**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 early exponential phase; we will also have another version for cultures at stationary 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 50mL 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
* 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

**Step by step procedure**

# Streaking:

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

# Batch Culture:

### Depending on whether the strain is slow/fast grower, the incubation duration in the tube should be decided in order to obtain the desired OD value.

### Fill Erlenmeyer flasks with 55 mL of sterile LB media. *Remember: add 10mL of LB to a 50mL Erlenmeyer flask as negative control!*

### For each strain, pick a colony and inoculate in the Erlenmeyer flask.

### Incubate for several hours at 28\*C, shaking at 250 rpm.

### Check the OD occasionally using the cuvette reader. *Given the volume, it may take about 6 hours to reach the desired OD.*

### When each batch culture reaches OD between 0.01-0.05, remove the Erlenmeyer from the incubator and use the observed OD to calculate how much volume is needed to re-suspend into 2.0mL fresh LB at an OD of 0.25 *The volume you need is:*

### Pipette the necessary volume into a 50mL Falcon tube.

### Use the ultracentrifuge to spin down the culture: 5000g for 5 minutes.

### Discard the supernatant and resuspend in 2.0mL of fresh LB. Vortex.

### 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.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **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/>