ELEGEN

APPLICATION NOTE

# ENFINIA™ Linear DNA Templates Drive Rapid Cell-Free Protein Expression


## SUMMARY

Cell-free transcription-translation (TXTL) systems can be used for rapid and flexible protein expression, powering foundational biology research, synthetic biology, biomanufacturing, and more. However, reliance on plasmid DNA templates for expression requires time-consuming cloning steps, limiting the speed and efficiency of protein production. Researchers often prefer using a linear DNA template as it eliminates the need for cloning, streamlines the expression process, and saves 1 to 2 weeks compared to *in vivo* protein expression workflows. Here, we demonstrate that ENFINIA Linear DNA templates, synthesized by Elegen, are functional in cell-free TXTL systems and drive reporter gene expression at levels comparable to plasmid DNA templates.


## INTRODUCTION

The academic and industrial applications of cell-free TXTL systems for protein expression have expanded in the past six decades, from answering fundamental biological questions to biomanufacturing, biosensing, biological computing, and more.[1] This technique employs extracts derived from cells, often bacterial or mammalian, which contain the necessary machinery (ribosomes, tRNAs, enzymes, etc.) to transcribe mRNA and translate it into protein using a linear or plasmid DNA template.

Cell-free TXTL systems typically support expression from plasmids and linear DNA if they contain elements recognized by the cell extract transcription and translation system. In the case of *E. coli* extract, for example, the expression template

### *Key Points*

- **All reporter genes synthesized as linear ENFINIA DNA templates were functional in cell-free TXTL reactions, with expression levels overall comparable to those from plasmid templates.**
- **Using linear ENFINIA DNA templates eliminates cloning steps, saves time, and reduces costs, making them an excellent choice for cell-free protein expression.**

must include a 5′ leader sequence, an inducible or constitutive promoter, a ribosomal binding site (RBS), a start codon, an open reading frame (ORF) of the protein of interest, a stop codon, a transcriptional terminator and a 3′ trailer sequence (for linear template only). Using linear templates as input for cell-free TXTL, as an alternative to plasmid templates, has been widely investigated, as it eliminates the need for cloning workflows, further streamlining the already rapid expression offered by TXTL systems.[3]

In the experiments described here, we combine a well-described, cell-free TXTL system, **myTXTL Pro® Cell-Free Expression Kit** manufactured by Daicel Arbor Biosciences, with ENFINIA DNA templates generated using Elegens' cell-free synthesis and cloning technology to deliver rapid protein expression in a single day. ENFINIA Linear DNA can reach lengths up to 7 kb, is NGS-verified, and shipped in 6 to 8 business days. Error rates in synthesis are as low as 1:70,000 per base pair, and ENFINIA DNA can be used in a broad range of applications, including cloning large multi-gene constructs, as templates for *in vitro* transcription (IVT), and cell-free TXTL. We used the myTXTL Pro kit to compare the expression yields for three reporter genes using a plasmid or ENFINIA DNA linear template and demonstrated that all of the DNA templates tested performed well. For the reporter genes tested, expression from ENFINIA Linear DNA templates was comparable to those seen with plasmid templates, supporting their use to shorten protein expression workflows.

The results indicated that ENFINIA Linear DNA is a suitable alternative to plasmids for cell-free protein expression, offering the potential to eliminate the time and costs of cloning longer and more complex constructs into plasmids.

*TXTL systems offer many advantages and overcome the limitations of cell-based expression systems, including:*

1. Rapid protein production within a day by skipping cloning and purification steps, making it ideal for high-throughput screening.
2. Automation-friendly and scalable protocols, compatible with small-scale (µL) volumes.
3. An ability to express toxic proteins that might be challenging to express in cell-based systems.
4. Incorporation of post-translational modifications.
5. High yields of target proteins.
6. Customizable protein expression with different promoter strengths, use of various RNA polymerases, incorporation of non-natural amino acids, and generation of antibody conjugates.
7. Simplified labeling and purification, delivering purer proteins.[2]

*Overall, the flexibility and efficiency of cell-free protein expression make it attractive to a wide range of industries and research areas.*

## MATERIALS AND METHODS

### Preparation of ENFINIA Linear DNA and Plasmid Templates for Cell-Free TXTL

Three DNA templates for cell-free TXTL of the mCherry and two codon-optimized mNeon Green (GFP-like) were designed with the required template elements (e.g., 5′ leader sequence, T7 promoter, terminator, RBS, 3′ leader sequence, etc.), according to the **manufacturer manual** ***(Figure 1A)***. Elegen synthesized the ENFINIA Linear DNA templates, and lyophilized ENFINIA DNA products were resuspended in nuclease-free water ***(Figure 1B)***.

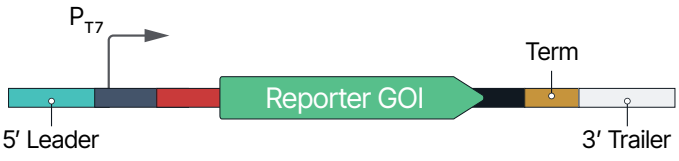


***Figure 1A.*** *General reporter construct design for all linear and plasmid DNA used in myTXTL reporter expression assays. GOI, gene-of-interest.*

The ENFINIA Linear DNA products were quantified and evaluated for purity according to the **manufacturer's manual** by UV absorbance and absorption wavelength ratios ($ABS_{260/230}$ and $ABS_{260/280}$) using DeNovix DS-11 FX spectrophotometer/Fluorometer. Linear ENFINIA DNA at a 1 to 3 µg scale was typically sufficient for screening; however, users should account for molar concentration and purity requirements. In cases where further purification is needed, purification columns can be used (Zymo Research DNA Clean & Concentrator-5 (#D4013).

Each template was also produced as ENFINIA Plasmid DNA with a pUC19 backbone. Plasmids were transformed into *E. coli* 10-beta competent cells (NEB). Six colonies per gene were picked and cultured overnight in TB, and plasmids were purified for sequencing and downstream applications. All six colonies per gene passed sequence QC, and the quantity and plasmids purity were evaluated for use in myTXTL reactions.

## Cell-Free TXTL Reactions

ENFINIA Linear DNA templates and ENFINIA Plasmid DNA concentrations were adjusted to 96 nM for the linear template and 24 nM, respectively. The final reaction concentrations of ENFINIA Linear and Plasmid DNA constructs were 20 nM and 5 nM, respectively, as the manufacturer's instructions recommended.

The T7 deGFP control plasmid, provided with the Daicel Arbor Bio myTXTL Pro Cell-Free Expression Kit, was used as a positive reaction control. Additionally, we used the control plasmid to generate a linear DNA template by PCR with the primers listed in the manufacturer's manual to obtain a linear expression control. The concentrations of the T7 deGFP controls were adjusted to the recommended reaction controls.

The Daicel Arbor Bio myTXTL Pro Cell-Free Expression reactions were set up according to the manufacturer's instructions in a total volume of 12 µL at 27°C overnight (16h) without agitation. The reactions were stopped by placing them on ice and then spun down at 16,000 x g for 3 minutes ***(Figure 1B)***.

## Protein Quantification

Reporter protein expression was quantified using VictorNivo plate reader with the following filters: For GFP, Ex/Em 488nm/510nm, measured at 480nm/530nm (-/+30); for mNeonGreen, Ex/Em 506nm/517nm, measured at 488nm/533nm (-/+30); for mCherry, Ex/Em 587nm/610nm, measured at 530nm ( -/+30)/ 600nm (-/+10).

Additionally, reactions were photographed for visual analysis of reporter gene expression, and 3-11 µL of the reaction was precipitated with ice-cold acetone, pelleted, and photographed for visual analysis. Protein was analyzed by SDS-PAGE, and overexpression of full-length reporter genes was confirmed visually using Coomassie brilliant blue staining ***(data not shown)***.

| Reporter GOI | Plasmid DNA Source | Linear DNA Source |
|---|---|---|
| deGFP | myTXTL Positive Control *(included with kit)* | PCR using plasmid template |
| mNeonGreen CO-1 | Cloned into pUC19 using ENFINIA DNA as insert | ENFINIA DNA |
| mNeonGreen CO-2 | Cloned into pUC19 using ENFINIA DNA as insert | ENFINIA DNA |
| mCherry | Cloned into pUC19 using ENFINIA DNA as insert | ENFINIA DNA |

***Table 1.*** *DNA source and names of constructs used as input for myTXTL reactions.*

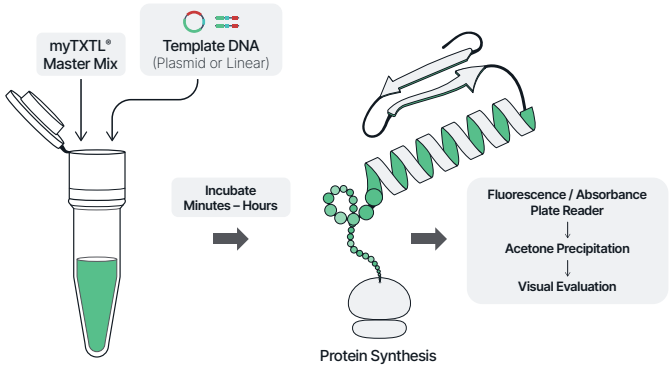


***Figure 1B.*** *Experimental workflow for myTXTL reporter assay. Briefly, linear or plasmid DNA templates were added to myTXTL reactions and incubated for 16 hours to allow reporter gene expression. Reporter expression was measured using fluorescence or absorbance plate reader (depending on the reporter) or acetone precipitation followed by visual evaluation of the protein pellet.*

# RESULTS

## ENFINIA Linear DNA Templates Perform Well in Cell-Free TXTL Reactions

Using linear DNA in cell-free TXTL saves researchers significant time by circumventing the cloning process required to generate plasmid templates. With such a benefit in mind, we examined the expression levels of several fluorescent reporter proteins from linear or plasmid DNA templates using the Daicel Arbor Bio myTXTL Pro Cell-Free Expression Kit from Arbor Biosciences.

We generated ENFINIA Linear DNA expression constructs of two codon-optimized mNeonGreen genes (mNeonGreen CO-1 and mNeonGreen CO-2). We also cloned these expression constructs into pUC19 to serve as a plasmid-based comparison of reporter expression ***(Figure 1A and B)***. Cell-free TXTL was done according to the DNA final concentrations for linear and plasmid DNA templates recommended in the manufacturer's instructions (20 nM and 5 nM, respectively).

For both codon-optimized mNeonGreen ENFINIA Linear DNA templates, we detected the same level of reporter expression compared to the ENFINIA Plasmid DNA template ***(Figure 2A and B)***. The relative fluorescence units (RFUs) detected for the mNeonGreen linear and plasmid DNA templates were on the same order of magnitude as the GFP plasmid (included as a positive control with the Daicel Arbor Bio myTXTL Pro Cell-Free Expression Kit), and a linear fragment of the GFP expression cassette generated by PCR ***(Figure 2A)***. Visual assessment of fluorescence and full-length reporter protein expression was confirmed by acetone precipitation and protein SDS-PAGE gel, respectively ***(Figure 2B and data not shown, respectively)***.

We performed an identical experimental setup and workflow for other commonly used reporter proteins and generated an ENFINIA Linear DNA expression cassette for mCherry and an ENFINIA Plasmid DNA template using pUC19 ***(Figure 1A & Figure 1B)***. We observed that similar to results generated for mNeonGreen CO-1 and CO-2, both linear and plasmid templates expressed equivalent levels of mCherry, supporting the use of ENFINIA Linear DNA templates as a lower cost and more rapid alternative to plasmid templates ***(Figure 3)***.

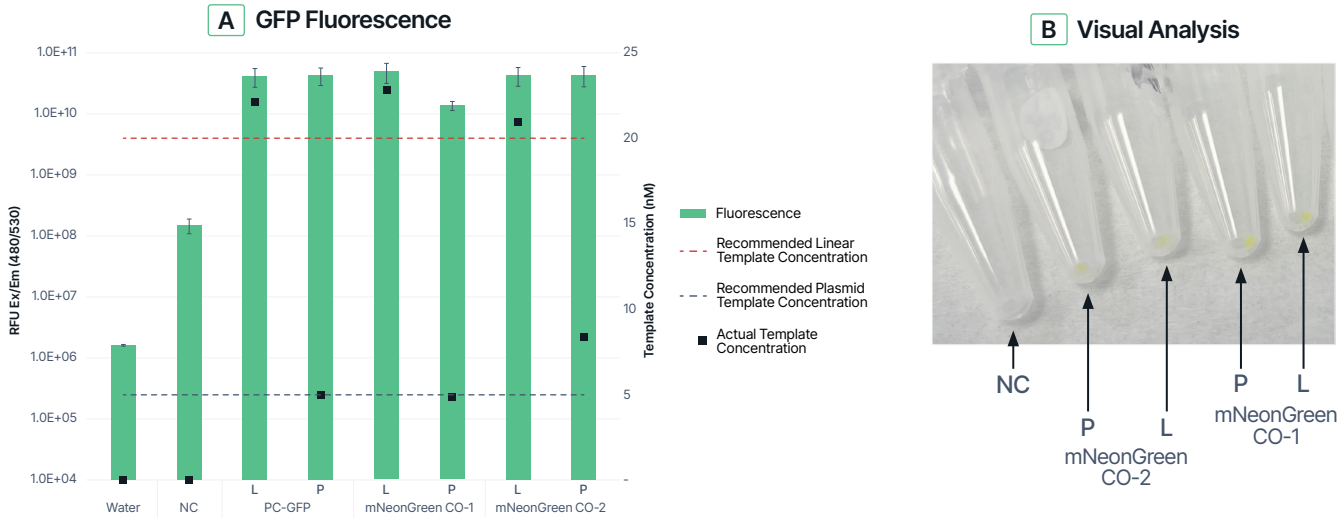


***Figure 2.*** *Expression of mNeonGreen CO-1 and CO-2 from plasmid or linear DNA templates.* ***(A)*** *myTXTL reactions were incubated with either plasmid or linear DNA templates encoding the mNeonGreen CO-1, mNeonGreen CO-2, or deGFP (as a positive control) reporter gene at the indicated concentrations. Water and a myTXTL reaction incubated without a template (NC for Negative Control) were assayed in parallel. Following 16 hours of incubation, reporter protein expression was quantified using VictoNivo plate reader. Error bars represent the SEM from 3 technical replicates.* ***(B)*** *The myTXTL reaction was acetone precipitated, pelleted by centrifugation and photographed for visual analysis of mNeonGreen expression. P, plasmid DNA template; L, linear DNA template.*

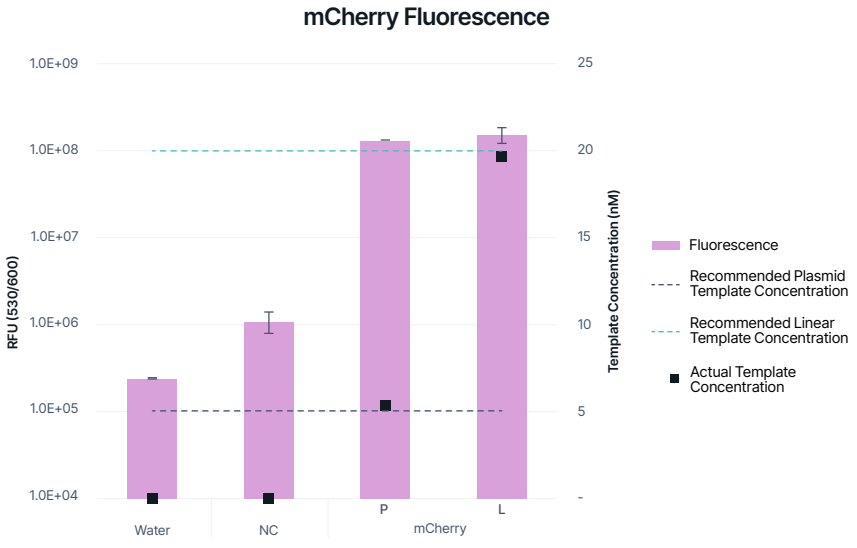


***Figure 3.*** *Expression of mCherry from plasmid or linear DNA templates. myTXTL reactions were incubated with either plasmid or linear DNA templates encoding the mCherry reporter gene at the indicated concentrations. Water and a myTXTL reaction incubated without a template (NC for Negative Control) were assayed in parallel. Following 16 hours of incubation, reporter protein expression was quantified using VictoNivo plate reader. Error bars represent the SEM from 3 technical replicates.*

*Reduced gene expression from linear templates compared to plasmids is a well described finding in cell-free TXTL systems, and myTXTL has been formulated to support linear template design without any further modifications. The mechanism behind this observation relies on an endogenous nuclease, RecBCD, a bidirectional, ATP-dependent single-stranded (ss)-DNA endonuclease activity, ss- and double-stranded (ds)-DNA exonuclease activity, present in E. coli extracts. To control the activity of this nuclease during cell-free TXTL, strategies such as recB disruption, nuclease inhibition (protein- and DNAbased), and template modification with noncanonical DNA backbones have been employed and shown to increase expression from linear templates, each with its pros and cons.[4,5] While not directly tested, these strategies are likely compatible with ENFINIA Linear DNA template in the myTXTL reaction.*

## CONCLUSION

The study described here demonstrated that Elegen's linear ENFINIA DNA templates perform well with the Daicel Arbor Bio myTXTL Pro Cell-Free Expression Kit for rapid protein expression, achieving levels comparable to plasmid templates for the reporter genes tested. If using ENFINIA DNA templates for cell-free TXTL, we recommend following the manufacturer's instructions to order the correct amount of linear DNA based on the required molar concentration of the gene of interest, measuring the template purity, and performing additional cleanup step if necessary. Researchers can fully leverage high-quality linear DNA templates to streamline workflows, reduce costs, and accelerate protein expression in cell-free systems. This approach eliminates cloning steps and opens new avenues for rapid protein production.



## References


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