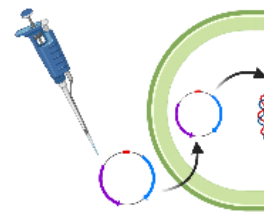


Oct 24, 2020

pYCR cloning strategy

DOI

dx.doi.org/10.17504/protocols.io.bnvxme7n



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iGEM Groningen 2020



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DOI: <https://dx.doi.org/10.17504/protocols.io.bnvxme7n>

Protocol Citation: Andreea S 2020. pYCR cloning strategy . protocols.io <https://dx.doi.org/10.17504/protocols.io.bnvxme7n>

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Protocol status: Other

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

Created: October 23, 2020

Last Modified: October 24, 2020

Protocol Integer ID: 43671

Keywords: pycr cloning strategy, crispr vector, synthetic dna for yqcG, using crispr, nlp14a in the genome, pcr product of gamy-fw, crispr in place, crispr, synthetic dna, using t4 ligase, pycr-gamy, switch cloning, backbone of pycr, cytoplasmic plasmid, pycr-gamy vector, gtrpeyqcG plasmid, t4 ligase, antitoxin mechanism, nlp14a sequence, genome, bacillus mycoides by use, pycr-gtrp, bacillus mycoide, resulting pcr product, pycr-gtrpe vector, pycr, introducing nlp14a

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 Sfil. 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 proven that no such operator is present, we suggest only 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 YqcG_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).

Troubleshooting



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 μ L Nuclease Free water
 -  10 μ L Oligo DNA annealing buffer 10x ($[M]$ 100 millimolar (mM) Tris  8 ,
 $[M]$ 500 millimolar (mM) NaCl, $[M]$ 10 millimolar (mM) EDTA)
 -  10 μ L DNA oligos A and B ($[M]$ 10 micromolar (μ M) 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
- 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 pre-cut pYCR:
Mix:
 -  10 μ L μ L of annealing product
 -  60 ng of digested pYCR
 -  1 μ L T4 ligase
- 9 Transform the ligation mixture into *E. coli* MC1061 using the heat-shock method.



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 Sfil cloning sites.

Expected result

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 Sfil restriction sites compatible with the backbone of pYCR
- 13 Digest the pYCR(+sgRNA) and the repair fragments with Sfil
Inactivate Sfil by PCR clean up
- 14 Ligate the components using T4 ligase
Heat inactivate the ligase (incubate  00:20:00  60 °C)
- 15 Perform Smal digestion to get rid of the original pYCR
- 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 Sfil cloning sites.
- 18 Miniprep and sequence the constructs

20m



Transform final construct in *B. mycoides* M2E_15

- 19 Transform using an electroporation protocol using  5 μ L plasmid DNA



Protocol



NAME

Cloning of *Bacillus mycoides*

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- 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 store at -80 °C














19.9 For the PCR mix pipette:

- 5 μL primers mixture Fw + Rv (5 μM) (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 appropriate 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 incubating 20 min  65 $^{\circ}\text{C}$
**SfiI can't be heat inactivated so in order to inactivate it a PCR clean up is necessary
- 19.16 Ligate the digested insert and vector.
Ligation mixture :
-10 μL digestion product
-1 μL T4
-2 μL T4 ligase buffer
-7 μL 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 μF , 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
- 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