

Nov 14, 2023

DatsenkoWannerCportucalensis

DOI

<https://dx.doi.org/10.17504/protocols.io.ewov1q5e7gr2/v1>

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Protocol Citation: Dianne K Newman, Lev Tsypin, Scott Saunders, Allen W Chen 2023. DatsenkoWannerCportucalensis. protocols.io <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: November 04, 2023



Last Modified: November 14, 2023

Protocol Integer ID: 90421

Keywords: datsenkowannercportucalensis protocol, wanner deletion, performing datsenko

Funders Acknowledgements:

NIH

Grant ID: 1R01AI127850-01A1

Doren Family Foundation

NSF GRFP

Abstract

Protocol for performing Datsenko-Wanner deletions of *C. portucalensis* MBL genes based on

<https://doi.org/10.1073/pnas.120163297>

Amplify the resistance cassette for knocking out a genetic region of interest using the appropriate primer pairs. Use a high-fidelity polymerase, such as Q5, KAPA, or Phusion according to manufacturer instructions.

- 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 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 °C.
- 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 °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 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 °C shaking horizontally for an hour.
- 5 Plate 300 µL of recovered cell suspension on LB + 50 µg/mL kanamycin and grow at 37 °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 λ Red (primers in λ Red gene on pKD46).
- 6.5 Prepare -80C stocks for good strains in 35% glycerol from cured overnight cultures grown in LB + 50 μ g/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 °C for 1 hour.
- 7.3 Plate 100 μ L to grow overnight on LB/Carb or Amp plate at 30 °C.
- 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.