**Construction of dual gRNA expression vectors**

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**Lentiviral dual gRNA expression vectors**

 pKLV2.2-h7SKgRNA5(SapI)-hU6gRNA5(BbsI)-PGKpuroBFP-W (#72666)

pKLV2.2-mU6gRNA5(SapI)-hU6gRNA5(BbsI)-PGKpuroBFP-W (#72667)

**gRNA design for the hU6gRNA5(BbsI) cassette**

Genome 5’-tggcgtgTAAGAGAGCATCATGGGCCACGGcagagaa-3’

Guide RNA 5’-GAAGAGAGCATCATGGGCCA-3’

Top strand oligo 5’-CACCGAAGAGAGCATCATGGGCCA-3’

Bottom strand ologo 3’-CTTCTCTCGTAGTACCCGGTCAAA-5’

**gRNA design for the h7SKgRNA5(SapI) cassette**

Genome 5’-tggcgtgTAAGAGAGCATCATGGGCCACGGcagagaa-3’

Guide RNA 5’-GAAGAGAGCATCATGGGCCA-3’

Top strand oligo 5’-CTCGAAGAGAGCATCATGGGCCA-3’

Bottom strand ologo 3’-CTTCTCTCGTAGTACCCGGTCAA-5’

**gRNA design for the mU6gRNA5(SapI) cassette**

Genome 5’-tggcgtgTAAGAGAGCATCATGGGCCACGGcagagaa-3’

Guide RNA 5’-GAAGAGAGCATCATGGGCCA-3’

Top strand oligo 5’-TTTGAAGAGAGCATCATGGGCCA-3’

Bottom strand ologo 3’-CTTCTCTCGTAGTACCCGGTCAA-5’

Note that the length of 5’ overhang produced by BbsI and SapI are different. Furthermore, each promoter needs specific 5’ overhang for ligation. Therefore, different sequences (highlighted in red) need to be added. In addition, if the first nucleotide is not G, replace the first nucleotide to G as exemplified above (G+N19 configuration) or append G at the 5’ end (G+N20 configuration). Order oligos individually (at 100 µM, desalt purification in water) or in a mix form (at 50 µM each, desalt purification in water).

With these vectors, two gRNAs need to be cloned sequentially. Pay attention when designing gRNAs. The first gRNAs should not have the restriction enzyme site for the second gRNA cloning.

**Oligo phosphorylation and annealing**

**1**| Mix the followings in a PCR plate.

 100 µM Top strand oligo 1 µl

 100 µM Bottom strand oligo 1 µl

 10x T4 ligation buffer (NEB) 1 µl

 T4 PNK (NEB M0201) 0.5 ul

 dH2O 6.5 µl

 or

 Mixed oligos (50 µM each) 2 µl

 10x T4 ligation buffer (NEB) 1 µl

 T4 PNK (NEB M0201) 0.5 ul

 dH2O 6.5 µl

**2**|Place the plate in a PCR machine and run the programme:

 37 °C, 30min → 95 °C, 5min → ramp down to 25 °C at 0.1 °C/sec → 4 °C

**3**|Store annealed oligos in -20 °C.

**1st guide RNA cloning into the hU6 (BbsI) cassette**

 **Vector linearization**

**1**| Mix the following reagents in a 1.5ml tube and incubate for 2 hr at 37 °C.

 Lentiviral plasmid 2 µg

 BbsI (NEB, R0539S/L) 3 µl

 10x NEB2.1 5 µl

 dH2O up to 50 µl

**2**| Run the sample on 1% agarose gel, excise the linearized fragment and purify the DNA.

**3**| Quantify the purified DNA using NanoDrop and adjust concentration at 20 ng/µl.

**Ligation & transformation**

**1**| Dilute ds-oligos (10 µM) for the hU6 cassette in EB buffer (Qiagen) on ice as follows.

 ∙ 1st dilution (142 fmol/ul) : 139 µl EB buffer + 2 µl 10 µM ds-oligo

 ∙ 2nd dilution(7.1 pmol/ul) : 57 µl EB buffer + 3 µl 1st dilution

**2**| Mix the following in a PCR tube on ice.

|  |  |
| --- | --- |
| 20 ng/ul linearized lentiviral vector | 1 µl (=3.7 fmol) |
| 7.1 fmol/µl ds-oligo | 2 µl (=14.2 fmol) |
| 10x ligase buffer (NEB M0202S) | 1 µl |
| T4 ligase (NEB M0202S) | 1 µl |
| dH2O | 5 µl |

**Note**: Make a negative control by adding 2 µl H2O instead of ds-oligo.

**3**| Incubate at 16 °C for 2 hr to overnight.

**4**| Add 5 µl of the ligation mixture to a 1.5ml microtube and keep it on ice.

**5**| Add 50 µl of DH5 chemical competent cell (Library efficient, Invitrogen), vortex for 1 sec and incubate for 10 min on ice.

**6**| Incubate the mixture at 42 °C for 30 sec and then incubate it on ice for 2 min.

**7**| Add 250 µl SOC (provided) and incubate the transformed bacteria at 37 °C for 30 min.

**Bacteria culture**

**(a) Bulk plasmid preparation**

Since double-stranded oligonucleotide cloning is so efficient that liquid selection can work very well. However, cloning efficiencies sometimes vary and impure vector could give rise to high background. This method is faster and easier, but caution must be taken as described below.

**8a**| Add 300 µl bacteria+SOC directly into 2 ml 2xTY (+amp, 50 µg/ml) in a 15-ml falcon tube and shake at 37 °C overnight.

**9a**| Check bacterial growth between 16 hr and 20hr post inoculation.

**Note**: There should be no or a very little bacterial growth in the vector-only control. If the insert+ bacteria clearly grow much better than the vector-only control, the cloning is successful. This difference is clear between 16-20hr incubation. Later, bacteria in the vector-only control quickly catch up and no difference can be observed.

**10a**| Culture the bacteria until it reach plateau.

**Note**: It requires much longer incubation than bacterial culture from a colony.

**11a**| Mini-prep, measure the DNA concentration and store at -20 °C.

**Note**: In general, sequence verification is not required as long as there is a clear difference in bacterial growth. Even if cloned plasmids are analysed by capillary sequencing, no background trace can usually be observed, meaning that the purity is high enough. However, please note that plasmids isolated by this way are bulk, not clones. This method should be used when you are confident on the accuracy of oligo synthesis, no cross-contamination and high cloning efficiency over background.

**(b) Plasmid from clones**

**8b**| Plate transformed bacteria on LB+amp plates (50µl, 5µl and 0.5 µl per LB+amp plate.).

**9b**| Culture colonies & mini-prep. Sequence MUST be verified by capillary sequencing.

 Sequencing primer: AGATAATTAGAATTAATTTGACTG for the hU6 cassette

**2nd guide RNA cloning into the h7SK or mU6 (SapI) cassette**

Follow the procedure for the 1st guide RNA cloning. Note that vectors need to be linearized with SapI as follows.

**Vector linearization**

**1**| Mix the following reagents in a 1.5ml tube and incubate for 2 hr at 37 °C.

 Lentiviral plasmid 2 µg

 SapI (NEB, R0569S/L) 3 µl

 10x NEB-CutSmart 5 µl

 dH2O up to 50 µl

**Sequencing gRNA**

When plasmids are purified from individual clones, analyse the sequence by capillary sequencing using the following primers.

 h7SK cassette: ATGATATTTGCTATGCTGGTTAAAT

 mU6 cassette: GGATTTCTATAAGAGATACAAAT