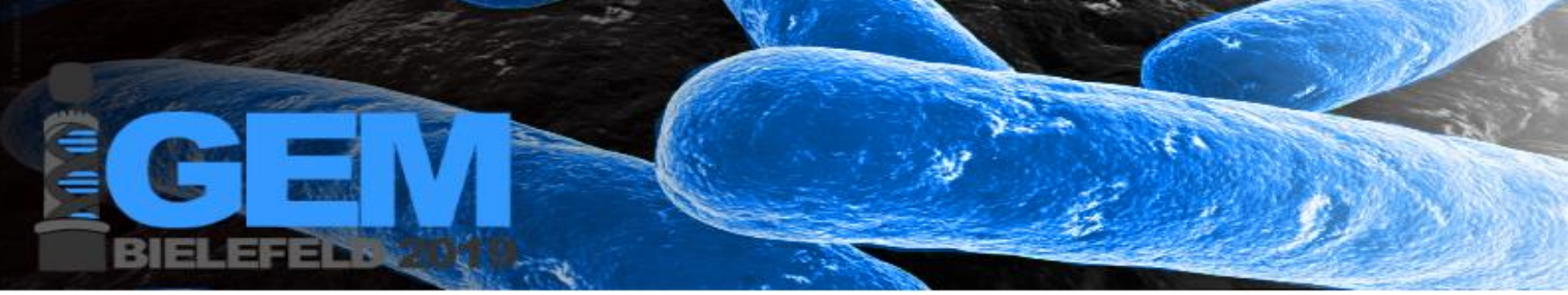


Plasmid Assembly Protocol with Golden Gate Assembly

- 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).

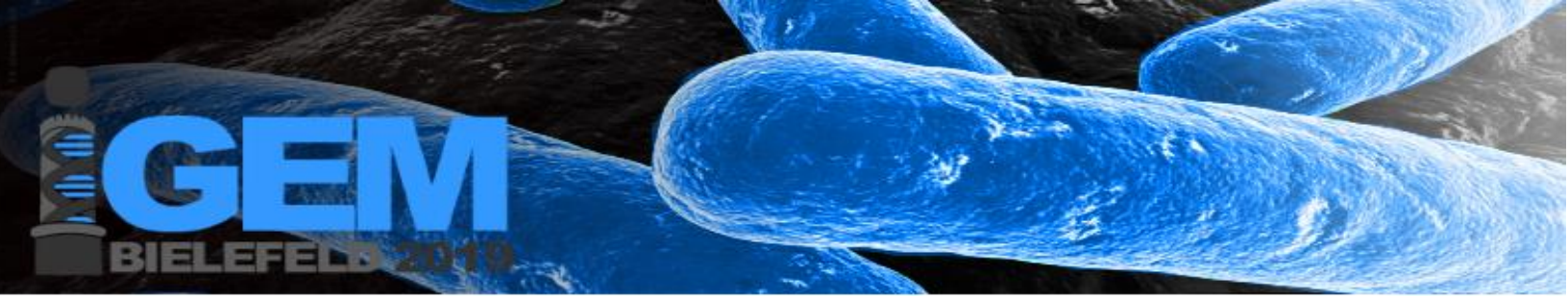
- Design two 24 nt oligonucleotides (4 nt 5' sticky end + 20 nt spacer sequence) with the sticky ends ACGC on the forward primer and AAAC on the reverse primer.

- For single spacers, anneal spacer oligos as follows:
    - Resuspend both oligos to 100µM in water
    - Mix 5 µL forward primer + 5 µL reverse primer + 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) o 1 µL BbsI (NEB)
    - Fill up to 20 µL with H2O

- 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* DH5α 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 Blue-gal (in DMSO).
- Pick white colonies to selective LB and recover plasmid.

From: iGEM Bielefeld-CeBiTec