Plasmid Assembly Protocol with Golden Gate Assembly

- Select a 20 nt protospacer of interest. The 3' protospacer adjacent sequence (PAM) must be NGG, where N is any nucleotide. Preference is given to:
  - ◊ Sequences with purines occupying the last four (3') bases of the protospacer.
  - ◊ Sequences on the non-coding strand.
  - ◊ Sequences in which the last 12 nt of protospacer + 3 nt PAM (15 nt total) are unique in the genome (check by BLAST with all four possible NGG sequences).
- Design two 24 nt oligonucleotides (4 nt 5' sticky end + 20 nt spacer sequence) with the sticky ends ACGC on the forward primer and AAAC on the reverse primer.
- For single spacers, anneal spacer oligos as follows
  - ◊ Resuspend both oligos to 100µM in water
  - ◊ Mix 5 µL FOR + 5 µL REV + 90 µL 30 mM HEPES, pH 7.8
  - ◊ Heat to 95 °C for 5 min, then ramp to 4 °C at 0.1 °C/sec
- Insert annealed spacer (or dual+spacer synthetic construct) by Golden Gate assembly.
  - ◊ X µL Backbone (100 ng)
  - ◊ 0.3 µL Insert
  - ◊ 2 µL T4 Ligase Buffer (NEB)
  - ◊ 1 µL T4 ligase (NEB)
  - ◊ 1 µL BbsI (NEB)
  - ◊ Fill up to 20 µL with H_2O

- Golden Gate Program:

	°C	min
9x	37.0	10
	16.0	10
	50.0	5
	65.0	20
	4.0	PAUSE

- Transform 3 µL of each reaction to E. coli NEB5alpha by heat shock
- Plate 10% of recovery culture on selective plates with 10 µL of 0.5 M IPTG and 40 µL of 20 mg mL^{-1} Bluo-gal (in DMSO).
- Pick white colonies to selective LB and recover plasmid

Cobb, R.E., Wang, Y., and Zhao, H. (2014). High-Effi ciency Multiplex Genome Editing of Streptomyces Species Using an Engineered CRISPR/Cas System.: 1–12.