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Protocol status: Working
We use this protocol and it's working

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CportucalensisOligoMediatedRecombineering

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ABSTRACT

A protocol for introducing translational knockouts via oligo-mediated recombineering in *C. portucalensis* MBL.

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Design oligos for recombineering translational knockouts in *C...*

1 Identify where the gene of interest is on the chromosome relative to the origin of replication and determine which strand of DNA will be the lagging strand (the mutating oligo needs to be complementary to the lagging strand in order to stand in for an Okazaki fragment).

2 Design the mutating oligo to introduce a minimal number of single nucleotide substitutions to change three consecutive codons into the three different stop codons (TAA, TGA, TAG) within the first third of the coding sequence.

3 Convenient codons to look out for are tyrosine, lysine, and glutamine for converting to TAA or TAG; tryptophan or cysteine for converting to TGA; leucine, proline, or arginine, depending on the synonym of the original codon in the gene.

4 The substitutions should take place near the middle of the oligo, which should be 90 nucleotides long, and the folding energy of the oligo should be more positive that -12.5 kcal mol-1.

5 If the folding energy is too negative, you can try sliding the oligo sequence to move the nucleotide substitutions toward the 5' end, but you may need to design a different oligo at a different locus.

6 When ordering the MAGE oligo, add phosphorothioated bonds between the first two nucleotides at the 5' end to stabilize the oligo.

Design the oligos for screening the candidate mutants (label...

7 Design one primer that matches the substituted nucleotides at its 3' end (the mut primer) and one that matches the wildtype nucleotides at its 3' end (the wt primer) to both have an annealing temperature of ~60 ºC. If the genotype is wildtype, only the wt primer will anneal; if the genotype is mutated, only the mut primer will anneal; if the cell has mismatched DNA or there is a mixed culture of mut and wt cells, both may anneal.

8 Design a primer that is reverse to both the wt and mut primers above that would give an amplicon of 200-400 bp. Design it such that it also anneals at ~60 ºC and does not dimerize with either the mut or wt primers with an energy more negative than -10 kcal mol-1. Tag the name with the expected amplicon length.

9 Benchmark primers by testing, for example, Fmut/R200 and Fwt/R200 on a strain that is wildtype for the gene of interest. Only Fwt/R200 should amplify by PCR.

9.1 Example PCR protocol with GoTaq Green 2x Master Mix:

Per reaction: 25 µL Master Mix, 1.25 µL 10 µM forward primer, 1.25 µL 10 µM reverse primer, 0.5 µL 100% DMSO, 21 µL nuclease-free water, 1 µL of colony resuspended in 20 µL sterile water (gently touch pipette tip to colony to get a minimal, but visible, amount of cells and pipette up and down in 20 µL sterile water to suspend)

1) 95 ºC 5 min
2) 95 ºC 30 sec
3) 60 ºC 30 sec
4) 72 ºC 30 sec
5) Go to (2) 24 times
6) 72 ºC 5 min
7) Hold at 22 ºC until refrigeration/freezing or use

10 Also design primers to sequence the edited region

Electroporate helper plasmid (pHelper_Ec1_V1_gentR) into th...

11 In the genetic background that you want to mutate, prepare electrocompetent cells as above to electroporate in the pHelper_Ec1_V1_gentR plasmid: dx.doi.org/10.17504/protocols.io.kqdg3x7r7g25/v1

11.1 Add 10-60 ng plasmid to 50 µL electrocompetent aliquot on ice (plasmid should be in ultrapure water, and you should aim to add no more than 1 µL to the cells).

11.2 Gently mix the DNA and cells by tapping the tube a couple times.

11.3 Transfer to ice-cold electroporation cuvette.

11.4 Zap: 2.5 kV, 200 Ω, 25 µF if using 2 mm gap cuvette; 1.25 kV, 200 Ω, 25 µF if using 1 mm gap cuvette.

11.5 Pulse length should be approximately 5 ms.

12 Recover electroporated cells

12.1 Recover in a total of 1 mL LB by adding 950 µL to the cuvette, mixing, and transferring as much volume as possible to a microcentrifuge tube.

12.2 Incubate shaking horizontally for one hour at 30 ºC

12.3 Plate serial dilution on selective LB agar (in this case, gentamicin)

13 Once the plasmid-bearing strain is attained, grow overnight in 5 mL LB under selection and prepare a -80 ºC freezer stock in 35% glycerol.

Induce the helper plasmid and electroporate in the MAGE oligo

14 Two days before, streak frozen stock carrying pHelper_Ec1_V1_gentR on LB/agar gentamicin (15 µg/mL) plate, grow over night at 37 ºC.

15 One day before, inoculate patch of struck cells into 5 mL culture overnight, shaking at 250 rpm, slanted, aerobically at 37 ºC, in LB + gentamicin (15 µg/mL).

16 Day of electroporation: inoculate 1 mL of overnight culture into 100 mL flask (this protocol can be scaled up or down according the how many reactions you want), add gentamicin to 15 µg/mL, grow shaking at 250 rpm at 37 ºC for ~2 hours until OD600 = 0.3. Check OD every 30 minutes starting at the 1-hour mark.

17 When culture reaches OD600 = 0.3, induce with 1 mM m-toluic acid (100 µL of 1 M solution or 125 µL of 800 mM solution), continuing to shake at 37 ºC for 30 minutes.

18 After induction, chill on ice for 10 minutes.

19 Split culture into two 50 mL conical tubes (pre-chilled), balance, and wash three times by spinning 10 minutes at 5000x(g) at 4C, using slow deceleration. After first spin, resuspend each pellet in 10 mL ice-cold NanoPure water and combine in one conical tube. After the second spin, resuspend in 10 mL ice-cold 10% glycerol and then add another 10 mL. After the third spin, resuspend with ice-cold 10% glycerol to a final volume of 1 mL (depending on how well you decant, this will take 500-750 uL). All resuspensions are done by gentle tapping and pipetting.

20 Aliquot 81 µL volumes of induced, electrocompetent cells into ice-cold microcentrifuge tubes.

21 For aliquots that are to be transformed immediately, add 9 µL of transformation oligo and mix by gently tapping.

22 Transfer to prechilled electroporation cuvette(s) and make sure that the culture fully spans the cuvette gap.

23 Zap: 2.5 kV, 200 Ω, and 25 µF in 2 mm cuvette (should give a ~5 ms pulse) (1.25 - 1.8 kV in 1 mm cuvettes might work, but I have not really tested this).

24 Add 900 µL LB to cuvette(s), mix by pipetting, and transfer as much as possible to an Eppendorf(s).

25 Flash-freeze unused aliquots in liquid nitrogen and store in -80 ºC freezer (can start at step (h) if thawing previously frozen induced, electrocompetent aliquots).

26 Rescue transformants, shaking horizontally at 250 rpm at 37 ºC for 1 hour.

27 Transfer the ~1 mL rescue cultures to 4 mL LB with 1.25x gentamicin for a final volume of 5 mL with 1x gentamicin (15 µg/mL) and outgrow (slanted, shaking, 37 ºC) for another 3 hours.

28 Plate serial dilution series on LB/agar + gentamicin, expect good separation of colonies at ~10-5 dilution.

29 Incubate at 37 ºC overnight.

Screen candidate mutants

30 Pick at least eight colonies for a given desired genotype.

31 PCR amplify with the wildtype and mutant primer combinations (MASC primers) described above in separate tubes.

32 Select colonies that give only mutant bands.

33 If you have colonies that give both wildtype and mutant bands, you can restreak them and screen individual colonies from the restruck plate.

34 If you have a lot of PCRs to run, a more efficient approach is often to PCR first with only the mutant primer pair and then confirm that positive colonies do not give a wildtype band, rather than screening each colony with two primer pairs from the outset.

Cure mutant strains of helper plasmid

35 Inoculate selected mutant strain into 5 mL LB with no antibiotics and grow overnight at 30 ºC in slanted tubes shaking at 30 ºC.

36 Serially dilute the overnight culture and plate on no-salt, 5% sucrose LB agar.

37 Grow overnight at 30 ºC.

38 Rescreen a couple colonies by PCR with the wt and mut MASC primers.

39 In parallel, patch mutant colonies onto non-selective and gentamicin LB agar and inoculate into 5 mL non-selective LB.

40 Incubate plates at liquid cultures at 30 ºC, shaking the liquid cultures at 250 rpm in slanted tubes.

41 If the patch test shows that the mutants lost gentamicin resistance and the PCR screen shows only mutant bands, prepare 35% glycerol -80 ºC freezer stocks of the mutants.

42 If you need the mutant strain to still carry the pHelper_Ec1_V1_gentR plasmid for adding more mutations into the same background, you can include a gentamicin LB liquid culture in step (e) and prepare a -80 ºC freezer stock from it directly.