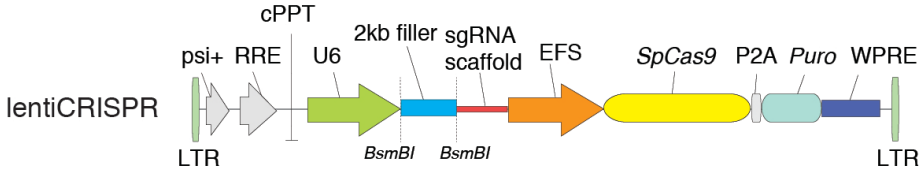
LentiCRISPR lentiviral CRISPR/Cas9 and single guide RNA

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. Using lentivirus, we delivered the type II CRISPR nuclease system to facilitate genome editing in mammalian cells (Shalem*, Sanjana*, *et al.*, Science 2014). The CRISPR/Cas system can be implemented in lentivirus capable of infecting a broad variety of mammalian cells by co-expressing the a mammalian codon-optimized Cas9 nuclease along with a single guide RNA (sgRNA). A protocol for cloning into the lentiviral transfer plasmid and general considerations for producing lentivirus are described below.

Separate protocols are available for the entire genome-scale CRISPR knock-out (GeCKO) library. This protocol is for creating individual lentiCRISPR targeting a single genomic locus.

LentiCRISPR (pXPR_001): 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.



Lentiviral production: Since this vector enables lentiviral delivery of both Cas9 and sgRNA for targeted gene knock out, it is important to perform these experiments in a lab with the appropriate biosafety level and controls, which can vary between different institutions. Before starting any lentiviral work, please ensure compliance with your Environmental Health and Safety office and government/organization/university. Briefly, to make lentivirus, lentiCRISPR (with sgRNA cloned) 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: 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 tools.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 website: www.genome-engineering.org .

Other questions? Many questions about using CRISPR for genome engineering are addressed here: Genome-engineering using the CRISPR-Cas9 system. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F (2013). *Nature Protocols*, 8, 2281-2308. DOI: 10.1038/nprot.2013.143

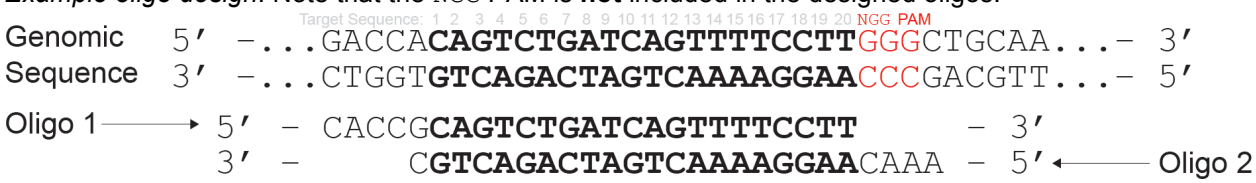
Citation: Please reference the following publication for the use of this material.

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 lentiCRISPR backbone, synthesize two oligos of the form:

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 100μM in sterile water or TE.

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

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

5 ug	lentiCRISPR plasmid
3 ul	FastDigest *BsmB*I (Fermentas)
3 ul	FastAP (Fermentas)
6 ul	10X FastDigest Buffer
X ul	ddH_2O
60 ul	total

2. Gel purify digested pLentiCRISPR 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 large (~11kb) band**. Leave the 2kb band.

3. Phosphorylate and anneal each pair of oligos:

1 ul	Oligo 1 (100μM)
1 ul	Oligo 2 (100μM)
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 and phosphorylated olgios 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

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.