



CRISPR 101

Your Guide to Understanding CRISPR

SYNTHEGO

Introduction to Genome Editing

Genome editing involves the deletion, insertion, or modification of specific DNA sequences in the genome. For many years, researchers had been trying to develop easy and cost-effective genome editing tools to address problems across a wide spectrum of fields. For instance, gene therapy in humans could progress rapidly if one could simply eliminate the gene responsible for a certain genetic disorder. In agriculture, manipulating plant DNA could be used to optimize crop yields and control plant diseases. Similarly, bacterial genomes could be fine-tuned to increase their product yields in several industrial applications.

Finally, the efforts of researchers paid off with the development of CRISPR, a robust molecular tool that can edit DNA at virtually any locus. CRISPR technology is igniting a revolution across the life sciences and is quickly becoming a standard tool in many labs. Given its ease-of-use and versatility, CRISPR is already being used for a variety of applications and holds a lot of promise for the future.

Read on for a crash course in everything you need to know about the fundamentals of CRISPR.

Genome Editing Tools Before CRISPR

Although CRISPR has now become synonymous with gene editing, it is not the first technology developed to edit DNA. Genome editing techniques initially emerged with the discovery of restriction enzymes and meganucleases. The possibility of precision gene editing was made clearer with the discovery of zinc-finger nucleases (ZFNs).

The ZFN method involves engineering an enzyme with both a zinc finger DNA-binding domain and a restriction endonuclease domain. The zinc finger domain is composed of 3-base pair site on DNA designed to target and bind to specific sequences of DNA, and the nuclease domain cleaves the DNA at the desired site. Although ZFN editing represented the first breakthrough in site-specific genome engineering, they have several limitations. In addition to exhibiting off-target effects, ZFNs are expensive and time-consuming to engineer. Furthermore, their inefficiency limits their practical application to only one genomic edit at a time.

Many years after ZFNs made their debut, a similar method known as transcription activator-like effector nucleases (TALENs) was developed. The TALENs method utilizes engineered enzymes containing a DNA binding domain and a separate DNA-cleaving domain, similar to the ZFNs method. However, TALENs have an advantage over ZFNs because they are more flexible; their DNA-binding domains can target a wider range

of sequences. Although they are easier to design than ZFNs, TALENs are expensive to produce.

An additional genome editing technique uses engineered restriction enzymes in concert with recombinant adeno-associated viruses (rAAVs). AAV is a non-pathogenic virus that infects mammalian cells at all stages of the cell cycle and integrates into the host genome at predictable sites. The AAV genome can be modified to target specific sequences in the host genome and integrate desired DNA sequences. However, the AAV approach also has several limitations. For instance, the vectors are difficult to produce and can only accommodate a small amount of genetic material.

Up until now, the field of genome engineering has provided researchers with a few gene editing technologies, all of which have limitations (Table 1). Because ZFNs and TALENs require complex protein-DNA interactions, they are challenging to design and manipulate. AAV vectors are also difficult to work with and have limited applications given their small packaging capacity. CRISPR, which relies on well-understood interactions between DNA and RNA, offers a far simpler way of editing genes and has completely changed the face of genomic engineering.

Table 1. Comparison of gene editing technologies.

	ZFNs	TALENs	AAV	CRISPR
Cost	High	High	Moderate	Low
Complexity	Difficult	Difficult	Difficult	Easy
Multiple Edits	Difficult	Difficult	Difficult	Easy

What is CRISPR?

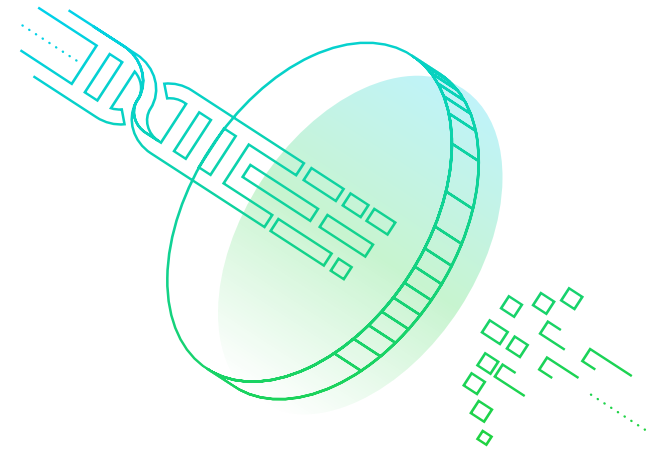
CRISPR is a simple two-component system that can be used to target a specific genomic sequence and then make a cut in the DNA. This powerful tool enables scientists to manipulate a specific gene of interest. For instance, one may knock out a gene (making it inactive) or knock in a particular sequence that repairs a mutation or adds a characteristic. Below, we describe how CRISPR was discovered and developed into a powerful genome editing tool.

The History of CRISPR

The foundational discoveries that led to the development of CRISPR-Cas9 technology can be traced back to 1993 when repetitive palindromic segments of DNA interspaced with other fragments of genetic material were identified in prokaryotes (1). These repeated segments of genetic code were named **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats, or CRISPR. However, the segments of DNA between the repeats were really the interesting part.

In 2007, after years of study, researchers concluded that CRISPR's function is related to prokaryotic immunity (2). It took the combined efforts of several research groups over the next 5 years to elucidate the underlying molecular mechanism behind the CRISPR system.

As it turns out, bacteria and archaea use the CRISPR-Cas9 system to defend themselves against invading viruses (called bacteriophages). Upon encountering a viral infection, the prokaryotic cell employs a special CRISPR-associated nuclease (Cas9) to snip-off a piece of viral DNA by creating a double-strand break (DSB) in its target loci.



How does the Cas9 protein recognize the target DNA? It is directed to the target sequence by a short RNA fragment known as a guide RNA (gRNA). The guide RNA is complementary to a segment of the viral genome, which allows Cas9 to cleave DNA with a high degree of specificity.

Not only does cleaving the DNA destroy the virus, but a fragment of foreign DNA, called a “spacer,” may be stored between the palindromic sequences of the CRISPR array as a way of retaining a genetic memory of past infections. If the virus were to re-invade, the CRISPR-Cas9 system could quickly target and destroy it. This library of viral fragments is thus essentially equivalent to our immune system, which stores antigens to prepare for future infections.

Once scientists figured out the mechanism of CRISPR in prokaryotes, it did not take long for them to realize the potential for engineering the genomes of microbes, plants, and animals. Today, CRISPR is utilized for a variety of applications and its adoption continues to increase in laboratories throughout the world.

CRISPR: *a set of DNA sequences involved in the prokaryotic immune system that has been adapted for genome engineering.*

The Components of CRISPR

The CRISPR system comprises two components: a **guide RNA (gRNA)** that is specific to the target DNA sequence and a **CRISPR-associated endonuclease (Cas)**. For CRISPR experiments, the guide RNA and Cas endonuclease are combined to form a ribonucleoprotein (RNP) complex.

The Cas protein functions as a pair of molecular scissors, while the gRNA is the GPS that guides it to the appropriate site. In prokaryotes, the gRNA guides the nuclease to viral DNA, but as a biotechnological tool, the design specifications of the gRNA can be altered to target any organism's genome at virtually any location.

For many genome engineering applications, the Cas9 protein (from *Streptococcus pyogenes*) is used. Binding of the gRNA to the genomic target is contingent upon the presence of a short **protospacer adjacent motif (PAM)** located directly downstream of the target (on the opposite DNA strand; Fig 1). The Cas proteins from different prokaryotic species recognize different PAM motifs. The PAM for Cas9 is 5'-NGG-3', where N is any nucleotide.

If a correct PAM match is made and if the gRNA successfully binds to the target, then Cas9 cleaves both DNA strands 3-4 nucleotides upstream of the PAM site. In nature, this short genetic element only occurs in invading viruses (not the bacterial genome), and thus ensures that Cas9 does not cleave its own CRISPR locus.

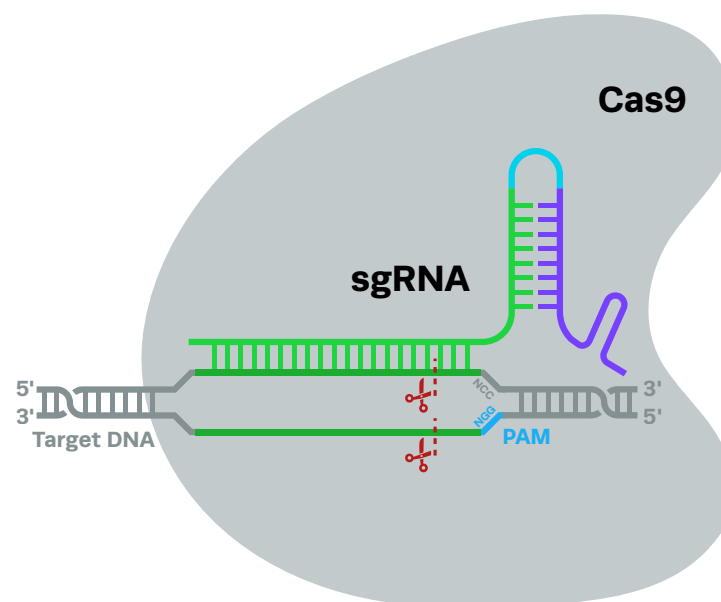


Figure 1. The CRISPR-Cas9 system.

The CRISPR-Cas9 system comprises a guide RNA (gRNA) and Cas9 nuclease, which together form a ribonucleoprotein (RNP) complex. The presence of a specific protospacer adjacent motif (PAM) in the genomic DNA is required for the gRNA to bind to the target sequence. The Cas9 nuclease then makes a double strand break in the DNA (denoted by the scissors). Endogenous repair mechanisms triggered by the double strand break may result in gene knockout via a frameshift mutation or knock-in of a desired sequence if a DNA template is present.

Guide RNA (gRNA): the RNA component of the CRISPR-Cas9 genome engineering tool. Contains a ~20 base pair sequence that is complementary to the genomic target.

CRISPR-associated endonuclease protein (Cas): a family of proteins that is able to cleave nucleic acids. Cas9 is the most commonly used Cas protein in CRISPR experiments.

Protospacer adjacent motif (PAM): a short sequence of nucleotides that must be present downstream of the target site in order for the nuclease to make a double strand break.

Guide RNA Formats

In its natural form, the gRNA consists of two distinct segments of RNA: CRISPR RNA (crRNA) and transactivating CRISPR RNA (tracrRNA). The crRNA is an 18-20 bp sequence that binds to the genomic target. The tracrRNA functions as a scaffold for the crRNA-Cas9 interaction. In natural contexts, guide RNAs form a duplex molecule, with the crRNA and tracrRNA segments annealed together (denoted as cr:tracrRNA). However, guide RNAs can now be synthetically produced with crRNA and tracrRNA connected by a linker loop. These seamless molecules are called a single guide RNAs (sgRNAs) (Fig 2).

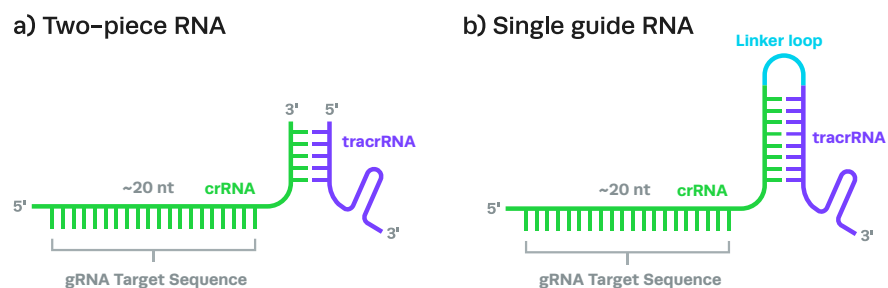


Figure 2. Two-piece and single guide RNA formats.

Guide RNAs can be constructed in two formats. a) the crRNA (green) and tracrRNA (purple) components may be annealed together to form a two-piece guide (cr:tracrRNA) or, b) they may be connected by a linker loop (blue) to form a continuous molecule (single guide RNA or sgRNA).

Creating Double-Strand Breaks

For many CRISPR-Cas experiments, Cas9 facilitates the gene editing process by creating a DSB in the DNA (Fig 3), similar to what occurs in bacteria and archaea. Generating a DSB is the initial step in the CRISPR editing pathway. The repair mechanism that follows will dictate the type of gene editing that occurs and will influence your desired editing outcome. In the next section we will explore two main types of repair: non-homologous end joining (NHEJ) and homology-directed repair (HDR).

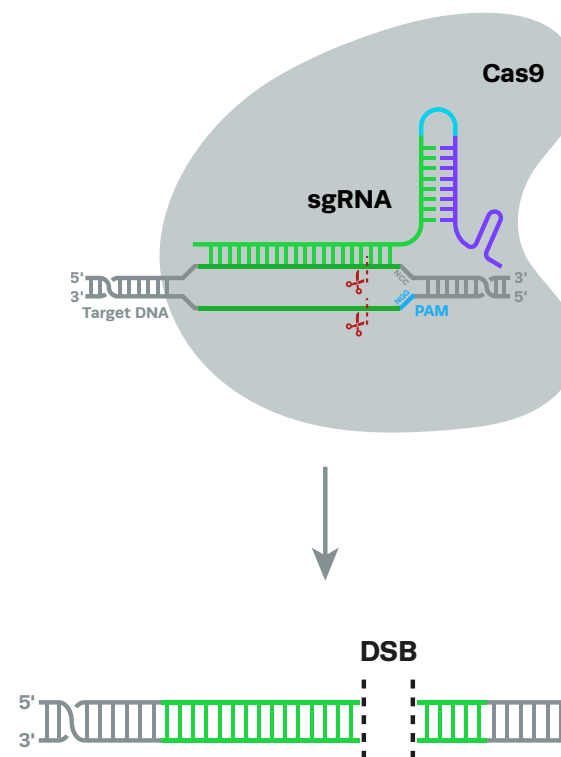


Figure 3. CRISPR-Cas components create a double strand break.

The sgRNA and Cas9 complex forms a ribonucleoprotein. The sgRNA binds to the genomic target and Cas9 makes a double-strand break (DSB) in the DNA.

Repairing CRISPR-Induced Breaks

As described above, CRISPR facilitates the generation of DSBs at specific locations in the genome. However, this action alone does not cause a gene to be edited. Rather, the process of repairing the break enables changes to be made to the target gene. After a DSB is made, innate DNA repair mechanisms are automatically triggered within the cell. Below, we will discuss two main types of repair pathways that are often used to edit genes: non-homologous end joining (NHEJ) and homology-directed repair (HDR).

Non-Homologous End Joining (NHEJ)

If the experimental objective is to permanently disrupt a gene so that no functional protein is made (a knockout), then the non-homologous end joining (NHEJ) repair mechanism can be exploited (Fig 4, left). NHEJ ligates the DNA ends back together, but it is prone to error and may insert or delete nucleotides (called **indels**) in the process. If the number of nucleotides inserted or deleted is not divisible by three, then it will induce a frameshift mutation and likely terminate the resulting protein's function.

Homology-Directed Repair (HDR)

If the objective of the experiment is to replace the targeted genetic element with a different sequence (a knock-in), the cell can be directed towards the homology-directed repair (HDR) pathway (Fig 4b). A **DNA donor template** bearing the desired sequence flanked by regions of homology must be introduced along with the CRISPR components to the cells. The cells will use this template to repair the broken sequence via homologous recombination, thereby incorporating the desired changes into the target region (Fig 4, right).

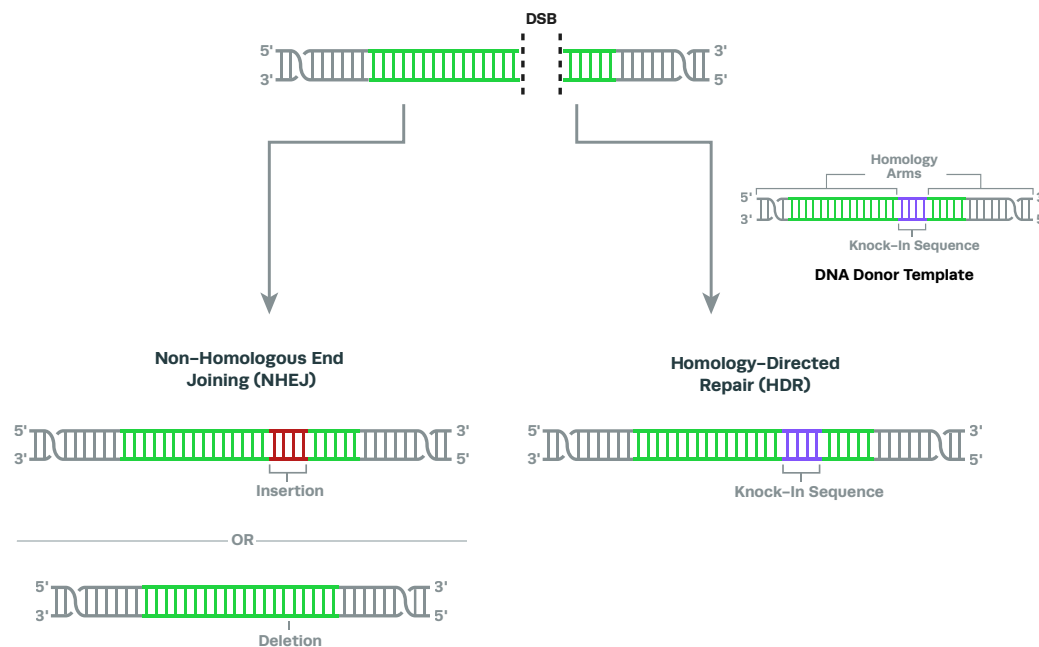


Figure 4. CRISPR repair mechanisms.

The two most common repair mechanisms facilitating CRISPR-Cas editing are non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ results in either insertions or deletions of nucleotides to repair the DSB. HDR repairs the DSB by incorporating a DNA donor template into the target sequence.

Indels: the insertion or deletion of nucleotides in the genome. Often caused by non-homologous end joining following a double-strand break in DNA.

DNA donor template: a specified DNA sequence desired to be knocked in to the genome that is flanked by homology arms to facilitate homologous recombination.

Components and Mechanism of CRISPR-Cas9 Editing

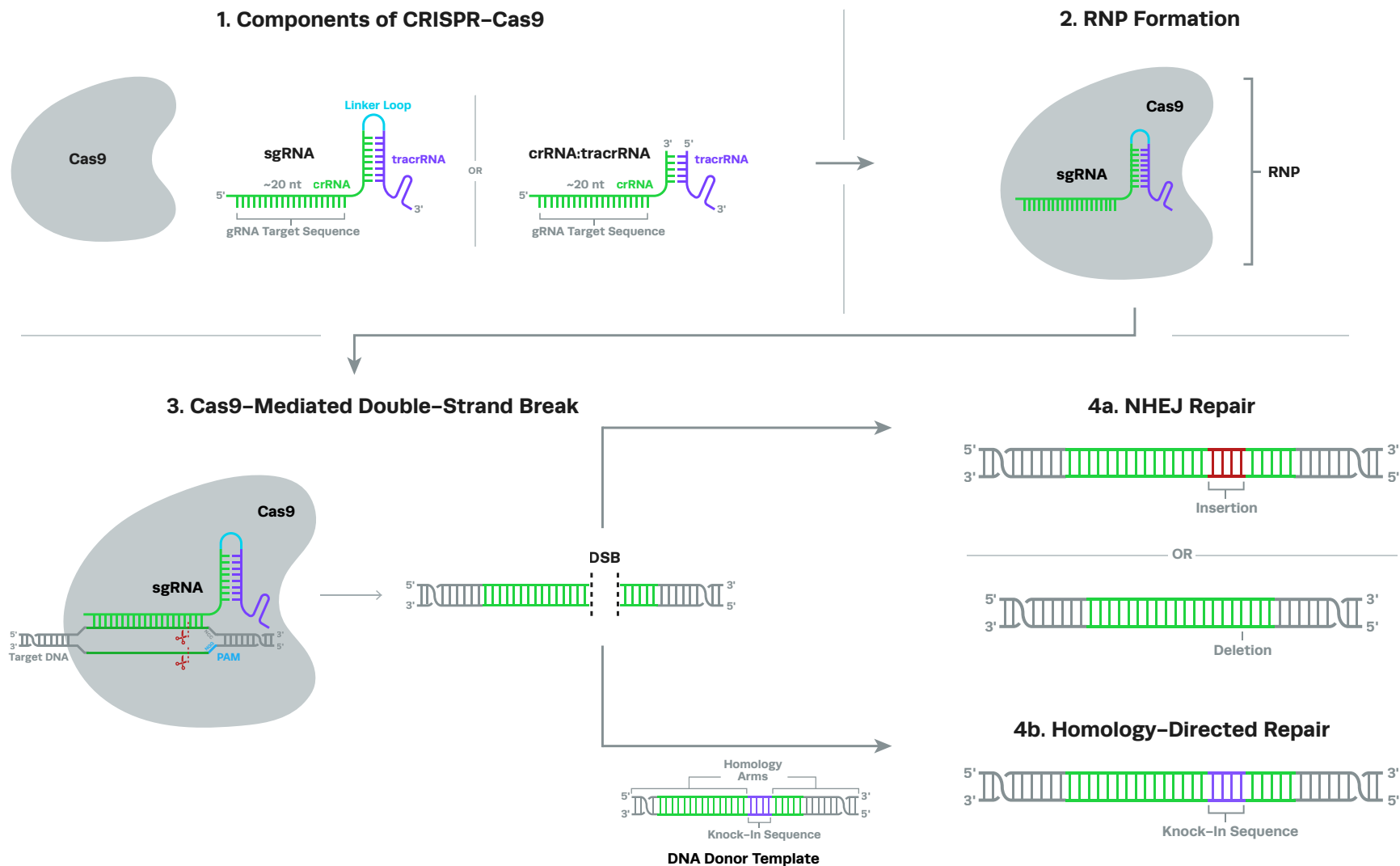


Figure 5. CRISPR-Cas9 genome editing.

1) The components of CRISPR are a guide RNA (either sgRNA or cr:tracr) and a Cas9 endonuclease. 2) The guide RNA and Cas9 form a ribonucleoprotein (RNP). 3) Inside the cell, the RNP creates a double-strand break (DSB) at the genomic target. One of two endogenous repair mechanisms may mend the break. 4a) Non-homologous end joining (NHEJ) repair is an error-prone pathway that often inserts or deletes nucleotides (indels). 4b) If a DNA template is provided, then the homology-directed repair (HDR) pathway may mend the break through homologous recombination.

What Can We Achieve Using CRISPR?

Once scientists realized the potential of CRISPR, the field of genome engineering rapidly grew. Since then, CRISPR has been used in a wide range of cells and organisms, including plants, fungi, and mammals. The technology has been used to manipulate genes in different ways, such as altering their nucleotide sequences or changing their expression. Figure 6 depicts some of the current uses of CRISPR technology. In this section, we will discuss these uses in greater detail.

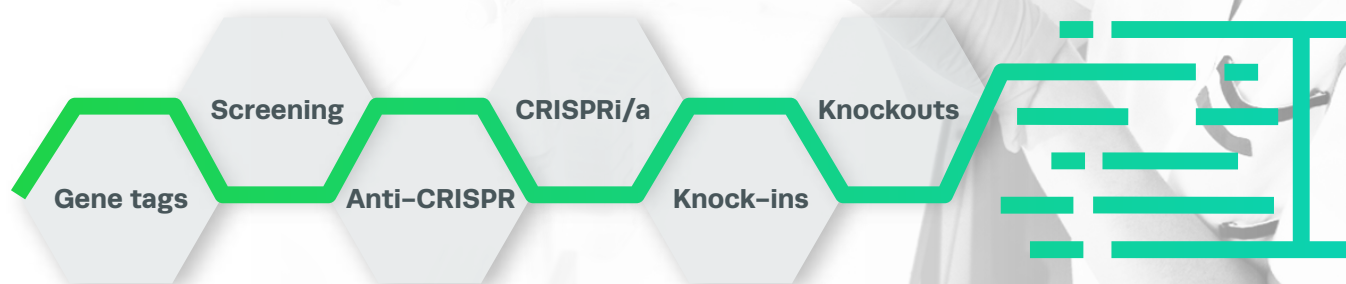


Figure 6. Current uses of CRISPR technology.

CRISPR technology is now being used in various methods other than the traditional gene knockouts and knock-ins. Advancements in CRISPR methods and research has given rise to technologies including CRISPRi and CRISPRa, anti-CRISPR proteins, CRISPR screens, and using CRISPR to tag genes for tracking and visualization.

Knockouts

Making a gene permanently inoperative (does not encode a functional protein) is called a **knockout**. CRISPR's ability to disrupt gene function relies on the error-prone nature of the NHEJ mechanism. As described above, indels that cause shifts in the reading frame of a gene will likely terminate the gene's function. This is especially true for frameshifts that cause premature stop codons.

A knockout can also be achieved using **multi-guide sgRNA** (multiple gRNAs that target the same gene). By making multiple simultaneous cuts in the DNA, the sgRNAs induce one or more large fragment deletions in target genes. Because these deletions remove several amino acids, they often render the targeted gene completely inoperative. Synthego's [Gene Knockout Kit v2](#) and [Screening Libraries](#) utilize this multi-guide approach to robustly knock out genes.

By purposefully disrupting the sequence of a gene (and corresponding mRNA and protein), researchers can elucidate the impact of the knockout on the phenotype of a cell or organism. This technique is thus useful for a variety of applications, including the identification and validation of potential drug targets, the analysis of cellular pathways, and the validation of antibodies.

To learn about how to experimentally make knockout cells, download our [Gene Knockouts eBook](#).

Knockout: *a mutation in a genetic sequence that causes it to be inoperative (i.e., no functional protein is made).*

Knock-in : *the integration of a foreign genetic sequence into a cell's genome.*

Multi-guide sgRNA: *multiple gRNAs designed to target the same gene and are introduced to cells simultaneously.*

Knock-ins

The incorporation of genetic material into a cell's genome is referred to as a **knock-in**. These edits are achieved by inducing cells to repair DSBs through HDR.

For HDR experiments, a DNA template containing the knock-in sequence flanked by regions of homology must be introduced into cells along with the CRISPR components. The template is used to precisely repair the severed target sequence, incorporating the knock-in sequence in the process. HDR enables countless genomic rewriting applications from introducing single point mutations to inserting selectable markers. While the HDR technique requires further refinement, researchers have already employed the method to correct a genetically-encoded mutation causing cataracts in mice (3), demonstrating proof of concept for HDR as a method for correcting genetically-based diseases.

Interested in doing CRISPR knock-ins but not sure where to start? Download our [Design and Optimization of CRISPR Knock-in Experiments Tips & Tricks](#) for recommendations and advice on how to design guide RNA and a DNA donor template for homology-directed repair.

Synthego's Tips and Tricks series provides useful information on how to enhance experimental techniques and protocols for CRISPR applications.

CRISPR interference (CRISPRi)

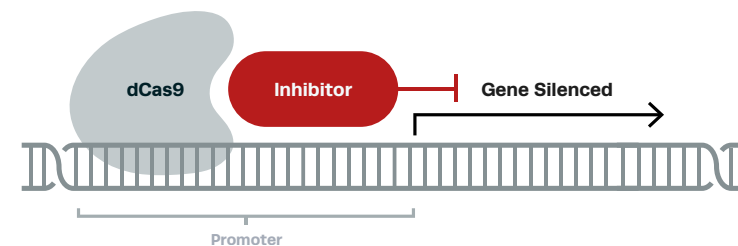
While gene knockouts can be achieved by disrupting a genetic sequence, gene expression can be repressed without altering the corresponding DNA. In 2013, Qi et al. made a Cas9 variant by mutating the endonuclease domains so that the enzyme no longer cuts DNA (called dead Cas9 or dCas9) (4). Researchers have used dCas9 to develop a technique wherein the CRISPR complex binds to its DNA target but does not cleave it. The binding of the dCas9 prevents the cell's transcription machinery from accessing the promoter, thereby inhibiting the gene's expression (Fig 7a). Fusing a transcriptional repressor domain (such as KRAB) to dCas9 allows reversible and fine-tuned reduction in gene expression.

In a nod to the precursor gene-silencing technique, **RNA interference (RNAi)**, the CRISPR silencing technique has been termed CRISPR interference, or **CRISPRi** (4). Whereas RNAi represses genes by destroying RNA transcripts, CRISPRi represses genes at the DNA-level. Compared to RNAi, CRISPRi is associated with higher efficiency, greater versatility, and fewer off-target effects.

CRISPR activation (CRISPRa)

While dCas9 endonucleases can be used to silence gene expression, they can also be modified to activate target genes (called CRISPR activation or **CRISPRa**). By fusing a transcriptional activator (such as VP64 or VP16) to dCas9, scientists are now developing systems that can overexpress genes of interest (Fig 7b). Polstein and Gersbach (2015) created one such system by fusing light-inducible proteins to mutated Cas9, causing gene expression to be activated in the presence of blue light and repressed in its absence (5). Other teams of researchers, such as Zalatan et al. (2015), are building more complex systems for multiplexed gene activation and repression at as many as three loci simultaneously (6).

a) CRISPRi



b) CRISPRa

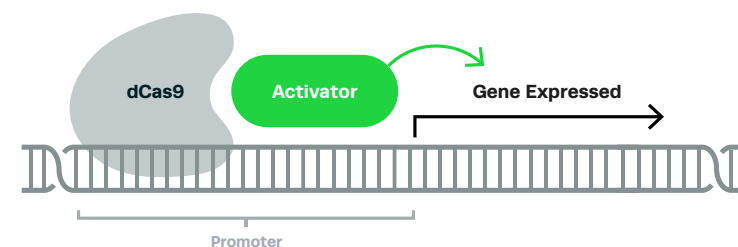


Figure 7. Mechanisms of CRISPR interference and activation.

CRISPRi and CRISPRa methods use catalytically dead Cas9 (dCas9) to alter the gene expression of targets without changing the genetic sequence. Depending on the transcriptional regulator used, gene expression can be a) inhibited or b) activated.

RNA interference (RNAi): a reversible and transient biological process whereby double-stranded RNA can inhibit gene expression.

CRISPR interference (CRISPRi): a technique using inactive Cas9 to silence or turn off gene expression without changing DNA sequence.

CRISPR activation (CRISPRa): a technique using inactive Cas9 to activate or turn on gene expression without changing DNA sequence.

CRISPR Screens

Another application of CRISPR is genome-wide functional screening. Until recently, RNAi was the primary approach for loss-of-function screens, where genes are systematically inhibited across the genome to determine their function. However, as mentioned previously, RNAi is plagued with problems related to low efficiency and high off-target effects. Now with CRISPR, gRNA libraries are being developed to knock out hundreds of genes in a single screen with high efficiency (Fig 8). Synthego now offers several [arrayed sgRNA libraries](#) that target one gene per well of a multiwell plate. These screens can be used for various applications, including the identification of potential drug targets.

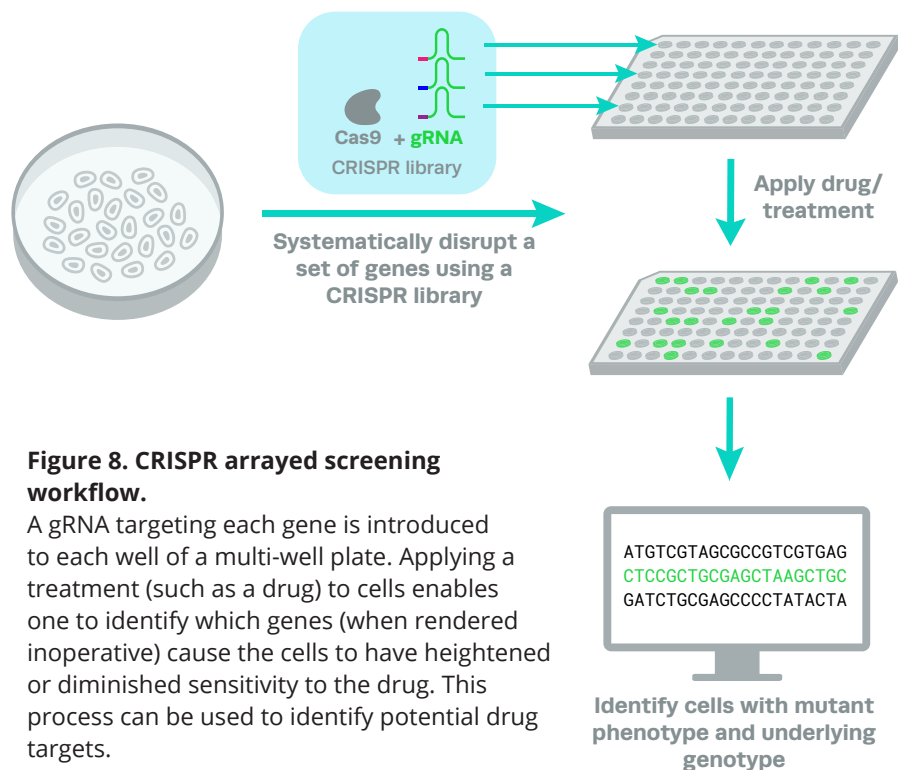


Figure 8. CRISPR arrayed screening workflow.

A gRNA targeting each gene is introduced to each well of a multi-well plate. Applying a treatment (such as a drug) to cells enables one to identify which genes (when rendered inoperative) cause the cells to have heightened or diminished sensitivity to the drug. This process can be used to identify potential drug targets.

Anti-CRISPR

Although the CRISPR-Cas9 system enables fine-tuning of genomic DNA, one downside is the risk of off-target effects (cutting DNA in the wrong place). One solution to this issue is to harness **anti-CRISPR** proteins that inhibit Cas9 activity. In nature, bacteriophages use these proteins to evade the CRISPR machinery of the prokaryotes. Pawluk et al. (2016) discovered anti-CRISPR protein inhibitors that were effective against Cas9 nuclease from *Neisseria meningitidis* (Nme). In fact, the team showed inhibition of the Nme-Cas9 activity in bacterial and mammalian cells using these anti-CRISPR proteins (7). Ultimately, this technology can be used to reduce editing errors. It turns out that adding anti-CRISPR proteins after editing takes place only partially reduces cleavage at on-target sites, but greatly reduces cleavage at off-target sites.

Gene Visualizations

The CRISPR system can also be used to visualize genomic regions. This is achieved by attaching **fluorescent proteins** (like green fluorescent protein; GFP) to dCas9 proteins or guide RNAs and using the CRISPR system to tag desired parts of the genome (8,9). This technology now enables scientists to visualize nucleic acids in real time, a process that previously was extremely difficult to perform.

Anti-CRISPR: Proteins capable of inhibiting Cas endonuclease activity by blocking target DNA binding or cleavage

Fluorescent proteins: proteins with bioluminescent properties commonly used to visualize cellular and molecular components.

Full Stack Genome Engineering

Synthego's Approach to CRISPR

At Synthego, we are providing support at every step of CRISPR experiment workflow, which involves three basic steps: **Design, Edit, and Analyze**. The end-to-end workflow represents a “full stack” approach to genome engineering.

Design

The first step of a CRISPR experiment involves designing the components and parameters you need for your particular experiment. You must design your gRNA and choose an appropriate Cas nuclease. Then, you will need to choose the format of the CRISPR components, select a transfection method, and optimize the transfection conditions for your cell type. These early steps will ensure that you obtain successful gene editing in your experiment.

Edit

Once you have designed your experiment and optimized your transfection conditions, you are ready to deliver your target-specific gRNA (and Cas nuclease) to your cells. Transfect your cells and allow CRISPR editing to take place.

Analyze

After editing has taken place, the targeted genomic sequences must be analyzed to assess the frequency and type of edits made. There are a number of analysis tools that evaluate genomic data and determine editing efficiency. Synthego's free analysis tool, [Inference of CRISPR Edits \(ICE\)](#), assesses Sanger sequence data. Protein and phenotypic assays can also be used to assess the effect of gene editing.

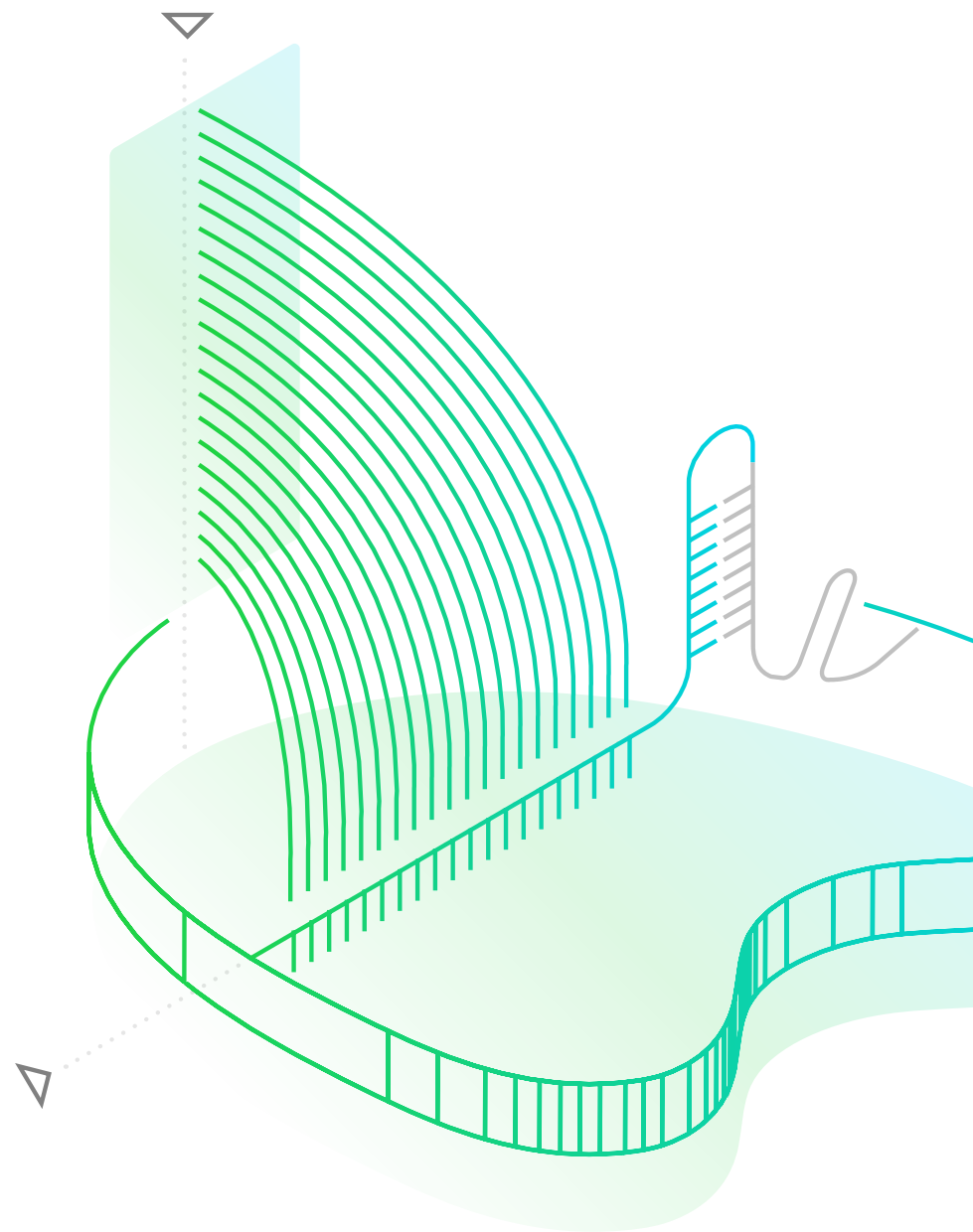
Looking to design, edit, and analyze your own CRISPR experiment? Please refer to our [How To Perform Successful CRISPR Experiments eBook](#) for more detailed instructions and specific experimental considerations you need to think about before starting.

CRISPR in the Future

CRISPR has received a lot of attention primarily due to its specificity and feasibility as a gene editing tool. Consequently, CRISPR has quickly revolutionized the field of genome engineering. The advances that CRISPR has facilitated in just the past few years are truly remarkable. Now, researchers have begun tinkering with the technology to unlock its vast potentials that go beyond the applications discussed so far.

Scientists are now using a modified version of CRISPR to explore epigenomics—the genome-wide set of chemical groups that adorn DNA and its associated histone packaging proteins. Previously, researchers were merely able to catalog the correlation between epigenetic markers and gene expression in cells. Now, a CRISPR complex that is capable of acetylating histone proteins at precise locations dictated by the complex's gRNA has been developed (10). Such technologies can shed light on the causal relationship between epigenetic markers and gene expression in the future.

CRISPR is also enabling the elucidation of large portions of the human genome, previously with unknown function. Scientists have long been trying to identify the location and function of 'non-gene' genetic elements that do not code for proteins but are thought to have important regulatory roles in expression. CRISPR is allowing researchers to knock



out these previously uncharted regions to study their role in the cell (11).

CRISPR is also playing an increasingly important role in the biomedical industry. The process of drug discovery is notoriously long, arduous, and expensive. CRISPR, however, is likely to expedite the pre-clinical stage of drug development. For instance, [CRISPR screening libraries](#) are now available to facilitate the discovery of new drug targets. CRISPR can also be used to develop accurate disease models to validate potential drugs. In parallel, research using CRISPR for *in vivo* and *ex vivo* therapies is on the rise. There is substantial optimism that gene editing will enable the development of better therapies and more personalized medicine in the near future.

CRISPR is not only paving the way for researchers to solve the most difficult of problems in the biomedical sciences, but it is also enabling advances in other scientific fields. CRISPR technology promises to deliver some truly stunning advances within the coming decades, particularly in relation to human therapeutics, agricultural biology, biofuels, and basic scientific research.

Additional Information

For an up-to-date list of all Synthego Application Notes and other resources, please visit:

[Synthego.com/resources](https://www.synthego.com/resources)

For technical assistance, contact our Scientific Support Team:

Ph: 888.611.6883

Email: support@synthego.com

About Synthego

Synthego is the leading genome engineering innovation company. The company's automated, full stack genome engineering platform enables broader access to CRISPR to accelerate basic scientific discovery, uncover cures for diseases, and develop novel synthetic biology applications.

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