Protocol for surrogate construction (Annealing method)

1. Prepare surrogate cloning vector:

Digest 5~10 μg of surrogate expressing vector with ***Kpn*I** and ***Age*I**, incubate at 37°C for overnight, then separate the restricted plasmid by electrophoresis using 1% agarose gel. The resulting surrogate cloning vector bearing two sticky ends, as depicted below, (~9.3kb) is then recovered by gel extraction kit. The purified vector is ready for ligation of annealed oligos (or a larger DNA fragment generated by PCR containing *Kpn*I / *Age*I protruding ends):

EGFP CDS - *Kpn*I - *Pme*I - *Afe*I - *Age*I - mCherry CDS

5'- gaagcttggtac cgtttaaacagcgcta ccggtatggtg **-3'**
3'- cttcgaac catggcaaatttgtcgcgatggcc ataccac **-5'**

2. Prepare annealed oligos (sgRNA targeting sequence) for cloning:

1) Design sgRNA targeting sequence according to the genomic sequence:

To mimic sgRNA targeting to its authentic DNA template, the base-pairing region of sgRNA including PAM sequence (5'-N_{20}-NGG-3'; N=A, C, G or T) are cloned into the *Kpn*I-*Age*I sites in between EGFP and mCherry CDS. **For generation of a reporter to detect CRISPR-mediated indel, please be sure that the mCherry CDS is out of frame relative to the open reading frame (ORF) of EGFP after cloning. Also, avoid potential in-frame STOP codon formation that may be generated after indel reaction in the spacer region of EGFP and mCherry genes.**

To create oligomer pair containing sticky-ends for ligating to the *Kpn*I & *Age*I-digested surrogate cloning vector, add c at the 5' end of the sense oligo and put a at the 3' end of the sense oligo; in antisende strand oligo, add ccggt at the 5' end and put ggtac at the 3' end as depicted below:

Sense oligo: 5'-c-N_{20}-NGG-a-3'

Antisense oligo: 5'-ccggt-CCN-N_{20}-ggtac-3'

For example, if the target site is GGGGCCACTAGGGACAGGATTGG (5'-N_{20}-NGG-3'), the guide oligos will be:

Sense guide oligo: 5'-c-GGGGCCACTAGGGACAGGATTGG-a-3'

Antisense guide oligo: 5'-ccggt-CCAATCCTGTCCCTAGTGGCCCC-ggtac-3'

2) Anneal 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 oligos 100 X with autoclaved distillated water(for ligation)

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

*Kpn*I & *Age*I-digested surrogate vector (100 ng)	2 μl
diluted oligos	2 μl
10X ligation buffer	1 μl
ligase (1 unit/μl)	1 μl
ddH_2O final volume to	10 μl

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

Note: To generate a reporter for CRISPRi, the inserted DNA fragment should be in-frame relative to EGFP ORF and/or mCherry ORF. (The design strategy is different from the reporter used for detection of CRISPR-mediated indel).