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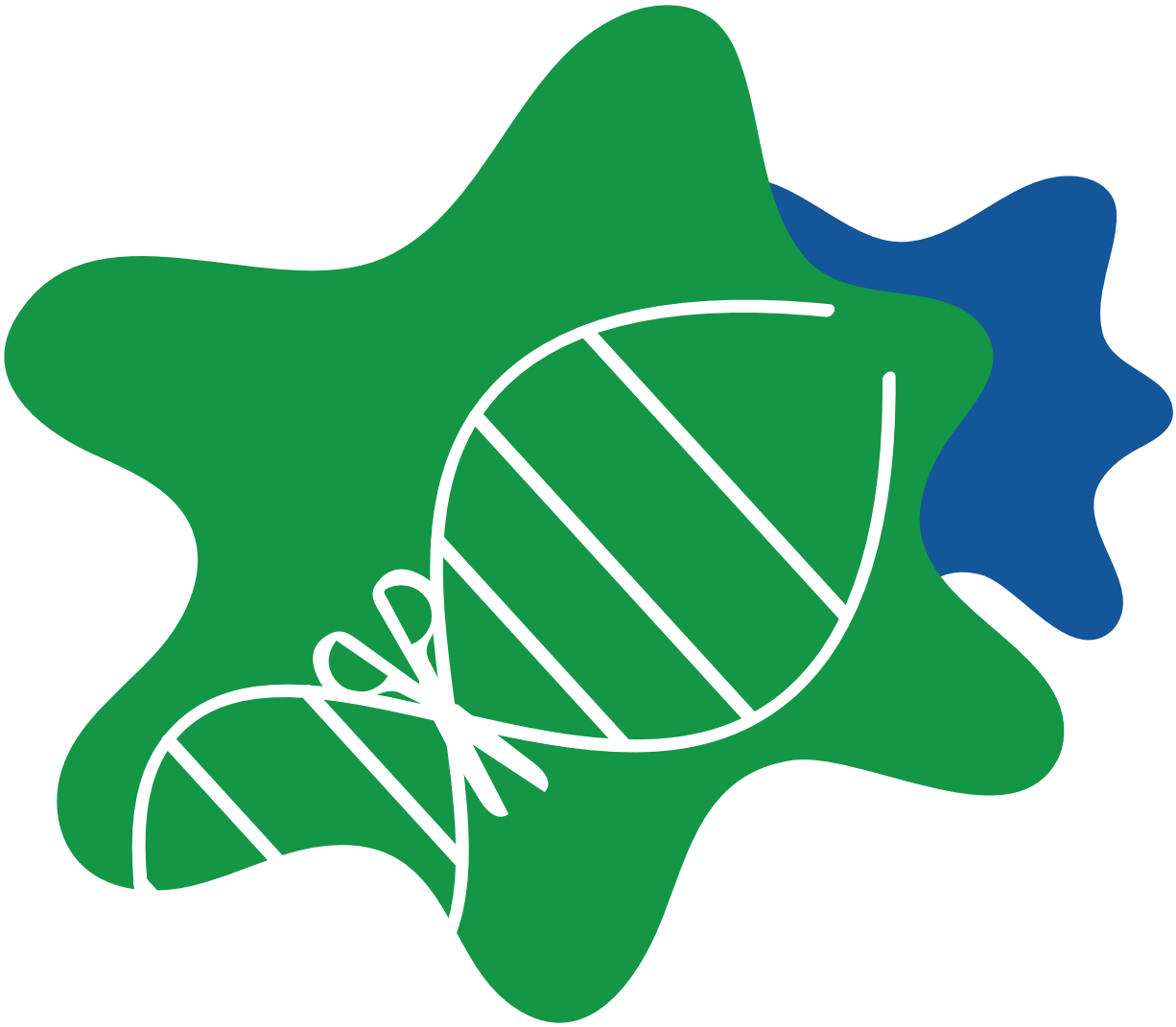

CRISPR/Cas9 gRNA Vector
Design Guide

GenScript CRISPR gRNA/Cas9 Plasmids

Genome Editing Has Never Been Easier!

Our Advantages

Never Miss a Deadline Again!
CRISPR plasmids in just 10 days.

Use What Works!
Broad Institute pre-validated CRISPR plasmids.

Guaranteed Quality
All plasmids are sequence validated.

- **Price starting at only $99!**
- **Licensing partnership with the Feng Zhang Lab** at the Broad Institute of MIT and Harvard
- **Guide RNA design and optimization** using algorithms created at the Broad Institute
- **Comprehensive plasmid options**, including eSpCas9, SpCas9, SpCas9 Nickase, SaCas9, and Transcription Activation (SAM) plasmids
- **24/7 Ph.D. level** service representatives to assist with your CRISPR related questions

GenCRISPR Plasmids

Vector Selection	Plasmid (Non-viral) Lentiviral AAV
Deliverables/Cas9 Selection	**Enhanced Specificity Sp Cas9 Plasmids** ***NEW!***; Sp Cas9 Plasmids; Sp Cas9 Nickase Plasmids; Sa Cas9 Plasmids; Transcription Activation Plasmids
Model Organism	Mammalian Cells
Quality Control	Sequence Chromatogram Quality Assurance Certificate
Ready for Transfection	✓
Multiplex Editing Versatility	✓
Gene Editing/Expression Options	Knock-out/Knock-in Transcription Activation
Delivery Time	10 days ***Fastest in the Industry!***
Pricing	Starting from $99

Have questions? GenScript's Ph.D.-level service representatives are available 24 hours a day, Monday through Friday, to assist you.

Toll-Free: 1-877-436-7274
Email: gene@genscript.com

Putting CRISPR into Practice:

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 1:

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 2).

Table 2:

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	In vitro transcribed Cas9 mRNA and gRNA	Transient expression of CRISPR gene editing components
sgRNA/Cas9 Ribonucleoprotein (RNP) Complexes	Purified Cas9 protein and chemically synthesized single guide RNA	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. 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. 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.
- Developing a split-Cas9 system using split-inteins.
- 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.

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.

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 1).

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 3). 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 3:

Cas Variant	PAM Sequence
eSpCas9/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, eSpCas9/SpCas9 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 4:

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).

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. 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. 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 genes42.

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.

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