How to perform gene knock-in by homology-directed repair in your research

With the Alt-R™ CRISPR-Cas System

OVERVIEW

A quick way to make a precise genome edit is by using a Cas enzyme, a guide RNA (gRNA), and a donor DNA template.

The following list provides the main requirements for CRISPR gene knock-in HDR experiments (see also **Figure 1**):

1. Cas enzyme
2. Guide RNA
3. HDR donor template
4. Cells
5. Delivery method (choose 1)
   a. Electroporation
   b. Lipofection
   c. Microinjection
6. Positive control guide RNA
7. (Optional) Alt-R Genome Editing Detection Kit

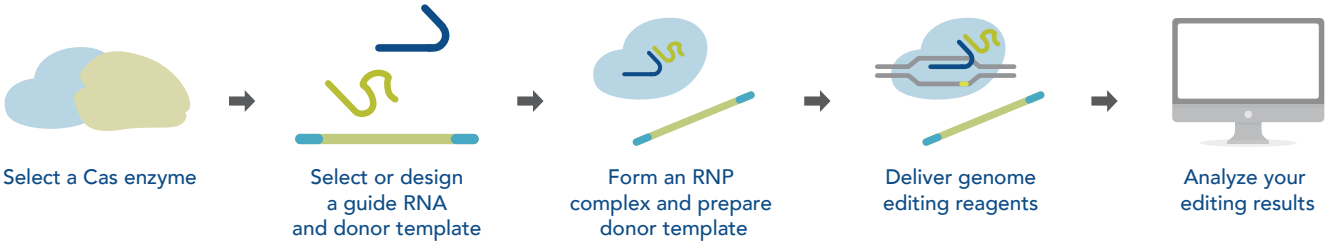


Figure 1. CRISPR HDR workflow.

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 use 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 the genome you want to study. Cas9 recognizes an NGG PAM, while Cas12a recognizes TTTV (V = A/C/G) as its PAM. 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 research application.

When using Cas9

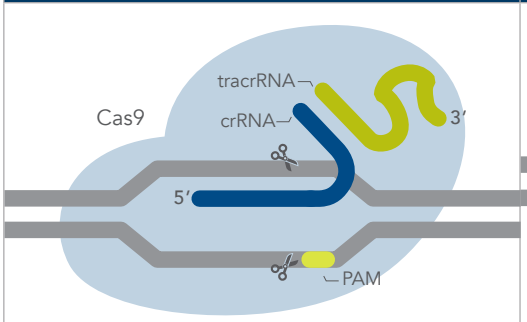
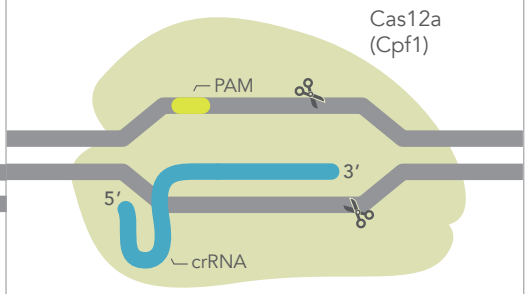
For Cas9, you can pick between the IDT Alt-R *S.p.* Cas9 Nuclease V3 (available with or without glycerol, labeled (GFP or RFP) or unlabeled formats), or Alt-R *S.p.* HiFi Cas9 Nuclease V3 as the enzyme for targeting genomic regions with NGG sequences. For most experiments, the Alt-R *S.p.* Cas9 Nuclease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

IDT also offers two Cas9 nickases: Alt-R *S.p.* Cas9 D10A Nickase V3 and Alt-R *S.p.* Cas9 H840A Nickase V3. The D10A nickase creates a single-strand cut in the targeted strand of DNA, and the H840A nickase creates a single-strand cut in the non-targeted strand of DNA. To use in genome editing experiments, a single nickase type is used with two guide RNAs to create a staggered double-strand break. While both nickases can be used for generating knockouts, the D10A nickase is recommended for HDR applications. To learn how to use nickases, review our DECODED article, "When and how to use nickases for efficient gene editing."

When using Cas12a (Cpf1)

Considering Cas12a, you can pick between the IDT Alt-R *A.s.* Cas12a (Cpf1) V3, Alt-R *A.s.* Cas12a (Cpf1) *Ultra*, or Alt-R *L.b.* Cas12a (Cpf1) *Ultra* as the enzyme for targeting genomic regions with TTTV sequences. Both Cas12a *Ultra* enzymes are the result of protein engineering and directed evolution. The *A.s.* and *L.b.* Cas12a *Ultra* enzymes are separate mutants, made by separate directed evolution efforts; the mutations in the two enzymes are not the same. Our *L.b.* Cas12a *Ultra* enzyme is an enhanced mutant that improves editing efficiency 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 (available with or without glycerol) • HiFi (reduces off-target editing) • GFP or RFP-labeled wild-type • D10A, H840A nickases	• *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.* of Cas9: 162,200 g/mol • M.W.* of Cas9-GFP or Cas9-RFP: 189,200 g/mol • Endonuclease domains: RuvC-like and HNH	• Class 2, Cas type V • M.W.* of *A.s.* Cas12a: 156,400 g/mol • M.W.* of *L.b.* Cas12a: 148,900 g/mol • Endonuclease domain: RuvC-like only
Double-stranded DNA cleavage	• Wild-type and HiFi: blunt-ended cut three 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	• 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 1 of 2 main 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* for guaranteed editing.

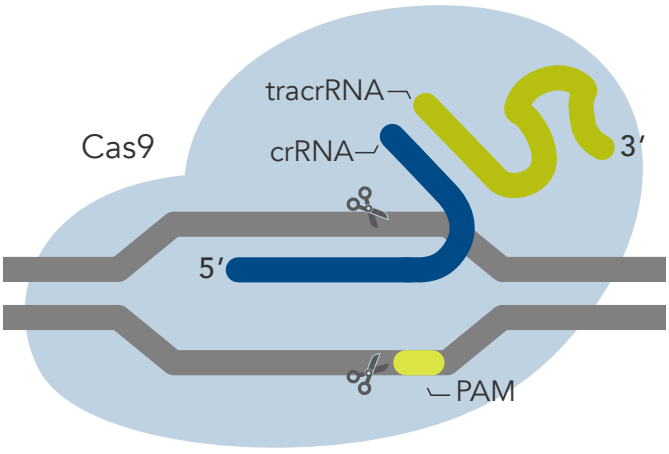
Notes:

- If a predesigned guide RNA for the genome you want to look at is not available, you can use the IDT Design Tool to custom-design your own guide RNA.
- To learn more about the repair pathways, check out this FAQ.

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.

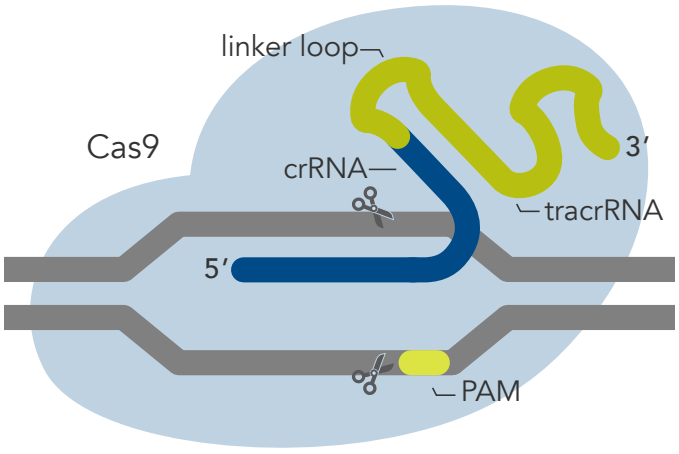
2-part guide RNA consists of crRNA and tracrRNA. crRNA (dark blue in Figure 2A) provides the sequence specificity through an approximately 20 nt region, known as the spacer, that is complementary to the target DNA. The remaining sequence of the crRNA (16 nt) is complementary to tracrRNA (green in Figure 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 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 aid in prevention of 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.

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 CRISPR genome editing DECODED article.

When selecting a gRNA for HDR experiments there are several considerations. It is imperative that the selected guide has high editing efficiency to enable high frequency of HDR. In addition, you may want to select a guide with low potential off-target cleavage to avoid undesired off-target effects. The choice of which nuclease to use should be dependent on where the relative genomic locations of the desired mutation reside in relation to the available CRISPR-Cas guides. For Cas9 nuclease, the guide should cut as close as possible to the desired HDR mutation. For Cas12a, the optimal insertion occurs at positions 12–16 of the guide sequence. When using the Cas9 D10A nickase, the HDR mutation should be placed between the two nick sites.

Design a donor template

IDT offers 2 formats for donor templates:

1. Alt-R HDR Donor Oligos—chemically modified single-stranded DNA up to 200 bases
2. Alt-R HDR Donor Blocks—chemically modified double-stranded DNA from 201 to 3000 base pairs long

To enable homology-based recombination, the HDR repair mechanism requires that the donor DNA contains regions of homology to both sides of the double-strand break. This donor DNA harboring overlaps of sufficient lengths must be delivered simultaneously with the Cas ribonucleoprotein (RNP) complex (formed by the Cas endonuclease and the targeting RNA system). We recommend 30–60 nt lengths for homology arms for short single-stranded oligo deoxynucleotide (ssODN) donors (e.g., Alt-R HDR Donor Oligos; <200 nt total length). For longer dsDNA HDR donor templates (e.g., Alt-R HDR Donor Blocks; 201–3000 bp total length), we recommend 100–300 bp for homology arms.

To enable design of optimal gRNA and HDR donors, IDT has developed the Alt-R HDR Design Tool. This tool provides a customized donor template design and Cas9 guide RNA selection. The higher HDR rates result from clear design rules based on extensive wet bench testing and customer experimentation. Simply provide basic information about your desired target site, then use the HDR Design Tool to design and visualize your desired edit within the sequence. The HDR Design Tool provides the recommended gRNA(s) and HDR donor template to your desired specifications, and is a gateway to order your custom designs.

The addition of phosphorothioate bonds (PS) on each end of an ssODN template increases HDR efficiency over unmodified ssODN templates. These modifications (see Figure 3) are beneficial in some circumstances. We have further improved HDR efficiency by developing Alt-R HDR Donor Oligos, which include two PS linkages and an IDT proprietary end-blocking modification at each end to provide increased stability and HDR rates.

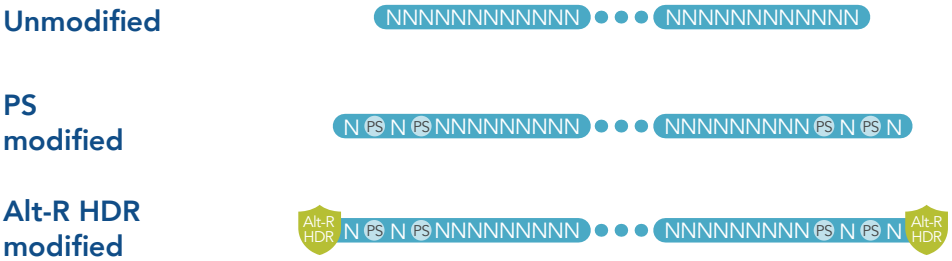


Figure 3. Diagram representing donor oligo modification formats.

Use of unmodified linear dsDNA templates can lead to unintended edits through non-homologous blunt integration at any CRISPR-Cas9 or endogenous double-strand break (DSB). To address the need for fast, cost-effective solutions for large HDR knock-ins, IDT has developed Alt-R HDR Donor Blocks. These modified linear dsDNA HDR templates add universal terminal sequences to the ends of the donor that contain a proprietary modification pattern. These modifications are designed to increase HDR rates and reduce the occurrence of non-homologous integration at on- or off-target DSBs, while the universal terminal sequences allow for consistent synthesis with a faster turnaround time and are not incorporated into HDR edits.

The introduction of silent blocking mutations into the protospacer sequence or mutating the PAM site itself when an intact CRISPR recognition site is present on the HDR template prevents the HDR template from reconstituting the gRNA recognition site, avoiding unwanted re-cutting at the same locus after the repair. The Alt-R HDR Design Tool allows for silent mutation incorporation using empirically defined rules to provide improved HDR rates.

Deliver genome editing reagents

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 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 reliable 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 the 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. Alt-R Electroporation Enhancer is specifically designed 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 electroporation.

See our protocol for a detailed description of the steps involved for Cas9 RNP delivery with an Alt-R HDR Donor Oligo or Block.

We generally do not recommend use of the Alt-R Cas9 Electroporation Enhancer when using Alt-R HDR Donor Blocks. The DNA template is typically sufficient to act as a carrier for improved Cas9 delivery, while the addition of Electroporation Enhancer may increase cytotoxicity. However, ideal delivery conditions may vary for individual cell lines, so some optimization may be required—particularly with cells that are difficult to transfect.

Lipofection

Lipofection can be used for delivery, especially if you already have an efficient 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.

Alt-R HDR Enhancer V2

For HDR experiments, we recommend using the Alt-R HDR Enhancer V2, which is a small molecule compound that can increase the rate of HDR. While the efficiency of HDR and relative improvement in HDR rates varies by cell line, editing site, and the desired insert, we have guidelines for how to improve HDR potential and limit cytotoxicity often associated with the delivery of HDR Enhancer V2 and genome editing reagents into cells.

Guidelines when using HDR Enhancer V2

- Use a maximum of 1% by volume DMSO in the final media.
- Use a control sample with DMSO, but no HDR Enhancer V2, in the final media to monitor toxicity.
- Use a concentration of 0.5–2 µM of HDR Enhancer V2 in the final media.
- Change to growth media without HDR Enhancer V2 12–24 hours after electroporation.

Important! The optimal concentration for Alt-R HDR Enhancer V2 will be cell-type dependent and may require a dose titration. Toxicity should be monitored closely when used at concentrations higher than 2 µM.

Analyze your editing results

All three workflow options result in the delivery of RNP complex and HDR donor template 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.

Positive controls

To ensure your experimental success, IDT recommends the use of controls. IDT offers controls that consist of both a guide RNA (available as the two-part crRNA:tracrRNA or the single guide sgRNA) and a single-stranded HDR donor template, which are intended to serve as positive controls in your HDR experiments. See this **document** for more information about using CRISPR-Cas9 HDR positive controls.

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™ CRISPR Analysis System** that enables singleplex or multiplex analysis of CRISPR edits by next generation sequencing (NGS) using IDT proprietary **rhAmp PCR technology**.

Workflow summary

Cas9: 2-part guide RNA workflow

1. Prepare a 2-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 at 95°C for 5 minutes, then cooling it to room temperature (15–25°C) on the benchtop.
2. Dilute the donor template to the desired concentrations in Nuclease-Free IDTE.
3. 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.
4. Transfect cells of interest by electroporation, or your delivery method of choice (Figure 4).

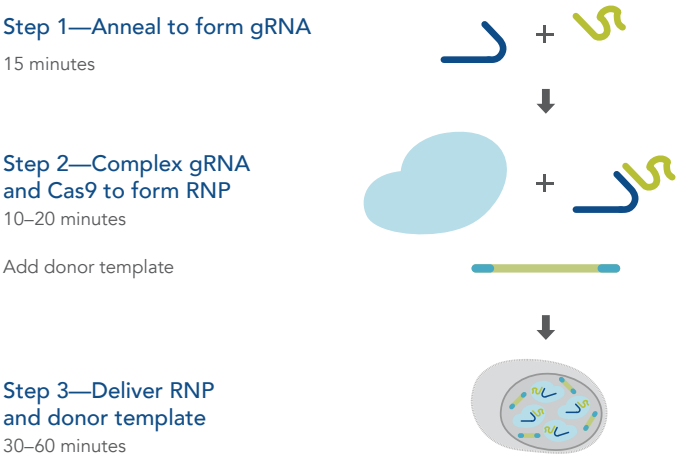


Figure 4. RNP delivery with Cas9 nuclease and 2-part guide RNA.

Cas9: Single guide RNA workflow

1. Dilute the sgRNA to the desired concentration in Nuclease-Free IDTE.
2. Dilute the donor template to the desired concentration in Nuclease-Free IDTE.
3. 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.
4. Transfect cells of interest by electroporation, or your delivery method of choice (Figure 5).

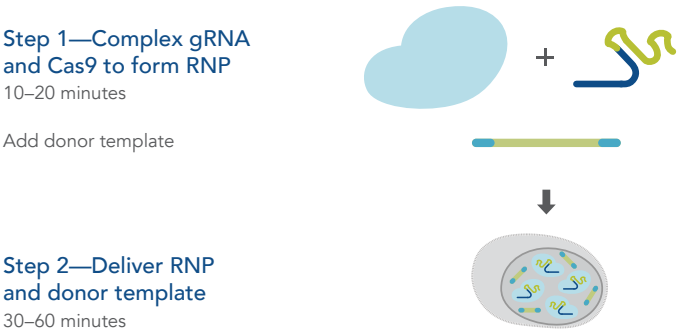


Figure 5. RNP delivery with Cas9 nuclease and sgRNA.

CRISPR-Cas12a workflow

1. Resuspend Alt-R CRISPR-Cpf1 crRNA to the desired concentration in Nuclease-Free IDTE.
2. Dilute the donor template to the desired concentration in Nuclease-Free IDTE.
3. Prepare the RNP complex by mixing gRNA with Cas12a nuclease of choice (see **Select a Cas enzyme** for available options), then incubate at room temperature for 10–20 minutes.
4. Transfect cells of interest by electroporation, or your delivery method of choice (Figure 6).

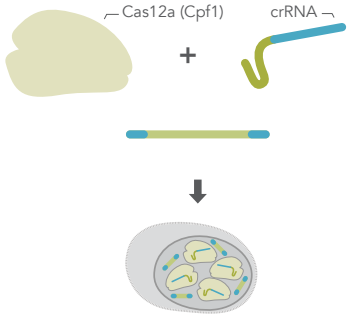


Figure 6. RNP delivery with Cas12a nuclease and crRNA.

Additional resources

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

- Video: Getting started with CRISPR: a review of gene knockout and homology-directed repair.
- 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
- Protocol: Homology-directed repair using the Alt-R CRISPR-Cas9 System and Alt-R HDR Donor Blocks
- Protocol: Homology-directed repair using the Alt-R CRISPR-Cas9 System and HDR Donor Oligos
- 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.

Perform gene knock-in by homology-directed repair

Technical support: applicationsupport@idtdna.com