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Complementation, Selection Assays (Coaux-Seq)

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We use this protocol and it's working

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Abstract

Strain preparation and subsequent complementation and selection assays in preparation for BarSeq for Coaux-Seq.

Preparation of auxotrophic strain

1 After determining the biochemical activity for which you want to screen, choose an appropriate auxotrophic strain and medium condition combination. For the following example, we will be using an *Escherichia coli* Keio single gene knock out strain and M9 minimal medium with 1% glucose as the sole carbon source.

NOTE: Be sure to validate the knockout strain before use.

2 Streak out selected strain (from glycerol stock or other source) on appropriate medium (here LB 1.5% agar + 25 µg/mL kanamycin). Incubate at 37°C overnight.

3 The following morning, pick a colony and inoculate into 3 mL of LB + 25 µg/mL kanamycin. Incubate at 37°C, 150 rpm overnight.

4 (The next steps are typical preparation of electrocompetent *E. coli* and will be assuming the use of the Keio knockout strain.) The following morning, subculture 1:100 into 50 mL of fresh LB + 25 µg/mL kanamycin using a non-baffled 250 mL shake flask. Grow until an OD_{600} of 0.4-0.6 is reached, measuring OD_{600} periodically.

NOTE: One can scale this depending on the number of aliquots of electrocompetent cells you would like to prepare.

5 Once desired OD_{600} is obtained, pellet cells by centrifugation (4,000 rpm, 5 minutes, 4°C).

NOTE: Run subsequent competent cell preparation steps on ice.

6 Wash cells 4-5x with sterile, cold (4°C) 10% glycerol. Wash with a volume of at least 5 mL of 10% glycerol each time. For each was step, gently re-suspend by pipetting in the fresh, sterile, cold 10% glycerol. After, centrifuge cells (4,000 rpm, 5 minutes, 4°C). Repeat process until the cells have been washed 4-5x.

NOTE: Keep materials on ice, pay close attention to sterile technique. This step is to remove salts and other medium components that would causes issues during electroporation.

7 After final wash, gently re-suspend cells in 1:100 of original volume in 10% glycerol. (If you had a 50 mL culture, re-suspend in ~500 µL of 10% glycerol.)

8 In sterile 1.5 mL Eppendorf tubes, pre-chilled on ice, aliquot 30 µL of resuspended cells. **These cells are now prepared for electroporation.** They can either be used now

(recommended for best transformation efficiency) or flash frozen and stored at -80°C for later use.

Electroporation, initial rich medium recovery

9 Add 1-10 µL of highly purified cloned genomic fragment plasmid library (single genome library or combination) to 30 µL aliquot of electrocompetent cells. Flick tube gently 4-5 times to mix. Let sit on ice for 5 minutes.

NOTE: Continue to conduct steps on ice. The higher the purity of the transforming DNA, the greater volume you can add without issues during electroporation. If high efficiency is needed, one can perform an ethanol precipitation on the DNA and concentration highly before addition to competent cell mixture.

10 Transfer entire contents (mixed cells + plasmid library) to chilled (4°C, on ice) electroporation cuvette.

11 Electroporate. Here we used a BTX Electro Cell Manipulator (PrecisionPulse) at setting 1750 V, 200 ohms resistance, and 25 µF capacitance.

12 If electroporation did not "arch," immediately added 975 µL of SOC recovery medium (NEB) and recover (37°C, 1 hour, 150 rpm). If electroporation "arched" discard cells.

13 After initial recovery, transfer to 100 mL of LB medium + 25 µg/mL kanamycin + 17 µg/mL chloramphenicol in a 500 mL non-baffled shake flask. Grow for 4-8 hours (37°C, 150 rpm) until culture becomes turbid.

NOTE: Kanamycin is to select for Keio knock out. Chloramphenicol is to select for the plasmid.

14 Make multiple (4-10) glycerol stocks of rich medium recovered library to use for future selections. Use 1:1 culture and 50% glycerol (500 µL each) for a 1 mL final volume. Store in cryovials at -80°C. Plasmid miniprep ~10 mL of culture for BarSeq to determine initial state of library (t_0) prior to selection. Use GeneJET Plasmid Miniprep Kit (Thermo Scientific, K0502) or equivalent for miniprep. Store t_0 miniprepped plasmid DNA in nuclease free water at -20°C until needed for BarSeq.

Selection

15 Take a glycerol stock of rich medium recovered library and inoculate entirety into 50 mL of LB + 25 µg/mL kanamycin + 17 µg/mL chloramphenicol in a 250 mL non-baffled shake flask. Incubate for 4-8 hours (37°C, 150 rpm) until culture becomes turbid.

16 Take culture, centrifuge to pellet (4,000 rpm, 5 minutes, 4°C). Wash 3x with sterile 1x PBS. For each wash, re-suspend in 5 mL of sterile 1x PBS and centrifuge (4,000 rpm, 5 minutes, 4°C).

17 After washing steps, re-suspend in 2 mL of sterile 1x PBS. Add 330 µL of re-suspended cells to selection plates (here M9 minimal medium, 1.5% agar, 1% glucose with either 1x [100 ng/mL] or 5x [500 ng/mL] adenotetracycline [aTc] + 25 µg/mL kanamycin + 17 µg/mL chloramphenicol). Plate out one replicate per condition (aTc concentration). Use glass bead to spread evenly. Allow plates to dry in sterile environment (e.g., Biosafety cabinet).

NOTE: aTc is used to induce the synthetic promoter.

18 Wrap plates with parafilm to prevent dehydration. Incubate plates at 37°C until colonies are observed (this can take up to 1 week time).

19 Optional: Pick a few (~4) colonies to grow up in 5 mL M9 minimal medium, 1% glucose, 5x aTc, 25 µg/mL kanamycin + 17 µg/mL chloramphenicol (overnight) to miniprep and send for Sanger sequencing to "spot check" and confirm complementation success (meaning, do the genes identified make sense).

20 Once colonies are observed, use a colony scraper and scape the colonies into 5 mL of sterile 1x PBS. Pooled plates as desired. Once pooled, centrifuge to pellet the cells (4,000 rpm, 5 minutes, 4°C). Decant PBS.

21 Plasmid miniprep pooled pelleted cells using GeneJET Plasmid Miniprep Kit (Thermo Scientific, K0502) or equivalent. Store miniprepped plasmid in nuclease free water at -20°C until preparation for BarSeq.

22 Run BarSeq on desired samples. Use protocol as described by Huang et al., *bioRxiv*, 2022, **Barcoded overexpression screens in gut Bacteroidales identify genes with new roles in carbon utilization and stress resistance.** Utilize https://github.com/morgannprice/BobaseqFitness.