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Gene Expression from Linear DNA in Cell-Free Transcription–Translation Systems

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Cell-free transcription–translation (TXTL) systems are increasingly employed in synthetic biology and protein engineering efforts because they have multiple advantages over traditional laboratory methods, including the ability to produce proteins that are toxic to living cells. In this work, we attempted to use TXTL to produce portions of native *Escherichia coli* transcriptional machinery with the intention of characterizing engineered variants of these proteins. Our original experimental plan relied on producing these genes in plasmids in living cells, which we were unable to achieve. To circumvent cloning difficulties, we produced linear genes via polymerase chain reaction (PCR) and expressed them in TXTL. This report summarizes our results comparing linear and plasmid gene expression in two different TXTL systems and highlights advantages and disadvantages of using linear DNA in TXTL. These results will help guide future research efforts involving TXTL systems.

15. SUBJECT TERMS

Transcription–translation (TXTL)	Linear DNA	Cell-free	Protein expression
Gene expression	Cloning	Plasmid	Green fluorescent protein (GFP)

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PREFACE

The work described in this report was authorized under the fiscal year 2019 Innovative Development of Employee Advanced Solutions Program entitled "Exploiting Designed Biological Recognition Elements for On-Target Detection" using Section 2363 funds. The work was started in May 2019 and completed in December 2020.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for public release.

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GENE EXPRESSION FROM LINEAR DNA IN CELL-FREE TRANSCRIPTION–TRANSLATION SYSTEMS

1. INTRODUCTION

Cell-free transcription–translation (TXTL) systems are being increasingly employed in synthetic biology and protein engineering efforts because they have multiple advantages over traditional laboratory methods. TXTL systems typically consist of two main components: a lysate derived from a living organism,[1] usually *Escherichia coli*, that includes the macromolecular machinery required for transcription and translation to occur, and a reagent mixture to buffer the reaction and provide energy molecules and biopolymer precursors that would ordinarily be produced by cellular metabolism.[2] TXTL thus mirrors a living system but is not constrained by factors that are essential to keep whole cells alive and replicating. For this reason, TXTL is amenable to a variety of applications that are not accessible using whole cells, such as the expression and engineering of proteins that are toxic to cells and gene expression directly from linear polymerase chain reaction (PCR)-generated DNA, both of which we explored in this work.

We initially sought design sensing schemes that employed TXTL because TXTL has been shown to be shelf-stable when lyophilized, tolerant to organic solvents, and a generally promising platform for low-burden field detection.[3] We began by engineering multi-protein fusions that sensed with a designed binding protein and transduced that binding event using native *E. coli* proteins that are critical for gene regulation in cells. Daicel Arbor Biosciences (Ann Arbor, MI) has a commercially available catalog of plasmids that express native *E. coli* sigma factors and corresponding plasmids that express a green fluorescent protein (GFP) reporter from the sigma factor's representative promoter. In addition to plasmids, Daicel Arbor Biosciences sells several varieties of TXTL kits, coined myTXTL, that have been demonstrated to robustly express the plasmids in their library. This system of factors and reporters seemed an ideal basis for this effort. However, scale-up production and purification of these plasmids for use in TXTL requires expansion of them within living *E. coli* cells. We found this to be problematic, primarily because any expression of the factors on these plasmids led to breakdown of genetic control in the host cells.

As previously stated, one of the key advantages of using TXTL systems is that they are not generally susceptible to interference from the genes or gene products of the factors we sought to engineer. We chose to employ a strategy that circumvented the use of plasmids for TXTL-based expression of cytotoxic proteins; we simply used linear (non-plasmid) DNA for gene expression. Linear DNA can be assembled and amplified using PCR and therefore does not require cell growth for its production. In this report, we describe experiments investigating methods and considerations for using linear DNA in TXTL systems. We anticipate that this will be an important technique for our future efforts because it removes time- and labor-intensive work associated with subcloning and propagating genes in plasmids.[4]

2. MATERIALS AND METHODS

2.1 Strains and Plasmids

DH5α cells were used for transformation and propagation of several plasmids used in this study. The first sample of *E. coli* strain KL740 cI857+ was received from the Yale University (New Haven, CT) Coli Genetic Stock Center (catalog number 14222); the second sample was generously donated by Dr. Evelyn Eggenstein (Daicel Arbor Biosciences). Chemically competent stocks of KL740 cI857+ were generated according to Daicel Arbor Bioscience's protocol.

Plasmids were purchased from the Daicel Arbor Biosciences myTXTL Toolbox 2.0 Plasmid Collection (plasmids and catalog numbers are summarized in Table 1). Plasmids were purified using the PureYield Plasmid Midiprep System (Promega; Madison, WI; catalog number A2492) following the manufacturer's protocol.

2.2 Linear DNA Amplification

Custom synthesized primers for the PCR-based amplification of the p70a-deGFP linear DNA constructs were purchased with and without biotinylated 5′ modifications from Integrated DNA Technologies (Coralville, IA). The primer sequences were as follows:

- forward short overhang: 5′-GTTCCGCTGGGCATGC-3′;
- forward long overhang: 5′-GACATGGTGAAGACTATCGCAC-3′; and
- reverse for short and long overhang: 5′-CACAGAAAAGCCCGCC-3′.

PCR amplification of the p70a-deGFP linear DNA fragments used plasmid pTXTL-p70a-deGFP as the template. Briefly, 100 µL PCR reactions containing 0.5 µM of each forward and reverse primer, 1 ng/µL template plasmid, and 1× final concentration of Q5 High Fidelity 2X Master Mix (New England Biolabs [NEB]; Ipswich, MA; catalog number M0492L) were assembled and amplified according to NEB's protocol. After amplification, the PCR products were purified using the Wizard SV Gel and PCR Clean-Up system (Promega; catalog number A9281), and the final DNA concentration was determined by measuring the sample's absorbance at 260 nm. Purity of the linear DNA product was assessed by agarose gel electrophoresis.

2.3 Cell-Free TXTL Assays

Two kits, the myTXTL Sigma 70 Master Mix kit and myTXTL Linear DNA Expression kit, were purchased from Daicel Arbor Biosciences (catalog numbers 507024 and 508024, respectively) and used according to the manufacturer's protocols.

Plasmid or linear DNA was used at a final concentration of 10 nM in each 12 µL TXTL reaction. Reactions were placed in a 384 well black/clear bottom microplate (Corning; Corning, NY; catalog number 4588), covered with an adhesive microtiter plate sealing film, and

incubated at 29 °C in a Synergy H1 Hybrid Multi-Mode Microplate Reader (Biotek; Winooski, VT). deGFP fluorescence was measured every 5 min for 8 h at 485 nm excitation and 528 nm emission wavelengths. The TXTL reaction data presented in this report are the averages and standard deviations of three biological replicates, each performed on a different day.

3. RESULTS AND DISCUSSION

3.1 TXTL for Plasmid-Based Expression of Native *E. coli* Genes

TXTL systems have shown utility in the expression and characterization of genetic circuits from *E. coli* promoters that are generally silenced inside a living cell under optimal growth conditions.[5] This promising capability is accessible by TXTL because TXTL allows for the manipulation and relative overexpression of proteins that would otherwise be detrimental to the growth of a living cell, such as toxins, insoluble proteins, and essential native *E. coli* machinery.

Plasmids are frequently used to express genetic elements in TXTL systems. Although TXTL enables the expression of proteins that are toxic to living cells, the propagation of plasmids encoding these genes relies on traditional molecular biology techniques, where the plasmid is expressed and purified from a genetically optimized *E. coli* strain, such as DH5α. If the gene or gene product being carried via plasmid in such a strain is detrimental to the cell, then even minor leaky expression of the gene product can slow or stop cell growth. Cells carrying the plasmid with a loss-of-function mutation in the gene are at an advantage and will replicate faster than their counterparts. The result is a heterologous population with a bias toward organisms carrying the mutated plasmid, yielding a product that is a variation of what was intended. In some cases, when the plasmid is very toxic, cells carrying it are unable to replicate at all.

In this work, we sought to use TXTL to express native and engineered variants of *E. coli* sigma factors, essential proteins that are difficult to manipulate using standard techniques that require living cells. Sigma factors are subunits of the *E. coli* RNA polymerase enzyme that are essential for cellular gene expression. Plasmids that express these sigma factors are commercially available from Daicel Arbor Biosciences and have been demonstrated for use in the company's myTXTL kits. Once purchased, these plasmids require amplification and subsequent purification from *E. coli* strain KL740 cI857+. This strain overexpresses a repressor protein that turns off expression of the plasmids' toxic gene products, which allows the plasmid to replicate inside the cells without hindering cell growth.

We purchased a series of plasmids from Daicel Arbor Biosciences and transformed them into KL740 cI857+ or DH5α according to the company's recommendations. Multiple attempts to transform the sigma factor-encoding plasmids into KL740 cI857+ failed, whereas plasmids carrying less harmful genes transformed well into KL740 cI857+ or a standard cloning strain (Table 1). Upon consulting with Daicel Arbor Biosciences personnel, we learned that the KL740 cI857+ strain has a tendency to mutate in such a way that it is rendered unable to suppress expression of the toxic genes that are carried on the plasmid. Dr. Evelyn Eggenstein

from Daicel Arbor Biosciences kindly sent us a new sample of KL740 cI857+. We were able to successfully transform the plasmids into this new sample (Table 1).

Table 1. Summary of Daicel Arbor Biosciences Plasmids and Transformation Success in the Manufacturer's Recommended Cell Lines*

Plasmid (pTXTL-_)	Arbor Biosciences Catalog No.	*E. coli* Strain	Transformation 1	Transformation 2
P70a-T7rnap	502082	KL740 cI857+	Fail	Success
P70a-deCFP	502055	KL740 cI857+	Success	Success
P70a-ntrC	502071	KL740 cI857+	Fail	Success
P70a-S54	502079	KL740 cI857+	Fail	Success
P70a-S24	502069	KL740 cI857+	Fail	Success
P24a-deGFP	502010	DH5α	Success	N/A
P54a-deGFP	502048	DH5α	Success	N/A

*Transformation 1 used a KL740 cI857+ strain that we speculate contained a mutation that rendered it unable to propagate the plasmids. Transformation 2 used a fresh KL740 cI857+ strain that was able to carry the plasmids. N/A, not applicable.

Once the plasmids had been successfully transformed into the appropriate cell lines, we grew the transformed cells in liquid culture and performed midiprep purifications. The plasmid yields were unexpectedly low and of seemingly poor quality. They showed little to no activity in positive-control TXTL reactions (data not shown). We then decided to explore other options for the expression of genes in TXTL without the requirement of cloning to propagate genes of interest.

3.2 Properties of Linear DNA in TXTL

TXTL systems do not require the use of plasmid DNA for gene expression. In fact, linear DNA is compatible with TXTL and offers several advantages over plasmids, including the ability to create and amplify genes without the requirement of cloning and propagation inside living cells. It is also relatively easy to produce and engineer linear DNA using common PCR techniques. Producing linear DNA via PCR is agnostic to the toxicity of the gene product and was therefore advantageous for this work. However, linear DNA is generally less stable than plasmids because nucleases that degrade linear DNA over time are naturally present in the *E. coli* cell extract portion of the TXTL reaction.

Several publications have discussed additions to TXTL that increase the stability of linear DNA in these systems; the two most prominent are Chi6 DNA[6] and GamS[7] protein. Both of these additives inhibit activity of the native *E. coli* exonuclease complex RecBCD. In addition, Daicel Arbor Biosciences sells a TXTL kit optimized for linear DNA expression, the myTXTL Linear DNA Expression kit. This kit has undisclosed and presumably proprietary properties that are advertised to increase protein expression from linear DNA templates.

Because linear DNA in TXTL would be a useful tool for multiple synthetic biology and protein engineering efforts underway within our team, including the work proposed for our Innovative Development of Employee Advanced Solutions project, we decided to assess

the functionality of linear DNA in TXTL systems. We compared the standard myTXTL Sigma 70 Master Mix kit and the myTXTL Linear DNA Expression kit for performance in protein expression from a linear template. We also assessed whether chemical modifications of the ends of the DNA or longer 5′ DNA extensions would improve expression from linear DNA.

In all of the experiments described in Section 3.2, linear DNA that expresses deGFP from the P70a promoter was generated by PCR amplification of this gene from plasmid pTXTL-P70a-deGFP. This plasmid was provided in the myTXTL Sigma 70 Master Mix kit as a positive control and was used as a control in the experiments described herein.

3.2.1 Assessment of myTXTL Linear DNA Expression Kit

We initially assessed the expression of deGFP from the native *E. coli* promoter p70a in linear and plasmid format using the standard myTXTL Sigma 70 Master Mix kit and the myTXTL Linear DNA Expression kit. The concentration of linear and plasmid DNA was held constant at 10 nM in each TXTL reaction so that comparisons could be made between the two DNA types.

Expression of deGFP from the linear DNA construct failed in the standard Sigma 70 Master Mix myTXTL reaction but was successful when the Linear DNA Expression kit was used (Figure 1). This demonstrates that the proprietary additives included in the Linear DNA Expression kit are essential for gene expression from linear DNA fragments. It was surprising that the standard myTXTL formulation produced no deGFP, but this result can probably be attributed to native *E. coli* exonucleases in the cell lysate that degrade linear DNA. The differences in deGFP expression do not seem to be caused by variation in overall TXTL performance because the expression of deGFP from p70a in plasmid format was similar for both TXTL formulations (Figure 2).

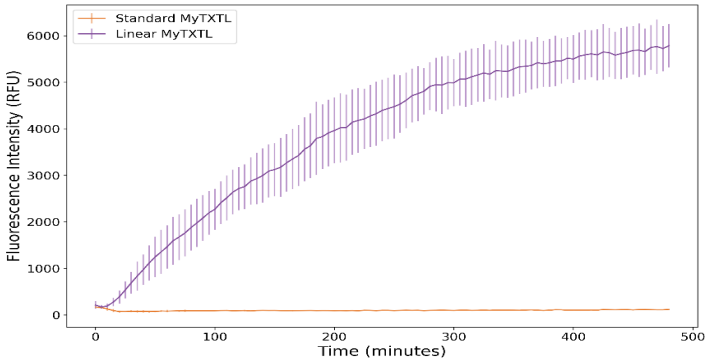

Figure 1. Linear expression of p70a_deGFP in myTXTL Sigma 70 Master Mix kit and myTXTL Linear DNA Expression kit. Production of deGFP was only observed in linear myTXTL formulation.

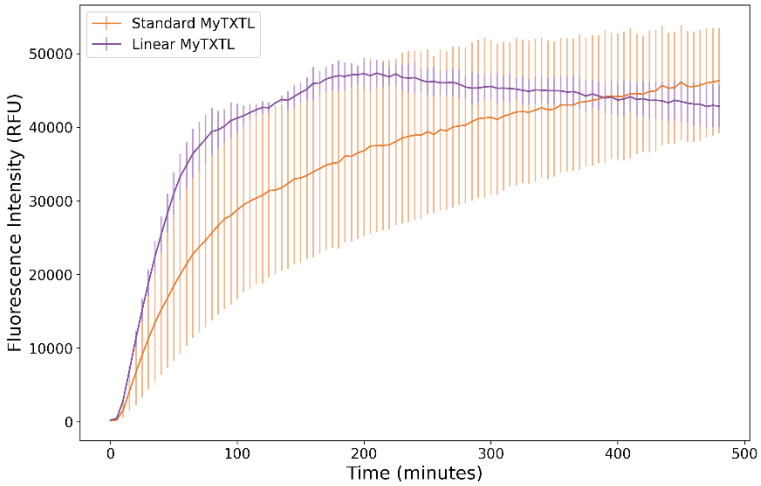

Figure 2. Plasmid expression of p70a_deGFP in myTXTL Sigma 70 Master Mix kit and myTXTL Linear DNA Expression kit. Production of deGFP was comparable in both versions of the myTXTL kit.

The expression of deGFP from the plasmid was much higher than that from the linear format. This indicates that either the stabilizing additives in the myTXTL Linear DNA Expression kit do not completely protect the linear DNA from degradation, or that expression is increased in plasmid format due to other factors, such as supercoiling.

3.2.2 Evaluation of 5′ Overhang Length on the Expression of Linear DNA in TXTL

The inhibition of exonucleases has been shown to improve gene expression from linear DNA in TXTL systems.[8] If exonuclease activity is one of the primary mechanisms preventing robust linear DNA expression in TXTL, then it is possible that including extra DNA base pairs on either side of the gene sequence may insulate the gene from degradation and lead to greater protein expression. The inclusion of longer DNA lengths on the 5′ end of the linear DNA construct may also allow the RNA polymerase to bind to the promoter or initiate transcription more efficiently.[9] With this in mind, we next evaluated whether adding extra DNA 5′ of the p70a promoter region in the linear p70a_deGFP construct would lead to greater deGFP expression in the standard and linear DNA myTXTL kits.

The original linear DNA construct described in Section 3.2.1 was 856 base pairs long and had 16 base pairs 5′ of the promoter. We extended the length of the linear construct by using a different PCR primer and amplifying the gene from the plasmid template. The new version of the linear DNA fragment had 132 base pairs 5′ of the promoter, which was 116 more than the original version.

The inclusion of a longer 5′ region improved expression of linear p70a_deGFP when the myTXTL Linear DNA Expression kit was used (Figure 3). Despite this improvement, there was no expression of deGFP from either version of the linear construct in the myTXTL

Sigma 70 Master Mix kit. This indicates that the longer 5′ extension was not sufficient to overcome the exonuclease activity in the standard TXTL reaction.

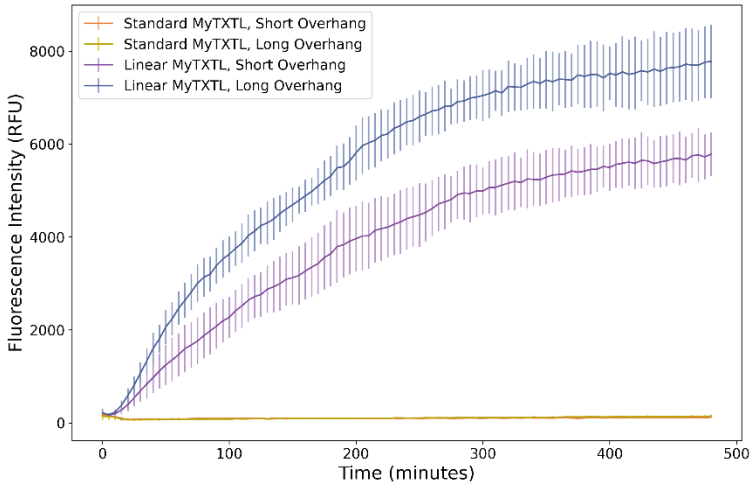

Figure 3. Expression of linear p70a_deGFP with different DNA lengths 5′ of the promoter. The longer 5′ end improved deGFP expression in the myTXTL Linear DNA Expression kit reaction but not in the myTXTL Sigma 70 Master Mix kit reaction.

3.2.3 Effects of Biotinylation on the Expression of Linear DNA in TXTL

Finally, we assessed whether including chemical modifications to the ends of the linear p70a_deGFP constructs would improve deGFP expression in TXTL. Biotinylation was previously reported to protect linear DNA from exonuclease activity.[10] To determine whether such modifications would have an impact on linear p70a_deGFP TXTL expression, we added biotin to the 5′ ends of the forward and reverse PCR primers used to generate the linear DNA fragments from the plasmid template. This modification was made to p70a_deGFP fragments both with and without an extended 5′ region.

Biotinylation did not lead to deGFP production in the myTXTL Sigma 70 Master Mix reaction for either the long or short version of linear p70a_deGFP (Figure 4). In myTXTL Linear DNA Expression kit reactions, biotinylation slightly improved deGFP production for the longer version of p70a_deGFP by the end of the reaction period, suggesting that this modification may have increased the lifetime of the DNA in the TXTL reaction (Figure 5). The opposite effect was observed for the shorter version of the DNA construct in the linear DNA-optimized TXTL reaction, where the biotinylated construct produced less deGFP than the unmodified version. It is possible that the proximity of the biotin modification to the promoter region may have interfered with RNA polymerase binding to the DNA.

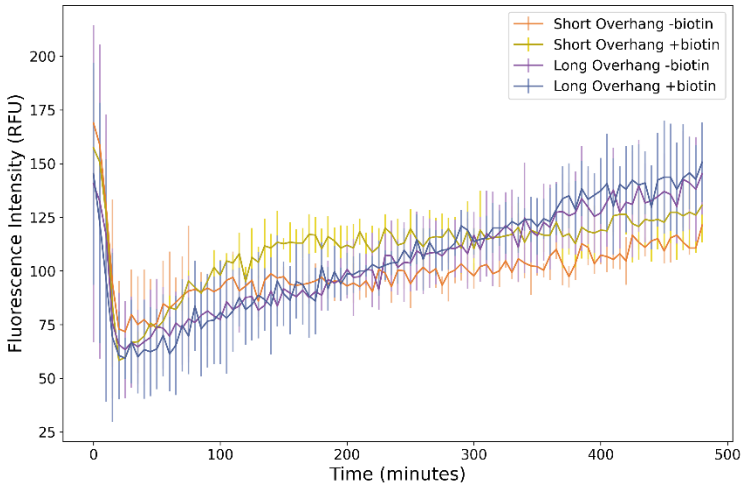

Figure 4. Expression of deGFP in biotinylated and unmodified linear DNA constructs in myTXTL Sigma 70 Master Mix kit reactions. No deGFP production was observed using any of the linear DNA variants.

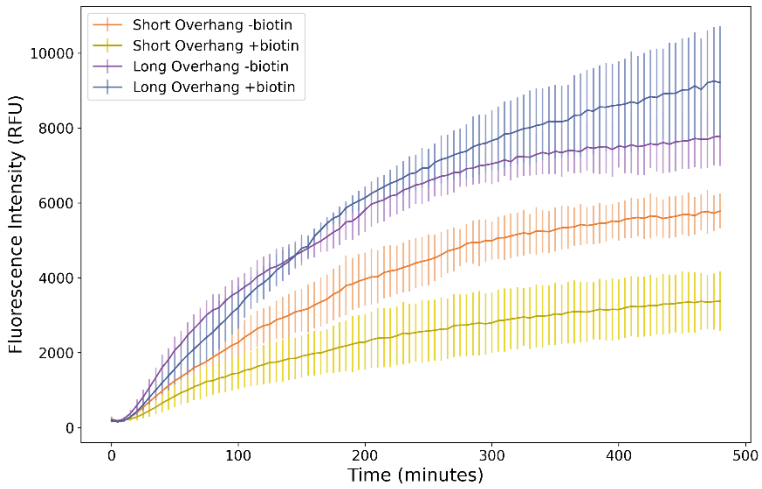

Figure 5. Expression of deGFP in biotinylated and unmodified linear DNA constructs in myTXTL Linear DNA Expression kit reactions. Biotin modification slightly improved deGFP expression in the long version of the p70a_deGFP fragment but had the opposite effect for the shorter fragment.

3.3 Discussion

Initially, the intention of our project was to use TXTL to screen engineered variants of essential *E. coli* proteins. We purchased plasmids containing genes that expressed these proteins; however, after some difficulty transforming cells with the plasmid, we were unable to sufficiently scale up the plasmid production for use in TXTL. TXTL is advantageous because it can express gene products that are toxic to living cells, but growing plasmid DNA for use in TXTL still relies on living cells.

Linear DNA can be produced and amplified without growing it inside a living cell. We investigated strategies for the expression of linear DNA in TXTL and found that using a TXTL system optimized for linear DNA expression, such as the myTXTL Linear DNA Expression kit, is essential for gene expression in linear format (Figure 1). Presumably, this kit contains additives, such as GamS protein, that inhibit the exonuclease-induced degradation of the linear fragment. Although this kit robustly expressed deGFP from the linear DNA fragment, expression of the same gene from a plasmid was still considerably higher (Figures 1 and 2).

We next looked at additional factors that could improve gene expression from linear DNA in TXTL. The inclusion of longer DNA overhangs on the linear DNA fragment improved gene expression for the linear-optimized TXTL system but not for the standard system (Figure 3). In addition, modification of the ends of the DNA fragment via biotinylation may improve gene expression when longer overhangs are present (Figure 5). In the standard DNA fragment size, biotinylation appeared to have a negative impact on gene expression. This may be because of some interference of the modification with RNA polymerase binding to the promoter.

In conclusion, we found that linear DNA could be used in TXTL without the use of plasmids. However, using a TXTL system that is optimized or engineered for linear DNA expression appears to be essential, and additional factors such as DNA length and end modification can help further optimize gene expression. These findings will be useful in future work with TXTL, such as in cases where the gene product is cytotoxic or rapid screening is desirable.

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