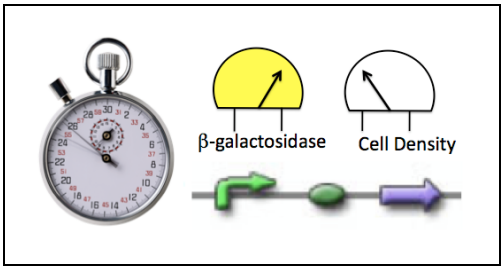
SYNTHETIC BIOLOGY AND THE HIGH SCHOOL CURRICULUM: LAB 2
http://openwetware.org/wiki/Synthetic_Biology_and_the_High_School_Curriculum:_Lab_2




**Lab 2: iTUNE device**
Evaluating promoter and RBS combinations to maximize beta-galactosidase output.

**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 spec 20.

**Procedure**

**Part 1: Culturing Bacteria**

We will be receiving our bacteria with the plasmid already inserted. This culture will come in the form of a "stab" or "slant", a test tube with a small amount of bacteria on a slanted media. To continue the experiment we will have to further culture the bacteria.

**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 cultures in a 37°C incubator overnight.

This video illustrates the technique used for this transfer:
http://www.youtube.com/watch?v=QydH5ZoD_Aw

**Day 2:**

1. Using a sterile inoculating loop, transfer a bacterial colony from the petri dish to a large sterile culture tube containing 5 ml of Luria Broth and 5 µl of ampicillin.
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.

This video illustrates the general technique for setting up overnight liquid cultures, though you'll be transferring cells from the petri dish to the Luria Broth.
http://www.youtube.com/watch?v=0odxJy0nR9s&NR=1

**Part 2: Beta-galactosidase assay**

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 plastic cuvettes. If you are reading the absorbance values in a Spec 20 that uses glass tubes, you will have to adjust all the volumes used in this assay to have a large enough volume to read (0.8 ml of Z buffer, 0.2 ml of cells, 0.2 ml of ONPG, 0.5 ml of $Na_2CO_3$), but can leave the volume of SDS and $CHCl_3$ as is.

1. Make 1.0 ml of a 1:10 dilution of each cell sample, using Zbuffer as the diluent
2. Measure the Absorbance at 600 nm of this dilution. Record the value X 10 in the data table. This is the density of the undiluted cells.
3. Add 400 μl of Zbuffer to 11 eppendorf tubes labeled 0-10.
4. Add 100 μl of the cells (undiluted) to each tube. Add 100 μl of LB to tube 0, to serve as your blank.
5. Next you will lyse the cells by add 20 μl of 0.1% SDS and 30 μl of $CHCl_3$ to each eppendorf. Wear gloves when you add the $CHCl_3$ and cap the tubes when you're done.
6. Still wearing your gloves, 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.
7. Start the reactions by adding 100 μl of ONPG to each tube at 15 second intervals, including your blank.
8. After 60 minutes, stop the reactions by adding 250 μl of $Na_2CO_3$ to each tube at 15 second intervals once sufficient yellow color has developed. "Sufficient" is defined as 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 $Na_2CO_3$ makes the reactions look more yellow. The reactions are now stable and can be set aside to read another day.
9. When all your samples have been stopped, add 250 μl of $Na_2CO_3$ to the blank.
10. If a microfuge is available, spin all the tubes for 1 minute at 13,000 RPM to pellet any cell debris. If a microfuge is not available then you can expect the absorbance values at 550 nm to be higher due to cell debris in the reactions. This difference will be corrected in the final calculation of Miller units.
11. Move 0.7 ml of each reaction to plastic cuvettes and read the absorbance at 420 nm. These values reflect the amount of yellow color in each tube.
12. Read the absorbance of each at 550 nm. These values reflect the amount of cell debris and differences in the plastic cuvettes themselves.
13. Calculate the beta-galactosidase activity in each sample according to the formula below. Note that if the 550 values, when multiplied by 1.75, give you a value that's greater than the 420 value, then simple set the 550 value to zero.

**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 | Abs600 | Start time | Stop time | Time elapsed (minutes) | Abs420 | Abs550 | β-gal activity Miller Units |
|---|---|---|---|---|---|---|---|---|
| 0 = blank | none | | 0:00 | | | | | |
| 1 | Reference strain | | 0:15 | | | | | |
| 2 | 2-1 | | 0:30 | | | | | |
| 3 | 2-2 | | 0:45 | | | | | |
| 4 | 2-3 | | 1:00 | | | | | |
| 5 | 2-4 | | 1:15 | | | | | |
| 6 | 2-5 | | 1:30 | | | | | |
| 7 | 2-6 | | 1:45 | | | | | |
| 8 | 2-7 | | 2:00 | | | | | |
| 9 | 2-8 | | 2:15 | | | | | |
| 10 | 2-9 | | 2:30 | | | | | |

**Calculations**
The β-gal production is reported in Miller Units

1 Miller Unit = $$1000 * \frac{(Abs420 - (1.75 * Abs550))}{(t * v * Abs600)}$$

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.
Abs 550 is the Spec 20 absorbance at 550 nm. It is a measure of cell debris.
Abs 600 is the Spec 20 absorbance at 600 nm. It is a measure of the cell density.
t is the reaction time in minutes.
v is the culture volume in mls.

**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<br>Miller Units (class data, may have >1 entry here) | class mean<br>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 | | |

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

**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.

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

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

**Discussion:**
- Draw a conclusion: Were we able to tune this system?
- Describe the results: How do each of the promoter/RBS pairs compare?
- 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?