA HiFi HotStart DNA Polymerase (1U/L)	0.15 U
Total volume	**10 µL**

Optimal annealing temperatures for the Alt-R HPRT PCR Primer Mixes have been determined for this protocol using KAPA HiFi HotStart DNA Polymerase. Use the annealing temperature listed below for your Alt-R HPRT PCR Primer Mix.
You may need to optimize for other polymerases. The expected amplicon and digested product sizes are listed in Figure 5.

Alt-R HPRT PCR Primer Mix	Annealing temperature (°C)
Human	67
Mouse	67
Rat	64

2. Run the PCR using the following cycling conditions:

Step	Temperature (°C)	Time (min:sec)	Cycles
Denature	95	5:00	1
Denature	98	0:20	
Anneal	Template optimized	0:15	30
Extension	72	0:60	
Extension	72	2:00	1

3. Combine the following in an appropriate PCR tube:

Component	Amount
PCR (from Step B2)	10 µL
10X NEBuffer 2*	1.5 µL
Nuclease-Free Water	1.5 µL
Total volume	**13 µL**

* Supplied with T7 Endonuclease I.

4. Heat and cool in a thermal cycler, using the following settings, to form heteroduplexes:

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

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

Component	Amount
PCR heteroduplexes (from Step 4 , this section)	13 µL
1 U/µL T7 Endonuclease I*	2 µL
Total volume	**15 µL**

* Dilute T7 Endonuclease I to a concentration of 1 U/µL in 1X NEBuffer 2.

6. Incubate at 37°C for 60 min.

C. Visualize T7EI mismatch endonuclease 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 (Figure 4, representative results).

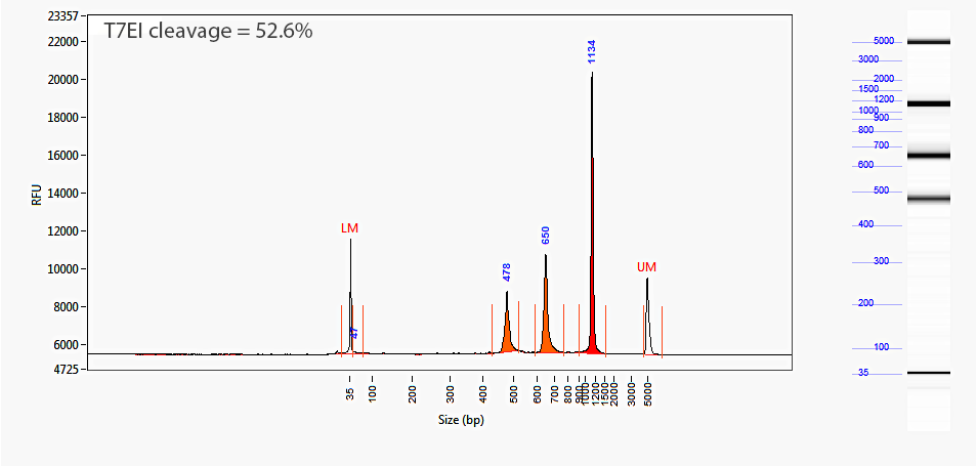
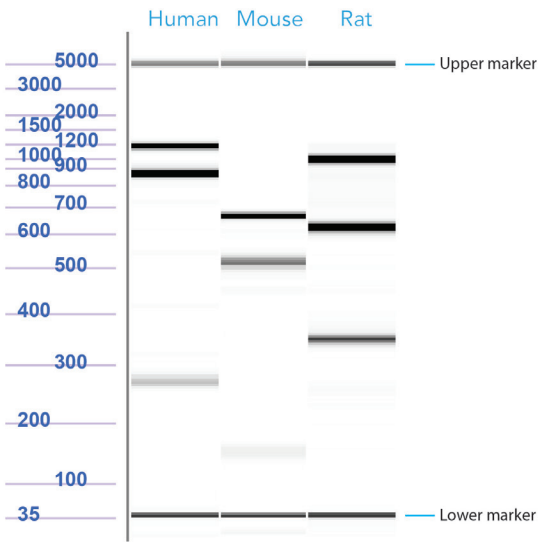


Figure 4. Representative results of a digestion from a Fragment Analyzer™ system. Percent cleavage = Average molar concentration of the cut products / (Average molar concentration of the cut products + Molar concentration of the uncut band) × 100. LM = lower marker; UM = upper marker.



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

Figure 5. T7EI sample data for Alt-R CRISPR HPRT Positive Controls. Genomic DNA from CRISPR-Cas9 edited human (Hs), mouse (Ms), and rat (Rn) HPRT controls were PCR amplified, digested using T7 Endonuclease I, and run on the Fragment Analyzer™. Reference standard bands at 5000 and 35 bp 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.

References

1. Jinek M, Chylinski K, et al. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science, 337(6096):816–821.
2. Makarova KS, Grishin NV, et al. (2006) A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. Biology direct, 1:7.
3. Vouillot L, Thélie A, et al. (2015) Comparison of T7E1 and Surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases G3: GenesIGenomesIGenetics, 5(3):407–415.
4. Mean RJ, Pierides A, et al. (2004) Modification of the enzyme mismatch cleavage method using T7 endonuclease I and silver staining. Biotechniques, 36(5):758–760.

Appendix A

Resuspension of Alt-R™ CRISPR crRNA controls and tracrRNA

For the protocols in this guide, we suggest that you resuspend Alt-R CRISPR crRNA and tracrRNA oligos in IDTE buffer or Nuclease-Free Duplex Buffer to 100 µM stock concentrations, and prepare working concentrations from those stock resuspensions. Additional instructions for the resuspension and dilution sof the crRNA and tracrRNA are provided in *Part 2: Delivery of Alt-R CRISPR RNAs* (page 11).

Your unique application may require dilution of the crRNA and tracrRNA oligonucleotides to different concentrations. Use the free Resuspension Calculator at **www.idtdna.com/scitools** to calculate other concentrations. For calculating dilutions of Alt-R CRISPR crRNA controls and Alt-R CRISPR tracrRNA, the molecular weights are provided in Table A1.

Store resuspended RNAs at –20°C.

Table A1. Molecular weights for Alt-R™ CRISPR crRNA controls and tracrRNA

Alt-R™ CRISPR RNA product	Molecular weight (g/mol)
Alt-R CRISPR Human HPRT Positive Control crRNA	11.854
Alt-R CRISPR Mouse HPRT Positive Control crRNA	11,750
Alt-R CRISPR Rat HPRT Positive Control crRNA	11,892
Alt-R CRISPR Negative Control crRNA #1	11,750
Alt-R CRISPR Negative Control crRNA #2	11,741
Alt-R CRISPR Negative Control crRNA #3	11,836
Alt-R CRISPR tracrRNA	22,182.3

Appendix B

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

The following tables will help you determine how much Alt-R CRISPR crRNA and tracrRNA oligos you need, depending on the number and size of your transfections. These numbers are only estimates to help you plan experiments. The estimates are based on our optimized experimental conditions, and assume no wasted material or instrument variation for pipetting volumes.

Table B1 shows the estimated number of transfection reactions that can be performed with the available scales of Alt-R CRISPR oligos when Cas9 is already expressed in your cells (via stable cell line or previous delivery of Cas9 plasmid or mRNA).

The amounts of the Alt-R CRISPR oligos used to estimate the total number of reactions can be found in Table B2.

Table B1. Estimated Number of transfections of Alt-R™ CRISPR oligos into Cas9-expressing cells by product scale.

Plate format	Number of transfection reactions for cells expressing Cas9*				
	crRNA, 2 nmol	crRNA, 10 nmol	tracrRNA, 5 nmol	tracrRNA, 20 nmol	tracrRNA, 100 nmol
6-well plate	26	130	65	260	1300
12-well plate	54	270	135	540	2700
24-well plate	110	550	275	1100	5500
48-well plate	222	1110	555	2220	11100
96-well plate	444	2220	1110	4440	22200

* Conditions were optimized for transfection using RNAiMAX, using cells with previous delivery of Cas9 plasmid or mRNA or stable expressing cells. Further optimization may be required for alternative transfection reagents.

How much Alt-R CRISPR oligos are used in each transfection?

Table B2 shows the estimated amounts of the major transfection components for transfection of Alt-R CRISPR crRNA and Alt-R tracRNA into Cas9-expressing cells, in various plate formats. The quantities of Alt-R CRIPSR crRNA and tracrRNA required for each well are shown, along with the estimated volumes for cells resuspended in culture media and transfection complexes.

Table B2. Reagent amounts required for transfection of Alt-R™ CRISPR oligos with Cas9-expressing cells, or cells that were transfected with a Cas9 expression plasmid or mRNA at least 24 h previously*.

Plate format	crRNA (pmol)	tracrRNA (pmol)	Transfection complex volume (µL)	Resuspended cell volume (µL†)	Total volume (µL)
6-well plate	72	72	800	1600	2400
12-well plate	36	36	400	800	1200
24-well plate	18	18	200	400	600
48-well plate	9	9	100	200	300
96-well plate	4.5	4.5	50	100	150

* Listed conditions were optimized for transfection using RNAiMAX. Further optimization may be required for alternative transfection reagents.

† Resuspended cell volume for obtaining ~75% confluency at the time of Alt-R CRISPR oligo transfection.

Revision history

Version	Date released	Description of changes
1	October 2015	Original protocol
2	November 2015	Added detail for experimental controls throughout protocol; added appendix A and B relating amount of material supplied to number of transfection reactions.
3.1	April 2016	Updated molecular weights of Alt-R CRISPR Controls. Updated Figure 6 to reflect changes in amplicons and cleavage products for Alt-R HPRT PCR assays.

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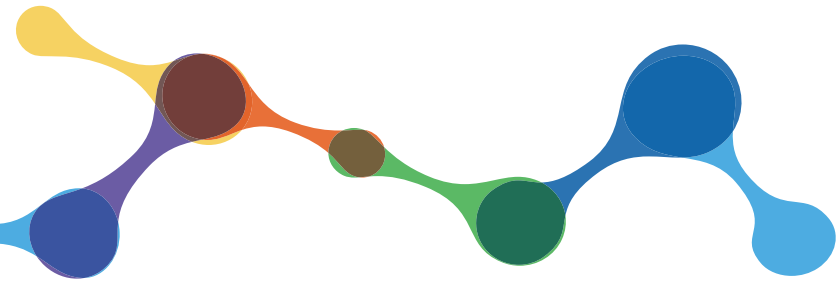

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