SYNTHEGO

CRISPR 101

Your Guide to Understanding CRISPR

INTRODUCTION TO GENOME EDITING

Genome editing involves the deletion, insertion, or modification of the genome at a specific site in a DNA sequence. For many years, researchers had been trying to develop easy and cost-efficient 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 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.

CRISPR IS IGNITING A REVOLUTION

Genome Editing Tools Before **CRISPR**

Although CRISPR has now become synonymous with gene editing, it is not the first technology developed to edit DNA. Rather, the pioneers of the genome engineering field were 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 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.

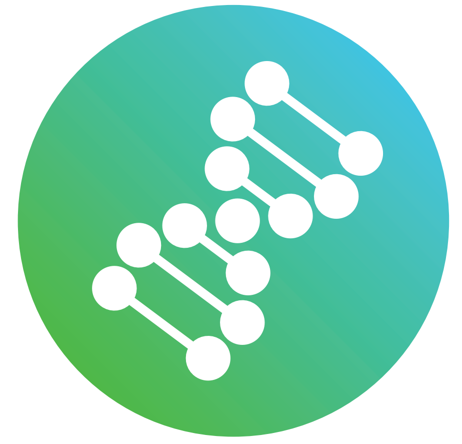
Many years after ZFNs made their debut, a similar method known as transcription activator-like effector nucleases (TALENs) was developed. The TALENs method utilizes engineered enzymes containing a DNA-binding domain and a separate DNA-cleaving domain, similar to the ZFNs method. However, TALENs have 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 modifications. 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 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, has offered a far simpler way of editing genes and has completely changed the face of genomic engineering.

Table 1 Comparison of Gene Editing Technologies

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



What is **CRISPR?**

History of CRISPR

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

In 2007, after years of studying CRISPR genetic motifs, researchers concluded that CRISPR's function is related to immunity. It took 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 that destroy the virus, but the fragment of foreign DNA may be stored between the palindromic CRISPR sequences 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:__ Clustered Regularly Interspaced Short Palindromic Repeats. A set of DNA sequences involved in the prokaryotic immune system and has been adapted for genome engineering.

What is CRISPR?

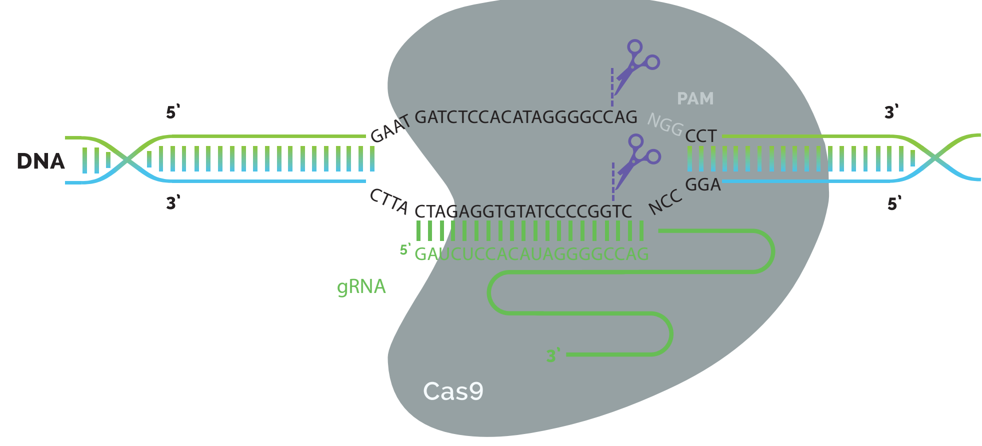


Figure 1. ***CRISPR Components.*** *The CRISPR-Cas9 system comprises a guide RNA (gRNA) and Cas9 nuclease, which together form a ribonucleoprotein (RNP) complex. The gRNA binds to the genomic target upstream of a protospacer adjacent motif (PAM), enabling the Cas9 nuclease to make a double-strand break in the DNA (denoted by the scissors).*

CRISPR Components

The CRISPR system comprises two components: a ***guide RNA (gRNA)*** that is specific to the target DNA sequence and a non-specific ***CRISPR-associated endonuclease protein (Cas9)***.

The Cas9 protein functions as a pair of molecular scissors, while the gRNA is the GPS that guides it to the appropriate site. In bacteria, the gRNA guides nuclease to viral DNA, but as a biotechnological tool, the design specifications of the gRNA can be altered to target the nuclease to cleave any host organism's genome at virtually any location.

Recognition of the the target DNA by the Cas9 enzyme is subject to the presence of a short protospacer adjacent motif (PAM) sequence located directly downstream on the untargeted DNA strand (Fig 1). If a correct match is made, 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.The PAM sequence varies for Cas9 proteins from different species: the PAM for the most widely used Cas9 from *Streptococcus pyogenes* is 5'-NGG-3', where N is any nucleotide.

In its natural form, the gRNA consists of two distinct segments of RNA: CRISPR RNA (crRNA) and transactivating CRISPR RNA (tracrRNA). The crRNA is complementary to the target DNA sequence, and thus recognizes the sequence to be cleaved. In nature, the RNA consists of sequences complementary to viral DNA. When used for gene editing, however, the crRNA can be programmed to target virtually any genetic sequence. The tracrRNA functions as a scaffold for the crRNA-Cas9 interaction. Guide RNAs naturally form a duplex molecule, with the crRNA and tracrRNA segments annealed together (crRNA:tracrRNA). Synthetically, they can be engineered as one seamless fusion sequence called a single guide RNA (sgRNA) (Fig 2).

Guide RNA (gRNA): *a programmable component of CRISPR, used to guide Cas9 to the targeted site of the genome.*

CRISPR-associated endonuclease protein (Cas9): *A nuclease from the bacteria, Streptococcus pyogenes, commonly used for CRISPR gene editing.*

What is CRISPR?

Repairing CRISPR-Induced Breaks

The magic of CRISPR is in its ability to cleave both strands of DNA. Cells must repair DSBs, or risk dying. Thus, all of the editing that comes from CRISPR is due to the cell's innate ability to repair itself. There are two kinds of repair pathways, each of which can be exploited to make desired edits (Fig 2).

Non-Homologous End Joining

If the objective of an experiment is to permanently disrupt gene function so that no protein is made (knockout), then one can exploit the cell's ***non-homologous end joining (NHEJ)*** repair mechanism. NHEJ binds the double stranded break 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 gene's function.

Homology-Directed Repair

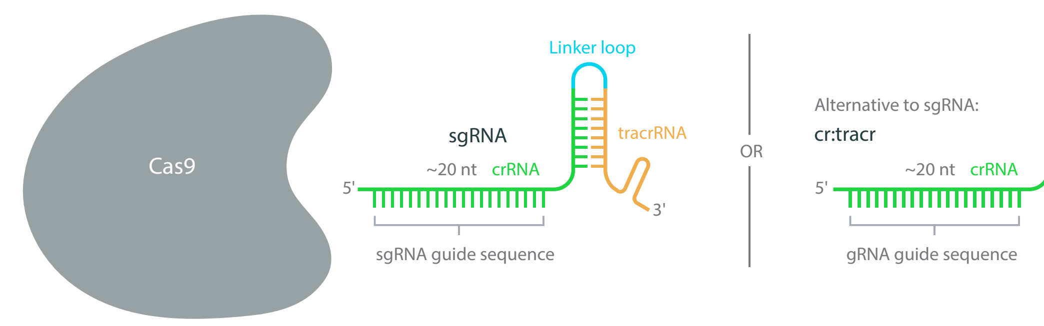
Alternatively, if the objective of the experiment is to replace the targeted genetic element with a different sequence (e.g., gene insertion, single-base editing, etc.), the cell can be directed towards an alternative repair pathway, ***homology-directed repair (HDR)***. To accomplish this, a homologous DNA template bearing the desired sequence must be introduced in the cell, along with the CRISPR components. A certain number of cells will use this template to repair the broken sequence via homologous recombination, thereby incorporating the desired edits into the genome.

Non-homologous end joining (NHEJ): *an error-prone cellular mechanism that repairs double strand breaks in DNA, but often inserts or deletes nucleotides (indels) in the process. Can be used to induce knockouts.*

Homology-directed repair (HDR): *a cellular mechanism that repairs double strand breaks in DNA by using a homologous DNA template. Can be used to induce knock-ins.*

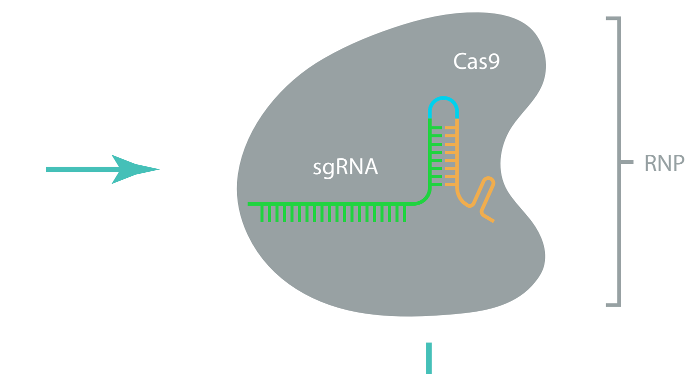
1. Components of CRISPR-Cas9

Cas9 protein and guide RNA (either single guide RNA (sgRNA) or cr:tracr)



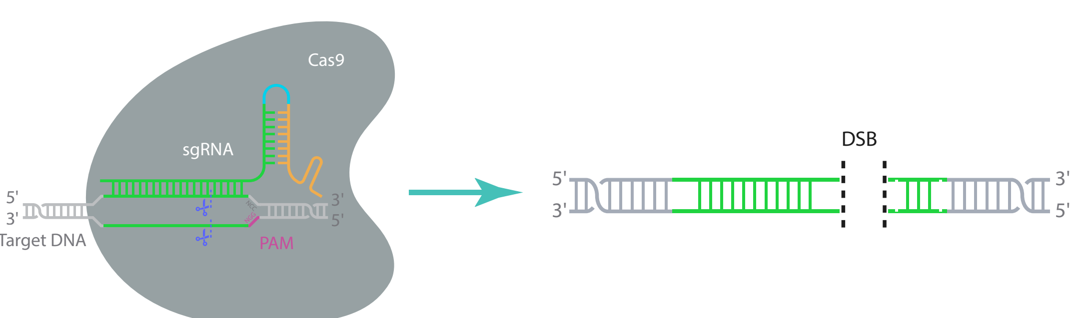
2. RNP formation

Cas9 and sgRNA form a ribonucleoprotein (RNP)



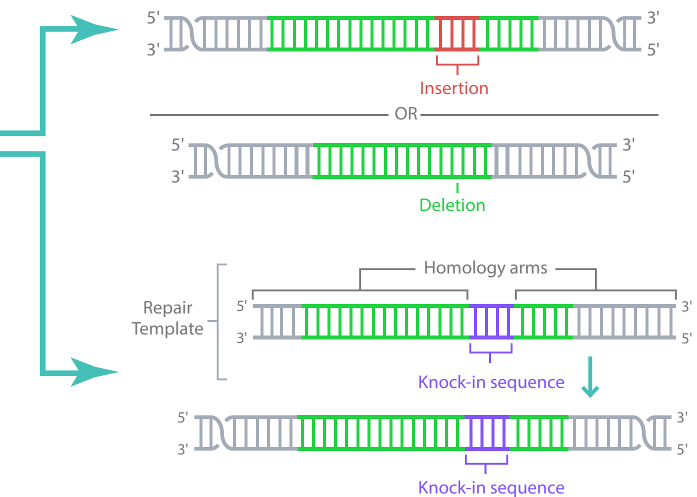
3. RNP-mediated DSB

Cas9 cleaves targeted DNA sequence, causing a double-strand break (DSB)



4a. NHEJ Repair

Error prone repair that leads to the insertion or deletion of nucleotides (indels) at the DSB, potentially resulting in a gene knockout.



4b. HDR

The addition of a DNA repair template with a new sequence flanked by homology arms leads to a knock in of the sequence into the genome.

Figure 2. 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 error-prone pathway that often inserts or deletes nucleotides (indels). If an indel causes a frameshift mutation, then the target gene may lose function (knockout). 4b) If a DNA template is provided, then the homology-directed repair (HDR) pathway may mend the break through homologous recombination. This pathway can be used to knock in a desired sequence of DNA.*

What Can We Achieve Using CRISPR

The field of genome engineering expanded quickly once scientists realized the potential of CRISPR. While mice have traditionally been the most popular model organism for transgenic experiments, CRISPR applications have also been demonstrated in a wide range of cells and organisms, including human embryos. In Figure 3, we have summarized the current techniques that use CRISPR-Cas9 technology.

Knockouts

The process of making a gene permanently inoperative (e.g., does not encode functional protein) is called a ***knockout (KO)***. 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 frameshift that cause premature stop codons. By purposefully disrupting the function of a gene, researchers can elucidate the impact of the knockout on cellular structure and function. In addition to knocking out gene function, CRISPR can be used to delete large DNA fragments from the genome. These genomic deletions can be achieved by using two gRNAs that direct Cas9s to simultaneously create DSBs at opposite ends of the DNA fragment to be excised. Scientists at Caribou Biosciences recently discovered that the size and nature of the errors made during NHEJ are in fact not random, but depend on the target sequence (1). This knowledge could allow us to further exploit the error-prone repair machinery to predict repair outcomes and edit DNA precisely.

To learn about how to experimentally make knockout cells, download our Cell Engineering 101 eBook.

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

What Can We Achieve Using CRISPR

Knock-ins

The incorporation of genetic material into a cell's genome is referred to as a ***knock-in (KI)***. CRISPR KI applications are achieved by inducing cells to repair breaks in DNA through HDR. To increase the likelihood of HDR, cells must be provided with copies of homologous DNA, which the cell then uses as a template to repair the severed target sequence. HDR enables countless genomic re-writing applications, from introducing single point mutations to inserting entire 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 (2), demonstrating proof of concept for HDR as a method for correcting genetically-based diseases.

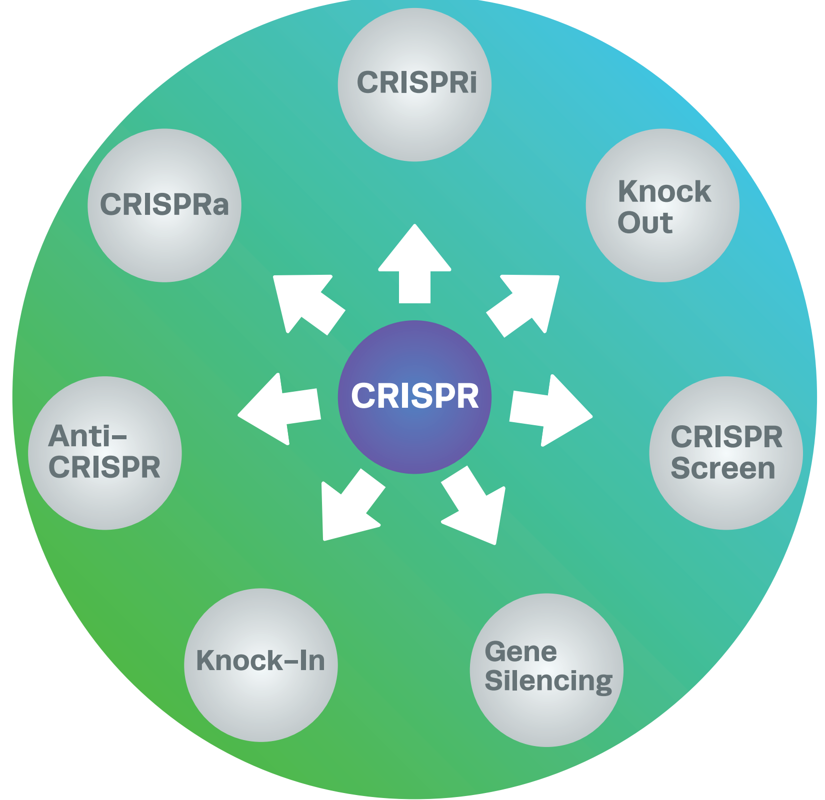
Knock-in (KI): *the integration of a foreign genetic sequence into a cell's genome.*

CRISPR interference (CRISPRi)

While genetic knockouts can be achieved by editing and disrupting a gene's constituent sequence, gene expression can also be tuned without altering the target sequence. By mutating the endonuclease domain of the Cas9 protein, researchers have created a system wherein the CRISPR complex binds to its DNA target but does not cleave it. The binding of the dead Cas9 (dCas9) interferes with gene expression by preventing the cell's transcription machinery from accessing the gene, thereby silencing its expression. Fusing a transcriptional repressor protein to dCas9 allows reversible and fine-tuned reduction in gene expression.

In a nod to the precursor gene silencing technique known as RNA interference, or RNAi, the technique has been termed CRISPR interference, or CRISPRi (3). Compared to other gene silencing techniques such as RNAi, CRISPRi is associated with higher efficiency, lower off-target effects, greater ease of design, and greater flexibility.

Figure 3. Current Techniques using CRISPR Technology



What Can We Achieve Using CRISPR

CRISPR activation (CRISPRa)

While "broken" Cas9 endonucleases can be used to silence gene expression, researchers have further modified the CRISPR apparatus to enable fine-tuned control over the activation of target genes (called CRISPR activation or CRISPRa). By complexing additional activator proteins to an inactive Cas9, scientists are now developing systems that toggle the expression of a gene on and off at their signal. Polstein and Gersbach 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 (4). Other teams of researchers, such as Zalatan et al., are building more complex systems for multiplexed gene activation and repression at as many as three loci simultaneously (5).

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, phages use these proteins to evade the CRISPR machinery of the bacteria. Recently, Pawluk et al. 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 and binding in bacterial and mammalian cells using these anti-CRISPR proteins (6). 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.

CRISPR Screens

Another application of CRISPR technology is in genome-wide functional screening. Until recently, RNAi was the primary approach for performing such screens, whereby genes are systematically inhibited across the genome in order to determine their associated function and phenotype. However, as mentioned previously, RNAi is plagued with problems related to low efficiency and high off-target effects. With the advent of CRISPR, genomic screening libraries are now being developed and applied to knockout thousands of genes in a single screen with high efficiency. Schmidt and colleagues recently developed a CRISPR screening library based on HEK293T cells that targets nearly every known protein-coding gene in the human genome and that they are making freely available to the research community (7).

Gene Visualization

The CRISPR system can also be used to visualize genomic regions. This is achieved by attaching fluorescent proteins (like GFP) to dead Cas9 proteins and using the CRISPR system to tag desired parts of the genome. (8,9).

Other Applications

The CRISPR system has more applications than those mentioned above. For instance, multiplex genome engineering using CRISPR allows encoding multiple guide RNAs in a single CRISPR array to simultaneously edit several genes or delete large sections of genomic DNA (10,11).

CHOOSING THE RIGHT CRISPR COMPONENTS

The design of gRNA and choice of the nuclease depends on the desired application. The right choice of components is crucial for a successful experiment.

Designing the Guide RNA

The guide RNA is the programmable component of the CRISPR-Cas9 system that directs the Cas9 nuclease to the target site. Therefore, it is important to ensure that the guide sequence yields minimum off-target (unintended) cuts while also providing maximum on-target activity.

Stringency of additional parameters for RNA sequence design further varies with the intended applications. For instance, knock out procedures rely on cells forming frameshifts in the DNA during repair of the DSB by NHEJ. The faulty reading frame disrupts transcription of the gene, and thereby downstream protein coding. These experiments offer some flexibility in terms of sequence selection and target location of the guide RNA. Nevertheless, the following criteria should be met when designing guide RNAs for KO experiments:

- Choose guide sequences that target exons that are present in all transcript variants.
- Choose sequences at the 5′ end of the gene or that encode essential protein domains, as these are more likely to interfere with protein function.

For knock-in, CRISPRa, and CRISPRi experiments, gRNAs have additional requirements. For knock-in experiments, the template DNA should be carefully checked for PAM sequences to ensure that it is not treated as a target. For CRISPRa/ CRISPRi applications, the gRNA sequence should be complementary to the promoter regulating the target gene, rather to sequence of the gene itself

Design Tools

Several online tools are available to help design guide RNAs by predicting their on-target and off-target activity. We have listed some of these below:

- **Synthego CRISPR Design Tool**
  Synthego's free CRISPR design tool is one of the fastest and most efficient design tools available for researchers. The tool offers easy design of synthetic sgRNAs with up to 97% editing efficiency and the lowest off-target effects. It includes a library of more than 100,000 genomes and 9,000 species, and offers a convenient way to order your guides within the tool. You can also use the tool to validate gRNAs designed using other platforms.
- **MIT CRISPR designer**
  The MIT CRISPR designer is another free platform that helps users design gRNAs with high target selectivity and low off-target effects.
- **sgRNA Scorer 2.0**
  The sgRNA scorer 2.0 from the Dr. George Church's lab identifies target sites for gRNA from an input DNA sequence. The tool currently allows selection from six different nucleases, and predicts the activity of Cas9 nucleases derived from *S. aureus* and *S. thermophilus* 3 especially well.
- **Benchling**
  Benchling's cutting edge design tool conveniently integrates other tools so that scientists can use a single interface for designing their experiment. While the basic plan is free, users need to pay fees for accessing their Startup and Enterprise plans.

CHOOSING THE RIGHT CRISPR COMPONENTS

SELECTING A NUCLEASE

Several types of nucleases are now available for CRISPR applications. Selecting one depends on the specific goals and limitations of the experiment. The four nucleases described below facilitate many types of genome editing projects. As additional nucleases are discovered, we can expect CRISPR editing to become even more flexible and more powerful than it is today.

S. pyogenes Cas9 (SpCas9)

The Cas9 nuclease from the bacteria *Streptococcus pyogenes* (SpCas9) is the most commonly used nuclease in CRISPR genome engineering assays. This nuclease recognizes the PAM motif 5'-NGG-3' (N signifies any nucleotide) and creates a DSB with blunt ends at the target site. It was the first nuclease engineered for CRISPR editing and is used for a variety of knockout and knock-in applications.

Cas9 Nickase

Cas9 nickase is a modified version of the Cas9 protein. Instead of creating a DSB, this nuclease nicks a single DNA strand. Because a nick in each DNA strand is needed to create a DSB, there are typically fewer off-target effects relative to using an unmodified Cas9 nuclease. Additionally, this nuclease makes a staggered cut that leaves long overhangs instead of a blunt ends at the cut site. This enables more control when inserting a DNA segment for HDR repair. For this reason, Cas9 nickase is often used in knock-in experiments.

Cpf1

Another common nuclease is Cpf1, short for 'CRISPR from Prevotella and Francisella after the bacterial species from which it originates. Cpf1 functionally differs from SpCas9 in three key aspects: 1) Cpf1 recognizes and binds to the PAM motif, 5'-TTN-3'. It therefore can be a better choice for targeting DNA regions with high AT-content than Cas9. 2) Cpf1 creates a staggered double-stranded (overhangs), rather than the blunt-end cut generated by its SpCas9 counterpart. Thus Cpf1 is preferred for experiments relying on the HDR repair outcome. 3) Cpf1 is a smaller protein than SpCas9 and does not require a tracrRNA. Thus, the gRNA required by Cpf1 is shorter in length and cheaper to generate than the gRNA required by SpCas9.

S. aureus Cas9 (SaCas9)

Cas9 from *Staphylococcus aureus* (SaCas9) is an ortholog of the Cas9 family. SaCas9 recognizes the a PAM sequence of 5'-NNGRRT-3'. However, the SaCas9 protein is much smaller than SpCas9, the sequences that encode them differ by length of about 1kb. Its small size allows SaCas9 to be packaged into an AAV vector for cellular delivery, an approach that is difficult with the large SpCas9.

Different Formats of CRISPR Guides

A CRISPR genome editing experiment can be orchestrated in several different ways. The essential CRISPR machinery (gRNA and Cas9 protein) can be delivered in one of the three formats: plasmid DNA, *in vitro*-transcribed RNA, or ribonucleoprotein (RNP) complex.

DNA

One approach is to deliver CRISPR components into the cells as plasmid DNA. This approach requires a researcher to clone both the Cas9 protein and desired gRNA DNA fragments into a plasmid, and then introducing the plasmid into target cells. Commercial plug-and-play plasmids are now available for this purpose, allowing researchers to insert a gRNA sequence with their design specifications.

The first step involves designing the DNA template to encode the gRNA and Cas9 protein.Once the DNA template sequence has been designed, an oligo can be ordered and cloned into a plasmid. Engineering a CRISPR plasmid follows the same protocol as a typical cloning assay. The DNA template insert must be amplified, digested, and ligated with the plasmid before being transformed into cells. After screening for the recombinant plasmid and verifying its sequence, the plasmid can be delivered to the target cell.

Overall, the process of preparing a custom CRISPR plasmid in-house consumes 1–2 weeks of time before the actual editing assay can be undertaken. In addition to the excessive time investment, the plasmid approach is also prone to o -target e ects due to the continual presence of Cas9 within the cell. Plasmids also run the risk of integrating into genome of the host cell in places that cause cytotoxicity. This is particularly problematic in applications related to human medicine and crop engineering.

RNA

The CRISPR components can be delivered into the cells in RNA format: gRNA and RNA encoding the Cas9 protein. The RNA can be derived in the following two ways.

In vitro transcription (IVT)

The RNA can be enzymatically transcribed from the corresponding DNA outside the cell in a process called in *vitro transcription (IVT).* The first step is same as that in plasmid cloning: design the DNA template based on the target sequence within the gene of interest. In this case, the template must additionally include an upstream

Different Formats of CRISPR Guides

promoter site (typically T7) for the RNA polymerase to use during transcription.

Once the DNA template has been obtained, it is then transcribed into a gRNA using one of multiple IVT kits available on the market (containing enzymes and reagents). After purification, the gRNA can be co-transfected into the cell alongside Cas9 mRNA. Alternatively, it can also be complexed with Cas9 protein and delivered to the target cell as a ribonucleoprotein (RNP). The IVT approach requires only 1-3 days to prepare the gRNA for a CRISPR assay.

Nonetheless, the approach remains labor-intensive and is not scalable for multiple CRISPR targets. In addition, IVT-derived gRNAs are prone to mistakes made by enzymes. For instance, RNA polymerase may insert the wrong nucleotide, or make a guide sequence too long or short. This may not only lead to highly variable editing efficiencies but also increase the possibility of off-target effects.

Synthetic guide RNA

A convenient substitute to the IVT-derived guide RNA involves synthetic polymerization of high quality RNA (up to 120-mer) sequences. These can either be in the form of separate crRNA and tracrRNA fragments that must be annealed together, or as seamless single guide RNA (sgRNA). Of these two forms, sgRNA generally produces a higher editing efficiency by avoiding the inefficient annealing of the two-piece system, and the tendency of tracrRNA fragments to form tetramers that interfere with the Cas9 protein.

Until very recently, the cost associated with producing synthetic sgRNA — even short, oligomeric strands — was high enough to make synthetic sgRNA a prohibitive expense for many laboratories. However, recent developments in technology enable affordable access and successful deployment of the CRISPR technology. One such advancement lies in Synthego's automated and scalable production methods, which allows these sgRNAs to be generated much more rapidly and at a much lower cost than traditional synthesis methods. The high-throughput synthesis technique also results in higher fidelity sgRNAs, as compared to IVT-derived guides, enabling more efficient and reproducible targeting of the desired genomic site.

In addition, Synthego's synthesis platform enables the chemical modification of sgRNAs. Such modifications protect the sgRNAs from degradation by exonucleases and intracellular immune responses within the cell. Synthetic gRNA may be co-transfected with Cas9 mRNA or they may be introduced with Cas9 protein as ribonucleoprotein complexes, as described below.

Ribonucleoprotein (RNP)

Deploying gRNA with Cas9 protein as ribonucleoprotein (RNP) complexes is the most effective strategy, as researchers have demonstrated higher editing efficiencies and fewer potential off-target effects using RNPs as compared to other delivery methods (11). Moreover, as the RNP exists transiently inside the cell (not inserted into the genome), there is low risk of cytotoxicity.

It has become clear that the RNP format is the most effective option for carrying out CRISPR gene editing assays and other applications, especially in embryos.

Different Formats of **CRISPR Guides**

Table 2. Comparison of Cost, Time, and Labor Associated with Different Guide RNA Formats.

	CRISPRevolution Modified sgRNA	*in vitro*-transcribed (IVT) sgRNA	Plasmid DNA
FLEXIBILITY	✓ stem cells, ✓ primary cells, ✓ cell lines	✗ stem cells, ✗ primary cells, ✓ cell lines	✗ stem cells, ✗ primary cells, ✓ cell lines
PREPARATION STEPS	3	5	5
PREPARATION TIME	LOW	HIGH	MEDIUM
NUMBER OF CLONES TO SORT	FEW	MANY	MANY
EDITING EFFICIENCY	CONSISTENTLY HIGH	VARIABLE	VARIABLE
TOTAL COST TO GENERATE A KO	$	$$$	$$$

DELIVERING CRISPR COMPONENTS INSIDE CELLS

Several different transfection methods can be used to deliver CRISPR components to cells (Table 3). The method used largely depends on the cell type and format of the CRISPR components.

Lipid-Based Delivery

In a lipid-based delivery system, cationic lipid reagents facilitate delivery of biomolecules into cells. This method is high-throughput, has low cytotoxicity, and is applicable in various cell types. Traditionally used for delivering nucleic acids in cells, lipid-delivery method has recently been optimized for delivering RNPs

Electroporation and Nucleofection

Electroporation enables delivery of the CRISPR machinery in cell types that are difficult to transform using lipid-based delivery systems. Application of a controlled, short electric pulse to the cells forms pores in the cell membrane, allowing entry of foreign material. Nucleofection is a variant of electroporation, in which the electric pulse is optimized such that the nuclear membrane of the cells also forms pores. The CRISPR components are thus directly delivered inside the nucleus.

Microinjection

Microinjection is commonly used to inject the Cas9 and gRNA ribonucleoprotein complex in embryos, although it can also be used in cells. Zebrafish, mouse, and most recently human embryos have been manipulated using this technique.

Table 3. Comparison of CRISPR Transfection Methods

	LIPID-BASED DELIVERY	ELECTROPORATION	NUCLEOFECTION	MICROINJECTION
PRINCIPLE	Lipid complexed with genetic material fuses with the cell membrane	Electric pulse forms pores in the cell membrane for entry of DNA/RNA/RNP	A electropotation-based optimized for nuclear deliver Pre-optimized for each cell type/	Microneedle injects CRISPR components inside cells, oocytes, or zygotes
ADVANTAGES	Cost-effective High throughput	Easy, Fast High efficiency	Easy, Fast High efficiency	High efficiency
LIMITATIONS	Less efficient	Requires optimization	Requires reagents & equipment	Time-consuming, Technically demanding, Low throughput
CELL TYPES	Few	Numerous	Numerous	Few

Analysis of **CRISPR Editing**

After transfecting the cells, the efficiency of DNA editing using CRISPR needs to be determined. The easiest way to do this is to Sanger-sequence the edited and control DNA at the cut site and a software analysis program, such as Synthego's Inference of CRISPR Edits (ICE) tool or Tracking Indels by DEcomposition (TIDE). Synthego's ICE tool is a free and easy to use program that will give results within minutes. From the ICE report, one can find out the overall editing efficiency, the specific indels generated, and the percentage of that the indels will likely cause a knockout.

Although purely qualitative and somewhat outdated, DNA mismatch detection assays are also an option. This approach involves the treatment of the CRISPR-edited DNA and the non-CRISPR edited DNA (control) with an enzyme that cuts DNA at mismatched sites. Gene deletions by the CRISPR system often result in mismatched bases during DNA repair. Thus, the CRISPR-edited DNA shows multiple small fragments after size-based separation in a gel, while the control shows a single band of uncut DNA. This method is a simple and crude way of estimating the CRISPR editing efficiency.

Laslty, next generation sequencing (NGS) is a gene sequencing method that can be used for accurate and quantitative analysis of the editing efficiency of CRISPR. It also provides additional information regarding off-target edits in the DNA. However, NGS is an expensive option, especially when Synthego's ICE tool gives NGS-quality results for free.

CRISPR IN THE FUTURE

CRISPR has received a lot of attention primarily due to its ability to genetically edit living organisms. However, while this side of CRISPR occupies the spotlight, 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 catalogue 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 (12). 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, the function of the vast majority of which is unknown. 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 (13).

CRISPR is not only paving the way for researchers to solve the most difficult of problems in the life sciences, but it is also enabling the scientific community to explore dimensions of the genome that we've been unable to study up until this point. Due to its adaptability across a wide range of species and its simplicity of use, CRISPR-Cas9 has quickly revolutionized genome engineering. CRISPR-Cas9 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.

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