**Protocol title:***Batch bacterial growth curves with cryostocking of over-night culture*

**Aim of the protoco****l:** *A high-throughput method for estimating growth curve parameters aka life history traits (specifically, growth rate & lag time) of 12 strains at different temperatures. The basic concept is that the inoculum is cryostocked at a fixed starting OD and then those cryostocks are used for inoculating a dilution series of replicate growth curves on different days, at different temperatures. This version of the protocol investigates cultures at stationary phase; we also have another version for cultures at early exponential phase. The protocol is adapted from a previously published version of the protocol:* [*https://app.jove.com/t/56197/precise-high-throughput-analysis-of-bacterial-growth*](https://app.jove.com/t/56197/precise-high-throughput-analysis-of-bacterial-growth)

**Materials**

* LB media
* LB agar petri dishes
* Biosafety cabinet
* Inoculation loop & Bunsen burner
* 100-250 mL Erlenmeyer flasks  
  *(50 mL Erlenmeyer ONLY for negative control with 10 mL of LB!)*
* Serological pipette & tips
* Sterile 15mL Falcon tubes
* Ultra-centrifuge
* Vortex
* P1000 pipette & sterile tips
* P200 pipette & sterile tips
* 3x PCR strip tubes with strip lids *(these are certified free of nucleic acids & DNase/RNase but not certified sterile. BEWARE!)*
* Trays for PCR strip tubes
* Sterile 0.22um single-use syringe filters (e.g., VWR 514-1273 25mm)
* Sterile 5-10mL single-use syringes (e.g., CODAN 62.6616)
* 50% glycerol
* Dry ice *(NOT liquid nitrogen because the PCR lids may snap off!)*
* -80 freezer
* Cuvette OD reader
* Multichannel P100 pipette & sterile tips
* 96 deep well plate (>1 mL)
* 2x U-bottom 96-well plates
* 2x Breathe-easy seals
* 2x Microplate OD readers

# Streaking:

### Streak agar plates using the cryostocks to obtain single (isolated) colonies of the particular strain following quadrant streaking (see references).

# Batch Culture:

### Fill test tubes with 3.0 mL of sterile LB media. *Remember: add 3.0mL of LB to a tube as negative control!*

### For each strain, pick a colony and inoculate in the test tube.

### Incubate for a day 28\*C, shaking at 250 rpm.

### Label 2.0mL microtubes with the strain number. Aliquot 1350uL of sterile PBS into each microtube.

### Incubate the cultures for a minimum of 24 hours. If after this time the cultures don’t yet look well saturated by eye, allow them to incubate for longer.

### Once the cultures look well saturated by eye, remove 150uL of culture and dilute into the PBS. (This will be referred to as the “10^-1 diluted culture” below.)

### Make a baseline control for the OD readings by combining 55uL of fresh LB media with 1045uL of PBS (final concentration is 5% LB in PBS). Aliquot 1.0mL into a cuvette reader and use for baselining.

### Get OD readings of each of the diluted cultures by combining 500uL of the 10^-1 diluted cultures with 500uL of PBS.

### Calculate how much volume of the 10^-1 cultures is needed to re-suspend into 2.0mL spent media at an OD of 0.25

### *The volume you need is:*

### Pipette the necessary volume of 10^-1 culture into a 15mL falcon tube. *Note: if you find that you don’t have enough volume of 10^-1 culture, you can take instead from the undiluted culture. However, this will be less precise.*

### Use the centrifuge to spin down the cultures: 3214rcf for 10 minutes. Discard the supernatant.

### Label 2.0mL microcentrifuge tubes.

### Open a fresh syringe and remove the piston from the top. Open a fresh filter and securely screw it into the narrow end of the syringe. Hold the syringe upside down and dump the first remaining test tube culture (i.e., the undiluted culture) into the syringe. Replace the piston onto the syringe and filter at least 2.0mL into the 2.0mL microcentrifuge tubes.

### Repeat step 12 for each of the cultures.

### For each pellet, add 2.0mL of its own filtered, spent media to the 15mL falcon tube. Vortex or pipette vigorously to resuspend.

### Add 500 uL of 50% glycerol to the cells (final concentration: 10% glycerol; final volume: 2.5 mL).

### Aliquot 100 µL into 3 sets of 8-strip PCR tubes.

### Snap freeze on dry iceand store at -80C.

# Setting up the plate:

### Take the LB out of the fridge and warm it to 28°C using the incubator.

### Prepare each of the two microplate readers. Turn on the instrument and its computer. Then open the protocol called “simple\_growth\_curve.prt”.

#### For each machine, set the total incubation time to 30-48 hours and gradient to 1°C.

#### For one machine, set the incubation temperature to 25 (/35)°C.

#### For the other machine, set the incubation temperature to 30 (/40)°C.

### Remove one tube from each PCR strip thus making a row of all 12 strains from the -80 C freezer and let thaw on the bench top. Arrange the tubes in the layout of the plate as designed.

### Aliquot 900µL LB into each well deep-well plate.

### Add 100uL thawed stock to row B using multichannel.

### Using 100 µL multi-channel pipette, now make a dilution series between rows C to G in the order mentioned in the layout.

### Using a fresh 200µL tip, pipette up and down to mix thoroughly. Then aliquot 100µL from the 1st well to the 2nd well (this is a 10-2 dilution). Discard the tip.

### Repeat step 7. 4x to make the 10-3, 10-4, 10-5, and 10-6 dilutions following the labels.

### Remove the packaging of each of the 2 U-bottom plates and mark one “25C” and the other “30C”.

### Load 200µL from the deep well plate into the 2 U-bottom plates.

### Seal the top of each plate with the Breathe-Easy film.

### Put each plate to incubate in the microplate reader that is set to the same temperature as written on the plate.

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Dilution** |  | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
|  | **A** | BLK (blank) | BLK | BLK | BLK | BLK | BLK | BLK | BLK | BLK | BLK | BLK | BLK |
| 10-1 | **B** | BSC002 | BSC003 | BSC001 | BSC005 | BSC008 | BSC009 | BSC006 | BSC010 | BSC004 | BSC007 | BSC015 | BSC019 |
| 10-2 | **C** | BSC002 | BSC003 | BSC001 | BSC005 | BSC008 | BSC009 | BSC006 | BSC010 | BSC004 | BSC007 | BSC015 | BSC019 |
| 10-3 | **D** | BSC002 | BSC003 | BSC001 | BSC005 | BSC008 | BSC009 | BSC006 | BSC010 | BSC004 | BSC007 | BSC015 | BSC019 |
| 10-4 | **E** | BSC002 | BSC003 | BSC001 | BSC005 | BSC008 | BSC009 | BSC006 | BSC010 | BSC004 | BSC007 | BSC015 | BSC019 |
| 10-5 | **F** | BSC002 | BSC003 | BSC001 | BSC005 | BSC008 | BSC009 | BSC006 | BSC010 | BSC004 | BSC007 | BSC015 | BSC019 |
| 10-6 | **G** | BSC002 | BSC003 | BSC001 | BSC005 | BSC008 | BSC009 | BSC006 | BSC010 | BSC004 | BSC007 | BSC015 | BSC019 |
|  | **H** | BLK | BLK | BLK | BLK | BLK | BLK | BLK | BLK | BLK | BLK | BLK | BLK |

In the schematic above, colours indicate the fluorescent protein (grey samples lack fluorophore).

**References:**

* *Here is the Jove protocol again:* [*https://app.jove.com/t/56197/precise-high-throughput-analysis-of-bacterial-growth*](https://app.jove.com/t/56197/precise-high-throughput-analysis-of-bacterial-growth)
* *And here is a paper that uses a dilution series and a “time-to-threshold” approach for estimating the net growth rate:* [*https://www.nature.com/articles/s41559-020-1126-5*](https://www.nature.com/articles/s41559-020-1126-5)
* *Quadrant Streaking* <https://microbeonline.com/streak-plate-method-principle-purpose-procedure-results/>