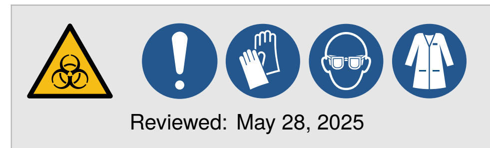
Design and cloning of CRISPR guide RNAs

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 2–6 bp specific protospacer adjacent motif (PAM).
Cas9 of *Streptococcus pyogenes* targets sites downstream 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 upstream of a 5'–`TTTV`–3' PAM 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.

This is a bench card. Full protocol available online.



Procedures

›› ***Guide RNA design***

(1.) Review transcript variants of the target gene using Ensembl or UCSC Genome Browser. Download the relevant genomic sequence in FASTA format if required by your design platform.

- For gene knockout, choose early coding exons common to all isoforms.
- For in-frame tagging, retrieve genomic sequences within 50–70 bp of the start or stop codon.

> ***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]

> ***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.

(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.

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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'–agatNNNNN···NNNNN–3'	5'–attcNNNNN···NNNNN–3'
SpCas9	pX260	Array	BbsI	30 bp	5'–aaacNNNNN···NNNNNgt–3'	5'–taaaacNNNNN···NNNNN–3'
SpCas9	pX330	sgRNA	BbsI	G + 19 bp	5'–caccGNNNN···NNNNN–3'	5'–aaacNNNNN···NNNNC–3'

(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 2 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'–CGTCTCAagatNNNNN···NNNNNtttttgaatCGAGACG–3'
SpCas9	pX330	sgRNA	BbsI	G + 19 bp	5'–GAAGACTGcaccGNNNN···NNNNNgtttAAGTCTTC–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'

(2.) Amplify each subpool from approximately 40 ng of oligonucleotide pool using the matching primer pair. Use an annealing temperature of 53 °C and limit the PCR to 20 cycles or less to avoid strand swapping between subpools.

(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.

›› *Ligation into U6-based vectors*

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

(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:

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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, 1:250 dilution	20 nM	2 nM	0.5 µL	5 µL
T4 DNA ligase	400 U/µL	20 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. 🕮

(6.) Transform into a competent bacterial host. 🕮

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🕮 Notes (available online)

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