TUNING DNA BINDING AND GENE EXPRESSION USING ZINC FINGER PROTEINS
AND ENGINEERED PROMOTERS

BY

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THESIS

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ABSTRACT

Synthetic biology provides an ideal approach to build functional biological devices by assembling biological parts. Using synthetic biology, efficient control of gene regulation may be achieved to a degree that is not possible using natural genetic structures.[1] However, previous studies on promoter engineering have focused on natural transcription factors (TFs),[2] including the *lac* repressor, which produces a switch-like "all-or-none" response.[3] In this project, we worked to develop a new system for transcriptional control based on tunable synthetic TFs, which are designed to yield programmable linear responses in gene expression. To accomplish this, we used zinc finger proteins (ZFPs) as regulators of engineered promoters assayed by green fluorescent protein (GFP) as a fluorescent transcriptional reporter probe. In particular, we designed strong-binding three finger ZFPs as proof-of-principle regulatory elements, with the intention of moving to weaker binding two finger ZFPs and the addition of the accessory binding module PAR (part of the protein Adr1).[4] To generate engineered promoters, we integrated ZFP binding sites into known promoters of varying strength. To analyze the engineered activity of each promoter, we cultured *E. coli* cells transformed with plasmids containing sequences for both ZFP production and our engineered promoters and measured the resulting fluorescence intensity. In this way, we constructed a novel method for tuning gene expression as well as testing the DNA binding affinity of synthetic TFs. We anticipate that this general approach could be used in the future for designing and characterizing synthetic TFs for gene therapy and gene regulation applications.

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CHAPTER 1 - INTRODUCTION

1.1 – Background

1.1.1 – What are ZFPs?

Zinc finger proteins (ZFPs) are proteins that fold by coordinating zinc ions and bind to DNA with high affinity and specificity. The most common type of ZFPs coordinate zinc with two cysteine and two histidine residues, called Cys_2His_2 ZFPs.[5] Cys_2His_2 ZFPs typically fold into a ββα structure, with the structure of the protein made up of two β-sheets and an α-helix held in formation by coordinating a zinc ion.[6] ZFPs recognize short segments of DNA, with each individual finger binding to a specific three base pair sequence.[5] ZFPs are modular such that multiple fingers can be appended together to build hybrid ZFPs with variable DNA binding affinities and specificities.[5] In addition, nonspecific DNA binding modules can be added to ZFPs to increase binding affinity without affecting specificity.[5] The ability to tune DNA binding affinity using ZFPs as synthetic TFs is the foundation of the project.

1.1.2 – Synthetic Biology Approach to TF and Promoter Design

In this work, we aim to combine carefully designed ZFPs and engineered promoters to create a completely synthetic system for characterization and tuning of TFs. In terms of designing ZFPs and creating libraries of possible DNA sequence binding sites, a popular method is the OPEN (Oligomerized Pool ENgineering) platform which involves randomized combination of individual fingers into three figure ZFPs and verification of binding via a B2H system.[7] These libraries of "designer" ZFPs can be used for wide ranging applications such as shown in this work and gene therapy[8] as well as drug delivery and antiviral therapy.[9] To characterize the binding affinity of tight binding ZFPs, fluorescence anisotropy can be used.[10] However, for weak binding ZFPs, this technique is not applicable due to the time required to

obtain fluorescence measurements. The general TF platform developed in this work can circumvent this problem and give quantitative analysis of binding for weak binding TFs through the use of engineered promoters.

Promoter engineering is a well developed practice in synthetic biology. Fully functional promoters have been engineered for strength based on consideration of consensus sequences[1] as well as TATA box strength.[11] Additionally, engineered promoter systems have been organized into genetic circuits,[12] furthering the design of biological systems for nontraditional applications. Often, these engineered promoter systems are used for gene regulation applications such as synthetic riboregulators[13] and using natural TFs.[2,14] Our TF testing system is not limited to natural TFs and does not require the complicated machinery of riboregulators for gene regulation.

The main motivation for this work stems from a combinatorial approach to promoter design for programming gene expression by the Elowitz group.[2] Four natural TFs (two activators and two repressors) were studied and a library of combinatorial promoters was created containing operators for each TF in three different regions of the promoter, labeled distal, core or proximal based on their location.[2] Design rules for promoter architecture and TF identity were developed based on characterization of over 200 unique promoters from the combinatorial library.[2] For example, after analyzing the effects of operator position and activator versus repressor it was found that activators function almost exclusively in the distal region while repressors are most effective in the core region, but function in all three regions (in the order core > proximal > distal).[2] The ability to define fundamental rules for designing synthetic biological systems demonstated by the Elowitz group is the driving force behind our work with combinatorial promoters. However, it was also observed that operator position had more effect

on gene regulation than number of operators within the promoter.[2] Because natural TFs usually function as an ON/OFF switch rather than a tunable knob, we hypothesize that our designed ZFPs will prove that increasing the number of operators can indeed affect fine control of promoter strength.

1.2 – Significance

Few publications exist describing two finger ZFPs.[15] Moreover, essentially no research on single fingers has been reported, as it is commonly believed that single fingers interact too weakly for most applications because they may only bind DNA for a few milliseconds. Our engineered promoter platform provides an ideal approach to study binding dynamics of any transcription factor, including TFs with off rates on the order of 1000 s^{-1} which may be the case for single finger ZFPs. TFs with weak DNA interactions can be assayed by arraying multiple binding sites in our engineered promoters and increasing the number of sites until a clear response in gene expression arises. After proof of principle experiments with three finger ZFPs, we will move to arraying two and single finger ZFP binding sites within the synthetic promoters as well as adding nonspecific DNA binding modules like PAR (part of the protein Adr1)[4] to two finger ZFPs to increase binding affinity without affecting sequence specificity.   With this method, we will directly measure the effect of two and single finger ZFPs as control elements for gene expression in comparison to three finger ZFPs. Using this method to characterize weak TFs will increase the known range of available tools for gene regulation machinery as well as give the ability to elucidate the activities and functions of naturally weak TFs.

1.3 – Plasmid List

Please refer to Table 1.1 for all plasmids used in this work. Plasmids are listed in the order that they appear in the text.

Table 1.1. List of all plasmids used in this work. Plasmids were designed from a basis of either pUA66 or pQE-80L

Plasmid Identifier	Basis	Replication Origin	Comments
pUA66	pUA66[18]	SC101	Without modification.
pKTB4x	pUA66[18]	SC101	pUA66 with B4 based promoter containing x ZFP binding sites inserted between XhoI and BamHI sites.
pKT104	pQE-80L (Qiagen)	ColE1	pQE-80L with the gene encoding for ZFP 104 inserted between XmaI and AgeI sites.
pKT10	pUA66[18]	SC101 and ColE1	pUA66 with ColE1 replication origin inserted between XhoI and BamHI sites.
pKT11	pUA66[18]	SC101 and ColE1	pUA66 with modified pQE-80L segment inserted at the PstI site and the ColE1 replication origin inserted between XhoI and BamHI sites.
pKT12	pUA66[18]	SC101 and ColE1	pUA66 with modified pQE-80L segment inserted at the AatII site and the ColE1 replication origin inserted between XhoI and BamHI sites.

CHAPTER 2 – REPRESSION OF ENGINEERED BACTERIAL PROMOTERS

2.1 – Promoter Design

Based on previous work with combinatorial promoter design,[2] we chose to use a strong combinatorial promoter as a base for engineered ZFP repression. The chosen promoter is denoted as B4 in the original work by the Elowitz group.[2] We designed a modular program for promoter architecture containing ZFP binding sites (Table 2.1). Each promoter is constructed from three individual segments: a distal, core and proximal region. Segments are separated by the -35 (between distal and core) and -10 (between core and proximal) sites recognized by σ factors of bacterial RNA polymerase.[16] The -35 and -10 sites are universal for all segments and overhangs span these sites, ensuring that each segment's overhangs are compatible with adjacent segments (distal/core pairs and core/proximal pairs) for ligation. This allows for the creation of a library of 27 (3^3) unique promoters from nine unique segments. Within a promoter sequence, the necessary components for gene expression include a core region approximately 17 base pairs in length and intact -10 and -35 sites.[17] Aside from the regulatory components known to be essential, we modified the remainder of the promoter by inclusion of ZFP binding sites, with the goal of limited impact on natural promoter function. We inserted ZFP binding sites in each segment (distal/core/proximal) for the ability to compare repression of transcription of target genes by ZFP binding depending on the segment containing the binding sites. For initial experiments, we constructed three specific promoters: B40 with no ZFP binding sites (unchanged), B43 with one binding site in each segment (three total), and B46 with two binding sites in each segment (six total). These correspond to the first, second and third rows of Table 2.1 respectively. B40 (the original base promoter) was compared both to reported fluorescence activity values[2] and to the experimental fluorescence measurements from the engineered

promoters B43 and B46. Promoters were synthesized as described in section A.3 in Appendix A, and verification of successful ligation can be seen in Figure 2.1.   Native agarose gel electrophoresis was preformed and bands of the correct length (approximately 109 bp) were seen, verifying that ligation had proceeded successfully.   Additional bands indicate both incomplete (only two segments) and nonspecific (unwanted overhang association) ligations, but this is not uncommon in a multiple segment ligation scheme.

Table 2.1. Engineered promoter segments based on promoter B4.[2] The lac operators in the original promoter are shown in red. Each segment contains either 0, 1, or 2 binding sites for ZFP 104 (highlighted). The leftmost overhang of the distal segments corresponds to that of the restriction site XhoI, the inner overhangs of each segment overlap the -35 and -10 sites of the promoter, and the rightmost overhang of the proximal segments corresponds to that of the restriction site BamHI.

|	Distal	Core	Proximal
0 sites (B40)	TCGAGTACAACGTCGTGTTAGCTGCAATTGTGAGCGGATAACAATT CATGTTGCAGCACAATCGACGTTAACACTCGCCTATTGTTAACTG	GACTTGTGAGCGGATAACAATG AACACTCGCCTATTGTTACTATGA	ATACTTCGTGCAATTTTTAAAATTAAAGGCGTTACCCAACG AGCACGTTAAAAATTTTAATTTCCGCAATGGGTTGCCTAG
1 site (B43)	TCGAGTACAACGTCGTGTTAGCTGCAATTGTGAGGCTGATACAATT CATGTTGCAGCACAATCGACGTTAACACTCCGACTATGTTAACTG	GACTTGTGAGGCTGATACAATG AACACTCCGACTATGTTACTATGA	ATACTTCGTGCAATTTGAGGCTGATAAGGCGTTACCCAACG AGCACGTTAAACTCCGACTATTCCGCAATGGGTTGCCTAG
2 sites (B46)	TCGAGTACAACGTCGTGAGGCTGATAATTGTGAGGCTGATACAATT CATGTTGCAGCACTCCGACTATTAACACTCCGACTATGTTAACTG	GACTGAGGCTGATATGAGGCTG ACTCCGACTATACTCCGACTATGA	ATACTTCGTGCAATTTGAGGCTGATAAGGCGGAGGCTGATG AGCACGTTAAACTCCGACTATTCCGCCTCCGACTACCTAG
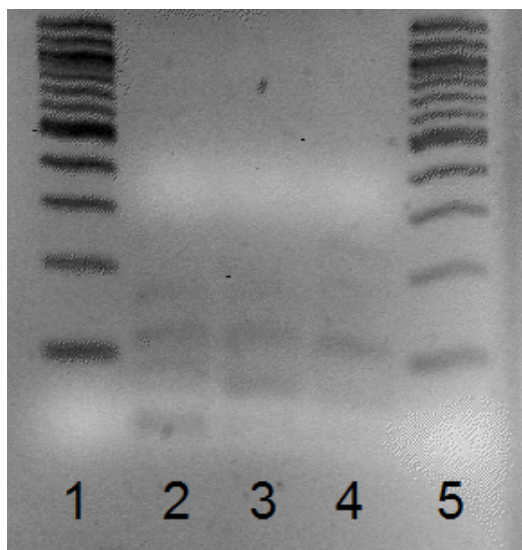
Figure 2.1. Native agarose gel electrophoresis used to verify successful promoter ligation. Lanes 1 and 5 show 100 bp DNA ladder (NEB Catalog #N0467L) with the lowest rung corresponding to 100 bp. Lanes 2, 3, and 4 show samples of no binding site segment ligation, one binding site segment ligation, and two binding site segment ligation, respectively. Ligated promoters should have total length of 109 bp and the strongest band in the ligation samples can be seen just above the 100 bp rung of the DNA ladder.

Engineered promoters were cloned into the plasmid pUA66 (Figure 2.2) upstream of the gene GFPmut2 that encodes for a fast folding green fluorescent protein (GFP),[18] which is used as a transcriptional reporter probe to directly measure gene expression using fluorescence. This cloning resulted in the plasmids pKTB40, pKTB43, and pKTB46 (referred to collectively as pKTB4x). In initial work, we used three finger ZFPs for proof-of-principle due to their strong binding affinities and sequence specificity.[5] In particular, we used ZFP 104 which binds to the sequence "GAGGCTGAT" (highlighted in Table 2.1). ZFP 104 was expressed using a second plasmid, pKT104 (Figure 2.3), for ZFP production controlled by IPTG induction of the T5 system.[19] A circuit diagram of the two plasmid system is shown in Figure 2.4. We hypothesized that arraying ZFP binding sites inside a promoter would generate a linear decrease in fluorescence with increasing number of sites, thereby allowing for a wide range of expression levels for gene regulation applications. We transformed *E. coli* cells with pKTB4x and pKT104, and obtained fluorescence measurements from cell cultures.

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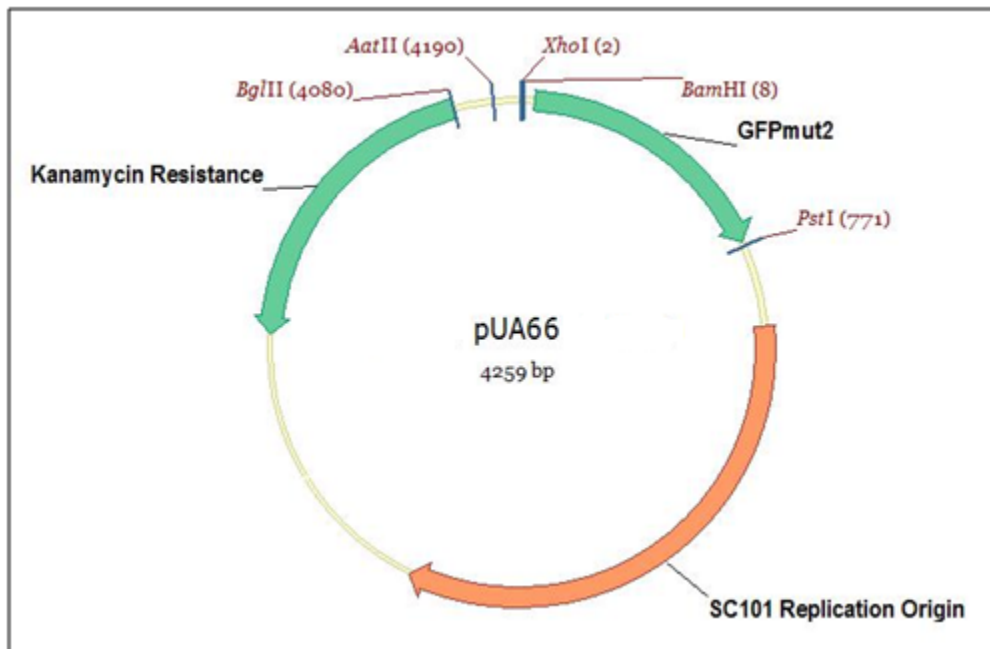
Figure 2.2. The plasmid pUA66 without modification.[18] Engineered promoters are inserted between the XhoI and BamHI restriction sites.
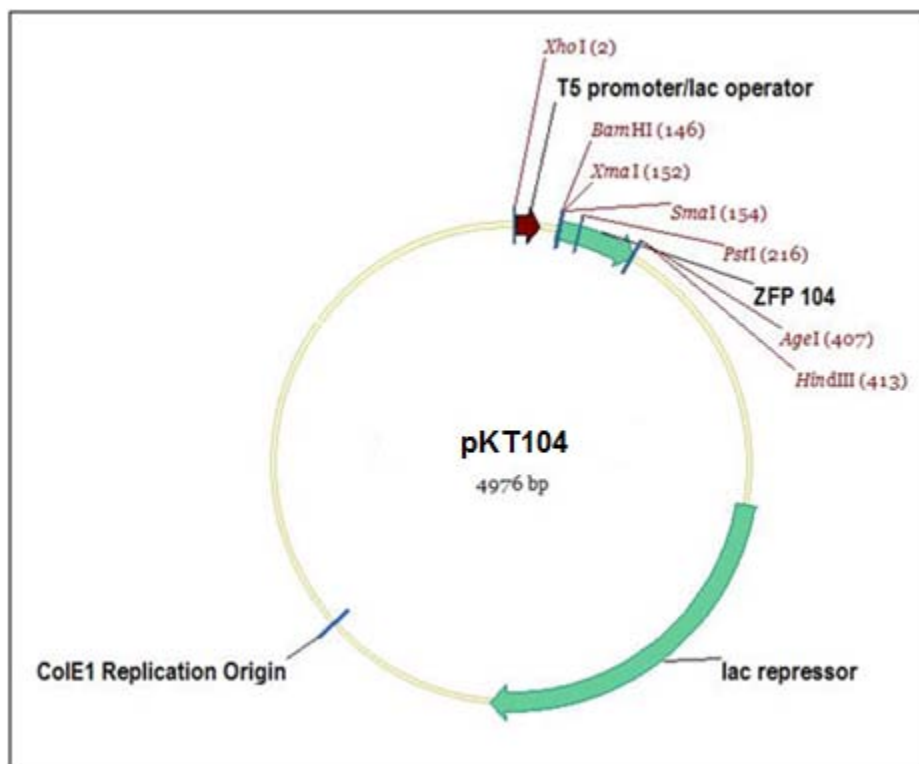

Figure 2.3. The plasmid pKT104. This plasmid is based on pQE-80L with the gene encoding for ZFP 104 between the XmaI and AgeI restriction sites. ZFP production is under inducible control of the T5 system.[19] ZFP 104 can be replaced by any ZFP using the BamHI or XmaI and HindIII or AgeI restriction sites.
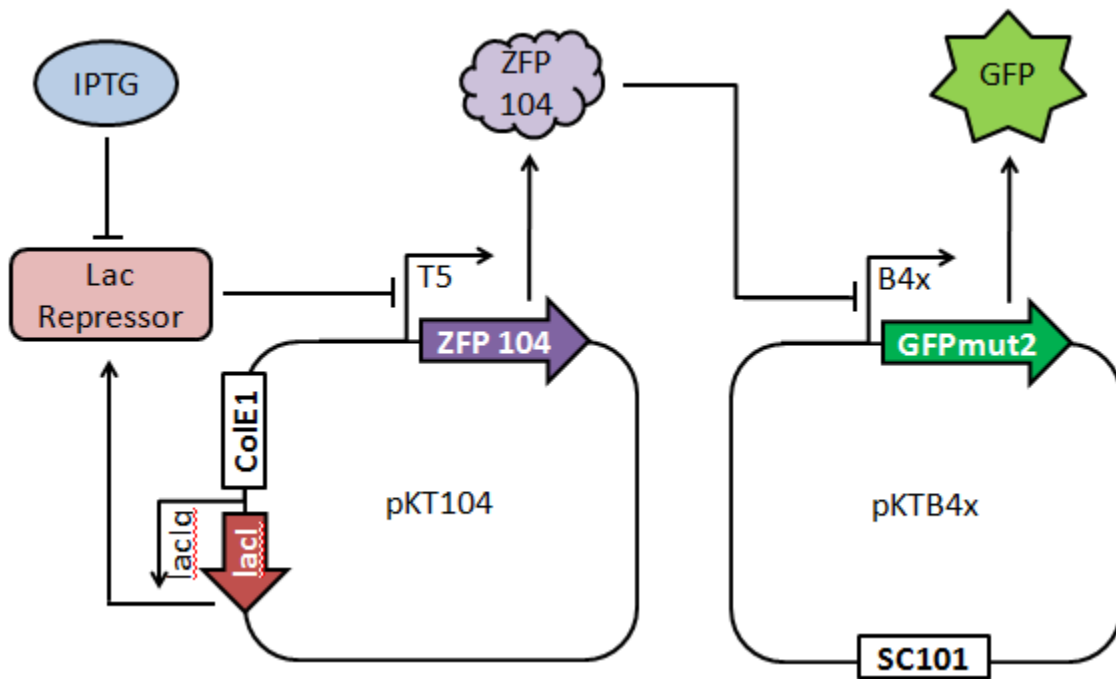
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Figure 2.4. Circuit diagram of the two plasmid system. ZFP 104 expression is regulated by the IPTG inducible T5 system (T5 promoter and lacI gene). After induction with IPTG, ZFP 104 is expressed and binds to the B4x promoter (where x can be 0, 3, or 6) in pKTB4x, repressing expression of GFP.

2.2 – Fluorescence Assay for Promoter Activity

The two plasmid system was transformed into BLR competent cells,[20] a strain of *E. coli* designed for increased protein expression compared to wild type *E. coli* or the DH5α strain.[21] Next, we obtained fluorescence measurements with and without IPTG induced ZFP production (Figure 2.5). Across all conditions, the control plasmid with the GFPmut2 gene regulated by the araC promoter showed no fluorescence peak, which was expected because araC is not activated by IPTG induction. Similar to the control, no GFP expression was seen for pKTB46 regardless of the conditions. We hypothesize that the significant modification to the original promoter sequence (including the -35 and -10 sites) caused loss of promoter function. For pKTB40 and pKTB43, it became apparent that the lac operators in the original B4 promoter sequence convoluted the fluorescence results. Figure 2.5A shows that inducing ZFP production represses GFP expression completely even for pKTB40 which contains a promoter without ZFP binding

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sites. We hypothesized that because the lac operator sequence (contained twice in the original promoter) contains an eight base pair sequence similar to the binding site of ZFP 104 (**GAG**C**GGAT** versus **GAG**GCT**GAT**) and that the T5 system expressed ZFP 104 in such high quantities[19] that even weak binding to the promoter was enough for significant repression. In Figure 2.5C, pKT104 was not transformed into the cells such that no ZFP expression was possible. Upon induction with IPTG, fluorescence increases eight times for pKTB40 because the lac operators in the original promoter repress GFP expression until induction. In the case of pKTB43, approximately the same level of GFP is expressed between Figure 2.5B, C and D regardless of induction. We hypothesized that the modification of the lac operator sequence in the original promoter with the ZFP binding site sequence caused some leaky expression of GFP.[22] Based on these results, we decided to 1) reduce the number of binding sites inserted into the promoter sequence to avoid fluorescence reduction based on sequence modification rather than repression by ZFP binding and 2) discarded promoter B4 for future studies to remove the complication of the lac operators within the promoter.
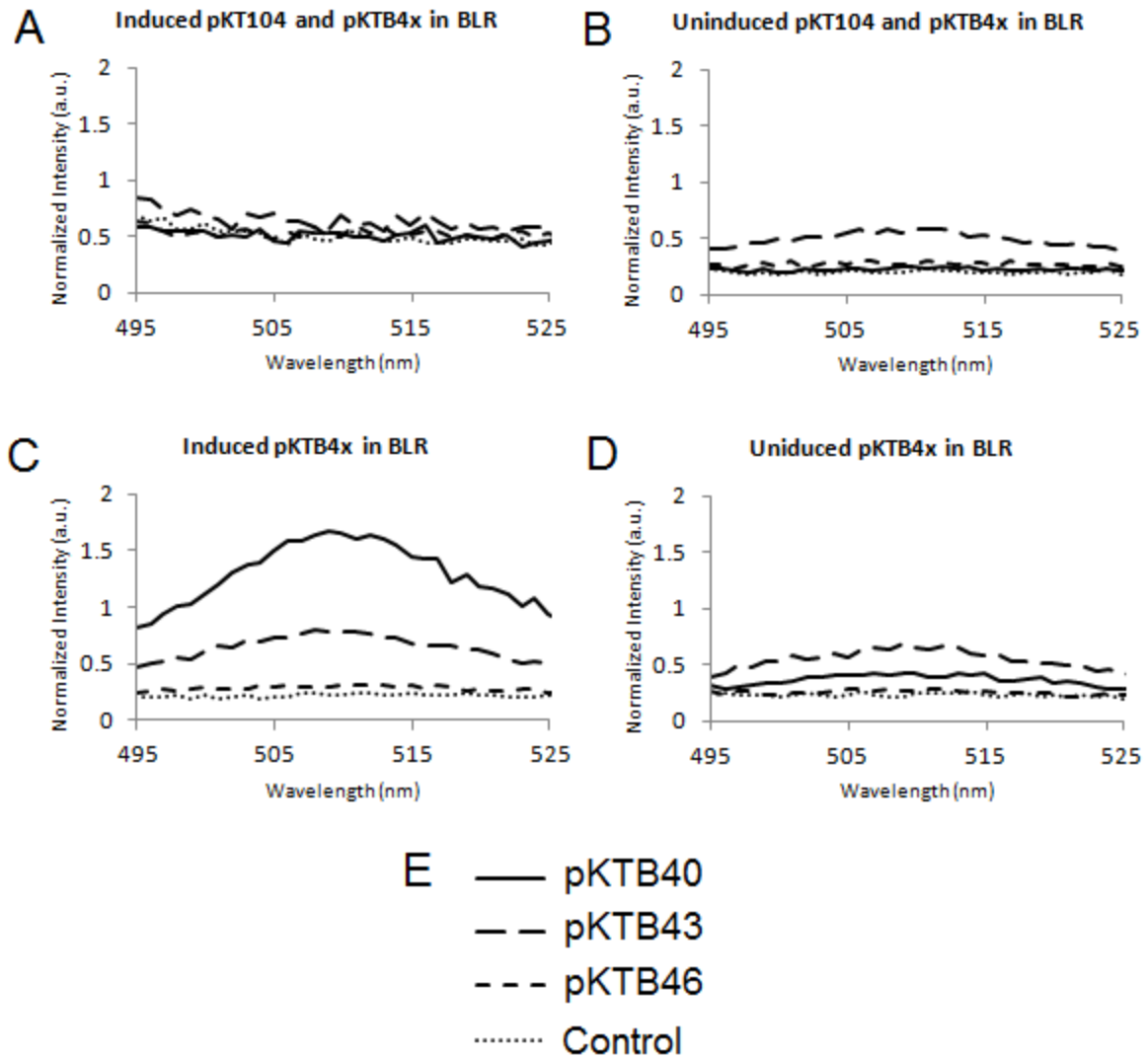
Figure 2.5. GFP fluorescence from A) induced BLR cells containing both pKTB4x and pKT104, B) uninduced BLR cells containing both pKTB4x and pKT104, C) induced BLR cells containing only pKTB4x, and D) uninduced BLR cells containing only pKTB4x. E displays a legend for A-D. BLR cells were induced with 0.7 mM IPTG. The control is the araC promoter which is unaffected by IPTG induction.

2.3 – Outcomes

Based on whole-cell fluorescence assays, it was clear that continued use of promoter B4 added too many complications to the system. The lac operators contained in the promoter sequence result in cross-reactivity with real time IPTG induction and display similarity to the binding site of ZFP 104. To overcome these problems, we chose to replace promoter B4 with constitutive promoters so that gene regulation would be the result of only ZFP binding. To

combat potential problems associated with substantial modifications to the promoter sequence, we decided to include only one binding site in all engineered promoter.[23] However, we aimed to maintain the segment containing the binding site (distal, core, proximal) variable so that the binding site location could be varied to potentially modulate the level of repression.

As an aside from promoter design, another difficulty arose from using pUA66, which is a low copy number vector, as a backbone for engineered promoters. The SC101 replication origin produces approximately five copies per cell which is valuable for quantitative gene regulation studies, but complicated the production of high concentrations of plasmid DNA. To save time and materials, we sought modify pUA66 so that the copy number would be high during cloning steps, but low during gene regulation experiments. In total, these modifications would make future promoter cloning more manageable.

CHAPTER 3 – REPRESSION OF CONSTITUTIVE PROMOTERS

3.1 – pUA66 Copy Number

As mentioned previously, the very low copy number (approximately five copies per cell) of the SC101 replication origin in pUA66 is valuable for quantitative gene regulation studies, but complicates sequential cloning steps. To increase the copy number of pUA66 temporarily during cloning, but allow for return to low copy number during gene regulation experiments, we inserted a new replication origin between the same restriction sites are used for promoter insertion (XhoI and BamHI). We chose the ColE1 replication origin which has a copy number estimated at 10 to 30 because it was readily available from pQE-80L. Cell machinery favors the mechanisms of high copy replication origins (thus their higher copy number), so ColE1 dominates plasmid replication over SC101. The ColE1 replication origin was amplified from pQE-80L by PCR with primer addition of XhoI and BamHI sites, followed by restriction digestion and ligation into pUA66 to create pKT10 (Figure 3.1). To confirm that copy number increase was successful for the new plasmid, we cultured both pKT10 and pUA66 and compared the final plasmid concentration obtained. A twofold increase between pUA66 and pKT10 was seen (108.85 ng/µl versus 204.24 ng/µl), which was consistent with copy number estimates for the two replications origins.
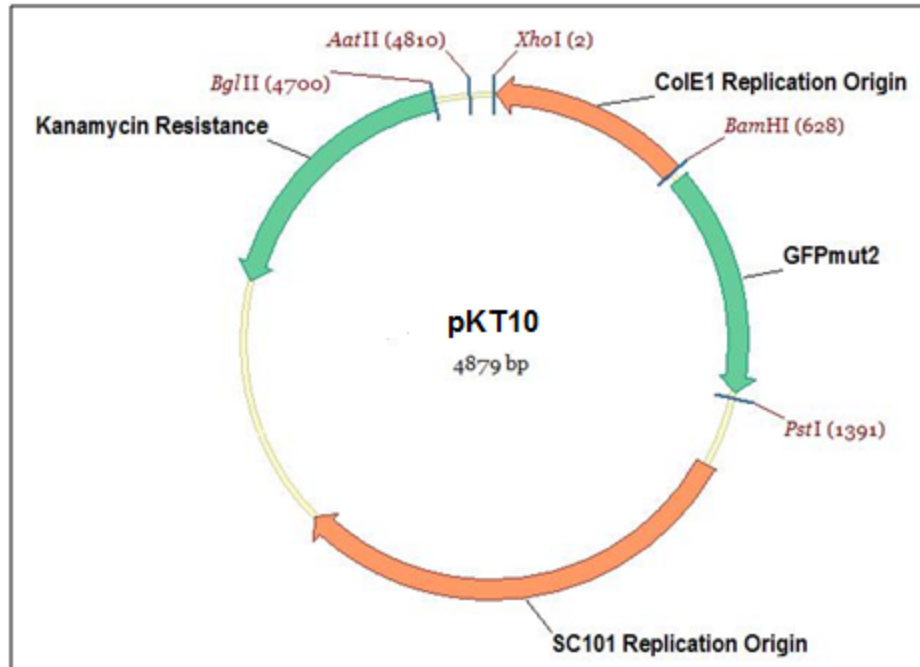
Figure 3.1. The plasmid pKT10. The ColE1 replication origin is inserted between the XhoI and BamHI restriction sites of pUA66.

3.2 – Promoter Design

Based on previous experiments, we decided to only engineer constitutive promoters in order to avoid conflicts with existing regulatory mechanisms in previously engineered promoters. For this work, we chose several constitutive promoters of different strengths for an added tunable variable aside from number of ZFP binding sites. For a weak promoter, we chose lacIp which regulates expression of the lac repressor in *E. coli*.[24] An additional benefit of choosing lacIp is that replacing the first cytosine in the -35 site with a thymine results in a promoter called lacIq, which has tenfold increased promoter strength compared to lacIp.[25] For a strong promoter in addition of lacIq, we chose the promoter rpoDp1, which is involved in the production of σ^{70}.[26] The bacterial RNA polymerase holoenzyme requires a subunit called the σ factor for specificity to the -10 and -35 sites of promoters.[16] σ^{70} is the most common σ factor required for natural cell growth[27] and promoters involved in its production are often constitutive and strong.

We designed a similar modular program for promoter architecture to that of the original

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promoter B4,[2] instead using the chosen constitutive promoters (Table 3.1). Because previous experiments showed that substantial modification of the promoter sequence could result in loss of functionality, we began by combining segments such that only one ZFP binding site would be contained within any one promoter. We constructed four specific promoters for initial experiments: 1) no ZFP binding sites (labeled 000), 2) one binding site in the proximal segment (labeled 001), 3) one binding site in the core segment (labeled 010), and 4) one binding site in the distal segment (labeled 100). We varied the location of the binding site to compare repression by ZFP binding depending on the segment containing the binding sites. An additional letter identifier based on the promoter was added to the beginning of each numerical identifier: "p" for lacIp, "q" for lacIq, and "r" for rpoDp1. For example, the promoter based on lacIq containing one binding site in the core segment would be labeled "q010". Promoters labeled "_000" functioned as a control for comparison both to reported promoter strengths[24,25,26] and to the experimental fluorescence measurements from the other promoters. Promoters were synthesized as described in section A.3 in Appendix A, and verification of successful ligation can be seen in Figure 3.2. Native agarose gel electrophoresis was preformed and bands of the correct length (approximately 87 bp) were seen, verifying that ligation had proceeded successfully. However, Figure 3.2 shows that ligation of the promoter segments resulted in a much lower yield than with the original promoter B4.[2] Ligation results did not improve even with fresh ligation mix components and longer ligation times, leading to the conclusion that the difficulty stemmed from segment annealing or phosphorylation. Because of this, attempts to clone the engineered promoters into pUA66 resulted in repeated failures. However, increase in segment length (to increase annealing efficiency) may have improved the percentage of successful ligations to the same level of the B4 promoter segments (Figure 2.1).

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Table 3.1. Engineered promoter segments based on the constitutive promoters lacIp[24] and rpoDp1.[26] The first cytosine in the -35 site of the lacIp promoter (in red) can be replaced with a thymine to create the lacIq promoter.[25] Each segment contains either 0, 1, or 2 binding sites for ZFP 104 (highlighted). The leftmost overhang of the distal segments corresponds to that of the restriction site XhoI, the inner overhangs of each segment overlap the -35 and -10 sites of the promoter, and the rightmost overhang of the proximal segments corresponds to that of the restriction site BamHI.

|		Distal	Core	Proximal
lacIp	0 sites	TCGAGCATTTACGTTGACACCATCGAATGGCG CGTAAATGCAACTGTGGTAGCTTACCGCGTT	CAAAACCTTTCGCGGTATGGCAT TTGGAAAGCGCCATACCGTACTA	GATAGCGCCCGGAAGAGAGTCAATTCAGGGTG TCGCGGGCCTTCTCTCAGTTAAGTCCCACCTAG
	1 site	TCGAGCATTTACGTTGAGAGGCTGATATGGCG CGTAAATGCAACTCTCCGACTATACCGCGTT	CAAAACGAGGCTGATTATGGCAT TTGCTCCGACTAATACCGTACTA	GATAGCGCCCGAGGCTGATTCAATTCAGGGTG TCGCGGGCTCCGACTAAGTTAAGTCCCACCTAG
	2 sites	TCGAGAGGCTGATTTGAGAGGCTGATATGGCG CTCCGACTAAACTCTCCGACTATACCGCGTT	CAAGAGGCTGATCGGAGGCTGAT CTCCGACTAGCCTCCGACTACTA	GATAGCGCCCGAGGCTGATTCAGAGGCTGATG TCGCGGGCTCCGACTAAGTCTCCGACTACCTAG
rpoDp1	0 sites	TCGAGAGAGCGGCGTTTCTCGCCCTGTTCCGCAG CTCTCGCCGCAAAGAGCGGGACAAGGCGTCGAT	CTAAAACGCACGACCATGCGTAT TTTGCGTGCTGGTACGCATATGA	ACTTATAGGGTTGCTGGTGCAAAATCCAG ATATCCCAACGACCACGTTTTAGGTCCTAG
	1 site	TCGAGAGAGCGGCGTTTCTGAGGCTGATCCGCAG CTCTCGCCGCAAAGACTCCGACTAGGCGTCGAT	CTAAAACGGAGGCTGATGCGTAT TTTGCCTCCGACTACGCATATGA	ACTTATAGGAGGCTGATTGCAAAATCCAG ATATCCTCCGACTAACGTTTTAGGTCCTAG
	2 sites	TCGAGAGGCTGATGTTTCTGAGGCTGATCCGCAG CTCCGACTACAAAGACTCCGACTAGGCGTCGAT	CTAGAGGCTGATACGAGGCTGAT CTCCGACTATGCTCCGACTATGA	ACTTATAGGAGGCTGATTGGAGGCTGATG ATATCCTCCGACTAACCTCCGACTACCTAG
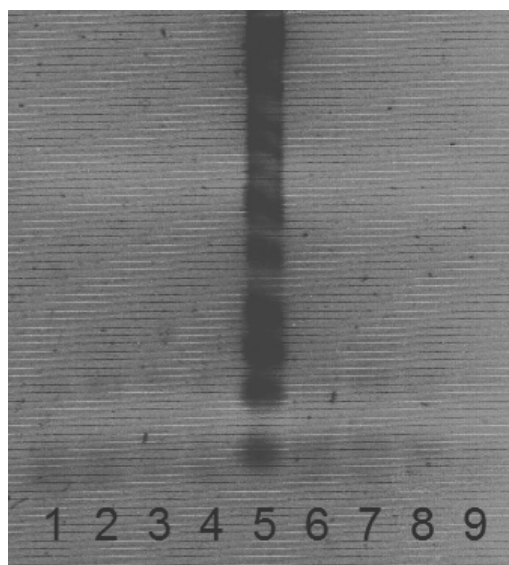
Figure 3.2. Native agarose gel electrophoresis used to verify successful promoter ligation. Lanes 1, 2, 3, and 4 show promoters q000, q001, q010, and q100 respectively. Lane 5 shows low molecular weight DNA ladder (NEB Catalog # N3233L). Lanes 6, 7, 8, and 9 show promoters p000, p001, p010, and p100 respectively. Ligated promoters should have total length of 87 bp and bands in the ligation samples can be seen near the 25 bp rung (individual segments), near the 50 bp rung (two segments), and just above the 75 bp rung of the DNA ladder (full promoters). The band above the 75 bp rung is the weakest, indicating inefficient ligation.

3.3 – Outcomes

Despite repeated failures to clone the new promoters into pUA66, we believe that the constitutive promoter approach was superior in concept to previous work. The chosen constitutive promoters were well documented in literature,[24,25,26] and the minimal extent of sequence modification due to promoter engineering should have mediated potential issues with the three and six binding site promoters previously. Moreover, the higher copy number replication origin modification made pUA66 production more manageable.

In future cloning attempts, a redesign of the constitutive promoter segments is suggested. Increasing the total promoter length from approximately 90 bp to 110-120 bp would allow for longer segments and stronger interactions between oligonucleotides during segment annealing. This could be accomplished by including more of the sequence surrounding the constitutive promoters in the segments and would increase the efficiency of segment annealing.

CHAPTER 4 – INTEGRATED TRANSCRIPTION FACTOR TESTING PLATFORM

4.1 – Integrated Plasmid Design

In previous experiments, two separate plasmids (pKTB4x and pKT104) were used to assay ZFP repression of engineered promoters. Because the copy numbers of SC101 (pKTB4x) and ColE1 (pKT104) varied by at least twofold during protein expression and the T5 system[19] expresses high levels of proteins when induced, concerns about possible oversaturation of the engineered promoters with ZFP binding arose. For quantitative repression experiments, the SC101 replication origin is desirable because of the relative certainty in the number of copies per cell as compared to higher copy number replications origins like ColE1. However, replacing the ColE1 replication origin in pKT104 with SC101 would not solve the problem. Two identical replication origins cannot coexist in a single cell for repeated generations because the cell cannot distinguish between them, leading to uneven division between daughter cells and eventual plasmid imbalance. As an alternative approach, we aimed to synthesize a hybrid plasmid containing the necessary portion of pKT104 for ZFP production into pKT10, thereby creating a single modular plasmid with sites for both engineered promoter insertion and ZFP variation. The pKT104 segment needed to contain the T5 system with the gene encoding for the lac repressor and XmaI and HindIII sites (for ZFP insertion), but the BamHI site before ZFP 104 needed to be removed to keep the engineered promoter insertion sites unique. This was accomplished by using a short "dummy" insert beginning with a BglII overhang followed directly by an XmaI site, a nonsense sequence and terminating with a HindIII overhang. BglII and BamHI sites have compatible overhangs (A**GATCT** and **GGATC**C, respectively) such that ligating the dummy insert into the BamHI site of pKT104 would produce the nonsense sequence GGATCT, thereby destroying the BamHI site functionality. The dummy insert was ligated into pKT104 after

restriction digestion by BamHI and HindIII, and the pKT104 segment was ready for amplification and ligation into pKT10.

Unfortunately, the original plasmid pUA66 was not designed for substantial modification. There is no multiple cloning site and the few restriction sites contained in the plasmid sequence are either rare such that their respective restriction enzymes are expensive, inefficiently cut by their respective restriction enzymes, or not unique. Additionally, only a few are located in the sequence such that they would not ablate function if digested. The two sites that could be used for insertion of the necessary pKT104 segment were the PstI site just after the GFPmut2 gene and the AatII site between the Kanamycin resistance gene and the XhoI site (Figure 2.2). We began by designing a modification scheme based on pKT104 segment insetion at the PstI site and the resulting plasmid (named pKT11) can be seen in Figure 4.1.
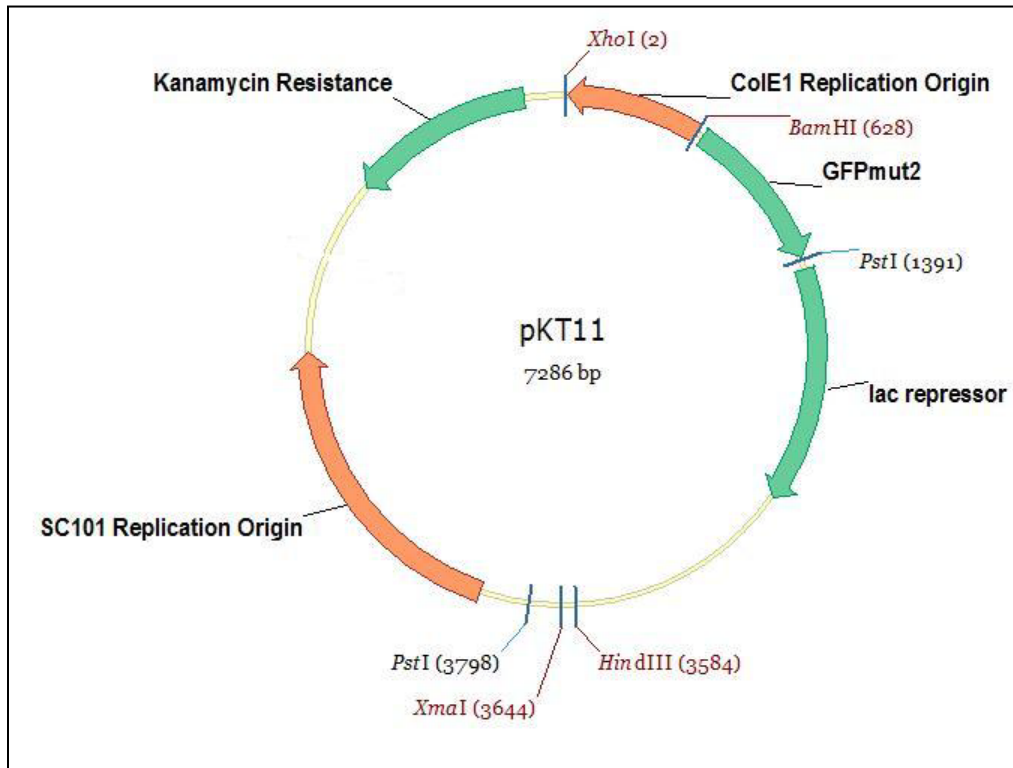

Figure 4.1. The plasmid pKT11. The XhoI and BamHI sites are unique and can be used for engineered promoter insertion and XmaI and HindIII are unique and can be used for ZFP variation.

After modification of pKT104 with the dummy insert, the ZFP production segment needed to be amplified via PCR with primer addition of PstI sites to both ends. Because the segment was over 2000 bps long, general PCR with Taq DNA Polymerase (Appendix A, section A.4.1) was likely to produce errors during segment amplification[28] so sequencing of the pKT11 plasmid was necessary after pKT104 segment ligation. However, an oversight in the planning of pKT11 made it necessary to redesign. Insertion at the PstI site would separate the GFPmut2 gene from the stop codon directly afterward. This was circumvented by primer addition of AatII sites to both ends of the pKT104 segment instead of PstI sites. The resulting plasmid (named pKT12) is shown in Figure 4.2.
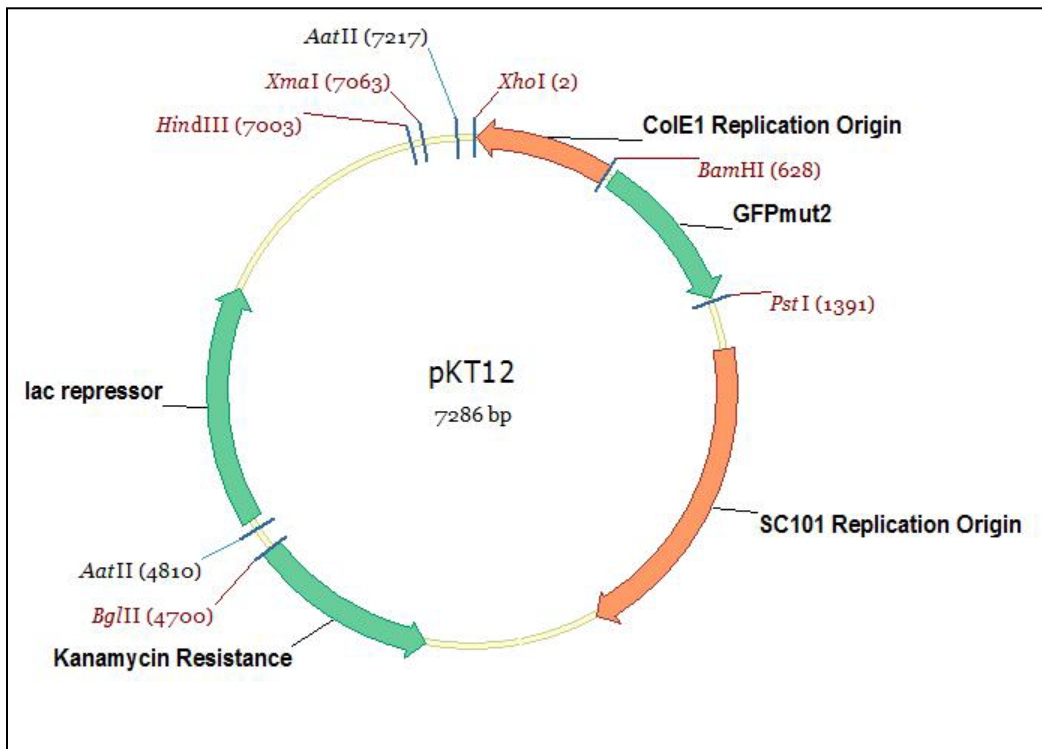
Figure 4.2. The plasmid pKT12.

4.2 – Outcomes

Successful ligation of the pKT104 segment into pKT10 to create pKT12 removed the concerns with copy number mismatch of the two separate plasmids during quantitative

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repression experiments. Plasmid production was streamlined because both engineered promoters and ZFPs could be cloned into the same plasmid rather than optimizing two separate plasmids. Overall, pKT12 represents a modular integrated TF platform suitable for assaying both promoters of interest as well as a variety of TFs which provides versatility in possible applications and ease of use.

CHAPTER 5 – CONCLUSION

5.1 – Summary of Outcomes

In this work, we developed a novel method for tuning gene expression as well as testing the DNA binding affinity of synthetic TFs. In the initial ZFP repression studies, we progressed through the approach of "reengineering" engineered promoters and moved to constitutive promoters. Constitutive promoters eliminate difficulties stemming from preexisting regulatory machinery and provide a "clean slate" for promoter engineering. Additionally, we designed these engineered constitutive promoters with an emphasis on limiting sequence modification to preserve promoter functionality after ZFP binding site addition. These modifications to the combinatorial promoter scheme in initial work create a more straightforward system to extract only the effect of TF binding on gene expression.

To facilitate modularity of both engineered promoters and ZFPs, we created the plasmid pKT12. We combined the IPTG inducible T5 system and unique ZFP insertion sites (XmaI and AgeI) from pKT104 with the very low copy number replication origin SC101 and modular promoter insertion sites (XhoI and BamHI) from pKT10 to create an optimized plasmid for quantitative assay of TF binding characteristics. Integrating both engineered promoters and ZFP production onto the same plasmid resolved the copy number difference between pKTB4x and pKT104 in initial work while maintaining the temporary medium copy number of pKT10. Overall, pKT12 is suitable for testing both promoter strength and TF binding, making it a versatile tool for studying gene regulation systems quantitatively.

5.2 – Suggested Alternative Approaches and Future Work

The most problematic step in this work was the ligation and subsequent cloning of engineered promoters. Every additional overhang that must be created by annealing

oligonucleotides, phosphorylated, and ligated reduces the ultimate efficiency of the overall cloning process. As a suggestion for future work, rather than engineering promoters with specific binding sites for a particular ZFP, ZFPs should be chosen and produced such that they bind to a certain sequence within an already existing constitutive promoter. This approach would remove the need to ligate multiple segments to create an engineered promoter before ligating into a plasmid backbone. pKT12 would still provide the perfect platform for this approach without modification and using constitutive promoters would keep the focus on the repression ability of ZFPs rather than other gene regulation factors.

APPENDIX A – EXPERIMENTAL METHODS

A.1 – Recipes

A.1.1 – Lysogeny Broth (LB) Medium
1. Measure out the following:
    10 g tryptone – provides amino acids for the growing bacteria
    5 g yeast extract – provides vitamins and other nutrients for the growing bacteria
    5 g NaCl – provides a more natural environment for bacterial growth
2. Suspend the solids in around 800 ml of $_{dd}H_2O$.
3. After the solids are dissolved, add $_{dd}H_2O$ to make a total volume of 1 liter.
4. Autoclave the mixture, making sure that it is in a large enough container so that the liquid volume is less than 50% (preferably around 25%) of the total volume. Failure to leave a generous amount of free space in the container will result in the mixture boiling out of the top during the autoclaving cycle.
5. After cooling, swirl the flask to ensure mixing, and the LB is ready for use.

A.1.2 – $CaCl_2$ Solution for Competent Cells
1. Add 75 ml glycerol to 1 L bottle.
2. Add 4.4106 g $CaCl_2 \cdot 2H_2O$.
3. Add 1.5119 g PIPES.
4. Fill to 500 ml mark with ddH_2O and shake to mix.
5. Check that pH is 7 and adjust if necessary.

A.2 – Cloning Protocols

A.2.1 – Cell Pellet Formation
1. Under flame, fill at least 6, preferably 8, culture tubes approximately 1/3 of the way up (usually 2-5 ml) with LB containing the appropriate antibiotic for your plasmid. It is best to denote this by the color code system used in the lab: red for Ampicillin and blue for Kanamycin.
2. Pick a single colony and place this same picking tool into each culture tube from a Petri dish harboring cells with the plasmid of interest. Do not let your picking tool (toothpick, pipette tip, etc…) touch more than one colony.
3. Incubate the tubes with gentle agitation in a 37ºC environment for 16-20 hours. After this time, the liquid in the tube should appear opaque and murky from the cell growth.
4. Aliquot each culture tube contents into 2 ml centrifuge tubes.
5. Centrifuge for 7 minutes at 6000 x g.
6. Discard supernatant to bleach solution and let stand for one hour.
7. Cell pellet is ready for storage at -80ºC, Miniprep, or Midiprep.

A.2.2 – Miniprep
This protocol is designed for cell pellets obtained from 2 ml samples. If cell pellets are from larger samples, scale steps 7, 8 and 9 accordingly. If anything is missing that cannot be created from components in the lab immediately take necessary steps to order more. The concentration of the final sample mixture should be between 100-200 ng/µl for medium to high copy number

plasmids.

1. Locate Buffer P1 used for resuspension. This buffer solution should be kept at 2-8˚C so it will be in the 4˚C refrigerator. Shake vigorously before use. If none is available it can be created with the composition:
   50 mM Tris-Cl (pH 8.0) – stabilizes pH
   10 mM EDTA – protects DNA from breakdown by metal containing enzymes
   100 µg/ml RNase A – breaks down bacterial RNA
   1µl/ml LyseBlue (if desired) – indicates completion of lysis and neutralization reactions
2. Locate Buffer P2 used for cell lysis. Do not shake vigorously at any point. Close bottle quickly after use to avoid reaction with CO_2. If salts are present, redissolve by warming to 37˚C. If none is available it can be created with the composition:
   200 mM NaOH – alkaline destruction of cell membrane and denatures DNA
   1% sodium dodecyl sulfate (SDS) –dissolves cell membranes and denatures proteins
3. Locate Buffer N3 used for neutralization of the lysis reaction. If none is available it can be created with the composition:
   4.2 M guanidinium hydrochloride – denatures proteins and DNA and shuts down nuclease activity
   0.9 M potassium acetate (pH 4.8 which can be adjusted with glacial acetic acid) – used to precipitate SDS after lysis reaction
4. Locate Buffer PB used for washing collection tubes. Use the large bottle of clear Buffer PB rather than the smaller bottle of yellow Buffer PB which is used for PCR protocols. If none is available it can be created with the composition:
   5 M guadinium hydrochloride – denatures proteins and DNA and shuts down nuclease activity
   30% EtOH – washing agent
   10 mM Tris-Cl (pH 6.6) – stabilizes pH
   It is used to remove trace nuclease activity when using *endA*+ bacterial strains.
5. Locate Buffer PE used for washing collection tubes. If none is available it can be created with the composition:
   75% EtOH – washing agent
   25 mM NaCl – stabilizes DNA
   5 mM Tris-Cl (pH 7.5) – stabilizes pH
6. Locate Buffer EB used for DNA elution. If none is available water can be used or it can be created with the composition:
   10 mM Tris-Cl (pH 8.5) – provides proper pH to elute DNA from silica membrane within collection tubes
7. Resuspend previously harvested bacterial cells in 100 µl Buffer P1 and transfer to a micro-centrifuge tube. Mixture should be homogeneous whitish solution with no clumps visible.
8. Add 100 µl of Buffer P2 and mix by gently inverting tube 4-6 times. Do not shake vigorously or vortex because this could result in shearing of genomic DNA. If LyseBlue reagent is present in Buffer P1 the mixture should turn blue. Continue inverting until blue color is homogenous throughout. Do not allow lysis reaction to proceed for more than 5 minutes.
9. Add 140 µl of Buffer N3 and mix by inverting the tube 4-6 times. Mix immediately and thoroughly to avoid localized precipitation of SDS. If LyseBlue reagent is present in

Buffer P1 the mixture should be mixed until all traces of blue disappear and the suspension is colorless. A white chunky suspension indicates that the SDS has been precipitated.

10. Centrifuge the mixture for 10 minutes at 17,000 x g. The actual protocol suggests 17,900 x g but our centrifuge only goes up to 17,000 x g. After centrifuge a compact white pellet should be visible.
11. Using the blue collection tubes with silica membranes, apply no more than 850 µl of supernatant (liquid after centrifuging) directly to the silica membrane. Do not touch the tip of the pipette to the membrane. The silica membrane attracts and collects DNA at the pH created by the added buffer solutions.
12. Centrifuge for 30-60 seconds at 17,000 x g. Discard the flow-through collected in the bottom portion of the tube by dumping the tube and then gently banging the rim of the tube on a paper towel to remove the remainder.
13. Repeat steps 11 and 12 using the same collection tube until all of the mixture has been centrifuged and the waste discarded.
14. A wash with Buffer PB is recommended. This step is not necessary for XL-1 Blue or DH5α bacterial strains. To wash the collection tube, apply 0.5 ml Buffer PB and centrifuge as in step 12.
15. Wash the collection tube by adding 0.75 ml Buffer PE and centrifuging as in step 12.
16. After discarding flow-through, centrifuge for an additional 60 seconds to remove residual wash buffer. Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Ethanol from Buffer PE may undesirably inhibit subsequent enzymatic reactions.
17. Heat Buffer EB to 55-65˚C prior to elution.
18. Remove the top portion of the collection tube and discard the bottom portion. Place the top portion in a clean micro-centrifuge tube. To elute DNA from the silica membrane, apply 50 µl Buffer EB to the silica membrane and let stand for 5 minutes. Elution efficiency is dependent on pH and the maximum efficiency is achieved within the range of 7-8.5. Centrifuge for 60 seconds at 17,000 x g. If water is used for elution, store at -20˚C because DNA may degrade in the absence of a buffering agent.

A.2.3 – Midiprep
This protocol is designed for cell pellets obtained from 2 ml samples. If cell pellets are from larger samples, scale steps 7, 8 and 9 accordingly. If anything is missing that cannot be created from components in the lab immediately take necessary steps to order more. The concentration of the final sample mixture should be between 100-200 ng/µl if you prepared 6-8 tubes of culture containing low copy plasmid.

1. Locate Buffer P1 used for resuspension. This buffer solution should be kept at 2-8˚C so it will be in the 4˚C refrigerator. Shake vigorously before use. If none is available it can be created with the composition:
   50 mM Tris-Cl (pH 8.0) – stabilizes pH
   10 mM EDTA – protects DNA from breakdown by metal containing enzymes
   100 µg/ml RNase A – breaks down bacterial RNA
   1µl/ml LyseBlue (if desired) – indicates completion of lysis and neutralization reactions
2. Locate Buffer P2 used for cell lysis. Do not shake vigorously at any point. Close bottle quickly after use to avoid reaction with CO_2. If salts are present, redissolve by warming

to 37˚C. If none is available it can be created with the composition:
200 mM NaOH – alkaline destruction of cell membrane and denatures DNA
1% sodium dodecyl sulfate (SDS) –dissolves cell membranes and denatures proteins

3. Locate Buffer P3 used for neutralization of the lysis reaction. If none is available it can be created with the composition:
3M potassium acetate (pH 5.5) – precipitates SDS after lysis reaction

4. Locate Buffer QBT used for equilibration. If none is available it can be created with the composition:
750 mM NaCl – stabilizes DNA
50 mM MOPS (pH 7.0) – stabilizes pH
15% isopropanol (v/v) – aggregates DNA together
0.15% Triton® X-100 (v/v) – detergent which reduces surface tension of the column

5. Locate Buffer QC used for washing collection tubes. If none is available it can be created with the composition:
1.0 M NaCl – stabilizes DNA
50 mM MOPS, pH 7.0 – stabilizes pH
15% isopropanol (v/v) – aggregates DNA together

6. Locate Buffer QF used for DNA elution. If none is available it can be created with the composition:
1.25 M NaCl – stabilizes DNA
50 mM Tris-Cl, pH 8.5 – provides proper pH to elute DNA
15% isopropanol (v/v) – aggregates DNA together

7. Resuspend previously harvested bacterial cells in 100 µl Buffer P1 and transfer to a micro-centrifuge tube. Mixture should be homogeneous whitish solution with no clumps visible.

8. Add 100 µl of Buffer P2 and mix by gently inverting tube 4-6 times. Do not shake vigorously or vortex because this could result in shearing of genomic DNA. If LyseBlue reagent is present in Buffer P1 the mixture should turn blue. Continue inverting until blue color is homogenous throughout. Do not allow lysis reaction to proceed for more than 5 minutes.

9. Add 140 µl of chilled Buffer P3 with the tube on ice and mix by inverting the tube 4-6 times. Mix immediately and thoroughly to avoid localized precipitation of SDS. If LyseBlue reagent is present in Buffer P1 the mixture should be mixed until all traces of blue disappear and the suspension is colorless. A white chunky suspension indicates that the SDS has been precipitated. Allow to incubate on ice for 5 minutes after mixing.

10. Centrifuge the mixture for 10 minutes at 4˚C and maximum speed. After centrifuge a compact white pellet should be visible.

11. Using the syringe-like columns with open tips, set up the column over an appropriate waste collection vessel and apply 1 ml of Buffer QBT. Allow the column to empty completely by gravity flow.

12. Apply the supernatant from step 9 to the column. Make sure that the supernatant is clear of precipitated material and do not touch the tip of the pipette to the white portion of the column. The environment created by the equilibration buffer will trap the DNA within the column. Allow the column to empty completely by gravity flow.

13. Wash the column twice with 2 ml of Buffer QC. Allow the column to empty completely by gravity flow between each wash.

14. Heat Buffer QF to 55-65°C prior to elution.
15. Place the column in a DNA collection vessel. To elute DNA, apply 0.8 ml of Buffer QF to the column and allow the column to empty completely by gravity flow.
16. Add 5 volumes of yellow Buffer PB to the eluted DNA sample. The mixture should turn yellow. If the mixture is orange or purple, add 10 µl of 3 M sodium acetate (pH 5.0) and mix until the color is yellow.
17. Using the blue collection tubes with silica membranes, apply the entire sample directly to the silica membrane. Do not touch the tip of the pipette to the membrane.
18. Centrifuge for 60 seconds at 17,000 x g. Discard the flow-through collected in the bottom portion of the tube by dumping the tube and then gently banging the rim of the tube on a paper towel to remove the remainder.
19. Wash the collection tube by adding 0.75 ml Buffer PE and centrifuging as in step 18.
20. After discarding flow-through, centrifuge for an additional 60 seconds to remove residual wash buffer. Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation.
21. Heat elution buffer to 55-65°C prior to elution.
22. Remove the top portion of the collection tube and discard the bottom portion. Place the top portion in a clean micro-centrifuge tube. To elute DNA from the silica membrane, apply 50 µl Buffer TE to the silica membrane and let stand for five minutes. Centrifuge for 60 seconds at 17,000 x g.

A.2.4 – Double Digest
1. Determine the proper restrictive enzymes to separate the vector from the plasmid and the NEBuffer needed for the process. This can be done by entering the enzymes into the Double Digest Calculator at http://www.neb.com/nebecomm/DoubleDigestCalculator.asp or by looking them up on the table available at http://www.neb.com/nebecomm/tech_reference/ restriction_enzymes/double_digests.asp. Also determine whether or not bovine serum albumin (BSA) is required to stabilize the enzymes to be used. BSA does not adversely affect enzymes that do not require it.
2. Mix the following:
   ~50 µl plasmid Miniprep DNA sample (should contain about 1 µg of DNA based on concentration)
   5 µl 10x NEBuffer
   1 µl enzyme 1
   1 µl enzyme 2
   100 µg/ml BSA (if needed)
   Make sure to add the enzymes last. Failure to do so could result in the permanent deactivation of the enzymes. The total volume of the mixture should be around 50 µl. Supplement the mixture with water if additional volume is needed.
3. Incubate mixture for 1-2 hours at 37°C.

A.2.5 – PCR Cleanup
1. Locate Buffer TE for DNA elution. If none is available it can be created with the composition:
   10 mM Tris-Cl (pH 8.0) – stabilizes pH
   1 mM EDTA – protects DNA from breakdown by metal containing enzymes

Note that EDTA may inhibit subsequent enzymatic reactions.

2. Add 5 parts yellow Buffer PB (indicates that pH indicator I has been added) to one part double digest product. If the double digest product volume is 50 µl, add 250 µl of buffer. The mixture should turn yellow. If the mixture is orange or purple, add 10 µl of 3 M sodium acetate (pH 5.0) and mix until the color is yellow.
3. Using the blue collection tubes with silica membranes, apply the entire sample directly to the silica membrane. Do not touch the tip of the pipette to the membrane.
4. Centrifuge for 60 seconds at 17,000 x g. Discard the flow-through collected in the bottom portion of the tube by dumping the tube and then gently banging the rim of the tube on a paper towel to remove the remainder.
5. Wash the collection tube by adding 0.75 ml Buffer PE and centrifuging as in step 3.
6. After discarding flow-through, centrifuge for an additional 60 seconds to remove residual wash buffer. Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation.
7. Remove the top portion of the collection tube and discard the bottom portion. Place the top portion in a clean micro-centrifuge tube. To elute DNA from the silica membrane, apply 50 µl Buffer TE to the silica membrane and let stand for five minutes. Centrifuge for 60 seconds at 17,000 x g.

A.2.6 – CIP Treatment

Calf intestinal alkaline phosphatase (CIP) is used to prevent the vector from self ligating, which improves yield and reduces the background of improperly self ligated contaminants.

1. Mix the following:
   ~50 µl double digest product
   5 µl NEBuffer 3
   0.5 µl CIP
   Make sure to add the CIP last. Failure to do so could result in the permanent deactivation of the enzyme.
2. Incubate the mixture for 15 minutes at 37˚C.
3. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel. Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to the table below to identify the dyes according to migration distance and agarose gel percentage and type.

%TAE (TBE) agarose gel	Xylene cyanol (light blue)	Bromophenol blue (dark blue)	Orange G (orange)
0.8	5000 bp (3000 bp)	800 bp (400 bp)	150 bp (<100 bp)
1.0	3000 bp (2000 bp)	400 bp (250 bp)	<100 bp (<100 bp)
1.5	1800 bp (1100 bp)	250 bp (100 bp)	<100 bp (<100 bp)
2.0	1000 bp (600 bp)	200 bp (<100 bp)	<100 bp (<100 bp)
2.5	700 bp (400 bp)	100 bp (<50 bp)	<50 bp (<50 bp)

A.2.7 – Gel Electrophoresis

1. Locate TAE Buffer for gel electrophoresis. If the jug is empty more buffer can be made using the 20 ml of the 50x TAE Buffer and 980 ml of $_{dd}H_2O$.

2. Mix the correct percentage of electrophoresis grade agarose in the necessary volume of TAE Buffer. See the table below to determine these values, where the percentage is by volume. Melt the agarose in a microwave oven and swirl to ensure even mixing. Plug the top of the container with paper towels to prevent excess vapor from escaping. The vapors from the mixture are **toxic** if inhaled. Allow the mixture to cool to 55°C (no vapor escaping from the flask and not painful to touch) before pouring onto the gel platform.
3. Prepare gel platform and choose correctly sized comb for the number of samples to be run. Pour the melted agarose onto the platform and insert the comb, making sure that no bubbles are trapped below the comb or appear on the surface of the gel.
4. Once the gel has hardened, remove the comb carefully by pulling straight up and without rocking the comb back and forth in the gel. Rocking or wiggling the comb could tear the wells and ruin the gel. If the gel is of a low percentage agarose, it may be necessary to cool it to 4°C before attempting to remove the comb for added rigidity.
5. Place the gel platform into the electrophoresis tank. Add sufficient TAE Buffer to cover the gel to a depth of around 1 mm or until the tops of the wells are submerged. Make sure there are no air pockets within the wells.
6. Prepare DNA samples that will not overflow the wells by addition of the appropriate amount of loading buffer if it has not already been added according to the table given. Loading buffers are available in the lab and contain a variety of dyes for visual confirmation of migration as well as a heavy component such as glycerol to keep the sample within the well rather than allow it to mix with the TAE Buffer. Load the samples into the wells using a pipettor, making sure not the mix the samples between wells.
7. Attach the leads, red to red and black to black, to the voltage source and attach the lid of the electrophoresis tank. A standard protocol is 100 V for 30 minutes. Make sure to set the timer on the voltage source if automatic operation is preferred over turning off the voltage manually.
8. Verify that the voltage source is turned off and remove the gel from the gel platform. Place the gel into a large enough container to accommodate its size. Add enough TAE Buffer to cover the gel and one drop (10 µl) of ethidium bromide to stain the DNA. Gently agitate the gel in the mixture for 15 minutes. Note that ethidium bromide is a mutagen and potential carcinogen so handling time should be kept as short as possible and nitrile gloves should be used when handling it. Ethidium bromide wastes must be placed in the respective waste containers in the waste cabinet and NEVER pour down the sink drain.
9. Remove the gel from the mixture and image over UV light. Make sure to wear protective gear over eyes and exposed skin that blocks UV radiation.

Agarose (%)	Effective range of resolution of linear DNA fragments (kb)
0.5	1 to 30
0.7	0.8 to 12
1.0	0.5 to 10
1.2	0.4 to 7
1.5	0.2 to 3

A.2.8 – Gel Extraction

1. Locate Buffer QG for solubilization. If none is available it must be ordered because the composition is proprietary.
2. Excise the DNA fragment for the agarose gel with a clean, sharp scalpel. Excise a band as small as possible.
3. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to one volume of gel. 100 mg of gel is approximately 100 µl in volume.
4. Incubate at 50°C for 10 minutes or until the gel has completely dissolved. Make sure to solubilize agarose completely. For greater than 2% gels, increase incubation time. After the gel is completely dissolved, the color of the mixture should be yellow. If the mixture is orange or purple, add 10 µl of 3 M sodium acetate (pH 5.0) and mix until the color is yellow.
5. Add 1 gel volume of isopropanol to the sample and mix by inverting the tube several times. Do not centrifuge the sample at this point because it will break up the DNA as it is aggregating together in the alcohol environment.
6. Using the blue collection tubes with silica membranes, apply the entire sample directly to the silica membrane. Do not touch the tip of the pipette to the membrane.
7. Centrifuge for 60 seconds at 17,000 x g. Discard the flow-through collected in the bottom portion of the tube by dumping the tube and then gently banging the rim of the tube on a paper towel to remove the remainder.
8. Wash the collection tube by adding 0.75 ml Buffer PE and centrifuging as in step 3.
9. After discarding flow-through, centrifuge for an additional 60 seconds to remove residual wash buffer. Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation.
10. Remove the top portion of the collection tube and discard the bottom portion. Place the top portion in a clean micro-centrifuge tube. To elute DNA from the silica membrane, apply 50 µl Buffer TE to the silica membrane and let stand for 5 minutes. Centrifuge for 60 seconds at 17,000 x g.

A.2.9 – Nanodrop

1. Before starting the software module, clean the sample surfaces with DI water to remove any dried sample that might be present. Alternatively, you can clean the sample surfaces with a Kimwipe moistened with ethanol.
2. Open the Nanodrop program and the appropriate module.
3. Wipe off the top and bottom sensors of the instrument with a Kimwipe. These are just

the polished ends of fiber optic cable, so wiping is sufficient to prevent carryover.

4. Pipette 2 μL of water onto the sensor. Bring down the lever arm very slowly and carefully to prevent damage to the instrument.
5. Follow the onscreen prompts to calibrate.
6. Wipe the sensors and pipette on 2 μL of the corresponding blank (Buffer EB or whatever solution your prep is in). Bring down the lever arm very slowly and carefully to prevent damage to the instrument.
7. Follow the onscreen prompts to blank.
8. Wipe the sensors and pipette on 2 μL of your sample. Bring down the lever arm very slowly and carefully to prevent damage to the instrument.
9. Click "Measure" and record the concentration measured.
10. For DNA, the peak should be at 260 nm, and as a general rule, the 260/280 ratio should be between 1.8 and 2.0. A ratio in this range indicates a relatively pure sample of DNA. Because proteins absorb UV light at 280 nm, a sample contaminated with proteins will have a 260/280 ratio < 1.8.
11. To test multiple samples, wipe the sensor in between measurements with a Kimwipe. Recalibration or re-blanking is not necessary.
12. Clean the sample surfaces when finished.
13. Verify that concentrations of your samples are high enough to proceed with ligation.
14. Make sure to print results for record purposes.

A.2.10 – Ligation

1. Mix the following in a 1.5 ml tube:
   10 μl of vector and insert mixed in a ratio of 1:2 or 1:10
   1.2 μl NEB ligase buffer
   1 μl T4 ligase
   Make sure to add the T4 ligase last. Failure to do so could result in the permanent deactivation of the enzymes.
2. Incubate for 30 minutes at room temperature.
3. Insert tube into ice.

A.2.11 – Transformation

This protocol should be carried out under the flame. Be careful to keep all tubes and pipettes near the flame to maintain sterile conditions. The competent cells are VERY fragile. They have been punctured to make them more susceptible to the insertion of plasmids and should never be shaken vigorously.

1. Add 25 μl of competent cells (found in the -80˚C freezer) and 1 μl of ligation mix to a 2.0 ml sterilized tube.
2. Leave the tube on ice for 10 minutes.
3. Take the tube off of the ice and insert it into the 42˚C bath for exactly 45 seconds. This "wakes up" the cells so that they can begin to recover.
4. Return the tube to the ice.
5. Add 175 μl of SOC. This acts as the food source for the weak recovering cells. SOC stands for Super Optimal Broth (SOB) with Catabolite repression which is SOB with added glucose. SOB is a nutrient rich bacterial growth medium with the composition: 2% w/v bacto-tryptone (20 g)

0.5% w/v bacto-yeast extract (5 g)

8.56mM NaCl (0.5 g) or 10mM NaCl (0.584 g)

2.5mM KCl (0.186 g)

$_{dd}H_2O$ to 1000 ml

SOC additionally contains:

10 mM $MgCl_2$ (0.952 g)

20mM $MgSO_4$ (2.408 g)

20mM glucose (3.603 g)

To make SOC, add all reagents except glucose to 500 ml of water and autoclave. Then add the glucose to 500 ml of water and autoclave. Mix the two solutions under the flame to prepare the final SOC solution.

6. Incubate cells with agitation for 1 hour at 37ºC.
7. Obtain Petri dishes with the correct antibiotic added to the agar. Plate out 100 µl of sample on each dish and sprinkle in several autoclaved beads. Close dish and shake for several seconds until sample is spread across agar surface. Discard beads into used bead container. Incubate plates at 37˚C for 16-20 hours.

A.3 – Promoter Creation

A.3.1 – Promoter Segment Annealing
  1. Mix the following in a PCR tube:
     2.5 µl of 100 µM oligonucleotide 1
     2.5 µl of 100 µM oligonucleotide 2
     45 µl of EB Buffer
  2. Using either a heat block or a PCR machine, heat tube to 95˚C. For a heat block, turn off heat and allow tube to cool to room temperature. For a PCR machine, ramp down to room temperature in 5˚C increments for 5 minutes each.

A.3.2 – Promoter Segment Phosphorylation
  1. Mix the following in a PCR tube:
     15.5 µl $_{dd}H_2O$
     2 µl T4 DNA Ligase Buffer
     2 µl annealed promoter segment
     0.5 µl T4 Polynucleotide Kinase
  2. Incubate at 37˚C for 2 hours.

A.3.3 – Promoter Ligation
  1. Mix the following in a PCR tube:
     3.33 µl of each of the desired distal, core, and proximal segments for the promoter
     1.2 µl *E. coli* DNA Ligase Buffer
     1 µl *E. coli* DNA Ligase
  2. Incubate at 16˚C for 30 minutes.

A.3.4 – Polyacrylamide Gel Electrophoresis
  1. Add 15 ml 8% polyacrylamide to a 50 ml Falcon tube.
  2. Put under vacuum for 20 minutes to remove air bubbles.

3. Line up a front plate and a back plate for each gel in gel holsters. Do this on a flat surface to ensure that there will be no leaks from the bottom of the gel as it hardens.
4. Snap holsters shut and lock into gel platform.
5. Add 10 µl TEMED and 75 µl 10% APS to the Falcon tube and gently rock back and forth 5 times.
6. Place the lip of tube at the top of the front plate of the gel holster and pour until filled.
7. Insert comb and let stand overnight.
8. Remove gel plates from holsters and load into gel electrophoresis basket.
9. Fill gel stand to the top with TBE Buffer and check that the liquid level does not go down.
10. After making sure no leaks are present, fill basket to the proper level for the number of gels with TBE Buffer.
11. Remove comb and secure the basket lid.
12. Run gel for 20 minutes at 80V without samples to remove impurities.
13. Fill wells and run gel for 40 minutes at 100V. The time and voltage may vary depending on the sample.
14. Verify that the voltage source is turned off and remove the gel from the gel stand. Place the gel into a large enough container to accommodate its size. Add enough TAE Buffer to cover the gel and one drop (10 µl) of ethidium bromide to stain the DNA. Gently agitate the gel in the mixture for 15 minutes. Note that ethidium bromide is a mutagen and potential carcinogen so handling time should be kept as short as possible and nitrile gloves should be used when handling it. Ethidium bromide wastes must be placed in the respective waste containers in the waste cabinet and NEVER pour down the sink drain.
15. Remove the gel from the mixture and image over UV light. Make sure to wear protective gear over eyes and exposed skin that blocks UV radiation.

A.4 – PCR

A.4.1 – General PCR
1. Make master mix:
   80 µl $_{dd}H_2O$
   10 µl ThermoPol Buffer
   4 µl 5 mM dNTP mix
   0.8 µl 50 µM primer mix
   0.8 µl Taq DNA Polymerase
2. Aliquot in 25 µl volumes into 4 PCR tubes.
3. Add 1 ul 100-200 ng/µl DNA template to each.
4. Immediately place in PCR machine and run the following program:
   1. 95°C for 2 minutes.
   2. 95°C for 30 seconds.
   3. Annealing temperature of primers for 1 minute.
   4. 72°C for 1 minute.
   5. Repeat steps 2-4 30 times.
   6. 72°C for 5 minutes.
   7. Hold at 4°C.

A.4.2 – Colony PCR
1. Make master mix:
   315 µl $_{dd}H_2O$
   37.5 µl ThermoPol Buffer
   15 µl 5 mM dNTP mix
   3.75 µl 50 µM primer mix
   3.75 µl Taq DNA Polymerase
2. Aliquot in 25 µl volumes into 15 PCR tubes.
3. Touch one colony on a plate with a pipette tip and dip pipette tip into one tube. Repeat for each colony of interest.
4. Immediately place in PCR machine and run the following program:
   1. 95˚C for 2 minutes.
   2. 95˚C for 30 seconds.
   3. Annealing temperature of primers for 1 minute.
   4. 72˚C for 1 minute.
   5. Repeat steps 2-4 30 times.
   6. 72˚C for 5 minutes.
   7. Hold at 4˚C.

A.5 – Competent Cell Protocol

A.5.1 – Competent Cell Production
1. Under flame, fill a 50 ml Falcon tube approximately 1/3 of the way up (usually 15 ml) with LB containing the appropriate antibiotic.
2. Pick a single colony of your cell strain (DH5α, BLR, etc.) from a Petri dish and place this same picking tool into the Falcon tube.
3. Incubate the tubes with agitation in a 37ºC environment for 16-20 hours. After this time, the liquid in the tube should appear opaque and murky from the cell growth.
4. Dilute a sample of culture (approximately 4 ml) 1 to 100 and incubate with agitation at 37˚C until it reaches an OD of 0.4. This generally takes about 2 hours, but depends on your cell strain and the incubation conditions.
5. Aliquot culture volume into 50 ml Falcon tubes.
6. Prechill all culture and $CaCl_2$ solution on ice.
7. Centrifuge Falcon tubes at 1600 x g for 7 minutes at 4˚C.
8. Discard supernatant to bleach.
9. Add 10 ml chilled $CaCl_2$ solution and shake gently to resuspend the cell pellets.
10. Centrifuge Falcon tubes at 1100 x g for 5 minutes at 4˚C.
11. Discard supernatant to bleach.
12. Add 10 ml chilled $CaCl_2$ solution and shake gently to resuspend the cell pellets.
13. Keep Falcon tubes on ice for 30 minutes.
14. Centrifuge Falcon tubes at 1100 x g for 5 minutes at 4˚C.
15. Discard supernatant to bleach.
16. Add 2 ml $CaCl_2$ solution to each and shake gently to resuspend the cell pellets.
17. Aliquot into desired volumes in 2 ml tubes and flash freeze in an ethanol/dry ice bath.
18. Store at -80C. The competent cells are ready for use.
19. *Great Job.*

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