WCSB, Wageningen University	EPP-SOP-WU03
	Version 1.0

EPP - Standard Operating Procedure

(only for selected experiments intended to transfer results from one lab to the other)

Title: Design and clone spacers in pSEVA231-CRISPR

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Desig and clone spacers in pSEVA231-CRISPR	Christos Batianis Stamatios Damalas		18/4/2018

Instruction

Design and cloning spacers in pCRISPR

1. Introduction / Purpose

The purpose of this protocol is the design of CRISPR spacers ready to be ligated in the pSEVA231-CRISPR cloning vector.

2. Equipment and chemicals

2.1. Equipment

- Thermomix
- PCR Cycler

2.2. Chemicals

- BsaI (no HF)
- T4 ligase
- T4 ligase buffer
- BSA
- Kanamycin
- NaCl (1 M)

2.3. Bacterial strains and plasmids

- *E. Coli* DH5a
- pSEVA231-CRISPR (Fig. 2)

2.4. Other materials

- Primers for colony PCR:

  86: CTGGATTCTCACCAATAAAAAACG
  87: TCTAGGGCGGCGGAT

3. Media and Buffers

- Super optimal Broth with added Glucose (SOC) medium
- Luria Bertaini (LB) medium
- Meta Assembly Mix:
    - BsaI (non HF) (10 µl)
    - T4 ligation buffer
    - T4 ligase
    - BSA (1.5 µl)
    - H20 (13.5 µl)

4. Procedures

4.1. Spacer design

- Use the online tool BPROM (Softberry) to define the gene promoter and sequence between the promoter and the ATG
- Find the available PAM (-NGGs) and choose the most suitable sequence (usually the closer to the promoter the higher the down-regulation)
- Select 30 nucleotides (5'-3') before the the PAM of the template or non-template strand (Fig.1)
- Copy and paste the appropriate flanks and order the single stranded DNA sequences as primers (Table 1)

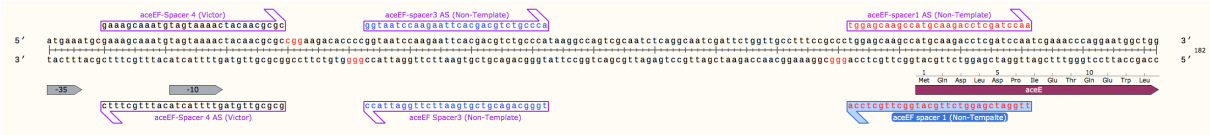

Figure 1

Spacer	Flanks
PAM-Spacer	*aggtctcaaaac--gttttgagacca*
Anti-PAM Spacer	*tggtctcaaaaac--gttttgagacct*

Table 1

4.2. Oligo annealing and Ligation

Annealing

1 ul oligo 1 (100 uM)
1 ul oligo 2 (100 uM)
2.5 ul NaCl (1 M)
45.5 ul ddH2O

Incubate for 5 minutes at 95˚C and slowly cool down to room temperature (using a Thermocycler). Alternatively, use the heat block, taking the block out of the heater and letting it cool for 2 hours. Dilute the annealed oligonucleotides 10 times.

The annealed oligonucleotides should have the structure shown in figure 3.

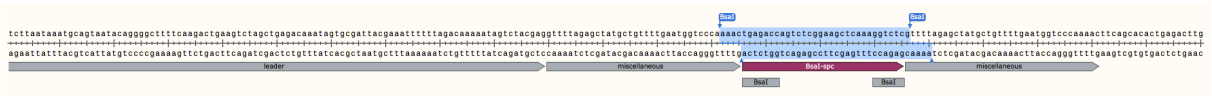

Figure 2

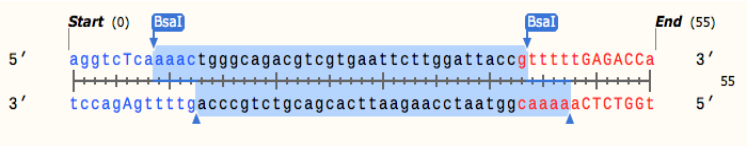

Figure 3

Ligation

2 ul diluted annealed oligo
2 ul Plasmid (10-15ng/ul)
2 ul Meta Assembly Buffer

Ligation, transformation and screening is performed according to the following steps:

- 37°C, 5 min → (16°C, 4 min → 37°C, 3 min) x 15 → 37°C, 5 min → 85°C, 15 min
- Transform *E.coli* DH5a
- Screen colonies by colony PCR with primers 86/87
- Select the colonies containing the DNA insert and send for sequencing

5. Biosafety

No biosafety issues are associated with this protocol. The protocol was developed and performed at an MLI /BSLII laboratory by using the commercial cloning strain E. coli Dh5αλpir.

6. Acknowledgements

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