Supplemental Material

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Characteristics of Synthetic Biology

Characteristics of synthetic biology are abstraction, mathematical modeling, modularity, and standards (*16*).

1. *Abstraction* is a way of thinking about parts that make up devices that can in turn be combined to make systems. Abstraction means users do not need to know the function of every nucleotide in their DNA devices. It is acceptable to understand a DNA part by its function and not its mechanism of action. For example, synthetic biologists can build DNA devices with well characterized promoters even if they do not understand the molecular interactions regulating transcription.
2. *Mathematical modeling* allows investigators to quickly analyze many more constructs than could be built and tested in the lab. Once a device is tested in the wet lab, those data are channeled back into the mathematical model to be refined for the next iteration.
3. *Modularity* means that a part or device functions independently. A power cord or a cup holder can be thought of as modular since its function is independent of any particular application and is easily added on to other devices. Promoters or ribosome binding sites are examples of modular DNA parts.
4. *Standardization* has a dual meaning in synthetic biology. One meaning is "standards of performance" that researchers can use to evaluate modules. Saying a promoter is 3.5 fold stronger than a standardized comparison promoter is much more informative than saying a promoter is medium strong. "Standards of assembly" refers to agreed-upon methods for connecting DNA parts and devices so that different labs can consistently assemble their modular parts. With standardization of assembly methods, synthetic biology is amenable to large scale teaching labs.

Methods

Synthetic Promoter Design and Synthesis

Thirty Introductory Biology students at Davidson College started their lab by conducting literature research on promoters that are regulated in interesting ways. For example, they found promoters that initiate transcription in bacteria in response to a sugar, an amino acid, a salt, and tetracycline. One hundred and three Genetics students at Missouri Western studied the control elements found in the Ptac promoter (*22*) and generated their own specific mutational hypotheses. In both cases, the students were responsible for converting the DNA sequence for the promoter they want to study into oligonucleotides that can be ordered from a DNA synthesis company and used in Golden Gate Assembly (GGA). The top strand oligonucleotide starts with CGAC sticky end and continues with a given promoter sequence, resulting in 5' CGACNNNNN…3', where the N's are the promoter sequence. The bottom strand begins CCGC and continues with the promoter sequence, resulting in 5' CCGCMMMMM…3', where the M's are the reverse complement of the promoter sequence. Production of both oligonucleotide sequences for promoters is automated by a convenient online tool called Oligator, developed by Dr. Laurie Heyer and her undergraduate students at Davidson College (*21*). Once our students generated their sequences, we placed an order for synthesis of the top and bottom strand oligonucleotides at Integrated DNA Technologies. Oligos that are less than 70 bp are delivered in about 3 business days. We ordered the oligos as "Lab Ready" which means that they arrive in solution with a concentration of 100 µM, instead of a dry pellet. If oligos arrive dry, they should be resuspended in water to 100 µM and stored frozen.

Annealing Oligonucleotides

The top and bottom strand oligos were annealed prior to GGA. A 20 µL annealing reaction was prepared using 16 µL of H_2O, 2 µL of 10X annealing buffer (1 M NaCl, 100 mM Tris-HCl pH 7.4), 1 µL top strand promoter oligonucleotide, and 1 µL bottom strand promoter oligonucleotide. The annealing reaction was boiled in 400 mL of water for 4 minutes and allowed to slowly cool for at least two hours (preferably overnight) in the same water bath. In preparation for GGA, the annealed oligonucleotides were diluted with water to the same concentration as the destination vector. This provides a 1:1 molar ratio of promoter insert to

pClone vector in the GGA reaction. For GGA, we use a vector concentration of 40 nM (60 ng/μL of pClone basic, 73 ng/μL of pClone Green, or 73 ng/μL of pClone Blue). We diluted the annealed oligonucleotides to 40 nM as well. The Lab Ready 100 μM oligonucleotides were diluted to 5 μM (5000 nM) in the annealing reaction. After annealing, we further diluted the annealed oligos 125-fold (5000 nM/40 nM) by adding 124 μL of H_2O to 1 μL of the cooled oligonucleotides.

Traditional Cloning Using BioBrick Assembly

The first widely used DNA assembly standard was the BioBrick method, developed by Tom Knight (*16*). BioBrick assembly was required of iGEM teams beginning in 2004 and is still a commonly used approach among teams (Campbell 2005). The Registry of Standard Biological Parts contains over 7,100 parts (*28*), and the vast majority of them use BioBrick assembly. BioBrick assembly uses traditional type II restriction enzymes XbaI and SpeI, which produce compatible sticky ends. For example, EcoRI binds to the sequence shown in the top of Figure 1A, and produces the 5' overhang sticky end of 5' AATT 3'. BioBrick parts carry EcoRI and XbaI sites upstream of the insert SpeI and PstI sites downstream. Two DNA parts are ligated together via their shared XbaI and SpeI compatible ends, producing a mixed site that does not contain binding site for either XbaI or SpeI. The newly joined parts retain a complete prefix and suffix, thus producing a new BioBrick part composed of two subparts. However, BioBrick parts cannot be disassembled once they are ligated together and the 6 base pair mixed site forms a scar between subparts that may prove problematic. BioBrick assembly works very well in the undergraduate research lab, where students develop proficiency in gel purification and ligation of DNA fragments, but this approach does not translate well into the course environment because of time constraints and limited student expertise.

Golden Gate Assembly

On the day before the student lab, the top and bottom strand oligonucleotides for each promoter were annealed using a simple process of boiling and cooling. The oligonucleotides were diluted further to facilitate efficient ligation into the pClone vectors. On the day when Golden Gate Assembly was to be performed, a master mix was prepared for the students that included BsaI, T4 DNA Ligase, and Ligase Buffer. Student research groups were given their annealed and

diluted oligonucleotides, the pClone vector, and sufficient master mix for two reactions. Each team assembled a negative control ligation with the vector but no annealed oligos and an experimental ligation with the vector and annealed oligos.

Students performed GGA reactions in a total volume of 10 µL containing 6 µL H_2O, 1 µL 10X T4 DNA Ligase Buffer (supplied by the company as 300mM Tris-HCl (pH 7.8 at 25°C), 100 mM $MgCl_2$, 100 mM DTT and 10 mM ATP), 1 µL of 40 nM pClone destination vector, 1 µL of 40 nM annealed promoter oligonucleotides, 0.5 µL HF (high fidelity; New England BioLabs) Bsa I (10 units), 0.5 µL T4 DNA Ligase (New England BioLabs; 50 Weiss units). For each lab group, we prepared a 8 µL master mix containing all of the components except the annealed oligonucleotides and the pClone destination plasmid before class and stored it in the refrigerator until needed later in the day. Students mixed the master mix, annealed oligos and pClone destination plasmid. They placed their GGA reactions in a thermal cycler set for 20 cycles of 1 minute at 37° C followed by 1 minute at 16°C. The 37°C temperature favors digestion by BsaI-HF while the 16°C favors ligation by T4 DNA ligase. GGA terminates with a 15 minute incubation at 37° C to cleave any remaining BsaI sites. Students also performed a negative control GGA in which the annealed oligos are replaced by water.

Transformation

Students transformed their completed GGA reactions (experimental and negative control) into Z-competent *E. coli* JM109 cells (Zymo Research). Students also performed a positive control transformation with J04450 plasmid DNA which contains a standard construct of a promoter, ribosomal binding site (RBS) and red fluorescent protein (RFP) reporter gene. According to the supplier's directions, students added competent cells to the three DNA solutions, placed all three on ice for 5 minutes, and plated each one directly onto a separate LB agar plate with 50 µg/ml ampicillin. Plates were incubated overnight at 37°C in an incubator. Instructors removed the plates and stored them for students to observe and photograph during their next laboratory meeting.

Phenotype Analysis

Students took photos of the plates to document the colors of their colonies. They transferred colonies with a sterile toothpick to LB + ampicillin media with added potential inducers as

appropriate for overnight growth. Depending on the promoter being studied, inducers included sugars, salts, amino acids, or tetracycline. Promoter function was tested in pClone Basic or pClone Green by quantifying the RFP output of clones with a Biotek Synergy fluorometer set for 585 nm excitation and 615 nm emission and absorption set at 590 nm. If schools already have a spectrophotometer that can measure cell density at 590 nm, a less expensive approach than buying a combination machine like Synergy is to buy one of the many fluorometers on the market.

Alternatively, students can obtain promoter function data by using the freely available software ImageJ (*20*) to analyze pClone colonies. The simple procedure begins with opening a picture of a pClone Basic or pClone Green transformation plate taken on a UV light box or a picture of pClone Blue transformation plate taken on a light box or dissecting microscope. Alternatively, clones from the transformation plate can be isolated with a sterile toothpick, grown overnight in LB broth with ampicillin, and spotted on plates for photography. The next step is to draw circles within the colonies or spots to be analyzed, identifying regions of interest (ROIs). The image is then split into red, green, and blue channels and a measurement is made by ImageJ of the intensity of color for each channel. A measure of RFP output can be obtained by dividing the red channel intensity by the green channel intensity; a blue chromoprotein output measure is obtained by dividing the blue channel intensity by the green channel intensity. The ratio of the RFP or blue chromoprotein expression measurements for two different promoters yields an expression ratio. The expression ratio shows the relative strengths of the two promoters compared. This procedure is illustrated in Supplemental Figure 2 using student-generated mutants of the Ptac promoter.

Promoter function in pClone Blue was determined qualitatively by visually comparing a dilution series of cultures using a standard promoter and the blue chromoprotein, or more quantitatively with a Spec20 set to measure absorbance at 600 nm and absorbance at 400 nm. The dilution series was prepared by mixing a positive control strain containing a known promoter cloned into pClone Blue with a negative control strain of bacteria lacking pClone Blue to keep the total number of cells nearly equal. Visual comparison of a student-built experimental promoter to this standard dilution series would provide a qualitative assessment of promoter strength. Supplemental Figure 3 shows the dilution series and quantitative measurements of pClone Blue

using a Spec20. Absorbance at 400 nm quantifies the reflectance of the blue pigment. As expected, absorbance decreases with increase in blue color of the cultures since more blue is reflected and not absorbed. Absorbance at 600 nm is a measure of cell density. The ratio of absorbance at 400 nm to absorbance at 600 nm decreases with increase in blue color of the cultures. Any spectrophotometer can be used to quantify the strength of student-generated promoters cloned into pClone Blue. The final values for RFP fluorescence and blue chromoprotein reflectance are given as a ratio with cell density values in the denominator.

Genotype Verification

Verification of successful assembly synthetic promoters into pClone plasmids can be done by PCR or DNA sequencing. PCR results in products that can be analyzed by agarose gel electrophoresis, as demonstrated by the results from the Davidson Introductory Biology (Supplemental Figure 1). PCR verification works if the size difference between the transcriptional terminator in pClone Basic and the synthetic promoter is enough to be reliably detected by gel electrophoresis.

Students verified successful assembly using PCR primers that bind just outside of the synthetic promoter insert site in pClone Basic. The forward primer is 5' GAATTCGCGGCCGCTTCTAG 3' and the reverse primer is 5' TTTGATAACATCTTCGGAGG 3'. For pClone Green and pClone Blue cloned plasmids, the forward primer is 5' CACTGACAGAAAATTTGTGC 3' and the reverse primer is 5' GCATGATTAAGATGTTTCAGTAC 3'. Amplification reactions included 10 µL of 2X Promega GoTaq Green Master Mix, 8 µL of H_2O, 1 µL of 10 µM forward primer , and 1 µL of 10 µM reverse primer. One bacterial colony was added to the reaction with a sterile yellow tip. The sterile tip was also used to start an overnight culture for subsequent storage and analysis. The thermal profile was one cycle of 5 minutes at 95ºC, 30 cycles of 30 seconds at 95ºC, 30 seconds at 43ºC, 20 seconds at 72ºC. Products were analyzed by 2% agarose gel electrophoresis in 0.5 X TBE. Unsuccessful assembly products are expected to be 251 bp for pClone Basic and successful assemblies will be 142 bp plus the size of the synthetic promoter cloned. For pClone Green and pClone Blue, the size of unsuccessful assembly products is 267 bp and the size of successful products is 196 bp plus the size of the synthetic promoter cloned. An example of pClone Basic PCR verification results is available in Supplemental Online Materials.

When students use pClone Green or pClone Blue, the phenotype is sufficient to indicate the genotype. The original purpose of pClone Green and Blue was to eliminate the need to screen by PCR to find clones where the transcriptional terminator had been removed. Colonies that are not green have lost their original left-facing promoters. Red colonies obviously have a new and functional promoter driving expression of RFP. However, white colonies using pClone Blue or Green could be the result of two possible ligation events. First, the original promoter might be excised but the plasmid recircularized without including a new promoter. Second, a new promoter could have been cloned but it is not functional as designed or requires an inducer not added to the growth media. If the student-designed promoter is the same size as the existing promoter, it would be helpful to have PCR primers that distinguish between the old and the new promoters. We have used a forward PCR primer that binds to the original promoter so that white clones that produce a band of the expected size indicate clones that should not be tested further since they lack the student-designed promoter. Using PCR to discriminate between the three types of white colonies will prevent students from measuring the wrong constructs.

The experiments presented in Figures 2B, 3B and 4B (center) used promoter mutations of `TTWACA` for the -35 region, so the identity of W had to be determined by DNA sequencing. A close examination of Figure 3B reveals green fluorescent, red fluorescent and uncolored colonies. DNA sequencing results showed that in the red fluorescent colonies, the identity of W is thymine. The identity of W in the uncolored colonies was adenine. This specific example demonstrates the ease with which students can conduct interesting and sophisticated promoter analyses.

Reagents and Suppliers

The following is a list of the reagents and suppliers that we used for the pClone system: Z-competent *E. coli* JM109 cells (Zymo Research Z-competent cells, #T3005); BsaI-HF (New England BioLabs, #R3535L); T4 DNA Ligase (Promega, #M1804); and 2X GoTaq Green Master Mix (Promega, #M7123). Oligonucleotides were purchased from Integrated DNA Technologies (http://www.idtdna.com/) as "lab ready" oligos at 100 µM concentration.

Online Resources

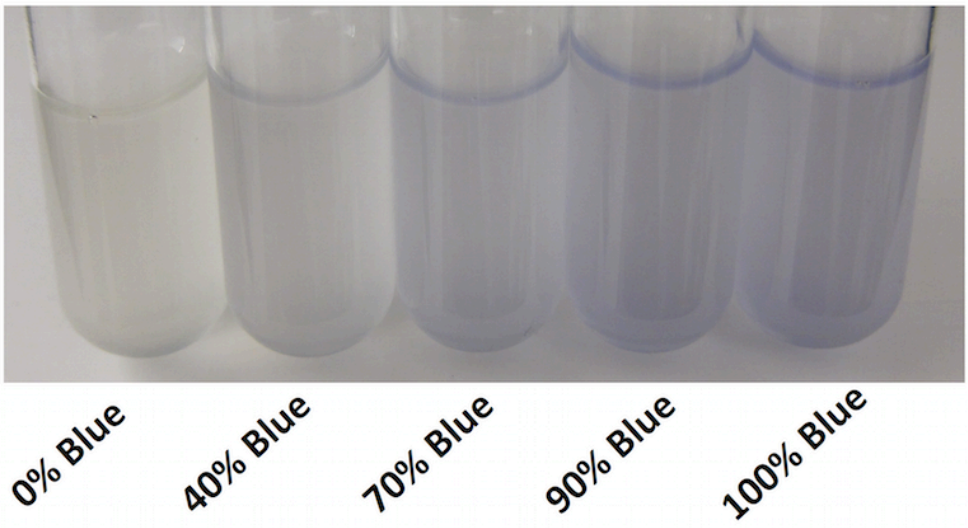
The following is a list of online resources in support of the pClone system. Annotation and sequences for the destination vectors can be found in the Registry of Standard Biological Parts (*28*). Plasmid pClone Basic is part number J100091 (http://partsregistry.org/Part:BBa_J100091); pClone Green is part number J119137 (http://parts.igem.org/Part:BBa_J119137); pClone Blue is part number J119313 (http://parts.igem.org/Part:BBa_J119313). Online protocols for all the experimental steps are freely available online (http://gcat.davidson.edu/GcatWiki/index.php/Davidson_Protocols#Bio113_Lab_Protocols).

Registry of Functional Promoters

We have established an online database for student promoter results called the Registry of Functional Promoters database (http://gcat.davidson.edu/RFP/). Faculty who want to use RFP database should sign up as faculty members and direct their students to self-register as students under their teacher's name and home institution. Faculty can edit any of their student's submitted information but students can only edit their own submission.

Once users are registered to submit their research findings, they will be prompted to submit information that allows others to compare results. For example, students will be asked to provide a part number from the Registry of Standard Biological Parts (http://parts.igem.org/Main_Page) or RFP database will assign a GCAT-named part number for them. Students will also supply information about the literature source of their promoters, growth conditions for their experiments and information about their comparison promoter. Ideally, everyone would use the same comparison construct of J04450 (http://parts.igem.org/Part:BBa_J04450?title=Part:BBa_J04450) in *E. coli* strain JM109 grown in LB + ampicillin media. Using a common standard construct facilitates easy comparison of new promoters cloned into any of the three pClone plasmids.

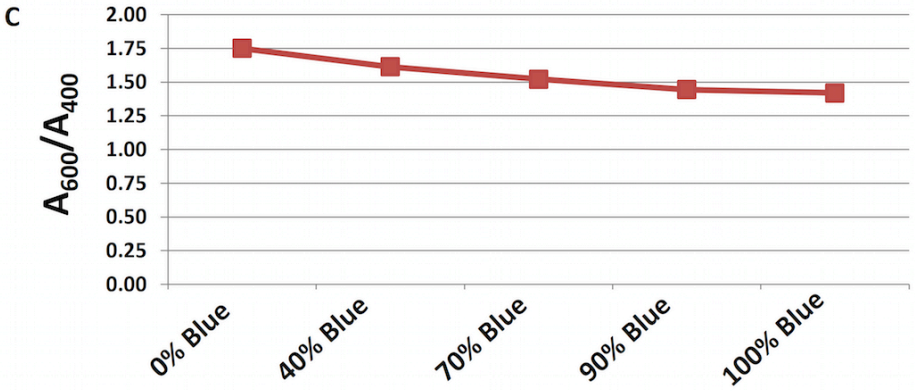
A



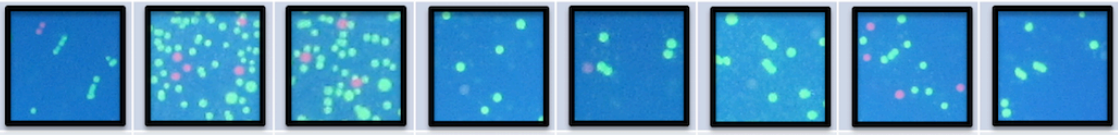
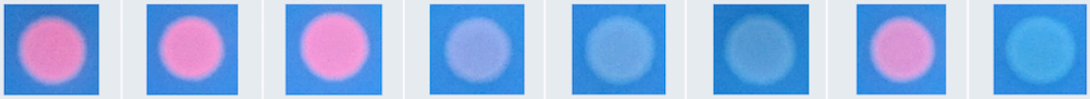
B

	0% Blue	40% Blue	70% Blue	90% Blue	100% Blue
Abs 400 nm	0.909 +/- .016	0.978 +/- .010	1.021 +/- .012	1.029 +/- .013	1.022 +/- .010
Abs 600 nm	0.519 +/- .010	0.606 +/- .010	0.671 +/- .014	0.712 +/- .010	0.720 +/- .021
A_{400}/A_{600}	1.750	1.614	1.521	1.445	1.420

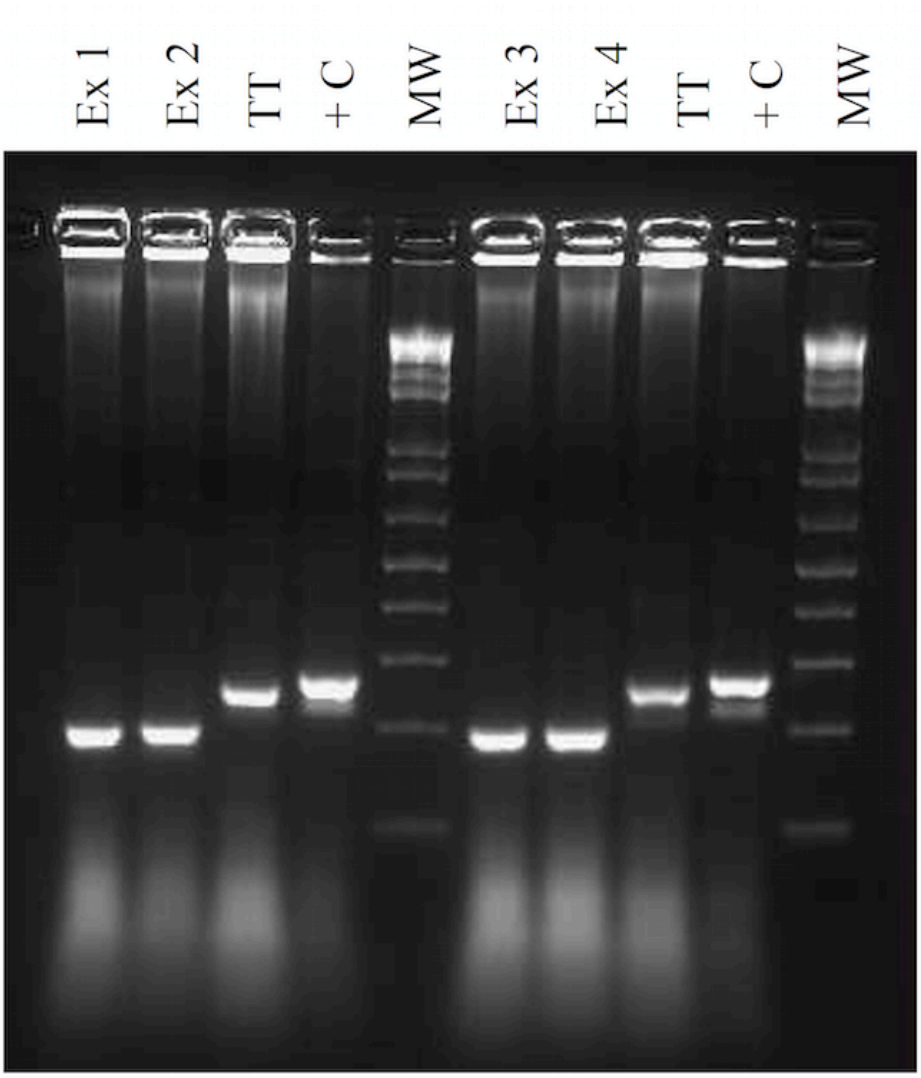
C



Supplemental Figure 1. Quantifying pClone Blue transcription. A. Tubes contain cultures of *E. coli* of the indicated dilutions with increasing levels of AmilCP Blue to illustrate a standard curve for qualitatively measuring promoter activity with pClone Blue. B. Absorbance values for the cultures were obtained with a Spec20. Absorbance at 400 nm is a measure of AmilCP Blue reflectance and absorbance at 600 nm measures cell density. C. The ratio of the absorbance value at 400 nm to absorbance at 600 nm is plotted for the five standard cultures.

Mutant	J119319	J119320	J119321	J119322	J119323	J119324	J119325	J119326
pClone Green plate								
Isolated clones								
Expression Ratio	4.09	3.94	3.84	2.04	1.54	1.34	3.52	1.00

Supplemental Figure 2. Quantifying pClone Green transcription. Students designed experiments to mutate the Ptac promoter and generated the plates shown. They isolated clones and entered them into the Registry of Biological Parts and the Registry of Functional Promoters. The pictures of isolated clones were analyzed with the open source software ImageJ to quantify RFP production. The expression ratios were generated by dividing each of the RFP measurement ratios by the RFP measurement ratio for the clone with the weakest promoter, J119326. This low tech approach uses free software so students can generate quantitative data to characterize the relative strength of their cloned promoters.



Supplemental Figure 3. PCR verification of plasmid genotype. PCR primers flanking the cloning site in pClone Basic were added to student colonies (Ex1, Ex2, Ex3 and Ex4). The negative control was the original pClone Basic plasmid that still contained the transcriptional terminator (TT). The positive control (+C) was part number J04450 which contained the known promoter. During the lab period, PCR products were separated on a 2% agarose gel in 0.5X TBE.

Using Synthetic Biology to Study Promoter Mutations

Assessment Quiz (correct answers provided upon request)

LO1) Which portion of a gene has the greatest effect on the timing and amount of protein produced in a cell?

a. introns

b. exons

c. promoter

d. codon

e. poly-A tail

f. they are all the same

g. I don't know for sure.

LO2 intro) Draw a genetic circuit diagram showing a gene with reduced transcription due to the presence of a repressor.

LO2 genetics) Draw a diagram of a bacterial promoter showing the -35 and -10 elements and the transcription start site.

LO3 intro) Draw a genetic circuit diagram showing a gene with increased transcription due to the presence of an activator.

LO3 genetics) What is the effect of mutation of a promoter on gene expression?

a. increase in rate of transcription

b. decrease in rate of transcription

c. no effect on rate of transcription

d. could be any of the above

e. change in the amino acid sequence of the protein

f. I don't know for sure.

LO4 intro) Use the letters from the list below to order the steps required to identify a promoter from literature, clone it and test its function. You can use steps more than once and some steps may not be used at all.

First step Last step

___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___ ___

List of possible steps to choose from:

a. add restriction enzyme to purified promoter

b. boil and cool oligos to anneal them

c. design oligos that will self-assemble into the desired promoters with appropriate sticky ends

d. add restriction enzyme and ligase to the DNA of interest

e. search PubMed for papers describing promoters

f. add ligase to *E. coli* genome to clone the promoter

g. transform bacteria with plasmid DNA containing promoter upstream of RBS and RFP

h. grow bacteria containing the cloned promoter in appropriate conditions

i. quantify fluorescence from bacterial cells

j. grow control bacteria containing similar DNA construct but with a known promoter

k. purify receiving plasmid fragment from a gel

l. transform bacteria with annealed oligos

m. mix receiving plasmid with annealed oligos

n. present promoter sequence and fluorescence results to class

LO4 genetics) not assessed by this instrument

LO5) Which statement best describes the process of transforming *E. coli* with a plasmid?

a. Put DNA in small syringe and inject host E. coli cells.

b. Load DNA in a gel and turn on the electricity to transform cells.

c. Grow bacteria and perform a miniprep on the cells.

d. Incubate cells with DNA for 5 minutes and put on petri dish.

e. I don't know for sure.

LO6) Which of the following procedures is used to verify that a new promoter has been cloned into the receiving plasmid?

a. Isolate bacterial genomic DNA and analyze by gel electrophoresis

b. Conduct PCR using the new promoter oligos

c. Perform a restriction digest and verify the correct fragment sizes by gel electrophoresis

d. Sequence the bacterial genome

e. Count the number of colonies on the transformation plate

f. I don't know for sure.

LO7) Which of these is the best experimental design to measure the strength of a promoter?

a. Put the promoter in *E. coli* and quantify the phenotype of the transformed cells.

b. Clone the promoter upstream of RBS+coding DNA and PCR the product.

c. Put the promoter with functional reporter and compare output to a known promoter.

d. Put the promoter with functional reporter and quantify the amount of reporter.

e. Use oligos to assemble a promoter and then golden gate assembly to clone the promoter.

f. I don't know for sure.

LO8) What distinguishes type IIs restriction enzymes from type II restriction enzymes.

a. type IIs enzymes don't have a specific recognition sequence.

b. type IIs enzymes cut within their recognition sequences.

c. type IIs enzymes cut once outside their recognition sequences.

d. type IIs enzymes cut on both sides of their recognition sequences.

e. type IIs enzymes recognize a palindromic DNA sequence.

f. I don't know for sure.

LO9) Which of the following choices best describes how Golden Gate Assembly worked when you tested your new promoters?

a. Use type IIs restriction enzymes to remove the terminator and use ligase to insert the new promoter into the receiving plasmid.

b. Use PCR to produce a new promoter using oligos that self-assemble and then clone these into the receiving plasmid.

c. Combine oligos and PCR product with Bsa I to ligate the new promoter into a receiving plasmid upstream of the terminator.

d. Use PCR and Bsa I with a type IIs ligation enzyme to combine the promoter and the reporter into the receiving plasmid.

e. Isolate *E. coli* genomic DNA, mix the DNA with a type IIs restriction enzyme so you can clone the promoter into the receiving plasmid.