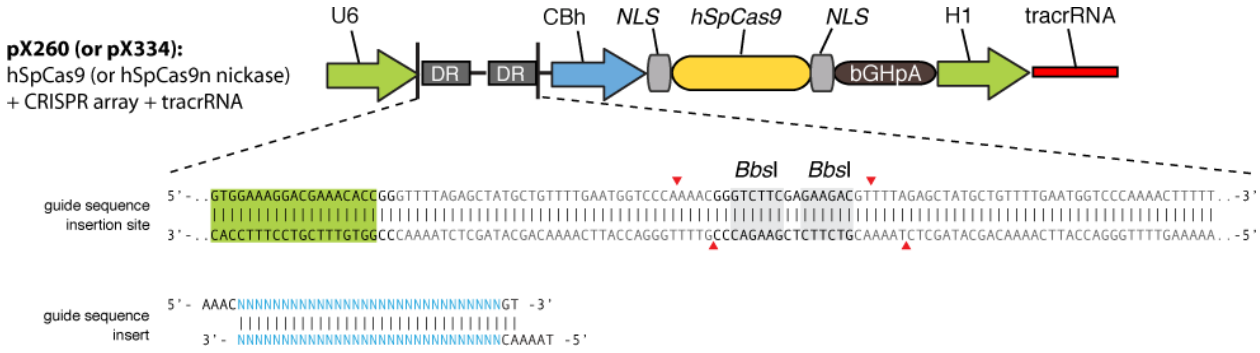
CRISPR | GENOME ENGINEERING TOOLBOX

ZhangLab

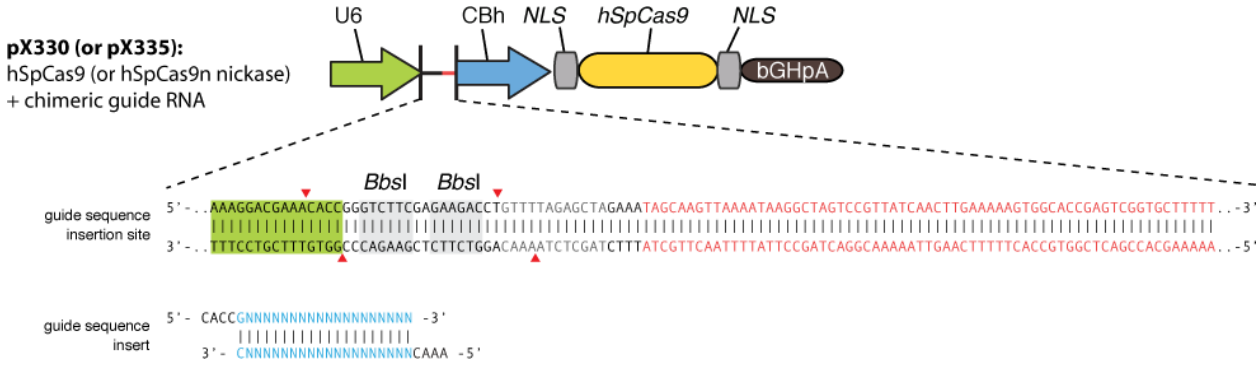
CRISPR Reagent Description

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. We have recently harnessed the type II CRISPR nuclease system to facilitate genome editing in mammalian cells (Cong *et al.*, Science 2013). The CRISPR/Cas system can be implemented in mammalian cells by co-expressing the bacterial Cas9 nuclease along with the guide RNA. Two forms of guide RNAs can be used to facilitate Cas9-mediated genome cleavage, using a CRISPR RNA array and tracrRNA or a synthetic guide RNA fusing the CRISPR RNA with the tracrRNA. These two systems are described below.

1. pX260 (or pX334): *S. pyogenes* Cas9 (or Cas9 D10A nickase) + CRISPR RNA array + tracrRNA: This plasmid contains three expression cassettes. In order to target a given site, the plasmid can be digested using *Bbs*I, and a pair of annealed oligos (design is indicated below) can be cloned into the CRISPR array. The oligos is designed based on the target site sequence (30bp) and needs to be flanked on the 3' end by a 3bp NGG PAM sequence.



2. pX330 (or pX335): *S. pyogenes* Cas9 (or Cas9 D10A nickase) + chimeric guide RNA containing +85nt of tracrRNA: This plasmid contains two expression cassettes, hSpCas9 and the chimeric guide RNA. The vector can be digested using *Bbs*I, and a pair of annealed oligos (design is indicated below) can be cloned into the guide RNA. The oligos is designed based on the target site sequence (20bp) and needs to be flanked on the 3' end by a 3bp NGG PAM sequence. We have found that increasing the length of the chimeric guide RNA can increase targeting efficiency; therefore this version of the backbone contains a longer fragment of the tracrRNA (+85nt).



Application 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. These sites are viewable as UCSC Genome Browser tracks for the human, mouse, rat, zebrafish, *C. elegans*, and *D. melanogaster* genomes. Sites are selected such that the seed sequence for each SpCas9 target site, 5'-NNNNNNNNNNNN-NGG-3', is specific to the relevant genome. A protocol for oligo cloning is included here and the plasmid sequences and additional information can be found at Zhang Lab's website (www.genome-engineering.org).

Citation Information: please reference the following publication for the use of these material.

Multiplex Genome Engineering using CRISPR/Cas Systems
Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. Science. 2013 Jan 3. DOI: 10.1126/science.1231143

CRISPR | GENOME ENGINEERING TOOLBOX

ZhangLab

Target Sequence Cloning Protocol

(standard de-salted oligos are sufficient)

pX260 (or pX334) – hSpCas9 (or hSpas9n nickase) + CRISPR array + tracrRNA:

In order to clone the target sequence into the pX260 backbone, synthesize two oligos of the form:

```
5′ – AAACNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGT     – 3′
3′ –     NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCAAAAT – 5′
```

pX330 (or pX335) – hSpCas9 (or hSpCas9n nickase) + chimeric guideRNA:

In order to clone the target sequence into the pX260 backbone, synthesize two oligos of the form:

```
5′ – CACCGNNNNNNNNNNNNNNNNNNN     – 3′
3′ –     CNNNNNNNNNNNNNNNNNNNCAAA – 5′
```

* * * * *

Oligo annealing and cloning into backbone vectors (original version, new version on next page):

1. Digest 1ug of pX260 or pX330 with *Bbs*I for 30 min at 37C:

1 ug	pX260 or pX330
1 ul	FastDigest *Bbs*I (Thermo)
1 ul	FastAP (Thermo)
2 ul	10X FastDigest Buffer
X ul	ddH_2O
20 ul	total

2. Gel purify digested pX260 or pX330 using QIAquick Gel Extraction Kit and elute in EB.

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)
10 ul	total

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

X ul	*Bbs*I digested pX260 or pX330 from **step 2** (50ng)
1 ul	phosphorylated and annealed oligo duplex from **step 3** (1:250 dilution)
5 ul	2X Quickligation Buffer (NEB)
X ul	ddH_2O
10 ul	subtotal
1 ul	Quick Ligase (NEB)
11 ul	total

5. (optional but highly recommended) Treat ligation reaction with PlasmidSafe exonuclease to prevent unwanted recombination products:

11 ul	ligation reaction from **step 4**
1.5 ul	10X PlasmidSafe Buffer
1.5 ul	10mM ATP
1 ul	PlasmidSafe exonuclease
15 ul	total

Incubate reaction at 37C for 30 min.

6. Transformation

Oligo annealing and cloning into backbone vectors (new version with single-step digestion-ligation)

The following protocol is a new version that is simpler to set up and increase cloning efficiency (higher percentage of correct colonies).

[IMPORTANT NOTE] Due to the simultaneous digestion-ligation step, the guide oligos CANNOT contain any *BbsI* enzyme site (i.e. the nucleotide sequence 'GAAGAC' or 'GTCTTC'). Please double-check your guides!

1. 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)
10 ul	total

Anneal 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

Dilute the annealed oligo 1:250 (250-fold).

2. Set up digestion-ligation reaction:

X ul	pX330 or other backbone vector (100ng)
2 ul	phosphorylated and annealed oligo duplex from **step 1** (1:250 dilution)
2 ul	10X Tango buffer (or FastDigest Buffer)
1 ul	DTT (10mM to a final concentration of 1mM)
1 ul	ATP (10mM to a final concentration of 1mM)
1 ul	FastDigest *Bbs*I (Thermo Fisher Fermentas)
0.5 ul	T7 DNA ligase
Y ul	ddH_2O
20 ul	total

Incubate the ligation reaction in a thermocycler:

37°C	5 min
23°C	5 min
Cycle the previous two steps for 6 cycles (total run time 1h)	
4 °C	hold until ready to proceed

3. (optional but highly recommended) Treat ligation reaction with PlasmidSafe exonuclease to prevent unwanted recombination products:

11 ul	ligation reaction from **step 4**
1.5 ul	10X PlasmidSafe Buffer
1.5 ul	10mM ATP
1 ul	PlasmidSafe exonuclease
15 ul	total

Incubate reaction at 37C for 30 min.

4. Transformation with 1-2 ul of the final product into competent cells

5. Pick colony and sequence verify the clones.