CRISPR-Cas target site cloning

1. **sgRNA design:**

   http://crispr.cos.uni-heidelberg.de

   recommended off-target settings:
   PAM type NRG; core length: 2; max core mismatches: 2; max total mismatches: 3 or 4,

   http://www.genome-engineering.org/crispr/?page_id=41

   both tools have similar quality from our point of view with some more flexibility for CCTop and distances given to neighboring genes

2. order primers (smallest amount, no special purification) with respective overhangs (underlined) like following example. Please note that the sgRNA has to start with a 'G' (marked bold) that serves as the transcriptional initiation for the U6 Promoter. If the target site does not naturally start with a 'G', add a 5' 'G'. Some very efficient sgRNAs have been generated that way strongly indicating that the additional 5' 'G' does not disturb the targeting. Avoid poly-T stretches (four or more) since 5xT serves as RNA-Pol-III termination signal.

CACC **G**CGCTCCCTGGGGGCAGTTCA
AAAC TGAACTGCCCCCAGGGAGCGC

3. **Primer Phosphorylation:**

Sense Primer (100µM)	1µl
Antisense Primer (100µM)	1µl
T4 DNA Ligase buffer	1µl
T4 PNK	0.5µl
H20	6.5µl
Total	10µl

PCR Program:

37C	45:00min
95C	2:30min
cool down at 0.1C/s to 22C	pause

4. **Vector Digest:**

2µg vector	x µl
2µl FastDigest buffer	2 µl
1µl FastDigest Esp3I (BsmBI)	1 µl
2µl DTT (10mM)	2 µl
H20	x µl
Total	20µl

Incubate at 37C for 45min.
Then add 1ul of Fermentas FastAP and incubate for an additional 15min. Gel purify the vector.

5. **Ligation:**

Dilute annealed and phosphorylated Oligo **1:500** in H20.

~30-50ng vector	x µl
Annealed & phosphorylated Oligo duplex (1:500)	1 µl
T4 DNA Ligase buffer	0.5µl
T4 DNA Ligase	0.5µl
H20	x µl
Total	5µl

Set up one additional ligation without insert oligo as control.
Incubate according to manufacturers instructions. In general 60-120min at RT yields good results.

6. **Transformation** into competent E.coli (XL1-blue, Top10, DH5a)

7. If control ligation without Insert is 'clean', pick two colonies each and send plasmid directly for sequencing

pLKO_U6_SEQ_fw	TTTGCTGTACTTTCTATAGTG