2023 iGEM InterLab study

Experiment 3 – Cell measurement protocol (growth in test tubes vs. growth in 96 well-plates)

This year we plan to test protocols that will eventually be automated. For this reason, we will use 96-well plates in addition to test tubes for culturing. This approach will enable an evaluation of results from the plate culturing protocol relative to growing cultures in test tubes (e.g. 50mL falcon tubes) on a global scale.

At the end of the experiment, you will have two plates to be measured - one for which the cells were grown in the plate, and the other used to make measurements of the cells grown in tubes. You will measure both fluorescence and absorbance in each plate.

Before performing the cell measurements, you need to perform all the calibration measurements. Please do not proceed unless you have completed the calibration protocol. Completion of the calibrations will ensure that you understand the measurement process and that you can take the cell measurements under the same conditions as the calibration. For consistency and reproducibility, we are requiring all teams to use E. coli K-12 DH5-alpha. If you do not have access to this strain, you can request streaks of the transformed devices from another team near you. If you are absolutely unable to obtain the DH5-alpha strain, you may still participate in the InterLab study by contacting the Engineering Committee (interlab [at] igem [dot] org) to discuss your situation.

For all below indicated cell measurements, you must use the same type of plates and the same volumes that you used in your calibration protocol. You must also use the same settings (e.g., filters or excitation and emission wavelengths) that you used in your calibration measurements. If you do not use the same type of plates, volumes, and settings, the measurements will not be valid.

Protocol summary: You will transform the eight devices listed in Table 1 into E. coli K-12 DH5-alpha cells. The next day you grow duplicate cultures of each transformation reaction by picking two colonies from each plate and inoculating each colony into 12ml media in tubes (for a total of 16 cultures). Each of these 16 overnight cultures will be used to inoculate four wells in a 96-well plate (200uL each, 4 replicates) and one test tube (12mL), and for growth in tubes and plate.

You will measure fluorescence and absorbance at 0 hours and after 6 hours of incubation both in tubes and 96 well-plate.

Protocol Outputs:

- 0 hr absorbance time point measurements of plate 1
- 0 hr green fluorescence time point measurements of plate 1
- 6 hr absorbance time point measurements of plate 1
- 6 hr green fluorescence time point measurements of plate 1
- 6 hr absorbance time point measurements of plate 2
- 6 hr green fluorescence time point measurements of plate 2

Protocol Materials:

- *E. coli* DH5 alpha competent cells
- Negative control
- Positive control (I20270)
- Test Device 1 (J364000)
- Test Device 2 (J364001)
- Test Device 3 (J364002)
- Test Device 4 (J364007)
- Test Device 5 (J364008)
- Test Device 6 (J364009)
- LB Broth + Chloramphenicol (34 ug/mL)
- LB Agar + Chloramphenicol (34 ug/mL)
- Chloramphenicol stock solution (34 mg/mL)*
- Ice
- Plate reader
- 96-well microplate orbital incubator (Some plate readers come directly with the ability to also shake and incubate).
- Shaking incubator (normal for culture tubes)
- Petri dish (x 8)
- 14mL culture tube (x16)
- 50mL conical tube (x32)
- 96 well microplate black with flat bottom (x 2)
- microplate adhesive sealing film

*NOTE: Antibiotics stock solutions, in this case, Chloramphenicol (Cm), are normally 1000X (34mg/mL for Cm), so to make a tube with 3mL LB broth + Cm, you should add 3mL of LB broth + 3uL of Cm stock solution (which is at 1000X) resulting in a final antibiotic concentration of 1X = 34ug/mL.

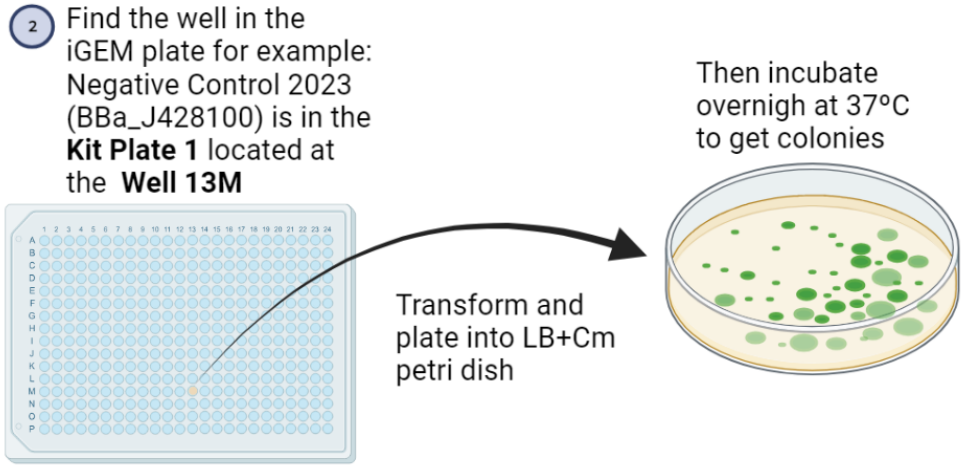
Table 1: Part Locations in Distribution Kit

Device	Part Name	Coordinate
Negative Control	BBa_J428100	Kit Plate 1 Well 13M
Positive Control	BBa_I20270	Kit Plate 1 Well 11A
Test Device 1	BBa_J364000	Kit Plate 1 Well 11C
Test Device 2	BBa_J364001	Kit Plate 1 Well 11E
Test Device 3	BBa_J364002	Kit Plate 1 Well 11G
Test Device 4	BBa_J364007	Kit Plate 1 Well 11I
Test Device 5	BBa_J364008	Kit Plate 1 Well 11K
Test Device 6	BBa_J364009	Kit Plate 1 Well 11M

Protocol Steps:

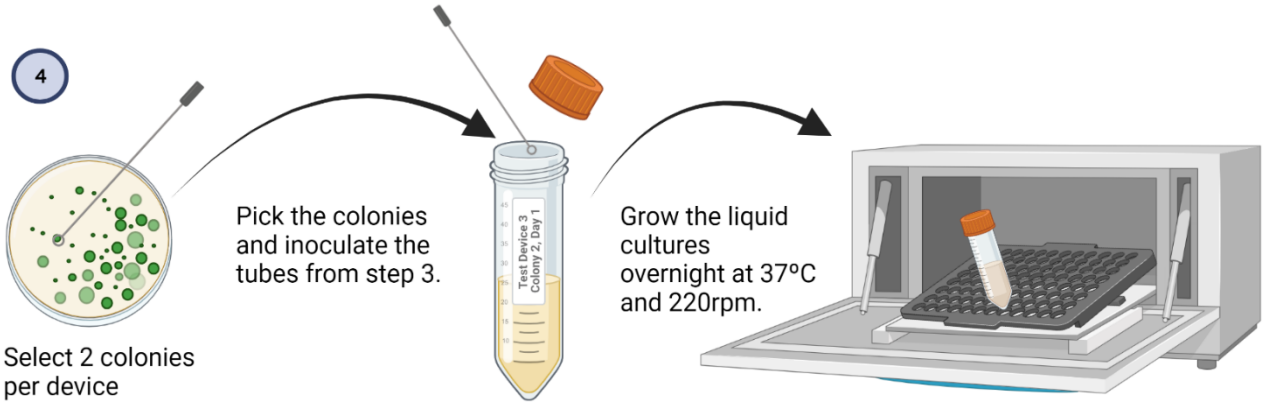
Day 0

1. Prepare 8 Petri dishes with LB Agar + Chloramphenicol (Cm) growth medium for culturing transformant strains.
2. Transform each one of the eight devices shown in Table 1 into *E. coli* DH5 alpha competent cells following your own protocol. Plate transformants on the LB Agar + Cm plates from Step 1. Incubate overnight (for at least 16 hours) at 37.0°C.



Day 1

3. Prepare 16 culture tubes (50mL) with 12.0mL of LB Broth + Cm. There, you will grow the colonies from the previous day into liquid cultures. You will need two tubes for each of the 8 devices, as you will pick two colonies per device. Label the tubes, accordingly, naming these tubes with the following convention: Test Device 3, Colony 2, Day 1.
4. Select 2 colonies per device (16 colonies in total). Inoculate (by picking the colonies) into each one of the tubes from Step 3. Grow all 16 tubes overnight for 16 hours at 37°C and 220 rpm in a shaker incubator.

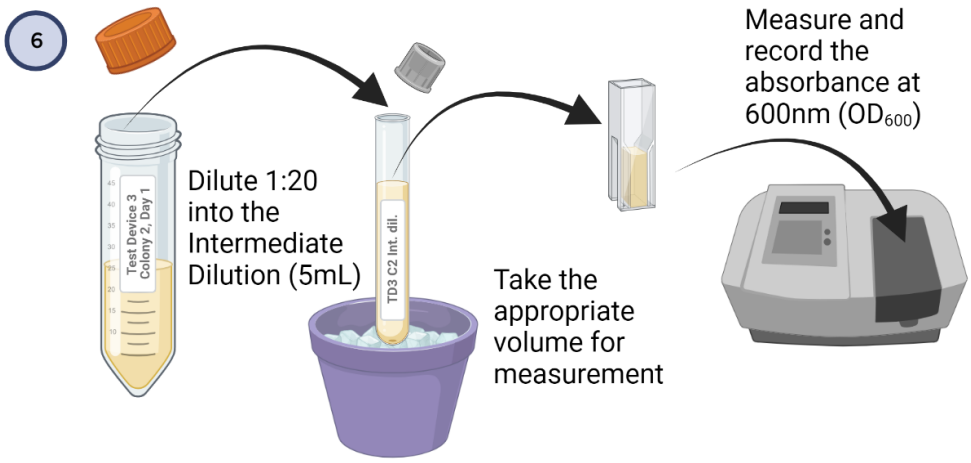


Day 2

Here you want to perform a dilution from the previous day's cultures to start the experiment with a known cell density (OD_{600}). For this you need to perform an intermediate dilution to measure OD_{600} before performing the final dilutions.

Preparation of the Intermediate dilution and OD measurement

5. Prepare sixteen (16) new 14mL tubes to make the intermediate dilutions from the cultures from Step 4. We will name these new tubes with the same convention as Step 4, changing the day number as follows: i.e., Test Device 3, Colony 2, Intermediate Dilution.
6. Dilute each of 16 culture (day 1, Step 4) samples at a 1:10 to 1:20 ratio into LB Broth + Cm (tubes prepared in Step 5). NOTE: As an example, to make a 1:20 dilution, you need to add 4750uL of LB Broth + Cm, and then add 250uL of the day 1 culture. Maintain at 4.0°C while performing dilutions. (You can also do this on ice). Take the appropriate volume from the Intermediate Dilutions (tubes made in this Step 6) and read the absorbance at 600nm in a spectrophotometer, i.e., the OD_{600}.

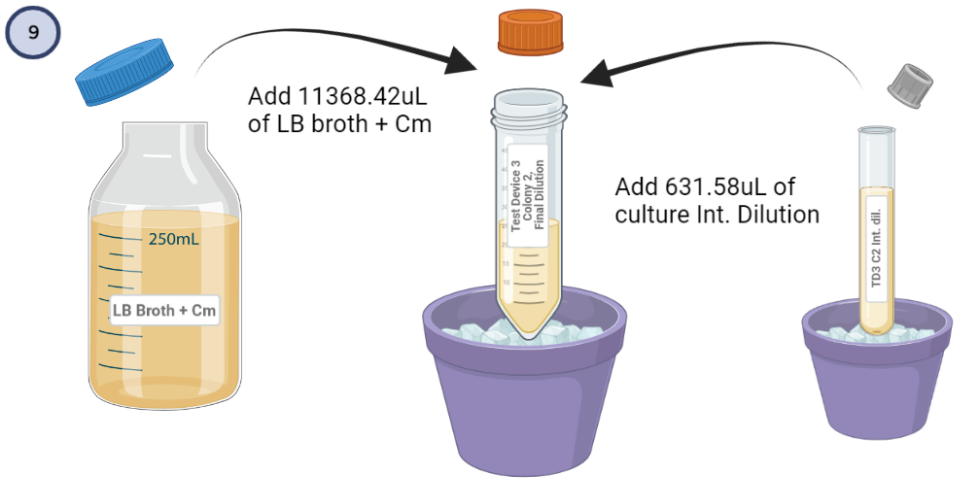


Preparation of the Final Dilution

7. Prepare 16 new 50mL tubes to perform the final dilutions using the Intermediate dilutions from Step 6. We will name these new tubes with the same convention as Step 4 as follows: i.e. Test Device 3, Colony 2, Final Dilution.
8. Use the **culture-dilution-calculator-template.xlsx** sheet (or any other means) to calculate the dilutions by inserting the measured OD (from Step 6) in the second column (Initial OD source). The needed volumes to make the dilutions will appear in the corresponding columns *culture volume* and *media volume*: As an example, to make 12mL dilution at 0.02 OD_{600} from a culture at 0.38 OD_{600}, (sample 1), you will need to add 631.58uL of culture (from the Intermediate Dilution) to 11368.42uL of LB broth + Cm media as shown here.

Sample	Initial OD	Final OD	Culture volume(uL)	Media Volume(ul)	Total Volume(ul)
1	0.38	0.02	632	11368	12000

9. Following the indications from Step 8, make appropriate dilutions (on ice) of each culture to achieve an OD_{600} of 0.02 in 12 ml LB Broth + Cm. These Final Dilution tubes are the starting point of the experiment: they will be used for measurement of the 0hs time-point (Plate 1) and to grow the cultures at 37ºC and 220rpm for 6 hrs (for Plate 2).

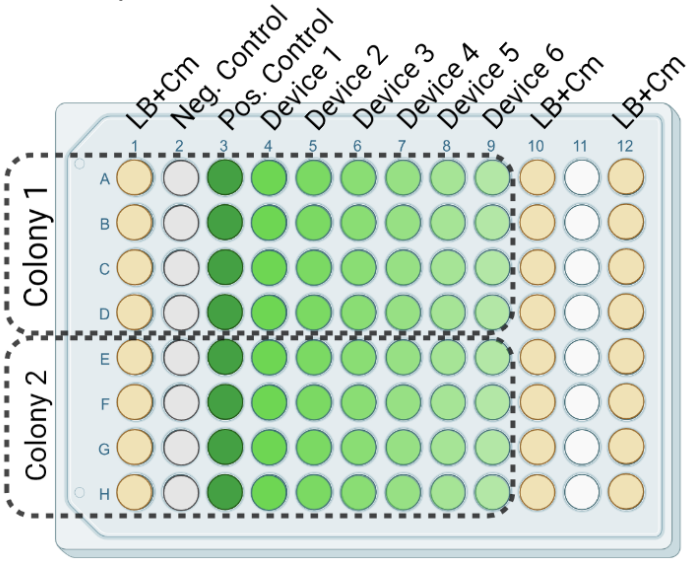


Note: Volumes are examples from the previous table. You need to adjust to your own volumes calculated from the OD measurement

Preparing Plate 1 (0hr)

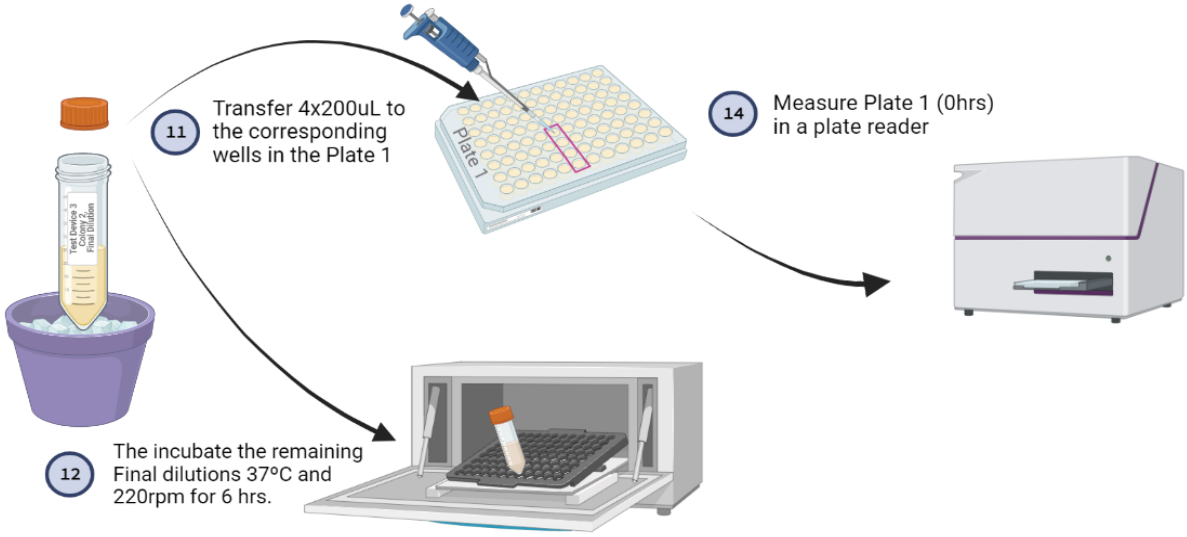
The 0hr bacterial cultures are already prepared to perform the experiment.

10. Prepare a 96 well-plate for measurement of the 0hr time-point. Label this 96 well-plate as Plate 1. You need to fill out the plate using the following plate map:



Note: For each device, you will end up with four (4) replicates per colony. The four replicates of Colony 1, will go in the rows spanning A:D and the corresponding column, and the four replicates of Colony 2, will go in the rows spanning E:G and corresponding column as indicated in the plate map.

11. Transfer 4 times 200uL (4x200uL) from the Final Dilution tubes made in Step 9 and into the appropriate wells of Plate 1 (from Step 10). As indicated in the plate map, you will have 4 technical replicates coming from each Final Dilution tube. Maintain both the Final Dilution tubes (from Step 9) and the Plate 1 at 4.0°C during transfer.
12. While keeping Plate 1 on ice, place the Final Dilution tubes (from Step 9 after removing the 4x200uL for measurement) in the orbital incubator (shaker) and incubate for 6.0 hours at 37.0°C at 220 rpm. (See figure in next page)
13. Once the Final Dilution tubes are incubating, Plate 1 is almost ready for measurement of the 0hrs time-point. You need to complete the blanks. For this, add 200.0uL of LB Broth + Cm to wells A1:H1, A10:H10, A12:H12 of Plate 1. Maintain at 4.0°C during transfer. These samples are blanks.



Absorbance and fluorescence measurements (Plate 1 0hrs)

14. Take Plate 1 to the plate-reader and set-up a measurement of:
    1. Absorbance at 600.0nm
    2. Fluorescence (green) with an excitation wavelength of 488.0nm, an emission wavelength of 530.0nm, and a 30.0nm bandpass.

Incubation of Plate 1

15. After measurement, seal (with foil or plate lid) the Plate 1 with a material of your choice in order to prevent evaporation. Then incubate in an orbital shaker incubator the Plate 1 for 6.0 hours at 37.0°C at 220 rpm.

Absorbance and fluorescence measurements (Plate 1 6hrs)

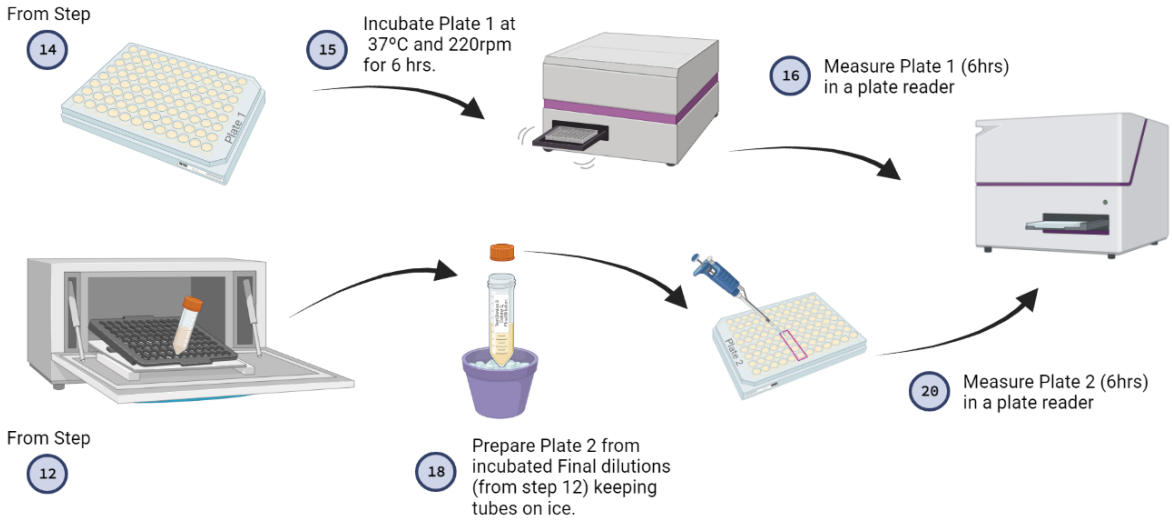
16. After 6hrs incubation, take Plate 1 again to the plate-reader and measure:
    1. Absorbance at 600.0nm
    2. Fluorescence (green) with an excitation wavelength of 488.0nm, an emission wavelength of 530.0nm, and a 30.0nm bandpass.

Preparing Plate 2 (6hr)

After 6hrs incubation, the Final Dilution tubes are ready to prepare Plate 2. This will follow the same pattern as Plate 1.

17. Prepare a 96-well microplate as Plate 2.
18. Transfer 4 times 200uL (4x200uL) from the incubated Final Dilution tubes from Step 12 and into the appropriate wells of Plate 2 (from Step 18). As indicated in the plate map, you will have 4 technical replicates coming from each Final Dilution tube. Maintain both the Final Dilution tubes (from Step 12) and the Plate 2 at 4.0°C during transfer.

19. Complete Plate 2 (as you did with Plate 1 in Step 13) by transferring 200.0uL of LB Broth + Cm to wells A1:H1, A10:H10, A12:H12 of Plate 2. Maintain at 4.0°C during transfer. These are the blanks.



Absorbance and fluorescence measurements (Plate 2)

20. Take Plate 2 (6hr time-point plate) to the plate-reader and measure:
    1. Absorbance at 600.0nm
    2. Fluorescence (green) with an excitation wavelength of 488.0nm, an emission wavelength of 530.0nm, and a 30.0nm bandpass.

Importing Experimental Data

21. Import data for all the measurements into the provided Excel sheet **interlab-2023-exp3.xlsx**. You need to include:
    - 0 hr absorbance time point measurements of Plate 1,
    - 0 hr green fluorescence time point measurements of Plate 1,
    - 6 hr absorbance time point measurements of Plate 1,
    - 6 hr green fluorescence time point measurements of Plate 1,
    - 6 hr absorbance time point measurements of Plate 2,
    - 6 hr green fluorescence time point measurements of Plate 2,
22. Congratulations, you have finished the 2023 iGEM InterLab study Experiment 3! Please read through the 2023 Interlab page for instructions on how to complete your data submission.

Protocol version: 3.0

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