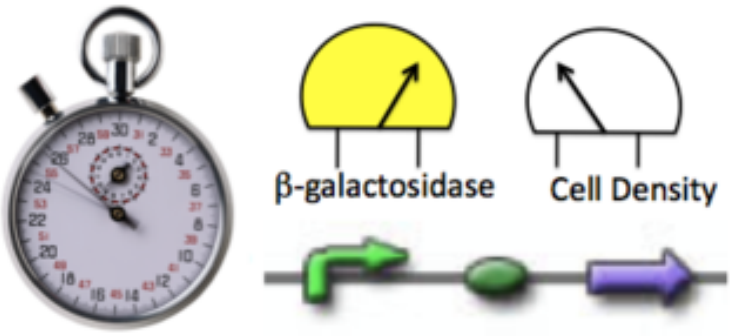
Lab 2: iTune Device



Teacher Considerations

This lab examines the role of parts, such as promoters and ribosome binding sites, in predicting the output of a genetic device. The students measure b-galactosidase enzymatic activity as the device's output, thereby looking through the lens of molecular genetics to predict and then evaluate a device's behavior. It provides an excellent way to study the transcription, translation, the lac operon and enzyme function within a synthetic biology context.

Part 1 of the lab offers an opportunity for the students to develop microbiology skills, though this procedure may be done by the teacher if time is an issue.

In **Part 2**, the students will lyse the cells to release the β-galactosidase enzyme and then carry out the reaction. It is important that the timing of each step be as precise as possible. You may want them to rehearse this procedure prior to the actual experiment. All of **Part 2** can be done in one lab period. However, the reaction mixture is stable once the reaction has been stopped. If you prefer, you can store these tubes overnight and read the OD 420 the next day. Just allow the tubes to warm to room temperature so condensation isn't collecting on the tubes when they are being read in the spectrophotometer.

If a spectrophotometer is not available, the cell density and β-gal activity can be easily measured using the McFarland Turbidity methodology, as explained below. If a microfuge is available for the students to use and cuvettes for the spectrophotometer, then we recommend transferring the stopped reactions to microfuge ("eppendorf") tubes, spinning the solutions for a minute to pellet the debris and then transferring the yellow supernatant to cuvettes to read the OD420. When the cleared liquid is measured in this way, then the OD420 measurement is no longer confounded by the cell debris that is present in the glass tubes.

An introductory power point for the lab can be found here.

Needed Materials

Teacher Provides

- Consumables
  - Luria Broth (LB), 25 ml
  - Sterile toothpicks, innoculating loops or sterile tips
  - Sterile tubes (16x150mm)+ loose caps (10)
  - Glass test tubes (13x100mm) (22)
  - Cuvettes for the spectrophotometer, if needed (22)
  - Pipet tips
  - Latex or nitrile gloves
  - Arm & Hammer baking soda (1g)
  - Clear liquid dish soap, like "SoftSoap" (one squirt)
  - Soda Ash, like what's used to tie dye T-shirts, (5.3g)
  - 50 ml conical tubes for bicarb, soap and soda ash soln's (3)
  - Distilled water, or bottled like Poland Spring
- Equipment
  - Roller drum or shaker at 37° for growing liquid overnights
  - Spectrophotometer OR turbidity stds (1.75 ml $BaCl_2$+ 80 ml 1% H_2SO_4)
  - Vortex
  - Pipets (5 ml) + bulbs or pipet-aids
  - Pipetmen (P1000, P200, P20)
  - Timer
  - Sharpie pens
  - Test tube rack for holding small tubes during rxn

Kit Provides
10 strains (see table below)

- Store stabs at room temp
- Store plates and liquid cultures at room temp or 4° (= fridge) for longer times (on the order of a few weeks).

Strain #	Promoter	Promoter #	Artificial Units	RBS	RBS #	efficiency
2-R (= NB424)	Reference promoter	J23115	387	Reference RBS	B0035	?
2-1 (= NB425)	weak	J23113	21	weak	B0031	0.07
2-2 (= NB426)	weak	J23113	21	medium	B0032	0.3
2-3 (= NB427)	weak	J23113	21	strong	B0034	1
2-4 (= NB428)	medium	J23106	1185	weak	B0031	0.07
2-5 (= NB429)	medium	J23106	1185	medium	B0032	0.3
2-6 (= NB430)	medium	J23106	1185	strong	B0034	1
2-7 (= NB431)	strong	J23119	consensus	weak	B0031	0.07
2-8 (= NB432)	strong	J23119	consensus	medium	B0032	0.3
2-9 (= NB433)	strong	J23119	consensus	strong	B0034	1
NOTE: these promoter/RBS combinations have not been sequence verified but perform ~ as expected						

Chemicals

4° (fridge)

- 40 mg [ONPG], needs to be diluted to 4 mg/ml in water)
- 100 mg [Amp], use at final concentration of 100 mg/liter LB
- 24 mg [IPTG], needs to be diluted to 24 mg/ml in water and used at a final concentration of 1:10 in LB

Workflow

Classroom Content

- BioBuilder material that sets up this lesson starts here
- **Day 1:** streak strains from stabs onto plates
- **Day 2:** grow strains from plates as liquid overnights
- **Day 3:** b-gal assays
- **Day 4:** calculations of units and comparison of class data (could also be Day 3 if time allows)

When they have finished the experiments, students should upload their data to the link on the BioBuilder site that's here. They'll be able to compare what they've measured to what other BioBuilders around the country have seen.

Annotated Laboratory Procedure

TEACHERS: Note that "Part 1: Culturing Bacteria" can be done by the students or by you (the teacher) depending on how much time and preparation you intend to take on/delegate.

TEACHERS: Clean-up instructions. Provide containers at each work-station for student biological waste such as pipet tips, eppendorf tubes, spreaders, innoculating loops, and plates. Be sure to follow hazardous waste procedures as set forth by your school or municipality. Generally, it is safe to soak the material in each container with a 10% bleach solution for 2 hours. Materials can then be discarded into the regular trash. You can find more information about microbiology lab safety here

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:

TEACHERS: The volume of cells you'll need to grow will depend on how you are setting up your student's work. If each student or student team is to test every strain, then 2.5 ml of each culture for each of them will be more than enough. If you would like students/student teams to share the cultures that are grown when they perform "Part 2: Beta-galactosidase assay," then insure that there is at least 1 ml of bacteria for every assay to be performed.

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 2.5 ml LB+Amp supplemented with 25 µL IPTG. This volume is more than enough for each strain that each student or team of students 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).

*TEACHERS: In advance of lab you should prepare the three solutions necessary to run these reactions. The **bicarbonate buffer** is made by mixing 1 g of Arm & Hammer baking soda into 50 ml of distilled or bottled water. The **ONPG** is made by adding 10 ml of distilled or bottled water to the 40 mg of ONPG that was sent to you with the kit. Finally, the **soda ash solution** is prepared by mixing 5.3 g of soda ash with 50 ml distilled or bottled water. All solutions are stable on the bench or in the fridge for at least a month.*

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.

TEACHERS: If a microfuge is available, you can transfer some of the reaction mixture to a microfuge tube, spin the eppendorf tube for one minute, and then transfer that cleared solution to a cuvette to read the OD 420. However, the microfuge tube will not hold enough of the reaction mixture to read the absorbance

using the larger glass tubes. If you must use the larger glass tubes or do not have a microfuge, you can skip this step, though allow time for the debris to settle. It is possible that the remaining cell debris will result in some negative values. These can be set to zero for calculation purposes.

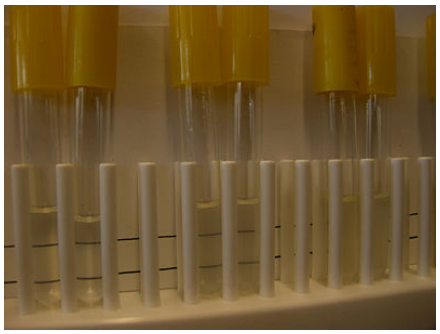
Procedure if a Spec 20 is not available

TEACHERS: If a Spec 20 is not available, your students can conduct the protocol presented below. While these results will not be as precise, they do provide accurate data for analysis.

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

TEACHERS: For this procedure it is not necessary to use a centrifuge.

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		

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.

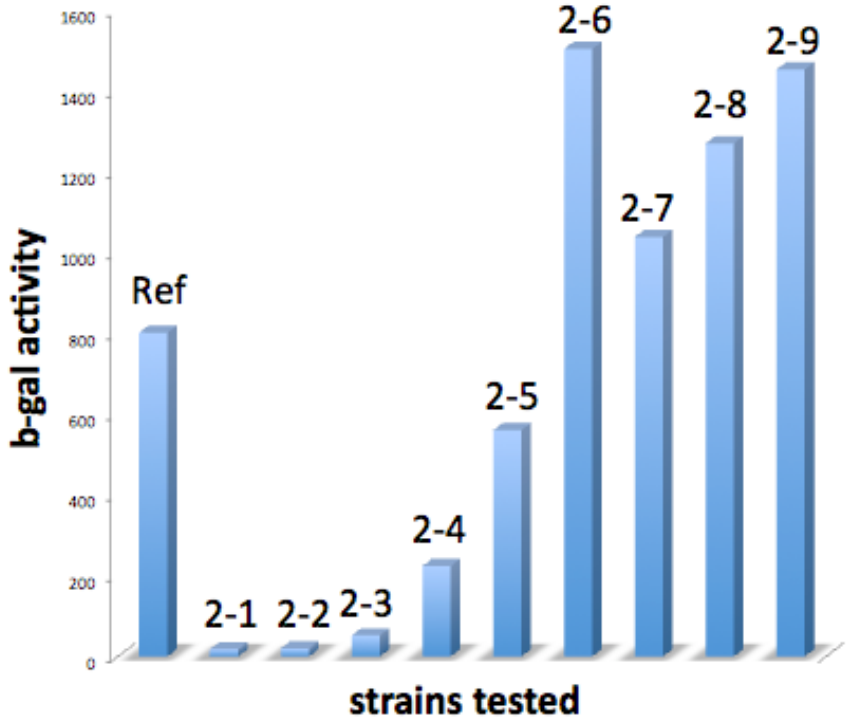
III. Results

- 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?

Sample Data Set



Assessment

Lab Report Rubric

Download doc or pdf

Lab Report ScoreSheet

Download doc or pdf

Survey

To help us improve the labs, you can

1. send the students here, where they can upload their data.
2. "join a discussion" from the BioBuilder homepage
3. email us: "info AT biobuilder DOT org"

Thanks!

Variations to try

- Try testing cells grown to log phase rather than stationary phase?
- Try growing in the absence of IPTG? It's not entirely clear what lac repressor is doing in the cells anyway but the output for the devices may be different if it's not included in the growth media.
- Try growing the cells at different temperatures? or running reactions at different temperatures?
- If you are using the McFarland standard, would more precise or subtle standards be useful?

Feedback

We're always looking to hear back from you if you've thought about this unit, tried it, or stumbled across it and want to know more. Please email us through BioBuilder, info AT biobuilder DOT org.