Perform gene knockout in your research

With the Alt-R™ CRISPR-Cas system

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OVERVIEW

The quickest way to make a gene knockout is by using a Cas enzyme and a guide RNA (gRNA).

The following list provides the main requirements for CRISPR gene knockout experiments (see also Figure 1):

1. Cas enzyme
2. Guide RNA
3. Your cells
4. Delivery method
    a. Electroporation
    b. Lipofection
    c. Microinjection
5. Positive control guide RNA
6. (Optional) Alt-R Genome Editing Detection Kit or rhAmpSeq™ CRISPR Analysis System

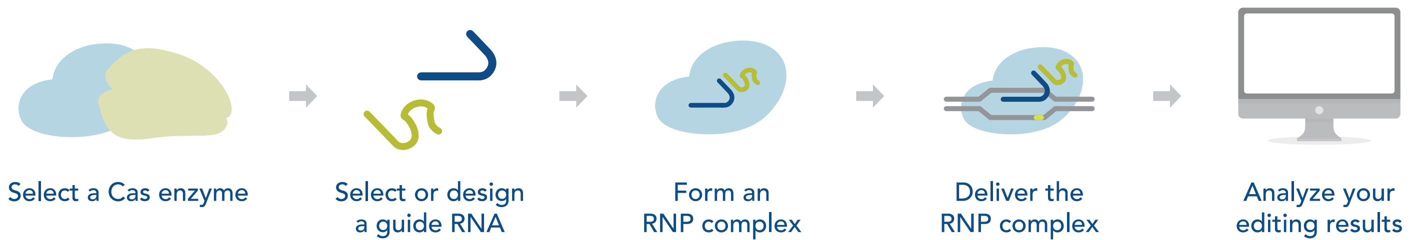


Figure 1. Gene knockout workflow.

PROTOCOL GUIDE

Select a Cas enzyme

The CRISPR-Cas system has two components:

1. **Cas enzyme**—an RNA-guided endonuclease (e.g., Cas9 or Cas12a)
2. **Guide RNA**

Cas enzymes require an RNA molecule, known as the guide RNA, to guide the enzyme to a specific location in the genome, which is referred to as the target DNA. The guide RNA contains an approximately 20-nucleotide sequence called the spacer that is complementary to the target DNA protospacer. Downstream to the complementary sequence in the target DNA is the protospacer-adjacent motif (PAM), which is recognized by the Cas enzyme. Both a guide RNA and a PAM are required for the Cas enzyme to bind to the target DNA and subsequently create a double-strand break (DSB).

Whether you use Cas9 or Cas12a will depend on the PAM sites that are available in the target region of your genome. Cas9 recognizes an NGG PAM, while Cas12a recognizes TTTV (V = A/C/G). Cas9 is better suited for GC-rich regions of the genome, while Cas12a is better for AT-rich regions. See Table 1 for a direct comparison between the two enzymes to help you decide which to use in your application.

When using Cas9

For Cas9, you can pick between the IDT Alt-R *S.p.* Cas9 Nuclease V3 (available with or without glycerol, and in fluorescent-labeled—GFP or RFP—or unlabeled formats), or Alt-R *S.p.* HiFi Cas9 Nuclease V3 as the enzyme for targeting genomic regions with NGG sequences. For most experiments, the Alt-R *S.p.* Cas9 Nuclease V3 sufficiently provides efficient genome editing. If you are concerned about off-target effects, use the HiFi Cas9 enzyme for the most precise editing.

IDT also offers two Cas9 nickases: Alt-R *S.p.* Cas9 D10A Nickase V3 creates a single-strand cut in the targeted strand of DNA, and Alt-R *S.p.* Cas9 H840A Nickase V3 creates a single-strand cut in the non-targeted strand of DNA. To use in genome editing experiments, a single nickase is used with two guide RNAs to create a double-strand break.
To learn how to use nickases, review this DECODED article.

When using Cas12a

Considering Cas12a, you can pick between Alt-R *A.s.* Cas12a (Cpf1) V3, Alt-R (Cpf1) *A.s.* Cas12a *Ultra,* or Alt-R *L.b.* Cas12a *Ultra* as the enzyme for targeting genomic regions with TTTV sequences. Both *A.s.* and *L.b.* Cas12a (Cpf1) *Ultra* enzymes are the result of protein engineering and directed evolution. With these enhancements, these enzymes are as reliable as the Cas9 nuclease. Our *L.b.* Cas12a *Ultra* enzyme is an enhanced mutant that is optimized to improve performance at lower culture temperatures, such as those required for plant cultures.

Table 1. Comparison of CRISPR genome editing using Cas9 vs. Cas12a (Cpf1).

	Cas9 system	Cas12a system
Applications	• General genome editing	• General genome editing when additional flexibility is needed and Cas9 is not a suitable choice • For species with AT-rich genomes
Ribonucleoprotein components	• gRNA options: 1. crRNA and tracrRNA 2. sgRNA • Cas9 endonuclease	• crRNA • Cas12a endonuclease
Alt-R CRISPR enzymes	• Wild-type (with or without glycerol) • GFP- or RFP-labelled wild-type • HiFi	• *A.s.* Cas12a wild-type • *A.s.* Cas12a *Ultra* • *L.b.* Cas12a *Ultra*
Cas9 crRNA:tracrRNA	crRNA • Native: 42 nt • Alt-R: 35–36 nt tracrRNA • Native: 89 nt • Alt-R: 67 nt	—
Cas9 sgRNA	• Alt-R: 99–100 nt	—
Cas12a crRNA	—	• Native: 42–44 nt • Alt-R: 40–44 nt
CRISPR enzyme	• Class 2, Cas type II • M.W.*: 162,200 g/mol (189,200 g/mol for Cas9-GFP, or Cas9-RFP) • Endonuclease domains: RuvC-like and HNH	• Class 2, Cas type V • M.W.*: *A.s.* Cas12a: 156,400 g/mol *L.b.* Cas12a: 148,900 g/mol • Endonuclease domain: RuvC-like only
Double-stranded DNA cleavage	• Wild-type and HiFi: blunt-ended cut 3 bases upstream of the protospacer sequence • D10A nickase with paired crRNAs: 5′ overhang • H840A nickase with paired crRNAs: 3′ overhang • PAM site often destroyed during genome editing	• 5′ overhanging cut on the 5′ side of the protospacer sequence • PAM site may be preserved after genome editing
PAM sequence†	• NGG for wild-type, HiFi, and both nickases	• TTTV for *A.s.* Cas12a, *A.s.* Cas12a *Ultra*, and *L.b.* Cas12a *Ultra*
Current recommendations for Alt-R RNP delivery	• Electroporation with (optional) Alt-R Cas9 Electroporation Enhancer • Microinjection • Lipid-mediated transfection	• Electroporation with Alt-R Cas12a Electroporation Enhancer (recommended) • Microinjection

* Molecular weight of Alt-R nuclease
† N = any base; V = A, C, or G

Select or design a guide RNA

The guide RNA will direct the Cas enzymes to the target site where the enzyme generates a DSB. The break is repaired by a cellular DNA repair mechanism that follows one of two pathways:

1. **Non-homologous end joining** (NHEJ), in which broken ends of the DNA are efficiently joined, but often with insertions or deletions at the breakpoint, which mainly leads to gene knock-out (on rare occasions, indels could occur in-frame, leading to the production of proteins with partial function)
2. **Homology-directed repair** (HDR), where cells use a DNA template, provided along with CRISPR components, to repair the DNA break via homologous recombination

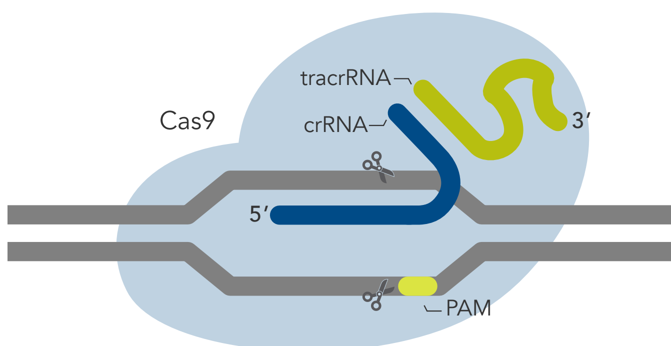
IDT provides predesigned guide RNAs for Cas9 for the genomes of 5 species: human, mouse, rat, zebrafish, and *C. elegans*, with guaranteed performance.

Note: If a predesigned guide RNA for your genome is not available, you can use our design tool to custom design your own guide.

The guide RNA can be ordered as two separate parts (crRNA and tracrRNA) and combined before the experiment, or as a single guide RNA (sgRNA) that has both parts already combined. All types of Alt-R RNAs are chemically modified to prevent immune stimulation and nuclease degradation.

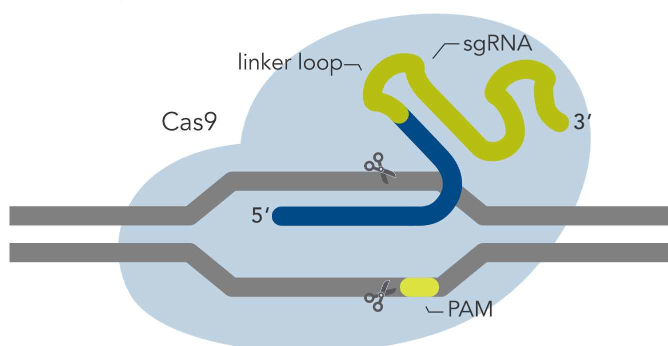
Two-part guide RNA consists of crRNA and tracrRNA. crRNA (dark blue in Figure 2A) provides the sequence specificity through an approximately 20 nt region, known as the spacer, that is complementary to your target DNA. The remaining sequence of the crRNA (16 nt) is complementary to tracrRNA (green in Figure 2A). The tracrRNA is a universal RNA molecule that does not change for each new target sequence but must be bound to the crRNA to create a functional, targeting ribonucleoprotein (RNP) with the enzyme.

A. Alt-R crRNA:tracrRNA



67 nt universal tracrRNA
36 nt site-specific crRNA

B. Alt-R sgRNA



100 nt site-specific sgRNA

Figure 2. Comparison between two-part guide RNA delivery system and a single guide RNA.

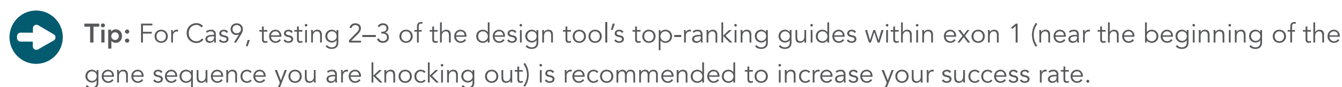
Alternatively, you can use a single guide RNA (sgRNA), a fusion of the crRNA and tracrRNA molecules (Figure 2B).

When deciding between the two-part versus the sgRNA, there are several differences to consider. The Alt-R two-part guide RNA is composed of two shorter RNA molecules, a 35–36 nucleotide crRNA, and a 67 nucleotide universal tracrRNA. Both molecules are chemically modified to reduce immune stimulation and nuclease degradation.

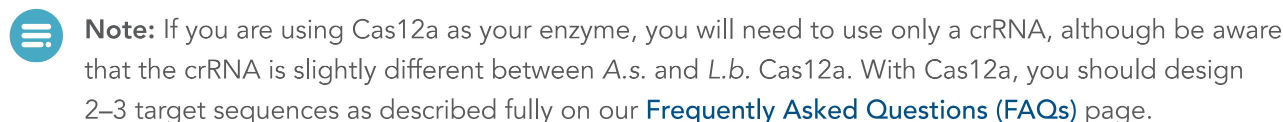
Tip: Shorter RNA molecules cut costs because you only need to order this 35–36 nucleotide site-specific crRNA for every new sequence that you would like to target.

For sgRNA, you have a 100-nucleotide, site-specific sgRNA for every sequence that you want to target. These guide RNAs are chemically modified to prevent immune stimulation and nuclease degradation.

With both two-part and single guide RNA, similarly high editing levels are achieved. For a smaller number of target sites, one or the other type of guide RNA may provide better editing efficiency.



Tip: For Cas9, testing 2–3 of the design tool's top-ranking guides within exon 1 (near the beginning of the gene sequence you are knocking out) is recommended to increase your success rate.



Note: If you are using Cas12a as your enzyme, you will need to use only a crRNA, although be aware that the crRNA is slightly different between *A.s.* and *L.b.* Cas12a. With Cas12a, you should design 2–3 target sequences as described fully on our Frequently Asked Questions (FAQs) page.

Deliver RNP complex

Once you have chosen your CRISPR reagents, you must choose a method of delivery. Transfection efficiency and subsequent cell viability are essential considerations when making your choice between methods.

For the most efficient genome editing, we recommend using an RNP consisting of Cas9 or Cas12a nuclease in complex with guide RNA (crRNA:tracrRNA duplex or sgRNA, or crRNA, respectively). Using this combination provides high editing efficiency across most target sites and addresses issues (e.g., inconsistent Cas enzyme expression levels and incorporation of DNA expression constructs) that can be problematic with other CRISPR-Cas editing methods. For more information about using RNP for CRISPR editing, see this protocol for Cas9 and this protocol for Cas12a.

With the RNP complex formed, deliver the complex to your cells with one of the following methods:

- Electroporation
- Lipofection
- Microinjection

Electroporation

Electroporation is the most commonly used method with a standard CRISPR workflow. IDT also provides an enhancer to increase electroporation editing efficiency. The Alt-R Electroporation Enhancers are specifically manufactured either for Cas9 or Cas12a (Cpf1) to act as a carrier to transport the RNP complex more efficiently into the cells.

Include Alt-R Electroporation Enhancer (Cas9 or Cas12a) with the RNP complex and run your electroporation. See this protocol for a detailed description of the steps involved for Cas9 RNP delivery.

Lipofection

Lipofection can also be used for delivery, especially if you already have a high-efficiency lipofection protocol for delivering molecules into cells. Lipofection is primarily suitable for CRISPR experiments in easy-to-transfect cells, such as some adherent, immortalized eukaryotic cell lines. Refer to this protocol for more details on using lipofection for delivery.

Microinjection

Microinjection is another method of delivering CRISPR components. Usually this method is used for delivery to embryos. In comparison to electroporation and lipofection, it is a more labor-intensive, time-consuming, and costly method that requires highly skilled lab personnel and specialized equipment.

Analyze your editing results

All 3 workflow options result in the delivery of RNP complex into cells. Upon entry, your guide RNA leads the Cas enzyme to the target site where the enzyme cuts the cellular DNA. Once the double-strand break is introduced by the CRISPR system, the genomic DNA repair mechanism may follow either the NHEJ or HDR pathway. Then, the genomic DNA can be isolated from the cells to verify the editing events.

Measuring success with the CRISPR process

Did CRISPR successfully knock out your gene? In some cases, you may know in advance that an obvious phenotype will occur in your cells when you knock out a gene, and that may be all you need to confirm that CRISPR worked. However, in most cases, sequencing is the best way to look for mutations.

How can IDT help?

IDT offers reagents for both traditional Sanger sequencing and for next-generation sequencing (NGS). We also offer the **T7EI assay** (Alt-R Genome Editing Detection Kit), which may help to confirm mutagenesis has occurred. After you have nominated the likely hotspot sites of off-target effects, you can identify off-target editing events using the IDT **rhAmpSeq CRISPR Analysis System** (a proprietary targeted sequencing method of hotspot regions).

Workflow summary

Cas9: Two-part guide RNA workflow

1. Prepare a two-part gRNA complex by annealing the two oligos:
    a. Dilute the crRNA and tracrRNA to the desired concentrations in Nuclease-Free IDTE.
    b. Prepare the gRNA by combining crRNA and tracrRNA at equimolar ratio to the desired concentration, heating the mixture 95°C for 5 minutes, then cooling it to room temperature (15–25°C) on the benchtop.
2. Prepare the RNP complex by mixing gRNA with Cas9 nuclease of choice (see **Select a Cas Enzyme** for available options), then incubate at room temperature for 10–20 minutes.
3. Transfect cells of interest by electroporation, or your delivery method of choice (**Figure 3A**).

Cas9: Single guide RNA workflow

1. Dilute the sgRNA to the desired concentration in Nuclease-Free IDTE.
2. Prepare the RNP complex by mixing sgRNA with Cas9 nuclease of choice (see **Select a Cas Enzyme** for available options), then incubate at room temperature for 10–20 minutes.
3. Transfect cells of interest by electroporation, or your delivery method of choice (**Figure 3B**).

Cas12a workflow

1. Resuspend Alt-R CRISPR-Cas12a crRNA to the desired concentration in Nuclease-Free IDTE.
2. For each well undergoing electroporation, dilute the crRNA and Cas12a nuclease of choice (see **Select a Cas Enzyme** for available options) in PBS, then incubate at room temperature for 10–20 minutes.
3. Transfect cells of interest by electroporation, or your delivery method of choice (**Figure 3C**).

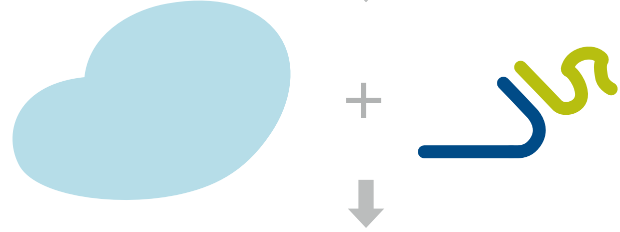
A. RNP delivery with Cas9 nuclease and two-part guide RNA (crRNA + tracrRNA)

Step 1—Anneal to form gRNA

15 minutes

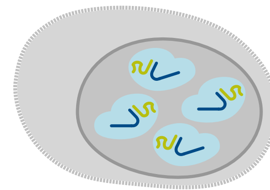
Step 2—Complex gRNA and Cas9 to form RNP

10–20 minutes



Step 3—Deliver RNP

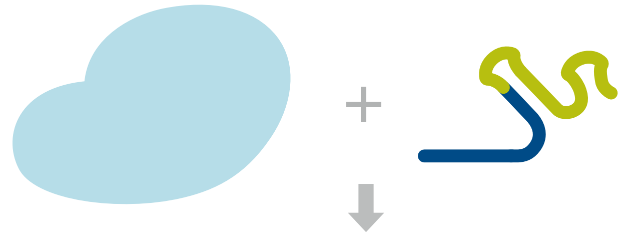
30–60 minutes



B. RNP delivery with Cas9 nuclease and sgRNA

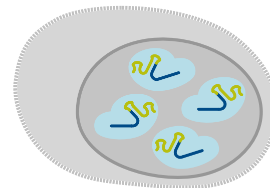
Step 1—Complex sgRNA and Cas9 to form RNP

10–20 minutes



Step 2—Deliver RNP

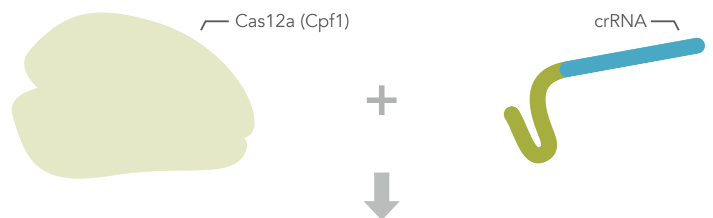
30–60 minutes



C. RNP delivery with Cas12a (Cpf1) nuclease and crRNA

Step 1—Complex crRNA and Cas12a to form RNP

10–20 minutes



Step 2—Deliver RNP

30–60 minutes

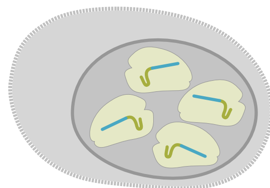


Figure 3. Overview of Alt-R CRISPR-Cas9 and Cas12a system workflow options for RNP complex formation and delivery using your transfection method of choice.

ADDITIONAL RESOURCES

See these additional resources for more information on performing and refining your CRISPR experiments:

- Protocol: Alt-R CRISPR-Cas9 System-RNP transfections
- Protocol: Alt-R CRISPR-Cas9 System-RNP electroporation, Nucleofector system
- Protocol: Alt-R CRISPR-Cas9 System-RNP electroporation, Neon Transfection system
- Protocol: Alt-R CRISPR-Cpf1-RNP electroporation, Nucleofector system
- Protocol: Alt-R CRISPR-Cas12a (Cpf1) System-RNP electroporation, Neon Transfection system
- DECODED article: Do you have the best guide RNA (gRNA) for your CRISPR-Cas9 genome editing?
- IDT's FAQ support web page on CRISPR genome editing

This information and more is available at www.idtdna.com/CRISPR.

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Doc ID: RUO21-0217_002.1 12/22