Generating targeting chiRNAs

A. Selecting a target sequence

The genomic target sequence should fulfill the following requirements:

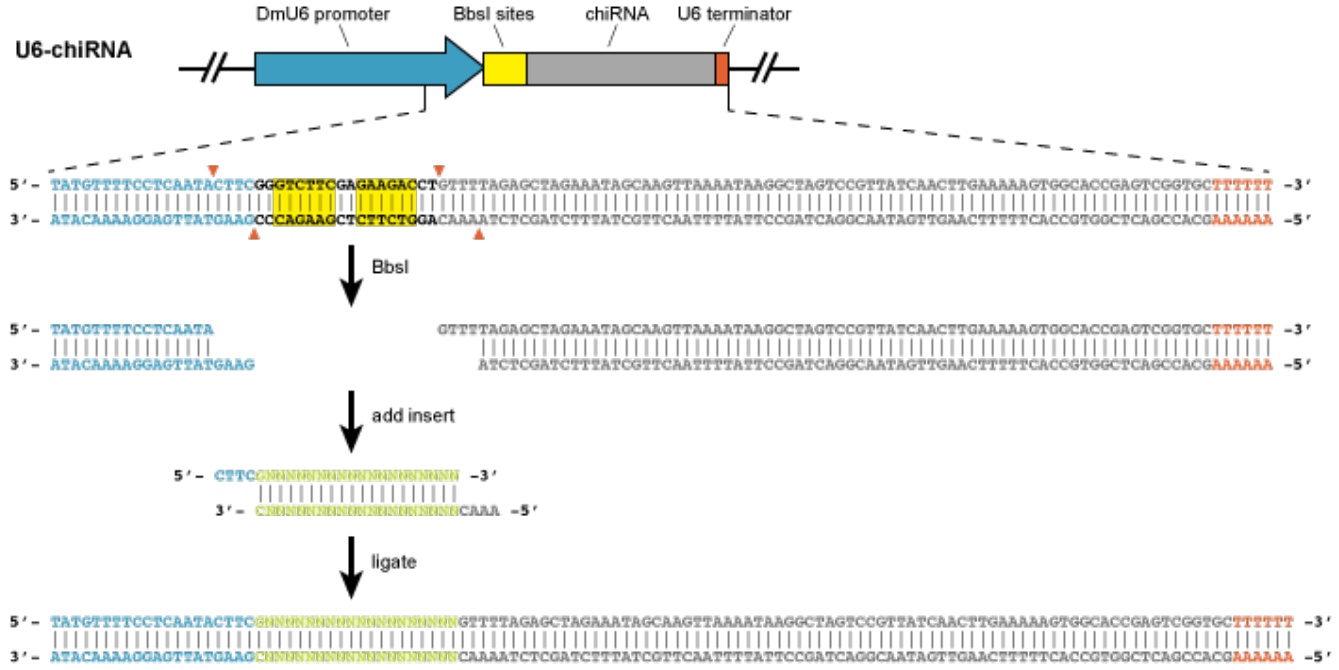
- 20-nt long
- followed by a 3-nt PAM sequence: **NGG**
- begin with a **G** to optimize U6-driven transcription

5' – GNNNNNNNNNNNNNNNNNNNNGG – 3'

Cas9 nuclease cuts 3-nt upstream of the PAM site (cleavage site indicated by red arrowhead).

To avoid off-target cutting, the 12-nt upstream of the PAM site (underlined above) should be unique in the genome. Predicted unique target sites have been identified by the Zhang lab.

B. Cloning

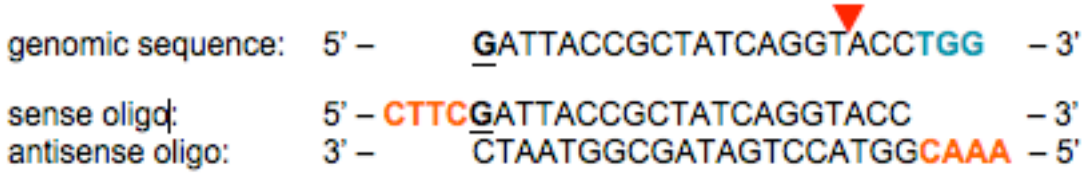


Targeting chiRNAs are easily cloned by annealed oligos into the pU6-BbsI-chiRNA plasmid via the BbsI restriction sites:

1. Design oligos based on the following template:

sense oligo: 5' – **CTTCG** (19 nt) – 3'
antisense oligo: 3' – C (19 nt) **CAAA** – 5'

example:



- The overhang sequences 5' – **CTTC** – 3' (sense oligo) and 3' – **CAAA** – 5' (antisense oligo) are complementary to the BbsI cut site.
- The **G** in the sense oligo corresponds to the first nt of the chiRNA and is necessary for efficient U6-driven expression. This G is the first nt in the 20-nt targeting sequence.
- The sense oligo19-nt sequence is the EXACT same sequence as the genomic target sequence. Do not include the 3-nt NGG PAM sequence.
- Red arrowhead indicates Cas9 cut site.

Oligos should be ordered 5' phosphorylated or phosphorylated using T4 PNK.

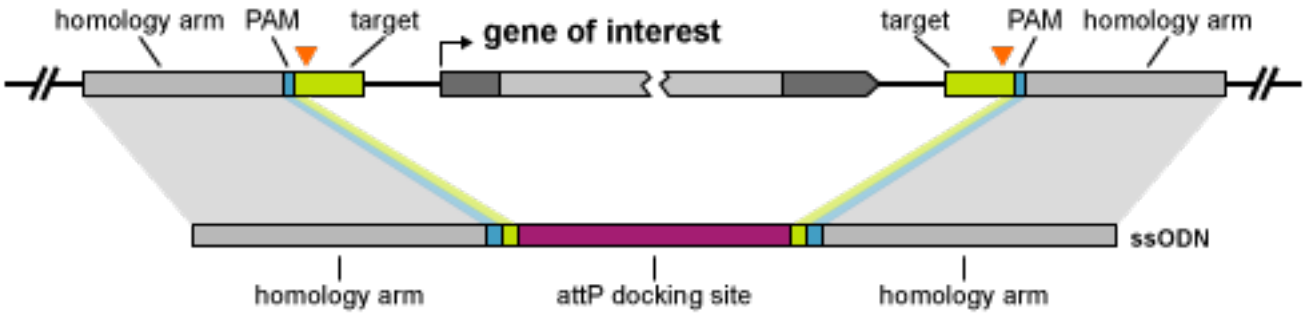
2. Anneal oligos
Dilute oligos in TE or 1X ligation buffer, then run the following thermocycler program:

95°C for 5 min, then ramp to 25°C at a rate of -5°C/min.

3. Cut the pU6-BbsI-chiRNA plasmid with BbsI enzyme and de-phosphorylate.

4. Ligate the annealed oligos with cut pU6-BbsI-chiRNA and transform E. coli.

Designing an ssODN template for HR-mediated repair



5’— (~60-nt 5’ homology) **CCNNNN** (**attP sequence**) **NNNNGG** (~60-nt 3' homology) – 3’

- **PAM**
- **NNN** corresponds to the 3-nt adjacent to the **PAM** site that remain following Cas9 cleavage. In this example, the 5’ PAM site is on the antisense strand and the 3’ PAM site is on the sense strand.
- **attP sequence (50-nt):**
  CTACGCCCCCAACTGAGAGAACTCAAAGGTTACCCCAGTTGGGGCACTAC

Single-stranded oligonucleotides (ssODNs) of up to 200-nt can be ordered from Integrated DNA Technologies and other companies. Do not have the oligonucleotide PAGE-purified, as this can introduce trace chemical contaminates that reduce embryo viability.

Injection Mix

phsp70-Cas9 and a single chiRNA plasmid can be coinjected to generate mutations via imperfectly repaired DSBs. Coinjection of phsp70-Cas9 and plasmids encoding two chiRNAs can induce defined deletions between the two cleavage sites. The addition of an ssODN donor template can mediate gene replacement by homologous recombination.

Dilute plasmids in dH20 to the following concentrations and inject following standard embryo injection protocols:

phsp70-Cas9: 500 ng/µL

pU6-BbsI-chiRNA: 500 ng/µL total (single chiRNA: 500 ng/µL, two chiRNAs: 250 ng/µL each)

(ssODN: 100 ng/µL)