The CRISPR basics handbook

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ABBREVIATIONS

Abbreviation	Definition
A.s.	*Acidaminococcus* species
Cas	CRISPR-associated (enzyme)
Cpf1	Another name for Cas12a. Technically, Cpf1 stands for "CRISPR from *Prevotella* and *Francisella 1*."
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPRa	CRISPR-mediated gene activation
CRISPRi	CRISPR interference
crRNA	CRISPR RNA
dCas9	Dead Cas9 (catalytically dead Cas9)
DSB	Double-strand break (double-strand DNA break)
dsDNA	Double-stranded DNA
FACS	Fluorescence-activated cell sorting
FASTA	A file format for DNA and protein sequences (it stands for Fast Alignment and Search Tool-All)
gRNA	Guide RNA
GUIDE-seq	Genome-wide unbiased identification of DSBs evaluated by sequencing
HDR	Homology-directed repair
IFN	Interferon
IVT	In vitro transcription
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
NLS	Nuclear localization sequence
PAM	Protospacer-adjacent motif
PCR	Polymerase chain reaction
RNAi	RNA interference
RNP	Ribonucleoprotein
S.p.	*Streptococcus pyogenes*
sgRNA	Single guide RNA
ssDNA	Single-stranded DNA
ssODN	Single-stranded oligodeoxynucleotide
T7EI	T7 endonuclease I
TALEN	Transcription activator-like effector nuclease
tracrRNA	Transactivating CRISPR RNA
ZFN	Zinc finger nuclease

INTRODUCTION TO GENOME EDITING

Genome editing is the use of various technologies to make permanent changes in the genomic DNA sequence of a cell or organism. Early methods that use zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs) are expensive, slow, and difficult in comparison to newer techniques based on CRISPR, which stands for **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats. CRISPR genome editing is remarkably reliable and flexible for many applications. This system, which offers scaling far beyond the capacities of ZFN- and TALEN-based methods, has proven to be amenable to efficient site-directed genome editing in a wide variety of biological systems.

A BRIEF HISTORY OF CRISPR

CRISPR genome editing is based on a natural immune process used by bacteria to defend themselves against invading viruses. CRISPR sequences, which were first identified in 1987 as short genomic DNA sequences in *E. coli* [1], are now known to be part of an adaptive defense mechanism that also involves Cas (CRISPR-associated) enzymes.

It wasn't until about 2003 that CRISPR functions were first associated with microbial cellular immunity [2]. CRISPR systems can recognize and cleave complementary DNA sequences, allowing bacteria to remember and destroy viral invaders. In 2013, researchers demonstrated that they could adapt CRISPR/Cas-dependent genome editing for use in mammalian cells [2]. Nowadays, PubMed lists thousands of CRISPR-related publications, many of which show its adaptability across a wide range of species and applications. The past few years have also seen tremendous advances in improving CRISPR's specificity, orthogonality, and multiplexibility.

Cas enzymes are part of a bacterial immune system that incorporates short, viral DNA sequences into the bacterial genome. This is a complicated process that is not entirely understood [3]. What is well characterized is that these viral sequences are found at regular intervals, short distances from one another in the bacterial genome. The bacterial DNA in between these sequences has palindromic repeating patterns, hence the name, clustered regularly interspaced short palindromic repeats. The incorporated viral DNA sequences can be transcribed into guide RNA (gRNA) when needed—that is, if the same kind of virus tries to infect the bacterium again, the CRISPR system can cut the invading viral DNA through use of the gRNA and Cas enzyme. This last step of the bacterial immune process, when the gRNA is combined with Cas and cleaves the target DNA, is what has been adopted for genome editing in laboratories. With CRISPR technology at our fingertips, we have entered a golden age of genome engineering.

HOW DOES CRISPR WORK?

At the most basic level, CRISPR genome editing requires 2 components: a Cas enzyme and a guide RNA. These components associate to form a ribonucleoprotein (RNP) complex. Then, the guide RNA can base pair with a protospacer (i.e., the target genomic sequence of about 20 bases in length), which is adjacent to a genomic sequence called a protospacer-adjacent motif (PAM). The PAM is only a few bases long, and its sequence depends on the Cas enzyme used. Once the RNP binds to the target DNA (protospacer), the Cas enzyme makes a precise cut in the target. Again, the type of cut—blunt-ended or staggered double-strand break (DSB)—depends on the Cas enzyme used.

Definitions: location of protospacer, spacer, and PAM sequences

Protospacer and spacer

Terminology can be confusing! In the context of most laboratory genome editing, the **protospacer is DNA**: the target DNA sequence. The **spacer is RNA**: the section of the guide RNA which base pairs with the target.

Confoundingly, "protospacer" and "spacer" are sometimes used in other ways. For example, in contexts of some bacterial immune systems, both of these terms can sometimes refer to strands of DNA or RNA.

Protospacer-adjacent motif

Within a genomic DNA sequence, the PAM site is directly adjacent to the ~20 base pairs of the protospacer (target). This is why it is named "protospacer-adjacent motif." But again, terminology can be confusing. Remember that DNA has 2 strands. Your PAM is actually adjacent to the 20 bases across from the "targeted" strand of the DNA (Figure 1).

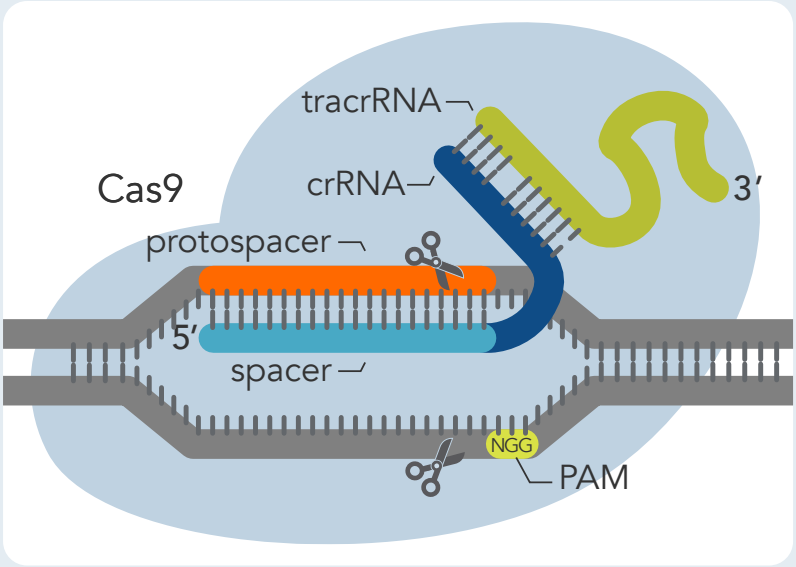


Figure 1. Spacer, protospacer, and PAM. The protospacer is the target DNA; the PAM is adjacent to the targeted double-stranded DNA (dsDNA). The spacer is the section of the guide RNA that anneals to the protospacer.

Tip: Are you thinking about experimental cells and wondering where RNP formation takes place?

RNP formation may occur either inside or outside the cells to be edited, depending on the experimental setup. It is possible to deliver plasmids or viruses to allow cellular machinery to synthesize Cas enzymes and gRNA, allowing RNP formation to occur inside cells. However, this is not optimal. Instead, we recommend that you form the RNP outside the cells and then deliver it into the cells (Figure 2). Once the RNP is inside the cell, it can base pair with the target sequence. Techniques for RNP formation and delivery will be discussed later in this handbook.

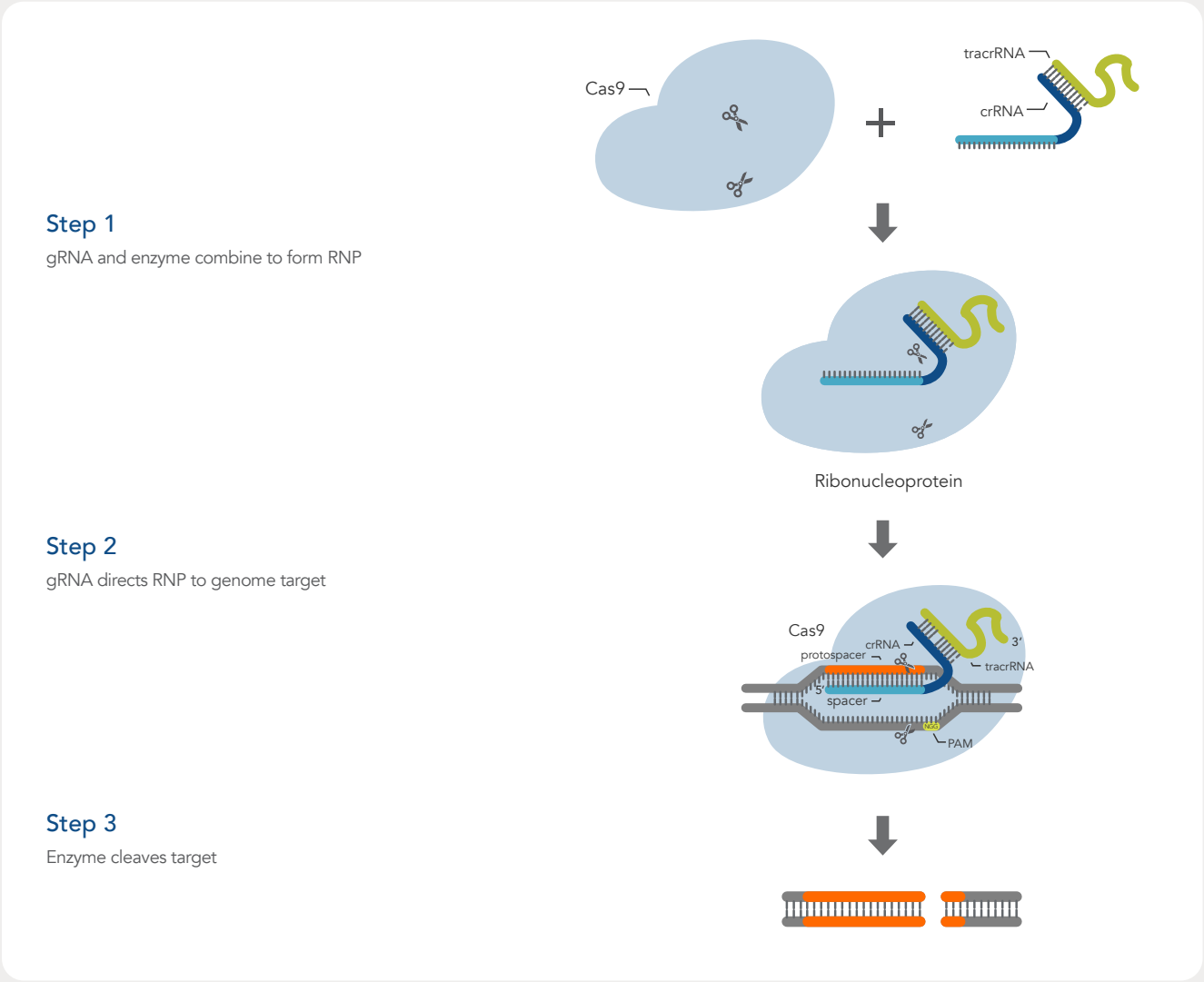


Figure 2. CRISPR-Cas9 gene editing. Forming of RNP (step 1) can occur either inside of cells or on the bench. First, CRISPR RNA (crRNA) and transactivating CRISPR RNA (tracrRNA) anneal together, forming a complete guide RNA. Next, gRNA binds the Cas enzyme, forming ribonucleoprotein (RNP). If RNP formation is performed on the lab bench, RNP must then be delivered to the cells. In step 2, the gRNA directs RNP to the target inside the cells. Then, the target is cleaved (step 3).

There are many Cas enzymes found in bacteria, but this guide focuses on Cas9 and Cas12a, which are part of the 2 Cas systems most commonly used for genome editing. The Cas9 enzyme is an endonuclease, which is often derived from *S. pyogenes*. The Cas12a enzyme, also known as Cpf1, is often derived from *Acidaminococcus* or *Lachnospiraceae* for genome editing purposes.

Either Cas9 or Cas12a can be used to cleave target DNA, resulting in a DSB. Each Cas enzyme is directed by the gRNA to a user-specified cut site in the genome. By providing the Cas protein with an RNA guide, you can program the nuclease to cut at virtually any location in any model organism's genome. There are good reasons to choose between Cas9 and Cas12a in any specific experiment. Making this choice will be discussed later in this handbook.

Natural DNA repair mechanisms can sometimes introduce mutations

Cells will try to repair DSBs, because unrepaired breaks can lead to cell death due to loss of genome stability. However, repair of the DSB doesn't necessarily mean perfect repair. If perfect repair took place every time, the most common application of CRISPR genome editing—gene knockout—would not work in laboratory settings. In reality, there can be insertions and deletions (indels) that introduce stop codons and gene knockouts, or there can even be a new sequence inserted that comes from a donor DNA introduced by the researchers (Figure 3).

Non-homologous end joining (NHEJ)

Non-homologous end-joining is the best known and probably the most common repair mechanism that makes changes (mutations) in the DNA [4]. In addition, other pathways, such as alternative end-joining [5], single-strand annealing [6], and other cellular mechanisms, can also result in DNA repair with mutations. As the name NHEJ implies, the NHEJ pathway joins DSB ends without the need for a homologous template. During the processes of ligating the cut ends of the DNA, NHEJ can often introduce small indels at the break point (Figure 3). Often an indel causes a frameshift mutation, which can disrupt a gene's expression and function, which is very useful when you want to knock out a target gene.

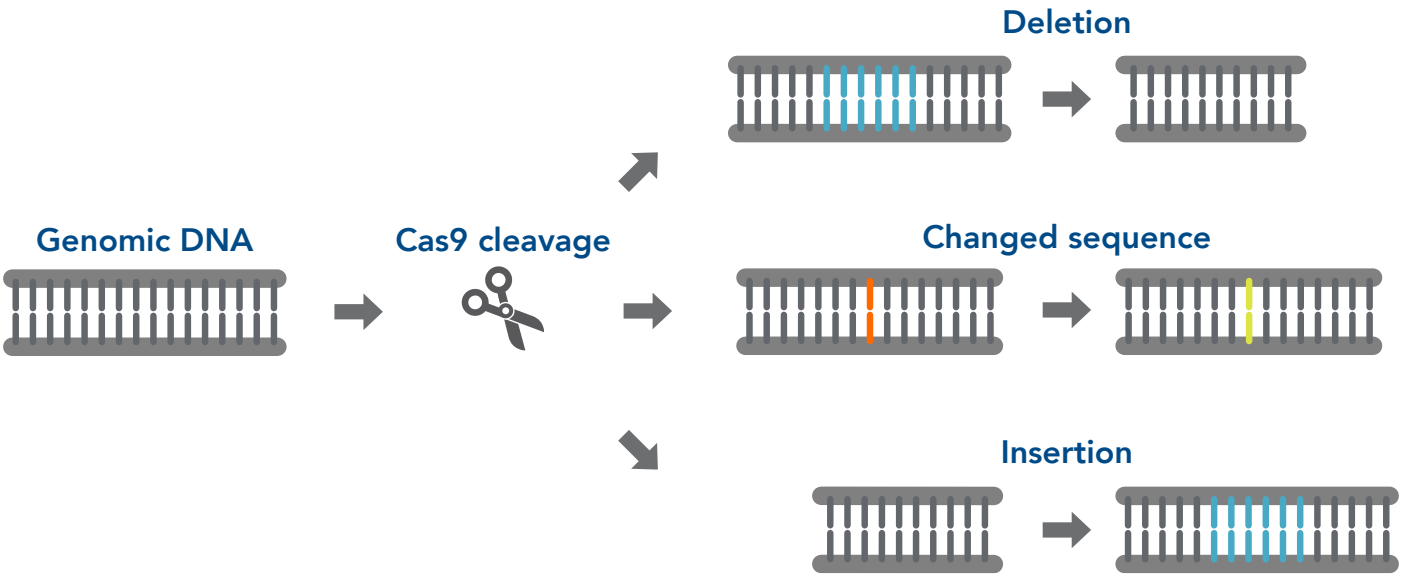


Figure 3. Possible outcomes after genomic DNA cleavage. Cellular DNA repair pathways such as NHEJ can lead to deletions, changed sequences, and small insertions.

Homology-directed repair (HDR)

In addition to mutagenic pathways for DSB repair, cells may use HDR, a non-mutagenic mechanism. HDR is a broad category that includes many distinct pathways leading to recombination [7–9]. Most of the HDR pathways lead to perfect copying of a donor strand into the genomic sequence being repaired. HDR seals a DSB in an error-free fashion, but not necessarily with the original sequence. Gene knock-in may be desired by researchers aiming to understand gene function or model disease-causing mutations. Generally speaking, knock-in experiments involve HDR for effectively and precisely introducing an exogenous DNA fragment.

HDR relies on a DNA template (donor) with sections homologous to the DNA that will be repaired or changed. When there is a DSB due to CRISPR cutting of DNA, a donor must have ends that are homologous with the DNA near the ends of the DSB. The new sequence between the 2 homologous ends will be incorporated into the genome (Figure 4).

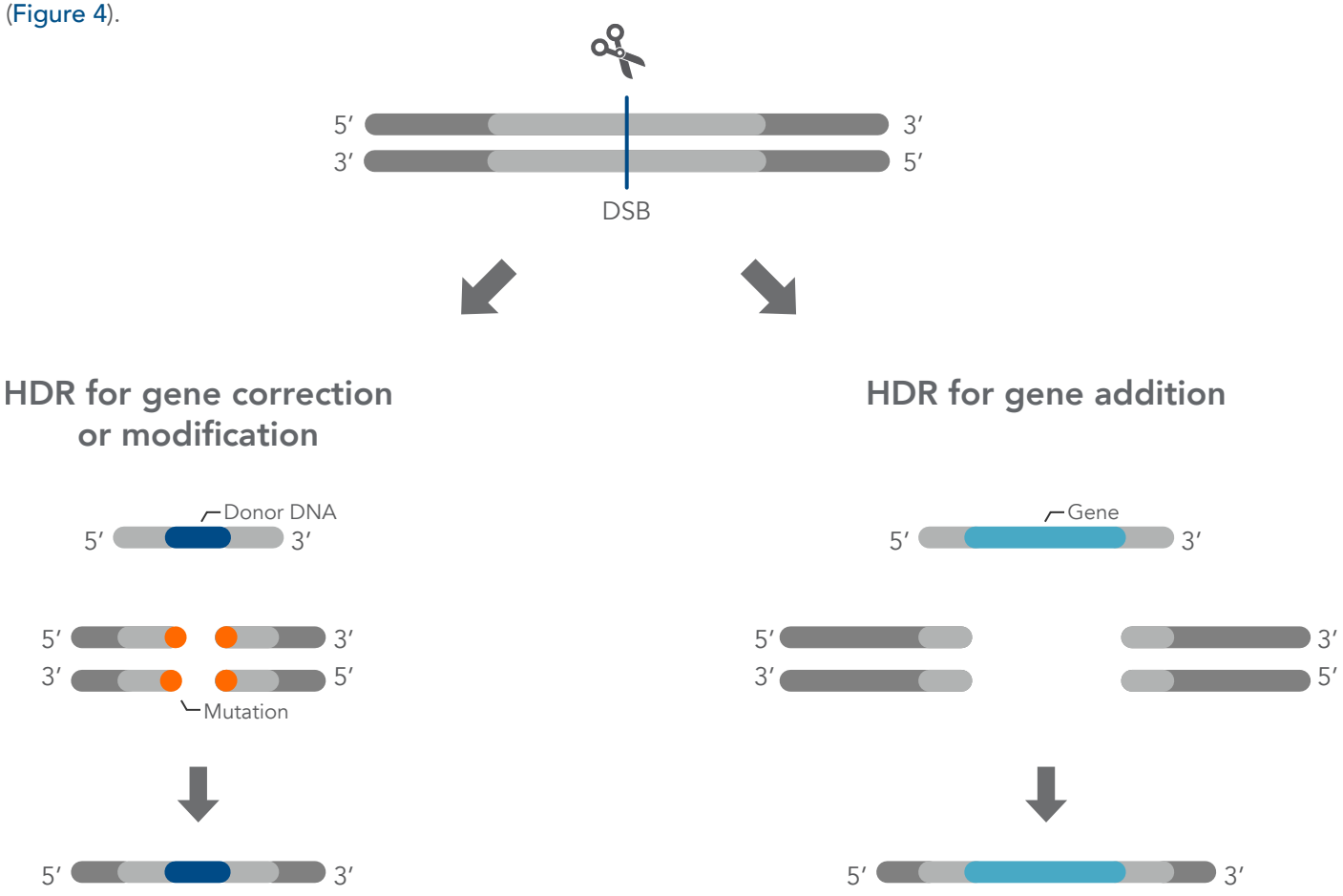


Figure 4. HDR can incorporate new sequences into genomic DNA. Small sequences can be changed for purposes of gene correction or modification (left), and large sequences such as whole genes can be added coincidentally (right).

Tip: More about rates of NHEJ vs. HDR in CRISPR genome editing

The HDR donor template must be delivered along with the guide RNA:Cas nuclease ribonucleoprotein complex. The reason for supplying a donor DNA sequence is to introduce new genetic material (a new DNA sequence) through HDR in the vicinity of the DSB. (If scientists do not supply a donor DNA strand, NHEJ will be the predominant or only mechanism of DSB repair.) However, even when a donor DNA strand is supplied, the error-free HDR pathway is inefficient compared to NHEJ.

Seamless insertion of foreign genetic material via HDR has long been a challenge. The rates of HDR can be compared experimentally with the rates of the mutagenic DSB repair mechanisms such as NHEJ [10]. HDR rates vary widely between cell types but usually are much lower than the rates of NHEJ. IDT has developed several reagents and protocols that greatly enhance error-free HDR rates, as described later in this handbook.

Fun fact about HDR

HDR is primarily a method of repairing DSBs in DNA. Interestingly, HDR can also occur to a minimal extent even when there is no DSB, mostly occurring in the S and G2 phases of the cell cycle [9].

CRISPR APPLICATIONS BEYOND SIMPLE EDITING

Screening

CRISPR screening is a scientific experiment designed to find the equivalent of a few needles in a haystack. That is, CRISPR screening identifies a small number of genes (out of the whole genome) involved in a specific physiological effect. Most CRISPR screening is done in cell culture, although some methods have been devised for use in animal models. In CRISPR screening, scientists usually knock out every gene in the genome that could be important, knocking out only one gene per cell. During this process, some cells die, but others survive and become the predominant cell types. Then, the scientists do next generation sequencing (NGS) on the surviving cells to find out which sequences are still present. RNA interference (RNAi) is sometimes used to screen the genome for genes of interest but is limited by its low efficiency and specificity. CRISPR screens circumvent these limitations by targeting almost every gene in any genome systematically to determine the roles genes play in specific physiological effects. For more information, see this DECODED article.

CRISPR without DSBs!

Even though the major, novel finding of CRISPR was that it was a quick and easy way to create a DSB at almost any desired genomic location, scientists rapidly discovered very useful ways to use CRISPR without making a DSB at all. Gene silencing and gene activation are major applications, but other non-editing functions are important, too.

Gene silencing

Almost immediately after the discovery of CRISPR genome editing, some researchers produced a mutated Cas9 that could not cut DNA. This catalytically inactive enzyme, dCas9 (dead Cas9), could still be targeted to a specific genomic site. Interestingly, simply by binding to a target site, dCas9 was able to inactivate gene transcription at that site by preventing binding of the cellular transcription machinery to the gene [11]. Without transcription, the relevant protein is not produced, and the gene is effectively silenced until the cell naturally eliminates the dCas9 enzyme. This approach, whereby CRISPR is used to temporarily silence a gene without cutting the DNA, was named CRISPR interference (CRISPRi). In some ways, CRISPRi is similar to RNAi, a widely used technology for studying gene function that also does not involve altering the DNA.

Interested in trying dCas9?

Alt-R™ S.p. dCas9 Protein is a high-purity, recombinant *S. pyogenes* protein that contains nuclear localization sequences (NLSs, to increase targeting efficiency) and C-terminal 6-His tags.

Gene activation

CRISPRa is a category of methods that use the same dead Cas9 mutant (dCas9) as CRISPRi. However, in CRISPRa, the RNP is used to carry transcriptional activators, which overcome the transcription-blocking effect caused by dCas9, and turn on transcription at target promoter regions within the target gene. Some CRISPRa systems use activating proteins connected to the gRNA itself rather than to the Cas enzyme [12]. Like CRISPRi, CRISPRa methods have been extensively developed so that transcriptional activity can be tightly modulated, such as, in response to light [13]. Also, both CRISPRi and CRISPRa can be used to perform powerful genome-wide screens.

Other non-editing functions with dCas9-containing RNP

As mentioned previously, because dCas9 can target specific genomic sites without cutting the target, dCas9 is useful when fused to other proteins. As part of an RNP, dCas9 can transport activators, inhibitors, fluorescent proteins, and epigenomic modifier enzymes to specific sites in the genome to achieve numerous desired effects at those sites. For example, CRISPRi can work by coupling an effector domain (such as KRAB) to dCas9 to repress transcription [14]. It is possible to modulate the silencing caused by dCas9 with various molecular switches [15].

Nuclear organization and epigenetic modification

You can use fluorescently labelled CRISPR components to help study nuclear organization, enabling easy visualization of target genes within the nucleus. Numerous systems using modifications of CRISPR components have been designed for this purpose [16].

In the nuclei of cells, DNA is usually tightly wound around histone proteins. The term “epigenetics” refers to post-translational modifications of these histone proteins, as well as methylation of DNA itself. These modifications affect nuclear and DNA behavior and are regulated by many enzymes. You can use CRISPR technology to direct such enzymes to particular sites in the genome to regulate epigenetic modifications [13].

WHAT DO I NEED TO START MY GENOME EDITING EXPERIMENT?

You may be asking yourself, how do I choose the right enzymes and reagents? Well, to start a CRISPR genome editing experiment you need a guide RNA, a Cas nuclease, and a delivery system. For CRISPR experiments that use HDR, you also need a donor DNA. This section will help you choose the format you need.

Guide RNAs: What is available and which is best for my experiment?

Expressed gRNAs—the old, hard way of doing things

Plasmids: When CRISPR technology was first developed, it was reasonable to construct plasmid vectors to express Cas enzyme plus the gRNA, because long synthetic RNAs were expensive to make and not widely available. However, genome editing with plasmids is often laborious, requiring time-consuming cloning steps before an editing experiment can be undertaken. If you want to use plasmids or viruses to produce a gRNA, you must allow extra days or weeks of preparation before you can start your CRISPR experiment. First, you will need to design a DNA template from which the gRNA will be transcribed. After you produce a plasmid, it must be amplified, purified, and transfected into the cells in which you would like to carry out your CRISPR experiment. (Producing a virus to express the gRNA is even more complicated.)

Of course, you also have to deliver the Cas enzyme, not just the guide RNA, into the cells. In the past, this has been accomplished by designing a single plasmid that carries both the gene for the gRNA and the Cas enzyme. Alternatively, some researchers use a plasmid to carry the gRNA and a second plasmid for the Cas gene. Then, they co-transfect the cells with both plasmids. These are not the currently recommended methods, because, besides being time-consuming, they can often lead to much lower editing efficiencies and a higher degree of off-target effects.

Still need expression methods? Let us help.

Although expression of Cas enzyme and gRNA (for example, using plasmids or in vitro transcription) is a suboptimal approach to most experiments, you may need to do this for unusual projects. In this case, IDT has many helpful reagents you can use. For example, for cloning into a plasmid, you can use our gBlocks™ or gBlocks HiFi Gene Fragments, which are double-stranded DNA available in lengths of 125–3000 bp. They can be assembled into functional genes or longer constructs by seamless assembly techniques or traditional restriction cloning. They can also be used directly in IVT. For most genome editing, though, expressing the guide RNA and Cas enzyme would not be recommended, as described below.

In vitro transcription (IVT): In vitro transcription is another method used to produce guide RNA. It was relatively popular in the early days of CRISPR genome editing and is still sometimes used. Similar to the plasmid approach, the IVT method requires a DNA template to be designed based on the target sequence of the gene of interest. The template must also include an upstream promoter region—typically T7 or U6—to enable RNA polymerase-dependent transcription. Unlike plasmid, IVT gRNA—typically a single guide RNA (sgRNA) format—is made outside of the cell. This requires a DNA template and a recombinant RNA polymerase. IVT sgRNA must be properly prepared before transfection into cells. It also requires the addition of the Cas enzyme, which can be supplied either as an mRNA or plasmid encoding a Cas enzyme, requiring co-transfection (of both the IVT sgRNA and the Cas mRNA/plasmid), or as a recombinant Cas enzyme, requiring complex formation with the IVT-produced sgRNA.

This may seem similar to buying chemically synthesized guide RNA, complexing it with Cas to form RNP, and transfecting the cells. However, the source of the guide RNA makes a big difference. In vitro transcribed guide RNA, unlike chemically synthesized and modified guide RNA, can trigger an interferon (IFN) response that causes cell toxicity. For more information, see this collaborative publication by IDT scientists and researchers at the Frederick National Laboratory for Cancer Research.

Chemical modifications—a big improvement for gRNAs

In general, expressing the guide RNA and Cas enzyme is a lot of work and takes time. That alone might not be an overriding issue for you, but there are other considerations. If you express guide RNA molecules by any of the means described above, they won't have chemical modifications that decrease cellular toxicity, enhance stability, and improve results. In contrast, when you purchase chemically-synthesized guide RNA, these modifications can be added during synthesis. All of the IDT Alt-R™ CRISPR RNA molecules contain chemical modifications, which protect from cellular endogenous nuclease activity. In addition, if you are still worried that high levels of endogenous, cellular nucleases might destroy the crRNA in your experiment, you can choose to use our XT modification on crRNA. Our XT crRNA contains additional chemical modifications, making it longer-lived, nuclease resistant, safe for your cells, and still just as functional for CRISPR as regular crRNA. (Our standard crRNA is end-blocked and is referred to as Alt-R crRNA, whereas Alt-R crRNA-XT has both end-blocking and internal chemical modifications.) Similarly, the Alt-R tracrRNA is heavily modified. When you use our 2-part, Cas9 guide RNA, both the crRNA and tracrRNA have stabilizing chemical modifications that increase nuclease resistance.

Chemically synthesized guide RNAs

Using one of the synthetic gRNA options has several advantages over expressing the gRNA, including improved editing efficiency and reduced labor intensity. Preformed guide RNA also does not continue to be expressed over a long period of time like plasmid-encoded guide RNA. By minimizing how long the gRNAs spend in the target cells, synthetic options help decrease the chance of toxicity. Despite that, chemical modifications also increase the amount of time the gRNA is in the cells. The goal is using the optimum time so that the gRNA is in the cells long enough to be effective, but not so long that the gRNA causes toxicity or increases off-target effects. IDT provides chemically synthesized guide RNAs with optimized formats. In some models, our chemical modifications may even decrease the risk of an immune response to the guide RNA [17].

Expressed vs. recombinant Cas enzymes

If you express the Cas enzyme in the cells you hope to edit, you have the disadvantage of waiting until it is expressed and the disadvantage that the enzyme is expressed over a fairly long period of time, potentially leading to off-target effects. In addition, you miss the benefits of our improved Cas variants. Our recombinant Cas enzymes have several mutations to improve editing efficiency and decrease off-target events, as described in more detail later.

Advantages of ribonucleoprotein delivery

For the majority of CRISPR experiments in most cell types, RNP delivery by electroporation works very well. Some advantages of delivering a preformed RNP directly to cells are as follows:

- **Protection against nucleases**. The Alt-R guide RNAs contain chemical modifications to protect against cellular RNase degradation.
- **The RNP complex can function immediately upon delivery.** The Cas enzyme within the cells does not need to wait for guide RNA to bind to it, because this is done before delivering into cells.
- **Recombinant Cas9 protein does not last long.** The use of RNP complexes mitigates the risk of off-target cleavage by shortening the duration of Cas9 in cells, a major determinant of precision genome editing. This shortened duration is due to turnover of the protein by endogenous cellular machinery.
- **Less waiting.** You do not have to wait one or more days for transfected cells to start expressing Cas enzyme and guide RNA. Once the RNP is delivered into the cells, CRISPR genome editing starts immediately. However, we still typically wait 48–72 hrs before harvesting cells.
- **No vector molecular footprint.** The cells will not permanently express the guide RNA, Cas enzyme, or any other plasmid sequences that could have confounding effects on your experiment. This is an occasional issue with plasmids that occurs only if plasmid DNA integrates into the genome. However, a benefit with plasmids is that depending on their design and the intentions of your experiment, you can put selection pressure on your cells if you want plasmid DNA integration.
- **The editing efficiency can be close to 100%.** This means that in some experiments, approximately 100% of the cells and 100% of the targeted genes may be edited. This may surpass the editing efficiency seen with expression of the guide RNA and Cas enzyme using plasmids or IVT, but is not guaranteed, because editing efficiency rates are cell-type dependent. For many commonly-used cell types, this high rate of editing is frequently observed with electroporation of RNP.

These advantages are true for CRISPR experiments that use either Alt-R Cas9 or Cas12a *Ultra*.

For Cas9 CRISPR experiments, sometimes there are advantages to splitting the guide RNA into a tracrRNA and a crRNA molecule, as found in nature. One obvious advantage is that tracrRNA is always the same across all experiments. Another is that gRNAs are relatively inexpensive, even with chemical modifications. Only the crRNA varies, based on the target sequence. Since the crRNA is short, it is relatively inexpensive. If you are targeting many sequences, you can simply get one large order of tracrRNA and use it with any of your crRNA sequences, saving cost compared to sgRNA, which is more expensive since it is longer. The 2-part guide RNA system is compared in more detail to the single guide RNA system in Table 1 and in the next section.

When using the 2-part guide RNA system in high nuclease environments, a more modified version of the guide RNA may be required. This is not common, but if you observe low editing efficiency with our regular gRNAs, the cells may be degrading the guide RNA. In this case, consider using one of our extra-modified gRNAs: the Alt-R 2-part XT guide RNA or Alt-R sgRNA, which both have high stability. Table 1 gives some basic information, while this DECODED article provides more detail.

Are there any disadvantages to using RNP that includes chemically synthesized guide RNA? Yes, even with the above cost savings, the price can still be higher than using plasmids or IVT. However, this disadvantage is offset by your experiments being quick, clean, and possibly even more fun! You get your results reliably, while saving days or weeks of preparation time.

Table 1. Differences in types of guide RNAs.

	Cas9 guide RNAs			Cas12a guide RNAs
Guide RNAs	Alt-R 2-part	Alt-R 2-part XT	Alt-R sgRNA	Alt-R Cas12a crRNA
Structure				
gRNA format	Alt-R CRISPR-Cas9 crRNA & tracrRNA	Alt-R CRISPR-Cas9 crRNA XT & tracrRNA	Alt-R CRISPR-Cas9 sgRNA	Alt-R CRISPR-Cas12a crRNA
Components	crRNA tracrRNA	crRNA XT tracrRNA	sgRNA	crRNA
Size (nt)	36 67	36 67	100	40–44 (41 nt recommended)
Annealing	Required	Required	None	None
Stability	++	+++	++++	+++
Applications	• Cas9-expressing cells • RNP in most cell types	• Co-delivery with Cas9 plasmid/Cas9 mRNA • RNP under difficult experimental conditions (e.g., high nuclease environments)		• KO/KI, RNP in most cell types • Cas12a-expressing cells

General considerations for guide RNA design

On- and off-target activity

The goal when designing a guide RNA is achieving the highest possible on-target activity of your CRISPR gene editing complex, while minimizing off-target activity. Off-target activity causes unwanted phenotypes, potentially including cell death.

Cas9 guide RNA design considerations

For Cas9 guide RNA designs, the target sequence must be next to a PAM sequence, NGG, where N is any base. It is important that you **do not** include the PAM sequence in the actual guide RNA design. The guide RNA recognizes and binds to 20 nucleotides on the DNA strand opposite from the NGG PAM site. The "N" of the NGG is immediately adjacent to the most 3′ base of the non-targeted strand side of the protospacer (see Figure 5).

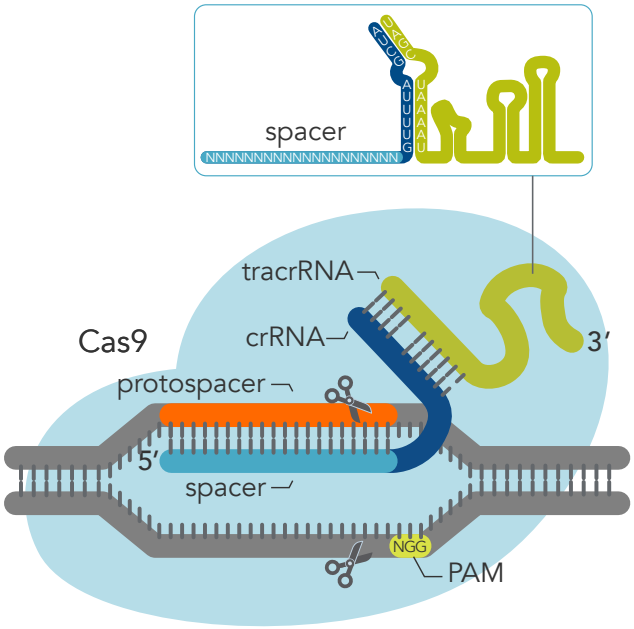
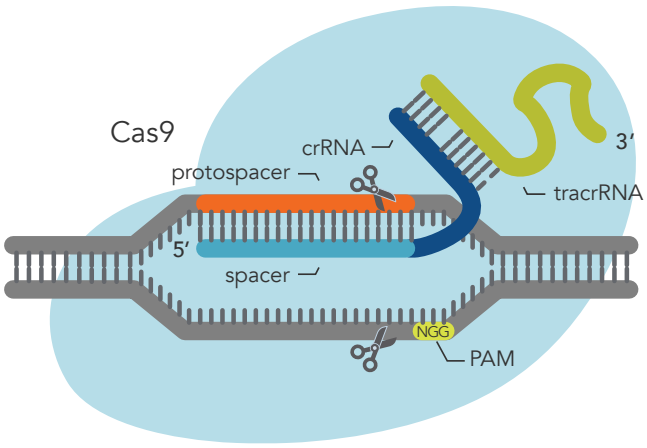


Figure 5. PAM site and Cas9 gRNA. The PAM site is on the non-targeted strand of DNA. The guide RNA binds to the targeted strand. The guide RNA in this figure is a 2-part guide RNA, consisting of crRNA (light blue and dark blue) and tracrRNA (green).

Cas9 guide RNA length considerations

If you decide to use Cas9 and chemically synthesized guide RNA, you have the choice of 2-part or single guide RNA (Figure 6). Both guide RNA formats are commonly delivered to cells as Cas9 RNP.

A. Cas9 and 2-part gRNA



B. Cas9 and sgRNA

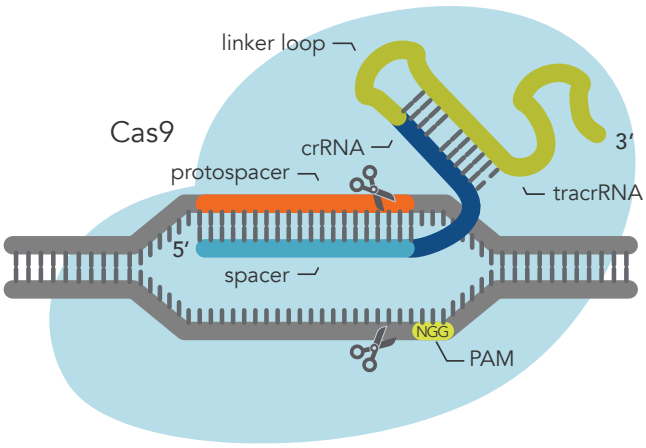


Figure 6. Comparison of 2-part gRNA (A) and sgRNA (B). Both kinds of gRNA can be added to Cas9 to form RNP, which can then be delivered to cells.

- When you are using 2-part guide RNA, there are crRNA and tracrRNA components.

  crRNA: The crRNA is composed of a target-specific spacer region (light blue in Figure 6) and another domain (dark blue) that hybridizes to the tracrRNA (green). The target-specific spacer sequence is 20 nucleotides long; if it is shorter than this, the on-target activity will be negatively impacted. IDT researchers have found that the optimal total length of the crRNA (the target-specific spacer region plus the domain that hybridizes to tracrRNA) is 36 nucleotides.

  tracrRNA: Part of the tracrRNA molecule hybridizes (base pairs) to the crRNA, and another part of it binds to Cas9. IDT scientists have found that the optimal, total length for tracrRNA is 67 nucleotides. This is shorter than the tracrRNA found in nature, but IDT scientists have found that when using 2-part guide RNA, shortening the tracrRNA to 67 nucleotides increases on-target activity.

- When using sgRNA, the crRNA and tracrRNA are all part of one longer RNA molecule joined by a hairpin-like loop (linker-loop, Figure 6). Cas9 sgRNA for genome-editing purposes are typically 100 nucleotides in length.

Caution: Cas9 guide RNA design

The CRISPR-Cas9 complex can tolerate several mismatches between the guide sequence and the target sequence, meaning it is fairly easy to end up with undesirable off-target effects. So, we recommend using high quality guide RNA design software, as described later in this handbook.

If you need help choosing which type of guide RNA is best for your Cas9 experiment, this DECODED article provides more discussion and guidance about which gRNA format to use (quick tip: it usually does not make a difference).

Cas12a guide RNA design considerations

For Cas12a guide RNA designs, just like for Cas9 guide RNA, the target sequence must be next to a PAM sequence. But for Cas12a, the PAM sequence is TTTV, where V can represent A, C, or G. In addition, if you are using Alt-R Cas12a *Ultra*, a TTTT PAM sequence may also work but may not be as potent. (See the discussion later in this guide for how to choose your Cas enzyme).

The "V" of the TTTV is immediately adjacent to the most 5′ base of the non-targeted strand side of the protospacer element (Figure 7). As for Cas9 guide RNA designs, it is important that you **do not** include the PAM sequence in the guide RNA design when ordering.

The guide RNA for Cas12a is relatively short at only approximately 40–44 bases long. The part that base pairs to the protospacer is 20–24 bases in length, and there is also a constant 20-base section that binds to Cas12a (Figure 7). Our scientists have found the optimal total length to be 41 bases (21 target-specific bases that will base pair with the protospacer, and 20 constant bases for binding the Cas12a enzyme).

Note: gRNA nomenclature

Guide RNA for Cas12a is often referred to as crRNA, even though there is no tracrRNA.

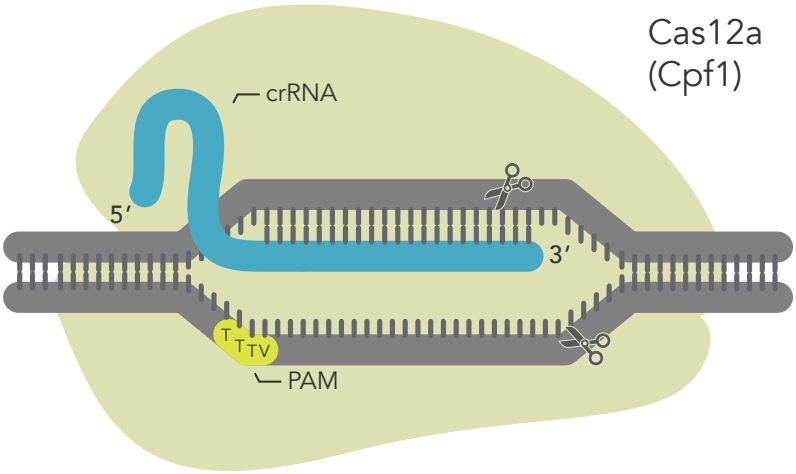


Figure 7. Cas12a and its crRNA. The crRNA for Cas12a is relatively short and does not require tracrRNA.

Cas ENZYMES

Cas proteins are nucleases: enzymes which can cut nucleic acids (DNA or RNA). There are 2 main types of Cas enzymes used for most CRISPR genome editing, Cas9 and Cas12a (also known as Cpf1), both of which cut dsDNA. In addition to the wild-type forms of these enzymes, several mutant forms of these enzymes have been engineered by IDT to improve and expand their functions.

Background: Cas vs. TALENs/ZFNs

Cas enzymes, unlike other genome editing proteins such as TALENs or ZFNs, do not need to be individually designed for each experiment. Instead, the guide RNA is designed for each experiment. This is much easier than designing whole proteins. The guide RNA ensures that the Cas enzyme finds its way to the target site within the genome.

To choose between Cas9 and Cas12a, you need to look at the genome and the specific gene you are going to edit, and decide whether the target is an AT-rich or GC-rich region. Typically, if you merely want to knock out a gene, you have a lot of design space to examine. In this case, either Cas9 or Cas12a can likely be used, unless the region is very GC-rich or AT-rich. However, if you want to perform HDR, the region to examine is approximately ±10 bp around your desired cut site. The choice of Cas enzyme depends on what PAM sites are in your target region. A GC-rich sequence is more likely to contain an NGG PAM, which can be targeted with Cas9. An AT-rich sequence is more likely to contain a TTTV PAM, which can be targeted with Alt-R Cas12a *Ultra*, a mutant form of Cas12a that works extremely well with TTTV PAMs.

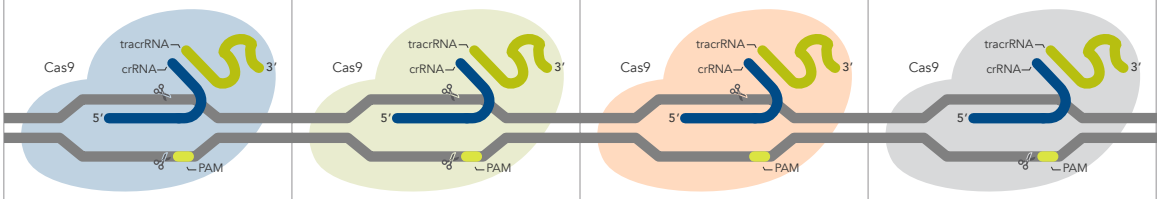
Cas9

Cas9, the best-known and most popular CRISPR-associated nuclease, can target any ~20-nucleotide DNA sequence with an adjacent NGG PAM sequence (where N is any base). There are 3 main types of Alt-R S.p. Cas9 enzymes available from IDT: wild-type (WT), HiFi, and nickase variants (Table 2).

Tip: All gRNAs are not equal!

Although any ~20-nucleotide DNA sequence with an adjacent NGG PAM sequence can theoretically be targeted with Cas9, not all guide RNAs are equally efficient. Choosing another nearby Cas9 or Cas12a target site can be the perfect solution. Sometimes this problem can be addressed using Cas9 nickases (as described below). Also, be sure to look at the sequences of both strands of the DNA when looking for PAM sites.

Table 2. Cas9 nuclease variants.

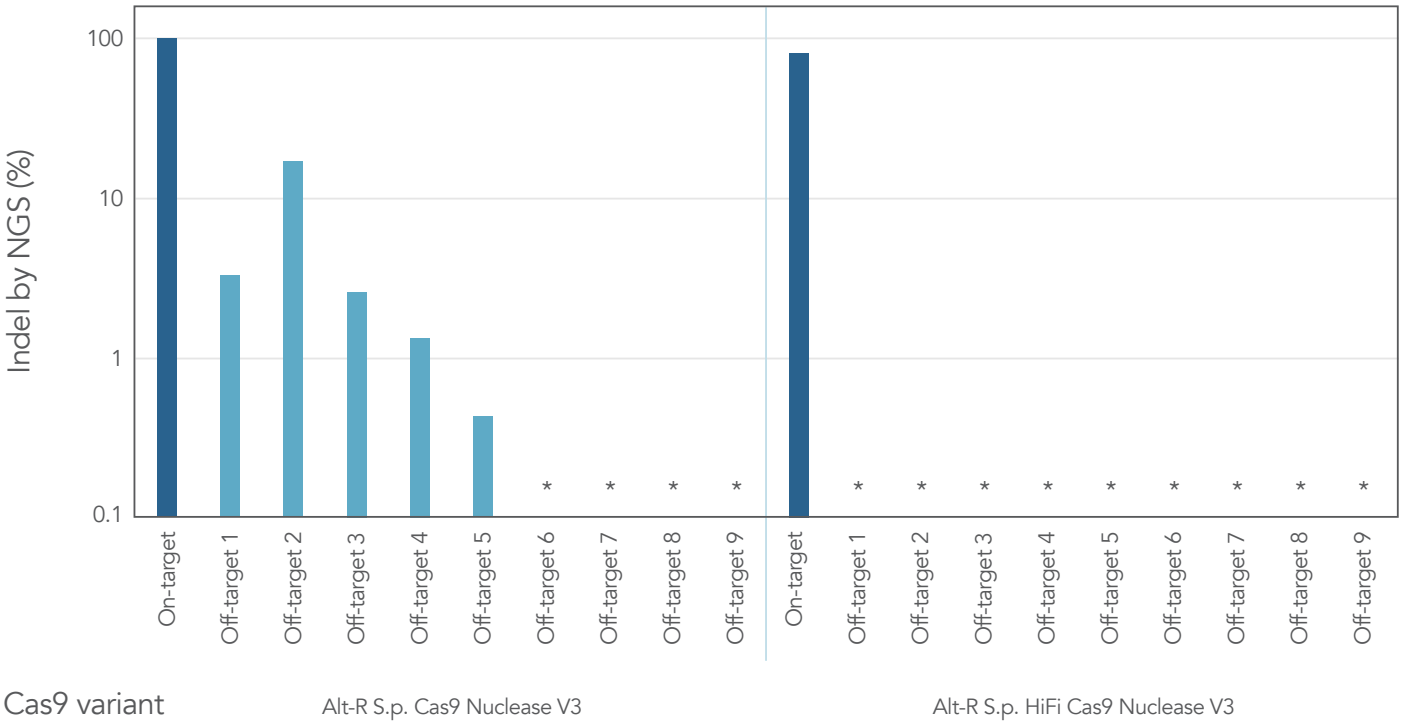
	Alt-R S.p. Wild-type Cas9 Nuclease	Alt-R S.p. HiFi Cas9 Nuclease	Alt-R S.p. Cas9 D10A Nickase	Alt-R S.p. Cas9 H840A Nickase
				
Description	Wild-type Cas9 with high genome editing potency that is simple to use and economical	Cas9 variant that effectively reduces off-target effects and preserves high on-target activity	Cas9 variant with a mutation in the RuvC domain that disables cleavage of the non-target strand	Cas9 variant with a mutation in the HNH domain that disables cleavage of the target strand
DNA cleavage	Both strands	Both strands	Target strand	Non-target strand
Suggested use	First choice for most CRISPR genome editing projects	Ideal for experiments that are sensitive to off-target events and require a high level of editing efficiency	May be beneficial for homology-directed repair (HDR) experiments, but requires two suitable cutting sites within an optimal distance of each other	
Molecular weight	162,200 g/mol			
Amount provided	100 µg, 500 µg, or 5 mg		100 µg or 500 µg	
Concentration	10 mg/mL (62 µM) in 50% glycerol			
Shipping conditions	Dry ice			
Storage conditions	–20°C at stock concentration			
Dilution	Dilute in Opti-MEM® medium (Thermo Fisher Scientific) or PBS before use			

Wild-type Cas9

IDT's wild-type Alt-R Cas9 enzyme is a recombinant *S. pyogenes* Cas9 enzyme, containing the wild-type sequence of Cas9 (that has been used in the majority of CRISPR genome editing research), plus an optimized nuclear localization signal (NLS) to improve performance (i.e., enhanced cleavage). This is suitable for most basic genome editing work.

High-fidelity Cas9

Alt-R HiFi Cas9 Nuclease has the natural potency of the wild-type Cas9 enzyme but with significantly reduced off-target effects. This makes the HiFi Cas9 enzyme well-suited to genome editing experiments where off-target effects are a serious concern. In such experiments, this enzyme still maintains its high on-target editing activity (Figure 8). HiFi Cas9 also contains an optimized NLS. For applications that are sensitive to off-target events, we highly recommend combining the Alt-R S.p. HiFi Cas9 Nuclease V3 with optimized Alt-R guide RNA.



* Indel formation <0.1% as measured by multiplexed amplicon sequencing

Figure 8. Alt-R HiFi Cas9 Nuclease V3 facilitates near-WT on-target editing potency and significantly reduces off-target site editing. RNP complexes were formed with either Alt-R Cas9 Nuclease V3 or Alt-R HiFi Cas9 Nuclease V3, combined with an Alt-R crRNA:tracrRNA complex targeting the *EMX1* gene where *n* = 1. RNP complexes (4 µM) were delivered into HEK-293 cells via the Nucleofector™ system (Lonza). Indels at the on-target locus, as well as 9 known off-target sites, were measured by NGS (indicated on the y-axis in log scale).

Cas9 nickases

Nickases are enzymes that produce single-strand breaks, as opposed to the double-strand breaks produced by WT and HiFi Cas9. Cas9 nickase variants have an alanine substitution within either of the 2 key catalytic domains, RuvC and HNH, of the WT Cas9 endonuclease. The RuvC mutant, D10A nickase, cuts on the targeted strand. The HNH mutant, H840A nickase, cuts the non-targeted strand.

The trick to producing a DSB using nickases is to combine a Cas9 nickase—either D10A or H840A, together with 2 gRNA sequences–targeting sites that are close together but on opposite DNA strands. This creates a staggered DSB. The distance between cleavage sites is important for optimal performance:

- Alt-R Cas9 D10A Nickase: 40–70 bp
- Alt-R Cas9 H840A Nickase: 50–70 bp

Why would you want to use a nickase? One benefit of using Cas9 nickases to introduce DSBs is that this can minimize unwanted off-target editing and support high on-target efficiency. The nickase method requires 2 different guide RNAs to bind and function at the same time. As a result, this increases precision: the chance of making a DSB at an off-target site is very slim because it is unlikely that both guide RNAs will bind in the same off-target vicinity. It is far more likely that when you have two guide RNAs, you will only get on-target production of DSBs.

However, you could just use HiFi Cas9 to achieve lower off-target editing, if that is your only concern.

Note: Increased experimental complexity is not always needed!

Using a nickase to introduce DSBs is more complex than using a regular nuclease, because you have to design 2 guide RNAs. Therefore, the simple fact that the nickase approach decreases off-target editing is often not enough of a reason to choose this approach. After all, using HiFi Cas9 is easier: HiFi Cas9 reduces off-target editing with only one guide RNA.

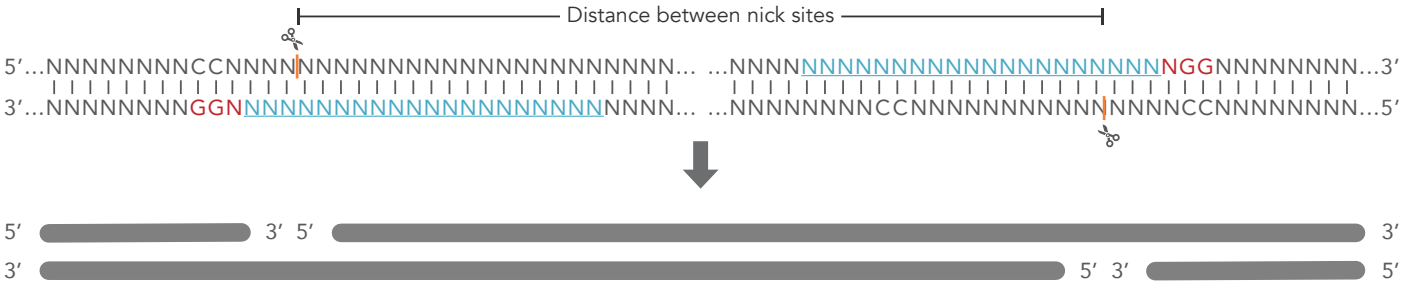
Even if you are not particularly worried about reducing the risk of off-target editing, there is another reason to consider nickases in your experiments: some Cas9 guide RNAs are less efficient than others. That is, sometimes there is a poor editing level with a certain guide RNA (no matter whether you use WT or HiFi Cas9). If you determine experimentally that there are no efficient Cas9 guides that target the site you want to mutate, then using a nickase with paired guides targeted at short distances from both sides of the desired mutation site may be a better approach. Alternatively, you can use another nearby protospacer target sequence or switch to the Cas12a system.

Preparation is the key to a successful nickase-based CRISPR genome editing experiment. Before the experiment, you should test pairs of gRNAs in combination with the nickase to determine their editing efficiency. The pair of gRNAs with the highest editing efficiency can then be selected for use in the experiment.

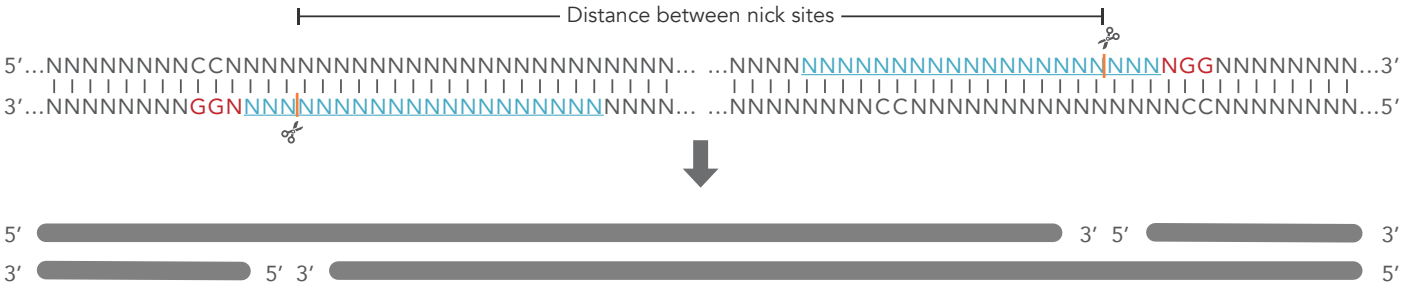
As shown in Figure 9, the NGG PAM sequences of the 2 guide RNAs can either face outwards (PAM-out) or towards each other (PAM-in). Both Cas9 nickases, D10A and H840A, have higher editing efficiency in combination with gRNAs that have PAM-out orientation (read the application note for details).

gRNA in PAM-out orientation

A. Double nicking by Cas9 D10A

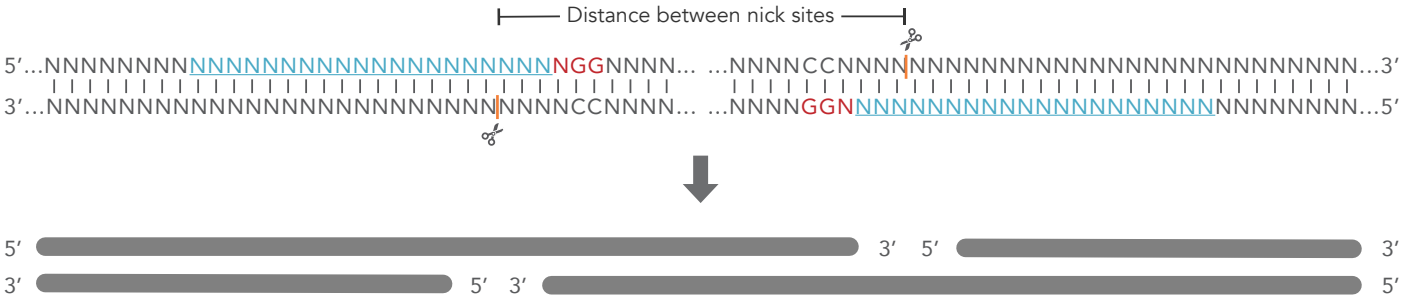


B. Double nicking by Cas9 H840A



gRNA in PAM-in orientation

C. Double nicking by Cas9 D10A



D. Double nicking by Cas9 H840A

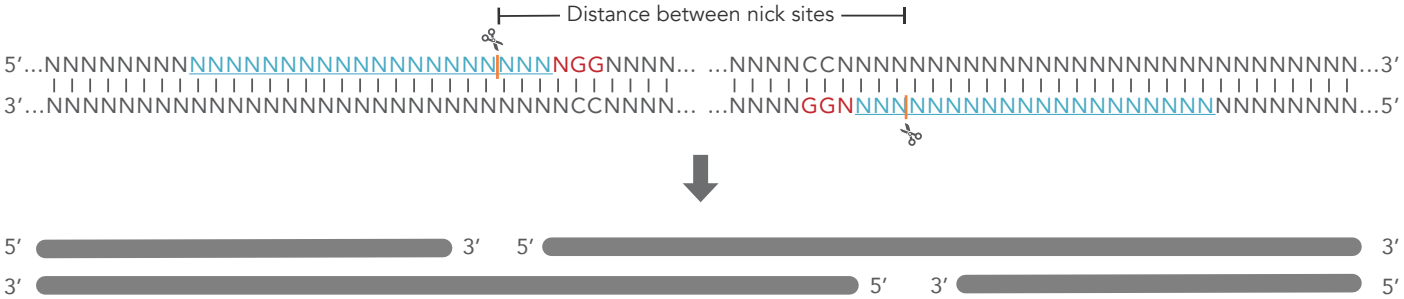


Figure 9. PAM-out vs. PAM-in gRNA with nickases. Guide RNA works better with PAM-out orientation than with PAM-in orientation when using either one of the nickases. PAM sites are shown in red.

Tip: You need only 1 nickase!

Nickase experiments for general genome editing use 1 nickase but 2 guide RNAs. A DSB is produced when 2 nicks on opposite DNA strands are made by the nickase. The 2 nicks are made because you use 2 guide RNAs—one targeted to a target site on one DNA strand, the other targeted to a different target site on the other DNA strand.

IDT sells 2 nickases, D10A and H840A. You would need only one or the other of these nickases for a general genome editing experiment. Typically, you do not need to buy them both. You do, however, need to buy 2 guide RNAs.

Choosing a nickase: Cas9 D10A and H840A are equally potent at nicking dsDNA, although the D10A form is more efficient at mediating HDR events. Generally, when you choose a nickase, you probably want the D10A form. The H840A form is mostly useful for very specialized experiments. For example, it was recently used in the development of a new technology known as CRISPR prime editing [18], described in this DECODED article.

To use a nickase for HDR-based knock-in experiments, single-stranded oligodeoxynucleotide (ssODN) donor templates complementary to both strands of the target DNA usually need to be designed and tested before the experiment. Donor templates are described in more detail later in this handbook.

Tip: Make 2 separate RNPs!

To maximize activity of your nickase, form separate RNP complexes for *each* gRNA before delivery, rather than attempting to make the RNPs in a single mixture containing both gRNAs.

dCas9

If you want to study a gene's function without editing the genome, dCas9 may be the perfect solution. dCas9 is a mutated Cas9 that does not cut DNA even though it still binds specifically to its target when directed by a guide RNA. By binding to a gene, dCas9 prevents other enzymes, such as transcription activators, from binding to that site, and in effect, blocking transcription and temporarily silencing the gene [11]. When dCas9 is naturally cleared by the cell, transcription can begin again. In this convenient way, dCas9 is useful for reversible gene-silencing experiments. If you want to try a quick gene-silencing experiment, IDT sells recombinant dCas9 so you do not even have to express it from plasmid.

Cas12a

In addition to the Cas9 system, there is the Cas12a (Cpf1) system, which was first published in 2015 [19]. The Cas12a enzyme recognizes a unique PAM site, made up of bases TTTV (where V is A, C, or G). This greatly expands the number of possible editing sites beyond those targeted by Cas9, which requires an NGG PAM site. Because of this, Cas12a systems can be used in genome editing experiments in organisms with AT-rich genomes, such as plants and *C. elegans*.

Unlike Cas9, Cas12a produces a staggered double-strand DNA cut, leaving a 5′ overhang in the target DNA sequence. On the strand which contains the PAM, the cut is 18–19 bases from the PAM in the 3′ direction. The cut on the opposite strand of the DNA is 23 bases from the PAM, resulting in a 5′ overhang of 4–5 bases.

As mentioned earlier, the gRNA used by Cas12a is simpler than the crRNA for Cas9, as there is no tracrRNA required for Cas12a.

Cas12a *Ultra*

In general, we recommend using Alt-R Cas12a *Ultra* rather than wild-type. As shown in **Figure 10**, Cas12a *Ultra* has higher on-target potency than the wild-type Cas12a, and Cas12a *Ultra* reaches or even exceeds the performance of Cas9. Cas12a *Ultra* can access an even wider range of target sites than WT Cas12a, as Cas12a *Ultra* recognizes not only TTTV PAM sites but also many TTTT PAM site sequences. Another benefit is that Cas12a *Ultra* has high HDR efficiency, making it suitable for both CRISPR knock-in and CRISPR knockout experiments.

Cas12a *Ultra*, unlike WT Cas12a, remains active across a wide range of temperatures, making Cas12a *Ultra* (or L.b. Cas12a *Ultra*) useful in experiments held at cooler temperatures, such as genome editing in fish and plants.

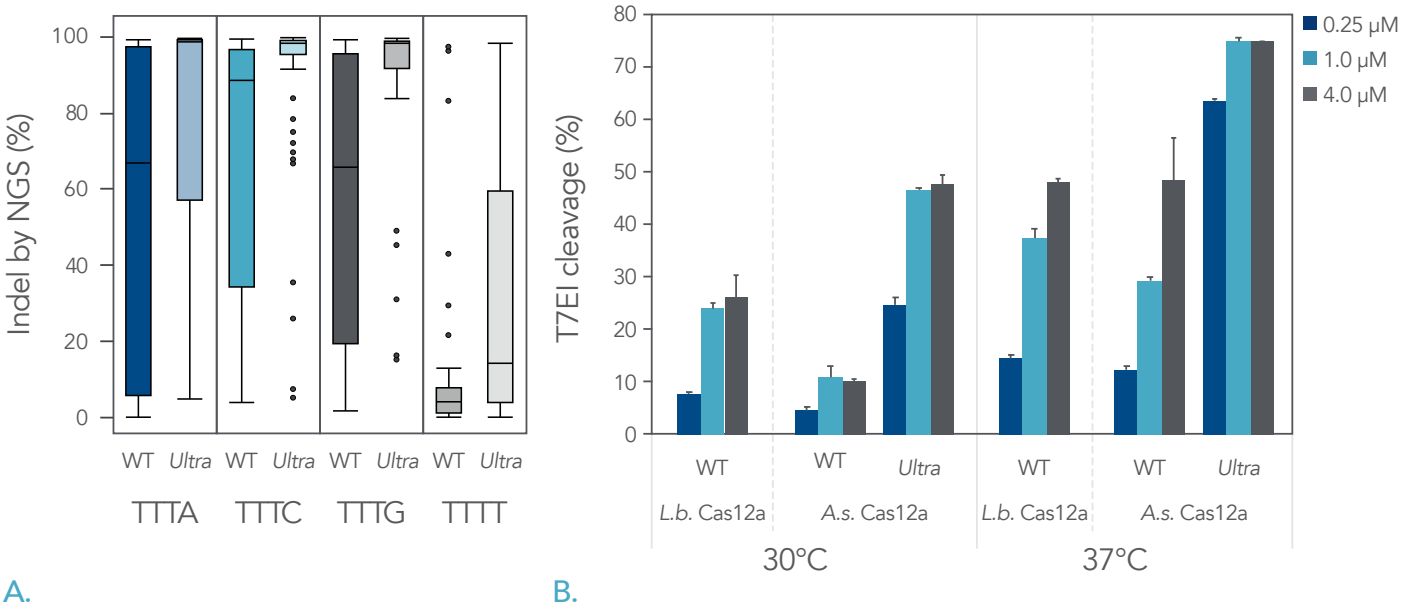


Figure 10. (A) Alt-R A.s. Cas12a (Cpf1) *Ultra* exhibits increased genomic editing efficiency in Jurkat and HEK-293 cells. Ribonucleoprotein complexes were formed with wild type (WT) or Alt-R A.s. Cas12a (Cpf1) *Ultra* (*Ultra*), combined with crRNAs synthesized for 120 genomic loci to be delivered in Jurkat cells and 96 genomic loci to be delivered in HEK-293 cells. RNP complexes (4 µM) were delivered into Jurkat and HEK-293 cells via a Nucleofector system (Lonza) in the presence of Alt-R Cas12a (Cpf1) Electroporation Enhancer. Genome editing efficiencies were determined by target amplification followed by next generation sequencing on an Illumina instrument. The Cas12a-associated PAM sequences are indicated below the graph. **(B) A.s. Cas12a *Ultra* is a superior low temperature solution.** This enzyme was compared with wild-type Cas12a enzymes from *Acidaminococcus* species and *Lachnospiraceae* bacterium. At both 30 and 37°C, Cas12a *Ultra* outperformed both of the wild-type enzymes.

Delivery methods

Having designed the guide RNA and selected a Cas enzyme, how do you get them into the cells?

There are several methods for delivering CRISPR components into cells: electroporation or transfection (lipofection) of either actual reagents or plasmids encoding the reagents, microinjection, and viral vectors encoding the reagents.

Reminder: plasmid vs. RNP

It is now widely accepted that the safest delivery method for CRISPR is to bind the Cas enzyme to the gRNA outside of the cell and deliver it as a complete RNP.

The reason? Well, if you use a plasmid or viral vector, the plasmid or vector stays in the cells longer than an RNP complex does. This means the CRISPR components are produced continually over a period of time, increasing the risk of triggering an immune response or off-target editing of the genome. Indeed, long-term activity is a major cause of off-target effects. By delivering the Cas enzyme and gRNA as a preformed RNP complex, the amount of time that the complex stays in the cells is reduced, minimizing these risks.

The 2 techniques usually used to deliver RNP into cells are electroporation and lipofection. Microinjection is a less common method used primarily in oocytes or embryos, although electroporation has successfully been used in embryos, too. In most non-embryo experiments, you will choose between electroporation and lipofection. Electroporation generally requires higher RNP concentrations (2–4 µM RNP) than lipofection (often as low as 10 nM RNP). We usually recommend electroporation over lipofection for most experiments.

Electroporation

Electroporation uses a short pulse of electrical current to increase cell permeability temporarily, allowing molecules to be delivered into the cell.

We recommend that you test a range of RNP concentrations before your experiment, starting with the concentrations suggested in our protocols, to establish optimal electroporation conditions for the experimental cell line, as well as help maximize cell viability. We sell positive and negative control guide RNA that are useful for these optimization experiments.

There are 2 electroporation enhancers available from IDT. One electroporation enhancer has been developed to work with Cas9 RNP, the other with Cas12a RNP. These electroporation enhancers increase CRISPR genome editing efficiency when added to an RNP electroporation solution and are only for electroporation, not microinjection or lipofection.

Both of the electroporation enhancers are single-stranded DNA molecules designed to have no homology to human, mouse, or rat genomes, and are unlikely to incorporate into the genomes of mammalian experimental models. Although not all the mechanistic details are understood, these electroporation enhancers may act as carrier molecules to improve the rate of RNP delivery into cells during electroporation [20]. Both of the electroporation enhancers have been shown to enhance overall gene editing efficiency with the Neon™ (Thermo Fisher Scientific) and Nucleofector (Lonza) systems. **This is especially useful if you want to do genome editing in primary cells or cells that are difficult to transfect.**

Using the appropriate electroporation enhancer brings a great improvement in the efficiency of gene editing, and may reduce the amount of RNP complex required. **This, in turn, may reduce the possibility of off-target genome editing and may help improve cell survival.**

The optimal amount of electroporation enhancer to include differs by the electroporation instrument. For example, we recommend 3 µM of Alt-R Cas12a (Cpf1) Electroporation Enhancer with the Nucleofector System, and 1.8 µM of Alt-R Cas12a (Cpf1) Electroporation Enhancer with the Neon System. Because we have observed that dose-response curves vary for different guide RNA sequences (depending on the potency of the guide RNA), we recommend titrating the amount of RNP, while keeping the amount of enhancer fixed. Otherwise, toxicity may be observed when using high concentrations of electroporation enhancer.

You have many instrument options when it comes to electroporation. IDT scientists frequently use the Lonza 96-well shuttle (AAM-1001S) with the Lonza 4D-Nucleofector Core Unit (AAF-1002B). Several other electroporation systems are also available to help simplify and improve the efficiency of your electroporation experiment. Electroporation devices successfully used with IDT reagents and a wide variety of cell types include instruments from Bex, Lonza (Nucleofector system), Thermo Fisher Scientific (Neon system), Nepa Gene, and Bio-Rad (Gene Pulser™ Xcell™ system). We have many IDT-verified and/or user-submitted protocols using these instruments on our website.

Transfection by lipofection

In general, electroporation is the preferred delivery method rather than lipofection. However, if you already have a high-efficiency lipofection protocol for delivering molecules into cells, you may want to try your lipofection protocol for delivery of RNP. If you do not have a lipofection protocol, electroporation is our recommended choice, as long as you have an appropriate electroporation instrument (see above).

Lipofection is primarily suitable for CRISPR experiments in easy-to-transfect cells, such as some adherent, immortalized eukaryotic cell lines. Compared to electroporation, lipofection tends to be cheaper and requires lower levels of RNP. For example, as little as 10 nM Alt-R CRISPR RNP has been used successfully in HEK-293 cells via lipofection.

Like electroporation, lipofection usually requires some optimization to suit your cell line. Again, the goal is to minimize cell toxicity and maximize genome editing efficiency.

Not all traditional plasmid or small RNA delivery reagents work well with RNPs. We recommend trying Lipofectamine® RNAiMAX or CRISPRMAX™ reagent (Thermo Fisher Scientific) for performing lipofection.

Hint: Using HEK-293 cells for your CRISPR experiment?

You are in luck. Our team of scientists at IDT have already prepared an optimized lipofection protocol.

Microinjection (for embryos)

Microinjection is another method of delivering CRISPR components. It is usually used for mouse or rat embryos. In comparison to electroporation and lipofection, it is a labor-intensive, costly, and time-consuming method that requires skilled lab workers and specialized equipment. The main obstacle is that each embryo needs to be injected individually.

In some experiments, electroporation, instead of microinjection, can be an alternative high-throughput method for delivering CRISPR components into embryos. Electroporation removes the need for specialist-handling skills and has less impact on embryo development.

Delivery methods for other organisms

There are many published protocols available for delivery of large molecules, such as DNA and proteins, to a wide variety of cell types. For many organisms, including zebrafish embryos and *C. elegans*, IDT can provide delivery protocols by users of our products.

Note: Authors welcome!

We always welcome fellow researchers to share protocols with us that may be helpful to other scientists. If you have a novel protocol that uses Alt-R CRISPR RNA or nucleases that you would like to share with the research community, please email us at CRISPR@idtdna.com to start the discussion.

Donor DNAs for homology-directed repair (HDR)

Donor DNAs are DNA templates used to repair double-strand breaks by homology-directed repair, or HDR. For donor DNA, researchers frequently use a single- or double-stranded DNA sequence. In either case, the donor DNA consists of the desired mutation sequence flanked by homology arms matching (i.e., complementary) to sequences adjacent to the target cut site. The choice of ssDNA or dsDNA depends largely on the size of the insertion, and the ability to synthesize a donor of the required length. For donor templates under 200 nt, IDT recommends ssDNA HDR Donor Oligos. For longer donors up to 3 kb, we recommend dsDNA Alt-R HDR Donor Blocks. Both options offer chemical modifications shown to improve HDR rates compared to unmodified donors. For more details, see this DECODED article.

Tip: Consider HDR for many kinds of experiments!

HDR can be used for knock-in, protein tagging, making single-nucleotide variants, introducing restriction sites for molecular biology purposes, and many other types of experiments.

Single-stranded oligodeoxynucleotides harboring the desired exogenous sequences have become common in HDR applications, due in combination to their high HDR efficiency, low toxicity, and the ease of chemically synthesizing diverse sequences [21,22]. For sequence replacements or small insertions shorter than 120 bp (e.g., addition of stop codons, protein functional sites, antibody reactive epitopes, and detectable tags), IDT recommends using ssODN templates such as Alt-R HDR Donor Oligos.

For longer insertions, Alt-R HDR Donor Blocks are available up to 3000 bp in length. Alt-R HDR Donor Blocks are modified linear dsDNA fragments generated from clonally purified DNA, which offers the greatest purity available. Utilizing the same high-fidelity synthesis process as IDT gBlocks™ HiFi Gene Fragments, Alt-R HDR Donor Blocks incorporate advanced chemical modifications placed within terminal universal sequences. These modifications boost HDR rates and inhibit the occurrence of non-homologous (blunt) integrations. Additionally, the use of terminal universal sequences allows for increased consistency and speed of production.

We recommend 30–60 nt lengths for homology arms for short ssODN donors (e.g. Alt-R HDR Donor Oligos;

<200 nt total length). For longer dsDNA HDR donors (e.g., Alt-R HDR Donor Blocks; 200–3000 bp total length), we recommend 200–300 bp lengths for homology arms. The Alt-R HDR Design Tool can design both short and long donors, and automatically selects either Alt-R HDR Donor Oligos or Alt-R HDR Donor Blocks in the output, depending on the final donor length.

Tip: You can use the Alt-R HDR Design Tool to make your HDR experiment much easier!

Designing an HDR donor template at the same time as designing your Cas9 guide RNA is easy when you use the IDT HDR design tool. The Alt-R HDR Design Tool enables greatly increased HDR rates by providing optimized 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 validation. You can use the tool by entering a gene name, accession number, genomic coordinates, or sequence in FASTA format for multiple species, including human, mouse, rat, zebrafish, and nematode. The tool also supports custom designs, as well as single, or multiple, entries.

In experiments with Cas9, when aiming for insertions of <2 kb in length, IDT scientists have shown that ssODN donors make it possible to include shorter homology arms, while providing a higher efficiency of insertion than similar templates in unmodified dsDNA form. More recently, chemically modified dsDNA donors have been developed that improve the rate of perfect HDR so as to be comparable to ssDNA formats. For donors over 200 nt, chemically modified dsDNA donors are generally recommended due to their strong performance and lower costs compared to similarly long ssDNA donors.

Recommendation: Consider the size of the HDR donor!

When you are designing the HDR donor template, differentiating between small indels/mutations and long insertions is helpful. For more information, see our HDR application note.

We currently recommend:

- **Small mutations and short insertions**: Use Alt-R HDR Donor Oligos that include two flanking, homology arms (30–60 nt each) and your insertion/mutation sequence (maximum template length is 200 nt). The Alt-R HDR Design Tool is very helpful for these designs. Alternatively, if you already have a template design, you can order it as an Alt-R HDR Donor Oligo (i.e., as a template with the same modification options that are available when selecting templates from our HDR design tool).
- **Mid-length insertions**: Use HDR Donor Blocks that include two flanking homology arms (100–400 bp each) and your insertion sequence (maximum template length is 3000 bp). The Alt-R HDR Design Tool is very helpful for these designs. Alt-R HDR Donor Blocks are linear dsDNA fragments containing chemical modifications designed to improve HDR and reduce unintended blunt integrations.
- **Very long (greater than ~2500 nt) insertions**: Use plasmids or linearized plasmids containing your homology arms (generally at least 500 nt) and sequence of interest, but monitor the cells for toxicity. Use of circular dsDNA with a minimal plasmid backbone, such as Nanoplasmids, may help improve HDR when toxicity is a limiting factor.

Additionally, look for new methods in the future from IDT scientists, who are constantly making improvements to inducing HDR with longer insertions.

Plasmids and linearized plasmids have been used as donor DNA molecules even for short insertions/mutations by some researchers in the past. However, these approaches can be associated with significant toxicity, and are not currently recommended for insertions <2.5 kb in length. Instead, for HDR donors, IDT offers Alt-R HDR Donor Oligos (up to 200 bases long, including the homology arms) and Alt-R HDR Donor Block (up to 3000 bases in length, including the homology arms).

If you are using Cas9 nuclease for HDR, it is usually best to test ssODN donors with homology arms matching both strands of the target DNA, as the preferred strand varies by site and insertion position relative to the DSB (Figure 5). However, if you are using Cas12a (Cpf1), we recommend you use the non-targeted strand sequence (Figure 7) for the homology arms in the ssDNA donor for the best results.

When using either Cas9 or Cas12a systems, avoid recreating the PAM and protospacer sequence after the desired HDR repair takes place. When designing the HDR template (donor) sequence, introduce a silent mutation into the protospacer sequence and/or PAM site, so that the repaired sequence cannot be recognized by the RNP. Otherwise the Cas enzyme may re-cut the repaired DNA, allowing formation of NHEJ-mediated indels in addition to the desired HDR mutation. For more information, see our HDR application note.

For Cas9, our DECODED articles contain additional tips for improving HDR efficiency for short donors and for long donors. For Cas12a (Cpf1), we have recommendations for improving HDR efficiency in this DECODED article.

HDR Enhancer

The IDT Alt-R HDR Enhancer V2 is a small molecule compound which blocks NHEJ, effectively tilting the balance of repair pathways in favor of HDR. The Alt-R HDR Enhancer can be used with either Cas9 or Cas12a systems and works in multiple cell lines, as described in this DECODED article.

Alt-R HDR Enhancer should not be confused with the Alt-R Electroporation Enhancers. There are two different Alt-R Electroporation Enhancers (one for Cas9 and one for Cas12a), but the HDR Enhancer is a universal reagent that can be combined with both Cas9 and Cas12a (Table 3).

Table 3. Comparison of enhancers.

Product	Alt-R HDR Enhancer V2	Alt-R Electroporation Enhancers
Type of molecule	Low-molecular-weight compound	ssDNA, not homologous to any sequence in rat, mouse, or human genomes
Intended purpose	To block NHEJ, effectively tilting the balance of the cell's DNA repair systems in favor of HDR.	To improve the overall amount of gene editing when electroporation is used as the delivery method.
Application	The Alt-R HDR Enhancer is usually recommended for HDR experiments no matter whether you use Cas9 or Cas12a.	There are 2 Alt-R Electroporation Enhancers: one for Cas9 and one for Cas12a. These enhancers are useful whether you want NHEJ or HDR.

Summary

Here is a quick review of what you need for your CRISPR experiment. First, you decide which area of the genome you are targeting and whether you will be aiming for simple knockout (via indel) or HDR. Then, you choose which Cas enzyme would be best for your experiment and which guide RNA to use. If you have decided to do HDR, you will design a donor DNA template, and you may opt to include the Alt-R HDR enhancer, which is beneficial in most HDR experiments. Once you have decided on your system, you will have to consider how to get it into the cells. For most applications, we recommend electroporation, in combination with the appropriate electroporation enhancer.

HOW DO I DESIGN GUIDE RNAs?

Designing guide RNA for Cas9

Designing a guide RNA for Cas9 may feel tricky or intimidating, but design tools can help. A good design tool uses optimized rules for maximizing on-target editing, while checking for (and minimizing) off-target effects, based both on experimental data and bioinformatics.

In the early days of CRISPR, design tools for guide RNAs were generously made available online by several labs. Some of these academic tools are still available, but their longevity and reliability is variable. Fortunately, free CRISPR guide RNA design tools are available at the IDT website.

How does the IDT gRNA design tool work?

The IDT guide RNA design tool can be used to design gRNA sequences for use with Cas9 with high on-target and low off-target activity in human, mouse, rat, zebrafish, and *C. elegans*. The tool is based on an on-target model, which was built using machine learning to predict whether a guide RNA design will work. The model was trained to predict Cas9 editing efficiency using >1400 features in 560 guide sequences. Predictive features include the composition and position of bases throughout the 20-nucleotide guide RNA sequence and the probability of self-hybridization between the crRNA and tracrRNA. High on-target scores indicate a guide is likely to work (working is defined as >40% editing efficiency). The model was validated using editing activity data for 215 guide RNAs with high on-target scores.

The IDT guide RNA design tool has 3 modes (accessible using the tabs at the top), as shown in Figure 11.

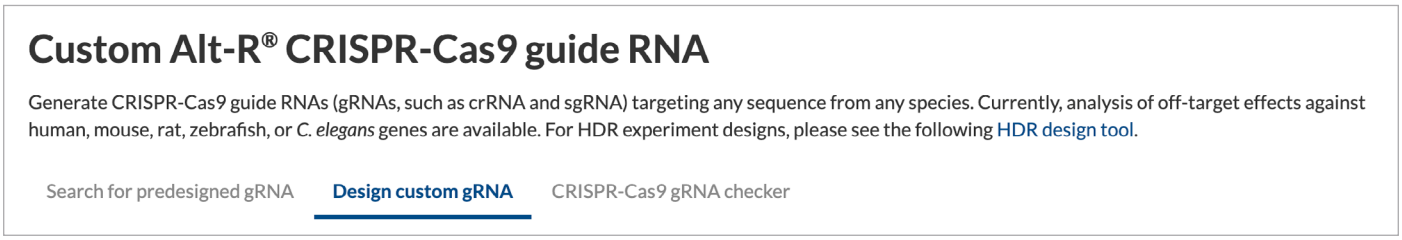


Figure 11. Screen capture of guide RNA design tool options. There are 3 options: "Search for predesigned gRNA," "Design custom gRNA," and "CRISPR-Cas9 gRNA checker."

1. **Predesigned gRNAs** (quickest and most convenient)

   A library of predesigned guide RNAs for 5 species can be accessed using the first tab. We have included CRISPR-Cas9 guide RNAs (such as crRNA and sgRNA) targeting human, mouse, rat, zebrafish, and *C. elegans* gene targets. You simply enter your gene symbol of interest; then, select the species you are working with and the number of designs you would like. The benefit of ordering predesigned crRNAs is that they are guaranteed to edit the target site with high efficiency. Still, we recommend testing 3 guides to ensure the best results.

2. **Custom designs**

   Custom guide RNA designs allow you to target any sequence from any species. The custom guide RNA design tool accepts FASTA-formatted target sequences, and the guide RNAs that are designed can be checked for potential off-target activity against any of the five supported species. You may also design against any other species not available in the tool; it will simply skip the off-target analysis in such a case. We recommend designing and testing three guides to find the best guide for your experiment.

Tip: How to use the custom-design mode to design gRNA

To design your custom gRNAs for Cas9 genome editing, first have the FASTA sequence for the region that you would like to edit. You can find this by performing a search for the ***genomic*** sequence of interest on the NCBI website, then select a portion of the genome (23–1000 bp) to use as input. We do ***not*** recommend starting with a ***transcript*** sequence, because of the risk that a returned guide RNA sequence will span a splice junction, which will not occur in the genome.

In the IDT design tool, select the desired species from the dropdown menu, copy and paste the FASTA sequence, then click "Design." The tool will return a list of the guide RNA sequences along with off- and on-target scores for each guide RNA. If the chance of off-target activity is too high, the tool will warn you (if you are analyzing a sequence from one of the 5 species mentioned above). The tool also will give you on-target scoring for a guide RNA sequence targeting any species and will warn you if the on-target score is low, indicating a risk of poor performance. Select a gRNA with the highest on- and off-target scores for optimal performance:

- A high on-target score is good. It means you are likely to have high editing efficiency at the target site.
- A high off-target score is *also good*. It means you are ***not*** likely to have many off-target effects in the cells. Off-target effects are what you want to avoid, so a high score is good.

3. **Design checker**

   The IDT Cas9 gRNA design checker tool allows you to assess on- and off-target potential of guide RNA sequences you have designed yourself, or have obtained from publications, before ordering. As with the custom-design mode, analyses of off-target effects against human, mouse, rat, zebrafish, and *C. elegans* genes are available, as well as on-target analysis for any species. You can copy the gRNA sequence into the IDT tool. The sequence must be 20 bases long and directly upstream (in the 5′ direction) of a PAM site (NGG, where N can be any nucleotide). The tool will return the off-target and on-target score for the sequence.

Other species: off-target analysis

If off-target analysis in the species of interest is not covered by IDT's design tools, you can find other guide RNA design tools currently available online from various academic research laboratories. We cannot guarantee the validity of the results from outside design tools. However, if you have questions about our tools, our support team is happy to help you. Email us at crispr@idtdna.com.

Important: You may notice that our ordering tool allows 19- and 20-nucleotide target sequences for use with Cas9. Our research has found that 19 nucleotides can provide similar editing efficacy to 20 nucleotides, but that 20 nucleotides is optimal. When using Alt-R *S.p.* HiFi Cas9 Nuclease, 20 nt protospacer sequences provide the greatest amount of genomic editing.

After using any of the 3 tool modes, scroll down the page to find your results. There's a convenient link to click and order your Cas9 guide RNA.

Designing guide RNA for Cas12a (Cpf1)

Simple instructions:

Look at your DNA sequence of interest. Find any PAM site close to your cut site. The PAM site is TTTV, where V is A, C, or G. Be sure to look at both strands of the DNA, because a PAM on either strand can work just as well. Then, go to our ordering page. Enter the 20–24 (preferably 21) bases downstream of the PAM site, in the 5′ to 3′ direction, when ordering your crRNA (Figure 12). And that's it!

Genomic target sequence	5′ …CATTCTTTGATACGCGATTCGACTAGGCATATCCTATTAGC… 3′
Correct ordering sequence	5′ ATACGCGATTCGACTAGGCAT 3′
Common errors	
Including PAM site	~~TTTGATACGCGATTCGACTAGGCAT~~
Entering reverse complement	~~ATGCCTAGTCGAATCGCGTAT~~
Entering reverse of target	~~TACGGATCAGCTTAGCGCATA~~
Entering as RNA	~~rArUrArCrGrCrGrArUrUrCrGrArCrUrArGrGrCrArU~~

Figure 12. Designing crRNA for Cas 12a. Both the correct sequence and some common errors are shown.

Detailed explanation of the simple instructions:

For crRNAs used with Cas12a, identify locations in your target region with a PAM sequence, TTTV, where V is A, C, or G. The PAM can be on either strand of the DNA sequence. If you are using Cas12a *Ultra*, some TTTT sites will work, although TTTV sites generally have a higher likelihood of efficient editing.

The Alt-R CRISPR-Cas12a crRNA is a single, 40–44 base guide RNA. It has a 20-base constant region (loop domain) and a 20–24 base target-specific region (spacer domain), which consists of the sequence 3′ of the TTTV PAM in the target DNA. The spacer recognizes (is complementary to) 21 nucleotides on the strand that does not contain the PAM site (Figure 7). We recommend a 21-base spacer domain for optimal activity. The cleavage site is specified by the spacer domain.

After you identify a 20–24 base target sequence, you can enter it into the ordering page. Then, 20 additional bases and the necessary modifications will automatically be added by our order-entry system for a total of 40–44 RNA bases. These additional bases and modifications are necessary to create a complete Alt-R CRISPR-Cas12a (Cpf1) crRNA. The system automatically converts the final sequence to RNA, so enter DNA bases into the ordering tool (Figure 12), but do not include the PAM site itself. Be sure to select either "L.b" or "A.s Cas12a" when ordering your crRNA as the fixed sequence varies slightly.

> **Fun fact about Cas12a**
>
> Cas12a does not require a tracrRNA. So you won't need to worry about this!

KNOCKOUT: HOW DO I DISRUPT A GENE IN A CELL LINE USING A CRISPR SYSTEM?

The first step in any CRISPR experiment is to decide which CRISPR system is best for your application. There are 2 main CRISPR systems to choose from: Cas9 and Cas12a. Each of these systems is made up of 2 central parts: the Cas enzyme, which is responsible for that all-important cut in the DNA, and the guide RNA, which helps the Cas enzyme find the right part of the genome to cut. When you have made these decisions, you can proceed with the experiment. For more information, see our reference guide.

CRISPR can be used to knock out a gene using the NHEJ repair pathway. As described earlier, NHEJ is an error-prone process, which usually results in indel formation. This typically produces a frameshift mutation, which in most cases will disrupt your gene of interest sufficiently to knock out expression (e.g., by introducing a premature stop codon).

Workflow overview of a knockout experiment

Step 1	**Note**: this step only applies to Cas9 experiments using a 2-part guide RNA: Mix your crRNA and tracrRNA together to form your guide RNA. Heat and slowly cool to form an annealed gRNA complex.
Step 2	Mix your Cas enzyme with your gRNA (when using Cas12a, this is only the crRNA) to form the RNP complex.
Step 3	Deliver your RNP complex to the cells or model organism.
Step 4	Allow the cells to incubate and grow (often this takes about 24–48 hours, depending on the cell type).
Step 5	Analyze the samples to determine the success of your genome editing.

KNOCK-IN: HOW DO I CORRECT A GENE MUTATION OR CREATE A FUSION PROTEIN IN A CELL LINE USING A CRISPR SYSTEM?

In cell lines, gene knock-in usually takes advantage of the intracellular HDR pathway, which can make a desired insert, such as a restriction site or a protein tag, introduce a SNP, or make other precise changes. Alternatively, some experimental systems use a new technology known as CRISPR prime editing, as discussed in this DECODED article. This handbook will focus on describing the HDR approach, which is currently more common than CRISPR prime editing.

High HDR rate is key

Although knock-in and knockout experiments have much in common, there are also important differences, such as the need for a donor template and the mechanism for DNA repair. As mentioned previously, NHEJ is good at disrupting the reading frame and silencing a gene, whereas HDR helps to ensure error-free editing and create functional, modified genes (e.g., introduction of a restriction site, protein tag, or single-nucleotide variant). A high rate of HDR is critical to mediate a successful repair in a bulk cell population, because DSBs not repaired by HDR will be repaired by competing mutagenic pathways such as NHEJ, as described earlier. As a general principle, efficient editing by NHEJ at a specific site is a great predictor of an efficient site for HDR. Here are 2 reagents that can improve HDR rates:

- HDR enhancer

  When a DSB occurs, HDR and NHEJ compete to repair it. The Alt-R HDR Enhancer V2 is a small molecule that increases the rate of HDR by blocking the NHEJ pathway, thereby biasing repair towards HDR. This is highly recommended for most HDR experiments. However, some delicate cell types may not respond well to this compound, so if in doubt, we recommend using appropriate controls such as a no-HDR enhancer control.

- Chemically modified HDR donors

  Another important step toward improving the HDR rate is choosing the right donor DNA. The addition of chemical modifications can help improve stability and overall HDR rates. In the case of the Alt-R HDR Donor Blocks, chemical modifications can also reduce unwanted repair events. Using an unmodified double-stranded template is associated with higher rates of undesirable outcomes such as blunt-end incorporation by NHEJ with duplication of the homology arms. This is described in more detail in our Application Note.

Recommendation: Use chemical modifications on HDR donor oligos

Phosphorothioate linkages (Figure 13) at the 5′ and 3′ ends of a short ssDNA donor act as stabilizing modifications that protect the donor from exonuclease activity. This protection means that more DNA is available for insertion, especially in cells such as iPS and Jurkat cells, which have high levels of nucleases.

Another improvement to HDR donor DNA is the addition of the IDT proprietary Alt-R modification, which further stabilizes the donor ssDNA, greatly increasing HDR.

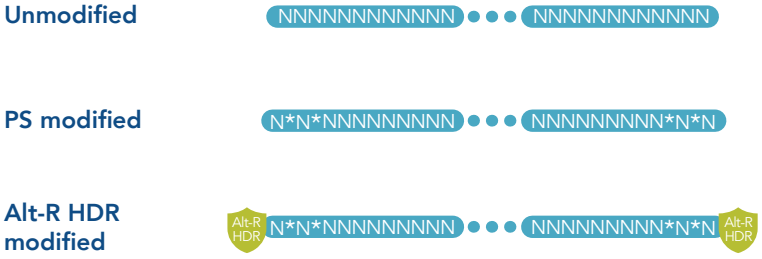


Figure 13. Chemical modifications of HDR donor oligos. Phosphorothioate and Alt-R modifications increase stability and improve performance.

N = DNA base
* = phosphorothioate linkage
Alt-R HDR = IDT proprietary end-blocking modification

Workflow overview of an HDR experiment

Step 1	**Note**: this step only applies to Cas9 experiments using a 2-part guide RNA: Mix the crRNA and tracrRNA together to form the guide RNA. Heat and slowly cool to form an annealed gRNA complex.
Step 2	Mix the Cas enzyme with the guide RNA (when using Cas12a, this is only the crRNA) to form the RNP complex.
Step 3	Deliver the RNP complex and **HDR donor** to the cells or model organism.
Step 4	Allow the cells to incubate and grow (often this takes about 24–48 hours, depending on the cell type). During this time, cells may be incubated with **HDR Enhancer V2** for 12–24 hours to boost the rates of HDR.
Step 5	Analyze the samples to determine the success of your genome editing.

HOW DO I ISOLATE CELLS THAT CONTAIN RNP?

First, isolating cells that have taken up RNP is not necessarily the same as identifying and isolating cells that have the mutation of interest. Some cells may not take up the RNP at all. Even when cells take up the RNP, you may or may not get 100% editing efficiency; that is, some cells with RNP may not undergo editing. And even when the cells with RNP show 100% editing efficiency, it is likely that not all cells will have the exact mutation you want. You may observe different indels occurring at different frequencies (often called the "indel profile" or "indel fingerprint"). If you are only interested in knockout by NHEJ, the exact mutation may not be important as long as your gene of interest is knocked out. However, if you want a specific change resulting from HDR, you may need to isolate cells with your specific edit.

Create a monoclonal cell culture: This results in a cell population with the precise edit you want (if this edited form exists among the cells).

How to do this: manually clone the cells in one of the following ways:

1. Grow cells in a large plate and isolate them with glass cloning rings. That is, the transfected cells are plated at a very low concentration so that they form colonies in little patches. Then, glass cloning rings, dipped in sterile vacuum grease, can be placed around the colonies to keep them separate.
2. Without using cloning rings, use a pipette tip to lift individual colonies from a large plate into the wells of multiwell plates.
3. (Recommended) Dilute transfected cells and systematically transfer them into 96-well plates with less than 1 cell per well. Then, grow the cells until you see colonies in some of the wells. The reason this is frequently the best method is because there is less possibility of cross-contamination between clones and less complexity in pipetting than the other methods. We have a detailed protocol for this technique here with a description of the pros and cons.

After obtaining monoclonal populations, grow the colonies until you have enough cells to split them to more or larger wells, and then use an analysis method (described later in this handbook) to determine which clones contain the mutation of interest.

CONTROLS

As you plan your experiment, we recommend including positive and negative controls, so your downstream analysis will be as informative as possible. Some of the most important controls are gRNA sequences. A positive control should lead to cutting of a known target site. A negative control should not specifically cut genome sequences. Other negative controls can include samples with only vehicle, Cas protein, gRNA, or cells, as well as any other negative controls that are appropriate in your particular experiment.

If you do not see editing of the target gene, the positive control gRNA can help determine whether the problem is with the design of the gRNA or something else. The negative controls help identify phenotypic changes that may be artifacts of the experimental conditions.

Cas9 controls

When you are using Cas9 with human, rat, or mouse cells, you can use one of the **Alt-R CRISPR-Cas9 Control kits**. These kits contain species-appropriate positive control crRNA, negative control crRNA, tracrRNA, and PCR primers for T7EI analysis of editing from control samples. You also have the flexibility to buy kit components separately if you already have some of the items in the kit. If using sgRNA, please use **Contact us** for the equivalent control sequences.

Cas12a controls

As previously mentioned, when the target has a TTTV PAM site, we highly recommend using Cas12a *Ultra*. This is such a potent enzyme that you might wonder if you really need any positive controls. Yes, even with this enzyme, you should plan to use positive controls. In particular, you will benefit by using positive control gRNA for titrating your Cas12a *Ultra* RNP. Too much Cas12a *Ultra* can be detrimental to HDR (see this **DECODED article**), so you should use an optimized amount of RNP (i.e., the minimum useful concentration) for your experiment. Positive and negative control gRNA target sequences for Cas12a for human, rat, and mouse genomes are listed on our **website**.

General advice for positive and negative controls

For both Cas9 and Cas12a, if the cells are from a species other than mouse, rat, or human, you may be able to design positive controls, based on previously published, successful gRNA sequences. For negative controls, design a gRNA sequence without homology to the genome of your model species, or at least use a sequence without a nearby PAM site.

HOW DO I ANALYZE GENE EDITING AT ON- AND OFF-TARGET LOCATIONS?

There are different ways to test the percentage of cells edited and to check on- and off-target editing. Here, we look at 3 methods: PCR-based assays, Sanger sequencing, and NGS.

PCR-based assays provide a quick overview of on-target editing

The T7 endonuclease I (T7EI) mismatch cleavage assay is used in the **Alt-R Genome Editing Detection Kit**. This assay is useful for a quick, qualitative assessment of genome editing efficiency in CRISPR-treated cells. T7EI is an enzyme that cleaves double-stranded DNA with small mismatches between the two strands, so-called heteroduplexes.

To execute the T7EI assay, we recommend isolating DNA 48 hours after addition of the RNP and amplifying the target locus using PCR. The PCR products are melted at 95°C and slowly cooled, creating a mixture of homoduplexes (between complementary wildtype sequences) and heteroduplexes (between strands of DNA from PCR products that contain wildtype or CRISPR-edited sequences) (**Figure 14**). The duplexes can then be digested with T7EI endonuclease to determine total editing.

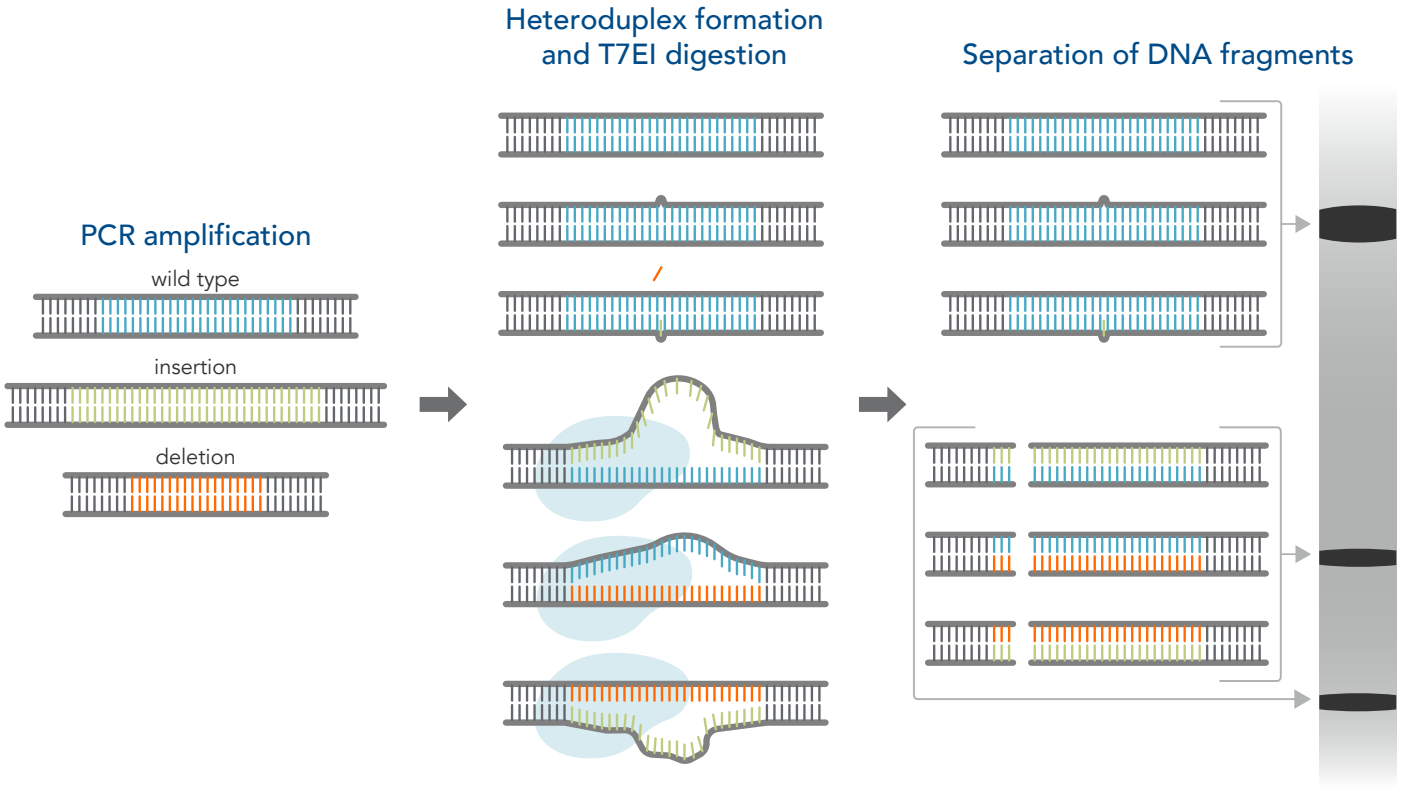


Figure 14. Overview of the T7 endonuclease I (T7EI) mismatch cleavage assay to determine total editing. (Left) The isolated DNA is amplified by PCR. (Center) After heating and cooling, the reannealed strands of the PCR products are treated with T7EI endonuclease (light blue oval), which recognizes mismatches that are ≥2 bases. (Right) Digested fragments are separated on a gel to visualize their different sizes. The sizes of the fragments should agree with your predictions, which are based on your experimental design.

Tip: How to design your T7EI assay

Design the PCR primers so that they amplify both the target sequence and flanking sequences on each side of the target. We recommend amplifying a region of 600–1000 bp with at least 100 bp at each side of the cut site. The cut site should be off-center so that the two digestion products have different sizes that are distinguishable on a gel.

The T7EI method is simple and fast and provides clean electrophoresis results. The T7EI assay is also compatible with a broad range of polymerases and PCR buffers, and does not usually require purification of PCR products before digestion. The disadvantages of the T7EI assay compared to sequencing are that it is not a quantitative method and that it cannot detect 1 bp indels, which means it underestimates CRISPR editing efficiency. It is noteworthy that in some cases T7EI may also give erroneous results [21]. Read more about the T7EI assay in this DECODED article.

Indel Detection by Amplicon Analysis (IDAA), another PCR-based assay, does not depend on mismatch cleavage, but instead uses capillary electrophoresis to separate PCR-amplified CRISPR repair products by size [22]. Again, this assay only provides indel sizes and cannot tell you what bases were deleted or inserted. IDAA is typically used for determining on-target editing efficiency alone, because scale-up to multiple loci is challenging.

Sanger sequencing assesses a single locus

Sanger sequencing is a straightforward and cost-effective method to check on-target editing at the single-nucleotide level at a single locus. This method also can be used to look at off-target activity, but it is hard to scale up to multiple loci in a way that would give meaningful off-target assessment. In addition, the population of edited cells will likely contain different sequences in the area of the targeted cut, because NHEJ introduces random indels in the cut site. Even if you use a monoclonal isolation method as described above, the clonal populations of cells may have different sequences at each of the corresponding alleles of a gene.

Various software programs are available online to analyze complex Sanger sequence traces representing samples of mixed edits. **T**racking of **I**ndels by **De**composition (TIDE) is a popular program that uses a Sanger trace deconvolution approach to calculate the insertions, deletions, and percent editing. However, this approach has its challenges, as insertions are represented as "N" bases, not specific bases. Also, the ability of the program to determine the length of an insertion is limited. Inference of CRISPR Edits (ICE) is similar to TIDE, as both use Sanger sequencing inputs that are deconvoluted to calculate the indels. The ICE and TIDE programs have limited capacity to determine the exact repair that has occurred, because they do not offer the same depth of sequencing afforded by next-generation sequencing technologies; therefore, Sanger-based methods are not as sensitive.

TIDE and other programs may be updated over time, so we recommend that you check recent literature for information about the best analytical programs for Sanger sequencing data. However, for greatest sensitivity and specificity, you will get the most complete and accurate picture of editing by using NGS, as described below.

Next generation sequencing: the gold standard for analyzing multiple edits

There are many reasons that NGS is the gold standard for analyzing CRISPR edits. The discussion below will highlight some of the most common methods that rely upon the robust sensitivity and specificity of NGS for 1) unbiased detection and nomination of off-target sites across the genome and 2) accurate evaluation of CRISPR editing events. A combination of assays rooted in this technology allows for an efficient, quantifiable, and comprehensive approach to measuring the levels of on- and off-target editing.

In general, NGS can be used to look at either the entire genome (whole genome sequencing) or a specific region of interest by targeted enrichment techniques (e.g., hybridization capture sequencing and amplicon sequencing). The most quantitative methods for CRISPR applications rely on targeted enrichment (typically involving amplicon sequencing), which is also the least labor-intensive and most cost-effective approach.

NGS is the only method that can fully characterize indel profiles. Amplicon sequencing by NGS can identify and quantify the frequency of the insertions and deletions that result from NHEJ following a CRISPR-generated DSB. Furthermore, with amplicon sequencing, you also can quantify the correct HDR events and determine what percent of the targeted alleles successfully underwent perfect HDR, thus defining a well-resolved picture of editing events at the target site. For this reason, NGS is superior to all methods used to analyze Sanger traces, including such methods as TIDE and ICE.

An additional advantage to amplicon sequencing is the ability to evaluate multiple genomic targets at a time—this can happen on 2 levels. First, individual samples are uniquely barcoded for deconvolution of individual experimental treatments, which allows you to combine hundreds or even thousands of samples on a single flow cell. Second, you can use IDT rhAmpSeq™ technology with Illumina sequencing platforms to generate multiplexed amplicons on a per-sample basis to investigate on- and off-target sites in a single reaction (see below).

Genome-wide NGS to determine off-target editing

Although it is possible to do targeted sequencing of the on-target edited site without knowing anything about off-target effects (OTEs), it is often critical to understand the OTEs in your experiment. Before you can properly do targeted sequencing to assess editing at off-target sites, it is necessary to identify potential sites of off-target effects. This is often called "nominating hotspots" and can be done effectively using unbiased techniques such as genome-wide NGS methods.

Many techniques have been and are being developed for empirical nomination of hotspots. These techniques include "breaks labeling and enrichment on streptavidin and sequencing" (BLESS) [23], DigenomeSeq [24], "circularization for in vitro reporting of cleavage effects by sequencing" (CIRCLE-seq) [25], "selective enrichment and identification of tagged genomic DNA ends by sequencing" (SITE-seq) [26], genome-wide unbiased identification of DSBs evaluated by sequencing (GUIDE-seq) [27], and "discovery of in situ Cas off-targets and verification by sequencing" (DISCOVER-Seq) [28]. The best method for your project depends on your experimental system and especially on which Cas enzyme you have chosen to use for genome editing.

When you use a Cas9 enzyme such as Alt-R Cas9 V3 or Alt-R HiFi Cas9 for genome editing, we recommend that you nominate the hotspots with GUIDE-seq [27], which is uniquely suited for the discovery phase of off-target identification. This method will generate a semi-quantitative assessment of the accumulation of on- and off-target editing across the genome. With GUIDE-seq, DSB sites across the genome are reported. There are controls included in the assays to mitigate false-positive detection of non-CRISPR breaks. In addition, there are guide RNA alignments to the nominated loci to help identify CRISPR-specific breaks. While GUIDE-seq provides a quantitative measurement of total reads aligned to a CRISPR-targeted site, the output does not perfectly correlate to the frequency of editing at each nominated site. However, quantification of Cas9-mediated edits at these sites can be accomplished with targeted amplicon sequencing (e.g., rhAmpSeq system, as described below).

With Cas12a genome editing, some of the above techniques for nominating hotspots may work better than others in your experimental system. DISCOVER-Seq may be a good starting point for nominating hotspots in many Cas12a experiments [28]. However, you may need to do empirical validation in your own experimental system to be confident about which technique you use for unbiased investigation of off-target Cas12a genome editing. Like with Cas9 experiments, once you have nominated the hotspots in your Cas12a experiment, we recommend targeted sequencing with the rhAmpSeq system.

rhAmpSeq targeted sequencing for analysis of on- and off-target editing

As mentioned above, nominated hotspot sites for off-target effects can be verified with a targeted NGS library prep method, such as the IDT **rhAmpSeq amplicon sequencing system**. The rhAmpSeq system utilizes primers that reduce non-specific amplification for characterizing editing events by amplifying the targeted site for sequencing after you have performed genome editing. You can generate amplicons of the targeted site and of the nominated hotspots in a single reaction, and then you use NGS to sequence the amplicons.

The rhAmpSeq rapid library preparation protocol allows amplification of up to 5000 targets in a single PCR sample. The method therefore enables simultaneous testing of multiple editing sites, both on- and off-target. The rhAmpSeq system enables verification of edits with reduced off-target amplification and can be used in conjunction with Illumina instruments. The resulting data can be analyzed using the rhAmpSeq CRISPR Analysis Tool.

rhAmpSeq technology relies on blocked primers that require enzymatic unblocking to initiate amplification (Figure 15). The enzyme which performs the unblocking step, RNase H2, gives high specificity to the amplification reaction.

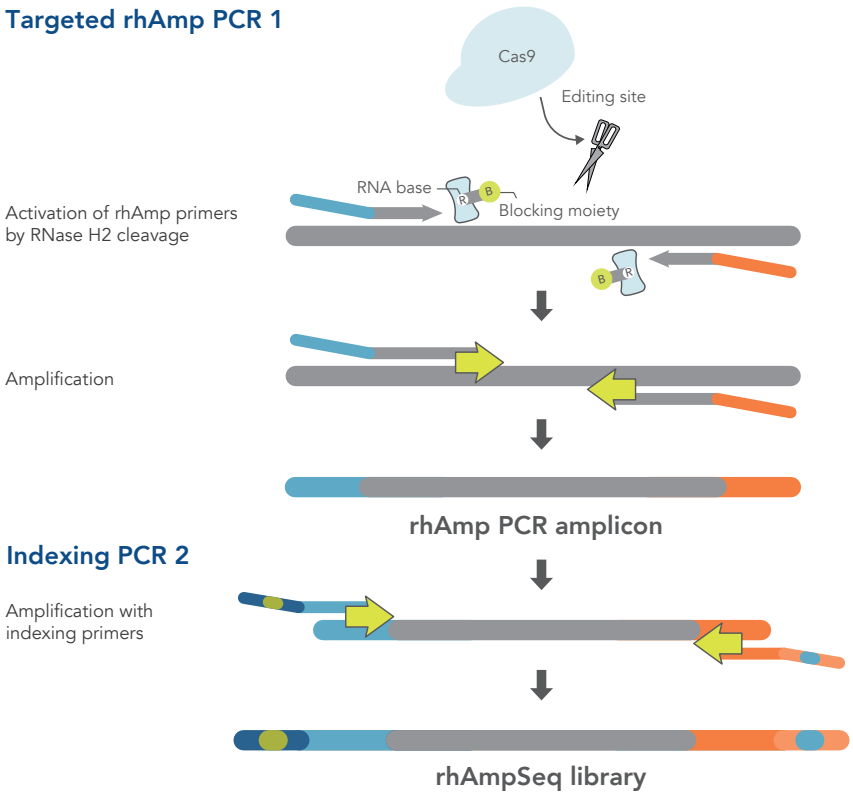


Figure 15. rhAmpSeq workflow after CRISPR genome editing. RNase H2 activates rhAmp primers by target-specific cleavage of the RNA base within the DNA:RNA duplex, removing a 3' blocker. RNase H2 activity is highly specific, thus reducing the amount of amplification from non-specific hybridization and primer dimers. Only activated rhAmp primers can be extended to generate target amplicons. Then, during Indexing PCR 2, Illumina sample barcodes and P5/P7 sequences are incorporated. The resulting rhAmpSeq library is then sequenced on an Illumina sequencer.

Summary: Two major steps in the analysis of on- and off-target sites

1. Use a validated method (e.g., GUIDE-Seq for Cas9 editing) to nominate hotspots.
2. Use targeted sequencing (e.g., rhAmpSeq system) to quantitate editing at both the on-target site and the nominated hotspots.

Rationale: All empirical methods have a false-positive rate. So, scientists often use the word "nominate" to allow for the possibility that some of the predicted sites are not real. In all cases, you want to validate nominated sites using an orthogonal, targeted technology. Amplicon sequencing allows you not only to validate off-target sites but also to quantify the on- and off-target editing events.

You can read more about NGS or our NGS products on our website. For additional questions or assistance, Contact us.

SUMMARY OF RELATED PRODUCTS

How can I increase the editing efficiency while reducing off-target effects in genome editing? IDT has optimized the Alt-R line of reagents to help you with this (Figure 16). Whether you choose Alt-R HiFi Cas9 or Alt-R Cas12a *Ultra*, you can be confident that you are getting our most recent genome editing methodology improvements with the Alt-R guide RNAs, Cas9 or Cas12a Electroporation Enhancer, HDR Enhancer, and controls.

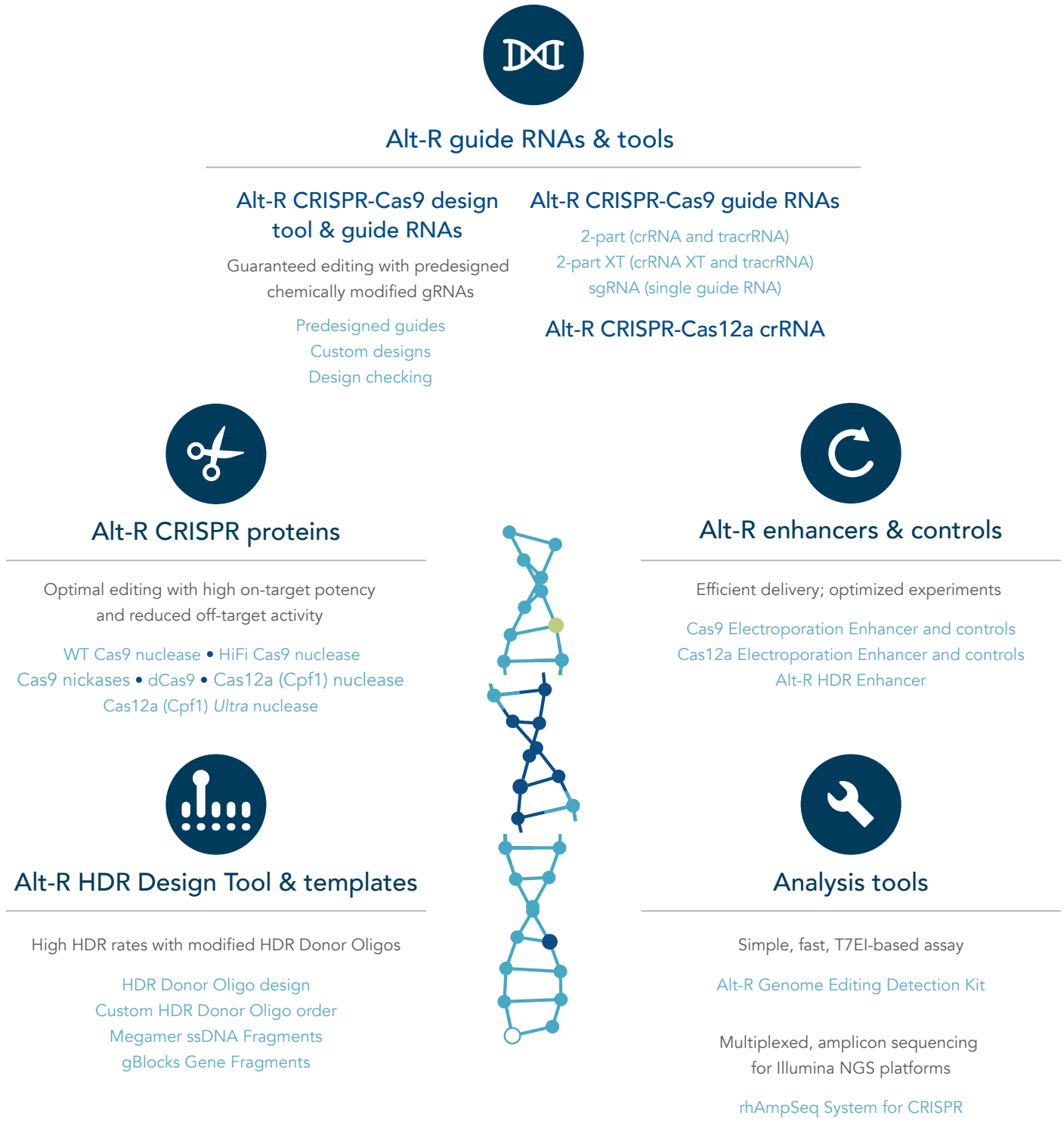


Figure 16. Overview of CRISPR genome editing reagents from IDT.

Custom CRISPR solutions

We are continually expanding our CRISPR product line, and we may have what you need. If you are interested in custom libraries, other CRISPR enzymes, formulations, or other CRISPR tools, email our CRISPR experts to discuss customized solutions for your research: CRISPR@idtdna.com.

For more information, visit www.idtdna.com/CRISPR.

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REVISION HISTORY

Version	Release date	Description of changes
2	September 2022	Revised document as per internal MAPSS review compliance
1	July 2020	Initital release.

The CRISPR basics handbook

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