

## BioE 498 Lab: In-Fusion BioBrick Assembly and Re-engineering

### I. GENETIC CIRCUIT CHARACTERIZATION: TRANSFER CURVE

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In this lab we will quantitatively characterize the GFP and RFP circuits at the population level with a transfer curve experiment and qualitatively at the single cell level using fluorescence microscopy. A transfer curve describes how the output of a genetic circuit (in this case either GFP or RFP fluorescence) changes with its input (in this case IPTG concentration which activates the circuit). It is expected that low concentrations of IPTG will lead to low output and high concentrations of IPTG will lead to high output. When the input vs. output is plotted, it is expected that this relationship will be a sigmoidal function. This is because there is a threshold at which the circuit is switched on, otherwise it would be a linear function.

#### Materials and Equipment

Glycerol stock of DH5 $\alpha$  lacI<sup>q</sup> strain with transformed GFP plasmid  
Glycerol stock of DH5 $\alpha$  lacI<sup>q</sup> strain with transformed RFP plasmid  
LB+Amp media  
GFP Teams: Mid-log culture of DH5 $\alpha$  lacI<sup>q</sup> strain with transformed GFP plasmid in LB+Amp media  
RFP Teams: Mid-log culture of DH5 $\alpha$  lacI<sup>q</sup> strain with transformed RFP plasmid in LB+Amp media  
Shaking incubator  
Spectrophotometer  
Spectrophotometer cuvettes  
1.5 mL tubes  
1M IPTG  
Molecular grade water  
Bunsen burner  
50 mL Falcon tubes  
Black clear-bottom 96-well plate  
Biotek Synergy HT plate reader

#### Protocol

1. *Instructors will do the following steps before lab starts:* Inoculate GFP and RFP circuit strains from freezer stocks into LB+Amp media and grow overnight at 37°C shaking at 250 RPM. Dilute overnight culture 1:10 in LB+Amp and grow until OD (optical density) of approximately 0.1 at 250 RPM. Measurement of OD is a common method used to estimate the cell density of a culture.
2. Remove your team's GFP or RFP circuit mid-log culture from the shaking incubator and bring it back to your bench.
3. Vortex the culture.
  - a. Measure the OD<sub>600</sub> of the culture in the spectrophotometer.
  - b. Set the spectrophotometer to measure absorbance at a wavelength of 600 nm.
  - c. Pipet 1 mL LB+Amp media into a plastic cuvette and blank the spectrophotometer.
  - d. Remove the blank cuvette and put into the Biohazard waste.

- e. Pipet 1 mL of your GFP or RFP culture into another cuvette and measure the OD.
  - f. Record the OD.
4. If the OD is at least 0.1, then continue to the next step. Otherwise, put the culture tube back in the incubator until it reaches 0.1.
  5. Label eight 1.5 mL tubes #1-8 according to the table below to mix your culture with varying concentrations of IPTG (this will be added to a 96-well plate later). Note: 1M = 1000 mM and 1 mM = 1000  $\mu$ M. For example, in Tube #2 in the table below, diluting a 1 mM stock concentration of IPTG 1:1000 in culture gives a 1  $\mu$ M IPTG concentration. Tube #8 below is media only and serves as the negative control for the plate reader experiment.

Tube#	Name	Media	[IPTG] ( $\mu$ M)	Culture Volume ( $\mu$ l)	Volume IPTG ( $\mu$ l)	Add to plate ( $\mu$ l)
1	LB - 0	LB+Amp	0	1000	0	200 X 2
2	LB - 1 X 10 <sup>-7</sup>	LB+Amp	0.1	999	1 (0.1 mM)	200 X 2
3	LB - 1 X 10 <sup>-6</sup>	LB+Amp	1	999	1 (1 mM)	200 X 2
4	LB - 1 X 10 <sup>-5</sup>	LB+Amp	10	999	1 (10 mM)	200 X 2
5	LB - 1 X 10 <sup>-4</sup>	LB+Amp	100	999	1 (100 mM)	200 X 2
6	LB - 5 X 10 <sup>-4</sup>	LB+Amp	500	995	5 (100 mM)	200 X 2
7	LB - 1 X 10 <sup>-3</sup>	LB+Amp	1000	990	10 (100 mM)	200 X 2
8	Blank	LB+Amp	0	0	0	200 X 2

6. Dilute the 1M IPTG via serial dilutions.
  - a. Take the 1M IPTG tube out of the fridge.
  - b. Label the tops of four 1.5 mL tubes: 100 mM, 10 mM, 1 mM, and 0.1 mM.
  - c. Add 18  $\mu$ l of water to each of the four tubes.
  - d. Vortex the 1M IPTG tube.
  - e. Add 2  $\mu$ l of the 1M IPTG to the 100 mM tube (to make a 1:10 dilution) and vortex.
  - f. Add 2  $\mu$ l of the 100 mM IPTG to the 10 mM tube (to make a 1:10 dilution) and vortex.
  - g. Add 2  $\mu$ l of the 10 mM IPTG to the 1 mM tube (to make a 1:10 dilution) and vortex.
  - h. Add 2  $\mu$ l of the 1 mM IPTG to the 0.1 mM tube (to make a 1:10 dilution) and vortex.
7. Pipet the amount of culture indicated in the table above into your tubes labeled #1-8.
8. Using the IPTG dilutions you just prepared, pipet the correct volume and concentration of IPTG into each tube. Vortex.
9. Transfer 200  $\mu$ l from each tube into the **two** appropriate wells in the black clear bottom 96-well plate (labeled in the table below as team letter, tube number, and replicates #1 and #2):

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1#1	A1#2	B1#1	B1#2	C1#1	C1#2	D1#1	D1#2	E1#1	E1#2	F1#1	F1#2
B	A2#1	A2#2	B2#1	B2#2	C2#1	C2#2	D2#1	D2#2	E2#1	E2#2	F2#1	F2#2
C	A3#1	A3#2	B3#1	B3#2	C3#1	C3#2	D3#1	D3#2	E3#1	E3#2	F3#1	F3#2
D	A4#1	A4#2	B4#1	B4#2	C4#1	C4#2	D4#1	D4#2	E4#1	E4#2	F4#1	F4#2
E	A5#1	A5#2	B5#1	B5#2	C5#1	C5#2	D5#1	D5#2	E5#1	E5#2	F5#1	F5#2
F	A6#1	A6#2	B6#1	B6#2	C6#1	C6#2	D6#1	D6#2	E6#1	E6#2	F6#1	F6#2
G	A7#1	A7#2	B7#1	B7#2	C7#1	C7#2	D7#1	D7#2	E7#1	E7#2	F7#1	F7#2
H	A8#1	A8#2	B8#1	B8#2	C8#1	C8#2	D8#1	D8#2	E8#1	E8#2	F8#1	F8#2

For example, Team A will transfer their #1 tube sample into the wells labeled A1#1 and A1#2 (A1 and A2), #2 tube sample into the wells labeled A2#1 and A2#2 (B1 and B2), etc. through H1 and H2. Normally we would want more replicates for each IPTG concentration, but we need to be able to fit the samples from all teams on to this one plate.

10. When you're finished loading your samples in the 96-well plate, clean up and put your samples in your freezer box, then put the box in the fridge until one of the instructors can put it in the freezer. Move to section 2 below.
11. When all teams have finished transferring their samples to the 96-well plate, the instructor will seal it with a gas-permeable membrane and put it in the plate reader to measure OD and fluorescence over time. The parameters for this experiment are to measure OD<sub>600</sub> and fluorescence (GFP excitation: 485 nm, emission: 516 nm; RFP excitation: 590 nm, emission 645 nm) every 15 minutes for 24 hours and to shake in between measurements. As explained in section 3, from this data we can plot the dynamic response of the fluorescence/OD (circuit function normalized by cell density) vs. time and the fluorescence/OD at steady-state (when the change in fluorescence over time is closest to zero) vs. IPTG concentration to get our transfer curve.

## II. GENETIC CIRCUIT CHARACTERIZATION: FLUORESCENCE MICROSCOPY

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Characterization of a genetic circuit at the population level allows us to determine the average behavior of the circuit. However, using averages hides the variation between single cells in the population and it is often useful to understand this variation when programming bacteria to perform a particular function. The variation between cells can arise from differences in small numbers of molecules (such as the LacI repressor protein) or differences in plasmid copy number. We will use fluorescence microscopy to observe GFP and RFP fluorescing cells that have been induced with IPTG. A movie can be constructed from photos taken of the cells over time and gives us an idea of the dynamic behavior of genetic circuit function.

### Materials and Equipment

LB+Amp plate

Mid-log culture of DH5 $\alpha$  lacI<sup>q</sup> strain with transformed GFP plasmid in LB+Amp 1mM IPTG media

Mid-log culture of DH5 $\alpha$  lacI<sup>q</sup> strain with transformed RFP plasmid in LB+Amp 1mM IPTG media

Willco microscope well

Fluorescence microscope

### Protocol (Instructors will do the following steps – all you have to do is view the cells under the microscope)

1. Cut out an agar slab from an LB+Amp plate.
2. Pipet 5  $\mu$ l of mid-log culture on to the agar slab and place in Willco microscope well.
3. Turn on fluorescence microscope and position well on the viewer.
4. Set the microscope to the GFP or RFP excitation/emission filter.
5. View the GFP or RFP-expressing cells.
6. Make a movie starting from a single cell that divides into a microcolony.

### III. TRANSFER CURVE DATA ANALYSIS

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1. Take your data and organize it in an Excel or Matlab spreadsheet.
2. You will have 16 measurements of OD and fluorescence taken at different timepoints during the experiment. Average the 2 replicates for your #1-8 samples for each timepoint and use these for subsequent data analysis.
3. Subtract the averaged blank (#8 sample) OD from each of the averaged #1-7 OD samples separately for each timepoint. Now you have 7 OD datapoints for every timepoint.
4. Subtract the averaged blank (#8 sample) fluorescence from each of the averaged #1-7 fluorescence samples separately for each timepoint. Now you have 7 fluorescence datapoints for every timepoint.
5. Plot **OD vs. time** for all 7 samples (time on the x-axis, OD on the y-axis). You should have 7 curves on the same graph. The timepoint where the OD has stopped increasing is where the cells have reached stationary phase and are no longer growing.
6. Plot **fluorescence vs. time** for all 7 samples (time on the x-axis, fluorescence on the y-axis). You should have 7 curves on the same graph.
7. Divide your fluorescence by OD for each timepoint. This will give a normalized average fluorescence measurement per cell.
8. Plot **fluorescence / OD vs. time** for all 7 samples (time on the x-axis, fluorescence / OD on the y-axis). You should have 7 curves on the same graph.
9. How does **fluorescence / OD vs. time** graph differ from the **fluorescence vs. time** graph?
10. To make the transfer curve, find the timepoint where the **rate of change** of fluorescence / OD is closest to zero (i.e. steady-state value). This should be where the peak of the curve is.
11. Take the fluorescence / OD datapoints at this timepoint for each of the seven IPTG concentrations. Plot **fluorescence / OD vs. IPTG concentration** for all 7 samples (IPTG concentration on the x-axis, fluorescence / OD on the y-axis).
12. Does this graph look sigmoidal? If not, why do you think it doesn't?