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

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DatsenkoWannerCportucalensis

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ABSTRACT

Protocol for performing Datsenko-Wanner deletions of *C. portucalensis* MBL genes based on https://doi.org/10.1073/pnas.120163297

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Amplify the resistance cassette for knocking out a genetic re...

1 Design primers for replacing the gene locus of interest with a Kanamycin resistance casette via homologous recombination.

1.1 Design primers with 36 bp homology upstream to/downstream from region you want to knock out.

1.2 Append GTGTAGGCTGGAGCTGCTTC to 3' end of upstream primer.

1.3 Append CATATGAATATCCTCCTTAG to 3' end of downstream primer.

1.4 Use pKD4 for KanR cassette template.

2 Use Phusion polymerase for PCR: approx. 15 ng plasmid template per reaction

1) 98 ºC 30 sec.
2) 98 ºC 5 sec.

3) 55 ºC 30 sec.
4) 72 ºC 54 sec.
5) Go to step 2 24 times
6) 72 ºC 5 min.
7) hold at 22 ºC or refrigerate/freeze until use

3 Purify PCR products using NEB PCR cleanup kit (Cat. No. T1030) after gel verification.

Deletion of genomic region

4 Electroporate the antibiotic resistance casette (PCR product from Step 3) into *C. portucalensis* MBL carrying pKD46 (the λRed plasmid)

4.1 Two days prior, streak out *C. portucalensis* MBL/pKD46 on 50 µg/mL ampicillin (Amp) or carbenicillin (Carb) LB agar at 30 ºC.

4.2 One day prior, grow overnight culture in 5 mL LB with 50 µg/mL Amp/Carb.

4.3 Morning of, inoculate 2 mL overnight culture into 100 mL (25 mL per transformation) LB with 50 µg/mL Amp/Carb and 0.2% L-arabinose.

4.4 Grow at 30 ºC to OD600 approx. 0.6-0.8 (around 2 hours).

4.5 Wash at 2000 x g (slow deceleration) at 4 ºC into ice-cold 10% glycerol three times, combining final aliquot in one tube during last wash.

4.6 Resuspend into 400 µL final vol 10% glycerol (assuming four reactions--100 µL per reaction).

4.7 Aliquot 100 µL reaction volumes into ice-cold microcentrifuge tubes.

4.8 Add approximately 100 ng resistance cassette to cell aliquot and gently mix by tapping.

4.9 Electroporate in ice-cold cuvettes using 2.5 kV, 250 Ω, and 25 µF (confirm approx. 5 ms pulse for each), assuming 2 mm gap cuvettes.

4.10 Add 500 µL LB to each cuvette.

4.11 Transfer cell suspension to microcentrifuge tube and recover at 37 ºC shaking horizontally for an hour.

5 Plate 300 µL of recovered cell suspension on LB + 50 µg/mL kanamycin and grow at 37 ºC. Leave remaining cell suspension standing overnight and plate the next day if nothing grows from the first attempt.

6 Cure pKD46 plasmid and confirm deletion

6.1 Pick a couple individual colonies for each transformation and re-streak on non-selective LB to grow at 42 ºC overnight (pKD46 has a temperature-sensitive origin of replication).

6.2 From each re-streak, pick a single colony to patch onto non-selective LB, LB/Kan, and LB/Carb or Amp. Grow at 30 ºC.

6.3 Check for colonies that are kanamycin-resistant and carbenicillin/ampicillin-sensitive.

6.4 PCR verify correct insertion of kanamycin resistance cassette (forward primer in KanR and reverse downstream from knocked out region and loss of λRed (primers in λRed gene on pKD46).

6.5 Prepare -80C stocks for good strains in 35% glycerol from cured overnight cultures grown in LB + 50 µg/mL kanamycin.

7 FLP out resistance cassette

7.1 Electroporate pCP20 (AmpR, ts-origin) into cured deletion strains. See: dx.doi.org/10.17504/protocols.io.kqdg3x7r7g25/v1

7.2 Recover in LB at 30 ºC for 1 hour.

7.3 Plate 100 µL to grow overnight on LB/Carb or Amp plate at 30 ºC.

7.4 Streak several colonies on non-selective LB and grow at 42 ºC overnight (the pCP20 plasmid also has a temperature-sensitive origin of replication).

7.5 Screen for loss of all antibiotic resistance by patching on non-selective and selective LB plates.

7.6 Confirm FRT scar by PCR using primers in flanking regions (e.g., Δnap_check_F and Δnap_check_R) and sequencing.