



NOV 14, 2023

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DOI:
[dx.doi.org/10.17504/protocol
s.io.ewov1q5e7gr2/v1](https://dx.doi.org/10.17504/protocols.io.ewov1q5e7gr2/v1)

Protocol Citation: Dianne K Newman, Lev Tsypin, Scott Saunders, Allen W Chen 2023.
DatsenkoWannerCportucalensis. **protocols.io**
[https://dx.doi.org/10.17504/p
rotocols.io.ewov1q5e7gr2/v1](https://dx.doi.org/10.17504/protocols.io.ewov1q5e7gr2/v1)

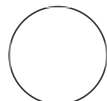
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Protocol status: Working
We use this protocol and it's working

Created: Nov 04, 2023

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>

Last Modified: Nov 14,
2023

PROTOCOL integer ID:
90421

Funders

Acknowledgement:

NIH

Grant ID: 1R01AI127850-
01A1

Doren Family Foundation

NSF GRFP

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

- 1 Design primers for replacing the gene locus of interest with a Kanamycin resistance cassette 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 °C 30 sec.
 - 2) 98 °C 5 sec.

- 3) 55 °C 30 sec.
- 4) 72 °C 54 sec.
- 5) Go to step 2 24 times
- 6) 72 °C 5 min.
- 7) hold at 22 °C 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 cassette (PCR product from Step 3) into *C. portucalensis* MBL carrying pKD46 (the λ Red plasmid)
 - 4.1 Two days prior, streak out *C. portucalensis* MBL/pKD46 on 50 μ g/mL ampicillin (Amp) or carbenicillin (Carb) LB agar at 30 °C.
 - 4.2 One day prior, grow overnight culture in 5 mL LB with 50 μ g/mL Amp/Carb.
 - 4.3 Morning of, inoculate 2 mL overnight culture into 100 mL (25 mL per transformation) LB with 50 μ g/mL Amp/Carb and 0.2% L-arabinose.
 - 4.4 Grow at 30 °C to OD600 approx. 0.6-0.8 (around 2 hours).
 - 4.5 Wash at 2000 x g (slow deceleration) at 4 °C into ice-cold 10% glycerol three times, combining final aliquot in one tube during last wash.

- 4.6 Resuspend into 400 μ L final vol 10% glycerol (assuming four reactions--100 μ L per reaction).
- 4.7 Aliquot 100 μ L 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 μ F (confirm approx. 5 ms pulse for each), assuming 2 mm gap cuvettes.
- 4.10 Add 500 μ L LB to each cuvette.
- 4.11 Transfer cell suspension to microcentrifuge tube and recover at 37 $^{\circ}$ C shaking horizontally for an hour.
- 5 Plate 300 μ L of recovered cell suspension on LB + 50 μ g/mL kanamycin and grow at 37 $^{\circ}$ C. 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 °C 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 °C.
- 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](https://doi.org/10.17504/protocols.io.kqdg3x7r7g25/v1)
- 7.2** Recover in LB at 30 °C for 1 hour.
- 7.3** Plate 100 µL to grow overnight on LB/Carb or Amp plate at 30 °C.

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- 7.4** Streak several colonies on non-selective LB and grow at 42 °C 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., Δ nap_check_F and Δ nap_check_R) and sequencing.