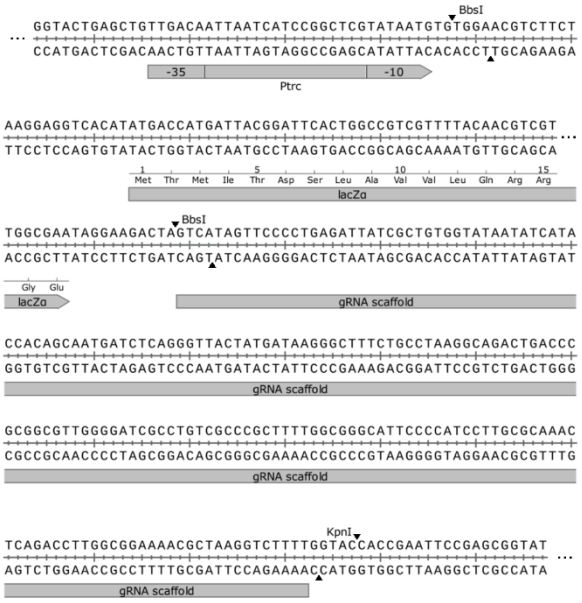
Protocol for cloning spacers into pTTCC*

Protocol overview:

1. Design oligonucleotides containing a desired spacer sequence. Add 4bp overhangs complimentary to BbsI restricted vector pTTCC:

   5'-TGGANNNNNNNNNNNNNNNNNNNNNNNNNNNNN -3'
   3'- NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCAGT-5'

2. Anneal and phosphorylate oligos
3. Digest pTTCC with BbsI
4. Ligate annealed and phosphorylated oligos into BbsI restricted pTTCC
5. Transform ligation mix into NEBStable competent cells; culture O/N on L media agar plates with 30 µg/ml kanamycin
6. Culture a few clones in liquid media O/N in L+ Kan (30 µg/ml), miniprep, and confirm spacer insert by restriction and/or sequencing



Protocol details:

1. Design oligonucleotides containing a desired spacer sequence adjacent to a PAM.

   The top-strand oligo should contain an overhang 5'-TGGA followed by the 5'-3' spacer sequence; The bottom-strand oligo should contain an overhang 5'-TGAC followed by the 5'-3' reverse complement of the spacer sequence; gRNAs tested in the present study contained a 30 nt spacer sequence. Other lengths may be suitable but have not been tested.

2. Anneal and phosphorylate the oligos:

   Mix:
   1 µl top-strand oligo (10 µM)
   1 µl bottom-strand oligo (10 µM)
   2 µl T4 DNA ligase buffer (M0202 NEB)
   0.5 µl T4 PNK (M0236 NEB)
   15.5 µl H_2O

   Incubate mix:
   37°C for 60 minutes
   95°C for 5 minutes
   Cool to 10°C in steps, at a rate of -5°C/min

3. Digest pTTCC

   Mix:
   1 µg pTTCC
   1 µl BbsI-HF (NEB R3539)
   5 µl CutSmart buffer (NEB)
   $H_2$0 to 50 µl

   Incubate mix:
   37°C for 1 hour
   65°C for 20 minutes

   Purification of the vector is not neccessary, but may reduce background in the following steps

4. Ligate vector and oligos

   Mix:
   10-40 ng digested vector (step 3)
   1 µl annealed oligos (step 2)
   1 µl 10X T4 DNA ligase buffer (NEB)
   0.5 µl T4 DNA ligase
   H_2O to 10 µl

   Incubate mix:
   24°C for 10 minutes

5. Transform NEBStable (C3040, NEB) according to manufacturer's instructions; plate on L agar media with 30 µg/ml kanamycin.

*The protocol is adapted from instructions for spacer cloning into vector MLM3636 (addgene 43860) from the Joung lab (J. Keith Joung, Harvard Medical School)