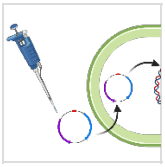

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pYCR cloning strategy

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ABSTRACT

Introducing NLP14a in the genome of B. mycoides: In order to create plasmid pYCR-gamyNLP, the backbone of Pycr will be digested with the PCR product of gamy_Fw and gamy_Rv to produce the pYCR_gamy (CRISPR vector containing the gRNA). The NLP14a sequence will be ordered from Twist Biosciences And PCR-amplified with the primers NLP_Fw and NLP_Rv. The resulting PCR product, as well as the pYCR_gamy vector will be digested with SfiI. The digested mixtures will be ligated using T4 ligase.

Kill switch cloning: The suggested kill switch mechanism is based on Trp auxotrophic strains as well as a toxin-antitoxin mechanism. The toxin will be introduced in the genome using crispr in place of trpE. The antitoxin will be maintained as a cytoplasmic plasmid (pAD-YqcF) and expressed only in the presence of solanine. Unfortunately, to the best of our knowledge, no operator that binds solanine of B. mycoides has been described in literature. If it is provebn that no such operator is present, we suggest on oly keeping the tryptophan dependence strategy.

In order to create plasmid pYCR-gtrpEYqcG plasmid, the backbone of Pycr will be digested with BsaI and ligated with the annealing product of gtrpe_Fw and gtrpe_Rv to produce the pYCR_gtrp (CRISPR vector containing the gRNA). Synthetic dna for *yqcG* will be ordered from Twist Biosciences and PCR-amplified with the primers Yqcg_Fw and Ygcg_Rv. The resulting PCR product, as well as the pYCR_gtrpE vector will be digested with SfiI. The digested mixtures will be ligated using T4 ligase

Protocol was adapted after a paper of Yi et al., 2018

1. Yi, Y., Li, Z., Song, C. & Kuipers, O. P. Exploring plant-microbe interactions of the rhizobacteria Bacillus subtilis and Bacillus mycoides by use of the CRISPR-Cas9 system. Environ. Microbiol. 20, 4245–4260 (2018).

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Clone sgRNA sequence into pYCR

1 Design sgRNA spacer sequence (~20 nts) using "Benchling"' and choose B. mycoides M2E_15 genome
Choose the gRNA with the highest on-target score and the lowest off-target score.

2 Order the 2 complementary oligos flanked by overhang containing BsaI restriction site

3 Anneal oligos by mixing the following components:

- **70 µl** Nuclease Free water
- **10 µl** Oligo DNA annealing buffer 10x ([M] **100 Milimolar (mM)** Tris pH **8** , [M] **500 Milimolar (mM)** NaCl, [M] **10 Milimolar (mM)** EDTA)
- **10 µl** DNA oligos A and B ([M] **10 Micromolar (µM)** each)

4 Mix well and incubate for **00:05:00** at **90 °C** 5m

5 Cool slowly to room temperature (aprox. **01:00:00**) by removing the heat block from the apparatus 1h

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6 Run agarose gel (1%) to check the annealing product

7 Digest pYCR with BsaI and inactivate the enzyme by PCR clean up

8 Ligate the annealing product with the precut pYCR:
MIx:
- **10 µl** l of annealing product
- **60 ng** of digested pYCR
- **1 µl** T4 ligase

9 Transform the ligation mixture into E. coli MC1061 using the heat-shock methos.
Grow the cells on LB agar plates + Erithromycin [M]**0.1 mg/ml**

10 To select the transformants perform a colony PCR with Fw primer binding upstream of PvanP and Rv primer downstream SfiI cloning sites.

Succesful transformation will have a size of 427 pb

11 Miniprep and sequence the constructs

Clone flanking regions into pYCR containing sgRNA

12 Select the repair fragment of choice:
-for knock-out: PCR amplify aprox. 1 kpb upstream and downstream of the sgRNA targeted region
-for knock-in: sequence of the desired inserction sequence flanked by SfiI restriction sites compatible with the backbone of pYCR

13 Digest the pYCR(+sgRNA) and the repair fragments with SfiI
Inactivate SfiI by PCR clean up

14 Ligate the components using T4 ligase 20m
Heat inactivate the ligase (incubate **00:20:00** **60 °C**)

15 Perform SmaI digestion to get rid of the original pYCR

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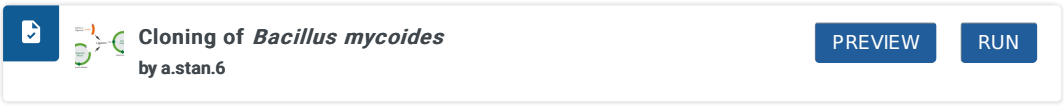
16 Transform the ligation mixture into E. coli MC1061 using the heat-shock methos.
Grow the cells on LB agar plates + erythromycin

17 To select the transformants perform a colony PCR with Fw primer binding upstream of PvanP and Rv primer downstream SfiI cloning sites.

18 Miniprep and sequence the constructs

Transform final construct in B. mycoides M2E_15

19 Transform using an electroporation protocol using **5 µl** plasmid DNA



19.1 Pick 1 colony of B. mycoides M2E_15 and inoculate it in BHIS

19.2 When OD600nm reached 0.85 add 2% glycine and 2% threonine in order to weaken the cell wall.

19.3 Grow the cells overnight at **200 rpm, 30°C**

19.4 Dilute the overnight culture 50 times in LBSP medium until the OD600nm reaches 0.65

19.5 Collect the cells by **4000 x g, 4°C, 00:10:00** . Discard supernatant

19.6 Wash cell pellet with pre-chilled electroporation buffer (10% glycerol, 0.25 M sorbitol, 0.25M trehalose) x4

19.7 Suspended in 1 ml electroporation buffer (10% glycerol, 0.25 M sorbitol, 0.25M trehalose).

19.8 Snap-freeze the electrocompetent cells in liquid nitrogen and sore at **-80 °C**

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19.9 For the PCR mix pipette:

- **5 µl** primers mixture Fw + Rv (5 uM) (see table "Primers")
- **10 µl** HF buffer
- **1 µl** dNTP (10mM)
- DNA template (0.1 ng)
- **0.25 µl** phusion polymerase
- MIliQ to **50 µl**

19.10 PCR reaction is performed with the following protocol:

Temperature (*C)	Time (mm:ss)
98	05:00
98	10:00
Variable	00:30
72	00:30
repeat above steps	30x
72	10:00
11	Infinite

19.11 Load **5 µl** of sample plus **1 µl** of staining solution 6x. Check the length of the band (bp).
Purify sample : PCR cleanup/ gel extraction according to manufacturer.

19.12 Digest the polymerized insert and the desired vector with 2U of apropriate restriction enzyme. See table "Primers" in order to choose the enzyme.

19.13 Digestion mixture (**20 µl**):

-SfiI 2 U
- Plasmid (**50 ng**)
-Insert (**20 ng**)
- **2 µl** CutSmart 10x (or appropriate buffer)
-MiliQ to **20 µl**

19.14 Incubate **01:00:00** at the temperature recommended by the manufacturer of the restriction enzyme

19.15 Inactivate the restriction enzyme by incubationg 20 min **65 °C**
**SfiI can't be heat inactivated so in order to inactivate it a PCR clean up is necesary

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19.16 Ligate the digested insert and vector.
Ligation mixture :
-10 ul digestion product
-1 ul T4
-2 ul T4 ligase buffer
-7 ul MQ

19.17 Incubate **02:00:00** **Room temperature**

19.18 Thaw on ice **100 µl** of electrocompetent cells

19.19 Add **2 µg** of plasmid and the aliquot of electrocompetent cells to ice-cold electroporation cuvettes

19.20 Electroporate 25 uF, 10 kV/cm, 200 Ohms

19.21 Add 1 ml of BHIS and incubate **100 rpm, 30°C, 05:00:00** for recovery

19.22 Plate on LB + Agar (1.5%) + 100 µg/ml spectinomycin.
For crispr cloning, to activate cas9 expression, add 0.2% mannose.

19.23 Incubate at **30 °C** **Overnight**

19.24 Randomly pick potential mutants and test by colony PCR (for primers use the ones that flank the region of insertion sites).

19.25 After selecting colonies that show the expected band size, purify the desired plasmid (miniprep) and transform into B. mycoides.

20 Plate on LB agar containing erythromycin
Grow at **28 °C** overnight

21 Pick colonies and test them by colony PCR
If the expected band size is present, purify the plasmid and sequence

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22 Grow the colonies with the expected sequence on liquid LB + erythromycin + manose
Incubate **200 rpm, 28°C, 12:00:00**

23 Next day pick colonies in BHI medium (without antibiotic) and grow at **37 °C** overnight

24 Dilute the liquid cultures and plate them on LN (no antibiotic)
grow overnight st **37 °C**

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