Engineering Scalable Combinational Logic in Escherichia coli Using Zinc Finger Proteins

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Abstract

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Available to synthetic biologists are a wide range of genetic devices. Many of these devices are able to either sense or alter local conditions. The ability to sense a multitude of inputs combined with diverse outputs could enable engineered organisms that interact with their environment in new and complex ways. Currently the complexity of such systems has been limited by our ability to integrate several inputs into a desired output. Simple combinational logic functions, containing 1 to 3 logic gates, have been constructed in *Escherichia coli*, but more complex logic networks are needed to fully exploit the opportunities presented by these sensors and actuators. Use of genetic logic gates is constrained by the specific molecular interactions that are used to implement each gate. These interactions involve diffusible molecules that can move within the cytoplasm of the cell and therefore are not spatially separated from other gates. To make larger logic blocks, sets of gates that use unique molecular interactions with minimal crosstalk are required.

Zinc finger proteins (ZFPs) can be used to predictably create a large number of unique protein-DNA interactions. These proteins can then be used to build transcriptional activators or repressors in *E. coli*, but these methods are not well defined. Attempts at using ZFPs to make one-hybrid transcriptional activators have failed to give a fold activation of higher than 2. ZFP repressors based on steric hindrance of RNA polymerase performed better with fold repressions values of up to 300. The positional dependence of the ZFP operator site within the promoter was investigated, and both position and dissociation constant were found to play important roles in determining the level of repression.

ZFP based repressors without cooperativity cannot be used to create logic gates. A new inverter topology using both ZFP based repressors and sRNA was designed. This topology uses a reference promoter to set the switching threshold of the gate. There are no cooperative interactions in this topology, but the maximum slope of the transfer function is similar to a Hill-equation with a coefficient of 10. The high slope and excellent transfer function of these gates make them robust to many types of parameter variation and noise.

A set of 27 validated ZFP repressors and 27 promoters with ZFP operator sites were created and tested for non-orthogonal interactions. A sub-set of 5 repressor-promoter pairs were found to have a high degree of orthogonality where the cognate pairs resulted in more than 73% attenuation of the promoter and non-cognate pairs gave less than 19%

attenuation. The ZFPs and promoter used in this task were far from optimal and these attenuation values could readily be improved.

The combination of these orthogonal repressor-promoter pairs and the new logic gate topology should enable more logic gates to be implemented in a single *E. coli* cell.

This dissertation is dedicated to my late father, who taught me the value of a strong work ethic. I know he would have been very proud to see this work come to completion.

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Introduction

Synthetic biology questions if the complexities of the cell, with all of its interacting and closely coupled elements, can be made manageable by the application of the principles of engineering? Over the past ten years the synthetic biology community has formally presented this question and tried to answer it. The output of the community has already demonstrated that design of biological systems can be made easier though the use of engineering fundamentals. Obviously, there is not a clear line where biology crosses into the realm of an engineering discipline. But now the question has evolved to focus on streamlining the design and implementation of these systems. Perhaps a better way to approach the question is to look at the rate of change in our ability to create more complex biological systems.

The field of synthetic biology is rife with analogies, and the majority of these analogies compare synthetic biology to the field of electrical engineering. There are many reasons for this choice including a desire to be associated with an industry that has been highly successful in building complex systems using abstraction, hierarchies, standards, modeling and automation. For the goals of attracting research funding and investment capital, it is useful to draw similarities to an industry that transformed the world, improved lives and created vast amounts of wealth. There are also similarities to the networks of interactions that are central to the work of electrical engineers and synthetic biologists; thus, analytical frameworks and tools developed by electrical engineers can be adopted to quickly solve problems in synthetic biology. But all analogies have their limits and there is a danger in trying to follow them too far.

As an electrical engineer entering into the field of synthetic biology, I have tried to be careful not to overstate what lessons can be applied across both of these areas. Determining where the crossover of ideas can be productive has been an exercise in comparing systems, methodologies, and assumptions from both fields and evaluating where they converge or diverge. Once it has been determine how well the concept transfers between the domains, an evaluation of the utility of the concept in the new domain is essential. One way to assess this utility is to consider the impact the implemented idea may have on the previously mentioned rate of change in our ability to create more complex biological systems. If the idea can withstand the transfer between domains and has utility in the new domain, then it does not over-extend the analogy between the fields and is a good candidate with which to proceed.

Digital logic has been one of the most powerful abstractions to come out of electrical engineering. By working with many instances of only a handful of highly characterized devices, extremely complex systems can be made with minimal understanding of the details of the inner workings of the devices. Some of the assumptions inherent to making useful digital logic are access to many instances of the device, globally defined states of high and low, and that the inputs and output of all devices must be compatible. Of these assumptions, the creation of many instances of a device provides the largest hurdle. The ongoing development of synthetic zinc finger proteins offered a possible route. If successful the utility of digital logic in synthetic biology could be huge. Every organism is filled with networks that combine multiple input signals into changes in gene expression, so there must be value in performing such functions. However, natural systems have been created

via evolution. Faster and better understood methods are required for us to engineer these types of functions. The abstractions of digital logic could provide these methods.

By working through the analysis outlined above, it appears that there is an opportunity to advance synthetic biology by applying concepts from digital logic in electrical engineering.

Acknowledgements

I have had a lot of time in graduate school to accumulate people to whom I am indebted for their advice, assistance, support and friendship.

None of this would have been possible without the enthusiastic support of my primary advisor, Jay Keasling. I have been fortunate to work in a lab where students are allowed to run with their own ideas and don't have to worry about when funding will dry up. Your attitude has been inspirational to me and I doubt I could have pushed past my failed projects without your unwavering belief in the value of my creativity and ideas.

My co-advisor, Michel Maharbiz, has played the ever changing role in my graduate career: from mentor, to friend, and finally to co-advisor. Thank you for filling in for this latest role and buffering me from a department that I barely fit into anymore. Your humor has brightened many a difficult day of graduate school. Most of all I appreciate the fearlessness you bring to your research. Not enough researchers have your willingness to go after difficult projects that are unlikely to reach their final goal.

My initial advisor, Roger Howe, had to leave Berkeley before I finished my degree, but I greatly enjoyed our time together. Thank you for putting up with my adventures in biology.

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The Howe group was full of great people who were always willing to help each other out. Together Frank Zendejas and Noel Arellano worked with me through prelims, qualifying exams, and the transition when Roger Howe left Berkeley. Sunil Bhave, Elliot Hui and Uthara Srinivasan taught me much through our discussions.

Turi Aytur and Emmanuel Quevy were always interesting and entertaining whether debating science, current events or hanging out after a long day in the lab.

I joined the Keasling Lab with minimal biology knowledge and no molecular or microbial biology experience. Many Keasling Lab members have taken their time to teach me including Tim Ham, Mario Oullette, Jack Newman, Sung Kuk Lee, David Laidlaw, Aindrila Mukhopadhyay, James Carothers, Jim Kirby, John Dueber, Eric Luning, Suzan Yilmaz, Brian Pfleger, Kai Wang, Josh Gilmore, Nathan Hillson and Wes Marner. Many Keasling members contributed to making graduate school a more pleasant place by just being a fun and friendly group of people. These people include Karen Wong, John Haliburton, Greg Bokinsky, Clem Fortman, Adrienne McKee, Jeffery Dietrich, Sarah Rodriguez, Becky Rutherford, Eric Steen, Lane Weaver, John Thorne, Tina Mahatdejkul, Sam Meyers, Rachel Eachus, Rumi Asano, Doug Pitera, Sydnor Withers, Bernardo da Costa, Eric Paradise, Trina McMahon, Afshan Shaikh, Sandra Villa, Sundiep Tehara and Matt Mattozzi.

Julius Lucks of the Adam Arkin Lab directly contributed to this research by giving me his sRNA devices and advice on how to best use them.

Ruth Gjerde of the EECS staff always had a friendly and reassuring smile that matched her ability to help me through any administrative mess.

I never told my mother the cardinal rule of graduate school – never ask a graduate student how long until they are done. But she figured it out on her own. Thank you for your love and support.

Finally, to my fiancée, Kier, meeting you was the best thing that happened to me in graduate school. I greatly appreciated your understanding of both the time commitments and emotional burdens of doing research. Thank you for reading and commenting on my dissertation draft.

Chapter 1 - Synthetic Biology

Biology as an engineering discipline

The goal of synthetic biology is to make engineering biological systems easier. By taking general engineering principles and applying them to the design of biological systems, one can hope that there will be improvements in the ability to predict system performance before construction, the time and money needed to deliver working systems, and the maximum size and complexity of systems created. General engineering principles include the following concepts: abstraction, standardization and decoupling [1].

Abstraction in engineering deals with removing complexity. In systems there are often various hierarchies of components. A designer who builds components on one level of the hierarchy only needs to know the interface specifications for the sub-components on the next lower level of the hierarchy. They do not need to know all the details of how the sub-components operate internally, only the information to interface sub-components together. By hiding the details of all the lower levels of the system, the total amount of knowledge the component designer must master to be successful is reduced. Additionally, the amount of information about a specific project that a designer must be able to mentally juggle is also lowered. Abstraction can play a large role in automating design processes, as abstracted systems are often simple enough that complete sets of formal rules can be enumerated. This then allows for computer algorithms to find optimal solutions for a given set of constraints without the intervention of someone with an expert level of knowledge.

Standards leverage the work of a community by making it easier for each individual to integrate the advances others have made into their own work. Standards can be made for many types of things, information or processes. Standards have been made for interfaces between objects, formats for data exchange, measurement methods, and even the creation of new standards. In terms of organizational structure, standards reduce the effort required for a party to integrate the work of another party into their own. Without standards, integrating an element into N systems requires order N times more work than if the systems utilized a common standard. When these savings are multiplied by a large number of exchanges in a community, the effort saved can be transformative and enable tasks that otherwise would be cost prohibitive.

Decoupling is the ability to break a task into smaller tasks that can be worked on independently. Decoupling can occur on several different levels within an engineering project. On a high level, a division of labor, where people with specialized skill sets perform different functions such as design and quality control, is an example of decoupling. Decoupling can also be applied to the design of components. By making components that function independently of the context in which they are used, it becomes possible to split the design of components into to individual problems that can be easily addressed in parallel.

The synthetic biology community is still in the early stages of fully integrating engineering principles into their workflows. Abstraction is evident in the hierarchy of parts, devices, chassis, and systems that has become common. However there are not yet clear boundaries between these levels and most practitioners do not currently work on only one level of this hierarchy.

Progress on the creation and adoption of standards is clearer. Numerous standards on DNA assembly methodology have been created and used including BioBricks[2], which has been widely used in the International Genetically Engineered Machine competition (iGEM), and BglBricks[3], which has been used in several labs at UC Berkeley. An informal standard exists for how parts that utilize transcription events should have their interface on the DNA between the promoter and the start of the transcribed region. This also led to a standardized unit of transcriptional activity, Polymerase Per Second (PoPS)[4]. Some work has also been done on standard measurement methods that can be used to characterize components including a promoter measurement technique [5]. There has also been the adoption of standards developed by related communities. For example, the Systems Biology Markup Language [6] (SBML) has become the primary format for moving models of synthetic biology systems between various design and simulation tools.

Decoupling of job functions is in its infancy within synthetic biology, but some instances can be found. In many projects there are multiple genetic networks that are involved. The work to create or modify each of these genetic networks can often take place in parallel. Creating components that are decoupled from their context has proven to be a difficult problem. It is probably not possible to create components that function independent of all contexts, and therefore the problem should probably be restated as components that function properly within some defined range of contexts. Some of the most promising work thus far on component decoupling has been on the introduction of orthogonal transcription and translation networks into *E. coli* [7].

Functional composition

One of the biggest challenges facing synthetic biology is enabling functional composition. Functional composition is the ability to take multiple characterized elements that have been combined into a larger unit and be able to predict how the larger unit will behave.

An example of where functional composition has not yet been achieved is the interface between a 5' un-translated region (5'-UTR) and a gene coding sequence. Most of the control of translation rates is encoded within the 5'-UTR and therefore it could be expected that the 5'-UTR will contribute to the translation rate and the coding sequence will contribute the primary sequence of the protein; however, if the translation rate of an mRNA consisting of a 5'-UTR and a gene coding sequence is measured and then the coding sequence is swapped to contain a different gene, then not only will the resulting protein sequence change but the rate of translation will also change. This finding is mostly due to the mRNA secondary structure playing a significant role in determining the translation rate; therefore both the 5'-UTR and gene coding sequence can contribute to the mRNA secondary structure. The secondary structure of an mRNA influences translation rates primarily by modulating the ribosome's ability to access its binding site on the mRNA. When the ribosome binding site (RBS) of the mRNA is bound to other bases of the mRNA strand and is not single stranded, then the ribosome losses its ability to bind to the mRNA and initiate translation. Predicting the translation rate of an mRNA may be possible with the complete sequence of that mRNA, but it is unlikely that this prediction can be done with just a few characterized parameters from both the 5'-UTR and the gene coding sequence [8].

While functional composition may not be possible for the general case of any pair of 5'-UTR and gene coding sequence, there may be sets of 5'-UTRs and gene coding sequences that are amenable to functional composition. If such a set does exist, then it is likely that through creative design work the size of the set can be expanded. 5'-UTRs that contain strong hairpin structures may be less likely to form secondary structures that cross the 5'-UTR to gene coding sequence boundary, and therefore make it possible to design for functional composition between some subset of components. This loss of generalizability, where a specification only holds when the component is used within a range of conditions, is common throughout engineering. These tradeoffs can be made because the loss of flexibility is offset by gains in designer productivity.

Orthogonality

In many domains of engineering, designing the first instance of a component is significantly harder than every additional instance. For example, designing a new ball bearing is a significant undertaking, but when a system requires four ball bearings each with the same specifications, then the first ball bearing design gets reused four times with minimal additional design work. In molecular biology, re-use is often more complicated because of diffusion and the limited constraints placed on the positions of molecules. All molecules in a cell have some ability to move relative to other molecules in that cell. There may be constraints placed on the relative movement of two molecules due to diffusion barriers such as cell or vesicle membranes, or the formation of a molecular complex may greatly restrict the movement of a molecule. Because genetic networks are primarily composed of molecular binding interactions and enyzmatic reactions, the elimnation of unwanted interactions and reactions must largely come from choosing or designing molecules that do not have compatiable interfaces for binding. Spatial separation either with membranes[9] or scaffolds[10], [11] has also been demonstrated as methods of keeping reactions orthogonal, but it is unclear how to scale these methods to more complex systems.

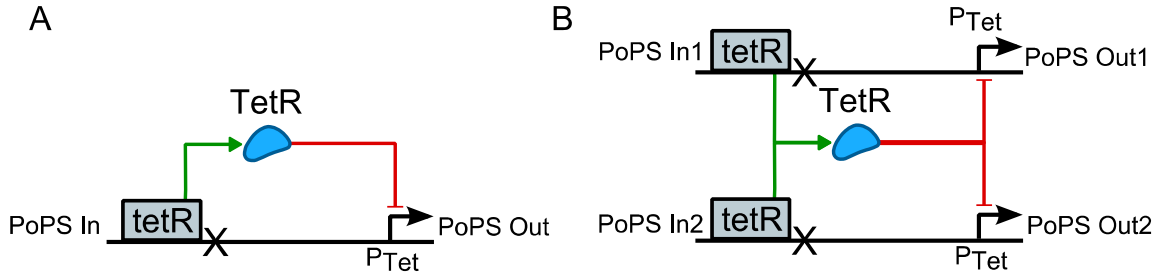


Figure 1-1 A) An inverter based on the tetR repressor only produces the protein TetR when the PoPS In level is high. When TetR is present it binds to the P_{Tet} promoter and turns it off. B) If two tetR based inverters are used within the same cell, the TetR protein produced by either input being high will bind to both output promoters and results in both outputs switching to a low PoPS level. Because the inverters are not orthogonal, the system behaves as a single NOR gate instead of two independent inverters.

Chapter 2 - C_2H_2-type Zinc Finger Proteins

Properties

Zinc finger proteins (ZFPs) contain a fold coordinated by a zinc ion and commonly bind DNA, but some are able to bind other molecules such as RNA, proteins or small molecules. Zinc fingers are frequently found in transcription factors, especially in eukaryotes. There are several classes of zinc finger proteins and the most commonly used class in engineered systems are the C_2H_2-type. C_2H_2-type zinc fingers contain a Zn(II) ion coordinated by two cysteine and two histidine residues and a single C_2H_2-type zinc finger binds to 3-4 bases of double-stranded DNA (dsDNA). Natural C_2H_2-type zinc finger proteins generally contain 3 or more zinc fingers, allowing them to bind to 9 or more base pairs (bp) of DNA with dissociation constants commonly in the nanomolar range.

Synthetic Zinc Finger Proteins

Several techniques have been used to create synthetic zinc finger proteins that can bind to a specified operator sequence. These methods can be broken into two broad classifications: screening or selection methods and rational methods. In the screening or selection methods a collection of coding sequences for individual fingers is created and then a large library of multi-finger coding sequences is generated with all or some of the fingers being randomly assigned from the collection. These coding sequences are then subjected to screening or selection by phage display[12-14] or an *in-vivo* binding assay[15], [16] to pick the best zinc finger protein in the library.

Rational design based on individual fingers

In the rational methods individual zinc fingers have been characterized to determine the DNA sequence they bind[15], [17-20]. This characterization is usually done in the context of a 3-finger with the middle finger being the site investigated. Because some fingers interact with 4 base pairs of DNA, the identity of the neighboring fingers becomes important. When the middle finger interacts with 4 base pairs of DNA, the 4th base pair is also interacting with the neighboring finger. Fingers that bind to GNG sequences are known

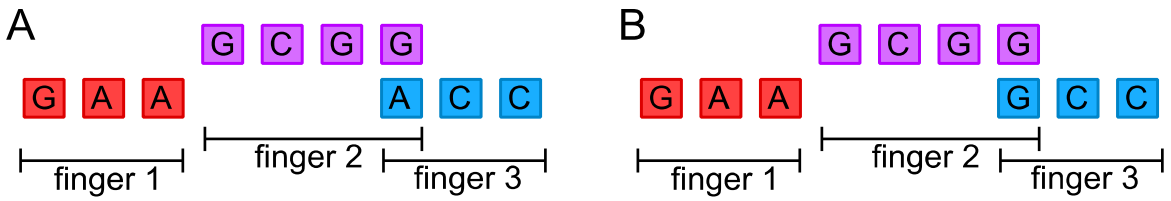


Figure 2-1 A) The DNA binding site for a zinc finger protein containing three fingers can clash if a GNG site is followed by an ANN or CNN site, because GNG site interact with a fourth base pair. A protein created with these fingers would likely have poor binding characteristics. B) By changing the third finger to a GNN site the clash has been relieved and the protein is more likely to be a functional DNA binder.

to interact with 4 base pairs, and the 4th base pair is required to be either G or T for a low dissociation constant and good specificity [21], as shown in Figure 2-1. Otherwise the final G of the middle finger and the initial A or C of the following finger will clash and result in a protein with a high dissociation constant and poor specificity.

In order to be able to rationally design zinc finger proteins that can bind to any arbitrary DNA sequence, fingers for each of the 4^3=64 triplets would need to be found and characterized. Thus far only 49 of the 64 possible triplets have high quality fingers[15], [17-20]. With 49 triplets, zinc finger proteins with 3 fingers can rationally be designed for $(49/64)^3$=~45% of all possible sites and 6 finger proteins can rationally be designed for $(49/64)^6$=~20% of all sites are possible.

Because rationally designed zinc finger proteins are easy to create, there was significant momentum behind their use, despite the limitations on the sequence space that can be targeted by rationally designed zinc finger proteins. Initial reports indicated that between 100%[22] and 60%[23] of rationally designed 3-finger proteins had the desired functionality. However these success rates were not universal. In 2008 a paper demonstrated that the initial reports were biased in the sequence space they targeted, and that only 24% of a less biased set of 3-fingers proteins worked correctly [24]. Extrapolating their data set to the completely unbiased case yields a 13% success rate on sites that can be targeted with the current set of good fingers. The rate of failure for a zinc finger protein was strongly correlated with the number of GNN fingers in the protein. All zinc finger proteins with no GNN fingers failed and 88%, 71%, and 41% the proteins containing 1, 2 and 3 GNN fingers respectively failed [24]. While this information reduced the expected utility of rationally designing zinc finger proteins, it also provided guidance on how to select binding sites with an increased likelihood of success. It is unclear if new sets of zinc fingers will be found that can overcome the current limitations that restrict the choice of operator site or require GNN fingers.

Pair wise finger design methods

Because zinc finger proteins can interact with more than three base pairs of DNA, rational design based on individual fingers can have a high failure rate when neighboring fingers clash. Alternative design methods have been developed that use pairs of fingers in order to reduce these problematic interactions. Some of these methods are rational and use characterized pairs of fingers to build specific zinc finger proteins [12], [25]. Other methods use phage based selections to find the desired zinc finger protein from a large library of randomized candidates[26]. The phage based methods have been shown to produce high quality zinc finger proteins, but the methods are very time intensive. Rational pair based design is known to work better than rational methods based on single fingers, but public information about characterized pairs is sorely lacking. Only a few characterized pairs have been published. In order to match the diversity possible using single fingers, a total of 49^2=2401 pairs of fingers would need to be characterized. A proprietary dataset of characterized finger pairs exists and is owned by Sangamo. They will provide custom zinc finger proteins for a fee, but the costs are significant.

DNA Assembly

PCR based methods

Individual fingers can be assembled into multi-finger constructs using a PCR overlap extension method [21]. A schematic of the process is shown in Figure 2-2. Single fingers or groups of contiguous fingers are PCR amplified with primers containing 5' regions not homologous to the template DNA. These primer defined segments are designed to be homologous with regions added to PCR products for adjacent fingers. Purified PCR products for two neighboring fingers are then placed in a PCR mix and thermocycled in order to extend the two templates into a single product. This process can be iterated to join any number of fingers together.

BglBricks

The majority of the zinc finger proteins used in this work were assembled using restriction enzymes within the context of the BglBrick standard. First, a set of plasmids each, containing a single zinc finger between BglBrick cloning sites, were constructed. These plasmids were made using the "Round-the-horn site-directed mutagenesis" method, which consists of a PCR using a plasmid template and phosphorylated primers with non-homologous 5' ends followed by a blunt end ligation [27]. This method results in the non-homologous region of the primers being inserted into the vector. The common sequence shared across all fingers along with the protein N and C terminal sequences are given in Table 2-1. The completed zinc finger proteins have ligation scars flanking each finger and the scar sequence (GGATCT) is not included in Table 2-1. The sequence of the variable region can be found in Table 2-5. The proteins constructed in this work all consisted of 6 zinc fingers. Because each zinc finger protein consisted of an N-terminal module, 6 finger modules, and a C-terminal module, a total of 7 junctions were ligated in order to create one zinc finger protein.

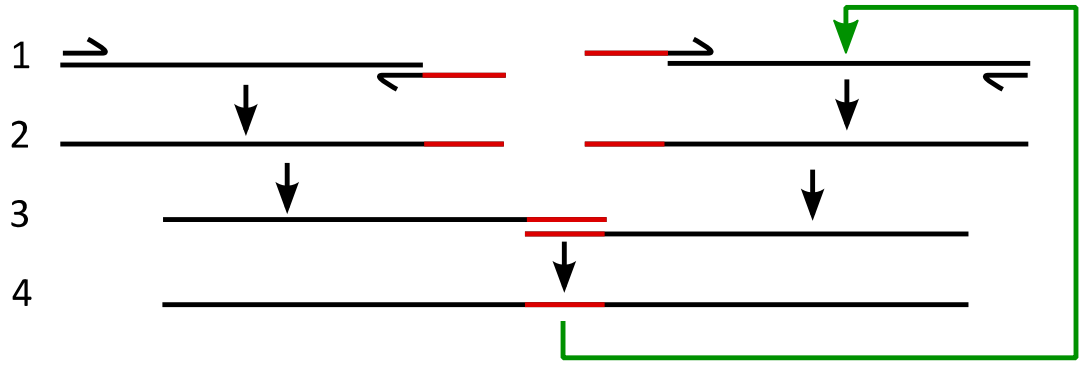


Figure 2-2 PCR based assembly of zinc finger proteins. 1) Individual fingers or contiguous groups of fingers are PCR amplified with primers that contain a non-annealing region shown in red. 2) PCR products generated contain matching red regions. 3) Thermocycling allows single stranded DNA from the two products to anneal in the red region and extend to form a double stranded product. 4) The resulting double stranded DNA contains fingers from the two initial templates. This process can be iteratively performed as shown by the green arrow.

Table 2-1 Sequences of segments of zinc finger proteins for assembly with BlgBrick cloning

Region	Coding Sequence	Amino Acid Sequence
N-terminus	CTGGAACCA	LEP
Finger, 5' of variable region	AAACCGTACAAATGTCCGGAATGT GGTAAATCCTTCTCC	KPYKCPECGKSFS
Finger, variable region	21 base pairs	7 amino acids
Finger, 3' of variable region	CATCAACGTACTCACACT	HQRTHT
C-terminus	AAAACCTCT	KTS

By organizing the assembly as a full binary tree, the protein can be generated in 3 parallel assembly steps as shown in Figure 2-3. A best case time frame for completing such a 6 finger assembly would involve DNA digest, ligation and transformation on the first day, colony PCR and colony picking into liquid media on the second day, and DNA purification, digesting, ligation and transformation on the third day. If verification by sequencing is desired before proceeding to the next level of the assembly tree, then an additional day must be added for each level of the tree. Therefore if there are no failures and sequencing is not required, assembled and purified DNA could be in hand on the seventh day for a 6 finger assembly. If sequencing is required at each step, then the best case time to go from individual fingers a fully assembled 6 finger protein with verified sequence is 10 days.

The frequency of sequence verification should be related to the expected error rate, and for restriction enzyme based sub-cloning this rate can be relatively low. However the error rate is not only dependent on the method, but also on the sequence being

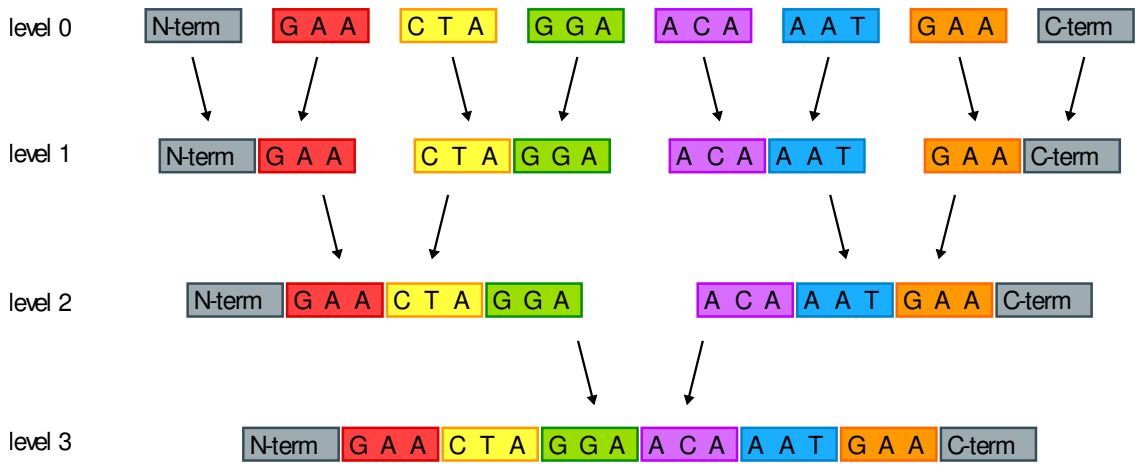


Figure 2-3 Assembly of 6-zinc fingers into a single protein using restriction enzyme based sub-cloning. By maximizing the steps performed in parallel, the depth of the tree, and therefore the duration of the process, can be minimized. Each box represents a segment of DNA. The boxes containing 3 letters each correspond to one zinc finger and the letters indicate the sequence that finger binds to, not the sequence of the DNA being assembled.

constructed. Constructs that are toxic, impose a high metabolic burden on the cell, or contain repeated sequence are likely to rapidly mutate away from the desired sequence[28], [29]. Initial construction of the zinc finger proteins was not done with a promoter present and therefore toxicity or metabolic burden were not an issue. Deletions due to recombination were a concern because a 63 base pair segment of DNA was repeated 5 times within each protein. To lower the rate of recombination, all work was done in *recA*- strains, primarily DH10B, DH5α and BLR(DE3). However *recA* dependent recombination only occurs with repeats of 200 base pairs or more, and thus it is questionable if the *recA*- strains altered the stability of these zinc finger protein constructs [29]. Given the possibility of recombination, a hybrid sequencing strategy was employed. Colony PCR was always utilized and was able to catch most failures. Sequencing was also performed after every ligation step, but downstream cloning continued in parallel with the sequencing. With this process the majority of the sequences were good, and no time was spent waiting for sequencing results. In the rare case when a sequence came back incorrect, no more than a few hours had been wasted on further cloning with the bad DNA.

Golden Gate

The Golden Gate cloning method allows for the rapid assembly of multiple pieces of DNA in a single pot [30], [31]. Type IIS restriction enzymes, which cut DNA a fixed distance away from their operator site, are essential to this method. Because the cut site does not overlap the operator site, it becomes possible to use one enzyme to generate many different DNA overhangs. Golden Gate cloning requires more design work and is more prone to failure than using BglBricks, but because it is not limited to joining only two pieces of DNA, it can considerably lower the time needed to assemble DNA. With aid of computer

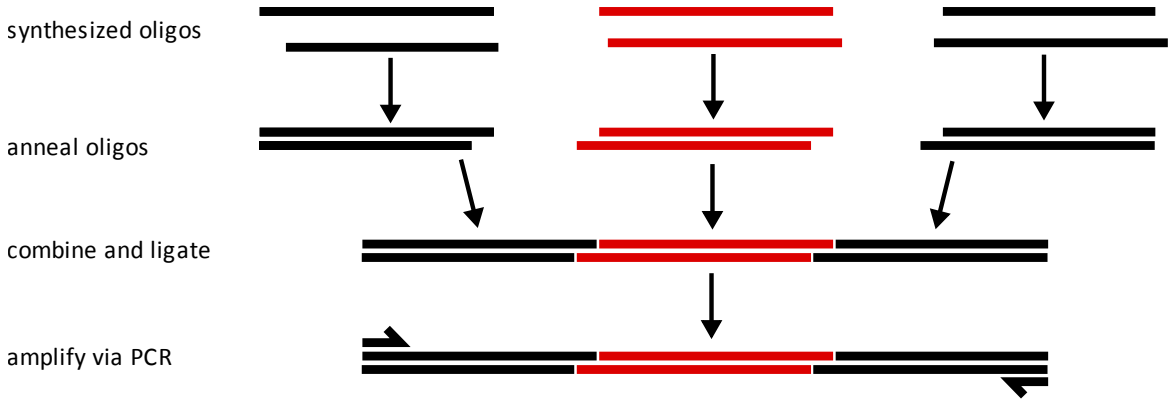


Figure 2-4 Individual zinc fingers for Golden Gate cloning have been constructed from oligonucleotides. Black oligonucleotides are shared across all zinc fingers that are designed for the same position within the final protein. Red oligonucleotides are shared across all zinc fingers that bind to the same DNA sequence. To use 49 binding triplets in 6 different finger positions requireds making 6x49=294 unique zinc fingers. Given the assembly strategy shown here, only 124 oligonucleoties were needed (2x3x6 black + 2x49 red) to construct and amplify all 294 zinc fingers.

Table 2-2 Nucleotide sequence of the PCR products that go into the Golden Gate cloning reaction. These sequences were digested by BsaI before they were ligated.

Finger Position	Nucleotide Sequence of PCR Products
1	TTTTTTGGTCTCATGCTCGAACCAGGAGAAAAACCATACAAATGCCCAGAATGCGGCAAAAGCTT TAGCNNNNNNNNNNNNNNNNNNNNCATCAACGAACACACACAGGCGATGAGACCAAAAAA
2	TTTTTTGGTCTCAGCGAAAAACCATACAAATGTCCAGAATGCGGAAAGAGCTTTAGCNNNNNNN NNNNNNNNNNNNNNCATCAACGCACACACACAGGAGAAATGAGACCAAAAAA
3	TTTTTTGGTCTCAGAAAAACCCTATAAATGCCCAGAATGCGGAAAAAGCTTTAGCNNNNNNNNN NNNNNNNNNNNNCATCAGCGAACACACACCGGATGAGACCAAAAAA
4	TTTTTTGGTCTCACGGAGAAAAACCATATAAATGCCCAGAGTGCGGAAAAAGTTTTAGCNNNNN NNNNNNNNNNNNNNNNCATCAACGAACCCACACATGAGACCAAAAAA
5	TTTTTTGGTCTCACACAGGAGAAAAACCGTACAAATGCCCAGAGTGCGGGAAAAGCTTTAGCNN NNNNNNNNNNNNNNNNNNCATCAACGCACCCACACAGGTGAGACCAAAAAA
6	TTTTTTGGTCTCACAGGAGAAAAGCCATACAAATGCCCAGAGTGTGGAAAAAGTTTTAGCNNNN NNNNNNNNNNNNNNNNNCATCAGCGAACCCACACAGGGAAGATGAGACCAAAAAA

aided design (CAD) tools, such as j5[32], the additional design effort can be reduced.

The setup costs (labor and oligonucleotide synthesis) for assembling zinc finger proteins using Golden Gate cloning are significantly higher than the setup costs for BglBricks. Setup for BglBrick cloning involves making one plasmid for each zinc finger to be used. Each of these fingers can then be used in any finger position within the protein. When using Golden Gate cloning, a unique version of each finger is needed for each position. Thus for making 6-finger proteins, Golden Gate cloning has 6 times the setup costs as BglBrick cloning. The zinc fingers for Golden Gate cloning were constructed from oligonucleotides using a multi-step protocol as shown in Figure 2-4. Because these fingers are not re-used at each position, it was possible to reduce the amount of repeated sequence in a protein relative to the BglBrick assembled proteins. A genetic algorithm was used to search through alternative coding sequences for the sections of the fingers that do not specify the binding site (or the flanking overlaps) in order to minimize the length of the longest direct repeat. The algorithm was not allowed to use any rare codons (coding for less than 5% of that amino acid in the *E. coli* genome). Additionally, sequences for the following restriction sites were prohibited: EcoRI, BamHI, BglII, AlwNI, BsaI and XhoI. Many of the codons were fully constrained, but a few of them were not, and some diversity was introduced. The sequences of the PCR products, each containing a zinc finger, are given in Table 2-2.

Initial attempts at assembling zinc finger proteins using Golden Gate cloning had low success rates, and by modifying the published protocol, higher success rates were obtained. The published Golden Gate protocol[30], [31] uses plasmids as the source for all DNA segments[30], [31], but PCR products were used in this work. PCR products were utilized because it was less labor intensive to generate PCR products than plasmids. However the initial low success rates may have been related to the use of PCR products instead of plasmids. The PCR products were designed to have a 6 base pair poly-T region at the 5' end of each DNA strand, directly flanking both BsaI restriction enzyme binding sites. These extensions beyond the BsaI sites were included to help BsaI bind and increase the

Table 2-3 Effect of important parameters for Golden Gate cloning.

Source of Variation	Effect	P-value
Digest duration	40	0.12
Digest temperature	-32	0.20
Ligation duration	23	0.35
Number of cycles	13	0.59

degree of cutting. Due to the low local melting temperature (T_m) at the ends of the PCR products, fraying of the PCR product may occur, and this could lead to less cutting than would have occurred if the segment was contained in a plasmid.

The first attempts at Golden Gate assembly of zinc finger proteins resulted in re-circularized vector backbone with none of the zinc fingers inserted. The size of the resulting plasmid was slightly smaller than the BsaI digested PCR product containing the vector backbone. The two ends of the digested PCR product did not have compatible overhangs, so circularization should not have occurred. Several of these resultant plasmids with no zinc fingers were sequenced. All of them were smaller than the digested PCR product for the vector backbone, and the missing base pairs were always from one or both of the ends of the digested PCR product. Because the digested PCR product was AT-rich at both ends, the digested PCR product may have frayed at the ends and created an opportunity for annealing and ligation within the frayed regions. The T_m of the 13 terminal *double stranded* bases on each end of the product were found to be 34°C and 33°C [33]. Because the Golden Gate cloning protocol includes a 37°C incubation step, it is likely that significant fraying of the digested DNA occurs (see protocol outline in Table 2-4).

An experiment was performed to determine if lowering the digestion temperature would result in higher efficiency cloning. It was hoped that lowering the digestion temperature would stop the ends of the digested DNA from fraying and give fewer incorrect ligations. A 2^{4-1} factorial experiment [34] was designed to investigate the effects of digestion temperature, digestion duration, ligation duration, and number of cycles. Digestion was tested at 37°C and 27°C for 5 minutes or 2 minutes. Ligation duration was also tested at 5 minutes and 2 minutes. Number of cycles was tested at 12 and 24. The ligation temperature was uniformly reduced to 22°C, as this is the temperature recommended by Fermentas, the manufacturer of the T4 ligase used. In order to extract as much useful information from this experiment as possible, the objective function used was based on the total number of zinc fingers successfully inserted into the vector across all colonies. This metric could be problematic if some conditions were extremely efficient at inserting some but not all of the 6 fingers, while conditions that inserted all 6 fingers were less efficient. Such results were not observed; the number of colonies roughly correlated with the number of zinc finger successfully inserted.

Table 2-4 Protocols for one pot Golden Gate cloning.

	Published protocol	Modified Protocol
BsaI digestion	37°C for 5 minutes	27°C for 5 minutes
Ligation (T4)	16°C for 5 minutes	22°C for 5 minutes
	Repeat above 2 steps 25 times	Repeat above 2 steps 25 times
BsaI digestion	50°C for 5 minutes	50°C for 5 minutes
Enzyme inactivation	80°C for 5 minutes	80°C for 5 minutes

This experiment indicated that digest duration is the most important parameter followed by digest temperature as shown in Table 2-3. This type of experiment only yields the local slope of the parameter space. Therefore it does not give an optimal set of parameters, but instead gives a direction to move towards. The absolute value of the effect indicates the strength of the dependence between the parameter and the objective function, and the sign of the effect indicates the direction the parameter should be moved to increase the objective function. The number of colonies that grew per experimental condition was low; therefore, it is not surprising that none of the p-values fell below the usual threshold of 0.05. Some of the conditions tested still gave more correct colonies than the original set of parameters, indicating these results are worth following up on even if the p-values were over the typical 0.05 threshold. The modified protocol that resulted from this experiment is shown in Table 2-4.

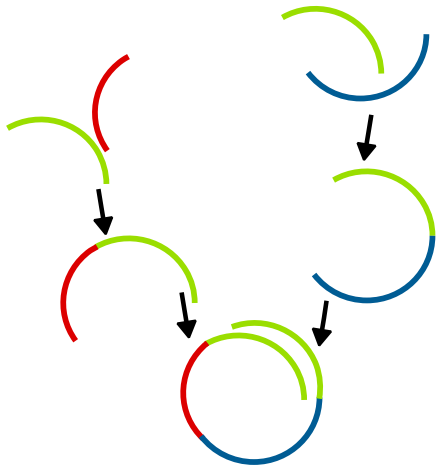


Figure 2-5 A three part Golden Gate assembly is shown where each part is in a different color. If parts 1 and 2 ligate together, parts 2 and 3 ligate together, and then these two products ligate to form a 4 part assembly. The resulting product is then larger than the desired product. This 4 part assembly could circularize into a plasmid that is an integer number of times larger than the desired product.

The simultaneous digestion and ligation of Golden Gate cloning along with the formation of irreversible junctions is supposed to drive all of the DNA to the final assembly product; however, it is possible for products to form that cannot contribute to the desired product. A simplistic example of this is shown in Figure 2-5.

Simulations were performed to determine the relation between number of pieces of DNA to assemble and the fraction of DNA segments that result in correct assemblies. Because of the many possible states in a multi-part assembly, analytical analysis methods were difficult to implement. To get past these limitations, a stochastic simulation method was used. Simulations were configured with assemblies of 2, 5 and 10 parts, and all parts present at equal molar concentrations. The primary parameters investigated in were the kinetic rates for two pieces of DNA joining and for a single piece of DNA to circularize and form the desired product. The rate of circularization is independent of the concentration of parts, but the rate of parts joining will increase with concentration; therefore, the ratio of these two parameters can be adjusted by setting the per-part concentration. The simulations were performed using the Stochastic Simulation Compiler v0.6 [35]. 1000 instances of each part were simulated in a single volume (no diffusion) until no further reactions were possible. The results are shown in Figure 2-6. All parts that did not end up in a complete plasmid were in assemblies larger than the desired plasmid.

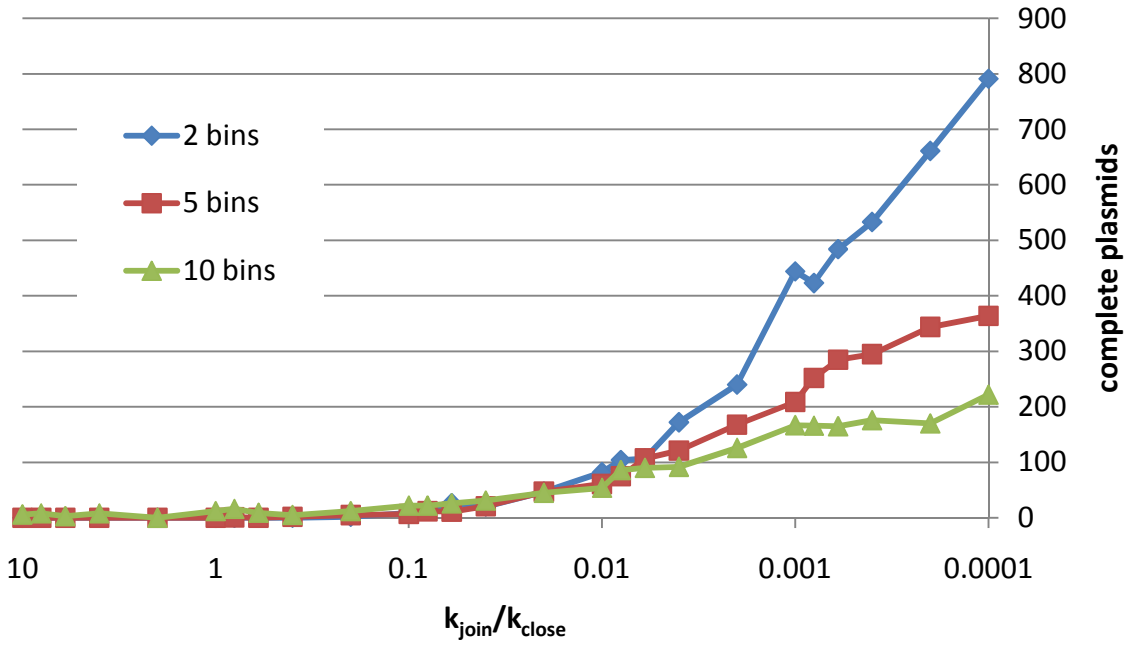


Figure 2-6 Stochastic simulations of a Golden Gate assembly showing how the relative rates of intra-molecular (k_{join}) and inter-molecular (k_{close}) reactions influence the number of correct plasmids created. Complete plasmids are out of a possible 1000. All reactions have perfect fidelity. The fraction of correct plasmids increases as the concentrations of parts decrease.

Adjusting the concentration of parts resulted in the majority of parts going into the desired product for 2 part assemblies, but was not able to achieve similar results for the 5 or 10 part cases. A modified assembly method was investigated to increase the number of correct plasmids formed for the assemblies with more than 2 parts. To prevent the formation of assemblies larger than the desired plasmid, one of the restriction enzyme sites was changed to recognize a second type IIS restriction enzyme but create the same overhang has before. This second enzyme would not be added to the reaction mix until late in the reaction. Simulations showed that over 99% of the parts went into linear products of the desired size before the second restriction enzyme was added. Once the second restriction enzyme is added, both circularization and joining of segments can occur. By performing a dilution when the second restriction enzyme is added, the ratio of k_{join}/k_{close} can be independently set for the linear growth phase and the circularization phase.

Table 2-5 Sequence of variable regions used in zinc fingers to determine binding specificity.

Binding Site	Amino Acid Sequence	Nucleotide Sequence used in BglBrick Assemblies	Nucleotide Sequence used in Golden Gate Assemblies
AAA	QRANLRA	CAACGTGCAAATCTGCGTGCT	CAGCGCGCGAACCTGCGCGCG
AAC	DSGNLRV	GACTCTGGTAATCTGCGTGTT	GATAGCGGCAACCTGCGCGTG
AAG	RKDNLKN	CGCAAAGATAACCTGAAAAAC	CGCAAAGATAACCTGAAAAAC
AAT	TTGNLTV	ACTACTGGTAATCTGACCGTT	ACCACCGGCAACCTGACCGTG
ACA	SPADLTR	TCTCCAGCTGATCTGACTCGT	AGCCCGGCGGATCTGACCCGC
ACC	DKKDLTR	GACAAAAAGGACCTGACTCGT	GATAAAAAAGATCTGACCCGC
ACG	RTDTLRD	CGTACTGATACTCTGCGTGAT	CGCACCGATACCCTGCGCGAT
ACT	THLDLIR	ACTCATCTGGATCTGATTCGT	ACCCATCTGGATCTGATTCGC
AGA	QLAHLRA	CAACTGGCTCATCTGCGTGCA	CAGCTGGCGCATCTGCGCGCG
AGC	ERSHLRE	GAGCGTTCTCATCTGCGTGAA	GAACGCAGCCATCTGCGCGAA
AGG	RSDHLTN	CGTTCCGATCATCTGACTAAT	CGCAGCGATCATCTGACCAAC
AGT	HRTTLTN	CATCGTACTACTCTGACTAAC	CATCGCACCACCCTGACCAAC
ATA	QKSSLIA	CAGAAATCTTCCCTGATTGCC	CAGAAAAGCAGCCTGATTGCG
ATG	RRDELNV	CGTCGTGATGAACTGAATGTT	CGCCGCGATGAACTGAACGTG
ATT	HKNALQN	CATAAAAACGCACTGCAAAAC	CATAAAAACGCGCTGCAGAAC
CAA	QSGNLTE	CAATCTGGTAACCTGACTGAA	CAGAGCGGCAACCTGACCGAA
CAC	SKKALTE	TCTAAGAAAGCGCTGACTGAA	AGCAAAAAAGCGCTGACCGAA
CAG	RADNLTE	CGTGCAGATAACCTGACTGAA	CGCGCGGATAACCTGACCGAA
CAT	TSGNLTE	ACCTCTGGTAATCTGACTGAA	ACCAGCGGCAACCTGACCGAA
CCA	TSHSLTE	ACCTCTCATTCTCTGACTGAA	ACCAGCCATAGCCTGACCGAA
CCC	SKKHLAE	TCTAAGAAACACCTGGCCGAA	AGCAAAAAACATCTGGCGGAA
CCG	RNDTLTE	CGCAATGATACTCTGACTGAA	CGCAACGATACCCTGACCGAA
CCT	TKNSLTE	ACTAAAAACTCCCTGACCGAA	ACCAAAAACAGCCTGACCGAA
CGA	QSGHLTE	CAATCTGGCCATCTGACTGAA	CAGAGCGGCCATCTGACCGAA
CGC	HTGHLLE	CATACTGGTCATCTGCTGGAA	CATACCGGCCATCTGCTGGAA
CGG	RSDKLTE	CGTAGCGATAAACTGACTGAA	CGCAGCGATAAACTGACCGAA
CGT	SRRTCRA	TCTCGTCGTACTTGTCGTGCA	AGCCGCCGCACCTGCCGCGCG
CTA	QNSTLTE	CAAAACTCTACCCTGACTGAA	CAGAACAGCACCCTGACCGAA
CTG	RNDALTE	CGTAATGATGCACTGACTGAA	CGCAACGATGCGCTGACCGAA
CTT	TTGALTE	ACTACTGGTGCTCTGACTGAA	ACCACCGGCGCGCTGACCGAA
GAA	QSSNLVR	CAATCTTCCAATCTGGTTCGT	CAGAGCAGCAACCTGGTGCGC
GAC	DPGNLVR	GATCCAGGTAATCTGGTTCGT	GATCCGGGCAACCTGGTGCGC
GAG	RSDNLVR	CGTTCTGATAATCTGGTACGT	CGCAGCGATAACCTGGTGCGC
GAT	TSGNLVR	ACTTCTGGTAATCTGGTACGT	ACCAGCGGCAACCTGGTGCGC
GCA	QSGDLRR	CAATCTGGTGATCTGCGTCGT	CAGAGCGGCGATCTGCGCCGC
GCC	DCRDLAR	GATTGTCGTGATCTGGCTCGT	GATTGCCGCGATCTGGCGCGC
GCG	RSDDLVR	CGTTCTGATGATCTGGTTCGT	CGCAGCGATGATCTGGTGCGC
GCT	TSGELVR	ACTTCTGGTGAACTGGTACGT	ACCAGCGGCGAACTGGTGCGC
GGA	QRAHLER	CAGCGTGCTCATCTGGAACGT	CAGCGCGCGCATCTGGAACGC
GGC	DPGHLVR	GATCCAGGCCATCTGGTTCGT	GATCCGGGCCATCTGGTGCGC
GGG	RSDKLVR	CGTTCTGATAAACTGGTTCGT	CGCAGCGATAAACTGGTGCGC
GGT	TSGHLVR	ACTTCTGGTCATCTGGTACGT	ACCAGCGGCCATCTGGTGCGC
GTA	QSSSLVR	CAATCTTCTTCTCTGGTTCGT	CAGAGCAGCAGCCTGGTGCGC
GTC	DPGALVR	GATCCTGGTGCACTGGTTCGT	GATCCGGGCGCGCTGGTGCGC
GTG	RSDELVR	CGCTCTGATGAACTGGTACGT	CGCAGCGATGAACTGGTGCGC
GTT	TSGSLVR	ACTTCTGGTTCTCTGGTTCGT	ACCAGCGGCAGCCTGGTGCGC
TAG	REDNLHT	CGTGAAGATAACCTGCATACT	CGCGAAGATAACCTGCATACC
TGA	QAGHLAS	CAAGCTGGTCATCTGGCATCT	CAGGCGGGCCATCTGGCGAGC
TGG	RSDHLTT	CGTTCTGATCATCTGACTACT	CGCAGCGATCATCTGACCACC

Chapter 3 - Synthetic Zinc Finger Protein Transcription Factors

Existing methods

Two-hybrid

Two-hybrid systems are tools for probing protein-protein interactions *in-vivo,* and they consist of two hybrid proteins and a reporter plasmid. One of the proteins contains a DNA binding domain fused to a protein interaction domain, and the second protein consists of a second protein-protein interaction domain fused to a transcriptional activation domain. An operator binding site corresponding to the DNA binding domain is placed upstream of a promoter with low basal activity. When both of the hybrid proteins are present, the DNA binding domain will bind the operator site, and the activating domain will bind part of an RNA polymerase. If the protein-protein interaction domains of the two hybrid proteins successfully bind, then physical connection between the DNA operator site and the RNA polymerase results in RNA polymerase being recruited to promoter and the onset of transcription. If the protein-protein interaction domains do not bind each other, then RNA polymerase will not be recruited to the promoter, and transcription will not occur.

Two-hybrid systems were first created in *Saccharomyces cerevisiae* [36] and they continue to be used extensively in this organism. It is not surprising that yeast was the first organism to be used in a two-hybrid assay because *S. cerevisiae* is a model organism with many genetic tools, and it has a well studied set of modular domains for DNA binding, transcription regulation, and protein-protein interactions.

Two-hybrid systems are easier to implement in eukaryotic systems than prokaryotic ones because of the increased modularity in eukaryotic systems and because eukaryotic organisms more frequently use regulation based on transcriptional activation. Being able to perform two-hybrid assays in prokaryotic organisms such as *E. coli* would have advantages including faster experiments due to higher growth rate, higher transformation efficiency yielding larger libraries, and a different background of proteins than yeast [37]. Nine years after the first two-hybrid system was published for yeast, an *E.*

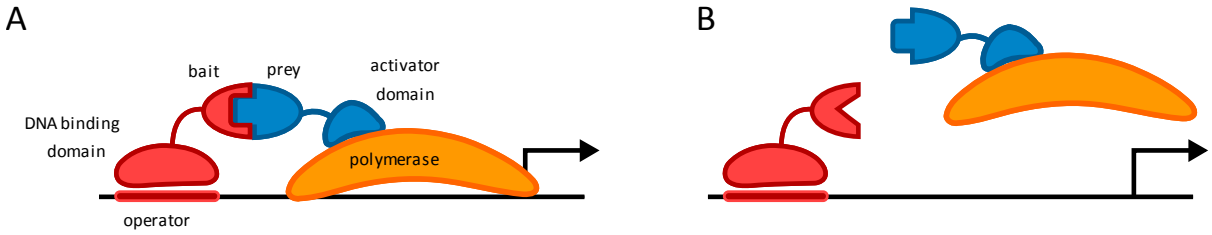


Figure 3-1 A two-hybrid system. A) When a protein-protein interaction exists between the bait and prey domains, then the polymerase is localized to the promoter region downstream of the operator site and transcription occurs. B) When a protein-protein interaction does not exist between the bait and prey domains, then the polymerase is not recruited to the promoter region and transcription does not occur.

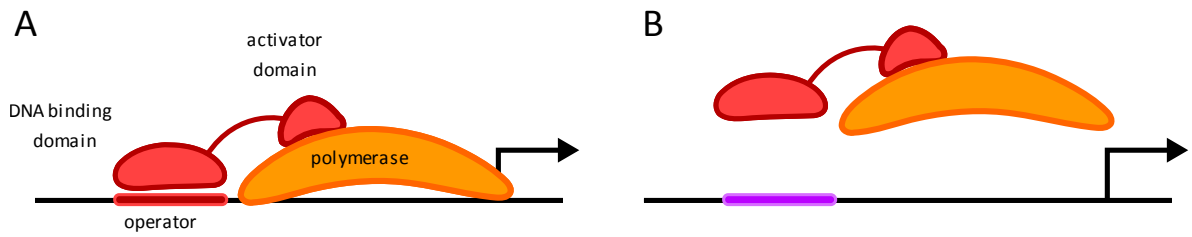


Figure 3-2 A one-hybrid system. A) If the DNA binding domain is able to bind to the operator site and the activator domain is able to bind an RNA polymerase, then the polymerase will be localized to the promoter region downstream of the operator site and transcription initiation occurs. B) If the DNA binding domain is not able to bind the operator site, then the polymerase is not localized and transcription does not occur.

coli version was published [38]. The duration between these publication dates should serve as an indication of the challenges involved in performing two-hybrid assays in *E. coli*.

One-hybrid

One-hybrid systems only contain a single hybrid protein that contains a DNA binding domain and an activation domain. These systems are typically used to investigate a protein-DNA interaction, but could also be used to assay for a protein-protein interaction between a protein and an RNA polymerase. One-hybrid assays have been developed in *E. coli* that use zinc finger proteins as the DNA binding domain [39], [40]. These one-hybrid proteins are synthetic transcriptional activators, and it may be possible to make sets of orthogonal transcriptional activators by modifying the zinc finger protein used for the DNA binding domain.

One-hybrid systems also have been developed for eukaryotic hosts, where a wider range of configurations are possible. Eukaryotic organisms have both transcriptional activation and repressions domains. Taking a DNA binding domain fused with an activation domain and swapping it for a repression domain will change the protein from a transcriptional activator to a transcriptional repressor. This swap cannot be done in prokaryotic systems because a modular repression domain has not yet been found.

Dimeric zinc finger proteins

Many DNA binding proteins form homo-dimers or higher order complexes in order to bind DNA. These structures can result in cooperative binding, where the binding affinity of the first monomer is higher than the binding affinity of the second half of the dimer. Cooperativity yields a system with higher sensitivity to the presence of the proteins. Dimerization also increases the size of the operator site, and therefore decreases the likelihood of the operator site occur elsewhere in the genome by random chance. For example, if a protein has a 9 base pair operator, then this operator would be expected to appear once in a random sequence of $4^9 \approx 2.6 \times 10^5$ base pairs, but if this protein can dimerize then the operator will likely be unique in a sequence of up to $4^{18} \approx 6.8 \times 10^{10}$ base pairs. Synthetic zinc finger protein dimers have been created by fusing a leucine zipper

domain to a zinc finger based DNA binding domain [41], [42]. Leucine zippers are a well studied class of protein-protein interaction domains.

Many prokaryotic transcriptional repressors function as dimers that sterically prevent the RNA polymerase from interacting with a promoter. Because zinc finger proteins are excellent DNA binders and can be made to dimerize, they are good candidates to use in engineering synthetic transcriptional repressors.

Such a system has been constructed, but the level of transcriptional repression obtained was 2-3 fold [43]; and this is significantly lower than that of other commonly used systems where the fold attenuation ranges from 17-5050 fold[44], [45]. Expression of the dimeric zinc finger proteins used by Shetty caused a significant decrease in growth rate, and this was hypothesized to be the result of non-specific binding of the zinc finger domains [43]. When non-specific binding exists, there is a possibility of transcriptional repression occurring at off-target sites. These off-target sites may be expressing genes that are important for maximizing the cell's growth rate in the current environment, and repression of these genes will therefore lead to a reduction in growth rate. Shetty noted that the 14 base pair operator for her dimeric zinc finger protein does not occur in the MG1655 *E. coli* genome, nor does any 1 base pair mismatch. However, it is highly likely that the operator for a monomeric zinc finger does exist within the MG1655 genome, as a 7 base pair operator should appear about every 4^7= 16384 base pairs. The MG1655 genome is more than 4.6 million base pairs, and therefore a 7 base pair sequence would be expected to occur more than 280 times. Alternatively, the toxicity may have been caused by the zinc finger protein being expressed to a concentration much higher than its dissociation constant. The protein needs to be expressed to a concentration above its dissociation constant in order to bind the majority of the operator sites, but as the concentration increases there is a tradeoff between tighter repression and minimizing non-specific binding. Even at concentrations well below the dissociation constant for non-specific binding there can be detrimental effects, as there are millions of non-specific sites and only a few key sites need to be occupied for the growth rate to be impacted.

Building repressors with dimeric zinc finger proteins has only had limited success so far, but they likely have potential that has not yet been completely explored. Further investigation into reducing the toxicity of dimeric zinc finger proteins by lowering expression levels may solve many of the current problems. But even if these problems are addressed, it is unclear how well dimeric zinc finger repressors will scale. Creating an orthogonal set of dimeric zinc finger repressors would require both sets of orthogonal DNA binding domains and orthogonal sets of protein-protein interaction domains. The human genome contains over 900 zinc finger proteins, and while these proteins may not all be orthogonal DNA binders, this provides a rough estimate for the productive limit of interacting zinc finger proteins. The human genome only contains 56 basic leucine zippers, and these zippers have been found to have a low degree of orthogonality [46]. Because of the low orthogonality of leucine zippers, it may be difficult to make more than 10 or 20 dimeric zinc finger proteins with strong orthogonality [47].

Steric hindrance of RNA polymerase

In prokaryotes there have been several demonstrations of attenuating transcription from a promoter by binding a protein near the promoter, specifically between -74 to +43

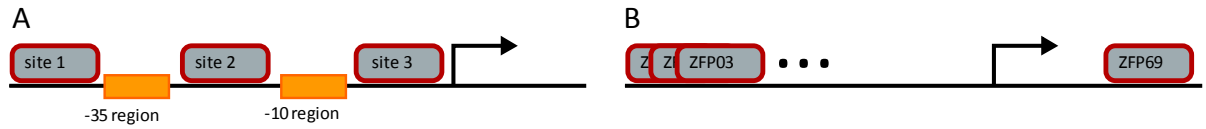


Figure 3-3 A) Previous work on elucidating positional effects in transcriptional attenuation was based on moving an operator site between three main sites that were minimally constrained. Site 1 is directly 5' of the -35 region. Site 2 is between the -35 and -10 regions. Site 3 is 5' of the -10 site. The sites can be moved a few base pairs to get a locally high resolution, but they cannot move into the important -35 and -10 regions, and thus the resolution near these regions is limited to 12-20 base pairs depending on the operator length. B) By using synthetic zinc finger proteins, it may be possible to target a unique zinc finger to every position along the promoter including the -35 and -10 regions, yielding a 1 base pair resolution.

relative to the transcriptional start site[44], [45], [48]. Despite the demonstrated ease of adding operator sites to existing promoters, there have been no reports of monomeric zinc finger proteins being used to sterically hinder RNA polymerase in prokaryotes. By using monomeric zinc finger proteins, the limitations on orthogonality due to the need for protein-protein interaction domains (leucine zippers) would be eliminated.

Previous work on the engineering of steric hindrance of RNA polymerase has been constrained by operator sites that cannot overlap with important promoter features. Because there was little or no flexibility in the sequence of the operator sites used, operators could only be introduced in non-critical sections of the promoter as shown in Figure 3-3-A. Replacing the -35 or -10 RNA polymerase binding sites with the sequence of an operator site would likely lead to a loss of activity for the promoter; thus, the insertion of operator sites has mainly been limited to 5' of the -35 region, between the -35 and -10 regions, or 3' of the -10 region [44], [45], [48]. Positions that overlap the -35 or -10 regions likely would yield high levels of transcriptional repression, but investigating these positions has not been possible with existing repressors. By using synthetic zinc finger proteins, it becomes possible to make a DNA binding protein that binds over the existing -35 or -10 sequence of a promoter. With this flexibility, new methods for investigating steric hindrance of RNA polymerase become feasible. Previous work on the effect of operator position on steric hindrance has used one repressor protein, and the operator for that protein was moved to several locations within a promoter [44], [45], [48]. With synthetic zinc finger proteins it becomes possible to keep the promoter sequence constant and design multiple zinc finger proteins that are targeted to unique operator sites at different positions along the promoter as shown in Figure 3-3-B. Ideally, every segment of DNA the size of an operator within the promoter would be targeted by a zinc finger protein, so that the position of the DNA binding protein could be investigated with single base pair resolution.

Due to the limitations on zinc finger protein operator sites that were discussed in Chapter 2, it is not possible to take an existing promoter and design zinc finger proteins that can bind at every position along that sequence. These limitations include a need for GNN binding fingers and that the operators only contain the well characterized pallet of 49

DNA triplets. Using bioinformatics, known promoter sequences could be searched for a promoter that contains a large number of high quality zinc finger protein binding sites. Given the data on GNN requirements [24] and that only 49 triplets have good fingers, the expected frequency of usable operator sites using 3 finger or 6 finger proteins respectively are:

$$\left[\left(\frac{64-16}{64}\right)^2\frac{16}{64}0.12+\frac{64-16}{64}\left(\frac{16}{64}\right)^2 0.29+\left(\frac{16}{64}\right)^3 0.59\right]^{-1} \approx 37.7 \text{ base pairs/site}$$

$$\left[\left(\frac{64-16}{64}\right)^5\frac{16}{64}0.12+\left(\frac{64-16}{64}\right)^4\left(\frac{16}{64}\right)^2 0.29+\left(\frac{49}{64}\right)^3\left(\frac{16}{64}\right)^3 0.59\right]^{-1} \approx 153.6 \text{ base pairs/site}$$

For this calculation, 64 is the total number of triplets, 49 in the number of triplets for which good fingers exist, and 16 is the number of GNN fingers. The 6 finger sites most likely occur more often than shown here as the work on GNN requirements only considered 3 finger proteins [24]. Proteins with 4 or more GNN sites probably have a higher than 59% chance of being functional. A search of promoters should yield some sequences that are significantly more enriched for good zinc finger protein operator sites than random sequence. Even with a 10-fold enrichment, the resolution using 3 finger or 6 fingers proteins would be 3.77 base pairs/site and 15.36 base pairs/sites respectively. Achieving single base pair resolution using an existing promoter appears unlikely.

Design of a synthetic promoter that can bind many zinc finger proteins

Since existing promoters were not expected to yield a high resolution of binding sites, a synthetic promoter was designed with single base pair resolution of high quality binding sites. The first step in the design of this promoter was deciding on the size of zinc finger proteins to use. With 4-finger proteins, about a quarter of all possible sequences would be found in the *E. coli* genome. Because the promoters have many constraints placed on their sequences, the operators needed to cover any promoter are probably over-represented in the genome. Therefore a factor of 4 in available sequence space did not seem adequate. By increasing the number of fingers to 5, the possible sequence space becomes 268 times the size of the *E. coli* genome. While 5 fingers would work well in the

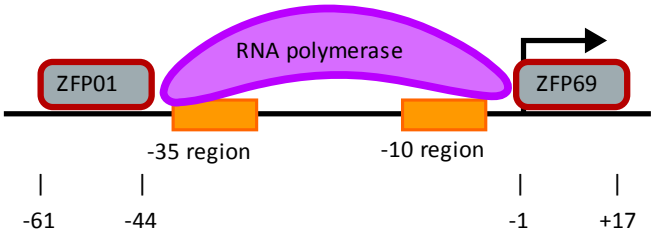


Figure 3-4 Defining the region to target with zinc finger proteins. The footprint of RNA polymerase extends from about -43 to -2 relative to the transcriptional start site. If zinc finger proteins with 18 base pair operator sites flank the RNA polymerase, then the region including these operator sites spans from -61 to +17. This region has been expanded by 4 base pairs on each end (-65 to +21) to accommodate any difference between the zinc finger protein operator site and footprint size and to give a margin of error.

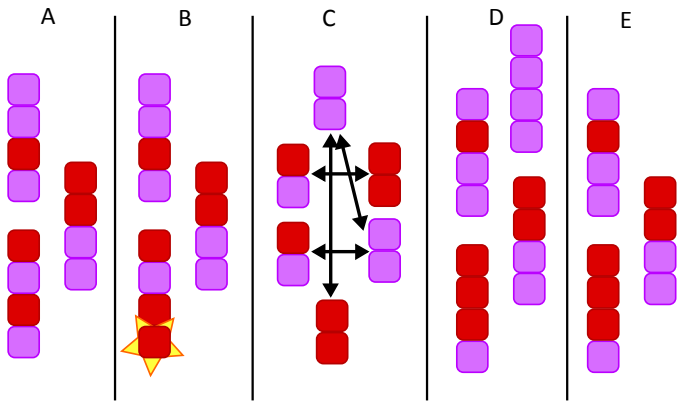


Figure 3-5 Description of a genetic algorithm. Genetic algorithms are useful for searching through a large parameter space that may not be smooth. A) A starting population with diversity is randomly generated. B) Mutations introduce additional diversity by randomly modifying an attribute for some fraction of the population. A yellow star marks the site of a mutation in the figure. C) Sexual reproduction takes the attributes from two members of the population and combines them to form new members. C) The population after the sexual reproduction step. The figure does not show the parents persisting, but in most implementations they are still included in the population at this step. E) The fitness of each member is evaluated through the objective function. The objective function is a quantitative metric and is application specific. Low fitness members of the population are eliminated. In this example fitness is proportional to the number of red squares. Steps B-E are repeated until the maximum fitness remains constant over many iterations. Tuning key parameters for tuning the genetic algorithm are the mutation rate, the rate of sexual reproduction, and the population size.

context of the *E. coli* genome, constructing a library of 5-finger proteins with restriction enzyme cloning requires the same number of steps as constructing a 6-finger protein library. 6-finger proteins will allow for more GNN fingers per protein, and the 18-finger proteins are large enough that their operators are unlikely to appear in the genome of any highly studied organism.

The next task was to determine how much flanking sequence around the core segment of -35 region through -10 region should be included. Footprinting measurements of *E. coli's* RNA polymerase have indicated that up to 41 base pairs can be covered by the polymerase [49]. If RNA polymerase is centered on the -35 to -10 regions, then a 41 base pair footprint would extend from about the -43 to -2 positions. To cover all positions that could interfere with this footprint, the most upstream 18 base pair operator needs to extend from -44 to -61 and the most downstream 18 base pair operator needs to extend from -1 to +17 as shown in Figure 3-4. To allow for a margin of error, and the possibility of the zinc finger protein having a footprint that is larger than its operator site, the promoter region to be targeted by zinc finger proteins was defined to go from -65 to +21 relative to the approximate transcriptional start site.

With the promoter region of interest defined to span from -65 to +21, the total possible sequence space is quite large at $4^{86} \approx 6\times10^{51}$ possibilities. Because the sequence space is large, a brute force approach, where every possible sequence is evaluated, is not possible. A genetic algorithm was used to rapidly search through the possible sequence space. For an overview of genetic algorithms see Figure 3-5. Properly defining the objective function is crucial to obtaining a useful result. Because genetic algorithms are conceptually based on evolution, the common phrase from directed evolution applies here also, "you get what you select for."

Creating the objective function

The most important constraint encoded in the objective function is to keep the sequence close to that of a consensus promoter, because if the sequence fails to have constitutive promoter activity, then transcription attenuation assays cannot be performed. A published consensus *E. coli* promoter sequence [50] was used to evaluate the potential sequences (sequence shown in Figure 3-8). This consensus sequence was constructed from a subset of *E. coli* promoters and includes both σ^{70} promoters and promoters that use alternative sigma factors. Because the majority of *E. coli* promoters are σ^{70} promoters, this consensus sequence is biased towards the consensus σ^{70} promoter sequence. This set of promoter is also biased by the fact they are studied promoters and therefore may not be a representative population. To evaluate the similarity between the consensus and a potential promoter, the number of matching base pairs between the consensus and potential promoter were counted (Ns in the consensus did not count as matches as they do not specify any reduction in degrees of freedom).

Scoring the number and quality of the zinc finger protein operator sites was based on several metrics. For all of these metrics, every possible 18 base pair sequence of the promoter was evaluated under the metric, and the results for all segments were summed.

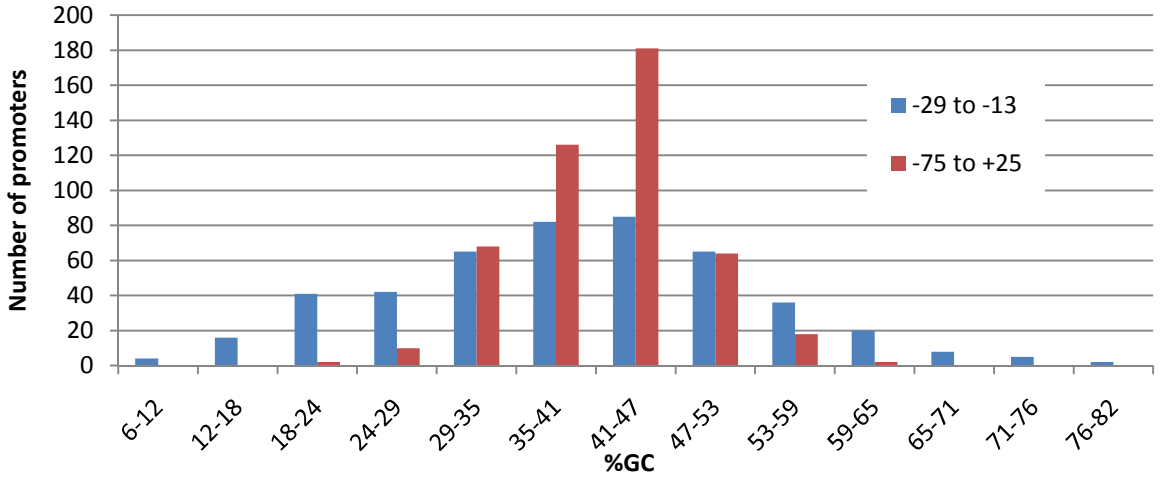


Figure 3-6 The %GC content of 472 E. coli promoters is shown in blue for the 17 bases that normally fall between the -35 and -10 regions and in red for the whole promoter defined as from -75 to +25 relative to the transcriptional start site. For both regions the distribution is centered slightly below 50%.

WTWNWNNNNAWWWNNTTCTTGACATNTTNNNNNNNNWTRTGRTATAATDNNNNNNRTWW
-35 region -10 region

Figure 3-8 A consensus *E. coli* promoter sequence[47]. This promoter sequence extends from -53 to +4 relative to the transcriptional start site. N can be any base pair. D can be A, C, or G. R can be A or G. W can be A or T.

The number of triplets that correspond to fingers that can be rationally designed for was counted. The number of GNN triplets was also tallied. Both of these counts were a positive contribution to the objective function. Each operator site was checked to see if it occurred elsewhere in the promoter sequence or to see if it occurred in the MG1655 *E. coli* genome. Checks were also done against the genome to see if changing 1 or 2 base pairs in the operator site would yield a match within the genome. Hits for repetition of sites contribute negatively to the objective function.

While the balance between consensus sequence and coverage with zinc finger proteins was the most important part of the objective function, there were several other minor factors that had to be included to get a useful result. Because the resulting promoter was to be constructed from oligonucleotides, restrictions from oligonucleotide synthesis had to be included in the objective function. Runs of consecutive Gs longer than 5 base pairs were penalized, because these are difficult to synthesize. Sequences tended to be very G-rich because of the GNN constraint, but E coli promoter sequences tend to be slightly AT rich [51]. The GC content of 472 E. coli promoters is shown in Figure 3-6. As the center of the GC content distribution is slightly below 50%, the objective function was set to target a GC content of 47% for the whole promoter.

A consequence of enforcing a specific AT content and rewarding GNN triplets were regions of AT at both ends of the promoter. A GNN triplet that was 17 base pairs or more away from the end of the promoter would occur in 6 different operators (each in a different finger position), but a GNN that was at either end of the promoter sequence would only contribute to one operator site. The objective function contains a term based on the number of GNN triplets summed across all operator sites. The genetic algorithm successfully found the optimum for these constraints and placed all the GNN triplets in the interior of the promoter and 17 base pairs of As and Ts at both ends of the promoter. While this solution is correct based on the objective function, the sequences are unlikely to function as promoters due to the high GC content between the -35 and -10 region where the DNA melting for open complex formation starts. Additionally, these sequences will not have uniform coverage with zinc finger protein operator sites, as they are more

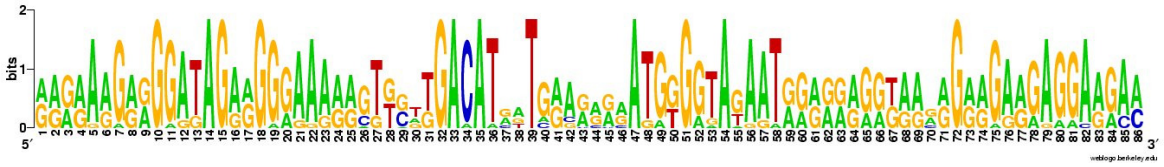


Figure 3-7 A sequence logo[49] showing the diversity in the promoter library generated by the genetic algorithm.

concentrated at the center of the sequence. Two approaches were taken to correct these issues. First, a new constraint was added to the objective function that targeted the percentage of GC content at 47% for the segment between the -35 region and -10 region. Second, a new penalty was placed in the objective function that is proportional to the highest percentage of GC content found in any 8 base pair segment of the sequence. Both of these corrections resulted in a more even distribution of the operator sites and a high likelihood of promoter activity.

The objective function used here primarily had to balance between keeping enough consensus promoter sequence that transcription still occurs in the absence of zinc finger proteins and including as many high quality zinc finger protein operator sites as possible. Promoters are generally rich in A and T bases because this lowers the energy needed to locally melt the DNA and allow for transcription, but a sequence optimized for the binding of multiple zinc finger proteins will have an elevated number of G bases. These two requirements for enrichment in both AT and G bases are in conflict and must be balanced. Predicting if a potential promoter sequence will have activity is difficult; thus, there was no good computational way to determine when the balance between consensus promoter sequence and zinc finger protein coverage was optimal.

A hybrid *in-silico*/*in-vivo* method was used to balance these two factors. The genetic algorithm was run several times with a range of weighting factors used in the objective function to tradeoff consensus promoter sequence verse zinc finger protein coverage. Each of these runs generated a promoter sequence, and this set of promoters was cloned 5' of green fluorescent protein (GFP, version mut3b) and assayed for constitutive promoter

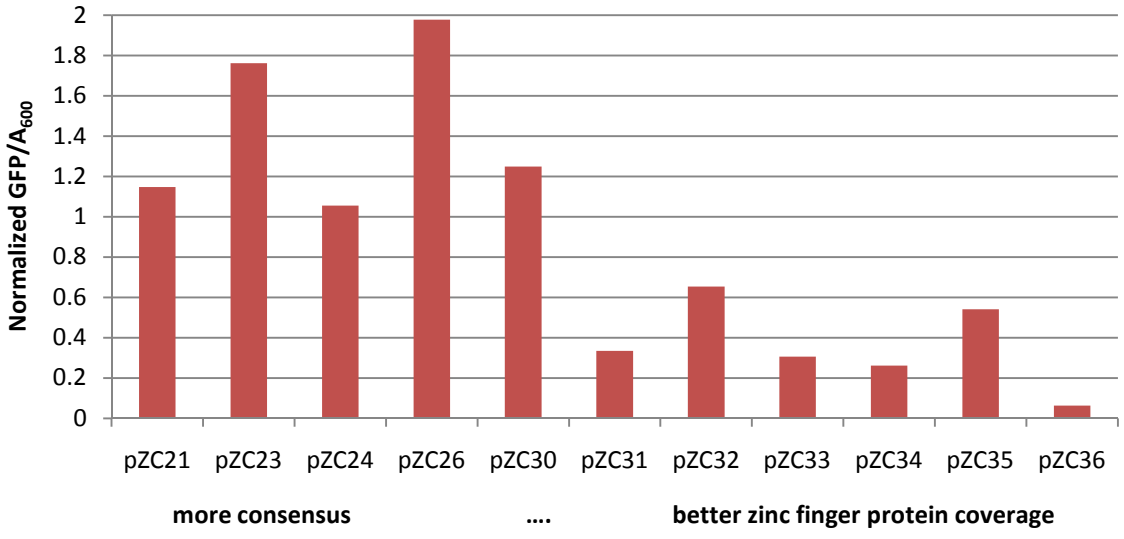


Figure 3-9 Evaluating constitutive activity of synthetic promoter library. Promoters are listed on the x-axis in rank order of their weighting toward either having sequence more similar to a consensus promoter sequence or having more high quality zinc finger protein operator sites with P_{ZC21} being closest to consensus and P_{ZC36} having the best set of operator sites. All values were normalized to the strength of $P_{Ltet0-1}$ in the absence of TetR.

AAAAAAGGGGAAGAGGGAAGAGGAGTGGTGGACATAGTGGAAGGAATGGGAGATAGTGGGAGAGAGGGAAGGAGAGGAGGAAAAA

-35 region -10 region

Figure 3-10 The sequence of the synthetic promoter P_{ZC35} is shown with the transcriptional start site (+1) marked by a purple dot. The base pairs shown in red are not in agreement with the E. coli consensus sequence given in Figure 3-8. 100% of the non consensus base pairs are Gs.

activity (no zinc finger proteins present). This library of candidate promoters has diversity at most positions outside of the -35 and -10 regions as shown in Figure 3-7. Adjusting the balance between the consensus promoter sequence and the coverage of zinc finger proteins caused alterations through out most of the promoter and did not involve just a few base pairs changing. This indicates that the two goals of having a completely consensus sequence and full coverage with zinc finger protein sites are conflicting goals.

Analysis of the synthetic promoter P_{ZC35}

Promoter P_{ZC35} was chosen for further analysis. Of the promoters that gave enough signal for easy detection, P_{ZC35} had the highest rating for potential zinc finger protein coverage as shown in Figure 3-9.

The naming convention adopted for the operator sites is opX where X is the position of the most 5' base pair of the operator relative to the transcriptional start site. X is prefaced with a "+" for positions downstream of the transcriptional start site. This means the operator site furthest upstream is op-65 and the operator furthest downstream is op+4.

All DNA triplets contained within P_{ZC35} are represented in the set of good zinc finger

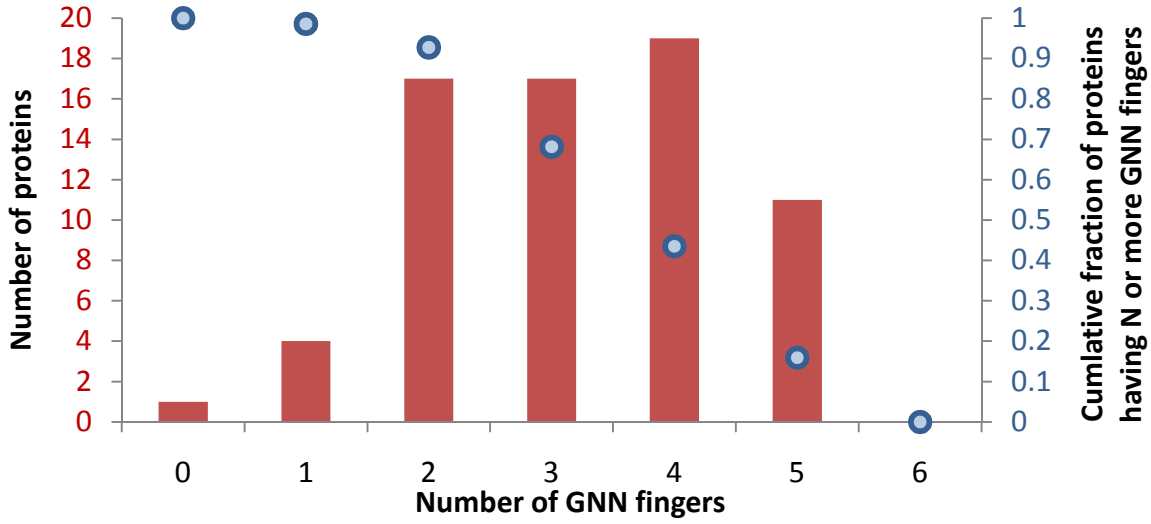


Figure 3-11 The distribution of GNN binding zinc fingers. Function zinc finger proteins constructed with rational methods are more likely to be function of they contain multiple GNN binding fingers. Over two-thirds of the proteins used in this experiment had 3 or more GNN binding fingers.

for rational design. The number of GNN targeting fingers used within each of the 69 operator sites ranges from 0 to 5, with the majority of operators containing 3 or more GNN sites as shown in Figure 3-11. The probability that a rationally designed zinc finger protein will bind its operator site is dependent on the number of GNN binding fingers in the proteins and has been studied for 3-finger proteins. If having more than 3 GNN binding fingers does not increase the probability of getting a functional zinc finger protein, then about 33 of the 69 proteins should be functional. Alternatively, the trend of additional GNN binding fingers contributing to proteins being function could continue above 3 GNN binding fingers. Extrapolating this trend with a second order polynomial predicts that 45 of the 69 proteins should be functional.

As mentioned previously, the objective function used to create P_{ZC35} initially was biasing the ends of the promoter sequence to have few GNN binding fingers, but additional constraints were added to mitigate this bias. To see if the quality of the operator sites near the ends of the sequence are worse, the difference from the median number of GNN binding fingers was plotted for each position as shown in Figure 3-12. The operator sites near the ends of the promoter do not appear to be systematically lower in quality than the operator sites for other regions of the promoter.

Of the 69 operator sites none of their sequences are found in the MG1655 *E. coli* genome and if any one base pair of any operator site is changed, the modified site will still not be found in the MG1655 *E. coli* genome. If any two base pairs of any operator are changed, then for only two of the operators (op-59 and op-28) will the modified sites be found in the MG1655 *E. coli* genome. Op-59's closest matching site on the genome is in a non coding region about 30 base pairs downstream of tsgA and about 200 base pairs upstream of nirB. If a zinc finger protein were to bind at this site, the transcription of tsgA

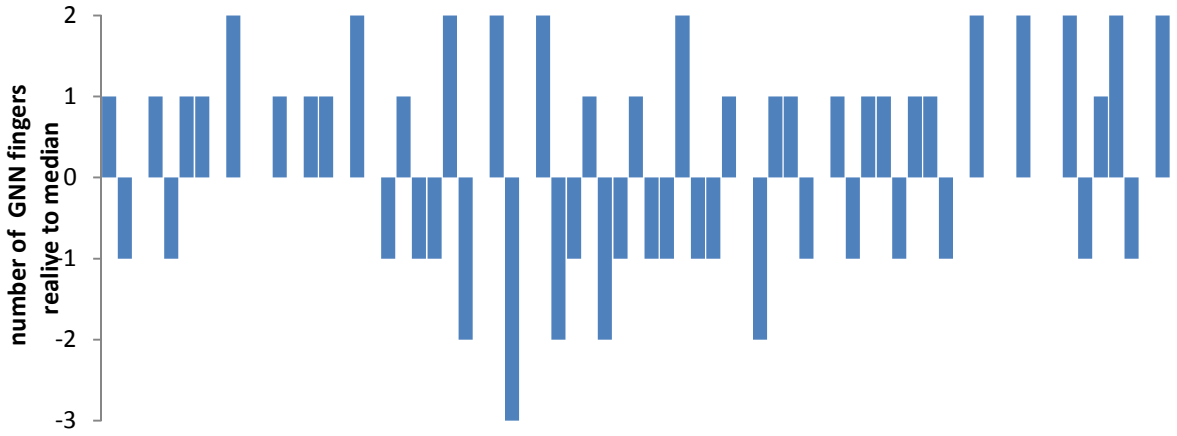


Figure 3-12 Quality of operator sites as a function of position. The median number of GNN binding zinc fingers this set of proteins was 3. The difference from that median value is plotted here. The left most bar is for op-65 and the right most bar is for op+4. The ends of the sequence did not end up biased to low quality operator sites. The distribution of quality appears even except for between op-60 and op-48 where no operator sites fall below the median number of GNN binding fingers.

should not be affected, but nirB transcription could be attenuated. Op-28's closest matching site on the genome is in the coding region of hcaR and about 225 base pairs upstream from hcaT. It is unclear if the binding of a zinc finger protein in the middle of gene can reduce transcription, so one or both of these genes could be affected by a protein binding at this site. Besides having limited hits in the genome, none of the 69 operator sites are with two base pair changes of any of the other 69 operators in P_{ZC35}.

Assay design for transcriptional attenuation experiment

With the P_{ZC35} promoter selected, the remainder of the attenuation assay could be built. The 69 zinc finger proteins were assembled using BglBrick based restriction cloning and placed on the plasmid pBbA5c-RFP. This plasmid has a p15A origin of replication, chloramphenicol resistance, constitutive expression of LacI, and the P_{lacUV5} promoter. A protein fusion was made between the zinc finger proteins and the red fluorescent protein (RFP), with the RFP on the C-terminal end of the protein. This fusion made it possible to easily verify that the induction system was working and that both transcription and translation of the zinc finger protein was occurring. A second plasmid contained the reporter operon of P_{ZC35} driving GFPmut3b along with a ColE1 origin of replication and kanamycin resistance. The reporter plasmid along with one zinc finger protein-RFP expression plasmid was transformed into DH10B.

To perform attenuation assays, 5mL overnight cultures were grown in LB medium, and then diluted by 20:1 into fresh M9 medium containing 1% glucose to a final volume of 155 µL in a 96 well plate. Inducer was added at the time of dilution into M9 medium. After growing to stationary phase at 37C, the absorbance at 600nm (A_{600}), RFP, and GFP were

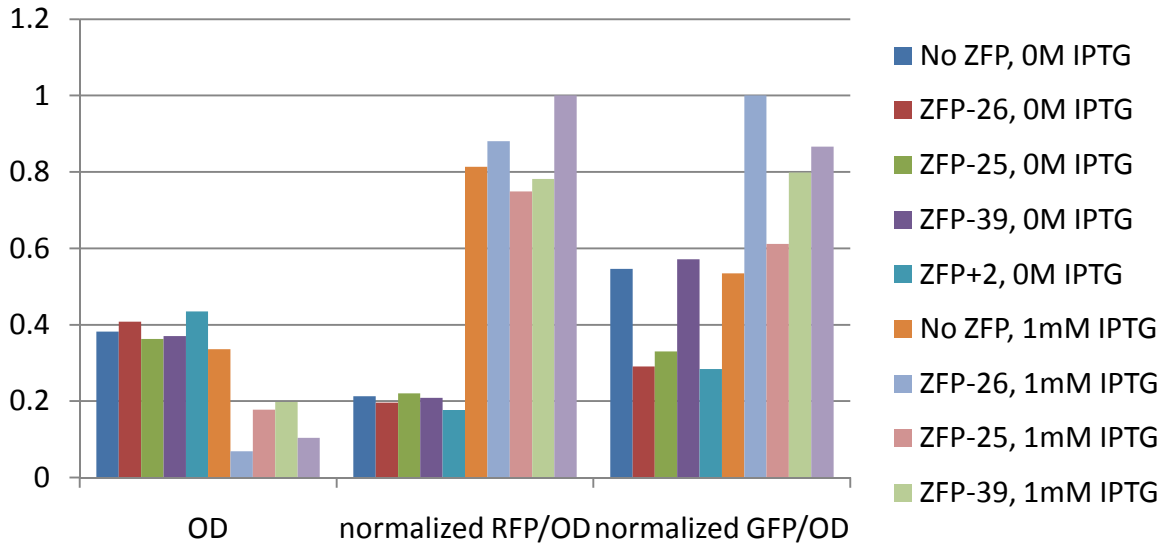


Figure 3-13 Initial experiment with ZFP-RFP fusion repressing GFP expressing operon. RFP/OD increased with induction indicating the ZFP-RFP fusions were expressed, but ZFP-RFP induction also lowered the OD and failed to repress the GFP expressing operon.

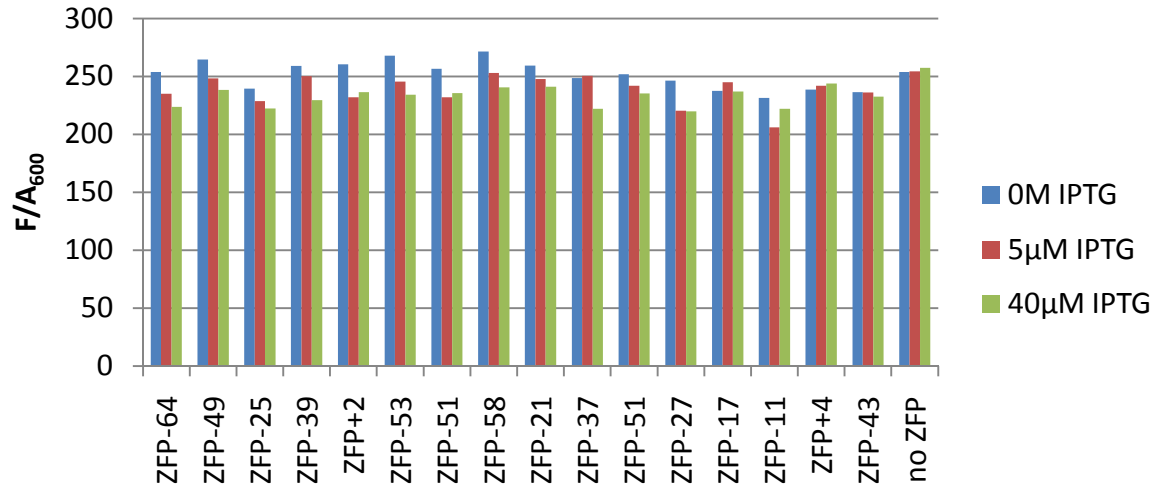


Figure 3-14 Early experiment with ZFP no longer fused to RFP. No growth inhibition was detected with these lower induction levels.

measured on a microplate reader.

Initial experiments with 1mM IPTG inductions showed that the zinc finger protein-RFP was expressed, because induction increased the RFP/A_{600} over 3-fold relative to the un-induced controls (Figure 3-13). However the expression of GFP on the reporter plasmid was not attenuated by the presence of the zinc finger protein-RFP fusion, and the cultures expressing the zinc finger protein-RFP fusion did not reach the same density as the un-induced cultures. The zinc finger protein-RFP fusions could have failed to fold correctly because they were in the context of a fusion, or they might have folded correctly but the

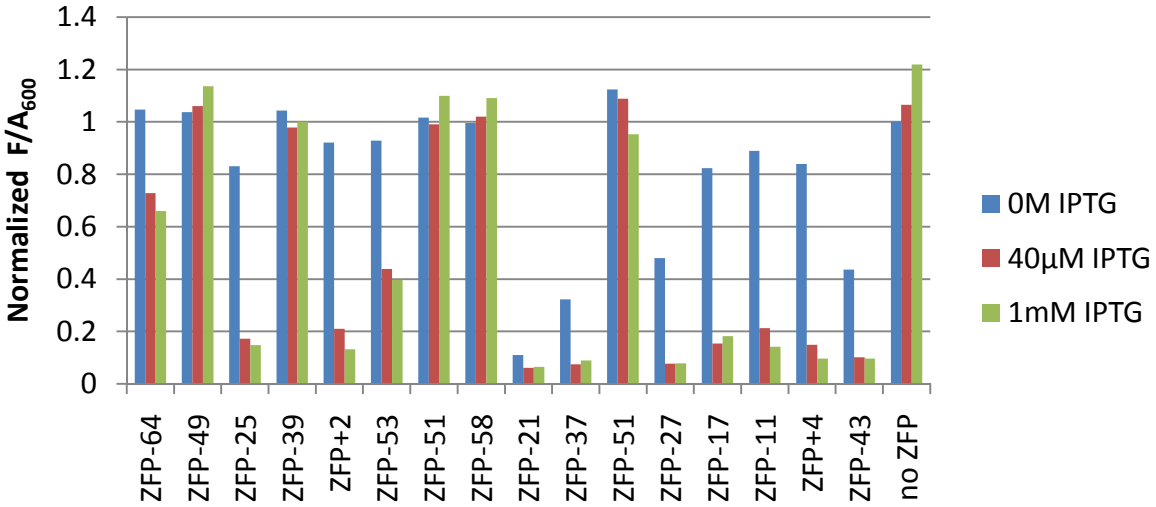


Figure 3-15 Demonstration of steric hindrance assay. Using mCherry as the reporter. All values have been normalized to the no ZFP 0M IPTG value.

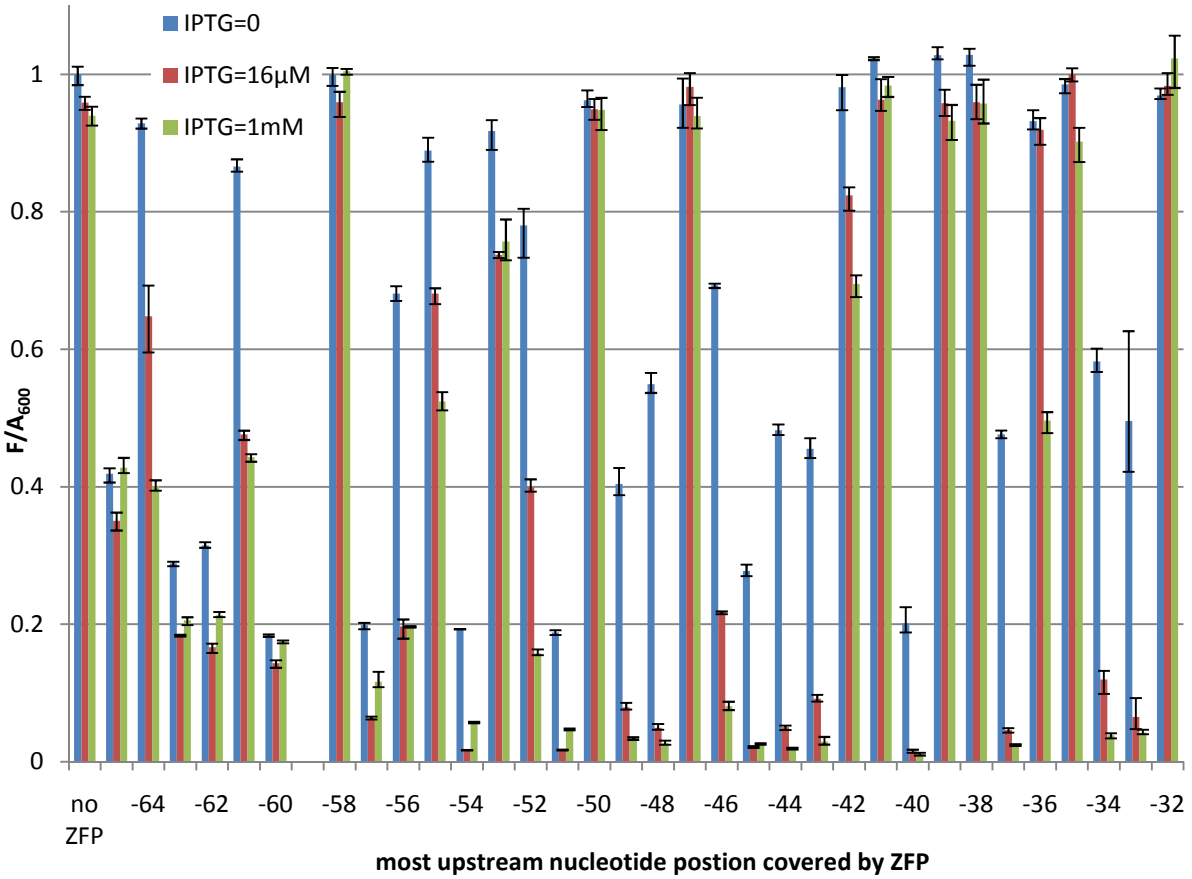


Figure 3-16 Transcriptional attenuation from the binding of zinc finger proteins to the promoter P_{ZC35} at various positions. The RFP/A_{600} values have been normalized to the un-induced (IPTG=0) no zinc finger protein control. Error bars are the minimum and maximum of 3 biological replicates. The positions listed on the x-axis are relative to the approximate transcriptional start site. No data was measured for position -59. For this data set the zinc finger proteins were expressed by a P_{lacUV5} promoter on plasmids containing the p15A origin of replication. Data for positions -32 through 4 is shown in the next figure.

zinc finger protein domain was prevented from binding to DNA because of the attached RFP. Since the expression of the RFP had already shown that the zinc finger proteins were successfully being transcribed and translated, the RFP was no longer needed. Thus RFP was removed from the zinc finger protein to eliminate the possibility that the fusion protein was causing the zinc finger protein domain to fail to bind the DNA. The slow growth of the zinc finger protein-RFP expressing cells may indicate the fusions are not folding properly or that the proteins are toxic.

The follow up experiment, without RFP, was performed with a range of sub-saturating inductions. Because the cultures grew slowly on M9 medium, the M9 medium was dropped in favor of EZ-Rich medium (Teknova). This medium has a low auto-fluorescence like M9, but also has amino acids and trace metals. All steric hindrance assays

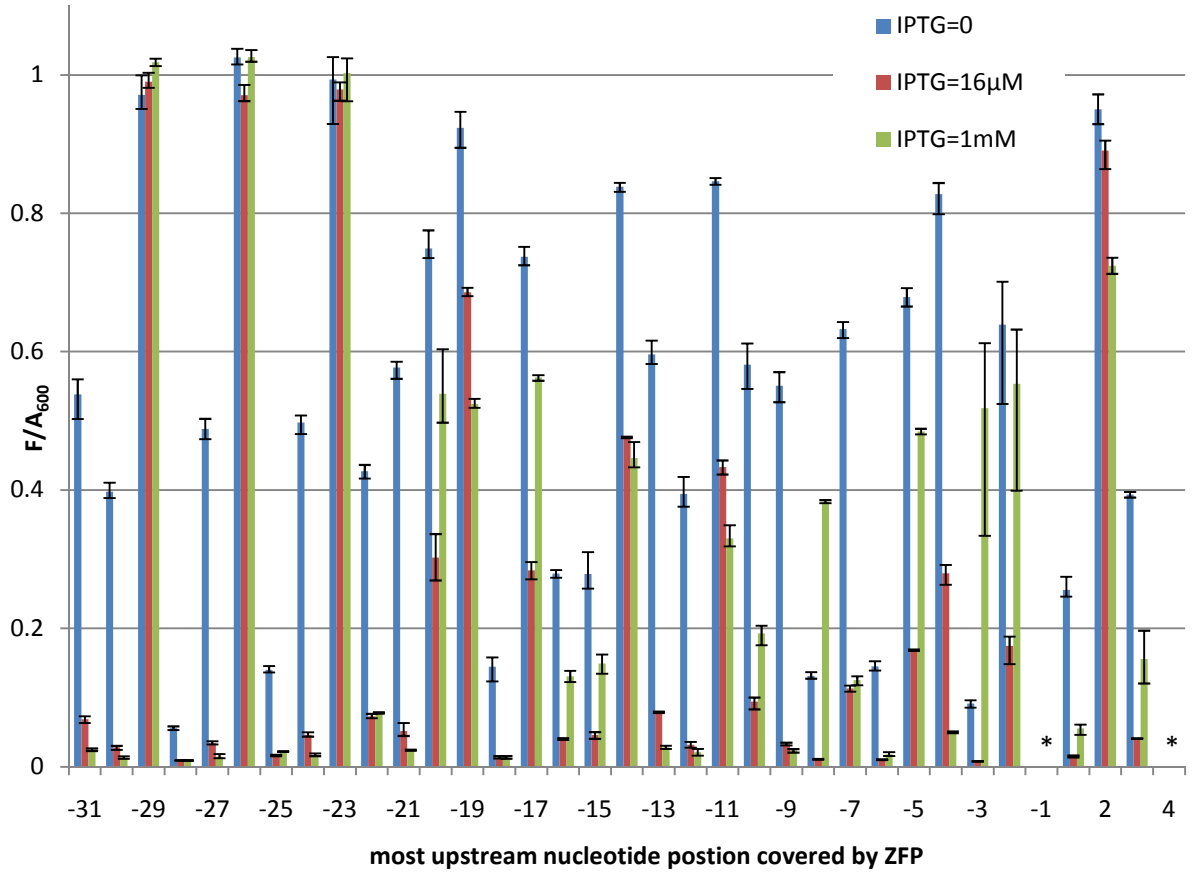


Figure 3-17 Transcriptional attenuation from the binding of zinc finger proteins to the promoter P_{ZC35} at various positions. The RFP/A_{600} values have been normalized to the un-induced (IPTG=0) no zinc finger protein control. Error bars are the minimum and maximum of 3 biological replicates. The positions listed on the x-axis are relative to the approximate transcriptional start site. No data was measured for positions -1 and 4. For this data set the zinc finger proteins were expressed by a P_{lacUV5} promoter on plasmids containing the p15A origin of replication. Data for the negative control (no ZFP) and positions -65 through -32 is shown in the previous figure.

done from this experiment on were done with EZ-Rich medium. The reduced induction levels did remove the growth inhibition, but the attenuation of GFP/A_{600} was only 10% or less (Figure 3-14). Unfortunately there was not a control without a GFP plasmid to reference as a minimum signal level.

Because *E. coli* fluoresces in the same range as GFP, GFP is not a good choice for measuring low signal levels. Therefore the GFP on the reporter plasmid was replaced with mCherry, a RFP variant that has been codon optimized for *E. coli*[52] and has less overlap with the fluorescence of *E. coli*. An experiment done with both saturating (1mM) and non-saturating (40μm) IPTG concentrations gave promising results. Saturating inductions of 5 of the 16 zinc finger proteins resulted in attenuations of over 90% (Figure 3-15).

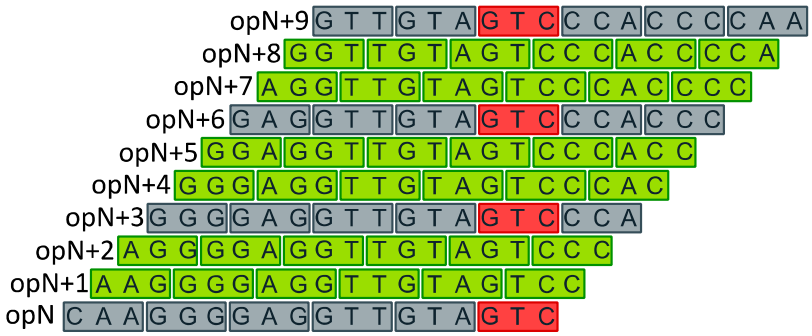


Figure 3-18 Zinc finger proteins that fail to attenuate transcription are often separated by multiples of 3. The set of 6 fingers that make up a single zinc finger protein are shown on each line and are labeled with the DNA triplet each finger targets. Adjacent rows contain operator sites that differ in position by one base pair, and the labels on the left give their relative positions. Proteins that only contain fingers with functional sets of fingers are shown in green, and proteins that contain a non-functional set of fingers are shown in grey with the non-binding finger in red. In every third row the non-binding finger occurs.

Additionally, the growth rate in exponential phase was similar for all cultures including the negative control, thus demonstrating no toxicity exists from the expression of zinc finger proteins at these levels.

Positional dependence in transcriptional attenuation experiment

At this point the transcription attenuation assay appeared to be working well enough to run an experiment with the full set of 69 zinc finger proteins done in biological triplicate. Three colonies were picked from each zinc finger protein transformant into 800µL of LB media in a deep 96 well plate. The plate was sealed with Aeroseal breathable seals (no. B-100, Excel Scientific) and incubated at 37°C and 300 RMP overnight. Each well of the LB plates were diluted 20:1 into new deep well plates containing 800µL of EZ-Rich media and either 0, 40µM, or 1mM of IPTG. After 24 hours 150 µL of culture was transferred to black walled, clear bottom 96 well plates for reading.

Interpretation of the data from this experiment involved some subtleties and resulted in additional questions being posed. The transcriptional attenuation data is split across Figure 3-16 and Figure 3-17. This data shows that over 100 fold attenuation of transcription is possible from zinc finger protein binding (position -3 for 16µM IPTG, position -28 for 16µM or 1mM IPTG). At many positions the leaky expression of the zinc finger protein from the P_{lacUV5} promoter was enough to cause the reporter to be attenuated by 50% or more, indicating that these zinc finger proteins likely have small dissociation constants. Several of the zinc finger proteins failed to give substantial attenuation at any expression level. These proteins either were targeted to insensitive locations on the promoter or these zinc finger proteins failed to bind their intended targets with a strong enough interaction.

Many of the zinc finger proteins that failed to attenuate transcription have operators separated by a multiple of 3 base pairs. This is not surprising given how rationally designed

zinc finger proteins are constructed. If a zinc finger is used in a context that renders it a poor binder, then there will be between 3 and 5 other proteins that also use the offending zinc finger protein in the problematic context (see Figure 3-18). The exact number of proteins affected will depend on the amount of context require for the finger to fail. If both flanking fingers are required for the detrimental context, then only 3 proteins will contain the stretch of three fingers necessary, but if only one neighboring finger contributes to the context, then there will be 5 proteins that have the problematic pair of fingers.

Several of the zinc finger proteins failed to attenuate transcription by more than 20% when fully induced and are separated by multiples of 3. Specifically the set op-41, op-38, op-35, op-32, op-29, op-26, and op-23 all gave minimal attenuation but one or more of their neighboring sites had over 90% attenuation. The strong repression at neighboring sites makes it unlikely that these minimally attenuating zinc finger proteins failed to cause more attenuation because proteins tightly bound at their positions do not sterically hinder RNA polymerase. Instead, the more likely senario is that this set of proteins has relatively high disociation constants for their intented operators sites and this weak binding was not able to hinder RNA polymerase. With the data presented here, it is not possible to prove this hypothesis, but it should be possible to make draw stronger conclusions once

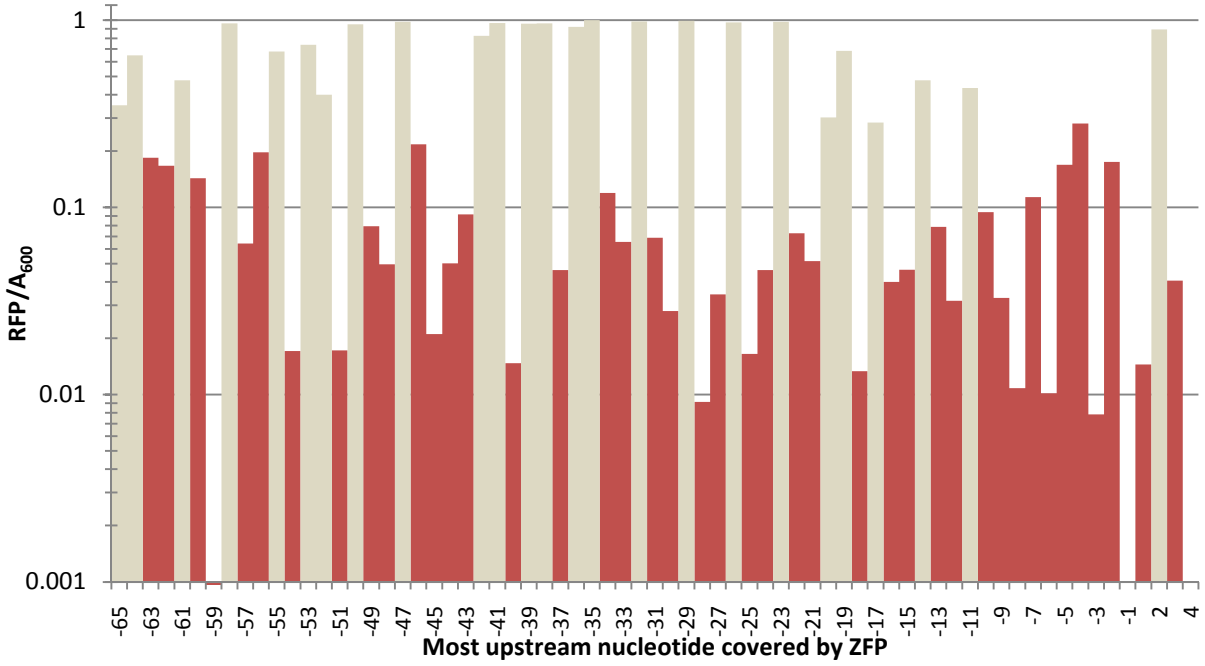


Figure 3-19 Transcriptional attenuation as a function of operator sites position relative to the approximate transcriptional start site with weak induction (16μM IPTG). Success rates for rationally designed zinc finger proteins indicate that 23 of the 66 zinc finger proteins assayed here should fail to bind their target operator site. Therefore the data for the 23 worst performing zinc finger proteins is shown in grey. The contour from the remaining red data points shows the attenuation as a function of position for the zinc finger proteins that are likely functional. Values were not measured for positions -59, -1, and 4.

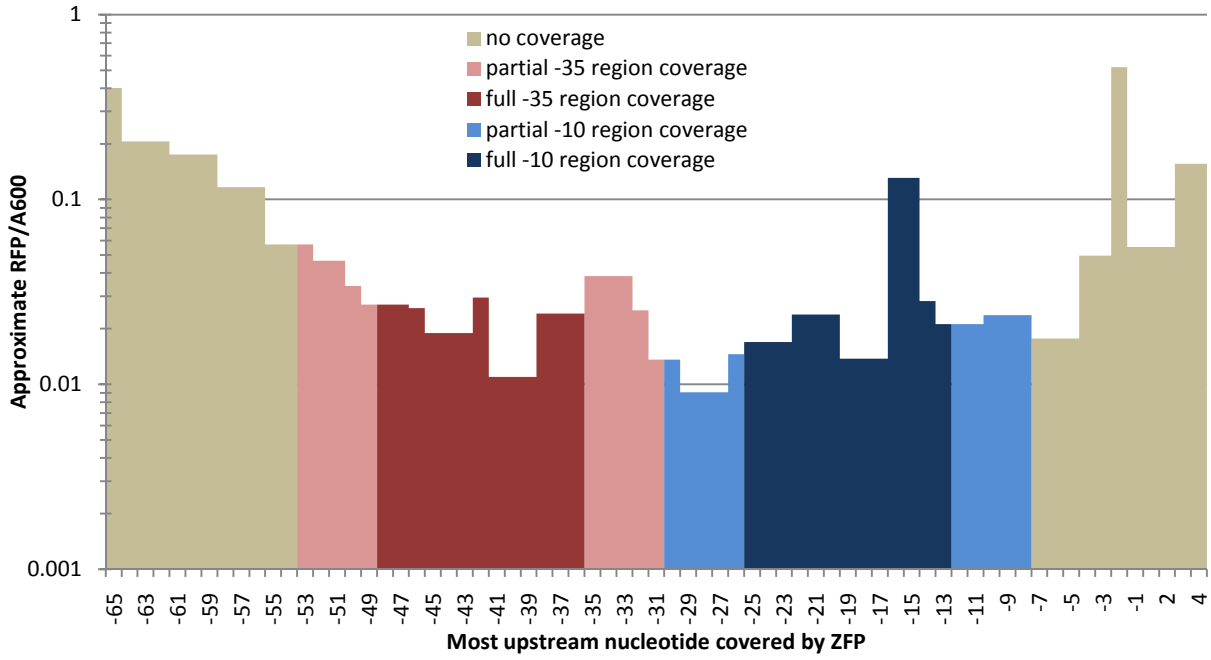


Figure 3-20 A lower resolution approximation of the attenuation as a function of position for saturating induction (IPTG=1mM). The value plotted at each position is the minimum of the value measured for that position and the two neighboring positions. The colors indicate if a zinc finger protein bound at that position would cover all 6 base pairs (full coverage) of either the -10 or -35 region of the promoter or 1-5 base pairs (partial coverage) of the promoter.

disocation constants have been measured.

If these poor attenuators are an artifact of non-functional zinc finger proteins, then they should not be considered when determining the amount of attenuation possible at a position due to the binding of a functional zinc finger protein. Earlier in this chapter it was estimated that 24 of the 69 zinc finger proteins that target P_{ZC35} would fail to bind their intended operator sites. Only 66 of the zinc finger proteins were used in this experiment, and thus the number of failures should scale to 23. The worst performing set of 23 zinc finger proteins can be found by using a threshold of less than 43% attenuation. Evaluating the results of the experiment minus the data from these 23 low attenuators will likely give a more informative view of how attenuation varies with operator position. A plot with these 23 low attenuators differentially colored is shown in Figure 3-19.

The local attenuation maxima (positions -51, -40, -28, -18) in this plot have some periodicty with an average spacing of 11 base pairs. The B-DNA geometry completes a turn in 10.5 base pairs and the Z-DNA geometry has 12 base pairs to a turn. B-DNA is the form most commonly found in cells, but the Z-DNA form occurs in DNA with alternating purine-pyrimidine sequences[53]. A short segment of alternative purine-pyrimidine sequence occurs in the P_{ZC35} promoter with the sequence ACATA from positions -33 to -29. Of the 3 periodic segments listed above, only the 12 base pair segment (positions -40 to -28) contains a possible Z-DNA region. The remaining two segments have an average spacing of

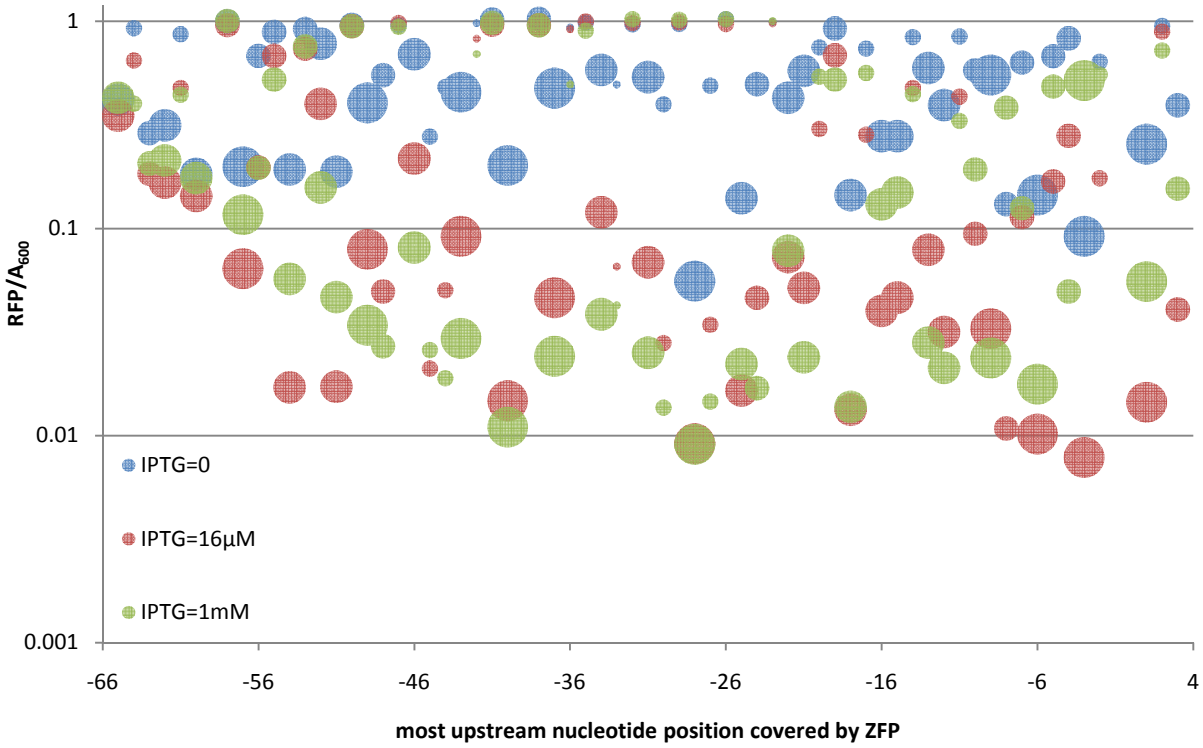


Figure 3-21 Transcriptional attenuation as a function of both position and number of GNN binding fingers in a zinc finger protein. The width of the bubble is proportional to the number of GNN binding fingers contained in each zinc finger protein. Many, but not all, of the weak attenuators have a low number of GNN fingers.

10.5 base pairs. A similar pattern, of approximately 10 base pairs extending from -54 to -6, has been observed in DNA footprinting analysis of RNA polymerase[54].

When the position of these local attenuation maxima are expressed as the most upstream base pair covered by the zinc finger protein, they occur at positions -34, -23, -11 and -1. Two of these maxima are near the center of the important -35 and -10 regions where contact between the σ factor and DNA occur. While it is not surprising the local maxima are spaced out by the length of a DNA twist, it is unexpected that the local maxima would have partial coverage of the -35 or -10 region. Maximum attenuation was expected to happen when the zinc finger protein was centered on the -35 or -10 region. This result may have to do with the kinetics of RNA polymerase scanning of the DNA and a potential directional bias, but this is well beyond the scope of this study.

An alternative way of thinking about which of the data points are likely to be non-functional zinc finger proteins is to consider how many GNN fingers each protein contains. In Figure 3-21 the average attenuation data for each position is shown in a bubble plot where the size of the width of the bubble is proportional to the number of GNN fingers.

Because of the non-functional zinc finger proteins, this experiment cannot give single base pair resolution of the attenuation as a function of position; however, an

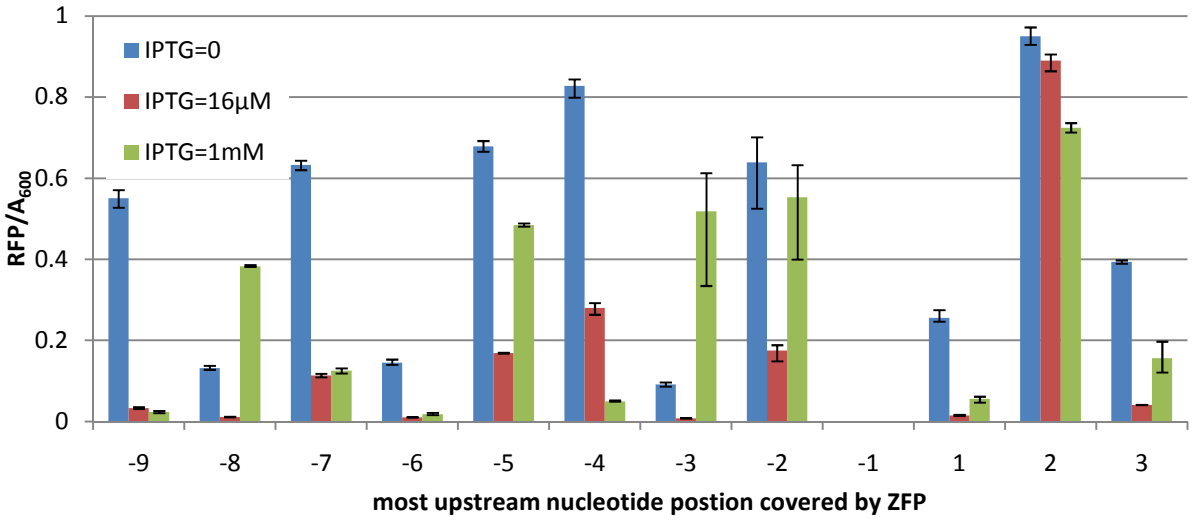


Figure 3-22 A portion of the transcriptional attenuation data showing the attenuation is not monotonic with induction when the zinc finger proteins are expressed from a P_{lacUV5}/p15A plasmid.

approximation of a lower resolution is possible. Neighboring positions will likely have similar attenuation levels when bound with zinc finger proteins having equal disociation constants. There are possible discontinuities in the attenuation function. For example, at the transition from the zinc finger protein binding just outside of the -35 region to having 1 base pair of the -35 region covered by the zinc finger protein. Even at these transition points, the protein footprint will probably extend beyond the operator site and the energy required to displace this protein overhang will likely drop off with distance from the operator site. Therefore, even at this very binary transition from covering the operator to partially covering the operator, there can be a gradient response spread over several base pair positions. To approximate a lower resolution attenuation function, the value for a position was set to the minimum of the values measured for that position or either of the neighboring positions. The reduced resolution data is shown in Figure 3-20. This approximation may overstate the attenuation possible at some positions, but if the actual attenuation possible at each position does not have large discontinuities, then this approximation, while not inherently conservative, should be at least be close to the actual value.

Some of the zinc finger proteins displayed a non-monotonic behavior where attenuation of the reporter did not always increase with increasing induction of the zinc finger protien. This is most prevelant from positions -20 to 3 and a portion of region is shown in Figure 3-22. Some of these non-monotonic zinc finger proteins appear toxic at high expression levels, but the two lowest A_{600} values are from zinc finger proteins with monotonic responses. The source of this non-monotonic behavior for the zinc finger proteins that do no display toxicity is unclear. Because only final A600 measurements were done and not growth rate measurements, it is possible that the non-monotonic zinc finger proteins do inhibit the growth rate but not the final cell density.

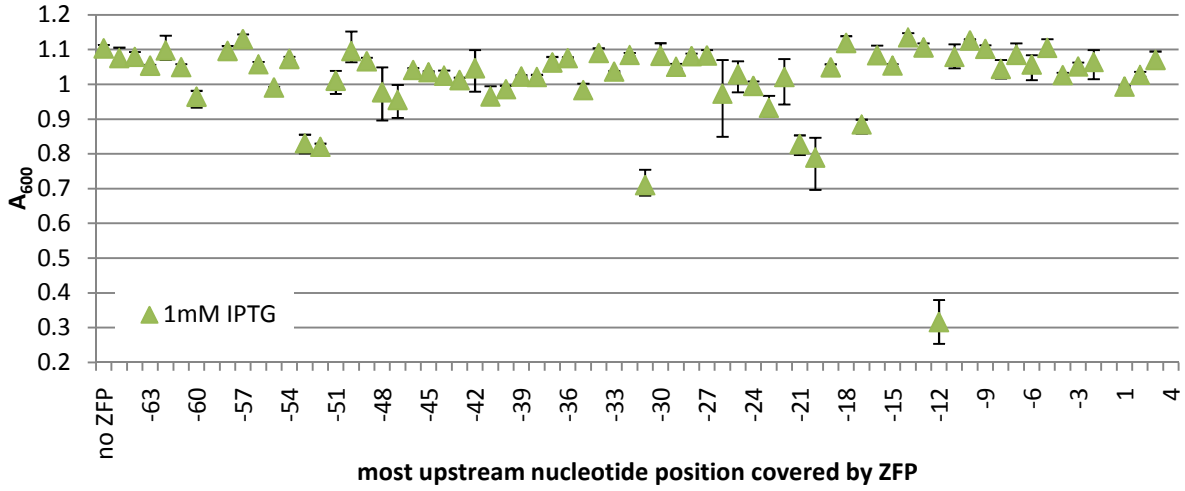


Figure 3-23 Final absorbance values with saturating (1mM IPTG) induction in a transcriptional attenuation experiment. Several of the zinc finger proteins appear to be toxic at high induction levels.

The combination of the toxicity issue combined with the high leakage of the P_{lacUV5} promoter motivated a move to different zinc finger protein expression plasmid. By moving from a P_{lacUV5} promoter on a p15A plasmid to a P_{BAD} promoter on an SC101** plasmid, it

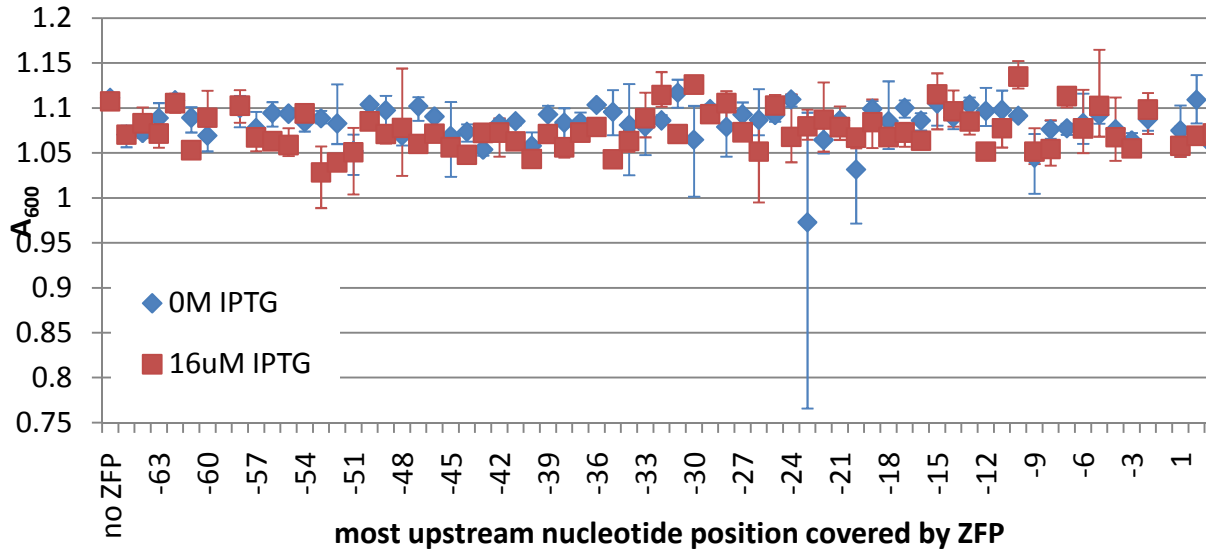


Figure 3-24 Final absorbance values for low inductions in transcriptional attenuation experiment. At these induction levels, there does not appear to be strong toxicity, although there may be a reduction in growth rate due to weak toxicity that would not be captured by this data. The low error bar for position -23 is only due to one data point and is likely not reproducible. The error bars are the maximum and minimum value for 3 biological replicates.

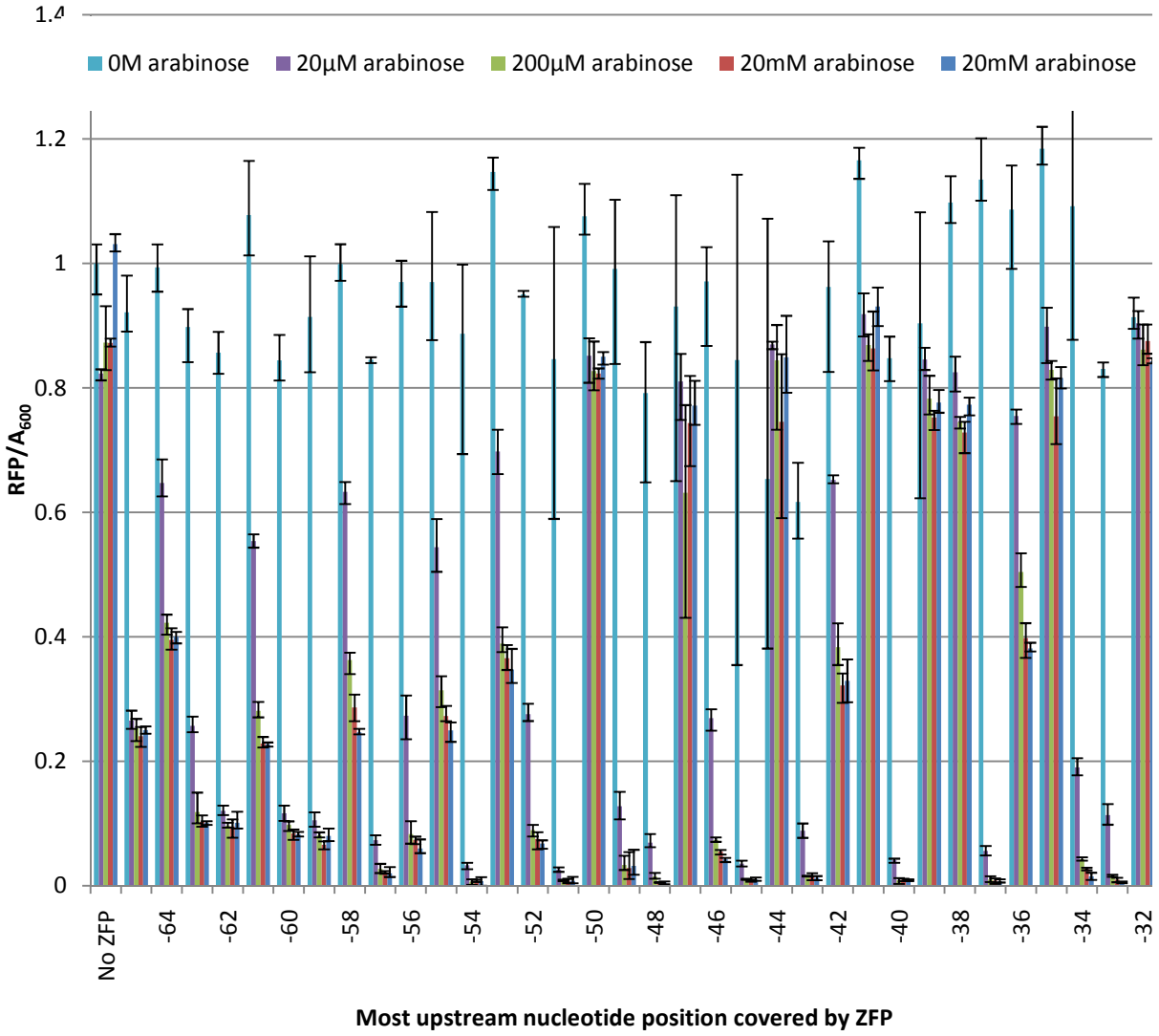


Figure 3-25 Transcriptional attenuation measurements with the zinc finger protein expressed from a P_{BAD}/SC101 plasmid. The other half of this data set can be found in the next figure. Error bars are min/max of 3 biological replicates.**

was hoped that no toxicity would be aparent , the reporter would be sensitive to a larger portion of the induction range, and that the non-induced state would be more similar to the negative control where no zinc finger protein is present. The host strain for P_{BAD} based experiments was DP10[55]. This strain has been modified to improve the linearity P_{BAD}'s induction.

The final A_{600} values for the P_{BAD}/SC101** plasmid are lower than from the P_{lacUV5}/p15A plasmid, but this is due to the plasmid backbone and not the expression of the zinc finger proteins (Figure 3-27). Even though the copy number of the plasmid was reduced, the concentration of chloramphenical was not reduced from 34 µg/mL, and this resulted in slower growth and lower final cell densities.

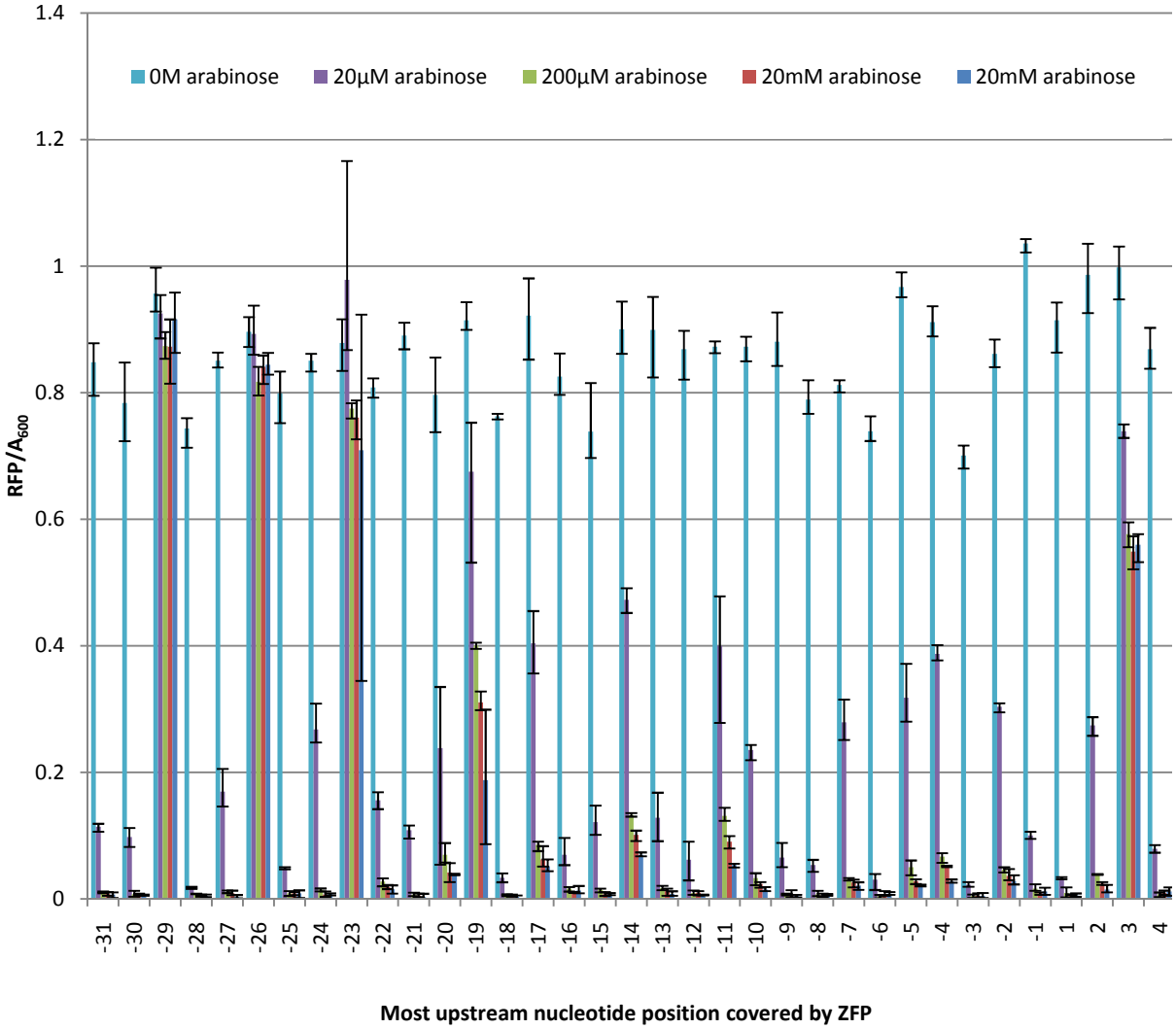


Figure 3-26 Transcriptional attenuation measurements with the zinc finger protein expressed from a P_{BAD}/SC101 plasmid. The other half of this data set can be found in the previous figure. Error bars are min/max of 3 biological replicates.**

The final cell density data contains a significant amount of noise that makes observing toxicity difficult. One 96 well plate of samples shows higher cell densities and larger variability between biological replicates. This plate likely was on the top of a stack of plates loaded onto the plate reader, and it was able to evaporate for about 30 minutes while the other plates in the stack were read. Future experiments using stacks of plates should be done with an empty plate placed on the top of the stack to prevent evaporation. Additional controls that are replicated across the plates would also help to clearly identify plate-to-plate variation and allow for systematic plate variations to be normalized out.

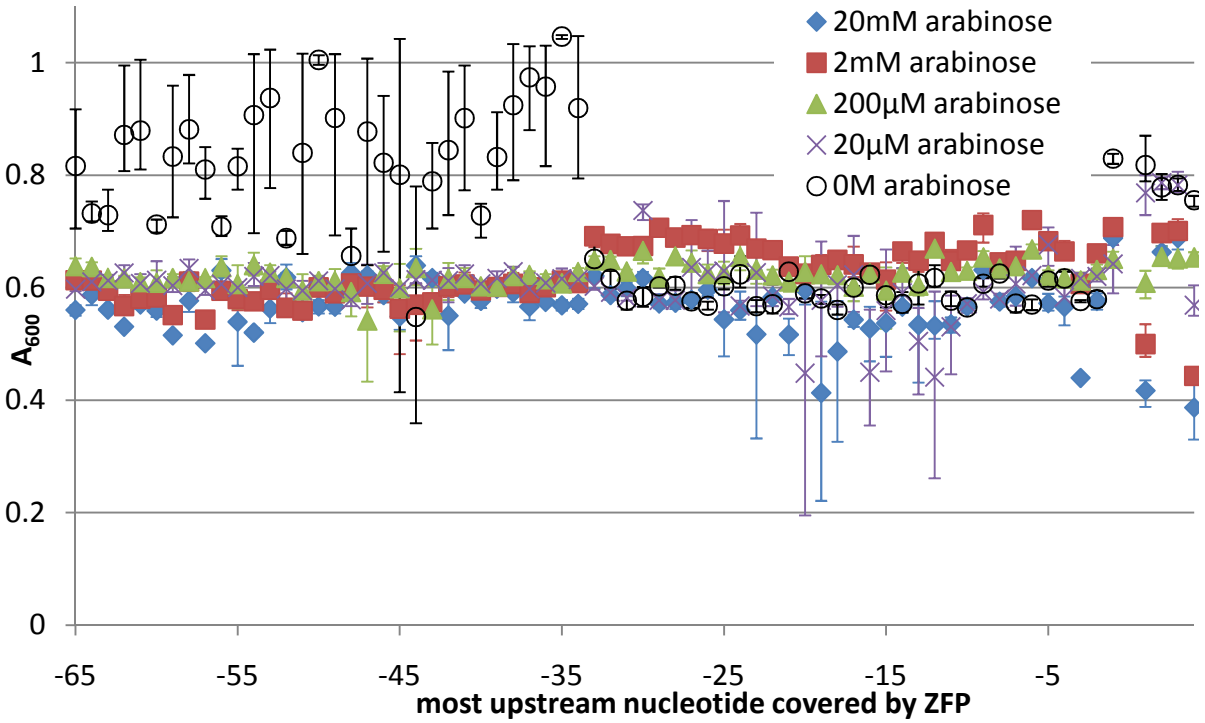


Figure 3-27 Final absorbance measurements with zinc finger proteins expressed from a P_{BAD}/SC101 expression plasmid. Despite the technical noise in this assay, it appears only three zinc finger proteins, at positions -3, 1, and 4, show significant toxicity. Error bars are min/max of 3 biological replicates.**

A saturating induction of the P_{BAD}/SC101** expression plasmids shows reduced toxicity relative to the previously used P_{lacUV5}/p15A expression plasmids. Significant toxicity was only observed for positions -3, 1, and 4, in contrast to the previous experiment where saturating induction of the PlacUV5 /p15A plasmid resulted in reduction of final A_{600} levels by over 10% for 8 different zinc finger proteins. Surprisingly, these two sets of zinc finger proteins that have been toxic in some contexts are non-overlapping. Such a result would be possible if a zinc finger protein was binding the origin of replication of a plasmid and causing a reduction in the copy number of that plasmid and the antibiotic resistance gene it contains. While this explanation is possible, the probability that 8 of the 69 zinc finger proteins all have binding sites within the ~4000 base pairs of the P_{lacUV5} expression plasmid is extremely low.

The transcriptional attenuation data generated by expressing the zinc finger proteins from P_{BAD}/SC101** plasmids is similar to the P_{lacUV5}/p15A data set. The attenuation data is shown in Figure 3-25 and Figure 3-26. The analysis done on the P_{lacUV5}/p15A data set was repeated with this new data set. The maximum attenuation was over 300 fold and was achieved at position -21. Some of the zinc finger proteins failed to give substantial attenuation, and because these low attenuation proteins have positions that differ by multiples of 3, they are likely due to a zinc finger not functioning in this context. Of the set of 69 zinc finger proteins tested, 24 of them are expected to fail.

In Figure 3-28 the data representing the 24 lowest attenuating zinc finger proteins are displayed in grey in order to highlight the remaining data points that have a higher probability of being from functional zinc finger proteins. Alternatively, by plotting the minimum of the value measured for a position and its two neighboring positions, a plot with a lower resolution approximation of the attenuation possible can be generated as shown in Figure 3-29. From this plot it is clear that if a functional zinc finger protein is used, then more than 80% attenuation is probably possible for positions -53 to 2 and attenuation of over 99% may be possible for the majority of these positions.

By considering the probability that each zinc finger protein is functional, it becomes easier to notice relationships within the data set. In Figure 3-30 it is striking that from -65 to -53 all of the zinc finger proteins result in over 59% attenuation. But from -50 to -23, 10 of the zinc finger proteins gave less than 30% attenuation. There are several possible explanations for this difference, but unfortuantely they can not be eluciated with the current data. One explanation is that the consensus promoter sequence is more constrained in the region covered by the -50 to -23 positions, and these sequence constraints resulted in a higher probability of non-functional zinc finger proteins. Another posibility is that poor binders may be able to cause over 59% attenuation in the region from -65 to -53 while similarly poor binders can only cause up to 30% attenuation in the region from -50 to -23. This would make mechanistic sense as -53 is the border between where there is no coverage of the -35 and -10 regions. Positions upstream of the -53 site may only be disrupting the RNA polymerase holoenzyme sliding along the DNA while

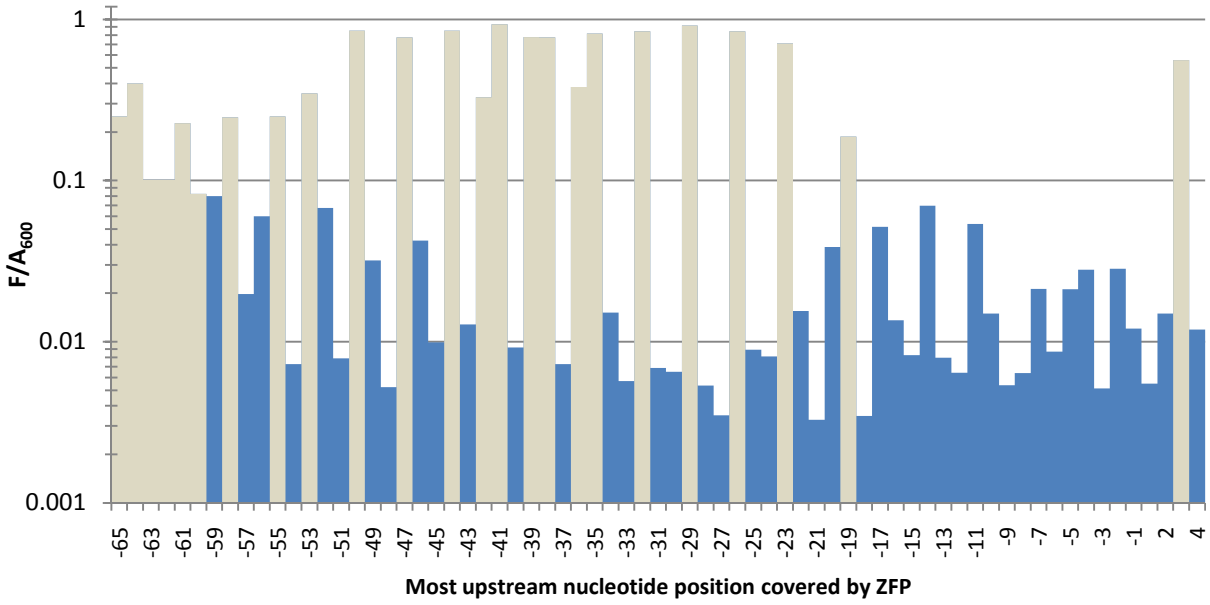


Figure 3-28 Transcriptional attenuation with zinc finger proteins expressed from the P_{BAD}/SC101 plasmid and a saturating induction (20mM arabinose). The 24 zinc finger proteins giving the least attenuation are displayed in grey because they have a high probability of being non-functional zinc finger proteins. For this data set, the cutoff attenuation value separating the 24 poor attenuators from the 45 good attenuators was 0.0825.**

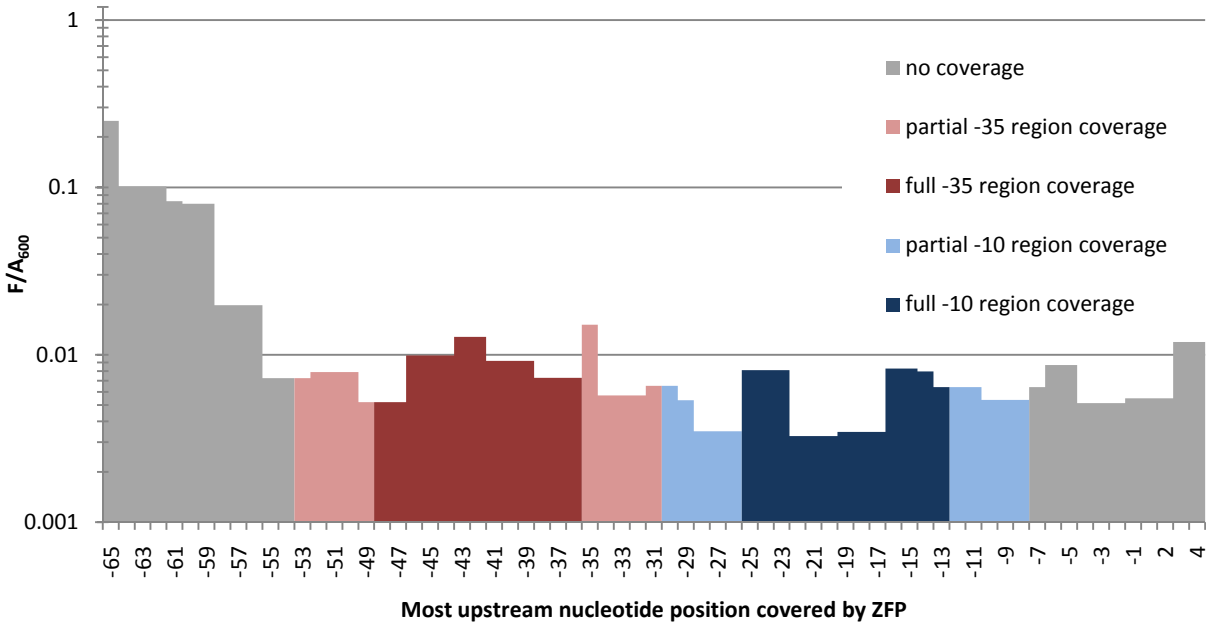


Figure 3-29 A lower resolution approximation of the attenuation as a function of position for saturating induction (20mM arabinose). The value plotted at each position is the minimum of the value measured for that position and the two neighboring positions. The colors indicate if a zinc finger protein bound at that position would cover all 6 base pairs (full coverage) of either the -10 or -35 region of the promoter or 1-5 base pairs (partial coverage) of the promoter.

weakly bound. This contrasts with the region from -50 to -23 where the zinc finger protein must compete with the stronger and specific binding of the σ^{70} portion of the RNA polymerase holoenzyme. If the weakly bound zinc finger protein can disrupt the non-specific interactions between the RNA polymerase holoenzyme but not the specific interaction with a promoter, then such a discontinuity would be expected.

The region from -20 to 2 also has a different behavior than either the -65 to -53 region or the -50 to -23 region. Within the -20 to 2 region all zinc finger proteins created over 80% of attenuation, and all but one zinc finger protein caused over 93% attenuation. These zinc finger protiens all have coverage within the -11 to 2 segment where DNA strand separation begins and the transciption initiation bubble occurs[56]. Because all 6 of the zinc finger proteins in the -20 to 2 region that contain exactly 2 GNN fingers were able to cause more than 93% attenuation, it is likely that even poor DNA binders are able to compete against the RNA polymerase attempting to form the transcription initiation bubble.

Measurement of dissociation constants for zinc finger proteins

The attenuation data in the previous section indicates there are many positions along a promoter where binding a zinc finger protein has the potential to attenuate transcription. Drawing stronger conclusions about the relative amount of repression possible at different positions will require more data. Measuring dissociation constants for

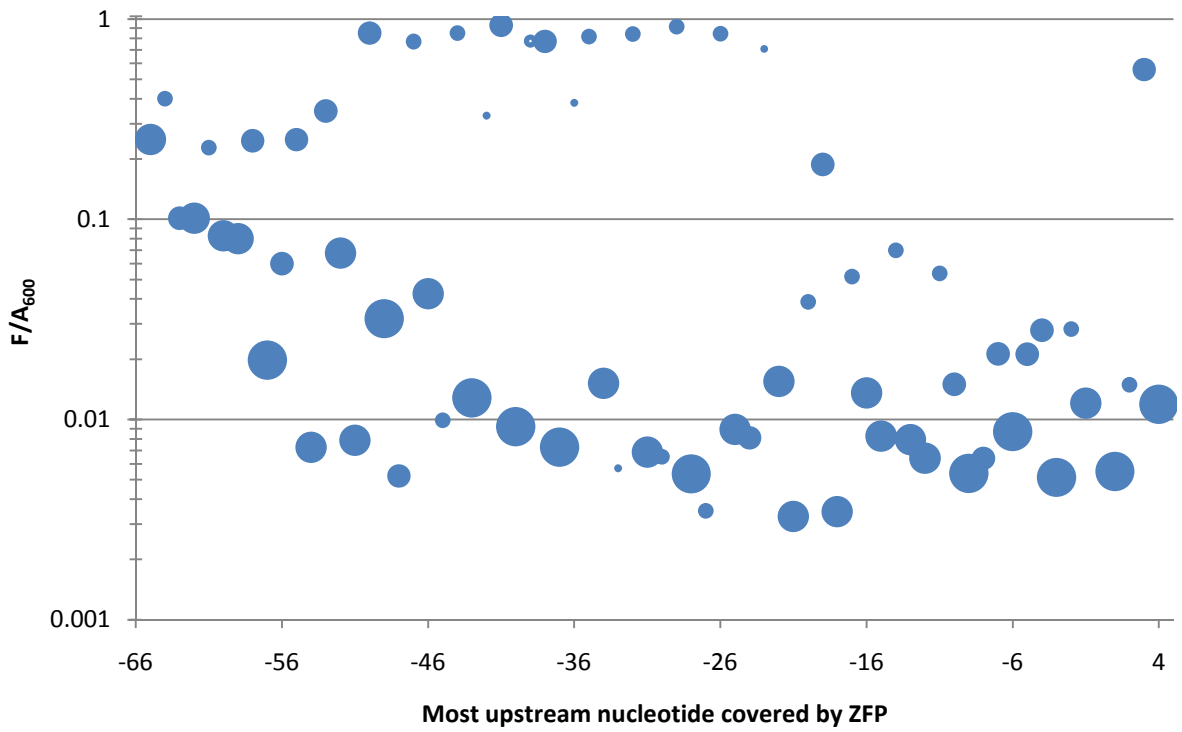


Figure 3-30 Transcriptional attenuation as a function of both position and number of GNN binding fingers in a zinc finger protein. The P_{BAD}/SC101 zinc finger protein plasmids were induced with 20mM arabinose. The width of the bubble is proportional to the number of GNN binding fingers contained in each zinc finger protein. Open circles represent proteins without any GNN fingers. Many, but not all, of the weak attenuators have a low number of GNN fingers.**

the zinc finger proteins with their target operator sites should yield additional insight. Measuring the dissociation constants for all 69 zinc finger proteins would be ideal, and this will require a fairly rapid method.

There are many methods for measuring dissociation constants. The gold standard is to use radioactively labeled DNA in an electrophoretic mobility shift assay (EMSA) where a DNA-protein complex runs slower on a gel than non-complexed DNA. These assays are relatively time consuming and the electrophoresis buffer and gel environment may not be representative of *in-vivo* conditions.

Fluorescence anisotropy assays use polarized light to measure the tumbling rate of fluorescently labeled DNA. When the protein binds to the DNA the mass increases and the tumbling rate slows. Fluorescence anisotropy assays can be performed in any buffer and can be rapidly performed on a plate reader. Published reports on using this assay with zinc finger proteins differ on how to setup the experiment with one source claiming the need for internal labeling of the operator site[57] and another source end labeling the DNA but fusing maltose binding protein to the zinc finger protein[58]. When internally labeling the operator site, it is unclear if the difference in the tumbling rate of the fluorophore is due to reduced tumbling of the DNA-protein complex or if the linker between the fluorophore and

DNA has reduced mobility when the zinc finger protein is bound. The cost to create a library of internally labeled DNA strands is high because a single primer with a fluorophore cannot be re-used to create many operators.

Equilibrium dialysis has been reported as a general method for measuring dissociation constants, but it has not yet been done with zinc finger proteins. The zinc finger protein is placed in a dialysis membrane which it cannot penetrate but can be crossed by DNA operators. Then a measurement of the equilibrium DNA concentration outside of the membrane can be used to calculate the dissociation constant. This method can be performed in any buffer and the does not require the use of labeled DNA.

A variation on equilibrium dialysis is equilibrium filtration where an equilibrated mixture of protein and DNA is placed in a spin filter that retains the protein, and ~10-20% of the solution is filtered [59]. The DNA concentration in both the eluted and retained volumes is measured and used to calculate the dissociation constant. Equilibrium filtration is better suited for small samples and potentially faster than equilibrium dialysis. The filtration method does assume that the inverse of the off rate will be much larger than the duration of the assay.

Some work has been done on a computational method for determining the dissociation constant of zinc finger proteins [58]. However this study was limited to 3-finger proteins, and it is unclear if the method will work equally well on 6-finger proteins, where the geometric constraints on the protein backbone are different [22].

Using an *in-vivo* method it is possible to distinguish high and low affinity zinc finger proteins [39]. By using a one-hybrid system to activate the transcription of an antibiotic, it was possible to determine if the dissociation constant of a zinc finger protein was above or below 1μM. Another study using a one-hybrid system found a strong correlation between transcription rates and zinc finger protein strength [60]; however because this work was performed with Zif268 and binding site variations, it is unclear if similar results would be found for rationally designed zinc finger proteins and their target operator sites.

Because the *in vivo* and computational methods are not yet well developed and tested enough to determine the dissociation constants of the zinc finger proteins in this study, *in vitro* methods were pursued. All of the *in vitro* methods require purifying the zinc finger protein first.

Purification of zinc finger proteins

Zinc finger proteins were cloned into a pET-29 expression plasmid. This vector contains a pBR322 origin, confers kanamycin resistance and the cloning site has an N-terminal S-tag along with a C-terminal 6xHis-tag. The 6xHis-tag enables fast purification of the protein using Qiagen's Ni-NTA spin columns. The S-tag can be used to quickly quantify the amount of protein in whole cell lysate using an enzymatic assay with a colorimetric output. Additionally, if the 6xHis-tag purification fails, a slower purification method can be done using a column with affinity for the S-tag.

Developing a purification protocol for these zinc finger proteins was difficult because they are toxic at high expression levels. The *E. coli* strain BLR(DE3) was used for protein over-expression because the zinc finger proteins contain repeated sequence and this strain is recA-; therefore this strain is less likely to delete repeated sequences through homologous recombination. The keys to obtaining substantial amounts of protein were to

minimize pre-induction expression and to induce near the relatively high cell density of A_{600}=1.6. Including the plasmid pLysS helped reduce leakage by producing T7 lysozyme, which inhibits T7 polymerase. This lysozyme is also helpful to have for the cell lysis step of the purification where a sonication step would be extremely time consuming. At all stages, both on solid media and for liquid culture, the cells were grown on LB supplemented with 1% glucose. The presence of glucose increases the inhibition of the Lac promoter driving expression of the T7 polymerase.

Care was taken to avoid having the cells enter stationary phase any more than necessary. A fresh transformation was made before each purification and was only grown long enough to obtain easy to pick colonies. Colonies were picked into 5 mL of LB with 1% glucose and grown for 8 hours at 37°C. Cultures were then placed at 4°C overnight. This was done to keep them from entering stationary phase. The following day a 50:1 dilution was done into 5mL of fresh LB with 1% glucose and the cultures were grown at 37°C until they reached A_{600}=1.6. Then they were induced with 1mM of IPTG. Non-saturating inductions done at lower cell densities were found to produce less of the desired protein. The cells were returned to 37°C shaker for 3 hours post-induction.

Cells were harvested and lysed using Novagen's Bugbuster according to the manufacturer's suggested protocol, including the optional benzonase and lysozyme additions. Protease inhibitors were found to be unnecessary, but if they are used they must be carefully selected to avoid chelating agents such as EDTA that may remove the zinc ions from the proteins.

The cell lysates were mixed in a 1:1 ratio with the Qiagen NPI-20 buffer and then purified using the manufacturer's suggested protocol. Using cell cultures of larger than 5mL was found to reduce the purity of the protein. Purified protein was stored at -20°C in a solution of 1mM DTT, 0.02% (w/v) sodium azide and 25% (w/v) glycerol. Once again, chelating agents are to be avoided.

Only 2 of the 69 zinc finger proteins have been purified thus far. Using a Bradford assay with BSA as the standard, the protein concentrations were measured. The cognate protein for op-39 has relatively low toxicity, and 6nmol was obtained from a 5mL culture. This is 50% of the maximum binding capacity of the spin column. The cognate protein for op+1 has relatively high toxicity, and 2 nmol was purified from a 5 mL culture. The calculated mass of the zinc finger protein for op-39 is ~24.6kDa, which includes 6 zinc atoms. SDS-PAGE using a MOPS buffer showed a strong band at 30kDa. These zinc finger proteins are proline rich, and proline rich proteins are known to migrate slowly on SDS-PAGE gels due to the reduced flexibility of the protein backbone near proline residues [61]. An S-tag assay using Novagen's S-tag rapid assay kit confirmed this 30kDa band was indeed the desired protein.

Measurements of the protein-DNA dissociation constants have not yet been completed but are needed to finish this work. Both equilibrium filtration and fluorescence anisotropy measurements will be attempted. To increase the mass of the zinc finger protein, it may be necessary to create a protein fusion with maltose binding protein. This increased mass is advantageous with both methods because both methods rely on the mass and size difference between the DNA molecule and the protein.

Creating repressors for other promoters

By designing zinc finger proteins that target native promoters, it should be possible to attenuate expression of native genes. If the zinc finger protein is introduced on a plasmid, then it should be possible to modulate gene expression from the chromosome without making any modifications to the chromosome. This would allow for the inducible and temporary attenuation of required genes that are lethal to knockout. Those promoters targeted by zinc finger protein repressors would still remain under the control of the native regulation when the zinc finger protein repressor was not present.

Similar results may be possible by designing sRNAs to target native genes, but currently there is not an efficient method for designing sRNAs to target a specific gene of interest. Additionally the metabolic burden of a zinc finger protein repressor approach differs from the burden imposed by an sRNA based system. For the zinc finger protein repressor system, the targeted gene does not get transcribed but there is the cost of translating the repressor protein. With an sRNA system the burden of the target transcript would still exist, but there would be no translational burden. There are advanced sRNA systems where the sRNA binds the target mRNA before it is completely transcribed and introduces an early terminator, thus reducing the transcriptional load [62]. However, engineering such a system to work with an arbitrary native mRNA would an impressive feat. Engineered sRNAs repressing the translation (and not transcription) of native mRNA is the more likely approach.

Whether an sRNA or protein repressor based system causes more overall metabolic burden will depend on the operon being targeted as shown in Figure 3-31. Weakly transcribed mRNA can be attenuated with less load by an sRNA, compared to highly transcribed genes where eliminating those transcripts could outweigh the cost of translating a repressor protein.

In metabolic engineering there are often native pathways that take away precursors or intermediate metabolites from an engineered pathway. These native pathways cannot be knocked out because they are required for growth. However, in metabolic engineering it is common practice to maximize the productivity of a culture by growing to a desired cell density without product formation and then inducing the engineered pathway. During the post-induction phase, the goal is push as much carbon through the product formation

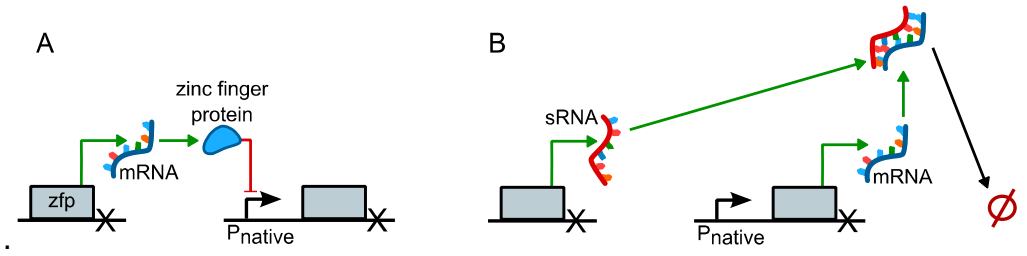


Figure 3-31 Differences in metabolic load for methods of repressing native genes. A) Use of a zinc finger protein repressor does not expend energy on the transcription of the target gene, but energy is used to translate the repressor. B) Regulation with sRNA does not use any energy for translation, but energy is used to translate the mRNA for the target gene. The system with the lowest metabolic burden depends on the rate of transcription from the native promoter.

pathway as possible and to divert as little carbon as possible to growth. By using zinc finger protein repressors the induction of the product formation pathway can also induce zinc finger proteins to turn off pathways that are related to cell growth. This may result in both higher productivities and increased yields.

Chapter 4 Topologies for building combinational logic gates

Combinational logic is defined by three attributes. The inputs and output are digital, meaning they can be represented by a series of two-state values. These two states are commonly referred to as "high" and "low" or 1 and 0. The second attribute is the relationship between the inputs and outputs consists of Boolean functions. These functions have binary (two-state) inputs and outputs and perform operations composed of AND, OR, and NOT operators. The third attribute is that the output of combinational logic is solely a function of the current input. There is no dependence on previous states of the system.

The primitive element of combinational logic is the logic gate. Logic gates take one or more binary inputs and produce a single binary output based on Boolean functions. The two simplest logic gates have only a single input and are called the buffer and the inverter. For a buffer gate the output state is the same as the input state. Because buffer gates only propagate an existing state, they are not needed in idealized systems; however, in real implementations they are used to limit the propagation of non-ideal effects. Inverters output the opposite state as the input. Inverters are also called a NOT gate. The basic logic gate symbols and their truth tables (input/output definitions) are shown in Figure 4-1. Both the NOR gate and the NAND gate are universal gates, meaning any combinational logic function can be implemented by only using one type of these gates. In Figure 4-1, NOR implementations of the basic logic gates are depicted in the right most column.

Desired properties of logic gates

A recent paper by Clancy and Voigt lists some important characteristics that a system of combinational logic should have [63]:

- Scalability – It should be possible to build many orthogonal gates, enabling the implementation of large logic functions.
- Extensibility – Making useful logic functions requires connecting the output of one gate to the input of another gate; therefore, the input and output signals must have compatible specifications.
- Modularity – In order for combinational logic to be useful in real applications, the inputs and output of the logic block must be easy to interface with other common devices such as sensors and gene expression devices.
- Robustness – Combinational logic blocks should produce the desired outputs over a range of contexts and environmental conditions and in the presence of typical amounts of noise. Additionally, the impact on the host organism should be minimized.
- Speed – Every application will have different constraints on speeds, but generally faster is better. As logic blocks get larger and the longest path from an input to an output grows, the speed for an individual gate to transition will become increasingly important.

Previous work

Implementing combinational logic in engineered organisms has been pursued by the synthetic biology community for over a decade. Some of the earliest work in this area was done by Ron Weiss during his Ph.D. including both simulation [64] and physical

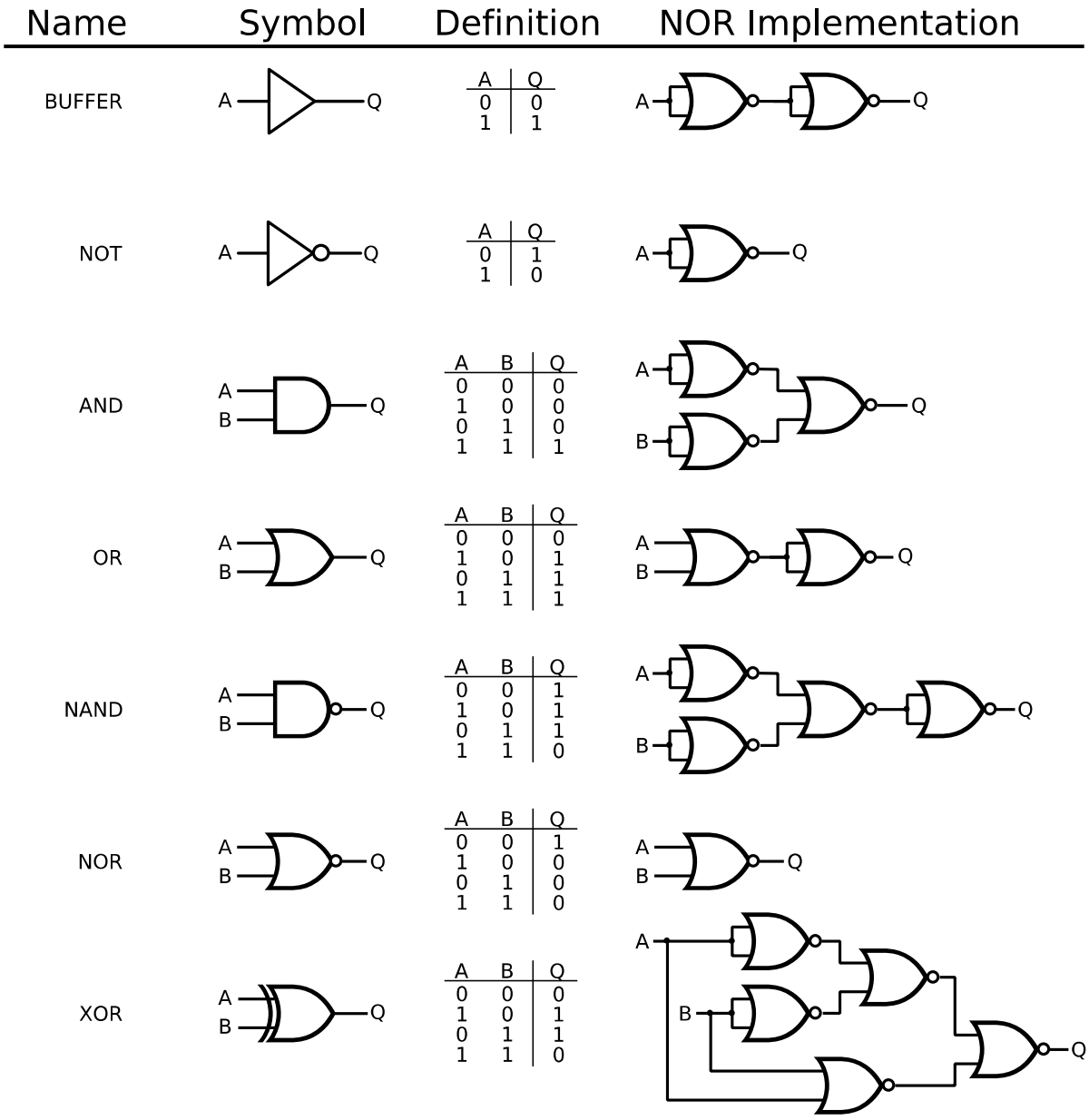


Figure 4-1 The basic set of two input logic gates. A and B are binary input signals, and Q is the binary output signal. The "Definition" column shows the truth table for each gate. This table defines the input/output behavior of each gate. The "NOR Implementation" column demonstrates how any of these basic gate functionalities can be implemented with a set of NOR gates. With enough NOR (or NAND) gates it is possible to implement any combinational logic function.

implementation [65]. The first synthetic inverters were design, built and characterized by Weiss using commonly studied repressor-promoter pairs such as LacI/P_{lac}, TetR/$P_{LtetO-1}$, and cI/P_R. By using repressor proteins with cooperativity (Hill coefficients>1) and small dissociation constants, Weiss' devices had transfer curves that were good enough for use in many applications. Since then several gates have been designed that use various small molecules as their inputs [66], [67]. Because of their dependence on specific small molecules, use of these gates is limited to applications where the small molecule used as inputs are relevant to the task at hand. Other work has strived to be more re-usable and has embraced the standard of PoPS based input and outputs [68], [69]. Having a standardized system of interconnect between gates, sensors, and output devices is essential to making devices re-usable in other applications. Libraries of mutant LacI repressors along with promoter variants have been engineered to create small sets of orthogonal gates [69]; however their device characterization left much to be desired as no transfer curves were reported. Tamsir reported that multi-strain circuits could allow for gate re-use by physically separating the multiple instances of the gate with cell membranes [9]. Repressor proteins have not been the only type of molecules used to create combinational logic. Several groups have created RNA based logic gates [70], [71]. While RNA has many appealing properties to use in logic gate design, the performance obtained so far has trailed behind protein based gates. Some groups are looking beyond the creation of gates and are designing software tools that can take a collection of components and design a gene network that will perform a given logic function[63], [72].

The previous work on creating combinational logic has been successful at demonstrating and advancing four of the five characteristics desired for combinational logic systems. Extensibility was demonstrated by Weiss' ability to connect gates together [65] Modularity was shown by Anderson when he was able to replace components connected to the input and output of his AND gate[68]. A degree of scalability was engineered by Zhan when he created his mutant LacI-promoter library [69]. Little has been published on testing the robustness of logic gates at the bench, but Tamsir does present some data on parameter variation and noise in the supplementary material [9]. The speed of logic gates has not yet received much attention. It is expected that RNA based gates have the ability to switch faster than gates based the combined transcription and translation of repressors proteins, but this has not yet been experimentally shown. Creating faster gates will likely remain a low priority until the other desired characteristics have all been successfully combined within a single system of logic gates.

Methodology for designing new logic gate topologies

For transcription-based logic systems the problems of extensibility and modularity have largely been solved through the use of PoPS based inputs and outputs. The next challenge to overcome was building logic systems that can scale to 5 gates and well beyond that while maintaining robust behavior.

Due to the advances in synthetic zinc finger protein engineering, it appeared that utilizing zinc finger proteins would allow for highly scalable logic; however, it was not obvious how to create logic gates from zinc finger proteins. For transcription-based logic, there will need to be a transcription factor, and there are multiple ways to create transcription factors from a DNA binding domain:

- A DNA binding domain can prevent RNA polymerase from binding or transcribing though steric hindrance
- Attaching a repression domain and binding upstream of a promoter to inhibit transcription
- Attaching an activation domain and binding upstream of a promoter to activate transcription

Creating logic gates from transcriptional repressors that operate through steric hindrance of the RNA polymerase requires cooperativity as shown in Figure 4-2. The transfer function obtained from the non-cooperative binding of a monomeric protein to a promoter is not a reverse sigmoid and therefore not suited for creating logic gates. Creating a logic gate from steric hindrance requires a Hill-coefficient greater than 1 and for reasonable performance should be at least 2. Because simple synthetic zinc finger proteins do not dimerize, they cannot be used as transcriptional repressors to create logic gates. Synthetic zinc finger proteins have been made to dimerize by adding leucine zipper domains [73], but due to the small number of orthogonal leucine zipper domains this topology has scaling limitations.

In eukaryotes, synthetic transcriptional repressors have been created by fusing a DNA binding domain to a domain that represses transcription. These repression domains

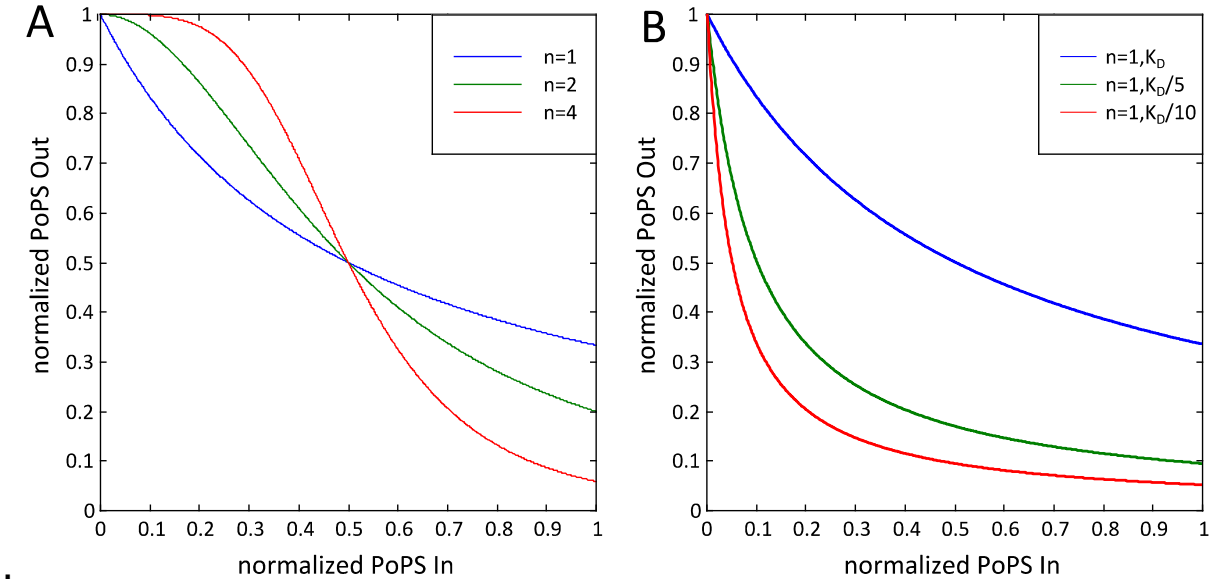


Figure 4-2 Idealized transfer curves for inverters constructed with transcriptional repressors. These are simply plots of the Hill equation A) Transfer curves are shown for three different amounts of cooperativity where n is the Hill-coefficient. Based on the number of monomers complexed, a TetR based repressor should have a Hill-coefficient of 2 and 4 for a LacI based repressor. Only once the Hill-coefficient is greater than 1 does the curve become a reverse sigmoid. B) Transfer curves where the Hill-coefficient is 1 but the dissociation constant is varied by a factor of 10. With the largest dissociation constant the off state is far from 0, but with the smaller dissociation constants the on state is too small.

from eukaryotes are unlikely to function in prokaryotic hosts, and similar modular repression domains have not yet been identified and studied in prokaryotes. Basic science work is needed for this approach to become feasible.

As discussed in earlier chapters, building transcriptional activators from zinc finger proteins in *E. coli* is a demonstrated technology. Using transcriptional activators, it is very easy to build OR and AND gates, but these gates do not allow for signal inversion. How to make a universal gate (NAND or NOR) from transcriptional activators is not obvious.

To determine how to make universal logic gates from zinc finger proteins the following process was followed:

1. Brainstorm possible topologies involving zinc finger proteins
2. Construct model using JDesigner
3. Run ODE based simulations using Systems Biology Workbench
4. Evaluate topologies heuristically
5. Eliminate topologies with poor evaluations
6. Solve ODE model for analytical solution at steady state
7. Evaluate sensitivity of topology to parameter variation
8. Eliminate topologies that are hyper-sensitive
9. Build and test topologies

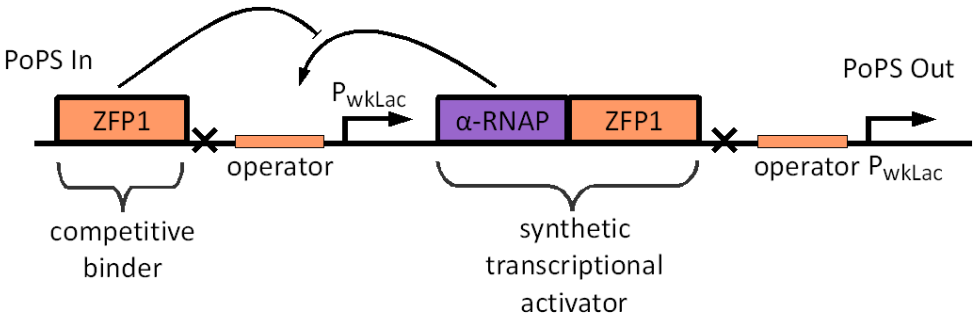


Figure 4-3 Positive feedback with competitive binder inverter topology. A synthetic transcriptional activator consisting of the α-subunit of RNA polymerase fused to a zinc finger based DNA binding domain is used to create a positive feedback loop by activating its own transcription. The same DNA binding domain without the α-subunit competes to bind to the same operator sites as the transcriptional activator. Because both proteins have the same DNA binding domain and thus the same dissociation constant, the output is proportional to the fraction of DNA binding domains with the α-subunit over the total number of DNA binding domains. Two copies of the P_{wklac} promoter are used to maintain a pure PoPS output. If a single P_{wklac} promoter was used to generate a multi-cistronic containing the transcriptional activator and the downstream gene connected to the output of the device, then it is possible for both RNA polymerase and ribosomes to cross the output interface. This possibility is eliminated by using a second promoter to drive the output. Additionally, the output of a PoPS device should ideally occur at the transcriptional start site, so that the PoPS generator is distinct from the mRNA coding.

The goal of this process was to rapidly test and eliminate topologies, thus allowing even farfetched or unusual ideas to be considered with a minimal investment of time. Using JDesigner from the Systems Biology Workbench [74], models of molecular interactions can quickly be designed using a graphical user interface to draw the molecules and their reactions. JDesigner can then create an ODE model from the drawn network. This was found to be faster and considerably less error prone than drawing a topology on paper, manually converting this to a system of ODEs and then putting these equations into simulation software.

At the time this work was performed, JDesigner and the Systems Biology Workbench were found to be the best tools for this task. Since then TinkerCell [75] has become available and has surpassed JDesigner and the Systems Biology Workbench for these tasks.

Positive feedback with competitive binder topology

Several rounds of iteration through the methodology listed above resulted in a candidate topology that seemed worthy of trying to implement. One of the keys to finding a candidate topology was realizing that it may be of use to have both transcriptional activators and pure DNA binding domains in the same device. This topology consists of a positive feedback loop and an inhibitor of the positive feedback loop. The positive feedback loop sets the inverter output value, and the inverter input is proportional to the inhibitor. Auto induction of a one-hybrid transcriptional activator creates the positive feedback loop, and a DNA binding domain competing for the same operator site as the one-hybrid activator creates the inhibition. The topology is shown in Figure 4-3.

Analysis of topology

Modeling and analysis

An ODE model was constructed for the purpose of solving for a steady state transfer function. Two ODEs were used to represent the total concentrations of activator and repressor. A third equality is used to define the output value in terms of the activator and repressor concentrations. It will be assumed that the concentrations of DNA-activator and DNA-repressor complexes are functions of the total concentrations of activator and repressor. This assumption may not be good for simulating dynamics of the system, but should be accurate for steady state results.

$$\frac{d\alpha}{dt} = PT_{\alpha}P_T\frac{\alpha}{K_D + \alpha + z} - D\alpha \quad \mathbf{1}$$

$$\frac{dz}{dt} = InputP_TT_R - Dz \quad \mathbf{2}$$

$$Output = P\frac{\alpha}{K_D + \alpha + z} \quad \mathbf{3}$$

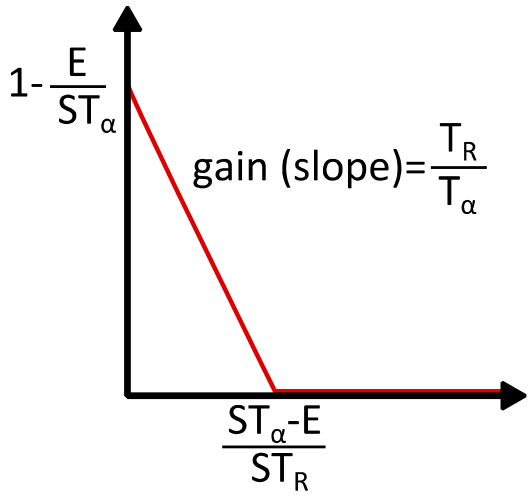


Figure 4-4 The transfer curve for the positive feedback with competitive binder inverter. The gain is determined by the ratio of strengths of the ribosome binding sites for the competitive binder and the transcriptional activator. This transfer function is not a reverse sigmoid, but because the gate has a gain of greater than one and the low state reaches zero, this gate can be used to amplify digital signals.

α is the concentration of transcriptional activator – α-subunit of RNA polymerase fused to a zinc-finger protein. z is the concentration of transcriptional repressor – a zinc-finger protein that is competitively binds to the same site as the activator. P is the maximum rate of production of mRNA from one copy of P_{wklac}. Units are t^{-1}. P_T is the concentration of the P_{wkLac}. K_D is the disassociation constant for the zinc-finger protein, both the activator and repressor, to the site upstream of the P_{wkLac} promoter. Input is the number of polymerases entering the device per unit of time. D is the degradation rate of both the activator and repressor proteins, with units of t-1. T_α and T_R represent the respective strengths of the activator and repressor RBSes. These variables are unit-less. Output is the number of polymerase exiting the device per unit of time. The variables of the system are non-dimensionalized as follows:

$$A = \frac{\alpha}{K_D}; R = \frac{z}{K_D}; S = \frac{P_T}{K_D}; \tau = tP; I = \frac{Input}{P}; E = \frac{D}{P}; O = \frac{Output}{P} \qquad \mathbf{4}$$

Substituting Equation 4 into Equations 1, 2, and 3 yields non-dimensionalized equations:

$$\frac{dA}{d\tau} = \frac{ST_\alpha A}{1 + A + R} - EA \quad \textbf{5}$$

$$\frac{dR}{d\tau} = IST_R - ER \quad \textbf{6}$$

$$O = \frac{A}{1 + A + R} \quad \textbf{7}$$

The steady state transfer function can then be found by setting the ODEs equal to zero, solving for A and R, and substituting those expressions into Equation 7.

$$O = \frac{\frac{ST_\alpha - E - IST_R}{E}}{1 + \frac{ST_\alpha - E - IST_R}{E} + \frac{IST_R}{E}} = 1 - \frac{E}{ST_\alpha} - \frac{T_R}{T_\alpha} I \quad \textbf{8}$$

For regions where Equation 8 is negative, the transfer function should be set equal to 0. The transfer function can also be calculated while taking into account the non-zero leakage of the P_{wklac} promoter, but the simplified equation derived above provide more insight into how the parameters shape the transfer function. To derive the transfer function with leakage included, P_L is defined as the rate of mRNA production by the P_{wklac} promoter while

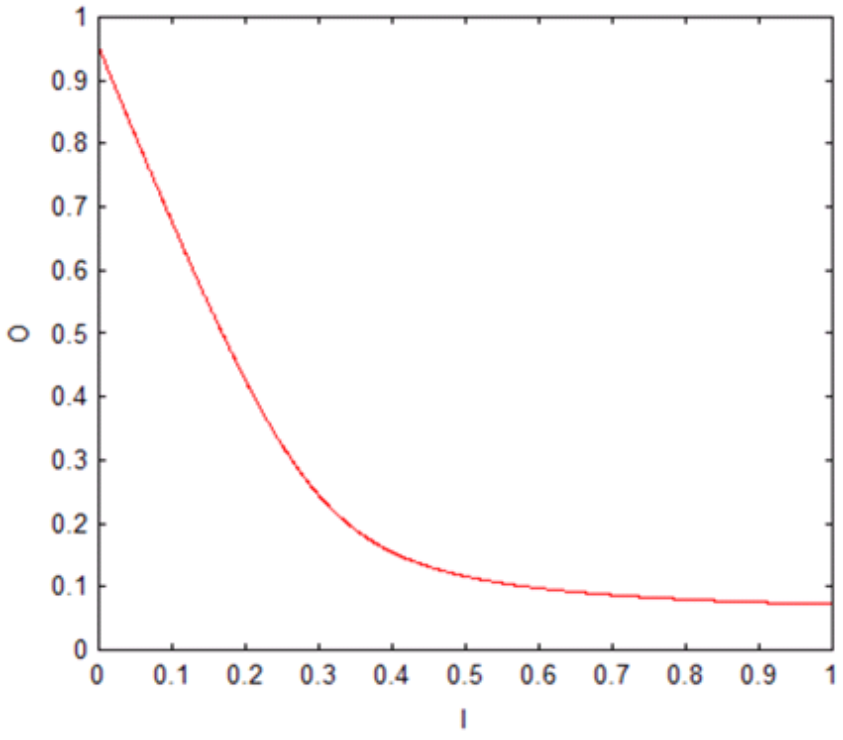


Figure 4-5 Transfer function of the inverter with leakage included. The parameter values used for this plot are: L=0.05; S=20; E=1; T_α=1; T_R=3.

in a non-activated state. Equation 1 is replaced with:

$$\frac{d\alpha}{dt} = PT_\alpha P_T \frac{\alpha}{K_D + \alpha + z} - D\alpha + \left[1 - \frac{\alpha}{K_D + \alpha + z}\right] P_L T_\alpha P_T \qquad \mathbf{9}$$

And Equation 1 is replaced by:

$$Output = P\frac{\alpha}{K_D + \alpha + z} + \left[1 - \frac{\alpha}{K_D + \alpha + z}\right] P_L \qquad \mathbf{10}$$

The new term P_L is non-dimensionalized as:

$$L = \frac{P_L}{P} \qquad \mathbf{11}$$

Proceeding as above, a closed form analytical solution for the transfer function can be found; however, it is a large expression and doesn't offer much intuition.

To demonstrate that this unusual transfer function can pass digital signals, an ODE model of three inverters in series was simulated with a pulse function as the input. The results of this simulation are shown in Figure 4-6.

Using the model with leakage included, the sensitivity of the steady state transfer curve to various noise sources and parameter variations was investigated as shown in Figure 4-7. The performance under these sets of conditions was mixed with some parameters having almost no effect on the transfer function and other parameters causing large changes. The inverter is very insensitive to the dissociation constant being made smaller, but increasing the dissociation constant can slightly alter the performance. If a set

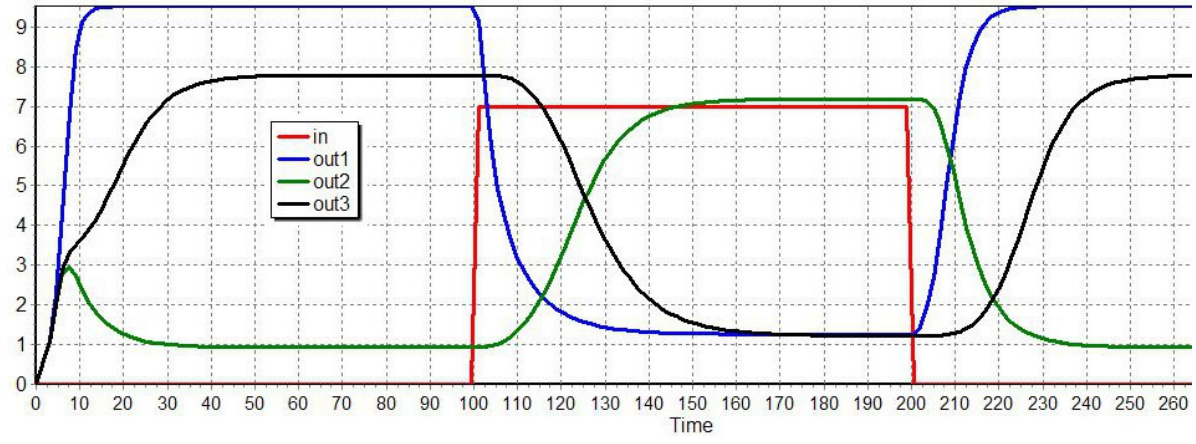


Figure 4-6 Simulation of three "positive feedback with competitive binder" inverters in series. An ODE based simulation was performed with L=0.05 and T_R=T_α. The input to the first gate was pulsed and while none of the inverter outputs are able to reach 0, there are two clearly defined regions for high and low signals.

of logic gates was constructed with this topology, it would be critical to have the dissociation value of all the zinc finger proteins be below some threshold value in order to ensure the gates have similar transfer functions. A change in the number of DNA copies of a gate has almost no effect on the transfer function. This is an important test because during every cell replication cycle, the DNA concentration in a cell must vary by a factor of 2.

For the copy number analysis, the transfer function is shown with the input and output units as Polymerase Per Second per DNA Copy (PoPSDC). This unit should always be used for transcriptional logic instead of just PoPS, but it is slightly more confusing and only becomes important when considering copy number effects. By using PoPSDC, the average

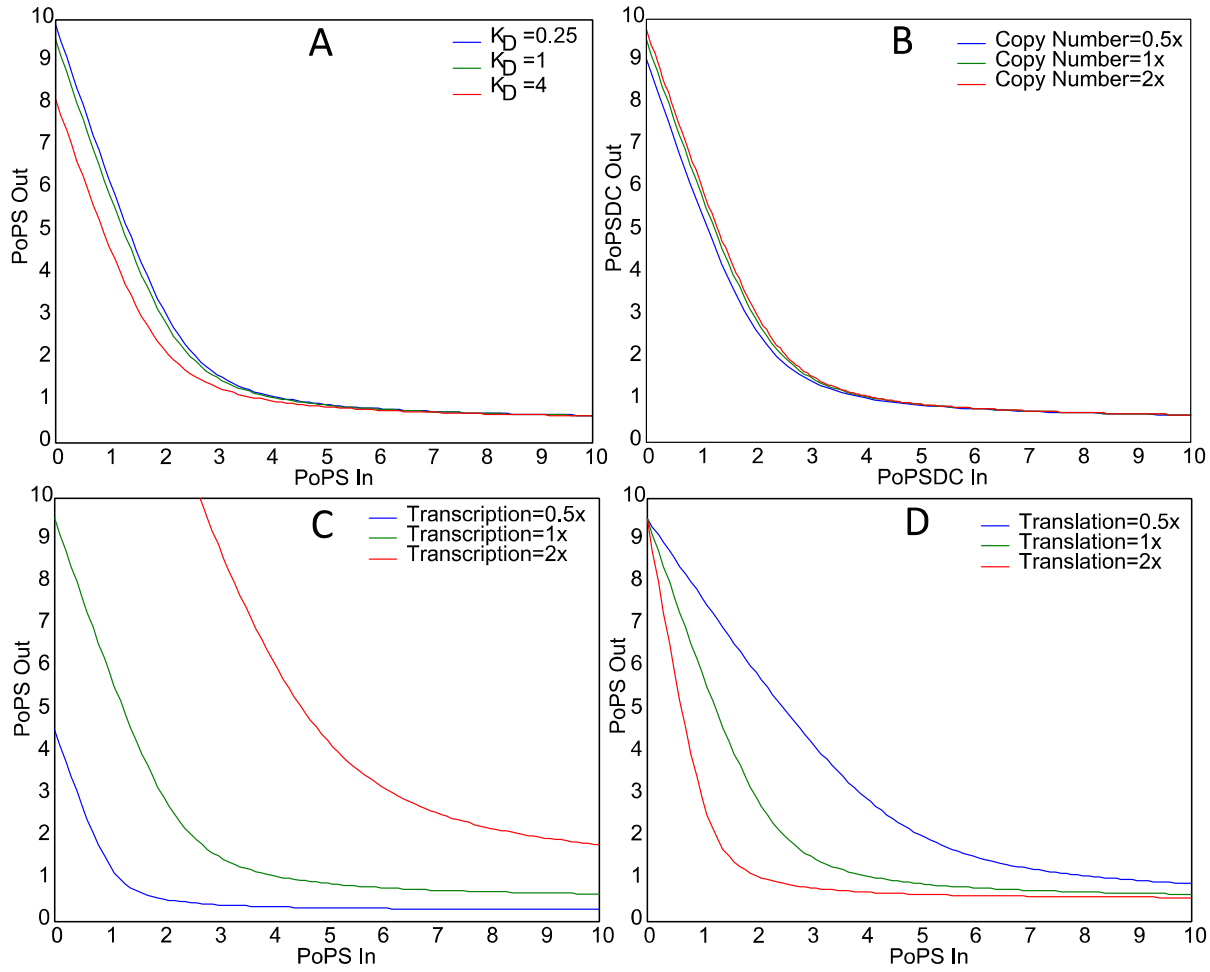


Figure 4-7 Transfer curves for the "positive feedback with competitive binder" inverters with various perturbations. A) The K_D of both the activator and competitive binder were simultaneously modified. B) The number of copies of DNA containing the circuit was varied. The units on this plot are Polymerase Per Second Per DNA Copy (PoPSDC) as measuring on a per copy basis becomes important when investigating copy number effects. C) Varying the rate of transcription has devastating effects on the transfer function. D) Translation rate changes also cause significant changes in the transfer function.

amount of polymerase crossing a junction on a piece of DNA is considered rather than the total amount of polymerase crossing all copies of a junction.

Changes to either the transcriptional or translational rates are quite detrimental to the transfer function. Altering the transcriptional rate shifts the whole transfer function and results in changes to the leakage level, the maximum output, and the position of the knee in the curve. Because changing the transcriptional rate scales both the input and output axes similarly, gates connected in series should be able to pass signals without a loss of fidelity. However the other devices connected to the logic block might react to a change in transcriptional rates differently than the gates, and thus signals may not properly propagate across these heterogeneous interfaces. Changes in translation rate only scale the transfer function along the input dimension. For small changes in translation rate, gates in series will still correctly propagate values, but their ability to tolerate noise will be reduced. If the reduction in translation rate is large enough that the gain of the gate drops below 1, then the gates will fail. For large increases in translation rate, the gain of the gate will increase while decreasing the input value needed to switch the output of the gate, and at high enough translation rates the width of the high state will vanish into the noise and the gates will fail.

Decreases in both transcriptional and translational rates are detrimental, and both can be caused by high metabolic burdens. For gates with these characteristics it will be

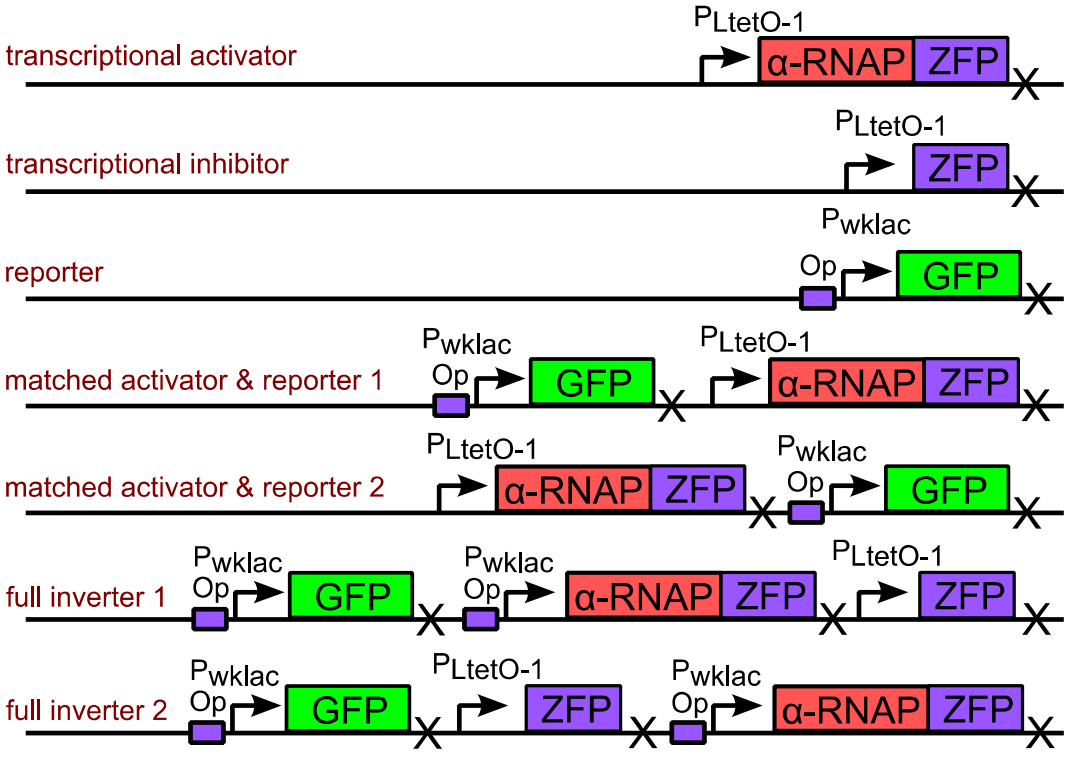


Figure 4-8 Inverter construct and associated controls. Each of these constructs is on a plasmid with a SC101 origin of replication and kanamycin resistance. A brief description of each construct is on the left in red text.

Table 4-1 Zinc finger proteins and their operator sites

Zinc finger protein name	Operator site used
ZFP02	ATA ATG GAC CTA GGA GCT
ZFP03	GGT AGA CGT CTA GTA ACT
Zif268	GCG TGG GCG
ZFP5475	ACC CGG GTT CCC CTC GGG

vital to design and test the gates at conditions that will have similar growth rates, and therefore similar metabolic loads, as the conditions these gates will be ultimately used in. Unfortunately theory, tools and metrics for quantifying metabolic burden are lacking, and growth rate is the only good quantitative metric for comparing metabolic loads.

Implementation and testing

The transcriptional activator architecture used was based on the work of Meng [40]. The α-subunit of RNA polymerase was attached to the N-terminus of a 6-zinc finger protein. Meng used a linker of AAAPRVRTGS, but in order to facilitate BglBrick based cloning this amino acid sequence was changed to GSAPRVRTGS. The promoter to be activated by this fusion was a weakened version of P_{lac} called P_{wklac}. The operator site was placed upstream of the -68 position relative to the transcriptional start site. This is the same promoter used by Meng except the bases from positions -68 to -62 were altered to allow for BlgBrick cloning of the operator.

A series of controls and inverters were constructed and tested. The constructs are shown in Figure 4-8. These construct were designed to verify the transcriptional activator was functional and to determine the amounts of transcriptional leakage and background

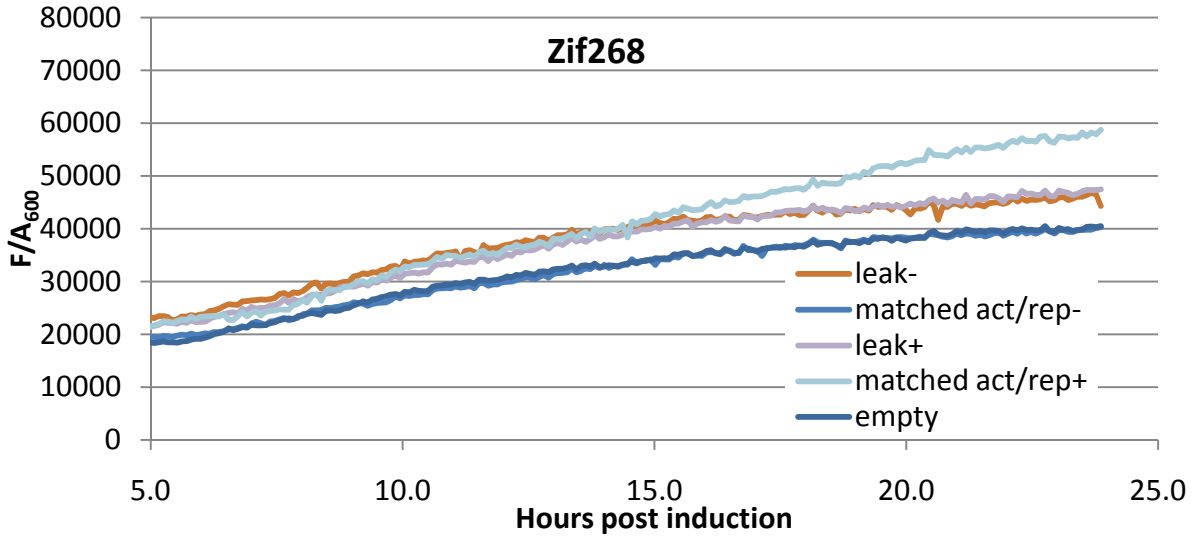


Figure 4-9 Results from Zif268 controls. In the legend, "-" indicates no induction, and "+" indicates a saturating induction with aTc. For a description of the constructs see Figure 4-8. The matched activator and reporter are of type 1.

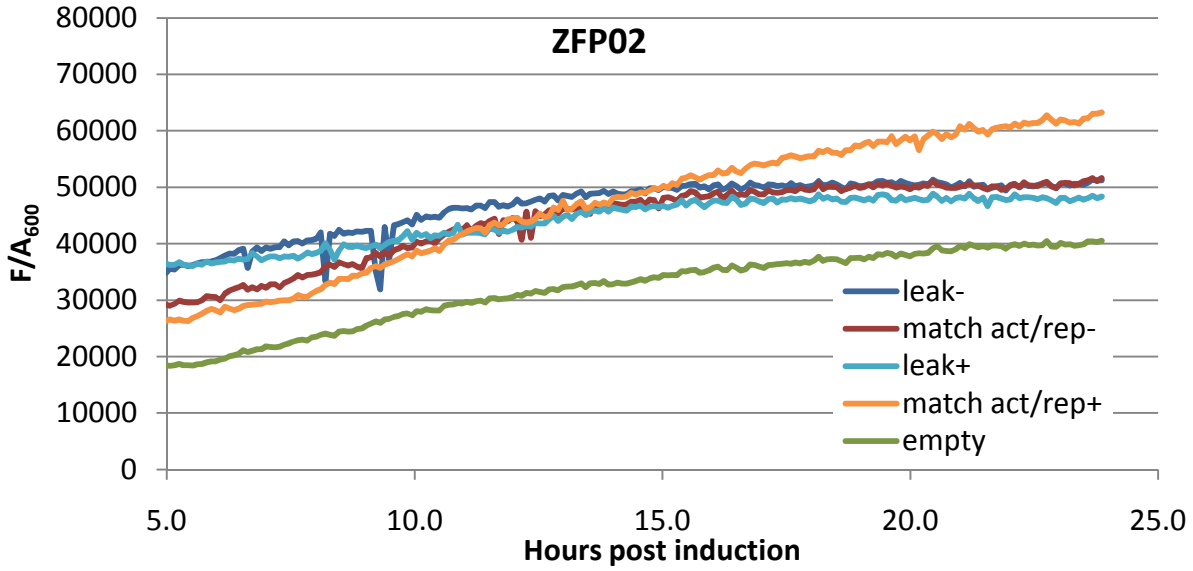


Figure 4-10 Results from ZFP02 controls. In the legend, "-" indicates no induction, and "+" indicates a saturating induction with aTc. For a description of the constructs see Figure 4-8. The matched activator and reporter are of type 2.

fluorescence that should be expected. The two controls that do not contain GFP were used to determine if the expression of the zinc finger protein or transcriptional activator had toxic effects on the cell. The reporter plasmid was used to find the baseline level of fluorescence due to leakage from the P_{wklac} promoter. The matched activator and reporter confirm the transcriptional activator is functional.

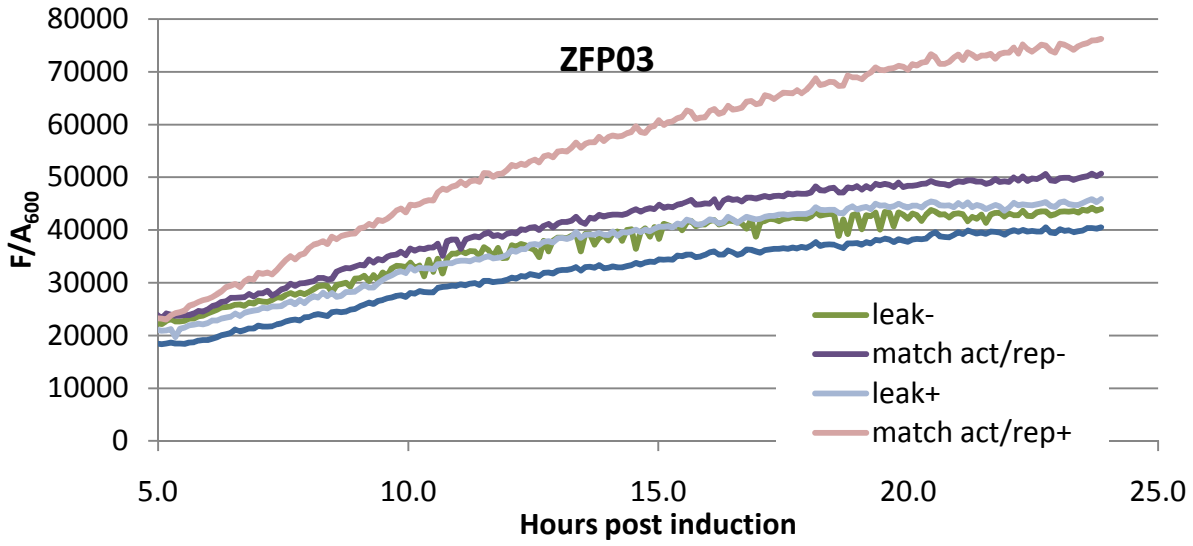


Figure 4-11 Results from ZFP03 controls. In the legend, "-" indicates no induction, and "+" indicates a saturating induction with aTc. For a description of the constructs see Figure 4-8. The matched activator and reporter are of type 1.

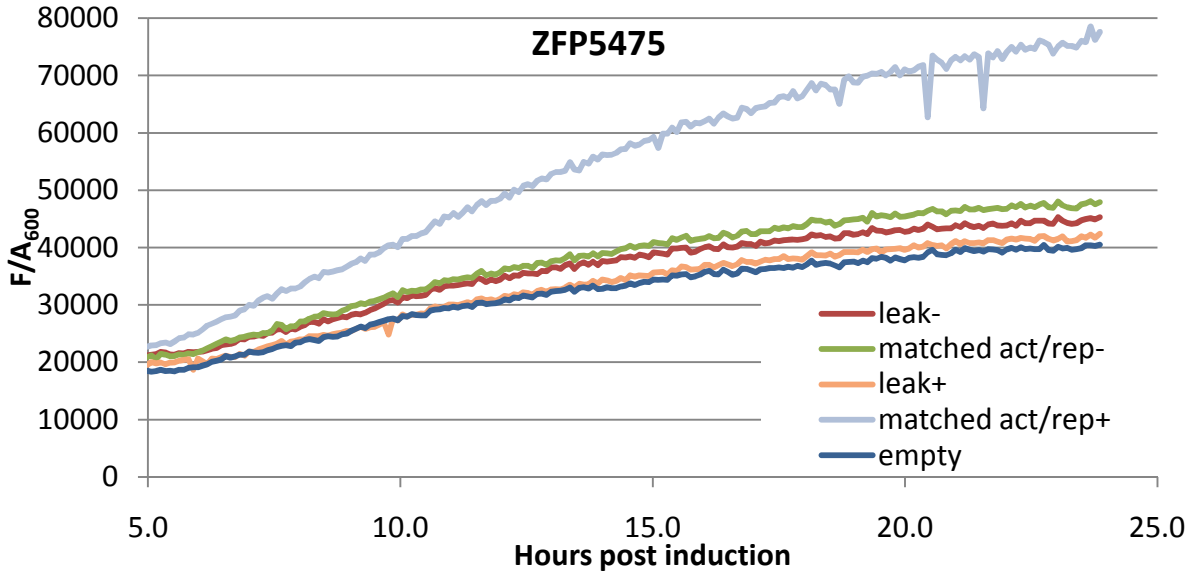


Figure 4-12 Results from ZFP5475 controls. In the legend, "-" indicates no induction, and "+" indicates a saturating induction with aTc. For a description of the constructs see Figure 4-8. The matched activator and reporter are of type 1.

These constructs were built using 4 different zinc finger proteins. ZFP02 and ZFP03 are 6-finger proteins designed as part of this work. ZFP5475 is a previously published 6-finger protein [76], and Zif268 is a heavily studied mammalian transcription factor with 3 fingers. The apparent dissociation constants for ZFP5475 and Zif268 are reported to be 70pM [76] and 19pM[60] respectively. The operator sites for these zinc finger proteins are listed in Table 4-1.

The first round of experiments presented here were done with a supplemented M9 media [40] in 24 well plates (Labnet, P9835) with a gas permeable seal (Thermo Scientific, AB-0718). The host DH5α-Z1 was used, and it expresses both LacI and TetR constitutively from the chromosome [44]. Induction was done by adding anhydrous tetracycline (aTc) at a saturating level (50 ng/mL).

Initial experiments indicated that some of the constructs were functional, but none of them performed as well as desired. The matched activator and reporter constructs containing ZFP03 and ZFP5475 both showed activation of the reporter, but in both cases the induced F/A_{600} was only about 50% higher than the F/A_{600} for the uninduced. For the constructs containing ZFP02 and Zif268, there may be slight activation but only when the cells are well into stationary phase (15 hours post-induction). The full inverter construct containing ZFP03 displayed no difference between the induced and uninduced cultures.The ZFP5475 inverter shows a slight inversion of signal, but it is unclear if this is significant or not as the induced and uninduced leakage constructs show about the same degree of difference. The F/A_{600} for the leakage constructs should be independent of the inducer levels, and therefore their difference can be used as a proxy for the noise in this assay.

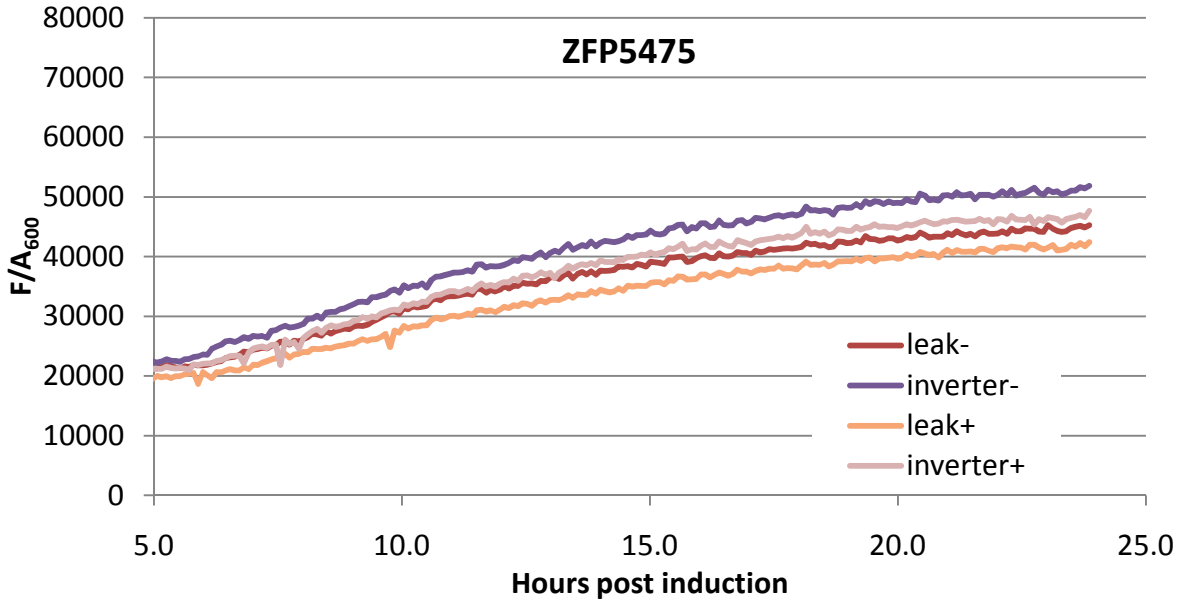


Figure 4-13 Results from ZFP5475 controls and inverter. In the legend, "-" indicates no induction, and "+" indicates a saturating induction with aTc. For a description of the constructs see Figure 4-8. The inverters are of type 2.

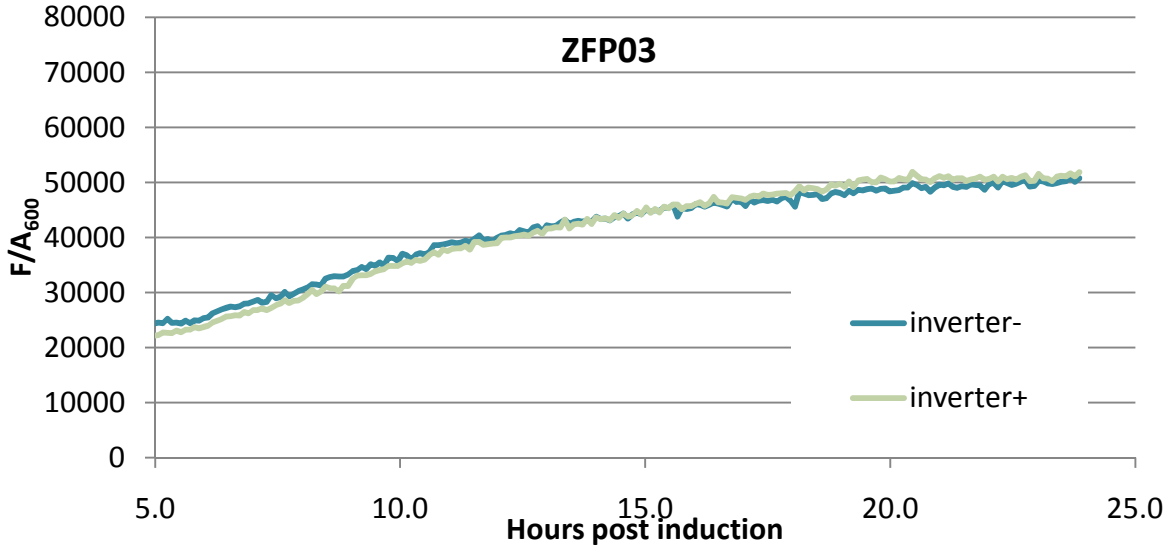


Figure 4-14 Results from ZFP03 inverter. In the legend, "-" indicates no induction, and "+" indicates a saturating induction with aTc. For a description of the construct see Figure 4-8. The inverters are of type 1.

The fold activation levels from the transcriptional activators did not seem to be large enough to use in making inverters. The measured activation was only about 1.5-fold, but 3-fold or more activation was desired. Additionally, the activation did not reach its highest levels until well into saturation. Ideally, gates will function in both exponential

Table 4-2 Alternate linkers for one-hybrid activators.

Number	Linker Amino Acid Sequence	Length	%G	%S	%A
1	AAADYKDDDDKFRTGSKTPPHRS	23	4	9	13
2	AAGGGGSGGGGSGGGGSTAAA	21	57	14	24
3	PAAGAAGAGAAP	12	25	0	58
4	PAAGAAGAGAAGAAP	15	27	0	60
5	PAAGAAGAGAAGAAGAAP	18	28	0	1
6	PAAGAAGAAGAGAAGAAGAAP	21	28	0	62
7	PAAGAAGAAGAGAAGAAGAAGAAP	24	29	0	63
8	PGSGSGGSGSGP	12	50	33	0
9	PGSGSGSGSGSGSGP	15	47	40	0
10	PGSGSGSGSGSGSGSGGP	18	50	39	0
11	PGGSGSGSGSGSGSGSGSGGP	21	52	38	0
12	PGGSGSGSGSGSGSGSGSGGSGGP	24	54	38	0

phase and stationary phase. Given a choice between functionality in only one growth phase, exponential would be preferred for ease of measurement and modeling.

Most one and two-hybrid systems are used as a selection in screening libraries.

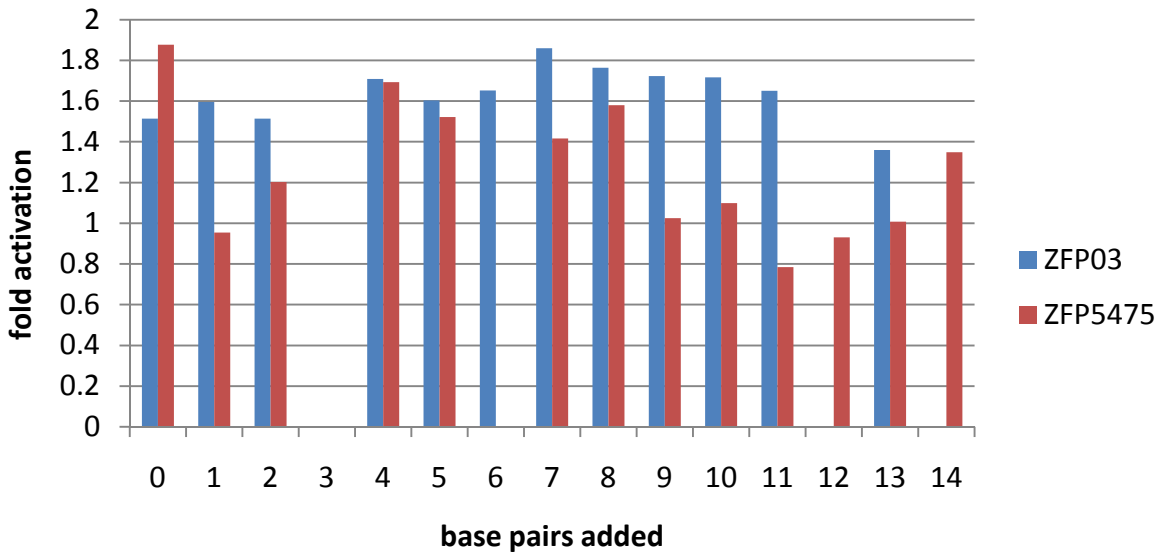


Figure 4-15 Modulating the distance between the promoter and operator. Fluoresce per A_{600} was measured after 17.5 hours, and the induced value divided by the uninduced value is given here.

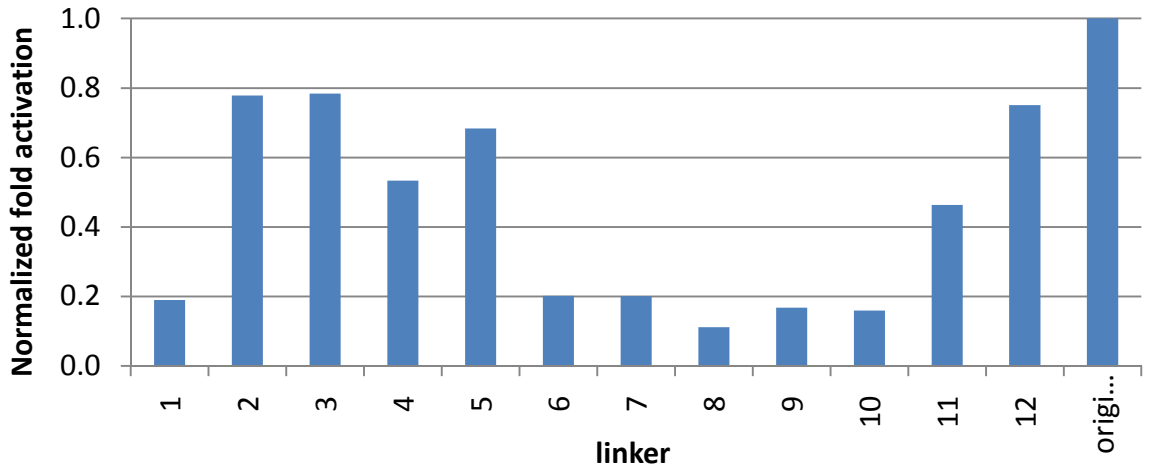


Figure 4-16 Fold activation as a function of linker. Twelve different linkers were used between the α-subunit of RNA polymerase and the DNA binding domain ZFP5475. All values are normalized to the fold activation of the original linker.

These selections are plate based, and only cells that that can activate transcription with the hybrid protein are able to grow. The results of these selections are reported as a number of colonies and not as fold activation. One paper has used a one-hybrid system in *E. coli* to drive the expression of GFP, and they claim fold activations of 8 to 22 fold [39]. But from the data presented it appears that 8 fold is their limit.

To attempt to achieve closer to 8-fold activation, the distance between the promoter and operator site was modulated. Because DNA completes a rotation about every 10.5 base pairs, by adding 0 to 14 base pairs of DNA, the operator site should complete more than 1 rotation around the DNA. One of those 15 positions should present the operator site on the same side of the DNA as the RNA polymerase. Because the previous experiment gave the best results with ZFP03 and ZFP5475, only these two DNA binding domains were used in this experiment. As shown in Figure 4-15, ZFP5475 did not have increased fold activation at other positions. ZFP03 did slightly increase its activation at several positions, with a maximum of 1.86-fold activation with 7 base pairs added, but this was still a lower fold activation than needed.

Another possible source of poor alignment between the one-hybrid activator and the RNA polymerase is the linker within the activator which connects the α-subunit to the zinc finger protein. Several new linkers were designed and tested. The linkers varied in not only length but also composition and can be found in Table 4-2. The first two linkers are from the one-hybrid literature[39], [40]. The remainder of the new linkers were designed to have different lengths and G/S or G/A ratios that promote stable folding of the linker region[77]. The fold activation for each linker was determined at 20 hours post induction. All of the linkers failed to give a higher fold activation than the original linker.

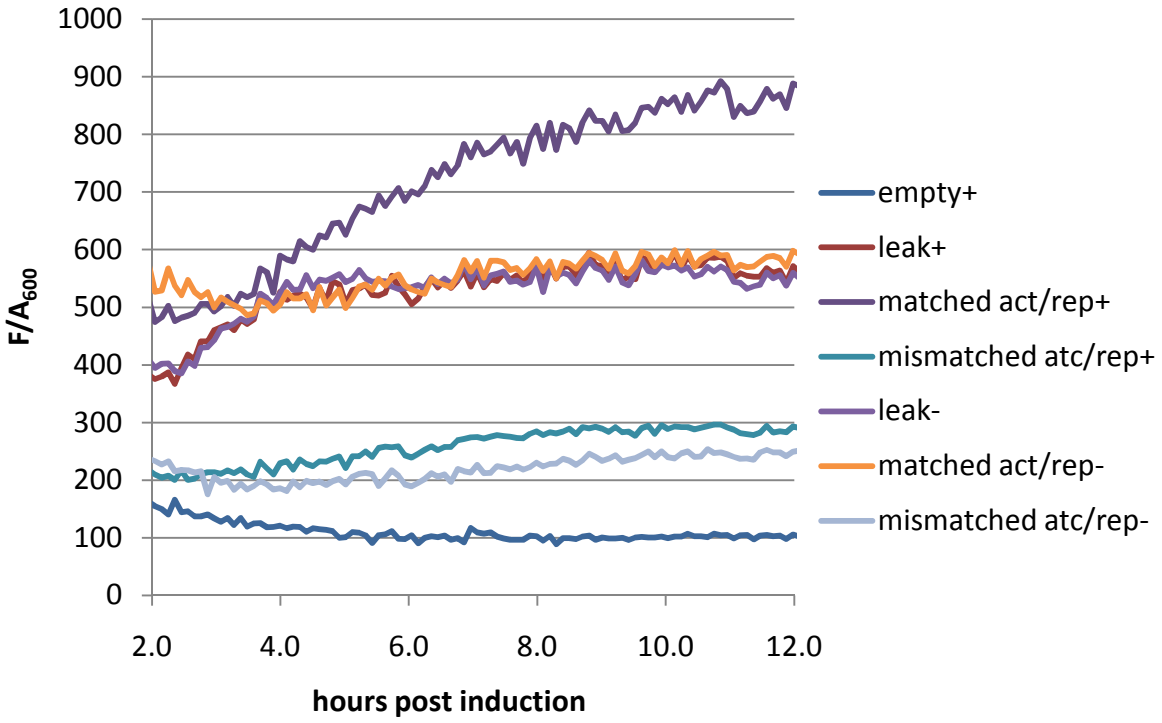


Figure 4-17 ZFP03 based controls on a p15A plasmid with mCherry as the reporter. In the legend, "-" indicates no induction and "+" indicates a saturating induction with aTc. For a description of the constructs see Figure 4-8. The matched/mismatched activator and reporter constructs are of type 1.

Neither moving the operator site nor changing the linker resulted in a large change in the fold activation, but these two variables are not indepenent. Finding the activation maximum probably requires a full search of these two variables and not just independent excursions along each axis. Because neither variable allowed for much improvement, the 2D search was not expected to find the large improvement that was needed. A poor fold activation can result from the activated state being too low or from the non-activated state being too high. Thus far the experiments have focused on pushing the activated state higher. A significant amount of auto-fluorescence from the *E. coli* made it difficult to assess the lower concentrations of GFP. Subtraction of auto-fluorescence can be tricky as it is not linearly related to A_{600}. Because non-accurate subtraction of background fluorescence can greatly inflate fold activation numbers, no subtraction of background fluorescence has been performed. F/A_{600} values have been calculated by dividing the raw F value by A_{sample}-A_{media}. This is quite conservative, but greatly reduces the subjectivity and the possibility of overstating a fold activation.

To reduce the auto-fluorescence and increase the reporter strength GFP was replaced with mCherry (a red fluorsecent protein) and the constructs were moved to p15A and ColE1 based plasmids with higher copy numbers. The exitation and emission filters used to measure GFP are similar to the auto-fluorsecent range for *E. coli*. mCherry has longer wavelengths for both exitation and emission, and these longer wavelengths have

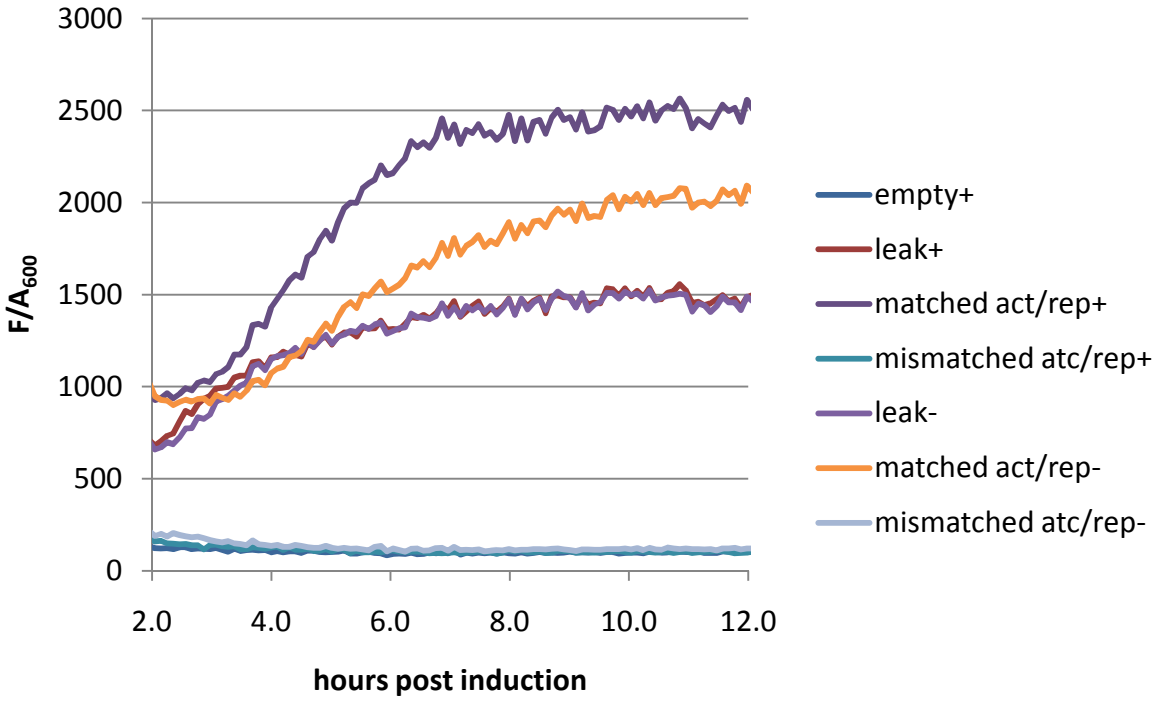


Figure 4-18 ZFP03 based controls on a ColE1 plasmid with mCherry as the reporter. In the legend, "-" indicates no induction, and "+" indicates a saturating induction with aTc. For a description of the constructs see Figure 4-8. The matched/mismatched activator and reporter constructs are of type 1.

less overlap with the *E. coli* auto-fluorsecene spectrum. Additionally, the constructs were moved to higher copy plasmids as this should increase the ratio between the low fluorsecent signal from the reporter and the cell's auto-fluorescence.

Moving from GFP to mCherry greatly reduced the backgrond fluorsence. In Figure 4-17 the data for ZFP03 constructs on a p15A based plasmid. The empty vector control, representing the auto-fluorescence of the cell, falls well below the leakage controls. This demonstrates the low fold induction was not an artifact of the high auto-fluorsence. The fold activation is still around 1.5. The uninduced matched activator and reporter construct nicely shows the same level of expression as the both the induced and uninduced leakage constructs. The mismatched activator and reporter did not behave as expected. First, if the mismatched activator and reporter did not interact, then for both the induced and uninduced cases the mismatched control should behave like the leakage control; but instead, the mismatch controls express mCherry at about half the strength as the leakage control, and there is a slight activation with induction.

The behavior of the ZFP5475 controls on p15A plasmids was similar to the behaviour of the ZFP03 controls on p15A plasmids (Figure 4-19). The mismatched activator and reporter still gave less expression than the leakage controls, but the difference between the uninduced and induced mismatched control was larger. The fold change for the mismatched control was about 1.5. This induction of the mismatched

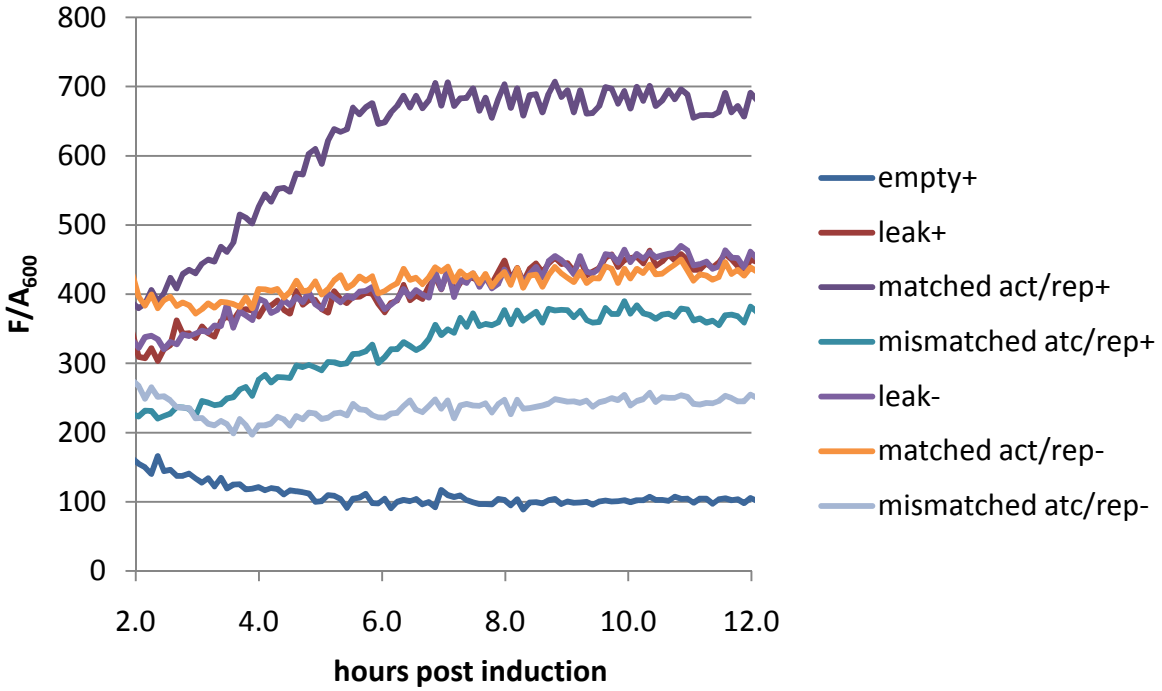


Figure 4-19 ZFP5475 based controls on a p15A plasmid with mCherry as the reporter. In the legend, "-" indicates no induction and "+" indicates a saturating induction with aTc. For a description of the constructs see Figure 4-8. The matched/mismatched activator and reporter constructs are of type 1.

activator and reporter could be due to some non-specific interaction with the α-subunit. To test this hypothesis a version of the mismatched activator and reporter construct was made without the zinc fingers. This should prevent the extra α-subunit from specifically activating the reporter. This "free" α-subunit control displayed a higher activation than the mismatched activator and reporter constructs.

Moving the constructs to a ColE1 plasmid proved rather detrimental to their performance. The ZFP03 constructs are shown in Figure 4-18. The fold activation for the matched activator and reporter decreased because the output from the uninduced culture became significantly higher than the output from the leakage construct. This simply could be that the leakage from the PLtetO-1 promoter, driving the one-hybrid protein, is now high enough to partially activate the reporter. Interestingly, the mCherry expression from both the uninduced and induced mismatched activator and reporter drop to the same level as the empty plasmid.

One-hybrid transcriptional activators continued to be problematic with low fold activation and unexpected behavior from non-cognate activators. Even if this set of controls were tweaked such that they behaved as desired, it is questionable if the system would be able to robustly scale to multiple gates. Transcriptional activator based gates were abandoned when a more promising topology was devised.

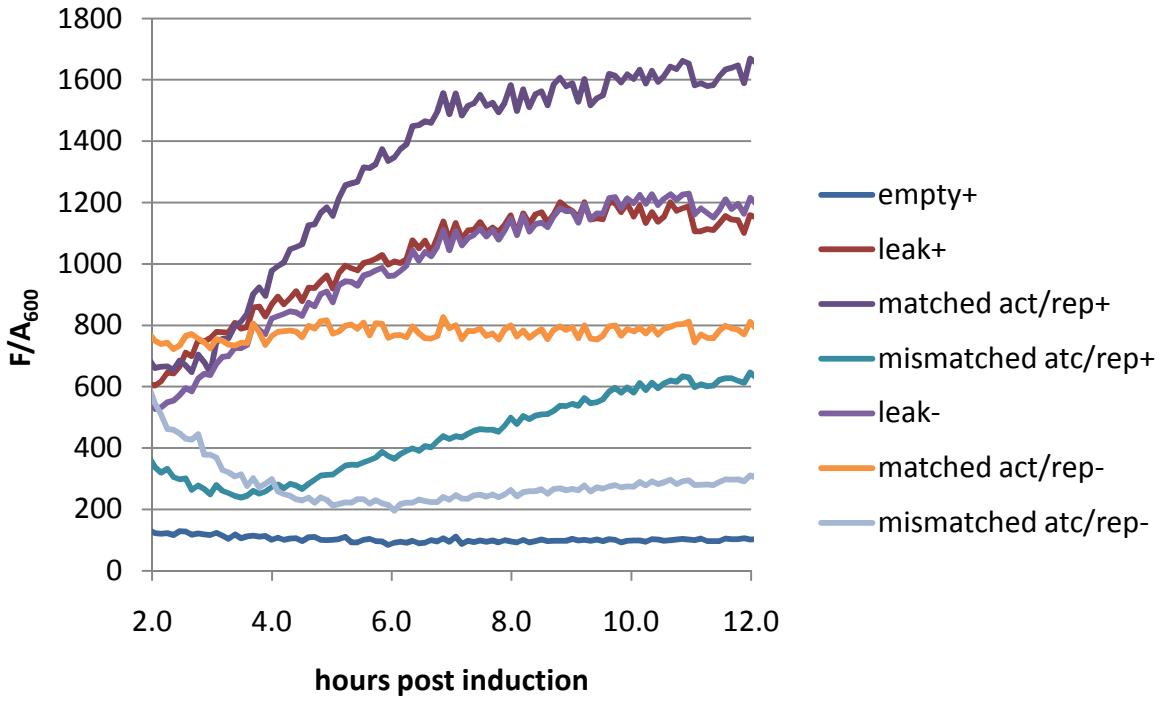


Figure 4-20 ZFP5475 based controls on a ColE1 plasmid with mCherry as the reporter. In the legend, "-" indicates no induction, and "+" indicates a saturating induction with aTc. For a description of the constructs see Figure 4-8. The matched/mismatched activator and reporter constructs are of type 1.

Threshold sensing topology using sRNA

Choice of reference levels

In electronic based combinational logic there are two power supply voltages, and signals are measured by their voltage relative to these supply voltage. The supply voltages span the range of voltage signals passed by the gates, and the voltage that represents the boundary between a high and low signal is typically close the mid-point between the supply voltages. In a PoPS based logic framework the reference low signal level is obvious, as there is a minimum PoPS value of 0. Defining a reference high level is not as straight forward. The maximum PoPS value of a promoter could be used. As the environmental conditions and metabolic load experienced by the cell varies, this maximum PoPS value could change. If the boundary between high and low states is half this maximum PoPS value, then gates have to be able to dynamically determine this half-way point. This is not an easy task.

Instead of using a promoter as a reference for approximately twice the switching value, the promoter could directly reference the switching value. Such an approach will reduce the complexity of highly robust gates and allows the switching value to be more precisely determined. By definition, a PoPS value should directly correspond to the

number of mRNA produced by a device. This mRNA then gets translated to protein by a stochastic process. Thus sensing protein levels can provide a relative estimate of the PoPS, but sensing mRNA creation directly gives the true PoPS value. Because of this difference, it would be better to use mRNA rather than protein levels to sense the strength of a promoter.

Using sRNAs

Recent work on small non-coding RNA (sRNA) indicates they might be well suited for the task of sensing differences in PoPS values. If a sRNA and mRNA contain enough complementary sequence, about 15 base pairs, then they can bind together and form a region of double stranded RNA [78]. If this bond is strong enough or if utilizes all base pairs on the mRNA, leaving no single stranded bases, then the mRNA cannot be translated because the ribosome cannot access the single coding strand. Double stranded RNAs are targeted for degradation and usually have a shorter half-live than their single stranded counterparts [79]. This pairing of mRNA with sRNA and then combined degradation results in a 1:1 removal of mRNA and sRNA. Because mRNA and sRNA binding can happen faster than translation initiation, this process should result in either the mRNA or sRNA existing in a single stranded form. The molecule present in the lower concentration will become completely complexed with the molecule present at the higher concentration; therefore, the presence or absence of single stranded mRNA can be used to indicate the relative strengths of the promoters producing the mRNA and sRNA.

The number of well studied prokaryotic sRNAs is small, and few synthetic sRNAs have been published. Many sRNAs work by binding within the 5'-UTR and altering the accessibility of the RBS. The functionality of these sRNAs is highly dependent on specific RNA secondary structures forming. Because RNA structure prediction has not been perfected, designing this type of sRNA would require some trial and error. A report indicates that sRNA do not have to bind within the 5'-UTR in order to cause reduced translation of the mRNA. Instead the sRNA can bind within the coding region of the mRNA [78]. Designing sRNA to bind within the coding region of an mRNA involves fewer constraints related to secondary structure.

Additionally, sRNAs that bind within the coding region are likely to be a scalable technology. Silent mutations within the coding regions enables the creation of orthogonal sRNA and coding sequence pairs even when the set of coding sequences all translate to the same amino acid sequence. The binding position of the sRNA must cover the 5th codon in the mRNA. There is a degree of freedom here too as the 5th codon can be placed at various positions within the sRNA. For a sRNA that binds a 15 base pair segment of the mRNA, there will be 13 positions that completely cover the 5th codon. Some tricks could also be played to increase the scalability of sRNA orthogonality. For example, additional codons could be added to the N-terminus of the protein so that there is greater possible sequence diversity in this critical region. Care would need to be taken to insure this addition does not disrupt the folding of the remainder of the protein. Knowledge of where the N-terminus of the unmodified protein folds and the inclusion of a glycine-serine linker between the original protein and the new N-terminal could help to maintain activity of the protein. While the scalability of sRNAs has not yet been demonstrated, it appears that 10s of

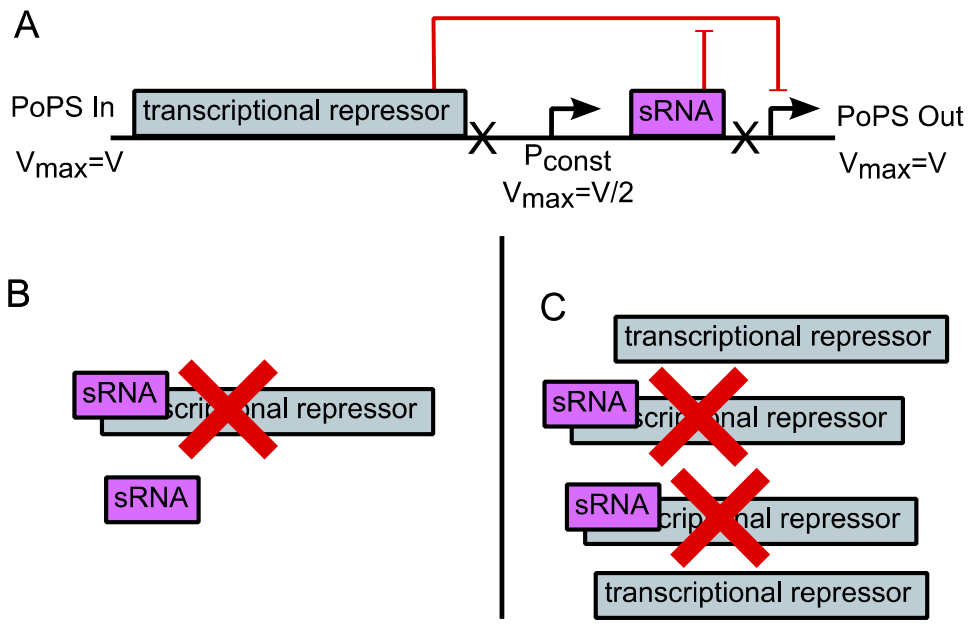


Figure 4-21 Overview of the threshold sensing topology using sRNA. A) Schematic of an inverter based on the threshold sensing topology using sRNA. A constitutive sRNA weakly attenuates translation of repressor mRNA. B) For low inputs there are more sRNAs than mRNAs and therefore no mRNA can be translated. C) For high inputs there are more mRNAs than sRNAs. Non-complexed mRNAs are translated.

orthogonal sRNAs should be possible. This is backed up by the fact that 62 native sRNAs have been found in *E. coli* and experimentally verified [80].

Overview of topology

The threshold sensing topology is based on a traditional transcriptional repressor based inverter with the addition of a constitutively expressed sRNA that attenuates expression of the transcriptional repressor. The topology is shown in Figure 4-21. The constitutive promoter will create a pool of sRNA. When the pool of mRNA is smaller than the sRNA pool, all of the mRNA will bind with sRNA and there will be no free mRNA available to be translated. When the pool of mRNA is larger than the sRNA pool, all of the sRNA will bind with mRNA and the excess mRNA will be translated. Only once the PoPS input is large enough to push the mRNA levels over the sRNA threshold does the output switch.

The constraints on the transcriptional repressor in this topology are fewer than the constraints when building a traditional repressor based inverter without sRNA. As stated previously, a Hill coefficient of greater than 1 is required to build a traditional repressor based inverter, and a Hill coefficient of 2 or more is needed for good performance. With the threshold sensing topology using sRNA, the sigmoidal shape of the transfer function mostly comes from the interaction between the mRNA and sRNA. Thus transcriptional repressors

with Hill coefficients of only 1 can be used to create well performing gates with this new topology. Therefore single zinc finger proteins, that do not form dimers, can be used to create orthogonal sets of these gates. It was hoped that creating repressor based gates would be easier than activator based ones because there is more of a precedent of wide spread use of engineered repressible promoters (such as $P_{Ltet0\text{-}1}$, $P_{Llac0\text{-}1}$).

Modeling and analysis

An ODE model was created to investigate the behavior of this topology.

$$\frac{dm}{dt} = \mathrm{In} - \gamma_m m - k_{on} ms \quad \textbf{12}$$

$$\frac{ds}{dt} = V_R n - \gamma_s s - k_{on} ms \quad \textbf{13}$$

$$\frac{dr}{dt} = mp - \gamma_r r \quad \textbf{14}$$

$$O = \frac{V}{1 + \frac{r}{K_D}} \quad \textbf{15}$$

m, s, and r are respectively the concentrations of the transcriptional repressor mRNA, the concentration of the sRNA, and the concentration of the transcriptional repressor protein. p is the maximum rate of production of repressor protein from one copy of mRNA. Units are t^{-1}. n is the concentration DNA containing the device. K_D is the disassociation constant for the transcriptional repressor, to the promoter. I is the number of polymerases entering the device per unit of time. γ_m, γ_s and γ_r are the degradation rate for mRNA, sRNA and repressor protein respectively with units of t^{-1}. k_{on} is the apparent on rate for mRNA-sRNA binding. V_R and V are the maximum rate of RNA production for the sRNA producing and output promoters respectively. O is the number of polymerase exiting the device per unit of time.

Generally V should be twice V_R in order to equalize the input ranges corresponding to high and low outputs. Such a configuration allows the inverter to maximally tolerate noise while in either state.

The complexes of mRNA and sRNA are assumed to be tightly bound and are rapidly degraded. This simplification means a differential equation is not needed for the complexes.

The equation for O (Equation 16) is based on a monomeric repressor protein. Modifying this equation for a dimeric repressor or one that forms higher order complexes is easy, but the analysis with a monomer better illustrates the advantages of this topology.

To find the steady state transfer function the ODEs are set equal to zero and then the variables m, r and s are eliminated. This results in:

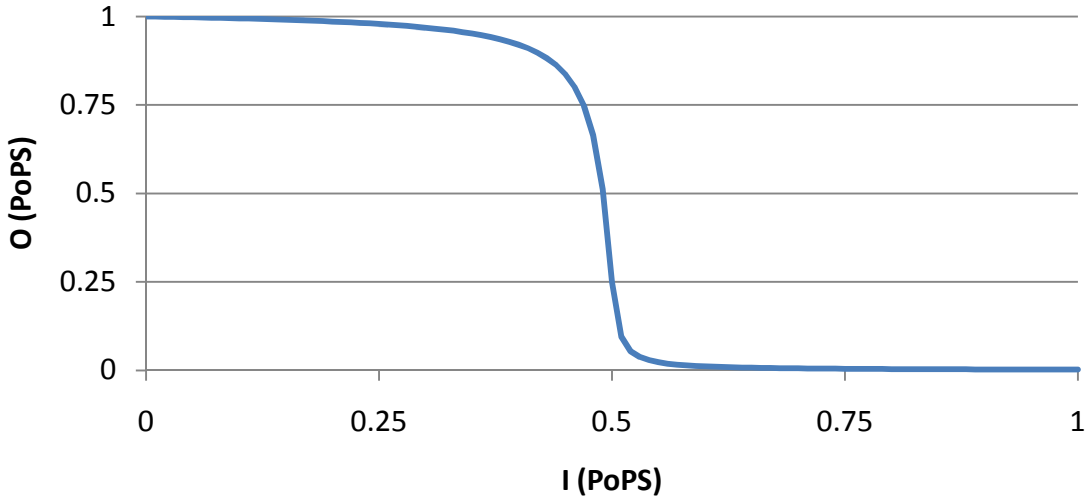


Figure 4-22 Example transfer function of threshold sensing topology using sRNA. Using parameters γ_s=0.0023 s^{-1}, γ_m=0.012 s^{-1}, γ_r=0.00039 s^{-1}, K_D=20 nM, k_{on}=69×10^6 $M^{-1}s^{-1}$, n=15 nM, p=0.005 s^{-1}.

$$O = \frac{2\gamma_m\gamma_r k_{on} V\, K_D}{p\sqrt{[k_{on}n(I-V_R)+\gamma_m\,\gamma_s]^2+4\gamma_m\gamma_s k_{on}n\,V_R}+k_{on}n(I-V_R)+\gamma_m(2\gamma_r k_{on}K_D-p\gamma_s)} \quad \textbf{16}$$

A plot of this function is shown in Figure 4-22. The k_{on} value is a published value for a specific sRNA-mRNA interaction occurring in the presence of the helper protein Hfq[81]. Hfq is a native *E. coli* protein that has been shown to increase sRNA-RNA annealing rates for several different sRNAs.

This gate topology is quite robust to parameter variations, environmental conditions and noise. This robustness is mostly due to the excellent shape of the transfer function. The transfer function has three characteristics that contribute to its robustness. First, the switching point can be set to the middle of the input range. This maximizes the range of possible reference values that will not lead to an incorrect output. Secondly, the maximum slope of the transfer function is large, and this prevents noise on a signal from propagating to downstream gates. Finally, for most of the input range, the output is close to the minimum and maximum values, and this also helps to suppress noise propagation.

The transfer function does not have the exact form of a Hill equation, but the Hill equation still provides a useful comparison. Traditional gates made with TetR or LacI are expected to have Hill-coefficients of about 2 and 4 because TetR functions as a dimer and LacI functions as a tetramer. With the right parameters, the threshold sensing topology using sRNA can far exceed the maximum slope of a Hill equation with a coefficient of 4 as shown in Figure 4-24.

To demonstrate the robustness of the transfer function to parameter variations, the transfer function was plotted with each parameter at the nominal value shown in Figure 4-22 and twice and half that value. The sensitivity to mRNA degradation rate variation is

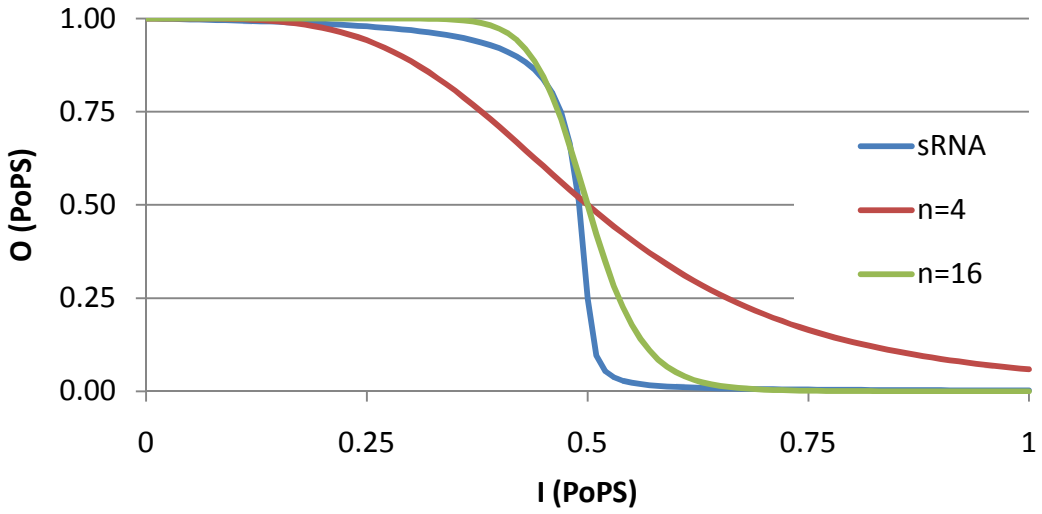


Figure 4-24 Comparison of Hill-equations with threshold sensing topology using sRNA. Lines labeled with a n parameter are Hill-equations where the n value is the Hill-coefficient used. The line labeled with sRNA represents the threshold sensing topology using sRNA. The parameters used for the sRNA line are the same as in Figure 4-22..

shown in Figure 4-23. The mRNA half-life must be kept small in order to have a strong off output state.

The sensitivity to sRNA degradation rate variation is shown in Figure 4-25. The sRNA half-life must be large relative to the mRNA half-life for the inverter to have a strong

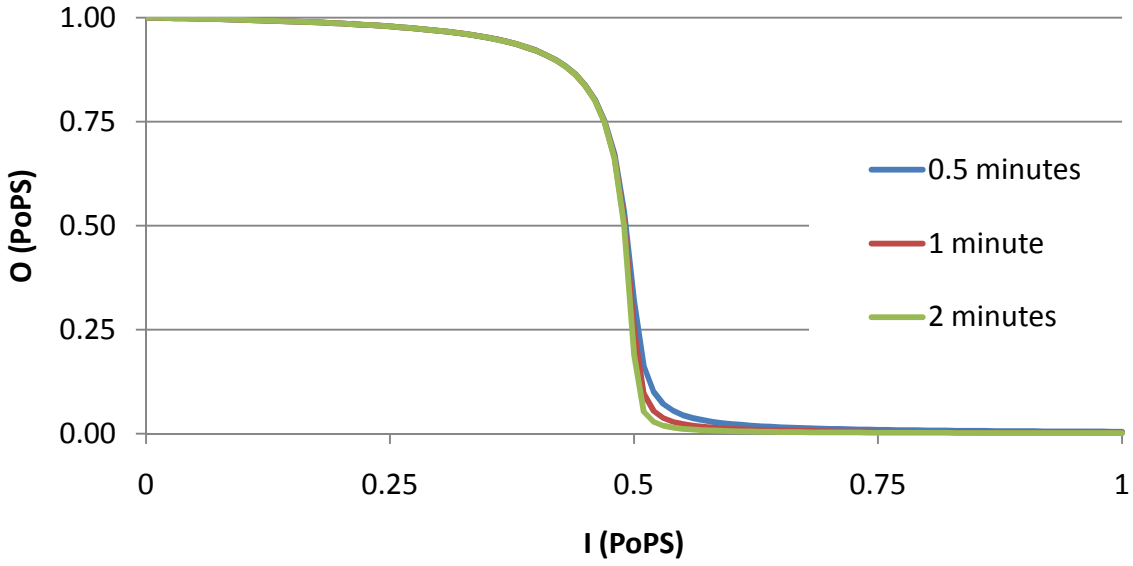


Figure 4-23 Sensitivity of transfer function to mRNA half-life variation.

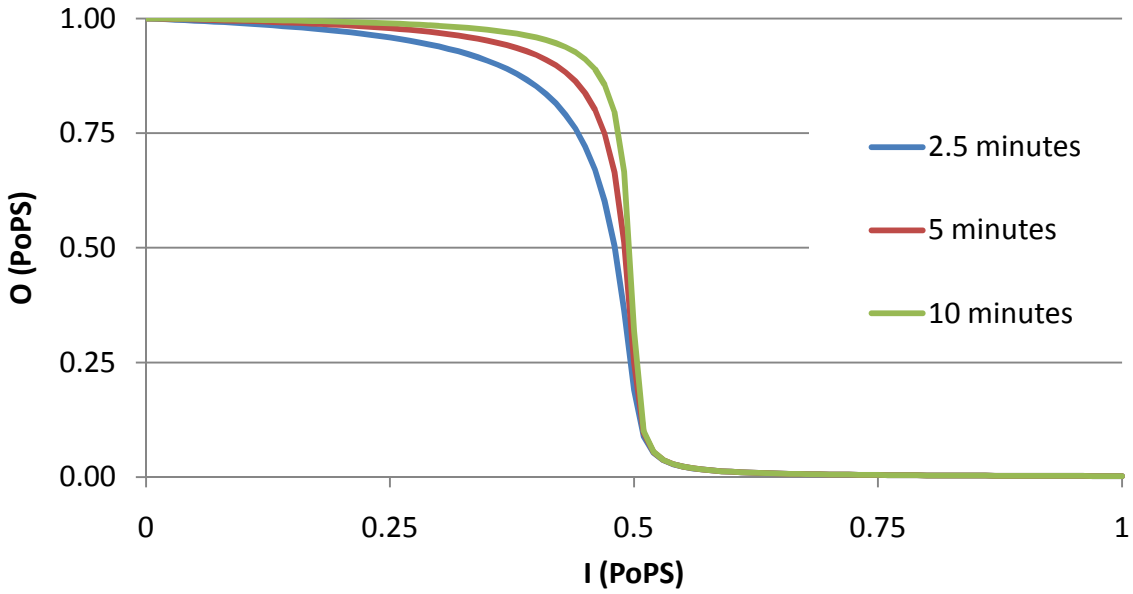


Figure 4-25 Sensitivity of transfer function to sRNA half-life variation.

on output state.

The sensitivity to protein degradation rate variation is shown in Figure 4-26. The protein degradation rate affects the steady state transfer function by modulating the size of the steady state pool of repressor protein. A small amount of repressor can get translated when the input is low, and this repressor will stick around longer with low degradation rates.

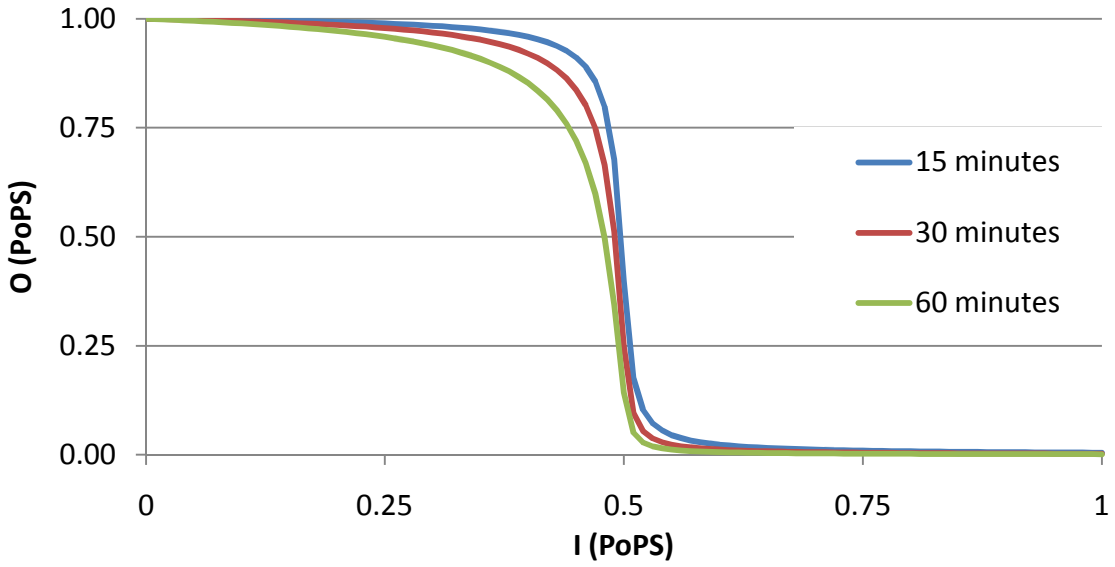


Figure 4-26 Sensitivity of transfer function to protein half-life variation.

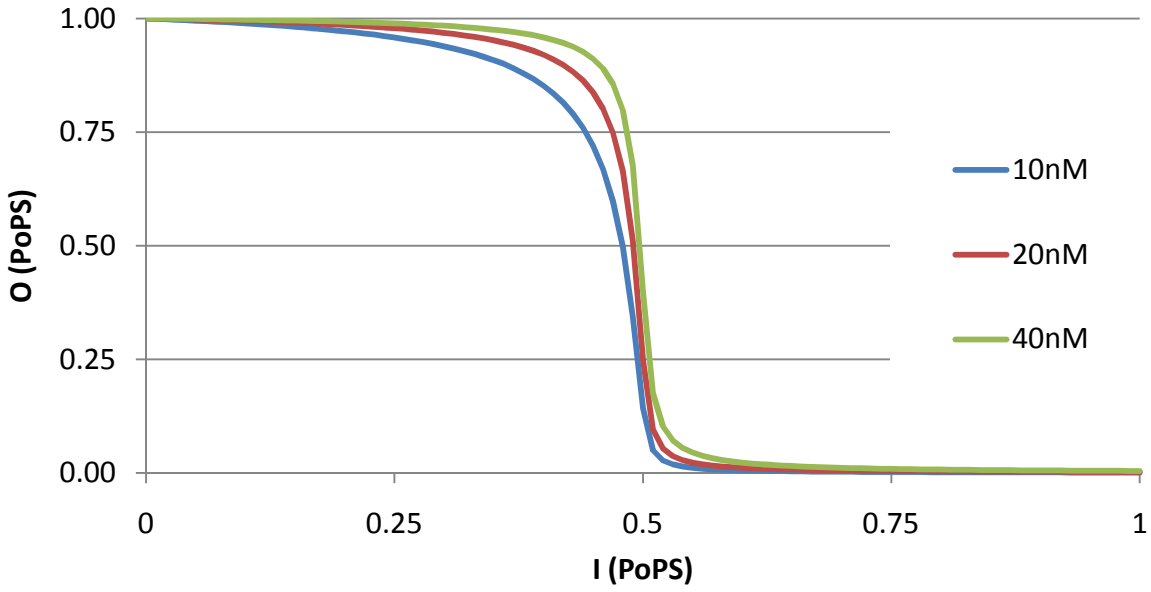


Figure 4-28 Sensitivity of transfer function to transcriptional repressor K_D variation.

The sensitivity to K_D (protein-DNA disociation constant) variation is shown in Figure 4-28. Larger K_D values result in a strong on output state, but slightly weaken the strength of the off output state. A large K_D value can mitigate a small amount of repressor being translated while the input is low because a larger pool of repressor is required to cause down regulation of the output promoter. A large K_D weakens the off output state for

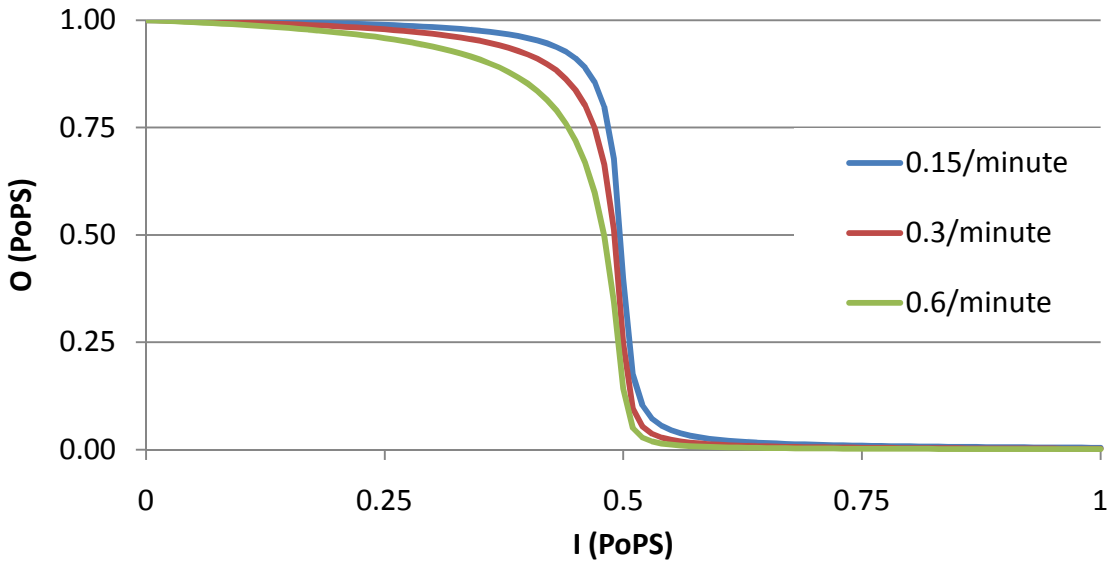


Figure 4-27 Sensitivity of transfer function to translational initiation rate variation. The values are rates of translation initiation for a single mRNA.

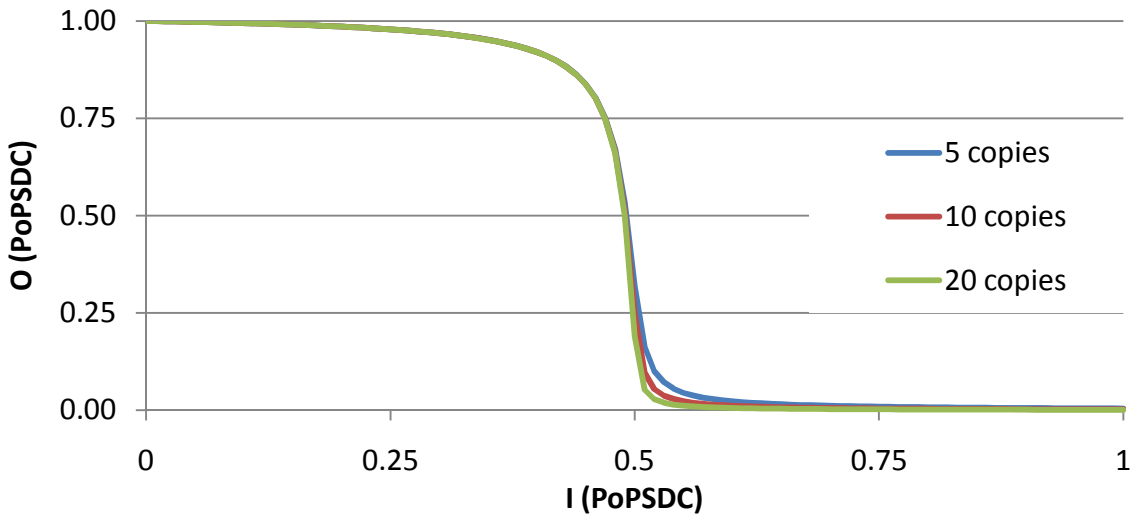


Figure 4-29 Sensitivity of transfer function to DNA copy number variation. The number of copies listed are per cell values.

the same reason, but the impact is less because the ribosomes are no longer competing with the sRNA to bind with mRNA.

The sensitivity to translation initiation rate variation is shown in Figure 4-27. Because these transfer functions are based on steady state conditions, an increase in the protein production rate is equivalent to a decrease in the protein degradation. Hence the arguments given above about the protein degradation rates also apply here, but with the impacts on the transfer function reversed.

The sensitivity to DNA copy number variation is shown in Figure 4-29. As discussed in the in the previous chapter, the axes of this plot are in PoPSDC (Polymerase Per Second per DNA Copy) to remove any anbiguity. Copy number has almost no impact on the transfer function. This is because the number of operator sites to repressor is equal to the copy number, but the number of promoters producing mRNA is also equal to the copy number. Because the number of repressors produced per copy number is constant and the number of operator sites per copy number is also constant, thus the PoPS output per copy number will also be constant. Because a continous ODE model was used here, stocastic effects due to small number of DNA copies are not modeled. In the limit as the copy number approaches 1 DNA copy per cell, these stochastic effects can have a large impact on performance.

The sensitivity to k_{on} variation is shown in Figure 4-30. The parameter k_{on} is one of the most imporant parameters of this system, but also is poorly understood. The sharpness of the transfer function depends on the competition between sRNA and the ribosomes to bind with the mRNA. A higher k_{on} value leaves less opportunity for the ribosomes to interact with the mRNA. Values of k_{on} have been measured for some sRNA [62] and many are near 10^6 $M^{-1}s^{-1}$, but the presence of Hfq can increase some kon rates by 100 fold [81].

The transfer curves presented thus far are an important part of the topology characterization, but they are only representative of steady state conditions. The dynamics of the gate are also a crutial element of the performance. The primary dynamics parameter

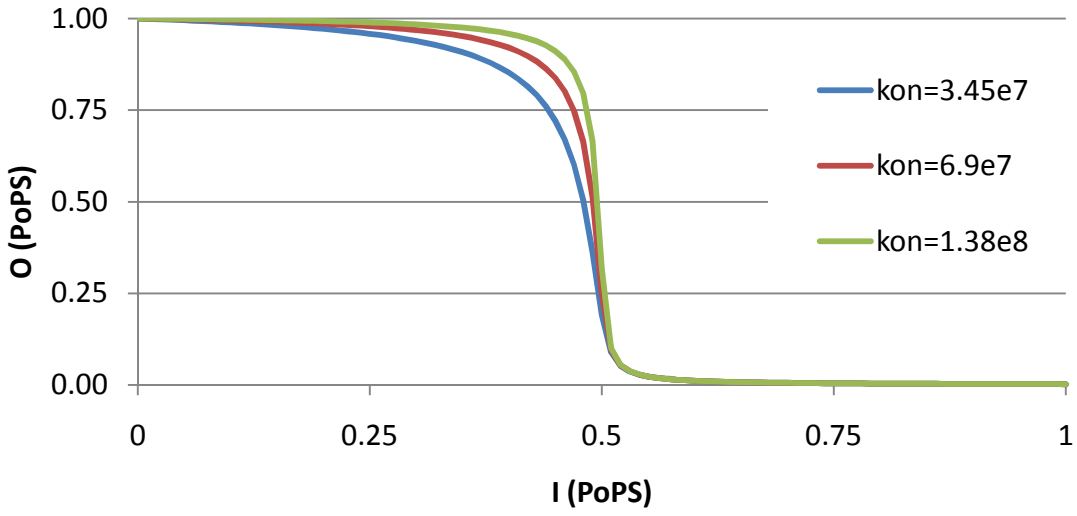


Figure 4-30 Sensitivity of transfer function to k_{on} (mRNA-sRNA annealing rate) variation. Units for the legend are $M^{-1}s^{-1}$.

to consider is the delay between the input changing and the output updating to reflect this change. For the this topology the dynamics of switching the output in a downward direction is quite different from switching in an upward direction. When the output is falling, enough mRNA must be produced to overcome the pool of sRNA and then the mRNA must be translated to create repessor protein in excess of the K_D. When the output is rising, any free mRNA must be degraded or bound to sRNA in order to stop the translation of new repressor proteins. Then the pool of existing repressor proteins must be degraded. For most reasonable cases the duration for protein degradation to occur will dominate the switching time.

When a promoter is fully repressed, the concentration of repressor protein concentration will be 100 or more times the K_D; therefore the time to lower the repression concentration to below the K_D can be many times the half-life of the protein. Increasing the protein degradation rate can be expensive to the cell because the protein product rate has to be increased by the same factor in order to maintain the same steady state levels of protein. Thus there is a direct trade off between the speed these gates can propagate signals and the metabolic burden they impose on the cell. This issue can be slightly mitigated when cooperative repressors are used because they can more tightly attenuate a promoter for the same multiple of concenration above the K_D. In an advance gate design feedback could be implemented to limit the maximum concentration of repressor protein that can accumulate and thereby reduce the duration needed to degrade the repressor protein when switching the output of the gate.

Implementation and testing

This gate topology was first implemented using TetR as the transcriptional repressor. The topology was conceived with zinc finger based repressors in mind, but as of that time a zinc finger based repressor had not been demonstrated to work in *E. coli*.

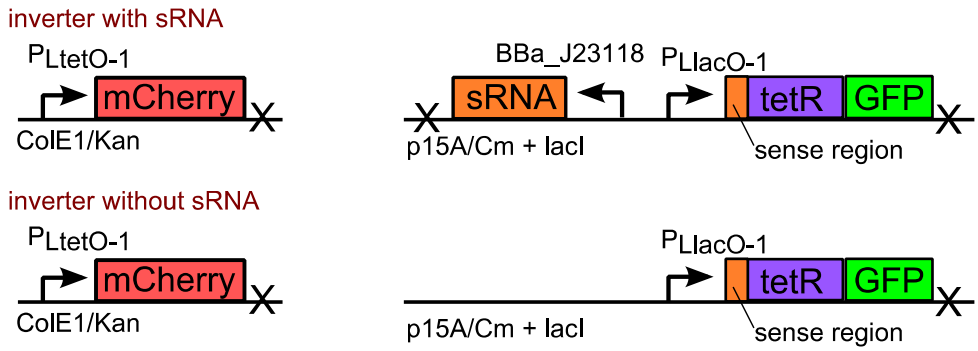


Figure 4-31 sRNA based Inverter construct and sans-sRNA control. BBa_J23118 is a synthetic constitutive promoter made by Chris Anderson. The sRNA binds to the sense region of the tetR-gfp mRNA.

An sRNA device and its cognate mRNA was obtained from Julius Lucks and Adam Arkin (not yet published). This device is based on the pT181 sense-antisense pair [62]. The previous description of the topology describes the sRNA as stopping translation of the mRNA, but for pT181 the presence of the sRNA causes an early terminator to form within the mRNA. This early terminator results in transcription stopping before the coding sequence can be transcribed. While this mechanism is different than the one given earlier, the previous ODE model does correctly encompass both mechanisms. The model only requires that mRNA and sRNA bind and then are never translated.

The approach used to find parameters that gave inverter behavior was as follows:

1. Construct TetR based inverter with an inducible $P_{Llac0-1}$ on the input and mCherry on the output. GFP was also fused to TetR. So that input levels could be monitored.
2. Create diversity in the RBS for TetR-GFP and then screen for an inverter that switches with upon induction of the $P_{Llac0-1}$ promoter with IPTG.
3. Express the sRNA from a library of constitutive promoters and screen for inverters that retain the ability to switch upon induction of the $P_{Llac0-1}$ promoter with IPTG.
4. Measure full transfer curves for the inverters that passed the previous screen and pick the one with the best transfer curve.

In the context of the models, it is easy to produce transfer functions of PoPS in to PoPS out. Directly measuring PoPS in a cell is quite difficult, and so fluorescent reporters are used as proxies for PoPS. If the fluorescence of a single reporter molecule is known along with its degradation rate and average number of proteins produced per mRNA, then it is possible to calculate the a PoPS value from the fluorescence values. A more direct route would be to use RT-qPCR to measure the mRNA concentration and then use the mRNA degradation to determine the rate of mRNA production. For this proof of concept work, only relative PoPS values were needed. The extra precision that may be achieved with RT-qPCR was not deemed worth the additional effort required.

Constructs were created for a traditional TetR based inverter and a TetR-sRNA based inverter. The constructs are shown in Figure 4-31. These constructs were exactly the same except for the addition of the sRNA producing operon. The TetR-GFP mRNA is the

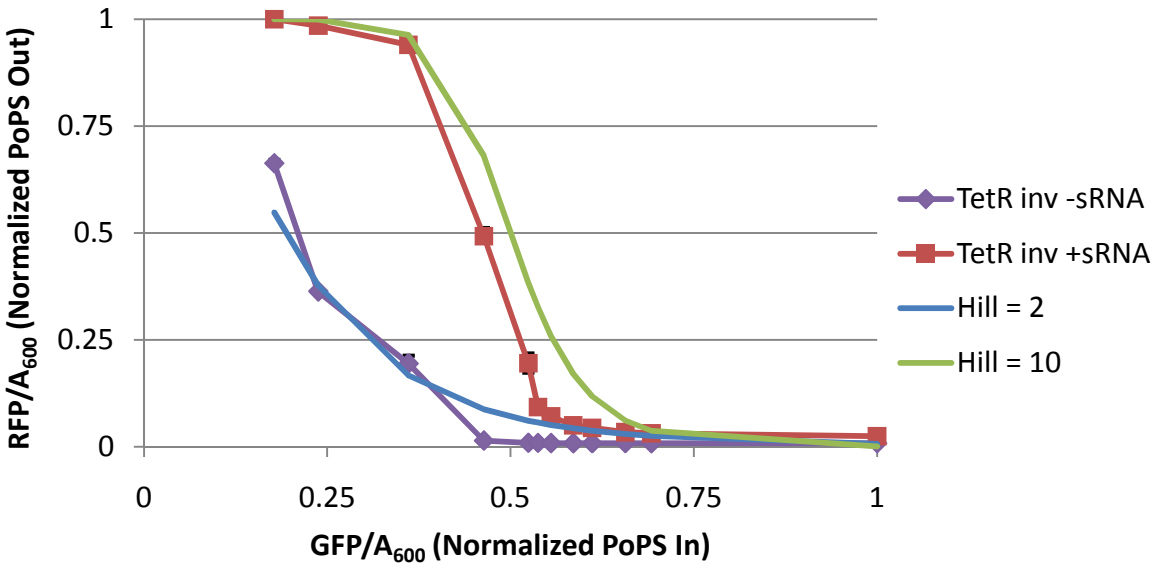


Figure 4-32 Measured transfer function for inverter made with tetR and sRNA. "TetR inv –sRNA" does not contain the sRNA operon, and the "TetR inv +sRNA" construct does contain the sRNA operon. The Hill=2 and Hill=10 lines are Hill equations where the Hill coefficient are 2 and 10 respectively. Due to the leakiness of the $P_{Llac0-1}$ promoter, inputs below 0.17 could not be generated with these constructs.

same for both constructs and includes a sense region in the 5'-UTR where the sRNA can bind. The host strain for these experiments was DH10B.

Cultures for this experiment were grown overnight in 5mL LB medium and then diluted 20:1 into fresh EZ-Rich (Teknova) medium containing 1% glucose, 50 mg/L kanamycin and 33 mg/L chloramphenicol for a total volume of 800 µL in a deep 96-well plate. Plates were sealed with Aeroseal breathable seals (no. B-100, Excel Scientific). Induction with IPTG was performed at the same time as the dilution. After 24 hours of growth at 37°C, 150 µL of culture was transferred to a black wall, clear bottom 96-well plate (Costar) for reading on a SpectraMax M2 plate reader (Molecular Devices).

The measured transfer curve shows the presence of the sRNA shifts the switching point to a higher input as expected (Figure 4-32). The maximum slope of the sRNA-TetR inverter is similar to the slop of a Hill-equation with a Hill-coefficient of 10. The low corner of this curve is sharper than the equation for the Hill-coefficient of 10, but the high corner of the curve performs slightly worse than the equation for the Hill-coefficient of 10. The TetR inverter without sRNA control should have a Hill-coefficient of 2 or less because TetR functions as a dimer. However, the TetR inverter without sRNA had a higher slope than expected. This may be due to nonlinearities in the GFP response that result in distortions of the input axis.

The next step in this work is to demonstrate that a zinc finger protein based transcriptional repressor can be used in place of TetR. Being able to use a zinc finger protein will allow this topology to scale ten or more gates. For at least the near term the limiting factor for scaling will be the number of orthogonal sRNAs.

Chapter 5 Creating orthogonal sets of repressor-promoters pairs

Orthogonality defined

Orthogonal sub-systems have behaviors that are uncorrelated to each other. In other words, the state of one sub-system does not impact the state of another orthogonal sub-system. In synthetic biology it is difficult to declare any two sub-systems within a single cell to be completely orthogonal. All sub-systems in a cell draw energy and common co-factors from the same pool, and depleting any of these shared resources will impact all other sub-systems in that cell. Therefore in synthetic biology, orthogonality must be stated in terms of the conditions of the test and the degree of correlation observed. When considering orthogonality for groups of more than two sub-systems it is often useful to define a threshold of orthogonality, where a set of sub-systems is considered orthogonal if all possible pairwise correlation coefficients for that set are below some defined threshold value.

Why orthogonality is needed

The level of orthogonality required between sub-systems is highly dependent on the functions performed by the sub-systems and the application context they are used in. Some sub-system, such as metabolic pathways, may be able to perform their intended function even when used in tandem with non-orthogonal sub-systems. Generally, the performance of the metabolic pathway will degrade, but not stop functioning completely in the presence of competing pathways. However, the class of sub-systems that contains cell signaling and information processing sub-systems are not as robust to non-orthogonal interactions. These sub-systems generally have a bimodal behavior where the performance is minimally impacted by increasing amounts of non-orthogonality up to a limit. After this limit has been exceeded the performance drops off rapidly and often catastrophically.

Orthogonality not only enables functional ensembles of sub-systems, but also plays an important role in reducing complexity. When sub-systems have material amounts of non-orthogonality an interaction term must be included between every pair of sub-systems. As the number of sub-systems increases, the number of interaction terms goes up with the square of the number of sub-systems, therefore, if non-orthogonal interaction terms can be kept small enough that they can be safely ignored, then a large amount of complexity can be dropped from the system. Removing this complexity gives an engineer a greater ability to think about the behavior of a larger system than would be possible if the interaction terms had to be considered. The reduced complexity also simplifies the design process, resulting in faster design cycles.

Desired specification for sets of orthogonal promoter-repressor pairs

The primary goal of creating orthogonal promoter-repressor pairs is to use them to implement logic gates. In this context orthogonality mainly implies that the transcriptional repressor from one promoter-repressor pair will not attenuate transcription from the promoter of a second promoter-repressor pair. The degree of orthogonality necessary for logic gates is not fixed. Generally a logic gate can tolerate a specific amount of non-orthogonal interactions. The total amount of non-orthogonal interactions a gate is

subjected to is the sum of the individual non-orthogonal interactions the gate has with every other gate in the system. This implies that the strength of the individual non-orthogonal interactions must decrease as the number of gates in the system increases.

The degree of orthogonality required is also a function of the fidelity required from the logic block being implemented. If the logic block specifications allow for the output to be incorrect for some percentage of inputs, then the orthogonality requirements can be reduced. Consider the case of a system involving 10 promoter-repressor pairs. The worst case scenario in terms of orthogonality is that 9 of the 10 repressors are being expressed. The promoter who's cognate repressor is not being expressed will be subjected to the non-orthogonal interactions with the 9 other repressors. There are a total of 2^{10}=1024 states that can be encoded by the presence or absence of 10 repressor proteins. Of these 1024 states, 10 of them, or less than 1%, are of this worst case class where 9 of 10 repressors are expressed. If the system is designed where it produces an incorrect output if 9 of the 10 repressors are expressed at once, but gives the correct output for cases where 8 of the 10 repressors are expressed at once, then the amount of orthogonality required for each gate can be decreased.

This tradeoff between orthogonality and fidelity can be taken further, but the number of states that give incorrect outputs grows faster than the orthogonality requirements decrease. For example, if the system is allowed to give incorrect outputs for cases when 9 or 8 of the 10 repressors are expressed, then the number of states where 9 or 8 of the repressors are expressed is given by:

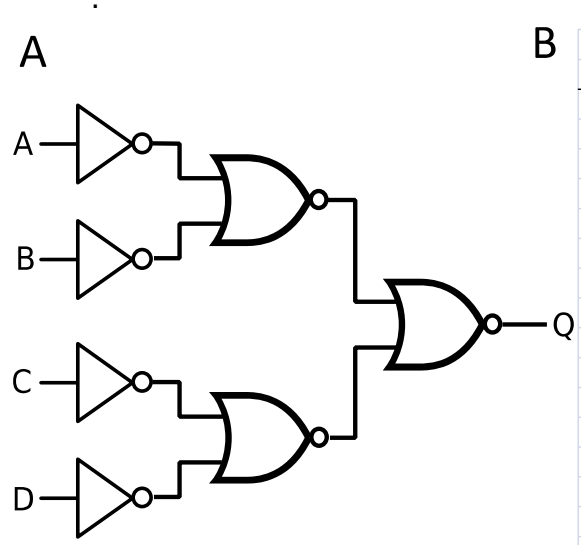
$$\binom{10}{9} + \binom{10}{8} = 10 + 45 = 55 \qquad 17$$

This is over 5% of the possible 1024 states.

Additionally, in most logic networks the combination of states taken on by the individual gates is a subset of the possible states the individual gates can take on when not connected. These unutilized states can allow for reduced orthogonality between a set of gates without a reduction of fidelity. For example, for the 4 inverter, 3 NOR gate circuit shown schematically in Figure 5-1A and as a genetic network in Figure 5-1C, no more than 5 of the 7 repressors proteins will ever be expressed simultaneously as shown in the table in Figure 5-1B. The worst possible condition for a generalized 7 repressor circuit is 6 of the 7 repressors being simultaneously expressed, but for this specific circuit that case cannot happen. With this knowledge, the allowable strength of interaction between non-cognate pairs can be increased.

In summary, the degree of orthogonality required for logic circuits will depend on the fidelity required by the application and the specific logic network being implemented. Generally the strength of non-cognate interactions must decrease as the size of the logic network increases. When designing sets of orthogonal promoter-repressor pairs for general use, the strength of the non-cognate interactions relative to the cognate interactions should be as small as possible.

For the specific goal of constructing a 5 or more gate logic block as a technology demonstration, the ceiling for the allowable orthogonality can be estimated. If the sum of non-cognate attenuation values for a promoter exceeds 50% of the promoter's maximal activity, then the output of the gate may switch incorrectly. The choice of a 50% limit



B

Input				Repressor Expressed							Total Repressors
A	B	C	D	1	2	3	4	5	6	7	Expressed
0	0	0	0	0	0	0	0	1	1	0	2
1	0	0	0	1	0	0	0	0	1	0	2
0	1	0	0	0	1	0	0	0	1	0	2
1	1	0	0	1	1	0	0	0	1	0	3
0	0	1	0	0	0	1	0	1	0	0	2
1	0	1	0	1	0	1	0	0	0	1	3
0	1	1	0	0	1	1	0	0	0	1	3
1	1	1	0	1	1	1	0	0	0	1	4
0	0	0	1	0	0	0	1	1	0	0	2
1	0	0	1	1	0	0	1	0	0	1	3
0	1	0	1	0	1	0	1	0	0	1	3
1	1	0	1	1	1	0	1	0	0	1	4
0	0	1	1	0	0	1	1	1	0	0	3
1	0	1	1	1	0	1	1	0	0	1	4
0	1	1	1	0	1	1	1	0	0	1	4
1	1	1	1	1	1	1	1	0	0	1	5

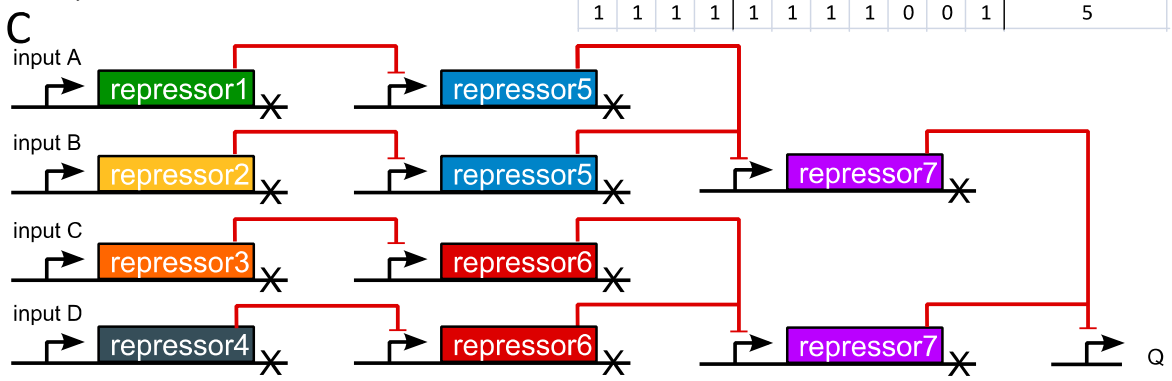


Figure 5-1 Not all combinations of repressor states can be reached by a logic circuit. A) An example logic circuit with 4 inverters and 3 NOR gates. B) The truth table for the circuit in A along with the state of each of the repressors proteins for every possible set of inputs. C) A genetic network that implements the same circuit as A.

assumes the gate's switching threshold is centered in the gate's input range and that none of the cognate repressor is present. Neither of these assumptions is likely to be true, but other parts of this analysis are overly conservative and offset these liberal assumptions. For a 5 repressor system, there are 4 non-cognate repressors. For each promoter the sum of the attenuation percentages from the non-cognate repressors should sum to less than 50%. Assuming all non-cognate interaction strengths are equal, then a non-cognate repressor should cause 12.5% of attenuate or less.

The above calculation does not place any constraints on the strength of cognate repression. There is a simple constraint of the cognate repressor causing over 50% repression in order for the output to switch. Beyond orthogonality, the output range of each gate ideally would be equal. This implies the maximum activity of each promoter in the set should be similar.

The strength of the promoters also should be strong enough to produce pools of proteins 10 times larger than the K_D of the repressors (~10nM) while cells are growing exponentially (30 minutes/cell division→effective degradation rate of $3.9\times10^{-4}s^{-1}$).

$$\frac{10K_D\gamma}{cell\ volume} = \frac{10(10^{-8}\text{M})3.9\times10^{-4}\text{s}^{-1}}{1.5\times10^{15}\text{L}} = 0.026\ \text{proteins/second/cell} \quad \textbf{18}$$

Depending on the number of proteins generated per mRNA, the required PoPS per cell should also be near this number. This represents a weak promoter. Too strong of a promoter is also detrimental as the metabolic burden on the cell will increase with promoter strength and proteins are more likely to be toxic at high expression levels. Placing an upper limit on the promoter strength is hard because there are limited analytical tools for investigating metabolic burden.

Creating orthogonal sets

Testing for orthogonality between N pairs of items generally requires N^2 evaluations. Because the number of evaluations grows quickly, there is a tradeoff between keeping the number of evaluations in the orthogonality experiment small and searching a large enough library of pairs to find the needed orthogonality. Assaying promoter-repressor pairs for orthogonality was done on 96-well plates, therefore it made sense to pick a library size based on how many pairs can be tested by an A×B number of 96-well plates. To reduce pipetting errors, the plates were tiled and the utilized wells were all in an N×N square. Therefore 9×9 test would use 2 96-well plates (because they are limited to 8 wells in one direction) even though one plate contains enough wells. Based on plate geometry, 8×8, 16×16, 24×24, 32×32 and 40×40 sized squares are efficient choices. Setting up a 40×40 sized experiment without using robotics would be fatiguing and push the limits of what one person can complete on each day of the experiment. A 32×32 square (12 plates) experiment appeared as though it could be carried out by a single person and hopefully would be large enough to get 5 pairs with good orthogonality.

Choosing zinc finger proteins

32 zinc finger proteins from the steric hindrance experiment were selected based on their ability to strongly attenuate transcription in that experiment. This provided experimental validation that these zinc finger proteins are able to bind to the operator site they were designed for and that they are not highly toxic when expressed at low levels. Because pre-existing zinc finger proteins were used, the set of operator sites was also fixed. This limits the placement of the operator sites to areas mostly outside of the -35 and -10 regions.

Choosing promoters

A set of orthogonal promoter-repressor pairs could be constructed from a set of promoters that only differ by the operator site for the zinc finger protein. However, if the end goal is to use the orthogonal promoters within a single cell, then minimizing sequence homology between the promoters is important for reducing homologous recombination and maintaining the desired DNA sequence. To achieve some sequence diversity and

Table 5-1 BioFAB promoters

RPL #	Promoter sequence
66	TTGACAATTAATCATCGGCTCATATGGTCTGTGGA
69	TTGACAATTAATCATCGGCTCGTAGGGTATGTGGA
81	TTGACAATTAATCTCCGGCTCTTACGGTATGTGGA
82	TTGACAATTAATCATCGGCTCCTATGGTGTGTGGA
83	TTGACAATTAATCATCGGCTCATAACCTTTGTGGA
104	TTGACAATTAATCATCGGCTCTTAGGTTCTGTGGA
106	TTGACAATTAATCATCGGCTCGTAGATTTTGTGGA
125	TTGACAATTAATCATCCGGCTCATAGCGTGTGTGGA
126	TTGACAATTAATCATCGGCTCGTAGGCTGTGTGGA

obtain similar maximum promoter activities, a set of promoters were picked. These promoters came from the BioFAB's Randomized Promoter Library (RPL) version 1. A set of 9 promoters with a similar but low constitutive activities were selected. These promoters are listed in Table 5-1. The 32 operators corresponding to the pool of 32 zinc finger proteins were cloned into the 9 promoters directly upstream of the -35 region. These constructs were built on the BioFAB plasmid pFAB217. The complete combinatorial set of 9×32=288 promoter-operators was attempted. A randomly picked colony from each cloning reaction was sequenced and 120 correct insertions were found, representing 30 of the 32 operator sites. This was deemed a large enough pool to move forward with.

The decision to place the operator just upstream of the -35 region was made before the results of the steric hindrance experiments had concluded. In the steric hindrance experiments this corresponds to the operator at position -54. The ZFP-54 was able to cause strong attenuation at this position, but in general the region around -54 had only moderate repression. Better approaches are certainly possible if a new set of zinc finger proteins were constructed, as then they could overlap the -35 region and extend downstream from there. Alternatively, it may be possible to use the current set of zinc finger proteins and place the operators between the -35 and -10 regions. This may involve extending this region from 17 to 18 base pairs. From the steric hindrance data, it appears that binding directly downstream of the -10 region could give good results. However, placing an 18bp operator downstream of the -10 region means the operator will extend into the sequence coding for the mRNA. This is not desirable from an engineering perspective because it restricts the flexibility to place any mRNA coding sequence downstream of the promoter. This also violates the spirit of a PoPS interface as nothing but the polymerase should cross the interface between the two connected components.

Constitutive activity of the promoter library was measured and the results are shown in Figure 5-2. From this data 30 promoters were chosen. This set contains all 30 of the zinc finger protein operator sites that were successfully cloned and has a low level of variation in promoter strength.

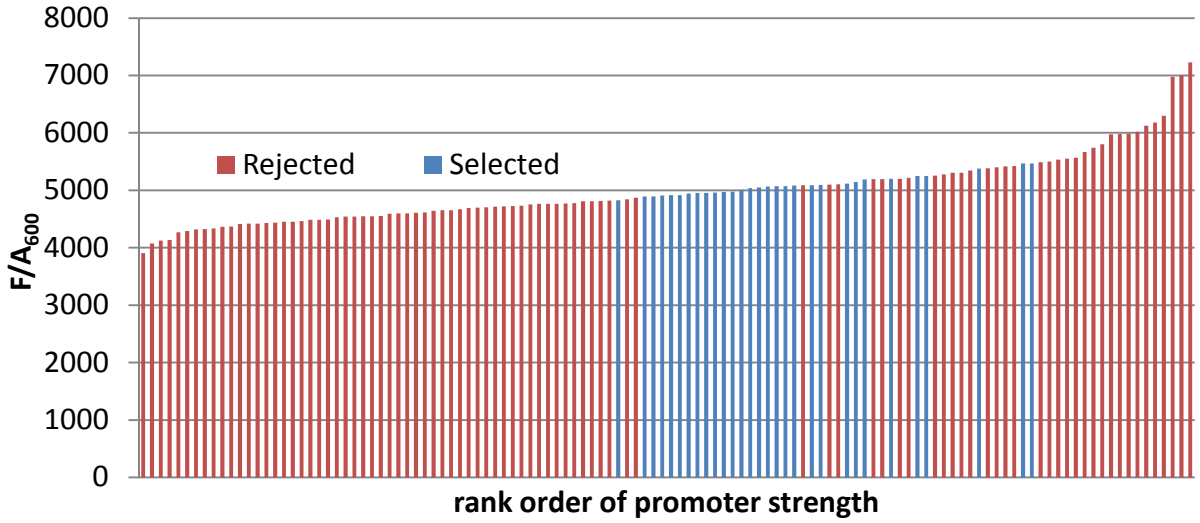


Figure 5-2 Strength of 120 promoters were measured. 30 promoters with similar strength were selected such that none of these 30 promoters contain the same zinc finger protein operator site.

Orthogonality assay

The selected promoters were moved to the same mCherry-based reporter plasmid that was used for the steric hindrance of RNA polymerase experiments. 3 of the 30 promoters failed to clone into this reporter plasmid and were dropped from the study. Once again the DP10 strain was used [55]. Even with a saturating induction, the DP10 strain is useful because it removes the up regulation of arabinose import upon induction. This positive feedback loop can result in a heterogeneous induction when a subset of the population sequesters all the arabinose.

Each evaluation of a promoter-repressor paring required transforming a zinc finger expression plasmid and a reporter plasmid into *E. coli*. For a 27×27 size orthogonality experiment, this required 729 transformations. To save time and effort these transformations were performed without plating on solid media. The sources of the plasmids going into the transformations were from mini-preps of plasmids that had been previously sequence verified, thus there was no need to obtain single colonies.

Initially a double transformation of the reporter with a ColE1 origin and the zinc finger protein expression plasmid with a SC101** was attempted with chemically competent DP10 cells. After growing the transformation in LB and antibiotics overnight, the A_{600} of the cultures was measured to verify the cultures were growing and the transformations were successful. This method was tried several times with various tweaks to the transformation protocol, but the transformation success rate never exceeded 65%. This was likely due to the SC101** plasmid. When plasmids based on this vector were transformed and plated on solid media, colonies would not appear until 18 to 24 hours after the transformation. Other plasmids often show colonies in as little as 12 hours. This

difference may be due to the antibiotic cassette on the SC101** plasmid not having been optimized for such low copy numbers.

The successful method was to perform single plasmid transformations to place the SC101** plasmid in DP10 to make 27 strains, and then make these 27 strains competent and transform in the ColE1 reporter strain. The transformation protocol used to do the ColE1 transformations without solid media was performed as follows:

1. Prepare and freeze chemically competent cells[82]
2. Place sterile deep 96-well plates on ice
3. Thaw competent cells on ice
4. Dispense 100µL into each well of a deep 96-well plate
5. Pipette 100ng of plasmid into each well
6. Leave 96-well plates on ice for 10 minutes

Table 5-2 27×27 orthogonality matrix, left hand half.

		zinc finger protein											
promoter	op	-57	-54	-51	-49	-48	-45	-44	-43	-40	-37	-33	-31	-30
83	-57	.12	.24	.12	.92	.37	.73	.89	.88	.95	.89	.81	.9	.38
82	-54	.44	.31	.35	1.	.33	.71	.85	.99	.89	.78	.82	.83	.68
125	-51	.37	.44	.3	.76	.39	.52	.85	.73	.82	.78	.82	.8	.67
125	-49	.56	.57	.49	.63	.43	.57	.85	.73	.87	.82	.79	.81	.74
82	-48	.45	.59	.38	.81	.43	.41	.7	.56	.75	.77	.84	.85	.73
83	-45	.42	.26	.52	.75	.59	.52	.8	.59	.73	.73	.71	.72	.75
126	-44	.67	.75	.59	.77	.6	.62	.69	.57	.62	.74	.67	.67	.8
69	-43	.66	.77	.71	.72	.67	.69	.69	.3	.64	.7	.75	.73	.75
125	-40	.67	.74	.71	.81	.7	.88	.75	.52	.11	.45	.71	.76	.78
69	-37	.7	.77	.73	.75	.65	.7	.81	.71	.47	.2	.7	.61	.94
83	-33	.77	.83	.72	.81	.73	.75	.78	.8	.82	.77	.6	.5	.79
83	-31	.84	.86	.85	.9	.8	.81	.91	.86	.88	.88	.52	.42	.51
83	-30	.78	.82	.75	.84	.74	.75	.8	.83	.82	.78	.6	.47	.14
83	-28	.89	.88	.79	.9	.74	.87	.86	.93	1.	.81	.89	.67	.9
83	-27	.8	.77	.63	.73	.58	.79	.69	.7	.8	.8	.66	.63	.38
104	-25	.84	.86	.81	.73	.74	.87	.78	.89	.96	.88	.64	.72	.33
82	-24	.48	.89	.77	.88	.85	.86	.87	.82	.84	.97	.83	1.	.49
106	-22	.61	.95	.79	.96	.77	1.	.87	.85	.88	.9	.84	.89	.59
69	-18	.96	.91	.71	.92	.8	.87	.97	.93	.92	.92	.97	.83	.87
83	-16	.96	.89	.7	.82	.72	.87	.87	.96	.88	.88	.9	.85	.92
83	-13	1.	.98	.75	.97	.76	.91	.86	.93	.94	.91	.9	.94	.92
125	-12	.96	.94	.76	1.	.76	.93	.94	.95	.95	.96	.87	1.	.96
104	-10	.99	1.	.45	.89	.57	.92	.62	.9	.89	.95	.67	.74	.56
83	-9	.89	.92	.81	.91	.78	.83	.92	.95	.91	.87	.87	.84	.95
66	-6	.56	.69	.55	.46	.54	.58	.57	.52	.64	.49	.65	.51	.59
83	-3	.78	.82	.74	.99	.67	.76	.95	.97	.95	.97	.94	.91	.88
126	+4	.69	.77	.65	.89	.61	.69	.92	.79	.9	.94	.9	.89	1.

7. Seal 96-well plate with Aeroseal breathable seals (no. B-100, Excel Scientific)
8. Incubate at 37°C with shaking for 1 hour
9. Add 900µL of LB media and antibiotics to each well
10. Re-seal plates and incubate at 37°C with shaking overnight

From this point on the assay is the same as the steric hindrance assay. Only saturating inductions were performed with 20mM arabinose, and F/A_{600} levels were measured 24 hours after dilution and induction into EZ-Rich media (Teknova).

Results and analysis

The data is shown in Table 5-2 and Table 5-3. Each row of these tables corresponds to a single promoter and each column belongs to a zinc finger protein. The promoter

Table 5-3 27×27 orthogonality matrix, left hand half.

														zinc finger protein	
-28	-27	-25	-24	-22	-18	-16	-13	-12	-10	-9	-6	-3	4	promoter	op
.62	.58	.88	.71	.77	.89	.85	.84	.85	.85	1.	.71	.5	.58	83	-57
.86	.73	.93	.63	.84	.91	.89	.86	.81	.77	.92	.77	.75	.76	82	-54
.87	.66	.78	.6	.78	.73	.74	.76	.76	.76	.92	1.	.65	.73	125	-51
.92	.74	.83	.68	.72	.8	.82	.79	.78	.78	.86	1.	.65	.87	125	-49
.67	.74	.72	.54	.83	.82	.5	.8	.66	.8	.97	1.	.62	.93	82	-48
.74	.79	.81	.81	.42	.81	.7	.77	.71	.76	.92	1.	.7	.88	83	-45
.69	.67	.66	.7	.66	.72	.74	.68	.71	.78	.85	.98	.76	1.	126	-44
.77	.78	.82	.75	.71	.66	.74	.79	.66	.79	.9	.92	.67	1.	69	-43
.83	.92	.82	.89	.76	.8	.84	.75	.82	.85	1.	.94	.76	.87	125	-40
.8	.93	.91	.86	.85	.81	.83	.83	.78	.79	.9	1.	.85	.89	69	-37
.64	.75	.76	.78	.75	.75	.75	.83	.81	.74	.89	1.	.79	.84	83	-33
.68	.6	.88	.57	.86	.86	.84	.85	.81	.82	1.	.94	.83	.87	83	-31
.15	.36	.73	.24	.77	.76	.83	.83	.8	.85	1.	.99	.76	.64	83	-30
.27	.7	.75	.62	.87	.81	.93	.88	.84	.92	.99	.98	.73	.73	83	-28
.29	.42	.35	.46	.64	.75	.8	.75	.71	.74	.83	1.	.78	.78	83	-27
.14	.53	.4	.37	.74	.92	.81	.82	.85	.82	.93	1.	.9	.91	104	-25
.73	.57	.71	.38	.65	.73	.73	.67	.73	.78	.99	.99	.84	.85	82	-24
.86	.64	.8	.52	.63	.7	.82	.86	.82	.85	.97	.98	.91	.91	106	-22
.89	.88	.85	.82	.88	.2	.8	.86	.86	.84	.95	1.	.88	.94	69	-18
.84	.87	.76	.78	.82	.78	.64	.83	.72	.8	.8	1.	.82	.97	83	-16
.91	.83	.83	.89	.78	.8	.81	.62	.22	.81	.4	.86	.84	.88	83	-13
.8	1.	.89	.93	.87	.84	.9	.89	.25	.81	.42	.86	.87	.93	125	-12
.83	.62	.69	.74	.72	.67	.6	1.	.48	.64	.39	.59	.54	.57	104	-10
.83	.94	.84	.84	.79	.79	.76	.81	.4	.61	.27	.62	.81	1.	83	-9
.69	.67	.66	.66	.66	.59	1.	.55	.49	.53	.46	.27	.2	.57	66	-6
.95	1.	.94	.85	.87	.84	.87	.84	.78	.79	.66	.37	.08	.27	83	-3
.94	.76	.88	.83	.91	.85	.83	.91	.63	.86	.87	.94	.33	.29	126	4

column lists the base promoter that the operator was added to. These number reference Table 5-1. The operator column lists the operator that is upstream of the -35 region of the promoter. These values are from the P_{ZC35} promoter in Chapter 3 and indicate the most upstream base pair of the operator site relative to the transcriptional start site. The numeric column headings also reference the P_{ZC35} promoter. These numbers tell what operator size along P_{Z35} the zinc finger protein was designed to bind.

The numeric data points at the intersection of a row and column are normalized F/A_{600} values, and they are normalized to the maximum value found in that row. This assumes that for each promoter tested, at least one of the 27 zinc finger proteins tested will not interact with that promoter. This is likely true, but a no zinc finger protein control should be included for each promoter if this experiment is repeated. Each cell of the table is shaded proportionally to the value it contains. The shading for a value of 1 is completely white and for a value of 0 the shading is dark blue/grey. Locations on the table that represent a cognate pair have a black boarder. These locations all fall on a diagonal line. Ideally this diagonal line would only contain values of 0 (dark shading) and the off diagonal locations should all contain values of 1 (white).

In the steric hindrance experiment where the zinc finger protein was expressed using P_{BAD}, one third of the zinc finger proteins were able to reduce the mCherry expression to below 1% of the nominal value. In the orthogonality experiment the strongest cognate pair repression only reduced expression to the 8% level. The cognate pair repression would probably increase if the operator sites were moved downstream.

At several regions along the diagonal there are groups of non-cognate high repression. For example the group -57, -54 and -51 all have more than 50% repression from the non-cognate pairings. These three proteins also have a four finger segment in common. It appears these common segments of fingers can weakly bind to a portion of their cognate operator and cause attenuation. If new zinc finger proteins were designed for the purpose of making sets of orthogonal promoter-repressor pairs, then sharing segments of several zinc fingers between proteins would not be advised.

Metrics for orthogonal sets

This set of orthogonality data begs the question, if a set of N orthogonal promoter-repressor pairs are needed, then how should a subset of pairs be selected from the full set? A metric is needed for evaluating the quality of a set of orthogonal pairs. Such a metric can be quite simple. For example the expression difference between the lowest non-cognate pair and the highest cognate pair could be used. This is a very conservation metric that only considers the worst case. In fact, this metric cannot differentiate between great set with one bad interaction and a set where all the interactions are bad. A less conservative metric would be to take the average expression level for non-cognate pairs and from this subtract the average expression level for cognate pairs. Such a metric encapsulates information about every interaction within the set. Variations on this metric can also be done where the difference is between one standard deviation below the average expression level for non-cognate pairs and one standard deviation above the average expression level for cognate pairs. This metric is more conservative than simply subtracting the means, but still includes information from all of the data points.

For more fine tuned control of the metric, the three methods listed above can be combined. These three methods are difference between minimum non-cognate and maximum cognate, difference between means, and difference between a standard deviation below the mean to a standard deviation above the mean. By using a weighted sum the three differences can be rolled into a single metric. The relative weighting of these three components in the summation can be modified to suit the application.

At the beginning of this chapter, the orthogonality requirements for logic gates hinted at some useful metrics of orthogonality. With logic gates, the sum of non-cognate interactions for an individual promoter is important. This can be expanded to a whole set of orthogonality data by taking the maximum of these per-promoter sums to arrive at a single orthogonality metric for the data set. Alternatively, some of these interactions may not be relevant to a specific logic network, as depicted in Figure 5-1. For these situations the orthogonality data related to a promoter can be evaluated by considering each possible set of inputs and then summing the non-cognate interaction strengths for only the repressors that can be simultaneously present in that case. Thus each promoter will have a sum for every possible input. The maximum of these sums is then the orthogonality term for that promoter. The orthogonality metric for the whole logic network would be the maximum of the per-promoter orthogonality terms. To take this optimization to its conclusion, the orthogonality metric for the whole logic network is a function of where each promoter is used in the logic network, and therefore this metric can calculated for all permutations of promoter assignments to find the optimal configuration. This optimal configuration will be the one with the lowest orthogonality metric.

Reduced sets

Using metrics like the ones described above, useful subset of promoter-repressor pairs can be pulled out of the orthogonality experiment data. A set of 7 orthogonal promoter-repressors pairs is shown in Table 5-4. This data comes from Table 5-2 and Table 5-3 and the labels and values have the same meaning here. The data has been re-normalized to the maximum in each row. This set has a few strong non-cognate interactions that will limit its usefulness. However, for applications where some combinations of repressors will never co-exist, this set of repressors may be usable.

Table 5-4 Orthogonal set of 7 promoter-repressor pairs

		zinc finger protein						
promoter	op	-57	-40	-37	-30	-18	-12	-3
83	-57	0.12	1.00	0.94	0.40	0.93	0.90	0.53
125	-40	0.81	0.14	0.55	0.95	0.97	1.00	0.93
69	-37	0.75	0.50	0.21	1.00	0.87	0.83	0.91
83	-30	0.95	1.00	0.94	0.17	0.93	0.97	0.93
69	-18	1.00	0.96	0.96	0.91	0.20	0.90	0.92
125	-12	1.00	0.99	1.00	0.99	0.87	0.26	0.90
83	-3	0.81	0.98	1.00	0.91	0.87	0.81	0.08

Table 5-5 Highly orthogonal set of 5 promoter-repressor pairs

		zinc finger protein				
promoter	op	-40	-30	-18	-12	-3
125	-40	0.14	0.95	0.97	1.00	0.93
83	-30	1.00	0.17	0.93	0.97	0.93
69	-18	1.00	0.95	0.21	0.94	0.96
125	-12	1.00	1.00	0.88	0.26	0.91
83	-3	1.00	0.92	0.88	0.82	0.08

For applications with more stringent requirements, a set of 5 promoter-repressor pairs has considerably better orthogonality and is shown in Table 5-5. This data comes from Table 5-2 and Table 5-3 and the labels and values have the same meaning here. The data has been re-normalized to the maximum in each row.

Chapter 6 - Multi-gate logic, putting it all together

This project came very close to attempting to assemble a multi-gate logic network in a single cell. Key components towards this task were developed including:

- A method for creating synthetic transcriptional repressors in *E. coli*
- A set of orthogonal promoters and transcriptional repressors
- A robust topology for making logic gates with high Hill-coefficients

Additionally an important lesson was learned about the relative difficulty level of engineering transcriptional activators compared to engineering transcriptional repressors in prokaryotes.

Even though a multi-gate logic network was not built, a considerable amount of time was spent thinking about the challenges that will exist in implementing and testing combinational logic networks.

Architecture

A multi-gate logic network will contain many operons and these operons must function independently of context on the DNA. Neighboring operons will need to be well terminated to prevent transcriptional read through. There are currently few strong and well proven transcriptional terminators. Basic design rules about terminators are also lacking. For example, when two operons are placed one after another on the same strand of DNA, how much space should be left between the terminator of the first operon and the promoter of the second operon?

There are some simple strategies that can be used to minimize the number of good terminators used. Divergent operons are the best way to maintain independence between operons; however with any more than two operons, terminators are required to maintain operon independence. For the threshold sensing topology using sRNA, the constitutive sRNA operon can be placed upstream of the gene it regulates and on the same strand as shown multiple times in Figure 6-1. A terminator should be used to separate the sRNA operon from the repressor it regulates. If the terminator is not 100% efficient then some additional mRNA will be produced, but this extra mRNA is proportional to the sRNA produced and therefore should be eliminated through mRNA-sRNA binding. Because the extra mRNA gets eliminated, the poor terminator does not result in a change in the repressor concentration. Instead the sRNA concentration is lowered from binding to the extra mRNA. The sRNA operons are driven by constitutive promoters, thus the lowering of sRNA concentration due to the termination can be compensated for with a stronger constitutive promoter.

Figure 6-1 An eight operon construct with 1 strong terminator and 6 weak terminators. Operons expressing sRNA are placed upstream of the gene they regulate. The terminators between the sRNAs and zinc finger proteins can be poor and will have minimal impact on the circuit. The only terminator that needs to be strong in this construct is the middle terminator depicted with a large X.

4 of these 8 operon pieces shown in Figure 6-1 could be constructed, with each one reusing the same 6 weak terminators and 1 strong terminator. These four segment of DNA could them be placed in 3 compatible plasmid and the 4th segment could easily be placed on the chromosome. This method would yield 32 synthetic operons, which should enough to construct 10 2-input NOR gates.

Methodology

Individual gates will need to be built and characterized. Once proven these gates can then be assembled into multi-gate structures. Care should be taken to design the single gate structures so that they can easily be used as parts in a multi-gate assembly. The multi-gate assembly process will be performed with either CPEC [83] or Golden Gate cloning [30], [31]

Testing of logic blocks with many inputs can be challenging because there are only a handful of inducible promoter that can be used to rapidly titrate input levels. Constitutive promoters can also be used as fixed inputs, but they should be used to mimic the output of imperfect gates and not as ideal high or low signals. Several inputs can be held by consultative promoters while a single inducible input is wiggled and the output observed.

Challenges

Construct stability

The individual gates that make up a multi-gate logic system should have similar characteristics so that they are fairly interchangeable. The easiest way to make gates have similar behavior is to make them out of similar components and thus similar DNA. But if many similar sequences need to be placed in the cell to build a logic network, then the probability of homologous recombination and loss of function increases. Codon optimization can give new sequence space and relieve some of this pressure, but a better understanding of homologous recombination is needed too. Some sources claim repeats need to be separated by 10 kbp to avoid homologous recombination [29] while other sources say only 1 to 2 kbp are needed between repeats to prevent homologous recombination [84]. Solid rate information on homologous recombination as a function of repeat separation distance is needed if stable multi-gate constructs are to be stable.

The metabolic burden of a logic network could be quite large if expression levels are not carefully tuned to minimize the load placed on the host organism. The rate mutations take over a population is exponentially related to the metabolic burden removed by the mutation[28], therefore the metabolic burden must be kept in check. Often engineering low metabolic burden systems can be annoying because the signal to noise ratio of assays drop as the amount of gene expression decreases. Using high expression levels may appear to make experiments easier, but in the long run systems with lower mutation rates are easier to understand and work with. Currently there are few metrics for measuring metabolic burden besides growth rate and no good methods for predicting metabolic burden from a DNA sequence. Due to this lack of tools, engineers are mostly reacting to effects of metabolic burden instead of anticipating and avoiding them.

Signal fidelity

Much has already been said on orthogonality and crosstalk between promoter-repressor pairs. As logic blocks increase in size there will be continuing pressure to reduce the orthogonality of gates.

Most electrical logic circuits exist as part of a state machine. These circuits are synchronous, meaning that a global clock signal indicates when the output of the circuit has completed transitioning and may be reliably sampled. Biological logic circuits have not yet been designed with such a clock and this exposes the system to temporary outputs that are not representative of the input state. Some techniques known as hazard-free logic have been developed for electrical circuits to deal with this issue, but they will not be cheap to implement in biological systems.

Chapter 7 – Bibliography

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Chapter 8 – Appendix: sequence information

The following sequence information is in GenBank format. While GenBank is currently the most widely supported annotated sequence format, the file format has not been well standardized. The files listed here were created with the tool GENtle. This tool is freely available at http://gentle.magnusmanske.de/.

In Chapter 3, several constructs are tested on a p15A/Kan backbone and a ColE1/Kan backbone. Only the p15A/Kan versions are listed below. The ColE1 version can be obtained by transferring the sequence between the BglII and BamHI sites from the p15A plasmid to between the BglII and BamHI sites on the "Empty vector ColE1/Kan" plasmid listed below

Empty vector ColE1/Kan

```
LOCUS       17-36                2051 bp    DNA     circular

FEATURES             Location/Qualifiers
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   rep_origin        complement(287..969)
                     /gene="ColE1"
   terminator        25..153
                     /gene="double term"
   terminator        975..1080
                     /gene="Terminator"
BASE COUNT     481 a    590 c    545 g    435 t
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       61 actgggcctt tcgttttatc tgttgtttgt cggtgaacgc tctctactag agtcacactg
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ZFP03 leak on SC101/Kan

LOCUS 12-10 5022 bp DNA circular

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CDS 1628..2422
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and Thermal Tm unaffected"
terminator complement(2443..2558)
/gene="Terminator"
terminator 4767..4812
/gene="Terminator"
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/codon_start="0"
terminator 1..46
/gene="term BBa_B0011"
prot_bind 145..159
/gene="RBS"
prot_bind 53..70
/gene="ZFP03 binding site"
promoter 77..138
/gene="pwklac"
BASE COUNT 1516 a 1075 c 1168 g 1263 t

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121 accgagcgga taacaattgg atctattaaa gaggagaaag gatctatgcg taaaggagaa
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ZFP5475 matched act/rep on SC101/Kan

LOCUS 12-16 6497 bp DNA circular

FEATURES Location/Qualifiers

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ZFP02 matched atc/rep on SC101/Kan

LOCUS 12-27 6472 bp DNA circular

FEATURES Location/Qualifiers
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ZFP5475 inverter on SC101/Kan

LOCUS 12-28 7233 bp DNA circular

FEATURES Location/Qualifiers
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ZFP03 matched atc/rep on SC101/Kan

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ZFP02 leak on SC101/Kan

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Zif268 leak on SC101/Kan

LOCUS 12-31 4963 bp DNA circular

FEATURES Location/Qualifiers

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     prot_bind      86..100
                    /gene="RBS"
     promoter       18..79
                    /gene="pwklac"
     prot_bind      1..11
                    /gene="Zif268 binding site"
BASE COUNT    1492 a   1068 c   1163 g   1240 t
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       61 gaccgagcgg ataacaattg gatctattaa agaggagaaa ggatctatgc gtaaaggaga
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Zif268 matched act/rep on SC101/Kan

```
LOCUS       12-33                6216 bp    DNA     circular

FEATURES             Location/Qualifiers
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   promoter       937..990
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ZFP03 inverter on SC101/Kan

LOCUS 12-34 7125 bp DNA circular

FEATURES Location/Qualifiers
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ZFP5475 leak on SC101/Kan

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ZFP03 activator p15A/Kan

LOCUS 21-61 3569 bp DNA circular

FEATURES Location/Qualifiers
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     terminator      2698..2803
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     terminator      244..289
                     /gene="term BBa_B0011"
     prot_bind       356..370
                     /gene="RBS"
     promoter        296..349
                     /gene="pLtetO-1"
     CDS             1127..1144
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     terminator      1761..1889
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BASE COUNT     900 a    963 c    871 g    835 t
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      121 caagaaagcc atccagttta ctttgcaggg cttcccaacc ttaccagagg gcgccccagc
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ZFP5475 activator p15A/Kan

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LOCUS       21-62                3593 bp    DNA     circular

FEATURES             Location/Qualifiers
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                  /gene="Terminator"
   terminator     244..289
                  /gene="term BBa_B0011"
   prot_bind      356..370
                  /gene="RBS"
   promoter       296..349
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   CDS            1127..1144
                  /gene="linker"
                  /codon_start="0"
   CDS            complement(2853..3593)
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   terminator     1785..1913
                  /gene="double term"
   CDS            377..1120
                  /gene="RNAP alpha subunit"
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   CDS            1151..1708
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                  /codon_start="0"
   terminator     1715..1760
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BASE COUNT     893 a    988 c    923 g    789 t
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ZFP03 mismatched atc/rep on p15A/Kan

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   terminator    2605..2733
                 /gene="double term"
   terminator    3542..3647
                 /gene="Terminator"
   promoter      282..343
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   promoter      1140..1193
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   terminator    206..251
                 /gene="term BBa_B0011"
   CDS           1971..1988
                 /gene="linker"
                 /codon_start="0"
   prot_bind     258..275
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   prot_bind     350..364
                 /gene="RBS"
   CDS           complement(3673..4413)
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   terminator    2605..2733
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   terminator    1088..1133
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3061 cgtttgtctc attccacgcc tgacactcag ttccgggtag gcagttcgct ccaagctgga
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3361 tcgaaaaacc gccctgcaag gcggtttttt cgttttcaga gcaagagatt acgcgcagac
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3661 ccagagtccc gctcagaaga actcgtcaag aaggcgatag aaggcgatgc gctgcgaatc
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4321 gacaaaaaga accgggcgcc cctgcgctga cagccggaac acggcggcat cagagcagcc
4381 gattgtctgt tgtgcccagt catagccgaa tag
//

ZFP5475 mismatched act/rep on p15A/Kan

LOCUS 22-22 4437 bp DNA circular

FEATURES Location/Qualifiers
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/codon_start="0"
rep_origin complement(2764..3475)
/gene="p15A"
gene 371..1081
/gene="mCherry"
terminator 2629..2757
/gene="double term"
terminator 3566..3671

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     promoter       1140..1193
                /gene="pLtetO-1"
     terminator     206..251
                /gene="term BBa_B0011"
     CDS            1971..1988
                /gene="linker"
                /codon_start="0"
     prot_bind      258..275
                /gene="mismatched binding site (V2)"
     prot_bind      350..364
                /gene="RBS"
     promoter       282..343
                /gene="pwklac"
     CDS            complement(3697..4437)
                /gene="KanR"
                /codon_start="0"
     CDS            1995..2552
                /gene="ZFP5475"
                /codon_start="0"
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BASE COUNT    1128 a   1171 c   1147 g    991 t
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     61 cgatcctcat cctgtctctt gatcagatca tgatcccctg cgccatcaga tccttggcgg
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    301 tgcttccggc tcgtatgttg tgtcgaccga gcggataaca attggatcta ttaaagagga
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    481 cgagggccgt ccgtatgaag gcacccagac cgccaaactg aaagtgacta aaggcggccc
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    661 ggaacgcgta atgaattttg aagatggtgg tgtcgtgacc gtcactcagg actcctccct
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    901 tgacgctgaa gtgaaaacca cgtacaaagc caagaaacct gtgcagctgc ctggcgcgta
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   1021 atatgagcgc gcggagggtc gtcattctac cggtggcatg gatgaactat acaaataata
   1081 aggatctaga gaatataaaa agccagatta ttaatccggc ttttttatta tttggatctt
   1141 ccctatcagt gatagagatt gacatcccta tcagtgatag agatactgag cacggatcta
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4261 gcaaggaacg cccgtcgtgg ccagccacga tagccgcgct gcctcgtcct gcagttcatt
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//

ZFP5475 leak on p15A/Kan

LOCUS 22-2 2970 bp DNA circular

FEATURES Location/Qualifiers
rep_origin complement(1297..2008)
/gene="p15A"
CDS 375..1085
/gene="mCherry"
/codon_start="0"
terminator 1162..1290
/gene="double term"
terminator 2099..2204
/gene="Terminator"
promoter 286..347
/gene="pwklac"
terminator 206..251
/gene="term BBa_B0011"
prot_bind 258..279
/gene="ZFP5475 binding site"
prot_bind 354..368
/gene="RBS"
promoter 286..347
/gene="pwklac"
CDS complement(2230..2970)
/gene="KanR"
/codon_start="0"
terminator 1162..1290
/gene="double term"
terminator 1092..1137
/gene="term BBa_B0011"
BASE COUNT 749 a 778 c 761 g 682 t
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61 cgatcctcat cctgtctctt gatcagatca tgatcccctg cgccatcaga tccttggcgg
121 caagaaagcc atccagttta ctttgcaggg cttcccaacc ttaccagagg gcgccccagc

181 tggcaattcc gaattcatga gatctagaga atataaaaag ccagattatt aatccggctt
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2821 cgatagccgc gctgcctcgt cctgcagttc attcagggca ccggacaggt cggtcttgac
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2941 tgtctgttgt gcccagtcat agccgaatag
//

ZFP03 leak on p15A/Kan

LOCUS 22-3 2966 bp DNA circular

FEATURES Location/Qualifiers
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CDS 371..1081
/gene="mCherry"
/codon_start="0"
terminator 2095..2200
/gene="Terminator"
terminator 206..251
/gene="term BBa_B0011"
prot_bind 258..275
/gene="ZFP03 binding site"
prot_bind 350..364
/gene="RBS"
promoter 282..343
/gene="pwklac"
CDS complement(2226..2966)
/gene="KanR"
/codon_start="0"
terminator 1158..1286
/gene="double term"
terminator 1088..1133
/gene="term BBa_B0011"
BASE COUNT 752 a 772 c 759 g 683 t
ORIGIN
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61 cgatcctcat cctgtctctt gatcagatca tgatcccctg cgccatcaga tccttggcgg
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ZFP5475 matched atc/rep on p15A/Kan

LOCUS 22-4 4441 bp DNA circular

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ZFP03 matched atc/rep on p15A/Kan

LOCUS 22-5 4413 bp DNA circular

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Reporter for TetR-sRNA inverter

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FEATURES             Location/Qualifiers
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Reporter for steric hindrance (P_{ZC35})

LOCUS pWH24-30 2927 bp DNA circular

FEATURES Location/Qualifiers
gene complement(2063..2857)
/gene="KanR"
rep_origin complement(1244..1926)
/gene="ColE1"
terminator 982..1110
/gene="terminator"
terminator 1932..2037
/gene="Terminator"
CDS 195..899
/gene="mCherry"
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gene 174..188
/gene="RBS"
promoter 82..167
/gene="PZC35"

misc_feature 147..147
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terminator 912..957
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BASE COUNT 752 a 749 c 798 g 628 t
ORIGIN
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61 aattccgaat tcatgagatc taaaaaaggg gaagagggga agaggagtgg tggacatagt
121 ggaaggaatg ggagatagtg ggagagaggg aaggagagga ggaaaaagga tctattaaag
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TetR and sRNA inverter (+sRNA)

LOCUS 24-81 5517 bp DNA circular

FEATURES Location/Qualifiers
gene complement(4418..5500)
/gene="lacI"
CDS 1784..2500
/gene="GFPmut3b"
/codon_start="0"
rep_origin complement(2661..3372)
/gene="p15a"
gene complement(3584..4243)
/gene="KanR"
CDS 1157..1777
/gene="tetR"
/codon_start="0"
terminator complement(6..299)
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terminator 2526..2654
/gene="double term"
terminator 3463..3568
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gene 1041..1136
/gene="repC miniCistron"
misc_feature 854..944
/gene="UT sense RNA"
misc_feature complement(321..411)
/gene="sRNA"
promoter 800..853
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prot_bind 1143..1150
/gene="RBS"
promoter complement(412..446)
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BASE COUNT 1508 a 1327 c 1290 g 1392 t
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//

RFP-ZFP fusion expression from P_{lacUV5}

LOCUS 26-51 4700 bp DNA circular
DEFINITION 4160 bp DNA circular pBbA5c-RFP
pBbA5c-RFP.1
.

holtz

FEATURES Location/Qualifiers
CDS complement(3323..4405)
/gene="LacI"
/codon_start="0"
rep_origin complement(1566..2277)
/gene="p15a"
CDS 189..866
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misc_feature 1431..1559
terminator 2368..2473
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promoter 74..149
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prot_bind 169..188
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gene complement(2489..3148)
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TetR inverter (-sRNA)

LOCUS 26-58 5391 bp DNA circular

FEATURES Location/Qualifiers
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CDS 506..1126

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ZFP expression from P_{lacUV5} for steric hindrance experiments

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   rep_origin      complement(885..1596)
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   misc_feature    750..878
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   promoter        74..149
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   prot_bind       169..188
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   CDS             189..719
                   /gene="ZFP-65"
                   /codon_start="1"
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ZFP-65 expression from P_{BAD} for steric hindrance and orthogonality testing

LOCUS 29-15 5072 bp DNA circular

FEATURES Location/Qualifiers
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ZFP-3 expression from P_{BAD} for steric hindrance and orthogonality testing

LOCUS 29-77 5072 bp DNA circular

FEATURES Location/Qualifiers
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ZFP-39 expression from pET-29 for protein purification

LOCUS 30-15 5871 bp DNA circular

FEATURES Location/Qualifiers
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Reporter for orthogonality testing: operator-3 and BioFAB promoter 83

LOCUS 32-32 3039 bp DNA circular

FEATURES Location/Qualifiers
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                /gene="ZFP-3 binding site"
     promoter       1519..1553
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     misc_feature   1554..1581
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     misc_feature   1357..1500
                /gene="insulator"
BASE COUNT     773 a    794 c    799 g    673 t
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     61 ggtatctgcg ctctgctgaa gccagttacc ttcggaaaaa gagttggtag ctcttgatcc
    121 ggcaaacaaa ccaccgctgg tagcggtggt ttttttgttt gcaagcagca gattacgcgc
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