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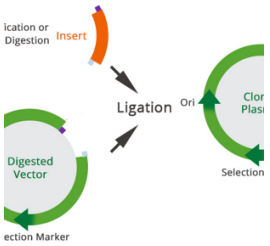
Cloning of *Bacillus mycoides*



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Protocol status: Other
Protocol has been developed based on literature, but hasn't been tested yet.

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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.

The plasmid pAD-KPS12-Pman will used as a starting point for introducing the antitoxin gene (ygcF) in *B. mycoides.* The vector will be cut with XbaI and SphI and ligated with the PCR amplified ygcF (using ygcF_Fw and ygcF_Rv as primers). In order to make the antitoxin expression inducible by solanine we plan on replacing the mannose inducible promoter with a promoter induced by solanine. To accomplish this, we will use the primers Psol_Fw and Psol_Rv to amplify the solanine promoter. The pAD-ygcFvector will be digested with EcoRI and XbaI, and ligated with the solanine promoter PCR product that will be digested as well with the up mentioned restriction enzymes.

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.

Making electro competent cells

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

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

3 Grow the cells overnight at 200 rpm, 30°C

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

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

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

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

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

PCR - Phusion cloning

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

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

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.

Cloning

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

13 Digestion mixture (20 µL):
-Sfil 2 U
- Plasmid (50 ng)
-Insert (20 ng)
- 2 µL CutSmart 10x (or appropriate buffer)
-MiliQ to 20 µL

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

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

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

17 Incubate 02:00:00 Room temperature

Transformation (electroporation)

18 Thaw on ice 100 µL of electrocompetent cells

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

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

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

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

Mutant selection

23 Incubate at 30 °C Overnight

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

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