Chromosomal Gene Replacements via lambda Red recombinase
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Overview

Single-gene knockouts using lambda-red system(Reference Datsenko & Wanner 2000 PNAS)

(Carb is the Ampicilin antibiotic derivative that we use)

Materials

Plasmids	Marker	Comments
pKD46*	T°C and Carb sensitivity	λ-recombinase
pKD13*	Carb sensitive	FLP-Kan-FLP
pCP20*	Carb sensitive	FRT recombinase

Use Kan (25 mg/mL) and Amp (50 mg/mL) (final concentrations) for LB media and allow all transformed cells to incubate on plates at least 20-24hrs, so that a critical mass of bacteria cells are used for liquid culture inoculation unless otherwise is mentioned. Overall you need:

- LB plates, LB media
- LBK (20mg/mL and 50mg/mL) plates,
- LBCarb (LBA) plates
- SOC and SOB media
- 0.5M Arabinose (Filter sterilized, stored at -20C, thaw not more than twice)
- Saline solution
- 10% Glycerol (ice-cold)
- Sterile ice-cold water

Preparing Cells

Day 1
1. Streak your bacteria of choice on LB plates 2. Prepare all other necessary media and plates. And make sure you have the linear DNA product with proper homology binding site (see Construction of the linear DNA for more).
Day 2
1. Inoculate a single REL606 colony in LB media
Day 3
1. Prepare electrocompetent REL606 by inoculating 50uL seed media into 50 mL LB. (Grow till OD_{600}: 0.5 at 37°C, wash 2x with sH_2O and 2x with 10% Glycerol, ice-cold) 2. Transform 50ng pKD46 into 50uL electrocompetent bacteria by electrophoresis (2mm ice-cold cuvettes, 2.5V) 3. Store leftover competent cells at -80C for future use 4. Recover the transformant in 250uL SOC media at 30°C for 1hour 5. Plate 20-50uL of the cells on LBCarb plates, grow at 30°C overnight
Day 4
1. Pick a single colony, inoculate in 5mL LBCarb, grow at 30°C overnight 2. Make sure your linear DNA is pure, concentrated and ready to go.
Day 5 (Long day, plan accordingly)
Prepare electrocompetent bacteria hosting pKD46: 1. Inoculate 50uL seed into 50mL SOB+Carb (3 Flasks in total) 2. Add arabinose to each culture for a final concentration of 1mM 3. Grow cultures at 30°C till OD_{600} is 0.3 4. Add the same amount of arabinose to cultures 5. Increase the temperature to 37°C (for the expression of the recombinase) 6. Grow until OD reaches ~0.45-0.5 7. Spin down the pellets at 4000rpm, 10min at 4C 8. Wash 2x with 50mL ice-cold sH_2O, 2x with 25mL sterile ice-cold 10% Glycerol (Keep everything under 4 degrees) 9. Dissolve the final cell pellet in 200uL 10% glycerol. Divide into 50uL cold tubes 10. Transform 0 (this is your control) 25, 50, 100 and 250ng linear DNA (i.e. Kan cassette) into 50uL of cells through electrophoresis (2mm ice-cold cuvettes, pulse at 2.5V) 11. Recover in 600uL SOB media at 30°C for ~3 hours 12. Plate 100uL media on LBK plates (25 mg/mL), grow at 37°C for 20-24 hours -- I usually plate 500uL cells tonight (5 plates per transformation) and leave the remaining cells on my bench O/N (in case the experiments don't work, re-plate using these cultures, sometimes recombinants are obtained this way, odd but true)
Day 6
1. Cross fingers for positive transformants! (You should not see anything on the plate that contains 0 DNA…)
If you spot colonies on the plates…
1. Select colonies from the LBK plates- Screen using the appropriate primers & colony PCR amplification. I also select colonies from the control plate (from no transformant. If a positive band is obtained, PCR purify and send for sequence confirmation.

Eliminating the FRT flanked Antibiotic Marker (origin: pKD13, pKD3 or pKD4)

Day 1
1. Streak the bacteria containing the to-be-eliminated antibiotic marker on LBK (in the case of pKD13 sourced Kan marker) to make them electrocompetent
Day 2
2. Inoculate a single colony in LBK. Grow at 37°C, Overnight 250rpm
Day 3
6. Prepare electrocompetent cells by inoculating 50uL seed media into 50 mL LBK. (Grow till OD_{600}: 0.5 at 37°C, wash 2x with sH_2O and 2x with 10% Glycerol, ice-cold) 7. Transform 50ng pCP20 into 50uL cells by electrophoresis 8. Store leftover competent cells at -80°C, 9. Recover in 250uL SOC media at 30°C for 1hour 10. Plate 20-50uL culture on LBCarb+Kan plates, grow at 30°C for >24 hours
Day 4
11. Select ~10 colonies, inoculate in 5mL nonselective LB media 12. Grow at 30°C for 3 hours 13. Increase the temperature to 42°C, grow for 6 hours 14. Parallel streak on LB, LBK and LBCarb
Day 5
15. Choose the colony that doesn't grow on LBK and LBCarb 16. Colony PCR (see Primer List for the appropriate primers) 17. If a deletion has occurred, send for sequencing and make -80°C stock