

Perform gene knockout

With the Alt-R CRISPR-Cas system

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

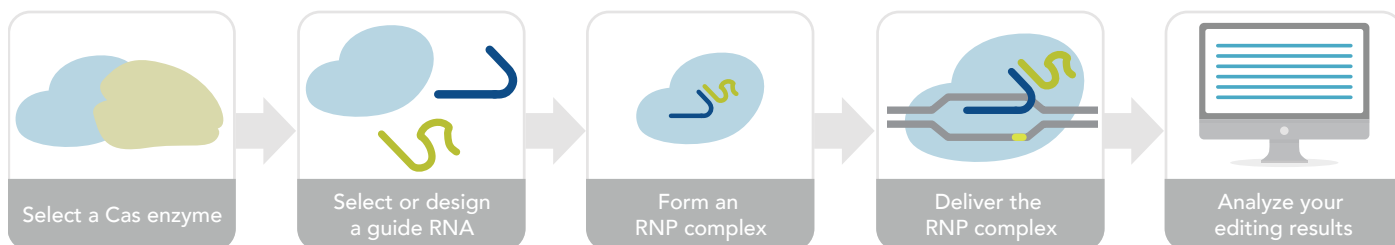


Figure 1. Gene knockout workflow.

PROTOCOL GUIDE

Select a Cas enzyme

The CRISPR-Cas system has 2 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 2 enzymes to help you decide which to use in your application.

When using Cas 9

For Cas9, you can pick between IDT's Alt-R S.p. Cas9 Nuclease V3 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 2 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 2 guide RNAs to create a double-strand break. To learn how to use nickases, review this [DECODED article](#).

When using Cas 12

Considering Cas12, you can pick between IDT's Alt-R A.s. Cas12a (Cpf1) V3 or Alt-R (Cpf1) Cas12a *Ultra* as the enzyme for targeting genomic regions with TTTV sequences. Cas12a (Cpf1) *Ultra* enzyme is the result of protein engineering and directed evolution. With these enhancements, this enzyme is as reliable as the Cas9 nuclease.

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

	Cas9 system	Cas12a system
Applications	General genome editing	<ul style="list-style-type: none"> • General genome editing when additional flexibility is needed and Cas9 is not a suitable choice. • For species with AT-rich genomes
Ribonucleoprotein components	<ul style="list-style-type: none"> • gRNA options: <ol style="list-style-type: none"> 1. crRNA and tracrRNA 2. sgRNA • Cas9 endonuclease 	<ul style="list-style-type: none"> • crRNA • Cas12a endonuclease
Alt-R CRISPR enzymes	<ul style="list-style-type: none"> • Wild-type • HiFi (reduces off-targeting editing) [1] 	<ul style="list-style-type: none"> • Wild-type • <i>Ultra</i> (improves performance)
Cas9 crRNA:tracrRNA	crRNA <ul style="list-style-type: none"> • Native: 42 nt • Alt-R: 35–36 nt tracrRNA <ul style="list-style-type: none"> • Native: 89 nt • Alt-R: 67 nt 	—
Cas9 sgRNA	<ul style="list-style-type: none"> • Alt-R: 99–100 nt 	—
Cas12a crRNA	—	<ul style="list-style-type: none"> • Native: 42–44 nt • Alt-R: 40–44 nt
CRISPR enzyme	<ul style="list-style-type: none"> • Class 2, Cas type II • M.W.*: 162,200 g/mol • Endonuclease domains: RuvC-like and HNH 	<ul style="list-style-type: none"> • Class 2, Cas type V • M.W.*: 156,400 g/mol • Endonuclease domain: RuvC-like only
Double-stranded DNA cleavage	<ul style="list-style-type: none"> • 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 	<ul style="list-style-type: none"> • 5' overhanging cut on the 5' side of the protospacer sequence • PAM site may be preserved after genome editing
PAM sequence[†]	NGG	<ul style="list-style-type: none"> • TTTV for Cas12a V3 and Cas12a <i>Ultra</i>
Current recommendations for Alt-R RNP delivery	<ul style="list-style-type: none"> • Electroporation with (optional) Alt-R Cas9 Electroporation Enhancer • Microinjection • Lipid-mediated transfection 	<ul style="list-style-type: none"> • 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 DBS. The break is repaired by a cellular DNA repair mechanism that follows 1 of 2 pathways:

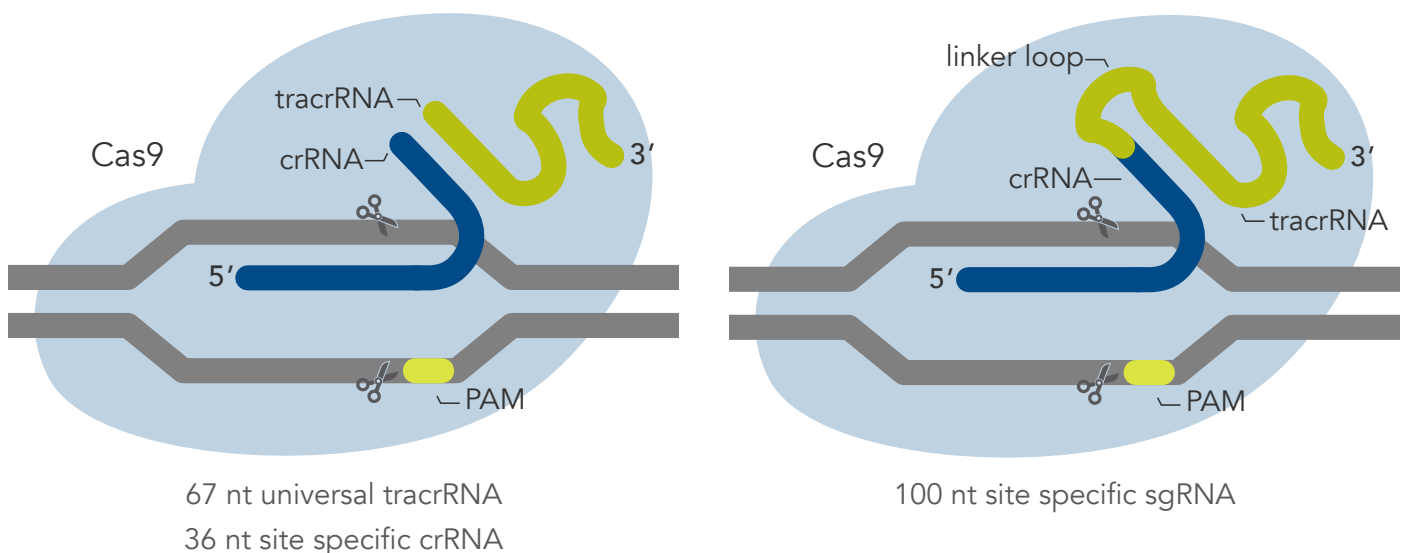
1. Non-homologous end joining (NHEJ), where broken ends of the DNA are efficiently joined, but often with insertions or deletions at the breakpoint, which mainly leads to gene knock-out (on rare occasions, indels could occur in-frame leading to the production of proteins with partial function)
2. Homology-directed 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 IDT's [design tool](#) to custom design your own guide.

The guide RNA can be ordered as 2 separate parts (crRNA and tracrRNA) and combined before the experiment, or as a single guide RNA (sgRNA) that has the 2 parts already combined. All types of Alt-R RNAs are chemically modified to prevent immune stimulation and nuclease degradation.

2-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

B. Alt-R sgRNA

Figure 2. Comparison between 2-part guide RNA delivery system and a 1-part guide.


Alternatively, you can use an sgRNA, a 1-part fusion of the crRNA and tracrRNA molecules ([Figure 2B](#)).


When deciding between the 2-part versus the sgRNA, there are several differences to consider. The Alt-R 2-part guide RNA is composed of 2 shorter RNA molecules, a 35–36 nucleotide crRNA and a 67 nucleotide universal tracrRNA. Both molecules are chemically modified to prevent 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 are chemically modified guide RNAs to prevent immune stimulation and nuclease degradation.

With both 2-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. For an excellent reference on what to keep in mind when picking the location of your target site, see this [DECODED article](#).

 **Note:** If you are using Cas12a as your enzyme, you will need to use only a crRNA. With Cas12a, you should design 2–3 target sequences as described in our [Frequently Asked Questions \(FAQs\)](#) page.

Deliver RNP complex

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

For the most efficient genome editing, we recommend using a RNP consisting of Cas9 or Cas12a nuclease in complex with guide RNA (crRNA:tracrRNA duplex or sgRNA, or crRNA respectively). Using this combination provides very 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 1 of the following methods:

- Electroporation
- Lipofection
- Microinjection

Electroporation

Electroporation is the most commonly used method and the one we recommend for most experiments performing a standard CRISPR workflow. IDT also provides an enhancer to increase electroporation editing efficiency. Alt-R Electroporation Enhancer is specifically designed either for Cas9 or Cas12a (Cpf1) and acts 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

Although electroporation is the preferred delivery method, lipofection can 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 follows the NHEJ or HDR pathway. At this point, the genomic DNA is 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. Though in most cases, sequencing is the best way to look specifically 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 can quickly confirm mutagenesis has occurred, but this assay is not as sensitive or specific as NGS. After you have nominated the likely hotspot sites of off-target effects, you can then verify off-target editing events using the IDT [rhAmpSeq amplicon sequencing system](#) (a proprietary targeted sequencing method of hotspot regions).

Workflow summary

Cas9: 2-part guide RNA workflow

1. Prepare a two-part gRNA complex by annealing the 2 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 Alt-R S.p. Cas9 or HiFi Cas9 Nuclease V3, 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 Alt-R S.p. Cas9 or HiFi Cas9 Nuclease V3, 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-Cpf1 crRNA to the desired concentration in Nuclease-Free IDTE.
2. For each well undergoing electroporation, dilute the crRNA and Cas 12a protein 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**).

Step 1—Anneal to form gRNA

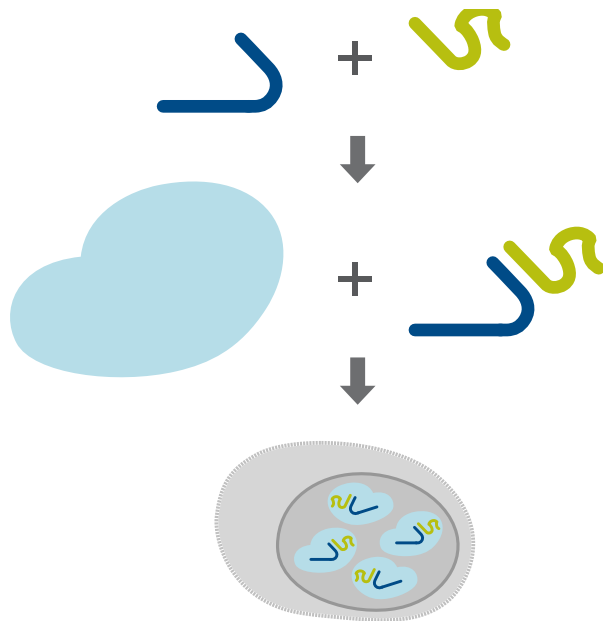
15 minutes

Step 2—Complex gRNA and Cas9 to form RNP

10–20 minutes

Step 3—Deliver RNP

30–60 minutes



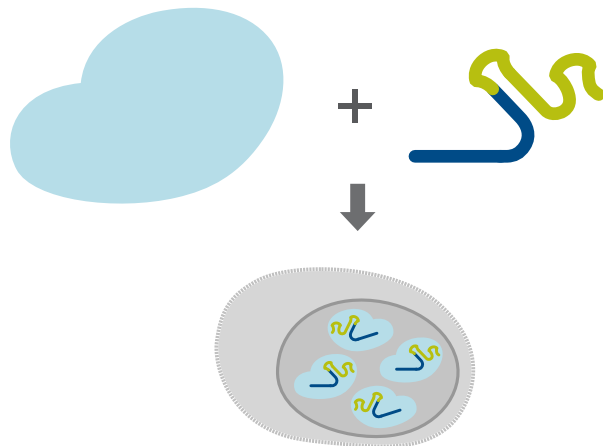
A. RNP delivery with Cas9 nuclease and two-part guide RNA (crRNA + tracrRNA)

Step 1—Complex sgRNA and Cas9 to form RNP

10–20 minutes

Step 2—Deliver RNP

30–60 minutes



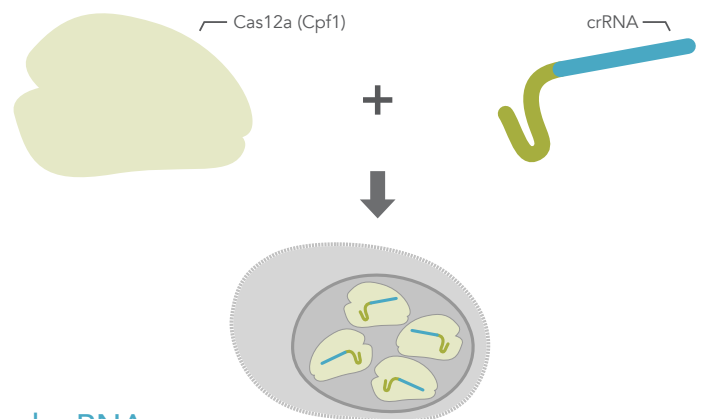
B. RNP delivery with Cas9 nuclease and sgRNA

Step 1—Complex crRNA and Cas12a to form RNP

10–20 minutes

Step 2—Deliver RNP

30–60 minutes



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

Figure 3. Overview of Alt-R CRISPR-Cas9 and Cas 12a system experiments for RNP delivery in the various ways (transfection, electroporation, or microinjection).

REFERENCES

1. Vakulskas C, Dever D, et al. (2018) **A novel high-fidelity Cas9 delivered as a ribonucleoprotein complex enables high frequency gene editing in human haematopoietic stem and progenitor cells.** Nat Med 24(8):1216–1224. DOI 10.1038/s41591-018-0137-0.

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-Cpf1-RNP electroporation, Neon Transfection system](#)
- DECODED article: [Do you have the best guide RNA \(gRNA\) for your CRISPR-Cas9 genome editing?](#)
- Analysis guidelines: [Evaluate CRISPR DNA editing with rhAmpSeq sequencing data](#)
- [IDT's FAQ support web page](#) on CRISPR genome editing

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

Technical support: applicationsupport@idtdna.com

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