Cloning of oligonucleotides to S2 CRISPR vector
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1. Pick 23 nt sequence upstream of NGG in the genome
2. Remove NGG sequence to leave 20 nt target sequence
3. If target sequence does not begin with G, add G to the 5' end of it to allow transcription from the U6 promoter
4. For the forward oligonucleotide, add TTC to the 5' end of the target sequence
5. For the reverse oligonucleotide, reverse compliment the target sequence (including any additional G nucleotide), and add AAC to the 5' end
6. Synthesise two oligonucleotides

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F = 5'- TTCGNNNNNNNNNNNNNNNNNNN(N) -3'
R = 5'- AAC(N)NNNNNNNNNNNNNNNNNNNC -3'
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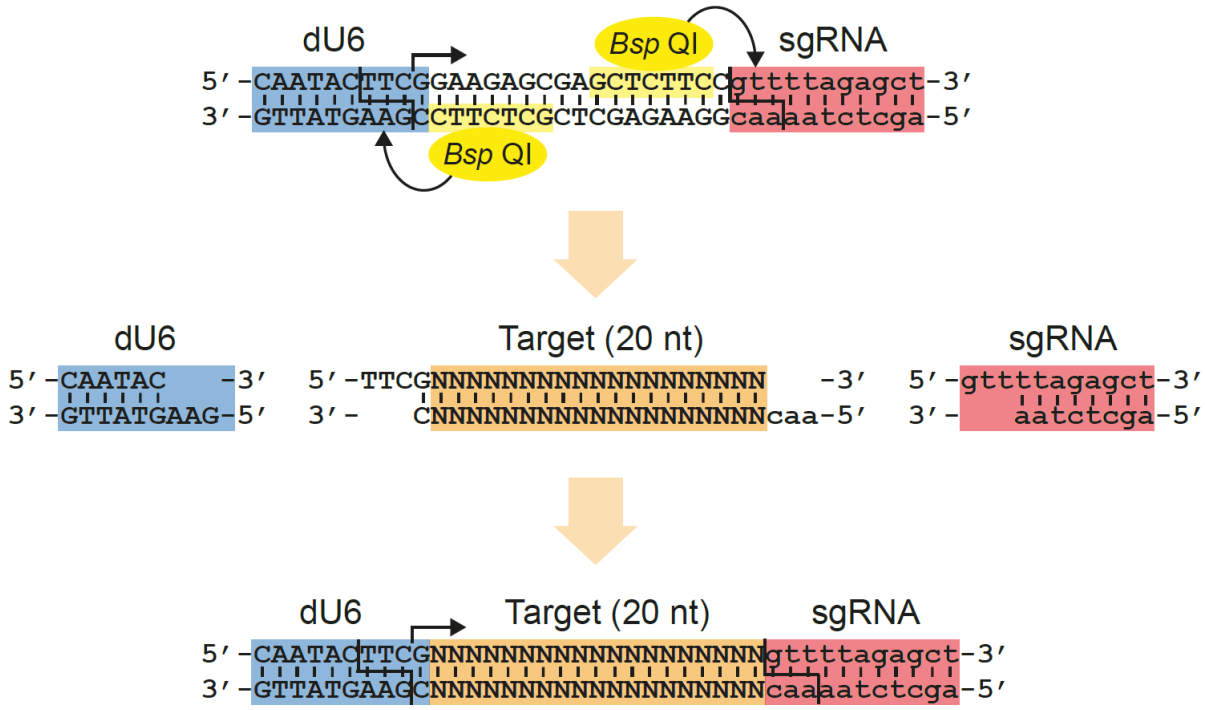
Schematic of cloning process



7. Anneal forward and reverse oligos as follows
8. Mix 10µl of 2x annealing buffer (20mM Tris, 2mM EDTA, 100mM NaCl, pH 8.0) with 5 µl each of 100 uM forward and reverse oligos.
9. Anneal in PCR machine on the following program

98˚C	1 min
98-88˚C	5 s, decrease 0.1˚C/cycle x99 cycles
88-78˚C	10 s, decrease 0.1˚C/cycle x99 cycles
78-68˚C	10 s, decrease 0.1˚C/cycle x99 cycles
68-58˚C	10 s, decrease 0.1˚C/cycle x99 cycles
58-48˚C	10 s, decrease 0.1˚C/cycle x99 cycles
48-38˚C	10 s, decrease 0.1˚C/cycle x99 cycles
38-18˚C	1 s, decrease 0.2˚C/cycle x99 cycles
18˚C	Forever

10. Phosphorylate oligos in the following mixture

1 µl	Annealed oligo
1 µl	Roche T4 DNA ligase buffer (contains ATP)
1 µl	T4 PNK
7 µl	ddH_2O
10 µl	Total

11. Incubate for 30 min at 37˚C
12. Dilute 10x in ddH_2O
13. Meanwhile, digest 2 µg pAc-sgRNA-Cas9 vector for 1 h at 37°C

2 µg	pAc-sgRNA-Cas9-Puro vector
5 µl	10x NEBuffer 4
X µl	ddH_2O
2 µl	Bsp QI (NEB)
50 µl	Total

14. Add 1ul CIP (NEB) and incubate for 10 min at 37°C
15. PCR purify to 30 µl EB
16. Ligate vector and oligo in the following mixture

1 µl	Vector
2 µl	10x diluted oligo
1 µl	Roche T4 DNA ligase buffer
4 µl	ddH_2O
1 µl	T4 DNA ligase
10 µl	Total

17. Incubate at 18˚C for 2 h, then transform 2 µl to a 50 µl aliquot of chemically competent DH5-α cells
    a. Incubate 20 min on ice with plasmid
    b. Heat shock for 30 s at 42˚C
    c. Cool for 2 min on ice
    d. Add 150 µl SOC and plate entire mixture to LB-carbenicillin plate (100 µg/ml)
    e. Incubate at 37°C overnight