Protocol for sgRNA construction (Annealing method)

1. Prepare sgRNA cloning vector:

Digest 5~10 μg of sgRNA expressing vector (pU6-gRNA.Ppuro) with ***Bsm*BI** 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--*Bsm*BI----------(1.9kb Stuffer)----------*Bsm*BI--sgRNA scaffold

5'-aaa **caccggagacggtgttgtaaa----(1.9kb Stuffer)----tttttgtacgtctct gtttaagagc-3'**
3'-ttt**gtgg cctctgccacaacattt----(1.9kb Stuffer)----aaaaacatgcagagacaaa** 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'-N_{20}-NGG-3' (N=A, C, G or T) and the sequence of N_{20} 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-N_{19}-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-N_{20}-3' (or 5'-cacc-G-H-N_{19}-3') (H=A, C or T)
Antisense guide oligo: 5'-aaac-N_{20}-3' (or 5'-aaac-N_{19}-D-C-3') (D=A, G or T)

For example, if the target site is CCGTCATGCATGACTGACTGTGG (5'-N_{20}-NGG-3'), the guide oligos will be:
Sense guide oligo: 5'-cacc-GCCGTCATGCATGACTGACTG-3' (an extra G at 5' end of N_{20})
Antisense guide oligo: 5'-aaac-CAGTCAGTCATGCATGACGGC-3' (an extra C at 3' end)

2) Anneal guide oligos:

(1) Dissolve oligonucleotides into 100 μM with autoclaved distillated 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
Antisense guide oligo	9 μl
10X annealing buffer	2 μl

(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 ddH_2O (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 μl
10X ligation buffer	1 μl
ligase (1 unit/μl)	1 μl
d.water final volume to	10 μl

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

Appendix 1: Free sgRNA design algorisms/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-offinder/)
(14) GT-Scan (http://gt-scan.braembl.org.au/gt-scan/)
(15) WGE (http://www.sanger.ac.uk/htgt/wge/)