InterLab Experiment

Project: NU iGEM 2017 Shared Project
Authors: Karen Taylor
Dates: 2017-06-26 to 2017-07-13

MONDAY, 6/26

Goal of InterLab Experiment: Establish a GFP measurement protocol based on engineering principles that anyone with a plate reader can use in their lab. Teams will use the same exact protocol around the world to produce common, comparable units for measuring GFP with different plate readers.

FRIDAY, 7/7

Goal: Perform Parts 1 and 2 of the Interlab - Calibration measurements

Calibration measurements (Parts 1 and 2)
The InterLab Plate reader Protocol was followed.

Modifications:

- The plate was covered using aluminum foil prior to avoid contact with light prior to placing in the plate reader
- The plate reader took measurements 3 times and the results were averaged

Raw Data

Raw Data - InterLab Part 1

	A	B	C	D	E
1		1	2	3	4
2	LDX - HS40 100%	0.043	0.047	0.045	0.046
3		0.043	0.047	0.045	0.047
4		0.043	0.047	0.045	0.046
5	H2O	0.036	0.036	0.036	0.036
6		0.036	0.035	0.036	0.036
7		0.036	0.036	0.036	0.036

Raw Data - InterLab Part 2

	A	B	C	D	E	F	G
1		1	2	3	4	5	6
2	A	OVRFLW	OVRFLW	OVRFLW	OVRFLW	83331	4763
3		OVRFLW	OVRFLW	OVRFLW	OVRFLW	81361	4661
4		OVRFLW	OVRFLW	OVRFLW	OVRFLW	81215	4639
5	B	OVRFLW	OVRFLW	OVRFLW	OVRFLW	84505	4749
6		OVRFLW	OVRFLW	OVRFLW	OVRFLW	82862	4663
7		OVRFLW	OVRFLW	OVRFLW	OVRFLW	82738	4647
8	C	OVRFLW	OVRFLW	OVRFLW	OVRFLW	84474	4749
9		OVRFLW	OVRFLW	OVRFLW	OVRFLW	83244	4677
10		OVRFLW	OVRFLW	OVRFLW	OVRFLW	83057	4666
11	D	OVRFLW	OVRFLW	OVRFLW	OVRFLW	78379	4060
12		OVRFLW	OVRFLW	OVRFLW	OVRFLW	77456	4011
13		OVRFLW	OVRFLW	OVRFLW	OVRFLW	77326	4006

Emailed Jake from iGEM regarding OVERFLOW: need to change sensitivity and redo this part

Data Analysis

The data was analysed by following instructions found in the InterLab protocol.

Microplate Reader Data - InterLab Part 1

	A	B	C
1	**Microplate Reader Measurements**		
2		**H2O**	**LDX-HSO4 100%**
3	**Replicate 1**	0.036	0.043
4	**Replicate 2**	0.0356666	0.047
5	**Replicate 3**	0.036	0.045
6	**Replicate 4**	0.036	0.0463333
7	**Average**	0.03591665	0.045333325
8	**Corrected Abs600**		0.009416675
9	**Reference OD600**		0.0425
10	**Correction factor**		0.2215688235

TUESDAY, 7/11

Transformations

Goal: Complete transformations of 6 Testing Devices and 2 controls

Materials

- Resuspended DNA to be transformed
    - Resuspend DNA Distribution Kit wells with 10uL dH20. Pipet up and down several times, let sit for a few minutes. Resuspension will be red from cresol red dye
- 10pg/µl Positive transformation control DNA
- Competent Cells (50µl per sample)
- 2ml Microtubes
    - One tube per transformation
    - Label tubes with part name or well location before starting
- SOC Media (200µL per sample)
- Petri plates w/ LB agar and antibiotic (2 per sample)
    - 2 plates per transformation
    - CAM

Equipment

- Floating Foam Tube Rack
- Ice & ice bucket
    - Fill bucket, pre-chill 2mL tubes for 5 min
    - Thaw competent cell stock on ice for 10-15 min
- Lab Timer
- **42°C water bath**
- 37°C incubator
- Sterile spreader or glass beads
- Pipettes and Tips (10µl, 20µl, 200µl tips and pipettes recommended)

Transformation Protocol

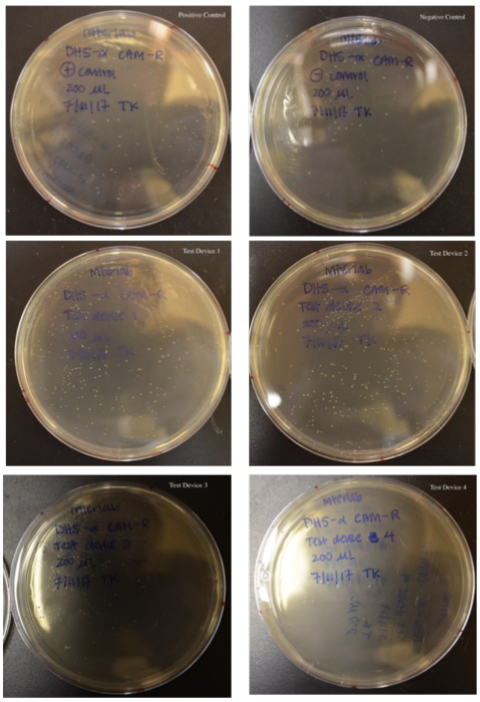
Resuspend DNA in selected wells in the Distribution Kit. Label 2ml tubes with part name or well location. Fill lab ice bucket with ice, and pre-chill 2ml tubes (one tube for each transformation, including your control) in a floating foam tube rack.

1. **Thaw competent cells on ice:** This may take 10-15min for a 260µl stock. Dispose of unused competent cells. Do not refreeze unused thawed cells, as it will drastically reduce transformation efficiency.
2. **Pipette 50µl of competent cells into 2ml tube:** 50µl in a 2ml tube per transformation. Tubes should be labeled, pre-chilled, and in a floating tube rack for support. Keep all tubes on ice. Don't forget a 2ml tube for your control.
3. **Pipette 1µl of resuspended DNA into 2ml tube:** Pipette from well into appropriately labeled tube. Gently pipette up and down a few times. Keep all tubes on ice.
4. **Pipette 1µl of control DNA into 2ml tube:** Pipette 1µl of 10pg/µl control into your control transformation. Gently pipette up and down a few times. Keep all tubes on ice.
5. **Close 2ml tubes, incubate on ice for 30min:** Tubes may be gently agitated/flicked to mix solution, but return to ice immediately.
6. **Heat shock tubes at 42°C for 1 min:** 2ml tubes should be in a floating foam tube rack. Place in water bath to ensure the bottoms of the tubes are submerged. Timing is critical.
7. **Incubate on ice for 5min:** Return transformation tubes to ice bucket.
8. **Pipette 200µl SOC media to each transformation:** SOC should be stored at 4°C, but can be warmed to room temperature before use. Check for contamination.
9. **Incubate at 37°C for 2 hours, shaker or rotor recommended:**
10. **Pipette each transformation on two petri plates for a 20µl and 200µl plating:** Pipette 20µl and 200µl of the transformation onto appropriately labeled plates. Spread with sterilized spreader or glass beads immediately. This helps ensure that you will be able to pick out a single colony.
11. **Incubate transformations overnight (14-18hr) at 37°C:** Incubate the plates upside down (agar side facing up). If incubated for too long, colonies may overgrow and the antibiotics may start to break down; un-transformed cells will begin to grow.

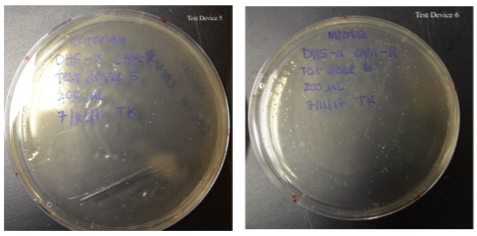
WEDNESDAY, 7/12

The 200µL plates were selected for the overnight culture since more colonies were present.

InterLab Plates (1/2)



InterLab Plates (2/2)



Kit plate 6:
B (positive control) I20270
D (negative control) R0040
F Test Device 1 J364000
H Test Device 2 J364001
J Test Device 3 J364002
L Test Device 4 J364003
N Test Device 5 J364004
P Test Device 6 J364005

Plasmids have pSBIC3 plasmid backbone

Purpose: Create overnight cultures from the plated bacteria.

Procedure:
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 - **STEP COMPLETE**

Note: 5mL of LB were added

THURSDAY, 7/13

Purpose: 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 m l 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.

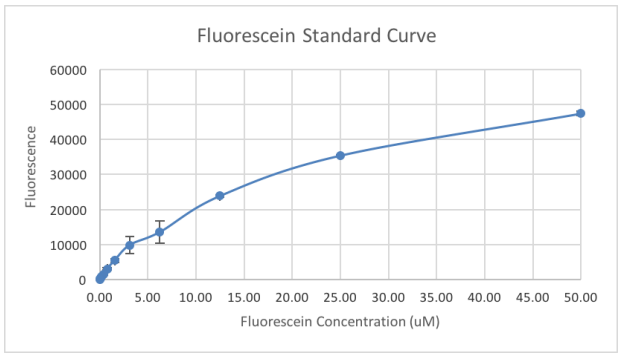
☐ Record data in your notebook

☐ Import data into Excel (cell measurement tab) Sheet_1 provided

- Calibration measurements were redone on 7/13 to account for overflow. Instrument sensitivity was changed
- A gain of 35 degrees was used.

Calibration curve:

Standard Curve



iGEM Sheet to record all measurements:

Northwestern_Interlab_Dilution_Calculation_Sheet (1).xlsx

Northwestern_University_InterLab_2017_Measurements.xlsx

All measurements are complete and forms have been submitted to iGEM (Saturday July 15th 2017)

INTERLAB COMPLETE

iGEM Registry: Transformation Protocol

Estimated bench time: 1 hour
Estimated total time: 3 hours (plus 14-18 hour incubation)

Transformations are essential to using the DNA Distribution Kits. However, they can also be one of the more fickle laboratory techniques.

At iGEM HQ, we run test transformations of the DNA Distribution Kit with the following protocol. We have found that it is the best protocol to use with the DNA Distribution Kit and ensures high efficiency transformations.

- At iGEM HQ, we make our own stocks of NEB 10b competent cells. Competent cells purchased from vendors will have better efficiency.
- Make sure to test the competency of your cells with the provided Competent Cell Test Kit.
- **Read through the entire protocol before starting!**

Materials

Resuspended DNA	Resuspend DNA Distribution Kit well(s) with 10µl dH20. Pipet up and down several times, let sit for a few minutes. Resuspension will be red from cresol red dye.
10pg/ul Control DNA	**1µl for control transformation.** pSB1C3 w/ BBa_J04450, RFP on high-copy chloramphenicol resistant plasmid. Located in the Competent Cell Test Kit.
Competent Cells	**50µl per transformation.** iGEM HQ stores competent cells in aliquots of 260µl (5rxns total) at -80°C.
2ml Microtubes	**One tube per transformation.** Label tubes with part name or well location before starting.
Floating Foam Tube Rack	Place 2ml tubes in floating tube rack for better support when working on ice and for the heat shock in the water bath.
Ice & ice bucket	Fill bucket with ice, and pre-chill 2ml tubes (5min). Thaw competent cell stock on ice (10-15min).
Lab Timer	
42°C water bath	Set water bath to 42°C before starting.
SOC Media	**200µl per transformation.** SOC Media is better than LB Media for higher transformation efficiency. SOC Media should not contain antibiotics, and can be easily contaminated.
37°C incubator	Preferably with a rotor/shaker for 2ml tubes. Incubate petri plates overnight (non-agitated).
Petri plates w/ LB agar and antibiotic	**2 plates per transformation:** for 20µl and 200µl platings. Make sure to use appropriate antibiotic. Label with part name or well location before starting.
Sterile spreader or glass beads	Used to spread transformation across petri plates. Be sure to use sterile technique in between platings.
Pipettes and Tips	10µl, 20µl, 200µl tips and pipettes recommended

Setup:

When transforming competent cells, both timing and temperature are very important. Use a lab timer, follow the incubation temperatures closely, and keep materials on ice when required.

Resuspend DNA in selected wells in the Distribution Kit. Label 2ml tubes with part name or well location. Fill lab ice bucket with ice, and pre-chill 2ml tubes (one tube for each transformation, including your control) in a floating foam tube rack.

1. Thaw competent cells on ice
   This may take 10-15min for a 260µl stock. Dispose of unused competent cells. Do not refreeze unused thawed cells, as it will drastically reduce transformation efficiency.
2. Pipette 50µl of competent cells into 2ml tube
   50µl in a 2ml tube per transformation. Tubes should be labeled, pre-chilled, and in a floating tube rack for support. Keep all tubes on ice. **Don't forget a 2ml tube for your control.**
3. Pipette 1µl of resuspended DNA into 2ml tube
   Pipette from well into appropriately labeled tube. Gently pipette up and down a few times. Keep all tubes on ice.
4. Pipette 1µl of control DNA into 2ml tube
   Pipette 1µl of 10pg/µl control into your control transformation. Gently pipette up and down a few times. Keep all tubes on ice.
5. Close 2ml tubes, incubate on ice for 30min
   Tubes may be gently agitated/flicked to mix solution, but return to ice immediately.
6. Heat shock tubes at 42°C for 1 min
   2ml tubes should be in a floating foam tube rack. Place in water bath to ensure the bottoms of the tubes are submerged. Timing is critical.
7. Incubate on ice for 5min
   Return transformation tubes to ice bucket.
8. Pipette 200µl SOC media to each transformation
   SOC should be stored at 4°C, but can be warmed to room temperature before use. Check for contamination.
9. Incubate at 37°C for 2 hours, shaker or rotor recommended
10. Pipette each transformation on two petri plates for a 20µl and 200µl plating
    Pipette 20µl and 200µl of the transformation onto appropriately labeled plates. Spread with sterilized spreader or glass beads immediately. This helps ensure that you will be able to pick out a single colony.
11. Incubate transformations overnight (14-18hr) at 37°C
    Incubate the plates upside down (agar side facing up). If incubated for too long, colonies may overgrow and the antibiotics may start to break down; un-transformed cells will begin to grow.
12. Pick single colonies
    Pick single colonies from transformations: do a colony PCR to verify part size, make glycerol stocks, grow up cell cultures and miniprep.
13. Count colonies for control transformation
    Count colonies on the 20µl control plate and calculate your competent cell efficiency. Competent cells should have an efficiency of $1.5x10^8$ to $6x10^8$ cfu/µg DNA.

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.

Prepare a column of 4 wells with 100 µl 100% LUDOX and 4 wells containing 100 µl H_2O. Repeat the measurement in all relevant modes used in your experiments (e.g. settings for orbital averaging).

Materials:

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

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.

Materials:

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

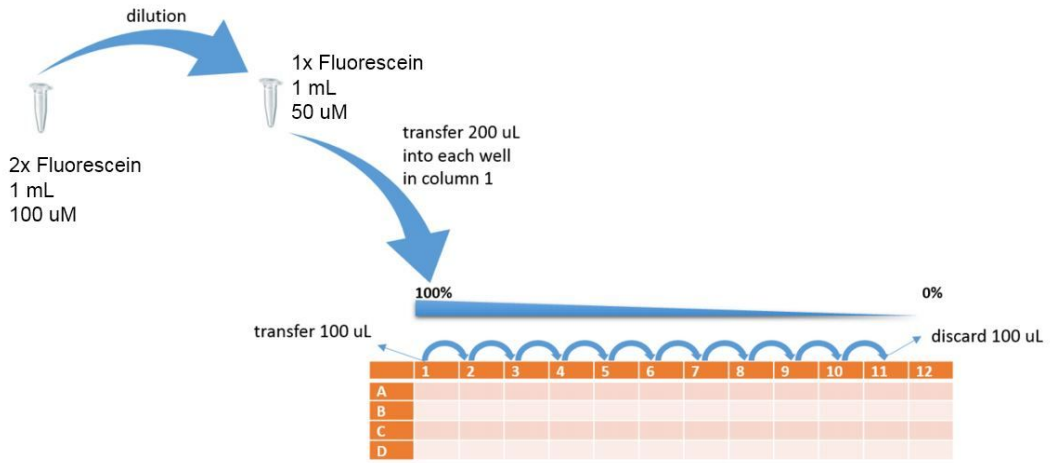
Method

Prepare the fluorescein stock solution:

- ☐ Spin down fluorescein stock tube to make sure pellet is at the bottom of tube.
- ☐ Prepare 2x fluorescein stock solution (100 µM) by resuspending 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 fluorescein stock solution with 1xPBS to make a 1x 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) 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 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.



Overview of 96-well plate setup

- ☐ **Add 100 µl of PBS** into wells A2, B2, C2, D2....A12, B12, C12, D12
- ☐ **Add 200 µl of fluorescein 1x stock** solution into A1, B1, C1, D1
- ☐ Transfer 100 µl of 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

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.

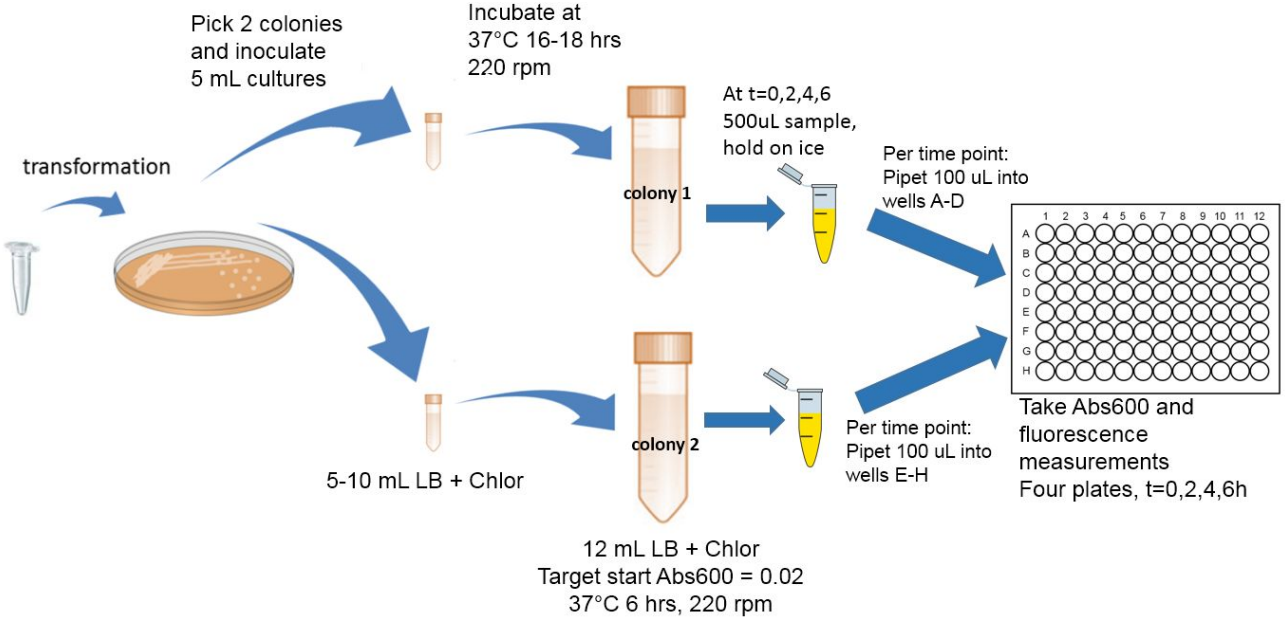
Materials:

Competent cells (*Escherichia coli* strain DH5 α)
LB (Luria Bertani) media
Chloramphenicol (stock concentration 25 mg/mL dissolved in EtOH - working stock 25 ug/mL)
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+I13504
- Test Device 2: J23106+I13504
- Test Device 3: J23117+I13504
- Test Device 4: J23101.BCD2.E0040.B0015
- Test Device 5: J23106.BCD2.E0040.B0015
- Test Device 6: J23117.BCD2.E0040.B0015

Workflow



Method

Day 1: transform *Escherichia coli* DH5 α with these following plasmids:

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

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. This includes using the sample volume (100 ul) you used for the fluorescein measurement.

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. 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.