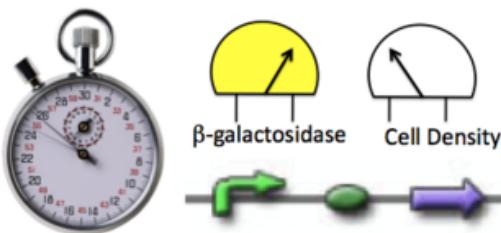


Lab 2: iTune Device



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 β -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.

Needed Materials

Teacher Provides

- Tubes to grow cells
- Eppendorf tubes or small glass tubes for running reactions
- Cuvettes to measure absorbances if spectrophotometer is not fitted for glass tubes
- Pipetmen and tips (P1000, P200)
- Pipets (10 ml and 5 ml) and bulbs
- Timers or stopwatches
- Sharpies
- Nitrile or Latex gloves
- rollerwheel at 37° for growing overnight cultures of bacteria
- vortex
- microfuge (optional)
- fume hood for measuring CHCl₃

Kit Provides

10 strains (see table below)

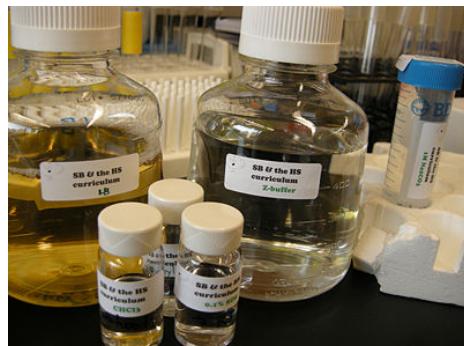
- Store stabs at room temp
- Store plates and liquid cultures at room temp or 4° (= fridge) for longer times.

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

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Chemicals



Room Temperature

- 500 ml LB (= 10 g Tryptone, 5 g Yeast Extract, 10 g NaCl per liter, plus 20g of Agar for plates). **Keep sterile.**
- 500 ml Z-buffer (= 8.05 g Na₂HPO₄·7H₂O, 2.75 g NaH₂PO₄·H₂O, 0.375 g KCl, 0.123 g MgSO₄·7H₂O in 500 ml H₂O)
- 50 ml 1M Na₂CO₃ (= 10.6 g in 100 ml)
- 10 ml 0.1% SDS (100 ul of 10% in 10 ml H₂O)

4° (fridge)

- 30 ml [[ONPG](#)] (4 mg/ml in Z-buffer)
- 2 ml [[Amp](#)] (100 mg/ml in H₂O, filter sterilized)
- 20 ml [[IPTG](#)] (0.1 M in H₂O, filter sterilized)

Chemical Hood

- 1 ml CHCl₃

Workflow

Classroom Content



[BioPrimer #2 pdf](#)

- [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 you are done with this lab, [here](#) is a link to survey that you can offer the students. Thank you for helping us improve this content.

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. The only exception is the aliquot of $CHCl_3$ (Day 3) that should be done in the fume hood by the teacher.



TEACHERS: Clean-up instructions. Provide containers at each work-station for student biological waste such as pipet tips, eppendorf tubes, spreaders, inoculating 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 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 ml of Luria Broth, 20 μL IPTG and 2 μl of ampicillin. 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).

1. Make 3.0 ml of a 1:10 dilution of each cell sample, using Zbuffer as the diluent.
2. 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.
3. Add 1.0 ml of Zbuffer to 11 glass spectrophotometer tubes labeled B (blank), R (reference), and 1 through 9 (the samples).
4. Add 30 μ l of the cells (undiluted) to each tube. Add 30 μ l of LB to tube B, to serve as your blank.
5. Next you will lyse the cells by add 60 μ l of 0.1% SDS and, in the hood, add 50 μ l of CHCl₃ to each tube. Wear gloves when you add the CHCl₃ 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 300 μ l of ONPG to each tube at 15 second intervals, including your blank.
8. After 7 minutes, stop the reactions by adding 750 μ l of Na₂CO₃ to each tube at 15 second intervals. Seven 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 Na₂CO₃ makes the reactions look more yellow. The reactions are now stable and can be set aside to read another day. Your teacher will inform you if that is the case.
9. Read the absorbance of each sample tube at 420nm (OD 420). These values reflect the amount of yellow color in each tube.
10. 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.

If a Spec 20 is not available

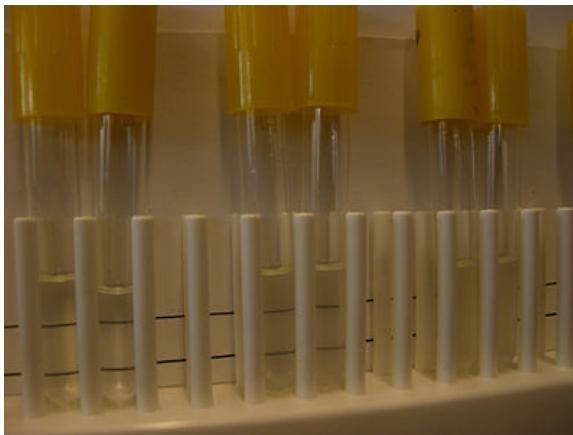


TEACHERS: If a Spec 20 is not available, your students can conduct the protocol presented above but substitute these procedures for steps 2 and 9. While these results will not be as precise, they do provide accurate data for analysis.

Estimate the OD 600

The OD 600 can be estimated using Turbidity Standards. This method uses suspensions of a 1% BaCl₂ in 1% H₂SO₄ 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. 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.
6. 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.
7. Use the table below to determine the comparable OD 600.

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8. 1 OD 600 unit equals approximately 1×10^9 cells.

Turbidity Scale	OD 600	1% BaCl ₂ / 1% H ₂ SO 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.

Once the reactions have been stopped with Na₂CO₃, 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.

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

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

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

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 culture volume in **mls**. (Not μl !) Use the volume of cells added to the reactions, not the final volume of the reactions themselves.

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.

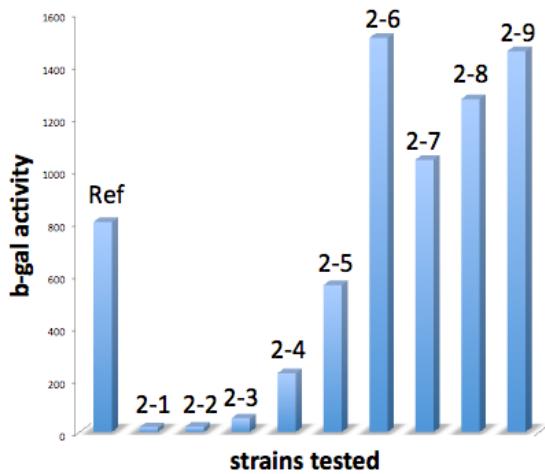
III. Results

- 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 Monkey Link

To help us improve the labs, you can send the students [here](#) where they can offer anonymous feedback. 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.