Growth Curve Protocol M63 1% Lactose Media/ CelB Mutant Selection

Purpose: To select for the best CelB mutants after T7 directed mutagenesis on an X-gal plate. Mutants with the highest beta-galactose activity will produce dark blue colonies on an X-gal topped LB+ aTc + antibiotic plate. It is important to note that most colonies on the plate will be a pale blue, but only those that are the darkest of blues are valuable. To confirm that these dark blue colonies have the best beta-galactose activity, they will be grown in nutrient deficient minimal media M63 containing 1% lactose as the carbon source. If the strain has a beneficial lactose utilization mutation, it should be able to grow significantly better than the wild type under these conditions.

Materials:

- LB + Antibiotic Plate (Contains ~ 20 mL Agar)
- aTc Stock in Water or DMSO (Current Stock is 20 µg/mL in water)
- M63 0.2% Glucose Media
- M63 1% Lactose Media
- Clear 96 Well Plate
- X-gal stock (20 mg/mL in DMSO)

Protocol:

Time: 5 - 18 hours

1. Inoculate 3 mL of LB + 10 ng/mL aTc + antibiotic media with a single colony from the T7 directed mutagenesis plate, or from a large scoop of freezer stock. Allow to grow to mid-exponential phase if cultures are fresh (~4-5 hrs), or overnight if cultures are old at 37 °C and 220 rpm.

Time: 18-20 hours

2. One hour prior to plating, top your LB antibiotic plate with 0.8 mg/mL X-gal and 10 ng/mL aTc.
    a. Create a plating solution containing your aTc and your X-gal. To a 1.5 mL tube, add 10 µL of your 20 µg/mL aTc in water stock, and 40 µL (500x dilute) of your X-gal stock. Bring the final volume up to 150 µL with sterile ddH_2O or LB media.
    b. Using glass beads or a plate spreader, plate all 200 µL and allow to stand at room temperature lid side up.
3. Depending on the density of your exponential phase or overnight culture you may have to dilute 10^3 to 10^8-fold in sterile LB. It is ideal to have no more than 150-200 colonies on your plate and anything higher than that is difficult to isolate or count.
    a. Plate 100-200 µL of your diluted culture on your plate and grow ON at 37 °C.

Time: 20 hours

4. The next day, circle approximately 30 isolated colonies that have the darkest blue color. Inoculate those colonies in 3 mL of M63 0.2% Glucose media + antibiotic without aTc. Grow ON at 37 °C and 220 rpm. Also include the WT strain as a control.

Time: 3-16 hours

5. If the cultures have grown dense enough, begin the washing steps.
    a. Spin cultures down for 5 minutes at 4000 rpm. Pour off supernatant.
    b. Resuspend pellets in 1 mL PBS-F. Vortex to mix. Spin cultures down as before and repeat the process two more times. Three washes total.
    c. After the last wash, pour off the PBS and resuspend the pellets in 1 mL M63 1% lactose media containing 10 ng/mL aTc and antibiotics.
6. Load 120 µL of each resuspended culture and your media as a blank into a clear 96 well plate. Measure the OD600 twice.
7. Blank the absorbance and calculate how much of each culture you will need to add to your M63 1% lactose, antibiotic, 10 ng/mL aTc media to get a final volume of 600 µL and an OD of 0.2.
8. Make up your dilutions then load 120 µL of each culture into a new 96 well plate in triplicate. Also load your blank media in triplicate. This should fill the plate.
9. Grow for 16 hours in the plate reader at OD600 without shaking. Measure the OD in 10-minute intervals.
10. Transfer blanked kinetic data to an Excel sheet and plot the OD over time. (Optional: Also take the Ln of the OD600 to determine the actual growth rates.)