Before You Begin

Read through this entire protocol sheet carefully before you start your experiment and prepare any materials you may need. This year, in order to improve reproducibility, we are requiring all participating teams to use plate readers to take their measurements. If you do not have access to a plate reader, you may collaborate with another team. If the plate reader requirement is a significant barrier for your team, you can still participate in the InterLab study. Contact the iGEM Measurement Committee at measurement at igem dot org to discuss your situation.

Calibration Protocols

1. OD_{600} Reference point

You will use LUDOX-S40 as a single point reference to obtain a ratiometric conversion factor to transform your absorbance data into a standard OD_{600} measurement. This has two key objectives. With standard 1 cm pathlength spectrophotometers, the reading is still instrument dependent (see above). With plate readers the path length is less than 1 cm and is volume dependent. In this instance the ratiometric conversion can both transform Abs_{600} measurements (i.e. the basic output of the instrument and not standardised optical density with 1 cm pathlength) into OD_{600} measurements, whilst simultaneously accounting for instrument differences.

[**IMPORTANT NOTE**: many plate readers have an automatic path length correction, this is based on volume adjustment using a ratio of absorbance measurements at 900 and 950 nm. Because scattering increases with longer wavelengths, this adjustment is confounded by scattering solutions, such as dense cells. **YOU MUST THEREFORE TURN OFF PATHLENGTH CORRECTION**.]

To measure your standard LUDOX Abs_{600} you must use the same cuvettes, plates and volumes (**suggestion**: use 100 µl for plate reader measurement and 1 mL for spectrophotometer measurement) that you will use in your cell based assays. The LUDOX solution is only weakly scattering and so will give a low absorbance value.

1ml LUDOX (provided in kit)
H_20 (provided by team)
96 well plate, black with clear flat bottom preferred (provided by team)

Method

- ☐ Add 100 µl LUDOX into wells A1, B1, C1, D1 (or 1 mL LUDOX into cuvette)
- ☐ Add 100 µl of H_2O into wells A2, B2, C2, D2 (or 1 mL H_2O into cuvette)
- ☐ Measure absorbance 600 nm of all samples in all standard measurement modes in instrument
- ☐ Record the data in the table below or in your notebook
- ☐ Import data into Excel (**OD600 reference point tab**) Sheet_1 provided

	LUDOX 100%	H_2O
replicate 1		
replicate 2		
replicate 3		
replicate 4		

Example Data:

	reference spectrophotometer		microplate reader	
	H20	LDX-HS40 100%	H20	LDX-HS40 100%
replicate 1	0	0.043	0.036	0.112
replicate 2	0	0.043	0.034	0.078
replicate 3	0	0.042	0.033	0.095
replicate 4	0	0.042	0.035	0.101
average	0	0.0425	0.0345	0.0965
corrected Abs600		0.0425		0.062
reference OD600		0.0425		0.0425
correction factor		1		0.685483871

x

Table shows the data for OD_{600} measured by a spectrophotometer and a plate reader for the H_2O and LUDOX. The corrected Abs_{600} is calculated by subtracting the H_2O reading. The reference OD_{600} is defined as that measured by the reference spectrophotometer (you should use this value too). The correction factor to convert measured Abs_{600} to OD_{600} is thus the Reference OD_{600} divided by Abs_{600}. ***All cell density readings using this instrument with the same settings and volume can be converted to OD_{600} by multiplying by (in this instance) 0.685.***

2. Protocol fluorescein fluorescence standard curve

You will prepare a dilution series of fluorescein in 4 replicates and measure the fluorescence in a 96 well plate in your plate reader. By measuring these in all standard modes in your plate reader, you will generate a standard curve of fluorescence for fluorescein concentration. You will be able to use this to correct your cell based readings to an equivalent fluorescein concentration. You will then be able to convert this into a concentration of GFP.

Before beginning this protocol ensure that you are familiar with the GFP settings and measurement modes of your instrument. Note, the GFP used in the devices (GFP mut3b) is one that has an excitation maximum at 501 nm and an emission maximum at 511 nm.

Materials:

Sodium fluorescein (provided in kit)
10ml 1xPBS (phosphate buffered saline; provided by team)
96 well plate, black with clear flat bottom preferred (provided by team))

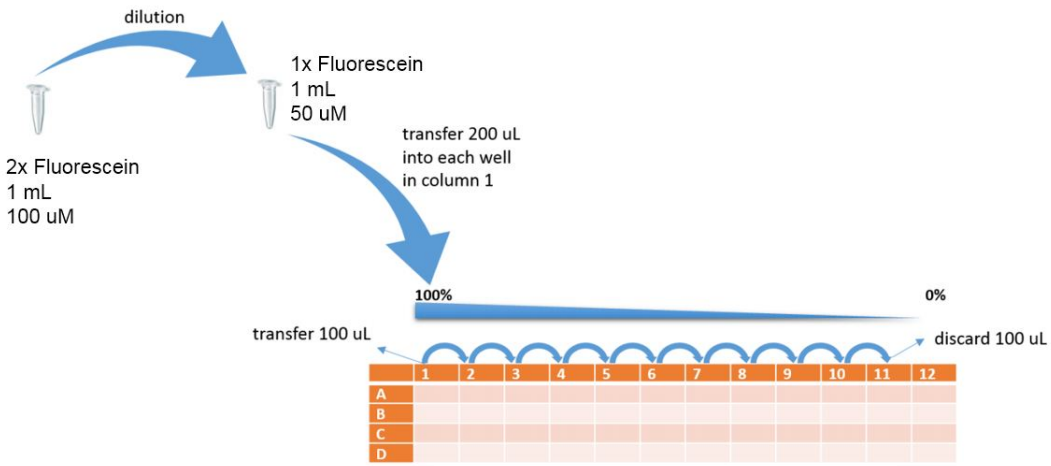
Method

Prepare the fluorescein stock solution:

- ☐ Spin down sodium fluorescein stock tube to make sure pellet is at the bottom of tube.
- ☐ Prepare 2x sodium fluorescein stock solution (100 µM) by resuspending sodium fluorescein in 1 mL of 1xPBS. [**Note**: it is important that the fluorescein is properly dissolved. To check this, after the resuspension you should pipette up and down and examine the solution in the pipette tip – if any particulates are visible in the pipette tip continue to mix the solution until they disappear.]
- ☐ Dilute the 2x sodium fluorescein stock solution with 1xPBS to make a 1x sodium fluorescein solution and resulting concentration of fluorescein stock solution 50 µM (500µL of 2x fluorescein in 500 µL 1x PBS will make 1 mL of 50 µM (1x) sodium fluorescein solution.)

Prepare the serial dilutions of fluorescein:

Accurate pipetting is essential. Serial dilutions will be performed across columns 1-11. COLUMN 12 MUST CONTAIN PBS BUFFER ONLY. Initially you will setup the plate with the sodium fluorescein stock in column 1 and an equal volume of 1xPBS in columns 2 to 12. You will perform a serial dilution by consecutively transferring 100 µl from column to column with good mixing.



- ☐ **Add 100 µl of PBS** into w ?
- ☐ **Add 200 µl of sodium fluorescein 1x stock** solution into A1, B1, C1, D1
- ☐ Transfer 100 µl of sodium fluorescein stock solution from A1 into A2.
- ☐ Mix A2 by pipetting up and down 3x and transfer 100 µl into A3…
- ☐ Mix A3 by pipetting up and down 3x and transfer 100 µl into A4...
- ☐ Mix A4 by pipetting up and down 3x and transfer 100 µl into A5...
- ☐ Mix A5 by pipetting up and down 3x and transfer 100 µl into A6...
- ☐ Mix A6 by pipetting up and down 3x and transfer 100 µl into A7...
- ☐ Mix A7 by pipetting up and down 3x and transfer 100 µl into A8...
- ☐ Mix A8 by pipetting up and down 3x and transfer 100 µl into A9...
- ☐ Mix A9 by pipetting up and down 3x and transfer 100 µl into A10...
- ☐ Mix A10 by pipetting up and down 3x and transfer 100 µl into A11...
- ☐ Mix A11 by pipetting up and down 3x and transfer 100 µl into **liquid waste**

TAKE CARE NOT TO CONTINUE SERIAL DILUTION INTO COLUMN 12.

- ☐ Repeat dilution series for rows B, C, D
- ☐ Measure fluorescence of all samples in all standard measurement modes in instrument *
- ☐ Record the data in your notebook
- ☐ Import data into Excel (fluorescein **standard curve tab**) Sheet_1 provided

*** see 'Measurement Notes' below**

Measurement Notes

You must now measure the plate in your plate reader. The machine must be setup with the standard GFP settings (filters or excitation and emission wavelengths) that you will use when measuring your cells (if you change them you will not be able to use this standard curve). It is therefore a good idea

to repeat the measurement a number of times with different settings. You will then have a series of standard curves to choose from. Most important it is necessary to use a number of settings that affect the sensitivity (principally gain and/or slit width). Be sure to also consider other options (orbital averaging, top/bottom optics). As before, TURN OFF path length correction if available.

Make sure to record all information about your instrument (wavelengths, etc.) as these will be required in the Plate Reader Form.

Cell measurement protocol

Prior to performing the measurement on the cells you should perform the **calibration measurements**. This will ensure that you understand the measurement process and that you can take the cell measurements under the same conditions. This year, for the sake of 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, and this can count as a collaboration as long as its appropriately documented on both team's wikis. If you are absolutely unable to obtain the DH5-alpha strain, you may still participate in the InterLab study by contacting the Measurement Committee (measurement at igem dot org) to discuss your situation.

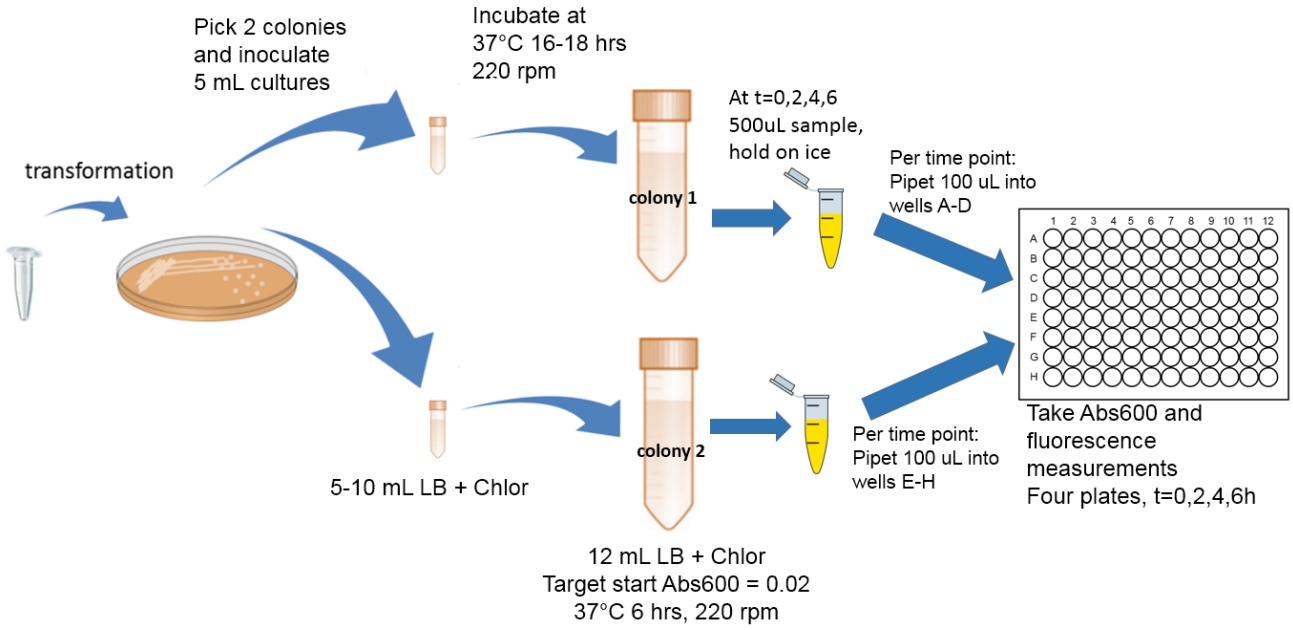
Materials:

Competent cells (*Escherichia coli* strain DH5α)
LB (Luria Bertani) media
Chloramphenicol (stock concentration 25 mg/mL dissolved in ethanol)
50 ml Falcon tube (or equivalent, preferably amber or covered in foil to block light)
Incubator at 37°C
1.5 ml eppendorf tubes for sample storage
Ice bucket with ice
Pipettes

Devices (from InterLab Measurement Kit):

- Positive control
- Negative control
- Test Device 1: J23101.BCD2.E0040.B0015
- Test Device 2: J23106.BCD2.E0040.B0015
- Test Device 3: J23117.BCD2.E0040.B0015
- Test Device 4: J23101+I13504
- Test Device 5: J23106+I13504
- Test Device 6: J23117+I13504

Workflow



Method

Day 1: transform *Escherichia coli* DH5α with these following plasmids:

- Positive control
- Negative control
- Test Device 1: J23101.BCD2.E0040.B0015
- Test Device 2: J23106.BCD2.E0040.B0015
- Test Device 3: J23117.BCD2.E0040.B0015
- Test Device 4: J23101+I13504
- Test Device 5: J23106+I13504
- Test Device 6: J23117+I13504

Day 2: Pick 2 colonies from each of plate and inoculate it on 5-10 mL LB medium + Chloramphenicol. Grow the cells overnight (16-18 hours) at 37°C and 220 rpm.

Day 3: Cell growth, sampling, and assay

- ☐ Set your instrument to read OD600 (as OD calibration setting)
- ☐ Measure OD600 of the overnight cultures
- ☐ Record data in your notebook
- ☐ Import data into Excel (**Dilution Calculation**) Sheet_1 provided
- ☐ Dilute the cultures to a target OD_{600} of 0.02 (see the volume of preloading culture and media in Excel (**Dilution Calculation**) Sheet_1) in **12 ml** LB medium + Chloramphenicol in 50 mL falcon tube (amber, or covered with foil to block light).
- ☐ Incubate the cultures at 37°C and 220 rpm.
- ☐ Take 500 µL samples of the cultures at 0, 2, 4, and 6 hours of incubation. (At each time point, you will take a sample from each of the 8 devices, two colonies per device, for a total of 16 samples per time point)
- ☐ Place samples on ice.
- ☐ At the end of sampling point you need to measure your samples (OD and Fl measurement), see the below for details.
- ☐ Record data in your notebook
- ☐ Import data into Excel (**cell measurement tab**) Sheet_1 provided

Measurement

It is important that you use the same instrument settings that you used when measuring the fluorescein standard curve. <u>This includes using the sample volume (100 ul) you used for the fluorescein measurement.</u>

Samples should be laid out according to Fig. 2. Pipette 100 µl of each sample into each well. Replicate samples of colony #1 should be pipetted into wells in rows A, B, C and D. Replicate samples of colony #2 should be pipetted into wells in rows E, F, G and H. Be sure to include

8 control wells containing 100uL each of only LB+chloramphenicol on each plate in column 9, as shown in the diagram below. Set the instrument settings as those that gave the best results in your calibration curves (no measurements off scale). If necessary you can test more than one of the previously calibrated settings to get the best data (no measurements off scale).

Hint:
No measurement off scale means the data you get does not out of range of your calibration curve.

Layout for Abs600 and Fluorescence measurement

At the end of the experiment, you should have four plates to read. Each plate should be set up as shown below. You will have one plate for each time point: 0, 2, 4, and 6 hours.