

Protocol for sgRNA construction (Annealing method)

1. Prepare sgRNA cloning vector:

Digest 5~10 µg of sgRNA expressing vector (pU6-gRNA.Ppuro) with **BsmBI** at 55°C overnight, then separate the digested plasmid by electrophoresis using 1% agarose gel. The resulting sgRNA cloning vector bearing two **5' protruding ends, as depicted below**, (~7.7kb) is then recovered by gel extraction kit and ready for the cloning of annealed guide oligos:

U6 promoter--BsmBI----- (1.9kb Stuffer) -----BsmBI--sgRNA scaffold

5'-aaa **cacc**ggagacggtgttgtaaa---- (1.9kb Stuffer) ----ttttgtac**cgctctc** **gttt**aagagc-3'
3'-ttt**gtgg** **cctctgc**cacaacattt---- (1.9kb Stuffer) ----aaaaacatgcagagac**aaa** ttctcg-5'

2. Prepare annealed guide oligos for cloning:

1) Design guide oligos using available algorism(s) (please see Appendix 1):

To target the template DNA, search for 5'-N₂₀-NGG-3' (N=A, C, G or T) and the sequence of N₂₀ will be used directly as the base-pairing region of the sgRNA. NGG is the PAM sequence that is recognized by the *S. pyogenes* Cas9 protein. Please note that U6 promoter requires a G at the 5' end for efficient transcription. To maximize U6 promoter activity for sgRNA expression, add an extra **G** at the 5' end of the sense guide oligo if the first nucleotide of the transcribed sgRNA is not a G, e.g., 5'-G-H-N₁₉-3' (H=A, C or T), and in this case also put an extra **C** at the 3' end of the antisense guide oligo. (For maximize T3 or T7 promoter activity for *in vitro* sgRNA expression, add two extra **G** at the 5' end of the sense guide oligo.)

Add **cacc** at the 5' end of the sense guide oligo, and put **aaac** at the 5' end of the antisense guide oligo to create 5' protruding ends for cloning as shown below:

Sense guide oligo: 5'-**cacc**-N₂₀-3' (or 5'-**cacc**-**G**-H-N₁₉-3') (H=A, C or T)

Antisense guide oligo: 5'-**aaac**-N₂₀-3' (or 5'-**aaac**-N₁₉-D-**C**-3') (D=A, G or T)

For example, if the target site is CCGTCATGCATGACTGACTG**TGG** (5'-N₂₀-NGG-3'), the guide oligos will be:

Sense guide oligo: 5'-**cacc**-**G**CCGTCATGCATGACTGACTG-3' (an extra **G** at 5' end of N₂₀)

Antisense guide oligo: 5'-**aaac**-CAGTCAGTCATGCATGACGG**C**-3' (an extra **C** at 3' end)

2) Anneal guide oligos:

(1) Dissolve oligonucleotides into 100 μ M with autoclaved distilled water.

(2) Prepare 10X annealing buffer:

1 M K-acetate

0.3 M HEPES-KOH pH7.4

20 mM Mg-acetate

(3) Set up annealing mixture:

Sense guide oligo	9 μl
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Antisense guide oligo 9 μl

10X annealing buffer 2 μ l

(4) Anneal mixture by PCR machine using the following parameters:

95°C for 5 min then decrease to 4°C slowly (0.01°C / sec)

dilute annealed mixture 100 X with autoclaved ddH₂O (for ligation)

(5) Set up ligation reaction mixture and ligation for O/N:

*Bsm*BI-digested sgRNA cloning vector (100 ng) 2 µl

diluted guide oligos 2 μ l

10X ligation buffer	1 μl
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ligase (1 unit/ μ l) 1 μ l

d.water final volume to 10 μ l

(6) Transform into Stbl3 competent cells (optimal for lentivector).

Appendix 1: Free sgRNA design algorithms/web tools:

- (1) ZiFiT (<http://zifit.partners.org/ZiFiT/>);
- (2) Optimized CRISPR Design (<http://crispr.mit.edu/>)
- (3) CRISPRdirect (<http://crispr.dbcls.jp/>)
- (4) Cas9 online designer (<http://cas9.wicp.net/>)
- (5) CHOPCHOP (<https://chopchop.rc.fas.harvard.edu/>)
- (6) E-CRISP (<http://www.e-crisp.org/E-CRISP/designcrispr.html>)
- (7) sgRNAcas9 (www.biootools.com)
- (8) sgRNA Designer (<http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design>)
- (9) CRISPRseek (<http://www.bioconductor.org/packages/release/bioc/html/CRISPRseek.html>)
- (10) CRISPR MultiTargeter (<http://www.multicrispr.net/>)
- (11) CCTop (<http://crispr.cos.uni-heidelberg.de>)
- (12) CasOT (<http://eendb.zfgenetics.org/casot/>)
- (13) Cas-OFFinder (<http://www.rgenome.net/cas-offfinder/>)
- (14) GT-Scan (<http://gt-scan.braembl.org.au/gt-scan/>)
- (15) WGE (<http://www.sanger.ac.uk/htgt/wge/>)