CRISPR-Cas target site cloning

1. sgRNA design: CCTop
   http://crispr.cos.uni-heidelberg.de/
   settings: core length=off, max mismatch=3, S. *pygones* Cas9, PAM = NGG; off-target: NGG-NRG, don't forget to choose the right species (Default is somewhat unusual)

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 hU6 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, also when interspaced by just one other nt) since they serve as RNA-Pol-III termination signal. Preferentially use balanced GC content.

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
NEB 3.1	3 µl
NEB BsmBI	1 µl
H20	x µl
Total	30µl

Incubate at 55C for 30min.
Then add 1ul of Fermentas FastAP (works in NEB buffer) and incubate for an additional 10-15min at 37C. Gel purify the vector.Do not exceed recommended times!

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

Prepare an additional control ligation without insert oligo as control.
Incubate 30-90min at RT

6. Transform into competent E.coli
7. If control without Insert is 'clean', pick two colonies each. Control digest with SalI can be performed to exclude undigested backbone, which is the most common false-positive background seen. Send plasmids for sequencing

mU6seq_fw1	AAAGGAAACTCACCCTAACTG
DH119_U6int_fw	AGTACAAAATACGTGACGTAG

For cloning with the mU6 promoter:

Oligo overhangs must be changed (note the additional T (sense) and compl. A (antisense):

TGTT **TG**CAGCCTCGTGACTGGGCGTG
AAAC CACGCCCAGTCACGAGGCTGC**A**

Generation of dual sgRNA vector

1. Insert spacers individually in a L40C-CRISPR… backbone (hU6) and a SGL40C.mU6… (also feasible with 7SK promoter)
2. Excise sgRNA with mU6 promoter form the SGL40C.mU6 with EcoRI/XhoI digest (approx. 450bp)
3. Open L40C-CRISPR with EcoRI/XhoI
4. Ligate, pick colonies, minipreps
5. check clones via NotI/XhoI digest