Design and cloning of CRISPR guide RNAs

CRISPR endonucleases such as Cas9 and Cas12a enable targeted genome editing in a wide range of organisms. A short guide RNA (gRNA) directs the endonuclease to a genomic site based on sequence complementarity, typically requiring a short, enzyme-specific protospacer adjacent motif (PAM).

Cas9 of *Streptococcus pyogenes* targets sites immediately upstream of a 5′–`NGG`–3′ PAM and accepts a synthetic single guide RNA (sgRNA) that fuses the variable protospacer to the constant tracrRNA scaffold. Cas12a of *Acidaminococcus sp.*, by contrast, cleaves downstream of a 5′–`TTTV`–3′ PAM, distal to it, using a shorter crRNA without a tracrRNA, while Cas12a of *Lachnospiraceae sp.* also accepts 5′–`TYCV`–3′ PAMs.

Depending on the vector and Cas variant, guide RNAs may be cloned as individual expression units or as arrays of direct repeats and spacers. This protocol describes single (*Alternative A*) and pooled (*Alternative B*) guide RNA subcloning into U6 promoter–driven plasmids. The included examples are not exhaustive but illustrate distinct cloning architectures.

Risk assessment

- Risk of creating a self-propagating gene drive
- ▷ DO NOT combine Cas endonucleases with homology-directed repair templates in organisms capable of sexual reproduction or gametogenic cell lines
- ▷ Wear gloves, safety glasses, lab coat



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Procedures

›› *Guide RNA design*

(1.) Review transcript variants of the target gene using Ensembl or UCSC Genome Browser. Then, depending on the editing goal, identify the target region:

- For gene knockout, choose early coding exons common to all isoforms.
- For in-frame tagging, retrieve genomic sequences within 40–60 bp of the start or stop codon, which is the homology a single-stranded donor carries on each side [SSB15].

> ***Critical:*** Make sure the target sequence is absent in the transgene to prevent re-cleavage after integration.

(2.) Design or select multiple candidate protospacer sequences using an online design tool.

Name	Purpose	Cas	Species range	Design mode	Validation	Reference
CRISPRDB	Knockout	SpCas9	Human, Mouse	Fixed library, Custom prediction	Post-hoc	[CW22]
GeCKO	Knockout	SpCas9	Human, Mouse	Fixed library	Empirical	[SSZ14]
E-CRISP	Knockout	SpCas9	Broad	Custom prediction	In silico	[HKB14]
PITCh designer	Tagging	User-specified	Broad	Custom prediction	In silico	[NNT+17]
CCTop	Any	Many	Broad	Custom prediction	In silico	[STD+15]
CHOPCHOP	Any	Many	Broad	Custom prediction	In silico	[LMK+19]

> ***Note:*** The table lists common CRISPR guide design tools. Some provide validated gRNA libraries like GeCKO, while others support custom protospacer prediction across species and Cas types. Choose based on experimental context and organism.

> ***Critical:*** Empirical validation remains essential. Design multiple candidate guides to ensure at least one high-efficiency clone.

(3.) Choose guides with high predicted on-target scores and low off-target potential. Avoid sequences with low sequence complexity, strong self-complementarity, or long homopolymer tracts.

> ***This is why:*** Typical guide lengths range from 20 bp for SpCas9 to 23–24 bp for Cas12a. These lengths are intrinsic to the enzyme system and should not be arbitrarily extended. Mismatches near the PAM (proximal) end are less tolerated than distal ones. Off-target scoring tools incorporate mismatch positions and genome-wide alignments.

> ***Hint:*** Avoid targeting splice junctions or sites near intron-exon boundaries unless specifically required. These regions may affect transcript stability or yield incomplete knockouts. For pooled or multiplexed designs, ensure that guides do not share off-targets or overlap in a way that complicates cloning or barcoding.

(4.) Determine the required oligonucleotide format for the Cas variant and expression vector. Make sure the protospacer adjacent motif (PAM) is *not included* in the guide RNA sequence.

> ***Hint:*** U6 promoters require a guanine as the +1 transcription start site. For Cas9 single guide RNA (sgRNA) formats, design protospacers that begin with 5′–G–3′ when possible, or add a leading guanine if necessary. For Cas12a, the crRNA includes a fixed direct repeat upstream of the protospacer, and no adjustment is needed.

A › *Making individual guide RNA sequences*

(1.) For each guide RNA, synthesize a pair of complementary oligonucleotides with ends designed to create overhangs compatible with the vector's U6 cloning site. The examples below cover commonly used Cas variants and U6-based expression vectors:

Cas	Plasmid	Format	Enzyme	Guide	Forward oligo	Reverse oligo
AsCas12a	pRDA052	Array	BsmBI	23 bp	5′–agatNNN···NNN–3′	5′–attcNNN···NNN–3′
SpCas9	pX260	Array	BbsI	30 bp	5′–aaacNNN···NNNgt–3′	5′–taaaacNNN···NNN–3′
SpCas9	pX330	sgRNA	BbsI	G + 19 bp	5′–caccGNNN···NNN–3′	5′–aaacNNN···NNNC–3′

> ***This is why:*** Vectors such as pX330, pX458–pX463 use a single guide RNA (sgRNA) format that fuses the protospacer to a constant scaffold. Vectors like pRDA052, pX260, or pX334 express CRISPR arrays consisting of alternating direct repeats and variable spacers. The tracrRNA is expressed from a distinct locus.

> ***Hint:*** Standard desalted oligonucleotides suffice. The linearized vector supplies the 5′ phosphates, so the oligonucleotides need not be phosphorylated as long as the vector is not dephosphorylated.

(2.) Anneal the oligonucleotide pairs at 10 µM in 10 µL of 1 × DNA duplex buffer (or T4 DNA ligase buffer). Heat to 95 °C for 3 min, then cool slowly to room temperature.

(3.) Dilute 1 µL of the annealed product in 500 µL water for downstream ligation.

B › *Making pooled guide RNA libraries*

(1.) For each guide RNA, flank the protospacer with restriction sites that generate overhangs compatible with the vector's U6 cloning site.

Cas	Plasmid	Format	Enzyme	Guide	Oligo sequence
AsCas12a	pRDA052	Array	BsmBI	23 bp	5′–CGTCTCAagatNNN···NNNttttttgaatCGAGACG–3′
SpCas9	pX330	sgRNA	BbsI	G + 19 bp	5′–GAAGACTGcaccGNNN···NNNgtttAAGTCTTC–3′

Add one pair of unique primer binding sites to each construct. This allows selective amplification of individual subpools from a combined oligonucleotide pool if desired.

No.	Forward primer (= 5′ Flank)	3′ Flank	Reverse primer
1	5′–AGGCACTTGCTCGTACGACG–3′	5′–TTAAGGTGCCGGGCCCACAT–3′	5′–ATGTGGGCCCGGCACCTTAA–3′
2	5′–GTGTAACCCGTAGGGCACCT–3′	5′–GTCGAAGGACTGCTCTCGAC–3′	5′–GTCGAGAGCAGTCCTTCGAC–3′
3	5′–CAGCGCCAATGGGCTTTCGA–3′	5′–CGACAGGCTCTTAAGCGGCT–3′	5′–AGCCGCTTAAGAGCCTGTCG–3′
4	5′–CTACAGGTACCGGTCCTGAG–3′	5′–CGGATCGTCACGCTAGGTAC–3′	5′–GTACCTAGCGTGACGATCCG–3′
5	5′–CATGTTGCCCTGAGGCACAG–3′	5′–AGCCTTTCGGGACCTAACGG–3′	5′–CCGTTAGGTCCCGAAAGGCT–3′
6	5′–GGTCGTCGCATCACAATGCG–3′	5′–CGTCACATTGGCGCTCGAGA–3′	5′–TCTCGAGCGCCAATGTGACG–3′

> ***Note:*** The reverse primer anneals to the reverse complement of the 3′ flank. Use the same primer pair consistently for amplification and cloning of a given subpool.

(2.) Amplify each subpool from approximately 40 ng of oligonucleotide pool using the matching primer pair. Use an annealing temperature of 53 °C and 24 cycles.

> ***Critical:*** For combinatorial constructs that carry two guide RNAs, cut the amplification to 3 cycles. More cycles swap strands between constructs and scramble the pairings.

(3.) Purify the amplicons over a silica membrane.

(4.) Digest the purified PCR product with the matching Type IIS restriction enzyme under standard conditions. No inactivation is required before ligation.

[DSS+21]

›› ***Ligation into U6-based vectors***

(1.) Linearize 1 µg entry vector with the designated restriction enzyme in 20 µL 1 × restriction digest buffer.

> ***Hint:*** If unsure, examine the vector map for a Type IIS restriction site downstream of the U6 promoter. Type IIS enzymes cut outside their recognition site and remove the site from the vector during digestion, leaving non-palindromic overhangs. As a result, the enzyme does not need to be heat-inactivated or removed before ligation.

(2.) *Optional:* When cloning guide RNA libraries, purify the linearized product from an agarose gel.

(3.) Assemble guide RNA constructs by ligating the annealed oligonucleotide into the linearized vector at room temperature for 10 min:

Ingredient	Stock	Final	Individual	Library
Water, reagent-grade			3.5 µL	35 µL
T4 DNA ligase buffer	10 ×	1 ×	0.5 µL	5 µL
Linearized vector, unpurified	50 ng/µL	3 ng/µL	0.3 µL	3 µL
Annealed duplex, 1:500 dilution	20 nM	2 nM	0.5 µL	5 µL
T4 DNA ligase	400 U/µL	8 U/fmol	0.2 µL	2 µL

(4.) *Optional:* To enhance ligation efficiency during library construction, add 10 U of the matching restriction enzyme and cycle five to ten times between 37 °C and 16 °C.

(5.) *Optional:* Treat with exonuclease V (Plasmid-Safe™ DNase) to remove residual linear DNA.

> ***This is why:*** Exonuclease V treatment reduces background from unligated linear vector. This is particularly helpful in pooled cloning or low-efficiency transformations.

(6.) Transform into a competent bacterial host.

> ***Hint:*** For pooled libraries, precipitate the ligation product with ethanol and electroporate.

Analyses

○ Analyze two to three clones for loss of the cloning enzyme's restriction site.

> ***This is why:*** A Type IIS site is removed with the fragment it releases, so a correct clone resists the enzyme used for cloning—BbsI for pX330 and pX260, BsmBI for pRDA052. You may verify by restriction digest or sequencing.

> ***Quality assurance:*** The success rate of this protocol is exceptionally high; more than 80% of clones should have the desired genotype.

○ Verify two to three clones by Sanger sequencing using a universal U6 sequencing primer such as 5′–`GACTATCATATGCTTACCGT`–3′.

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Change log

2025-05-28 **Ewelina Bolcun, Benjamin C. Buchmuller** Version 1, 2024 to 2025. Design and cloning of pX330-based plasmids, after protocols from the Feng Zhang lab and the transgenic core at Cornell University.

2026-11-02 **Benjamin C. Buchmuller** Version 2. Cloning conditions change: 8 enzymeunit of ligase per femtomol where the text said 20, and a 40–60 bp homology arm after Singh et al. (2015). Credits: DeWeirdt et al. (2021) for the subpool cycle number. Also corrected the PAM orientations, the duplex dilution and the reason oligonucleotides need no phosphorylation; the clone screen now names the cloning enzyme.

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