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	EPP-SOP-WU03
WCSB, Wageningen University	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

- Bsal (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:
  - Bsal (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)

		aceEF-Spacer 4 (Victor) gaaagcaaatgtagtaaaactacaacgcgc	aceEF-spacer3 AS (Non-Template) ggtaatccaagaattcacgacgtctgccca	aceEF-spacer1 A5 (Non-Template) [tggagcaagccatgccatgccatgatccaa	
5′	atgaaat	gcgaaagcaaatgtagtaaaactacaacgcgccggaa	gacaccccggtaatccaagaattcacgacgtctgcccataaggccagtcg	caatctcaggcaatcgattctggttgcctttccgccctggagcaagccatgcaagacctcgatccaatcgaaacccaggaatggctgg	3'
3'	tacttta	cgctttcgtttacatcattttgatgttgcgcggcctt	ctgtggggccattaggttcttaagtgctgcagacgggtattccggtcagc	gttagagtccgttagctaagaccaacggaaagg <mark>cggg</mark> acctcgttcggtacgttctggagctaggttagctttgggtccttaccgacc	5' 182
	-35	-10		1 5 1 10 Met Gin Asp Leu Asp Pro IIe Giu Thr Gin Giu Trp Leu Accte	
		ctttcgtttacatcattttgatgttgcgcg aceEF-Spacer 4 AS (Victor)	ccattaggttcttaagtgctgcagacgggt aceEF Spacer3 (Non-Template)	acticgtlcggtacgtlctggagctaggtt ccEFspace1(NonTempolo)	

## Figure 1

Spacer	Flanks		
PAM-Spacer	aggtctcaaaacgtttttgagacca		
Anti-PAM Spacer	t <b>ggtctc</b> aaaaacgtttt <b>gagacc</b> t		

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.

	Bial		Bsal	
contrasta a a cycay can a cycyy contrasta a cycy a cycy a can cycy cycy a can cycay contrasta a cycay cycy a cycy	ayyttitayayotatyotyttityaatyyttotaaaa	cryagaccagrercygaagercaaaggr	cogreetagagerargergerergaarggreeeaaaaere	Jaycacactyayactty
		nact ct ant canancet t coant t t cca		tcatataectotaeec
agaararrangenarargennoogaaagennogennogenargerraagenaagenaagen gebeure gebeure gebeure gebeure gebeure gebeure g		A	Jugennue eregurungunnune erenengiggereregun	leodededageoroedaa
leader	> miscellaneous	Bsal-spc	miscellaneous	
		BsaI Bs	I	

#### Figure 2

	Start (0)	Bsal Bsal	<b>End</b> (55	6)
5′	aggtcTca	aaactgggcagacgtcgtgaattcttggattaccg <mark>tttttGAGACCa</mark>	3	, EE
3′	tccagAgt	tttgacccgtctgcagcacttaagaacctaatggcaaaaaCTCTGGt	5	,

#### 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 Dh5 $\alpha\lambda$ pir.

#### 6. Acknowledgements



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