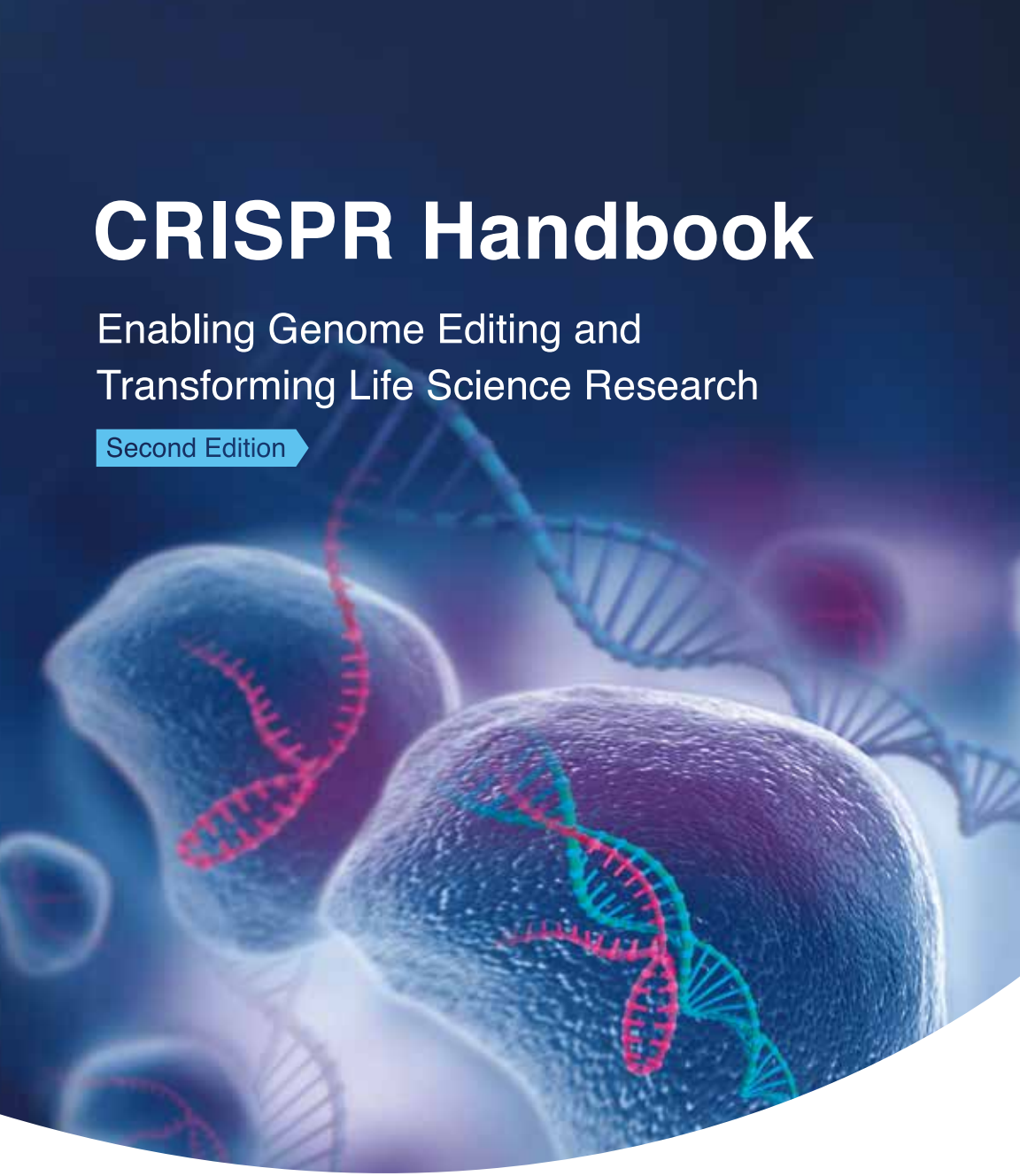
CRISPR Handbook

Enabling Genome Editing and Transforming Life Science Research

Second Edition



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*Updated September 2016 to include new CRISPR library and ribonucleoprotein technology and workflows.

GenScript
Make Research Easy

CRISPR Reagents and Services from GenScript

CRISPR Plasmids

Validated all-in-one, dual, non-viral and viral vectors for Cas9 & gRNA constructs

CRISPR RNA/Cas9 Protein

Synthetic custom crRNAs/tracrRNAs and Cas9 proteins for high specificity gene editing.

CRISPR Library Screens

Broad Institute designed GeCKO v2 and SAM libraries for genome-scale loss-of-function and gain-of-function screens. Pathway-focused gRNA libraries for molecular pathway screening.

Mammalian Cell Line Gene Editing

CRISPR gene editing to generate knock-out mammalian cell lines.

Microbial Gene Editing

Bacterial and yeast gene knock-in/out editing using CRISPR technology.

Genome-wide gRNA Databases

Search for Broad Institute pre-validated gRNA sequences in humans and mice using our free online database.

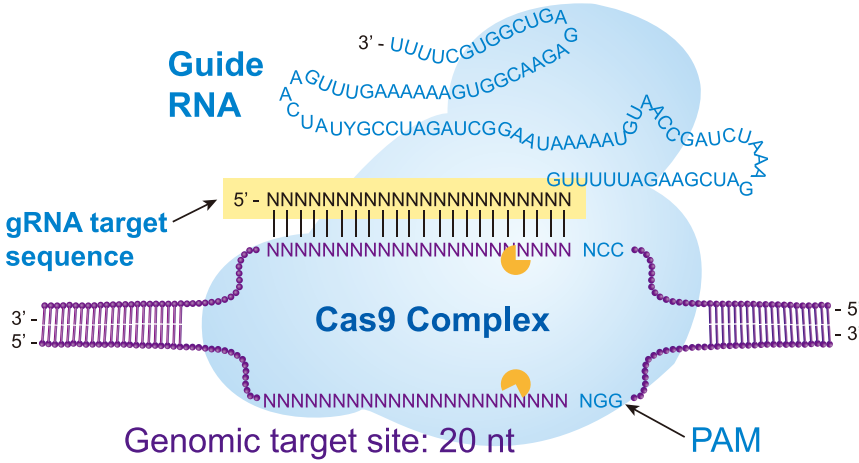
gRNA Sequence Design Tool

Construct your own gRNAs using our free online design tool developed by the Broad Institute. Sequence design is compatible with humans, pigs, dogs, rabbits, mice, rabbits, chickens, zebrafish, C. elegans, D. melanogaster, and more species.

To download this handbook as a PDF, please visit

www.genscript.com/CRISPR-handbook.html

Overview of CRISPR/Cas9 Technology



The guide RNA (gRNA) complex consists of a target recognition sequence and tracrRNA motif, which recruits the Cas9 complex to the target sequence. The protospacer adjacent motif (PAM) sequence is defined as NGG for S. pyogenes-derived Cas9. Cas9 will cut 3-4 nucleotides upstream of the PAM sequence.

Double-stranded breaks (DSB) on target sequence activates repair pathways in a cell. Non-homologous end joining (NHEJ) causes random insertion or deletions at the site of repair. This results in frameshift mutations which cause the gene to no longer be expressed, thus resulting in a knock-out. Homologous recombination (HR) can allow a piece of exogenous DNA to be integrated, or “knocked-in” to the break site, thus allowing for the addition of an exogenous sequence, or a precise point mutation on the targeted gene.

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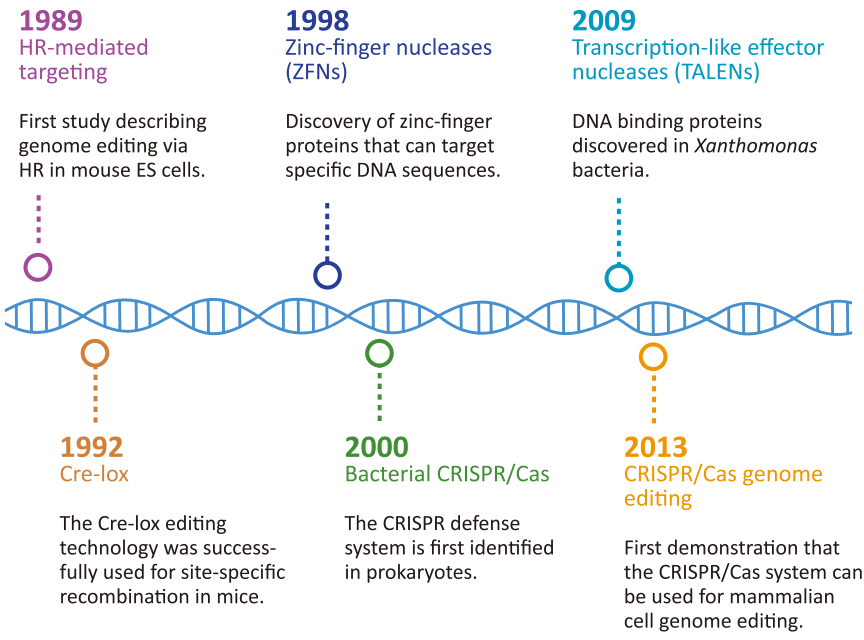
The CRISPR Genome Editing Revolution

The ability to manipulate DNA is a biologist's greatest tool – making it possible to define the relationship between a gene, its mRNA and protein products, and their functions. By inhibiting gene function or altering gene expression, genome editing can provide tremendous insight into the basis of disease or identify new targets for medical intervention. Over the last 20 years, advances in genome editing technologies have evolved to allow precise genome manipulation in cell lines and animal models. Of these new technologies, perhaps the most exciting is CRISPR/Cas9, a gene editing system adapted from the bacterial immune system that is efficient, rapid, and easy-to-use. CRISPR/Cas9 technology allows targeted knock-in and knock-out of any gene within the genome. In this handbook, we will discuss how CRISPR technology has fueled a genome editing revolution and how it has been adapted for other biological applications.

Evolution of Genome Editing Technology

Genome editing technology allows for the direct manipulation of the genetic code, giving researchers the ability to delete, insert or replace DNA. While the

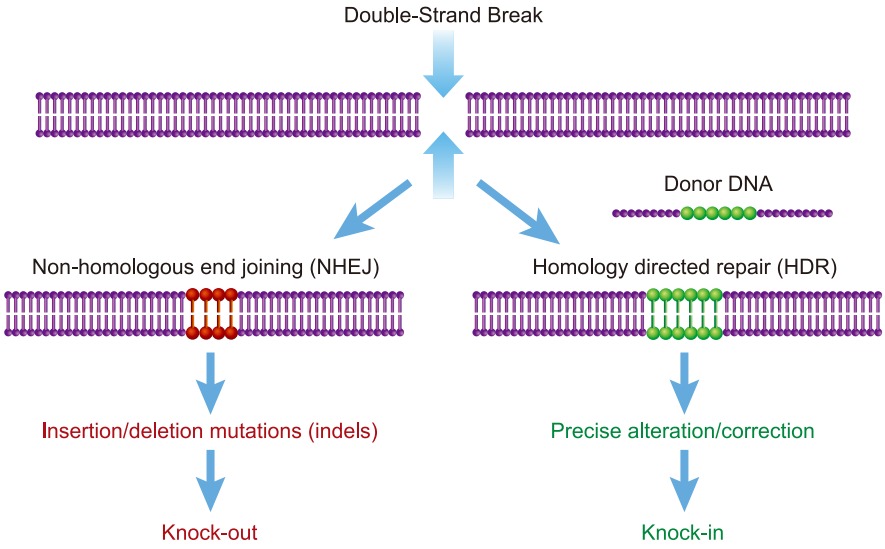
Figure 1: Advancements in genome editing



use of genome editing was first widely popularized in the early 2000s, the concept of genome editing was originally born more than a decade earlier. Gene editing was first used in 1989, when researchers targeted specific genes in mouse embryonic stem cells using homologous recombination (HR) to generate knock-in and knock-out mutations[1] (Figure 1). Since HR occurs infrequently in mammalian cells, the initial targeting frequencies were low (1 per 3×10^4 cells); however, this work provided a foundation for how genes can be targeted and altered.

While HR alone rarely results in gene integration in mammalian cells, the introduction of double-strand breaks (DSBs) into the genome can increase recombination rates significantly[2]. DSB resolution occurs by either or error-prone non-homologous end joining (NHEJ) or homology directed repair (HDR) (Figure 2). If no donor DNA is present, resolution will occur by NHEJ, resulting in insertion or deletion mutations (indels) that will ultimately knock-out (KO) gene function. Alternatively, if donor DNA sequences are available, the DSBs will be repaired by HR, resulting in gene knock-in (KI)[3].

Figure 2: DNA repair by targeted genome editing



As the need for relevant animal disease models rose, so did the need for more sophisticated and efficient genome editing tools. Cre-Lox technology came into use in the early 1990s, contributing greatly to the development of transgenic mouse models[4,5]. The Cre-Lox system allows scientists to control gene expression both spatially and temporally, using a site-specific DNA recombinase Cre, which

recognizes 34-bp loci called *loxP*[6]. Cre-mediated recombination leads to the knock-out of targeted genes between *loxP* sites. But while easier to control than homologous recombination, the Cre-Lox system becomes less efficient as the genetic distance increases between *loxP* sites[7].

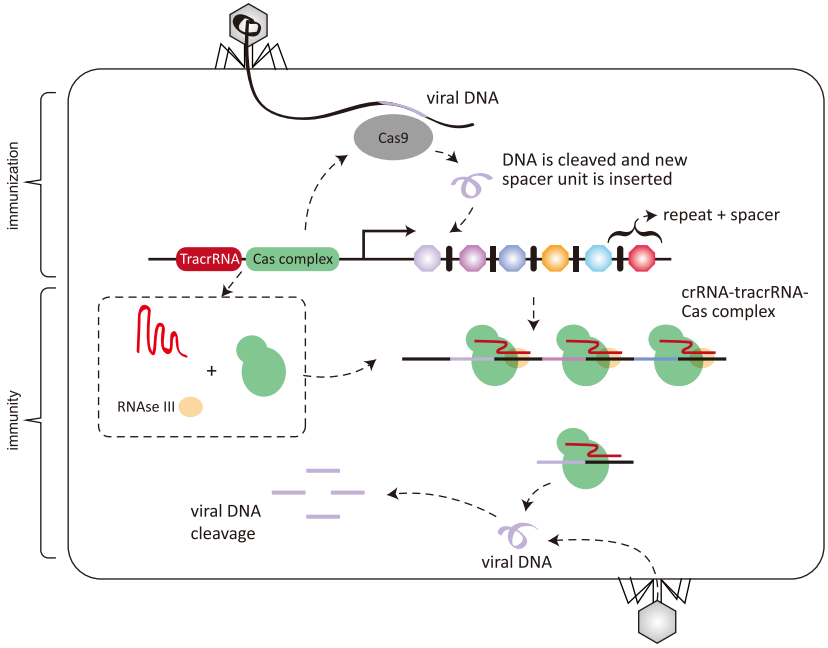
Starting in the late 1990s, newer and more effective genome editing techniques employing DSB-mediated repair came into use, including zinc-finger (ZF) and transcription activator-like effector (TALE) nucleases[8-10]. Both of these systems use DNA-binding proteins with nuclease activity that bind to DNA and create site-specific DSBs. While effective, these methods require extensive expertise in protein engineering, which has been a bottleneck for many research labs using this technology[11]. These advances in genome editing technology revealed specific prerequisites required for broader genome editing applications in research: the technology needs to be efficient, effective, affordable, and be easy-to-use.

Discovery of CRISPR in Bacterial Immune Systems

In the late 1980s, researchers discovered the clustered DNA repeats, that would become known as CRISPRs. In 1987, while studying the *iap* gene in *Escherichia coli*, researchers at Osaka University also accidently cloned a series of interrupted clustered repeats in the gene's 3' flanking region[12]. By 2000, additional sequencing studies had shown that these interrupted clustered repeats were widespread in both bacteria, archaea, and mitochondria[13].

In 2002, researchers dubbed these repeats, CRISPRs (clustered regularly interspaced short palindromic sequences), and identified nearby CRISPR-associated or *cas* genes[14]. These *cas* genes were consistently found adjacent to CRISPR loci and displayed motifs characteristic of both helicases and endonucleases[14]. The presence of multiple chromosomal CRISPR loci and invariably adjacent endonucleases suggested both that CRISPRs are mobile elements and that Cas proteins play a role in their genomic integration. In 2005, researchers from three independent groups confirmed that CRISPR spacers are derived from viral and extrachromosomal sources[15-17]. Together, these findings led researchers to conclude that the CRISPR/Cas system functions in bacterial adaptive immunity against foreign genetic elements.

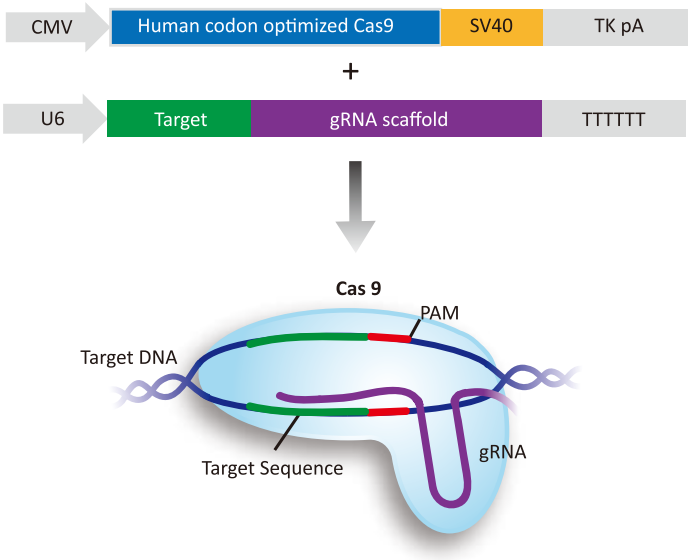
Figure 3: Mechanism of CRISPR-mediated immunity in bacteria



Experimental work in recent years has revealed the mechanism of action for CRISPR/Cas-mediated immunity. The CRISPR/Cas adaptive immune system is composed of two main phases: immunization and immunity (Figure 3). In the immunization phase, Cas proteins form a complex which cleaves foreign, viral DNA[18]. This foreign DNA is then incorporated into the bacterial CRISPR loci as repeat-spacer units[18]. In the immunity phase, following re-infection, the repeat spacer units are transcribed to form CRISPR RNA (crRNA) precursors[19]. The Cas9 endonuclease is then guided by trans-activating crRNAs (tracrRNA) to bind with crRNA precursors[20]. A mature crRNA-Cas-tracrRNA complex is formed following cleavage by RNAse III[20]. The mature crRNA serves as a small guide RNA, which upon infection will pair with viral DNA, triggering Cas cleavage and interference of the viral DNA[19,21,22].

The specificity of CRISPR/Cas for foreign DNA is triggered by the presence of a short 3-5 base pair sequence known as a protospacer adjacent motif (PAM)[23]. PAM sequences lie adjacent upstream of "protospacers," the foreign DNA genomic target sequences[23]. These motifs allow Cas endonucleases to discern between bacterial self DNA and invading non-self DNA, and are essential for Cas-mediated cleavage and targeting[24,25].

Figure 4: CRISPR/Cas system for genome editing in mammalian cells



Since their initial discovery, five different CRISPR/Cas system types have been characterized in bacteria[26]. In 2013, researchers adapted type II *Streptococcus pyogenes* Cas9 (SpCas9) for genome editing in mammalian cells[25,27]. But, instead of interfering with invading DNA, the CRISPR/Cas9 system can be used to create DSBs in endogenous genes to either trigger a KO mutation via NHEJ, or if cleaved in the presence of donor DNA, a KI mutation via HDR.

The CRISPR/Cas9 system is relatively simple and requires only two components, the Cas9 endonuclease and a fused crRNA-tracrRNA transcript, known as a guide RNA (gRNA)[25]. The gRNA combines the functionality of its crRNA-tracrRNA components, acting as both a targeting apparatus to DNA target sequences and a

GenScript offers Broad Institute-validated **gRNA/SpCas9 Plasmids** for gene editing in mammalian cells. SpCas9 gene editing allows for knock-out or knock-in mutation of any gene.

scaffold for the Cas9 protein[21] (Figure 4). Targeting and specificity is determined by the 20 nucleotides in the 5'-terminus of the gRNA[28]. By modifying this sequence, it is possible to edit any site in the genome adjacent to a SpCas9 PAM, encoded by 5'-NGG-3', which occurs approximately every 8 to 12 nucleotides in humans[29].

Expanding CRISPR/Cas9 Recognition Sequences

One limitation of the first CRISPR genome editing protocol was the constraint on genomic sequences that could be targeted. The SpCas9 enzyme requires the presence of a PAM sequence at the end of the gRNA 20-mer recognition sequence[29]. Guide RNA expression was driven by the U6 human pol III promoter due to its transcription initiation efficiency. But U6 initiates transcription from a guanosine (G) nucleotide, forcing U6-expressed gRNAS to be selected from genomic sequences that fit the pattern $GN_{19}NGG$ – which might occur infrequently in a gene of interest.

One strategy for expanding CRISPR/Cas9 sequence recognition was to drive gRNA expression using a different promoter. The H1 promoter can initiate transcription from A or G; therefore, H1-driven gRNAs can also target $AN_{19}NGG$ sequences, which occur 15% more frequently than $GN_{19}NGG$ within the human genome[29]. This small change in the gRNA expression cassette more than doubles the number of targetable sites within the genomes of humans and other eukaryotes.

Another strategy has been to remove restrictions on the PAM sequence, as the SpCas9 requirement for NGG presents a tight constraint. One approach has been to use protein engineering techniques to create novel engineered Cas mutants that recognize alternative PAM sequences[30]. By analyzing structural information, bacterial selection-based directed evolution, and combinatorial design, researchers developed several Cas9 variants that can recognize alternative PAM sequences, including NGA and NGCG, NNGRRT, and NNHRRT[30].

In addition to alternative PAM variants, there has also been increased interest in Cas9 alternatives for CRISPR-mediated gene editing. One example is the exploration of *Francisella* Cpf1 (FnCpf1), a type 2 nuclease that lacks the HRH domain present in SpCas9[31]. Unlike traditional Cas9 nucleases, this enzyme recognizes a different PAM sequence (5'-TTN-3'), features a shorter crRNA, and does not require a tracrRNA[31]. In addition, Cpf1 cleaves DNA is a staggered pattern, which can be beneficial for primary cell editing[31]. And due to the smaller size of Cpf1, the enzyme is easier to package into vectors, making it ideal for in vivo gene editing applications[31].

Improving Cleavage Specificity of the CRISPR/Cas9 System

While specificity of the CRISPR/Cas9 system is governed by gRNA sequence complementation, CRISPR/Cas9 complexes are tolerant of several mismatches with their targets[32]. DSBs have been observed at sites containing five or more mismatched nucleotides relative to the guide RNA sequence[33]. Due to the variable activity of the CRISPR/Cas9 system major efforts have been undertaken in recent years to improve cleavage specificity.

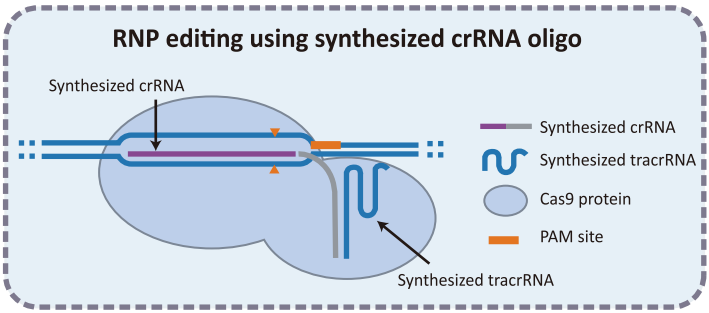
In 2014, the Broad Institute of MIT and Harvard pioneered one of the earliest studies for modeling gRNA binding specificity[34]. Researchers examined all possible targetable sites present in 6 mouse and 3 human genes, creating 1,841 gRNAs in total, and quantified their ability to knock out gene expression[34]. Based on the results a predictive model of gRNA activity was constructed. This model enabled the development of a gRNA design tool publicly available at: http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design.

Later in 2016, a second generation model was designed by the Broad Institute in collaboration with Microsoft[35]. The second generation model utilizes an algorithm that better models on-target activity, as well as a Cutting Frequency Determination (CFD) score to predict off-target activity[35]. These new tools are also publicly available at:
http://portals.broadinstitute.org/gpp/public/software/sgrna-scoring

Another strategy used to improve the cleavage specificity of the CRISPR/Cas9 system is the direct transfection of crRNA/Cas9 ribonucleoproteins (RNPs) into the cell. Recent experiments have observed that off-target binding of Cas9 is concentration-dependent[36]. By delivering optimized concentrations of Cas9 protein, as opposed to ubiquitously or differentially expressing DNA plasmids, the persistence and expression levels of Cas9 can be limited. When Cas9 is combined with duplexed crRNA:tracrRNA and delivered into cells the RNP complex is able to cleave chromosomal target DNA immediately after delivery (Figure 5)[37,38]. The RNP complex is then quickly removed by the cell's endogenous degradation machinery, further limiting off-target cleavage.

GenScript's **synthetic CRISPR RNA/Cas9 Protein** service delivers pre-duplexed crRNA:tracrRNA oligos and Cas9 protein ready-for-use. This service offers high-efficiency gene editing, with low off-target effects.

Figure 5. Gene editing with synthetic CRISPR RNA/Cas9 Protein

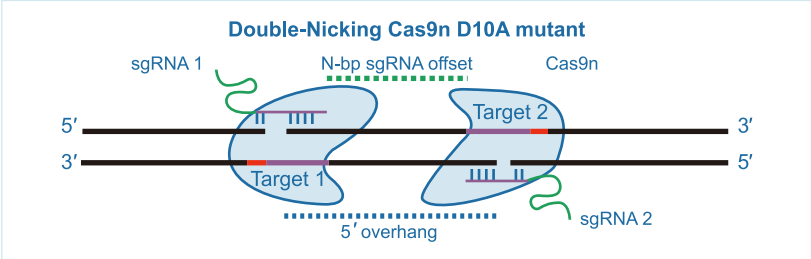


New strategies to improve CRISPR efficiency and specificity involve modifications to the Cas9 endonuclease itself. Cas9 contains two catalytic domains, RuvC and HNH. Broad Institute researchers have recently shown that mutations to catalytic residues D10A in RuvC and H840A in HNH, cause Cas9 to create single strand nicks as opposed to double strand breaks[28].

By utilizing nickase mutants (Cas9n) with paired gRNAs, it is possible to obtain target specific knock out mutants (Figure 6). Targeting Cas9n to two loci within close proximity, but on opposite strands, causes the endonuclease to nick both strands and create a DSB, eventually resulting in a mutation. Although each gRNA used might have off-target binding sites throughout the genome, Cas9n only catalyzes single strand breaks (SSBs) at each of those locations. SSBs are preferentially repaired through HDR rather than NHEJ, which will decrease the frequency of unwanted indel mutations. Paired nicking has been demonstrated to reduce off-target activity by 50-1,000 fold in cell lines[39].

GenScript offers Broad Institute-validated **gRNA/Cas9 Nickase Plasmids** for gene editing in mammalian cells. Use paired nicking for maximized on-target cutting efficiency.

Figure 6. Increasing cleavage specificity through paired nickase



Upon further analysis of the Cas9 protein structure, Broad Institute researchers discovered a positively charged groove between the HNH and RuvC domains, which likely stabilizes non-target DNA within the enzyme and ultimately increases the likelihood of off-target cleavage[40]. Working under the hypothesis that neutralizing this interaction would reduce the attraction between Cas9 and non-target DNA, the group replaced the positively charged amino acids within the groove to alanine[40]. This Cas9 mutant, named "enhanced" SpCas9 or eSpCas9, was also able to reduce off-target effects without compromising on-target cleavage efficiency[40].

Inducible Cas9 Expression

In order to make Cas9 active only at specific times or in specific tissues, several research groups have engineered CRISPR/Cas9 systems that are inducible or conditional. For example, spatial and temporal control of genome editing can be accomplished using a photoactivatable Cas9 (paCas9), created by splitting Cas9 into two fragments each fused to a photoinducible dimerization domain[41]. Upon blue light irradiation, paCas9 dimerizes and becomes active, creating targeted genome edits via NHEJ or HDR only while the optical stimulus is present[41].

Tissue-specific genome editing can be accomplished by using tissue-specific promoters to drive Cas9 expression. Many mouse strains have been developed that stably express Cre recombinase under the control of tissue-specific specific promoters. These can easily be crossed with mice harboring a Cre-driven Cas9 cassette to enable tissue-specific genome editing upon delivery of guide RNAs[42]. Heritable tissue-specific Cas9 expression has also been achieved in a variety of model animals other than mice, including zebrafish, sea squirts, and fruit flies[43-46]. Tissue-specific promoters are also useful for constraining Cas9 activity after *in vivo* delivery via adeno-associated virus (AAV) vectors,, which can infect many different cell types[47].

Advantages of CRISPR Genome Editing

The advent of CRISPR has revolutionized genome editing – not only for its cost effective specificity, but also for its ease-of-use in any lab, regardless of molecular biology expertise. Unlike ZF and TALE nucleases, CRISPR/Cas9 does not require protein engineering for each gene being targeted. The CRISPR/Cas9 system requires only a DNA construct encoding the target specific gRNA and Cas9, and if knock-in is being performed, the donor template for HDR. In addition, multiple genes can be edited simultaneously with CRISPR, vastly increasing the efficiency of your experiments. The key differences and advantages between the most common DSB-mediated genome editing technologies are summarized below (Table 1).

Table 1: Key differences between TALENs, ZFNs, and CRISPR/Cas

	TALEN (transcription activator-like effector nucleases)	ZFN (zinc finger nucleases)	CRISPR/Cas
Target	Protein: DNA	Protein: DNA	(gRNA-Cas9): DNA
Construct	Proteins containing DNA-binding domains that recognize specific DNA sequences down to the base pair	Zinc finger DNA binding motifs in a ββα configuration, the α-helix recognizes 3 bp segments in DNA	20nt crRNA (CRISPR RNA) fused to a tracrRNA and Cas9 endonuclease that recognize specific sequences to the base pair
Design feasibility	**Difficult:** -Need a customized protein for each gene sequence -Low delivery efficiency		**Easy:** - all-in-one gRNA-Cas9 vector system - multigene editing is feasible

Genome-wide and Pathway-specific Screens Using CRISPR Libraries

In addition to single- and multi-gene targeting, CRISPR has been adapted for genome-wide screening to discover genes whose inhibition or aberrant activation can drive phenotypes implicated in disease, development, and other biological processes.

Genome-scale CRISPR knock out libraries (GeCKO v2 libraries) in mouse and humans enable rapid screening of loss-of-function mutations[48]. GeCKO v2 libraries express a mixed pool of CRISPR guide RNAs that target every gene and miRNA in the genome. Each gRNA is cloned into a lentiviral vector optimized for high-titer virus production and high efficient transduction of primary cells or cultured cell lines. After transduction, deep sequencing can be performed to assess gRNA representation in the cell pool prior to screening. After selection, a second round of sequencing is performed to identify the gRNAs that were lost or enriched over the course of the screen. Genes identified with multiple gRNAs enriched represent positive hits. A detailed GeCKO screening protocol may be found on the GeCKO Genome Engineering website: http://genome-engineering.org/gecko/

GenScript offers Broad Institute sequence validated **CRISPR GeCKO v2 Libraries** to accelerate your genome-wide screening efforts.

GeCKO v2 libraries were designed to contain 6 gRNAs targeting each gene within the genome, 4 gRNAs targeting each miRNA within the genome, and 1,000 control (non-targeting) gRNAs. GeCKO v2 libraries are also available as two half-libraries, A and B, each containing 3 gRNAs per gene. The gRNA sequences are distributed over three or four constitutively expressed exons for each gene and are selected to minimize off-target genome modification.

Molecular pathway-focused gRNA libraries have also been developed for targeted screening of specific molecular pathways. Pathway-specific gRNA libraries were designed using gene targets identified through the Drug Gene Interaction Database by the McDonnell Genome Institute at Washington University in St. Louis. All gRNA sequences have been pre-designed and validated by the Broad Institute.

GenScript offers Broad Institute sequence validated **Pathway-specific gRNA Libraries** to assist in screening specific molecular pathways.

Expanding the Research Applications for CRISPR

CRISPR/Cas9 technology has been adapted for many research applications beyond than genome editing, such as:

- CRISPR/Cas9-mediated Chromatin Immunoprecipitation
- CRISPR Technologies for Transcriptional Activation and Repression
- Epigenetic Editing with CRISPR/Cas9
- Live Imaging of DNA/mRNA with CRISPR/Cas9
- CRISPR/Cas9 Therapeutic Applications

CRISPR/Cas9-mediated Chromatin Immunoprecipitation

Purification of specific genomic loci is vital for the characterization of chromatin-associated proteins and RNAs. Modifications to the CRISPR/Cas9 system allow for flexible targeting and isolation of these genomic regions[52]. A nuclear localization signal and epitope tag can be introduced into catalytically inactive Cas9 (dCas9) to create a DNA-binding protein that can be targeted by CRISPR guide RNAs[49]. Existing CRISPR gRNA databases and design tools allow for targeting to any gene of interest. The CRISPR/Cas9-chromatin complex can then be purified with traditional chromatin immunoprecipitation (CHiP) techniques and exposed to mass spectrometry for further characterization.

CRISPR-mediated CHiP techniques have been utilized to identify proteins associated with the interferon regulatory factor-1 (IRF-1) promoter region in response to interferon γ stimulation[53]. In this study, researchers purified 15 associated proteins including histone deacetylase complex proteins, which have previously been implicated in interferon γ-mediated gene expression, as well as transcription factors, histones and other DNA-associated proteins[50].

CRISPR-mediated CHiP holds a number of advantages over traditional CHiP methods. While large scale assays require the use of multiple antibodies against each DNA-binding protein or the creation and expression of epitope-tagged proteins, the modular nature of the CRISPR/Cas9 system requires only a single antibody against the tagged-Cas9 protein for purification. In addition, the CRISPR/Cas9 system is unaffected by issues stemming from low, differential or toxic gene expression.

CRISPR Technologies for Transcriptional Activation and Repression

Several research groups have harnessed the specificity and easy re-programmability of the CRISPR/Cas9 system to create targetable CRISPR/Cas9 ribonucleoprotein complexes that can either activate (CRISPRa) or interfere (CRISPRi) with transcription of any desired coding region within a genome[51-53].

These systems fuse dCas9 to a well-characterized transcription-regulatory domain, using pre-designed guide RNAs to direct the complex upstream of the transcription start site. By using inactivated dCas9 protein, the complex can be targeted to specific loci without cleaving or altering the genomic DNA. After Cas9 binds the targeted DNA sequence, the fused transcription-regulatory domains are then able to recruit repressive or activating effectors to modify gene expression.

The laboratory of Feng Zhang at the Broad Institute has pioneered use of the CRISPR/Cas9 Synergistic Activation Mediator (SAM) system in gene activation assays. The SAM system enables robust transcriptional activation of endogenous genes targeted by guide RNAs that bind within 200 bp upstream of the transcription start site[54].

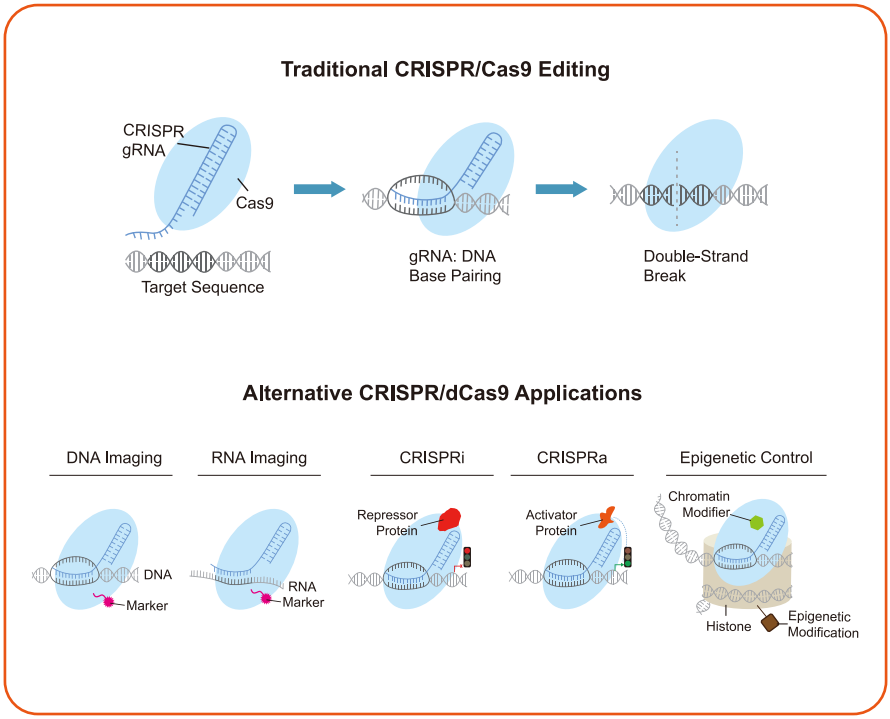
GenScript offers Broad Institute-validated **SAM Vectors** for transcriptional activation in mammalian cells.

Studies using the SAM system to activate gene expression show an increase in transcription of up to 3,000[54,55]. The SAM system has multiplexing gene activation ability, and has been shown to activate the transcription of as many as 10 genes concurrently[57]. In addition, SAM has been demonstrated to activate non-coding elements, such as long intergenic non-coding RNAs[54].

SAM can also be used for discovery research to identify the genes that drive phenotypes of interest in disease models or developmental/differentiation process by using a genome-wide SAM gRNA library for gain-of-function screening[54]. The screening process is similar to the GeCKO loss-of-function library screening techniques, but the library is designed to activate transcription rather than edit the genome. The human genome-wide SAM library contains 3 distinct guide RNAs each targeting one of 23,430 coding gene isoforms with a unique transcription start site in the human reference genome, for a total of 70,290 guides.

GenScript offers Broad Institute sequence-validated **CRISPR SAM Libraries** to promote your genome-wide activation screening efforts.

Figure 7. CRISPR/Cas9 *In Vivo* Applications



Epigenetic Editing with CRISPR/Cas9

Epigenetic modifications to genomic DNA and histone proteins have been shown to play increasingly critical roles in biological processes. Epigenetic marks, such as methylation or acetylation, at specific genomic loci and histone residues can either be inherited or acquired, and can influence gene expression. Recent studies have used CRISPR/Cas9 genome editing to investigate the roles and targets for these epigenetic marks. In one such study, researchers performed CRISPR-mediated knock out of all three active DNA methyltransferases present in human embryonic stem cells, to characterize viable, pluripotent cell lines and study the distinct effects on the DNA methylation landscape[56].

But researchers increasingly need methods for introducing epigenetic modifications at desired genomic loci, in order to model diseases and test hypotheses regarding potential therapeutic strategies. For example, specific

epigenetic alterations are often necessary and sufficient to drive the transformation of normal cells into cancerous cells, and play roles in later steps of carcinogenesis[57].

Using the CRISPR/Cas9 system, epigenetic editing has now become feasible. Utilizing inactive dCas9 as a DNA-binding domain platform, fused enzymes such as DNA methylases, histone acetyltransferases, and deacetylases, can be targeted to alter the epigenetic state at precise locations within the genome. Researchers have used this approach, to fuse the catalytic core of human acetyltransferase p300 with dCas9, and shown this system to be sufficient for acetylation of histone H3 lysine 27 at specific target sites and to robustly activate transcription of target genes[58]. Cas9 epigenetic effectors (epiCas9s) can also be used for genome-wide screening to discover novel relationships between epigenetic modifications, chromatin states, and phenotypes such as, cellular differentiation or disease progression[59].

Live Imaging of DNA/mRNA with CRISPR/Cas9

DNA visualization is an important application in understanding a variety of cellular processes, such as replication, transcription, and recombination, and the interactions between DNA and associated proteins and RNA. Two techniques are commonly used for DNA imaging, fluorescence *in situ* hybridization (FISH) and fluorescent tagging of DNA-binding proteins. FISH uses fluorescently tagged nucleic acid probes to bind and visualize DNA[60,61]. While this technique offers the flexibility to target specific sequences through base pairing of the nucleic acid probes, it cannot be used for live imaging because of the requirement for sample fixation. Conversely, proteins tagged with a fluorescent label can be used for live imaging, but are limited by their fixed target sequences, restricting their use mostly to repetitive DNA sequences, such as telomeres[62].

New advances in CRISPR/Cas9 technology offer the benefits of both live imaging and easy target sequence customization and flexibility. Inactivated dCas9 can be tagged with fluorophores for imaging both repetitive DNA elements and protein-encoding genes, enabling us to observe chromatin organization throughout the cell cycle[63]. In addition to live DNA imaging, the CRISPR/Cas9 system can be used for live RNA imaging as well. Modifications to the gRNA sequence allow for mRNA recognition and tracking[64]. Using CRISPR-mediated RNA imaging techniques, researchers have been able to visualize the accumulation of ACTB, CCNA2 and TRFC mRNAs in RNA granules[64]. These new applications improve existing methodologies for live imaging within cells allowing for the study of dynamic cellular processes involving DNA and RNA.

CRISPR/Cas9 Therapeutic Applications

Both well-established pharmaceutical companies and new start-up biotech companies are racing to create CRISPR-based therapeutics. Compared to other strategies for gene therapy, CRISPR genome editing is thought to be faster, less expensive, and potentially far safer. Autologous CRISPR cell therapies that use genome editing to correct a mutation in a patient's own cells hold promise in circumventing the rejection issues present with transplant therapies that require donor matching. CRISPR genome editing is especially promising for diseases that can be tackled by modifying cells that can easily be removed from a patient, which allows for additional screening to ensure no off-target genome modifications during genome-editing.

Table 2:

Cancer Immunotherapy	New studies using CRISPR/Cas9-mediated immunotherapy are being used to combat metastatic lung cancer at Sichuan University and myeloma at the University of Pennsylvania[65,66]. Researchers will be using the CRISPR/Cas9 system to knock out the PD-1 gene in T-cells extracted from patients. PD-1 is an important down-regulator in T-cell activation and functions as an immune checkpoint. After knockout of PD1, the modified T-cells will be released into the bloodstream to target cancer. PD-1 inhibition offers a promising approach for cancer treatment. Last year alone, the FDA approved two new antibody-based therapies which target PD-1, nivolumab and pembrolizumab.
Tissue Regeneration	Recent reports indicate that CRISPR/Cas9 may be an essential tool to improve cell differentiation. CRISPR technology and has been used to derive a variety of cell types for transplantation, including muscle cells for the treatment of muscular dystrophy and hematopoietic stem cells for the treatment of sickle cell anemia[67,68]. Together these results demonstrate that CRISPR/Cas9 technology can be applied for directed cell differentiation and implantation.
Gene Therapy	Huntingtons disease is an inherited neurological condition caused by accumulation of mutant Huntingtin protein within the brain which results in cognitive impairment, dementia and death. Using mouse models, researchers have shown that CRISPR/Cas9 gene editing can knock-out production of these mutant proteins[69]. CRISPR/Cas9 editing is sufficient to reduce mutant protein production by up to 90% and shows promise as a therapeutic solution. Additional studies are ongoing using humanized huntingtin genes.

Malaria and Insect-borne Diseases	Insect-borne diseases such as malaria and zika pose enormous health concerns across the world. To combat the spread of insect-borne diseases, researchers have modified CRISPR/Cas9 into highly efficient "gene drive" systems which can spread disease resistance genes to entire populations. To create a gene drive, researchers have package disease resistance genes together with CRISPR gRNA and Cas9 components into a single DNA construct. After insertion, the gene drive autonomously replicates into both parental chromosomes, and is inherited by ~99.5% of progeny[70,71]. Advances in gene drive technology offer immediate solutions for the eradication of these diseases.
HIV and Viral Diseases	Gene editing can provide new strategies and therapeutic applications against infectious viral diseases. HIV has been effectively eliminated in patients using gene therapy to delete receptors essential for viral cell entry and infection. Recent studies using CRISPR technology have shown that mutations in CCR5 and CXCR4 receptors in both induced pluripotent stem cells (iPSCs) and primary $CD4^+$ cells can lead to HIV resistance in lineages derived from these cells[72,73].
Obesity and Metabolism	FTO is one of the most strongly linked genes to obesity. Certain FTO genetic variants correlate significantly with obesity and heavier weight. Researchers have shown that CRISPR/Cas9-mediated knock-in techniques can convert obesity-promoting FTO variants to normal variants in adipocyte precursor cells[74]. Treated cells display increased metabolic activity and reduced expression of *IRX3* and *IRX5*, genes which determine cell fate as white adipocytes for fat storage.

Putting CRISPR into Practice: Workflows and Case Studies

With CRISPR/Cas9 genome editing, modified clonal cell lines can be derived within 2-3 weeks starting from the gRNA design stage, while transgenic animal strains can be created in a single generation. The following workflows and case studies describe best practices on how to use CRISPR in your laboratory.

CRISPR/Cas9 Vector Design Workflow

CRISPR/Cas9-mediated gene editing can be performed in the following steps:

1. Determine Genetic Modification
Select the application for your experiment (Table 3).

Table 3:

Genetic Modification	Application	Nuclease Activity	gRNA
Knock-out	Permanently remove gene function	Cas or Cas9n	gRNA targeting 5′ exon or essential protein domains
Knock-in	Generate a specific sequence change	Cas or Cas9n	gRNA targeting region of interest
Interference	Reduce gene expression	dCas-repressor	gRNA targeting gene promoter elements
Activation	Increase gene expression	dCas-activator	gRNA targeting gene promoter elements

2. Select Expression System
CRISPR/Cas9 system components can be delivered *in vivo* using modified non-viral plasmid or viral vector or delivery systems (Table 4).

Table 4:

Expression System	Components	Application
Plasmid Vector	Constitutive or inducible Cas9 Constitutive or inducible gRNA Reporter/selection marker	Expression of Cas9 and gRNA
Lentiviral Vector	Constitutive or inducible Cas9 Constitutive or inducible gRNA Reporter/selection marker	Expression of Cas9 and gRNA For infection of difficult-to-transfect cell types
AAV Vector	Constitutive or inducible Cas9 Constitutive or inducible gRNA Reporter/selection marker	Transient or stable expression of SaCas9 and/or gRNA For non-toxic infection of dividing and non-dividing cells
Cas9 mRNA and gRNA	Transcription reactions *in vitro* to generate Cas9 mRNA and gRNA Delivery via microinjection or electroporation	Transient expression of CRISPR gene editing components
crRNA/Cas9 Ribonucleoprotein Complexes	Purified Cas9 protein and *in vitro* transcribed gRNA Delivery via microinjection or electroporation	Transient expression of CRISPR gene editing components

Modified recombinant AAV particles are attractive for transduction because of their low immunogenicity and capability to infect both quiescent and dividing cells[75]. While AAV vectors are a preferred vehicle for *in vivo* gene delivery, the size of the SpCas9 gene (>4 kb) exceeds the typical cargo limit of AAV vectors.

GenScript offers Broad Institute-validated **gRNA/SaCas9 Vectors** for gene editing in mammalian cells. Use SaCas9 for AAV packaging and transduction.

Solutions that have been developed to date include:

- Creating transgenic animal lines that express Cas9, either constitutively or in an inducible manner, and then to deliver only the guide RNAs and any necessary inducer at the time of the experiment[42].
- Developing a split-Cas9 system using split-inteins[76].
- Use smaller Cas9 orthologues from other species, such as *Staphylococcus aureus* (SaCas9), which are small enough to be packaged along with a single guide RNA expression cassette into a single AAV vector[77].

Some of the most widely-used model systems for biomedical research are primary mammalian cell cultures or hard-to-transfect cell lines in which transfection efficiency can be quite low. For these cell types lentiviral vectors are preferred.

Guide RNAs may be also introduced via U6-gRNA cassette expression, but the cleavage efficiency is typically lower than when gRNA is expressed from a plasmid. However, U6-gRNA cassettes may be used for rapid comparison of gRNA cleavage efficiencies so that the most optimal gRNA sequences can be identified before subsequent cloning into your vector[28].

3. Gene Sequence Analysis
It is highly advisable to sequence the region of interest for the cell line or animal model you are using, rather than assuming it matches with the NCBI reference sequence for your species/strain. Allele number may also vary depending on species/strain. Variations can result in reduced cleavage efficiency.

4. Select Sequence for Modification
Select specific genetic sequences for modification depending on your application (Table 3).

For loss-of-function mutations, design gRNAs against early exons to optimize expression disruption and prevent the expression of truncated protein isoforms. Alternatively, targeting a functional site can generate a loss-of-function mutant. For genes with multiple splice variants, care should be taken to ensure that a constitutive exon is targeted if the goal is to knock out all splice variants.

For applications using paired Cas9n, opposite strands of the genomic DNA should be targeted, with a 40-60 bp offset between PAM sequences.

For interference and activation applications, promoter elements within 200 bp of the transcription start site should be targeted.

5. Determine On/Off-Target Activity
Identify all PAM sequences within the region of interest: the PAM sequence will vary depending on the Cas variant being used in the experiment (Table 5). A PAM sequence is required for targeting, so if none are present, considering targeting a different location. The next 20 bps upstream of the PAM will correspond with your putative gRNA sequence. Be sure to check for off-target sites, locations within the genome where partial homology is present, which can result in off-target cleavage.

Table 5:

Cas Variant	PAM Sequence
SpCas9	NGG
SpCas9 VRER Variant	NGCG
SpCas9 EQR Variant	NGAG
SpCas9 VQR Variant	NGAN or NGNG
SaCas9	NNGRRT
Cpf1	TTN

GenScript hosts free online human and mouse genome-wide databases developed by researchers at the Broad Institute. These databases can be searched and accessed here:
http://www.genscript.com/gRNA-database.html

GenScript also host a free online gRNA design tool developed by researchers at the Broad Institute. The design tool can be accessed here:
http://www.genscript.com/gRNA-design-tool.html

6. Designing Knock-in Constructs

To introduce specific changes within the genome it is necessary to supply a donor template that can be used for HDR after Cas9 creates a DSB. HDR templates may be delivered as plasmids or as single-stranded oligos (ssODN). To detect successful HDR and quantify knock-in efficiency, donor templates are often designed to include several synonymous mutations that can be distinguished from the wild-type through sequencing. To prevent the cleavage of donor DNA after successful HDR, the donor template should be designed with mutations in the PAM sequence.

As a general rule, SpCas9 is more efficient at mediating homologous recombination than Cas9 nickase.

7. Synthesize gRNA/Cas9 Vectors

Once you have determined your expression system, Cas nuclease and gRNA sequence your customized vectors can be cloned or commercially ordered.

CRISPR-mediated Gene Editing in Model Systems

CRISPR/Cas9-mediated genome editing has been successfully conducted in many different species and models (Table 6). Although the basic CRISPR/Cas9 components are the same regardless of the model organism, the delivery method varies widely, and choosing the most appropriate vector for your host is critical for success.

Table 6:

Host	PAM Sequence
Mammalian Cells	Lipofection-based transfection of DNA plasmids Electroporation of DNA plasmids or RNP Lenti or AAV virus-based transfection of DNA plasmids
Bacteria	Transformation of plasmids into competent cells
Yeast	Electroporation of plasmids and galactose induction of Cas9
Mouse: **Germline Mutations**	Direct injection into embryos Electroporation into zygotes
Mouse: **Somatic Mutations**	Direct injection of AAV into tissue of interest
Danio rerio	Direction injection into one-cell embryos
Drosophila melanogaster	Direct injection into embryo germline
Danio rerio	Direction injection into one-cell embryos
Caenorhabditis elegans	Direct injection into hermaphrodite germline
Plants	*Agrobacterium*-mediated transformation of gRNA/Cas9 vector

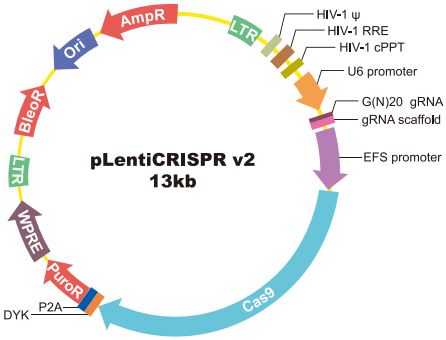
***In vitro* genome editing:**

For easy-to-transfect cell lines, plasmids encoding gRNAs and Cas9 can be delivered with high efficiency via lipofection. CRISPR plasmids typically contain selection markers such as genes conferring antibiotic resistance, or fluorescent proteins for easy visualization via FACS.

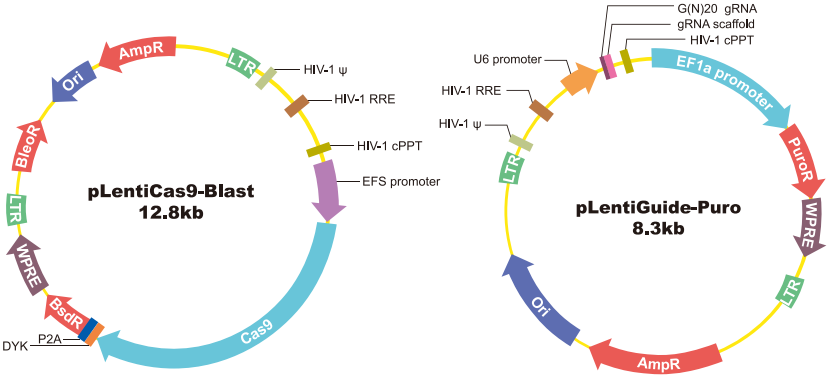
For difficult-to-transfect cell lines or primary cells, lentiviral vectors are preferred. Guide RNAs may be delivered either via an all-in-one plasmid that also encodes the Cas9 nuclease, or a separate plasmid that can be delivered into cells already expressing Cas9 (Figure 8).

Figure 8: Optimized Lentiviral Vectors for CRISPR genome editing in mammalian cells

Option 1: An all-in-one vector, pLentiCRISPRv2, enables CRISPR editing in any cell type of interest without generating stable Cas9-expressing cell line first.



Option 2: A two-vector system; sequential transduction with, and selection for, pLenti-Cas9-Blast followed by pLentiGuide-Puro, shows 10-fold higher efficiency compared to pLentiCRISPRv2.



***In vivo* genome editing:**

CRISPR/Cas9 system components can be delivered to germ line cells to create heritable mutations. Stable, homozygous mutations at multiple loci can be achieved in a single generation in mice[78]. CRISPR-mediated genome editing can similarly be used to generate precise mutations in somatic tissues of adult animals, and to modify multiple genes at once in the same cells[25,27]. These tools

are especially valuable for creating clinically relevant *in vivo* cancer models, because human tumors often contain a combination of gain-of-function mutations in oncogenes and loss-of-function mutations in tumor suppressor genes[42].

Verification of KO/KI and Off-target Effects

To identify successful cases of CRISPR-mediated KO/KI and determine whether a loss-of-function or gain-of-function mutation has occurred, mRNA and protein gene products should be analyzed. Techniques such as quantitative PCR, Northern blotting, and Western blotting can be utilized to determine whether mRNA and protein concentrations are depleted or molecular weights are changed.

For difficult-to-transfect cells, it can be sufficient just to show that high KO/KI efficiency has been achieved, without isolating clones for confirmation. For these cases genome editing efficiency is typically assayed by next generation sequencing. A range of unique insertions and deletions will likely be observed.

Best practices for managing off-target Cas9 activity:

- Use at least two independent gRNA sequences in parallel to derive distinct clones. Models created through genome editing with distinct gRNAs that share the on-target locus, but do not share off-target loci are an excellent way to create independent replicates.
- Isolate multiple, independent clonal cell populations for each gRNA used. The likelihood off-target DSBs occurring at the same loci in independent clones is very low.
- Although few labs have the resources to do statistically powerful whole genome sequencing verification protocols such as gUIDEseq, it is relatively easy to select the few predicted off-target sequences for each gRNA you use and then sequence around those loci to ensure that off-target indels have not been introduced.

To determine off-target effects, it is recommended to sequence predicted off-target sites, particularly those with matches in the “seed” region of the 20 mer recognition site, which lies adjacent to the PAM. More rigorous reviews of off-target cleavage can be performed using whole-genome sequencing.

CRISPR/Cas9 Library Screening Workflow

CRISPR/Cas9-mediated library screening can be performed in the following steps:

1. Select CRISPR Library
Genome-wide and pathway-focused libraries for humans and mice are commercially available online. CRISPR libraries are commercially available online for both genome-wide and pathway-specific screening.

2. Amplify CRISPR Library
CRISPR libraries can be transformed into bacteria via electroporation for amplification. Maxiprep DNA isolation can be used for library production and backup stocks.

3. Verify Library Coverage
Next generation sequencing of amplified CRISPR library DNA is highly recommended to ensure full library coverage. Incomplete library coverage can result in false negatives and positives.

4. Package Library in Lentiviral Vectors
Identify the desired multiplicity of infection (MOI) and infectious forming unit (IFU) titer to determine the amount of library reagent required for infection.

5. Infection of Target Cells
Cells should be infected at low MOI to ensure that only one plasmid is absorbed. In addition, a large number of cells should be transfected in comparison to the number of plasmids in your library (~200), in order to minimize false positives. Media selection should at a minimum continue until a non-transduced control population shows 100% death.

6. Screening
To reduce background effects from the screen, it is recommended to include transduction replicates and gRNA redundancy in your experiments. Transduction replicates will reduce false positives due to background enrichment/depletion using an expanded cell population. Guide RNA redundancy can be used to discriminate between off-target false-positives.

To identify screen positives, amplify the integrated, genomic lentiviral construct via PCR, and use next generation sequencing to determine the relative representation for each gRNA.

Case Study 1: Using CRISPR to Generate Knock-out Cell Lines

The *KRAS* gene encodes for a protein called K-Ras, an important regulator of cell division. *KRAS* is a proto-oncogene which has been implicated in various malignancies, including leukemia, pancreatic, colon, and lung cancers. In this case study, the *KRAS* locus was knocked-out in the human colon cancer cell line, HCT116 (Figure 9).

Using GenScript's **Mammalian Cell Line Gene Editing Services**, any gene can be targeted in any mammalian cell. All clones are target sequence validated and a detailed report on clone generation is provided.

For this case, exon 4 was targeted for CRISPR/Cas9-mediated gene knock-out. For delivery, gRNA and Cas9 encoding components were packaged into lentiviral vectors. In the absence of donor DNA, the ensuing DSB was repaired by NHEJ to create an indel. Sanger sequencing and Western blotting were used to confirm successful knock-out of the KRAS gene (Figure 10).

Figure 9: Knock-out targeting strategy for *KRAS*

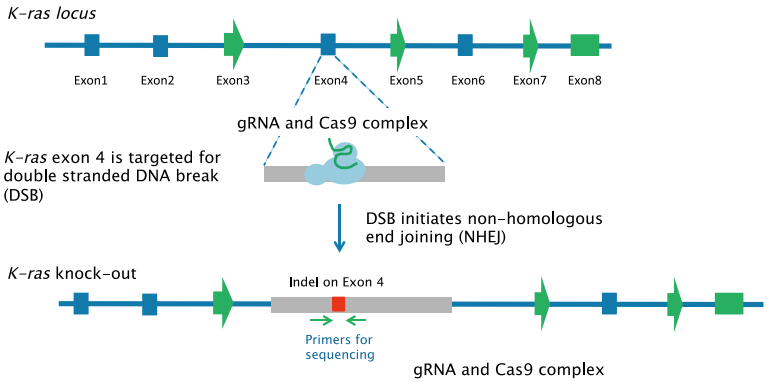
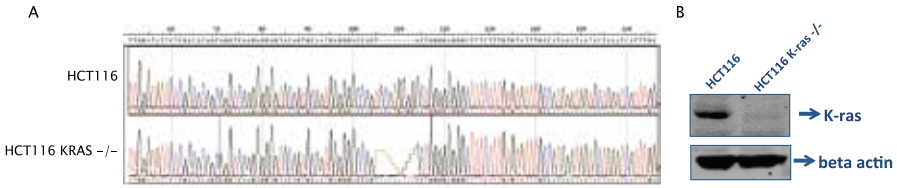


Figure 10: *KRAS* loss-of-function determined by Sanger sequencing and Western blotting



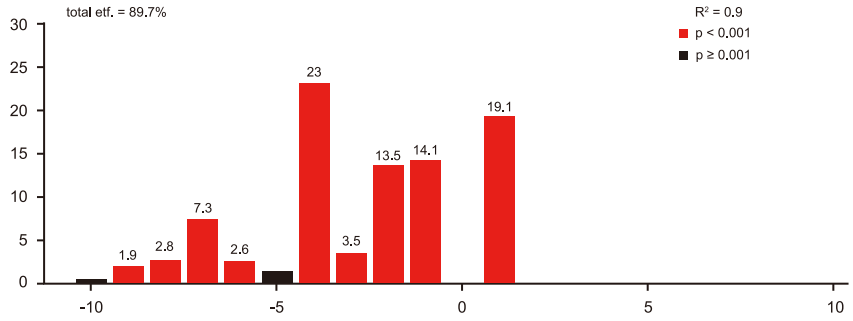
Case Study 2: Using CRISPR to Generate a Knock-out Cell Pool

Stable CRISPR cell pools, in which Cas9 and gRNA encoding components are integrated into the cell genome, are commonly used in screening applications or when generating single cell clones is difficult. In this case study, the *dnajc3* gene, a member of the DNAJC3 heat shock protein family, was targeted via cell pool knock-out in the HEK293 cell line.

GenScript's CRISPR **Cell Pool Gene Editing Service** can be used to knock-out genes in difficult-to-transfect mammalian cell lines

To create the cell pool, five gRNAs were designed to target shared exons in the *dnajc3* locus. These gRNAs were cloned into an all-in-one gRNA/Cas9 carrying puromycin resistance. HEK293 cells were transfected and selected by antibiotic resistance. After transfection, cells from the pool were examined by Sanger Sequencing to confirm nucleotide mismatching. Using sequencing trace analysis, an 89.7% indel mutation efficiency could be verified (Figure 11).

Figure 11: Indel mutation efficiency of *dnajc3* CRISPR cell pool



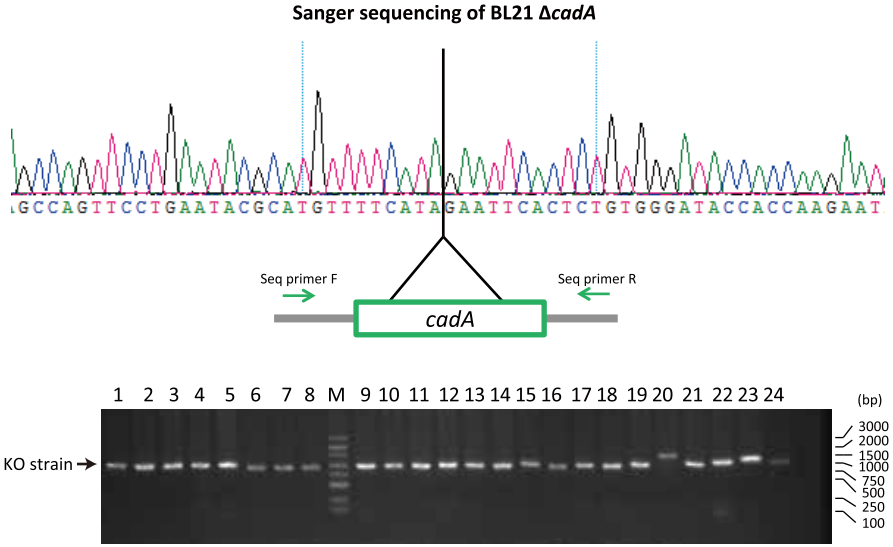
Case Study 3: Using CRISPR for Microbial Genome Editing

As in mammalian cell lines, CRISPR/Cas9 technology can be used to generate knock-in and knock-out mutations in microbes. While HR frequency is generally lower in microbes than mammalian cells, alternative techniques can be utilized to improve recombination efficiency. Optimized gene editing in microbes can be achieved by coupling CRISPR/Cas9 technology with λ Red recombination techniques. The λ Red system utilizes bacteriophage recombinases to mediate recombination of homologous sequences as short as 30 base pairs[79].

GenScript's **Microbial Gene Editing Services** uses λ Red – CRISPR/Cas editing technology. This technique is the most precise, efficient, and cost effective recombineering method on the market.

In this case study, λ Red – CRISPR/Cas9 was used to knock-out cadA in the BL21 *E. coli* strain. The CadA protein is a component of lysine decarboxylase, an enzyme that helps bacteria survive in acidic environments[80]. After editing, Sanger sequencing and colony PCR screening was used to confirm that the cadA gene was successfully knocked-out (Figure 12).

Figure 12: Knock-out of *cadA* verified by Sanger sequencing and colony PCR



Future of CRISPR

CRISPR/Cas9 has revolutionized genome editing with its ease of use and broad applicability to mammalian cells, microbes, and animal models. Not only does CRISPR have the potential to enhance our ability to analyze and understand gene function, but this new tool is also rapidly reforming the biomedical industry. Accessible genome editing techniques can be used to correct genetic mutations that are responsible for inherited disorders or diseases, and also for large-scale screening of new drugs. In 2016, CRISPR/Cas9 gene editing was tested in human patients for the first time ever, used to treat aggressive forms of lung cancer. Additional trials for bladder, prostate, and renal-cell cancer are slated for 2017.

Considering how recently the CRISPR system has been applied to mammalian and microbial gene editing, there is still room for improvement. As the structure of Cas9 becomes better understood, smaller and more efficient Cas9 variants can be created for improved delivery and cleavage efficiency. Along the same vein, as researchers elucidate the mechanism for how Cas9 binds to DNA, more effective Cas9-gRNA constructs can be designed.

In the future, CRISPR/Cas9 technology will play a significant role in innovating thelife science research and industry fields. CRISPR/Cas9 gene editing remains the easiest, most cost-efficient, and most exciting technology in genome engineering. There is no doubt that this is just the beginning of a revolutionary technology that can be used by generations of scientists to come.

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