InterLab study

Overview

The experiment will take 3 days and the aim is to transform the plasmids we received from iGEM HQ into *E.coli* and then measure GFP with a **Plate Reader**. (This year iGEM requires every team to do measurements with plate reader and not with a flow cytometer. You can do test with flow cytometer in addition to plate reader if you really want to.)

Day 1 – Overview

- Transform plasmids into E.coli DH5-alpha
- Calibrate the Plate Reader, using Calibration Protocol

Day 2 – Standard curve

- Inoculate 2 colonies from each transformation in 5 mL LB + Chloramphenicol
- Make a standard curve, using Protocol fluorescein fluorescence standard curve.

Day 3 – Cell measurements

- Cell growth, sampling and assay. (Measure GFP with Plate Reader)

The protocol for the whole InterLab Study from iGEM is here:
http://2017.igem.org/wiki/images/8/85/InterLab_2017_Plate_Reader_Protocol.pdf

InterLab iGEM webpage: http://2017.igem.org/Competition/InterLab_Study

Preparation

The vector which we are going to use in this experiment have chloramphenicol resistance, which mean that we have to use LB Agar plates and LB medium containing chloramphenicol. This is not in the Sysbio stock's which mean we have to make them by ourselves, or ask a research engineer to do this.

We need:

- LB Agar plates + chloramphenicol (MAKE!)
- LB medium + chloramphenicol (working stock - conc. Chloramphenicol will be 25 µg/mL). This we should be able to prepare. We will need at least 300 mL.
- 1 x PBS (Phosphate Buffered Saline). Should be able to make, our be in stock. Check this!

Chloramphenicol should be located in the Balance Room (according to KLARA). Did not fick any stock in the freezer -> we have to make it ourselves.

Risk declaration for chloramphenicol is made, decide which one will do the InterLab: read it and send in!

Don't know where the Plate Reader is in the Sybio lab (or if there is one), but there is one in the IndBio lab. Remember to book. To use the Plate Reader you must take a course. So either:

- ask someone that took that course to help with the experiment (Amanda took the course, but she is on vacation w32)
- contact the responsible person and take the course!
Before starting take a look at these Plate Reader forms which will be filled out each day.

Day 1:
https://docs.google.com/forms/d/e/1FAIpQLSdgUQs4AhljgGSzRZ8FxjXtCNOU2Sf6chHFKX7U2bJVnTIcNg/viewform?c=0&w=1

Day 2:
https://docs.google.com/forms/d/e/1FAIpQLSd1mb7vF0AvavsCW5COL6cKkwD87VSS1j6i9t7fo5TkAulzxg/viewform?c=0&w=1

Day 3:
https://docs.google.com/forms/d/e/1FAIpQLSegxVuDFmDobXY7_v7uCqhLiqCnxXGB3g5-nMbdOi93gyu2QA/viewform?c=0&w=1

Make chloramphenicol stock (25 mg/mL)
OBS super important to read the risk declaration before doing this and use the proper protection!
Weigh 0.25 g of chloramphenicol. Put in tube.
Add 10 ml 95 % EtOH.
(If we don't want 10 ml stock, just redo calculations ☺)

Make Agar plate LB + Chloramphenicol.
LB plates: 1 L
Peptone from casein (Tryptone) 10g
NaCl 10 g
Yeast Extract 5 g
Set pH to 7.0
Agar agar 16 g
Autoclave.
Add Chloramphenocol, 25 g/ml

Check how much we will need to make. At least 8 plates.

Make PBS 1 L
For 1 liter of 1X PBS, prepare as follows:

1. Start with 800 ml of distilled water:
2. Add 8 g of NaCl.
3. Add 0.2 g of KCl.
4. Add 1.44 g of Na_2HPO_4.
5. Add 0.24 g of KH_2PO_4.
6. Adjust the pH to 7.4 with HCl.
7. Add distilled water to a total volume of 1 liter.

Dispense the solution into aliquots and sterilize by autoclaving (20 min, 121°C, liquid cycle). Store at room temperature.

Day 1 – Overview

Transform plasmids into *E.coli*

The 8 plasmids will be transformed into *E.coli* DH5-alpha. First they need to be resuspended with 10 µL of distilled water (diH_2O). The liquid will turn red to indicate you have successfully resuspended the plasmid (iGEM dry down the plasmids with cresol red dye).

We recommend using our Transformation Protocol, but any plasmid transformation protocol should work well if you have an alternative.

Kit Plate 7 InterLab Part Locations

- Positive Control (BBa_I20270): well 21B
- Negative Control (BBa_R0040): well 21D
- Test Device 1 (BBa_J364000): well 21F
- Test Device 2 (BBa_J364001): well 21H
- Test Device 3 (BBa_J364002): well 21J
- Test Device 4 (BBa_J364003): well 21L
- Test Device 5 (BBa_J364004): well 21N
- Test Device 6 (BBa_J364005): well 21P

Antibiotic Resistance: Each device is in the pSB1C3 plasmid backbone, so transform all of the devices onto plates containing ***chloramphenicol***. Plate on LB + chloramphenicol agar plates, (8 plates in total), **make sure the plates are done before starting the experiment since they are not in the common stock.**

Calibrate Plate Reader

Using protocol Calibration Protocol from iGEM. Copy pasted into this document.

Materials:
1ml LUDOX (provided in kit)
H20 (provided by team)
96 well plate, black with flat, transparent/clear bottom preferred (provided by team)

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. 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 Abs600 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 H2O. Repeat the measurement in all relevant modes used in your experiments (e.g. settings for orbital averaging).

- Add 100 µl LUDOX into wells A1, B1, C1, D1 (or 1 mL LUDOX **into cuvette**)
- Add 100 µl of H2O into wells A2, B2, C2, D2 (or 1 mL H2O **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. (Uploaded into Box, InterLab Study. The name is: iGEMChalmersGothenburg_InterLab_2017_Measurments.xlxs)

Day 2 – Standard curve

Inoculate *E.coli* cells

Inoculate 2 colonies from each transformation into 5 mL LB + Chloramphenicol. Cover in foil to block light. Put in 37 ˚C shaker 16-18 h.

Fluorescein fluorescence standard curve

Protocol from iGEM will be used. Copy paste in to this document.

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

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.

Prepare the fluorescein stock solution:

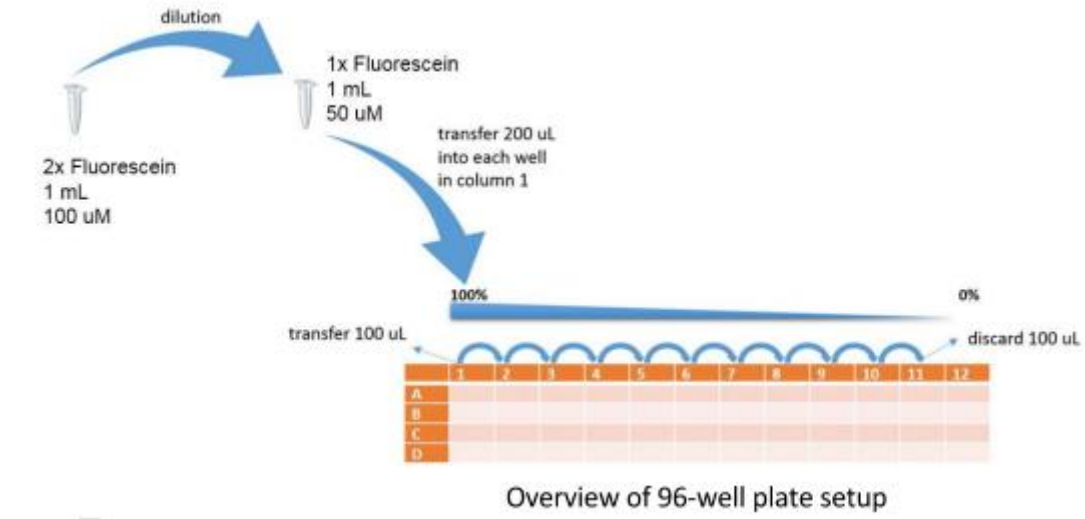
- Spin down fluorescein stock tube to make sure pellet is at the bottom of tube.
- Prepare **2x fluorescein stock solution** (100 µM) by resuspending fluorescein in 1 mL of 1xPBS.

[**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

Workflow



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 (Uploaded into Box, InterLab Study. The name is: iGEMChalmersGothenburg_InterLab_2017_Measurments.xlxs)

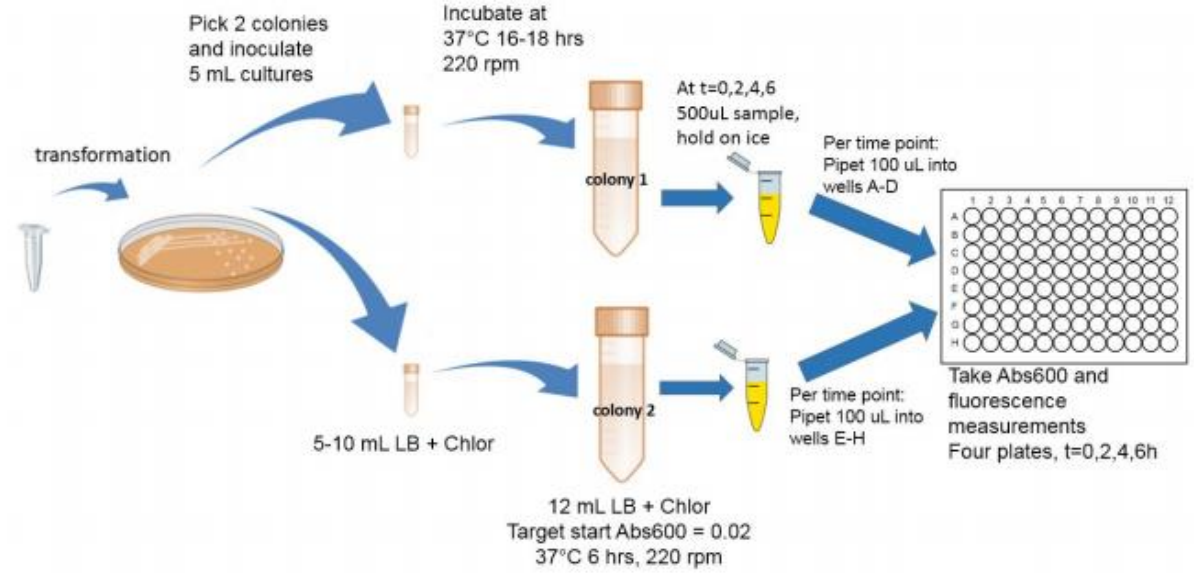
Measurement Notes
You must now measure the plate in your plate reader. The machine must be setup with the standard GFP settings (filters or excitation and emission wavelengths) that you will use when measuring your cells (if you change them you will not be able to use this standard curve). It is therefore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

Recommended filters:
- Excitation 485nm
- Emission 530/30 (or as close to this as possible)

Day 3 – Cell measurement

The cells are transformed, inoculated. The machine has been calibrated and a standard curve has been done. Now, let's do the cell measurements! :D

Workflow

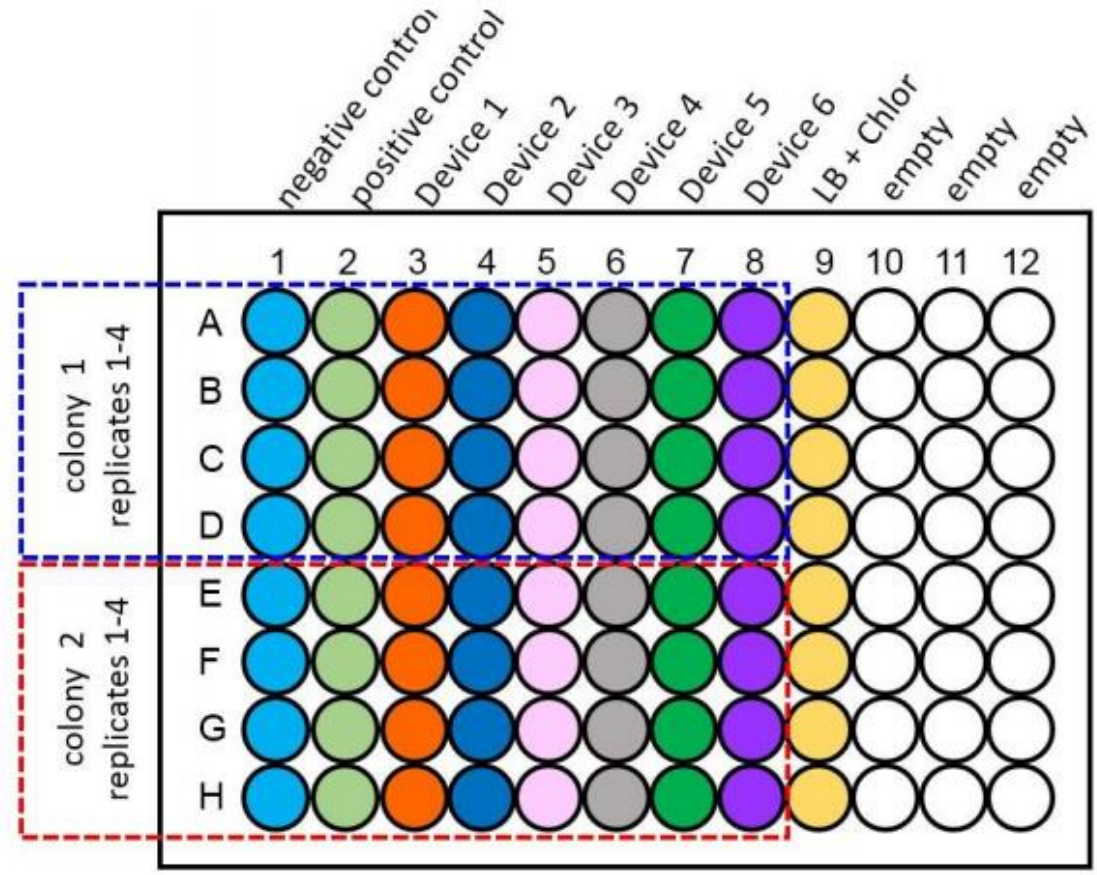


As before, protocol from iGEM.

- 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. Uploaded into Box, name: 2017_Interlab_Dilution_Calculation_Sheet.xlsx.
- Dilute the cultures to a target OD600 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. Uploaded into Box, InterLab Study. The name is: iGEMChalmersGothenburg_InterLab_2017_Measurments.xlxs

You will have one plate for each time point (0, 2, 4, and 6 hours of incubation). So in totalt 4 plates for the cell measurements.



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 the

picture above. 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

Analysis

You are almost done. Hang in there! To finish the InterLab Study you have to:

1. Download the Excel File below (TeamName_InterLab_2017_Measurements.xls) and fill it out with your data. Rename the file [TeamName] to reflect your team's name. (**Done**, uploaded in Box)
2. Email your completed Excel files to *measurement AT igem DOT org*
3. Fill in the three **Plate Reader Forms (Day 1, Day 2, and Day 3)** provided at the bottom of this page
4. Edit the InterLab study page on your team wiki (edit this page in your wikis: *http://2017.igem.org/Team:[TeamName]/InterLab*

Plate reader forms

Day 1:
https://docs.google.com/forms/d/e/1FAIpQLSdgUQs4AhljgGSzRZ8FxjXtCNOU2Sf6chHFKX7U2bJVnTIcNg/viewform?c=0&w=1

Day 2:
https://docs.google.com/forms/d/e/1FAIpQLSd1mb7vF0AvavsCW5COL6cKkwD87VSS1j6i9t7fo5TkAulzxg/viewform?c=0&w=1

Day 3:
https://docs.google.com/forms/d/e/1FAIpQLSegxVuDFmDobXY7_v7uCqhLiqCnxXGB3g5-nMbdOi93gyu2QA/viewform?c=0&w=1