**Construction of gRNA expression vectors**

**V2015-8-25**

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**Empty gRNA expression vectors**

● V1 vectors:

 Lentiviral gRNA expression: pKLV-U6gRNA(BbsI)-PGKpuro2ABFP (#50946)

● V2 vectors:

 Lentiviral gRNA expression: pKLV2-U6gRNA5(BbsI)-PGKpuro2ABFP-W (#67974)

 pKLV2-U6gRNA5(BbsI)-PGKpuro2AZsG-W (#67975)

 pKLV2-U6gRNA5(BbsI)-PGKpuro2AmAG-W (#67976)

 pKLV2-U6gRNA5(BbsI)-PGKpuro2AmCherry-W (#67977)

 pKLV2-U6gRNA3(BbsI)-PGKpuroBFP (#67990)

 pKLV2-U6gRNA5(BbsI)-PGKpuroBFP (#67991)

**gRNA design (for v1 vectors only)**

Genome 5’-tggcgtgTAAGAGAGCATCATGGGCCACGGcagagaa-3’

Guide RNA 5’-GAAGAGAGCATCATGGGCCA-3’

Top strand oligo 5’-CACCGAAGAGAGCATCATGGGCCAGT-3’

Bottom strand ologo 3’-CTTCTCTCGTAGTACCCGGTCAAAAT-5’

**gRNA design (for v2 vectors only)**

Genome 5’-tggcgtgTAAGAGAGCATCATGGGCCACGGcagagaa-3’

Guide RNA 5’-GAAGAGAGCATCATGGGCCA-3’

Top strand oligo 5’-CACCGAAGAGAGCATCATGGGCCA-3’

Bottom strand ologo 3’-CTTCTCTCGTAGTACCCGGTCAAA-5’

Note that v1 and v2 vectors produce different overhangs at the 3’ side. Therefore, different sequences (highlighted in red) need to be added at the 3’ side of a guide sequence. In addition, if the first nucleotide is not G, replace the first nucleotide to G as exemplified above or append G at the 5’ end. Order oligos individually (at 100 µM, desalt purification) or in a mix form (at 50 µM each, desalt purification)

**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.

 **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) in EB buffer (Qiagen) on ice as follows. Keep the diluted ds-oligo on ice.

 ∙ 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 fragments give rise to high background. This method is much 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. However, please note that plasmids isolated by this way are bulk population, not clones. This method should be used when you are confident on accuracy of oligo synthesis, no cross-contamination and high cloning efficiency over background.

**(b) Isolation of 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