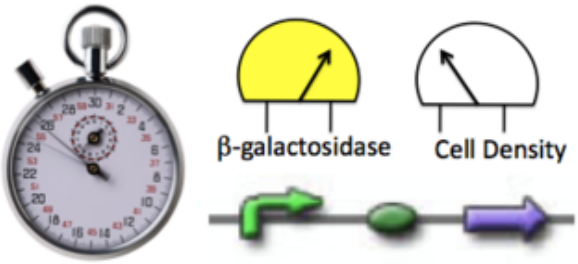
LAB 2: iTune device

- Evaluate promoter and RBS combinations to optimize beta-galactosidase output



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Objectives

By the conclusion of this laboratory investigation, the student will be able to:

- Explain how synthetic biology as an engineering discipline differs from genetic engineering.
- Explain the engineering paradigm and the role of tuning a system.
- Explain the functioning of the lac operon and relate it to this lab.
- Culture bacteria using proper microbiology methods.
- Measure a kinetic chemical reaction.
- Define and properly use synthetic biology terms: [Part.][Device.] [Measurement.]
- Define and properly use molecular genetics terms: [Promoter.][ribosome binding site ("RBS").] [open reading frame ("ORF").] [Terminator.] [Plasmid.]

Introduction

As engineers, synthetic biologists engage in the **"design--> build--> test"** process. They **design** genetic devices by coupling together promoters, ribosome binding sites (RBS), open reading frames (ORF), and terminator sequences. They then **build** devices using techniques such as DNA synthesis, gel electrophoresis, polymerase chain reaction, and cloning. The synthetic biologists then **test** the function of the devices they've built, characterizing the cells that bear the devices through enzyme activity assays, fluorescent protein measurements or phenotype analysis. Depending on the device that's being characterized, measurements may evaluate the speed of a device's response, its sensitivity to environmental signals, or the level of a protein made by the device. It's tempting to think that a strong quick level of response is always desired when

designing genetic devices. However, depending on the role that the device will play in a system, it may be desirable to be able to tune the output to intermediate levels, or even to slow and low outputs in some cases.

Tuning genetic devices may be accomplished in many ways. One method controls the rate of transcription initiation by choosing a promoter of a particular efficiency or that's active only under some conditions, for example. Another method involves translation control, modifying the strength of the ribosome binding site to increase or decrease the translation initiation rate. Finer tuning can be achieved by rational combination of the promoter and RBS elements. Predictable design, however, is confounded by the fact that some devices are not fully insulated from others in the cell and so might be affected by the system in which they must perform. Other devices demand a lot of the cell's resources to run and so might slow a cell's growth or protein production rates. These problems would be like a car in which the volume button on the radio also turned the steering wheel, or like a car in which the louder you played the radio, the slower the car could run. Problematic to say the least!

Thus measuring the performance of a device, even a rationally designed one, is still needed. As a starting point, we will consider a "reference device" that includes a strong log phase promoter, a strong RBS, a lacZ ORF that produces beta-galactosidase, and a transcriptional terminator sequence. Variants of this device are also available. All contain the same lacZ ORF and terminator sequence, but the devices vary in the efficiency ("strength") of the promoters and RBSs. We will measure the output of each device, presuming that any difference in beta-galactosidase activity level will be due to the combination of promoter and RBS. The lacZ ORF provides us with an easy method to measure the activity level of each promoter/ORF combination since the beta-galactosidase that is produced by the lacZ ORF allows the bacteria to metabolize lactose (see lac operon). Normally lactose is cleaved into two monosaccharides, galactose and glucose. However, we will provide the cells with ONPG (o-nitrophenyl-β-D-galactoside) rather than lactose. ONPG will be metabolized by the beta-galactosidase into galactose and o-nitrophenol, a yellow compound. The intensity of the yellow color formed will be proportional to the amount of beta-galactosidase enzyme that the device produced in the cell. We measure intensity of yellow color using a spectrophotometer, like a Spec 20, or with visual comparisons to turbidity standards.

Procedure

Part 1: Culturing Bacteria

We will be receiving our bacteria with the plasmid already inserted. This culture may come in the form of a "stab" or "slant," a test tube with a small amount of bacteria on a slanted media, in which case you will have to streak out the bacteria

onto a petri dish to continue the experiment. If the bacteria have arrived on petri dishes, you can proceed to "Day 2."

Day 1:

1. Using a sterile toothpick or inoculating loop, gather a small amount of bacteria from the stab and transfer it to a petri dish containing Luria Broth (LB) agar plus ampicillin medium.
2. Repeat with the remaining stab samples, streaking out each onto a different petri dish.
3. Place these petri dishes media side up in a 37°C incubator overnight.

A video of this procedure is here.

Day 2:

1. Using a sterile inoculating loop or toothpick or pipet tip, transfer a bacterial colony from the petri dish to a large sterile culture tube containing 2 ml of Luria Broth, 20 µL IPTG and 2 µl of ampicillin. This volume is more than enough for each strain that you or your team must grow.
2. Repeat for each strain you will inoculate.
3. Place the culture tubes in the roller wheel in the incubator at 37°C overnight. Be sure to balance the tubes across from each other to minimize stress on the roller wheel.

A video of this procedure is here.

Part 2: Beta-galactosidase assay

Procedure using a Spec 20

With this assay you will determine the amount of beta-galactosidase activity associated with each sample of cells. As a class you should try to perform replicate assays of each sample (so each strain gets measured two or three times) and then pool your class data to gain some confidence in the values you measure. A data table is included to help you organize your assay, but you can make one of your own if you prefer. Note that the volumes here are given for spectrophotometers that use glass test tubes (13x100 mm).

1. Make 3.0 ml of a 1:10 dilution (300 µL of cells in 2.7 ml of bicarbonate buffer) of each cell sample.
2. If you made the dilution in glass spectrophotometer tubes, you can proceed to the next step. If not, you will need to transfer some of this diluted cell mixture to a cuvette or glass spectrophotometer tube. The exact amount to transfer will depend on the size of the cuvette you use. Your teacher will provide further instructions.
3. Measure the Absorbance at 600 nm (OD 600) of this dilution. Record the value **X 10** in the data table. This is the density of the undiluted cells. If you

do not have a spectrophotometer and are using Turbidity Standards instead, follow the instructions in the next section.

4. You can now dispose of these dilutions and tubes as instructed by your teacher.
5. Add 1.0 ml of bicarbonate buffer to 11 test tubes labeled B (blank), R (reference), and 1 though 9 (the samples). These are the reaction tubes.
6. Add 100 µl of the cells (undiluted) to each tube. Add 100 µl of LB to tube B, to serve as your blank.
7. Next you will lyse the cells by add 100 µl of dilute dish soap to each tube.
8. Vortex the tubes for 10 seconds each. You should time this step precisely since you want the replicates to be treated as identically as possible.
9. Start the reactions by adding 100 µl of ONPG to each tube at 15 second intervals, including your blank.
10. After 10 minutes, stop the reactions by adding 1 ml of soda ash solution to each tube at 15 second intervals. Ten minutes is sufficient time to provide results that are yellow enough to give a reliable reading in the spectrophotometer, best between 0.1 and 1.0. Usually this color is approximately the same as that of a yellow tip for your pipetman. Don't be surprised when the soda ash makes the reactions look more yellow. The reactions are now stable and can be set aside to read another day.
11. If you conducted the reaction in glass spectrophotometer tubes (your teacher will tell you this), you can skip to the next step. If not, you will need to transfer some of the reaction mixture from the reaction tubes to a cuvette or glass spectrophotometer tube. The exact amount to transfer will depend on the size of the cuvette you use. Your teacher will provide further instructions.
12. Read the absorbance of each sample tube at 420nm (OD 420). These values reflect the amount of yellow color in each tube. If you do not have a spectrophotometer and are comparing the color to paint chips instead, follow the instructions in the next section.
13. Calculate the beta-galactosidase activity in each sample according to the formula below.

Procedure if a Spec 20 is not available

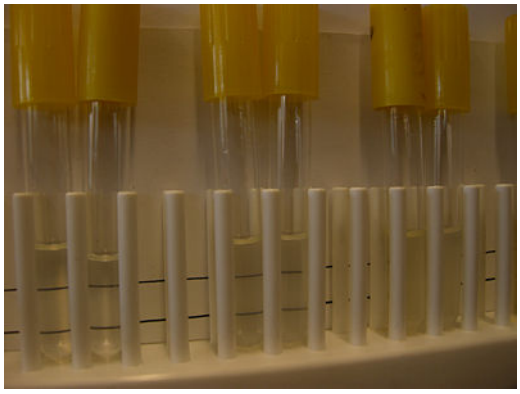
Estimate the OD 600

The OD 600 can be estimated using Turbidity Standards. This method uses suspensions of a 1% $BaCl_2$ in 1% H_2SO_4 at various concentrations and is modeled after the McFarland Turbidity Scale. These suspensions appear visually similar to suspensions of various populations of *E coli*.

1. Following your teacher's instructions, obtain small clear test tubes containing the turbidity standards. The tubes should contain enough standard in each to fill the tube to a height of about 1 inch (2.5 cm) from the

bottom. Make sure each tube is properly labeled with its turbidity standard number. If you are filling the tubes from stock bottles of the standards, use small tubes and place enough standard in each to fill the tube to a height of about 1 inch (2.5 cm) from the bottom.

2. Place them in a test tube rack that allows you to view them from the side. Use small tubes and place enough standard in each to fill the tube to a height of about 1 inch (2.5 cm) from the bottom.
3. On a blank index card or paper use a marker to draw two thick black lines. These lines should be within the height of the standards.
4. Place the card with the lines behind the standards.



5. Make 3.0 ml of a 1:10 dilution of each cell sample, using bicarbonate buffer as the diluent.
6. To compare your bacterial cultures to the standards, you will need to place the bacterial sample in a test tube of the same size and equal volume as the standards. Be sure to label these sample tubes.
7. Place the sample tube next to the standard tubes. You should move the sample to compare it to the standard tubes with the most similar turbidity. You can make this assessment more precise by looking for a standard that most similarly obscures the black lines on the background card.
8. Use the table below to determine the comparable OD 600.
9. 1 OD 600 unit equals approximately 1 x 10^9 cells.

Turbidity Scale	OD 600	1% $BaCl_2$/ 1% H_2SO_4 (mL)
0	0	0.0/10
1	0.1	0.05/9.95
2	0.2	0.1/9.9
3	0.4	0.2/9.8
4	0.5	0.3/9.7
5	0.65	0.4/9.6
6	0.85	0.5/9.5
7	1.0	0.6/9.4

Estimate the OD 420

The OD 420 can be estimated using Benjamin Moore paint chips. Color chips will be provided by your instructor.

Benjamin Moore color	Paint Chip	OD 420
0330 Palm Coast Pale		0.1
0331 Lemon Souffle		0.15
0332 Banan-appeal		0.2
0333 Pineapple Grove		0.5
0334 Limon		0.7
0335 Delightful Yellow		1.0
0336 Bold Yellow		2.0

1. Once the reactions have been stopped with soda ash solution, allow the debris to settle for a few minutes and then compare the solution's meniscus to the color samples provided. The approximate OD 420 value that corresponds to each color is listed in the table below.
2. Calculate the beta-galactosidase activity in each sample according to the formula below.

Data Table

In your lab notebook, you will need to construct a data table as shown below. If you are testing only a subset of the promoter and RBS collection, be sure to note which ones you are investigating:

- Tested Promoter (circle the experimental sample(s) you are measuring):
  - **weak**
  - **medium**
  - **strong**
- Tested RBS (circle the experimental sample(s) you are measuring):
  - **weak**
  - **medium**
  - **strong**

Sample number	Strain	Abs 600	Start time	Stop time	Time elapsed (minutes)	Abs 420	β-gal activity Miller Units
B = blank	none		0:00				
R	Reference strain		0:15				
1	2-1		0:30				
2	2-2		0:45				
3	2-3		1:00				
4	2-4		1:15				
5	2-5		1:30				
6	2-6		1:45				
7	2-7		2:00				
8	2-8		2:15				
9	2-9		2:30				

Calculations

The β-gal production is reported in Miller Units

β-gal production in Miller Units = $$1000 * \frac{Abs420}{(t * v * Abs600)}$$

Where:
Abs 420 is the Spec 20 absorbance at 420 nm. It is a measure of the yellow color produced by the β-gal activity. It is a unitless number.
Abs 600 is the Spec 20 absorbance at 600 nm. It is a measure of the cell density. It is a unitless number.
t is the reaction time in **minutes.**
v is the volume of cells added to the reaction in **mls.** (Not µl!).

Summary Data Table

In your lab notebook, you will need to construct a data table as shown below. Fill in as many values as possible.

Strain	Promoter	RBS	β-gal activity Miller Units (class data, may have >1 entry here)	class mean β-gal activity Miller Units
2-R	Reference promoter	Reference RBS		
2-1	weak	weak		
2-2	weak	medium		
2-3	weak	strong		
2-4	medium	weak		
2-5	medium	medium		
2-6	medium	strong		
2-7	strong	weak		
2-8	strong	medium		
2-9	strong	strong		

When you've finished your experiments, upload your data to the BioBuilder site that's here. You'll be able to compare what you've measured to what other BioBuilders around the country have seen.

Lab Report

As you write, be sure to define and properly use all highlighted terms throughout the introduction and other parts of the lab.

I. Introduction

- Provide a brief introduction describing the field of synthetic biology.
- Briefly describe the purpose of the lab. What are we trying to do here? Presume that a reader of your lab report has not read the assignment.
- Discuss the function of the promoter and the RBS. Relate your discussion to the function of the lac operon.

II. Methods

- You do not have to rewrite the procedure.
- Explain why you did each step of the protocol.

III. Results

- Present the data tables in clear format.
- Create a graph summarizing the results.

IV. Discussion

- Draw a conclusion: Were we able to tune this system?
- Describe the results: How do each of the promoter/RBS pairs compare? Did changing the promoters and changing the RBS have the same effect?
- Analyze the data: Be sure to discuss how each part of the experiment adds to your conclusion.
- Discuss errors and other reasons for data variability.
- How might experiments like this one help us learn about evolution?