Growth assay measurements using either the plate reader or TECAN robot
Sept. 26, 2017

GOAL: Quantitatively measure growth of *E. coli* by monitoring culture density (optical density @600nm) over time.

INSTRUMENTATION: These measurements can be made in a high-throughput way using either the Reynolds lab plate reader (Victor) or the Toprak lab TECAN robot.

It is important to note that these two instruments provide different growth conditions, and so data collected in one instrument is not directly comparable to the other. In the TECAN, the 96-well plates are housed in a humidified incubator with the lid on, typically set to 30°C. In the Victor plate reader, the plate is warmed by a heating coil beneath the plate (also typically set to 30°C), the lid remains off for the duration of the assay, the plate is intermittently shaken (to provide aeration), and sterile water is periodically added (to counteract evaporation and to provide additional mixing to the culture). Some key considerations:

- If you plan to make quantitative comparisons across multiple experiments, you should use one instrument for all of them).

- If you are using Victor plate reader and choose to change the temperature of your assay from 30°C, you will also need to re-calibrate and edit the amount of water being added to the 96-well plate to compensate for the change in evaporation rate.

- If you are using the Victor plate reader, you should not use the edge wells of the plate (columns 1/12 and rows A/H) as these evaporate faster (edge effects).

PROTOCOL:
Week before (or as soon as you are ready):
Sign up for equipment.
Reynolds lab plate reader: outlook calendar
Toprak lab TECAN: slack group

2 days before:
Transform plasmids of interest into *E. coli* strains OR streak out a relevant glycerol stock. Plate on selective media as necessary. If you are using an auxotroph strain (e.g. ER2566 *ΔfolA ΔthyA*) you will also need to supplement your media with the relevant nutrients to ensure good growth (usually either 50 μg/ml thymidine, or folA mix).

**If* you have recent plates (within the last two weeks) you can omit this step and use the existing plates (re-transforming every time is not essential, but having reasonably new plates is).

1 day before:
Set up 2 ml overnight cultures in LB (+folA mix or thymidine, if using) and appropriate antibiotic(s). Grow overnight (16h*) at 37°C.
**to ensure good reproducibility, grow for a fixed amount of time for each experiment.*

Plan plate layout (see template below); for plate reader, outer edge wells should be undesignated. For robot, all wells can be used.

Prepare all media and buffers necessary. (see **REAGENTS** section for folA mix, amicase and M9 recipes)

Day of assay:

1. Pellet overnight cultures (5000 rpm, 5 min, in *room-temp* centrifuge), resuspend in 1ml M9 medium (or assay media if different) with antibiotics.
2. Repeat pellet/resuspend step two more times, resuspending in 1ml M9 (or assay media if different)
3. Adaptation: The goal of the adaptation is two-fold: (1) to give the cells an opportunity to adapt to new media conditions (so that any diauxic shift/change in growth rate in response to different nutrients occurs prior to your experiment), (2) to deplete any nutrient stores in the cells from the rich media when shifting to minimal media and (3) to ensure your cells are growing in exponential/log phase prior to the beginning of your experiment. *If the adaptation phase is not done properly, it can result in an absence of selection during the actual time course of your experiment (e.g. all your mutants may grow the same), and/or substantial experiment-to-experiment variability in the lag phase (and sometimes even growth rates)*. In general, it is worth noting that the lag phase is most sensitive to experimental variation – this variability can come from improper adaptation, or experiment-to-experiment variation in inoculation density. The adaptation should typically be performed in the same media and temperature conditions to be used for the experiment.
    a. Back-dilute washed cultures in assay media (typically M9 without supplements and with antibiotics). Here the goal is to get your cells down to an OD=0.1 (often this corresponds to a dilution of about 1:100 of the washed, resuspended overnight culture). Adaptation cultures are typically ~2ml total, but just make sure you have enough for setting up the plate (i.e. 2 ml of OD=0.1 cells is enough to inoculate 200 wells at a starting OD of 0.005).
    b. Incubate at temperature of selection conditions for 4 hours. After this point you will want to make every effort to keep your cells near the selection temperature. This avoids possible variation in lag phase coming from temperature shock.
    c. <u>During adaptation</u>: prepare plate reader
        i. <u>For plate reader</u>: empty waste cup, wash pump lines with 70% EtOH and sterile water, fill lines with sterile water. Set plate temperature control.
        ii. <u>For robot</u>: check water levels in incubator, check/set the temperature, do a test run with an empty plate using the code you will use for experiment.
        iii. <u>Prepare plate</u>: Dispense 190ul media per well in Corning 96-well plate, incubate plate at appropriate temperature to prewarm.
        iv. <u>Prewarm additional media</u>: prewarm media (usually ~25 ml) alongside the plate for making any later dilutions (so you do not add room temp media to your cells).
4. Measure the OD_{600} of adapted cultures (in plate reader or spec). If measuring in a plate reader, measure the OD_{600} for 200µL of cells and multiply by a factor of 4.2 to get the

OD_{600} as measured in the spec. Normalize to a fixed OD (typically the starting OD in the well for plate assays is 0.005):

   a. Dilute all cultures to OD_{600} = 0.1 in 1ml
   b. Inoculate wells with 10µL diluted cells. (if you decide to inoculate with more than 10µL, you will need to adjust your media recipe so that you do not dilute too much). *(** for plate reader, be sure to leave the outer edge wells uninoculated – all wells should contain 200µl of culture or media; edge wells should be media only*).

5. Run assay for ~24 hours:
   a. Plate reader: Use standard protocol
   b. Robot: 'Main_LidHandling_MultiplePLates_SlowCode_Mathis'

   POST RUN:
   c. Plate reader: Remove plate, empty the pump lines, flush and fill with 70% EtOH. Empty waste cup. Be sure to turn off temperature control.
   d. Robot: Remove plate, save data, and reset the temperature.

6. Data analysis: There are several ways in which the data from these experiments can be analyzed. The lab has several pieces of standardized code that implement these, but the basic approaches are: (1) linear fit to a
   a. Plate reader: standardized lab code
   b. Robot: code from Andrew Mathis.

COMMONLY USED MEDIA REGIMENS FOR OVERNIGHT/ADAPTATION/EXPERIMENTAL CONDITIONS

Crispr-I regimen. This protocol is designed to minimize "escapers" (mutations) prior to the beginning of selection. Currently being used by Andrew Mathis, Judith Boldt, James McCormick.
Overnight (15.5h): LB+folA mix + thymidine + antibiotics
Adaptation (4h): M9 media+antibiotics
Selection (24h): M9 media+antibiotics

Mutant library regimen. This protocol is designed to remove the increased growth rate "bump" that we often observe at the beginning of turbidostat experiments and which seems to be caused by overnight culture in LB. Currently being used by Thuy Nguyen.
Overnight (15.5h): M9+folA mix + thymidine +antibiotics
Adaptation (4h): M9 media + antibiotics
Selection (24h): M9 media + antibiotics

REAGENTS

folA mix recipe
by Andrew Mathis
Adapted from Samuel Thompson, UCSF.
24 August 2017

Reagents	1x (ug/mL)	250x stock (mg/mL)	50 mL 250x solution (mg)
Glycine	38	9.5	475
Methionine	75.5	18.88	943.8
Pantothenate	1	0.25	12.5
Adenosine	20	5	250

250x solution is made in ddH_2O, filter-sterilized, and aliquoted into 1 mL volumes and stored at -20 °C. Always use a fresh aliquot – freeze/thaw cycles are not well tolerated.

M9 media
Stocks:
5X M9 salts (per liter)
- 33.9g Na_2HPO_4
- 15g KH_2PO_4
- 5g NH_4Cl
- 2.5g NaCl

(autoclaved)

20% glucose
(filter sterilized)

1M $MgSO_4$
(autoclaved or filter sterilized)

2.5% amicase (if using)
(autoclaved – powder will not go into solution prior to autoclaving).

Media recipes:

No amicase:
200ml 5X M9 salts
20ml 20% glucose
2ml 1M $MgSO_4$
bring to ~950ml with ddH2O
pH to 6.5 and adjust volume to 1L
filter sterilize

With amicase:
200ml 5X M9 salts
20ml 20% glucose
2ml 1M $MgSO_4$
80ml 2.5% amicase
bring to ~950ml with ddH2O
pH to 6.5 and adjust volume to 1L
filter sterilize

Plate template:

96-Well Plate Template

Date: __/__/__ Experiment/Plate #: _____

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12