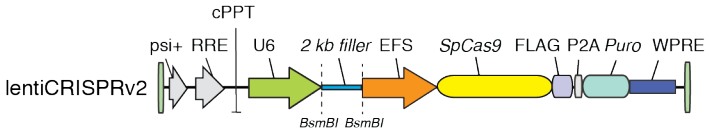
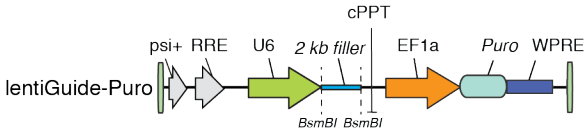
LentiCRISPRv2 and lentiGuide-Puro: lentiviral CRISPR/Cas9 and single guide RNA

CRISPR (**C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats) is a microbial nuclease system involved in defense against invading phages and plasmids. CRISPR loci in microbial hosts contain a combination of CRISPR-associated (Cas) genes as well as non-coding RNA elements capable of programming the specificity of the CRISPR-mediated nucleic acid cleavage. Lentiviral CRISPR/Cas can infect a broad variety of mammalian cells by co-expressing a mammalian codon-optimized Cas9 nuclease along with a single guide RNA (sgRNA) to facilitate genome editing (Shalem*, Sanjana*, *et al.*, Science 2014). Protocols for cloning into the lentiviral transfer plasmid and general considerations for producing lentivirus are described below. **Separate protocols are available for amplifying the genome-scale CRISPR knock-out (GeCKO) libraries. This protocol is for creating individual lentiviral CRISPR plasmids targeting a single genomic locus.**

lentiCRISPRv2 (one vector system): This plasmid contains two expression cassettes, hSpCas9 and the chimeric guide RNA. The vector can be digested using *BsmB*I, and a pair of annealed oligos can be cloned into the single guide RNA scaffold. The oligos are designed based on the target site sequence (20bp) and needs to be flanked on the 3' end by a 3bp `NGG` PAM sequence, as shown on the next page.



lentiGuide-Puro (two vector system): This plasmid expressed only the chimeric guide RNA. It does *not* contain Cas9. Please use lentiCas9-Blast (a separate lentiviral construct that delivers hSpCas9 and blasticidin resistance) to first integrate Cas9 into your cell line. The lentiGuide-Puro vector can be digested using *BsmB*I, and a pair of annealed oligos can be cloned into the single guide RNA scaffold. The oligos are designed based on the target site sequence (20bp) and needs to be flanked on the 3' end by a 3bp `NGG` PAM sequence, as shown on the next page.



Which vector to use: lentiCRISPRv2 is identical to the original lentiCRISPRv1 but produces nearly 10X higher titer virus. lentiGuide-Puro produces >100X higher titer virus over lentiCRISPRv1 and should be used in cell lines where Cas9 has already been integrated in (e.g. using the separate lentiCas9-Blast lentivirus). For applications where Cas9 cannot first be introduced (e.g. primary cells), lentiCRISPRv2 is recommended. After transduction, use puromycin to select for cells with lentiCRISPRv2 or lentiGuide-Puro.

Lentiviral production: Before starting any lentiviral work, please ensure compliance with your Environmental Health and Safety office and government/organization/university. Briefly, to make lentivirus, a transfer plasmid (e.g. lentiCRISPRv2 or lentiGuide-Puro) must be co-transfected into HEK293(F)T cells with the packaging plasmids pVSVg (AddGene 8454) and psPAX2 (AddGene 12260). As a positive control for viral production, we often use a CMV-EGFP lentiviral transfer plasmid (eg. AddGene 19319).

Target design notes and online resources: For application of Cas9 for site-specific genome editing in eukaryotic cells and organisms, we have computationally identified suitable target sites for the *S. pyogenes* Cas9 and calculated most likely off-targets within the genome. Please visit *http://www.genome-engineering.org* to access these Cas9 target design tools. Complete plasmid sequences, protocols, a discussion forum and additional information can be found at the Zhang Lab GeCKO website: *http://www.genome-engineering.org/gecko/* .

Citation: Please reference the following publications for the use of this material.

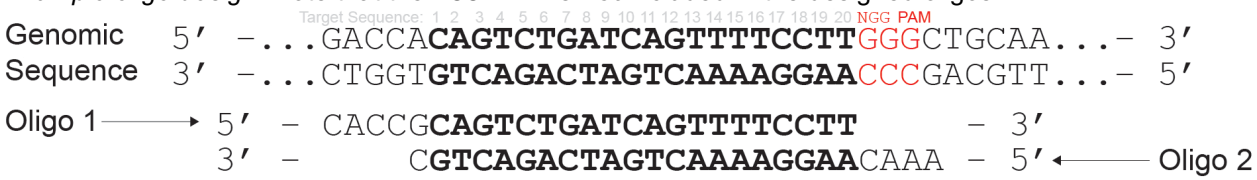
Improved lentiviral vectors and genome-wide libraries for CRISPR screening. Sanjana NE*, Shalem O*, Zhang F. *Nature Methods* (2014).

Genome-scale CRISPR-Cas9 knockout screening in human cells. Shalem O*, Sanjana NE*, Hartenian E, Shi X, Scott DA, Mikkelsen T, Heckl D, Ebert BL, Root DE, Doench JG, Zhang F (2014). *Science*, 343, 83-7. DOI: 10.1126/science.1247005

Target Guide Sequence Cloning Protocol

In order to clone the target sequence into the lentiCRISPRv2 or lentiGuide-Puro backbone, synthesize two oligos of the following form. **All plasmids have the same overhangs** after *BsmBI* digestion and the same oligos can be used for cloning into lentiCRISPRv2, lentiGuide-Puro or lentiCRISPRv1.

Example oligo design: Note that the NGG PAM is **not** included in the designed oligos.



Oligonucleotide ordering tips: Standard de-salted oligos (usually the most inexpensive synthesis) are sufficient for cloning. If not already resuspended, dilute each oligo to 100 μM in sterile water or TE.

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Lentiviral vector digestion, oligo annealing and cloning into digested vector:

1. Digest and dephosphorylate 5ug of the lentiviral CRISPR plasmid with *BsmB*I for 30 min at 37C:

5 ug	lentiCRISPRv2 **or** lentiGuide-Puro
3 ul	FastDigest *BsmB*I (Fermentas)
3 ul	FastAP (Fermentas)
6 ul	10X FastDigest Buffer
0.6 ul	100 mM DTT (freshly prepared)
X ul	ddH_2O
60 ul	total

2. Gel purify digested plasmid using QIAquick Gel Extraction Kit and elute in EB.

   If *BsmBI* digested, a ~2kb filler piece should be present on the gel. **Only gel purify the larger band**. Leave the 2kb band.

3. Phosphorylate and anneal each pair of oligos:

1 ul	Oligo 1 (100 μM)
1 ul	Oligo 2 (100 μM)
1 ul	10X T4 Ligation Buffer (NEB)
6.5 ul	ddH_2O
0.5 ul	T4 PNK (NEB M0201S)
10 ul	total

   Please use the T4 **Ligation** Buffer since the buffer supplied with the T4 PNK enzyme does not include ATP (or supplement to 1mM ATP).

   Put the phosphorylation/annealing reaction in a thermocycler using the following parameters:

37°C	30 min
95°C	5 min and then ramp down to 25°C at 5°C/min

4. Dilute annealed oligos from **Step 3** at a 1:200 dilution into sterile water or EB.

5. Set up ligation reaction and incubate at room temperature for 10 min:

X ul	*BsmB*I digested plasmid from **Step 2** (50ng)
1 ul	diluted oligo duplex from **Step 4**
5 ul	2X Quick Ligase Buffer (NEB)
X ul	ddH_2O
10 ul	subtotal
1 ul	Quick Ligase (NEB M2200S)
11 ul	total

Also perform a negative control ligation (vector-only with water in place of oligos) and transformation.

6. Transformation into **Stbl3 bacteria**. Lentiviral transfer plasmids contain Long-Terminal Repeats (LTRs) and must be transformed into recombination-deficient bacteria. We use homemade Stbl3 (propagated from Invitrogen C7373-03) and get excellent plasmid yields. Although other RecA- strains may work, we have found the most consistent transformations and yields using Stbl3.