Cloning of sgRNAs

This protocol recommends the usage of either plasmid pU6-(BbsI)sgRNA_CAG-Cas9-venus-bpA (Addgene ID 86986) or pU6-(BbsI)sgRNA_CAG-Cas9_EF1-TagRFP (Addgene ID 86987), encoding for sgRNA and Cas9 and a fluorescent Venus or RFP reporter, or usage of pU6-(BbsI)sgRNA_CAG-venus-bpA (Addgene ID 86985), carrying a sgRNA-cassette and a fluorescent reporter only. These vectors allow expression of the sgRNA by the human U6-promoter. This promoter requires a "G" base at the transcription start site. Hence, it is recommended using CRISPR/Cas9 target sites starting with a "G". Otherwise an additional "G" should be added at the start of the sgRNA sequence. It should be noted that wildtype Cas9 is amenable to the inclusion of an extra G but other RNA guided nucleases are not (Cas9-HF/eCas9).

3.1.1 Annealing of sgRNA oligos

The sgRNA oligos can be cloned into the above mentioned vectors by *BbsI* restriction enzyme overhangs, with N_1-N_{20} as the selected Cas9 target sequence:

sgRNA-oligo-F 5' –CACC(G)N_1NNNNNNNNNNNNNNNNNN$_{20}$ –3'

sgRNA-oligo-R 5' –AAACNNNNNNNNNNNNNNNNNNN(C) –3'

1. Resuspend oligos at 1 µg/µl in 1x TE-buffer.
2. Combine in a microcentrifuge tube
   - 1 µl oligo F (1 µg/µl)
   - 1 µl oligo R (1 µg/µl)
   - 98 µl 1x TE-buffer
3. Incubate 5 min at 98 °C in a heat block.
4. Switch off the heat block and cool down slowly to RT for 1-2 h.
5. Put the annealed oligos on ice or store at -20 °C.

3.1.2 Digestion of sgRNA expression vector

1. Set up digestion reaction as following:
   - X µl of the above mentioned vectors (5 µg)
   - 2.5 µl *BbsI* (store at -80 °C)
   - 10 µl NEB2 buffer
   - Fill up to 100 µl with nuclease free water
2. Incubate at 37°C for 1 h.
3. Inactivate the restriction enzyme for 20 min at 65 °C.
4. Load the digested vector on a 0.9% agarose gel and extract the linearized vector

using a DNA gel extraction kit. Expected fragment sizes are: pU6-(BbsI)sgRNA_CAG-Cas9-venus-bpA – 10.1 kb; pU6-(BbsI)sgRNA_CAG-Cas9-bpA_EF1-RFP – 11.6 kb; pU6-(BbsI)sgRNA_CAG-venus-bpA – 6.0 kb.

3.1.3 Ligation of sgRNA oligos

1. Set up ligation in a microcentrifuge tube
    - X µl linearized vectors (100 ng)
    - 1.5 µl annealed oligos
    - 1.5 µl fresh ligase buffer
    - 1 µl T4 DNA Ligase
    - Fill up to 15 µl with nuclease free water and incubate O/N at 16 °C.

3.1.4 Transformation

1. Thaw chemically competent *E. coli* on ice slowly.
2. Pipet the transformation reaction on ice
    - 50 µl chemically competent DH5 alpha *E. coli*
    - 5 µl of the ligation mix.
3. Incubate 30 min on ice.
4. Perform the heat shock in a water bath at 42 °C for 90 sec.
5. Incubate 3 min on ice.
6. Add 1 ml LB medium w/o antibiotics and incubate 30 min at 37°C at 200 rpm.
7. Plate the transformation on LB-Agar plates 50 µg/ml carbenicillin or ampicillin and incubate O/N at 37°C.

The next day pick up to five colonies and inoculate 5 mL LB medium containing 50 µg/ml carbenicillin or ampenicillin. Incubate O/N at 37 °C at 200 rpm. Perform plasmid mini preparations and subject for Sanger sequencing to verify the correct sequence using primer hU6-For: GAGGGCCTATTTCCCATG.