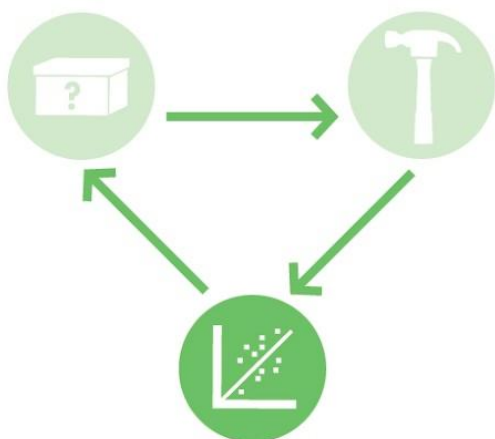


# BioBuilder

## Synthetic Biology for Students

### ▶ iTunes Device

This activity focuses on predictable biodesign and the composition of genetic “parts,” such as promoters and ribosome binding sites, to better understand how assembly influences the output of a genetic device.



# BioBuilder

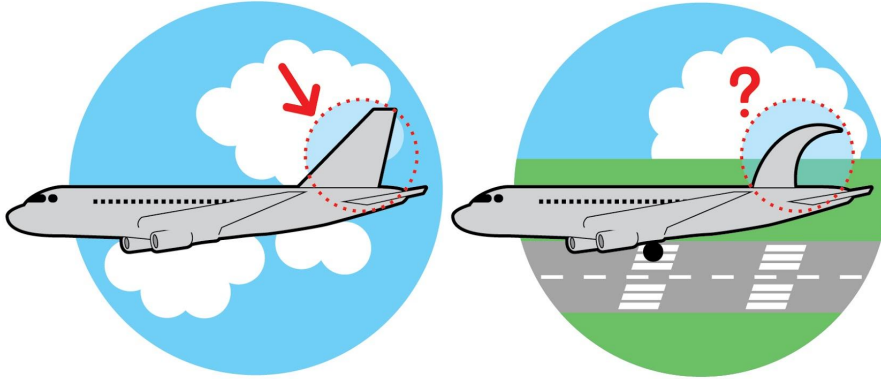
## iTunes Device

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# PREDICTABLE DESIGN

What would a company like Boeing or Airbus think if a new wing shape made the plane fly in unexpected ways? Designing and building in the face of such uncertainty would create huge expense and potentially put lives in danger.



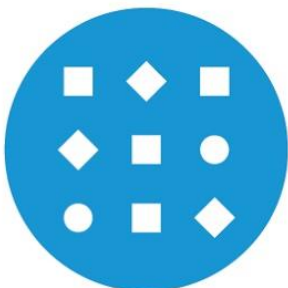
If an engineer modifies the standard tail of an airplane (left), then the novel design (right) must not introduce unintended behaviors that affect how the airplane can fly or land safely.

To predictably design new systems, engineers rely on modular components that can be functionally assembled in a variety of ways, customizing combinations according to an individual's needs. When the modular components are physically connected, the parts must function as expected. Significant differences between predicted and observed behavior are unacceptable.

At this point in the field of synthetic biology, biological engineers are still working toward such functional assembly of genetic parts. Researchers have characterized many cellular behaviors at the molecular level. In many cases it's possible to catalog the genetic elements necessary and sufficient to carry out a biological function. Yet, combining these genetic components in new ways often generates unexpected results.

To improve the functional assembly of biological parts, synthetic biologists have focused on the modularity, insulation, and measurement of genetic parts. These principles are more traditionally applied to the engineering of nonliving systems, but here they are tested in the context of an enzyme-producing genetic device. BioBuilder's iTune Device tests a variety of genetic circuits in cells, in order to compare their expected and their measured behaviors.

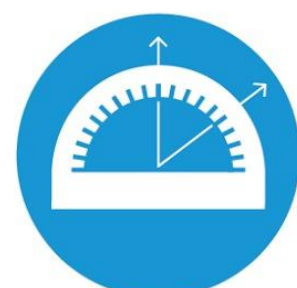
## Modularity



## Insulation

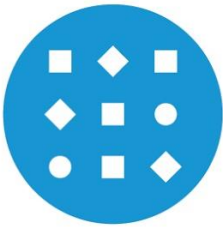


## Measurement

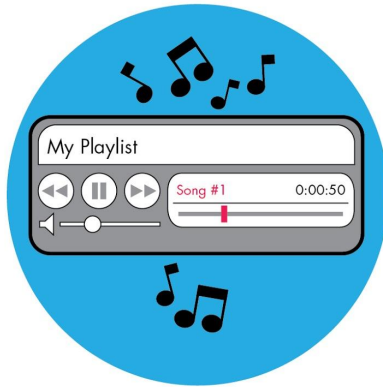


# Modularity, Insulation, Measurement

## Modularity



Modularity refers to the idea that engineers can design and generate systems by combining functional units, or “modules.” To understand the advantages gained from modularity, consider the way music distribution has changed. For much of the 20th century, the album was the standard unit for the music industry and its artists. Single songs were available but were significantly more costly, so even if people only liked a few of the songs on an album, they generally bought the album.



Once music was digitized, it became possible to download it. Suddenly, buying the physical album was no longer necessary, and individual songs from any digital album could easily be unbundled. The songs became independent modules that listeners could mix and match as desired and needed.

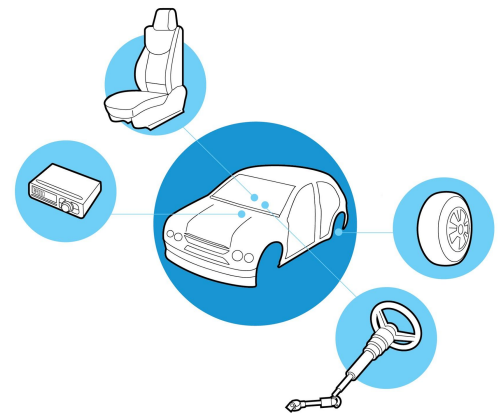


Modularity is sensible to apply to biology as well, because we can attribute discrete functions to particular snippets of DNA and protein domains. This notion of “genetic parts” is fundamental to synthetic biology.

## Insulation



Insulation keeps modules from interacting from with one another in undesirable ways. This is a familiar feature of many engineered objects. For example, the operation of a car’s stereo doesn’t affect the operation of the driver’s steering wheel. If it did, then twisting the knob on the radio might also turn the steering wheel -- presenting a real challenge for safe operation of the car.



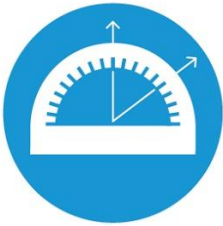
Unlike a car, the cell is a fluid environment. The molecules, proteins, and cellular structures are constantly mixing. How is it possible to insulate their behaviors when they encounter new partners and neighbors all the time?



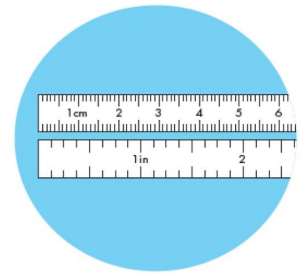
One of the major challenges in synthetic biology is to rationally design living systems with component parts that do not interfere with one another or with the basic cellular machinery needed to maintain growth and life.

# Modularity, Insulation, Measurement (con't)

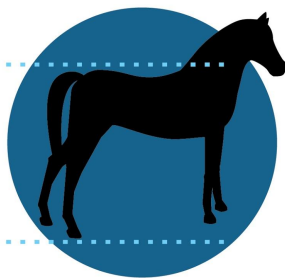
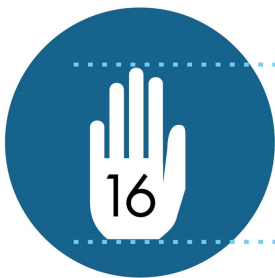
## Measurement



Whether it's the number of cards in a deck, the horsepower in a car engine or the score in a football game, measurements tell us about the state of the items, and their behaviors, relationships, or characteristics. Measurements allow us to compare items with one another, using an agreed upon unit for measuring them. Units might be taken for granted in something like sports, where teams get a standard number of points for each basket or goal. But some measurements are less clear and in some cases, like the English vs the Metric system, the units are not even agreed upon.



### English v. metric



You might think it would be easy to measure the height of a horse, for example. From hoof to wither (~shoulder) could be 67 inches or maybe 1.7 meters. In fact, such a horse would be 16.3 hands high, since King Henry VIII standardized a hand (his hand) to be 4 inches and the units after the decimal to reflect additional inches.

### The power of measurements

Scientists use measurement as a powerful tool for discovery. Mendel, for instance, uncovered the rules for inheritance by counting offspring with particular traits and looking for patterns. Engineers use measurement not only to describe systems but also to control, assemble, and improve the objects being measured. Some engineering measurements are described below.

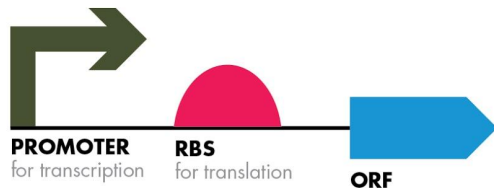
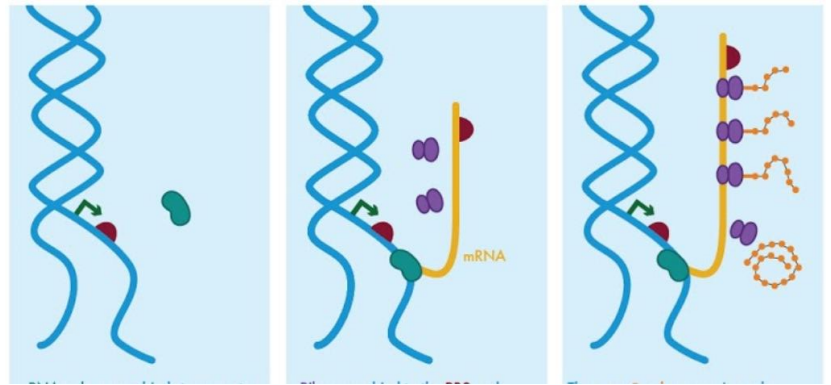
Measurement	Description	Utility
<b>Static performance</b>	Maps a range of controlled inputs to a part's measurable final output(s)	Helpful for ensuring one part's output will be sufficient to trigger the next part in a circuit
<b>Dynamic performance</b>	A part's output over time in response to a change in the input signal	Shows how a system will behave upon initial stimulation, which may differ from stabilized long-term behavior
<b>Input compatibility</b>	How a part responds to various inputs	Illustrates the part's flexibility for composition with various upstream parts/inputs
<b>Reliability</b>	Measured as Mean Time to Failure (MTF)	Used to determine how long the system can be expected to behave as originally specified
<b>Consumption of materials or resources</b>	Determines choice of power supply or resource pool	Affects chassis decisions among other things



By measuring the actual performance of synthetic living systems and comparing the measurements to what was predicted, synthetic biologists can assess their designs and move closer to correctly anticipating the success or failure of future designs.

# Introduction to Gene Regulation

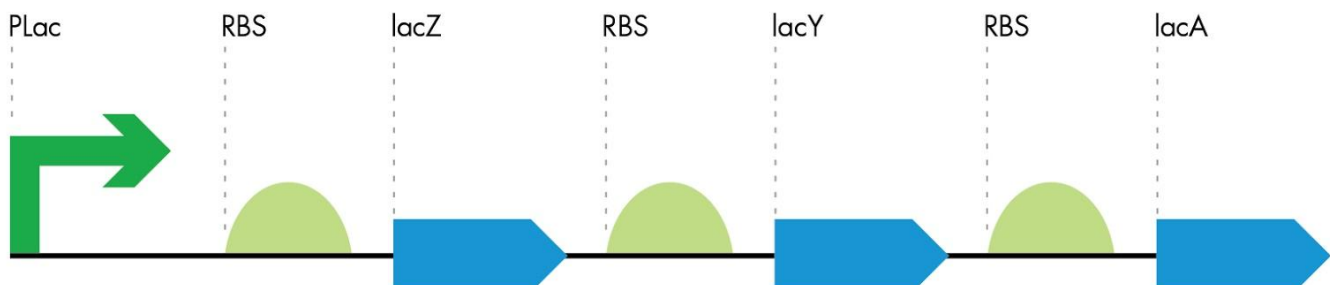
The mantra “DNA makes RNA makes protein” is shorthand for the knowledge that RNA sequences are transcribed from DNA sequences, and that those RNA sequences can be translated into the proteins that carry out many of the key jobs in a cell. Transcription and translation have been extensively studied so many of the core components that control these processes are known.



For example a promoter is the term for a DNA sequence where RNA polymerase binds and initiates transcription. Similarly, the sequence where translation initiates in bacteria is a site known as the ribosome binding site (RBS), defined as the DNA sequence that allows ribosomes to begin protein synthesis from the RNA copy. Additionally, an open reading frame, abbreviated “ORF,” represents a DNA sequence that encodes a protein. It is often illustrated as an arrow or a box. The direction of the arrows indicate the direction on the DNA strands in which the promoter or ORF are read.

## The lac operon

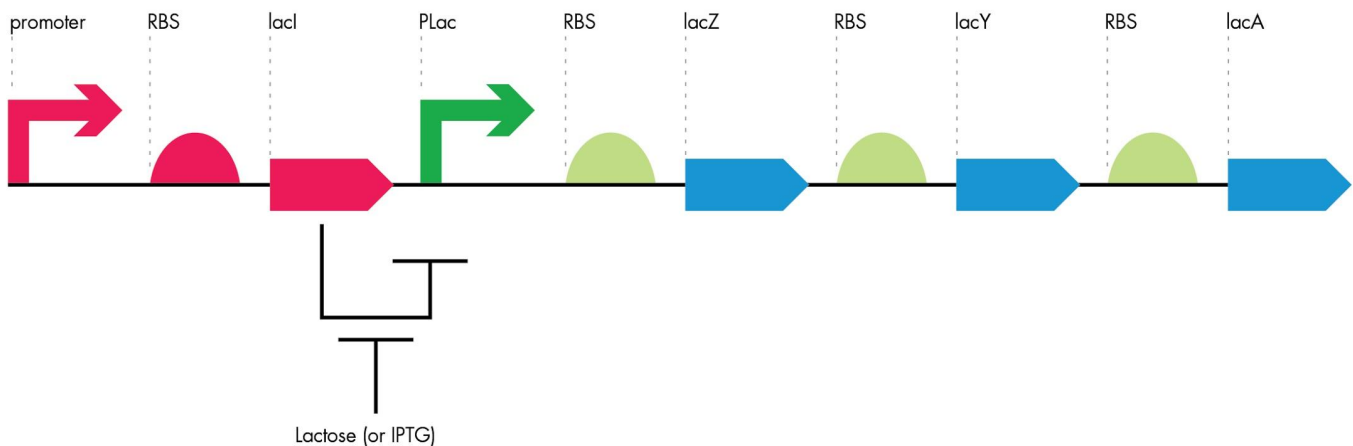
In the 1960s, Dr. Francois Jacob and Dr. Jacques Monod identified foundational principles of gene regulation through their studies of lactose transport and metabolism in bacteria. The genes for lactose metabolism are clustered in the lac operon. The lac operon consists of a single promoter (pLac, green arrow) controlling three downstream RBS–ORF pairs (green semicircles and blue arrows, respectively). A single mRNA is transcribed from the lac operon’s promoter, giving rise to the multiple protein products needed for lactose metabolism and transport. Translation of each product can occur from the single mRNA thanks to the RBSs that are associated with each ORF.



# Introduction to Gene Regulation (con't)

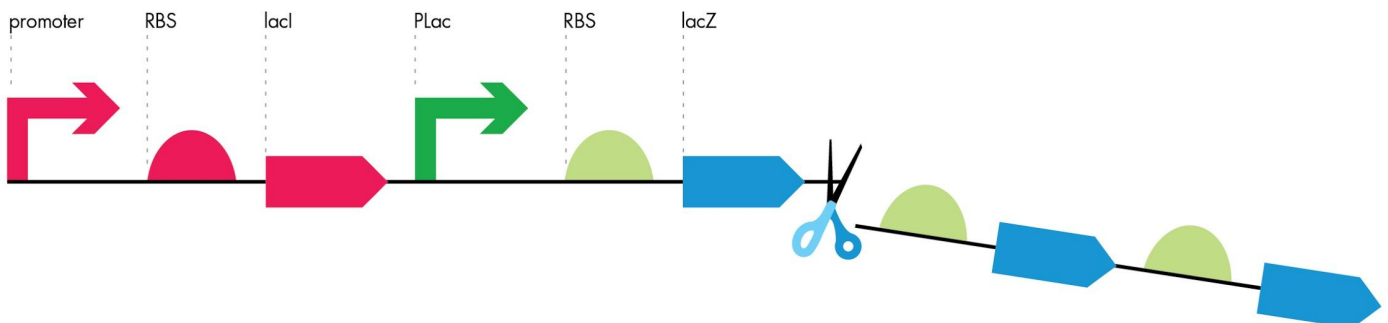
Bacteria conserve energy by transcribing the lac operon genes only when glucose is absent. When glucose is present, a transcriptional repressor protein encoded by the LacI gene turns off transcription of the lac operon. When lactose is present and glucose is absent, the lac repressor protein switches behaviors and allows for transcription of the operon so lactose can be transported into the cell and metabolized.

**In this lab, the cells will be grown in the presence of IPTG, which will artificially remove the lac repressor protein from the Plac promoter. In this way we can be sure that measurements reflect the fully de-repressed promoter.**



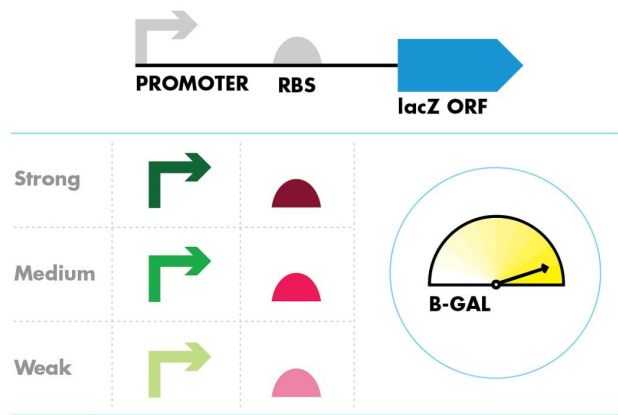
The key protein for lactose metabolism is an enzyme called  $\beta$ -galactosidase, often abbreviated  $\beta$ -gal, and it is encoded by the ORF called lacZ. The  $\beta$ -gal enzyme cleaves lactose into glucose and galactose, which can be used by the cell to power its other functions. Researchers found that  $\beta$ -gal reacts with a variety of molecules similar to lactose, including synthetic analogs such as ONPG, which you will use in the iTunes Device lab.

**In this lab, you will react the cells with ONPG, which will turn the solution yellow according to how much  $\beta$ -gal the cells are making. More yellow  $\rightarrow$  more  $\beta$ -gal!**



While the other ORFs in the lac operon, lacY and lacA, are important for lactose transport and metabolism in wild-type bacteria, they are dispensable for BioBuilder's iTunes Device experiment. Your measurements of lacZ expression in this experiment will be dependent only on the Plac promoter and ribosome binding site.

# BIOBUILDER'S iTUNE DEVICE ACTIVITY



BioBuilder's *iTune Device* activity emphasizes the "test" phase of the design-build-test cycle. Standard biological parts were combined to build several variants of an enzyme-generating genetic circuit. The circuits have small differences in their DNA sequences, which are expected to change the amount of enzyme the cells produce.

What predictions can be made about the output for the genetic circuits?

It seems reasonable that a "strong" promoter would give rise to more enzyme activity than a "weak" promoter. But can we predict just how much more? Examining the DNA sequences of the parts gives some clues to the activity of the parts because the most commonly found sequence for each part is called the "consensus sequence." Changes from the consensus will affect the efficiency of transcription for the promoter parts and of translation for the RBS parts -- though it's hard to know by how much.

Even more difficult to anticipate is how modular and how insulated the parts are from one another. Perhaps a medium promoter and a strong RBS will give greater activity than a strong promoter and a medium RBS. Luckily, the output of each genetic circuit can be measured with an enzyme assay, allowing predicted and actual behaviors to be compared quantitatively.

## MEASURING ENZYME ACTIVITY

The assay for  $\beta$ -gal activity is carried out on cells that have been grown in LB with Ampicillin and IPTG. The Ampicillin selects for the plasmids carrying the genetic circuits. The IPTG insures that the lac repressor protein is not binding to the promoter region of the circuits.

Once the cells are grown, their density is measured using a spectrophotometer set to 600 nm. The spectrophotometer measures the amount of light scattered by the cells. More cells lead to more scattering and a higher absorbance. If no spectrophotometer is available, then it is possible to use MacFarland standards, made with barium chloride and sulfuric acid.

The cells are then lysed with detergent to release the  $\beta$ -gal enzyme from the cell, reacted with ONPG for a measured amount of time, and then the reactions are stopped by changing the pH of the solutions which inactivates the enzyme. The amount of yellow color in the reactions is measured with the spectrophotometer set to 420 nm. If no spectrophotometer is available, then it is possible to use yellow paint samples, as described on the BioBuilder website.

## Reference measurements

To compare data collected by different laboratory groups, you will use a "reference" promoter:RBS:lacZ sequence. This reference is known to generate some intermediate amount of enzyme, so you can use it to calibrate all the other measurements you make. The reaction run without any cells can serve as one negative control as well as the blank for the spectrophotometer.



# PRE-LAB QUESTIONS

Briefly explain the goal of synthetic biology.

B-galactosidase ( $\beta$ -gal) is an enzyme that normally cleaves the disaccharide, lactose, into the monosaccharides, glucose and galactose. For the enzymatic measurements we make in this lab, we use ONPG rather than lactose. Why?

In this lab, we are varying the amount of enzyme that a cell can produce by varying the strength of two gene expression elements. Name them, the proteins they bind and processes they control.

Part	Binds	Process controlled

Why can't we just assume that the strongest promoter and the strongest ribosome binding site, when combined, will generate the most  $\beta$ -galactosidase?

To a synthetic biologist, what is a part?

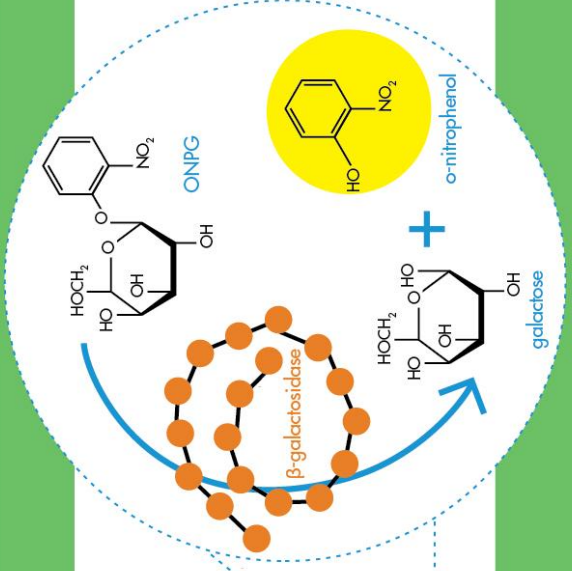
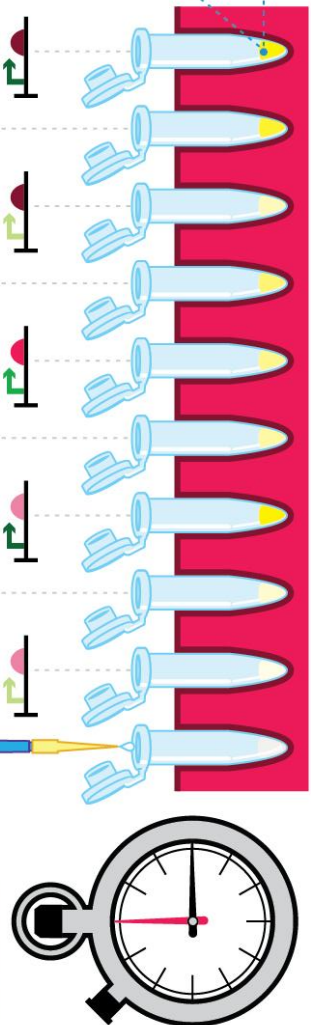
The reactions you will perform must be started at precisely timed intervals. Why do you not start all the reactions at the same time?

The reactions include a step when you add detergent. Why is it added?

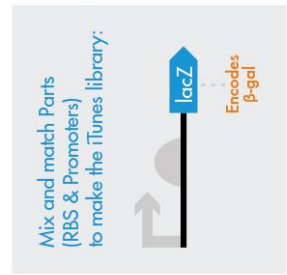
The reactions are stopped by the addition of sodium bicarbonate. Why does this work?

# MAKING THE INVISIBLE VISIBLE

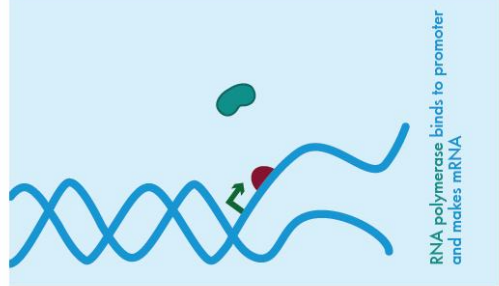
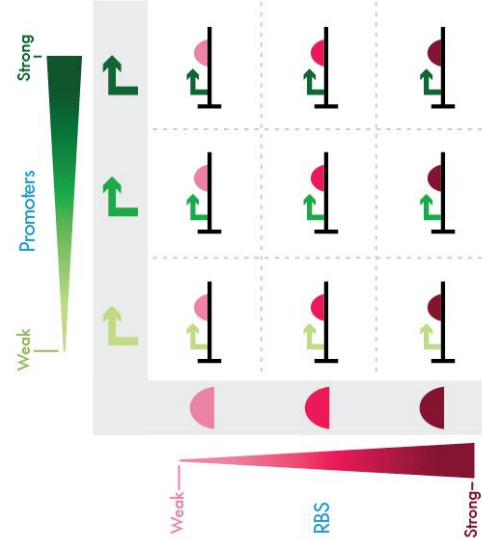
10-15 second intervals:  
stagger start times to run many  
samples in parallel!



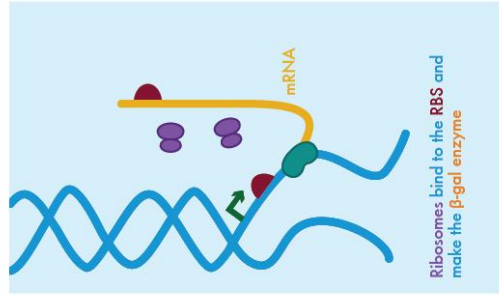
# UNDER THE HOOD



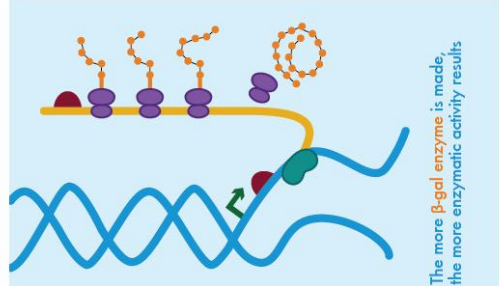
Mix and match Parts  
(RBS & Promoters)  
to make the iTunes library:



RNA polymerase binds to promoter  
and makes mRNA



Ribosomes bind to the RBS and  
make the beta-gal enzyme



The more beta-gal enzyme is made,  
the more enzymatic activity results



## IN ADVANCE

Grow liquid overnight cultures of the 10 strains to be tested\*\* Don't forget to add ampicillin and IPTG to growth media!

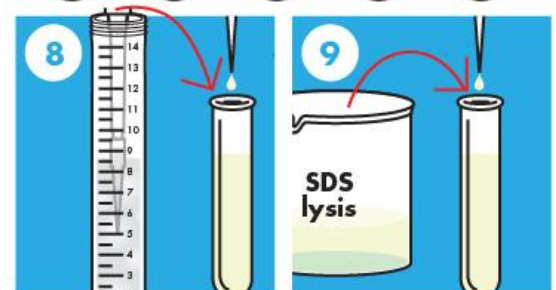
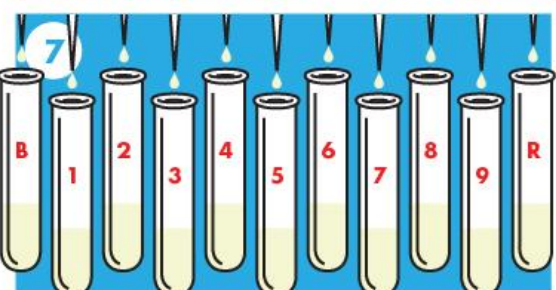
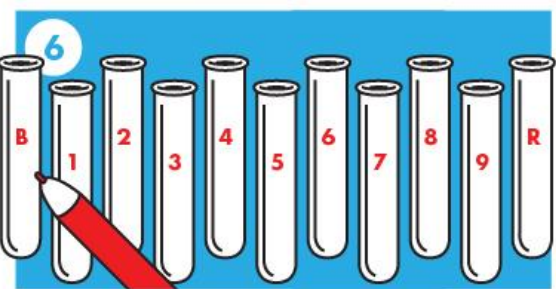
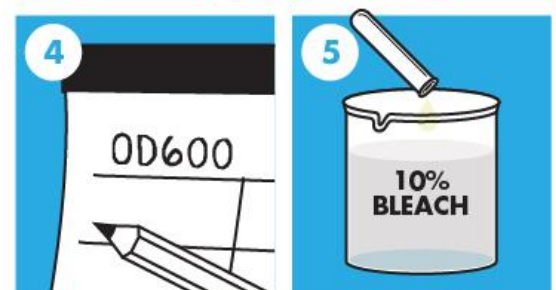
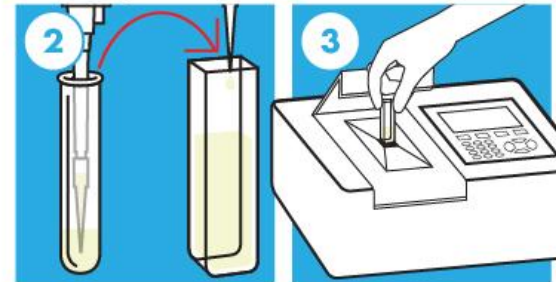
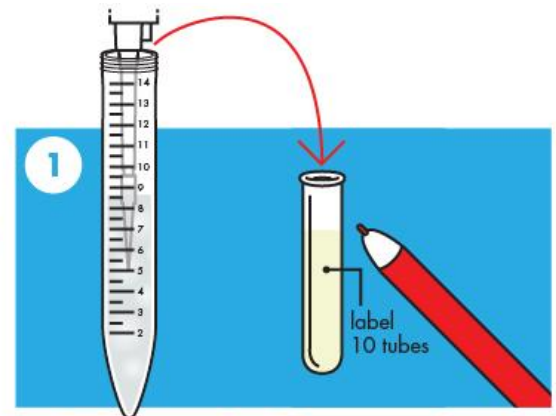
## DAY OF LAB

### Cell Density Measurements:

1. Label 10 glass tubes "1" through "9" and "R" for reference. Make a 1:10 dilution of each overnight culture by mixing 300  $\mu$ l of cells with 2.7 mL of bicarbonate buffer.
2. Transfer each sample to a cuvette, filling the cuvettes about three-quarters full.
3. Read and record the absorbance value for each sample. Start by zeroing the spectrophotometer set at **600** nm using bicarbonate buffer or water.
4. Record the value x 10 in your data table under the **OD600** heading.
5. Discard all dilutions in 10% bleach.

### Enzyme Reactions:

6. Label 11 glass tubes "1" through "9" as well as "B" for blank and "R" for the reference.
7. Add 1.0 mL bicarbonate buffer to each tube.
8. Transfer 100  $\mu$ l of cells from the UNDILUTED overnight cultures to the appropriate tube. Transfer 100  $\mu$ l of bicarbonate to the blank.
9. Add 100  $\mu$ l of SDS lysis solution to each tube, including the blank.

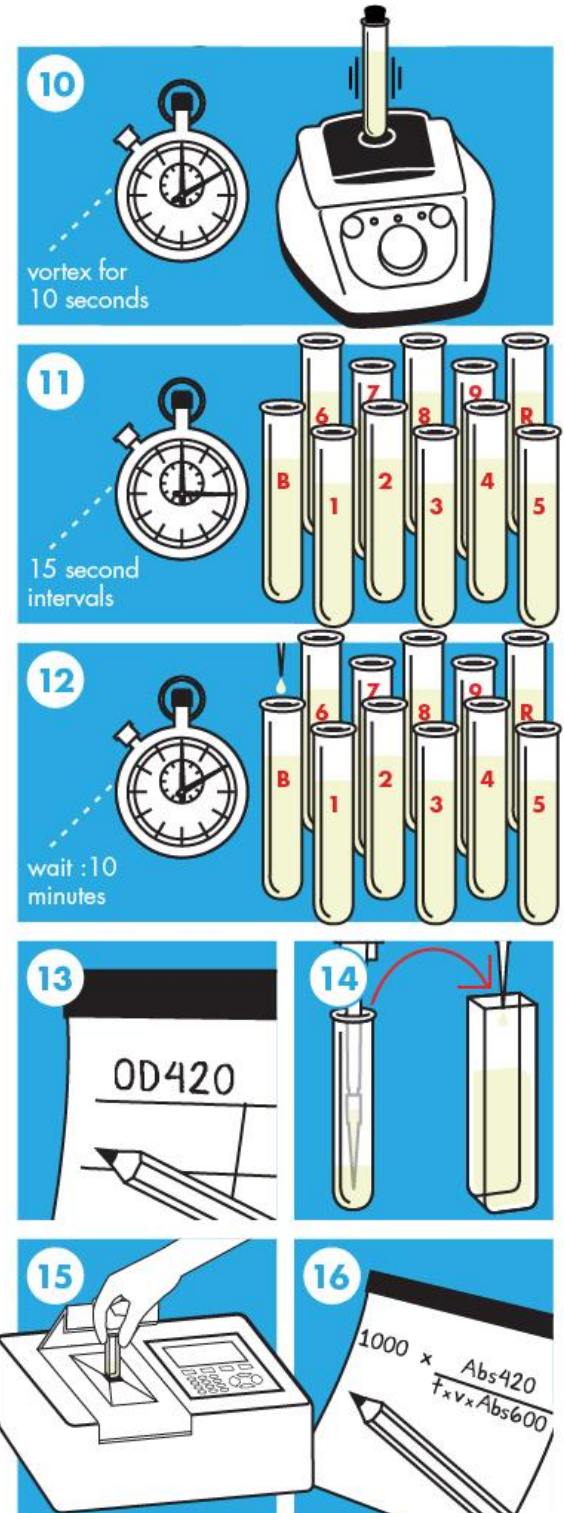


10. Vortex each tube, including the blank, for EXACTLY 10 seconds.
11. Start the timer when you add 100  $\mu$ l of ONPG solution to the first tube. Then at 15 second intervals, add 100  $\mu$ l of ONPG solution to each tube, including the blank.
12. When 10 minutes have elapsed from the time you started the reaction in the first tube, you should stop that reaction by adding 1 mL of the soda ash solution. Add the quench solution at 15 second intervals to all the tubes in the order that you started the reactions, including the blank.
13. Reactions can be refrigerated for later analysis or absorbance data can be collected immediately.
14. Transfer each sample to a cuvette, filling the cuvettes about three-quarters full. (OPTIONAL: Spinning down samples in a centrifuge to pellet cell debris will increase the reproducibility of your absorbance measurements.)
15. Read and record the absorbance value for each sample. Start by zero-ing the spectrophotometer set at **420** nm using the blank reaction.
16. Calculate Miller units for all of your sample data using the following formula:

$$\beta\text{-gal production in Miller units} = 1000 \times \frac{\text{Abs } 420}{t \times v \times \text{Abs } 600}$$

*t* is the time in minutes

*v* is the volume in mLs



\*\* VIDEO OF PROCEDURE AVAILABLE ONLINE

# POST-LAB QUESTIONS

What is expected for the enzymatic reactions in each of the following cases:

- a. We forgot to add detergent
- b. We added twice as many cells to one of the tubes but didn't include that change in the calculation of units
- c. We added twice as much ONPG to one of the tubes
- d. We measured these genetic devices in a strain with a wild type lac operon in the genome

What were some potential problems that may have affected the outcome of this experiment? List at least 2 problems.

If the reference strain behaved identically for each group, would it be sensible to compare the experimental strains that each group tested? What about if the reference strain didn't behave identically?

Why might a collection of well-characterized genetic parts that behave in a predictable way be a useful resource for synthetic biologists?

What is one thing you learned from this lab? What is the one thing that you are still confused about? Did you like the lab?



**Ideally, the interpretation of these results should encourage more experimentation, provide ideas for improved designs, and build excitement to explore and do more.**

STUDENT  
MANUAL

**BioBuilder**

Do you have an idea for improving and extending the units? Please email us: [info@biobuilder.org](mailto:info@biobuilder.org)