

A Python-Based Algorithm to Design Primers for One-Step-Assembly (OSA). A Python-based algorithm, denoted OSA Primer Finder (OPF), was used to search potential protospacers in DNA sequences flanking the *phiCDKH02/phi027* locus and design corresponding primers for constructing the plasmid through OSA. OPF is run with Python (version 3.11; <https://www.python.org/>). Detailed procedures for using OPF to design primers are described previously (Hong et.al, 2018).

Plasmid construction

To construct pEcCdH01, the small RNA promoter (sRNAP; promoter of sCbei_5830) from *C. beijerinckii* 8052 genome with the crRNA sequence (5'-TAATTCTACTCTTGTAGATCATC TGATAAGAAGGACTTAATA-3') were synthesized by GenScript and introduced into pUC19, generating pEcCdH01.

The plasmid pEcCdH07 was designed to delete the ~55.9-kb *phiCDKH02/phi027* locus (JBCJLD010000007.1, 93652-149601). The fragment sRNAP::crRNA-*phiCDKH02*, containing the specific spacer 5'-GCCACCCATTGCGACCGTATTA-3' (PAM sequence: 5'-TTTT-3'), was generated with primers YW3105/phi500_S1_0. Homology arms *phiCDKH02*-Up-arm and *phiCDKH02*-Down-arm were generated with primers phi500_S1_1/phi500_S1_2 and phi500_S1_1/phi500_S1_2, respectively. Then the three fragments were assembled with BtgZI-linearized pWH34 to generate pEcCdH07. The plasmid pEcCdH08 was also designed to delete the ~55.9-kb *phiCDKH02/phi027* locus, however, with two spacers (5'-GCCACCCATTGCGACCGTATTA-3' and 5'-TCAGCAACACCATTGCTAAGTC-3') in order to improve DSB efficiency for this extremely large gene deletion. The fragment sRNAP::crRNA-*phiCDKH02 double* was generated with primers YW3105/phi500_S1S2_0, in which a second spacer 5'-TCAGCAACACCATTGCTAAGTC-3' was added to the 3'-end of sRNAP::crRNA-*phiCDKH02*. Homology arms *phiCDKH02*-Up-arm and *phiCDKH02*-Down-arm were generated with primers phi500_S1S2_1/phi500_S1_2 and phi500_S1_1/phi500_S1_2, respectively. The rest of the plasmid construction process was similar to the construction of pEcCdH07.